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DANIELE SAPEDE ALVARENGA

INFLUÊNCIA DO CONSUMO DE SACAROSE E FRUTOSE SOBRE A MORFOLOGIA E FUNÇÃO DO SISTEMA REPRODUTOR MASCULINO DE RATOS WISTAR

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Tese apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina - UEL, como requisito para a obtenção do título de Doutora.

Orientadora: Profa. Dra. Glaura Scantamburlo Alves Fernandes

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"A tarefa não é tanto ver aquilo que ninguém viu, mas pensar
o que ninguém ainda pensou sobre aquilo que todo mundo vê".

(Arthur Schopenhauer)

ALVARENGA, Daniele Sapede. **Influência do consumo de sacarose e frutose sobre a morfologia e função do sistema reprodutor masculino de ratos Wistar.** 2020. 91 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2020.

RESUMO

O aumento do consumo de açúcar é responsável pela incidência de hipertensão, obesidade e diabetes em crianças e adolescentes. O estilo de vida pode resultar em alterações nesses indivíduos quando adultos, afetando o desenvolvimento pós-natal do sistema reprodutor e prejudicando a fertilidade. O estresse oxidativo é o desequilíbrio entre elementos antioxidantes e oxidantes como os radicais livres. Dessa forma, níveis elevados de radicais livres estão envolvidos em diferentes processos patológicos. Alimentos ricos em componentes antioxidantes estão associados com uma melhor qualidade de vida. Pterostilbeno é um componente antioxidante das blueberries e tem demonstrado benefícios terapêuticos em várias doenças. Sendo assim, nós levantamos as hipóteses: se o alto consumo de frutose no período puberal poderia afetar a fase adulta; e se a administração alta de sacarose na fase adulta afeta o sistema reprodutor e o uso de antioxidantes melhora os efeitos gerados. Para isso, ratos Wistar machos (30 dias de idade) foram distribuídos em quatro diferentes grupos: Fr30, que receberam frutose (20%) na água por 30 dias; Re-Fr60, que receberam frutose (20%) por 30 dias e permaneceram por mais 60 dias sem o tratamento; e dois grupos controles C30 e Re-C60, com água ad libitum. No segundo modelo experimental, ratos Wistar machos (60 dias de idade) foram tratados com solução de sacarose (40%) ou água por 150 dias e distribuídos em quatro grupos: grupo Controle; Pterostilbeno (40mg/kg por gavagem); Sacarose (40%) na água; Sacarose (40%) + Pterostilbeno (40mg/kg) por mais 45 dias. Frutose induziu um aumento de túbulos seminíferos anormais com vacúolos epiteliais, degeneração e células imaturas no lúmen, além disso, aumentou a produção diária de espermatozoides (DSP). Após descontinuar o tratamento, a DSP e o número de espermatozoides diminuiu significativamente. Sacarose induziu estresse oxidativo reduzindo túbulos seminíferos normais, células de Leydig e Sertoli e o número de espermatozoides normais. No entanto, o tratamento com pterostilbeno não foi eficiente em reduzir a peroxidação lipídica nos testículos. Nós concluímos que o consumo de frutose em ratos peribuberais leva a alterações no sistema reprodutor observadas na fase adulta; e o tratamento com pterostilbeno não foi eficiente em reduzir os danos oxidativos nos testículos causados pelo alto consumo de sacarose.

Palavras-chave: açúcares; pterostilbeno; testículo; epidídimo; espermatozoide.

ALVARENGA, Daniele Sapede. **Influence of sucrose and fructose consumption on morphology and function of male reproductive system of Wistar rats.** 2020. 91 p. Thesis (Doctorate in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2020.

ABSTRACT

The increase of sugar consumption is responsible for the incidence of hypertension, obesity and diabetes in children and adolescents. Lifestyle can result in changes when these individuals become adults, affecting the postnatal development of the reproductive system and impairing fertility. Oxidative stress is the imbalance between antioxidant elements and oxidants like free radicals. Thus, excess levels of free oxygen radicals have been implicated in different pathological process. Foods rich in antioxidant compounds are associated with better quality of life. Pterostilbene is an antioxidant component of blueberries and have been demonstrated therapeutic benefits in several diseases. Therefore, we raised the hypotheses: if high fructose intake in the pubertal period may affect the adulthood; and if the administration of high sucrose in adulthood affects the reproductive system and the use of antioxidants improves the effects generated. For this, male Wistar rats (30 days old) were assigned to four different groups: Fr30, that received fructose (20%) in water for 30 days; Re-Fr60, that received fructose (20%) for 30 days and resting for more 60 days without treatment; and two control groups C30 and Re-C60 with water ad libitum. In the second experimental design, male Wistar rats were treated with sucrose solution (40%) or water for 150 days and distributed into four groups: Control group, Pterostilbene (40mg/kg) by gavage; Sucrose (40%) in water; Sucrose (40%) + Pterostilbene (40mg/kg) for more 45 days. Fructose induced an increase of abnormal seminiferous tubules with epithelial vacuoles, degeneration and immature cells in the lumen, moreover, increasing daily sperm production (DSP). After discontinuing the treatment, DSP and sperm number decreased significantly. Sucrose induced oxidative stress decreasing normal seminiferous tubules, Leydig and Sertoli cells and normal sperm number. However, pterostilbene treatment was not efficient to reduce lipid peroxidation in testes. We concluded that fructose intake in peripubertal rats led to changes in the reproductive system observed in adulthood; and pterostilbene treatment was not efficient to reduce oxidative damage in the testes caused by high sucrose consumption.

Key words: sugars; pterostilbene; testis; epididymis; sperm.

LISTA DE ABREVIATURAS

BHT	Barreira Hematotesticular
DPN	Dia pó-natal
DSP	Daily sperm production
ERMO	Espécies Reativas do Metabolismo do Oxigênio
FSH	Hormônio Folículo Estimulante
HDL	High Density Lipoproteins
HOMA	Homeostatic Model Assessment
HTF	Human tubal fluid
IBGE	Instituto Brasileiro de Geografia e Estatística
iNOS	Óxido Nítrico Sintase induzível
LH	Hormônio Luteinizante
MDA	Malondialdehyde
NF-κB	Fator Nuclear Kappa B
OMS	Organização Mundial da Saúde
PDN	Postnatal day
POF	Pesquisa Nacional de Orçamentos Familiares
TBARS	Thiobarbituric acid reactive substances
TNF-α	Fator de Necrose Tumoral Alfa

SUMÁRIO

1. INTRODUÇÃO

1.1 SISTEMA REPRODUTOR MASCULINO

O Sistema reprodutor masculino humano, assim como nos ratos, é composto por testículos (gônadas), epidídimos, ductos deferentes, pênis e glândulas assessórias incluindo próstata, vesículas seminais e bulbouretrais (Figura 1A) (JUNQUEIRA, CARNEIRO, 2011; KOMÁREK, *et al*. 2000).

Os testículos, nos humanos, são morfologicamente divididos em lóbulos contendo cerca de 250 a 1000 túbulos seminíferos (Figura 1B), enovelados dentro de um tecido conjuntivo frouxo formando o tecido intersticial. Externamente, os testículos são envolvidos por uma camada de tecido conjuntivo denso, denominada túnica albugínea. Esta possui um papel importante na manutenção dos testículos, a uma temperatura abaixo da temperatura abdominal (Figura 1B) (GOLDSTEIN, SCHLEGEL, 2013; JUNQUEIRA, CARNEIRO, 2011). Em roedores adultos os testículos apresentam menos túbulos (cerca de 20 túbulos seminíferos) e não apresentam lóbulos, além do mais, o tecido intersticial é escasso (FOLEY, 2001).

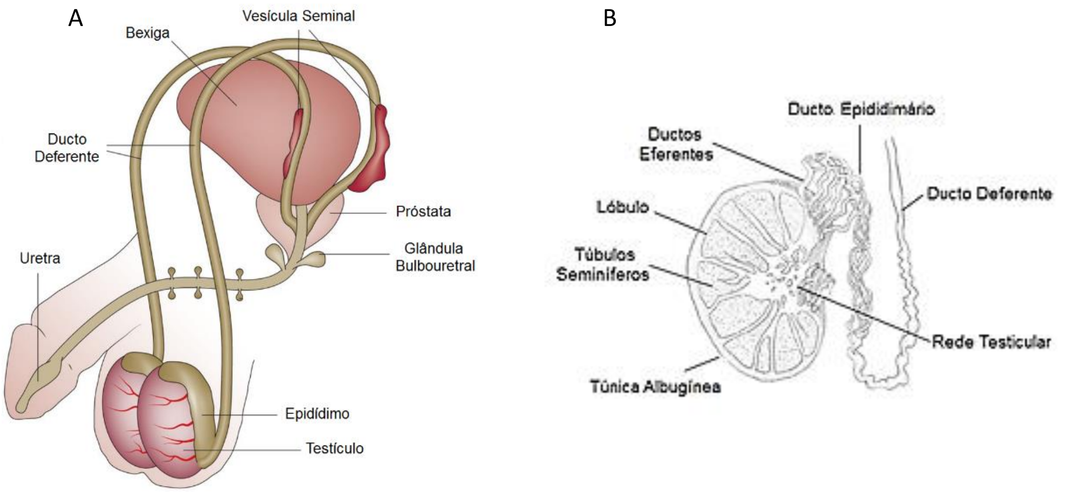

Figura 1. Esquema anatômico do sistema reprodutor masculino. **A** – Representação em humanos com a localização de componentes do sistema genital (adaptado de DRABOVICH *et al*. 2014). **B** – Corte transversal de um testículo, mostrando os lóbulos com os túbulos seminíferos, rede testicular, ductos eferentes, e epididimários (adaptado de GOLDSTEIN, SCHLEGEL, 2013).

Dentre as funções testiculares, destacam-se: produção de gametas (espermatozoides) e a produção do hormônio testosterona (AMORY, BREMNER, 2002; AMANN, 1989). Estas funções são guiadas pelo Sistema Nervoso Central, onde os hormônios Folículo Estimulante (FSH) e Luteinizante (LH) desempenham um papel importante para a atividade normal testicular. Enquanto o FSH age diretamente nas células de Sertoli promovendo a espermatogênese nos túbulos seminíferos, LH age no interstício e estimula a produção de testosterona pelas células de Leydig, sendo essencial para o desenvolvimento de células da linhagem espermatogênica (JUNQUEIRA, CARNEIRO, 2011; AMORY, BREMNER, 2002; CHRISTIAN *et al*., 1983).

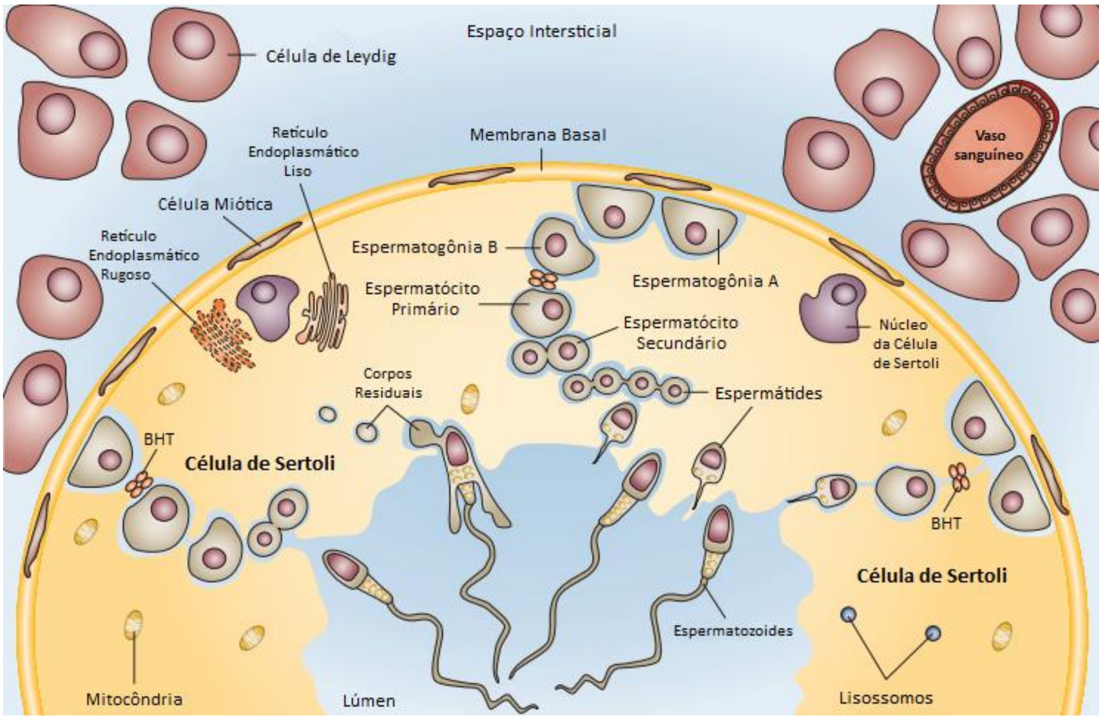

Figura 2. Representação de um túbulo seminífero e fases da espermatogênese. O epitélio seminífero é composto por células de Sertoli e células germinativas em diferentes estágios. As células de Leydig e os vasos sanguíneos estão localizados no interstício. As espermatogônias tipo A dividem-se e desenvolvem-se em espermatogônias do tipo B, que se dividem e diferenciam em espermatócitos primários e tardiamente em espermatócitos secundários. Os espermatozoides formados são liberados no lúmen. BHT – barreira hematotesticular (adaptado de RATO, *et al*. 2012).

As células de Sertoli estão localizadas no interior dos túbulos seminíferos, fornecem suporte estrutural e nutricional para o desenvolvimento das células germinativas. Cada célula de Sertoli pode suportar de 30-50 células germinativas em diferentes estágios da espermatogênese (CHENG *et al*., 2010; FOLEY, 2001; AMANN, 1989). Além da formação da barreira hematotesticular (BHT) (Figura 2), importante para proteger as células em divisão celular contra o sistema imunológico, as células de Sertoli também promovem a fagocitose de células germinativas e a produção de inibina e citocinas (FOLEY, 2001; AMANN, 1989).

A espermatogênese é um evento de sucessivas divisões celulares e citodiferenciação que culmina na formação do espermatozoide. O processo tem início a partir das células germinativas imaturas, que residem ao longo da membrana basal dos túbulos seminíferos e podem originar várias gerações de células-filhas por mitose (MRUK, CHENG, 2015; JUNQUEIRA, CARNEIRO, 2011). As células-filhas, por sua vez, podem continuar se dividindo mantendo-se como células-troco de outras espermatogônias, denominadas espermatogônias do tipo A, ou simplesmente diferenciarem-se em diversos ciclos de divisão celular para se tornarem espermatogônias do tipo B (Figura 2) (MRUK, CHENG, 2015; JUNQUEIRA, CARNEIRO, 2011; AMANN, 1989).

