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"Relação entre estresse oxidativo e desordens reprodutivas em ratos machos hiperglicêmicos: potencial antioxidante das vitaminas C e E"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Glaura Scantamburlo Alves Fernandes e aprovada pela Comissão Julgadora.

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“A fé e a ciência são as duas asas que conduzem o homem à verdade!”

Pe. Anderson

Aos meus pais Geni e Jonas...

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Antoine de Saint – Exupéry

SUMÁRIO

Lista de abreviaturas da introdução

GSH - Glutathione

SOD – Superoxide dismutase

GSH-Px - Glutathione peroxidase

TBARS – Espécies reativas ao ácido tiobarbitúrico

ERMO - Espécies reativas do metabolismo do oxigênio

FSH – Hormone folículo estimulante

LH – Luteinizing hormone

DHT - Dihidrotestosterone

GLUTs – Transportadores de glicose

RESUMO

A hiperglicemia está relacionada com desordens no sistema reprodutor masculino. Quando induzida quimicamente, ela pode causar diminuição nos níveis dos hormônios sexuais, no peso dos órgãos reprodutores, na concentração espermática, na fertilidade bem como alteração no comportamento sexual de ratos machos. No homem, a hiperglicemia pode levar a impotência sexual, diminuição da libido e da espermatogênese. Contudo, os principais fatores que ocasionam essas alterações ainda são discutíveis. Uma das hipóteses seria o aumento do estresse oxidativo, o qual pode causar peroxidação lipídica nas membranas biológicas, neuropatias hiperglicêmicas, danificar a molécula de DNA e comprometer a comunicação celular. No entanto, há escassez de informações que relacionem o efeito do estresse oxidativo aumentado com a morfofisiologia do sistema reprodutor masculino em indivíduos hiperglicêmicos. Diante disso, o objetivo deste estudo foi avaliar se o tratamento com antioxidantes (vitaminas C e/ou E), ao diminuir o estresse oxidativo, poderia diminuir os danos causados pela hiperglicemia no sistema reprodutor masculino de ratos. Para tanto, a hiperglicemia foi induzida quimicamente utilizando dose única do antibiótico *streptozotocin* em ratos machos adultos (90 dias de idade; 9 ou 10/grupo experimental). Esses animais foram divididos em grupos experimentais que receberam vitamina C e E (sozinhas ou associadas), ou apenas os veículos (grupo normoglicêmico) durante trinta dias consecutivos por via oral. No 31º dia do período experimental, após a eutanásia dos animais, foi coletado sangue para avaliação dos biomarcadores do estresse oxidativo e dos níveis hormonais, e órgãos reprodutores para avaliação dos parâmetros espermáticos e resposta noradrenérgica do ducto deferente. O delineamento experimental acima descrito foi empregado a um outro lote de animais para realização das análises histopatológicas e morfométricas do testículo e epidídimo. Os resultados mostraram que a hiperglicemia causou significativo aumento do estresse oxidativo

e da sensibilidade dos receptores α_1 noradrenérgicos, diminuição no peso corporal e dos órgãos reprodutores, redução na motilidade espermática, no nível de testosterona, aumento no número de espermatozóides malformados e um rearranjo nos componentes do tecido epididimário. As vitaminas reduziram significativamente o nível do estresse oxidativo e o número de espermatozóides malformados, mas em ambos os casos a vitamina C foi mais efetiva. As vitaminas também reduziram parcialmente a sensibilidade dos receptores α à noradrenalina. Com relação aos prejuízos da motilidade espermática a vitamina C sozinha amenizou parcialmente essas alterações. Também, a administração da vitamina C sozinha preveniu a redução da proporção dos componentes epididimários. Além disso, a vitamina C sozinha e associada com a vitamina E restabeleceram parcialmente os níveis de testosterona. Entretanto, a associação das vitaminas causou uma redução significativa nos níveis de LH. Contudo, a produção espermática diária, o tempo de trânsito espermático pelo epidídimo, o aspecto histológico dos testículos e epidídimos, o diâmetro dos túbulos seminíferos, os níveis de FSH e a resposta máxima noradrenérgica foram estatisticamente semelhantes entre os grupos experimentais. Em conclusão, essas vitaminas, atuando como antioxidantes, podem diminuir algumas alterações no sistema reprodutor masculino de ratos causados pela hiperglicemia.

ABSTRACT

The hyperglycemia is related to disorders in the male reproductive system. When induced chemically, it can reduce the sexual hormones' level, reproductive organs' weight, sperm concentration, and fertility as well as the male rat sexual behavior. In men, the hyperglycemia can lead to sexual impotence, libido and spermatogenesis reduction. However, the main factors that are responsible for these alterations are still controversial. One of the possible causes for this would be the increase of the oxidative stress, which can cause lipid peroxidation in the biological membranes, hyperglycemic neuropathies, injury in the DNA molecule and to compromise the cellular communication. However, there is a lack of information related to the increased oxidative stress effect with the morphophysiology from the male reproductive system in hyperglycemic subjects. In this sense, the aim of the present study was to evaluate if the treatment with antioxidants (vitamins C and/or E), lowering the oxidative stress, could somehow attenuate the damages caused by hyperglycemia in the male rat reproductive system. Therefore the hyperglycemia was induced chemically using a single dose of *streptozotocin* antibiotic in male adult rats (90 days old; 9 or 10/experimental group). These animals were divided into experimental groups that received vitamin C or E (isolated or in association), or only the vehicles (normoglycemic group) during thirty consecutive days orally. At the 31st day from the experimental period, after the euthanasia of the rats, blood was collected to evaluate biomarkers of oxidative stress and hormonal levels, and reproductive organs to evaluate the spermatic parameters and the noradrenergic response of vas deferens. The experimental design described above was applied in another amount of animals for the accomplishment of histopathological and morphometric analysis from testis and epididymis. The results showed that hyperglycemia caused significant increase in the oxidative stress and in the sensibility of noradrenergic α_1 receptors,

decrease in the body mass and in the reproductive organs' weights, reduction on the sperm motility, on epididymal tissue, in the testosterone level, an increase in the malformed sperm number and a rearrangement in the epididymal tissue components. The vitamins reduced significantly the level of oxidative stress and the number of malformed sperm, but in both cases vitamin C was more effective. These vitamins also reduced partly the sensibility to noradrenalin in the α receptors. In relation to the injuries in the sperm motility, the vitamin C alone attenuated partly these alterations. In addition, the vitamin C alone also prevented the reduction of the epididymal compartimental. Besides that, the vitamin C alone and in association to vitamin E reestablished partly the testosterone levels. Meanwhile, the association of the vitamins caused a significant reduction in the LH levels. Nonetheless, the daily sperm production, the transit time by epididymis, the histological aspect of the testes and epididymides, the seminiferous tubular diameter, the FSH levels and the maximum noradrenergic response were statistically equal among the groups. In summary, these vitamins, acting as antioxidants, may reduce some changes in the rat male reproductive system caused by hyperglycemia.

INTRODUÇÃO

Aspectos gerais da morfofisiologia do sistema reprodutor masculino

O sistema reprodutor masculino do rato, assim como na maioria dos mamíferos, é composto por testículos (gônadas), epidídimos, ductos deferentes, glândulas sexuais acessórias como próstata e vesícula seminal, e órgão copulador. Esses órgãos são andrógeno-dependentes sofrendo assim ação dos hormônios testosterona e diidrotestosterona - DHT. A DHT é formada a partir da testosterona por ação da enzima 5α-redutase sendo um andrógeno potencialmente mais ativo. Esses dois andrógenos se ligam ao mesmo tipo de receptor – o AR (Androgen Receptor), porém possuem diferentes afinidades de ligação e distintas funções fisiológicas (Robaire & Henderson, 2006).

Os testículos estão localizados fora do canal inguinal, dentro da bolsa escrotal, o que resulta numa menor temperatura no órgão em relação ao resto do corpo e proporciona seu estado funcional adequado. Cada testículo pode ser, funcionalmente e anatomicamente, dividido em duas partes: tecido intersticial e túbulos seminíferos, responsáveis pela esteroidogênese e pela espermatogênese, respectivamente (Rodrigues & Favaretto, 1999). Os túbulos seminíferos são constituídos pelo epitélio seminífero composto por células germinativas e células de Sertoli, e o tecido peritubular. Em animais sexualmente maduros, a população de células germinativas é constituída por espermatogônias, espermatócitos e espermátides. A espermatogênese é um processo elaborado pelo qual células-tronco espermatogoniais tornam-se células haplóides altamente diferenciadas e especializadas, os espermatozóides (Clermont, 1972). O tecido intersticial está localizado entre os túbulos seminíferos e é composto por vasos sanguíneos e linfáticos, nervos e as células intersticiais ou de Leydig responsáveis pela produção de testosterona (Russell et al., 1990). A função testicular é regulada por uma série de relações entre

os hormônios do hipotálamo (GnRH), da hipófise (FSH e LH), dos hormônios testiculares (testosterona) com o compartimento germinativo (Sokol, 1997). Assim, qualquer fator que atue desordenando a interação coordenada do eixo hipotalâmico – hipofisário – testicular pode refletir em alterações no sistema reprodutor masculino as quais podem levar a prejuízos nesse sistema, sobretudo na fertilidade do indivíduo (Sokol, 1997).

No mamífero adulto, o epidídimo é formado por um ducto único e enovelado que liga os dúctulos eferentes ao ducto deferente (Cosentino & Cockett, 1986; Hermo & Robaire, 2002). Sua extensão varia de maneira espécie-específica e atinge, por exemplo, cerca de 2m no rato e 6m no homem (Brooks, 1983). Anatomicamente, é dividido em cabeça, corpo e cauda (Reid & Cleand, 1957; Cosentino & Cockett, 1986; Gatti et al., 2004; França et al., 2005; Sullivan et al., 2005), sendo que a cabeça possui uma região inicial conhecida como segmento inicial, para onde convergem os dúctulos eferentes (Glover, 1982). Estas porções são subdivididas histologicamente em zonas que são designadas de acordo com a altura do epitélio e a distribuição e quantidade dos tipos celulares (Reid & Cleand, 1957). O epitélio é do tipo pseudoestratificado ciliado possui seis tipos celulares: basais, principais, estreitas, halo, claras e apicais (Hermo & Robaire, 2002). Há uma camada de músculo liso que envolve esse epitélio epididimário, apresentando-se mais fina na região da cabeça e corpo, e mais espessa na cauda. A contração dessas fibras musculares, as quais recebem inervação de fibras adrenérgicas do sistema nervoso simpático (Setchell, 2002), é importante para definir o trânsito espermático e a expulsão dos espermatozóides da região da cauda para o ducto deferente.

O ducto deferente é um órgão par que liga o epidídimo à uretra prostática. Suas funções estão relacionadas com o transporte e término do processo de maturação dos espermatozóides. Ele é caracterizado por apresentar um lúmen estreito e uma espessa camada de músculo liso que

sofre fortes contrações peristálticas que participam da expulsão do sêmen durante a ejaculação. Sua mucosa forma dobras longitudinais e a maior parte de seu trajeto é constituído por epitélio pseudo-estratificado com estereocílios. Antes de entrar na próstata, o ducto deferente se dilata formando uma região chamada ampola, onde o epitélio é mais espesso e muito pregueado. Em seguida o ducto deferente penetra na próstata e se abre na uretra prostática. O seguimento que entra na próstata é chamado de ducto ejaculatório, cuja mucosa é semelhante a do ducto deferente mas não é envolta por músculo liso (Junqueira & Carneiro, 2004).

A vesícula seminal consiste de um ducto único muito dilatado e enovelado. Este ducto é revestido por um epitélio pseudo-estratificado pregueado, constituído por células epiteliais secretoras e células basais. A camada muscular lisa que reveste o órgão é constituída por duas lâminas: uma interna, de fibras circulares, e outra externa, de fibras longitudinais. A luz é ocupada pelo produto de secreção, de aspecto hialino (Hayward et al. 1996a; 1996b).

A próstata é a maior glândula sexual acessória do sistema reprodutor masculino e está localizada na pelve inferiormente à bexiga, onde circunda a bexiga. Ela produz um líquido alcalino que neutraliza o conteúdo vaginal ácido, proporcionando nutrientes e transporte para os espermatozóides, e liquefaz o sêmen. Os produtos de secreção de natureza protéica incluem a fosfatase ácida específica da próstata, antígeno prostático específico (PSA), amilase e fibrinolisina (Kierszenbaum, 2008). No homem, a próstata é um órgão compacto e internamente dividido em três zonas ou regiões distintas denominadas de zona central, zona periférica e zona de transição (McNeal, 1981). Em ratos e camundongos, essa glândula não é compacta, sendo composta por um par de lobos ventrais, um par de lobos dorsolaterais e um par de lobos anteriores ou glândula de coagulação que estão associados às vesículas seminais (Abbout et al., 2003). Em termos de homologia com as zonas da próstata humana, os lobos anteriores são

considerados homólogos à zona central, enquanto os lobos dorsolaterais são considerados homólogos à zona periférica humana. Entretanto, os lobos ventrais de roedores não apresentam qualquer homologia à próstata humana, apesar de ser o lobo prostático mais estudado em modelos experimentais (Roy-Burman et al., 2004). Histologicamente, tanto no homem como nos roedores, ela é constituída por um epitélio pseudo-estratificado secretor, contendo células epiteliais colunares, células basais e células neuroendócrinas, sendo sustentado por um estroma fibromuscular (Marker et al., 2003). Na próstata, o hormônio dihidrotestosterona (DHT) é o andrógeno predominante (Soronen et al., 2004). É importante salientar que os produtos de secreção da vesícula seminal e próstata contribuem para a nutrição e suporte dos espermatozóides fora do trato genital masculino.

Estresse oxidativo

O estresse oxidativo provém de um desequilíbrio entre espécies reativas do metabolismo de oxigênio (ERMO) e antioxidantes do organismo. As ERMO, de modo geral conhecidas como radicais livres, são íons ou moléculas muito instáveis os quais são representados, principalmente, pelos radicais hidroxila (OH), superóxido (O_2^-), hidroperoxila (HO_2) e peróxido de hidrogênio (H_2O_2). Os elementos antioxidantes podem ser antioxidantes endógenos não-enzimáticos (vitamina E ou α-tocoferol, vitamina C ou ácido ascórbico, vitamina A, carotenos, bilirrubinas, ácido úrico e albumina) e enzimáticos (superóxido diamutase - SOD, glutationa peroxidase - GSH-Px e catalase) (Ceriello et al., 1997; Cay et al., 2001; Damasceno et al., 2002; Naziroğlu, 2003; Ozkan et al., 2005).

Sabe-se que altas concentrações de ERMO são extremamente tóxicas para as células causando danos na molécula de DNA, peroxidação lipídica, degradação protéica (Sun, 1990) e

podendo afetar diversos tipos de moléculas biológicas (Damasceno et al., 2002). Estes elementos também estão envolvidos com a etiologia de uma ampla variedade de doenças como diabetes, ateriosclerose, catarata, neurodegenerativas, neuromotoras, inflamatórias crônicas, cânceres, AIDS e envelhecimento (Makker et al., 2009). Porém, em condições fisiológicas, as ERMO estão envolvidas em processos bioquímicos normais, como controle da proliferação e sinalização celular (Finkel, 1998). Os primeiros estudos a respeito de "radicais livres" se deram por volta de 1924. No entanto, só nos anos setenta começaram a surgir trabalhos, relatando a importância desses elementos para os seres vivos aeróbicos. Os principais estudos relacionaram sua atuação junto a aspectos da Biologia Celular e Molecular, Fisiologia e Patologia Humana.

Em relação ao sistema antioxidante endógeno enzimático, a enzima SOD faz parte de um grupo de metaloenzimas, que têm a função de proteger o organismo contra a toxicidade do ânion superóxido. Em meio aquoso, a enzima SOD age sobre o radical superóxido para produzir oxigênio molecular e peróxido de hidrogênio. São descritos três tipos de SOD classificadas de acordo com metal associada a ela: a) cobre, zinco superóxido dismutase (Cu, Zn-SOD), presente no citosol, b) manganês-superóxido dismutase (Mn-SOD) localizada na matriz mitocondrial, c) ferro-superóxido dismutase (Fe-SOD) descrita em bactéria e não em seres humanos (Olszewer, 1995).

A GSH-Px é uma enzima antioxidante que contém selênio (Se) em seu sítio ativo e está presente no citoplasma e nas mitocôndrias. Estes locais também correspondem à presença de SOD, sugerindo uma ação seqüencial do sistema antioxidante para controlar a produção de ERMO. É responsável pela decomposição (redução) do peróxido de hidrogênio, usando glutationa reduzida (GSH) como substrato doador de elétron, transformando-a em glutationa oxidada (GSSG) (Olszewer, 1995).

A GSH é um dos agentes mais importantes do sistema de defesa antioxidante da célula, protegendo-a contra a lesão resultante da exposição a agentes como íons ferro, oxigênio, ozônio, radiação e luz ultravioleta. Uma vez oxidada a GSSG, esta é convertida em GSH através da enzima glutationa redutase (GSH-Rd) (Olszewer, 1995).

A catalase é uma metaloproteína que catalisa a formação de água e oxigênio a partir de peróxido de hidrogênio, formado pela ação da SOD (Grace, 1994). No seres humanos, está presente nos eritrócitos e em quase todos os tecidos, mas existe em maior quantidade no fígado, rim, baço e coração, provavelmente pelo metabolismo aeróbio intenso nesses órgãos e grande produção de radicais livres. É importante lembrar que o bom funcionamento da SOD deve ser simultâneo ao da catalase, caso contrário ocorrerá um acúmulo de grandes quantidades de peróxido de hidrogênio, que por sua vez poderá inativar a própria SOD e outros sistemas enzimáticos celulares. Assim, o sistema antioxidante deve funcionar em equilíbrio e de maneira seqüencial para impedir a formação excessiva de ERMO (Olszewer, 1995).

Contudo, apesar dos muitos estudos sobre a influência do estresse oxidativo no agravamento de várias doenças ainda é discutível se ele, como evento patogênico, atue tão logo que seja aumentado ou após um período mais longo de sua ação.

Vitamina C

A vitamina C ou ácido ascórbico, é o fator antiescorbútico que foi isolado em 1928 por Szent-Gyorgyi, em tecidos da supra-renal, laranjas e repolhos e foi chamado de ácido hexurônico. Em 1932, Szent-Gyorgyi e C. Glenn King demonstraram que o ácido hexurônico era a vitamina C. Apesar do escorbuto ter sido descrito pela primeira vez durante as Cruzadas (século XI a XIII)

e infestar comumente exploradores e viajantes, a relação específica entre o escorbuto, alimentos cítricos e ácido ascórbico não foi estabelecida até o século XX (Combs, 2002).

