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UNIVERSIDADE ESTADUAL PAULISTA
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PG-BGA

Programação Fetal por Sacarina Sódica: Impacto sobre a saúde materna e na capacidade reprodutiva da prole masculina

Alana Rezende Godoi

Dissertação apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Mestra no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia Celular, Estrutural e Funcional.

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PROGRAMAÇÃO FETAL POR SACARINA SÓDICA: IMPACTO SOBRE A SAÚDE MATERNA E NA CAPACIDADE REPRODUTIVA DA PROLE MASCULINA

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Palavras-chave: Adoçantes; Eficiência reprodutiva; Metabolismo de glicose; Programação fetal; Testículo.

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano de verdade continua misterioso diante de meus olhos”.

(Isaac Newton)

SUMÁRIO

RESUMO

Introdução: A hipótese da “programação fetal” defende que eventos ocorridos durante a vida intrauterina exerçam influência na patogênese de doenças na vida adulta. Fatores ambientais podem programar no indivíduo o surgimento precoce de doenças cardiovasculares e metabólicas. Atualmente, há um aumento no consumo de adoçantes artificiais associados a tratamentos para a perda de peso e no controle do diabetes, sendo a sacarina sódica um dos mais consumidos. Entretanto, durante a gestação e lactação, o uso de sacarina sódica é restrito, por ser permeável a placenta, interagindo com o concepto e, por compor o leite materno. Embora os efeitos do uso de adoçantes sobre o peso corpóreo e o metabolismo sejam bastante conhecidos, não há relatos de pesquisas que relacionam a programação fetal pelo uso de sacarina sódica com o desenvolvimento pós-natal do testículo. Desta forma, o presente estudo visa investigar a influência do uso da sacarina sódica e da glicose na saúde materna e reprodutiva dos descendentes machos. **Material e métodos:** Ratas *Sprague Dawley* foram alimentadas durante a prenhez e lactação com dieta padrão para roedores, água filtrada *ad libitum* e suplementadas com iogurte natural desnatado (Grupo Controle Iogurte, n= 9); iogurte natural desnatado adoçado com solução de glicose (Dinâmica®) a 5% (v/v) (Grupo Glicose, n= 10); iogurte natural desnatado adoçado com solução de sacarina sódica (Dinâmica®) a 0,3% (v/v) (Grupo Sacarina Sódica, n= 10). As dietas líquidas foram preparadas com a adição de 20mL de iogurte natural desnatado (Nestlé®) a 15mL de água filtrada, para ajuste de viscosidade. Durante a prenhez e lactação, Ratas *Sprague Dawley* receberam dieta líquida por 7 dias por semana. Após o desmame, entre o DPN70 aos DPN120, metade da prole recebeu as mesmas dietas durante 5 dias por semana.A outra metade recebeu dieta padrão para roedores, água filtrada *ad libitum*. Após a eutanásia, o fígado, o pâncreas, os testículos e os depósitos de gorduras foram removidos, dissecados e pesados. Os testículos foram processados de acordo com os protocolos histológicos, imunohistoquímicos e de Western Blotting. O desenvolvimento físico, a glicemia, a tolerância à glicose e à insulina, os níveis de enzimas antioxidantes, as concentrações plasmáticas de hormônios esteroides, a morfologia, produção diária e o tempo de trânsito dos espermatozoides também foram analisados. **Resultados:** Capítulo 2: Ratas *Sprague Dawley* expostas à sacarina sódica exibiram aumento na eficiência alimentar, resistência insulínica após o tratamento, diminuição no peso do fígado e aumento no peso do pâncreas e gorduras retroperitoneais. Os descendentes de mães expostas à sacarina sódica apresentaram adiantamento no crescimento de pêlos, atraso na abertura dos olhos, maior distância anogenital no DPN20, baixo peso do fígado e aumento das gorduras epididimárias, retroperitoneias e cardíaca. Capítulo 3: A exposição dos animais à glicose e à sacarina sódica durante a vida intrauterina resultou na descamação do epitélio seminífero. Animais que foram expostos durante a vida fetal e adulta à glicose apresentaram menor coeficiente de eficácia alimentar, menor depósito de gordura, diminuição do espaço luminal e no diâmetro tubular, aumento do espaço intersticial, do fluído testicular, das concentrações plasmáticas de testosterona e estradiol, além da expressão aumentada de AR, maior produção espermática, menor contagem de espermatozoides e menor tempo de trânsito no epidídimo. Os animais suplementados com sacarina sódica demonstraram diminuição no coeficiente de eficácia alimentar e no peso das gorduras retroperitoneais, maior depósito de gordura cardíaca, fluído testicular aumentado, diminuição da concentração plasmática de testosterona, aumento da expressão de AR e da quantificação de PCNA, diminuição da expressão de PCNA, aumento da produção espermática e menor tempo de trânsito na região de cabeça e corpo do epidídimo. **Conclusões:** As respostas adaptativas confirmam a existência de programação fetal por consumo de sacarina sódica e glicose. Essas adaptações foram demonstradas pelas alterações metabólicas e morfofuncionais confirmadas pela: predisposição ao diabetes; interferência da sacarina sódica nas alterações dos níveis plasmáticos de testosterona e estradiol; queda na produção espermática nos animais programados com sacarina sódica e glicose.

Palavras-chave: adoçantes não nutritivos, testículo, metabolismo de glicose, desenvolvimento físico, reprodução.

ABSTRACT

Introduction: The "fetal programming" hypothesis argues that events that occur during intrauterine life have an influence on the pathogenesis of diseases in adulthood. Environmental factors can program in the individual the early onset of cardiovascular and metabolic diseases. Currently, there is an increase in the consumption of artificial sweeteners associated with treatments for weight loss and diabetes control, with sodium saccharin being one of the most consumed. However, during pregnancy and lactation, the use of sodium saccharin is restricted, since it is permeable to the placenta, interacting with the conceptus, and for composing breast milk. Although the effects of the use of sweeteners on the body weight and metabolism are well known, there are no reports of researches which relate fetal programming through the use of sodium saccharin to the postnatal development of the testis. Thus, the present study aims to investigate the influence of the use of sodium saccharin and glucose on the maternal and reproductive health of male offspring. **Material and methods:** Dams Sprague-Dawley rats were fed during pregnancy and lactation with a standard chow for rodents, filtered water *ad libitum* and supplemented with low-fat plain yogurt (Yogurt Control Group, n = 9); low-fat plain yogurt sweetened with 5% (v/v) glucose solution (Dinâmica®) (Glucose Group, n = 10); low-fat plain yogurt sweetened with 0.3% (v/v) sodium saccharin solution (Dinâmica®) (Sodium Saccharin Group, n = 10). The liquid diets were prepared with the addition of 20mL of low-fat plain yogurt (Nestlé®) to 15mL of filtered water, for viscosity adjustment. During the pregnancy and lactation, Dams Sprague-Dawley received a liquid diet during 7 days a week. After weaning, between DPN70 to DPN120, half of the offspring received the same diets during 5 days a week. The other half received a standard diet for rodents, filtered water *ad libitum*. After euthanasia, the liver, pancreas, testis and fat deposits were removed, dissected and weighed. The testes were processed according to histological, immunohistochemical and Western Blotting protocols. Physical development, blood glucose, glucose and insulin tolerance, antioxidant enzyme levels, plasma concentrations of steroid hormones, morphology, daily production and sperm transit time were also analyzed. **Results:** Chapter 2: Dams Sprague-Dawley exposed to sodium saccharin exhibited an increase in feed efficiency, insulin resistance after the treatment, a decrease in liver weight and an increase in the weight of the pancreas and retroperitoneal fats. The offspring of mothers exposed to sodium saccharin showed an advancement in hair growth, delayed opening of the eyes, greater anogenital distance in the DPN20, smaller weight of the liver and increased epididymal, retroperitoneal and cardiac fats. Chapter 3: Exposure of animals to glucose and sodium saccharin during intrauterine life resulted in the desquamation of the seminiferous epithelium. Animals which were exposed during fetal and adult life to glucose had a lower coefficient of feed efficiency, smaller fat deposits, decreased luminal space and tubular diameter, increased interstitial space, increased testicular fluid, decreased testosterone and estradiol concentrations, in addition to increased expression of AR, higher sperm production, lower sperm count and shorter transit time in the epididymis. The animals supplemented with sodium saccharin showed a decrease in the coefficient of feed efficiency and in the weight of retroperitoneal fat, a greater deposit of cardiac fat, increased testicular fluid, a decrease in plasma testosterone concentration, an increase in the AR expression and in the quantification of PCNA, a decrease in expression of PCNA, increased sperm production and shorter transit times in the head and body region of the epididymis.**Conclusions**: Adaptive responses confirm the existence of fetal programming due to the consumption of sodium saccharin and glucose. These adaptations were demonstrated by the metabolic and morphofunctional changes confirmed by a predisposition to diabetes; interference of sodium saccharin in the changes in plasma testosterone and estradiol levels; decline in the sperm production in animals programmed with sodium saccharin and glucose.

Key-words: nonnutritive sweeteners, testis, glucose metabolism, physical development, reproduction.

CAPÍTULO 1

Introdução

A hipótese da “programação fetal”, primeiramente apresentada por David Barker, associa o surgimento de doenças cardiovasculares e metabólicas a uma origem fetal. Os estudos de Baker indicaram que regiões que possuíam altas taxas de mortalidade por doenças cardiovasculares, também apresentavam altas taxas de mortalidade entre os recém-nascidos. Assim, foi defendido que eventos ocorridos durante a vida intrauterina influenciam a patogênese de doenças cardiovasculares e metabólicas durante a vida adulta (BARKER, OSMOND, 1986; BARKER, OSMOND, 1987; BARKER et al., 1989; OSMOND et al., 1993; BARKER, 1994).

A falta ou a deficiência nutricional em períodos críticos do desenvolvimento dos sistemas fisiológicos tem consequências a curto e longo prazo alterando o desenvolvimento de tecidos específicos, modificando a secreção ou a sensibilidade destes tecidos a hormônios ao longo da vida. Nessas condições, há um ajuste ou uma resposta adaptativa que otimiza o crescimento dos principais sistemas orgânicos em detrimento de outros conduzindo o organismo a um metabolismo pós-natal alterado, que é projetado para melhorar a sobrevivência na vida pós-natal em condições de nutrição pobre ou intermitente (MCMILLEN & ROBINSON, 2005).

O *imprinting* metabólico é o termo utilizado para descrever essas respostas adaptativas do indivíduo sob condições nutricionais específicas no início da vida que são, sumariamente, caracterizadas pela susceptibilidade limitada do organismo a uma janela ontogênica crítica e pelo surgimento de efeitos duradouros sobre a estrutura ou função do corpo (MCMILLEN & ROBINSON, 2005). Atualmente, as condições relacianadas à programação fetal são apresentadas como “Origens fetais do desenvolvimento da saúde e da doença”, DOHaD (MCMILLEN, ROBINSON, 2005; RINAUDO, WANG, 2012).

Há trabalhos de pesquisa que observaram que filhos de mães expostas a dietas durante o período gestacional podem apresentar alterações morfofuncionais acentuadas quando expostos ao mesmo estímulo durante o desenvolvimento pós-natal (PENNINGTON et al., 2012; TSOULIS et al., 2016). Fatores ambientais, particularmente a má nutrição materna, podem programar no indivíduo o surgimento precoce de doenças cardiovasculares e metabólicas. Associados à estas doenças crônicas não transmissíveis, observa-se o aumento do risco de desenvolvimento de hipertensão arterial, de hiperlipidemia, do diabetes mellitus tipo 2, da resistência à insulina e da obesidade na vida adulta. Essas alterações físicas, metabólicas e teciduais caracterizam a síndrome metabólica humana (OZANNE, 2001; DRAKE & WALKER, 2004; MCMILLEN & ROBINSON, 2005; MCARDLE, 2006; BARKER, 2007; LEANDRO et al., 2009).

Por sua vez, nota-se um aumento exponencial da incidência da obesidade nos últimos anos, bem como a grande preocupação com relação ao aumento na ingestão de alimentos e bebidas com alto teor de açúcares e a possível associação ao ganho de peso e a instalação da obesidade (GUTHRIE, MORTON, 2000; KANT, 2000; KREBS-SMITH et al., 2000; JOHNSON, FRARY, 2001; KREBS-SMITH, 2001; LUDWIG, PETERSON, GORTMAKER, 2001; COULSTON, JOHNSON, 2002; PUTNAM, ALLSHOUSE, KANTOR, 2002). Dietas ricas em açúcares são capazes de elevar a carga glicêmica, uma das principais causas de obesidade e da incidência de síndrome metabólica (BRAND-MILLER et al., 2002; SCHULZE et al., 2004).

Açúcares simples, como a sacarose e a frutose, são encontrados em muitos produtos industrializados como refrigerantes e sucos. Além disso, seu consumo aumentado na adolescência e vida adulta (DANYLIW et al., 2012; JENSEN et al., 2012; NIKPARTOW et al., 2012) está relacionado ao surgimento e a prevalência da obesidade e do diabetes (MORENO & RODRIGUEZ, 2007; NIKPARTOW et al., 2012).

Neste sentido, com o intuito de auxiliar indivíduos em tratamentos para a perda de peso e no controle do diabetes, observa-se que nos últimos anos, o consumo de adoçantes artificiais, substitutos do açúcar de mesa, aumentou e ganhou popularidade em todo o mundo (CUMMINGS & OVERDUIN, 2007; POLYÁK et al., 2010). No entanto, surpreendentemente, verifica-se na literatura científica especializada relatos de estudos epidemiológicos e clínicos que associam o uso de adoçantes não nutritivos ao aumento de adiposidade (COLDITZ et al., 1990; FOWLER et al., 2008), ao surgimento do diabetes mellitus tipo 2, da síndrome metabólica e de doenças cardiovasculares (DHINGRA et al., 2007; LUTSEY, STEFFEN, & STEVENS, 2008). Parte dessas alterações orgânicas está relacionada ao fato dos adoçantes não nutritivos interferirem nos mecanismos reguladores do apetite promovendo o ganho de peso (ROGERS et al., 1988; POLYÁK et al., 2010; DAVIDSON et al., 2011). Esses adoçantes são pouco calóricos ou efetivamente não calóricos e, normalmente, excedem ao poder adoçante da sacarose (açúcar de mesa) por um fator adoçante entre 30 a 13.000 vezes (WHITEHOUSE, BOULLATA, MCCAULEY, 2008).

Há o entendimento de que o consumo dos adoçantes não nutritivos estimula a ingestão alimentar compensatória em resposta a consequente ausência de calorias associadas aos estímulos gustativos doces. Esta resposta estaria relacionada a percepção do sabor doce que envolve a ação de receptores gustativos doces orais e intestinais que, por meio de impulsos vagais aferentes levam esta informação ao tálamo e ao sistema de recompensa (BERTHOUD, 2002; SARIS, 2003; BELLISLE & DREWNOWSKI, 2007; CUMMINGS & OVERDUIN, 2007; RENWICK & MOLINARY, 2010; SMEETS, ERKNER, & DE GRAAF, 2010; YANG, 2010).

Adoçantes

Os adoçantes são compostos por edulcorantes (substâncias que adoçam) e por um agente de corpo. Grande parte dos adoçantes comercializados combinam dois ou mais edulcorantes. Essa combinação potencializa as propriedades de cada edulcorante e minimiza as desvantagens dos mesmos, principalmente a existência de sabor residual (TORLONI et al., 2007; BRUGNERA, BARUFFI, PANATTO, 2012).

Os agentes de corpo constituem substâncias derivadas do álcool ou do amido e sua associação com os adoçantes ajuda a disfarçar o sabor residual dos edulcorantes, melhorando a palatabilidade do produto final. Os agentes de corpo ainda inibem a cristalização, além de serem espessantes e anticongelantes (TORLONI et. al., 2007).

Os agentes de corpo derivados do álcool são chamados de polióis. Os mais usados são: manitol, sorbitol, xilitol, eritrol, lactilol, isomalte e maltilol. Os derivados do amido são carboidratos naturais sendo a lactose, a frutose, a maltodextrina, a dextrina e o açúcar invertido os mais utilizados (TORLONI et. al., 2007).

A Agência Regulatória de Drogas e Alimentos dos Estados Unidos da América (*U.S. Food and Drug Administration* - FDA) apresenta uma lista de adoçantes nutritivos e não nutritivos de alta intensidade, classificados como reconhecidamente seguros, são eles: a sacarina, o aspartame, o acessulfame-potássio, a sucralose, o neotame, o advantame, a estévia (*Stevia rebaudiana*) e o fruto monge (*Luo Han Guo fruit extracts ou monk fruit*) (SHARMA et al., 2016; FDA, 2020).

Dos adoçantes não nutritivos, a sacarina sódica é um dos mais consumidos em todo o mundo (Figura 1). A sacarina sódica, descoberta em 1878, foi o primeiro adoçante não nutritivo comercializado. Amplamente utilizada como substituto ao açúcar em dietas para diabéticos, entre os anos de 1970 a 1981, a sacarina sódica foi o único adoçante disponível nos Estados Unidos (KROGER, MEISTER, KAVA, 2006). Atualmente, o seu uso é destinado para adoçar vários produtos como refrigerantes, pães, geleias, goma de mascar, frutas em conserva, doces, coberturas de sobremesa e saladas, bem como produtos cosméticos (creme dental e enxaguante bucal), vitaminas e medicamentos (WHITEHOUSE, BOULLATA, MCCAULEY, 2008). Devido ao seu sabor amargo, passou a ser associada a outros edulcorantes, principalmente após a descoberta do ciclamato de sódio em 1950 (TORLONI et al., 2007).

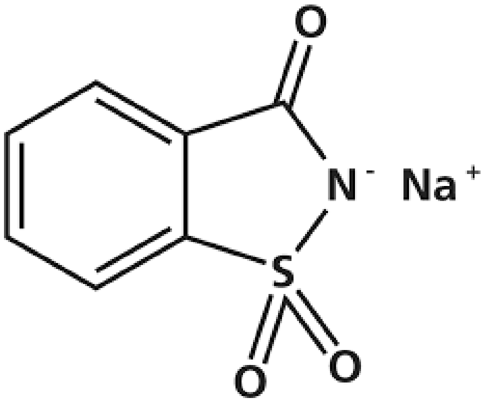

Figura 1: Estrutura química da sacarina sódica.

