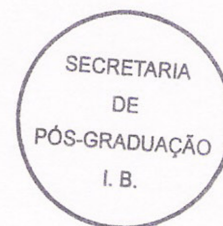
UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA



DAVI ABEID PONTES

"AVALIAÇÃO DOS MECANISMOS CAUSADORES DE DISTÚRBIOS REPRODUTIVOS EM RATOS DIABÉTICOS"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a)
DAVI ABEID PONTES
e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profa. Dra. Wilma De Grava Kempinas

Campinas, 2008

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

P777a

Pontes, Davi Abeid
Avaliação dos mecanismos causadores de distúrbios reprodutivos em ratos diabéticos / Davi Abeid Pontes. – Campinas, SP: [s.n.], 2008.

Orientador: Wilma De Grava Kempinas.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

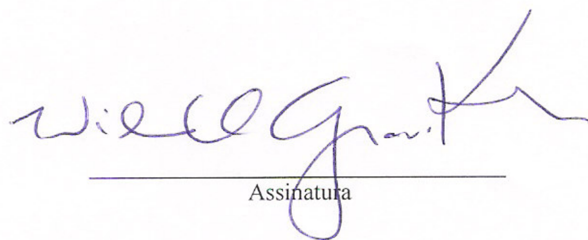
1. Diabetes. 2. Rato. 3. Testículos. 4. Testosterona. 5. Infertilidade. I. Kempinas, Wilma De Grava. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Título em inglês: Evaluation of the mechanisms responsible for reproductive damage in diabetic rats.
Palavras-chave em inglês: Diabetes; Rat; Testis; Testosterone; Infertility.
Área de concentração: Biologia Celular.
Titulação: Mestre em Biologia Celular e Estrutural.
Banca examinadora: Wilma De Grava Kempinas, Isabel Cristina Cherici Camargo, Catarina Segreti Porto.
Data da defesa: 14/02/2008.
Programa de Pós-Graduação: Biologia Celular e Estrutural.

Campinas, 14 de Fevereiro de 2008.

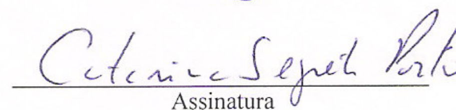
BANCA EXAMINADORA

Dra. Wilma De Grava Kempinas (Orientadora)



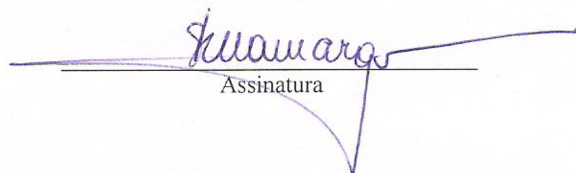
Assinatura

Dra. Catarina Segreti Porto



Assinatura

Dra. Isabel Cristina Cherici Camargo



Assinatura

Dr. Sérgio Luís Felisbino

Assinatura

Dra. Camila Contin Diniz de Almeida Francia

Assinatura

"A mente que se abre a uma nova idéia jamais volta ao seu tamanho original"

"Quanto mais eu observo o universo, mais ele se parece a um grande pensamento do que a uma grande máquina"

Albert Einstein
(1879-1955)

DEDICATÓRIA

À minha família, por seu amor incondicional, e pelos valores a mim ensinados pelos quais tanto prezo e me orgulho.

E ao meu amor, pelo amor...

AGRADECIMENTOS

“A Deus, pelo dom da vida e por nos mostrar através de Seu filho o que é o verdadeiro amor.”

“Aos meus pais e irmãos, por estarem sempre comigo pelo caminho que escolhi.”

“À Bruna, por seu sorriso e força. Por me mostrar que nada é impossível a alguém que realmente vai atrás daquilo o que realmente almeja.”

“À minha família em geral, pela alegria e união sempre presentes.”

“À Dra. Wilma, pelos conselhos e conhecimentos passados, e pela oportunidade a mim dada de inserção no meio científico, sendo a ciência umas das minhas grandes paixões”

“Ao CNPq e à FAPESP, pelo apoio dado em forma de bolsa científica para realização da pesquisa envolvida nesta dissertação de mestrado.”

“Ao Programa de Pós-Graduação em Biologia Celular e Estrutural da UNICAMP e seus Docentes, pelo excelência e pelo conhecimento disponibilizado a nós alunos.”

“À Secretária do Programa acima referido, Líliam Panagio, por sua gentileza e boa vontade em sempre ajudar quando necessário.”

“Aos profissionais do Laboratório de Neuroendocrinologia da Faculdade de Odontologia de Ribeirão Preto – USP, pelo auxílio na técnica de dosagens hormonais realizada neste trabalho.”

“Ao Prof. Dr. Oduvaldo C.M. Pereira, à Profa. Dra. Daniela C.C. Gerardin e à Pós-Graduanda Renata C. Piffer, do Laboratório de Farmacologia da Reprodução do Instituto

de Biociências de Botucatu – UNESP, pelo auxílio na técnica de contratilidade do ducto deferente realizada neste trabalho."

"Ao técnico de laboratório, e acima de tudo grande amigo e companheiro José Eduardo, pela ajuda, paciência, companhia, e pelas conversas e causos durante toda minha estadia no laboratório."

"Ao Departamento de Morfologia da UNESP de Botucatu e a seus Funcionários, pela excelência de suas instalações e serviços oferecidos."

"À Secretária do Departamento acima referido, Luciana, pela ajuda, disponibilidade e boa vontade em seu trabalho."

"Aos Professores presentes em minha Banca de Qualificação, Dr. Alexandre R.L. de Oliveira, Dra. Shirlei Maria Recco Pimentel, e Dra. Mary Anne Heidi Dolder, pelos conselhos e ensinamentos passados."

"À Dra. Isabel C.C. Camargo, à Dra. Débora C. Damasceno e à Dra. Catarina S. Porto, pela avaliação prévia deste trabalho."

"Aos amigos do Laboratório, e faço questão de citar todos pelo nome: Glaura (Glendinha), Juliana (Sapa), Marina (Má), Thaiane (Tuia), Denise (Kimera vaca véia), Ana Paula (Aninha), Fernanda (Cajá), Carla (Orgulho), Fabíola (Fá), Carla Teixeira (Carlinha), Wellerson (São Jorge), Gustavo (Prendado), Luiz (Pechocho), Daniela (Dani) e Ana Silvia (Ana), por todos os momentos compartilhados na famosa salinha dos estagiários. Nos momentos bons, obrigado pela alegria, naqueles não tão bons, obrigado pela força."

"Ao Ferinha, Luiz Gustavo de Almeida Chuffa, pelo grande amigo de todas as horas que você é. Obrigado pelo companheirismo, pela força, e por tudo garoto!"

"Aos amigos e colegas, aos que passaram por mim e aos que permanecem até hoje, por fazerem parte desta experiência maravilhosa que é a vida. Por cada momento, pois tudo é válido como ensinamento."

"A todos que esqueci, devido aos problemas que tenho com minha memória, embora ainda seja novo... rsrs. Meu muito obrigado!"

"Às críticas que recebi, recebo e receberei. Às positivas, pela sensação de que o esforço vale a pena; às negativas, pela força que me dão em ser alguém melhor."

"A todos, meu muitíssimo obrigado!"

"Procure ser uma pessoa de valor, em vez
de procurar ser uma pessoa de sucesso.
O sucesso é conseqüência."
Albert Einstein

"Os grandes sonhos e objetivos são o que
nos mantêm vivos; a jornada até lá é a vida.
Faça dela uma experiência memorável"
Autoria própria

SUMÁRIO

Resumo.. 01
Abstract.. 03
Introdução.. 05
Revisão Bibliográfica.. 09
Neuroendocrinologia do Sistema Reprodutor Masculino................................... 10
Testosterona.. 12
Ejaculação... 14
Justificativa e Relevância da Temática.. 16
Artigos 18
Artigo 1... 20
Artigo 2... 43
Conclusões Finais... 72
Referências da Introdução e da Revisão Bibliográfica.. 74

Resumo

RESUMO

O *Diabetes mellitus* geralmente se associa a algum tipo de disfunção sexual, provocando infertilidade, tanto em seres humanos, quanto em animais experimentais. Em trabalho anterior realizado em nosso laboratório, ratos machos que tiveram hiperglicemia induzida quimicamente pela administração de *streptozotocin* apresentaram diminuição da fertilidade através de múltiplos parâmetros analisados. O presente estudo teve como objetivo investigar os mecanismos envolvidos e o papel da testosterona no processo. Para tanto, ratos machos foram divididos em 3 grupos experimentais: normoglicêmico (controle), hiperglicêmico (*streptozotocin*), e hiperglicêmico com reposição hormonal (*streptozotocin*+testosterona) e foram avaliados parâmetros reprodutivos e espermáticos, níveis hormonais, a contratilidade do ducto deferente isolado *in vitro*, comportamento sexual e o número de espermatozóides ejaculados no útero. O ducto deferente de animais diabéticos apresentou um quadro de hipersensibilidade à metoxamina, um agonista sintético de adrenoceptores α_1. Estes mesmos animais apresentaram os seguintes resultados: alteração de comportamento sexual e ausência de espermatozóides ejaculados, redução dos níveis plasmáticos de testosterona, perda de peso corpóreo e de órgãos como epidídimo, ducto deferente, vesículas seminais e próstata ventral, perda de células germinativas na luz e desorganização epitelial aparente em túbulos seminíferos, além da aceleração do tempo de trânsito dos espermatozóides pelo epidídimo. Os dados apresentados neste trabalho indicam que os mecanismos responsáveis pela perda de fertilidade natural de ratos diabéticos envolvem comprometimento do processo espermatogênico, assim como desregulação do eixo reprodutivo masculino, juntamente com evidências para problemas no processo de maturação espermática, tendo como fator complicante o prejuízo da função ejaculatória, dependente da contratilidade da musculatura lisa dos ductos deferentes. A reposição de andrógeno não foi totalmente capaz de reverter os danos causados pelo diabetes no sistema reprodutivo masculino de ratos adultos.

Palavras-chave: diabetes, rato, testículo, testosterona, infertilidade

Abstract

ABSTRACT

Diabetes mellitus is usually related with some kind of sexual dysfunction, promoting infertility in humans as well as in experimental models. In a prior work from our laboratory, male rats, which had a diabetic-induced state of hyperglycemia caused by *streptozotocin* administration, demonstrated reduced fertility through several parameters analyzed. The present study aimed at investigating the mechanisms involved and the role of testosterone in the process. Male rats were randomly allocated in 3 experimental groups: control, hyperglycemic (*streptozotocin*), and hyperglycemic with hormone replacement (*streptozotocin*+testosterone) and the following parameters were analyzed: reproductive and spermatic parameters, hormone levels, sexual behavior, contractility of vas deferens in vitro, sexual behavior parameters and the number of sperm ejaculated in utero. The vas deferens of diabetic animals was hypersensitive to methoxamine, a synthetic agonist of α_1 adrenoceptors. The same animals showed the following results: alterations in sexual behavior and lack of sperm ejaculated, reduction in plasma testosterone levels, decreased body weight and epididymis, seminal vesicles, ventral prostate and vas deferens weights, loss of germ cells in the lumen and apparent epithelial disarrange in seminiferous tubules, and accelerated sperm transit time in the epididymis. The data presented herein indicate that the mechanisms underlying the reduced fertility through natural mating observed in diabetic rats involve impairment of the spermatogenic process, as well as a dysregulation of the male reproductive axis, together with evidence for problems in the sperm maturation process, which has as a complicant factor the impairment of the ejaculatory function, dependent on the vas deferens smooth muscle contractility. Androgen replacement was not totally capable of reversing the damage caused by diabetes on the male reproductive system of adult rats.

Key-words: diabetes, rat, testis, testosterone, infertility

Introdução

INTRODUÇÃO

O *Diabetes mellitus,* ou simplesmente diabetes, é uma doença que se caracteriza por uma deficiência na secreção ou na ação do hormônio insulina, sendo relativamente comum: aproximadamente 6% da população dos EUA mostram algum grau de anormalidade no metabolismo da glicose, indicativo de diabetes ou uma tendência a ele (Nelson & Cox, 2002). No Brasil, o único estudo estatístico a respeito foi realizado entre 1986 e 1989 e indicou uma prevalência de 7,6% de diabéticos na população entre 30 e 69 anos, sendo que 50% dos indivíduos não conheciam o diagnóstico; estudos mais atuais têm sido adiados devido ao alto custo para o governo, mas estimativas indicam cerca de 11 milhões de diabéticos no país (SBD, 2006). Esse quadro clínico pode se estabelecer devido a uma deficiência na produção de insulina (total ou parcial) pelas células beta (β)-pancreáticas. Há duas classes principais da doença: Tipo 1 e Tipo 2. Na primeira, a doença surge já nos primeiros anos de vida do indivíduo e torna-se grave rapidamente, deixando-o dependente da administração de insulina exógena e do controle do consumo de glicose segundo o nível de glicemia. A última é de aparecimento lento, moderado e freqüentemente passa despercebida, associando-se a problemas metabólicos como obesidade, tendo como principal característica a resistência insulínica.

Os sintomas característicos do diabetes são sede excessiva e micção freqüente (poliúria), levando à ingestão de grandes volumes de água (polidipsia). Essas alterações são causadas pela grande quantidade de glicose que se acumula na corrente sangüínea, gerando hiperglicemia que pode, por conseguinte, causar outros problemas metabólicos no organismo (Nelson & Cox, 2002).

O modelo de indução de diabetes Tipo 1 por *streptozotocin* (STZ) surgiu como uma ferramenta útil no estudo do diabetes em ratos e outros modelos experimentais (Like & Rossini, 1976), sendo amplamente utilizado em estudos sobre efeitos da doença até a data presente, mimetizando o quadro clínico da doença (Howland & Zebrowski, 1976; Hassan et al., 1993; Ballester et al., 2004; Scarano et al., 2006). STZ tem meia-vida de 24h, e apesar de não ter efeito em humanos, é altamente eficaz em lesar seletivamente células β pancreáticas de ratos e outros mamíferos (Like & Rossini, 1976).

Um dos efeitos freqüentemente associados ao diabetes em homens e em modelos experimentais é algum tipo de disfunção sexual (Fairburn, 1981; Calvo et al., 1984; Steger & Rabe, 1997; Scarano et al., 2006). Estes problemas geralmente resultam de mudanças nos sistemas nervoso periférico e vascular, além dos problemas periféricos resultantes do metabolismo deficiente de carboidratos, e evidências apontam que alterações no sistema nervoso central, que venham a afetar o controle endócrino e indiretamente o controle sexual do organismo podem gerar também algum tipo de disfunção sexual (McVary et al., 1997).