O ácido ascórbico é encontrado em tecidos vegetais e animais. Diferente de muitas espécies de animais que sintetizam grandes quantidades de vitamina C, os humanos, outros primatas, alguns morcegos e algumas espécies de aves perderam essa capacidade como resultado de uma série de mutações de inativação nos genes que codificam a enzima gulonolactone oxidase (enzima chave para biossíntese de vitamina C) durante a evolução (Li & Schellhorn, 2007). Assim, para essas espécies é necessário que a obtenção dessa vitamina se dê através de sua ingestão, ou seja, pela alimentação. Frutas, hortaliças e vísceras são excelentes fonte de vitamina C (Combs, 2002).

A vitamina C é absorvida pelo intestino e é distribuída para diferentes tecidos e órgãos mostrando concentrações significativas nos testículo, cérebro, glândulas adrenais e pulmões (Castro et al., 2008). Por ser uma molécula hidrossolúvel, ela não passa através da bicamada lipídica, assim o fluxo de vitamina C dentro e fora da célula é controlado por mecanismos específicos, incluindo difusão facilitada e transporte ativo, os quais são mediados por classes distintas de proteínas de membrana como transportadores de glicose (GLUTs - Glucose Transporters) ou co - transportadas com o íon sódio (SVCT - Sodium Dependent Active Transport), respectivamente (Li & Schellhorn, 2007).

A vitamina C atua em diversas funções do organismo. Ela é um co-fator para várias enzimas participando da hidroxilação pós-transcricional do colágeno, na biossíntese de carnitina e na conversão do neurotransmissor dopamina em noradrenalina por exemplo (Duarte & Lunec, 2005, Castro et al., 2008). Outra importante função da vitamina C é seu papel antioxidante no organismo protegendo as células da ação das ERMO (Lewis et al., 1997; Naziroğlu, 2003; Greco

et al., 2005a), e forma a primeira linha de defesa antioxidante contra a peroxidação lipídica. Devido às suas características, em ambiente aquoso, o ácido ascórbico é um eficiente doador de elétron, sendo capaz de substituir radicais altamente nefastos para o organismo por radicais pouco reativos, ou mesmo neutralizando-os (Duarte & Lunec, 2005). Vitamina C pode neutralizar o radical superóxido, radical hidroxila e peróxido de hidrogênio (Makker et al., 2009). Além disso, o ácido ascórbico também regenera a molécula de α-tocoferol (Vitamina E) ao reagir com o radical tocoferoxil, que é formado quando o α-tocoferol reage com um radical peroxil lipídico, portanto restaurando e aumentando a propriedade antioxidante intrínseca da molécula de α-tocoferol (Sen Gupta et al., 2004). O ácido ascórbico também recicla a molécula de glutationa (Duarte & Lunec, 2005).

Vitamina E

A vitamina E foi descoberta em 1922 por Evans e Bishop como um micronutriente necessário para prevenir a morte fetal e reabsorções em roedores, sendo considerada como elemento essencial para reprodução em roedores. Subseqüentemente, estudos com roedores e galináceos mostraram que certas patologias podiam se curadas com a administração de certos produtos, nos quais foram posteriormente identificados a presença de vitamina E (Traber & Atkinson, 2007). Esta vitamina foi primeiramente caracterizada em óleo de germe de trigo e alface. Em 1936 sua estrutura ativa foi quimicamente isolada por Evans e colaboradores, sendo chamada de tocoferol, do grego *tokos* (parto) e *pherein* (suportar). Posteriormente, descobriram-se quatro isoformas dessa molécula denominadas de α, β, γ ou δ tocoferol. Contudo, as formas α e γ são as mais importantes, e a α a predominante no organismo (Wang & Quinn, 1999; Combs,2002). Hoje a vitamina E é a representante de duas famílias de substâncias ativas: os

tocoferóis e tocotrienóis, contudo os tocotrienóis apesar de serem compostos ativos, são menos efetivos (Combs, 2002).

O tocoferol, ou vitamina E, é sintetizado apenas pelas plantas. Assim, animais podem obtê-la através da alimentação uma vez que é encontrada em altas concentrações em óleos vegetais (principalmente), grãos, vegetais e frutas, e em pequenas quantidades nos tecidos animais onde a fonte mais rica são os tecidos gordurosos e os de animais alimentados com grande quantidade de vitamina E. Devido ao seu caráter lipossolúvel, o tocoferol, na maioria das células está quase que exclusivamente distribuído pelas membranas biológicas das quais ele pode ser mobilizado (Combs, 2002). Os fosfolipídios presentes na membrana da mitocôndria, do retículo endoplasmático e da membrana plasmática, possuem alta afinidade pelo tocoferol pelo fato de ser lipossolúvel, permitindo movimentação entre as camadas das membranas (Wang & Quinn, 1999).

A vitamina E está envolvida numa variedade de eventos fisiológicos e bioquímicos cujos mecanismos moleculares não estão totalmente definidos. Desde a década de 1950 ela foi inserida na classe de antioxidante celular, sendo hoje considerada o principal antioxidante presente no interior das membranas celulares e uma das suas principais funções. Deste modo, a capacidade antioxidante da vitamina E despertou o interesse de muitos grupos de pesquisas interessados em avaliar sua capacidade de prevenir, ou amenizar, doenças crônicas como doenças cardiovasculares, ateriosclerose e câncer, nas quais o estresse oxidativo pode ser um dos principais agravantes (Brigelius-Flohé & Traber, 1999).

Esse evento ocorre através de reações em cadeia onde a vitamina E reage com elementos oxidantes como o íon superóxido produzindo moléculas estáveis, ou seja, evita que um elemento instável se forme e reaja com o oxigênio molecular produzindo novos oxidantes. Deste modo, ocorre a formação de um íon estável e inócuo para o organismo (Wang & Quinn, 1999) uma vez

que também doa elétrons para radicais peroxil lipídicos formados nas membranas biológicas após ação de ERMO. Assim, a vitamina E impede que o processo de peroxidação lipídica se propague por toda membrana previndo a perda de função ou até mesmo a morte celular em diferentes tecidos do organismo animal (Wang & Quinn, 1999). A vitamina E também reage com peróxido de hidrogênio e radical hidroxila (Wang & Quinn, 1999; Makker et al., 2009).

Além disso a sua deficiência no organismo animal, incluindo humanos, pode levar a manifestações clínicas nos sistemas reprodutor, neuromuscular e vascular, e em nível celular ocasionar o aumento da peroxidação lipídica nas membranas biológicas (Combs, 2002; Traber & Atkinson, 2007).

Streptozotocin

A *streptozotocin* é um antibiótico de amplo espectro de ação que foi isolado e identificado em colônias da bactéria *Streptomyces achromogenes,* em 1950 por cientistas da companhia farmacêutica Upjohn (agora parte da Pfizer) em Kalamazoo, Michigan a partir de amostras de solo da cidade de Blue Rapids, Kansas, a qual é considerada o berço da *streptozotocin* (Vavra et al., 1959). Em meados de 1960, a *streptozotocin* foi identificada como tóxico seletivo para as células β pancreáticas produtoras de insulina. Isto sugeriu que ela passasse a ser usada em modelos animal como indutora do estado hiperglicêmico, uma vez que mimetiza o quadro clínico do diabetes tipo I. Hoje é a droga mais utilizada para mimetizar o estado hiperglicêmico por promover uma curva-resposta bem definida e uma taxa de mortalidade muito baixa.

Essa toxicidade dirigida às células β é devida à similaridade estrutural entre as moléculas de *streptozotocin* e de glicose, o que permite que a *streptozotocin* seja internalizada pelo receptor GLUT2 (Wang & Gleichmann 1998; Schnedl et al., 1994). Rakieten et al. (1963), relataram

rompimento das ilhotas de Langerhans e diminuição ou ausência dos grânulos contendo insulina nas células β pancreáticas em animais diabéticos induzida por *streptozotocin.* Deste modo, Evans et al. (1965) associaram essas alterações, assim como os demais trabalhos realizados até 1965, apenas com inibição da síntese ou secreção de insulina pelas células β. Contudo havia dúvidas sobre esses resultados, uma vez que a pureza dessa substância até 1965, era incerta. Assim, Junod et al. (1967) utilizando *streptozotocin* com pureza certificada viram que ela causava lesão nas células β pancreáticas sedo considerado um agente citotóxico extraordinariamente efetivo para essas células, contudo não causa nenhum dano nas células α nem no pâncreas exócrino. Neste ínterim e ainda diante de algumas dúvidas, o laboratório de pesquisas Upjohn, de Kalamazoo, investiu em vários e longos estudos sobre o mecanismo de ação dessa droga e constatou que ela, pura, tem ação específica, rápida e irreversível sobre as células β pacreáticas.

Ela exerce ação sobre o pâncreas endócrino (Rakieten et al., 1963; Bolzan & Bianchi, 2002) especificamente nas células β onde exerce sua citotoxicidade impedindo a conversão da pro-insulina em insulina. Assim, ela é uma droga eficiente para a indução do estado hiperglicêmico crônico que mimetiza o quadro clínico do *Diabetes mellitus* tipo I do indivíduo (Rees & Alcolado, 2005).

Ao elevar o nível de glicose no sangue e diminuí-la no interior das células, a *streptozotocin* leva ao aumento de estresse oxidativo nos tecidos por induzir a maior formação de espécies reativas do metabolismo do oxigênio (ERMO) que acarreta em peroxidação lipídica e alquilação da molécula de DNA levando a mutações e sérias alterações no metabolismo do organismo.

No entanto, por ser um membro do grupo de drogas alquilantes, a *streptozotocin* começou a ser utilizada como droga antineoplásica no tratamento contra o câncer de pâncreas por inibir a

divisão de células β tumorais (Murray-Lyon et al., 1968). Sua aprovação como quimioterápico se deu em 1982 após muitas pesquisas realizadas pelo Instituto Nacional do Câncer dos EUA entre 1960 e 1970, e vem sendo utilizada até a atualidade com essa finalidade.

Hiperglicemia: ênfase no sistema reprodutor masculino e estresse oxidativo

A hiperglicemia pode ser ocasionada pela ausência ou diminuição nos níveis de insulina (hormônio produzido pelo pâncreas endócrino) circulante, ou ainda devido a alteração nos receptores de insulina nas células alvo. Essa modificação fisiológica pode advir de fatores como diabetes, obesidade, predisposição genética e/ou envelhecimento.

O pâncreas é uma glândula mista que exerce função endócrina e exócrina. A porção exócrina apresenta-se como uma glândula acinosa composta que secreta enzimas digestivas (amilases, proteases, nucleases e lipases) no duodeno. As ilhotas de Langerhans constituem a porção endócrina do pâncreas, representando cerca de 1,5% do seu volume total e apresenta-se sob a forma de aglomerados arredondados de células, imersos no tecido pancreático exócrino. O número de ilhotas no pâncreas humano é variável, oscilando ao redor de 1.000.000. Essas ilhotas são constituídas por quatro tipos principais de células: β, α, δ e PP (polipeptídeo pancreático). As células β constituem a maior população celular que representa 70% a 80 % da população celular das ilhotas, e se caracterizam pela presença de grânulos citoplasmáticos de depósito e liberação de insulina e outros peptídeos. A liberação de insulina é estimulada por altos níveis de glicose, bem como de glucagon, epinefrina e de aminoácidos. As células α (15 a 20% do total) secretam glucagon. As células δ que constituem aproximadamente 5% da população celular e secretam somatostatina. As células PP (1-2% do total), se distribuem tanto na ilhota quanto no pâncreas exócrino, estimulam a secreção gástrica das enzimas intestinais e inibem a motilidade do

intestino. Assim, o pâncreas é um órgão responsável pela digestão e homeostase de glicose no sangue. Por ser o único produtor de insulina no organismo humano, uma vez prejudicado, leva a um dos maiores problemas de saúde, o diabetes mellitus (Clever & Melton, 2005).

O diabetes é uma síndrome metabólica crônica que pode ser do tipo 1 (Diabetes tipo 1), a qual é causada pela falta de produção de insulina, ou do tipo 2 (Diabetes tipo 2) que ocorre devido a alteração nos receptores para insulina nas células-alvo. Em ambos os casos resultam em doença metabólica hiperglicêmica (Bennetti & Knowler, 2005). É progressiva, silenciosa, atinge vários órgãos e é considerada incurável até o presente.

Segundo Guyton & Hall (2006), no estado de ausência ou de distúrbio na ação e/ou secreção de insulina, os nutrientes ingeridos não são devidamente captados pelas células. Assim, a sensação de fome é constante nesses indivíduos, levando-os a ingestão de maior quantidade de alimento (polifagia). Como conseqüência, há um acúmulo de nutrientes na circulação sangüínea. Os rins, por sua vez, que reabsorveriam a glicose útil, eliminam seu excesso na urina (glicosúria). Isso leva à necessidade de maior quantidade de água para eliminação da glicose, aumentando a quantidade de urina excretada (poliúria). Assim, há perda de grande quantidade de água, o que estimula o aumento da ingestão de líquidos pelo organismo (polidpsia). Esses eventos levam a perda de peso e a falta de energia (astenia).

O estudo detalhado da interação dos inúmeros fatores envolvidos na síndrome diabética é muito complicado e difícil quando investigado na espécie humana principalmente por questões burocráticas, éticas e de carência de pacientes voluntários. Desta forma, modelos experimentais foram desenvolvidos com a expectativa de que estudos em animais diabéticos levem ao entendimento mais completo de sua etiologia, patogenicidade e tratamento, visando melhor aplicação clínica (Calderon et al., 1992).

Vários métodos são utilizados para indução do diabetes experimental, dentre eles pode-se destacar o uso de agentes beta – citotóxicos como aloxana (Dunn et al., 1943) e a *streptozotocin* (Rakieten et al., 1963; Calderon et al., 1992; Damasceno et al., 2002; Bolzán & Bianchi, 2002). O rato macho tem sido utilizado como modelo experimental para a avaliação da hiperglicemia sobre as funções reprodutiva, uma vez que apresenta quadro clínico semelhante ao observado em homens portadores dessa síndrome (Res & Alcolado, 2005).

Vem sendo descrito na literatura científica que a hiperglicemia causa alterações no sistema reprodutor masculino de homens e animais portadores de diabetes. Em ratos machos hiperglicêmicos, induzidos quimicamente por *streptozotocin*, as alterações reprodutivas incluem diminuição do peso dos órgãos reprodutores (Oksanen, 1975; Howland & Zerbrowski, 1976; Paz et al., 1978; Murray et al., 1981; Scarano et al., 2006; Suthagar et al., 2009), oligospermia (Nakhooda et al., 1977; Hassan et al., 1993; Scarano et al., 2006, Olivares et al., 2009), redução da fertilidade (Anderson et al., 1987; Singh et al., 2009), diminuição nos níveis de gonadotrofinas e testosterona (Howland & Zerbrowski, 1976; Murray et al., 1981; Cameron et al., 1990; Sudha et al., 2000; Olivares et al., 2009) e disfunção sexual (Steger et al., 1989; Calvo et al., 1984). No homem diabético as alterações reprodutivas e sexuais são diminuição da libido, da espermatogênese, dos hormônios sexuais e principalmente impotência sexual (Kolodny et al., 1974; Fairburn, 1981). Análises histopatológicas de biópsia testicular de homens e ratos diabéticos revelaram um aumento na espessura da parede dos túbulos seminíferos, depleção de células germinativas e vacuolização das células de Sertoli (Cameron et al., 1985).

Em experimento anterior realizado em nosso laboratório, ratos machos adultos receberam única injeção intravenosa de *streptozotocin* (40 mg/Kg) e apresentaram quadro hiperglicêmico após uma semana. Nesse estudo, verificaram-se alterações reprodutivas importantes como

diminuição na produção espermática diária, oligospermia epididimária, alterações no comportamento sexual e diminuição da fertilidade (Scarano et al., 2006).

Outra conseqüência da hiperglicemia é o estresse oxidativo, que neste caso é ocasionado pelo aumento de ERMO e diminuição nos níveis de antioxidantes (enzimáticos e não enzimáticos). Sabe-se que a qualidade ideal do sêmen humano está diretamente associada à concentração satisfatória de antioxidantes e níveis baixos de oxidantes (ERMO) no organismo (Lewis et al., 1997; Eskenazi, 2005), e que o aumento do estresse oxidativo está associado à infertilidade no macho (Lewis et al., 1997; Agarwal et al., 2005). A presença de altos níveis de ERMO leva a peroxidação lipídica na membrana do espermatozóide, o que pode diminuir a motilidade, a capacidade de fusão com o ovócito II (Aitken, 1994), a capacitação (Aitken et al., 2004), alterar a morfologia e aumentar os danos na cromatina (Lamirande et al., 1997; Aitken & Baker, 2006; Aitken, 2006). Esta última alteração pode levar a mutações no embrião ou mesmo torná-lo inviável ocasionando aborto.

Assim, é crucial para o sistema reprodutor masculino estar bem protegido contra as injúrias do estresse oxidativo. Como meio de se proteger do estresse oxidativo, o espermatozóide apresenta antioxidantes como glutationa reductase, glutationa peroxidase, superoxide dismutase e catalase (Lamirande et al., 1997; Zubkova & Robaire, 2004). Contudo essa quantidade não é muito alta o que faz dessa célula ser mais susceptível a ação de ERMO. O epidídimo e a vesícula seminal também desempenham papéis importantes na defesa do gameta masculino contra o estresse oxidativo por remover as ERMO e secretar antioxidantes no lumen epididimário (Zubkova & Robaire, 2004) e no fluido seminal, respectivamente (Tramer et al., 1998).

É conhecido que as vitaminas C e E têm papéis importantes na espermatogênese, esteroidogênese, atuando como protetores contra o estresse oxidativo em ratos e homens (Cooper,

1987; Moger, 1987; Bensoussan et al., 1998; Hsu et al., 1998; Sönmez et al., 2005). A administração oral de vitamina C ou E em homens inférteis, por causa desconhecida, levou à redução na incidência da fragmentação da molécula de DNA em espermatozóides do ejaculado (Greco et al., 2005a; Greco et al., 2005b). Tem sido documentado na literatura científica que a suplementação com antioxidantes diminui os efeitos sistêmicos do diabetes (Garg & Bansal, 2000; Cay et al., 2001; Naziroğlu, 2003; Ozkan et al., 2005; Garg et al., 2005; Ramesh & Pugalendi, 2006; Yatoh et al., 2006). Contudo, o impacto que esse tratamento teria sobre os danos reprodutivos no sexo masculino causados pela hiperglicemia foram muito pouco estudados.

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, existe no homem um processo chamado de ejaculação o qual possibilita a expulsão dos gametas do trato reprodutor masculino para o meio externo de modo que durante um ato sexual estes possam entrar no sistema reprodutor feminino e, se em período de ovocitação, fecundar um ovócito II, culminando na perpetuação da espécie.