Em 1958, com a aprovação da *Emenda de Aditivos Alimentares* à Lei Federal de Alimentos, Medicamentos e Cosméticos (*Federal Food, Drug and Cosmetic* – FDC), inciou-se o processo de regulamentação do uso de sacarina sódica. Embora considerada como uma substância reconhecidamente segura, em 1972 estudos associaram o seu uso ao surgimento de câncer de bexiga em ratos (TISDEL et al., 1974; TAYLOR et al., 1980; SCHOENIG et al., 1985). Dessa forma, a FDA removeu a sacarina sódica da lista de substâncias reconhecidamente segura. Mesmo assim, este aditivo dietético nunca foi retirado do mercado (KROGER, MEISTER, KAVA, 2006).

Após ameaças da FDA em proibir o consumo de sacarina sódica, foi aprovada uma lei que determinou a adoção de etiquetas de advertência em embalagens de alimentos e bebidas que continham sacarina sódica. Outros estudos em animais somados à relatos de caso não haver associações entre o consumo de sacarina sódica e o risco de câncer de bexiga em humanos (MORGAN, WONG, 1985; ELCOCK, MORGAN, 1993). Dessa forma, a sacarina sódica foi removida da lista de compostos cancerígenos e, uma nova legislação determinou a retirada dos avisos nos rótulos de produtos alimentares (KROGER, MEISTER, KAVA, 2006).

Atualmente, a FDA considera sacarina sódica um composto seguro, pois é excretada pelos rins de maneira inalterada (WHITEHOUSE, BOULLATA, MCCAULEY, 2008). A FDA recomenda para humanos (adultos ou crianças) a ingestão máxima de 15mg/kg corpóreo de sacarina sódica como dose diária aceitável (IDA) (FDA, 2020). Entretanto, a Agência Nacional de Vigilância Sanitária (ANVISA, Brasil), indica que a IDA de sacarina sódica deve ser 0,08g/100mL (líquido) e 0,50g/100g (sólido) (Resolução RDC n°24, de 15 de fevereiro de 2005).

Durante o período gestacional, indica-se a restrição ao seu uso, pois a placenta é permeável a sacarina sódica, podendo interagir com o concepto, permanecendo assim nos tecidos fetais devido a sua menor capacidade de excreção. Esta também pode compor o leite materno se ingerida durante a lactação (BRUGNERA, BARUFFI, PANATTO, 2012).

Embora os efeitos do uso de adoçantes sobre o peso corpóreo e sobre o metabolismo sejam bastante conhecidos, não há relatos de pesquisas que relacionam a programação fetal pelo uso destes adoçantes, incluindo a sacarina sódica, com o desenvolvimento pós-natal do testículo.

Estrutura e função do testículo

No desenvolvimento de parte do sistema genital masculino é importante considerar a existência de uma janela de “programação fetal masculinizante” (MPW), que nos ratos está compreendida entre o 15,5 – 18,5 dias pós-concepção. Neste período, os andrógenos atuam na formação, na proliferação e no crescimento normal dos órgãos e estruturas masculinas (Figura 1). A atuação dos andrógenos contínua ao longo da vida reprodutiva dos machos. Em humanos e em primatas não-humanos (PRAHALADA et al., 1997; HERMAN et al, 2000) a MPW está compreendida entre a 8ª e 14ª semana de gestação (WELSH et al., 2008) (Figura 2).

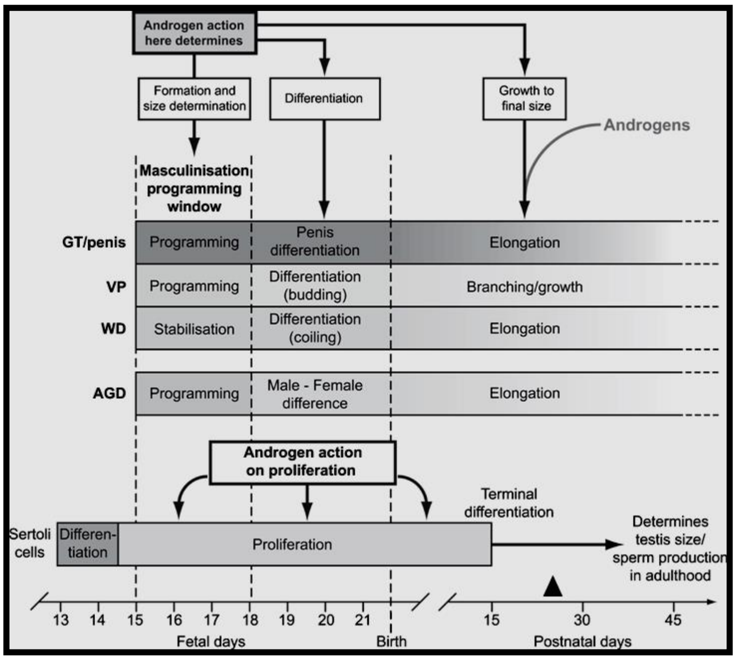

Figura 2: Esquema sobre a atuação dos andrógenos (tempo-dependente) na determinação da diferenciação, formação e crescimento dos órgãos do sistema genital masculino. Em ratos, a MPW ocorre entre 15,5 e 18,5 dias pós-concepção. Note que, nos períodos da MPW e pós-natal, os andrógenos atuam no desenvolvimento dos órgãos. GT, tubérculo genital; VP, próstata ventral; WD, duto de Wolff; AGD, distância anogenital (WELSH et al., 2008).

Os testículos se originam da crista genital, que sob estímulo adequado promove a programação de genes, a organização e a regulação celular distinta (SVIGEN AND KOOPMAN, 2013).

Sabe-se que o gene *Sry* é o fator determinante para o desenvolvimento do testículo em mamíferos, a partir da crista genital (GUBBAY, et al, 1990; SINCLAIR, et al.,1990; KOOPMAN, et al., 1991). De acordo com pesquisadores, o testículo é formado a partir da associação de células precursoras inatas e de células migrantes, incluindo as células germinativas. Entretanto, a diferenciação das linhagens de células que formam o testículo não ocorre de forma independente, mas segue através da diferenciação das células de Sertoli, que em seguida orquestra o comportamento de todos os outros tipos de linhagens de células do testículo (BURGOYNE, et al. 1995).

No rato, ao redor do 12º dia pós-natal (DPN), observa-se que o desenvolvimento dos túbulos seminíferos inicia quando as células de Sertoli fetais e as células mióides peritubulares cercam as células germinativas masculinas primordiais, determinando a formação de cordões sexuais primitivos e a migração de células germinativas primordiais para eles (SHARPE et al., 2003). Posteriormente ao período de diferenciação das células de Sertoli, as células de Leydig fetais e as células mióides peritubulares se diferenciam e ocorre a formação dos compartimentos do testículo com o estabelecimento dos túbulos testiculares e do espaço intersticial. Os túbulos testiculares, uma vez formados, constituem-se de células germinativas em mitose e ao seu redor as células de Sertoli. Os túbulos também apresentam uma camada de células mióides peritubulares e matriz extracelular que dá suporte estrutural (SVINGEN & KOOPMAN, 2013). Para proteger o testículo que está em desenvolvimento e estender essa proteção pós-natal, ainda na fase fetal, é formada a túnica albugínea que se constitui de membrana basal fibrosa que envolve o testículo. Além de proteção, a túnica contribui para cessar a migração das células extragonadais colaborando para a diferenciação precoce do testículo (KARL & KARPEL, 1998, citado por SVINGEN & KOOPMAN, 2013).

O desenvolvimento pós-natal dos testículos de ratos inclui vários estágios, com início no dia do nascimento, dia zero, até 70 DPN. Os estágios são: neonatal (0 a 7 DPN), infantil (8 a 20 DPN), juvenil (21 a 32 DPN), peripuberal (33 a 55 DPN) e “adolescência” (pós-puberal/adulto jovem) (56 a 70 DPN). A partir de 70 DPN, os ratos machos são considerados sexualmente adultos (PICUT, et al. 2014).

Desenvolvimento do testículo e Síndrome da Disgenesia Testicular (TDS)

Distúrbios na saúde reprodutiva masculina, em resposta à níveis anormais de andrógenos na MPW, podem se manifestar no nascimento (criptorquidia e hipóspadia) ou até mesmo na idade adulta jovem (câncer de células germinativas e baixa contagem de espermatozoides) compreendendo a Síndrome da Disgenesia Testicular (TDS) (SCOTT et al., 2008; SHARPE & SKAKKEBAEK, 2008; MACLEOD et al., 2010).

A incidência destas desordens muda conforme a região geográfica, afetando parte população do Ocidente, particularmente os caucasianos, sendo relacionadas à fatores ambientais e/ou estilo de vida. Além disto, têm-se notado que em regiões que possuem baixa ocorrência de casos, o desenvolvimento testicular é mais rápido quando comparado a locais de alta incidência de casos de TDS, indicando que os distúrbios podem ter origem na vida fetal (SHARPE & SKAKKEBAEK, 2008).

Sendo assim, o desenvolvimento anormal do testículo, que por sua vez pode possuir diversas causas primárias, leva, secundariamente, a uma disfunção das células de Leydig e de Sertoli, aumentando as chances de evolução das desordens que compreendem a TDS (SCOTT et al., 2008; SHARPE & SKAKKEBAEK, 2008; MACLEOD et al., 2010).

Justificativa e Objetivos Gerais

O excesso de gordura corporal que resulta no alto índice de massa corporal, característico da obesidade, está associado a diminuição na eficiência reprodutiva de homens (JENSEN et al., 2004; SALLMÉN et al., 2006; NGUYEN et al., 2007; RAMLAU-HANSEN et al., 2007; CHAVARRO et al., 2010). Esta condição é caracterizada por maiores índices de espermatozoides com morfologia anormal (ANDERSEN et al., 2015), maiores danos no DNA (SERMONDADE et al., 2013) e menor contagem de espermatozoides (CHAVARRO et al., 2010).

Além disso, está definido que a obesidade e o diabetes mellitus tipo 2 paterno é capaz de alterar o desenvolvimento e metabolismo embrionário (BINDER, MITCHELL, GARDNER, 2012; MITCHELL, BAKOS, LANE, 2011), a expressão gênica placentária, o *status* de metilação do DNA (BINDER et al., 2015), a qualidade do sêmen (NAVYA, YAJURVEDI, 2017) e a capacidade da fertilidade da prole masculina (FULLSTON et al., 2012). Neste mesmo sentido, a obesidade e o diabetes materno é capaz de afetar negativamente o metabolismo da prole masculina (ROONEY, OZANNE, 2011; TAMASHIRO, MORAN, 2010; WILLIAMS et al., 2014) e o epigenoma espermático da prole adulta (GE et al., 2014; DING et al., 2012), podendo influenciar assim sua eficiência reprodutiva.

Dessa forma, considerando que os altos consumos do açúcar de mesa e da sacarina sódica podem alterar a glicemia, modificar o metabolismo materno e causar efeitos danosos à saúde reprodutiva, o presente estudo propõe investigar o consumo de glicose e sacarina sódica durante a gestação e lactação e suas implicações sobre o metabolismo materno, a estrutura, função do testículo e o potencial de fertilidade dos descendentes machos.

Objetivos específicos

- Avaliar a saúde materna por meio do comportamento alimentar e do metabolismo de glicose;
- Avaliar a saúde reprodutiva dos descentes machos adultos aferindo o desenvolvimento físico, o metabolismo de glicose, a estrutura e função do testículo, bem como a eficiência reprodutiva.

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CAPÍTULO 2

Title of the manuscript: *Adaptive responses to the intake of sodium saccharin during gestation and lactation: Observations in the maternal and male offspring rats metabolism.*

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Abstract

Background: This study evaluated the influence of the intake of sodium saccharin during gestation and lactation on the maternal and male offspring rats metabolism.

Method: Dam Sprague-Dawley rats were randomly divided into two experimental groups: control (C) and saccharin (S). The saccharin dam rats received liquid diets prepared with 20 mL of plain yogurt, 15 mL filtered water and 0.3% sodium saccharin during pregnancy and lactation. The C group received diet liquid with 20 mL of plain yogurt and 15 mL of filtered water. In addition to the yogurt diet, all dam rats received standard chow and water *ad libitum* during the experimental protocol. After the weaning, the dam rats and male pups were sacrificed, and then the pancreas, liver, retroperitoneal, pericardial and visceral white adipose tissues were dissected and weighed. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed during pregnancy. Pregnancy duration, litter size and sex ratio, body weight, feed intake and efficiency during gestation and lactation were assessed.

Results: Sodium saccharin intake resulted in the increase of the feed efficiency, adiposity and pancreas weight and in the decrease of the liver weight. The saccharin dam rats presented higher blood glucose levels than the control dam rats. In the ITT, the saccharin dam rats showed a threshold value which characterizes the pre-diabetic state, as confirmed by AUC of the ITT, indicating insulin resistance. Also, possible changes in the intrauterine environment caused alteration in the somatic development of the male offspring.

Conclusion*:* The sodium saccharin intake during pregnancy and lactation leads to changes in maternal and male offspring parameters. The adaptive responses in the male offspring agree with the existence of fetal programming by sodium saccharin consumption, reflecting in the alteration of the physical development.

Keywords: fetal programming, sodium saccharin, gestation, lactation, glycemia, development, male offspring.

1. Introduction

In order to help people with diabetes mellitus and obesity control treatments, the consumption of artificial sweeteners, sugar substitutes, has increased worldwide [1,2]. The sweeteners are classified as nutritive and non-nutritive. The non-nutritive sweeteners are stimulators of compensatory food intake in response to the consequent absence of calories associated with sweet taste stimuli [3-8]. The use of non-nutritive sweeteners has been linked to increased adiposity and to the onset of type 2 diabetes mellitus, metabolic syndrome and cardiovascular disease [9-12]. Part of these changes is associated with interferences in the appetite-regulating mechanisms promoting weight gain [12-14]. Among non-nutritive sweeteners, sodium saccharin is one of the most consumed and recognized as safe by the U.S. Food and Drug Administration - FDA. However, it is recommended that the consumption of sodium saccharin be restricted during gestation and lactation because of its high placental permeability and because of its presence in breast milk [14-16].

In the intrauterine environment, the fetus is vulnerable to several external factors, especially during critical periods of development, and maternal nutritional status determines the probability of offspring disorders [17]. Thus, maternal metabolic changes, such as deficiency or excess of nutrient, may increase the propensity for metabolic diseases and lead to fetal programming which will affect the health of the individual in adulthood, this effect being called “Developmental origins of health and disease”, DOHaD [17-19]. The increase in the consumption of sweeteners stimulates the interest to investigate their use on maternal metabolism and the relationship with fetal programming, a subject not yet addressed by the specialized literature. Thus, the main objectives of this research were to evaluate the effects of the sodium saccharin intake during pregnancy and lactation on the maternal biometric parameters, glucose metabolism and offspring development to test the hypotheses: (1) maternal intake of sodium saccharin promotes insulin resistance and glucose tolerance, and (2) maternal intake of sodium saccharin interferes with the developmental of biometric parameters of the male offspring.

2. Materials and methods

2.1. Ethics committee approval

The experimental protocol (number 1078) was approved by the Committee of Ethics in Animal Experimentation at São Paulo State University (UNESP), Institute of Biosciences, Botucatu, Brazil.

2.2. Animal mating

Male and female Sprague-Dawley rats (90 days of life) were obtained from University of Campinas (UNICAMP), Campinas, São Paulo State, Brazil. They were maintained in an experimental room under controlled conditions with temperature (22±2°C), humidity (50±10%), and a 12h light/dark cycle. At the beginning of the experiment, two females and one male of reproductive age (12 weeks) were housed in polypropylene cages for mating. The females were evaluated for detection of sperm in vaginal smear, which together with the estrus phase, marked gestation day (GD) 0. The dam rats were transferred to individual cages to form experimental maternal groups.

2.3. Experimental design

All experimental procedures are summarized in figure 1.

2.3.1. Experimental groups (Dam rats)

Dam rats received a liquid diet *ad libitum* of skimmed natural yogurt (control dam rats, n= 9) or a liquid diet of skimmed natural yogurt sweetened with 0.3% sodium saccharin (saccharin dam rats, n= 10). Moreover, both groups were maintained with free access to food and filtered water. The gestation duration, litter size and the ratio of female and male offspring were evaluated in control and saccharin groups.

2.3.1.2. Liquid diet

Liquid diets offered to dam rats were prepared by adding 20mL of skimmed natural yogurt (Nestlé®) and 15mL of filtered water for viscosity adjustment. For the homogenization of the diet, a mixer was used. The 0.3% sodium saccharin solution (15mL) was mixed with the liquid diet prepared with skimmed natural yogurt (20mL). During all gestation and lactation periods, the dams received a liquid diet [20-22].

2.3.1.3. ***Body weight, feed intake and efficiency***

The body weight, feed intake (amount of food and water ingested) were evaluated weekly until weaning. The offspring was weighed on day 1 of birth.

2.3.1.4. Blood glucose

Blood glucose was measured at GD15 and 17, postnatal day (PND) 21 and 23. A drop of blood (50 µL) was collected by puncturing the tail vein and it was used to determine blood glucose by the glucose oxidase method. A blood glucose indication has been specified by the Accu-Chek Active® blood glucose monitor. Animals with fasting blood glucose ≥100mg/dLwill be considered pre-diabetic individuals [23].

2.3.1.5. ***Insulin tolerance test (ITT)***

The ITT was performed on the GD15 and PND21. After 6 hours of fasting, the animals received subcutaneous insulin injection (1U/mL/kg body). Then, blood samples of the tail vein (50 µL) were taken at 15, 30, 60, and 120 minutes after administration subcutaneous insulin injection to determine blood glucose by the glucose oxidase method. The reading was performed using Accu-Chek Active® blood glucose monitor. Changes in blood glucose during ITT were assessed by areas under curves (AUC) calculated by the trapezoidal method [24].

2.3.1.6. Glucose tolerance test (GTT)

The GTT was applied on GD17 and PND23. After 6 hours of fasting, the animals received, via gavage, glucose solution (2g/mL/kg body). Then, blood samples of the tail vein were collected at 15, 30, 60, and 120 minutes after administration of glucose solution to determine glucose by the glucose oxidase method. The reading was performed using Accu-Chek Active® blood glucose monitor. Changes in blood glucose during GTT were evaluated by AUC calculated by the trapezoidal method [24]. During the test, individuals with two or more curve points $\geq$140mg/dL will be considered pre-diabetic [23].

2.3.1.7. Organs and fat deposits collection

Three days after weaning, the dam rats were weighed after 6-hour fasting and anesthetized with sodium thiopental (40mg/Kg body) intraperitoneally and killed by decapitation. The abdominopelvic laparotomy was performed and the pancreas, liver, retroperitoneal, pericardial and visceral white adipose tissues and the adiposity index was calculated (the sum of all fat deposits divided by body weight×100) [25].