Mais especificamente, algumas complicações encontradas no quadro do diabetes descontrolado são disfunção erétil, infertilidade e ejaculação retrógrada, mas os mecanismos fisiopatológicos relacionados a estes sintomas ainda não são totalmente compreendidos (Melman et al., 1980; Andersson, 2003; Basu & Ryder, 2004; Chaiban & Azar, 2004; Fedele, 2005). Problemas relacionados à espermatogênese e à função testicular em animais têm sido demonstrados (Kuhn-Velten et al., 1978; Murray et al., 1983) e também alterações que poderiam modificar a qualidade espermática e o processo de fecundação, como redução no número de ATPases e na atividade enzimática de

fosfatases (Balasubramanian et al., 1991). Outro sintoma relacionado à doença é a redução dos níveis circulantes de testosterona no organismo (Arduíno, 1980; Scarano et al., 2006; Zhang et al., 2006), causada pela diminuição dos níveis de insulina que, quando baixos, prejudicam a função das células de Leydig testiculares que produzem o hormônio esteróide masculino, devido ao importe insuficiente de glicose para a célula.

Estudo realizado previamente em nosso laboratório (Scarano et al., 2006) verificou uma diminuição da fertilidade natural de ratos com diabetes induzido por *streptozotocin*. Entretanto, os mecanismos pelos quais a função reprodutiva se demonstra afetada no quadro de diabetes grave descontrolado ainda são desconhecidos.

Revisão Bibliográfica

REVISÃO BIBLIOGRÁFICA

Neuroendocrinologia do Sistema Reprodutor Masculino

Em mamíferos, a função reprodutiva é controlada por hormônios produzidos tanto em níveis centrais, em regiões do encéfalo e da hipófise, quanto em níveis periféricos, nas gônadas. Os neurônios hipotalâmicos participantes do eixo reprodutivo hipotalâmico-hipofisário-gonadal produzem GnRH (hormônio liberador de gonadotrofina), regulador que atua na hipófise estimulando a secreção de LH (hormônio luteinizante) e de FSH (hormônio folículo-estimulante). Estes por sua vez atuam na gônada masculina diretamente, controlando a função reprodutiva: o LH estimulando as células intersticiais de Leydig a produzir testosterona a partir de precursores esteróides, e o FSH atuando nas células de Sertoli que, por sua vez, controlam o processo de espermatogênese através do metabolismo de lactato (essencial como fonte de energia para as células germinativas) e produzem a ABP (*Androgen Binding Protein*), que se liga à testosterona produzida localmente para que ela atue de maneira parácrina, contribuindo para o ciclo espermatogenético de produção de gametas. As células de Sertoli produzem, além destes dois compostos, ativador de plasminogênio, transferrina e estrógeno mediante a ação de FSH. Outras substâncias que afetam a função de Sertoli são retinol, insulina e testosterona (Hutson, 1984; Plant & Marshall, 2001, Sharpe et al, 2003).

Este eixo reprodutivo descrito resumidamente acima se mantém sob controle de um refinado sistema de *feedback* negativo e positivo. Desta maneira, em mamíferos adultos, altas concentrações circulantes de FSH ou LH, por exemplo, são sinais

biológicos que levam o hipotálamo a diminuir a secreção de GnRH, e esta relação de *feedback* se estabelece durante o processo de maturação sexual animal, visto que ratos imaturos não têm esta relação bem consolidada (Payne et al, 1977).

A ação do hormônio FSH se dá de diferentes maneiras nos tecidos-alvo gonadais, dependendo do estágio da vida reprodutiva em que se encontra o animal. Em ratos neonatos, o FSH inibe a produção de testosterona em células de Leydig, de modo que essa secreção andrógena é postergada até o momento certo, que é a instalação da puberdade, quando o epitélio germinativo está suficientemente diferenciado para suportar o processo de espermatogênese – possivelmente esta inibição se dê de maneira indireta, através de estradiol ou de MIS (substância inibitória mulleriana) que seriam produzidas pelas células de Sertoli mediante o estímulo de FSH hipofisário. Estradiol e MIS modulam a cascata citoplasmática de produção de andrógeno em Leydig, reduzindo a atividade das enzimas que agem na produção de esteróides e diminuindo o número de receptores para hCG (Sriraman & Jagannadha Rao, 2004). À medida que o rato entra na puberdade, cessa a produção de estradiol e de MIS nas células de Sertoli. Em ratos pré-púberes adultos, o FSH começa a agir diferentemente no testículo, aumentando a produção de testosterona em células de Leydig. Esse aumento de produção deve-se, ao que tudo indica, ao fator IGF-I, produzido pelas células de Sertoli mediante o estímulo de FSH, que atua localmente nas células de Leydig estimulando-as a produzir testosterona.

Como se pode perceber, existe uma íntima correlação parácrina no testículo entre células de Sertoli e de Leydig mediada por citocinas, fatores de crescimento e esteróides (Lejeune et al., 1996), de modo a controlar assim direta e indiretamente o processo espermatogênico. A presença ou ausência de qualquer um dos tipos celulares presentes

no testículo, sejam as células germinativas, de Sertoli ou de Leydig, afeta todas as outras células, como pode ser observado em diversos estudos metabólicos, ultraestruturais, fisiológicos ou moleculares relacionados (Jégou et al., 1991; Jégou, 1993; Sharpe et al., 1993; Huleihel & Lunenfeld, 2004). Co-culturas de células de Leydig e Sertoli, quando em meios contendo FSH, apresentam células de Leydig com maior quantidade de receptores para LH e também retículo endoplasmático liso mais desenvolvido (Parvinen et al., 1984), o que demonstra uma inter-relação entre estas duas populações celulares até mesmo em termos de modulação da resposta a estímulos centrais.

Testosterona

A testosterona, no indivíduo masculino, é produzida principalmente pelas células de Leydig. A via metabólica de produção de andrógenos se inicia com o colesterol que se converte em pregnenolona (através da enzima desmolase ou P450ssc) nas mitocôndrias de células que possuem enzimas específicas para tal conversão, e as etapas seguintes de conversão de pregnenolona em andrógenos acontecem no retículo liso destas células, também por enzimas específicas deste processo. Isto ocorre também na glândula adrenal, nas zonas *fasciculata* e *reticularis*, local de principal produção de andrógenos nas mulheres. Como parte do controle homeostático animal, a testosterona promove *feedback* negativo nos neurônios hipotalâmicos liberadores de GnRH e também nas células da hipófise que produzem LH, diminuindo assim o estímulo para produção de mais andrógeno, pois se há testosterona livre na corrente sanguínea, não é necessária sua produção no testículo. Este é um marcador biológico para o organismo.

Bancroft (2005) atenta para um importante papel da prolactina como mediador do *feedback* negativo da testosterona. A presença de receptores para prolactina em células da hipófise corroboraria a hipótese de que altos níveis circulantes de testosterona estimulam a secreção de prolactina, que por sua vez agiria na hipófise desestimulando a produção de LH. O fato de que um quadro de hiperprolactinemia leva a uma redução nos níveis de testosterona circulantes também condiz com esta hipótese.

A testosterona tem muitas funções no homem, principalmente aquelas ligadas às características secundárias masculinas, exercendo estas funções através de mecanismos de ativação direta de genes específicos em células como fibras de musculatura esquelética e folículos pilosos, por exemplo, propiciando a estes tecidos o crescimento celular e o desenvolvimento relacionado à chegada da puberdade. Hormônios esteróides, como a testosterona, atravessam a membrana plasmática de células-alvo, ligando-se a proteínas do citosol e formando complexos protéicos que sofrem importação para o núcleo celular, e assim ativam genes específicos, modulando sua expressão. Por todo o trato reprodutor masculino, em células específicas, a testosterona é convertida através da ação da enzima 5α-redutase em DHT (dihidrotestosterona), um andrógeno cerca de 10 vezes mais potente. É o DHT que age nas glândulas acessórias como próstata e vesícula seminal, ativando a transcrição de genes específicos importantes para as funções destes órgãos (Dohle et al., 2003).

Os efeitos da testosterona no organismo se dão também em nível central, em várias regiões do cérebro, sendo que nas regiões hipotalâmicas, da amígdala e da estria terminal (região do tálamo) seu efeito está associado à ação da enzima aromatase, que converte o andrógeno em estradiol. Outras regiões cerebrais em geral mostram receptores

para testosterona, e são consistentes com a hipótese de que a testosterona está associada à libido e ao comportamento sexual masculino, além de outros efeitos como a masculinização cerebral e a estimulação de crescimento neuronal em regiões como o córtex cerebral, por exemplo, em humanos e macacos (Bancroft, 2005). Foi constatada também a ação de andrógenos na região lombossacral da medula espinhal, e receptores específicos foram observados no gânglio pélvico maior e em outros tecidos corporais e vasculares, regiões relacionadas à ação da testosterona nos processos de ejaculação e ereção (Gooren & Kruijver, 2002; Simonsen et al., 2002).

Ejaculação

O ciclo de resposta sexual humano consiste de quatro diferentes estágios, que são: desejo, excitação, orgasmo, e resolução, sendo o orgasmo o mais curto, porém mais intenso de todos. Em meio a estes quatro estágios, existem no homem processos que possibilitam a expulsão dos gametas do trato reprodutor masculino para que possam entrar em contato com o trato feminino durante o ato sexual; dentre estes processos, destaca-se a ejaculação (Giuliano & Clément, 2005).

O processo de ejaculação é de estimulação multifatorial, tendo como coadjuvantes a testosterona, a ocitocina, e principalmente a inervação autonômica simpática e parassimpática, onde ocorre um balanço de neurotransmissores estimulatórios (noradrenalina, adrenalina e metilnoradrenalina) (Rang et al., 2004) e relaxantes (acetilcolina perifericamente e serotonina em nível central) (Giuliano & Clément, 2005) da musculatura lisa dos ductos deferentes e epidídimos, órgãos pelos quais ocorre trânsito

de espermatozóides que neste momento ainda são imóveis, e necessitam tanto da emissão do fluido seminal quanto da contração muscular dos órgãos citados para continuarem seu trajeto pelo trato masculino.

A ejaculação pode ser dividida em duas fases: emissão e expulsão. A primeira fase – emissão – pode ser explicada como a ejeção de espermatozóides juntamente com produtos secretados pelas glândulas sexuais acessórias em direção à uretra posterior. Durante este processo, tanto a secreção epitelial quanto a contração da musculatura lisa acontecem pelo trato reprodutivo, e todos os órgãos participantes deste processo recebem intensa inervação tanto simpática quanto parassimpática, vinda principalmente do plexo pélvico. Durante a emissão propriamente dita, a inervação simpática toma lugar, liberando localmente noradrenalina. Outros fatores atuantes são acetilcolina, ocitocina, NPY – neuropeptídeo Y, VIP – *vasoactive intestinal peptide*, e NO – óxido nítrico. Já o processo de expulsão, seguinte à emissão, ainda permanece sem muitas explicações quanto aos mecanismos fisiológicos envolvidos. Representa a ejeção do esperma da uretra, e o mais aceito atualmente é que acontece devido a um reflexo da medula espinhal que ocorre quando o processo ejaculatório alcança um "ponto sem volta", onde as fibras musculares lisas se contraem prevenindo um fluxo retrógrado do sêmen à bexiga (Giuliano & Clément, 2005).

Justificativa e Relevância da Temática

JUSTIFICATIVA E RELEVÂNCIA DA TEMÁTICA

O número de homens diabéticos é muito alto e, além disso, muitos desses pacientes não seguem o tratamento devido (dieta, medicamentos, entre outros) ou mesmo desconhecem seu quadro patológico. Dentre as conseqüências do diabetes descontrolado estão os distúrbios reprodutivos, sendo que os mecanismos pelos quais a hiperglicemia provoca infertilidade ainda são pouco conhecidos. Diante disso, justifica-se a realização de um estudo experimental para averiguar os fatores que geram tais danos reprodutivos.

Artigos

ARTIGOS

O presente trabalho de Mestrado originou dois artigos científicos, intitulados: “Possible mechanisms involved in the impairment of adrenergic response of the vas deferens in streptozotocin-induced diabetic rats”, submetido à publicação no periódico internacional “Journal of Andrology” e “Impairment of spermatogenesis and reproductive axis dysregulation in streptozotocin-induced diabetic rats”, preparado para submissão ao periódico internacional “International Journal of Andrology”, que são apresentados a seguir.

ARTIGO 1

Possible mechanisms involved in the impairment of adrenergic response of the vas deferens in streptozotocin-induced diabetic rats

Running head: *diabetes and vas deferens contractility*

Davi A. Pontes[1], Glaura S.A. Fernandes[1], Thaiane A. Assumpção[2], Renata C. Piffer[3], Daniela C.C. Gerardin[3], Oduvaldo C.M. Pereira[3], Wilma D.G. Kempinas[2].

1 – Graduate Program in Cellular and Structural Biology, Institute of Biology, State University of Campinas – UNICAMP.
2 – Department of Morphology, Institute of Biosciences of Botucatu, São Paulo State University – UNESP.
3 – Department of Pharmacology, Institute of Biosciences of Botucatu, São Paulo State University – UNESP.

Corresponding author:
Wilma De Grava Kempinas
Departamento de Morfologia
Instituto de Biociências de Botucatu
Universidade Estadual Paulista – UNESP
Caixa-Postal 510
CEP 18618-000, Distrito de Rubião Jr., s/n, Botucatu, SP – Brazil.
Telephone number: +55 (14) 38116264, Fax: +55 (14) 38116264
Email: kempinas@ibb.unesp.br

Financial Support: FAPESP – The State of São Paulo Research Foundation and CNPq - National Council for Scientific and Technological Development.

Abstract

The hyperglycemic and hypoinsulinemic state caused by *Diabetes mellitus* is usually related with some kind of sexual dysfunction, resulting in infertility in humans as well as in experimental models, mostly due to its effects on the ejaculatory function. This study aimed at a deeper investigation of the possible mechanisms by which this damage occurs, and whether testosterone replacement has any effect in restoring this situation to normal parameters. Male Wistar rats were randomly allocated into 3 experimental groups: control, diabetic (streptozotocin), and diabetic with hormone supplementation (streptozotocin+testosterone). In vitro isometric contractions of the vas deferens were assessed, together with weight values of the organ, final body weight, circulating testosterone levels, and the number of spermatozoa ejaculated in utero through natural mating. Alteration of the ejaculatory process in diabetic animals was confirmed, showed by the hypersensitivity state of the alpha-1 adrenoreceptor to methoxamine, a synthetic agonist of norepinephrine, and by the lack of spermatozoa ejaculated in the uterus of receptive females; hormone replacement did not restore these parameters to their normal condition, as seen in controls. We conclude that the diabetic state damages the ejaculatory process dependent also of the contractility of the vas deferens resulting in no sperm ejaculation, mostly probably because of problems related to the functioning of norepinephrine release at the synaptic cleft, caused primarily by autonomic neuropathy.