A ejaculação, usualmente acompanhada pelo orgasmo, é composta por duas fases distintas: a fase de emissão e a fase de expulsão do sêmen. Os órgãos reprodutores envolvidos com a fase de emissão são o epidídimo, ducto deferente, vesícula seminal, próstata, uretra prostática e a região do istmo da bexiga urinária. E, os órgãos que participam da fase de expulsão são istmo da bexiga urinária, uretra e musculatura estriada pélvica . Todas essas situações estão sob controle do sistema nervoso autônomo (Giuliano & Clément, 2005). A ejaculação é um

processo complexo estimulado por uma série de eventos os quais são dependentes de serotonina, dopamina, ocitocina, GABA, adrenalina, acetilcolina (Giuliano & Clément, 2005), testosterona, neuropeptídio Y, peptídio intestinal vasoativo e óxido nítrico (Ralph and Wylie, 2005; Giuliano & Clément, 2006). O reflexo ejaculatório é controlado por áreas cerebrais e medulares, as quais formam uma rede altamente interconectada. Os centros espinais simpáticos, parassimpáticos e somáticos, sobre a influência de estímulos cerebrais e sensoriais genitais integrados e processados na medula, atuam em sinergia comandando os eventos neurofisiológicos envolvidos na ejaculação (Giuliano & Clément, 2006).

O componente eferente do reflexo nervoso ejaculatório responsável pela fase de emissão do ejaculado consiste em fibras simpáticas do nervo hipogástrico, as quais liberam principalmente noradrenalina, causando contrações propulsivas do epidídimo, ducto deferente, vesícula seminal e próstata, propelindo assim o sêmen até a uretra posterior. Com a chegada do fluido seminal na uretra, propioceptores desencadeiam reflexos através de eferentes parassimpáticos do nervo pudendo, promovendo a contração do músculo bulbocavernoso e bulboesponjoso, culminando na ejaculação propriamente dita (Ralph & Wylie, 2005). Portanto, o sistema nervoso autônomo simpático tem papel fundamental na fase de emissão do reflexo ejaculatório, através da liberação de noradrenalina de suas terminações nervosas nos ductos deferentes e vesícula seminal.

Neuropatia hiperglicêmica: disfunção ejaculatória e estresse oxidativo

A neuropatia hiperglicêmica, ou neuropatia diabética, é uma das mais comuns complicações do diabetes afetando 50 - 60% dos pacientes diagnosticados, e é a causa mais comum de amputação não-traumática e disfunção do sistema nervoso autônomo (Figueroa-

Romero et al., 2008; Kumar et al., 2007). Essa patologia afeta várias funções do organismo incluindo o sistema reprodutor. Disfunção erétil e ejaculatória, e diminuição da fertilidade estão entre os danos causados pela neuropatia hiperglicêmica (Günes et al. 2005).

Vários estudos foram conduzidos com o objetivo de explicar os mecanismos pelos quais a neuropatia hiperglicêmica afeta a função ejaculatória. Relata-se que no ducto deferente de animais hiperglicêmicos há uma hipersensibilidade dos receptores α ao seu agonista noradrenalina podendo assim prejudicar a função ejaculatória do indivíduo (Peredo et al., 1984; Tomlinson et al., 1982; Günes et al., 2005). De modo semelhante Öztürk et al. (1994a) relataram que um período curto de hiperglicemia induzida por aloxana (72h) causou aumento da resposta α-adrenérgica enquanto que um período longo de hiperglicemia (8 semanas) diminui essa resposta em ductos deferentes. De modo semelhante, Kamata et al. (1998) utilizando *streptozotocin* como indutor de hiperglicemia em ratos, mostraram diminuição na resposta noradrenérgica do ducto deferente após dez semanas de tratamento. Longhurst (1990) mostrou que a diminuição nos níveis de testosterona circulante prejudica a resposta contráctil do ducto deferente em animais hiperglicêmicos. Também foi relatado que o aumento ou diminuição no influxo de cálcio pela membrana do neurônio assim como diminuição nos níveis de calmodulina também interfere com a resposta contráctil do ducto deferente, a qual pode prejudicar a função ejaculatória (Öztürk et al., 1994b). Diminuição nos níveis de insulina também é apontada como responsável por desordens ejaculatórias, porém reposição de insulina não restabelece os níveis de calmodulina (Öztürk & Aydin, 2006).

Várias evidencias clínicas e pré-clínicas sugerem que o estresse oxidativo tenha papel central no desenvolvimento de complicações hiperglicêmicas incluindo neuropatias (Kumar et al., 2007). É conhecido que os axônios, e por conseguinte o sistema nervoso são susceptíveis a

ação danosa de ERMO devido a grande quantidade de mitocôndrias que possuem para produção de energia. Com a ação de ERMO essas mitocôndrias tornam-se progressivamente não funcionais deixando os axônios sem energia, que por sua vez, leva a degeneração axonal (Figuero-Romero at al., 2008). Contudo a morte de axônios por apoptose devido a ação de estresse oxidativo ainda não está esclarecida.

OBJETIVO

Diante da relevância clínica do assunto e da falta de informações na literatura especializada, este trabalho de tese teve por objetivo avaliar se a suplementação com as vitaminas C e/ou E, ao reduzirem o estresse oxidativo, seriam capazes de amenizar os danos reprodutivos causados pela hiperglicemia analisando os biomarcadores do estresse oxidativo, parâmetros reprodutivos e espermáticos, níveis hormonais e resposta noradrenérgica do ducto deferente de ratos adultos.

ARTIGO 1. Artigo submetido para a revista International Journal of Andrology

The antioxidants vitamins C and E partially attenuate the sperm deficts in hyperglycemic rats

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Short title: Antioxidants and male reproductive disorders

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Abstract

Oxidative stress caused by hyperglycemia can lead to impairment of the male reproductive system. The objective of this work was to evaluate whether three treatments with antioxidants (vit. C and/or E) that reduce oxidative stress can ameliorate reproductive insults in hyperglycemic male rats. Adult male rats received a single dose of streptozotocin and were divided into four groups (n=10 per group): hyperglycemic (Hy); Hy + 150 mg of vitamin C (HyC); Hy + 100 mg of vitamin E (HyE); Hy + vitamins C and E (HyCE; at aforementioned doses). The normoglycemic group received the vehicles (water and corn oil). At the end the rats were anesthetized and sacrificed for evaluation of oxidative stress biomarkers (TBARS, SOD, GSHt and GSH-Px), body and reproductive-organ weights, sperm parameters, plasma hormone levels (FSH, LH and testosterone), testicular and epididymal morphometry and histopathology. Both vitamins reduced oxidative stress in relation to the hyperglycemic control group, but vitamin C was more efficient. Hyperglycemia reduced the body, testicular, epididymal, ventral prostate and seminal vesicle weights in relation to normoglycemic rats. The sperm counts and seminiferous tubular diameter in the testis, FHS level and the testicular and epididymal histopathology were similar among groups. Moreover, vitamin C and E reduced the number of malformed sperm in relation to the hyperglycemic control group, while vitamin C alone produced this effect more efficiently. The vitamin C-treated rats showed improvement of sperm motility, while the hyperglycemic groups presented diminished sperm number in cauda epididymidis compared to normoglycemic groups. Hyperglycemia caused a rearrangement in the epididymal tissue components as demonstrated by the stereological analyze, which was partially prevented by vitamin C alone. Based on this model we conclude that vitamins C and E acted as antioxidants, and partially attenuated the sperm deficits in hyperglycemic rats.

Keywords: oxidative stress, vitamin C, Vitamin E, male reproductive system, testosterone, sperm, rats.

Introduction

Factors such as diabetes, obesity, genetic predisposition and ageing may lead to hyperglycemia. Male sexual dysfunctions related to hyperglycemia have been described in animal models (Altay et al., 2003; Scarano et al., 2006) and in hyperglycemic humans (Baccetti et al., 2002), since alterations to the central nervous and endocrine systems may play important roles. In *streptozotocin*-induced hyperglycemic male rats, the reproductive changes include reduction of male organ and body weights (Scarano et al., 2006; Suthagar et al., 2009), oligospermia (Hassan et al., 1993; Scarano et al., 2006, Olivares et al., 2009), diminished fertility (Singh et al., 2009), decreased testosterone and gonadotrophin levels (Sudha et al., 2000; Olivares et al., 2009), Sertoli cell vacuolation (Muralidhara 2007), depletion of spermatogesis and other testicular damage (Cai et al., 2000; Guneli et al., 2008).

However the mechanisms of these alterations to the male reproduction system are still controversial and unclear, one hypothesis for this would be increased oxidative stress (Cay et al., 2001; Naziroğlu, 2003; Young et al., 2004; Suthagar et al., 2009), which is a usual consequence of hyperglycemia (Ozkan et al., 2005). The oxidative stress imbalance between oxidants, known as reactive oxygen species (ROS), and antioxidants, is extremely toxic to cells. This imbalance exerts its devastating effects either directly, by damaging cellular proteins, lipids and DNA, or indirectly, by affecting normal cellular signaling and gene regulation (Vincent et al., 2004; Pop-Busui et al., 2006). However, in physiological conditions the very low and controlled

concentrations of ROS participate in signal transduction mechanisms such as sperm capacitation and acrosome reaction (Lamirande el al., 1997).

The sperm cell presents high sensitivity to increased oxidative stress. Its plasma membrane is very susceptible to the action of oxidative stress which can lead to sperm lipid peroxidation (Agarwal et al., 2005), that consequently may lead to lost motility, decreased sperm-oocyte fusion capacity (Aitken, 1994), capacitation (Aitken et al., 2004) and altered morphology. Oxidative stress also can lead to increased sperm chromatin damage (Lamirande et al., 1997; Aitken & Baker, 2006; Aitken, 2006) which, in turn, can cause damage in the zygote since the sperm antioxidant defense system is impaired.

It has been reported that therapy with the antioxidant vitamins C and E decreases the oxidative stress in hyperglycemic subjects (Garg & Bansal, 2000; Cay et al., 2001; Naziroğlu, 2003; Ozkan et al., 2005; Garg et al., 2005; Yatoh et al., 2006). However, data on potential contributors to the reduction of oxidative stress in the male reproduction system are limited (Young et al., 2004; Muralidhara, 2007). There is no detailed study in the scientific literature describing its effects on the male reproductive system.

Vitamin E (α-tocopherol) is a lipid-soluble vitamin present in biological membranes that efficiently protects against lipid peroxidation and is very important for male reproductive system functions. Vitamin C (ascorbic acid) is an essential vitamin for humans. It is involved in several biochemical process of the organism and despite possessing hydrophilic characteristics, vitamin C can also interact with the plasma membrane by donating electrons to the α-tocopheroxyl radical, a trans-plasma membrane oxidoreductase activity thus recycling the α-tocopherol. There is a synergic interaction between vitamins C and E that helps to protect membrane lipids from peroxidation (Cay et al., 2001).

Therefore, because of the clinical relevance of this matter and the lack of pertinent information in the scientific literature, the present study aims to ascertain whether oxidative stress could be the one mechanism in hyperglycemia-induced damage to the male reproductive system. Thus, the present study verified whether treatment with the antioxidant vitamins C and E (alone or associated) could decrease oxidative stress, and prevent some of its effects in the reproductive system of hyperglycemic adult male rats.

Material and Methods

Animals

Adult male Wistar rats (90 days old) were supplied by the Multidisciplinary Center for Biological Investigation, State University of Campinas (CEMIB – UNICAMP) and housed in polypropylene cages (43 x 30 x 15 cm) with laboratory-grade pine shavings as bedding. Rats were kept under controlled temperature (23+/-1°C) and lighting conditions (12L, 12D photoperiod, lights switched on 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 Ethics Committee for Animal Experimentation (022/06-CEEA) at the Biosciences Institute / UNESP, campus at Botucatu, Sao Paulo, Brazil. In order to evaluate reproductive system endpoints the study was conducted in two steps, denominated Experiment 1 and Experiment 2, described as follows.

Experiment 1 – assessment of the reproductive organ weights, oxidative stress analysis, plasma sexual hormone determinations and sperm parameters.

Hyperglycemic model and experimental protocol

Hyperglycemia was chemically induced in adult male rats (n = 40) using a single dose of 40mg/Kg BW streptozotocin (SIGMA Chemical Company, St. Louis, MO) injected into the tail vein. Streptozotocin had been diluted in citrate buffer 0.01M, pH=4.6. After five days glucose levels of all animals were obtained by blood-glucose monitoring test strips in a specific glucometer (One Touch Ultra, Johnson&Johnson®) and all animals presenting levels higher than 300mg/dL were considered hyperglycemic. These animals were randomly assigned to four experimental groups of 10 animals each: hyperglycemic control (Hy), hyperglycemic + 150mg/day of vitamin C (HyC), hyperglycemic + 100mg/day of vitamin E (HyE) and hyperglycemic + 150mg/day of vitamins C + 100mg/day of E (HyCE). These doses were adapted from Naziroğlu (2003). Another animal group (n=10) received no streptozotocin (normoglycemic control group = N group) and presented glycemic levels lower than 120mg/dL. Hyperglycemic treated animals received the vitamins by gavage (oral route) throughout 30 consecutives days. Normoglycemic and hyperglycemic groups received only the vehicles (corn oil and water).

- Preparation of vitamins

Vitamin C (L-Ascorbic acid; SIGMA-ALDRICH, St. Louis, MO, USA) was prepared daily by diluting the required quantity in the corresponding volume of warm water. The vitamin E (α- Tocopherol; SIGMA-ALDRICH, St. Louis, MO, USA) was wholly diluted in corn oil before experimental period then used throughout the experiment. Both oil and vitamin E were heated to 54ºC to become a homogeneous mixture. This was stored at 20°C and checked for stability by the HPLC technique, once a week, at the Center for Metabolism in Exercise and Nutrition (CeMENutri), UNESP School of Medicine, campus at Botucatu, Sao Paulo state, Brazil. Both vitamins were stored in a dark container to protect against light.

Body weight and weight of some reproductive organs

At the end of the treatment, 9 or 10 rats from each experimental group were weighed, slightly anesthetized with 3% sodium pentobarbital (Hyptonol® - 0.1mL/Kg weight) and killed by decapitation. Blood was collected (between 9:00 and 11:30am) from the ruptured cervical vessels for analyses of sexual hormones and oxidative stress. The right testis and epididymis, ventral prostate and seminal vesicle (without the coagulating gland and full of secretion) were removed and weighed on an analytical balance. The relative and absolute weights were determined.

Oxidative stress status analysis

All of the biochemical procedures to determine antioxidant status were performed according to Ferreira et al. (1999). Part of the blood was collected in a heparinized tube (Liquemine, Hoffman-La Roche, Switzerland) for this analysis. Thiobarbituric acid reactive substances (TBARS) and antioxidant substances were estimated in washed erythrocytes. Lipid peroxides were evaluated using thiobarbituric acid (TBA). Briefly 1.0mL of washed erythrocytes was added to the test tube containing 1.0mL of 3.0% sulphosalicylic acid, shaken for 10 seconds, centrifuged at 11.000 rpm for 3 minutes and kept at rest for 15 min. The sample was diluted to 500µl of 0.67% TBA solution. The mixture was heated to 80ºC for 30 minutes and its absorbance was measured at 535 nm wavelength. Results were expressed as nM of TBARS per gram of hemoglobin (nM/g Hb), thus indirectly representing the lipid peroxidation index.

Superoxide dismutase (SOD) activity was determined from its ability to inhibit the auto-oxidation of pyrogallol. The reaction mixture (1.0mL) consisted of 5.0mM Tris (hydroxymethyl) aminomethane (pH 8.0), 1.0mM EDTA, bidistilled water and 20 µL of the sample. The reaction

was initiated by the addition of pyrogallol (final concentration of 0.2mM), and the absorbance was measured by a spectrophotometer at 420 nm wavelength (25ºC) for 5 minutes. Enzymatic activity unit was defined as the number of SOD units able to produce 50% inhibition of pyrogallol oxidation. All data were expressed as units of SOD per milligram of hemoglobin.

Glutathione total content (GSHt), which consists of reduced and oxidized glutathiones, was enzymatically determined using 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and glutathione reductase in the presence of a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), forming 2-nitro-5-thiobenzoic acid. A mixture consisting of 1290 µL of distilled water, 200 µL of Tris/HCl buffer (1M, pH8.0, 5mM EDTA), 200 µL of 10UI/mL glutathione reductase (GSH-r) (SIGMA, St. Louis, USA), 200 µL of 2 mM NADPH (SIGMA, St. Louis, USA) and 100µL of 12mM of DTNB (SIGMA, St. Louis, USA) was added to 10µL of the sample. Activity was measured at 412nm on a spectrophotometer. One unit of activity was equal to the micromolar of substrate reduced per gram of hemoglobin.

Glutathione peroxidase content (GSH-Px) was assessed by monitoring NADPH oxidation. The mixture consisted of the addition of 1,300 µL of distilled water, 200 µL of Tris/HCl buffer (EDTA 1M; pH 8.0; 5mM), 200 µL of 10 UI/mL of glutathione reductase (GSH-Rd), 200 µL of NADPH (2mM), 40 µL of GSH (0.1 M) to 40 µL of hemolysate. The mixture was shaken in a vortex mixer for 10 seconds. Next, 20 µL of T-butyl hydroperoxide (7mM) was added and maintained at 37ºC for 10 minutes. Absorbance was determined by a spectrophotometer at 340 nm wavelength. GSH-Px activity was expressed as enzymatic-activity units per gram of hemoglobin (UI/gHb).

Hormone Assays

Plasma testosterone, LH and FSH levels

The remainder of the blood was collected in a heparinized tube (Liquemine, Hoffman-La Roche, Switzerland) to determine plasma testosterone, 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. The analyses were determined by double-antibody radioimmunoassay at the Neuroendocrinology Laboratory, Dental School of University of São Paulo - USP campus at Ribeirão Preto, Sao Paulo state, Brazil. Plasma testosterone level was determined by the kit TESTOSTERONE MAIA® (Biochem Immuno System). The LH and FSH levels were quantified by specific kits from the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK, USA). All samples were measured in the same assay to avoid inter-assay errors. The lowest detection limits and intra-assay errors for FSH, LH and testosterone were respectively: 0.09, 0.04 and 0.064 ng/mL and 2.8, 3.4 and 4.0%.

Daily sperm production per testis, sperm number, sperm concentration 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 described by Fernandes et al. (2007): the right testis, decapsulated and weighed soon after collection, was homogenized in 5mL of NaCl 0.9% containing TritonX100 0.5%, followed by sonication for 30 s. After a 10-fold dilution a sample was transferred to Newbauer chambers (four fields per animal), preceding a count of mature spermatids. To calculate 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, right epididymidis portions (caput/corpus and cauda) were cut into small fragments with scissors and homogenized, and sperm counted as described for the testis. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by DSP.