2.3.2. Offspring

2.3.2.1. Physical development

Pups from the control and saccharin dams were observed daily from PND 1 to 20 to analyze somatic development. Pinna unfolding, hair growth, incisors teeth eruption, and eyes opening also were evaluated.

2.3.2.2. *Body weight, naso-anal length (NAL) and anogenital distance (AGD)*

The offspring were weighed from birth to PND21. A digital pachymeter was used from PND 0 to 20 to measure, in millimeters, the anogenital distance (AGD), and the naso-anal length (NAL). The AGD corresponds to the distance between the anus and the external genitalia [26]. The anogenital index is the ratio of anogenital distance/body length.

2.3.2.3. Organs and fat deposits collection

The pups (PND 21) after 6-hour fasting were weighed and anesthetized with sodium thiopental (40mg/Kg body) intraperitoneally and killed by decapitation. The abdominopelvic laparotomy was performed and the pancreas, liver, retroperitoneal, pericardial and visceral white adipose tissues and the adiposity index was calculated (the sum of all fat deposits divided by body weight×100) [25].

2.4. Statistical analysis

The variables were analyzed by the GraphPad Prism software (version 7, GraphPad Software Inc., San Diego, CA). Firstly, the data were subjected to the Shapiro-Wilk normality and variability test. For parametric data, the variables were studied using t-tests and analysis of variance (ANOVA) complemented by Sidak's multiple comparison tests. For non-parametric data, we used the Mann-Withney tests. Results were expressed as mean ± SD (standard deviation) and presented in figures and tables, considering the 0.05% significance level.

3. Results

3.1. Dam rats

There were no statistically significant differences in the monitored parameters. The gestation duration was 21.89 ± 0.57 days to control dams and 21.70 ± 0.48 days to saccharin dams. There were no differences reported in the litter size (control = 12.80 ± 1.75; saccharin= 12.60 ± 2.22) and in the ratio of female and male offspring (data not showed).

3.1.1. Body weight, feed intake and efficiency during gestation and lactation periods

Sodium saccharin intake in the gestational and lactacional periods increased the body weight in the 3rd week of gestation (Figure 2A). Although the food and water consumption did not alter during the observation period (Fig. 2B-C), the feed efficiency was greater in the saccharin dam rats group.

3.1.2. Blood glucose, insulin and glucose tolerance tests

During gestational period, the insulin and glucose tolerance tests, as well as fasting blood glucose measurements were normal (Figure3).

In the weaning day, the saccharin dam rats presented higher blood glucose levels than the control dams. In the ITT, the saccharin dam rats showed a threshold value which characterizes the pre-diabetic state, as confirmed by AUC of the ITT, indicating insulin resistance. Two days after weaning, no significant changes were found despite the lowest AUC and blood glucose at 120 min (Figure 4).

3.1.3. Body weight, absolute and relative organs weights, white adipose tissues weights and adiposity index

Table 1 shows the body and organs weights of control and saccharin dams. There was a decrease in the liver weight and, the pancreas and retroperitoneal fat weights of the dam rats exposed to sodium saccharin increased.

3.2. Offspring

3.2.1. Physical development

Table 2 presents variables related to the physical development of the control and saccharin dam rat offspring. In the saccharin group, the hair growth was early and eye opening was delayed.

3.2.2. *Body weight, naso-anal length (NAL) and anogenital distance (AGD)*

There was no observed difference in the body weight between the groups at birth (Table 3).

The AGD, and NAL evolution are represented in figure 5. There was an increase in the absolute and anogenital distance indexes (Figure 5A-B) on PDN1 of male offspring, whose mothers were exposed to sodium saccharin. Regarding NAL, the saccharin offspring was greater on PND7 and shorter on PND20 than the control offspring (Figure 5C).

3.2.3. Body weight, absolute and relative organs weights, white adipose tissues weights and adiposity index

As observed in table 4, there was a decrease in the liver weight of the male offspring, whose mothers were exposed to sodium saccharin during gestation and lactation periods. The epididymis, retroperitoneal, and pericardial fat weights were lower, but the adiposity index did not alter.

4. Discussion

In the present study, we evaluated the effects of sodium saccharin intake during gestation and lactation on maternal parameters and on the development of the male offspring. Sodium saccharin intake resulted in an increase in feed efficiency, insulin resistance, adiposity, and pancreas weight and a decrease in liver weight. Moreover, the changes in the intrauterine environment possibly caused alterations in the somatic development of the male offspring.

The consumption of non-nutritive sweeteners has been associated with increased body weight and adiposity as a result of decreased satiety [20, 21, 27]. In our study, food and water consumption were similar in the studied groups. However, feed efficiency and body weight were increased in the saccharin dam rats group. In other words, saccharin-consuming rats have more adequately converted their caloric intake. Higher body weight gain, unrelated to feed intake, may be associated with a reduction in sweetener-induced caloric expenditure [21].

Ruud, Steculorum, and Bruning (2017) reported that agouti-related protein (AgRP)-neurons, present in the Hypothalamic Arcued Nucleus, are capable of detecting peripheral substances which indicate the body's energy status and, when activated, evoke feed intake and reduce body energy expenditure [28]. In addition, studies with sodium saccharin have shown improvement in energy efficiency and altered cephalic phase response [29, 30]. These responses send stimuli, which prepare the gastrointestinal environment for nutrient arrival, increasing the metabolic efficiency and decreasing possible homeostatic disturbances [30, 31]. Substances with sweetened flavors are identified as potent stimulators of cephalic reflexes in which energy regulation depends on signaling-induced cephalic stimulation [32]. Thus, the presence of sodium saccharin promotes calorie-free responses, interfering with homeostasis [20]. Therefore, we associate higher calorie conversion as the main factor responsible for retroperitoneal fat accumulation, which results in a positive energy balance with the intake of sodium saccharin by mothers [21]. Additionally, metabolic disorders may differentially express adipocyte secretion regulating genes, with a tendency to obesity and adiposity [33].

A recent study has shown that insulin resistance is the precursor to other diseases associated with metabolic syndrome. High-fat diets and high feed intake disturb the regulation of the transcription factor Foxo1, which is responsible for the activation of hepatic gluconeogenesis, which is inhibited by insulin. Obese rats lose the insulin sensitivity, leading to the activation of gluconeogenesis with glucose accumulation and inhibition of glucose transporters. This disorder causes hyperglycemia, increased insulin secretion which results in hyperinsulinemia, consequent activation of hepatic lipogenesis, and increased VLDL output, resulting in hyperlipidemia (obesity) [34]. Our study reports insulin resistance at the end of the treatment of dam rats of the sodium saccharin group and an acceptable glycaemia threshold. Also, these animals had lower liver weight and increased retroperitoneal fat. Therefore, we believe that these animals will develop glucose intolerance possibly resulting in type 2 diabetes mellitus and obesity.

Gallego et al. (2018) demonstrated that diabetic rats presented the pancreatic α-cell hyperplasia, responsible for glucagon release, and pancreatic β-cell hypoplasia, responsible for insulin release [35]. Thus, we conjecture that the increase in pancreas mass observed in the saccharin dam rats may be associated with increased α cell proliferation. In an experimental study related to long-term ingestion of aspartame, the researchers observed high fasting blood glucose levels, visceral fat accumulation, and improved energy efficiency, as well as insulin resistance [29], in agreement with our findings.

The nutrition maternal condition during pregnancy and lactation influences prenatal and postnatal development of offspring [36]. In the present study, the physical development of the male offspring, whose mothers were consumers of sodium saccharin showed alterations. Although the maternal saccharin group showed no change in the body weight, the results of the

GTT and ITT tests indicated a tendency for hyperglycemia and pre-diabetes. Diabetes and maternal hyperglycemia cause fetal adaptations and may induce hyperinsulinemia and, thus alter the developmental pattern in offspring [18, 36].

In addition, several hormones and growth factors are determinant for fetal development [17, 37]. Insulin is one of the major hormones which reflects fetal growth and deficiency or excess insulin-like growth factors (IGF) compromises signaling pathways and implies body growth [37]. Endothelial Growth Factor (EGF) is important for regulating the activity of epidermal and epithelial tissues and acts in the delay of the appearance of hair and anticipate eye opening [38, 39]. We believe that a possible decrease in EGF expression led to the advancement in the appearance of hair and a delay in the eye opening.

Regarding AGD, a predictor of androgen action and production during male fetal development, the increase of the AGD observed in PND1 in the offspring of saccharin consumers dam rats highlights possible androgenic amplification during the masculinization programming window, since the testosterone is responsible for the perineal muscle growth and is important for male development [26]. Considering we found progress in physical development and higher AGD and NAL in the neonatal phase, we hypothesized an increase in the testosterone in this phase followed by a decrease in the infant phase which resulted in lower NAL and developmental delay.

Nutritional interferences during lactation may predispose to metabolic changes in glucose in adulthood [34]. The evolution in the development of the pancreas is observed until the DPN17. High cell proliferation rates determine pancreatic adaptation as the somatic growth of the animal occurs. This important adaptive phase programs adult glycemic homeostasis [40, 41].

Czech (2017) reports that the dysregulation of glucose mechanisms influences adipocyte function by decreasing its responsiveness to insulin. There is also a decreased expression of perilipin and Cide proteins, which are responsible for retaining triglycerides in the mature adipocytes by inhibiting lipolysis when an increase in blood insulin occurs [34]. These disorders lead to a lower retention of triglycerides by the adipocytes with fat accumulation and lipotoxicity in the liver [34, 42]. We associate these metabolic failures with a decrease in the liver weight and in the adiposity.

5. Conclusion

In conclusion, sodium saccharin intake during pregnancy and lactation leads to changes in maternal and offspring parameters. Metabolic changes in dam rats sodium saccharin consumers were confirmed by the increase in the feed efficiency, fat accumulation, weight gain, and insulin resistance. Alterations in the liver weight might be associated with possible functional inefficiency. Alterations in the pancreas might be associated with the differentiated release of insulin and glucagon. The adaptive responses in the male offspring agree with the existence of fetal programming by sodium saccharin consumption, reflecting in the alterations of the physical development.

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Authors' contributions

PFFP designed the study. PFF, FEM, ARG and VCF performed the experiments and wrote the manuscript. PFF, ARG, VCF and RSN obtained and analyzed the data. All authors read and approved the final version manuscript.

Conflict of interest

There was no conflict of interest.

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Legends

Figure 1. Experimental design. Two groups of dam rats: (1) control group (C) subjected to diet with yogurt diet and (2) saccharin group (S) subjected to diet with yogurt diet sweetened with 0.3% sodium saccharin. Evaluation of physical development of the male offspring (PND 0 to 20). The insulin and glucose tolerance tests were applied in the dam rats and in the male offspring.

Figure 2. (A) Body weight gain; (B) water intake; (C) feed intake and (D) feed efficiency during gestation and lactation periods of control (C) and saccharin (S) dam rats. The values are expressed as the mean ± S.E.M. * $p < 0.05$. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

Figure 3. Glycemia on GD 15 (A) and GD 17 (D), ITT (B) and area under curve the ITT (AUC) (C) on GD 15, GTT (E) and area under curve the GTT (AUC) (F) on GD 17, during gestation period of control (C) and saccharin (S) dam rats. The values are expressed as the mean ± S.E.M. * $p < 0.05$. The values are expressed as the mean ± standard deviation * $p < 0.05$. T-tests were used to glycemia and ANOVA followed Sidak post-test to the ITT and GTT.

Figure 4. Glycemia in weaning (A) and two days after the weaning (D), ITT (B) and area under the ITT (AUC) (C) in weaning, and GTT (E) and area under curve the GTT (AUC) (F) on and area under curve the ITT, during lactation period of control (C) and saccharin (S) dam rats. The values are expressed as the mean ± standard deviation * $p < 0.05$. T-tests were used to glycemia and ANOVA followed Sidak post-test to the ITT and GTT.

Figure 5. Evolution (days) of absolute (mm) and anogenital distance indexes and naso-anal length (mm) of male offspring of control (C) and saccharin (S) groups. The values are expressed as the mean

± S.E.M. * p <0.05. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

Tables

Table 1 Body weight, absolute and relative organs weights, white adipose tissues weights and adiposity index of the dam rats.

Parameters	**Experimental groups**	
	C (n=9)	**S (n=10)**
Final body weight (g)	321.7 ± 22.48	331.0 ± 10.75
Liver (g)	11.33 ± 1.36	9.34 ± 0.77*
Liver (g/100g)	4.01 ± 0.38	3.25 ± 0.21*
Pancreas (g)	1.79 ± 0.33	2.12 ± 0.35*
Pancreas (g/100g)	0.63 ± 0.10	0.74 ± 0.12*
Retroperitoneal fat (g)	0.64 ± 0.11	0.92 ± 0.21*
Visceral fat (g)	2.48 ± 0,30	2.60 ± 0.27
Pericardial fat (g)	0.39 ± 0.05	0.41± 0.05
Adiposity index	1.34 ± 0.18	1.39 ± 0.17

Values are expressed as mean±standard deviation. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.
* p<0.05, statistically significant difference compared with control group.

Table 2 Mean day of physical development on male offspring.

Parameters	**Experimental groups**	
	C (n=16)	**S (n=16)**
Pinna unfolding	2.60 ± 0.46	2.67 ± 0.48
Hair growth	3.21 ± 0.45	2.70 ± 0.47*
Eye opening	12.9 ± 0.53	13.41± 0.50*
Incisor eruption	8.45 ± 1.26	8.10 ± 1.52

Values are expressed as mean±standard deviation. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.
* p<0.05, statistically significant difference compared with control group.

Table 2 Body weight at birth and naso-anal length and absolute and relative anogenital distance on male offspring at PND1.

Parameters	**Experimental groups**	
	C (n=16)	**S (n=16)**
Body weight (g)	6.56 ± 0.57	6.77 ± 0.73
Naso-anal length (mm)	54.37 ± 2.22	54.93 ± 3.79
Anogenital distance (mm)	3.85 ± 0.44	4.29 ± 0.51*
Relative anogenital distance	0.071 ± 0.01	0.08 ± 0.01*

Values are expressed as mean±standard deviation. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

⁎ $p<0.05$, statistically significant difference compared with control group.

Table 3 Body, absolute and relative organs weights, and white adipose tissues weights and adiposity index of male offspring (PND 21).

Parameters	**Experimental groups**	
	C (n=16)	**S (n=16)**
Body weight (g)	53.96 ± 5.55	50.22 ± 6.12
Liver (g)	1.80 ± 0.26	1.60 ± 0.17*
Liver (g/100g)	3.67 ± 0.11	3.42 ± 0.25*
Pancreas (g)	0.20 ± 0.04	0.19 ± 0.03
Pancreas (g/100g)	0.42 ± 0.10	0.41 ± 0.05
Epididymis fat (g)	0.06 ± 0.02	0.04 ± 0.01*
Retroperitoneal fat (g)	0.04 ± 0.01	0.03 ± 0,01*
Visceral fat (g)	0.25 ± 0.06	0.24 ± 0. 07
Pericrdial fat (g)	0.25 ± 0,06	0.20 ± 0.07*
Adiposity index	1.20 ± 0,16	1.10 ± 0.18

Values are expressed as mean±standard deviation. T-tests were used to assess the significance of differences in parametric data, and Mann-Whitney tests were used to assess the significance of differences in nonparametric data.

⁎ $p<0.05$, statistically significant difference compared with control group.

Figure 1

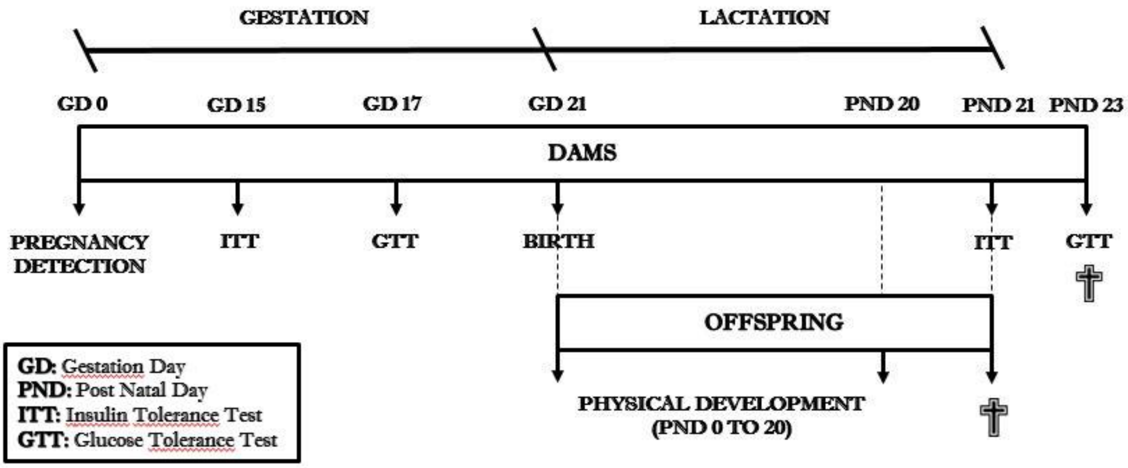

GESTATION
LACTATION
GD 0
GD 15
GD 17
GD 21
PND 20
PND 21
PND 23
DAMS
PREGNANCY DETECTION
ITT
GTT
BIRTH
ITT
GTT
OFFSPRING
PHYSICAL DEVELOPMENT (PND 0 TO 20)
GD: Gestation Day
PND: Post Natal Day
ITT: Insulin Tolerance Test
GTT: Glucose Tolerance Test

Figure 2

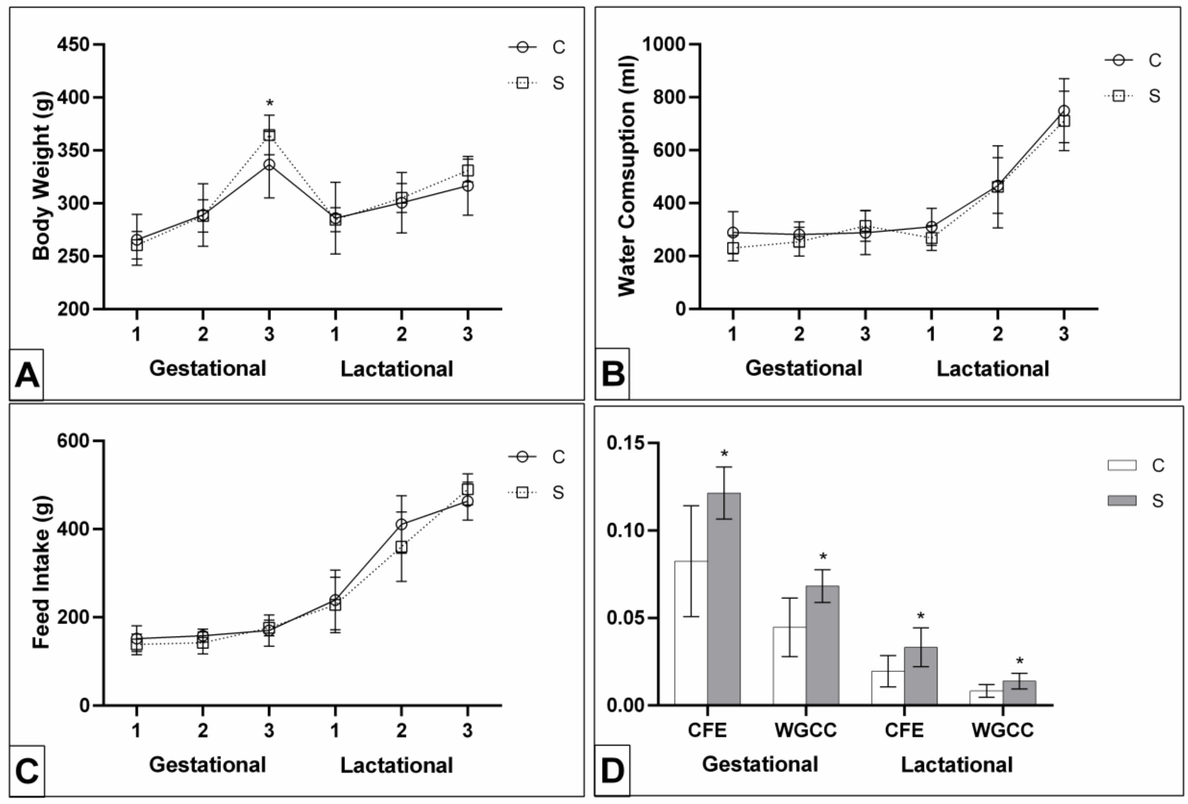

Body Weight (g)
450
400
350
300
250
200
*
C
S
1
2
3
1
2
3
Gestational
Lactational
A
Water Comsuption (ml)
1000
800
600
400
200
0
C
S
1
2
3
1
2
3
Gestational
Lactational
B
Feed Intake (g)
600
400
200
0
C
S
1
2
3
1
2
3
Gestational
Lactational
C
0.15
0.10
0.05
0.00
*
*
*
*
C
S
CFE
WGCC
CFE
WGCC
Gestational
Lactational
D

Figure 3

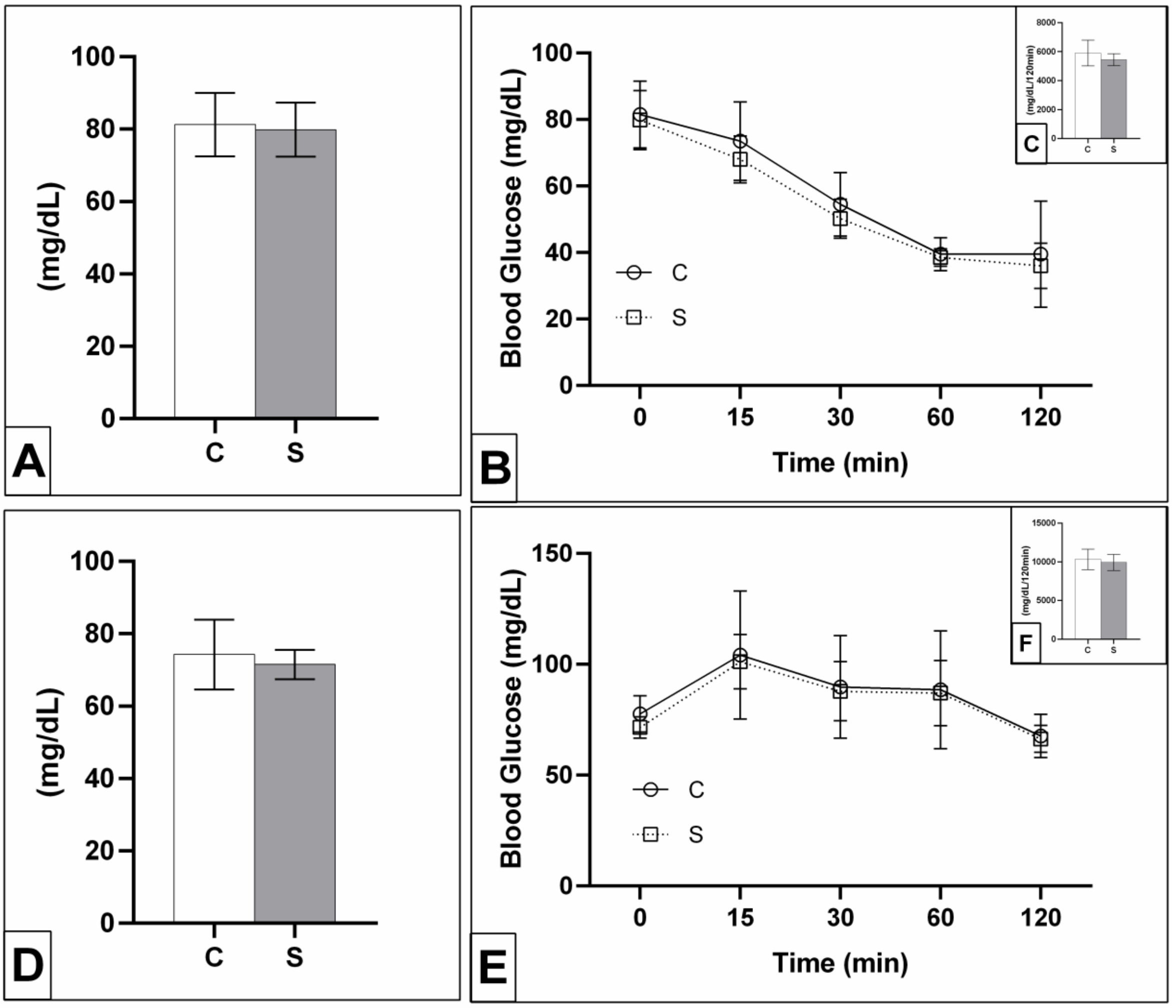

A
(mg/dL)
0
20
40
60
80
100
C
S
B
Blood Glucose (mg/dL)
0
20
40
60
80
100
0
15
30
60
120
Time (min)
C
S
C
(mg/dL/120min)
0
2000
4000
6000
8000
C
S
D
(mg/dL)
0
20
40
60
80
100
C
S
E
Blood Glucose (mg/dL)
0
50
100
150
0
15
30
60
120
Time (min)
C
S
F
(mg/dL/120min)
0
5000
10000
15000
C
S

Figure 4

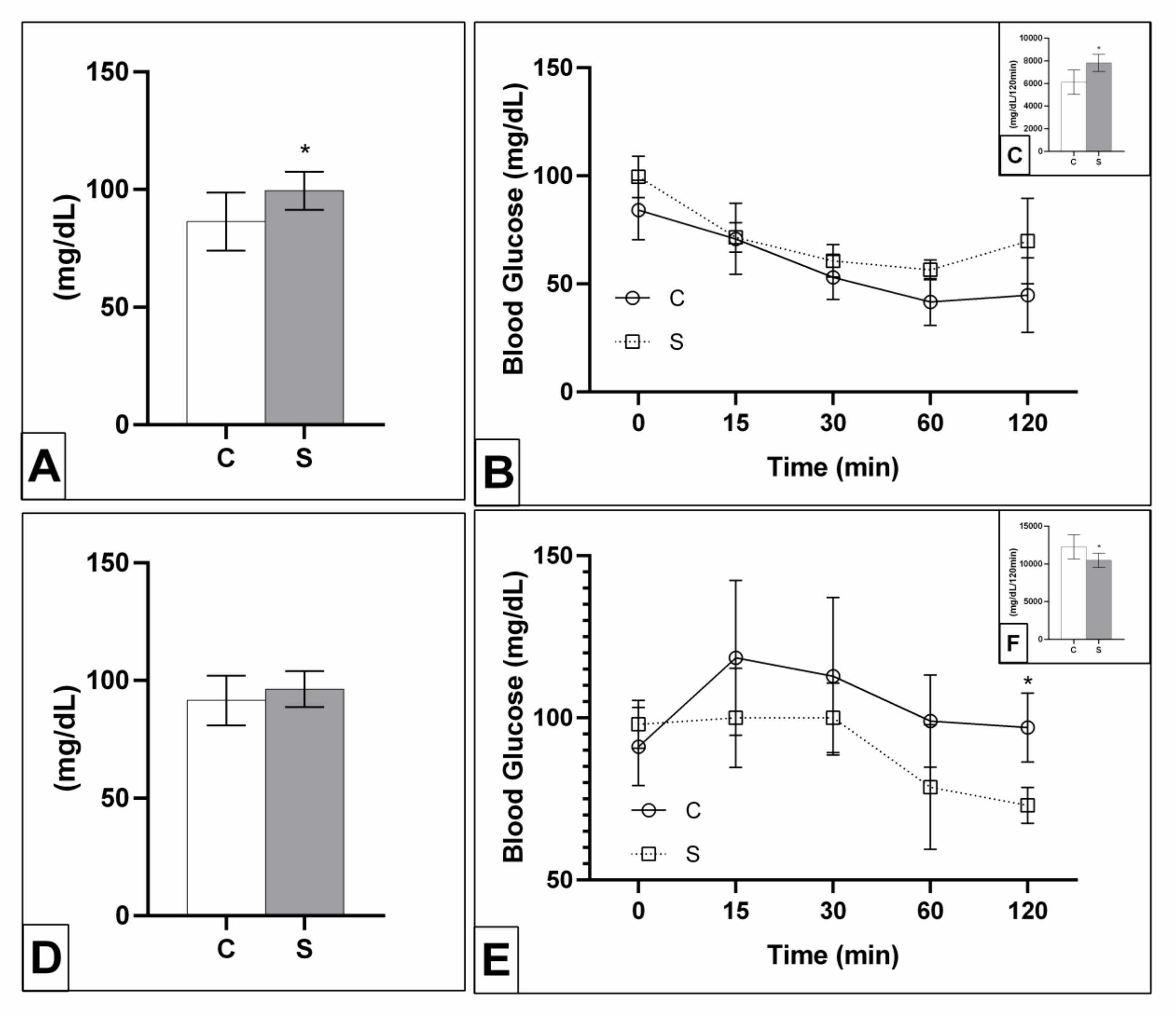
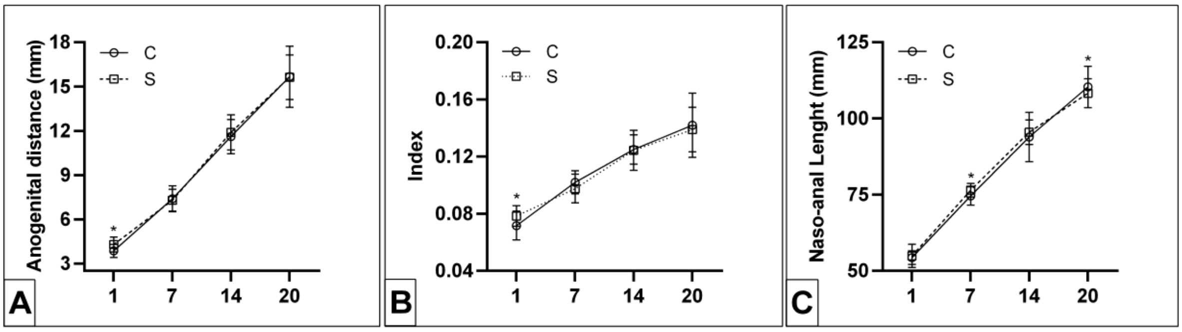

150
100
50
0
(mg/dL)
*
C
S
A
Blood Glucose (mg/dL)
150
100
50
0
C
S
0
15
30
60
120
Time (min)
B
10000
8000
6000
4000
2000
0
(mg/dL/120min)
*
C
S
C
150
100
50
0
(mg/dL)
C
S
D
Blood Glucose (mg/dL)
150
100
50
C
S
*
0
15
30
60
120
Time (min)
E
15000
10000
5000
0
(mg/dL/120min)
*
C
S
F

Figure 5


A
Anogenital distance (mm)
18
15
12
9
6
3
1
7
14
20
C
S
*
B
Index
0.20
0.16
0.12
0.08
0.04
1
7
14
20
C
S
*
C
Naso-anal Lenght (mm)
125
100
75
50
1
7
14
20
C
S
*
*

CAPÍTULO 3

Título do Manuscrito: *Fetal programming by glucose and sodium saccharin: Impact in the reproductive capacity male offspring Sprague Dawley rats.*

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Abstract

Due to the increased intake of foods and drinks with high sugar content and the possible association with weight gain and the onset of obesity, non-nutritive sweeteners have been the best choices as dietary additives. Although there are many reports on the use of the sweetener on body health, there are no clinical and experimental data on its use during the pre and postnatal periods and its implications for the reproductive health of the offspring. Yet, considering glucose, a constituent of table sugar, the most consumed sweet-tasting substance, this investigation also proposes, under the same conditions, to evaluate glucose consumption. For the investigation, two experiments were carried out using 20 pregnant rats which received during pregnancy and lactation periods, water and chow *ad libitum* and supplementation with liquid diet sweetened with glucose at 5% (n = 10) or sodium saccharine at 0.3% (n = 10). At weaning, male offspring was divided into two groups, for each experiment: those who received only water and feed *ad libitum* throughout the experimental period; and those who, in addition to water and food *ad libitum*, received supplementation from PND70 to PND120 similar to the one of their mothers. The adoption of sodium saccharin and glucose in the diet during adulthood caused a reduction in water consumption and feed efficiency. The supplemented glucose group showed higher levels of testosterone and estradiol while the supplemented sodium saccharin group showed only changes in the testosterone level, in addition to higher levels of AR expression in Sertoli cells and intense immunoreactivity of AR in Leydig cells. The supplemented sodium saccharin group also showed higher quantification of positive sperm for the PCNA and daily sperm production. The animals supplemented in adulthood showed an increase in testicular fluid and a decrease in epididymal transit time. Animals exposed only during fetal life to glucose and sodium saccharin exhibited desquamation of germ cells. It was concluded that the consumption of sodium saccharin and glucose interfered in the plasma levels of testosterone and estradiol and, programmed the decrease in sperm production in the supplemented animals.

Key-words: testis, sweetener nonnutritive, androgen receptor, proliferating cell nuclear antigen, reproduction.

Introduction

The increase in obesity has caused great concern among experts. There is a clear relationship between weight gain and the onset of obesity with a high consumption of foods and drinks with high levels of sugars [1-8].

Simple sugars, such as sucrose and fructose, are found in industrialized products such as soft drinks and juices. In addition, the increased consumption of these products in adolescence and adulthood [9-10] can trigger the onset and prevalence of several metabolic diseases of multifactorial origin, such as obesity and diabetes [9, 11].

Experimental studies have shown that these metabolic diseases may be related to prenatal exposures to inadequate diets, being able to program the individual's development. This programming is responsible for the “Developmental Origins of Health and Disease”, DOHaD [12-13]. There is a lot of research that supports this theory. In these studies, it was observed that children of mothers exposed to diets during gestation can cause marked morphofunctional changes when exposed to the same stimulus during postnatal development. This condition induces, in some situations, the onset of obesity and type 2 diabetes mellitus [14-15].

The excess body fat characteristic of obesity is due to the relationship between the high amount of energy ingested and decreased energy expenditure [1]. Associated with this, there is an important relationship between the high body mass index and the decrease in the reproductive efficiency of men [16-20], characterized by higher rates of abnormal sperm [21], the greater damage to spermatic DNA [22] and lower sperm count [17]. In addition, obesity and paternal type 2 diabetes mellitus are able to alter embryonic development and metabolism [23-24], placental gene expression, DNA methylation status [25], semen quality [26] and the fertility capacity of male offspring [27]. In this same sense, obesity and maternal diabetes are able to negatively affect the metabolism of male offspring [28-30] and the sperm epigenome of adult offspring [31-32], thus influencing their reproductive efficiency.

In order to help control body weight and treat metabolic diseases, such as diabetes, non-nutritive sweeteners are the best dietary additive choices because they do not provide any caloric intake in their composition, with sodium saccharin being the non-nutritive sweetener. most consumed worldwide [33-34]. Although safe, during gestational and lactational periods, restriction to consumption is indicated, as the placenta is completely permeable, remaining in the fetal tissues, in addition to composing breast milk [35-36].

Although there are many reports on the effects of sweetener consumption on body composition [37-40], there are no clinical and experimental data on the use of this food additive during the pre- and post-natal periods and its implications for the reproductive health of the offspring.

Thus, considering that high consumption of table sugar and sodium saccharin can alter glycemia and cause harmful effects on reproductive health, the present study proposes to investigate the consumption of glucose and sodium saccharin during pregnancy and lactation and its implications for structure, the function of the testis and the potential for the fertility of male offspring.

Materials and methods

1. Animals

1.1. Mating

Sprague-Dawley rats 90-day-old were obtained from the State University of Campinas (UNICAMP, Campinas, São Paulo, Brazil). Males (n = 10) and females (n = 20) were housed in maternity boxes, in the ratio of 2 females to 1 male for mating. During the mating period, the females were periodically evaluated, by means of vaginal smear, for the detection of sperm. After confirmation of pregnancy, the dam rats were placed in individual boxes for the formation of experimental groups. Throughout the experimental period, the rats were kept in the vivarium of the Department of Anatomy of the Biosciences Institute of Botucatu (IBB/UNESP, Botucatu, São Paulo, Brazil) under controlled conditions of temperature (22 ± 2°C), humidity (50 ± 10%) and 12h light / dark cycle. The Ethics Committee on the Use of Animals of IBB/UNESP (n ° 1078 CEUA) approved this protocol.

1.2. Experiments

1.2.1. Experiment 1

From gestational day 0, 10 rats received chow and water *ad libitum* and liquid diet containing glucose (Dinâmica$^{®}$) at 5% (v/v) during the gestational and lactational period. On the day of weaning, the offspring of male rats were divided into two experimental groups:

(1) Glucose (G) (n=10): animals which received feed and water *ad libitum* throughout the experimental period;

(2) Supplemented Glucose (SG) (n=10): animals which received food and water *ad libitum* throughout the experimental period and supplementation of liquid diet containing glucose at 5% (v/v) from 70 to 120 days of age [39-40].

1.2.2. Experiment 2

From gestational day 0, 10 rats received chow and water *ad libitum* during the gestational and lactational period and a liquid diet containing 0.3% sodium saccharin (Dinâmica®). On the day of weaning, the offspring of male rats were divided into two experimental groups:

(1) Saccharin Sodium (S) (n=11): animals which received feed and water *ad libitum* throughout the experimental period;

(2) Supplemented Saccharin Sodium (SS) (n=12): animals which received food and water *ad libitum* throughout the experimental period and supplementation of liquid diet containing saccharin sodium at 0.3% (v/v) from 70 to 120 days of age [37-40].

The experimental design is illustrated in figure 1.

1.3. Liquid diet

The liquid diet offered was prepared by adding 20mL of low-fat plain yogurt (Nestlé®) to 15mL of glucose solution (5%) or 15mL of sodium saccharin solution (0.3%). A mixer was used to homogenize the diet. Liquid diets were offered to the mothers, for 22h, throughout the gestational and lactational periods. A male offspring (described above) received from postnatal day (PND) 70 to PND 120, for 5 days a week, a liquid diet supplemented with glucose or sodium saccharin. Only animals which ate at least 70% of the diet were included in the study. [37-40].

2. Analysis

2.1. Body weight, food intake and feed efficiency

From weaning to the death of the animals, the body weight, water intake, and male offspring data were measured weekly. The records of these data were applied in two formulas to calculate feed efficiency.

$$\boldsymbol{CFE = \frac{(IBW - FBW)}{TAF}} \qquad \boldsymbol{WGCC = \frac{(FBW - IBW)}{Kcal}}$$

CFE= coefficient of feed efficiency

IBW= initial body weight (g) of the animal

FBW= final body weight (g) of the animal

TAF= the total amount of food (g)

WGCC= weight gain by caloric consumption

2.2. Collect of biological material

Prior to the collection of testes and fats, the animals were fasted for 6 hours. Then the rats were weighed and anesthetized with sodium thiopental (40mg / kg body), intraperitoneally and killed by decapitation. After blood collect, due to the rupture of the cervical blood vessels, an abdominal-pelvic laparotomy was performed and the testes and fats were dissected and weighed.

2.3. Determination of the adiposity index

The fats on the epididymides, cardiac, visceral and retroperitoneal fats were collected and the absolute weights were measured. The adiposity index was determined by the following formula:

$$\boldsymbol{Adiposity\ index} = \left[\frac{(\boldsymbol{fat\ epididymides} + \boldsymbol{cardiac} + \boldsymbol{visceral} + \boldsymbol{retroperitoneal})}{\boldsymbol{final\ body\ weight}}\right] * \mathbf{100}$$

2.4. Light Microscopy

The testes were fixed in a 10% formaldehyde solution in sodium phosphate buffer (PBS) (pH 7.4), washed for 48 hours in running water and placed in a 70% alcohol solution. Then they were dehydrated in growing solutions of alcohols (ethanol and butyl), included in paraplastic and cut to 4µm thick. Collections of slides stained with hematoxylin-eosin (HE) and PAS (histochemical reaction with periodic acid from Schiff) were obtained. The slides were analyzed and photographed using a BX 41 - 2 photomicroscope with a digital camera, model SIS-SC30, Olympus from the Department of Anatomy, Institute of Biosciences - UNESP – Botucatu, Brazil.

2.5. Immunohistochemistry

Histological sections of the testes were placed on silanized slides and remained for 1 hour in an oven regulated at 58°C. Then, the histological sections were deparaffinized in xylol and hydrated in decreasing alcohol (ethanol) solutions, running water for 10 minutes and in PBS buffer (pH 7.4). Then, the slides in 0.01M sodium citrate buffer (pH 6.0) were subjected to microwave oven heat (700-800W) during 15 minutes, divided into 3 5-minute cycles. The slides that were incubated with anti-androgen receptor antibody were placed in 0.01M sodium citrate buffer (pH 6.0) in a pressure cooker and subjected to high pressure and temperature for 60 minutes. Subsequently, the slides were placed in a 3% hydrogen peroxide solution in methanol during 15 minutes. To block nonspecific reactions, the slides were incubated in a 2% skimmed milk solution (Molico®) in PBS buffer (pH 7.4) during 1 hour. In the next step, the histological sections were subjected to reaction with anti-cell proliferation antibody (PCNA - PC-10 - Novocastra®, dilution 1: 100 in PBS, pH 7.4) and (AR - N-20 Santa Cruz Biotechnology Inc.®, dilution 1: 100 in PBS, pH 7.4) and incubated in a humid chamber overnight at 4ºC in a refrigerator. After incubation, the slides were washed in PBS buffer (pH 7.4) for 1h at room temperature, the sections were incubated with biotinylated IgG anti-mouse secondary antibodies, antibody produced in rabbit (E 0354 - Dako Cyt. Inc.®, 1: 100 dilution in PBS, pH 7.4) and biotinylated anti-rabbit IgG, antibody produced in goat (E 0432-1 - Dako Cyt. Inc.®, 1: 100 dilution in PBS, pH 7.4). After this step, the slides were washed with PBS (pH 7.4) and submitted to the avidin-biotin-peroxidase solution (ABC Kit - PK - 6100 - Vector Laboratories®) for 45 minutes. Then, the slides were washed in PBS (pH 7.4) and subjected to diaminobenzidine (DAB) (di-amino-benzidine-Sigma®) during 5 minutes. Then, the slides were washed in tap water and contrasted with Harris' hematoxylin. During the technique, positive and negative controls were obtained. The slides were analyzed and photographed using a BX 41 - 2 photomicroscope with a digital camera, model SIS-SC30, Olympus from the Department of Anatomy, Institute of Biosciences - UNESP - Campus de Botucatu.

2.6. Morphometric and stereological analysis

The PCNA$^+$ sperm and Sertoli AR$^+$ cells were measured. In determining the indices (total number of positive cells / seminiferous tubule), 180 tubules per animal were evaluated. The described variables were analyzed using the image analysis program Cellsens Standard (Olympus) from the Department of Anatomy, Institute of Biosciences - UNESP - Botucatu.

The diameter of the seminiferous tubules of the animals was evaluated in transverse tubular sections per slide. Three histological sections of each animal were evaluated. Sixty seminiferous tubules from each section were analyzed, totaling 180 tubules per animal [41].

For the stereological evaluation of the testis, 100 histological fields of each animal (5 animals/group) were analyzed. The images were evaluated using the method described by Weibel (1978) using a 168-point grid [42]. The counting of points on the epithelium, interstitium and the testicular lumen allowed to measure the relative volumes of these regions.

2.7. Protein extraction and Western Blotting

The testes were homogenized in extraction buffer containing, Triton-x-1%, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1mM Hepes pH 7.6, 0.2 mM PMSF and 10 µL / mL of protease inhibitor cocktail. Testicular extracts were obtained by centrifugation for 20 minutes at 4000 rpm at 4ºC. An aliquot of each sample was used to determine the protein concentration, using Bradford's reagent. The corresponding 60 micrograms of protein was applied to the SDS-polyacrylamide gel. After electrophoresis, the material was transferred electrically to nitrocellulose membranes. The membranes were then blocked with 3% skimmed milk (Molico®) diluted in TBS-T for one hour and incubated with the primary AR antibodies (AR N-20 Santa Cruz Biotechnology Inc.®, 1: 100 dilution in skimmed milk 1 %) and PCNA (PC-

10 - VP-P980 - Vector Laboratories® and PC-10 - Novocastra®, dilution 1: 500 in skim milk 1%). After washing with TBS-T buffer, the membranes were incubated for 2 hours with secondary anti-rabbit IgG peroxidase antibodies, antibody produced in goat (A0545 - Sigma®, 1: 20,000 dilution in 1% skimmed milk) and anti-mouse IgG peroxidase, antibody produced in goat (A9044 - Sigma®, 1: 20,000 dilution in 1% skimmed milk). After a new series of washes with TBS-T, the bands were developed with an Amersham ECL Prime Western Blotting Detection Reagent (RPN2236 - Amersham®) chemiluminescent substrate on the G-BOX equipment. B-actin was used as an endogenous control. The intensity of the marking obtained on the different targets was determined through pixel counting using the Image J® software (United States).

2.8. Sperm morphology

For the morphological analysis of the sperm, the volume of 10μL of washing of the vas deferens was used for the preparation of histological slides. Then, the slides were dried at room temperature for 10 minutes for observation with the aid of a phase-contrast light microscope (400X magnification) [43]. Two hundred sperm per animal were evaluated. The morphological abnormalities found in the sperm were classified into: Abnormalities of the head (straight and isolated) and abnormalities of the tail (curled, broken and isolated) [44].

2.9. Daily testicular production

The testicular parenchyma was thawed and placed in a 15mL test tube and weighed, adding 5mL of STM solution containing 0.9% NaCl and 0.05% Triton X100 and homogenized. A new mixture of the homogenate was obtained by dilution with STM in the proportion of 1:10. Then, a sample was transferred to two Neubauer chambers, divided into 2 antimers and spermatids in stage 19 were counted in 5 fields per antimer. The number of spermatids was obtained by the average of the counts multiplied by the dilution factors. In the sperm concentration (number of sperm/g of testis), the average number of sperm was divided by the weight of the testicular parenchyma. The calculation of daily sperm production consisted of dividing the sperm quantity by 6.1 (a factor that corresponds to the number of days that mature spermatids, stage 19 of spermatogenesis, are present in the germinal epithelium) [45].

2.10. Counting the number and transit time of sperm

The collected epididymides were divided into the head (H) + body (B) and tail (T), weighed and frozen. When evaluating the transit time of sperm in the epididymis, the H + B and T segments were prepared as follows: for each 200mg of H + B, 1ml of STM solution was added and for each 100mg of T, 1ml of STM. Then, the samples were submitted to the homogenizer. A new mixture of the homogenate was obtained by dilution with STM in the proportion of 1:20. The number of sperm in each segment was counted in a Neubauer chamber. Two chambers were prepared per animal divided into 2 antimers and 5 fields per antimere were counted. The transit time of sperm through the epididymis was calculated by dividing the number of spermatozoa by the value obtained in the daily production of sperm from each animal.

2.11. Hormonal Analysis

Plasma concentrations of testosterone (ng/mL) and 17β-estradiol (pg/mL) were determined by the competitive ELISA method. The ELISA kit: testosterone (Elabscience Biotechnology Co., Houston, Texas, USA, catalog nº E-EL-0155, 96T, sensitivity of 0.17 ng / mL, detection range between 0.31-20ng / mL, repeatability with coefficient of variation <10%, specificity proven with no significant cross-reaction or interference between T and analogs); estradiol (Elabscience Biotechnology Co., Houston, Texas, USA, catalog nº E-EL-0065, 96T, sensitivity of 25 pg / mL, detection range between 40-1500 pg / mL, repeatability with intra and inter coefficient of variation ≤15 %, proven specificity with no significant cross-reactivity or interference with progesterone and estriol). The samples were read on a spectrophotometer at 450 nm.

2.12. Statistical analysis

The variables were analyzed using the GraphPad Prism program (version 7, GraphPad Software Inc., San Diego, CA). First, the data were subjected to the Shapiro-Wilk normality and variability test. For parametric data, the variables were studied using t-test and analysis of variance (ANOVA) complemented with the Sidak multiple comparison test. For non-parametric data, the Mann-Whitney test was used. The results were expressed as mean ± SD (standard deviation) and presented in figures and tables, considering the level of significance of 0.05%. For the analysis of sperm morphology, the median and interquartile intervals (Q1 - Q3) were used.

Results

Experiment 1

Body weight, Food intake and feeding efficiency

In the evaluation of weight gain and feed intake, no significant differences were found. At weeks 8, 11 and 12, water consumption was higher in the group of animals not supplemented with glucose during adulthood. In addition, the feed efficiency coefficient of the supplemented animals was lower compared to those just programmed (Figure 2).

Table 1 shows the body weights of the rats, testes, and fats collected on the day of the animals' death. In animals supplemented with glucose, the retroperitoneal and visceral fats had lower weights compared to the records of the animals in the glucose group. The cardiac fat of the animals in the glucose supplementation group showed greater weight compared to the glucose group. The adiposity index was also lower in supplemented animals.

Morphological, Immunohistochemistry, Morphometric, Stereological Analysis of the Testis

Comparatively, the experimental groups presented tubules with a histological pattern of cellular differentiation typical of the adult albino rat seminiferous epithelium cycle (Figure 3). Tubular morphological changes characterized by the presence of a lumen containing spermatocytes, elongated spermatids desquamated and residual bodies were found in stages I-VI and VII-VIII in the testis of the animals in the glucose group (Figure 3 A e G). Glucose supplementation caused an increase in testicular fluid, as observed in several tubular sections (Figure 3 F).

The stereological analysis of the regions of the interstitium, epithelium, and testicular lumen indicated that the group supplemented with glucose had a higher relative volume of the interstitium and a lower relative volume of the lumen compared to the data presented by the glucose group (Figure 4).

As for the tubular diameter, the supplemented glucose group showed a 2% reduction compared to the glucose group (Table 2).

PCNA was present in the spermatogonia, primary and secondary spermatocytes nuclei of the studied groups (Figure 3 C-F). The semi-quantitative evaluation of PCNA$^+$ spermatogonia indicated no differences in the cell proliferation rates of the animals (Table 2).

Sertoli, Leydig, and myoid cells showed positive immunostaining for AR. The staining positive AR in these cells was more intense in the supplemented glucose group compared to the glucose group (Figure 3 G-J). Regardless of the experimental group, it was noted that the intensity of labeling of Sertoli cells was strong in seminiferous tubules in stages VII-VIII (Figure 3 G-H), moderate in seminiferous tubules in stages I-VI (Figure 3 H) and XIV (data not shown) and weak in seminiferous tubules in stages IX –XIII (data not shown). The analysis of the count of the number of Sertoli AR$^+$ cells by seminiferous tubule demonstrated that there were no differences between the groups studied (Table 2).

Hormonal Analysis

Regarding the hormonal evaluation, both 17β-estradiol and testosterone showed higher circulating levels in animals supplemented with glucose (Table 2).

Quantitative analysis of PCNA and AR expression in the testis

The quantification of the expression of PCNA and AR in the animals indicated that only the AR showed greater expression in the supplemented animals. The data were normalized by quantification of β-actin (Figure 5).

Analysis of sperm quality parameters

The potential of male fertility was assessed by studying the daily sperm production, the number of mature sperm cells, the sperm count and the transit time in the epididymis. In general, the supplemented glucose group had the highest values of all fertility indicators. Supplementation of animals with glucose in adulthood determined an increase in sperm production, a lower count and accelerated transit in the epididymis (Table 3).

Experiment 2

Body weight, Food intake and feeding efficiency

Figure 6 shows the data of animals exposed to saccharin. There were no differences in body weight gain and feed intake. In the assessment of water consumption, lower values were recorded at weeks 9, 10 and 11 in animals which were supplemented with sodium saccharin in adulthood. The coefficient of feed efficiency was lower in rats supplemented with sodium saccharin in adulthood (Figure 6).

Table 4 shows the body weights of rats, testes, and fats collected on the day of the animals' death. Supplementation of the diet with saccharin sodium offered to adult animals determined a decrease in retroperitoneal fat and an increase in cardiac fat. The other parameters evaluated showed no differences between the groups studied (Table 4).

Morphological, Immunohistochemistry, Morphometric, Stereological Analysis of the Testis

Similarly to the morphological evaluation of the testes of experiment 1, groups S and SS showed cell differentiation typical of the seminiferous epithelium of the adult albino rat (Figure 7). The sodium saccharin group had tubular sections with elongated spermatocytes and spermatids desquamated in the lumen (Figure 7 A, C e E). The supplemented group exhibited a greater number of tubular sections filled with testicular fluid compared to the sodium saccharin group (Figure 7 F).

As for the relative volumes of the regions of the interstitium, epithelium and lumen testicular, as well as the tubular diameter, it was observed that there were no differences between the groups studied in experiment 2 (Figure 7 B and Table 5).

Staining positive PCNA was verified in the spermatogonia, primary and secondary spermatocytes of the studied groups (Figure 7). In addition to the intense PCNA labeling observed in the supplemented group, the semi-quantitative evaluation of PCNA$^+$ spermatogonia indicated a higher proliferation rate (Table 5).

AR was found in Sertoli, Leydig, and myoid cells. The intensity of AR marking in Sertoli cells was found to be similar between the groups studied (Figure 7). However, Leydig cells of the supplemented group showed more intense immunoreactivity when compared to cells from the experimental saccharin sodium group (Figure 7 J). Similar to the observations indicated in experiment 1, the groups presented Sertoli cells with more intense immunoreactivity in seminiferous tubules in stages VII-VIII (Figure 7 H), moderate in seminiferous tubules in stages I-VI (Figure 7 J), and XIV (data not shown) and weak in seminiferous tubules in stages IX-XIII (data not shown). The analysis of the count of the number of Sertoli AR$^+$ cells by seminiferous tubule showed no differences between the groups studied (Table 5).

Hormonal Analysis

With regard to hormonal evaluation, animals supplemented with sodium saccharin had the lowest circulating testosterone levels (Table 5).

Quantitative analysis of PCNA and AR expression in the Testis

The result of the quantification of the expression of the PCNA was lower in the supplemented group, whereas for the same group, the study of the expression of the AR showed greater quantification (Figure 9).

Analysis of sperm quality parameters

In the evaluation of fertility parameters, there was an increase in daily sperm production and in the number of mature sperm cells in supplemented animals. In this same experimental group, transit time was reduced in the head and body of the epididymis. There were no differences in the other parameters evaluated (Table 6).

Discussion

Changes in maternal nutrition, during the critical periods of prenatal development and lactation, have a short and long-term impact by modifying the formation of specific tissues or the secretion of hormones throughout life [46, 47, 48-51]. In this context, the present work studied the morpho-functional response of the testis and the fertility potential of offspring exposed during the pre and postnatal periods to glucose and sodium saccharin, in addition to the additional supplementation of these compounds in adulthood.

The adoption of sodium saccharin in the diet during adulthood caused a reduction in water consumption and feed efficiency, without altering the animals' body weight, contrary to the records of Feijó et al. (2013) [38]. Scientific works describe that the consumption of non-nutritive sweeteners stimulates compensatory food intake in response to the absence of calories related to the perception of sweet taste by the action of sweet oral and intestinal taste receptors that, through afferent vagal impulses, take this information to the thalamus and the reward system [52-58]. The absence of differences in weight gain observed in experiments 1 and 2 may be associated with a short period of exposure to the consumption of sodium saccharin and glucose. The decrease in water intake in both experiments may have been offset by the consumption of the liquid diet.

The consumption of glucose and sodium saccharin determined changes in the levels of the steroid hormones studied. The supplemented glucose group had higher levels of testosterone and estradiol. Associations between circulating estrogen levels and body weight are reported in studies with rats and mice. In short periods, high levels of estrogen act to reduce body weight due to the decrease in food and water intake, in addition to reducing adiposity [59-60]. Although the supplemented glucose group had higher levels of estradiol, this result has no direct association, indicating a possible inhibition of body growth (lower weight), even with a lower rate of adiposity. In experiment 2, the supplemented saccharin group showed only a change in testosterone level. For this study, circulating levels of estradiol are compatible with the behavior of consumption of feed, water, the evolution of body weight and with the records of the weights of fat deposits.

The evaluation of the weight of organs in the male genital system is a useful parameter for verifying the influence of experimental protocols on circulating testosterone levels [61]. In experiments 1 and 2, changes in hormone levels did not directly influence the absolute and relative weights of the organs.

Testosterone, the most important androgen in the seminiferous tubule, is essential for the development and maintenance of spermatogenesis and male fertility [62]. To exert its effects, testosterone has classical, AR-dependent and non-classical mechanisms of action, with activated cellular signaling independent of AR [63]. Non-classical testosterone signaling in Sertoli cells is essential for the survival of spermatocytes and for the production of mature sperm. In the present study, it was observed that animals supplemented with sodium saccharin had the lowest testosterone levels to the detriment of the highest levels of AR expression. In addition, in the semi-quantitative evaluation of Sertoli AR^+ cells, no significant differences were observed between the sodium saccharin and supplemented sodium saccharin groups. Possibly, the difference between the analysis of AR expression and the quantification of its immunolocation in Sertoli cells may be associated with the fact that the expression evaluation also included Leydig cells and peritubular myoids [64-66]. Furthermore, reinforcing the quantitative data on the expression of AR, intense immunoreactivity of AR was observed in the Leydig cells of the animals in the group supplemented with sodium saccharin. It is still necessary to consider that testicular testosterone levels reach values ten times higher when compared to serum [67]. Thus, it is possible that the testicular testosterone levels of the supplemented sodium saccharin group may have influenced the increase in local expression of AR.

As for the study of cell proliferation profiles, only the animals in experiment 2 showed differences. PCNA expression was lower in the supplemented sodium saccharin group, while the quantification of PCNA positive sperm was higher. Furthermore, the daily sperm production of these animals was higher in relation to the sodium saccharin group. In this sense, the desquamation of germ cells observed in the sodium saccharin group and also in the glucose group could, in part, explain the increase in daily sperm production in the supplemented groups. It is possible that the highest expression of PCNA recorded in the sodium saccharin group is related to DNA repair processes, as described by Verderame, Migliaccio, and Scudiero (2018) [68]. In the germinal epithelium, in addition to its action on DNA replication, PCNA detected in non-proliferative pre-meiotic cells, has an important reparative action of post-replication DNA [69].

Regarding the desquamation of germ cells, one of the causes of exfoliation is the possible loss of adhesion between Sertoli cells [70]. This condition is also related to the testicular estrogenization, which induces an increase in the production of the plasminogen activating factor, responsible for loosening the occlusive junctions present in the hematotesticular barrier [71-72].

Another finding of this study was an increase in testicular fluid in animals supplemented with glucose or sodium saccharin. In these same animals, there was a decrease in epididymal transit time. Bearing in mind that the testicular fluid produced by Sertoli cells, includes the presence of androgen-binding protein (ABP), it is believed that a greater volume of testicular fluid would lead to the arrival of higher levels of testosterone in the epididymis, thus contributing to the improvement epididymal functions, including sperm transit [73-74].

Conclusion

The consumption of sodium saccharin and glucose interfered in the plasma levels of testosterone and estradiol and, programmed a drop in sperm production in the non-supplemented animals.

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Authors' contributions

PFFP designed the study. PFF, FEM, ARG and VCF performed the experiments and wrote the manuscript. PFF, ARG, VCF and BMS obtained and analyzed the data. All authors read and approved the final version manuscript.

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Legends

Figure 1: Experimental design.

Figure 2: Body weight, consumption chow, water and feeding efficiency of the male descendants of the experimente 1. Values expressed as mean ± SD. (n=12) * p<0,05.

Figure 3: Cross sections of seminiferous tubules. G (Glucose) and GS (Supplemented Glucose) groups (120 days old). HE (Hematoxilin and Eosin); staining positive PCNA and AR. Spermatogonia (ES), Sertoli Cell (S), Leydig Cell (L), Interstitium (I), Testicular fluid (F), Seminiferous tubules with cells desquamated (**). Seminiferous tubules – I-VI and VII-VIII stages. (A-D) Barra = 100µm, (E-H) Barra = 20µm, (I-J) Barra = 10µm. (n=5).

Figure 4: Relative volumes of the interstice, epithelium and testicular lumen of descendants of experiment 1. Values expressed as mean ± SD. (n=5) * p<0,05.

Figure 5: Optical densitometric analysis of PCNA and RA of the descendants of experiment 1. Values expressed as mean±SD. (n=4) * p<0,05.

Figure 6: Body weight, consumption chow, water and feeding efficiency of the male descendants of the experimente 1. Values expressed as mean ± SD. (n=12) * p<0,05.

Figure 7: Cross sections of seminiferous tubules. S (Sodium Saccharin) and SS (Supplemented Sodium Saccharin) groups (120 days old). HE (Hematoxilin and Eosin); staining positive PCNA and AR. Spermatogonia (ES), Sertoli Cell (S), Leydig Cell (L), Interstitium (I), Testicular fluid (F), Seminiferous tubules with desquamated cells (**). Seminiferous tubules – I-VI and VII-VIII stages. (A) Barra = 50µm, (B-D) Barra = 100µm, (E-H) Barra = 20µm, (I-J) Barra = 10µm. (n=5).

Figure 8: Stereological analysis of animals descendants from experiment 2. Values expressed as mean ± SD. (n=5) *p <0.05.

Figure 9: Optical densitometric analysis of PCNA and AR of the descendants of experiment 2. Values expressed as mean±SD. (n=4) *p<0.05.

Tables

Table 1: Body weight, absolute and relative weights of the testis, fats and adiposity index of males descendants from experiment 1.

Parameters	***Experimental groups***	
	Glucose (n = 12)	***Supplemented Glucose (n = 12)***
Final body weight (g)	479.50 ± 38.40	464.00 ± 25.66
Testis (g)	1.98 ± 0.27	1.91 ± 0.16
Testis (g/100g)	0.41 ± 0.06	0.41 ± 0.04
Epididymal fat (g)	3.99 ± 0.81	3.49 ± 0.73
Retroperitoneal fat (g)	5.26 ± 0.88	3.70 ± 0.72*
Visceral fat (g)	4.39 ± 0.95	3.68 ± 0.68*
Heart fat (g)	0.47 ± 0.07	0.56 ± 0.06*
Adiposity index	2.89 ± 0.49	2.50 ± 0.42*

Values expressed as mean ± SD. *p<0,05.

Table 2: Mean±SD of the cell proliferation index (number of PCNA+ sperm cells/seminiferous tubule), the number of Sertoli AR+ cells/seminiferous tubule, tubular diameter (µm) and plasma testosterone and 17β-estradiol levels of the descendants of the experiment 1.

Parameters	***Experimental groups***	
	Glucose	***Supplemented Glucose***
Cell proliferation index	34.17 ± 6.08	34.62 ± 21.75
Nº of Sertoli AR$^+$ cells/seminiferous tubule	12.07 ± 6.18	12.46 ± 6.82
Tubular diameter (µm)	276.20 ± 20.75	270.20 ± 24.51*
Testosterone (ng/mL)^1	2.14 ± 0.36	2.85 ± 0.47*
17β-estradiol (pg/mL)^1	156.10 ± 21.81	185.90 ± 11.52*

Values expressed as mean ± SD. *p<0,05.
^{1}Hormonal analysis (n=12).

Table 3: Sperm count in the testis and epididymis, transit time and sperm morphology of the descendants of the experiment 1.

Parameters	***Experimental groups***	
	Glucose (n = 8)	***Supplemented Glucose (n = 8)***
Sperm count in the testis		
Daily sperm production ($x10^6$/testis/day)	14.15 ± 2.47	24.25 ± 4.08*
Relative sperm production ($x10^6$/g/testis/day)	8.46 ± 1.03	14.77 ± 2.33*
Number of sperm cells in the testis ($x10^6$)	86.31 ± 15.09	147.90 ± 24.91*
Number of sperm in the testis ($x10^6$/g/day)	51.63 ± 6.30	90.12 ± 14.18*
Sperm count in the epididymis (head/body)		
Number of sperm ($x10^6$)	83.02 ± 11.62	51.87 ± 15.54*
Number of sperm ($x10^6$/g organ)	276.70 ± 48.47	209.80 ± 43.17*
Sperm transit time (days)	5.99 ± 0.91	2.17 ± 0.64*
Sperm count in the epididymis (tail)		
Number of sperm ($x10^6$)	177.90 ± 53.15	144.50 ± 55.23
Number of sperm ($x10^6$/g organ)	1050.00 ± 84.59	781.30 ± 245.30*
Sperm transit time (days)	12.83 ± 3.89	5.88 ± 1.64*
Total sperm transit time (days)	18.82 ± 3.99	8.05 ± 1.37*
Sperm morphology^1		
Normal sperm (%)	99.50 (99.00 – 99.63)	99.50 (99.50 – 99.63)
Abnormal sperm (%)	0.50 (0.38 – 1.00)	0.50 (0.38 – 0.50)

Broken tail	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)
Curled tail	0.00 (0.00 – 0.50)	0.00 (0.00 – 0.13)
Isolated tail	0.50 (0.00 – 0.50)	0.00 (0.00 – 0.13)
Tail with broken intermediate piece	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.13)
Isolated head	0.00 (0.00 – 0.13)	0.00 (0.00 – 0.50)
Straight head	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)

Values expressed as mean ± SD. *p<0,05.
[1] Values expressed as median and interquartile range [Q1 – Q3]. Teste Mann-Whitney. *p<0,05.

Table 4: Body weight, absolute and relative weights of the testis, fat and adiposity index of males descended from experiment 1.

Parameters	***Experimental groups***	
	Sodium Saccharin (n=12)	***Supplemented Sodium Saccharin (n=12)***
Final body weight (g)	*451.80 ± 24.61*	*452.10 ± 24.54*
Testis (g)	*1.78 ± 0.17*	*1.78 ± 0.15*
Testis (g/100g)	*0.39 ± 0.03*	*0.39 ± 0.02*
Epididymal fat (g)	*3.31 ± 0.48*	*3.46 ± 0.51*
Retroperitoneal fat (g)	*3.91 ± 0.66*	*3.25 ± 0.53**
Visceral fat (g)	*3.78 ± 0.50*	*3.57 ± 0.49*
Heart fat (g)	*0.55 ± 0.09*	*0.64 ± 0.11**
Adiposity index	*2.65 ± 0.37*	*2.68 ± 0.50*

Values expressed as mean ± SD. *p<0,05.

Table 5: Mean±SD the cell proliferation index (number of sperm PCNA+/seminiferous tubule), the number of Sertoli AR+ cells/seminiferous tubule, tubular diameter (µm) and the plasma levels of testosterone and 17β-estradiol of the descendants of experiment 2.

Parameters	***Experimental groups***	
	Sodium Saccharin	**Supplemented Sodium Saccharin**
Cell proliferation index	21.89 ± 5.50	27.12 ± 5.33 *
Nº of Sertoli AR^+ *cells/seminiferous tubule*	13.15 ± 5.30	13.01 ± 5.08
Tubular diameter (µm)	279.40 ± 18.60	276.50 ± 21.02
Testosterone (ng/mL)[1]	2.21 ± 0.46	1.38 ± 0.23*
17β-estradiol (pg/mL)[1]	141.00 ± 19.17	141.60 ± 18.70

Values expressed as mean ± SD. *p<0,05.
[1] Hormonal analysis (n=12).

Table 6: Sperm count in the testis and epididymis, transit time and sperm morphology of the descendants of experiment 2.

Parameters	***Experimental groups***	
	Sodium Saccharin (n = 8)	***Supplemented Sodium Saccharin (n = 8)***
Sperm count in the testis		
Daily sperm production (x10^6/testis/day)	15.67 ± 2.34	20.59 ± 4.30 *
Relative sperm production (x10^6/g/testis/day)	11.00 ± 2.02	13.37 ± 2.96
Number of sperm cells in the testis (x10^6)	95.58 ± 14.25	125.60 ± 26.25 *
Number of sperm in the testis (x10^6/g/day)	67.08 ± 12.29	81.57 ± 18.07
Sperm count in the epididymis (head/body)		
Number of sperm (x10^6)	83.23 ± 11.85	76.72 ± 14.10
Number of sperm (x10^6/g organ)	334.30 ± 36.77	312.50 ± 67.96
Sperm transit time (days)	5.50 ± 1.14	3.90 ± 1.18 *
Sperm count in the epididymis (tail)		
Number of sperm (x10^6)	161.10 ± 36.34	194.10 ± 68.85
Number of sperm (x10^6/g organ)	827.50 ± 106.60	903.60 ± 247.60
Sperm transit time (days)	10.24 ± 1.84	9.55 ± 3.41
Total sperm transit time (days)	15.74 ± 2.05	13.45 ± 3.37
Sperm morphology[1]		
Normal sperm (%)	100.00 (99.00 – 100.00)	99.75 (99.13 – 100.00)
Abnormal sperm (%)	0.00 (0.00 – 1.00)	0.25 (0.00 – 0.88)
Broken tail	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)
Curled tail	0.00 (0.00 – 0.50)	0.00 (0.00 – 0.00)
Isolated tail	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.38)
Tail with broken intermediate piece	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)
Isolated head	0.00 (0.00 – 0.50)	0.00 (0.00 – 0.00)
Straight head	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.00)

Values expressed as mean ± SD. *p<0,05.

[1] Values expressed as median and interquartile range [Q1 – Q3]. Teste Mann-Whitney. *p<0,05.

Figure 1

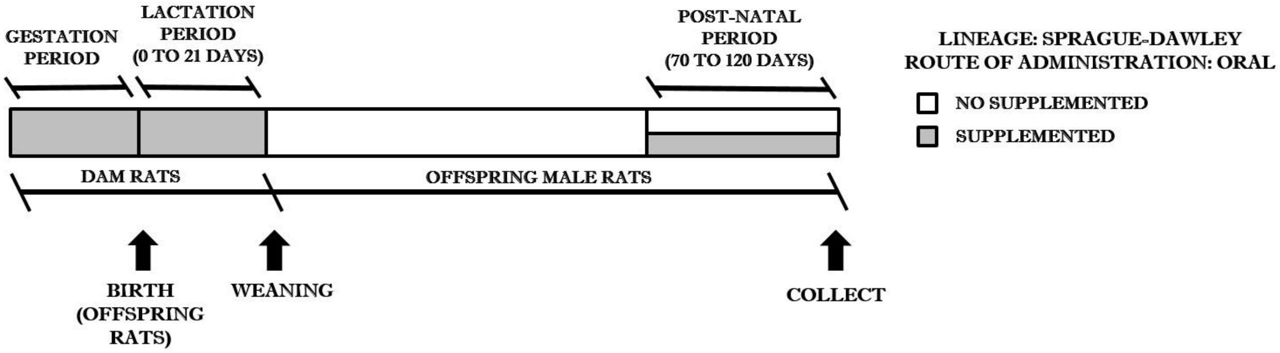

GESTATION PERIOD
LACTATION PERIOD (0 TO 21 DAYS)
POST-NATAL PERIOD (70 TO 120 DAYS)
LINEAGE: SPRAGUE-DAWLEY
ROUTE OF ADMINISTRATION: ORAL
NO SUPPLEMENTED
SUPPLEMENTED
DAM RATS
OFFSPRING MALE RATS
BIRTH (OFFSPRING RATS)
WEANING
COLLECT

Figure 2

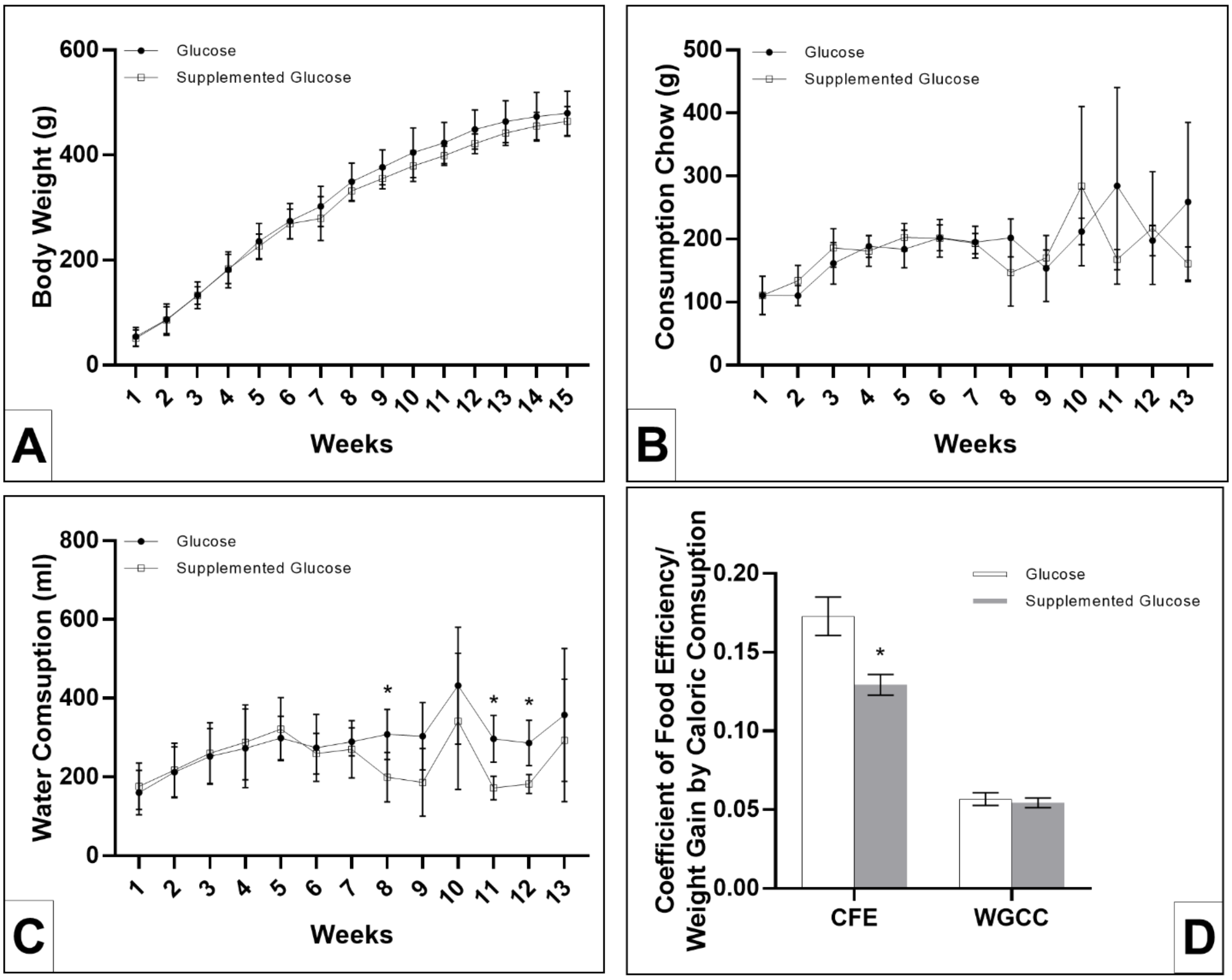

A
Body Weight (g)
600
400
200
0
Glucose
Supplemented Glucose
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Weeks
B
Consumption Chow (g)
500
400
300
200
100
0
Glucose
Supplemented Glucose
1 2 3 4 5 6 7 8 9 10 11 12 13
Weeks
C
Water Comsuption (ml)
800
600
400
200
0
Glucose
Supplemented Glucose
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12 13
Weeks
D
Coefficient of Food Efficiency/
Weight Gain by Caloric Comsuption
0.20
0.15
0.10
0.05
0.00
Glucose
Supplemented Glucose
*
CFE
WGCC

Figure 3

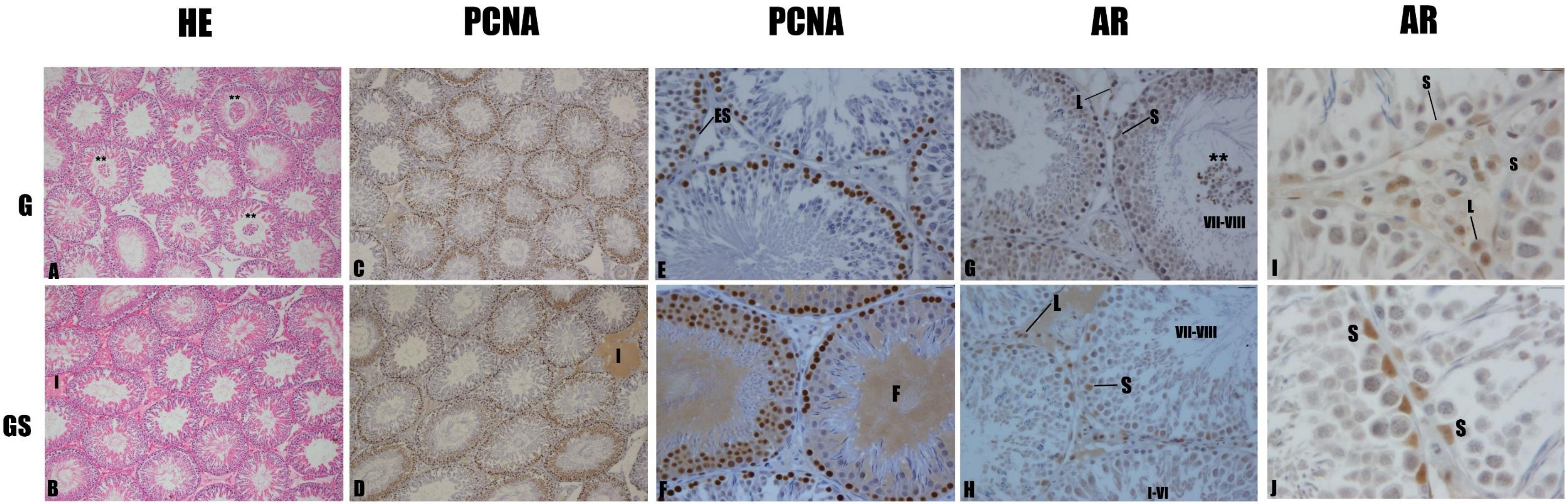

HE
PCNA
PCNA
AR
AR
G
GS
A
**
**
**
**
B
I
C
D
I
E
ES
F
F
G
L
S
**
VII-VIII
H
L
VII-VIII
S
I-VI
I
S
S
L
J
S
S

Figure 4

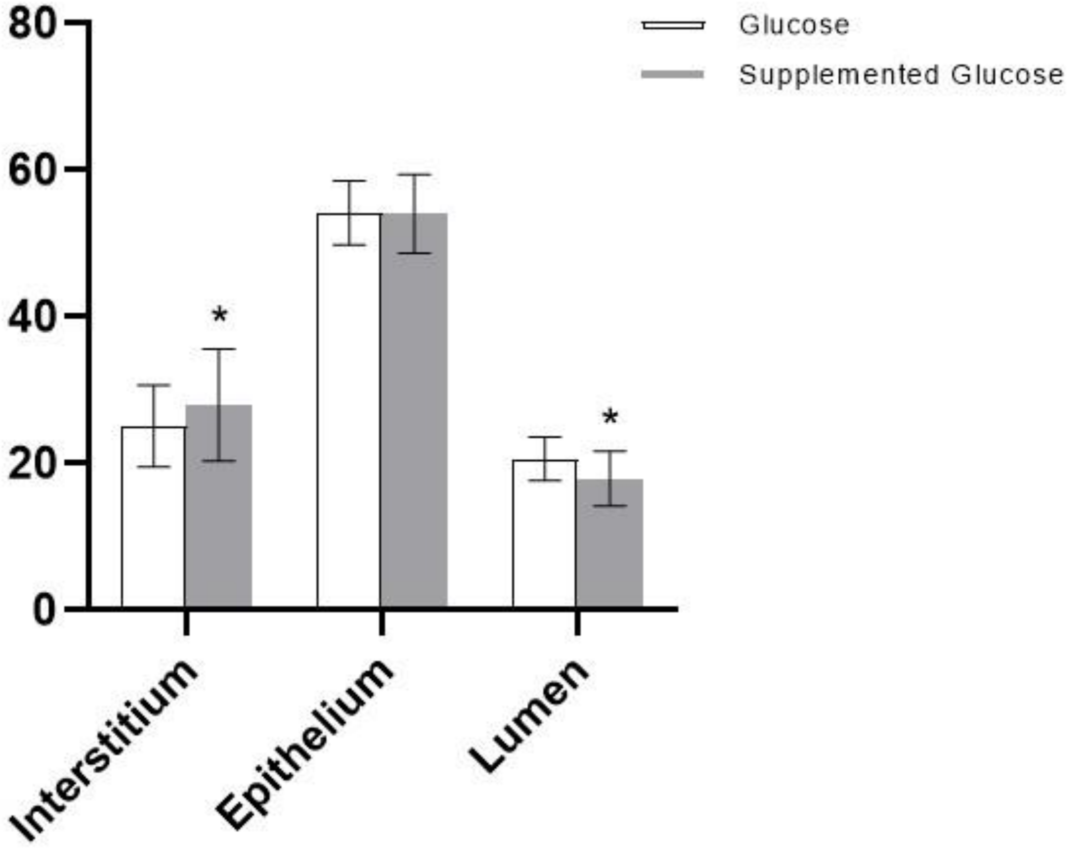

80
60
40
20
0
Glucose
Supplemented Glucose
*
*
Interstitium
Epithelium
Lumen

Figure 5

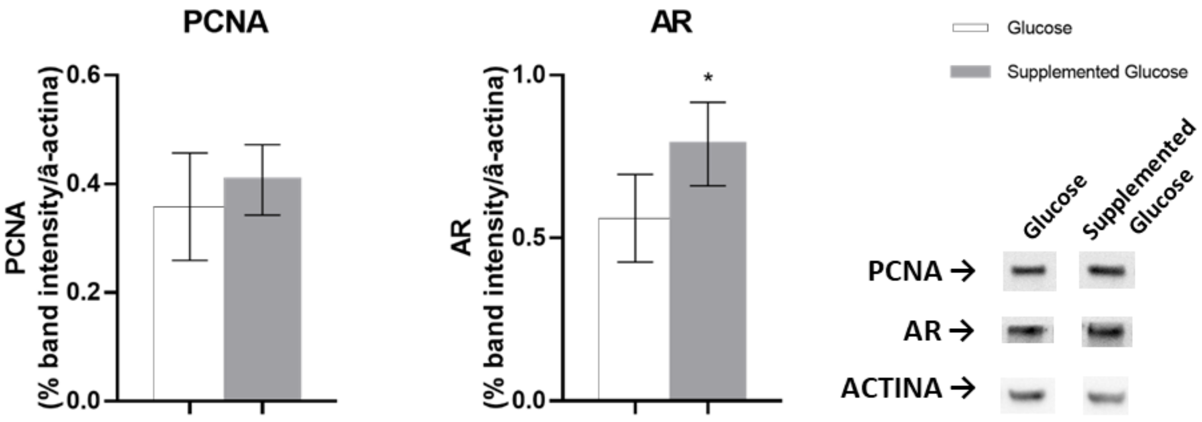

PCNA
PCNA (% band intensity/â-actina)
0.6
0.4
0.2
0.0
AR
AR (% band intensity/â-actina)
1.0
0.5
0.0
*
Glucose
Supplemented Glucose
Glucose
Supplemented Glucose
PCNA →
AR →
ACTINA →

Figure 6

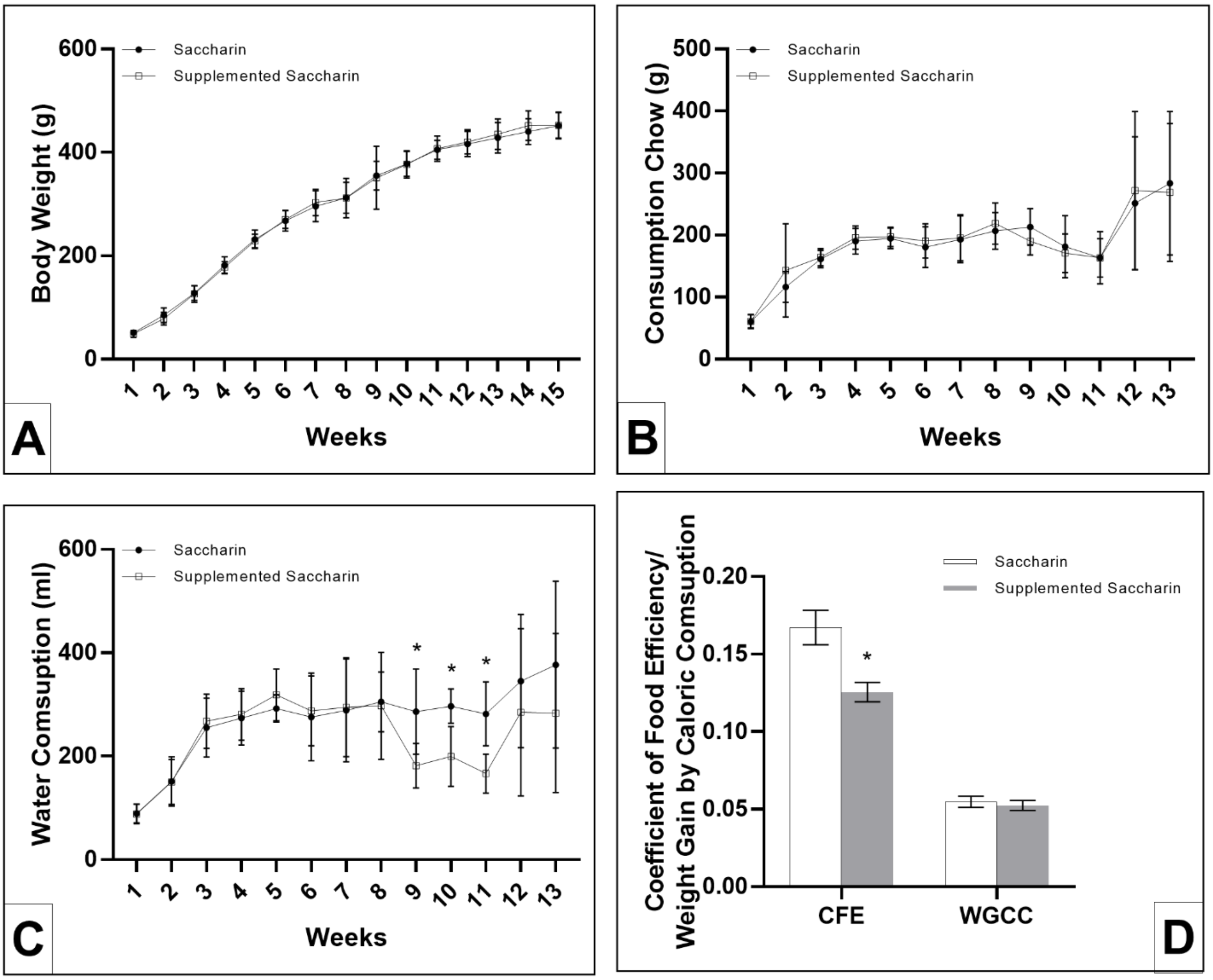

A
Body Weight (g)
600
400
200
0
Saccharin
Supplemented Saccharin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Weeks
B
Consumption Chow (g)
500
400
300
200
100
0
Saccharin
Supplemented Saccharin
1 2 3 4 5 6 7 8 9 10 11 12 13
Weeks
C
Water Comsuption (ml)
600
400
200
0
Saccharin
Supplemented Saccharin
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12 13
Weeks
D
Coefficient of Food Efficiency/
Weight Gain by Caloric Comsuption
0.20
0.15
0.10
0.05
0.00
Saccharin
Supplemented Saccharin
*
CFE
WGCC

Figure 7

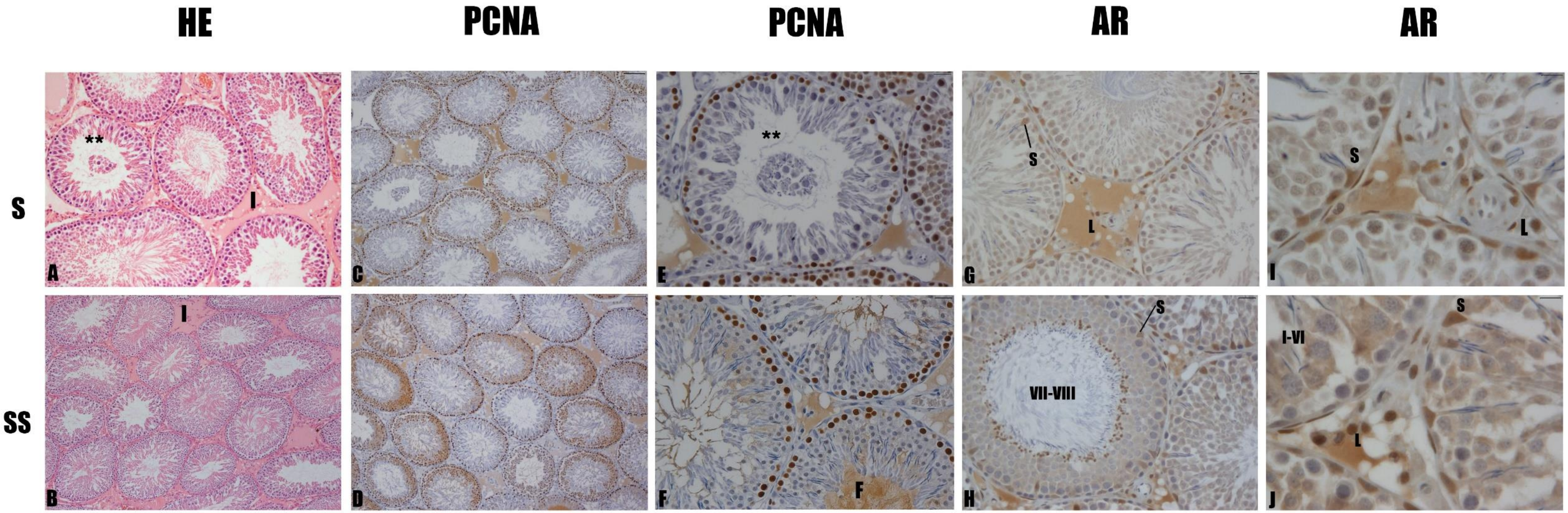


Figure 8

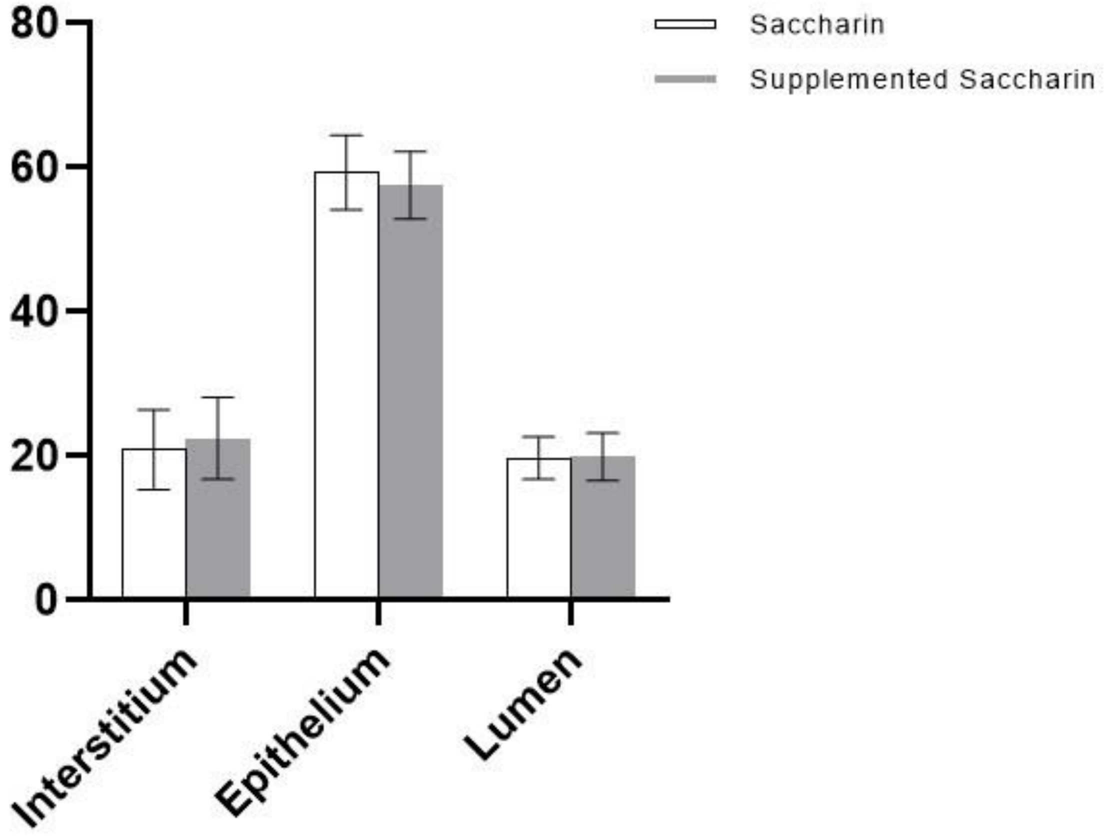
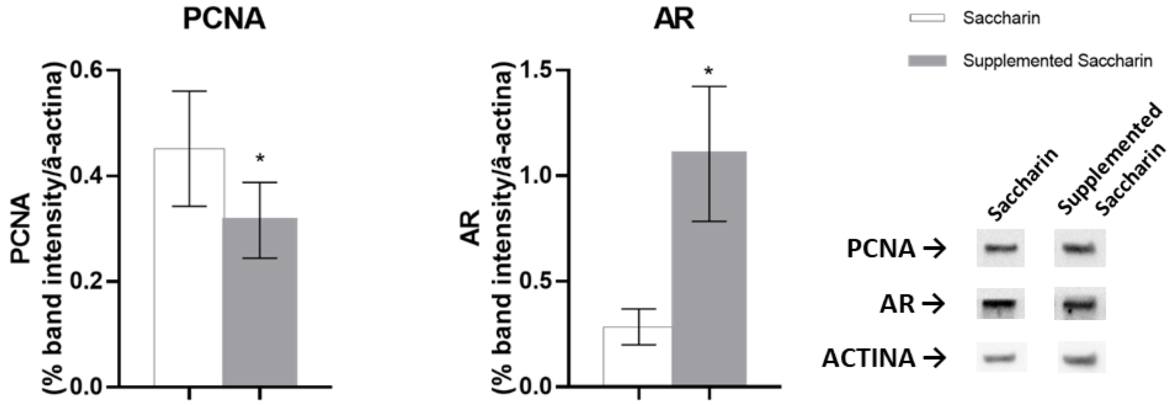

80
60
40
20
0
Saccharin
Supplemented Saccharin
Interstitium
Epithelium
Lumen

Figure 9


PCNA
PCNA
(% band intensity/â-actina)
0.6
0.4
0.2
0.0
*
AR
AR
(% band intensity/â-actina)
1.5
1.0
0.5
0.0
*
Saccharin
Supplemented Saccharin
Saccharin
Supplemented Saccharin
PCNA →
AR →
ACTINA →

CONCLUSÕES

As respostas adaptativas confirmam a existência de programação fetal por consumo de sacarina sódica e glicose. Essas adaptações foram demonstradas pelas alterações metabólicas e morfofuncionais confirmadas pela:

- predisposição ao diabetes;
- interferência da sacarina sódica nas alterações dos níveis plasmáticos de testosterona e estradiol;
- queda na produção espermática nos animais programados com sacarina sódica e glicose.

APÊNDICES

Estresse oxidativo

É bem conhecido que algumas das alterações morfofuncionais associadas à programação fetal por alterações na nutrição materna podem levar ao surgimento de doenças cardiovasculares e metabólicas. Em sua fase inicial, essas doenças apresentam elevação nos níveis das espécies reativas de oxigênio (ERO), causando o desequilíbrio entre as defesas oxidante/antioxidante caracterizando o estresse oxidativo (OSAWA & KATO, 2005; WU et al., 2006). O diabetes mellitus tipo 2, por exemplo, é uma doença metabólica com importantes alterações no estresse oxidativo, tanto na sua fase inicial quanto na fase de progressão (BAYNES, 1991). Neste sentido, há uma atenção voltada para a adoção de antioxidantes na dieta, com a finalidade de suprimir o estresse oxidativo e, assim, prevenir o diabetes mellitus (OSAWA & KATO, 2005). Ademais, sabe-se que a obesidade, também observada nos quadros de alterações metabólicas decorrentes da programação fetal por má nutrição materna, amplifica o cenário de desequilíbrio das defesas oxidante/antioxidante (WU et al., 2006; CHALLIER et al., 2008).

As ERO, como o superóxido (O2-.), a hidroperoxila (HO2.), a hidroxila (OH) e o peróxido de hidrogênio (H2O2), em condições fisiológicas do metabolismo celular aeróbio, resultam da redução do oxigênio (O2) com a formação de molécula de água (H2O). Neste contexto, pode-se dizer que todos os sistemas fisiológicos geram ERO, levando o organismo, por meio da ação de agentes antioxidantes, à neutralização destes compostos impedindo ou minimizando possíveis danos causados pelas ERO (FERREIRA; MATSUBARA, 1997).

Para proteger-se do ataque das ERO, a célula pode atuar em duas linhas de defesa, sendo uma anterior à lesão celular e outra atuante no reparo da lesão. A prevenção se dá pela ação da vitamina E e das enzimas glutationa reduzida (GSH), superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GHS-Px), enquanto que o mecanismo de reparo está condicionado à presença do ácido ascórbico, da glutationa-redutase (GSH-Rd) e da GSH-Px (FERREIRA; MATSUBARA, 1997). No testículo, as enzimas SOD e a GSH-Px são as mais importantes na defesa oxidante/antioxidante, sendo produzidas pelas células germinativas, células de Sertoli e células de Leydig (RODRÍGUEZ-GONZÁLEZ et al., 2014).

O estresse oxidativo é caracterizado pelo desequilíbrio entre a geração de radicais livres capazes de atacar as membranas por meio da lipoperoxidação e produção de enzimas combatentes conhecidas como agentes antioxidantes. A lipoperoxidação é um processo altamente tóxico que gera danos na estrutura e na função das membranas plasmáticas e do DNA, ocasionando morte celular por apoptose (FERREIRA, MATSUBARA, 1997). Este fenômeno é considerado importante na patogênese de algumas doenças e tem sido relacionado com o envelhecimento precoce do sistema genital masculino (SIKKA, 1996; RODRÍGUEZ-GONZÁLEZ et al., 2014; MCHUNU et al., 2019).

Ressalta-se que na literatura especializada há estudos clínicos e experimentais explorando o papel dos adoçantes no estresse oxidativo (MCHUNU et al., 2019). Os agentes antioxidantes mais importantes produzidos pelos testículos são a SOD e a GSH-Px (RODRÍGUEZ-GONZÁLEZ et al., 2014), responsáveis por reduzir o H2O2 aos seus respectivos álcoois. A GSH-Px catalisa H2O2 às custas da conversão de GSH a GSSG (FERREIRA, MATSUBARA, 1997). Dessa forma, através dos níveis de expressão da GSH, podemos estimar a atividade da GSH-Px. O GSH é um importante biomarcador da funcionalidade celular onde, a diminuição deste resulta em alteração significante no metabolismo celular (ASHOK et al., 2017).

Nesta pesquisa, a dieta líquida foi capaz de aumentar os níveis de enzimas antioxidantes SOD, GSH e CAT no testículo da prole de mães tratadas. Os animais adultos dos grupos tratados, de forma semelhante aos seus respectivos grupos maternos, exibiram aumento nos níveis enzimáticos comparado aos perfis de animais não tratados. Destaca-se que os níveis elevados dessas enzimas estão alinhados à predisposição ao estado diabético.

Apesar da dieta líquida configurar como fator significativo desencadeador no aumento da atividade das enzimas antioxidantes, quando foi realizada a adição da sacarina ou da glicose, estes níveis mostraram-se aumentados. A sacarina aumentou os níveis de CAT nos animais de DPN120 tratados. A glicose aumentou os níveis de GSH nos animais não tratados e de CAT nos animais tratados no DPN120. Além disso, o aumento do estresse oxidativo foi acompanhado do declínio nos níveis plasmáticos de testosterona e estradiol.

Em estudo com ratos Sprague-Dawley diabéticos, que foram desafiados ao consumo de aspartame, sacarina, sucralose e ciclamato de sódio, foram obtidas respostas diferentes em diversos órgãos. No entanto, de maneira geral, a sacarina foi capaz de diminuir os níveis de estresse oxidativo em comparação aos grupos controle e controle diabético (MCHUNU et al., 2019).

Neste estudo não foi possível determinar se nas idades avaliadas (DPN21 e DPN120) houve aumento nos níveis de estresse oxidativo, pois não foi realizada a quantificação da lipoperoxidação ou da produção de malonaldeído (MDA) para determinar se houve desequilíbrio na razão entre os agentes oxidantes/antioxidantes.

É importante ressaltar que o estresse oxidativo possui importante papel na função e qualidade espermáticas. Os agentes antioxidantes são responsáveis pela manutenção da funcionalidade celular, porém esse sistema é parcialmente eficaz quando a formação de radicais livres supera a de agentes antioxidantes (ASHOK et al., 2017).

Diferentemente dos resultados deste estudo, um estudo realizado com ratos Wistar consumidores de aspartame, os pesquisadores detectaram aumento nos níveis de lipoperoxidação e diminuição nos níveis de GSH, SOD, CAT, GSH-Px e vitamina C. Além disso, estes animais apresentaram menor viabilidade espermática e maiores defeitos morfológicos nos espermatozoides (ASHOK, et al. 2017).

Neste sentido, vale destacar que os espermatozoides são as células mais suscetíveis ao EO, pois possuem altos níveis de ácidos graxos insaturados em sua membrana, o que facilita o processo de lipoperoxidação. Ademais, por possuírem reduzido conteúdo citoplasmático, os espermatozoides produzem menor quantidade de agentes antioxidantes, dependendo apenas da maquinaria mitocondrial. No testículo também há grande produção de ERO porém, este órgão possui um sistema de defesa sofisticado que garante as funções espermatogênica e esteroidogênica. Sendo assim, alguns autores atribuem ao testículo a defesa antioxidante dos espermatozoides (RODRÍGUEZ-GONZÁLEZ, 2014).

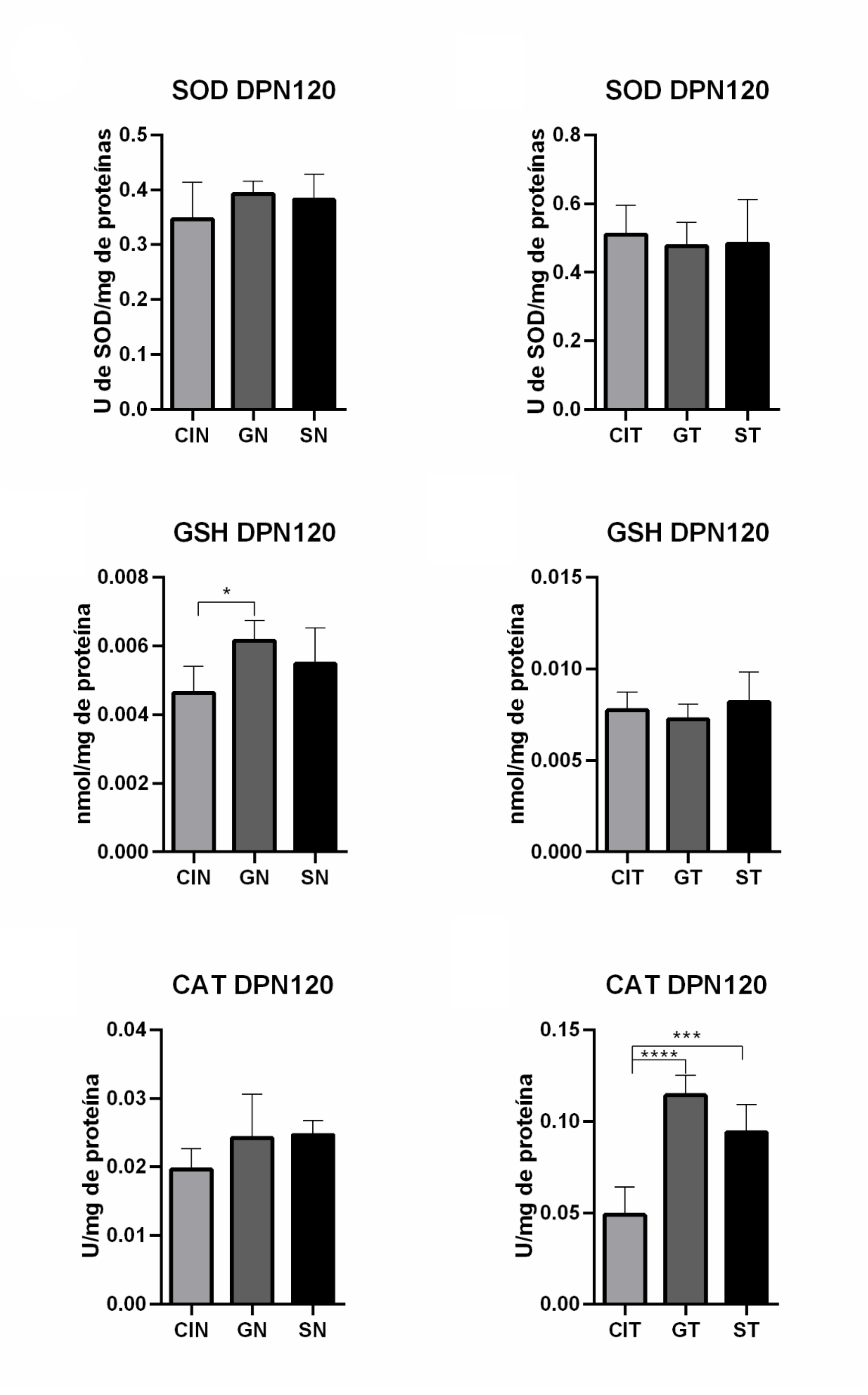


Figura 1: Média±SD dos níveis de SOD, GSH e CAT do DPN120. One-way ANOVA com pós-teste Tukey. SOD – Superóxido dismutase; GSH – Glutationa reduzida; CAT – Catalase; DPN – Dia pós-natal; CIN – Controle iogurte não tratado; CIT – Controle iogurte tratado; GN – Glicose não tratado; GT – Glicose tratado; SN – Sacarina não tratado; ST – Sacarina tratado; * p<0,05; ** p<0,005; *** p<0,0005; **** p<0,0001.

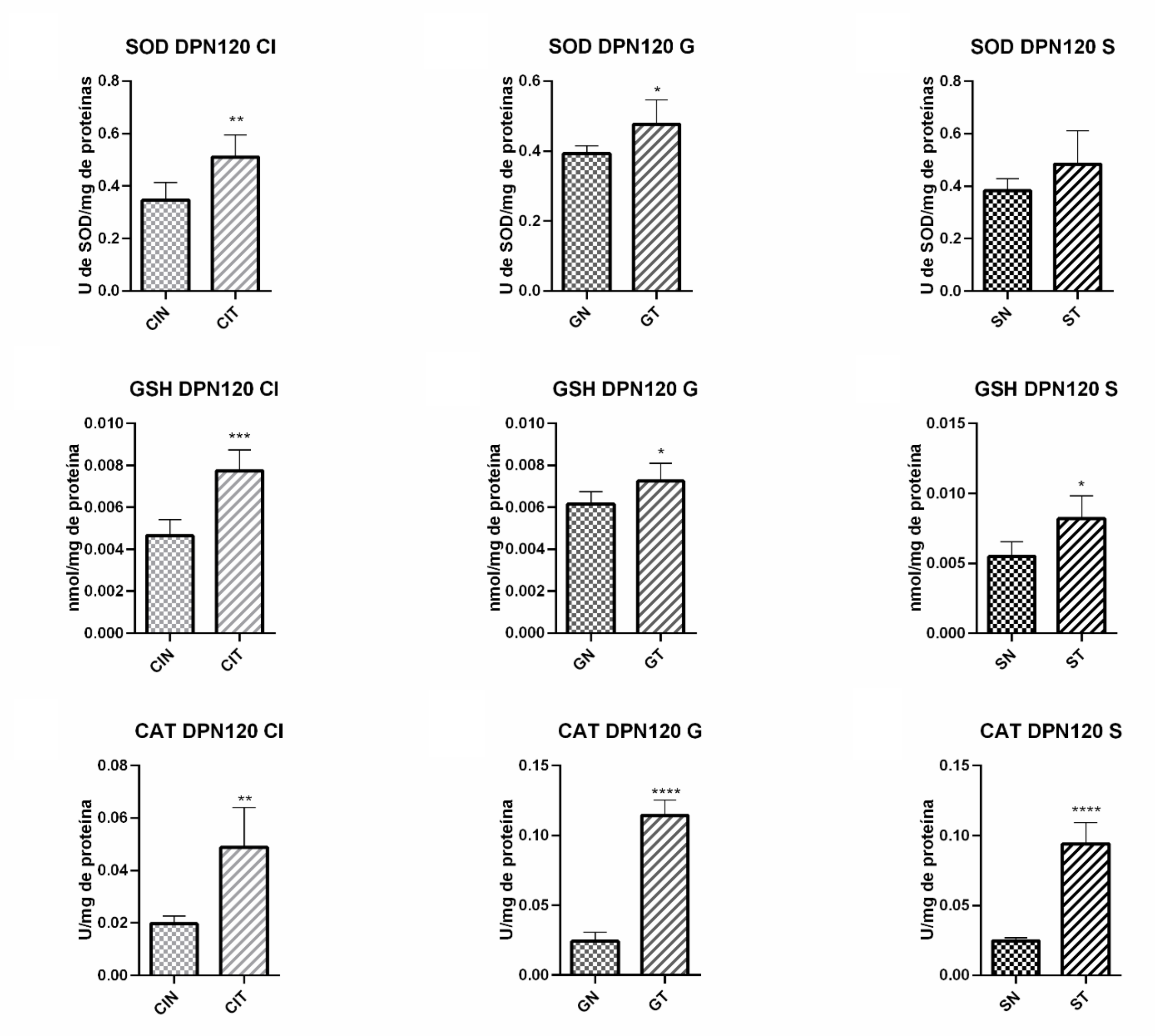


Figura 2: Média±SD dos níveis de SOD, GSH e CAT do DPN120. Teste t. SOD – Superóxido dismutase; GSH – Glutationa reduzida; CAT – Catalase; DPN – Dia pós-natal; CI – Controle iogurte; CIN – Controle iogurte não tratado; CIT – Controle iogurte tratado; G – Glicose; GN – Glicose não tratado; GT – Glicose tratado; S – Sacarina; SN – Sacarina não tratado; ST – Sacarina tratado; * p<0,05; ** p<0,005; *** p<0,0005; **** p<0,0001.

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