As espermatogônias do tipo B são as células progenitoras dos espermatócitos primários, possuem 46 cromossomos e, por sua vez, entram em divisão meiótica para formar os espermatócitos secundários já com 23 cromossomos. A divisão de cada espermatócito secundário leva a duas células-filhas, as espermátides (Figura 2) (GOLDSTEIN, SCHLEGEL, 2013; JUNQUEIRA, CARNEIRO, 2011).

As espermátides passam por um processo de diferenciação celular, onde ocorre reposicionamento das mitocôndrias, formação do acrossomo, condensação do material genético, formação do flagelo e perda do citoplasma, tornando-as alongadas e maduras (GOLDSTEIN, SCHLEGEL, 2013; JUNQUEIRA, CARNEIRO, 2011; CLERMONT, 1972).

A Espermiação ocorre no final da espermatogênese, onde as espermátides maduras são liberadas no lúmen do túbulo seminífero e assim, passam a ser denominados Espermatozoides (JUNQUEIRA, CARNEIRO, 2011; CLERMONT, 1972).

Embora sejam morfologicamente completos, os espermatozoides ainda são incapazes de fecundar um ovócito e ainda não possuem motilidade (DACHEUX; DACHEUX, 2014). Sendo assim, o epidídimo tem um papel essencial no transporte de espermatozoides, desenvolvimento da motilidade espermática, maturação através das atividades absortivas e secretoras do epitélio (ROBAIRE; HINTON, 2015; GOLDSTEIN, SCHLEGEL, 2013).

Os túbulos seminíferos culminam nos túbulos retos que seguem formando a rede testicular, situada no mediastino. Dessa rede partem os ductos eferentes que gradualmente se convergem formando os ductos epididimários (Figura 1B) (JUNQUEIRA, CARNEIRO, 2011; NISTAL; PANIAGUA, 1984).

O epidídimo nos humanos é composto por 3 regiões, formado por cabeça, corpo e cauda e os ductos epididimários podem chegar a 6 metros de comprimento. Enquanto que nos ratos, o órgão é dividido em segmento inicial, cabeça, corpo e cauda, com ductos de até 3 metros (ROBAIRE; HINTON, 2015; FOLEY, 2001; KOMÁREK, *et al*. 2000; HERMO, 1995; AMANN, 1989).

As primeiras alterações espermáticas ocorrem na cabeça do epidídimo, onde os espermatozoides adquirem a capacidade de se ligar à zona pelúcida do ovócito (DACHEUX; DACHEUX, 2014). Durante a passagem pelo epidídimo, os espermatozoides recebem um repertório de proteínas e enzimas (fatores de crescimento, proteínas-ligantes) que auxiliam na remoção de componentes celulares residuais (ROBAIRE; HINTON, 2015). A motilidade é adquirida pela passagem na região do corpo e cauda do epidídimo (DACHEUX; DACHEUX, 2014). Após a maturação, os espermatozoides são estocados na cauda do epidídimo até o momento da ejaculação (ROBAIRE; HINTON, 2015; AMANN, 1989).

Durante o ato sexual, os espermatozoides deixam o epidídimo e seguem o percurso pelo ducto deferente por peristaltismo até o ducto ejaculatório localizado na próstata (JUNQUEIRA, CARNEIRO, 2011; KOMÁREK, *et al*. 2000). As glândulas acessórias (vesículas seminais, próstata e glândulas bulbouretrais) são responsáveis pela produção do líquido seminal, essencial para o suporte nutricional dos espermatozoides (JUNQUEIRA, CARNEIRO, 2011). O sêmen segue pela uretra até o órgão copulador, o pênis.

O pênis é formado por 3 estruturas cilíndricas denominadas corpos cavernosos, revestidos por uma camada de tecido conjuntivo denso. Um deles localizado ventralmente envolve a uretra, chamado corpo cavernoso da uretra ou corpo esponjoso, se dilata formando a glande. Ao longo da uretra peniana, encontram-se glândulas de Littré secretoras de muco. (JUNQUEIRA, CARNEIRO, 2011; KOMÁREK, *et al*. 2000). Nos roedores, o pênis é formado pelo osso peniano localizado na parte central, que se conecta com a glande (PHILLIPS *et al*., 2015).

1.2 DESENVOLVIMENTO PÓS-NATAL DO SISTEMA REPRODUTOR MASCULINO

Os períodos de desenvolvimento pós-natal dos ratos compreendem as fases neonatal (dia pós-natal – DPN 1-7), infantil (DPN – 8-21), juvenil (DPN – 22-35), peripuberal (DPN 36 – 60) e adulto, onde a maturidade sexual é alcançada

(OJEDA *et al*., 1980). Estas fases são caracterizadas por mudanças hormonais do organismo (DAMGAARD *et al*., 2002). Os andrógenos primários como a androstenediona, 5-α-androstanediol e diidrotestosterona são produzidos entre as fases infantil e juvenil (DPN 8-35) (PODESTÁ; RIVAROLA; JYUJO, 1974). Na fase juvenil, entre os dias pós-natal 28 e 35, as células de Leydig imaturas passam por diferenciação, com pouca ou ausente atividade mitótica e alta síntese de testosterona (BENTON; SHAN; HARDY, 1995).

No epidídimo, entre os períodos infantil e peripuberal (DPN 16-44) as células colunares se diferenciam e passam por um processo de expansão (SUN; FLICKINGER, 1979). Este processo leva a um grande aumento no peso epididimário, entre os dias pós-natal 49 e 63 (SCHEER; ROBAIRE, 1980).

A puberdade é marcada pelo tempo em que a primeira espermatogênese completa todo o ciclo (KLINEFELTER *et al*., 1997). Durante esta fase, ocorre a maturação do eixo hipotalâmico-hipofisário-gonadal, com aumento da síntese e secreção de LH, FSH e de testosterona (OJEDA; SKINNER, 2006). A partir do dia pós-natal 40 é possível encontrar as primeiras espermátides maduras nos testículos, enquanto que os espermatozoides são apenas observados nos epidídimos no DPN 50 (ROBB; AMANN; KILLIAN, 1978). Após atingida a maturidade sexual, a produção máxima de espermatozoides no testículo ocorre a partir do dia pós-natal 75, e a concentração máxima de espermatozoides armazenados na cauda do epidídimo ocorre no DPN 100 (ROBB; AMANN; KILLIAN, 1978; ZANATO *et al*., 1994).

A fase peripuberal representa um período crítico quanto à exposição a agentes tóxicos, uma vez que a maturidade sexual e hormonal ainda não foram definidas e os testículos e epidídimos estão em desenvolvimento (JOHNSON; WELSH, WILKER, 1997). Assim, a exposição a estes agentes durante o período peripuberal podem prejudicar a fase adulta, como efeitos sobre o comportamento e riscos de câncer, alterando definitivamente o organismo (MANTOVANI; FUCIC, 2014). Dessa forma, estudos visando os agravantes ambientais ocorridos na vida fetal ou durante o desenvolvimento pós-natal que podem levar ao surgimento de doenças crônicas ou alterações maléficas vêm ganhando destaque.

1.3 ORIGENS DESENVOLVIMENTISTAS DA SAÚDE E DA DOENÇA (DOHaD)

O conceito DOHad (do inglês "*Developmental origins of health and disease*"), define os modelos de estudos nos quais fatores ambientais e exposição *in utero* ou após o nascimento podem afetar a saúde ou levar a susceptibilidade a doenças para o resto da vida (GAGE; MUNAFO; DAVEY SMITH, 2016).

Em 1976, foi realizado um estudo em uma população masculina, filhos de mulheres expostas a um longo período de escassez alimentar na gestação durante o cerco da Holanda pela Alemanha na Segunda Guerra Mundial. Esses indivíduos na fase adulta, mostraram padrões diferenciados na composição corporal relacionado ao período em que haviam sido expostos durante a vida intrauterina. Os indivíduos expostos no último trimestre da gestação, apresentavam uma baixa incidência de obesidade em relação aos demais indivíduos (RAVELLI; STEIN; SUSSER, 1976).

A hipótese de que exposições intrauterinas ou durante a infância poderiam levar ao desenvolvimento de doenças tardias foi criada por Barker e colaboradores em 1989 em Hertfordshire, Inglaterra. Neste estudo, associaram o peso neonatal e as condições ambientais durante a infância com a condição cardiovascular de indivíduos adultos (BARKER *et al*., 1989). Indivíduos nascidos com baixa massa corporal seriam biologicamente diferentes, persistindo até a fase adulta, apresentando pressão arterial elevada (BARKER *et al*., 2002), e propensão a desenvolver diabetes tipo 2 (ERIKSSON *et al*., 2002).

Desde a década de 1930, cresce o interesse científico sobre o impacto da nutrição no crescimento somático e de órgãos específicos através de manipulações alimentares (JACKSON *et al*., 1932; RUDIN *et al*., 1935). Atualmente é conhecido que a nutrição em períodos vulneráveis do desenvolvimento pode comprometer a estrutura da cromatina e a expressão gênica, assim como influenciar a saúde a longo prazo do indivíduo (MATHERS, 2007).

1.4 CARBOIDRATOS

Os carboidratos encontram-se amplamente distribuídos em plantas e animais, exercendo um importante papel estrutural e metabólico. Os animais podem sintetizar carboidratos a partir de aminoácidos, porém a fonte mais abundante é proveniente das plantas (BENDER, MAYES, 2007).

Estruturalmente, os carboidratos podem ser classificados em: Monossacarídeos, Dissacarídeos, Oligossacarídeos e Polissacarídeos. Os monossacarídeos constituem o tipo mais simples de carboidrato, apresentando como grupo funcional aldeído (aldoses) ou cetona (cetoses), como exemplo a glicose, galactose, ribose e frutose (BENDER, MAYES, 2007; MARZZOCO, TORRES, 2007).

Os dissacarídeos são o grupo formado a partir da condensação de duas unidades de monossacarídeos, tendo como exemplos mais comuns a sacarose (formada por glicose e frutose) e lactose (formada por galactose e glicose) (BENDER, MAYES, 2007; MARZZOCO, TORRES, 2007).

Os oligossacarídeos são representados pela junção de três a dez monossacarídeos, sendo poucos digeridos pelas enzimas digestivas, enquanto os polissacarídeos são polímeros caracterizados por mais de dez ou milhares de unidades, podendo formar cadeias lineares como a celulose ou ramificadas, como o amido ou o glicogênio (BENDER, MAYES, 2007; MARZZOCO, TORRES, 2007).

O amido é o carboidrato mais frequente na dieta dos seres humanos, seguido por sacarose e lactose. Àqueles com sabor adocicado como a glicose, sacarose e frutose são comumente chamados de açúcares (MARZZOCO; TORRES, 2007).

Os açúcares como frutose, glicose e sacarose estão presentes em uma diversidade de alimentos como frutas, legumes, hortaliças, grãos e mel (Tabela 1) (HALLFRISCH, 1990). A sacarose é o açúcar mais popular, conhecido como "açúcar de mesa", proveniente da cana-de-açúcar, enquanto a frutose é predominante em várias frutas, podendo conter de 1% a 2% de seu peso na forma de frutose livre e mais 3% de frutose sob a forma de sacarose (MATTHEWS; PEHRSSON; FARHAT-SABET, 1987).

A frutose vem sendo empregada como adoçante de bebidas industrializadas por ser mais solúvel e doce, constituindo de 4% a 8% de seu peso, cerca de 2 vezes mais doce que a sacarose (HALLFRISCH, 1990).

Segundo a Organização Internacional do Açúcar, a média mundial per capita de consumo aumentou 16% nos últimos 20 anos (GAINO; DA SILVA, 2011). Em um levantamento realizado pela Sucden em 2014, uma multinacional do ramo açucareiro, o Brasil representa o 4º maior consumidor de sacarose do mundo (Ministério da Saúde, 2016).

O açúcar consumido na alimentação brasileira se encontra no açúcar de mesa utilizado no preparo de refeições e bebidas, e também os açúcares adicionados aos alimentos industrializados, como refrigerantes e bebidas prontas para consumo, além do mel, xaropes e sucos de frutas com adição de açúcar. Boa parte dos açúcares está oculto nos alimentos processados, como refeições prontas, temperos, sucos industrializados e refrigerantes. Estes dados foram apresentados na última Pesquisa Nacional de Orçamentos Familiares (POF) realizada no ano de 2008/09 pelo IBGE.

Tabela 1. Percentual médio de frutose, glicose e sacarose nos alimentos *in natura*.

Alimentos	Frutose	Glicose	Sacarose
Frutas			
Maçã	6 – 8,0	1 – 4,0	1 – 5,0
Banana	2 – 4,0	3 – 6,0	6 – 14,0
Cereja	5 – 7,0	5 – 7,0	0,2
Uva	5 – 7,0	5 – 7,0	0,5
Laranja	2 – 3,0	2,3	4 – 7,0
Morango	1 – 3,0	1 – 3,0	1 – 2,0
Melão	2 – 4,0	1 – 2,0	1 – 5,0
Mel	41	34	2
Hortaliças e Vegetais			
Feijão	1 – 1,5	0,5 – 1,0	0,6
Repolho	1,6	1,7	0,4
Alface	0,6	0,5	0,1
Cebola	1	2	1
Batata	< 0,1	< 0,1	< 0,1

*Adaptado de HALLFRISCH, 1990 e BARREIROS *et al*., 2005.

A recomendação da Organização Mundial de Saúde (OMS) é que apenas 10% dos alimentos consumidos por dia venham do açúcar industrializado, mas no Brasil, o consumo chega a 16,3%. O açúcar consumido *in natura* em frutas, verduras, legumes e leite fresco não são considerados (OMS, 2015).

1.5 FATORES NOCIVOS ASSOCIADOS AO CONSUMO DE CARBOIDRATOS

O consumo exagerado de açúcares é alarmante e tem sido alvo de estudos por estar associado com a má qualidade da alimentação, sobrepeso cada vez mais frequente em crianças e adolescentes (DUBOIS *et al*., 2007), obesidade e doenças cardiovasculares (MALIK *et al*., 2010[a]), resistência à insulina (MEYDANLI *et al*., 2018), síndrome metabólica e diabetes tipo 2 (MALIK *et al*., 2010[b]).

A prevalência de diabetes do tipo 2 pode aumentar em até 1,1% pelo excesso de açúcar na alimentação em relação a 0,1% considerando a mesma quantidade energética originada de alimentos orgânicos (BASU *et al*., 2013). O consumo diário de bebidas industrializadas pode aumentar em 26% as chances de desenvolver a doença (MALIK *et al*., 2010[b]).