Key-words: diabetes, ejaculation, testosterone, norepinephrine, infertility.

Introduction

Diabetes mellitus is one of the most widespread diseases that represent threat to human's health in the modern world, affecting over 171 million people; an estimative for 2030 reaches 366 million people (WHO, 2007). Its incidence is increasing rapidly, and its effects on human's health are mostly due to the hyperglycemic and hypoinsulinemic state caused by the disease, usually affecting many organic functions, such as neurological, endocrine and reproductive. Obesity, genetic predisposition and ageing are thought to be the leading factors in this current worldwide scene. The most worrying aspect of diabetes is that it commits many men at reproductive age, and most of them are not aware of their ill state, whether at initial or late stages (SBD, 2007).

Sexual dysfunctions related to the diabetic state have been extensively described (Kuhn-Velten et al., 1978; Arduíno, 1980; Melman et al., 1980; Fairburn, 1981; Murray et al., 1983; Calvo et al., 1984; Ficher et al., 1984; Balasubramanian et al., 1991; Andersson et al., 2003; Chaiban and Azar, 2004; Fedele, 2005; Scarano et al., 2006; Zhang et al., 2006), but their pathophysiological pathways are yet to be clearly elucidated. These studies demonstrate that decreased levels of testosterone in diabetic men and experimental models lead to impairment of reproductive parameters, but whether these low levels correspond to a main factor leading to damage of the ejaculatory function dependent of vas deferens contractility remains unclear. It is believed that pelvic autonomic neuropathy contributes to impotence and retrograde ejaculation in the male. Gallego et al. (2003) stated that the catecholaminergic systems in the diabetic rat are affected in a highly specific manner, with decreased levels of dopamine in the

dopaminergic nigrostriatal system, increased levels of norepinephrine in cardiac ventricles and decreased levels in the stellate ganglia and the blood serum, and increased levels of epinephrine in the adrenal gland and decreased levels in the serum.

Ejaculation is a process stimulated by a series of complex events, which depend on coadjuvants such as testosterone, oxytocin, NPY – neuropeptide Y, VIP – vasoactive intestinal peptide, and NO – nitric oxyde. The main role is played by the sympathetic and parasympathetic autonomic neural transmission, leading to a balance of stimulatory (norepinephrine, epinephrine, among others) and relaxant neurotransmitters (acetylcholine locally, serotonin centrally) taking place on smooth muscle present in the vas deferens and the epididymides, organs in which the sperm transits. At this moment spermatozoa are still immotile, therefore need emission of the seminal fluid and local muscle contractions to continue their path through the male reproductive system (Rang et al., 2004; Giuliano and Clément, 2005).

The effects of diabetes on the ejaculatory process of rats, which can lead to a decrease of the fertility rate in vivo (Scarano et al., 2006), can be explained by a secondary complication of diabetes, the autonomic neuropathy syndrome (Hassan et al., 1993; Vinik et al., 2003). This pathological situation affects autonomic neurotransmission involved with ejaculation, among other important biological functions, acting both on the vas deferens and the epididymis, having largely negative impacts on the survival and life quality of diabetic individuals.

This syndrome is capable of impairing the whole autonomic system, and it is usually associated with advanced stages of diabetes, although subclinical symptoms can be noted at earlier stages. Hypotheses to the rising of this syndrome relate to damaging of

neuronal metabolism, vascular insufficiency, autoimmune damage, deficiency on neuro-hormonal growth factors, and increase of oxidative stress (Vinik et al., 2003). All these enumerated factors can lead to cellular necrosis and activation of genes related to neuronal damage, leading us to believe that any organ or tissue innervated by the autonomic system may have its function compromised, as in the case of the vas deferens and the epididymis, both important organs for sperm transit through the male reproductive tract and dependent of sympathetic and parasympathetic neurotransmission. In these cases, symptoms like decrease of ejaculatory potential, retrograde ejaculation and erectile dysfunction may appear. As to the mechanisms responsible for erectile dysfunction in diabetes, the main cause is the impairment of the vasodilator machinery dependent of the neurogenic and endothelial function of the corpora cavernosa, related to nitric oxyde (NO); more specifically, problems with BH4 – tetra-hydrobiopterin, which acts on the dimerization of NOS (NO sintase), a crucial process to the right functioning of the enzyme (Flora-Filho and Zilberstein, 2000; Simonsen et al., 2002; Vinik et al., 2003).

This study aimed at a deeper investigation of the mechanisms by which streptozotocin-induced diabetic rats have an impairment of the ejaculatory function, leading to problems regarding fertility in vivo, and whether testosterone replacement in diabetic animals has any effect on restoring these conditions to those observed in control animals.

Methods

Adult male and female Wistar rats (90-120 days old) were supplied by the Multidisciplinary Center for Biological Investigation, State University of Campinas (CEMIB – UNICAMP) and were housed in same sex polypropylene cages (43 x 30 x 15 cm) with laboratory-grade pine shavings as bedding. Rats were maintained under controlled temperature (23+/-1°C) and lighting conditions (12L, 12D photoperiod, lights switched off at 07:00am). Rat chow and filtered tap water were provided ad libitum. Experimental protocols followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and were approved by the Biosciences Institute Ethical Committee for Animal Experimentation/ UNESP Botucatu.

Experiment 1

Twenty-one male rats were randomly assigned to three experimental groups of seven animals each: control (vehicle), diabetic and diabetic+testosterone. Diabetes was chemically induced using a single dose of intravenous 40mg/Kg BW streptozotocin (SIGMA Chemical Company, St. Louis, MO) diluted in citrate buffer 0,01M, pH=4,6. Five days after the induction, glycemia of all animals was assessed using glucose test strips and a monitoring System (One Touch Ultra, Johnson&Johnson®) and all animals presenting levels higher than 120mg/dL were considered diabetic. On this same day, seven diabetic animals were operated receiving a sillastic capsule implant filled with testosterone in the subscapular space (Ewing et al., 1977); animals from the other groups were sham operated. Due to the mortality rate of one animal per diabetic group observed

during the treatment period, all experimental groups were standardized to six animals per group.

Final body weight and vas deferens weight

Three weeks after diabetes induction, the rats were weighed, and then slightly anesthetized with ether and killed by decapitation. The left vas deferens was removed and its weight was determined.

Pharmacological analysis: organ bath studies

The vasa deferentia isolated from rats of the experimental groups were individually set up in 10mL organ-baths containing continuously nutritive solution, aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 30ºC according to the methods previously described by Pereira (1987). The composition of the nutritive solution consisted of: NaCl 136.0 mM; KCl 5.7 mM; $CaCl_2$ 1.8 mM; $NaH_2PO_4.H_2O$ 0.36 mM; $NaHCO_3$ 15.0 mM; dextrose 5.5 mM, prepared in glass distilled water (Picarelli et al., 1962). A resting tension of 1.0g was applied to the tissue with changes in isometric tension measured via force-displacement transducers. After an initial resting period of 45min, complete concentration-response curves for norepinephrine (Sigma Co., USA), methoxamine (Sigma Co., USA) and norepinephrine in the presence of a cocktail containing cocaine and propanolol (Sigma Co., USA) were obtained by cumulative addition of molar concentrations of the agonists increasing geometrically (van Rossum and van Den Brink, 1963). The pD2 values, expressed as the negative of the logarithm for the agonist concentration producing 50% (ED50) of its maximum effects (Miller et al.,

1948) were determined. In addition, the maximal contractile responses (g of wet tissue) to all agonists were determined.

Hormone Assays

Testosterone levels. After decapitation, blood was collected (between 9:00 and 11:30am) from the ruptured cervical vessels in a heparinized tube for the determination of plasma testosterone levels. The plasma was obtained after centrifugation (2400rpm, 20min, 3.5ºC) in a refrigerated device and was frozen at –20ºC until the moment of hormonal determination. Plasma testosterone levels were determined by double-antibody radioimmunoassay, using the Testosterone Maia® kit (Biochem Immuno System), at the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo – USP. All the samples were dosed in the same assay, to avoid inter-assay errors. The lower detection limit for testosterone was 0.064ng/mL, with a 4% intra-assay error.

Experiment 2

Thirty male rats were tested for sexual behavior 15 days before the treatment period, and only sexually active ones were selected for the experiment. Rats were randomly assigned to three groups of ten animals each: control (vehicle), diabetic and diabetic+testosterone, and were made diabetic by the same protocol described for Experiment 1. All animals were placed individually in polycarbonate crystal cages, measuring 44x31x16cm, five minutes before the introduction of one adult female in natural oestrus (sexually receptive) determined by vaginal smear. The male rats were allowed to cohabit with the receptive female for 4 hours in the dark period of the cycle

between 8:00 and 12:00 am in a separate room under dim red illumination, and then the females were used as described below.

Ejaculated sperm counts after natural breeding

The procedure was the same conducted by Kempinas et al. (1998), with adaptations described as follows. After the 4-hour mating period, female rats were killed by decapitation and uterine sperm were enumerated. A fine curved forceps was used to elevate the cervix, and the cervix was ligated. The uterine horns were excised, trimmed, washed in Dulbecco's PBS, transferred to a 35mm Petri dish containing 2mL of warm Medium 199 (Gibco™, Auckland NZ), and opened using small scissors. The dish was shaken gently for 10min of dispersion and placed in the incubator at 35°C, thereby allowing the uterine sperm to disperse. The sperm suspension was then transferred to a 15mL conical tube, and then sonicated for 30s. A sample of 100µL was diluted 1:20 with fixative (10% formalin in PBS with 10% sucrose, pH 7.4), and spermatozoa were counted using a hemacytometer.

Statistics

For comparison of results among the three experimental groups, statistical tests for analysis of variance were utilized – ANOVA, with the “a posteriori” Tukey-Kramer test or the non-parametric Kruskall-Wallis test, with the “a posteriori” Dunn or Bonferroni test, according to the characteristics of each variable. Differences were considered significant when $p<0.05$.

Results

Experiment 1

Five days after the induction procedure, all animals in diabetic and diabetic+testosterone groups showed glycemia above 120mg/dL. Rats from the control group showed normal glycemia, below 120mg/dL. The same was verified 3 weeks after induction (Table 1).

Diabetic rats without hormone replacement showed decreased final body weight when compared to controls, and testosterone replacement was not capable of restoring these values to normal (Table 1). Vas deferens absolute weight was reduced in diabetic animals, and hormonal supplementation was only partially efficient in restoring these values to normal, comparing to controls (Table 1).

Testosterone levels were decreased in diabetic animals. In the androgen-supplemented diabetic group the levels of this hormone were statistically equal to those found in control animals (Table 1).

The in vitro biological assay for the vas deferens contractility showed no significant differences as to the organ response to norepinephrine (NE) among the three experimental groups, with or without the presence of the cocktail containing cocaine and propranolol. Methoxamine (MET) responses were altered, as diabetic animals alpha-1 receptor was more sensitive, indicated by the curve dislocation to the left (Figure 1) and by the increase of the pD2 value (Table 2). Testosterone supplementation was not capable of reversing this increased sensitivity observed in diabetic animals. There were no differences on the organ maximal contractile responses to NE whether in the presence of

the cocktail or not, among the Experimental groups, however the organ maximal response to MET was decreased in diabetic animals with hormone replacement when compared to the diabetic group without supplementation (Table 2, Fig. 1).

Experiment 2

The in utero sperm count demonstrated that the diabetic rats did not ejaculate, due to the absence of spermatozoa in the uterus of the females. Only 1 in 8 (12.5%) diabetic rats with testosterone supplementation ejaculated, and this animal had an amount of spermatozoa around 70.9% less than the mean of the control group (diabetic+testosterone: 12.50×10^6; mean±SEM of the control group: $42.95 \pm 5.39 \times 10^6$). In the control group, 8 in 9 animals (88.89%) ejaculated (*data not shown*).

Discussion

Diabetic rats showed a state of hypersensitivity of the post-junction alpha-1 adrenoceptor to the synthetic agonist methoxamine on the vas deferens, and a 2-week testosterone supplementation could not recover this situation. Most diabetes-related studies demonstrate reduction in plasma testosterone levels of these animals (Hassan et al., 1993; Steger & Rabe, 1997; Scarano et al., 2006), as seen in the present work, while some report normal levels of this hormone in a diabetic-induced state (Jelodar et al., 2007); these disparities are probably due to different experimental models and induction procedures. Steger (1990) reported that testosterone was not able to restore ejaculatory function. A study developed by Longhurst (1990) demonstrated a gain on the ejaculatory function in diabetic rats through a longer period of testosterone supplementation of 8 weeks. The result of the present study (increase of sensitivity of adrenergic receptors) was observed in other studies (Peredo et al., 1982; Tomlinson et al., 1982; Sakai et al., 1989), and indicate that probably the diabetic organism is trying to compensate the damage caused by the illness in terms of preserving or trying to preserve the capacity of leaving descendents, that is, to improve the contractility of the vas deferens and to facilitate the sperm release. Kamata et al. (2006) findings corroborate this hypothesis, since in heart ventricular muscle preparations, the methoxamine-induced smooth muscle contractions in 4-week diabetic rats were unchanged, but after 10 weeks the response was significantly enhanced, demonstrating the physiological change in the organ, which was supported by increases in the mRNA levels for both the α_{1a} adrenoceptor and the α_{1a} adrenoceptor protein.

Together, these results showed that the reproductive damage observed in diabetic animals was not directly related to reduced testosterone levels. In spite of this, testosterone replacement was able to restore the maximal contractile responses of the vas deferens of diabetic rats, as seen in control animals. Despite of this effort of the organism, in this work diabetic rats presented problems during ejaculation, as no spermatozoa could be observed in the uterus of receptive females after natural mating, as verified by Hassan et al. (1993), and testosterone supplementation was not efficient in reversing this situation.

Regarding the contractile machinery of the vas deferens, Oztürk and Aydin (2006) demonstrated that diabetes compromises the calcium-dependent contractile process. Contractile responses to KCl and calmidazolium were decreased, demonstrating that the organ's machinery is impaired in the diabetic model, which can be correlated with altered ejaculatory function. The authors also found that this situation could be only partially reverted by insulin supplementation, which suggests that other factors beside lower insulin levels are affecting the organ's function. The present work demonstrated that another possible factor involved on this situation could be the decrease in testosterone plasma levels, but this possibility does not explain by itself the alterations observed in the diabetic rat, as the androgen-supplementation could not recover normal alpha-1 receptor responses, suggesting that testosterone did not play a main role on the ejaculatory process on this study, most likely being an adjuvant on the process.