Sperm morphology

For the analysis, sperm were removed from the right vas deferens and smears were prepared on histological slides that were left to dry for 90 min and observed in a phase-contrast microscope (400X total magnification) (Seed, 1996). One hundred spermatozoids were analyzed per animal. Morphological abnormalities were classified into two general categories: head abnormalities (without curvature, without characteristic curvature, or isolated form, i.e., no tail attached) and tail abnormalities (broken, isolated, i.e., no head attached, or rolled into a spiral (Filler, 1993).

Experiment 2 – Analysis of morphometry and histopathology (testis and epididymis) and sperm motility

Another 50 animals were randomly assigned to five groups (n=10/group) following the same experimental protocol described for Experiment 1.

Morphometric and histopathological analyses

The left testis and epididymis (five per experimental group) were removed and fixed in Alfac fixing solution (80% ethanol, formaldehyde and glacial acetic acid, 8.5:1.0:0.5 v/v) for 24 h. The pieces were embedded in paraffin wax and sectioned at 7 μm. The sections were stained

with hematoxylin and eosin (HE) and observed by light microscopy for general histopathological examination and morphometric analysis as follows:

- *Seminiferous tubule diameters*

Using an imaging analysis system (Leica Q-win Version 3 software for WindowsTM) and a Leica microscope DMLB (200X, total magnification), ten random image cross-sections of seminiferous tubules per animal in stage IX of spermatogenesis were captured. In each seminiferous tubule the mean of four measures was calculated and used in the statistical analysis.

- *Spermatogenesis stages*

Two hundred cross-sections of seminiferous tubules per animal (200 X, total magnification) were randomly analyzed in order to evaluate the relative frequency of stages I-VI, VII-VIII, IX-XIII and XIV of the germinal epithelium cycle (Ferreira et al., 1967).

- *Epididymal stereological analysis*

Using an imaging analysis system (Leica Q-win Version 3 software for WindowsTM) and a Leica microscope DMLB (200X, total magnification), random images of 50 histological fields per experimental group were captured and analyzed by the stereological method, such that histological fragments of all animals were evaluated equally (10/animal). This analysis was performed by means of Weibel's multipurpose graticulate, with 120 points and 60 test lines (Weibel, 1963) to compare the relative proportion among the epididymal components (epithelium, stroma and lumen) in the experimental groups.

Sperm motility

The sperm collected from the right vas deferens was immediately diluted in modified HTF medium with gentamicin (Human Tubal Fluid, IrvineScientific ®), warmed to 34 ° C. An aliquot

of 10 µl was placed in a Makler chamber (Irvine, Israel) and analyzed using light microscopy (400X). One hundred sperm were evaluated per animal and classified according to their motility into one of the following three categories: type A: mobile, with progressive movement; type B: mobile, with non progressive movement; type C: immobile.

Statistics

The variance among the experimental groups was compared by ANOVA, with the "a posteriori" Tukey test or the non-parametric Kruskall–Wallis test, with the "a posteriori" Dunn test, according to the dada distribution. Differences were considered significant when $p < 0.05$.

Results

In both experiments, five days after the hyperglycemia induction, all streptozotocin – treated animals showed glucose levels above 300mg/dL and were, therefore, considered hyperglycemic. The rats of the normoglycemic group showed normal glucose levels, below 300 mg/dL. These glycemic states were maintained throughout the experimental period (Table 1). In addition, all streptozotocin-treated animals exhibited characteristic qualitative signs of hyperglycemia such as polyphagia, polydipsia and polyuria (data not shown).

In relation to oxidative stress analysis (Table 2) the lipidic peroxidation level (TBARS) was significantly increased in the hyperglycemic group in relation to the other groups. The treatments with vitamin C and E, isolated or in combination, were able to significantly reduce this parameter in relation to the hyperglycemic group. However, TBARS levels were comparable to the normoglycemic group only in the vitamin C treated group.

Similarly, the hyperglycemic control group showed significantly higher SOD activity than the other groups. However, this enzymatic activity was significantly recovered by vitamins treated in the hyperglycemic rats. On the other hand, GSHt concentrations were similar between hyperglycemic control and normoglycemic groups. Moreover, the GSHt concentrations were significantly increased in the vitamins-treated hyperglycemic groups in relation to the other groups Nevertheless, there was a significantly decrease of the GSH-Px activity in the hyperglycemic control group in relation to other groups. However, the activity of this enzyme was maintained in the vitamins-treated hyperglycemic groups which was similar to normoglycemic group.

Body and reproductive organ weights are shown in Table 1. Streptozotocin-induced hyperglycemia produced significant decreases in the final body, testicular, prostate and full seminal vesicle weights compared to normoglycemic group. However, the relative epididymal weight was similar among all groups and relative weight of ventral prostate was partially recovered in the vitamin C-treated group.

In relation to hormonal assays (Table 3), the FSH levels were similar among all experimental groups. There was a significant reduction in the plasma testosterone level in hyperglycemic control (73%) and vitamin E-treated hyperglycemic (85%) groups compared to the normoglycemic group. However, in vitamin C-treated groups (alone or in association) the androgen levels were partially recovered. The LH level was significantly lower in the group treated with the association of vitamin C and E than the other groups, which did not vary among themselves.

The daily sperm production, frequency of spermatogenesis stages and semineferous tubular diameters assay did not differ among experimental groups (data not showed). In addition,

epididymal sperm counts showed that the absolute number of sperm in the caput/corpus epididymis and sperm transit time (Table 4) did not differ among experimental groups. Regarding the sperm counts in the cauda epididymis (Table 4), absolute sperm numbers were significantly less in all hyperglycemic groups in relation to normoglycemic rats. Despite the acceleration in the sperm transit time in this region, the results were not statistically different.

Sperm morphology analyses (Table 5) showed that the percentage of abnormally shaped sperm was higher in all hyperglycemic groups (Hy=35%, HyC=23%, HyE=30%, HyCE=32%) compared to the normoglycemic group (9%). However, vitamin C alone was able to partially recover this parameter. The number of sperm presenting head without curvature was totally recovered by the treatment with both vitamins, alone or associated. However, the number of sperm with isolated head was only partially recovered in the vitamin C treated group. The other treatments failed to reverse this abnormality. In relation the sperm number with isolated tail, vitamin C and E, isolated, partially recovered this alteration.

The sperm motility assay results, shown in Table 6, reveal reduced Type A sperm (mobile, with progressive movement) in hyperglycemic groups compared to normoglycemic group, which were partially recovered by the treatment with vitamin C alone. The Type B sperm (mobile, with non progressive movement) did not differ significantly among the experimental groups. In addition, Type C sperm increased significantly in the hyperglycemic groups compared to normoglycemic group, which was also partially recovered by vitamin C.

The histopathology of the testis and epididymis (data not shown) was unaffected by hyperglycemia or hyperglycemia plus vitamins (alone or associated). In the testis, the spermatogenic cells, Sertolli cell and the arrangement of the seminiferous epithelium were structurally normal among the experimental groups. In addition, the Leydig cells and interstitial

connective tissue appeared to be uniform in size and shape among all experimental groups. In the epididymis, the stromal and ephithelial tissues appeared to be normal, as was the lumen which showed only sperm.

Nevertheless, the epididymal stereological analysis (Table 7) showed that there was a significant increase of epididymal stroma compartimental in animals from the hyperglycemic groups compared to normoglycemic except by the vitamin C group, in which there was a complete recovery. The epididymal duct epithelium was significantly higher in all hyperglycemic groups than in normoglycemic animals. This analysis also revealed a significant decrease in the epididymal lumen compartimental in the hyperglycemic group compared to normoglycemic group, but was also totally recovered by the administration of vitamin C alone.

Discussion

Little is known about the relationship between oxidative stress and dysfunction in the male reproductive tract of hyperglycemic rats. The results of the present study demonstrated that oral administration of vitamins C and E (alone or in association) produced a potent antioxidant effect that significantly reduced the oxidative stress level caused by hyperglycemia. Similarly, Cay et al. (2001), Baydas et al. (2002) and Naziroğlu (2003) reported a reduction of oxidative stress after treatment with vitamin C or E in *streptozotocin*-induced hyperglycemic rats. The assay employed in the present study showed that the significant increase of TBARS in the hyperglycemic group was significantly reduced by vitamin treatment, expressed by a reduction of lipid peroxidation in these groups. Thus, it was noted that vitamins C and E improved the antioxidant defenses of the organism against reactive oxygen species (ROS). But contrary to what was expected, the association of vitamins C plus E did not provide greater efficiency in reducing

lipid peroxidation. Notably, the treatment with vitamin C alone was slightly more efficient since the lipid peroxidation level of this group was equal to that of the normoglycemic group. Thus, vitamin C alone could promote better health in hyperglycemic subjects. In addition, the increases in GSHt and GSH-Px activity, promoted by vitamins, diminished the TBARS levels in these groups. On the other hand, in the hyperglycemic group without vitamins GSH and GSH-Px were found to impair antioxidant defense, which is characteristic of hyperglycemia, as previously reported (Cay et al., 2001; Naziroğlu, 2003). Interestingly, the SOD activity level in the hyperglycemic group was significantly higher than in the other groups, probably due to the body's attempt to defend itself against attack from ROS.

Reductions of body, testicular, epididymal and prostate weights have long been recognized in hyperglycemic animals (Hassan et al., 1993; Altay et al., 2003; Scarano et al., 2006; Muralidhara, 2007). In the present study, the decreased body and reproductive organ weights can be associated with hyperglycemic metabolic alterations such as decreased testosterone levels and absence or attenuation of insulin, but not with the reduction of oxidative stress. As reported by Naziroğlu (2003), vitamins C and E reduce oxidative stress, but do not prevent the loss of body weight in hyperglycemic rats. Under hyperglycemic conditions the insulin replacements restore body weight and reproductive organ weights, except that of the prostate, which can be restored by testosterone therapy (Seethalakshmi et al., 1987). However, Suthagar et al. (2009) reported that insulin replacement resulted in partial maintenance of body and ventral prostate weights in *streptozotocin*-induced hyperglycemic rats.

In this sense, the HyC and HyCE groups showed partial recovery in testosterone level, which did not prevent the loss of ventral prostate absolute weight. But, in the HyC group the ventral prostate relative weight was partially restored, probably due to changes in the body

weights in different experimental groups, and not to testosterone level. However, it is known that vitamin C (Biswas et al., 1996; Sönmez et al., 2005) and vitamin E (Chen et al., 2005) play key roles in the synthesis of testosterone. However, in the present study, this did not occur in the vitamin E-treated group, which indicates disturb in testosterone synthesis, in hyperglycemic conditions, may be related not only to increased oxidative stress, but also to other factors of hyperglycemia such as reduction of insulin level and impairment of cell signal transduction.

Surprisingly, the LH reduction in the HyCE group did not interfere with testosterone synthesis. Although we cannot explain this result, it is interesting to observe that vitamins C and E did not alter LH synthesis in rats (Uzun et al., 2009) and that ascorbic acid can be a vitaminergic transmitter that activates the release of both FSH and LH from the anterior pituitary gland (Karanth et al., 2001). In this sense, vitamin E stimulates the release of luteinizing hormone-releasing hormone (LHRH) (Karanth et al., 2003). In addition, in the present study, the unchanged LH level differs from previous studies that reported diminished LH release in hyperglycemic male rats (Steger et al., 1989, Olivares et al., 2009).

The FSH levels found in the present study corroborate data on testicular sperm counts and spermatogenesis stage frequencies, despite the testosterone reductions (significant or partially significant) in the hyperglycemic groups. However, Abou-Seif & Youssef (2001) reported FSH diminution in hyperglycemic rats.

Moreover, despite changes in testicular weights and testosterone levels, in the hyperglycemic groups, the daily sperm production and seminiferous tubular diameter were not altered. These results are corroborated by Ballester et al. (2004) reporting similarity between the seminiferous tubular diameters in hyperglycemic and normoglycemic rats. In contrast, Hassan et al. (1993) and Scarano et al. (2006) related the diminished sperm number in the testis in diabetic

rats to the decrease in testosterone level. Some other authors found reduction of seminiferous tubular diameter as a consequence of high glucose and low insulin levels in the blood (Cai et al., 2000; Altay et al., 2003; Muralidhara, 2007; Guneli et al., 2008).

In relation to the epididymis, the decrease in the absolute weight caused by hyperglycemia was related to a significant decrease in sperm number in the cauda epididymidis and apparent increase in the sperm transit time, and rearrangement of the tissue components as demonstrated by the stereological analyze. Furthermore, Soundamani et al. (2005) reported that *streptozotocin*-hyperglycemia induced on prepubertal rats (40 days old), caused in puberty (61st day of postnatal life) epididymal regression, leading to a decrease in the absolute weights of caput, corpus, and caudal regions, while histological studies also revealed a considerable reduction in the sizes of the tubule and lumen of these segments with an increase in interstitial stroma. However, in the present study, this regression in epididymal tissue was attenuated in the HyC group as shown by epididymal stereological analysis. This probably occurred since vitamin C may have acted in the synthesis of extracellular matrix components (Duarte & Lunec, 2005; Li & Schelldorn, 2007), although high glucose concentration inhibits the action of vitamin C on collagen and proteoglycans synthesis (Fischer, 1991). This finding may be due to the antioxidant effect of vitamin C alone, since it was more efficient at reducing lipid peroxidation than the other vitamin-treated groups, as evidenced by TBARS levels.

The lack of morphological alteration in the testis and epididymis observed in hyperglycemic animals corroborated a previous study in our laboratory (Scarano et al., 2006). However, it is known that hyperglycemic rats can present testicular and epididymal morphological changes such as the presence of germ cells in seminiferous tubule lumen in spontaneous (Wringht et al. 1982) or streptozotocin-induced (Muralidhara, 2007) diabetes. Sertoli

cell vacuolation (Muralidhara (2007), germ cells degeneration (Guneli et al., 2008) and presence of immature and degenerated germ cells in epididymal lumen of streptozotocin-induced hyperglycemic rats (Cai et al., 2000) were also reported.

In the current study, we can confirm that oxidative stress is directly related to the spermiogenesis process, whereas the sperm morphological abnormalities caused by hyperglycemia were prevented by vitamins. Interestingly, vitamin C was slightly more efficient at producing this preventive effect perhaps due to the lower lipid peroxidation level in this group, in relation to other vitamin-treated groups. In contrast, Scarano et al. (2006) found no damage to sperm morphology parameters of hyperglycemic adult rats after 15 days *streptozotocin*-induced hyperglycemia procedure.

Furthermore, the sperm motility analysis in the present study showed that as previously reported (Hassan et al., 1993; Singh et al., 2009), there was a decrease in sperm motility of hyperglycemic animals. However, only vitamin C, when administered in isolation, partially prevents the damage that can impair sperm motility in hyperglycemic conditions. In the scientific literature, it is known that vitamin C reduces oxidative stress and increases gamete mobility (Hsu et al., 1998). Although this phenomenon is also true vitamin E (Hsu et al., 1998; Latchoumycandane et a., 2002; Sönmez et al., 2007), it inexplicably did not occur in the present study. In this sense, Donnelly et al. (1999) showed that supplementation of prepared media with ascorbate and alpha-tocopherol, either singly or in combination, reduced oxidative stress, but was not beneficial to sperm motility in human semen. Thus, in the present study, oxidative stress may not be primarily responsible for damage to sperm motility.

In conclusion, the present study showed that oxidative stress is not solely responsible for the disorders in the male reproductive system of hyperglycemic rats. Moreover, vitamin C

showed greater effectiveness than vitamin E in reducing oxidative stress and minimizing alterations in the male reproductive system. Furthermore, we found an apparent lack of synergism between vitamins C and E, suggesting that, depending on the experimental design, non-enzymatic exogenous anti-oxidants, such as vitamins, may be more efficient if used separately.

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References

Abou-Seif, M. A. & Youssef, A. A. (2001) Oxidative stress and male IGF-1, gonadotropin and related hormones in diabetic patients. Clinical Chemistry and Laboratory Medicine, 39(7), 618-623.

Agarwal, A., Prabakaran, S. A. & Said T.M. (2005) Prevention of oxidative stress injury to sperm. *Journal of Andrology,* 26(6), 654-660.

Aitken, R. J. (1994) A free radical theory of male infertility. Reproduction, fertility, and development, 6(1), 19-23.

Aitken, R. J. & Baker, M. A. (2006) Oxidative stress, sperm survival and fertility control. Molecular and Cellular Endocrinology, 250(1-2), 66-69.

Aitken, R. J., Ryan, A. L., Baker, M. A. & McLaughlin, E.A. (2004) Redox activity associated with the maturation and capacitation of mammalian spermatozoa. Free radical biology & medicine, 82, 1684-1686.

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

Baccetti, B., La, 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.

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.

Baydas, G., Canatan, H. & Turkoglu, A. (2002) Comparative analysis of the protective effects of melatonin and vitamin E on streptozocin-induced diabetes mellitus. Journal of Pineal Research, 32, 225-230.

Biswas, N. M., Chaudhuri, A., Sarkar, M. & Biswas R. (1996) Effect of ascorbic acid on *in vitro* synthesis of testosterone in rat testis. Indian Journal of Experimental Biology, 43, 612-613.

Cai, L., Chen, S., Evans, T., Deng, D. X., Mukherjee, K. & Chakrabarti, S. (2000). Apoptotic germ-cell death and testicular damage in experimental diabetes: prevention by endothelin antagonism. Urological Research, 28, 342-347.

Cay, M., Naziroğlu, M., Simsek, H., Aydilek, N., Aksakal, M. & Demirci, M. (2001) Effects of intraperitoneally administered vitamin C on antioxidative defense mechanism in rats with diabetes induced by streptozotocin. Research in Experimental Medicine (Berl), 200 (3), 205-213.

Chen, H., Liu, J., Luo, L., Baig, M. U., Kim, J-M. & Zirkin B.R. (2005) Vitamin E, aging and Leydig cell steroidogenesis. Experimental Gerontology, 40, 728-738.

Donnelly, E. T., McClure, N. & Lewis, S. E. M. (1999) Antioxidant supplementation in vitro does not improve human sperm motility. *Fertility and Sterility*, 72 (3), 484-495.

Duarte, T.L. & Lunec, J. (2005) When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Radical Research, 39(7), 671-686.

Fernandes, G. S. A., Arena, A. C., Fernandez, C. D., Mercadante, A., Barbisan, L. F. & Kempinas, W. G. (2007) Reproductive effects in male rats exposed to diuron. Reproductive Toxicology, 23(1), 106-112.

Ferreira, A. L. A., Machado, P. E. A. & Matsubara, L. S. (1999) Lipid peroxidation, antioxidant enzymes and glutathione levels in human erythrocytes exposed to colloidal iron hydroxide in vitro. Brazilian Journal of Medical and Biological Research, 32(6), 689-694.