Além do mais, a incidência de doenças cardiovasculares associadas com a ingestão de açúcares também foi evidenciada (JOHNSON *et al*., 2013; MALIK *et al*., 2010[a]). Pessoas que consomem acima de 10% (acima do recomendado pela OMS) de alimentos com açúcares na dieta (10-24,9%) possuem risco de 30% de mortalidade por doenças cardiovasculares (YANG *et al*., 2014).

O excesso de açúcar também, principalmente frutose, está sendo diretamente relacionado à hipertensão arterial ao invés do sal, que era considerado seu principal causador. Isso se deve ao aumento sérico de insulina, levando a resistência à insulina, aumentando a resistência vascular e aumento da pressão arterial (DINICOLANTONIO; LUCAN, 2014). O aumento da pressão arterial e de triglicerídeos também foi observado em crianças entre 7 e 12 anos pela ingestão alta de açúcar (KELL *et al*., 2014).

No modelo animal, o tratamento com frutose em ratos também levou ao aumento de triglicerídeos e diminuição de HDL. Além do mais, o aumento de glicose sérica, insulina e índice HOMA também foram identificados, sendo um indicador para resistência à insulina (MEYDANLI *et al*., 2018).

1.6 CONSUMO DE CARBOIDRATOS E FERTILIDADE

A fertilidade humana vem diminuindo ao longo do tempo, fator que pode estar associado aos valores socioeconômicos, distúrbios hormonais, idade, agentes tóxicos como álcool ou tabaco (DEYHOUL; MOHAMADDOOST; HOSSEINI, 2017), e hábitos alimentares incluindo o consumo de açúcar e adoçantes artificiais (SETTI *et al*., 2018). Desse modo, a má alimentação pode contribuir negativamente para a fertilidade (SETTI *et al*., 2018; GASKINS; CHAVARRO, 2018).

A obesidade pode diminuir a taxa de fertilização *in vitro*, assim como aumentar a probabilidade de aborto espontâneo (FERREIRA *et al*., 2010). O consumo de bebidas adoçadas artificialmente pode diminuir a formação de blastocisto, implantação e a chance de gravidez (SETTI *et al*., 2018).

O alto consumo de açúcares na gestação pode afetar a fertilidade da prole, como foi visto por Mao e colaboradores (2018) em modelo animal. Esses autores mostraram que a prole masculina de fêmeas de camundongos que receberam excesso de sacarose durante a gestação, teve uma baixa contagem espermática. Quando a prole recebeu uma dieta hipercalórica pós-natal, os efeitos foram mais exacerbados, com diminuição dos níveis séricos de testosterona, diminuição do peso de órgãos, incluindo o epidídimo e supressão de apoptose de espermatogônias.

1.7 FERTILIDADE MASCULINA X CARBOIDRATOS

Nos últimos anos, pesquisas envolvendo fertilidade masculina e hábitos como alimentação vêm ganhando destaque. Estudos mostram que o alto consumo de açúcar está relacionado ao aumento da infertilidade entre homens reduzindo a qualidade do sêmen (GIAHI *et al*., 2016; CHIU *et al*., 2014). A obesidade masculina também pode aumentar o risco de infertilidade através de mecanismos de desregulação endócrina, reduzindo a concentração de LH e FSH e consequentemente prejudicando a espermatogênese (HAMMOUD *et al*., 2008).

Nas pesquisas envolvendo modelo animal, foi relatado que o consumo de sacarina (8,1g/Kg) durante 35 dias levou a uma baixa contagem espermática, aumento na taxa de anormalidade e diminuição na motilidade e viabilidade de espermatozoides em camundongos (GONG *et al*., 2016). Além do mais, foi demonstrado que o alto consumo de sacarose (30%) por 20 semanas pode levar a peroxidação lipídica, detectada no plasma e tecido testicular de ratos (MASEK; STARCEVIC, 2017). O tratamento com a mesma proporção de frutose (30%) por 8 semanas, foi associado à diminuição do diâmetro de túbulos seminíferos, acumulação lipídica em células de Sertoli, dano mitocondrial em células germinativas e anormalidades no acrossomo em células de linhagem espermatogênica em ratos (MEYDANLI *et al*., 2018).

Em outro estudo, também foi observado atrofia, degeneração e necrose em túbulos seminíferos, com perda celular e vacuolização de células de Sertoli em ratos após o tratamento com frutose na mesma concentração e tempo de administração (DOKUMACIOGLU *et al*., 2018).

Degeneração tubular também foi observada após a administração de frutose (20%) por 15 semanas, com inflamação testicular e aumento na expressão de TNF-α, iNOS e NF-κB e diminuição de testosterona (YILDIRIM *et al*., 2018).

Na dieta humana o consumo elevado de açúcares está crescendo de forma alarmante, aumentando os riscos de doenças cardiovasculares, metabólicas e infertilidade, sendo assim, a busca pelo conhecimento a respeito deste consumo devem crescer na mesma proporção. Tendo em vista que muitos fatores são afetados na fertilidade masculina, torna-se interessante investigar novos mecanismos e outros fatores envolvidos para melhorar a qualidade de vida e a saúde pública no geral.

1.8 ESTRESSE OXIDATIVO E ANTIOXIDANTES

O estresse oxidativo é uma condição fisiopatológica causada por um desequilíbrio entre espécies oxidantes e antioxidantes (MELCHIORRI *et al*., 1996). Na mitocôndria ocorre a principal geração de espécies reativas do metabolismo do oxigênio (ERMO) por meio da cadeia transportadora de elétrons (GREEN; BRAND; MURPHY, 2004). As principais espécies reativas de oxigênio são representadas principalmente pelo radical hidroxila ($^{\bullet}OH$), ânion superóxido ($O_2^{\bullet}$), peróxido de hidrogênio (H_2O_2), alcoxila ($LO^{\bullet}$) e peroxila ($LO_2^{\bullet}$) (BARBOSA *et al*., 2010).

A classe dos lipídios é um alvo biológico bastante comum de estresse oxidativo. A peroxidação lipídica é uma reação em cadeia dos ácidos graxos poliinsaturados, gerando radicais livres que alteram a permeabilidade, fluidez e integridade das membranas celulares (MAHATTANATAWEE *et al*., 2006). Um dos produtos da peroxidação lipídica é o malondialdeído (MDA), uma molécula altamente tóxica, cuja interação com ácidos nucleicos já foi sugerido como potencialmente mutagênico (DEL RIO; STEWART; PELLEGRINI, 2005).

O estresse oxidativo pode ser desencadeado por diversos fatores como metais pesados (DONG et al., 1998) ou o uso de etanol (HOEK; PASTORINO, 2002). O consumo elevado de açúcares por exemplo, pode levar ao aumento de estresse oxidativo e dano tecidual vascular (RUIZ-RAMÍREZ, 2013), renal (AMIN; KAMEL; ELTAWAB, 2011) e testicular (MASEK; STARCEVIC, 2017).

O desequilíbrio entre as espécies oxidantes e antioxidantes pode levar à oxidação de macromoléculas como proteínas, lipídios, DNA, e consequente perda de suas funções biológicas, prejudicando o funcionamento celular ou levar a dano tecidual (HALLIWELL; WHITEMAN, 2004; WU *et al*., 2003). Deste modo, o estresse oxidativo está envolvido em diversos processos patológicos (HAINES *et al*., 2012; REYNOLDS *et al*., 2007; AFANAS'EV, 2007), o que aumentou a busca e o uso de compostos antioxidantes em pesquisas científicas na tentativa de minimizar os danos patológicos gerados (COCUZZA et al., 2007; GUPTA *et al*., 2004; CHITRA; MATHUR, 2004).

O sistema de defesa antioxidante tem a função de inibir ou reduzir os danos causados pela ação das ERMO, ou impedir a ação desses, ou ainda, favorecer o reparo e a reconstituição das estruturas biológicas lesadas (KOURY; DONANGELO, 2003; CLARKSON; THOMPSON, 2000). Os elementos antioxidantes podem ser enzimáticos, como exemplo a enzima superóxido dismutase, glutationa peroxidase e catalase (VINCENT; INNES; VINCENT, 2007) ou antioxidantes não-enzimáticos que podem ser adquiridos através da alimentação, como a vitamina A (RODRIGO; GUICHARD; CHARLES, 2007), vitamina C (GUPTA *et al*., 2004), vitamina E (GUPTA *et al*., 2004; CHITRA; MATHUR, 2004), fitoquímicos como resveratrol, catequinas e quercetinas (COVAS, *et al*., 2009) e pterostilbeno (ROUPE *et al*., 2006).

O pterostilbeno é um antioxidante naturalmente encontrado em plantas medicinais e frutas, como uvas e *blueberries*. É também identificado como um composto fenólico e quando administrado oralmente rapidamente é conjugado no trato intestinal de humanos e roedores, ganhando facilmente acesso a circulação sanguínea (ASENSI *et al*., 2011; e MANACH *et al*., 2004) e no fígado é metabolizado, podendo sofrer metilação, glucuronidação e sulfatação (GAO; HU, 2010).

Estudos mostram que o pterostilbeno possui vários benefícios para o tratamento e prevenção de doenças devido a sua capacidade antioxidante, anti-inflamatória e antitumoral (REMSBERG *et al*., 2008; ROUPE *et al*., 2006; RIMANDO *et al*., 2002). Sua capacidade antioxidante consiste na redução do estresse oxidativo por diminuir a produção de espécies reativas de oxigênio como peróxido de hidrogênio e ânion superóxido (ADLY, 2010). Além disso, aumenta a expressão de enzimas antioxidantes como catalase, glutationa, glutationa peroxidase, glutationa redutase e superóxido dismutase (MCFADDEN, 2013), sendo um composto antioxidante em potencial.

2. JUSTIFICATIVA

Nas últimas décadas, o consumo de açúcares foi associado com o aumento de casos de hipertensão, obesidade e diabetes em crianças e adolescentes. Os hábitos alimentares adotados ainda neste período de desenvolvimento pós-natal são cruciais para a constituição e maturidade do organismo do indivíduo na fase adulta. Dessa forma, muitos componentes são adicionados à dieta na tentativa de obter uma melhor qualidade de vida, como os alimentos ricos em antioxidantes.

Considerando estudos na literatura evidenciando a relação do alto consumo de açúcares com alterações no sistema reprodutor e fertilidade nos direciona a investigar a correlação entre a ingestão de açúcares no período puberal e consequências na fase adulta, assim como a administração na fase adulta e o uso de antioxidantes para amenizar os possíveis efeitos no sistema reprodutor.

3. OBJETIVOS

3.1 OBJETIVOS GERAIS

Avaliar o consumo elevado de sacarose ou frutose na morfofisiologia do sistema reprodutor de ratos Wistar.

3.2 OBJETIVOS ESPECÍFICOS

- Experimento frutose:

Investigar as alterações espermáticas de ratos púberes ou adultos após o consumo de frutose durante o período peripuberal.

Avaliar as alterações histológicas no testículo e epidídimo de ratos púberes ou adultos após o consumo de frutose durante o período peripuberal.

- Experimento sacarose:

Investigar as alterações espermáticas de ratos adultos após o consumo de sacarose ou em associação com o antioxidante pterostilbeno.

Avaliar as alterações histológicas no testículo e epidídimo de ratos adultos após o consumo de com sacarose ou tratados com pterostilbeno.

Avaliar se ocorreu peroxidação lipídica após a administração de sacarose e sua relação no testículo e epidídimo após o tratamento com pterostilbeno.

4. PRODUÇÃO LITERÁRIA

4.1 ARTIGO 1

HIGH-FRUCTOSE DIET DURING PUBERTY ALTERS THE SPERM PARAMETERS, TESTOSTERONE CONCENTRATION, AND HISTOPATHOLOGY OF TESTES AND EPIDIDYMIS IN ADULT WISTAR RATS

High-fructose diet during puberty alters the sperm parameters, testosterone concentration, and histopathology of testes and epididymis in adult Wistar rats

Short Title: Fructose at puberty alters fertility in adult rats

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Abstract

The consumption of fructose has increased in children and adolescents and is partially responsible for the high incidence of metabolic diseases. The lifestyle during postnatal development can result in altered metabolic programming, thereby impairing the reproductive system and fertility during adulthood. Therefore, the aim of this study was to evaluate the effect of a high-fructose diet in the male reproductive system of pubertal and adult rats. Male Wistar rats (30-days-old) were assigned to four different groups: Fr30, which received fructose (20%) in water for 30 days and were euthanized at postnatal day (PND) 60; Re-Fr30, which received fructose (20%) for 30 days and were euthanized at PND 120; and two control groups C30 and Re-C30, which received water *ad libitum* and were euthanized at PND 30 and 60, respectively. Fructose induced an increase in abnormal seminiferous tubules with epithelial vacuoles, degeneration, and immature cells in the lumen. Moreover, Fr30 rats showed altered spermatogenesis and daily sperm production (DSP), as well as increased serum testosterone concentrations. After discontinuing high fructose consumption, DSP and sperm number decreased significantly. We observed tissue remodeling in the epididymis, with a reduction in stromal and epithelial compartments that might have influenced sperm motility. Therefore, we concluded that fructose intake in peripubertal rats led to changes in the reproductive system observed both during puberty and adulthood.

Keywords: Fructose diet, male fertility, puberty, developmental origins of health and disease

Introduction

Poor nutritional status can result in a decreased quality of life and influence a variety of effects on the endocrine system[1,2]. In adult rats, malnutrition can reduce the testes, epididymis, and prostate weights, and decrease the serum concentrations of LH, FSH, and testosterone[3-6].

The consumption of sugars has increased considerably during the past years, especially in the developed countries. A commonly consumed added sugar is fructose. Fructose is a monosaccharide and a natural component of fruits and vegetables. It is sweeter than glucose or sucrose; therefore, it is commonly used as a sweetener in corn syrup, soft drinks, fruits, and other derivatives. Its consumption in the form of soft drinks is considered a public health problem[7].

The consumption of fructose that is naturally present in food has beneficial effects and provides energy, while high intake as a sweetener in industrialized products is unhealthy. The increase in consumption of foods with high levels of fructose is considered one of the main factors responsible for development of metabolic syndrome, insulin resistance, obesity, and diabetes in children and adolescents[8-10]. Unlike glucose, the effect of fructose to stimulate insulin secretion from pancreatic β-cells is low, causing a decrease in leptin levels, thereby leading to a high caloric intake favoring weight gain[11-13].