According to Güneş et al. (2005), there are alterations in adrenergic nerves (autonomic nerval termination) of diabetic rats, including degenerative damage that could be responsible for an increase in the vas deferens contractile responses to electric

stimulation. This damage could compromise NE neuronal uptake and therefore explain the increase in sensitivity of the alpha-1 receptor in diabetes. However, there were not differences among the experimental groups as to the organ responses to exogenous NE in the presence of the cocktail containing cocaine and propranolol, showing the absence of participation of post-junctional adrenoceptor β- beta and of neuronal uptake process in the response of the organs from diabetic and diabetic plus testosterone animals.

Considering that, in the diabetic rat, degenerative problems were observed on nerve terminations (Tomlinson et al., 1982; Güneş et al., 2005), decreased levels of NE were measured in blood serum (Gallego et al., 2003) together with the results hereby presented (increase in sensitivity of the alpha-1 receptor in diabetes and lack of response to the cocktail), we can conclude that the nervous termination of the diabetic animal may be releasing a lower quantity of NE (endogenous mediator), and this factor may explain the increase in sensitivity of the post-junctional alpha-1 receptor on the vas deferens. Thus, the diabetic state damages the ejaculatory process dependent also of the contractility of the vas deferens resulting in no sperm ejaculation, due to a number of different mechanisms, probably including problems related to the functioning of norepinephrine release at the synaptic cleft, caused primarily by autonomic neuropathy.

Acknowledgements

We are grateful to Dr. Débora Cristina Damasceno and Dr. Gustavo Tadeu Volpato of the Laboratory of Experimental Research of Gynecology and Obstetrics, Department of Gynecology and Obstetrics, Botucatu Medical School, São Paulo State

University – UNESP, for the diabetes induction procedures. We are also grateful to Dr. Janete Aparecida Anselmo Franci of the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo – USP, for the hormone assays.

References

Andersson KE. Erectile physiological and pathophysiological pathways involved in erectile dysfunction. J. Urol. 2003; 170(2 Pt 2): S6-13.

Arduíno F. Sintomas, diagnóstico, prognóstico e mortalidade do diabetes. In: Diabetes Mellitus. Rio de Janeiro: Guanabara-Koogan, 1980: 78-94.

Balasubramanian K, Sivashanmugam P, Thameedheen S, Govindarajulu P. Effect of diabetes mellitus on epididymal enzymes of adult rats. Indian J. Exp. Biol. 1991; 29:907-909.

Calvo JC, Baranao JL, Tesone M, Charreau EH. Hypothalamic-hypophyseal-gonadal axis in the streptozotocin-induced diabetic male rat. J. Steroid Biochem. 1984; 20: 769-772.

Chaiban JT, Azar ST. Erectile dysfunction in diabetic patients. J. Med. Liban. 2004; 52(4): 217-9.

Ewing LL, Desjardins C, Irby DC, Robaire B. Synergistic interaction of testosterone and oestradiol inhibits spermatogenesis in rats. Nature. 1977; 269(5627):409-411.

Fairburn CG. The sexual problems of diabetic men. Br. J. Hosp. Med. 1981; 25:484-491.

Fedele D. Therapy Insight: sexual and bladder dysfunction associated with diabetes mellitus. Nat. Clin. Pract. Urol. 2005; 2(6):282-90, quiz 309.

Ficher M, Zuckerman M, Fishkin RE, Goldman A, Neeb M, Fink PJ, Cohen SN, Jacobs JA, Weisberg M. Do endocrines play an etiological role in diabetic and non-diabetic sexual dysfunction? J. Androl. 1984; 5:8-16.

Flora-Filho R, Zilberstein B. Nitric oxide: the simple messenger passing through complexity. Metabolism, synthesis and functions [Article in Portuguese]. Rev. Assoc. Med. Bras. 2000; 46(3):265-71.

Gallego M, Setién R, Izquierdo MJ, Casis O, Casis E. Diabetes-induced biochemical changes in central and peripheral catecholaminergic systems. Physiol. Res. 2003; 52:735-41.

Giuliano F, Clement P. Neuroanatomy and physiology of ejaculation. Annu. Rev. Sex Res. 2005; 16:190-216.

Güneş A, Ceylan A, Sarioglu Y, Stefek M, Bauer V, Karasu C. The Antioxidants in Diabetes-Induced Complications (AIDC) Study Group. Reactive oxygen species mediate abnormal contractile response to sympathetic nerve stimulation and noradrenaline in the vas deferens of chronically diabetic rats: effects of in vivo treatment with antioxidants. Fundam. Clin. Pharmacol. 2005; 19(1):73-9.

Hassan AA, Hassouna MM, Taketo T, Gagnon C, Elhilali MM. The effect of diabetes on sexual behavior and reproductive tract function in male rats. J. Urol. 1993; 149:148-154.

Jelodar G, Razmi N, Gholampour V. Arginase alteration in the reproductive system of alloxan-diabetic dogs. J. Reprod. Dev. 2007; 53(2): 317-21.

Kamata K, Satoh T, Matsumoto T, Noguchi E, Taguchi K, Kobayashi T, Tanaka H, Shigenobu K. Enhancement of methoxamine-induced contractile responses of rat

ventricular muscle in streptozotocin-induced diabetes is associated with α_{1a} adrenoceptor upregulation. Acta Physiol. 2006; 188: 173-83.

Kempinas WG, Suarez JD, Roberts NL, Strader L, Ferrell J, Goldman JM, Klinefelter GR. Fertility of rat epididymal sperm after chemically and surgically induced sympathectomy. Biol. Reprod. 1998; 59(4):897-904.

Kuhn-Velten N, Shermer R, Staib W. Effect of streptozotocin-induced hyperglycemia on androgen-binding protein in the testis and epididymis. Diabetologia 1978; 26:300-306.

Longhurst PA. The effects of testosterone or insulin treatment on contractile responses of the rat vas deferens following castration or streptozotocin-induced diabetes mellitus. Gen. Pharmacol. 1990; 21(4):427-34.

Melman A, Henry DP, Felten DL, O'Connor B. Effect of diabetes upon penile sympathetic nerves in impotent patients. South Med. J. 1980; 73(3):307-9,317.

Miller LC, Becker TJ, Tainter ML. Quantitative evaluation of spasmolytic drugs "in vitro". J. Pharmacol. Exp. Therap. 1948; 92:260-268.

Murray FT, Cameron DF, Orth JM. Gonadal dysfunction in the spontaneously diabetic BB rat. Metabol. Clin. Exp. 1983; 32:141-147.

Oztürk Y, Aydin S. Effects of streptozotocin-induced diabetes and insulin on calcium responsiveness of the rat vas deferens. Life Sci. 2006; 78(10):1084-90.

Peredo H, Agostini MD, Gimeno MF, Borda ES. Hypersensitivity to norepinephrine in vasa deferentia from diabetic rats: possible participation of metabolic products of arachidonic acid. Prostaglandins Leukot. Med. 1984; 15(2):229-39.

Pereira OCM. Some effects of cimetidine on the reproductive organs of rat. Gen. Pharmacol. 1987; 18:197-199.

Picarelli ZP, Hyppolito N, Valle JR. Synergistic effect of 5-hydroxytryptamine on the response of rats' seminal vesicles to adrenaline and noradrenaline. Arch. Int. Pharmacodyn. Ther. 1962; 138:354-63.

Rang HP, Dale MM, Ritter JM, Moore PK (eds). Farmacologia. 5 ed. Rio de Janeiro: Elsevier, 2004.

Sakai Y, Aihara K, Honda H, Inazu M. Calcium mobilization and phosphatidylinositol turnover in vas deferens smooth muscle of diabetic rats. Eur. J. Pharmacol. 1989; 162(3):475-81.

Scarano WR, Messias AG, Oliva SU, Klinefelter GR, Kempinas WG. Sexual behaviour, sperm quantity and quality after short-term streptozotocin-induced hyperglycaemia in rats. Int. J. Androl. 2006; 29(4):482-8.

Simonsen U, García-Sacristán A, Prieto D. Penile arteries and erection. J. Vasc. Res. 2002; 39:283-303.

SBD, Sociedade Brasileira de Diabetes. Dados sobre diabetes mellitus no Brasil. Available at <http://www.diabetes.org.br/imprensa/estatisticas>. Access on March 26th, 2007.

Steger RW. Testosterone replacement fails to reverse the adverse of streptozotocin-induced diabetes on sexual behavior in the male rat. Pharmacol. Biochem. Behav. 1990; 35:577-582.

Steger RW, DePaolo LV, Asch RH, Silverman AY. Interactions of delta 9-tetrahydrocannabinol (THC) with hypothalamic neurotransmitters controlling luteinizing hormone and prolactin release. Neuroendocrinol. 1983; 37:361-370.

Steger RW, Rabe MB. The effect of diabetes mellitus on endocrine and reproductive function. Proc. Soc. Exp. Biol. Med. 1997; 214(1): 1-11.

Tomlinson DR, Gillon KR, Smith MG. Axonal transport of noradrenaline and noradrenergic transmission in rats with streptozotocin-induced diabetes. Diabetologia 1982; 22(3):199-204.

Van Rossum JM, Van Den Brink FG. Cumulative dose-response curves. I. Introduction to the technique. Arch. Int. Pharmacodyn. Ther. 1963; 143:240-246.

Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. Diabetes Care 2003; 26(5):1553-1579.

WHO, World Health Organization. Prevalence of diabetes worldwide. Available at <http://www.who.int/diabetes/facts/world_figures/en/>. Access on August 20th, 2007.

Zhang XH, Filippi S, Morelli A, Vignozzi L, Luconi M, Donati S, Forti G, Maggi M. Testosterone restores diabetes-induced erectile dysfunction and sildenafil responsiveness in two distinct animal models of chemical diabetes. J. Sex. Med. 2006; 3(2):253-264.

Table 1. Glycemia 5 and 21 days after the beginning of the treatment, final body weight, vas deferens weight and testosterone levels of rats from the experimental groups (Mean±SEM).

	Experimental groups		
	Control (n=06)	**Diabetic (n=06)**	**Diabetic+Testosterone (n=06)**
Glycemia 5 days after induction (mg/dL)	88.33±2.96 [a]	526.89±22.92 [b]	520.38±30.65 [b]
Glycemia 21 days after induction (mg/dL)	81.11±1.89 [a]	541.00±10.17 [b]	544.37±15.83 [b]
Final body weight (g)	392.98±10.962 [a]	296.36±12.643 [b]	309.82±8.297 [b]
Vas deferens (mg)	112.13±5.99 [a]	89.44±5.60 [b]	99.68±5.98 [ab]
Vas deferens (mg/100g)	28.79±1.86 [a]	30.22±1.55 [a]	32.18±1.59 [a]
Testosterone levels (ng/mL)	1,91±0,53 [a]	0,32±0,09 [b]	1,42±0,21 [ab]

[a,b]: different letters indicate statistically different results ($p<0.05$). ANOVA test, with the Tukey-Kramer "a posteriori" test.

Table 2. pD_2 of the agonists (NE, MET, NE+Cocktail) and vas deferens maximal contractile response to the drugs, obtained from the pharmacological response of the isolated organ of the animals from the three experimental groups (Mean±SEM).

	Experimental groups		
	Control (n=06)	**Diabetic (n=06)**	**Diabetic+ Testosterone (n=06)**
pD_2[1]			
NE	5.43±0.09 [a]	5.55±0.06 [a]	5.70±0.05 [a]
MET	4.36±0.11 [a]	4.91±0.06 [b]	5.09±0.09 [b]
NE + Cocktail[2]	6.08±0.14 [a]	6.23±0.09 [a]	6.29±0.07 [a]
Emax[3]			
NE	1.48±0.06 [a]	1.60±0.12 [a]	1.37±0.06 [a]
MET	1.45±0.05 [ab]	1.62±0.12 [a]	1.24±0.09 [b]
NE + Cocktail	1.96±0.20 [a]	1.99±0.12 [a]	1.78±0.08 [a]

[1] pD_2 = - log [ED50]

[2] Cocktail: cocaine + propranolol

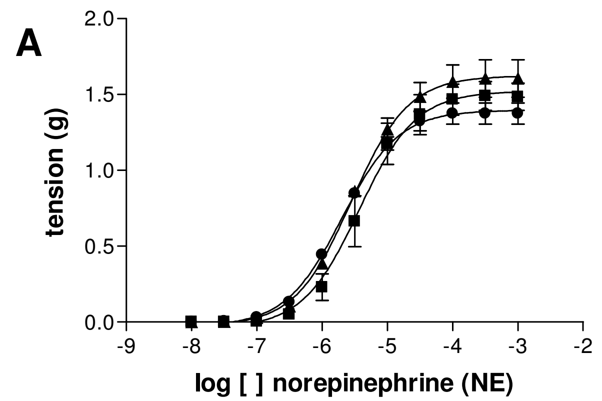
[3] Emax: maximal contractile responses

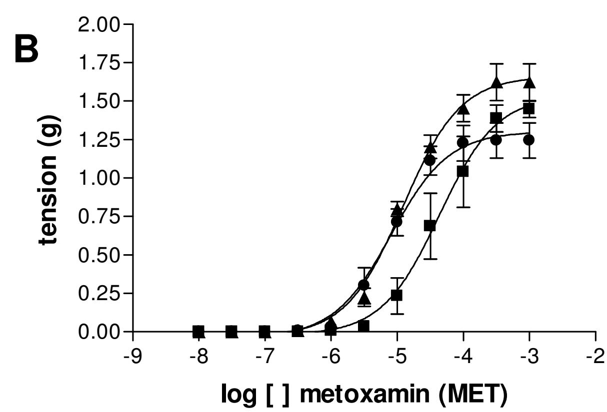
[a,b]: different letters indicate statistically different results ($p<0.05$). ANOVA test, with the Bonferroni "a posteriori" test.

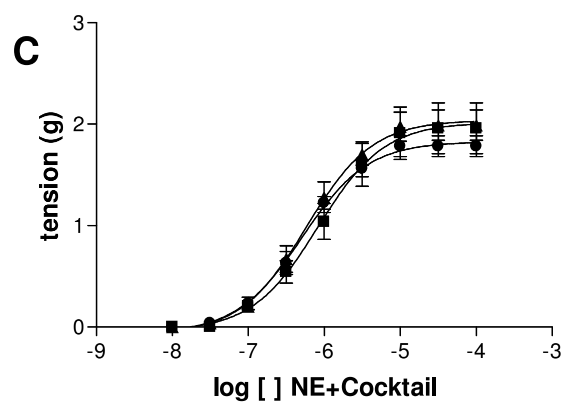
Figure Legends

Figure 1. Concentration-response curves for norepinephrine (A), methoxamine (B) and norepinephrine+cocktail (C), obtained from the vas deferens of male rats of the three experimental groups (n=06 per group). In abscises: molar concentration of the drug in logarithmic scale, in ordinates: maximum effect percentage. Vertical bars indicate the standard error of the mean.

Figure 1







Legend:
- ■ control
- ▲ diabetic
- ● diabetic+testosterone

ARTIGO 2

Impairment of spermatogenesis and reproductive axis dysregulation in streptozotocin-induced diabetic rats

Running head: diabetes induced spermatogenesis impairment

Davi Abeid Pontes[1], Glaura Scantamburlo Alves Fernandes[1], Thaiane Amanda Assumpção[2], Janete Aparecida Anselmo Franci[3], Wilma De Grava Kempinas[2].

1 – Graduate Program in Cellular and Structural Biology, Institute of Biology, State University of Campinas – UNICAMP.
2 – Department of Morphology, Institute of Biosciences of Botucatu, São Paulo State University – UNESP.
3 – Department of Morphology, Stomatology and Physiology, Dental School of Ribeirão Preto, University of São Paulo – USP.

Corresponding author:
Wilma De Grava Kempinas
Departamento de Morfologia - Instituto de Biociências de Botucatu
Universidade Estadual Paulista – UNESP
Caixa-Postal 510
CEP 18618-000, Distrito de Rubião Jr., s/n, Botucatu, SP – Brazil.
Telephone number: +55 (14) 38116264, Fax: +55 (14) 38116264
Email: kempinas@ibb.unesp.br

Key words: diabetes, testosterone supplementation, infertility, spermatogenesis, neuroendocrine dysregulation.

Abstract

Diabetes causes several adverse affects on male reproduction, eventually leading to infertility in many young and adult men still in fertile age. The aim of this study was to evaluate the mechanisms involved in the impairment of spermatogenesis in diabetic rats. Male Wistar rats, 90 days old and weighing around 300g, were randomly allocated into 3 experimental groups: diabetic (streptozotocin 40mg/Kg), diabetic+T (same protocol, receiving exogenous testosterone for 2 weeks) and control (vehicle). Three weeks after diabetes induction, the rats were sacrificed and blood was collected for plasma testosterone (T), LH and FSH levels measurement. Body weight and weights of the reproductive organs were recorded, and testis and epididymis were collected for histopathological analysis, sperm counts, and intratesticular T analysis. Statistical analyses were performed using the ANOVA test ($p<0.05$), and results were expressed as mean±SEM. Diabetic rats had lower levels of both plasma and intratesticular T than controls. Testosterone supplementation only recovered plasma T levels, while LH and FSH levels were lower in this group when compared to the other groups, which was correlated with a loss of weight in the testes of these animals. The testes of diabetic rats presented several histopathological alterations, including loss of germ and Sertoli cells in the lumen of seminiferous tubules. Also, a high amount of biological material, other than spermatozoa, were observed in the lumen of the epididymis of these animals, and T supplementation was not capable of recovering this situation to normal, as presented by control animals. These results indicate impairment of the spermatogenic process due to the dysregulation of the reproductive hormonal axis in diabetic rats, and suggest that T supplementation was not capable of recovering this situation.

Introduction

Diabetes mellitus is the name given to a group of disorders with different etiologies. It is characterized by disarrangements in carbohydrates, proteins and fat metabolism caused by the complete or relative insufficiency of insulin secretion and/or insulin action (ADA, 2007). Several secondary adverse effects are caused by this disease, including disorders related to the reproduction of males, which are known to provoke infertility in many young and adult men still in fertile age. This turns to be a worrying situation by the fact that most of them are not aware of their ill state, whether at initial or late stages, and therefore are not under insulin treatment (SBD, 2006).

The problems caused by the hyperglycemic and hypoinsulinemic state due to the uncontrolled disease may include ejaculatory and erectile dysfunction, neuroendocrine reproductive axis dysregulation, and spermatogenic damage (Melman et al., 1980; Fairburn, 1981; Murray et al., 1983; Calvo et al., 1984; Balasubramanian et al., 1991; Andersson, 2003; Chaiban & Azar, 2004; Scarano et al., 2006; Zhang et al., 2006, Pontes et al., 2007b). Most of these studies found in the scientific literature provide evidence to decreased testosterone levels in diabetic men and experimental models, but the specific mechanisms by which this lack of androgen affects the male reproduction are not yet clearly understood.

Despite of the fact that diabetes is considered one the most spread diseases all over the world nowadays, playing an important role as a threat to human's health in the modern world (WHO, 2007), little is known about the real mechanisms underlying its relationship with infertility. With a rapidly increasing incidence mostly due to correlated

risk factors such as obesity, genetic predisposition and ageing, the concern around diabetes is also growing fast. Therefore, it is important to evaluate the mechanisms behind this disease-related infertility.

The aim of this study was to evaluate the mechanisms involved in the impairment of spermatogenic function in diabetic adult male rats, and whether testosterone supplementation has any effect on restoring this condition to normal parameters.

Materials and Methods

Animals

Sixty male Wistar rats, 90 days old and weighing around 300g, were supplied by the Multidisciplinary Center for Biological Investigation, State University of Campinas (CEMIB – UNICAMP) and were housed in same sex polypropylene cages (43x30x15cm) with laboratory-grade pine shavings as bedding. All rats were maintained under controlled temperature (23+/-1°C) and lighting conditions (12L, 12D photoperiod, lights switched off at 07:00am). Rat chow and filtered tap water were provided ad libitum. Experimental protocols followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation and were approved by the Biosciences Institute Ethical Committee for Animal Experimentation/ UNESP Botucatu.

Experiment 1

Thirty rats were randomly allocated into 3 experimental groups of ten animals each: diabetic, diabetic+T (with testosterone supplementation) and control (vehicle).

Diabetes was chemically induced using a single dose of intravenous 40mg/Kg streptozotocin (SIGMA Chemical Company, St. Louis, MO) diluted in citrate buffer 0,01M, pH=4,6. Seven days after the induction, glycemia of all animals was assessed using glucose test strips and a monitoring System (One Touch Ultra, Johnson&Johnson®) and all animals presenting levels higher than 120mg/dL were considered diabetic. On this same day, twenty diabetic animals were operated receiving a sillastic capsule implant filled with testosterone in the subscapular space (Ewing et al., 1977); animals from the other groups were sham operated. Body weight was recorded at alternate days in all experimental groups, beginning five days after diabetes induction.

Three weeks after diabetes induction, the rats were sacrificed by decapitation and blood was collected from the cervical vessels for plasma testosterone (T), LH and FSH levels measurement. Body weight and testis, epididymis, ventral prostate, seminal vesicles (full and empty of secretion) and vas deferens weights were recorded. The left testis and epididymis were collected for histopathological analysis, and the right testis was collected and kept at -80ºC for intratesticular testosterone analysis.

Hormone Assays

Plasma T, LH and FSH levels. Blood collected (between 9:00 and 11:30am) from the ruptured cervical vessels in a heparinized tube was evaluated for the determination of plasma T, LH and FSH levels. The plasma was obtained after centrifugation (2400rpm, 20min, 3.5ºC) in a refrigerated device and was frozen at –20ºC until the moment of hormonal determination. Plasma hormone levels were determined by double-antibody radioimmunoassay, using the Testosterone Maia® kit (Biochem Immuno System) and

specific kits for FSH and LH, supplied by the National Institute of Arthritis, Diabetes and kidney Diseases (NIADDK, USA), at the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo - USP. All the samples were dosed in the same assay, to avoid inter-assay errors. The lower detection limit was 0.064ng/mL, with a 4% intra-assay error for testosterone; 0.04ng/mL, with a 3.4% intra-assay error for LH; and 0.09ng/mL, with a 2.8% intra-assay error for FSH.

Intratesticular testosterone levels (Kempinas et al., 1998). The testis of each animal was removed and decapsulated, and the parenchyma was sliced into ~50mg pieces. Each piece was weighed and placed into a 1.5mL microfuge tube containing 1.0mL Medium 199 (M199). The M199 was buffered with 0.71g/L sodium bicarbonate (NAHCO3) and 2.1g/L HEPES, and contained 0.1% BSA (Schwartz-Mann, Orangeburg, NY) and 25mg/L soybean trypsin inhibitor, pH 7.4. The parenchyma was incubated for 2h at 34°C (Laskey et al., 1994). After centrifugation (5min, 10000x g), medium was frozen at –70°C until testosterone assay, which was determined by double-antibody radioimmunoassay at the Neuroendocrinology Laboratory, Dental School of Ribeirão Preto, University of São Paulo - USP.

Histopathological analysis

The left testes and epididymides from the three experimental groups were removed, fragmented and fixed in Karnovsky’s solution (2.5% glutaraldehyde, 5% paraformaldehyde, 0.1 M phosphate buffer pH 7.2) for 24h. Fixed tissue samples were dehydrated in graded ethanol series and embedded in glycol methacrylate resin (Leica Historesin Embedding Kit). Histological sections (3-4µm) were subjected to

haematoxylin and eosin (H&E) for general studies. Histopathological analyses were performed on a Leica Photomicroscope and the microscopic fields were digitized using the software Leica Q-Win V3.

Experiment 2

Thirty male rats were randomly assigned to three groups of ten animals each: control (vehicle), diabetic and diabetic+testosterone, and were made diabetic by the same protocol described for Experiment 1. Three weeks after diabetes induction, all animals were sacrificed and their right testis and epididymis were frozen at –20°C for posterior germ cell sperm counts.

Daily sperm production per testis, sperm number and transit time in the epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were enumerated as described previously by Robb et al. (1978), with adaptations as following: the right testes, decapsulated and weighed soon after collection, were homogenized in 5mL of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 seconds. After a 10-fold dilution a sample was transferred to Neubauer chambers (4 fields per animal), proceeding an enumeration of mature spermatids. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle in which these spermatids are present in the seminiferous epithelium. In the same manner, caput/corpus and cauda epididymis parts were cut into small fragments with scissors and homogenized, and sperm enumerated as described for

the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each part of the organ by the DSP.

Statistics

For comparison of results among the three experimental groups, statistical tests for analysis of variance were utilized – ANOVA, with the “a posteriori” Tukey-Kramer test or the non-parametric Kruskall-Wallis test, with the “a posteriori” Dunn test. Differences were considered significant when $p<0.05$. All data are expressed as mean±SEM.

Results

Experiment 1

All the animals utilized in diabetes induction presented, seven days after induction, glycemia above 120mg/dL. Control rats presented normal glycemia, equal or below 120mg/dL. The same could be verified three weeks after induction (Figure 1).

Figure 1 also shows that diabetic rats had decreased plasma and intratesticular testosterone levels, and that testosterone supplementation could only restore plasma testosterone levels in these animals. LH and FSH levels were not altered in diabetic rats without supplementation, but they were decreased in the diabetic animals with hormone replacement (Figure 1).

Few days after the diabetes induction diabetic animals showed a significant decrease in the body weight when compared to the control group (data not shown), and

this difference remained until the end of the experimental period. Testosterone supplementation did not interfere on the body weight gain of these animals (Table 1).

As to the weights of reproductive organs, ventral prostate and seminal vesicles weights (absolute and relative to body weight) were lower in diabetic rats when compared to control animals; in this case, the treatment with exogenous testosterone was capable of restoring these values to normal levels (Table 1). Absolute weights of the epididymis and vas deferens were also decreased in diabetic rats; however, while hormone replacement was partially efficient in restoring the values of the vas deferens, it could not show the same efficacy for the epididymis (Table 1).

Testicular weight relative to body weight was increased in diabetic rats when compared to the other groups; the treatment with exogenous testosterone in diabetic rats resulted in a decrease in the absolute weight of this organ when compared to the other groups (Table 1).

The testes of diabetic rats with and without T supplementation presented several histopathological changes, including loss of germ cells in the lumen of seminiferous tubules, epithelial depletion, and loss of the characteristic histoarchitecture of these tubules (Figure 2). Both diabetic groups presented a high amount of biological material, other than spermatozoa, in the lumen of the epididymis, which was found just occasionally in controls (Figure 2).

Experiment 2

Sperm counts in the testis did not demonstrate any differences among the experimental groups, with the exception of sperm production per unit of mass, which was

higher in the diabetic rats with hormone supplementation (all values expressed as mean ± EPM, x10^6 - Control: 127.62±6.19, Diabetic: 142.06±6.51, Diabetic+T: 170.63±5.94). There were no differences as to daily sperm production (DSP) among the three experimental groups (Control: 33.04±1.36, Diabetic: 34.56±2.15, Diabetic+T: 32.05±1.76).

There was a significant acceleration of the sperm total transit time through the epididymis in diabetic rats with or without testosterone supplementation (Figure 3). The total amount of spermatozoa stored in the cauda epididymis was also reduced in both diabetic groups (Control: 203.84±10.25, Diabetic: 105.61±13.71, Diabetic+T: 142.09±17.83).

Discussion

This study uses an experimental model for severe diabetes that mimics the clinical aspects of an individual with an uncontrolled disease, that is, hyperglycemic and hypoinsulinemic. Glycemia results during the experimental period confirm the efficacy of the utilized method for diabetes induction by a single injection of streptozotocin (40mg/kg BW).

In the present study diabetic rats presented decreased levels of plasma testosterone, which corroborates with the scientific literature (Paz et al., 1978; Kuhn-Velten et al., 1978; Babichev, 1983; Jackson & Hutson, 1984; Benitez & Perez Diaz, 1985; Hassan et al., 1993; Steger & Rabe, 1997; Scarano et al., 2006). This is a direct consequence to Leydig cell reduced steroidogenesis, insulin-dependent biological process

(Paz et al., 1978; Blanco et al., 1981; Pérez Díaz et al., 1982; Julie et al., 2003). In this condition, it would be expected an increase in LH secretion by the hypophysis at normal conditions, by negative feedback mechanisms. However, in the present study the results revealed normal levels of LH and FSH in these rats when compared to control animals. As alterations in plasma levels of gonadotropins in diabetic animals are still discussed in literature (Babichev, 1983; Calvo et al., 1984; Jackson & Hutson, 1984; Benitez & Perez Diaz, 1985; Hassan et al., 1993; Sudha et al., 1999), we suggest that probably the problems in the reproductive axis of diabetic rats occur as a consequence of hypophyseal disorders, involving reduction of LHRH-stimulated and testosterone-stimulated LH secretion (with decreased number of androgen receptors) (Babichev, 1983; Jackson & Hutson, 1984; Hassan et al., 1993; Steger & Rabe, 1997). This condition could allow random moments of hyper or hyposecretion, which would explain the disparities found in literature. Exogenous testosterone supplementation in diabetic rats was not benefic in restoring the normal functioning of the reproductive axis in these rats, as plasma LH and FSH levels were decreased when compared to control animals.

The decrease in body weight gain of diabetic rats even after testosterone supplementation presented herein is in agreement with other studies (Jackson & Hutson, 1984; Soudamani et al., 2005) and can be attributed to metabolic alterations caused by the total absence or decrease of insulin levels of these animals.

According to Clegg et al. (2001), reproductive organ weights, such as from the testes, epididymides, seminal vesicles and ventral prostate, are also important parameters to better understand the mechanisms underlying infertility in rats. The decrease in weight observed in the epididymis and the vas deferens of diabetic rats, with or without

testosterone supplementation, can be explained mainly by the concomitant decrease of body weight in these animals, as a systemic effect of insulin absence in the organism. This decrease in the epididymal weight is also reported by others (Paz et al., 1978; Jackson & Hutson, 1984; Hassan et al, 1993; Scarano et al., 2006).

The evaluation of testes weights among the different experimental groups demonstrated that, although diabetic rats presented decreased final body weight when compared to controls, the testes of these animals resisted in a certain manner the damaging effects of uncontrolled diabetes. This is demonstrated by the increased relative testis weight in these rats. These results corroborate with an early study from our laboratory (Scarano et al., 2006), although other studies report weight loss in the testis of diabetic rats (Jackson & Hutson, 1984; Hassan et al., 1993). Testosterone replacement in diabetic rats led to a decrease in testicular absolute weight when compared to the other groups, demonstrating that in this experimental situation the testis lost its ability of keeping its weight intact, therefore losing weight along with the rest of the organism, as the relative weight of this organ in this group was similar to the results from control rats. This can be explained by the decrease in plasma LH and FSH observed in this group, which was probably harmful to the normal functioning of the organ. In spite of this decrease in weight, the testis in this group had a higher number of sperm per unit of mass, indicating that the organism is trying to compensate the damage suffered from the illness.

Another important factor associated is that plasma levels of testosterone do not necessarily reflect intratesticular levels of this androgen. This could be confirmed by the results presented herein, showing that diabetic rats with low intratesticular testosterone levels do not have these levels recovered after androgen supplementation, which agrees

with Jackson & Hutson (1984) findings. This result shows that the testis is extremely dependent on Leydig cell steroidogenesis. On this matter, low intratesticular testosterone levels could contribute to hypogonadism in the uncontrolled diabetes model, along with LH and FSH-linked mechanisms (Ballester et al., 2004), insufficient carbohydrate metabolism and increased local oxidative stress observed in these animals (Naziroğlu, 2003; Ozkan et al., 2005).

Histopathological alterations in the testis of diabetic rats have been described by other authors (Paz et al., 1978; Sainio-Pöllänen et al., 1997; Altay et al., 2003), along with reports of increased apoptosis (Koh, 2007a,b). These alterations together with the results presented herein suggest impairment of spermatogenesis in these animals. The disarrangement of seminiferous tubules histoarchitecture verified in the present study indicate altered Sertoli cell function in these animals, which is supported by the findings of Hutson (1984) showing decreased ABP levels in the testis of diabetic animals, found to be that directly controlled by insulin. The presence of biological material other than spermatozoa in the lumen of the tubules, which was also verified in the epididymis lumen in diabetic rats with and without testosterone supplementation, provide further evidence on spermatogenesis damage, probably indicating impaired quality of the gametes, since daily sperm production was not affected by diabetes in the present study.

The decrease in absolute and relative weights of the ventral prostate and the seminal vesicles of diabetic rats found in this study show is in agreement with prior studies (Paz et al., 1978; Jackson & Hutson, 1984; Seethalakshmi et al., 1987; Steger & Rabe, 1997; Scarano et al., 2006). However, it has not been verified whether the weight recovery achieved by androgen replacement reflects a recovery of function in these

organs, since diabetic rats also showed a variety of morphological alterations in the prostate and seminal vesicles (Paz et al., 1978; Soudamani et al., 2005). The decrease in weight found in these glands also suggests alterations in the levels of some semen contents produced locally, which together with the acceleration of transit time through the epididymis observed in diabetic rats in the present study, indicate damage to the maturation processes (Fernandez et al., 2007), which is associated with impaired sperm quality (Baccetti et al., 2002; Amaral et al., 2006; Agbaje et al., 2007; Shrilatha & Muralidhara; 2007).

Despite of this indication of altered sperm quality, Scarano et al. (2006) demonstrated that sperm from diabetic rats have the ability to fertilize eggs using artificial intra-uterine insemination. On this behalf, Agbaje et al. (2007) state that damages to sperm DNA do not necessarily affect the gamete fertilizing ability, since the egg has a limited but efficient capacity of repairing DNA damage from the male gamete, which is likely to be involved with Scarano et al. (2006) findings. Nevertheless, DNA damage might pass to the developing embryo, resulting in increased rates of embryonic development failure and gestational loss (Ahmadi & Ng, 1999a,b) and even spontaneous abortion in humans (Carrell et al., 2003a,b). Furthermore, the future health of the offspring has been also related to gamete quality of the parents (Brinkworth, 2000; Aitken et al., 2003a,b; Aitken, 2004). Thus, alterations in sperm quality of diabetic individuals are of extreme importance to their own reproductive health and even to their offspring's health.

In the present work, diabetic rats had a reduced amount of spermatozoa stored in the cauda epididymis, which corroborates with other studies' reports (Hassan et al., 1993;

Ballester et al., 2004; Amaral et al., 2006). Thus, this reduced availability of sperm for ejaculation in diabetic rats together with the alterations found in the ejaculatory function and sexual behavior of these animals (Hassan et al., 1993; Pontes et al., 2007a,b) may explain the decreased fertility rate of diabetic rats after natural mating observed by Scarano et al. (2006).

All the factors here enumerated related to sexual dysfunction in the diabetic rat, due to hyperglycemia and hypoinsulinemia, can be also observed in the aged rat, which lead us to the hypothesis that uncontrolled diabetes mimics a state of early senescence of the reproductive system. Aging leads to lower levels of steroidogenesis, decrease in LH receptor expression in Leydig cells and increased levels of circulating SHBG (sex hormone binding globulin) in male rats and humans, which result in lower testosterone levels, and dysregulated neuroendocrine reproductive axis functioning, besides local problems related to spermatogenesis and ejaculation or erection (Pirke et al, 1979; Larrea et al., 1981; Bedrak et al, 1983, Zirkin & Chen, 2000, Syed & Hecht, 2002; Bahia & Gomes, 2003; Gonzalez-Cadavid & Rajfer, 2004; Zubkova & Robaire, 2004), mainly caused by increased oxidative stress in these tissues (Naziroğlu, 2003; Ozkan et al., 2005; Shrilatha & Muralidhara, 2007).

The results discussed herein provide helpful information about the participation of the neuroendocrine reproductive axis dysregulation on spermatogenesis impairment in diabetic rats. Important evidence has been provided suggesting reduced sperm quality in these animals, even though more studies need to be carried out to better investigate this quality and the maturation process in similar experimental conditions. Testosterone supplementation was not capable of reversing most of the alterations seen in diabetic

animals, indicating that androgens did not play key roles in the mechanisms underlying diabetes-induced sexual dysfunctions. Overall, the secondary effects of diabetes affect the reproduction of males by many different mechanisms which are all primarily associated with four principal and important reproductive aspects: the neuroendocrine reproductive axis, spermatogenesis, sperm quality, and ejaculatory/ erectile functions.

Acknowledgements

We are grateful to Dr. Débora Cristina Damasceno and Dr. Gustavo Tadeu Volpato of the Laboratory of Experimental Research of Gynecology and Obstetrics, Department of Gynecology and Obstetrics, Botucatu Medical School, São Paulo State University – UNESP, for the diabetes induction procedures. We would also like to express our gratitude to The State of São Paulo Research Foundation – FAPESP, and National Council for Scientific and Technological Development – CNPq for financial support.

References

Agbaje I. M., Rogers D. A., McVicar C. M., McClure N., Atkinson A. B., Mallidis C. & Lewis S. E. (2007) Insulin dependant diabetes mellitus: implications for male reproductive function. *Human Reproduction*, **22**(7), 1871-1877.

Ahmadi A. & Ng S. C. (1999a) Developmental capacity of damaged spermatozoa. *Human Reproduction,* **14**(9), 2279-2285.

Ahmadi A. & Ng S. C. (1999b) Fertilizing ability of DNA-damaged spermatozoa. *The Journal of Experimental Zoology,* **284**(6), 696-704.

Aitken R. J. Founders' Lecture. (2004) Human spermatozoa: fruits of creation, seeds of doubt. *Reproduction, Fertility and Development,* **16**(7), 655-664.

Aitken R. J., Baker M. A. & Sawyer D. (2003a) Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reproductive Biomedicine Online*, **7**(1), 65-70.

Aitken R. J., Ryan A. L., Curry B. J. & Baker M. A. (2003b) Multiple forms of redox activity in populations of human spermatozoa. *Molecular Human Reproduction*, **9**(11), 645-661.

Altay B., Cetinkalp S., Doganavsargil B., Hekimgil M. & Semerci B. (2003) Streptozotocin-induced diabetic effects on spermatogenesis with proliferative cell nuclear antigen immunostaining of adult rat testis. *Fertility and Sterility*, **80**(2), 828-831.

Amaral S., Moreno A. J., Santos M. S., Seiça R. & Ramalho-Santos J. (2006) Effects of hyperglycemia on sperm and testicular cells of Goto-Kakizaki and streptozotocin-treated rat models for diabetes. *Theriogenology*, **66**(9), 2056-2067.

ADA, American Diabetes Association. (2007) Diagnosis and classification of diabetes mellitus. *Diabetes Care,* **30,** S42-S46.

Andersson K. E. (2003) Erectile physiological and pathophysiological pathways involved in erectile dysfunction. *The Journal of Urology,* **170**(2), S6-14.

Babichev V. N., Peryshkova T. A. & Adamskaia E. I. (1983) Status of the hypophyseal-gonadal system in male rats with diabetes (experimental study). *Problemy endokrinologii,* **39**(1), 42-45.

Bahia L. & Gomes M. B. (2003) Influência da idade e do diabetes sobre esteróides sexuais e SHBG em homens. *Arquivos Brasileiros de Endocrinologia e Metabologia*, **47**(3), 256-260.

Baccetti B., Marca A., Piomboni P., Capitani S., Bruni E., Petraglia F. & De Leo V. (2002) Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. *Human Reproduction,* **17**(10), 2673-2677.

Balasubramanian, K., Sivashanmugam, P., Thameedheen, S. & Govindarajulu, P. (1991) Effect of diabetes mellitus on epididymal enzymes of adult rats. *Indian Journal of Experimental Biology*, **29**, 907-909.

Ballester J., Muñoz M. C., Domínguez J., Rigau T., Guinovart J. J. & Rodríguez-Gil J. E. (2004) Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *Journal of Andrology,* **25**(5), 706-719.

Bedrak E., Chap Z. & Brown R. (1983) Age-related changes in the hypothalamic-pituitary-testicular function of the rat. *Experimental Gerontology*, **18**(2), 95-104.

Benitez A. & Perez Diaz J. (1985) Effect of streptozotocin-diabetes and insulin treatment on regulation of Leydig cell function in the rat. *Hormone and Metabolic Research*, **17**, 5-7.

Blanco F. L., Fanjul L. F., & Ruiz de Galarreta C. M. (1981) The effect of insulin and luteinizing hormone treatment on serum concentrations of testosterone and dihydrotestosterone and testicular 3 beta-hydroxysteroid dehydrogenase activity in intact and hypophysectomized diabetic rats. *Endocrinology*, **109**(4), 1248-1253.

Brinkworth M. H. (2000) Paternal transmission of genetic damage: findings in animals and humans. *International Journal of Andrology,* **23**(3), 123-135.

Calvo, J. C., Baranao, J. L., Tesone, M. & Charreau, E. H. (1984) Hypothalamic-hypophyseal-gonadal axis in the streptozotocin-induced diabetic male rat. *Journal of Steroid Biochemistry*, **20**, 769-772.

Carrell D. T., Liu L., Peterson C. M., Jones K. P., Hatasaka H. H., Erickson L. & Campbell B. (2003a) Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Archives of Andrology*, **49**(1), 49-55.

Carrell D. T., Wilcox A. L., Lowy L., Peterson C. M., Jones K. P., Erickson L., Campbell B., Branch D. W. & Hatasaka H. H. (2003b) Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. *Obstetrics and Gynecology,* **101**(6), 1229-1235.

Chaiban J. T. & Azar S. T. (2004) Erectile dysfunction in diabetic patients. *Le Journal médical Libanais,* **52**(4), 217-219.

Clegg E. D., Perreault D., Klinefelter G. R. (2001) Assessment of male reproductive toxicity. In: *Principles and Methods of Toxicology* (ed. A.W. Hayes), pp. 1263 – 1300. Taylor & Francis, Philadelphia.

Ewing, L. L., Desjardins, C., Irby, D. C. & Robaire, B. (1977) Synergistic interaction of testosterone and oestradiol inhibits spermatogenesis in rats. *Nature*, **269**(5627), 409-411.

Fairburn, C. G. (1981) The sexual problems of diabetic men. *British Journal of Hospital Medicine,* **25**, 484-491.

Fernandez C. D. B., Porto E. M., Arena A. C. & Kempinas W. G. (2007) Effect of altered epididymal sperm transit time on sperm quality. *International Journal of Andrology*. DOI: 10.1111/j.1365-2605.2007.00788.x.

Gonzalez-Cadavid N. F. & Rajfer J. (2004) Molecular pathophysiology and gene therapy of aging-related erectile dysfunction. *Experimental Gerontology,* **39**(11-12), 1705-1712.

Hassan, A. A., Hassouna, M. M., Taketo, T., Gagnon, C. & Elhilali M. M. (1993) The effect of diabetes on sexual behavior and reproductive tract function in male rats. *The Journal of Urology,* **149,** 148-154.

Hutson J. C. (1984) Altered biochemical responses by rat Sertoli cells and peritubular cells cultured under simulated diabetic conditions. *Diabetologia*, **26,** 155-158.

Jackson F. L. & Hutson J. C. (1984) Altered responses to androgen in diabetic male rats. *Diabetes,* **33,** 819-823.

Julie P. M., Arunakaran J., Malini T. & Balasubramanian K. (2003) The role of luteinizing hormone and prolactin in the regulation of insulin receptors in Leydig cells of the adult rat. *Endocrine Research*, **29**(3), 327-341.

Kempinas, W. G., Suarez, J. D., Roberts, N. L., Strader, L., Ferrell, J., Goldman, J. M., Narotsky, M. G., Perreault, S. D., Evenson, D. P., Ricker, D. D., Klinefelter, G. R. (1998) Fertility of rat epididymal sperm after chemically and surgically induced sympathectomy. *Biology of Reproduction,* **59**, 897-904.

Koh P. O. (2007a) Streptozotocin-induced diabetes increases apoptosis through JNK phosphorylation and Bax activation in rat testes. *The Journal of Veterinary Medical Sciences,* **69**(9), 969-971.

Koh P. O. (2007b) Streptozotocin-induced diabetes increases the interaction of Bad/Bcl-XL and decreases the binding of pBad/14–3–3 in rat testis. *Life Sciences,* **81**, 1079-1084.

Kuhn-Velten, N., Shermer, R. & Staib, W. (1978) Effect of streptozotocin-induced hyperglycemia on androgen-binding protein in the testis and epididymis. *Diabetologia*, **26**, 300-306.

Larrea G. A., Calvo J. C., Foglia V. G. & Libertun C. (1981) Effect of testosterone on the hypothalamic-pituitary-testicular axis of the old male rat. *Hormone and Metabolic Research,* **13**(7), 396-399.

Laskey J. W., Klinefelter G. R., Kelce W. R. & Ewing L. L. (1994) Effect of ethane dimethane-sulphonate on adult and immature rabbit Leydig cells: comparison with EDS treated rat Leydig cells. *Biology of Reproduction,* **50**, 1151-1160.

Melman A., Henry D. P., Felten D. L. & O'Connor B. (1980) Effect of diabetes upon penile sympathetic nerves in impotent patients. *The Southern Medical Journal,* **73**(3), 307-309, 317.

Murray, F. T., Cameron, D. F. & Orth, J. M. (1983) Gonadal dysfunction in the spontaneously diabetic BB rat. *Metabolism: clinical and experimental,* **32,** 141-147.

Naziroğlu M. (2003) Enhanced testicular antioxidant capacity in streptozotocin-induced diabetic rats. Protective role of vitamins C and E and selenium. *Biological Trace Element Research,* **94,** 61-71.

Ozkan Y., Yilmaz O., Ozturk A. I. & Ersan Y. (2005) Effects of triple antioxidant combination (vitamin E, vitamin C and alpha-lipoic acid) with insulin on lipid and cholesterol levels and fatty acid composition of brain tissue in experimental diabetic and non-diabetic rats. *Cell Biology International,* **29** (9), 754-760.

Paz G., Homonnai Z. T., Drasnin N., Sofer A., Kaplan R. & Kraicer P. F. (1978) Fertility of the streptozotocin-diabetic male rat. *Andrologia,* **10**(2), 127-136.

Pérez Díaz J., Benitez A. & Fernández Galaz C. (1982) Effect of streptozotocin diabetes on the pituitary-testicular axis in the rat. *Hormone and Metabolic Research*, **14**(9), 479-82.

Pirke K. M., Krings B. & Vogt H. J. (1979) Further studies on hypothalamic-pituitary-testicular function in old rats. *Acta Endocrinologica,* **92**(2), 358-369.

Pontes D. A., Fernandes G. S. A., Assumpção T. A. & Kempinas W. G. (2007a) Mechanisms involved with reduced fertility in streptozotocin-induced diabetic male rats. *Annual Review of Biomedical Sciences* (in press).

Pontes D. A., Fernandes G. S. A., Assumpção T. A., Piffer R. C., Gerardin D. C. C., Pereira O. C. M. & Kempinas W. D. G. (2007b) Possible mechanisms involved in the impairment of adrenergic response of the vas deferens in streptozotocin-induced diabetic rats. Submitted to *Journal of Andrology.*

Robb G. W., Amman R. P. & Killian, G. J. (1978) Daily sperm production and epididymal sperm reserves of pubertal and adult rats. *Journal of Reproduction and Fertility,* **54,** 103-107.

Sainio-Pöllänen S., Henriksén K., Parvinen M., Simell O. & Pöllänen P. (1997) Stage-specific degeneration of germ cells in the seminiferous tubules of non-obese diabetic mice. *International Journal of Andrology,* **20**(4), 243-53.

SBD, Sociedade Brasileira de Diabetes. (2007) Dados Sobre Diabetes Mellitus no Brasil. Available at: <http://www.diabetes.org.br/imprensa/estatisticas>. Access on March 26th 2007.

Scarano W. R., Messias A. G., Oliva S. U., Klinefelter G. R. & Kempinas W. G. (2006) Sexual behaviour, sperm quantity and quality after short-term streptozotocin-induced hyperglycaemia in rats. *International Journal of Andrology*, **29**(4), 482-488.

Seethalakshmi, L., Menon, M. & Diamond, D. (1987) The effect of streptozotocin-induced diabetes on the neuroendocrine-male reproductive tract axis of the adult rat. *The Journal of Urology*, **138**, 190-194.

Shrilatha B. & Muralidhara D. R. (2007) Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: Its progression and genotoxic consequences. *Reproductive Toxicology,* **23**(4), 578-587.

Soudamani S., Yuvaraj S., Malini T. & Balasubramanian K. (2005) Experimental diabetes has adverse affects on the differentiation of ventral prostate during sexual maturation of rats. *The Anatomical Record Part A*, **287**(A), 1281-1289.

Steger R.W. & Rabe M. B. (1997) The effect of diabetes mellitus on endocrine and reproductive function. *Proceedings of the Society for Experimental Biology and Medicine,* **214**(1), 1-11.

Sudha S., Valli G., Julie P. M., Arunakaran J., Govindarajulu P. & Balasubramanian K. (1999) Influence of streptozotocin-induced diabetes and insulin treatment on the pituitary-testicular axis during sexual maturation in rats. *Experimental and Clinical Endocrinology & Diabetes,* **107**, 14-20.

Syed V. & Hecht N. B. (2002) Disruption of germ cell-Sertoli cell interactions leads to spermatogenic defects. *Molecular and Cellular Endocrinology,* **186**(2), 155-157.

WHO, World Health Organization. (2007) Prevalence of diabetes worldwide. Available at <http://www.who.int/diabetes/facts/world_figures/en/>. Access on August 20th 2007.

Zhang X. H., Filippi S., Morelli A., Vignozzi L., Luconi M., Donati S., Forti G. & Maggi M. (2006) Testosterone restores diabetes-induced erectile dysfunction and sildenafil responsiveness in two distinct animal models of chemical diabetes. *The Journal of Sexual Medicine,* **3**(2), 253-264.

Zirkin B. R. & Chen H. (2000) Regulation of Leydig cell steroidogenic function during aging. *Biology of Reproduction,* **63**(4), 977-981.

Zubkova E. V. & Robaire B. (2004) Effect of glutathione depletion on antioxidant enzymes in the epididymis, seminal vesicles, and liver and on spermatozoa motility in the aging brown Norway rat. *Biology of Reproductoin,* **71**(3), 1002-1008.

Figure Legends

Figure 1. Glycemia at days 7 and 21 after diabetes induction, plasma testosterone, LH and FSH levels and intratesticular testosterone levels of the three experimental groups. Different number of asterisks indicate different levels of statistical significance (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Figure 2. Photomicrographs of testes (A, B and C) and epididymides (D, E and F) from the three experimental groups. The normal histoarchitecture of the seminiferous epithelium can be noted in control animals in A. In B (diabetic) and C (diabetic+T) the rats present several morphological alterations, such as epithelial depletion and loss of the morphological integrity (asterisks), and loss of cells (arrows) in the lumen of the tubules. In E and F, biological material, other than spermatozoa, is evidenced (arrows) in the lumen of the cauda epididymis, which can not be found in controls (D). This biological material is highlighted in a higher magnificence. H&E. st = seminiferous tubule.

Figure 3. Total sperm transit time through the epididymis of the three experimental groups. Different number of asterisks indicate different levels of statistical significance (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Table 1. Final body weight and weight of the reproductive organs of the animals of the three experimental groups (mean±SEM).

	Control (n=10)	**Diabetic** (n=07)	**Diabetic+T** (n=09)
Final body weight (g)	392.98±10.96 [a]	296.36±12.64 [b]	309.82±8.30 [b]
Testis (g)	1.72±0.03 [a]	1.61±0.05 [a]	1.22±0.05 [b]
Testis (g/100g)	0.44±0.01 [a]	0.55±0.03 [b]	0.39±0.01 [a]
Epididymis (mg)	556.01±15.73 [a]	430.21±23.19 [b]	419.16±12.88 [b]
Epididymis (mg/100g)	140.68±5.89 [a]	145.26±5.54 [a]	135.55±3.34 [a]
Ventral prostate (mg)	370.68±15.37 [a]	189.61±25.69 [b]	339.56±16.82 [a]
Ventral prostate (mg/100g)	94.55±3.69 [a]	64.87±9.67 [b]	109.70±4.88 [a]
Seminal vesicle (full) (g)	1.13±0.06 [a]	0.39±0.04 [b]	1.18±0.08 [a]
Seminal vesicle (full) (g/100g)	0.29±0.01 [a]	0.13±0.01 [b]	0.38±0.03 [a]
Seminal vesicle (empty) (g)	0.50±0.05 [a]	0.27±0.02 [b]	0.44±0.02 [a]
Seminal vesicle (empty) (g/100g)	0.13±0.01 [a]	0.09±0.01 [b]	0.14±0.01 [a]
Vas deferens (mg)	112.13±5.99 [a]	89.44±5.60 [b]	99.68±5.98 [ab]
Vas deferens (mg/100g)	28.79±1.86 [a]	30.22±1.55 [a]	32.18±1.59 [a]

[a,b]: different letters indicate statistically different results ($p<0.05$). ANOVA test, with the Tukey-Kramer “a posteriori” test.

Figure 1

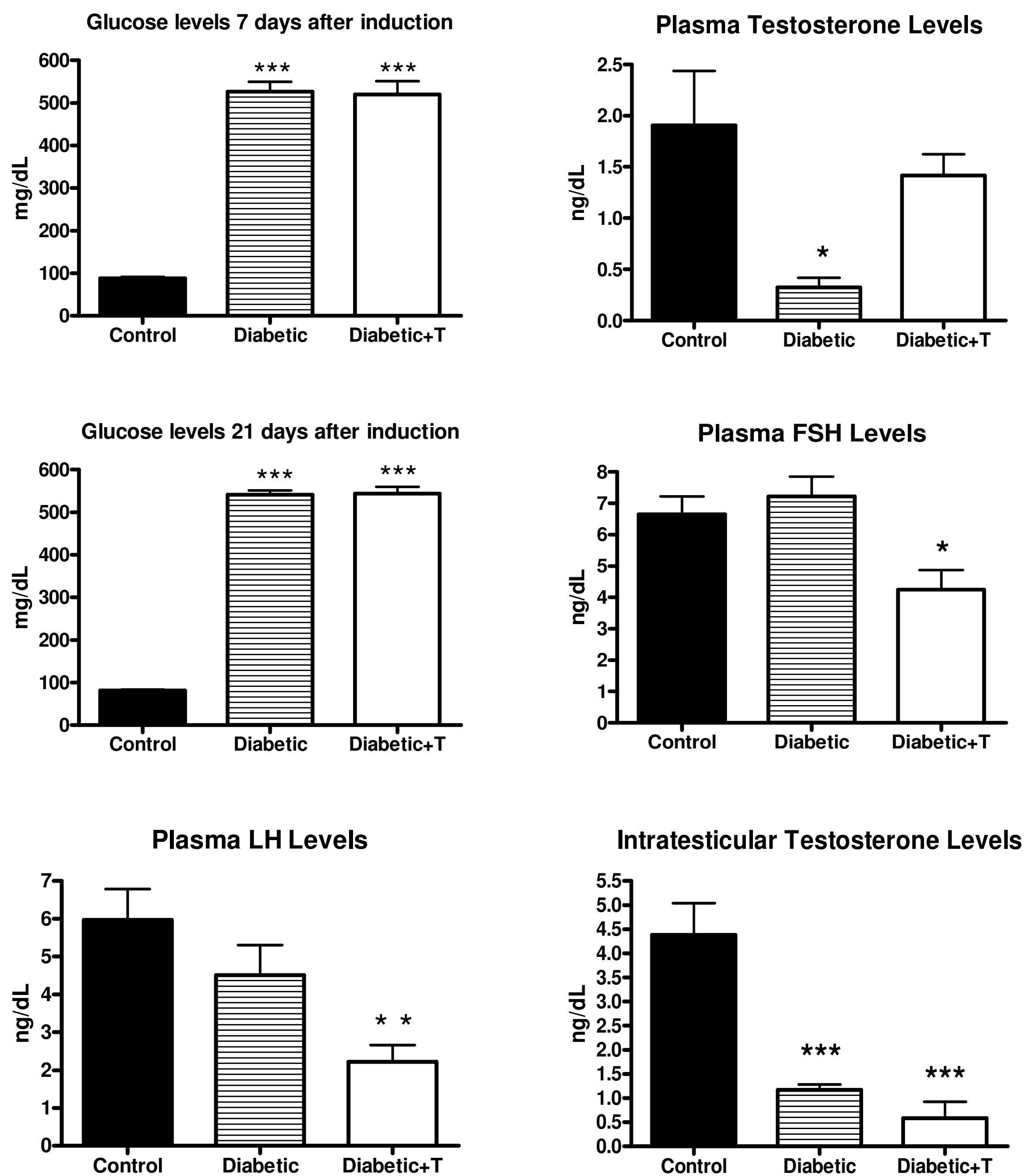


Figure 2

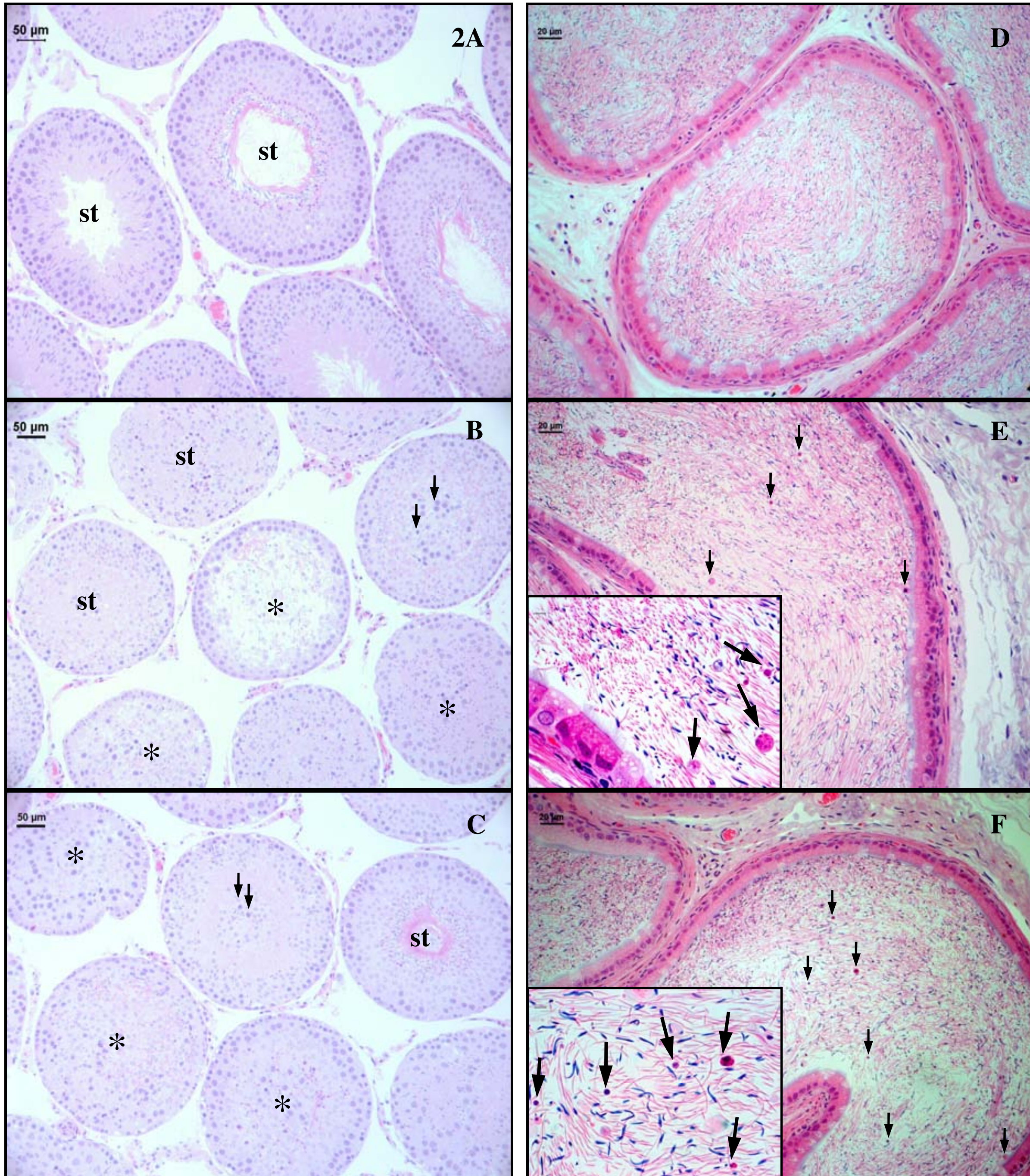
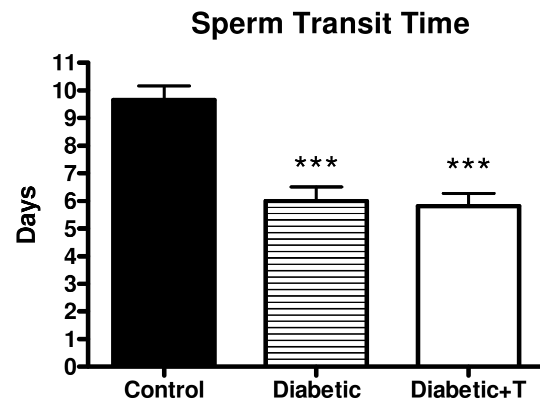


Figure 3



Conclusões Finais

CONCLUSÕES FINAIS

A indução do diabetes tipo 1, descontrolado, em ratos adultos, provocou diversos distúrbios reprodutivos, tais como níveis reduzidos de testosterona e mecanismos ineficientes de retroalimentação hormonal, provocando desregulação do eixo hipotalâmico-hipofisário-testicular. Estes distúrbios prejudicaram o processo espermatogênico, e sugere-se também prejuízo à maturação espermática. Como um fator complicante, os ensaios farmacológicos demonstraram que a função ejaculatória foi alterada, o que pode ajudar a explicar a diminuição de fertilidade de animais diabéticos em acasalamentos naturais observada em estudo anterior realizado neste laboratório. Estes danos se assemelham àqueles observados em animais senescentes, provavelmente devido ao fato de que o diabetes causa danos aos tecidos corporais similares aos processos naturais de envelhecimento como, por exemplo, aumento do estresse oxidativo. Sendo assim, os resultados deste trabalho revelam que o diabetes descontrolado afeta a reprodução masculina por causar descontrole neuroendócrino, danos à espermatogênese, diminuição da qualidade espermática e prejuízo do processo ejaculatório.

Referências da Introdução e da Revisão Bibliográfica

REFERÊNCIAS DA INTRODUÇÃO E DA REVISÃO BIBLIOGRÁFICA

Andersson K.E. Erectile physiological and pathophysiological pathways involved in erectile dysfunction. *The Journal of Urology* **170**(2): S6-14, 2003.

Arduíno, F. Sintomas, diagnóstico, prognóstico e mortalidade do diabetes. In: *Diabetes Mellitus*, pp. 78-94. Rio de Janeiro: Guanabara-Koogan, 1980.

Balasubramanian, K., Sivashanmugam, P., Thameedheen, S., Govindarajulu, P. Effect of diabetes mellitus on epididymal enzymes of adult rats. *Indian Journal of Experimental Biology* **29**: 907-909, 1991.

Ballester J., Muñoz M.C., Domínguez J., Rigau T., Guinovart J.J., Rodríguez-Gil J.E. Insulin-dependent diabetes affects testicular function by FSH- and LH-linked mechanisms. *Journal of Andrology* **25**(5): 706-719, 2004.

Bancroft J. The endocrinology of sexual arousal. *Journal of Endocrinology* **186**: 411-427, 2005.

Basu A., Ryder R.E. New treatment options for erectile dysfunction in patients with diabetes mellitus. *Drugs* **64**(23): 2667-88, 2004.

Calvo, J.C., Baranao, J.L., Tesone, M., Charreau, E.H. Hypothalamic-hypophyseal-gonadal axis in the streptozotocin-induced diabetic male rat. *Journal of Steroid Biochemistry* **20**: 769-772, 1984.

Chaiban J.T., Azar S.T. Erectile dysfunction in diabetic patients. *Le Journal médical Libanais* **52**(4): 217-219, 2004.

Dohle G.R., Smit M., Weber R.F.A. Androgens and male fertility. *World Journal of Urology* **21**(5): 341-345, 2003.

Fairburn, C.G. The sexual problems of diabetic men. *British Journal of Hospital Medicine* **25**: 484-491, 1981.

Fedele D. Therapy Insight: sexual and bladder dysfunction associated with diabetes mellitus. *Nature clinical practice. Urology* **2**(6): 282-90, quiz 309, 2005.

Giuliano F., Clément P. Physiology of ejaculation: emphasis on serotonergic control. *European Urology* **48**: 408-417, 2005.

Gooren L.J., Kruijver F.P. Androgens and male behavior. *Molecular and Cellular Endocrinology* **198**(1-2): 31-40, 2002.

Hassan, A.A., Hassouna, M.M., Taketo, T., Gagnon, C., Elhilali M.M. The effect of diabetes on sexual behavior and reproductive tract function in male rats. *The Journal of Urology* **149**: 148-154, 1993.

Howland, B.E., Zebrowski, E.J. Some effects of experimentally-induced diabetes on pituitary-testicular relationships in rats. *Hormone and Metabolics Research* **8**: 465-469, 1976.

Huleihel M., Lunenfeld E. Regulation of spermatogenesis by paracrine/autocrine testicular factors. *Asian Journal of Andrology* **6**(3): 259-68, 2004.

Hutson J.C. Altered biochemical responses by rat Sertoli cells and peritubular cells cultured under simulated diabetic conditions. *Diabetologia* **26**: 155-158, 1984.

Jégou B. The Sertoli-germ cell communication network in mammals. *International Review of Cytology* **147**: 25-96, 1993.

Jégou B., Le Magueresse B., Pineau C., Sharpe R.M. Germ cells and post-natal development of testicular function: in vitro studies (Article in French). *Bulletin de l'Association des anatomistes* **75**(228): 33-8, 1991.

Kuhn-Velten, N., Shermer, R., Staib, W. Effect of streptozotocin-induced hyperglycemia on androgen-binding protein in the testis and epididymis. *Diabetologia* **26**: 300-306, 1978.

Lejeune H., Chuzel F., Thomas T., Avallet O., Habert R., Durand P., Saez J. Paracrine regulation of Leydig cells. *Annales d'endocrinologie* **57**(1): 55-63, 1996.

Like A.A, Rossini A.A. Streptozotocin-induced pancreatic insulitis - new model of Diabetes-mellitus. *Science* **193**(4251): 415-417, 1976.

McVary, K.T., Rathnau, C.H. McKenna, K.E. Sexual dysfunction in the diabetic BB/WOR rat: role of central neuropathy. *American Journal of Physiology* **272**: R259-R267, 1997.

Melman A., Henry D.P., Felten D.L., O'Connor B. Effect of diabetes upon penile sympathetic nerves in impotent patients. *The Southern Medical Journal* **73**(3): 307-9,317, 1980.

Murray, F.T., Cameron, D.F., Orth, J.M. Gonadal dysfunction in the spontaneously diabetic BB rat. *Metabolism: clinical and experimental* **32**: 141-147, 1983.

Nelson, D.L., Cox, M.M. *Lehninger Princípios de Bioquímica*. Tradução: Arnaldo Antônio Simões, Wilson Roberto Navega Lodi. 3 ed. pp 692-693. São Paulo: Sarvier, 2002.

Parvinen M., Nikula H., Huhtaniemi I. Influence of rat seminiferous tubules on Leydig cell testosterone production in vitro. *Molecular and Cellular Endocrinology* **37**(3): 331-6, 1984.

Payne A.H., Kelch R.P., Murono E.P., Kerlan J.T. Hypothalamic, pituitary and testicular function during sexual maturation of the male rat. *The Journal of Endocrinology* **72**(1): 17-26, 1977.

Plant T.M., Marshall G.R. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. *Endocrine Reviews* **22**(6): 764-786, 2001.

Rang H.P., Dale M.M., Ritter J.M., Moore P.K. *Farmacologia*. 5 ed. Rio de Janeiro: Elsevier, 2004.

SBD, Sociedade Brasileira de Diabetes. *Dados Sobre Diabetes Mellitus no Brasil*. Disponível em: <http://www.diabetes.org.br/imprensa/estatisticas>. Acesso em 26 de Março de 2007.

Scarano W.R., Messias A.G., Oliva S.U., Klinefelter G.R., Kempinas W.G. Sexual behaviour, sperm quantity and quality after short-term streptozotocin-induced hyperglycaemia in rats. *International Journal of Andrology* **29**(4): 482-8, 2006.

Sharpe R.M., McKinnell C., Kivlin C., Fisher J.S. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction* **125**:769-784, 2003.

Sharpe R.M., Millar M., McKinnell C. Relative roles of testosterone and the germ cell complement in determining stage-dependent changes in protein secretion by isolated rat seminiferous tubules. *International Journal of Andrology* **16**(1):71-81, 1993.

Simonsen U., García-Sacristán A., Prieto D. Penile arteries and erection. *Journal of Vascular Research* **39**: 283-303, 2002.

Sriraman V., Jagannadha Rao A. Evaluation of the role of FSH in regulation of Leydig cell function during different stages of its differentiation. *Molecular and Cellular Endocrinology* **224**: 73-82, 2004.

Steger R.W., Rabe M.B. The effect of diabetes mellitus on endocrine and reproductive function. *Proceedings of the Society for Experimental Biology and Medicine* **214**(1), 1-11, 1997.

Zhang X.H., Filippi S., Morelli A., Vignozzi L., Luconi M., Donati S., Forti G., Maggi M. Testosterone restores diabetes-induced erectile dysfunction and sildenafil responsiveness in two distinct animal models of chemical diabetes. *Journal of Sexual Medicine* **3**(2): 253-264, 2006.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Mestrado** intitulada "Avaliação dos mecanismos causadores de distúrbios reprodutivos em ratos diabéticos":

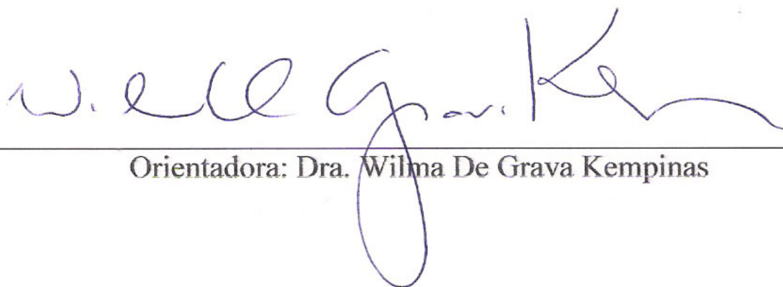
( ) não se enquadra no Artigo 1º, § 3º da Informação CCPG 002/06, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo nº ________), intitulado ________________________________

(X) tem autorização da Comissão de Ética em Experimentação Animal do Instituto de Biociências de Botucatu (Certificado 44/06 CEEA, 28/09/2006).

( ) tem autorização do Comitê de Ética para Pesquisa com Seres Humanos (?) (Protocolo nº ________).

Aluno: Davi Abeid Pontes



Orientadora: Dra. Wilma De Grava Kempinas

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Nome:
Função: Profa. Dra. ANA MARIA A. GUARALDO
Presidente
Comissão de Ètica na Experimentação Animal
CEEA/IB - UNICAMP

Universidade Estadual Paulista
Instituto de Biociências
CEEA – COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL
Caixa Postal 510 - 18.618-000 - Botucatu, SP **fone** (014) 38116013 **fax** (01438113744

CERTIFICADO

Certificamos que o Protocolo nº 44/06-CEEA, sobre *"Avaliação dos mecanismos causadores de infertilidade em ratos hiperglicêmicos"*, sob a responsabilidade de WILMA DE GRAVA KEMPINAS, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de 28/09/06.

Botucatu, 28 de setembro de 2006.

Prof. Dr. FRANCISCO DE ASSIS GANEO DE MELLO
Presidente - CEEA

NADIA JOVÊNCIO COTRIM
Secretária – CEEA