Ferreira, A. L., Lison, L. & Valeri, V. (1967) Caryometric study of spermatogenesis in the rat. Zeitschrift fur Zellforschung und mikroskopische Anatomie 76, 31-55.

Filler, R. (1993) Methods for evaluation of rats epididymal sperm morphology, in: Male reproductive toxicology (eds. R. E. Chapin and J. H. Heindel), pp. 334-43. Academic Press, Inc., San Diego, California.

Fisher, E., McLennan, S. V., Tada, H., Heffernan, S., Yue, D. K. & Turtle, J. R. (1991) Interaction of ascorbic acid and glucose on production of collagen and proteoglycan by fibroblasts. Endocrine Research, 31(2), 81-98.

Garg, M. C. & Bansal, D. D. (2000) Protective antioxidant effect of vitamins C and E in streptozotocin induced diabetic rats. Indian Journal of Experimental Biology, 38(2), 101-104.

Garg, M. C., Chaudhary, D. P. & Bansal D. D. (2005) Effect of vitamin E supplementation on diabetes induced oxidative stress in experimental diabetes in rats. Indian Journal of Experimental Biology, 43(2); 177-180.

Guneli, E., Tugyan, K., Ozturk, H., Gumustekin, M., Cilaker, S. & Uysal N. (2008) Effect of melatonin on testicular damage in streptozotocin-induced diabetes rats. European surgical research, 40, 354-360.

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.

Hsu, P-C., Liu, M-Y., Hsu, C-C., Chen, L-Y & Guo Y-L. (1998) Effects of vitamin E and/or C on reactive oxygen species-related lead toixicity in the rat sperm. Toxicology 128, 169-179.

Karanth, S., Yu, W. E., Walczewska, A., Mastronardi C. A. & McCann, M. (2001) Ascorbic acid stimulates gonadotropin release by autocrine action means of NO. Proceedings of the National Academy of Sciences of the United States of America, 98(20), 11783-11788.

Karanth, S., Yu, W. H., Mastronardi, C. A. & McCann, S. M. (2003) Vitamin E stimulates luteinizing hormone-releasing hormone and ascorbic acid release from medial basal hypothalami of adult male rats.

Lamirande, E., Jiang, H., Armand, Z., Kodama, H. & Gagnon, C. (1997) Reactive oxygen species and sperm physiology. Reviews of Reproduction, 2, 48-54.

Latchoumycandane, C., Chitra, K. C. & Mathur, P. P. (2002) The effect of methoxychlor on the epididymal antioxidant system of adult rats. Reproduction Toxicology 16(2), 161-172.

Li, Y. & Schellhorn, H. E. (2007) New developments and novel therapeutic perspectives for vitamin C. The Journal of Nutrition, 137, 2171-2184.

Muralidhara, S. B. (2007) Occurrence of oxidative impairments, response of antioxidant defences and associated biochemical perturbations in male reproductive milieu in the Streptozotocin-diabetic rat. Internatinal Journal of Andrology 30(6), 508-518.

Murray, F. T., Orth, J., Gunsalus, G., Weisz, J., Li, J. B., Jeferson, L. S., Musto, N. A. & Bardin C.W. (1981) The pituitary-testicular axis in the streptozotocin diabetic male rat: Evidence for gonadotroph, Sertoli cell and Leydig cell dysfunction. Internatinal Journal of Andrology, 4; 265-280.

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.

Olivares, A., Méndez, J. P., Cárdenas, M., Oviedo, N., Palomino, M. A., Santos, I., Perera-Marín, G., Gutiérrez-Sagal, R. & Ulloa-Aguirre, A. (2009) Pituitary-testicular axis function, biological to immunological ratio and charge isoform distribution of pituitary LH in male rats with experimental diabetes. General and comparative endocrinology, 161(3), 304-312.

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.

Pop-Busui, R., Sima, A. & Stevens, M. (2006) Diabetic neuropathy and oxidative stress. Diabetes/Metabolism Research and Reviews, 22(4), 257-273.

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

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. Internatinal Journal of Andrology, 29(4); 482-488.

Seed, J., Chapi, R. E., Clegg, E. D., Dostal, L. A., Foote, R. E., Hurtt, M. E., Klinefelter, G. R., Makris, S. L., Perreault, S. D., Schrader, S., Seyler, D., Sprando, R., Treinen, K. A., Veeramachaneni, D. N. & Wise, L. D. (1996) Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. Reproduction Toxicology, 10, 237-244.

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.

Sigh, S., Malini, T., Rengarajan, S., Balasubramanian, K. (2009) Impact of experimental diabetes and insulin replacement on epididymal secretory products and sperm maturation in albino rats. Journal of Cellular Biochemistry - doi: 10.1002/jcb.22337.

Sönmez, M., Türk, G. & Yüce, A. (2005) The effects of ascorbic acid supplementation on sperm quality, lipid proxidation and testosterone levels of male Wistar rats. Theriogenology 63(7), 2063-2072.

Sönmez, M., Yüce, A. & Türk, G. (2007) The protective effects of melatonin and Vitamin E on antioxidant enzyme activities and epididymal sperm characteristics of homocysteine treated male rats. Reproductive toxicology, 23(2), 226-231.

Soundamani, S., Malini, T. & Balasubramanian, K. (2005) Effects of streptozotocin-diabetes and insulin replacement on the epididymis of prepubertal rats: histological and histomorphometric studies. Endocrine Research, 31(2), 81-98.

Steger, R. W., Amador, A., Lam, E., Rathert, J., Weis, J. & Smith, M. S. (1989) Streptozotocin-induced deficits in sex behavior and neuroendocrine function in male rats. Endocrinology, 124, 1737-1743.

Sudha, S., Valli, G., Julie, P. M., Arunakaran. J., Govindarajulu, P. & Balasubramanian, K. (2000) Influence of streptozotocin-induced diabetes and insulin treatment on the pituitary-testicular axis during sexual maturation in rats. Experimental and clinical endocrinology & diabetes, 8(1), 14-20.

Suthagar, E., Soudamani, S., Yuvaraj, S., Ismail Khan, A., Aruldhas, M. M. & Balasubramanian, K. (2009) Effects of streptozotocin (STZ)-induced diabetes and insulin replacement on rat ventral prostate. Biomedicine & Pharmacotherapy, 63(1), 43-50.

Uzun, F. G., Kalender, S., Durak, D., Demir, F. & Kalender, Y. (2009) Malathion-induced testicular toxicity in male rats and the protective effect of vitamins C and E. Food and Chemical Toxicology, 47(8), 1903-1908.

Vincent, A. M., Russell, J. W., Low, P. & Feldman, E. L. (2004) Oxidative stress in the pathogenesis of diabetic neuropathy. Endocrine Reviews, 25(4), 612-628.

Weibel, E. R. (1963) Principles and methods for the morphometric study of the lung and other organs. Laboratory Investigation, 12, 131-155.

Wright, J. R. Jr., Yates, A. J., Sharma, H. M., Shim, C., Tigner, R. L. & Thibert, P. (1982) Testicular atrophy in the spontaneously diabetic BB Wistar rat. The American journal of pathology, 108(1), 72-79.

Yatoh, S., Mizutani, M., Yokoo, T., Kozawa, T., Sone, H., Toyoshima, H., Suzuki, S., Shimano, H., Kawakami, Y., Okuda, Y. & Yamada, N. (2006) Antioxidants and an inhibitor of advanced glycation ameliorate death of retinal microvascular cells in diabetic retinopathy. Diabetes/metabolism research and reviews, 22(1), 38-45.

Young, L., Yu, D., Bateman, R. & Brock, G. (2004) Oxidative stress and antioxidant therapy: their impact in diabetes-associated erectile dysfuction. Journal of Andrology, 25, 830-836.

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 Reproduction, 71(3), 1002-1008.

Table 1: Body and organ (absolute and relative) weights

	Normoglycemic (n = 10)	Hyperglycemic (n = 10)	Hyperglycemic + vitamin C (n = 09)	Hyperglycemic + vitamin E (n = 10)	Hyperglycemic + vitamins C and E (n = 10)
Initial BW (g)	366.00 ± 7.75	365.00 ± 5.80	366.80 ± 11.26	367.00 ± 8.24	366.00 ± 6.68
Final BW (g)	417.40 ± 9.02 [a]	303.40 ± 9.80 [b]	303.44 ± 7.27 [b]	298.40 ± 10.06 [b]	309.30± 9.00 [b]
Testis (g)	1.80 ± 0.05 [a]	1.64 ± 0.05 [b]	1.63 ± 0.06 [b]	1.53 ± 0.04 [b]	1.69 ± 0.04 [b]
Testis (g/100g)	0.43 ± 0.01 [a]	0.54 ± 0.01 [b]	0.54 ± 0.01[b]	0.52 ± 0.04 [b]	0.55 ± 0.05 [b]
Epididymis (mg)	569.84 ± 13.33 [a]	417.80 ± 26.37 [b]	436.11 ± 20.90 [b]	390,34 ± 15.73 [b]	441.15 ± 15.43 [b]
Epididymis (mg/100g)	137.00 ± 3.70	137.00 ± 6.11	143.32 ± 4.60	131.00 ± 3.13	143.09 ± 4.50
Seminal vesicle full (g)	1.37 ± 0.07 [a]	0.54 ± 0.12 [b]	0.58 ± 0.10 [b]	0.41 ± 0.05 [b]	0.49 ± 0.06 [b]
Seminal vesicle full (g/100g)	0.32 ± 0.02 [a]	0.14 ± 0.02 [b]	0.15 ± 0.04 [b]	0.13 ± 0.05 [b]	0.15 ± 0.02 [b]
Ventral prostate (mg)	418.91 ± 18.50 [a]	198.87 ± 19.62 [b]	239.38 ± 26.64 [b]	212.38 ± 17.00 [b]	234.75 ± 22.62 [b]
Ventral prostate (mg/100g)	97.61 ± 4.80 [a]	64.82 ± 5.65 [b]	77.81 ± 7.31 [ab]	70.67 ± 3.63 [b]	71.80 ± 7.01 [b]
Glycemia after 5 days from induction (mg/dL)	94.00 ± 3.33 [a]	401.60 ± 20.03 [b]	386.00 ± 18.30 [b]	378.70 ± 21.66 [b]	365.3 ± 10.35 [b]
Final Glycemia (mg/dL)	93.00 ± 2.12 [a]	555.40 ± 11.20 [b]	543.11 ± 17.56 [b]	562.40 ± 15.00 [b]	573.60 ± 10.25 [b]

Values expressed as Mean±SEM. [a,b] indicate statistically different results ($p<0.05$). ANOVA test, with the Tukey "a posteriori" test.

Table 2: Blood biomarkers of oxidative stress

Groups	TBARS (nM/gHb)	SOD (UI/mgHb)	GSHt (µM/gHb)	GSH-Px (UI/gHb)
Normoglycemic (n = 10)	39.04 [32.82 – 52.82][a]	1842.80 [1434.73 – 3481.02][a]	0.05 [0.04 – 0.07] [a]	0.33 [0.28 – 0.65] [a]
Hyperglycemic (n = 10)	456.78 [391.30 – 630.00][b]	3929.30 [3687.09 – 5044.50] [b]	0.08 [0.04 – 0.12] [a]	0.02 [0.01 – 0.09] [b]
Hyperglycemic + vitamin C (n = 09)	96.60 [75.02 – 132.20][ac]	2000.00 [1603.20 – 2257.96][a]	0.29 [0.23 – 0.33] [b]	0.13 [0.13 – 0.16] [a]
Hyperglycemic + vitamin E (n = 08)	120.40 [105.71 – 148.01][c]	2406.40 [1931.06 – 2678.19][ab]	0.31 [0.26 – 0.37] [b]	0.14 [0.12 – 0.17] [a]
Hyperglycemic + vitamins C and E (n = 09)	128.43 [105.51 – 142.20][c]	1728.50 [1599.80 – 2160.63][a]	0.20 [0.19 – 0.24] [b]	0.15 [0.13 – 0.16] [a]

Values expressed as median [Q_1 – Q_3]. [a,b,c] Different letters indicate groups that differ statistically ($p<0.05$). Kruskal - wallis test, with the Dunn "a posteriori" test.

Table 3: Hormone assay

Groups	Testosterone (ng/mL)	LH (ng/mL)	FSH (ng/mL)
Normoglycemic (n = 10)	1.87 ± 0.25 [a]	7.90 ± 1.50 [a]	7.00 ± 0.74
Hyperglycemic (n = 10)	0.51 ± 0.16 [b]	4.06 ± 1.40 [a]	6.80 ± 1.40
Hyperglycemic + vitamin C (n = 09)	0.81 ± 0.34 [ab]	3.64 ± 0.66 [a]	7.00 ± 0.45
Hyperglycemic + vitamin E (n = 09)	0.28 ± 0.05 [b]	4.60 ± 0.71 [a]	4.80 ± 0.56
Hyperglycemic + vitamins C and E (n = 09)	0.84 ± 0.48 [ab]	2.78 ± 0.35 [b]	5.54 ± 0.71

Values expressed as Mean±SEM. [a,b] indicate statistically different results ($p<0.05$). Kruskall-Wallis test, with the Dunn "a posteriori" test.

Table 4: Sperm parameters in the caput/corpus and cauda epididymis.

	Experimental groups				
	Normoglycemic (n = 10)	**Hyperglycemic (n = 10)**	**Hyperglycemic + vitamin C (n = 09)**	**Hyperglycemic + vitamin E (n = 09)**	**Hyperglycemic + vitamins C and E (n = 09)**
Caput/ corpus sperm number (x10^6)	59.93 ± 5.11	43.44 ± 7.00	67.11 ± 4.40	67.11 ± 7.08	56.00 ± 5.00
Caput/ corpus sperm transit time (days)	2.14 ± 0.21	1.67 ± 0.34	2.64 ± 0.31	2.61 ± 0.30	2.47 ± 0.46
Cauda sperm number (x10^6)	106.87 ± 12.00 [a]	51.03 ± 8.70 [b]	58.34 ± 6.68 [b]	56.88 ± 4.55 [b]	67.15 ± 7.30 [b]
Cauda sperm transit time (days)	3.85 ± 0.53	2.42 ± 0.59	2.20 ± 0.23	2.24 ± 0.21	3.03 ± 0.50

Values expressed as mean ± SEM. [a,b] Different letters indicate groups that differ statistically ($p<0.05$). ANOVA test, with the Tukey test "a posteriori" test.

Table 5: Sperm morphology

Groups	Head isolated	Head without curvature	Tail isolated	Normal sperm
Normoglycemic (n = 10)	4.70 ± 0.52 [a]	1.4 ± 0.30 [a]	2.30 ± 0.65 [a]	91.30 ± 1.03 [a]
Hyperglycemic (n = 10)	20.00 ± 3.60 [b]	3.8 ± 0.73 [b]	11.00 ± 3.06 [b]	65.30 ± 5.05 [b]
Hyperglycemic + vitamin C (n = 09)	14.00 ± 2.60 [ab]	1.30 ± 0.36 [a]	7.71 ± 1.15 [ab]	77.30 ± 2.24 [ab]
Hyperglycemic + vitamin E (n = 09)	21.5 ± 3.14 [b]	1.62 ± 0.42 [a]	6.12 ± 2.00 [ab]	70.75 ± 4.60 [b]
Hyperglycemic + vitamins C and E (n = 09)	20.20 ± 4.90 [b]	0.8 ± 0.33 [a]	10.4 ± 2.22 [a]	68.60 ± 6.00 [b]

Values expressed as mean ± SEM. [a,b] Different letters indicate groups that differ statistically ($p<0.05$). ANOVA test, with the Tukey "a posteriori" test.

Table 6: Sperm motility

Groups	Type A sperm	Type B sperm	Type C sperm
Normoglycemic (n = 10)	68.4 [64.25 – 69.75] [a]	8.5 [7 – 12.5]	22.1 [19.25 – 24.5] [a]
Hyperglycemic (n = 09)	36.2 [28 – 46] [b]	6.0 [4 – 12]	56.3 [46 – 65] [b]
Hyperglycemic + vitamin C (n = 09)	35 [17.25 – 51.75] [ab]	9.0 [5.75 – 25.5]	47.5 [38.75 – 50.25] [ab]
Hyperglycemic + vitamin E (n = 09)	31 [28 – 38.5] [b]	10 [8.5 – 13.5]	55.25 [50.75 – 62.25] [b]
Hyperglycemic + vitamins C and E (n = 09)	13 [2 – 17] [b]	11 [6 – 26]	70 [56 – 79] [b]

Values expressed as median [Q_1 – Q_3]. [a,b] Different letters indicate groups that differ statistically ($p<0.05$). Kruskal – Wallis test, with the Dunn "a posteriori" test.

Table 7: Epididymal stereological analysis (region 6A = proximal cauda) (n=50 histological fields / group)

	Experimental groups				
	Normoglycemic	**Hyperglycemic**	**Hyperglycemic + Vitamin C**	**Hyperglycemic + Vitamin E**	**Hyperglycemic + Vitamins C and E**
Epididymis (Cauda – region 6A)					
Stroma	15.75 [10.71 – 22.17] [a]	23.51 [20.39 – 26.80] [b]	14.30 [11.01 – 20.10] [a]	23.81 [16.67 – 31.70] [b]	28.28 [19.20 – 29.76] [b]
Epithelium	14.28 [11.16 – 17.85] [a]	20.24 [16.82 – 22.02] [b]	19.64 [17.41 – 23.00] [b]	19.64 [17.26 – 23.81] [b]	22.02 [19.78 – 26.20] [b]
Lumen	67.86 [64.29 – 75.16] [a]	56.25 [48.51 – 62.65] [b]	63.10 [58.63 – 70.24] [a]	54.46 [48.66 – 62.65] [b]	51.20 [45.54 – 58.63] [b]

Values expressed as Median ($Q_1 – Q_3$). [a,b] indicate statistically different results ($p<0.05$). Kruskal – Wallis test, with the Dunn "a posteriori" test.

ARTIGO 2. Artigo submetido para a revista Pharmacology Biochemistry and Behavior

Can vitamins C and E ameliorate androgen depletion and hypersensibility of the vas deferens of hyperglycemic rats?