The concept of developmental origins of health and disease (DOHaD) defines the environment *in utero* or during early development that can affect the health and increase the susceptibility to diseases in adulthood[14]. Previous studies by our research group reported that diet caloric restriction[15] or excess intake[16] during adolescence leads to cardiometabolic dysfunction in adult rats. The testis and epididymis mature due to postnatal development of the reproductive system during puberty[17]; therefore, an unhealthy lifestyle can result in alterations in adulthood leading to impaired fertility[18].

Some studies have shown negative correlations between fructose intake and fertility. In female rats, fructose consumption induces changes in the length of the estrous cycle, ovarian, and uterine histology[19]. In adult male rats, a high-fructose diet causes testicular degeneration with increased expression of inflammatory factors[20], destruction of the germinal epithelium, lipid accumulation in Sertoli cells[21], and necrosis and atrophy in the seminiferous tubules[22]. However, the role of a high-fructose diet and its effects on puberty and the reproductive system have not been explored.

Thus, the aim of the present study was to evaluate the influence of fructose intake (20%) on the reproductive system in pubertal rats and investigate the changes that can occur in adulthood even after ceasing consumption.

Methods

Ethics approval

The experimental procedures described in this study were performed in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee on Animal Use of the State University of Maringá (CEUA/UEM protocol no. 5669210917).

Animals and experimental design

Male Wistar rats (n = 60; 25-days-old) were obtained from the Central Animal House of the State University of Maringá. The rats were allowed to acclimatize to their new environment for 5 days. The animals were randomly divided into four experimental groups consisting of 15 animals each: two groups received fructose supplementation (20%) diluted in water for 30 days and were euthanized at postnatal day (PND) 60 (Fr30 group) or PND 120 (recuperation for 60 days without fructose treatment, Re-Fr30 group), while the other two control groups received only the vehicle (water) and were euthanized at PND 60 (C30 group) or PND 120 (Re-C30). Fructose was obtained commercially from Lighsweet Industries (Lowçucar, Paraná, Brazil) and the fructose solution (20%) was prepared as per Jearapong[23] and Yildirim[20]. The rats were weighed and then euthanized by guillotine decapitation, and blood was collected from the ruptured cervical vessels. The reproductive organs were removed and weighed. The testes and epididymis were used for histological analysis and sperm count.

Daily sperm production per testis and transit time in the epididymis

Decapsulated right testis and epididymis were weighed and homogenized as described previously by Robb[24], with the adaptations described previously[25]. After dilution of homogenates, a small aliquot of sample was transferred to a Neubauer chamber (4 fields per animal) for counting homogenization- resistant spermatids (that corresponds to the stage 19 of the spermatogenesis) in the testis, and spermatozoa in epididymis. To calculate the daily sperm production (DSP), the concentration of homogenization resistant spermatids per testis was divided by 6.1, which is the number of days during which mature spermatids remain in the seminiferous epithelium. To calculate sperm transit time through the epididymis, the number of sperm in each epididymal portion was divided by DSP.

Sperm motility

Sperm motility was evaluated using the methods of Siervo[25]. Briefly, the left vas deferens was rinsed with 1.0mL modified human tubal fluid (HTF) medium with gentamicin (Irvine Scientific) at 34°-37°C to obtain spermatozoa. At the same temperature, a Makler counting chamber (Sefi-Medical) was loaded with a 10μL aliquot of the sperm solution prepared previously. Sperm motility was assessed by visual estimation (100 spermatozoa per animal) under a light microscope (Motic) at 100X magnification and was performed by the same person throughout the study. Spermatozoa were classified as motile or immotile.

Sperm morphology

The contents of the vas deferens were removed via internal rinsing with 1.0 mL of 10% formol-saline. Histological slides were prepared from this solution and observed using an Opton photomicroscope (400X magnification). One hundred spermatozoa were analyzed per animal. Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached) and tail abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached). This analysis was performed as described by Fernandes[26].

Histopathological analyses

Testis and epididymis were fixed in methacarn solution (10% acetic acid, 60% methanol and 30% chloroform) and then put in alcohol 70%. Next, were embedded in Paraplast® wax and cut into 5μm sections and stained with haematoxylin and eosin (H&E). The tissue was evaluated using a Motic microscope (Motic®, Richmond, Canada) (100X and 400X magnification). For the testes, random seminiferous tubular sections (100 per animal) in three non-consecutive testis cross-sections were analyzed to detect the presence of abnormalities in these tubules: immature germ cells in the lumen, acidophil cells, vacuoles and tubular degeneration. In the epididymis, histopathological inspection was performed qualitatively in the caput–corpus and cauda of each animal as described by Favareto[27].

Number of Sertoli cells

The number of Sertoli cell nuclei was determined in 20 cross-sections of the seminiferous tubules (stage VII-VIII of spermatogenesis) per testis in each rat, under a light microscope at 400X magnification[28].

Kinetics of spermatogenesis

Random seminiferous tubular sections (100 per animal) in three non-consecutive testis cross-sections were classified into four stages: I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelial cycle, according to Leblond and Clermont[29] using a photomicroscope at 400X magnification[30]. This analysis allows the assessment of the proportion of staging of the seminiferous tubules.

Seminiferous tubule diameters and seminiferous epithelium height

For evaluation of the seminiferous tubule diameter and epithelium height, 40 random testicular cross-sections per animal in stage IX of the seminiferous epithelium cycle were examined. Seminiferous tubule diameters were measured using an Opton photomicroscope (400X magnification) and BELview software (version 6.2.3.0 for Windows). Likewise, the seminiferous epithelium height was measured using the same tubules and methodology as mentioned above. In each seminiferous tubule, the mean of four measures for the diameters and heights was calculated and used in the statistical analysis[27].

Stereology analyses of the epididymis

For stereological analysis of the epididymis, 10 random epididymal cross-sections per animal were captured and analyzed. This analysis was performed by means of Weibel’s multipurpose graticulate, with 168 points was superimposed on the images. By counting the overlapping dots in the seminiferous cord or interstitial compartment, it was possible to establish the respective proportions of each of these components in the epididymis for each experimental group as described by Favareto[27].

Testosterone Assay

Blood samples were collected in heparinized tubes and centrifuged at 2400× *g* for 20 min (4°C). The plasma was separated and stored at -20ºC until the assay. Commercial testosterone ELISA kit (Cayman Chemical, Ann arbor, MI, USA - #582701) was used for testosterone measurement, according manufacturer’s protocol. All samples were included in the same assay to avoid inter-assay errors. The intra-assay coefficient of variation is 4.4%.

Statistical analysis

All parameters were submitted to the Shapiro–Wilk test for normality and thus classified into parametric and nonparametric data. Data were compared using Unpaired t-test or the non-parametric Mann-Whitney test. Differences were considered significant when $p<0.05$. Statistical analyses were performed using GraphPad Prism (version 5.0).

Results

Body weight and weight of the reproductive organs

Body and reproductive organ (testis, epididymis, seminal vesicle, prostate, and vas deferens) weights are shown in Table 1. The Fr30 group had a significant increase in body weight compared to the C30 group ($p = 0.0446$). However, after discontinuing the treatment for 60 days (Re-Fr30), there was a reduction in body ($p = 0.0036$), epididymis ($p = 0.0002$), vas deferens ($p = 0.0013$), and prostate gland ($p = 0.0022$) weights compared with their respective controls.

Sperm morphology and motility

The percentage of abnormal sperms was significantly greater in the Fr30 and Re-Fr30 groups than in the control C30 ($p = 0.0031$) and Re-C30 ($p < 0.0001$) groups, respectively (Fig. 1*a*). The animals that received fructose and underwent a recovery time of 60 days had a significant increase in the percentage of immobile sperms compared to those in the control ($p = 0.0032$) (Fig. 1*b*).

Histopathological analysis, kinetics of spermatogenesis, and Sertoli cell count

Histopathological analysis of the testes from rats treated with fructose revealed a significant increase in the number of abnormal tubules in the Fr30 and Re-Fr30 groups compared with the control C30 ($p = 0.0410$) and Re-C30 ($p = 0.0002$) groups, respectively (Fig. 2*a*). Certain structural changes, such as immature germ cells in the lumen, tubular degeneration, and presence of vacuoles, were observed in the seminiferous tubules (Fig. 3). Moreover, fructose consumption led to a significant reduction in the Sertoli cell number per seminiferous tubule in the animals from the Fr30 and Re-Fr30 groups when compared to the animals from control groups C30 ($p < 0.0001$) and Re-C30 ($p < 0.0001$), respectively (Fig. 2*b*). Analysis of the kinetics of spermatogenesis (Table 2) revealed a significant increase in the number of seminiferous tubules in stages VII–VIII in the Fr30 group compared to the control group ($p = 0.0162$). Conversely, in the same groups, there was a significant reduction in stages IX–XIII ($p = 0.0027$) and XIV ($p = 0.0453$). In the Re-Fr30 group, there was an increase in the number of seminiferous tubules in stages I–VI ($p = 0.0286$) and a reduction in stages VII–VIII ($p = 0.0244$) compared with the control.

Seminiferous tubule diameter and epithelium height in the testis

Morphometric analysis revealed a significant increase in the diameter of seminiferous tubule in the Fr30 group ($p = 0.0130$) and the seminiferous epithelium height in the Re-Fr30 group ($p = 0.0269$) compared with their respective control groups (Table 3).

Stereological analysis

The results of the stereological analysis are presented in Table 4. There was a significant increase in the stromal compartment from the 2A region of the caput epididymis in the Fr30 group compared to the control group ($p = 0.0008$). Fructose treatment (Fr30) increased the luminal compartment from the 5A/B region of the cauda epididymis (p = 0.0005), followed by a decrease in the stromal (p = 0.0075) and epithelial (p = 0.0092) compartments, compared with the control group. Furthermore, after 60 days, a significant increase was observed in the luminal compartment (p < 0.0001), followed by reduction in the stromal (p = 0.0007) and epithelial (p < 0.0001) compartments from the 2A region of the caput epididymis in the Re-Fr30 group compared to the control group. Moreover, in the 5A/B cauda region, fructose treatment caused a significant increase in the luminal compartment in the Re-Fr30 group (p = 0.0087) and a significant decrease in the epithelial compartment when compared to the control (p = 0.0158).

Daily sperm production per testis and transit time in the epididymis

The sperm count parameters are shown in Table 5. Analysis of the sperm parameters showed that fructose treatment increased the daily sperm production (DSP) (p = 0.0024), number of sperm in the testis (absolute and relative) (p = 0.0051 and p = 0.0117, respectively), and caput and corpus of the epididymis (absolute) (p = 0.0114) in the Fr30 group. The sperm transit time through the epididymal caput and corpus remained unchanged. However, the Re-Fr30 group showed a decrease in the number of sperms (p = 0.0002) and DSP (p = 0.0003) in the testis, but the relative number (p = 0.0033) and sperm transit time in the epididymal caput, corpus (p = 0.0345), and cauda (p = 0.0252) were significantly higher than those in the control group.

Plasma testosterone concentrations

The plasma testosterone concentrations increased in the Fr30 group compared to the C30 group (p = 0.0017) but remained unchanged in the Re-C30 and Re-Fr30 groups (Fig. 4).

Discussion

The results from the present study indicate that high fructose consumption during puberty impairs testicular and epididymal development and the quality of male gametes in pubertal and adult life. Therefore, the consumption of sugar as fructose should be avoided during the postnatal developmental phase of the male reproductive system. Poor nutritional status can result in a decreased quality of life and influence a variety of effects on the endocrine system[1,2]. In adult rats, malnutrition can reduce the testes, epididymis, and prostate weights, and decrease the serum concentrations of LH, FSH, and testosterone[3-6].

Fructose is more lipogenic than glucose and can decrease leptin levels and favor weight gain[10,11]. However, ceasing consumption can alter this phenomenon, as shown by the increase in body weight after 30 days of fructose intake, followed by a reduction in the body weight and weight of few reproductive organs after 60 days without sugar consumption. Interestingly, this effect was not observed in the testes.

In humans, Chen[31] explained how the consumption of calories affects the body weight of adults. The authors associated the initial weight gain to a large amount of fructose in beverages that favor fat storage and an increase in lipogenesis. Although fructose is related to weight gain[10,11,32,33], this association was not observed in few studies[20,21].

In the present study, high fructose intake led to an increase in the percentage of abnormal seminiferous tubules, which corroborates the findings of Yildirim[20], Meydanli[21], and Dokumacioglu[22]. Yildirim[20] observed the presence of vacuolar and intratubular degeneration in the testes of Wistar rats (PND 30) treated with fructose (20%) for 15 weeks. The Wistar rats (6–8-weeks-old) treated with fructose (30%) for 8 weeks showed degeneration, necrosis, and atrophy of seminiferous tubules, vacuolization of Sertoli cells, and spermatogonia, as reported by Dokumacioglu[22]. In addition, under the same experimental conditions, Meydanli[21] observed disorganization and destruction of germinal epithelium with immature germ cells in the lumen, accumulation of lipids within the cytoplasm of Sertoli cells, and germ cell apoptosis.

The presence of epithelial vacuoles indicates the absence of Sertoli cells or loss of germ cells. Sertoli cells provide nutritional and structural support to the seminiferous tubules[34,35]. The absence of these cells impairs the structural organization of the epithelium, thereby releasing immature cells in the lumen. In the present study, this impairment was observed even after discontinuing the treatment in the Re-Fr30 group. The decrease in Sertoli cells may signal germ cell apoptosis[36], as well as, changes in the stages of spermatogenesis[35].

In the current study, the increase in the number of seminiferous tubules at stages VII–VIII of spermatogenesis in the Fr30 group is associated with an increase in sperm number and DSP in the testes. This increase in sperm production indicates a decrease in gamete quality since the sperm morphology is impaired after fructose consumption. After 60 days without fructose exposure, the number of seminiferous tubules at stages VII–VIII of spermatogenesis, as well as DSP, reduced significantly, which indicated the loss of gamete quality and persistence of this damage. In this context, it is noted that sperm motility is affected only after the recuperation period, indicating that some imminent factors associated with sperm motility were affected during fructose exposure; however, this damage was not observed upon immediate evaluation. Despite normalization of the testosterone concentrations after 60 days without treatment, the initial damage influenced by fructose in spermatogenesis persisted, thereby decreasing the DSP and sperm number.

The spermatogenic cycle in rats is approximately 58 days[37] and more than 8–10 days for the sperm transit time[38]. Therefore, the spermatozoa were stimulated since the beginning of the spermatogenic cycle, and the influence of fructose consumption on quality of gamete could have been different if the recuperation period was longer.

In the present work, fructose consumption for 30 days led to an increase in the absolute sperm number in the epididymis (caput/corpus) while the transit time and relative sperm number remained unchanged. This result is due to the high amount of sperm produced in the testes that travel to the epididymis with no significant increase in the cauda region. Nevertheless, after 60 days, the reduction in DSP and absolute sperm number in testes and epididymis and the increase in relative number in epididymis (caput and cauda) indicate the negative effects of fructose intake. Therefore, this suggests that the increase in relative sperm number in the caput and cauda of epididymis, in fact, was due to a reduction in the epididymis weight.