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Abstract

Hyperglycemic neuropathy can affect the reproductive system. The aim of this study was to evaluate whether the treatment with antioxidants (vitamins C and/or E) can ameliorate reproductive insults in hyperglycemic adult male rats. The animals were randomly assigned into four experimental groups: hyperglycemic control (Hy), hyperglycemic + 150mg/day vitamin C (HyC), hyperglycemic + 100mg/day vitamin E (HyE) and hyperglycemic + vitamins C and E (HyCE). Normoglycemic group (n=10) received vehicles. Testosterone level and noradrenergic response of vas deferens were analyzed. Both vitamins significantly decreased the TBARS level in the hyperglycemic groups. There was a significant reduction in the testosterone level in Hy and HyE groups when compared to normoglicemic group. However, HyC and HyCE groups the testosterone levels were partially recovered. In addition, an increased sensitivity of the vas deferens through the alpha-1 adrenoceptor was observed in the hyperglycemic control group. However, vitamins treated restored partially (vitamin E or in association) or totally (vitamin C alone) this alteration. Moreover, the maximum response values to norepinephrine were similar among all groups. Thus, we concluded that vitamin C, compared to vitamin E, is more efficient in ameliorating the effects of hyperglycemia on testosterone levels and on the vas deferens sympathetic neurotransmission in rats.

Keywords: Antioxidants, vitamin C, vitamin E, oxidative stress, hyperglycemia, testosterone, vas deferens, norepinephrine, male reproductive system, neuropathy, male rat.

Introduction

Diabetes, obesity, genetic predisposition, and aging are some factors that may lead to a hyperglycemic state. These factors may act by different action mechanisms and affect many human and animal bodily functions, including reproduction. Thus, sexual dysfunctions such as decrease in fertility, testosterone levels and sperm counts have been extensively described in the male hyperglycemic state (Calvo et al., 1984; Longhurst, 1990; Scarano et al., 2006; Zhang et al., 2006; Suthagar et al., 2009), and have prejudiced the ejaculatory process (Steger et al., 1989; Hassan et al., 1993; Scarano et al., 2006) by means of an autonomic nervous system neuropathy also known as hyperglycemic neuropathy (Tomlinson et al., 1982; Peredo et al., 1984; Öztürk et al., 1994; Longhurst, 1990; Vinik et al., 2003). Hyperglycemic neuropathy, affecting 50 - 60% of patients, is the most common complication in diabetes and causes autonomic failure (Kumar et al., 2007; Figueroa-Romero et al., 2008).

Ejaculation is a complex process stimulated by a series of events, depending on serotonin, dopamine, oxytocin, GABA, adrenaline, acetylcholine (Giuliano & Clément, 2005), testosterone, neuropeptide Y, vasoactive intestinal peptide and nitric oxide – and is under the control of sympathetic autonomic nervous system (Ralph and Wylie, 2005; Giuliano and Clément, 2006). The central ejaculatory neural circuit comprises the spinal cord and cerebral areas, which form a highly interconnected network. The sympathetic, parasympathetic, and somatic spinal centers, under the influence of sensory genital and cerebral stimuli integrated and processed at the spinal cord level, act in synergy to command physiological events occurring during ejaculation (Giuliano and Clément, 2006). The efferent reflex of the nervous system, responsible for the emission phase of ejaculation, consists of sympathetic efferent fibers of the hypogastric nerve which release, mainly, noradrenaline, causing propulsive contractions of the epididymis, vas

deferens, prostate and seminal vesicle, thus expelling sperm to the prostatic urethra (Ralph and Wylie, 2005). Many animal studies based on function, biochemistry, and sensitivity of α receptors to adrenergic agonists of the vas deferens show that the organ's machinery is impaired in the hyperglycemic model, which can be correlated with ejaculatory dysfunction (Longhurst, 1990; Öztürk et al., 1994; Öztürk and Aydin, 2006). Such changes may be related to the decrease of testosterone and/or insulin (Longhurst, 1990; Longhurst et al., 1989; Longhurst and Brotcke, 1989; Öztürk and Aydin, 2006).

Another usual consequence of the hyperglycemic state is increased oxidative stress (Ceriello et al., 1997; Cay et al., 2001; Naziroğlu, 2003; Ozkan et al., 2005), which is extremely toxic to cells and exerts its devastating effects by directly damaging cellular proteins, lipids, and DNA, or indirectly, by affecting normal cellular signaling and gene regulation (Vincent et al., 2004; Pop-Busui et al., 2006). This oxidative stress may cause hyperglycemic neuropathy (Günes et al. 2005; Kumar et al., 2007; Pop-Busui et al., 2006; Figueroa-Romero et al., 2008). These authors show a positive relationship between increased oxidative stress and functional, structural and biochemical abnormalities in the autonomic nervous system. However, their pathophysiological pathways have not yet been clearly elucidated. Günes et al. (2005) showed that using antioxidants (stobadine and vitamin E) in hyperglycemic rats may be an effective therapy for restoring sympathetic neurotransmission in the vas deferens. Different types of antioxidants are utilized to reduce the oxidative stress in hyperglycemia, and thus diminish, at least in part, the complications caused by oxidative stress.

On the basis in these considerations, this study aimed to verify whether treatment with two antioxidants, vitamin C and E (alone or associated), is able to ameliorate some effects of

oxidative stress on testosterone level and noradrenergic response of the vas deferens in hyperglycemic rats.

Methods

Animals

Adult male Wistar rats (90 days old) were supplied by the Multidisciplinary Center for Biological Investigation, State University of Campinas (CEMIB – UNICAMP) and were housed in 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 Ethics Committee for Animal Experimentation (022/06-CEEA) - UNESP Botucatu.

Hyperglycemic model and experimental protocol

Hyperglycemia (or diabetes) was chemically induced in adult male rats (n = 40) using a single dose of 40mg/Kg BW streptozotocin (SIGMA-ALDRICH, St. Louis, MO, USA) injected into the tail vein. Streptozotocin was diluted in citrate buffer 0.01M, pH=4.6. Five days after the induction, glucose levels of all animals were assessed using glucose test strips and a monitoring System (One Touch Ultra, Johnson&Johnson®) from which all animals presenting levels higher than 300mg/dL were considered hyperglycemic. These animals were randomly assigned on four experimental groups of 10 animals each: hyperglycemic (Hy), hyperglycemic + 150mg/day of

vitamin C (HyC), hyperglycemic + 100mg/day of vitamin E (HyE) and hyperglycemic + 150mg/day of vitamins C + 100mg/day of E (HyCE). These doses were adapted from Naziroğlu (2003). Another animal group (n=10) received no streptozotocin (normoglycemic group = N group) and presented glycemic levels lower than 120mg/dL. Hyperglycemic animals received the vitamins by gavage (oral route) throughout 30 consecutives days. Normoglycemic and hyperglycemic groups received only the vehicles (corn oil and water).

Preparation of vitamins

Vitamin C (L-Ascorbic acid; SIGMA-ALDRICH, St. Louis, MO, USA) was prepared daily by diluting the required quantity in the corresponding volume of warm water. The vitamin E (α- Tocopherol; SIGMA-ALDRICH, St. Louis, MO, USA) was wholly diluted in corn oil before the experimental period, and was used throughout the experiment. The oil and vitamin E were heated to 54ºC and combined into a homogeneous mixture. This was stored at 20 °C and its stability was verified by the HPLC technique, once a week, at the Center for Metabolism in Exercise and Nutrition (CeMENutri) of the Botucatu School of Medicine – UNESP. Both vitamins were stored in a dark container to protect against light.

Body weight and weights of some reproductive organs

At the end of the treatment, 9 or 10 rats from each experimental group were weighed, slightly anesthetized with sodium pentobarbital 3% (Hyptonol® - 0,1mL/Kg weight) and killed by decapitation. Both vas deferens and left seminal vesicle (without the coagulating gland) were

removed and freed of secretion. Their relative weights were determined on an analytical balance. The right vas deferens was immediately used for pharmacological analysis.

Plasma testosterone level

After decapitation, blood was collected (between 9:00 and 11:30am) from the ruptured cervical vessels in a heparinized tube (Liquemine, Hoffman-La Roche, Switzerland) 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 measurement. Plasma testosterone levels were ascertained 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 lowest detection limit for testosterone was 0.064ng/mL, with a 4% intra-assay error.

Oxidative stress status analysis (TBARS)

The remainder of the heparinized blood (3mL) was properly prepared and stored at -80ºC to measure the concentration of TBARS, which indicates the level of lipid peroxidation by oxidative stress. Lipid peroxides were estimated in washed erythrocytes using thiobarbituric acid (TBA). One mL of washed erythrocytes was added to the test tube containing 1.0mL of 3.0% sulphosalicilic acid, shaken for 10 seconds, centrifuged at 15,557 g for 3 minutes and kept at rest for 15 min. The sample was diluted to 500µl of 0.67% TBA solution. The mixture was heated to 80ºC for 30 minutes and absorbance was measured at 535 nm wavelength. Results were

expressed as nM of substances reactive to thiobarbituric acid (TBARS) per gram of hemoglobin (nM/g Hb), indirectly representing a lipid peroxidation index, according to Ferreira et al. (1999).

Pharmacological analysis of the isolated vas deferens

The right vas deferens isolated from 5 or 6 rats of each experimental group was individually set up in 10mL organ-baths containing a continuous nutritive solution, aerated with a mixture of 95% O_2 and 5% CO_2 and kept 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 (NE) (arterenol bitartrate, Sigma) and tyramine (Ty) (Sigma Co., USA) were obtained by cumulative addition of molar concentrations of the agonists, which are geometrically increased (van Rossum and van Den Brink, 1963). The pD2 values, expressed as the negative of the logarithm for the agonist concentration that produces 50% (ED50) of its maximum effects (Miller et al., 1948), were ascertained. In addition, the maximal contractile response (g of wet tissue) to norepinephrine was determined.

Statistical analysis

For comparison of results among the five experimental groups, statistical tests for analysis of variance were utilized – ANOVA, with the "a posteriori" Tukey test or the non-parametric

Kruskal-Wallis test with the Dunn “a posteriori” test, according to the characteristics of each variable. Differences were considered significant when $p<0.05$.

Results

Five days after the streptozotocin injection, the animals began to show qualitative characteristic signs of hyperglycemia such as polyphagia, polydipsia and polyuria, which persisted throughout the experimental period. In addition, these animals also exhibited glucose levels above 300mg/dL, characterizing the hyperglycemic state (Table 1). The normoglycemic group showed normal glucose levels, below 300 mg/dL (Table 1).

The lipidic peroxidation level (TBARS) (Fig. 1) was significantly increased in the hyperglycemic control group in relation to the other groups. The treatments with vitamin C and E, isolated or in combination, were able to significantly reduce this parameter in relation to the hyperglycemic group. However, TBARS levels were comparable to the normoglycemic group only in the vitamin C treated group.

Body and reproductive organ weights are shown in Table 1. There was a significant reduction in the body weight and weights of the empty seminal vesicle and its secretion in the hyperglycemic groups compared to the normoglycemic group. Vitamin C treatment was able to partially recover the empty seminal vesicle weight. On the other hand, the vas deferens relative weight increased significantly in the hyperglycemic group in relation to normoglycemic group. This alteration was completely reverted by the treatment with vitamin C alone or in combination with vitamin E, or partially with vitamin E isolated.

Plasma testosterone levels (Fig. 2) were reduced in all Hy groups compared to the normoglycemic group, and partially recovered by the treatment with vitamin C, alone or associated to vitamin E.

Moreover, although the maximum response values to norepinephrine were statistically similar among all groups (Table 2), the *in vitro* isometric contractions of the vas deferens revealed a significant increase in the pD2 value of norepinephrine in the hyperglycemic control group in relation to the normoglycemic group which is indicative of hypersensitivity of the post-junction alpha-1 adrenoceptor. On the other hand, vitamin C alone recovered totally this hypersensitivity since the pD2 value of norepinephrine was similar to the normoglycemic group (Table 2 and Fig. 3A). However, in the hyperglycemic groups treated with vitamin E (alone or associated) this parameter was only partially recovered (Table 2 and Fig 3A).

Moreover, the tyramine (Ty) curve dislocation to the left (Fig. 3B) and the increase of the pD2 value (Table 2) in the Hy, HyE and HyCE treated groups (Table 2 and Fig 3B) confirm this hypersensitivity. However, in the vitamin C treated group this event was partially recovered since tiramine pD2 values in this group were similar to all other groups (Table 2 and Fig. 3B).

Discussion

Among the various disorders arising in the hyperglycemic state are hyperglycemic neuropathies and oxidative stress (Vincenti et al., 2004; Pop-Busui et al., 2006, Figueroa-Romero, et al., 2008). Hyperglycemic neuropathy is the most common complication of hyperglycemic condition that can affect the reproductive system and the ejaculatory process. One of the mechanisms responsible for this may be increased oxidative stress (Günes et al., 2005). In the current study, the antioxidant potential of vitamin C and E in hyperglycemic animals was

achieved, since oxidative stress was significantly reduced in the vitamin-treated hyperglycemic groups. Thus, both vitamins C and E are described as expressing their antioxidant potential by reducing or eliminating the oxidative stress level in hyperglycemic subjects (Garg and Bansal, 2000; Cay et al., 2001; Baydas et al., 2002; Naziroğlu 2003, Young et al., 2004; Pop-Busui et al., 2006).

In the present study, the decreased body weight in hyperglycemic animals can be attributed to metabolic changes that arise from the absence or decrease of insulin in the blood, which is characteristic of hyperglycemic conditions. This finding is supported by studies from Seethalakshmi et al. (1987) and Suthagar et al. (2009) who found that administration of insulin restored the body weight of hyperglycemic rats. Thus, in the present study, body weight restoration may have been more dependent on recovery from insulin levels than the normalization of oxidative stress. It is known that vitamins C and E are able to reduce oxidative stress in stretozotocin-induced hyperglycemic rats, but do not prevent the loss of body weight (Naziroğlu, 2003). In addition, the increase of vas deferens relative weight in the hyperglycemic group differs from the prior study that reported this organ weight unchanged (Scarano et al., 2006). Nonetheless, in the present study, this can also be considered an expression of damage in the male reproduction system due to the hyperglycemic state. However, the vas deferens weight increase was completely prevented by vitamin C, both singly and in association with vitamin E, but only partially by vitamin E alone. Therefore, we cannot conclude that oxidative stress is responsible for this event, since it was statistically similar among all vitamin-treated groups. However, this alteration in the vas deferens may be a consequence of other metabolic changes arising from hyperglycemia that were not evaluated in this study.

The significant reduction of plasma testosterone in the hyperglycemic group is corroborated by previous studies (Cameron et al., 1990; Hassan et al., 1993; Scarano et al., 2006). These studies reported that testosterone diminished with insulin shortage. Nevertheless, the HyC and HyCE groups presented partially increased plasma testosterone levels. This is consistent with the previous finding that vitamin C (Biswas et al., 1996; Sönmez et al., 2005) and vitamin E (Chen et al., 2005) play key roles in the steroidogenic process that stimulates testosterone synthesis. However, in the HyE group neither this stimulation nor testosterone release occurred. Probably, the dose of vitamin E used in this group may have been inadequate to stimulate these processes.

Furthermore, the decrease of plasma testosterone level (total or partial) observed in hyperglycemic groups probably caused the reduction in the empty seminal vesicle and vesicle secretion weights, a change that could indicate hyperglycemia-induced functional impairment of these androgen-dependent organs (Balasubramanian et al., 1991; Scarano et al., 2006) Interestingly, in the present study, vitamin C attenuated the seminal vesicle relative weight loss relative to other hyperglycemic groups, probably due to body weight reduction. Moreover, this may have occurred because the oxidative stress level in the HyC group was similar to that in the normoglycemic group, despite the fact that the oxidative stress level remained similar among vitamin-treated groups.

In relation to the pharmacological response of vas deferens, the hypersensitivity of α adrenergic receptors to norepinephrine in the hyperglycemic group, evidenced in this study, is corroborated by previous studies (Tomlinson et al., 1982; Peredo et al., 1984; Günes et al., 2005). Our results suggest that the hyperglycemic organism is trying to compensate for the damage caused by the illness by preserving the contractile capacity of the vas deferens in order to

facilitate the sperm release. Therefore, this α adrenoceptor hypersensitivity to exogenous norepinephrine, in the hyperglycemic group vas deferens, may be due to diminished release of endogenous norepinephrine in the nerve endings of these animals. In the same manner, Öztürk et al. (1994) showed that short-term alloxan-induced hyperglycemia (72h) also caused enhanced α-adrenergic responses in the rat vas deferens while long-term diabetes (8 weeks) reduced the responses in this tissue. Similarly, Kamata et al. (1998) showed, in rats, that ten weeks of stretozotocin-induced hyperglycemia provoked decline of the contractile response of the vas deferens to norepinephrine. In the current study, it was also observed that these vitamins may be able to partially (vitamin E alone or associated) or totally (vitamin C) prevent the α-adrenoceptor's hypersensitivity to norepinephrine in hyperglycemic rats. Moreover, Günes et al. (2005) demonstrated that reactive oxygen species are responsible for impaired sympathetic neurotransmission and abnormal function of diabetic vas deferens, and that antioxidants may be more effective as a therapy against reproductive system disabilities in hyperglycemic male rats.

However, in relation to Ty response, vitamin C alone was more efficient in inducing norepinephrine release in hyperglycemic animals. Probably the stock of norepinephrine in the sympathetic nerve endings was partially increased, resulting in a greater Ty-induced release of norepinephrine. Thus, oxidative stress is not the only factor responsible for neuropathy in the male reproductive system of hyperglycemic rats, since the oxidative stress level also was reduced in the HyE and HyCE groups.

Therefore, in the current study, other factors resulting of the hyperglycemic state such as insulin level reduction, besides increased stress oxidative and low testosterone, may be acting. It was already demonstrated that the impairment of the vas deferens contractile response of

hyperglycemic animals is related to diminution of insulin level (Öztürk & Aydin 2006). In addition, Longhurst (1990) showed that replacement of testosterone and insulin may restore the function of that organ in hyperglycemic animals. However, in the present study, despite the change in the sensitivity of the organ to norepinephrine, the hyperglycemic rats did not show any damage in maximal contractile capacity induced by norepinephrine. In fact, previous studies using streptozotocin-induced hyperglycemia showed that at least part of the rats can ejaculate during sexual behavior tests (Steger et al., 1983; Scarano et al., 2006).

In conclusion, oxidative stress was responsible, at least in part, for the androgen depletion and damage in the sympathetic neurotransmission in the vas deferens of hyperglycemic rats. Moreover, vitamin C, compared to vitamin E, is more efficient in ameliorating the effects of hyperglycemia in these parameters in rats.

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References

Balasubramanian K, Sivashanmugam P, Thameemdheen S, Govindarajulu P. Effect of diabetes mellitus on epididymal enzymes of adult rats. Indian J Exp Biol 1991; 29(10):907-9.

Baydas G, Canatan H, Turkoglu A. Comparative analysis of the protective effects of melatonin and vitamin E on streptozocin-induced diabetes mellitus. J Pineal Res 2002; 32:225-30.

Biswas NM, Chaudhuri A, Sarkar M, Biswas R. Effect of ascorbic acid on *in vitro* synthesis of testosterone in rat testis. Indian J Exp Biol 1996; 43:612-3.

Calvo JC, Baranao JL, Tesone M, Charreau EH. Hypothalamic-hypophyseal-gonadal axis in the streptozotocin-induced diabetic male rat. J Steroid Biochem 1984; 20:769-72.

Cameron DF, Rountree J, Schultz RE, Repetta D, Murray FT. Sustained hyperglycemia results in testicular dysfunction and reduced fertility potential in BBWOR diabetic rats. Am J Physiol 1990; 259: E881-9.

Cay M, Naziroğlu M, Simsek H, Aydilek N, Aksakal M, Demirci M. Effects of intraperitoneally administered vitamin C on antioxidative defense mechanism in rats with diabetes induced by streptozotocin. Res Exp Med (Berl) 2001; 200(3):205-13.

Ceriello A, Bortolotti N, Falleti E. Total radical-trapping antioxidant parameter in NIDDM patients. Diabetes Care 1997; 20:194-7.

Chen H, Liu J, Luo L, Baig MU, Kim J-M, Zirkin BR. Vitamin E, aging and Leydig cell steroidogenesis. Exp Gerontol 2005; 40:728-38.

Ferreira ALA, Machado PEA, Matsubara LS. Lipid peroxidation, antioxidant enzymes and glutathione levels in human erythrocytes exposed to colloidal iron hydroxide in vitro. Braz J Med Biol Res 1999; 32(6):689-94.

Figueroa-Romero C, Sadidi M, Feldman EL. Mechanisms of disease: the oxidative stress theory of diabetic neuropathy. Rev Endocr Metab Disord 2008; 9(4):301-14.

Garg MC, Bansal DD. Protective antioxidant effect of vitamins C and E in streptozotocin induced diabetic rats. Indian J Exp Biol 2000; 38(2): 101-4.

Giuliano F, Clément P. Physiology of ejaculation: emphasis on serotonergic control. Eur Urol 2005; 48(3):408-17

Giuliano F, Clement P. Serotonin and premature ejaculation: from physiology to patient management. Eur Urol 2006; 50:454-66.

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; 149:148-54.

Kamata K, Kirisawa H. Changes in electrophysiological properties and noradrenaline response in vas deferens of diabetic rats. Eur J Pharmacol 1998; 350(2-3):237-41.

Kumar A, Kaundal RK, Iyer S, Sharma SS. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy. Life Sci 2007; 80(13):1236-44.

Longhurst PA, Brotcke TP, Burrell CL, Belis JA. Comparison of the effects of castration and streptozotocin-induced diabetes mellitus on contractile responses of the rat vas deferens. Pharmacology 1989; 38(4):253-62.

Longhurst PA, Brotcke TP. Effects of castration and diabetes mellitus on cholinergic responsiveness and muscarinic receptors in the rat vas deferens. J Urol 1989; 141(5):1225-9.

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

Mann T. Secretory function of the prostate, seminal vesicle and other male accessory organs of reproduction. J Reprod Fertil 1974; 37:179-88.

Miller LC, Becker TJ, Tainter ML. Quantitative evaluation of spasmolytic drugs "in vitro". J Pharmacol Exp Therap 1948; 92:260-8.

Naziroğlu M. 2003. Enhanced testicular antioxidant capacity in streptozotocin-induced diabetic rats. Protective role of vitamins C and E and selenium. Biol Trace Elem Res. 94; 61-71.

Ozkan Y, Yilmaz O, Ozturk AI, Ersan Y. 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 Biol Int 2005; 29(9):754-60.

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.

Oztürk Y, Yildizoğlu-Ari N, Altan VM. Altered alpha-adrenergic responses of vas deferens to noradrenaline and tyramine from rats with short- and long-term alloxan diabetes. Gen Pharmacol 1994; 25(7):1519-24.

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; 18:197-9.

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.

Pop-Busui R, Sima A, Stevens M. Diabetic neuropathy and oxidative stress. Diabetes Metab Res Rev 2006; 22(4):257-73.

Ralph DJ, Wylie KR. Ejaculatory disorders and sexual function. BJU Int 2005; 95:1181-6.

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.

Seethalakshmi L, Menon M, Diamond D. The effect of streptozotocin-induced diabetes on the neuroendocrine-male reproductive tract axis of the adult rat. J Urol 1987; 138:190-4.

Sönmez M, Türk G, Yüce A. The effects of ascorbic acid supplementation on sperm quality, lipid proxidation and testosterone levels of male Wistar rats. Theriogenology 2005; 63: 2063-72.

Steger RW, Amador A, Lam E, Rathert J, Weis J, Smith MS. Streptozotocin-induced deficits in sex behavior and neuroendocrine function in male rats. Endocrinology 1989; 124:1737 – 43.

Suthagar E, Soudamani S, Yuvaraj S, Ismail Khan A, Aruldhas MM, Balasubramanian K. Effects of streptozotocin (STZ)-induced diabetes and insulin replacement on rat ventral prostate. Biomed Pharmacother. 2009; 63(1):43-50.

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; 143:240-6.

Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev. 2004; 25(4):612-28.

Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev 2004; 25(4):612-28.

Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. Diabetes Care 2003; 26(5): 1553-9.

Young L, Yu D, Bateman R, Brock G. Oxidative stress and antioxidant therapy: their impact in diabetes-associated erectile dysfuction. J Androl 2004; 25:830-6.

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):253-64.

Table 1. Body weight; wet vas deferens, empty seminal vesicle and vesicle secretion weights and glycemia levels.

	Normoglycemic (n=10)	Hyperglycemic (n=10)	Hyperglycemic + vitamin C (n=09)	Hyperglycemic + vitamin E (n=10)	Hyperglycemic + vitamins C and E (n=10)
Body weight (g)	417.40 ± 9.02 [a]	303.40 ± 9.80 [b]	303.44 ± 7.27 [b]	298.40 ± 10.06 [b]	309.30± 9.00 [b]
Vas deferens (mg/100g)	24.64 ± 1.05 [a]	30.90 ± 1.08 [b]	26.60 ± 0.94 [a]	27.73 ± 0.38 [ab]	27.00 ± 1.19 [a]
Empty seminal vesicle (g/100g)	0.14 ± 0.01 [a]	0.08 ± 0.00 [b]	0.12 ± 0.01 [ab]	0.09 ± 0.01 [b]	0,10 ± 0,01 [b]
Seminal vesicle secretion (g)	0.81± 0.06 [a]	0.28 ± 0.11 [b]	0.30 ± 0.11 [b]	0.14 ± 0.04 [b]	0.18 ± 0.04 [b]
Glycemia after 5 days from induction (mg/dL)	94.00 ± 3.33 [a]	401.60 ± 20.03 [b]	386.00 ± 18.30 [b]	378.70 ± 21.66 [b]	365.3 ± 10.35 [b]
Final Glycemia (mg/dL)	93.00 ± 2.12 [a]	555.40 ± 11.20 [b]	543.11 ± 17.56 [b]	562.40 ± 15.00 [b]	573.60 ± 10.25 [b]

Values expressed as Mean±SEM. [a,b] indicate statistically different results ($p<0.05$). ANOVA test, with the Tukey "a posteriori" test.

Table 2: Responses to norepinephrine (NE) and tyramine (Ty) (pD_2), and maximal response to norepinephrine (Emax) of isolated vasa deferentia.

Groups	pD_2[1]		E_{max} to NE (n=06)
	NE (n=06)	Ty (n=05)	
Normoglycemic	5.31 ± 0.05 [a]	4.10 ± 0.12 [a]	1.52 ± 0.05
Hyperglycemic	5.63 ± 0.04 [b]	4.49 ± 0.04 [b]	1.54 ± 0.11
Hyperglycemic + Vitamin C	5.35 ± 0.08 [a]	4.43 ± 0.05 [ab]	1.44 ± 0.19
Hyperglycemic + Vitamin E	5.40 ± 0.07 [ab]	4.50 ± 0.08 [b]	1.50 ± 0.18
Hyperglycemic + Vitamins C and E	5.46 ± 0.08 [ab]	4.45 ± 0.08 [b]	1.67 ± 0.25

[1] pD_2= - log[ED50]

E_{max} = maximal contractile response (g/100g of tissue).

Values expressed as Mean±SEM. [a,b] indicate statistically different results ($p<0.05$). ANOVA test, with the Tukey "a posteriori" test.

Figure Legends

Figure 1 – Blood oxidative stress level. Values expressed as median, quartiles, minimum and maximum values (vertical bars). [a,b,c,d] indicate statistically different results ($p<0.05$); Kruskal-Wallis test, with the Dunn "a posteriori" test.

N= normoglycemic group; *Hy* = hyperglycemic group; *HyC* = hyperglycemic + vitamin C; *HyE* = hyperglycemic + vitamin E; HyCE = hyperglycemic + vitamin C + vitamin E.

Figure 2 – Testosterone level. Values expressed as mean and S.E.M. (vertical bars). [a,b] indicate statistically different results ($p<0.05$); Kruskal-Wallis test, with the Dunn "a posteriori" test.

N= normoglycemic group; *Hy* = hyperglycemic group; *HyC* = hyperglycemic + vitamin C; *HyE* = hyperglycemic + vitamin E; HyCE = hyperglycemic + vitamin C + Vitamin E.

Figure 3 - Cumulative concentration response for norepinephrine (A) and tyramine (B) in vas deferens isolated from rats of different experimental groups. Abscissas show the molar concentration of the drugs on a logarithmic scale. Ordinates show effects produced by the drug, expressed as grams per 100 mg tissue. Vertical bars indicate S.E.M.

N= normoglycemic group; *Hy* = hyperglycemic group; *HyC* = hyperglycemic + vitamin C; *HyE* = hyperglycemic + vitamin E; HyCE = hyperglycemic + vitamin C + Vitamin E.

Figure 1

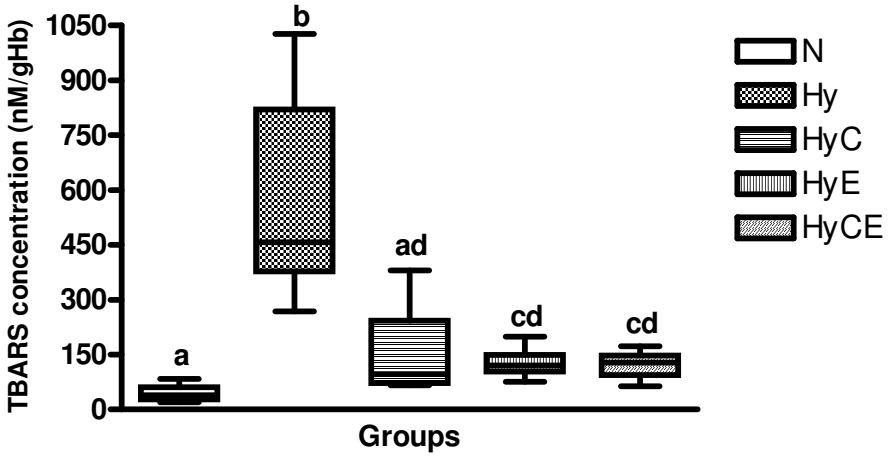


Figure 2

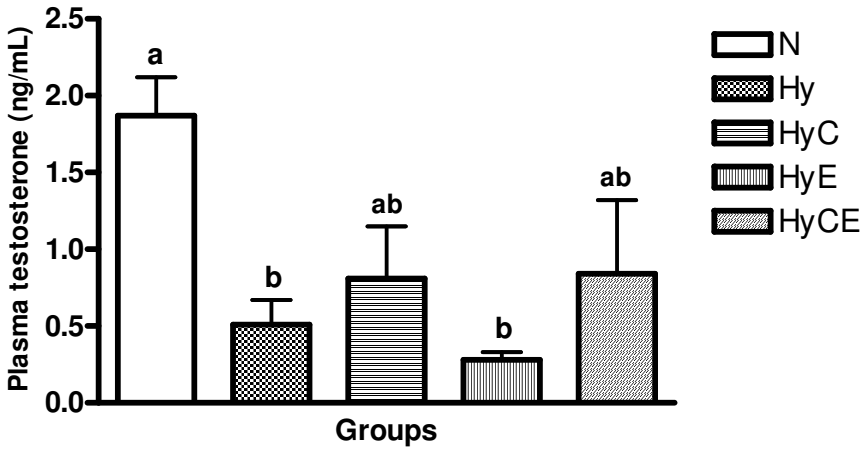
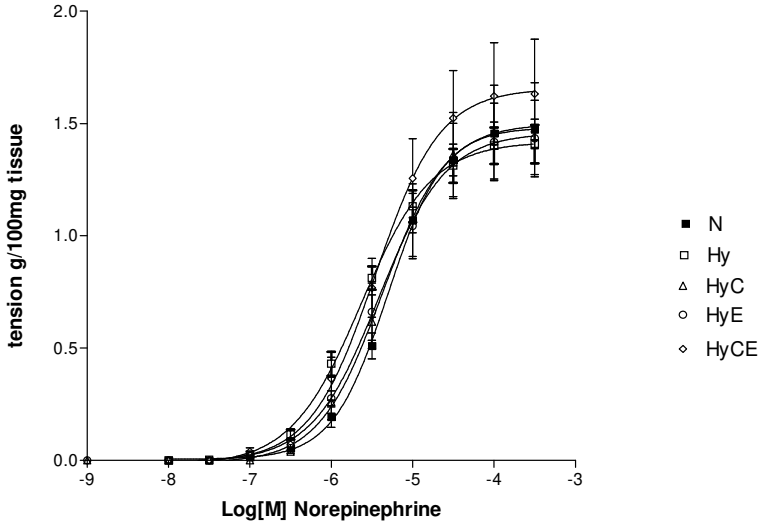
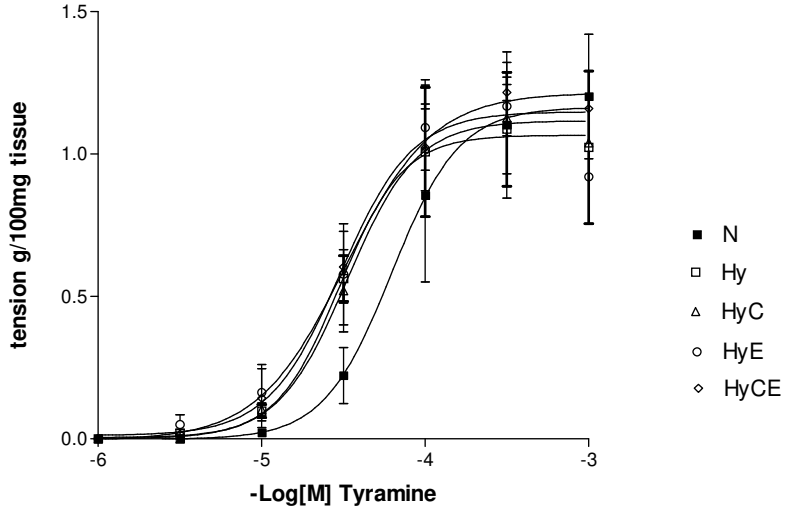


Figure 3

A


tension g/100mg tissue
2.0
1.5
1.0
0.5
0.0
-9
-8
-7
-6
-5
-4
-3
Log[M] Norepinephrine
N
Hy
HyC
HyE
HyCE

B


tension g/100mg tissue
1.5
1.0
0.5
0.0
-6
-5
-4
-3
-Log[M] Tyramine
N
Hy
HyC
HyE
HyCE

CONCLUSÕES FINAIS

Conclui-se com esse estudo que devido a eficiência do potencial antioxidante das vitaminas C e/ou E, o estresse oxidativo advindo do estado hiperglicêmico pôde ser reduzido significativamente e que, nessas condições experimentais a vitamina C foi mais eficiente tanto para diminuir o estresse oxidativo como para restaurar (total ou parcialmente) algumas alterações no sistema reprodutor masculino. Também pode se afirmar que o estresse oxidativo pode estar significativamente associado com algumas anormalidades no sistema reprodutor masculino hiperglicêmico como níveis de testosterona plasmática, sensibilidade dos receptores α_1 noradrenérgicos pós juncionais do ducto deferente, morfologia e motilidade espermática bem como a proporção dos componentes do tecido epididimário. Além disso, pode-se inferir que quanto mais reduzido estiver o estresse oxidativo, ou seja, quanto mais próximo dos níveis fisiológicos, menos ele pode interferir com a qualidade do sistema reprodutor masculino.

REFERÊNCIAS DA INTRODUÇÃO

Abbout D.E., Pritchard C., Clegg N.J., Fergunson C., Dumpit R., Sikes R.A., Nelson P.S. Expressed sequence tag profiling identifies developmental and anatomic partitioning of gene expression in the mouse prostate. Genome Biology 4; R79.

Agarwal A., Prabakaran S.A., Said T.M. 2005. Prevention of oxidative stress injury to sperm. J Androl 26(6); 654 – 60.

Aitken R.J. 1994. A free radical theory of male infertility. Reprod Fertil Dev 6(1); 19-23.

Aitken R.J. 2006. Sperm function tests and fertility. Int J Androl 29; 69-75.

Aitken R.J., Baker M.A. 2006. Oxidative stress, sperm survival anf fertility control. Mol Cell Endocrinol 250(1-2); 66-9.

Aitken R.J., Ryan A.L., Baker M.A., McLaughlin E.A. 2004. Redox activity associated with the maturation and capacitation of mammalian spermatozoa. Free Radic Biol Med 82; 1684-6.

Anderson J.E., Jones D., Penner S.B., Thliveris J.A. 1987. Primary hypoandrogenism in experimental diabetes in Long-Evans rat. Diabetes 36; 1104-10.

Bennetti P.H., Knowler W.C. 2005. Definition, diagnosis, and classification of diabetes mellitus and glucose homeostasis. In: Johlin´s Diabetes mellitus. (ed. Kahn C.R.), 14º edição., Lippincott Williams and Wilkins, Philadelphia. pp. 331-54.

Bensoussan K., Morales C.R., Hermo L. 1998. Vitamin E deficiency causes incomplete spermatogenesis and affects the structural differentiation of ephitelial cells of the epididymis in the rat. J Androl 19(3); 266-88.

Bolzán A.D., Bianchi M.S. 2002. Genotoxicity of streptozotocin. Mutat Res 512(2–3); 121-34.

Brigelius-Flohé R., Traber M.G. 1999. Vitamin E: function and metabolism. FASEB J 13(10):1145-55.

Brooks D.E. 1983. Epididymal functions and their hormonal regulation. Aust J Biol Sci 36; 205-21.

Calderon I.M.P., Rudge M.V.C., Brasil M.A.M., Henry M.A.C.A. 1992. Diabete e gravidez experimental em ratas I. Indução do diabete, obtenção e evolução da prenhez. Acta Cir Bras 7(1); 9-14.