In the current study, high fructose intake led to tissue remodeling in the epididymis at puberty and adulthood. Thus, after discontinuing fructose consumption for 60 days, the epididymal alteration remains associated with decreased sperm motility since epididymal epithelial cells produce components for sperm maturation[39].

Based on the results of testosterone concentration in the present study, we inferred that the increase in this hormone in animals from the Fr30 group is related to the damage observed in the sperm, histopathological, and morphometric parameters of the testes and epididymis. Furthermore, despite the normalization of testosterone concentrations after a period of 60 days without treatment (Re-Fr30 group), it is still possible to observe alterations in these parameters when compared to those observed in the Fr30 group. These results show the influence of testosterone on the testicular and epididymal postnatal development since the increase in plasma testosterone levels in the pubertal phase was crucial to cause immediate damage and reprogrammed the characteristics of these tissues in adulthood. Dokumacioglu[22] showed an increase in serum testosterone concentrations caused by high-fructose intake (30%) for 8 weeks in adult male rats. In conclusion, the present study shows the negative effects of high-fructose intake in peripubertal rats, leading to alterations in the spermatozoa, testis, and epididymis in the pubertal and adult periods by increasing testosterone concentrations in the pubertal phase.

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Conflicts of interest

The authors declare no conflicts of interest.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals, Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and has been approved by the institutional Ethic Committee on Animal Use of the State University of Maringá (CEUA/UEM) in the meeting of 12/13/2017.

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Tabela 1

Table 1 - Body and reproductive organs weight

	C30	Fr30	Re-C30	Re-Fr30
Initial bodyweight (g)	87.38 ± 17.90	87.79 ± 18.12	93.64 ± 15.19	94.79 ± 13.49
Final bodyweight (g)	251.5 ± 22.78	269.9 ± 25.18*	393.2 ± 20.25	360.4 ± 32.61**
Testicle (g)	1.34 ± 0.14	1.36 ± 0.09	1.45 ± 0.12	1.44 ± 0.12
Epididymis (g)	0.28 ± 0.032	0.30 ± 0.04	0.56 ± 0.03	0.48 ± 0.05***
Vas deferens (g)	0.06 ± 0.01	0.06 ± 0.01	0.10 ± 0.01	0.08 ± 0.01**
Prostate (g)	0.20 ± 0.04	0.19 ± 0.06	0.43 ± 0.13	0.30 ± 0.06**
Seminal vesicles (g)	0.39 ± 0.12	0.41 ± 0.08	0.75 ± 0.22	0.78 ± 0.18

Values are expressed as mean ± SD, Student t-test (*n* = 15 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. *Significantly different to the respective control ($P<0.05$); **$P<0.01$; ***$P<0.001$.

Figura 1

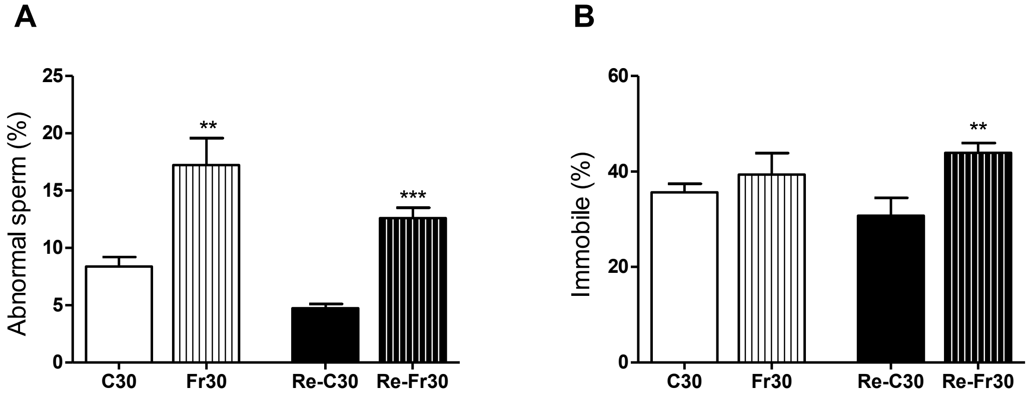

Figure 1. Sperm morphology and motility. (**A**) Percentual of abnormal sperm and (**B**) percentual of immobile sperm. Values are expressed as mean ± SD, Student t-test (*n* = 15 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. *Significantly different to the respective control (P<0.05); **P<0.01; ***P<0.001.

Figura 2

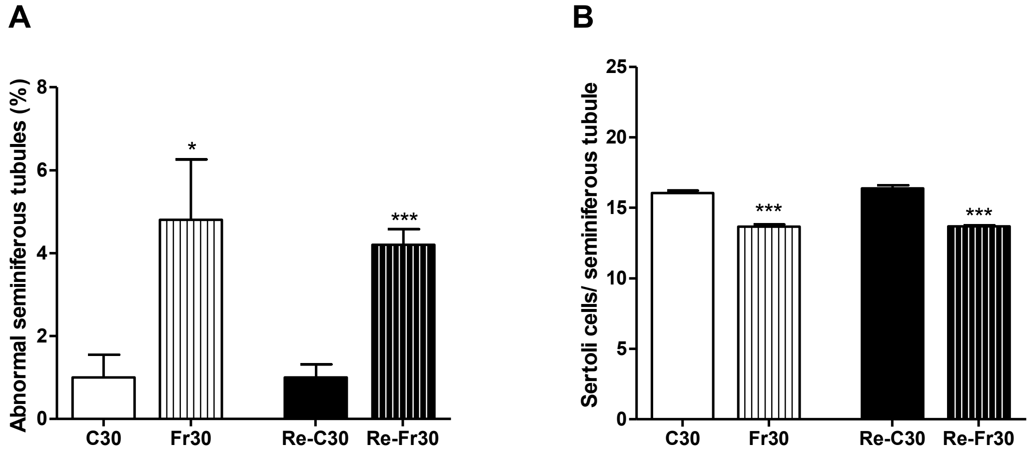

Figure 2. Testicular histopathological analysis and Sertoli cells counts. (**A**) Percentual of abnormal seminiferous tubules and (**B**) number of Sertoli cells per seminiferous tubules. Values are expressed as mean ± SD, Student t-test (*n* = 5 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. *Significantly different to the respective control (P<0.05); ***P<0.001.

Figura 3

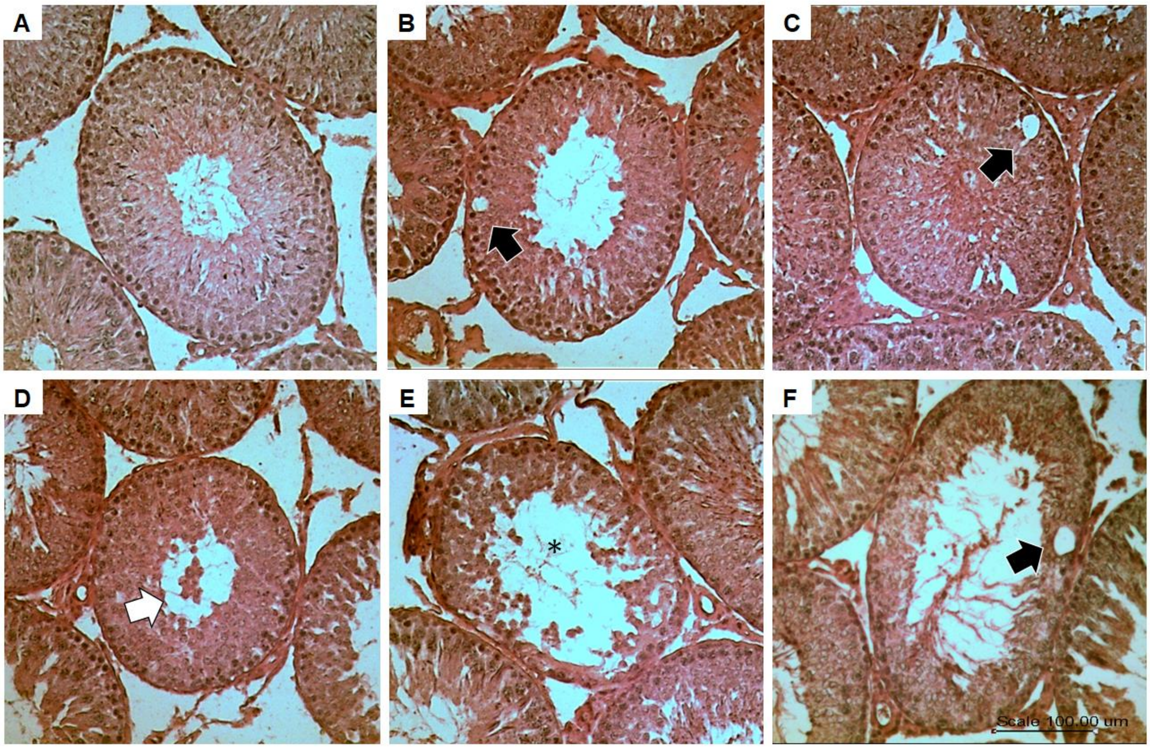

Figure 3. Testicular histopathological analysis. (**A**) C30 group; (**B-D**) Fr30 group, (**E-F**) Re-Fr60 group. Vacuolization (black arrows), immature germ cells in the lumen (white arrow) and epithelial degeneration (asterisks). Haematoxylin and eosin staining (original magnification x100).

Tabela 2

Table 2 – Spermatogenesis kinetics

	C30	Fr30	Re-C30	Re-Fr30
I-VI	28.45 ± 6.10	31.73 ± 4.51	32.43 ± 4.70	38.79 ± 2.50*
VII-VIII	50.83 ± 3.76	59.19 ± 4.87*	59.28 ± 3.73	53.58 ± 2.68*
IX-XIII	18.36 ± 4.50	8.53 ± 2.45**	5.63 ± 1.47	5.56 ± 2.38
XIV	2.35 ± 1.66	0.24 ± 0.48*	2.01 ± 1.22	2.07 ± 0.52

Values are expressed as mean ± SD, Student t-test (*n* = 5 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. *Significantly different to the respective control ($P<0.05$); **$P<0.01$.

Tabela 3

Table 3 – Seminiferous tubule diameter and seminiferous epithelium height

	C30	Fr30	Re-C30	Re-Fr30
Seminiferous tubule diameters (μm)	232.9 ± 18.96	242.2 ± 19.72*	248.5 ± 21.08	254.3 ± 21.11
Seminiferous epithelium height (μm)	66.41 ± 7.95	64.16 ± 5.66	64.31 ± 7.83	67.73 ± 8.09*

Values are expressed as mean ± SD, Student t-test (*n* = 15 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. *Significantly different to the respective control (P<0.05).

Tabela 4

Table 4 - Epididymal stereological analysis

	C30	Fr30	Re-C30	Re-Fr30
Caput (2A region)				
Lumen	58.9 [54.0-64.5]	57.1 [51.6-61.3]	58.3 [54.3-62.9]	66.0 [63.0-68.6]***
Stroma	10.1 [7.1-12.5]	13.0 [10.1-14.8]***	11.3 [9.5-13.6]	8.9 [7.1-11.3]***
Epithelial	30.9 [27.3-33.3]	30.3 [27.3-32.7]	30.3 [25.7-32.7]	24.1 [22.4-26.9]***
Cauda (5A/B region)				
Lumen	55.0 [48.8-61.3]	60.4 [57.4-63.6]***	60.1 [55.5-62.9]	64.2 [59.3-68.7]**
Stroma	16.6 [14.2-22.7]	13.9 [10.7-16.9]**	13.0 [10.1-15.3]	11.9 [8.3-14.8]
Epithelial	28.2 [22.9-31.9]	25.2 [20.6-27.5]**	27.3 23.3-30.2]	23.8 [21.2-27.3]*

Values are expressed as median [Q1-Q3], Mann-Whitney test (*n* = 5 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. *Significantly different to the respective control (P<0.05); **P<0.01; ***P<0.001.

Tabela 5

Table 5 - Sperm parameters

	C30	Fr30	Re-C30	Re-Fr30
Testis				
Sperm absolute number (x10^6)	63.83 ± 6.33	78.54 ± 10.26**	137.4 ± 9.98	117.3 ± 7.34***
Sperm relative number (per gram x10^6)	50.85 ± 7.41	61.74 ± 8.53*	96.46 ± 11.03	82.74 ± 9.44*
Daily sperm production (x10^6)	10.15 ± 1.30	13.37 ± 2.23**	22.52 ± 1.63	18.81 ± 1.73***
Caput /corpus epididymis				
Sperm absolute number (x10^6)	35.15 ± 13.81	50.22 ± 9.09*	73.64 ± 16.61	77.56 ± 9.04
Sperm relative number (per gram x10^6)	295.8 ± 96.84	302.5 ± 52.11	235.5 ± 47.59	302.3 ± 34.63**
Transit time (days)	3.79 ± 1.90	3.79 ± 0.65	3.29 ± 0.80	4.18 ± 0.81*
Cauda epididymis				
Sperm absolute number (x10^6)	33.23 ± 12.94	47.76 ± 17.40	105.0 ± 30.32	113.9 ± 14.31
Sperm relative number (per gram x10^6)	508.1 ± 157.3	462.0 ± 180.8	444.5 ± 118.2	570.6 ± 57.12*
Transit time (days)	3.55 ± 1.85	3.20 ± 1.45	4.69 ± 1.41	6.37 ± 1.57*

Values are expressed as mean ± SD, Student t-test (*n* = 10 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. *Significantly different to the respective control ($P<0.05$); **$P<0.01$; ***$P<0.001$.

Figura 4

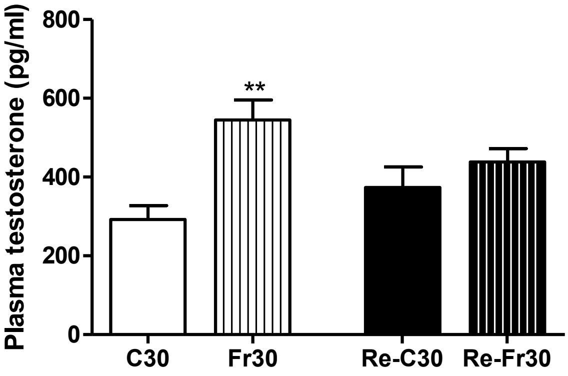

Figure 4. Serum testosterone concentrations. Values are expressed as mean ± SD, Student t-test (*n* = 9 per group). C30 – Control 30 days; Fr30 – Fructose intake for 30 days; Re-C30 – Control recuperation for 60 days; Re-Fr30 – Fructose recuperation for 60 days. **Significantly different to the respective control (P<0.01).

4.2 ARTIGO 2

SUCROSE CONSUMPTION IMPAIRS THE MALE REPRODUCTIVE SYSTEM IN WISTAR RATS AND PTEROSTILBENE TREATMENT PARTIALLY REVERSES THE EFFECTS

Sucrose consumption impairs the male reproductive system in Wistar rats and pterostilbene treatment partially reverses the effects

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Abstract

In recent decades, high sugar consumption has been associated with an increased prevalence of metabolic diseases and impairments in the male reproductive system. High sucrose consumption can induce oxidative damage in tissues, while dietary antioxidants such as pterostilbene can protect cells against oxidative stress. Pterostilbene, an antioxidant component of blueberries, has been demonstrated to exert therapeutic benefits in several conditions. In the present study, we investigated the effects of ingestion of sucrose solution (40%) and pterostilbene treatment (40 mg/kg) on the male reproductive system. We hypothesized that pterostilbene could normalize the changes caused by sugar consumption. Male Wistar rats were distributed into four groups: control, pterostilbene, sucrose, and sucrose + pterostilbene groups. Initially, rats were fed sucrose solution or water for 140 days. Next, the rats were treated with pterostilbene for 45 consecutive days. Pterostilbene treatment did not impair the testes or epididymis. In the testis, sucrose consumption induced oxidative stress, increased the percentual of abnormal seminiferous tubules and abnormal sperm cells, and reduced the testis weight and Leydig cell number. Pterostilbene treatment in the epididymis normalized the number of Leydig cells. Sucrose + pterostilbene treatment caused an increase in the percentual of abnormal sperm cells as well as tissue remodeling in the epididymis. In conclusion, sucrose consumption damaged the epididymis, testes, and sperm cells. Moreover, the testicles and epididymis presented different responses to pterostilbene in association with sucrose intake, and the damage was partially reversed.

Keywords: Sugar, sperm, oxidative stress, testis, epididymis, rats

1. Introduction

In recent years, the consumption of soft drinks, energy beverages, and industrialized juices has increased in parallel with the number of overweight and obese people [1,2]. Overconsumption of sugars is considered one of the main factors responsible for the development of diabetes mellitus type 2 [3], metabolic syndrome [4], hypertension [5] and hepatic disorders [2,6].

As the fertility rate has declined in recent years, eating habits, including the excessive consumption of sugar and artificial sweeteners, deserve deeper investigation [7]. High consumption of sucrose leads to lipid peroxidation and reduced testicular weight in rats [8]. Similarly, fructose solution administration for 8 weeks destroys the germinal epithelium and causes acrosomal abnormalities in spermatogenic cells [9].

Oxidative stress (OS) results from a disbalance between reactive oxygen species (ROS) generation and antioxidant defense, with OS being in favor of the former. ROS are able to react with macromolecules, including proteins, lipids, and DNA [10,11], impairing cell viability, and OS is implicated in many pathological processes [12,13]. Previous studies have shown that OS causes testicular tissue damage and infertility [8,14,15]. Therefore, the use of antioxidants that can prevent or minimize oxidative damage in the spermatozoa and reproductive organs is of great interest [16–18].

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is produced as an active secondary metabolite by plants and occurs naturally in *Vaccinium berries*, grapes, and *Pterocarpus marsupium* [19]. In comparison with resveratrol, pterostilbene is more liposoluble and shows higher biological activity [20]. The antioxidant activity of pterostilbene has been demonstrated in both *in vitro* and *in vivo* models, illustrating its potential preventative and therapeutic benefits [21]. Pterostilbene reduces OS and the production of ROS, such as hydrogen peroxide and superoxide anion [22] and increases the expression of antioxidant enzymes, including catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase [23]. Thus, pterostilbene has antioxidant, anti-inflammatory, anti-obesity, cholesterol-lowering, and antidiabetic therapeutic effects [24,25]. However, studies on the effects of pterostilbene on the male reproductive system and fertility are scarce. Our aim was not to investigate the supplementation of pterostilbene in the human diet, but to evaluate the effects of high sucrose consumption on the testes and epididymis in adult male Wistar rats and determine whether pterostilbene treatment can positively influence the parameters evaluated.

2. Methods and Materials

Ethics approval

The experimental procedures described herein were performed in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Animal Ethics Committee of North of Parana State University (CEUA/UENP protocol no. 4148).

Animals and experimental design

A total of 24 male Wistar rats (60 postnatal days (PND)) were housed in individual cages in an environmentally controlled, clean air room, under a standard temperature (22 ± 3 °C), 12 h light and dark cycles, and a relative humidity of 60 ± 5% at the North of Parana State University (UENP, PR, Brazil). The animals were acclimated to the environment for seven days in polypropylene cages (43 cm × 9.30 cm × 9.15 cm). The experiment was performed in two stages (Figure 1). Initially, 12 animals received filtered water or 40% sucrose solution (corresponding to 0.4 g/mL) *ad libitum* for 140 days. Next, the animals were randomly separated into four groups (n = 6): the control group that received filtered water *ad libitum* and vehicle solution at a volume same as that of pterostilbene daily via oral gavage; the pterostilbene group (Pter) that received water *ad libitum* and 40 mg/kg of pterostilbene daily via oral gavage; the sucrose group

(Sucr) that received 40% sucrose *ad libitum* and vehicle solution same as that for the control group; and the sucrose + pterostilbene group (SucrPter) that received 40% sucrose *ad libitum* and 40 mg/kg of pterostilbene daily via oral gavage. Pterostilbene treatment lasted for 45 consecutive days. The sucrose diet regimen was based on that as used by Souza Cruz *et al*. [2] and Roncal-Jimenez *et al*. [26]. Sucrose was obtained from Camil Alimentos Industries (União, São Paulo, Brazil) and pterostilbene (>98.0% - HPLC) from Tokyo Chemical Industry Co., LTD. Throughout the experimental period, the animals received standard chow (NuvilabCR1®, Nuvilab, Brazil) *ad libitum*. The composition of the standard chow diet is given in Table 1. Sucrose solution was prepared daily and pterostilbene was dissolved daily in a 0.5% methylcellulose solution containing 0.2% Tween 80 solution, which was considered the vehicle solution. At the end of the experimental period, rats were anesthetized with thiopental (2.5%). The total body, testis, epididymis, vas deferens, and prostate weights were measured. The testis and epididymis were used for histopathological and oxidative stress analyses. Spermatozoa isolated from the right vas deferens were used for sperm morphological analysis.

Sperm morphology

Contents of the vas deferens were removed by internal rinsing with 1.0 mL of saline formol 10%. Smears into histological slides were prepared from this solution and observed in a photomicroscope (Opton) at 400x magnification. Two hundred spermatozoa were analyzed per animal. Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached) and tail abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached). This analysis was performed as described by Fernandes *et al*. [27].

Histopathological process

Testis and epididymis were fixed in methacarn solution (10% acetic acid, 60% methanol and 30% chloroform) and then put in alcohol 70%. Next, were embedded in Paraplast wax and cut into 5-mm sections and stained with haematoxylin and eosin. The tissue was evaluated using a Motic microscope (Motic®, Richmond, Canada) (100x and 400x magnification). For the testes, random seminiferous tubular sections (100 per animal) in three non-consecutive testis cross-sections were analyzed to detect the presence of abnormalities in these tubules, such as immature germ cells in the lumen, acidophil cells, vacuoles and tubular degeneration. In the epididymis, histopathological inspection was performed qualitatively in the caput–corpus and cauda of each animal as described by Favareto *et al.* [28]. The analyzes were performed in a blinded fashion.

Sertoli and Leydig cells counts

The number of Sertoli cell nuclei was determined in 20 cross sections of seminiferous cords (stage VII-VIII of spermatogenesis) per testis in each rat under a light microscope at 400x magnification [29]. Leydig cells were counted on interstitial compartment in testis by 10 photomicrographs captured per animal using a light microscope (Leica; 400x magnification) equipped with a digital camera.

Spermatogenic Kinetics

Random tubular sections (100 per animal) in three nonconsecutive testis cross-sections were classified into four categories, namely Stages I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelial cycle, according to Leblond and Clermont [30] and using a photomicroscope at 400x magnification [31].

Seminiferous tubule diameters and seminiferous epithelium height in the testis

For evaluation of the diameter and height of the seminiferous tubules, forty random testicular cross-sections per animal in stage IX of the seminiferous epithelium cycle were examined. Seminiferous tubule diameters were measured using an Opton photomicroscope (400x magnification) and BELview software (version 6.2.3.0 for Windows). Likewise, the seminiferous epithelium height was measured using the same tubules and methodology as mentioned above. In each seminiferous tubule, the mean of four measures for the diameters and heights was calculated and used in the statistical analysis [31].

Stereological analysis

To determine the proportion of epididymal components: (epithelium, stroma and lumen) in each epididymis, 10 photomicrographs were captured per animal using a light microscope (Leica; 400x magnification) equipped with a digital camera. According to the method described by Favareto *et al*. [28], a graticule containing 168 points was superimposed on the images. By counting the overlapping dots in the seminiferous cord or interstitial compartment, it was possible to establish the respective proportions of each of these components in the epididymis for each experimental group.

Malondialdehyde levels and catalase activity in the testes and epididymis

Testes and epididymis samples were homogenized with 0.01 M sodium phosphate buffer (pH 7.4), using L-beader cell disrupter with 3 mm zirconium beads (1.315 x g, 1 min). The homogenate was centrifuged (9.503 x g) for 15 min at 4 °C as described previously by Pinafo *et al*. [32]. Malondialdehyde (MDA) was measured by the thiobarbituric acid reactive substances (TBARS) assay. Briefly, the samples homogenate was prepared with H_2SO_4, 0.05 M, centrifuged in trichloroacetic acid (10%), and then reacted in thiobarbituric acid (0.67%). Catalase activity was determined with phosphate buffer (pH 7.0) in an assay mixture containing 0.019 M hydrogen peroxide and buffer solution in a final volume of 0.3 mL. The samples were read in a spectrophotometer at 535 nm. The MDA content and the catalase activity were normalized, respectively, by the tissue weight and the protein content, which was determined by the Lowry method.

Statistical analysis

According to the normality test evaluation, the data distribution Data were compared using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test or by the non-parametric Kruskal–Wallis test followed by Dunn's post hoc test. Differences were considered significant when P<0.05. Statistical analyses were performed using GraphPad Prism (version 8.0).

3. Results

The body and reproductive organ weight

The sucrose and SucrPter groups showed an increase in body weight compared with that noted in the control and Pter groups (Table 2). The consumption of sucrose (Sucr group) induced a decrease in the testis weight compared with that observed in the control group; however, the other parameters were not altered by different treatments. Data on fat mass as well as liver parameters have been published elsewhere [33]; briefly, sucrose consumption significantly increased the fat depots in rats, and pterostilbene treatment could not mitigate this effect.

Sperm morphology and histopathological analysis

Animals from the sucrose and SucrPter groups showed an increase in the number of abnormal sperm cells compared with the control, Pter, and Sucr groups (Figure 2A). Histopathological analysis of the testes revealed a significant increase in the percentual of abnormal seminiferous tubules in the sucrose group compared with the control and Pter groups (Figure 2B). The most frequently observed abnormalities were head without the characteristic curvature and an isolated form, and broken or isolated tail. In the seminiferous tubules, immature germ cells in the lumen were found to be present, along with degeneration of seminiferous tubules, and presence of vacuoles (Figure 3).

Sertoli and Leydig cells counts

The number of Sertoli cells was not altered (Figure 4A). Sucrose consumption caused a significant reduction in the number of Leydig cells compared with that in the control, Pter, and SucrPter groups (Figure 4B).

Spermatogenic kinetics and seminiferous tubule assessment

Significant differences were not observed in the stages of spermatogenesis (Table 3) or in the seminiferous tubule diameter and epithelium height (Table 4).

Stereological analysis

There was a significant decrease in the luminal compartment of the epididymis caput region in the Sucr group compared with that in the control and Pter groups, as well as an increase in the stromal compartment compared with that in the Pter group (Table 5). In the epididymis cauda, the stromal compartment was reduced and the epithelial compartment was increased in the Sucr and SucrPter groups compared with that in the control and Pter groups.

Malondialdehyde (MDA) levels and catalase activity

The MDA levels in the testis were significantly higher in the Sucr and SucrPter groups than in the control and Pter groups (Table 6). Although a significant difference was not observed in the epididymis, sucrose diet induced an increase of 33.68% in the MDA level compared with that in the control group. The antioxidant activity of catalase was significantly increased in the epididymis of the Pter group, compared with that in the epididymis of the control, sucrose, and SucrPter groups. Moreover, an increase in the enzymatic activity in the testis was noted.

4. Discussion

This study revealed the negative influence of high-sucrose consumption on the testicular and epididymal tissues and demonstrated the effects of pterostilbene in normalizing some of the damages caused by sucrose intake.

Here, the weight gain observed upon high-sucrose consumption corroborates with that noted in previous studies [34–36]. However, the relationship between sucrose intake and weight gain is controversial because some previous studies have not reported weight gain [37–39]. Sucrose can increase leptin and insulin levels, affecting satiety [34]. This event can lead to an increase or decrease in food consumption, which could explain the discrepancy in previous findings. Furthermore, the administration of pterostilbene did not affect body weight, as observed in the SucrPter group.

It is well known that increased oxidative stress can cause testicular tissue damage [14,15]. The abundant presence of polyunsaturated fatty acids in the testes makes them prone to lipid peroxidation induced by ROS action [8]. Therefore, a decrease in antioxidant capacity could have a significant impact on testicular function [40]. Studies have shown the presence of oxidative damage in response to sucrose intake in the vascular [37] and renal tissues [36], but the possibility of OS caused by high consumption of sucrose in the male reproductive system remains undetermined. Pterostilbene and its metabolites have different lipophilicity/hydrophilicity, which may account for the variations in their relative abundance in tissues. In rats, the pterostilbene-4'sulfate is a predominant metabolite, which may be detected in the lungs, heart, liver, kidneys and testes after oral administration [41]. Therefore, the antioxidant capacity may be associated with different metabolites. Our results demonstrated that the effects of sucrose or pterostilbene are tissue-dependent. Pterostilbene treatment was not effective in normalizing lipid peroxidation caused by sucrose intake in the testes, even with increased catalase activity; in contrast, in the epididymis, high sucrose intake did not cause lipid peroxidation. Catalase is an important antioxidant and a crucial heme enzyme responsible for the conversion of hydrogen peroxide into water and molecular oxygen. In the current study, the increased antioxidant activity in the testes of rats from the SucrPter group may indicate an adaptive response to the elevated ROS production caused by sucrose consumption or may be a result of the pterostilbene treatment. Although sucrose consumption did not elevate lipid peroxidation in the epididymis, the antioxidant function of pterostilbene was evidenced by the increase in catalase activity in the Pter and SucrPter groups.

In the present study, OS induced by sucrose intake may have resulted in the reduction in the testicular weight. Similar findings were reported by Masek and Starcevic [8]. This study showed that consuming sucrose solution (30%) over a 20-week period causes lipid peroxidation in the rat testes and alters glucose levels and the testis lipogenesis, with an accentuated reduction in linoleic acid synthesis. Furthermore, this study suggests that this phenomenon may have caused an increase in oxidation. Additionally, the reduction in the testicular weight is related to a reduction in the number of Leydig cells.

Sertoli cells provide structural and nutritional support to germ cells in the seminiferous tubules [42]. The absence of these cells impairs the structural organization of the seminiferous epithelium, releasing immature cells in the lumen. A decreased number of Sertoli cells may trigger germ cell apoptosis [43]. Therefore, in the present study, the presence of vacuoles in the seminiferous epithelium indicated the absence of Sertoli cells or loss of germ cells. In addition, the presence of immature germ cells in the seminiferous lumen and the epithelial vacuoles in the testes of animals exposed to sucrose alone or along with pterostilbene implied that sucrose intake (40% for 45 days) impaired the tubular architecture and pterostilbene did not ameliorate this damage.

In this study, the altered sperm morphology suggests that sucrose overconsumption, alone or in combination with the polyphenol, impaired the spermiogenesis process. Interestingly, pterostilbene treatment after sucrose intake significantly affected sperm morphology compared with that of animals administered with sucrose alone. Treatment with pterostilbene intensified the unwanted effects of sucrose on sperm morphology. These results may be related to the significant increase in the levels of lipid peroxidation observed in the testicles of the Sucr and SucrPter groups.

The spermatogenic cycle in rats is approximately 58 days [44], and it requires more than 8–10 days for sperm transition [45]. In the current study, the spermatozoa were stimulated with pterostilbene for 45 days; therefore, not all gametes were affected since the beginning of the cycle, and the influence of pterostilbene treatment on the quality of spermatozoa could have been different if the period was longer or the treatment began at the same time as sucrose administration.

Although we have associated our results with OS, it is important to note that according to Adekunbi *et al*. [35], sperm of Sprague–Dawley rats administered with sucrose solution (30%) for six weeks exhibited reduced motility and viability. In contrast, changes in MDA levels, even at higher levels, were not statistically significant. In human studies, OS has been shown to increase abnormalities in the sperm middle piece and tail, and sperm motility, suggesting that the effects of OS on the epididymis have led to these alterations [46].

In relation to epididymal stereological analysis, it was possible to observe the region-specific effect of pterostilbene. In the epididymal caput region, pterostilbene was able to protect against the harmful effects of sucrose intake, while this was not observed in the epididymal cauda region. A plausible explanation is that the functions performed by the epididymal head are maintained and those the tail are not. Therefore, all sperm maturation processes that occur in the epididymal head region can be lost when these sperm are stored in the epididymal tail, thereby reducing the sperm quality.

We can still infer that fertility may be affected in the following situations: high consumption of sucrose alone or in combination with pterostilbene.

The data from the present study show that sucrose impairs the male reproductive system and pterostilbene treatment alone did not impair the testes, epididymis, or sperm. However, the reversal of some of the damage by pterostilbene cannot be correlated with OS levels, since the reversal or maintenance of these damages does not depend on the presence of OS. This can be evidenced by the percentage of abnormal seminiferous tubules, the number of Leydig cells, and the proportion of epididymal compartments in the caput region observed in this study.

In conclusion, the present work showed that high-sucrose consumption led to testicular, epididymal, and spermatogenic damages and pterostilbene treatment might reverse some of the damage in the testes and epididymis.

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Figura 1

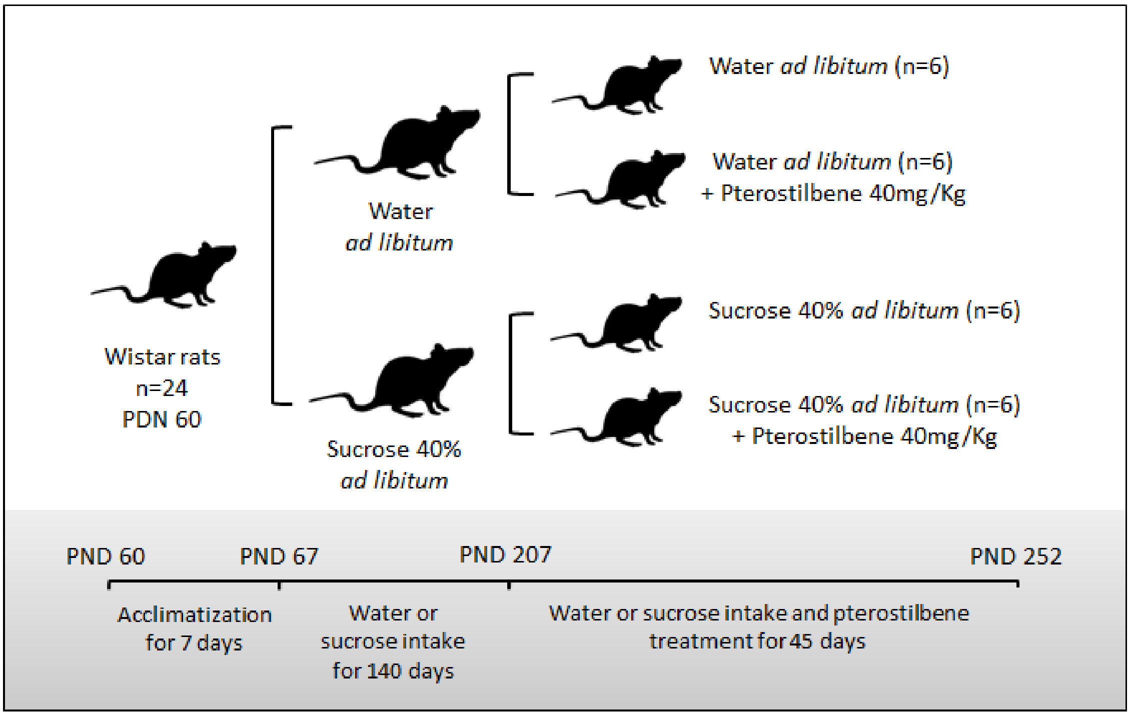

Figure 1. Experimental design. Twenty-four animals were divided and received filtered water or sucrose solution (40%) *ad libitum* for 150 days. Then, were separated in four groups (*n*=6): Control group (water *ad libitum*), Pterostilbene group (water *ad libitum* + 40mg/kg by gavage for 45 consecutive days), Sucrose (40% supplementation in water for 45 days) and Sucrose + Pterostilbene (40% supplementation in water + 40mg/kg by gavage) totalizing 192 days of experiment.

Tabela 1

Table 1 - Ingredient composition of the diet (g/kg)	
Carbohydrates	540
Proteins	220
Minerals*	90
Fibers	70
Lipids	40
Calcium	10 - 14
Vitamins**	--

*Minerals: Sodium: 2,700 mg, Iron 50.00 mg; zinc 60.00 mg; copper 10.00 mg; iodine 2.00 mg; manganese 60.00 mg; selenium 0.05 mg; cobalt 1.50 mg, fluorine (max) 80 mg / kg. **Vitamin A 25,500 IU; vitamin D3 2,100 IU; vitamin E 60.00 IU; vitamin K3 12.50 mg; vitamin B1 14.40 mg; vitamin B2 11.00 mg; vitamin B6 12.00 mg; vitamin B12 60.00 mcg; niacin 60.00 mg; pantothenic acid 112.00 mg; folic acid 6.00 mg; biotin 0.26 mg; choline 2,400 mg. Amino acids: methionine 5,000 mg; Lysine 14 g.

Tabela 2

Table 2 - Body and reproductive organs weight

	Control	Sucrose	Pter	SucrPter	D	T	I
Body weight (g)	433.96 ± 39.62	486.52 ± 59.11 ^{a} **	422.40 ± 34.31 ^{b} **	487.78 ± 42.22 ^{a,b} **	++	ns	ns
Testis (g)	1.63 ± 0.11	1.43 ± 0.05 ^{a} *	1.61 ± 0.11	1.60 ± 0.12	ns	+	ns
Epididymis (g)	0.62 ± 0.03	0.60 ± 0.05	0.60 ± 0.04	0.56 ± 0.05	+	ns	ns
Vas deferens (g)	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.02	0.09 ± 0.01	ns	ns	ns
Prostate (g)	0.32 ± 0.06	0.30 ± 0.06	0.36 ± 0.03	0.34 ± 0.11	ns	ns	ns

Values expressed as mean ± SD, ANOVA followed by Tukey's multiple comparisons test (*n* = 6 per group). ^{a}Significantly different from Control group and ^{b}Sucrose. *P <0.05 and **P<0.01. + P <0.05, ++ P<0.01, for the probability based on analysis of variance. D, effect of sucrose diet; T, effect of pterostilbene treatment; I, interaction between diet and treatment; ns, no significant difference.

Figura 2

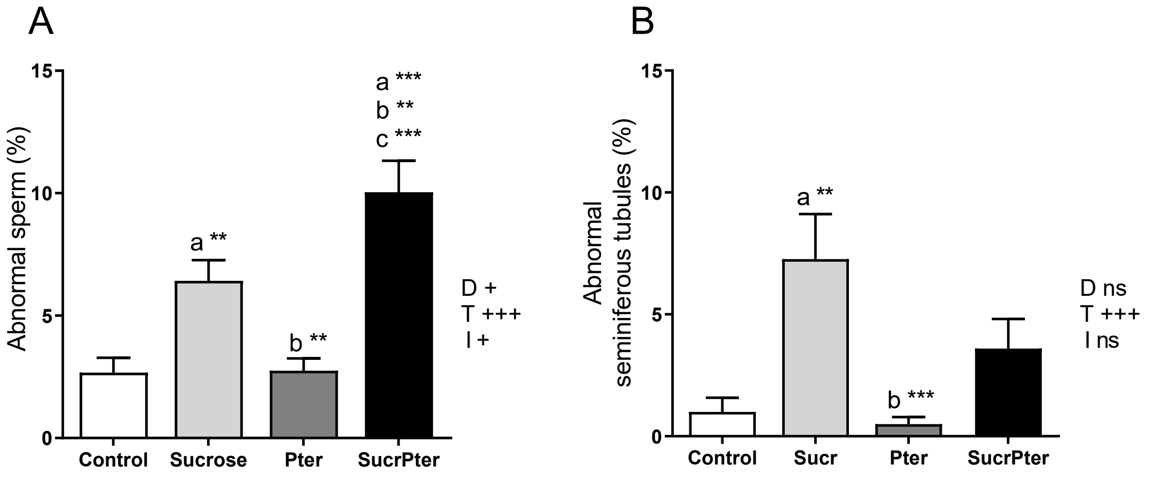

Figure 2. Sperm morphology and histopathological analysis of seminiferous tubules. (**A**) Percentual of abnormal sperm and (**B**) percentual of abnormal seminiferous tubules. Values expressed as mean ± SD, ANOVA followed by Tukey's multiple comparisons test (*n* = 6 per group). ^{a}Significantly different from Control group, ^{b}Sucrose and ^{c}Pterostilbene group. **P<0.01 and ***P < 0.001. + P <0.05, +++ P < 0.001 for the probability based on analysis of variance. D, effect of sucrose diet; T, effect of pterostilbene treatment; I, interaction between diet and treatment; ns, no significant difference.

Figura 3

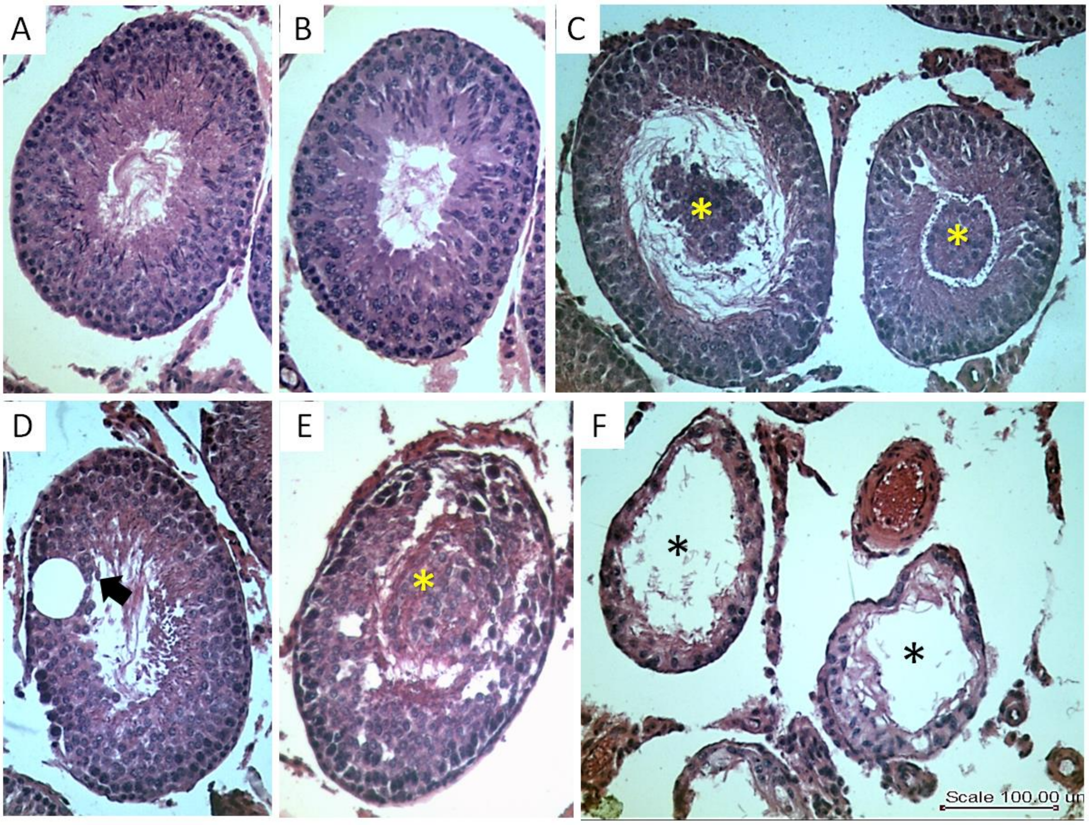

Figure 3. Testicular histopathological analysis. (**A**) Control group, (**B**) Pterostilbene group, (**C** and **D**) Sucrose group, (**E-F**) sucrose and pterostilbene association group. Photomicrographs indicates vacuolization (black arrows), immature germ cells in the lumen (yellow asterisks) and epithelial degeneration (black asterisks). Haematoxylin and eosin staining (original magnification x100).

Figura 4

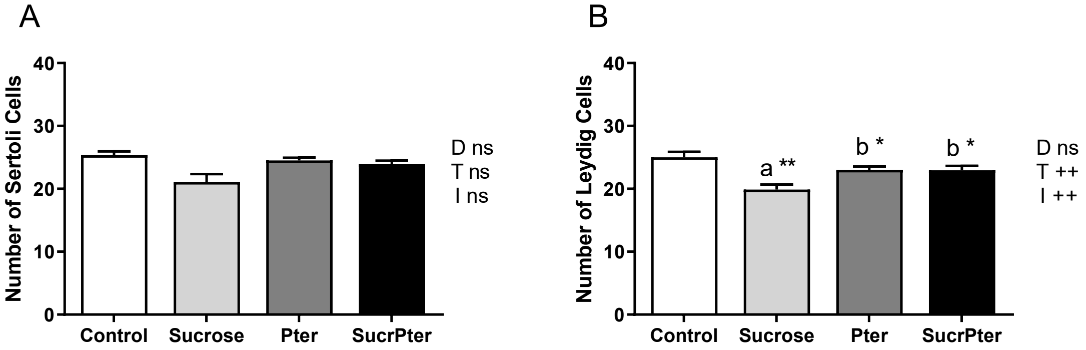

Figure 4. Sertoli and Leydig cells counts. (**A**) Mean of Sertoli cells per seminiferous tubule and (**B**) number of Leydig cells. Values expressed as mean ± SD, ANOVA followed by Tukey's multiple comparisons test (*n* = 6 per group). ^{a}Significantly different from Control and ^{b}Sucrose group. *P <0.05 and **P<0.01. ++ P<0.01 for the probability based on analysis of variance. D, effect of sucrose diet; T, effect of pterostilbene treatment; **I**, interaction between diet and treatment; ns, no significant difference

Tabela 3

Table 3 – Spermatogenesis kinetics

	Control	Sucrose	Pter	SucrPter	D	T	I
I-VI	31.44 ± 8.05	38.99 ± 6.76	38.03 ± 2.30	31.43 ± 3.54	ns	ns	+
VII-VIII	49.68 ± 6.61	41.48 ± 4.08	44.63 ± 3.37	51.56 ± 6.56	ns	ns	+
IX-XIII	15.44 ± 0.96	14.68 ± 4.02	14.41 ± 5.28	13.87 ± 5.69	ns	ns	ns
XIV	3.43 ± 1.92	4.85 ± 0.60	2.92 ± 1.47	3.14 ± 1.95	ns	ns	ns

Values expressed as mean ± SD, ANOVA followed by Tukey's multiple comparisons test (n = 6 per group). + $P < 0.05$ for the probability based on analysis of variance. D, effect of sucrose diet; T, effect of pterostilbene treatment; I, interaction between diet and treatment; ns, no significant difference.

Tabela 4

Table 4 – Seminiferous tubule diameter and seminiferous epithelium height

	Control	Sucrose	Pter	SucrPter	D	T	I
Diameter of seminiferous tubule (μm)	253.5 ± 21.92	247.7 ± 23.85	260.7 ± 23.88	254.4 ± 26.61	ns	ns	ns
Height of seminiferous epithelium (μm)	84.29 ± 7.22	79.14 ± 12.66	78.70 ± 11.99	82.63 ± 13.50	ns	ns	ns

Values expressed as mean ± SD, ANOVA followed by Tukey's multiple comparisons test (*n* = 6 per group). D, effect of sucrose diet; T, effect of pterostilbene treatment; I, interaction between diet and treatment; ns, no significant difference.

Tabela 5

Table 5 - Epididymal stereological analysis

	Control	Sucrose	Pter	SucrPter
Caput (2A region)				
Lumen	54.7 [47.6-61.4]	50.0 [41.5-55.5] ^{a} *	56.8 [50.4-62.5] ^{b} **	53.5 [43.0-59.5]
Stroma	22.3 [15.3-31.5]	26.7 [22.0-34.5]	20.5 [15.3-28.5] ^{b} *	24.1 [18.4-33.9]
Epithelial	22.9 [20.5-26.7]	22.6 [19.426.7]	21.4 [18.3-25.1]	21.4 [18.624.4]
Cauda (5A/B region)				
Lumen	49.7 [44.4-55.3]	53.8 [46.1-57.5]	52.0 [45.3-55.3]	49.1 [44.6-52.9]
Stroma	28.8 [21.8-37.3]	21.4 [16.2-27.3] ^{a} *	26.1 [22.1-33.1]	24.1 [18.0-27.3] ^{a} *
Epithelial	21.7 [17.8-25.1]	25.0 [22.0-28.4] ^{a} *	22.3 [19.0-26.1]	27.6 [23.8-30.2] ^{a,c} ***

Values are expressed as median [Q1-Q3]. Kruskal–Wallis test followed by Dunn's post hoc test (*n* = 6 per group). ^{a}Significantly different from Control group, ^{b}Sucrose and ^{c}Pterostilbene group. *P <0.05, **P<0.01 and ***P < 0.001.

Tabela 6

Table 6 - Malondialdehyde levels and catalase activity

	Control	Sucrose	Pter	SucrPter	D	T	I
Testis							
Malondialdehyde (nmol/mg tissue)	71.03 ± 21.91	121.8 ± 34.75 [a] *	62.32 ± 15.44 [b] *	121.1 ± 29.73 [a,c] *	++	ns	ns
Catalase (μmol/mg protein)	24.40 ± 9.73	24.44 ± 6.96	37.31 ± 17.38	46.14 ± 10.28	ns	++	ns
Epididymis							
Malondialdehyde (nmol/mg tissue)	57.9 ± 16.10	77.4 ± 13.96	62.7 ± 16.97	53.1 ± 17.59	ns	ns	ns
Catalase (μmol/mg protein)	4.06 ±1.23	3.43 ± 0.74	11.63 ± 2.63 [a,b] ***	5.44 ± 2.34 [c] **	++	++	+

Values expressed as mean ± SD, ANOVA followed by Tukey's multiple comparisons test (*n* = 6 per group). [a]Significantly different from Control, [b]Sucrose group and [c]Pterostilbene group. *P <0.05, **P<0.01 and ***P < 0.001. + P <0.05, ++ P<0.01, +++ P < 0.001 for the probability based on analysis of variance. D, effect of sucrose diet; T, effect of pterostilbene treatment; I, interaction between diet and treatment; ns, no significant difference.

5. CONSIDERAÇÕES FINAIS

O presente estudo mostrou que o alto consumo de açúcares causa alterações no sistema reprodutor masculino de ratos Wistar. A administração de frutose durante a puberdade levou a alterações no testículo e epidídimo, observadas na fase adulta mesmo após cessar o consumo, o que nos leva a refletir sobre a má alimentação de crianças e adolescentes e como isso, os prejuízos na saúde destes indivíduos na vida adulta. O alto consumo de sacarose na vida adulta levou ao aumento de peroxidação lipídica no testículo, alteração na morfologia espermática e dos túbulos seminíferos. No entanto, o uso de pterostilbeno não foi eficiente para normalizar os níveis da peroxidação lipídica mesmo com o aumento da atividade da enzima catalase, mas restaurou o número de células de Sertoli e Leydig. Assim, considera-se que o excesso no consumo de açúcares em diferentes fases da vida prejudica a morfofisiologia do sistema reprodutor masculino e que o uso de antioxidantes pode amenizar alguns dos efeitos danosos.

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7. ANEXOS

7.1 Declaração CEUA UENP

UNIVERSIDADE ESTADUAL DO NORTE DO PR
COMITÊ DE ÉTICA NO USO DE ANIMAIS- CEUA UENP
PORTARIA 266/2016

DECLARAÇÃO nº 05/2017

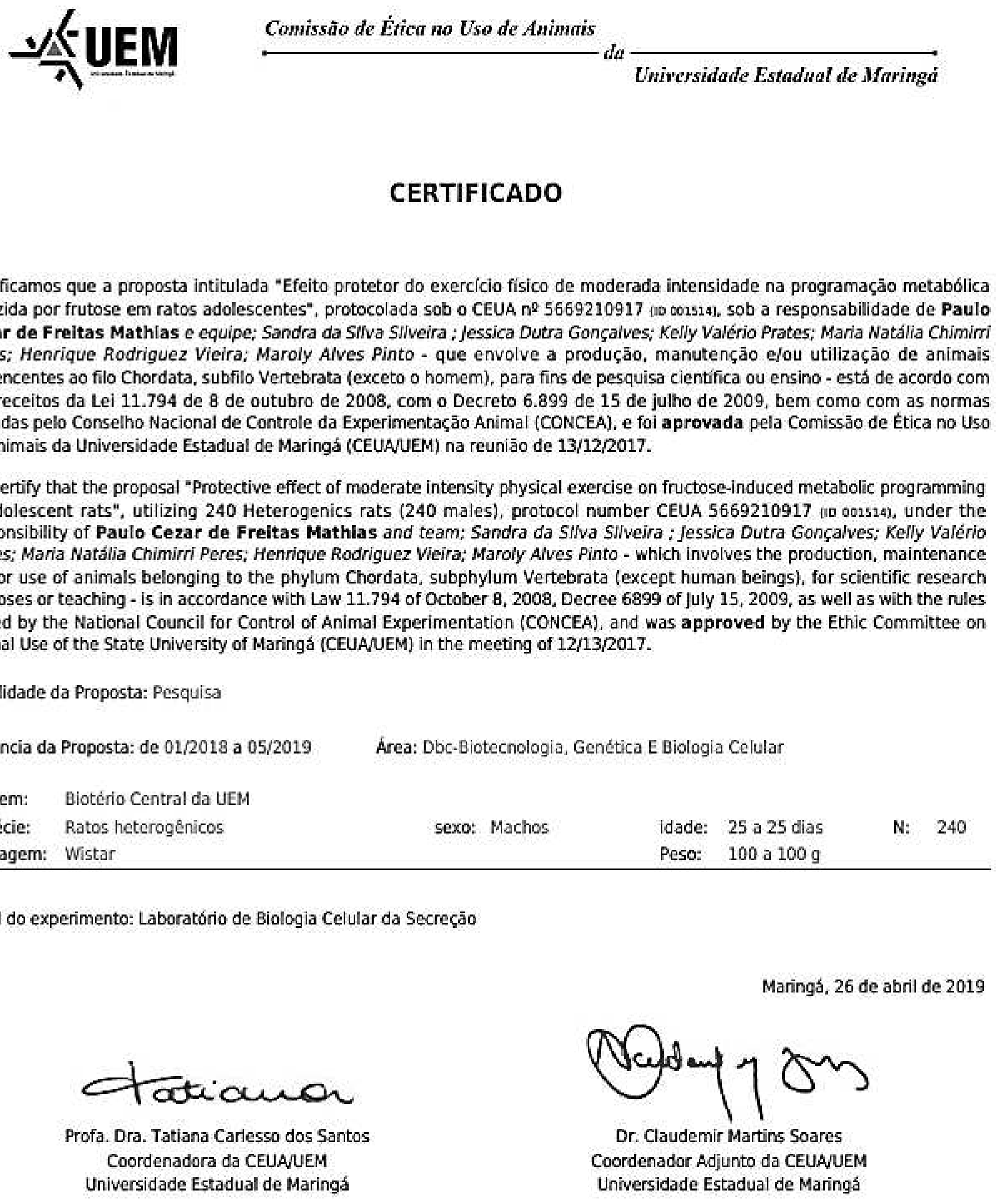
Certificamos que o Projeto registrado no SECAPEE nº 4148 EFEITOS DO PTEROESTILBENO EM RATOS SUBMETIDOS À DIETA RICA EM SACAROSE sob a responsabilidade de Prof. FABIO SEIVA está de acordo com os PRINCÍPIOS ÉTICOS DE EXPERIMENTAÇÃO ANIMAL vigentes na legislação Brasileira e foi aprovado pela Comissão de ética no uso de animais da Universidade Estadual do Norte do Paraná .

(We certify that the research : EFFECTS OF PTEROESTILBENE IN RATS SUBMITTED TO THE RICH DIET IN SACAROSE agrees with Brazilian ethical principles in animal research and was approved by the ETHICAL COMISSION OF UNIVERSIDADE ESTADUAL DO NORTE DO PARANÁ having been approved by its reporters.)

Bandeirantes, 20 de abril de 2017.

Prof. Dra. Mariza Fordellone Rosa Cruz
Coordenação CEUA/UENP

7.2 Certificado CEUA UEM



Comissão de Ética no Uso de Animais
da
Universidade Estadual de Maringá

CERTIFICADO

Certificamos que a proposta intitulada "Efeito protetor do exercício físico de moderada intensidade na programação metabólica induzida por frutose em ratos adolescentes", protocolada sob o CEUA nº 5669210917 (ID 001514), sob a responsabilidade de **Paulo Cezar de Freitas Mathias** *e equipe; Sandra da Silva Silveira ; Jessica Dutra Gonçalves; Kelly Valério Prates; Maria Natália Chimirri Peres; Henrique Rodriguez Vieira; Maroly Alves Pinto* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Maringá (CEUA/UEM) na reunião de 13/12/2017.

We certify that the proposal "Protective effect of moderate intensity physical exercise on fructose-induced metabolic programming in adolescent rats", utilizing 240 Heterogenics rats (240 males), protocol number CEUA 5669210917 (ID 001514), under the responsibility of **Paulo Cezar de Freitas Mathias** *and team; Sandra da Silva Silveira ; Jessica Dutra Gonçalves; Kelly Valério Prates; Maria Natália Chimirri Peres; Henrique Rodriguez Vieira; Maroly Alves Pinto* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the State University of Maringá (CEUA/UEM) in the meeting of 12/13/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 01/2018 a 05/2019

Área: Dbc-Biotecnologia, Genética E Biologia Celular

Origem:	Biotério Central da UEM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	25 a 25 dias	N:	240
Linhagem:	Wistar			Peso:	100 a 100 g		

Local do experimento: Laboratório de Biologia Celular da Secreção

Maringá, 26 de abril de 2019

Tatiana

Profa. Dra. Tatiana Carlesso dos Santos
Coordenadora da CEUA/UEM
Universidade Estadual de Maringá

Dr. Claudemir Martins Soares
Coordenador Adjunto da CEUA/UEM
Universidade Estadual de Maringá