Calvo J.C., Baranao J.L., Tesone M., Charreau E.H. 1984. Hypothalamic-hyposhyseal-gonadal axis in the streptozotozin-induced diabetic male rat. J Steroid Biochem 20; 769–72.

Cameron D.F., Murray F.T., Drylie D.D. 1985. Interstitial compartment pathology and spermatogenic disruption in testes from impotent diabetic men. Anat Rec 213; 53-62.

Cameron D.F., Rountree J., Schultz R.E., Repetta D., Murray F.T. 1990. Sustained hyperglycemia results in testicular dysfunction and reduced fertility potential in BBWOR diabetic rats. Am J Physiol 259; E881-9.

Castro M.A., Angulo C., Brauchi S., Nualart F., Concha I.I. 2008. Ascorbic acid participates in a general mechanism for concerted glucose transport inhibition and lactate transport stimulation. Pflugers Arch - Eur J Physiol 457; 519-28.

Cay M., Naziroğlu M., Simsek H., Aydilek N., Aksakal M., Demirci M. 2001. Effects of intraperitoneally administered vitamin C on antioxidative defense mechanism in rats with diabetes induced by streptozotocin. Res. Exp. Med. (Berl) 200(3); 205-13.

Ceriello A, Bortolotti N, Falleti E. 1997. Total radical-trapping antioxidant parameter in NIDDM patients. Diabetes Care 20; 194-97.

Clermont Y. 1972. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52; 198-236.

Clever O., Melton D.A. 2005. Development of the endocrine pancreas. In Joslin´s Diabetes Mellitus. (eds. Kahn C.R., Weir G.C., King G L., Jacobson A.M., Moses A.C., Smith R.J.) 14º edição. Lippincott Williams & Wilkins, Boston. pp. 21-39.

Combs Jr, GF. 2002. Vitaminas. In: Krause - Alimentos, nutrição e dietoterapia. (eds. Kathleen Mahan L and Escott-Stump S), Rocca, São Paulo. pp. 77-100.

Cooper D.R., Kling O.R., Carpenter M.P. 1987. Effect of vitamin E deficiency on serum concentrations of follicle-stimulating hormone and testosterone during testicular maturation and degeneration. Endocrinol 120(1); 83-90.

Cosentino M.J., Cokett A.T. 1986. Structure and function of the epididymis. Urol Res, 14(5); 229-40.

Damasceno D.C., Volpato G.T., Calderon I.M.P., Rudge M.V.C. 2002. Oxidative stress and diabetes in pregnancy rats. Animal Reprod. Sci. 72; 235-44.

Duarte T.L., Lunec J. 2005. When is an antioxidant not an antioxidant? A review of novel actions and reactions of vitamin C. Free Radical Research, 39(7); 671-86.

Dunn J.S., Sheenhan H.L., Mcletchie N.G.B. 1943. Necrosis of islets of Langerhans produced experimentally. Lancet 1; 484-7.

Eskenazi B., Kidd S.A., Marks A.R., Solder E., Block G., Wyrobek A.J. 2005. Antioxidant intake is associated with semen quality in healthy men. Hum Reprod 20; 1006-12.

Evans J.S., Gerritsen G.C., Mann K.M., Owen S.P. 1965. Antitumor and hyperglycemic activity of streptozotocin (NSC-37917) and its cofactor, U-15,774. Cancer Chemoth Rep 48; 1-6.

Fairburn C.G. 1981. The sexual problems of diabetic men. Br J Hosp Medic 25; 484 – 91.

Ferreira A.L.A., Matsubara L.S. 1997. Radicais livres: conceitos, doenças relacionadas, sistema de defesa e estresse oxidativo. Rev Assoc Med Bras 43; 61-8.

Figueroa-Romero C., Sadidi M., Feldman E.L. 2008. Mechanisms of disease: the oxidative stress theory of diabetic neuropathy. Rev Endocr Metab Disord 9(4); 301-14.

Finkel T. 1998. Oxygen radicals and signaling. Curr Opin Cell Biol 10; 248-53.

França L.R., Avelar G.F., Almeida F.F.L. 2005. Spermatogenesis and transit through the epididymis in mammals with empashis on pigs. Theriogenology 63; 300-18.

Garg M.C., Bansal D.D. 2000. Protective antioxidant effect of vitamins C and E in streptozotocin induced diabetic rats. Indian J Exp Biol 38(2); 101-4.

Garg M.C., Chaudhary D.P., Bansal D.D. 2005. Effect of vitamin E supplementation on diabetes induced oxidative stress in experimental diabetes in rats. Indian J Exp Biol 43(2); 177-80.

Gatti, J.L., Castella S., Dacheux F., Ecruyd H., Métayer S., Thimon V., Dacheux J. L. 2004. Post-testicular sperm environment and fertility. Anim Reprod Sci 82-83, 321-39.

Giuliano F., Clément P. 2005. Physiology of ejaculation: emphasis on serotonergic control. Eur Urol 48(3); 408-17.

Giuliano F., Clement P. 2006. Serotonin and premature ejaculation: from physiology to patient management. Eur Urol 50; 454-66.

Glover T.D. 1982. The epididymis. In: Scientific Foundations of Urology. (eds. Chisholm G D and Williams D I), William Heinemann Medical Books, London. pp. 544-55.

Grace P.A. 1994. Ischemia-reperfusion injury. Brit J Surg 81; 637-47.

Greco E., Iacobelli M., Rienzi L., Ubaldi F, Ferrero S., Tesarik J. 2005a. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. J Androl 26(3); 349 –53.

Greco E., Iacobelli M., Rienzi L., Ubaldi F, Ferrero S., Tesarik J. 2005b. ICSI in cases of sperm DNA damage: effect of oral antioxidant treatment. Hum Reprod 20(9);2590–4.

Güneş A., Ceylan A., Sarioglu Y., Stefek M., Bauer V., Karasu C. 2005. 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 19(1); 73-9.

Guyton A.C., Hall J.E. 1996. Insulina, glucagon e diabetes Mellitus. In: Tratado de fisiologia médica. (eds. Guyton A C and Hall J E), 9 ed., Guanabra Koogan, Rio de Janeiro. pp. 883-94.

Hayward S.W., Baskin L.S., Haughney P.C., Cunha A.R., Foster B.A., Dahiya R., Prins G.S., Cunha G.R. 1996a. Epithelial developmental in the rat ventral prostate, anterior prostate and seminal vesicle. Acta Anat (Basel) 155; 81-93.

Hayward S.W., Baskin L.S., Haughney P.C., Foster B.A., Cunha A.R., Dahiya R., Prins G.S., Cunha G.R. 1996b. Stromal developmental in the ventral prostate, anterior prostate and seminal vesicle of the rat. Acta Anat (Basel) 155; 94-103.

Hermo L., Robaire B. 2002. Epididymal cell types and their functions. In: The Epididymis – from molecules to clinical pratice. (eds. Robaire B and Hinton B T). Kluwer Academic/ Plenum Publisher, New York. pp. 81-102.

Howland B.E., Zerbrowski E.J. 1976. Some effects of experimentally-induced diabetes on pituitary-testicular relationships in rats. Horm Metab Res 8; 465-9.

Hsu P-C., Liu M-Y., Hsu C-C., Chen L-Y., Guo Y-L. 1998. Effects of vitamin E and/or C on reactive oxygen species-related lead toixicity in the rat sperm. Toxicol 128; 169-79.

Junod A., Lambert A.E., Orci L., Gonet A.E., Renold A.E. 1967. Studies on the diabetogenic action streptozotocin (32401). Proc Soc Exp Biol Med 126; 201-5.

Junqueira L.C., Carneiro J. 2004. Aparelho reprodutor masculino. In: Histologia básica. (eds. Junqueira L C and Carneiro J), Guanabara Koogan, Rio de Janeiro. pp 415-31.

Kamata K., Kirisawa H. 1998. Changes in electrophysiological properties and noradrenaline response in vas deferens of diabetic rats. Eur J Pharmacol 350(2-3); 237-41.

Kierszenbaum A.L. 2008. Transporte e maturação de espermatozóides. In: Histologia e Biologia Celular. (ed. Kierszenbaum A L), 2ª edição, Elsevier, Rio de Janeiro. pp. 601-39.

Kolodny R.C., Kahn C.G., Goldstein H.H., Barnett D.M. 1974. Sexual dysfunction in diabetic men. Diabetes 23; 306-9.

Kumar A., Kaundal R.K., Iyer S., Sharma S.S. 2007. Effects of resveratrol on nerve functions, oxidative stress and DNA fragmentation in experimental diabetic neuropathy. Life Sci 80(13); 1236-44.

Lamirande E., Jiang H., Armand Z., Kodama H., Gagnon C. 1997. Reactive oxygen species and sperm physiology. Rev Reproduc 2; 48-54.

Lewis S.E.M., Sterling E.S.L., Young I. S., Thompson W. 1997. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril 67; 142-47.

Li Y., Schellhorn H. E. 2007. New developmental and novel therapeutic perspective for vitamina C. J. Nutr 137; 2171-84.

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

Mann T. 1974. Secretory function of the prostate, seminal vesicle and other male acessory organs of reproduction. J Reprod Fert 37; 179-88.

Makker K., Agarwal A., Sharma R. 2009. Oxidative stress & male fertility. Indian J Med Res 129; 357-67.

Marker P.C., Donjacour A.A., Dahiya R., Cunha GR. 2003. Hormonal, cellular, and molecular controlos prostatic development. Dev Biol 253; 165-74.

McNeal J.E. 1981. The zonal anatomy of the prostate. Prostate. 2; 35-49.

Moger W.H. 1987. Uptake and release of ascorbic acid by rat Leydig cells in vitro. J Androl 8(6); 398-402.

Murray F.T., Orth J., Gunsalus G., Weisz J., Li J.B., Jeferson L.S., Musto N.A., Bardin C.W. 1981. The pituitary-testicular axis in the streptozotocin diabetic male rat: Evidence for gonadotroph, Sertoli cell and Leydig cell dysfunction. Int J Androl 4; 265-80.

Murray-Lyon I.M., Eddleston A.L., Williams R., Brown M., Hogbin B.M., Bennett A., Edwards J.C., Taylor K.W. 1968. Treatment of multiple-hormone-producing malignant islet-cell tumour with streptozotocin. Lancet 2 (7574); 895–8.

Nakhooda A.F., Like A.A., Chappel C.I., Murray F.T., Marliss E.B. 1977. The spontaneously diabetic Wistar rat. Metabolic and morphological studies. Diabetes 26; 100-12.

Naziroğlu M. 2003. Enhanced testicular antioxidant capacity in streptozotocin-induced diabetic rats. Protective role of vitamins C and E and selenium. Biol Trace Elem Res 94; 61-71.

Oksanen A. 1975. Testicular lesions of streptozotocin diabetic rats. Horm Res 6(3); 138-44.

Olivares A., Méndez J. P., Cárdenas M., Oviedo N., Palomino M. A., Santos I., Perera-Marín G., Gutiérrez-Sagal R., Ulloa-Aguirre A. 2009. Pituitary-testicular axis function, biological to immunological ratio and charge isoform distribution of pituitary LH in male rats with experimental diabetes. Gen Comp Endocrinol, 161(3), 304-12.

Olszewer E. 1995. Radicais livres em medicina. São Paulo (SP): Fundo Editorial.

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 Biol Int 29(9); 754-60.

Öztürk Y., Yildizoğlu-Ari N., Altan V.M. 1994a. Altered α-adrenergic responses of vas deferens to noradrenaline na tyramine from rats with short- and long-term alloxan diabetes. Gen.Pharmac 25 (7); 1519-24.

Öztürk Y., Aydin S., Altan V.M., Yildizoğlu-Ari N., Özcelikay A.T. 1994b. Effect of short and long term streptozotocin diabetes on smooth muscle calmodulin levels in the rat. Cell Calcium 16; 81-6.

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

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); 127-36.

Peredo H., Agostini M.D., Gimeno M.F., Borda E.S. 1984. Hypersensitivity to norepinephrine in vasa deferentia from diabetic rats. Possible participation of metabolic products of arachidonic acid. Prostaglandins Leukot Med 15(2); 229-39.

Rakieten N., Raikieten M.L., Nadkarni M.V. 1963. Studies in the diabetogenic action of streptozotocin. Cancer Chemother Rep 29; 91-4.

Ralph D.J., Wylie K.R. 2005. Ejaculatory disorders and sexual function. BJU Int 95; 1181-6.

Ramesh B., Pugalendi K.V. 2006. Antioxidant role of Umbelliferone in STZ-diabetic rats. Life Sci. 79(3); 306-10.

Rees D.A., Alcolado J.C. 2005. Animal models of diabetes mellitus. Diabetic medicine 22; 359 – 70.

Reid B.L., Cleand K.W. 1957. The structure function of the epididymis. I-The histology of the rat epididymis. Aust J Zool 5(3); 223-51.

Robaire B., Henderson N.A. 2006. Actions of 5 α- reductase inhibitors in the epididymis Mol Cell Endocrinol 250(1-2); 190-5.

Rodrigues J.A., Favaretto A.L.V. 1999. Sistema reprodutor. In: Fisiologia. (ed. Aires M M), Guanabara Koogan, Rio de Janeiro. pp. 877-917.

Roy-Burman P., Wu H., Powell W.C., Hagenkord J., Cohen M.B. 2004. Genetically defined mouse models that mimic natural aspects of human prostate cancer developmental. Endocr Relat Cancer 11; 225-324.

Russell L.D., Ettlin R.A., Sinhahikin A.T., Clegg E.D. 1990. Histological and histopathological evaluation of the testis. Cache River Press, Clearwater.

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. Int J Androl 29(4); 482-8.

Schnedl W.J., Ferber S., Johnson J.H., Newgard C.B. 1994. STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes 43 (11); 1326–33.

Sen Gupta R., Sen Gupta E., Dhakal B.K., Thakur A.R., Ahnn J. 2004. Vitamin C and vitamin E protect the rat testes from cadmium-induced reactive oxygen species. Mol Cells 17(1); 132-9.

Setchell B.P. 2002. Innervation and vasculature of the excurrent duct system. In: The Epididymis – from molecules to clinical pratice. (eds. Robaire B and Hinton BT), Kluwer Academic/ Plenum Publisher, New York. pp. 35-48.

Sigh S., Malini T., Rengarajan S., Balasubramanian, K. 2009. Impact of experimental diabetes and insulin replacement on epididymal secretory products and sperm maturation in albino rats. J Cell Biochem - doi: 10.1002/jcb.22337.

Sokol R.Z. 1997. The hypothalamic – pituitary – gonadal axis as a target for toxicants. In: Comprehensive toxicology. (eds. Sipes G, McQueen C A and Gandolfi A J), Cambridge University Press, Cambridge,. pp. 87-98.

Sönmez M., Türk G., Yüce A. 2005. The effects of ascorbic acid supplementation on sperm quality, lipid proxidation and testosterone levels of male Wistar rats. Theriogenology 63; 2063-72.

Soronen P., Laiti M., Torn S., Harkonen P., Patrikainen L., Li Y., Pulkka A., Kurkela R., Herrala A., Kaija H., Isomaa V., Vihko I. 2004. Sex steroid hormone metabolism and prostate cancer. J Steroids Biochem Mol Bio 92; 281-6.

Steger R.W., Amador A., Lam E., Rathert J., Weis J., Smith M.S. 1989. Streptozotocin-induced deficits in sex behavior and neuroendocrine function in male rats. Endocrinology 124; 1737 – 43.

Sudha S., Valli G., Julie P. M., Arunakaran J., Govindarajulu, P., Balasubramanian, K. 2000. Influence of streptozotocin-induced diabetes and insulin treatment on the pituitary-testicular axis during sexual maturation in rats. Exp Clin Endocrinol Diabetes, 8(1); 14-20.

Sullivan,R., Saez F., Girouard J. Frenette G. 2005. Role of exossomes in sperm maturation during the transit along the male reproductive tract. Blood Cells Mol Dis 35; 1-10.

Suthagar E., Soudamani S., Yuvaraj S., Ismail Khan A., Aruldhas M. M., Balasubramanian K. 2009. Effects of streptozotocin (STZ)-induced diabetes and insulin replacement on rat ventral prostate. Biomed Pharmacother, 63(1); 43-50.

Sun Y. 1990. Free radical, antioxidante enzymes, and carcinogenesis. Free Radical Biol Med 8; 583 -99.

Tomlinson D.R., Gillon K.R., Smith M.G. 1982. Axonal transport of noradrenaline and noradrenergic transmission in rats with streptozotocin-induced diabetes. Diabetologia 22(3) ; 199-204.

Traber M.G., Atkinson J. 2007. Vitamin E, antioxidant and nothing more. Free Radic Biol Med 43(1); 4-15.

Tramer F., Rocco F., Micali F., Sandri, G., Panfili E. 1998. Antioxidant systems in rat epididymal spermatozoa. Biol Reprod 59(4); 753-8.

Vavra J.J., Deboer C., Dietz A., Hanka L.J., Sokolski W.T. 1959. Streptozotocin, a new antibacterial antibiotic. Antibiot Annu 7; 230–5.

Wang X., Quinn P.J. 1999. Vitamin E and its function in membranes. Prog Lipid Res 38(4); 309-36.

Wang Z., Gleichmann H. 1998. GLUT2 in pancreatic islets: crucial target molecule in diabetes induced with multiple low doses of streptozotocin in mice. Diabetes 47 (1); 50–6.

Yatoh S., Mizutani M., Yokoo T., Kozawa T., Sone H., Toyoshima H., Suzuki S., Shimano H., Kawakami Y., Okuda Y., Yamada N. 2006. Antioxidants and an inhibitor of advanced glycation ameliorate death of retinal microvascular cells in diabetic retinopathy. Diabetes Metabol Res Rev 22(1); 38-45.

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. Biol Reprod 71(3); 1002-8.

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CERTIFICADO

Certificamos que o Protocolo nº 022/06-CEEA, sobre *"Antioxidantes (vitaminas C e E): uma alternativa para diminuir os danos reprodutivos causados pela hiperglicemia em ratos machos e adultos"*, 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 06/04/06.

Botucatu, 07 de abril de 2006.

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

NADIA JOVÊNCIO COTRIM
Secretária – CEEA

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada "Relação entre estresse oxidativo e desordens reprodutivas em ratos machos hiperglicêmicos: potencial antioxidante das vitaminas C e E".

( ) não se enquadra no Artigo 1°, § 3° da Informação CCPG 01/2008, referente a bioética e biossegurança.

( ) está inserido no Projeto CIBio (Protocolo nº _____), intitulado _____

(X) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº 022/06-CEEA).

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

_____

Aluna: Glaura Scantamburlo Alves Fernandes

_____

Orientadora: Wilma De Grava Kempinas

Para uso da Comissão ou Comitê pertinente:

( ) Deferido ( ) Indeferido

_____

Nome:
Função: