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IANNY BRUM REIS

"EFFECT OF *Ilex paraguariensis* (ST. HIL) ON CADMIUM INDUCED DAMAGE TO WISTAR RAT TESTICLES"

"ANÁLISE DOS TESTÍCULOS DE RATOS WISTAR SUBMETIDOS A TRATAMENTO COM ASSOCIAÇÃO DE CÁDMIO E *Ilex paraguariensis* (ST. HIL.)"

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RESUMO

O cádmio é conhecido pelos seus efeitos genotóxicos e espermiotóxicos, sendo um dos poluentes ambientais e industriais mais tóxicos. Considerando essas informações, o desenvolvimento de terapias naturais para diminuir os danos que esse metal pode causar é considerado altamente pertinente. *Ilex paraguariensis,* (St. Hil) conhecida como erva mate, tem sido utilizada na medicina tradicional e sua infusão apresenta propriedades hepatoprotetoras, hipoglicêmica, antiinflamatórias, dentre outras. Estes efeitos foram atribuídos aos compostos fenólicos presentes nesta planta. Nós hipotetizamos que a infusão da erva mate poderia minimizar o estresse oxidativo, modulando a defesa antioxidante enzimática. Para testar esta hipótese, foram realizados dois experimentos, um de curta duração (15 dias) e outro de longa duração (56 dias). O experimento de curta duração consistiu em: C-15. controle (água), M-15. erva mate, Cd-15. cádmio, CdM-15. cadmio seguido de erva mate e MCd-15. erva mate durante 15 dias seguido de cádmio no último dia. O experimento a longo prazo empregou grupos muito semelhantes, mantidos durante 56 dias, incluindo: C-56. controle (água), M-56. erva-mate, Cd-56 cádmio, MCd-56 cádmio seguido de erva-mate. Uma dose única de cloreto de cádmio (1,2 mg / kg de peso corporal) foi injetada i.p. em ratos adultos (grupos Cd-15, MCd-15, CdM-15, Cd-56, MCd-56). A erva-mate usada nesta pesquisa foi a erva comercial (110mg de erva-mate / 1000mL de água). Cada animal recebeu diariamente 0,5 mL de água (grupos C-15, Cd-15, C-56, Cd-56) ou infusão (grupos M-15, MCd-15, CdM-15, M-56, MCd -56). Após o tratamento, os animais foram anestesiados com xilazina e cetamina (5: 80mg / kg). Os testículos foram removidos, pesados, fixados e rotineiramente processados para microscopia de luz e eletrônica. O testículo esquerdo e o epidídimo foram congelados (-20 ° C) para o cálculo da produção espermática diária (DSP) e o número de espermatozóides no epidídimo e o trânsito espermático, respectivamente. Analisamos danos oxidativos lipídicos atráves da dosagem de MDA e as defesas antioxidantes nos testículos (SOD e CAT), bem como a dosagem de cádmio nos testículos. A análise histopatológica mostrou claramente danos induzidos pelo cádmio, tais como danos nas células germinativas. Diferenciação celular e desenvolvimento de espermatozóides não foram observados na maioria dos testículos deste grupo. O cádmio afetou a taxa de produção espermática e DSP durante os tratamentos. O cádmio diminuiu a espermatogênese e estimulou as atividades antioxidantes através do aumento de enzimas SOD e CAT, provavelmente devido à indução de uma resposta adaptativa, no modo de manutenção e / ou aumento das atividades fisiológicas. Concluimos que a ingestão de infusão de *Ilex paraguariensis* durante 15 dias é apenas parcialmente eficiente na proteção dos testículos contra os danos induzidos por cádmio. E a integridade do testículo foi parcialmente preservada no grupo tratado com infusão de cádmio e *Ilex paraguariensis* durante 56 dias, sugerindo um efeito protetor da planta sobre o parênquima testicular. Em contrapartida, os grupos que receberam apenas infusão da erva-mate, em ambos tratamentos (15 e 56 dias), não apresentaram alterações negativas em relação a dinâmica e integridade testicular, e em alguns parâmetros analisados, foi observado aumento da espermatogêneses e maior produção de enzimas antioxidantes, sugerindo melhora substancial. Mostrando não haver correlação entre o consumo da erva e danos testisculares.

Palavras-chave: estresse oxidativo; fisiologia; morfologia; chá mate; metal pesado.

ABSTRACT

Cadmium is known for its genotoxic and spermiotoxic effects and is one of the most toxic environmental and industrial pollutants. Considering these characteristics, the development of natural therapies to diminish the damage it can cause is highly pertinent. *Ilex paraguariensis* (St. Hil), known as yerba mate, has been used in traditional medicine and its infusion exhibits hepatoprotective, antirheumatic, and anti-inflammatory properties. These effects have been attributed to the phenolic compounds present in this plant. We hypothesized that yerba mate infusion could minimize oxidative stress, modulating enzymatic antioxidant defense. To test this hypotesis, two experiments were performed. The short-term experiment (15 days) consisted in the groups (n=5): C-15. control (water), M-15. yerba mate, Cd-15. cadmium, CdM-15. cadmium followed by yerba mate and MCd-15. mate during 15 days followed by cadmium on the last experimental day. The long term experiment employed similar groups maintained for 56 days, including: C-56. control (water), M-56. yerba mate, Cd-56 cadmium, MCd-56. cadmium followed by yerba mate. A single dose of cadmium chloride (1.2 mg/kg BW) was injected i.p. in adult rats (groups Cd-15, MCd-15, CdM-15, Cd-56, MCd-56). The yerba mate used in this research was commercial tea (110mg yerba mate/1000mL water). Each animal received, daily, 0.5mL of either water (groups C-15, Cd-15, C-56, Cd-56) or infusion (groups M-15, MCd-15, CdM-15, M-56, MCd-56). After the treatment, animals were anesthetized with Xylazine and Ketamine (5:80mg/kg). Testicles were removed, weighed, fixed and routinely processed for light and electron microscopy. The left testis and epididymis were frozen (-20°C) for the calculation of daily sperm production (DSP), the number of sperm within the epididymis and sperm transit, respectively. We analyzed lipid oxidative damage and antioxidant defense in testes tissue and cadmium acumulation in this tissue. Histopatological analysis clearly showed damage induced by cadmium, such as defective germ cells. Cell differentiation and spermatozoa development were not observed in some more strongly affected testicles. Cadmium affects DSP and sperm production rate over a period of time. Cadmium affected spermatogenesis and stimulated the antioxidant activities probably due to an adaptive response, in an effort to maintain and/or increase physiological activities. *Ilex paraguariensis* infusion ingestion during 15-days is only partially efficient in protecting the testicles from cadmium induced damage. Testis integrity was partially preserved in the group treated with both cadmium and *Ilex paraguariensis* infusion during 56 days, suggesting a protective effect of the plant on the testicular parenchyma. On the other hand, groups that received only mate, in both experiments, testicular dynamics and integrity were not altered, and substantial improvement was observed in some parameters analyzed, such as spermatogenesis and antioxidant enzime production. Therefore, no harmful correlations could be drawn associated between the use of plant infusion and testicle structure.

Key words: oxidative stress; phisiology; morphology; mate tea; heavy metal.

SUMÁRIO

1. INTRODUÇÃO GERAL

A consequência mais negativa do processo de industrialização e da agricultura em larga escala é a produção de resíduos, cujo manuseio e destino tornaram-se um grande problema, uma vez que são potencialmente deletérios à saúde humana (Asmus, 2008). Os principais resíduos são os metais pesados (peso atômico acima de 22) e os produtos químicos orgânicos (Pereira, 2008).

Os metais pesados podem interagir com a regulação endócrina e/ou componentes do sistema reprodutor, inclusive induzir estresse oxidativo (Kavlock et al., 1996; Gupta et al., 2004). Nos último anos, a infertilidade, tanto em humanos como demais espécies animais, tem sido motivo de preocupação.

A busca por terapias capazes de reverter tais danos vem aumentando com o passar do tempo, e diversos produtos naturais com propriedades que atuam em diversas etapas destas disfunções reprodutivas vem sendo estudados estudados (El-Missiry e Shalaby, 2000; Yang *et al.,* 2006).

1.1 Contaminação ambiental por metais pesados

No que diz respeito ao aumento dos níveis de metais pesados, atividades como a mineração, indústrias de galvanoplastia, e o despejo de efluentes domésticos tem importante papel (Blazquez, 2005; Zouboulis et al., 2004). A presença de metais muitas vezes está associada à localização geográfica, seja na água ou no solo, e pode ser controlada, limitando o uso de produtos agrícolas e proibindo a produção de alimentos em solos contaminados com metais pesados.

Os metais tendem a se acumular no ambiente, representando assim, risco potencial de contaminação ao longo do tempo (Oliveira et al., 2010). A contaminação do solo por esses elementos é uma preocupação mundial devido a alta toxicidade, persistência em longo prazo e a incorporação subsequente dos metais na cadeia trófica, o que pode levar a efeitos prejudiciais ao ambiente e à saúde humana (Venegas et al., 2015).

Os metais pesados podem ser adicionados ao solo por meio da aplicação de lodo de esgoto, adubação fosfatada (Hooda e Alloway, 1998), fundição, mineração,

uso de pesticidas, fertilizantes e lamas (Paz-Ferreiro et al., 2014), além de outros resíduos provenientes de indústrias e resíduos urbanos. A mineração e o processamento de metais têm causado contaminação ambiental diversa e difusa, com altas concentrações de vários metais, como cádmio, cobre, chumbo e zinco.

Adicionalmente há que se destacar a presença dos metais pesados na água para consumo humano. De acordo a Organização Mundial de Saúde (WHO, 2011), o nível de ingestão tolerável na água potável é de 3 µg L-1 para cádmio e 10 µg L -1 para chumbo. Esses elementos podem ser lixiviados dos solos até atingir águas subterrâneas, ampliando a probabilidade de adentrar na cadeia trófica em teor acima dos toleráveis.

1.2 Cádmio

O cádmio (Cd) é um metal pesado encontrado no ambiente, sendo metabolizado por plantas quando presente no solo e como resultado de atividade vulcânica (erupção e baixa atividade). Fontes antropogênicas, relacionadas principalmente com a mineração, incineração de resíduos e combustão de carvão mineral e óleo contribuem para a disseminação deste elemento (Robards e Worsfold, 1991). O cádmio é encontrado naturalmente na maioria dos minérios e solos, quase sempre associado ao zinco, sendo obtido como subproduto do refino do zinco e de outros minérios como chumbo-zinco e cobre-chumbo-zinco (Salgado, 1996).

O Cd constitui um importante poluente ambiental, pois é amplamente utilizado na indústria, estando presente em uma ampla gama de produtos agrícolas, fertilizantes, pigmentos, esmaltes, tinturas têxteis, baterias recarregáveis de níquel-cádmio, varetas para soldagem, tubos para televisores, plásticos (Robards e Worsfold, 1991; Salgado, 1996). Sais de cádmio são também usados como anti-helmínticos, antissépticos, acaricidas e nematicidas (Robards e Worsfold, 1991).

A toxicidade de um metal é dependente da dose ou do tempo de exposição, da forma física e química do elemento e da via de administração/absorção. O caráter tóxico de um determinado elemento depende do tipo de interação que este tem com o organismo, e ocorre em três estágios: a) entrada e absorção no corpo; b) transporte, distribuição, acumulação e biotransformação; c) efeito e saída do organismo (Tavares e Carvalho, 1992).

Estudos mostram que o cádmio, depois de absorvido, se distribui pelo organismo, sendo encontrado em células sanguíneas, ligado a proteínas do soro plasmático como albumina e outras glicoproteínas, ou ainda em metaloproteínas produzidas pelo fígado (Mattiazzo-Prezotto, 1994).

O cádmio é considerado um dos metais de transição mais tóxicos, pois causa graves danos a uma variedade de tecidos e órgãos incluindo fígado, rins e testículos (Gupta et al., 2004; Shimada et al., 2004; Predes et al., 2010). As principais rotas de exposição humana a este metal podem ser identificadas como exposição aguda em ambientes de trabalho (inalação de fumaça e poeira) e exposição aguda e crônica a pequenas doses, na população em geral, via alimentação, cigarros, ar e água (Robards e Worsfold, 1991; Hoyer, 2001). A exposição acentuada via oral pode resultar em sérias irritações no epitélio gastrointestinal, náuseas, vômitos, salivação, dor abdominal, cólica e diarréia (Labuska et al., 2000). A ingestão de alimentos contaminados é a fonte mais comum de de exposição para pessoas não-fumantes e não-expostas ocupacionalmente.

A literatura apresenta estudos que investigam a possibilidade de que a exposição a poluentes tem relação com o aumento da decorrência de distúrbios reprodutivos, como redução da espermatogênese (Dallinga et al., 2002; Mehta e Kumar, 2003) tanto em animais experimentais, quanto em humanos (Skarda, 2002).

Alguns estudos mostraram que o cádmio tem ação oxidante e é particulamente tóxico para os testículos reduzindo tanto o peso testicular (Predes et al., 2010) quanto o dos órgãos sexuais acessórios (Manna et al., 2008; Fouad et al., 2009). Nesta pesquisa, o cádmio foi utilizado como ferramenta para produzir um ambiente de estresse oxidativo.

Após a exposição aguda, constatou-se que danos testiculares induzidos pelo cádmio podem ser encontrados tanto nos túbulos seminíferos quanto no interstício (Blanco et al., 2007). Neste caso, a disfunção testicular pode ser resultante de distúrbios nas células de Sertoli, responsáveis pela formação da barreira hematotesticular, ou nas células de Leydig, comprometendo a produção de andrógenos sob controle do eixo hipotálamo-hipófise-testículo (Bizarro et al., 2003; Manna et al., 2008; Siu et al., 2009).Além disso, o Cd pode causar atrofia testicular, diminuição do lúmen do túbulo seminífero, espessamento de seu epitélio (Herak

Kramberger et al., 2000; Predes et al., 2010) e apoptose das células germinativas (Huang et al., 2005).

1.3 O testículo

O testículo é um órgão par, situado no escroto, localizado na região anterior do períneo, logo por trás do pênis. São circundados pela túnica albugínea, de natureza conjuntiva, branco-nacarada, que se projeta para o seu interior formando o mediastino testicular e dividindo o testículo em lóbulos (Netter, 2000). Esse órgão é constituído por dois compartimentos principais: o compartimento tubular e o compartimento intertubular ou intersticial (Russel et al., 1990).

O compartimento tubular constitui a maior parte do testículo, ocupando, na grande maioria dos mamíferos, de 70% a 90% do parênquima testicular (França & Russell, 1998). Neste compartimento encontram-se os túbulos seminíferos, que são alças contorcidas que têm suas extremidades conectadas na *rete testis* pelo túbulo reto (Russel et al., 1990). São constituídos por túnica própria, epitélio seminífero e lúmen.

Nos túbulos seminíferos formam-se os espermatozoides, em um processo complexo e bem organizado, conhecido por espermatogênese (Netter, 2000). Durante esse processo, no epitélio seminífero, são encontrados diferentes estágios de maturação das células espermatogênicas: espermatogônias, espermatócitos primários e secundários, espermátides e espermatozoides, além das células de Sertoli (Russel et al., 1990; França & Russel, 1998; Griswold, 1998). Na maioria dos mamíferos, a espermatogênese dura de 40 a 60 dias (Saunders, 2002). No lúmen tubular encontram-se o fluido secretado pelas células de Sertoli e os espermatozoides recém espermiados.

Os componentes do compartimento intersticial são as células de Leydig, os vasos sanguíneos e espaços linfáticos, além de nervos e de uma população celular variável constituída principalmente por fibroblastos, macrófagos e mastócitos, os quais estão diretamente associados ao funcionamento das células de Leydig (Russell et al., 1990; Hales, 2002).

A produção de andrógenos ocorre por meio de estímulos do hormônio luteinizante (LH) em receptores na membrana plasmática das células de Leydig.

Dentre os andrógenos sintetizados pelas células de Leydig, incluem-se a testosterona e a diidrotestosterona, responsáveis pela diferenciação do trato genital masculino e da genitália externa na fase fetal, pelo aparecimento dos caracteres sexuais secundários e pela manutenção quantitativa da espermatogênese a partir da puberdade (Sharpe, 1994; Pelleniemi et al., 1996). Adicionalmente, a diidrotestosterona é responsável pela manutenção funcional das glândulas sexuais acessórias e do epidídimo (Luke & Coffey, 1994).

Diversos estudos mostram potencial efeito protetor de agentes fitoterápicos contra o estresse oxidativo causado, em especial pelo cádmio, tanto no sistema reprodutor masculino, quanto em diversas doenças. A flora brasileira é particularmente rica em plantas com diversas aplicações terapêuticas que são comumente usadas como remédios populares e tônicos.

1.4 *Ilex paraguariensis*

Pertencente à família Aquifoliaceae, *Ilex paraguariensis*, descrita por Saint Hilarie, é uma árvore perene, com seis a oito metros de altura, sendo nativa da América do Sul. É de grande importância econômica para o sul do Brasil, nordeste da Argentina e todo Paraguai (Hussein et al., 2011).

Através da industrialização de suas folhas e ramos obtém-se o produto destinado à preparação de bebidas tônicas conhecidas como chá-mate, chimarrão ou tererê. Popularmente, a infusão da erva-mate é utilizada para diversas enfermidades como fadiga, digestão lenta e doenças hepáticas (Bastos et al., 2007). Entretanto, o interesse científico na utilização da erva mate para promoção da saúde é relativamente recente.

Em medos dos anos noventa, foi publicada a primeira evidência científica demonstrando a atividade antioxidante da erva-mate in vivo e in vitro (Gugliucci et al, 1996; Bastos et al, 2007).

Ilex paraguariensis é rica em compostos bioativos, entre eles os polifenois (ácido clorogênico), xantinas (cafeína e teobromina), purinas alcaloides, saponinas, flavonoides, aminoácidos, vitaminas (C, B1 e B2), minerais (P, Fe e Ca) (Pomilio et al., 2000; Zaporozhet et al., 2004; Heck e de Mejia, 2007).

O chá mate possui importantes propriedades farmacológicas: antioxidante (Bixby et al., 2005), anti-inflamatório (Burris et al., 2011), antimutagênico (Miranda et al., 2008), anti-obesidade (Arçari et al., 2008; Pang et al., 2008; Arçari et al., 2011) e atividade hipoglicêmica (Rodriguez et al.,2002). Alguns estudos mostraram que o chá da erva-mate pode, também, regular a quantidade de lipídios no sangue, tanto em humanos, quanto em animais (Morais et al., 2009; Stein et al., 2005).

Além disso, o extrato de *I. paraguariensis* possui ação quimiopreventiva (Ramirez-Mares et al., 2004), inibe a glicação (Lunceford e Gugliucci, 2005) e o estresse oxidativo (Gugliucci e Stahl, 1996; Gugliucci e Menini, 2002), tem efeito vasodilatador (Baisch et al., 1998), colerético e na propulsão intestinal (Gorzalczany et al., 2001).

Neste contexto, considerando-se as propriedades da erva-mate e o fato que não foram encontrados estudos relatando o efeito da erva no sistema reprodutor masculino, o presente estudo avalia o efeito da infusão de *Ilex paraguariensis* e de sua associação com o cádmio nos testículos de ratos Wistar adultos.

2. OBJETIVOS

O presente estudo teve como objetivos:

1) Comparar o efeito da erva-mate na morfologia dos testículos de ratos Wistar em idade reprodutiva em diferentes tempos de tratamento (15 e 56 dias), bem como sua associação ao cádmio, utilizando ferramentas para análises morfométricas e estereológicas, produção diária de espermatozóide (PDE) e reserva espermática.

2) Avaliar o possível efeito protetor da erva-mate quanto aos danos oxidativos causados pelo cádmio nos testículos durante 15 dias de tratamento através de análises hematológicas e bioquímicas, bem como dosagem da quantidade de cádmio nos testículos.

3. CAPÍTULO I

Será submetido para: **Journal of Histology and Histopathology**

Effects of *Ilex paraguariensis* (St. Hil) infusion in Wistar rat testicles: associated or not to cadmium- induced damage

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Running head: Effect of mate tea and cadmium in rat testis

Key words: oxidative stress;testis;stereologycal analysis; yerba mate tea; metal.

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ABSTRACT

Background: *Ilex paraguariensis* (St. Hil) known as yerba mate has been used in traditional medicine and its infusion exhibits hepatoprotective, antirheumatic, anti-inflammatory properties. Such effects have been attributed to the phenolic compounds present in this plant. Considering that cadmium, known for its genotoxic and spermiotoxic effects, is a common environmental contaminant, the development of natural therapies to diminish the damage it can cause is considered highly pertinent.

Material and Methods: To evaluate the effects and possible protective effects of *I. paraguariensis* infusion on the testis, two experiments were performed. The short-term experiment (15 days) consisted in: C-15. control (water), M-15. yerba mate, Cd-15. cadmium, MCd-15. cadmium and yerba mate. The long term experiment employed very similar groups maintained for 56 days, included: C-56. control (water), M-56. yerba mate, Cd-56 cadmium, MCd-56. cadmium and yerba mate. A single dose of cadmium chloride (1.2 mg/kg BW) was injected i.p. in adult rats (groups Cd-15, MCd-15, Cd-56, MCd-56). The yerba mate used in this research was commercial tea (110mg yerba mate/1000mL water). Each animal received, daily, 0.5mL of either water (groups C-15,Cd-15, C-56, Cd-56) or infusion (groups M-15, MCd-15, M-56, MCd-56). After the exposition, animals were anesthetized with Xylazine and Ketamine (5:80mg/kg). Testicles were removed, weighed, fixed and routinely processed for light and electron microscopy. The left testis were frozen (-20°C) for the calculation of daily sperm production (DSP).

Results: Histopatological analysis clearly showed damage induced by cadmium, such as, germ cell damage. Cell differentiation and spermatozoa development were not observed in most affected testicles. The sperm production rate and the DSP were reduced in MCd-15 and Cd-15 compared to other groups, and significant diferences were not observed in the groups after 56-days expositiont.

Conclusion: The testes were better preserved in the group treated with both cadmium and *Ilex paraguariensis* infusion during 56 days, suggesting a protective effect of the plant on the testicular parenchyma. However, after 15 days, the testicular parenchyma was not preserved by the association of *I. paraguariensis* with cadmium. In both experiments, testicular dynamics were not altered after treatment with only mate tea, suggesting that testicles structure was not affected by plant infusion consumption.

Key words: oxidative stress; testis; stereologycal analysis; yerba mate tea; heavy metal.

INTRODUCTION

Environmental and industrial pollution are associated with reproductive and developmental disorders [1]. The exposition to high levels of toxic chemicals, including heavy metals released into the environment and reaching the food chain, can affect health of living organisms, sometimes with fatal consequences. Cadmium (Cd) is a heavy metal that is associated with severe damages to organs, including the testes, where somatic and germ cells are heavily affected [2; 3]. Thus, testicular dysfunction may arise from spermatogenesis impairment due to disturbances of the hypothalamic-pituitary-testicular axis, altering Sertoli and Leydig cells morphophysiology and compromising male fertility [4].

The Brazilian flora is diverse and many plants can be used as natural tonics and remedies, however few plant species have been properly studied in controlled experimental designs. Among them, *Ilex paraguariensis* (Saint Hilaire) known as yerba mate, is a plant widely consumed in South America, being naturally grown and cultivated in Brazil, Argentina and Paraguay [5]. The leaves are used to prepare different drinks, including “chimarrão” (infusion of fresh or dried leaves with hot water), “tererê” (infusion of fresh or dry leaves in cold water) and mate tea (infusion of roasted leaves with hot water) [5]. Yerba mate infusions exhibit hypocholesterolemic, hepatoprotective, antirheumatic, vasodilatory and anti-inflammatory properties [6], which have been attributed to the phenolic compounds present within, such as phenolic acids (chlorogenic acid and derivatives) and flavonoids, especially rutin [5-8].

This study was undertaken to evaluate the effect of *I. paraguariensis* infusion on the testis of Wistar rats treated with cadmium using morphological analysis.

METHODS

Experimental Design

Forty male adult Wistar rats (*Rattus novergicus*) were obtained from the breeding house of Universidade Estadual de Campinas (Campinas, SP, Brazil). All experiments were performed in accordance to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and acknowledged by the institutional Ethical Committee for Animal Experimentation (Protocol no. 3898-1).

The animals were kept in polyethylene cages with sterile wood shavings in

a controlled room for light and temperature (12:12 light⁄dark cycles, 22 ± 2ºC), along with free access to food and filtered water. After acclimation, the rats were weighed and randomly distributed into eight groups (n=5 each). The short-term experiment (15 days) consisted in: C-15. control (water), M-15. yerba mate, Cd-15. cadmium, MCd-15. cadmium from day one and yerba mate. The long-term experiment employed the same groups maintained for 56 days, including: C-56. control (water), M-56. yerba mate, Cd-56 cadmium, MCd-56. cadmium followed by yerba mate. A single dose of cadmium chloride (1.2 mg/kg) was injected i.p. in adult rats (groups Cd-15, MCd-15, Cd-56, MCd-56). The yerba mate used in this research was commercial tea (110mg yerba mate/1000mL water) following Stein at al. methodology [9]. Each animal received daily, by gavage, 0.5mL of either water (groups C-15, Cd-15, C-56, Cd-56) or infusion (groups M-15, MCd-15, M-56, MCd-56).

After the treatment, animals were weighed and anesthetized with Xylazine and Ketamine (5:80mg/kg intramuscular injection). Testes were removed, weighed, fixed in Karnovsky fixative (formula/componentes) for 24 hours and routinely processed for light microscopy. Briefly, testicular fragments were dehydrated in ethanol and routinely embedded in glycol methacrylate resin (Leica Historesin, Leica Microsystems Nussloch, Germany). The histological sections (3µm thick) were made in a rotary microtome (Leica) and stained with haematoxylin and eosin. The left testis and epididymis were frozen (-20°C) for the calculation of daily sperm production (DSP), the number of sperm within the epididymis and sperm transit.

Yerba Mate Determination of Total Phenolic Content (TPC)

The Folin-Ciocalteu reagent was used to evaluate the TPC according to the method described by Roesler et al. [10] in yerba mate. The absorbance of the resulting blue color was measured at 760 nm in a spectrophotometer (DU-640™, Beckman-Coulter - Brea, CA, USA). The results were expressed in µg of gallic acid equivalents/mg of yerba mate. Estimation of the phenolic compounds was carried out in triplicate and the data are reported as mean ± SD.

Testicular morphometry

The testicular relative weight (gonadossomatic index, %) was calculated dividing testicular weight by body weight and multiplying the result by 100.

Images of the testicular parenchyma were taken with a digital camera coupled to the light microscope (Leica Microsystem DM500). The software Image Pro Plus (MediaCybernetics) was used to perform the analyses following previous protocols [11; 12]. To calculate proportions between parenchyma elements (seminiferous tubules and interstitium), a square grid (121 intersections) was placed on the digital image. Volumetric proportions (%) between seminiferous tubules (tunica propria, seminiferous epithelium and lumen) and interstitium were estimated by counting 2040 intersections (per animal) from random fields (400x magnification).

The volume of each component (mL) was estimated from the previous knowledge of the proportions occupied by each of the tissue types within the testicle. The testicular weight was considered equal to its volume, discounting the albuginea weight [13-16]. The seminiferous tubule diameters and the epithelium height were calculated after measuring cross sections of 30 tubules per animal. Total length of the seminiferous tubules was estimated from the volume they occupy within the parenchyma and the tubular diameter, as described by Attal & Courot and Dorst & Sajonski [17; 18].

Daily Sperm Production

Elongated spermatids, stage 19 [19] within the testis and sperm cells from the epididymis were counted using Neubauer chamber following the protocol described by Robb et al [20]. Thus, the Daily Sperm Production (DSP) was calculated dividing the sperm number by 6.1, which is the number of days in which the mature spermatids are still attached to the epithelium.

Statistics

For all values, the means and standard deviation (SD) were calculated. The comparison of control values and other groups was made using the ANOVA test, followed by Tukey's post test using Prism 6.0 software. The p value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Yerba Mate Total Phenolic Contents

The results of the total phenol content of *Ilex paraguariensis* used in this

experiment showed a concentration of 26.62µg (± 0.14 µg) gallic acid/mg of yerba mate, corresponding to 0.55% of the total polyphenol content in the tea used.

Biometric Data

Biometric data are shown in table 1. After 15-days exposition, the group exposed to cadmium and *Ilex paraguariensis* (MCd-15) gained less weight compared to the other groups. A significant reduction in the gonadosomatic index was observed comparing groups treated with cadmium (Cd-15) and cadmium and yerba mate infusion (MCd-15) in comparison with control (C-15) and mate (M-15) groups. After 56-days treatment, significant differences were observed in Cd-56 group accessory organs weight compared to the other groups, athough, GSI, testis weight and body weight gain were not altered.

Testicle Morphology

The volume and volumetric density of testicular parenchyma compounds were not altered after 15-days and 56-days of treatment **(Figure 2)**. Tubular diameter and tubular length were not altered in mate (M-15) group, as shown by the epithelium height **(Table 2)**, whereas in Cd-15 and MCd-15 the seminiferous epithelium was degenerated, and only the basal lamina was identified **(Figure 3)**.

Tubular diameter and tubular length were not altered in all groups after 56-days treatment. The epithelium height decreased in cadmium (Cd-56), whereas, comparing Cd-56 and MCd-56 groups, epithelium height was lower in the group that received only cadmium. Data are tabulated **(Table 3)**.

Statistically significant changes were not observed in Leydig cell stereology in all groups after 15-days treatment **(Figure 4)**, and histopathology confirms this, as shown in figure 3. After 56-days treatment, the interstitial space was very dense in the groups that received cadmium and it was not possible to identify Leydig cells by conventional analysis to compare between the groups **(Figure 5)**.

Histopatological analysis showed damages induced by cadmium, such as, germ cell damage. Testicles were clearly affected after cadmium exposure; in some seminiferous tubules cell differentiation and sperm development were not observed in both experiments. In some seminiferous tubules of the Cd-56 group, only Sertoli cells were observed and the testicular interstitium was very dense **(Figures 6 and 7)**.

Comparing testis images between Cd-56 and MCd-56 groups **(Figures 6 and 7)**, the integrity of the testis was somewhat protected in the group treated with both cadmium and *Ilex paraguariensis* infusion. However, after the treatment of 15 days, the testicular parenchyma was not preserved **(Figures 3 and 4)** by the association of *I. paraguariensis* with cadmium.

Daily Sperm Production and Sperm Production Rate

The sperm production rate and the DSP were reduced in MCd-15 and Cd-15 compared to other groups. Significant differences were not observed in the groups after 56-days treatment **(Figure 1)**.

DISCUSSION

Several studies on yerba mate have reported the presence of different phenolic compounds [5; 7; 21-24]. It has also been reported that yerba extracts have an in vitro antioxidant capacity which is due to the presence of chlorogenic acids and dicaffeoylquinic acid derivatives, which have an antioxidant capacity equal to or higher than that of ascorbic acid and vitamin E [21; 25].

Dudonné et al. [26] ranged aqueous extracts of yerba mate in the fifth place of plants (among thirty) with higher antioxidant activity. Chandra and Gonzalez de Mejía [23] reported a total polyphenol content (TP) in the range from 9.0 to 17.6 g gallic acid equivalent and from 23.6 to 49.0 chlorogenic acid equivalent per 100 g of dry mass (dm) for yerba mate beverages prepared as decoctions of nearly 3 g of mate leaves in 250 mL of boiling water.

In this experiment the results of the total phenolic content of *Ilex paraguariensis* showed a concentration of 26.62 ug gallic acid/ mg of yerba mate, (0.55 % of polyphenols in the used tea). This confirms that the infusion used here has an elevated phenolic content.

It is important to mention that in popular medicine, as also in this research, the infusion time between additions of water and consumption is comparatively short, so that these beverages ensure the total extraction of yerba mate polyphenol compounds [27]. Currently no standard is available for total extraction of yerba mate polyphenols, thus we referred the average TP of the beverages simulated to the total soluble solids. It is well known that total soluble solids represent the amount of solid

extracted in specific extraction conditions [27].

This study demonstrated that acute single i.p. administration of cadmium (1,2 mg/kg) induced severe damage to testicular tissue of exposed rats, and oral administration of *Ilex paraguariensis* infusion did not have a protective role when administered for 15 days, whereas when administered during 56 days, *I. paraguariensis* infusion contributes to the maintenance of the tubular epithelium. Since the molecular mechanisms of cadmium testicular toxicity have not been entirely elucidated, this work focused on the earliest morphological changes in the testis.

Our results agree with Predes et al. [12]. They did not observe alterations in body and testicular weight, and IGS after 56 days in animals that received a single i.p. dose of cadmium (1.2 mg/kg). Many authors agree that there is a direct relationship between reproductive organs weights and the availability of androgens [28-31]. In the present study, acessory organs weights were not significantly altered in 15-days experiment. These results could be due to the low dose (for morphological studies in reproductive biology) used which led to very precise changes that were not measured by these more general analyses. On the other hand, acessory organs weights were significantly altered after 56-days treatment.

Most testicular toxicants cause germ cell degeneration and depletion to a greater or lesser extent. Severe and prolonged insults result in lesion of the tubules to be lined only by Sertoly cells. Although Sertoli cells are very sensitive to functional perturbations, they are remarkably resistant to cell death [32]. The testis alterations, such as the absence of a lumen and seminiferous tubules completely filled with degenerated germ cells, and few developed sperm, were verified after 15 days. The progressive degeneration resulted in lesion of many tubules that were lined only by Sertoli cells after 56 days, indicating loss of spermatogenesis.

Predes et al. [33] also observed reduction of the seminiferous epithelium height in animals treated with cadmium. In the group treated only with infusion of *Ilex paraguariensis* during 56 days, this parameter remained unchanged after treatment. This suggests that the infusion of the plant associated to cadmium intoxication protected the seminiferous epithelium, to a certain degree from very severe cadmium damages.

In some seminiferous tubules, germ cells were totally degenerated, it being

possible to observe only Sertoli cells. This occurs in some tubules, but not in all. However, the sperm production was not zero, and not significantly lower related to the other groups. Possibly the tubules very close to blood vessels receive more Cd and were more strongly affected than tubules that were farther and received less, having a greater survival of the germ line cells that, being in constant division and differentiation, are more susceptible. The protection of the infusion of mate leaves showed less damage observed after 56 days of treatment and suggests the recovery of some germ cells under the positive influence of the tea.

CONCLUSION

We conclude that the testis was somewhat protected in the group treated with both cadmium and *Ilex paraguariensis* infusion during 56 days, suggesting a protective effect of the plant on the testicular parenchyma. However, after an acute treatment of 15 days, the integrity of the testicular parenchyma was not preserved by the association of *I. paraguariensis* with cadmium.

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APÊNDICE 1
Tables and Figures

Table 1: Biometric data: Body weight gain (g), testis weight (g), Gonadosomatic index (%), accessory organs weights (g) for 15-days and 56-days treatments.

Parameters	***15 days***				***56 days***			
Groups	**C**	**M**	**Cd**	**MCd**	**C**	**M**	**Cd**	**MCd**
Body weight gain	76.53± 5.52	73.13± 5.86	54.8± 8.84	50.13± 13.56[a]	151.6± 22.74	175.2± 3.82	132.9± 17.03[b]	144± 15.2
Testis weight	1.906± 0.058	1.864± 0.143	1.062± 0.48[b]	0.8202± 0.214[c]	1.568± 0.129	1.597± 0,169	1.434± 0.205	1.487± 0.239
IGS (%)	0.446± 0.036	0.438± 0.036	0.203± 0.070[b]	0.203± 0.032[c]	0.3502± 0.025	0.345± 0.045	0.3134± 0.027	0.3271± 0.030
Epididymis	0.612± 0.05	0.6258± 0.03	0.4988± 0.12	0.4861± 0.13	0.613± 0.07	0.64± 0.07	0.598± 0.06	0.617± 0.07
Seminal vesicle	0.8968± 0.2505	0.7698± 0.2086	0.7938± 0.3028	0.6426± 0.1466	1.28± 0.06[a]	1.234± 0.08	1.03± 0.17[a]	1.208± 0.11
Coagulation gland	0.195± 0.02	0.1408± 0.04	0.1892± 0.10	0.4006± 0.27	0.276± 0.034	0.283± 0.029[a]	0.214± 0.062[a]	0.279± 0.036
Ventral prostate	0.4268± 0.09	0.397± 0.12	0.3554± 0.15	0.2752± 0.12	0.697± 0.11	0.613± 0.11	0.672± 0.14	0.651± 0.10

Values are mean ± SD. Values followed by a letter are significantly different, [a]$p<0.05$ with C; [b]$p<0.02$ with C,and M; [c]$p<0.001$ with C and M; [d]$p<0.05$ with M.

Table 2: Tubular diameter (µm) and tubular length (µm) of groups after 15-days treatment.

Parameters	*C-15*	*M-15*	*Cd-15*	*MCd-15*
Tubular diameter (µm)	297.47±13.14	275.48± 22.11	280.86± 52.67	258.66± 34.96
Tubular length (µm)	$1.13x10^6$± $0.12x10^6$	$0.98x10^6$± $0.20x10^6$	$0.67x10^6$± $0.40x10^6$	$0.48x10^6$± $0.50x10^6$

Values are mean ± SD.

Table 3: Tubular diameter (µm) and tubular length (µm) of groups after 56-days treatment.

Parameters	*C-15*	*M-15*	*Cd-15*	*MCd-15*
Tubular diameter (µm)	215.82± 19.15	216.81± 35.73	216.95± 35.73	230.12± 50.50
Epithelium height (µm)	167.95± 17.26	172.76± 25.85	39.69± 10.12	135.25± 45.69
Tubular length (µm)	$1.499x10^6$± $0.60x10^6$	$1.691x10^6$± $1.0x10^6$	$1.185x10^6$± $0.80x10^6$	$1.422x10^6$± $0.80x10^6$

Values are mean ± SD. Values followed by a letter are significantly different, [a]p<0.05 with Cd.

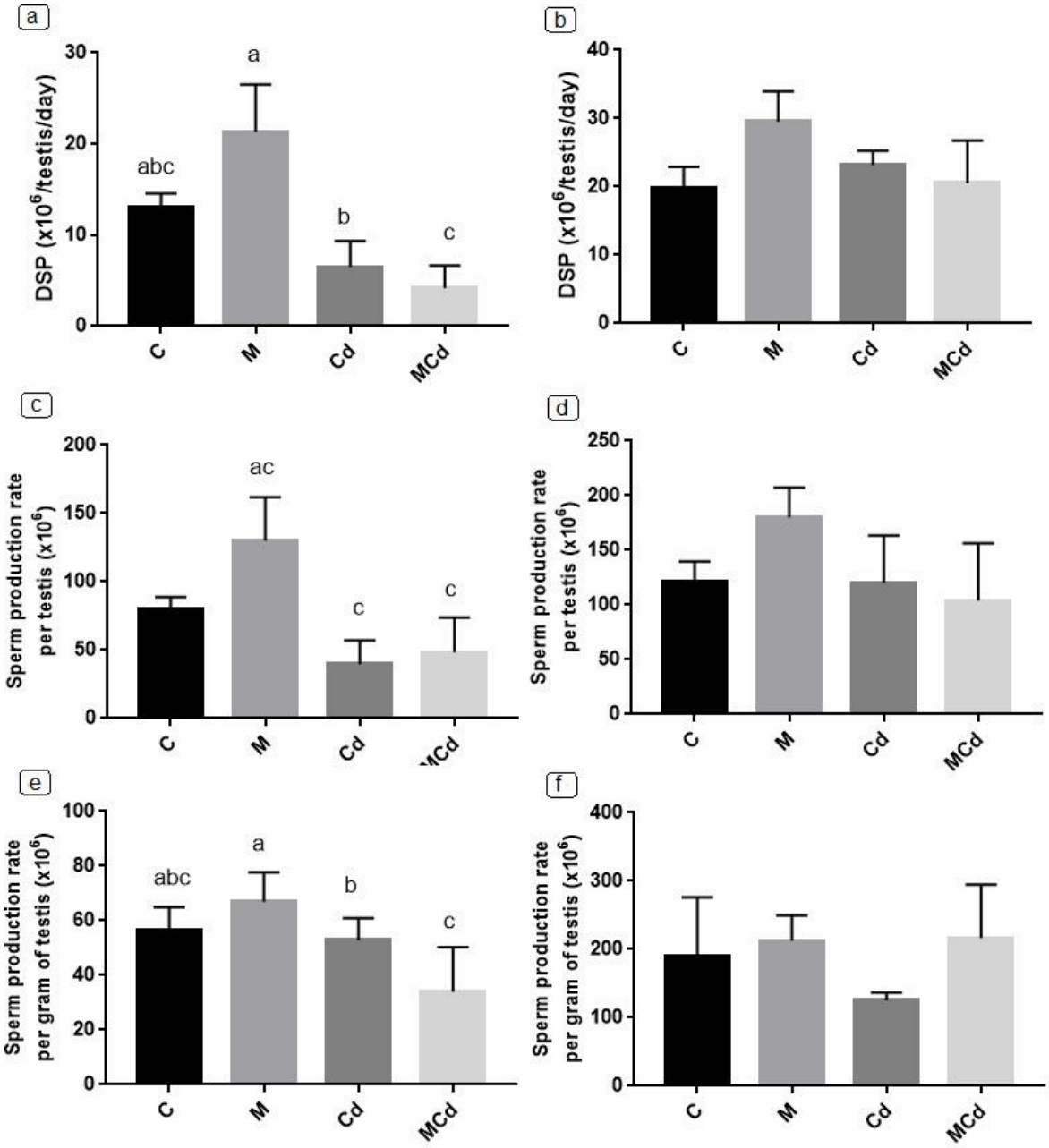

Figure 1: Spermatid count in the testis and daily sperm production. (a), (c) and (e): 15-days treatment; (b), (d) and (f): 56-days treatment. *[a]p= 0.019 with C; [b]p=0.0004 with M and C; [c]p=0.001 with M and C.*

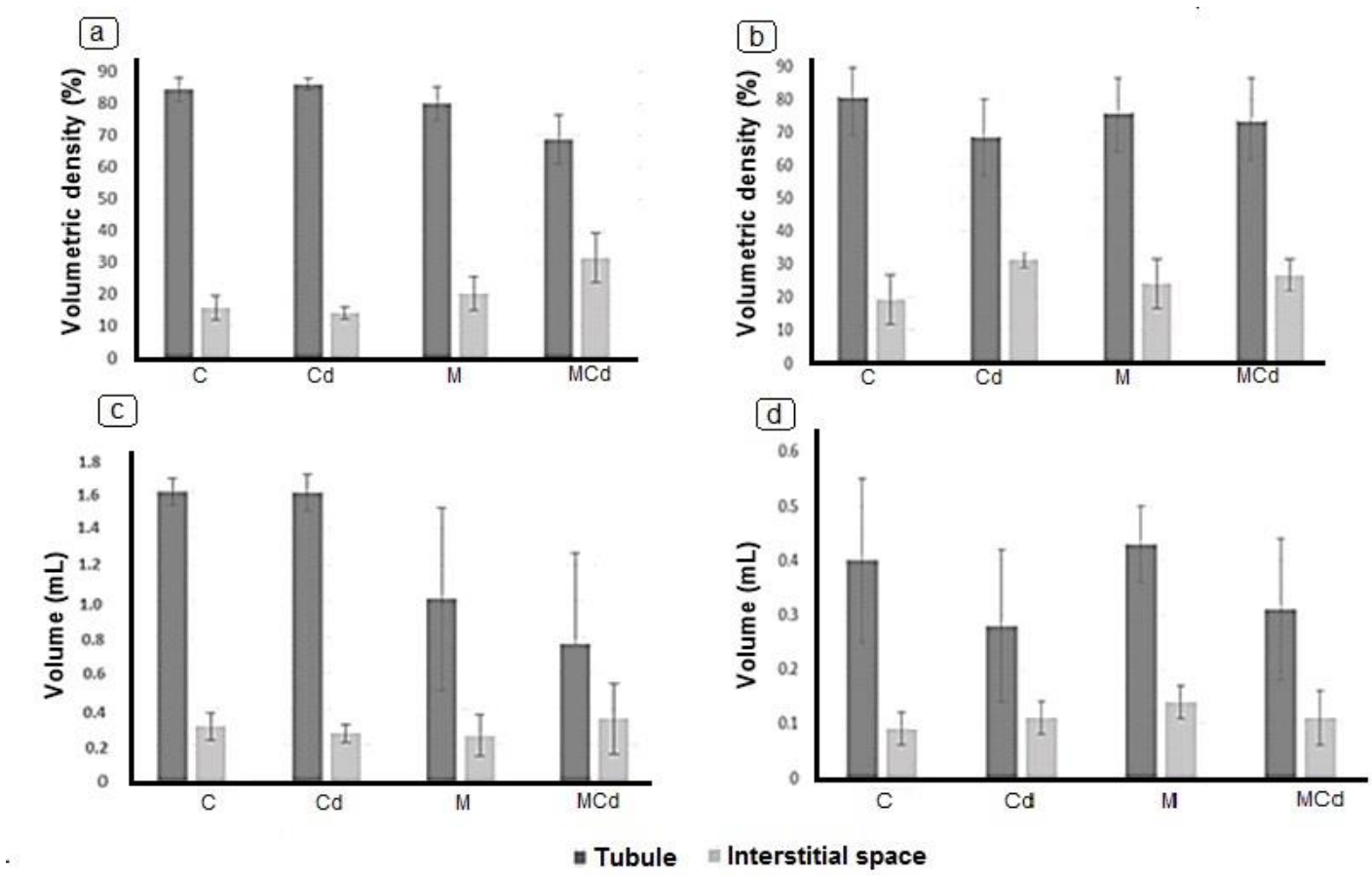

Figure 2: Volumetric density (%) and volume (mL) of testicular parenchyma compounds. (a) and (c): 15-days treatment; (b) and (d): 56-days treatment.

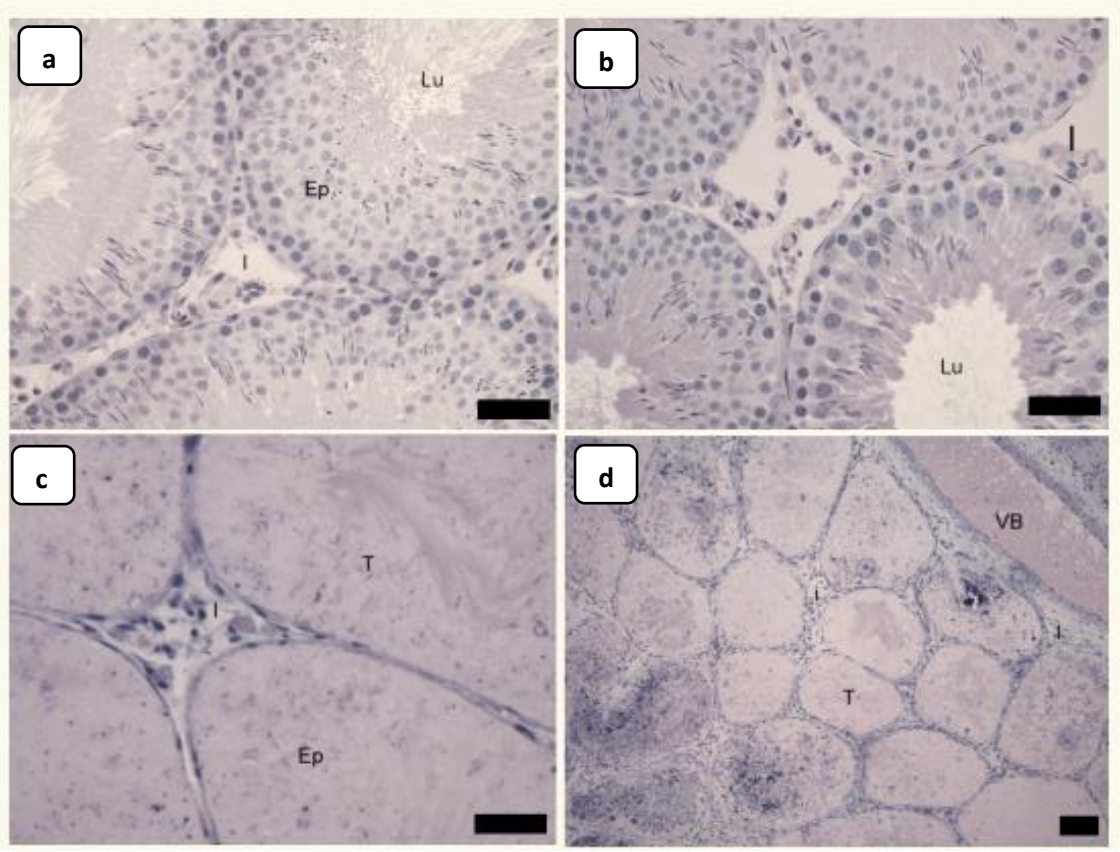

Figure 3: Ilustration of testicular tissue characteristics for 15-days treatment groups.(A) control group and (B) M group showing normal seminiferous tubules. (C) Cd and (D) MCd groups, showing degenerated tubules. Ep= seminiferous epithelium; L= lumen; I= interstitial space; T= seminiferous tubule; VB= vessel blood. Toluidine blue stain. Bar= 40 µm.

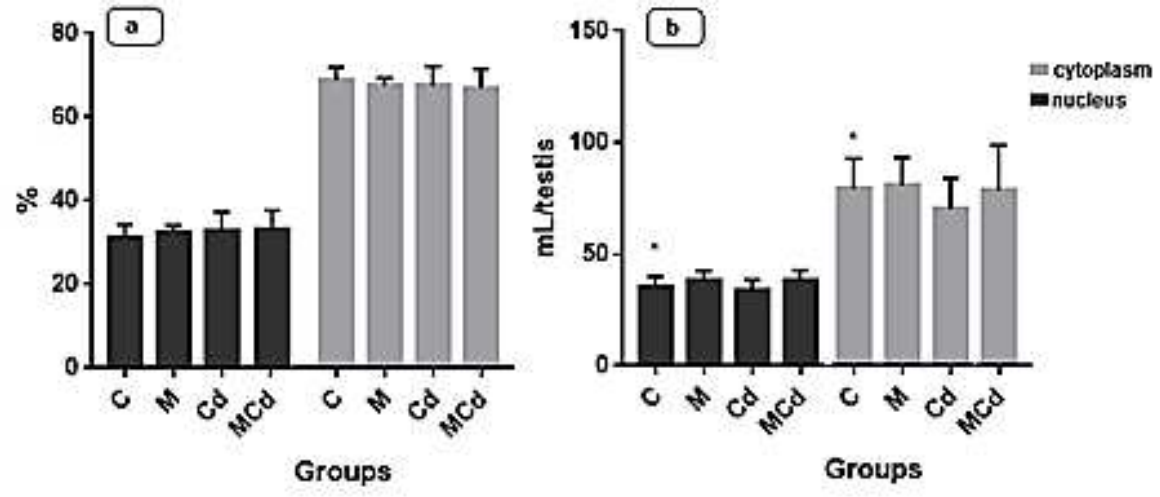

Figure 4: Leydig cell stereology 15-days treatment. (a) nucleus and cytoplasm volumetric density; (b) nucleus and cytoplasm volume. **p<0.0001 with M, Cd and MCd.*

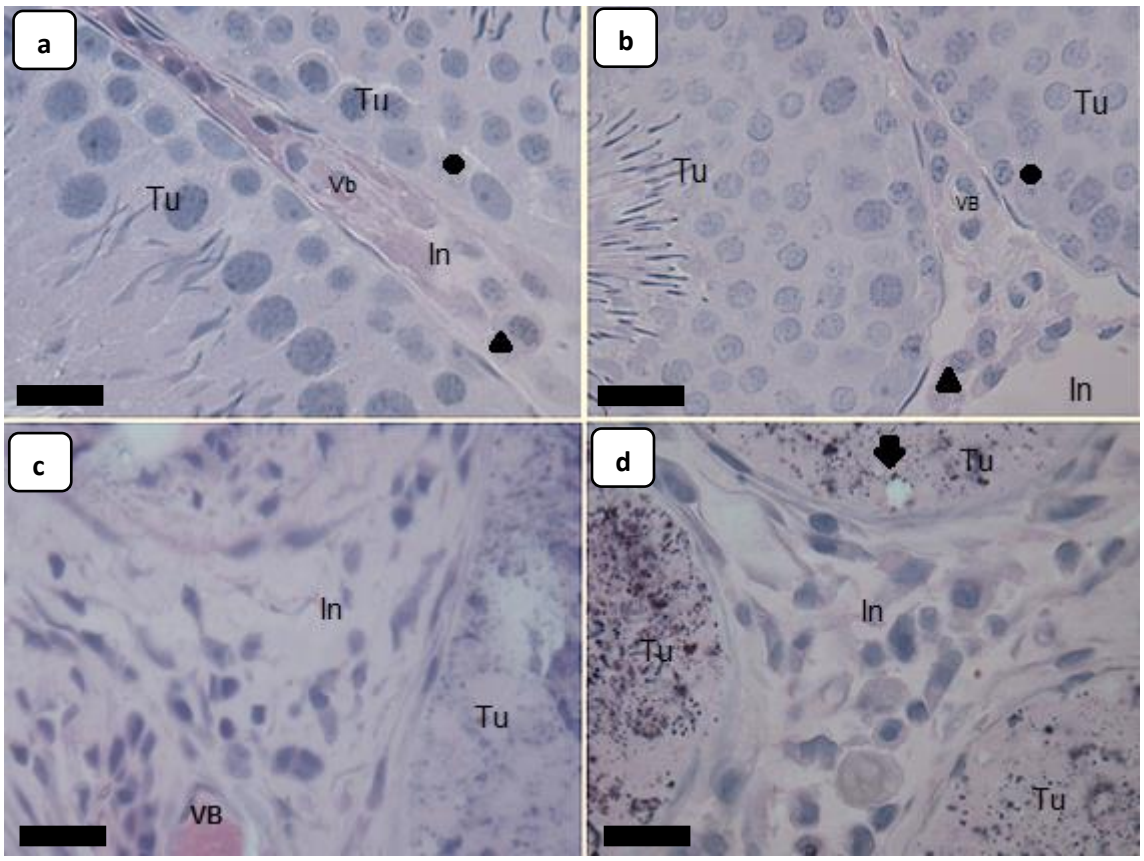

Figure 5: Interstitial space 15-days treatment groups, showing Leydig cell. (a) control; (b) mate; (c) cadmium and (d) cadmium followed by mate. Control and mate groups showed normal seminiferous tubules (Tu); and interstitial space (In). Leydig cells are pointed out with a tringle; and Sertoli cells with a circle. Cd and MCd groups showed degenerated tubules (Tu), with vacuolizations (indicated by an arrow. Tu= seminiferous tubule; In= interstitial space; T= seminiferous tubule; VB= vessel. Scale bar= 27,3 µm.

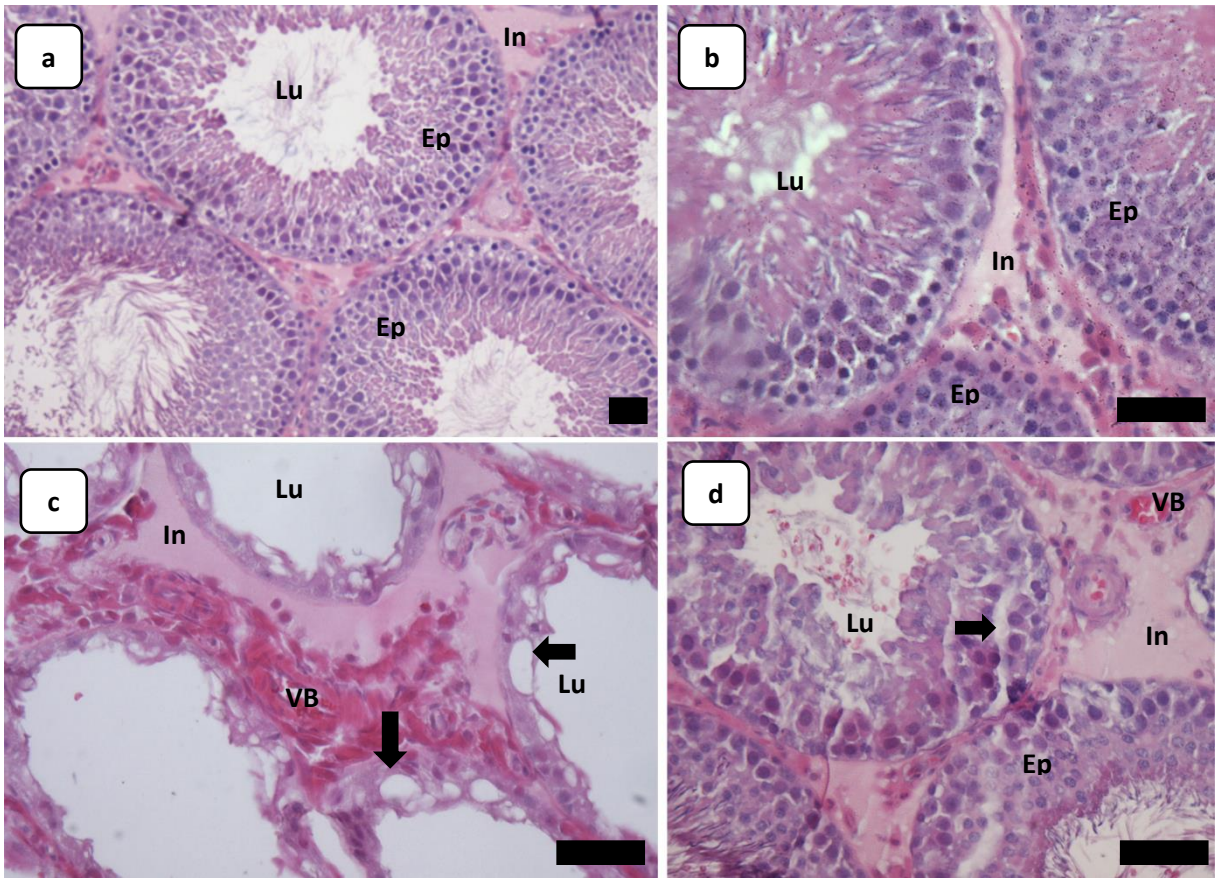

Figure 6: Illustration of tissue morphology long-term treatment. Groups: (a) control; (b) mate; (c) cadmium and (d) cadmium followed by mate. Control and mate groups showed normal tubular structure, normal epithelium (Ep) and interstitial space (In). Cadmium-exposed groups (MCd and Cd) showed degenerated tubules, interstitial tissue dense compared to control and mate groups. Cd and MCd groups showed degenerated tubules, with tubular vacuolization and intercellular spaces were observed in MCd group. MCd tubules were somewhat protected compared to Cd group. Ep= epithelium; In= interstitial space; Lu= lumen; VB= blood. vessel Arrows are pointing degenerations and intercellular spaces. Scale bar= 31,5 um

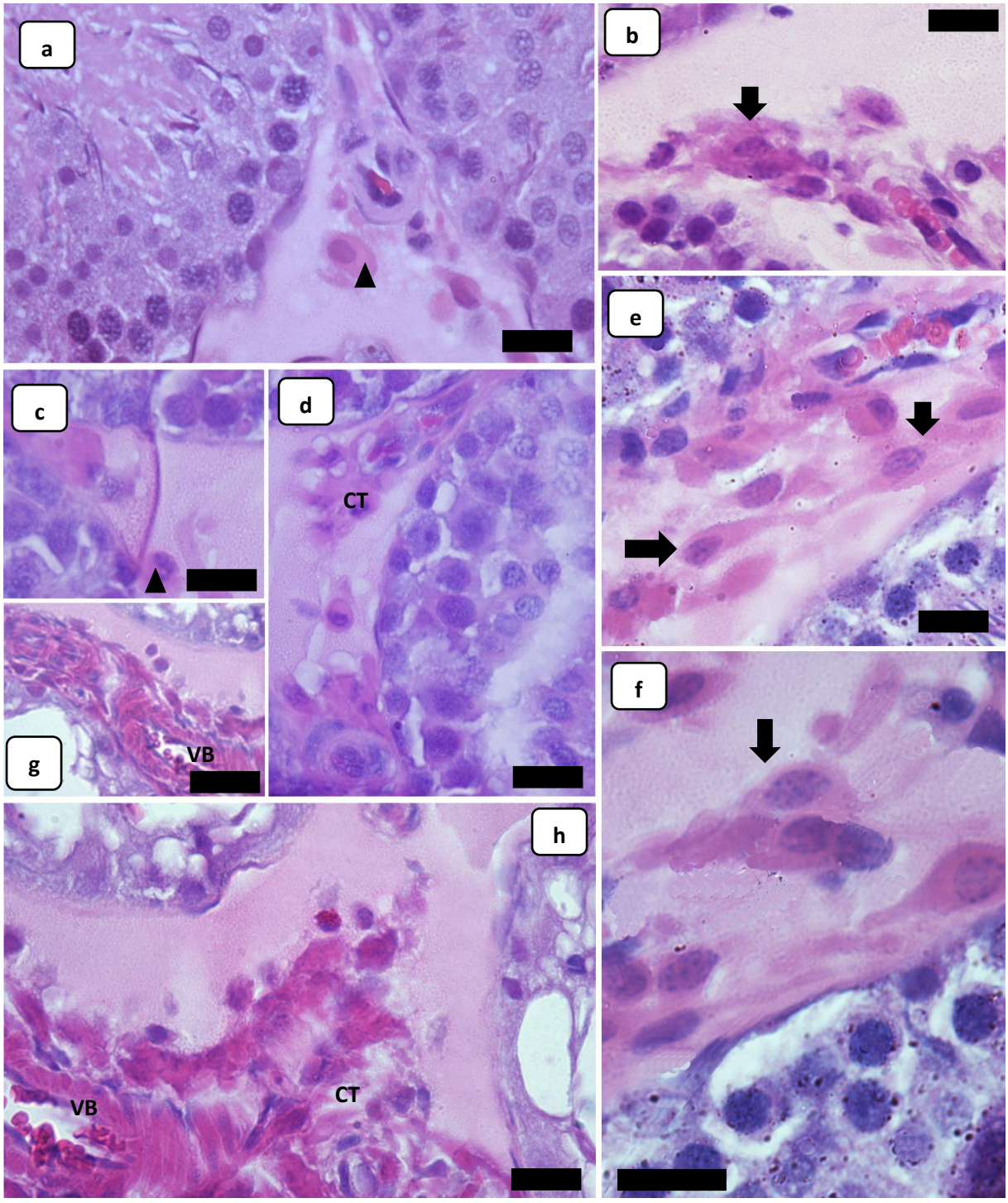

Figure 7: a) and (b) control group; (c) and (d) cadmium followed by yerba mate; (e) and (f) yerba mate (g) and (h) cadmium**.** Control group showed normal interstitium structure, normal Leydig cells around blood vessels. The mate group had normal Leydig cells, Cadmium-exposed groups (MCd and Cd) showed degenerated tubules, dense interstitial tissue compared to control and mate groups. Cd and MCd groups showedmore connective tissue (CT) than C and M, and more expanded vessels blood (VB). Arrows are indicate Leydig cell; triangle = macrophage. Hematoxilin-eosin stain. Scale bar= 29 um.

4. CAPÍTULO II

Será submetido para: **Reproductive Toxicology**

Could yerba mate protect testicles against cadmium chloride toxic effects?

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Running head: Effect of mate tea and cadmium in testis

Key words: oxidative stress; testicles; morphology.

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ABSTRACT

Cadmium is one of the most toxic environmental and industrial pollutants. Previous studies showed that cadmium exerts gonadotoxic and spermiotoxic effects. In the present study, we examined the possible protective effect of *Ilex paraguariensis* to diminish cadmium induced damages in this testis, a particularly sensitive organ. Known as yerba mate, it is a typical and inexpensive beverage consumed in Brazil, Argentina and Uruguay. We considered the hypothesis that yerba mate infusion could minimize oxidative stress, modulating enzymatic antioxidant defense. To test this concept, we analyzed the lipid oxidative damage and antioxidant defense in testes tissue, and to complete our research, hematological analyses and cadmium tissue concentration determination were performed. Twenty-five Wistar rats were divided into five groups (n=5) and treated during 15 days - C: control (water), M: yerba mate, Cd: cadmium, CdM: cadmium followed by yerba mate and MCd: yerba mate during 15 days and cadmium on the last day. A single dose of cadmium chloride (1.2 mg/kg BW) was injected i.p. in the adult rats (groups Cd, CdM and MCd). The yerba mate used in this research was commercial tea (110mg yerba mate/1000mL water). Each animal received, daily by gavage, 0.5mL of either water (groups C and Cd) or infusion (groups M, CdM and MCd). After the treatment, animals were anesthetized with Xylazine and Ketamine (5:80mg/kg). Testicles were removed, weighed, fixed and routinely processed for light and electron microscopy. The left testis and epididymis were frozen (-20°C) for the calculation of daily sperm production (DSP) and the number of sperm within the epididymis and sperm transit, respectively, and for antioxidant assays (-80°C). Cadmium affects DSP and sperm production rate over a period of time. Cadmium affected spermatogenesis and stimulated the antioxidant activities probably due to the induction of an adaptive response, in the way of maintenance and/or increase of physiological activities. *Ilex paraguariensis* infusion ingestion during 15-days is only partially efficient in the protection of the testicles from cadmium induced damage. Although treatment with only mate tea analyzed parameters were not altered, suggesting that testicles structure was not affected by plant infusion consumption.

Key words: oxidative stress; testicles; morphology.

INTRODUCTION

Several Brazilian plant species are often used as phytotherapic remedies in traditional medicine. Studies using medicinal plants with possible protective effects on reproduction have been investigated [1–4]. The Yerba Mate (*Ilex paraguariensis* St. Hill) is an Aquifoliaceae that occurs in South America, especially in Argentina, Paraguay and Brazil. The aerial parts of this plant are used to prepare well known infusion beverages, such as chimarrão and tereré, with its particular taste and stimulating properties [5, 6].

Active phytochemicals have been identified in yerba mate: polyphenols, xanthines, purine alkaloids, saponins, flavonoids, amino acids, vitamins, and minerals [7–9]. Many important pharmacological properties are attributed to mate tea, including: anti-inflammatory [10], antimutagenic [11], anti-obesity [12,13] and hypoglycemic activities [14], due to its antioxidant properties [15].

There are different heavy metals associated with oxidative damage, among them, Cadmium (Cd). According to several earlier studies, Cd-induced damage to the male reproductive organs was associated with oxidative stress in the testicles [16,17]. Some researchers have shown that pretreatment with antioxidants and free radical scavengers protected against Cd-induced testicular oxidative stress and male reproductive damage [18,19]

Reactive oxygen species (ROS) such as the superoxide anion radical (O_2S^-), hydroxyl ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) are produced in aerobic metabolism [20]. An imbalance between the production and detoxification of ROS results in oxidative stress. ROS has been implicated in the pathogenesis of certain diseases, including cancers [21–23]. ROS reacts with polyunsaturated fatty acids to induce the release of toxic and reactive aldehyde metabolites such as malondialdehyde (MDA), one of the end products of lipid peroxidation (LPO). MDA may be involved in tumor promotion because it can interact with the functional groups of a variety of cellular compounds [24].

To control the overproduction of ROS, the cells protect themselves against oxidative damage by antioxidant detoxifying mechanisms that help to lower ROS concentrations in the body. Different antioxidant systems, including nonenzymatic antioxidants such as glutathione (GSH), vitamins A, C, and E and various antioxidant

enzymes will defend against free radical attacks [20].

Therefore, the aim of this study was evaluate the possible protective effect of the infusion of Yerba Mate on the oxidative damage caused by Cd in Wistar rat testicles, using biochemical tools, hematological analyses and measurement of cadmium concentration in this tissue.

METHODS

Determination of Total Phenolic Content (TP) in *Ilex paraguariensis*

The Folin-Ciocalteu reagent was used to evaluate the TP according to the method described by Roesler et al. [25] in yerba mate. This method involves reduction of the reagent by phenolic compounds, with concomitant formation of a blue complex. The intensity of this complex increases linearly at 760 nm according to the concentration of phenols in the reaction medium. Yerba mate (leaves or infusion) was dissolved in methanol to obtain the appropriate concentration and sonicated for 2 h. Then, 0.5 mL of a known dilution of the yerba mate was added to the test tube and combined with 2.5 mL of 10% Folin-Ciocalteu reagent and 2.0 mL of 7.5% Na_2CO_3 solution. After the mixture had been allowed to stand for 5 min at 50° C, the absorbance of the resulting blue color was measured at 760 nm in a spectrophotometer (DU-640™, Beckman-Coulter - Brea, CA, USA). The results were expressed in µg of gallic acid equivalents/mg of yerba mate using the standard curve of gallic acid (10 – 100 µg/mL). Estimation of the phenolic compounds was carried out in triplicate and the data is reported as mean ± SD.

Antioxidant activity

$DPPH^{\bullet}$ Scavenging Assay

DPPH is a rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical, 2, 2-Diphenyl-1- picrylhydrazyl (DPPH). It is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity [26]. DPPH scavenging assay has been the most accepted model for evaluating the free radical scavenging activity of any new drug [27].

The antioxidant property of yerba mate to scavenge DPPH radicals was measured according to the method described by Brand-Williams, Cuvelier, & Berset

[28] with some modifications. Experiments were performed on freshly prepared ethanolic solutions of DPPH (0,004% w/v). In brief, 200µL of yerba mate solution in a known dilution was mixed with 1000µL of DPPH solution. Ethanol was used as a blank and DPPH$^{\bullet}$solution without test samples (1000 µL of DPPH$^{\bullet}$+ 200 µL of ethanol) was employed as a control. After 30minutes of reaction at 25°C, the absorbance of the remaining DPPH was measured at 517nm in a spectrophotometer (DU-640™, Beckman-Coulter - Brea, CA, USA) against blank. Free radical-scavenging activity was expressed as a percentage of the absorbance of the control DPPH solution, obtained from the following equation: % Activity = $[(A_{DPPH} - A_{Yerba\ mate})/A_{DPPH})] \times 100$, where A_{DPPH} is the absorbance value of the DPPH control, and $A_{Yerba\ mate}$ is the absorbance value of the test solution. Trolox was used as a standard using a calibration curve (15 – 250 µM), and the results of the assay were expressed as µmol Trolox equivalent (TE)/g of yerba mate. These results were obtained in triplicate and expressed as mean ± SD.

ABTS$^{\bullet+}$ Scavenging Capacity Assay

The ABTS$^{\bullet+}$ scavenging capacity assay was determined as described by Le, Chiu, & Ng [29] in yerba mate. The method is based on the decolorization of the ABTS radical cation to determine the antioxidant potential of samples. The solution of ABTS radical cation was prepared in advance by reacting aqueous ABTS solution (7 mM) with potassium persulfate (2.45 mM). In the analysis, diluted ABTS$^{\bullet+}$ solution with an absorbance of 0.70 ± 0.02 at 734 nm was employed. Briefly, 200 µL of optimal diluted extract was added in 1000 µL of ABTS$^{\bullet+}$ solution, followed by 6 minutes incubation at room temperature. The absorbance values were measured in a spectrophotometer (DU-640™, Beckman-Coulter - Brea, CA, USA) at 734 nm, in triplicate. Free radical-scavenging activity was expressed as a percentage of the absorbance of the control ABTS$^{\bullet+}$, obtained with the following equation: %Activity = $[(A_{ABTS\bullet+} - A_{Yerba\ mate})/A_{ABTS\bullet+}] \times 100$, where $A_{ABTS\bullet}$ is the absorbance value of the ABTS$^{\bullet}$ control, and $A_{Yerba\ mate}$ is the absorbance value of the yerba mate solution. A calibration curve was plotted of absorbance reduction and concentration of the Trolox (1 – 170 µM) and the results were expressed as µmol Trolox equivalent (TE)/g of yerba mate.

Animals and Experimental Design

All animal procedures were in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College for Animal Experimentation, according to the American Psychological Association Guidelines for Ethical Conduct in the Care and Use of Animals. Study protocols were approved by the Ethical Committee of the Universidade Estadual de Campinas (Protocol no. 3898-1).

Fifty male adult Wistar rats (*Rattus novergicus*) were used for this experiment. The animals were kept in polyethylene cages with wood shavings in a 12:12 light/dark cycle, at a temperature of about 22º C and with food and water *ad libitum*. After an adaptation period, animals were separated into five groups (n=10): control (C), receiving water; mate (M), receiving yerba mate infusion; single cadmium injection (Cd) and water from day one; mate/cadmium (MCd), receiving mate gavage and an cadmium injection on the 15th day; cadmium injection on day 1 and gavage with yerba mate for 15 days (CdM). *Ilex paraguariensis* infusion or water was given orally through gavage and a dose of 0.5mg cadmium (1.2 mg Cd/kg) was administered intraperitoneally, according to previously published research [30].

Twenty-four hours after 15-days treatment, animals were anesthetized with an intramuscular injection of 5mg/kg Xylazine and 80mg/kg Ketamine. A median laparotomy was performed and the chest cavity was opened to expose the heart. Blood samples were obtained by cardiac puncture. Testicles were removed and weighed. Half of the right testicle was fixed in Karnovsky fixative for 24 hours, testicular tissue blocks were dehydrated in ethanol (60% to 100%) and routinely embedded in glycol methacrylate resin (Leica Historesin, Leica Microsystems Nussloch, Germany). Histological sections (3µm thick) were stained with hematoxylin and eosin. The other testis (n=5 each group) was frozen (-80°C) for biochemical analyses. The left testis and epididymis were frozen (-20°C) for the calculation of daily sperm production (DSP), the number of sperm within the epididymis and sperm transit.

Biometric Data

The BW (body weight) gain was obtained by subtracting the animal's weights at the end of the experiment from their initial weights. The relative testis weight, known as the gonadosomatic index (GSI, %), was calculated by dividing the total testes weight by the final body weight, multiplied by 100 [31].

Hematological Parameter Determination

Hematological parameters measured were: total RBC (red blood cells), hemoglobin content (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), leucocyte (Leu) and platelet count (Pl). To estimate those parameters 0.08 mL blood was mixed with 0.02mL Ethylene Diamine Tetracetic Acid-EDTA (33.33 mg/mL) and fed to the auto analyzer (ANALYSER 6604,388, TKS COULTER, Hialeah, Florida, USA).

Lipid Peroxidation – TBARS Assay

The extent of lipid peroxidation was determined as the concentration of thiobarbituric acid reactive substances (TBARS). Thiobarbituric acid (TBA) assay is the most commonly used method for determination of the malondialdehyde (MDA) in biological fluids [32]. The assay is based on a condensation reaction of two molecules of TBA with one molecule of MDA, in which the reaction rate depends on temperature, pH and concentration of TBA. The reaction is carried out in acidic solution and temperature of ~ 100° C within one hour. Most of the MDA is produced during the decomposition of products of lipid peroxidation [33]. MDA was measured using the assay kit (Cayman, MI, USA) according to manufacturer's instructions.

Superoxide Dismutase Assay

Superoxide dismutase (SOD) activity was measured using an assay kit (Cayman, MI, USA), according to manufacturer's instructions. This kit employs a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical [34].

Catalase Assay

Catalase is a ubiquitous antioxidant enzyme that degrades hydrogen peroxide into water and oxygen [35]. Catalase activity was estimated by the reaction mixture that contained 2.0 mL of phosphate buffer (50 mM, pH 7.0) and 0.1 mg of sample protein, 100 µL of 10 volumes hydrogen peroxide. The decrease in absorbance was measured at 240 nm against a blank containing all the components except the

enzyme at 15s intervals for 2:30 min. The activity of the enzyme was expressed in mmol of hydrogen peroxide metabolized/mg protein/min [36].

Protein Determination

Small testicle fragments were weighed and homogenized in sodium phosphate buffer (pH=7.0, containing PMSF protease inhibitor) 5 mL/ g tissue, using IKA T10 Basic Ultra-Turrax homogenizer. Protein concentration was measured according to the biuret method described by Gornall et al. [37].

Determination of Cadmium Concentration in Tissue Samples

In order to determine Cd concentration in testicular tissue, 4 mL (per testicle) of deionized water were added to 0.2 g of the homogenized sample. Subsequently, 0.5 mL of tetramethylammonium hydroxide was added, the mixture was placed for 60 minutes in an ultrasound cleaning unit. The analysis was performed by graphite furnace atomic absorption spectrometry (GF AAS model AAnalyst 600, Perkin-Elmer, Norwalk, USA), and dilutions were made when necessary. The analytical calibration curve was obtained at concentrations of 1 – 5 μg L^{-1} using aqueous metallic element standards and the $NH_4H_2PO_4$ was used as the chemical modifier. The pyrolysis and atomization temperatures were 500°C and 1500°C, respectively. The same procedure was carried out with two certified reference materials (NIST 1577b and NIST 8414) to evaluate the methodological accuracy. All readings were performed at least in triplicate.

Daily Sperm Production (DSP) and Sperm Number within the Epididymis and in Sperm Transit

According to the protocol described by Robb et al [38], elongated stage 19 spermatids within the testis and sperm cells from the epididymis were counted using a Neubauer chamber. The DSP was calculated dividing the sperm number by 6.1, which is the number of days in which the mature spermatids are still attached to the epithelium. Then, to evaluate the sperm transit time within the head/body and cauda epididymis, the sperm numbers in each fragment were divided by the DSP [39].

Sperm Morphology

One centimeter of the portion of the deferent duct attached to the cauda

epididymis was removed, twisted with tweezers immersed in sodium phosphate buffer (0.1 mol/L, pH=7.2) for 15 min. The sperm freed in the buffer were placed in a Neubauer chamber for morphological analyses [40]. Sperm (200 per animal) were observed under light microscopy and classified in two categories: normal and abnormal [40-42]. The abnormal sperm were divided into categories as follows: abnormal tail (bent, coiled, absent and short) and abnormal head (amorphous or absent) [43-46].

Statistical Analyses

For all values, the means and standard deviation (SD) were calculated. The comparison of control values and other groups was made using the ANOVA test, followed by Tukey's post test using Prism 6.0 software. The *p* value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Ilex paraguariensis Total Phenolic Content and Antioxidant Assays

Ilex paraguariensis used in this experiment showed a concentration of 26.62µg gallic acid/ mg of yerba mate, results of the total phenol content.. Scavenging capacities were determined using DPPH, ABTS. DPPH concentration in the performed assay was 51.82 µmol Trolox equivalent (TE)/g of yerba mate and the result obtained for ABTS assay was 358.64 µmol Trolox equivalent (TE)/g of yerba mate. These results showed high total phenolic content and very strong antioxidant properties.

Biometric Data

Body weight and body weight gain were not altered in all groups when compared to the control. Testicular weight was reduced in all groups that received cadmium. Thus, GSI was significantly lower in groups exposed to cadmium as well as cadmium and mate association **(Figure 1)**.

Hematological Analyses

Treatment with Cadmium reduced hemoglobin content and hematocrit compared to control and mate groups, as also for the MCV, which were significantly altered in these groups. MCHC were not altered, although cadmium elevated leucocytes and platelet counts significantly. Comparing MCd and CdM groups - leucocytes significantly increase in CdM and platelet counts were significatively lower

in MCd compared to CdM group **(Table 1)**.

Lipid peroxidation (TBARS), SOD and Catalase Assays

Treatments with cadmium, mate, and both associations, significantly increased lipid peroxidation levels in the testis tissue compared to the control group and also elevated SOD and Catalase levels **(Figure 2)**. Protein concentrations, performed by the Biuret method, were not different between groups **(Figure 2-d)**.

Determination of Cadmium tissue concentration

The instrumental conditions for Cd quantification were optimized by evaluating the recovery of a certified reference material and the results showed that suitable recoveries were obtained (96 ± 6 % and 102 ± 1 % for the 1577b and 8414 materials, respectively), as well as adequate relative deviations, below than 10%. Limits of detection (LOD) and quantification (LOQ) were calculated and the values are 0.08 and 0.26 μg L^{-1}, respectively.

Cadmium tissue concentration values are shown in **figure 3**. Groups treated with cadmium showed the highest concentration of the metal. Cadmium tissue concentration in the groups treated with cadmium and *Ilex paraguariensis* infusion were not statistically different compared to the cadmium group.

Daily sperm production (DSP) and the number of sperm within the epididymis and sperm transit

The DSP and sperm production rate were significantly higher in the M group compared to the control group, while the MCd group showed a significant increase in the above parameters, in comparison to the control group. On the other hand, DSP and sperm production rate significantly decreased in Cd and CdM groups **(Table 2)**.

Sperm morphology

The Cd and CdM groups showed the highest proportion of sperm abnormalities **(Table 3)**. M showed the lowest proportion of amorphous heads, while Cd and CdM showed the highest proportion of absent heads **(Table 3)**. The main alterations in sperm morphology are represented in **figure 4**.

DISCUSSION

Antioxidant properties and total phenolic content in yerba mate used in this research showed very strong antioxidant properties and high total phenolic content, in agreement with previous studies [46-49]. Antioxidant activity is characterized by the inhibition of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·). The DPPH reduction test is based on the ability to react with hydrogen donors. When in the presence of antioxidant substances it receives H + and is reduced. The ability of the sample to reduce DPPH, to avoid oxidation, is indicated by the percentage of DPPH● remaining in the system. Therefore, the percentage of remaining DPPH● is proportional to the antioxidant concentration [28].

The inhibition of the [2,2'-azino-bis (3-ethylbenzothiazolin) 6-sulphonic acid] (ABTS●+) radical is used to characterize the antioxidant activity. The principle of the ABTS method is to monitor the decay of $ABTS^+$ radical cation, produced by the oxidation of ABTS, generated by the addition of a sample containing antioxidants. In the absence of phenols, $ABTS^+$ is stable, but it reacts energetically with a hydrogen atom donor, such as phenols, and is converted into a non-colorimetric form of ABTS [50].

The benefits of the consumption of yerba mate, a plant widely consumed in South America, have been extensively studied in recent years [51, 52]. Nevertheless, this is the first study investigating the effect of yerba mate infusion on the reproductive system, and protective effects against cadmium damages in testicles.

The mechanism of lipid peroxidation induction by cadmium is still poorly understood. As a transitional element, cadmium is unable to directly cause the formation of free radicals under physiological conditions [53]. So it probably acts through indirect mechanisms [54].

In most cases damage caused by cadmium is related to induction of oxidative stress which is demonstrated by alterations in the level of enzymes responsible for the endogenous antioxidant mechanism and lipid peroxidation levels [55, 56]. The intracellular concentration of ROS depends on its production and/or removal by the antioxidant system. Cells contain a large number of antioxidants to prevent or repair the damage caused by ROS, as well as to regulate redox-sensitive signaling pathways.

Three of the primary antioxidant enzymes contained in mammalian cells that are thought to be necessary for life in all oxygen metabolizing cells [57] are superoxide dismutase (SOD), catalase, and a substrate specific peroxidase, glutathione peroxidase (GPx). The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen (O_2), while the catalase and peroxidases convert hydrogen peroxide into water and in the case of catalase to oxygen and water. Cadmium is known to induce lipid peroxidation (LPO) by stimulating the production of superoxide anions [58]. Within the cells, cadmium accelerates LPO and suppresses antioxidants such as superoxide dismutase or glutathione peroxidase [59, 60]. Free radicals then accumulate, leading to cell damage, aging and the development of chronic diseases. Lipid peroxidation is considered the primary mechanism for Cd toxicity despite its inability to directly generate free radicals [53]. Decomposition products of lipid hydroperoxides such as malondialdehyde (MDA) can cause chaotic cross linkage with proteins and nucleic acids.

In this study, groups treated with cadmium, mate, and both associations of the elements, exhibited high levels of MDA in the testis tissue compared with the control group, which indicates an intensification of lipid peroxidation in this organ under the influence of Cd. This is in agreement with several previous studies [61-63]. Increased MDA levels found for the mate group suggest that the stimulating effect of the tea increased the metabolic rate of the rats, which led to higher lipid peroxidation. Very high LPO levels were found for the Cd and the CdM groups, as expected. The intermediate level found for MCd is interesting because it clearly demonstrates that the tea increased LPO somewhat and the Cd also contributed to a certain degree, but since this contamination occurred 24 hours before the end of the experiment, the full effect could not be demonstrated. It is possible that the rate would have increased had the test been executed after a longer period, possibly reaching the same level found for the other Cd groups tested after 16 post contamination days.

On the other hand, we reported an increase in SOD and Catalase activity in groups treated with cadmium. These results contradict those of several previous studies [64, 65], which noted a decrease in the activity of this enzyme in the kidneys and testes of rats treated with Cd. The increase in SOD activity may be interpreted as a protective response against cadmium toxicity in the testicles [66]. Indeed, it has been shown that the activity of antioxidant enzymes behaves in two different ways during

oxidative stress. At the beginning of stress, this activity increases, while in the long term, it is reduced due to the massive production of free radicals. This reduction is the result of damage to the molecular machinery that is required to produce these enzymes [65, 67]. In our acute experiment, it is possible that the molecular machinery had not yet been damaged and therefore the higher production of these enzymes was still occurring. Therefore the higher rate of catalase production in the groups that received Cd is expressive of this hypothesis.

In the case of SOD, the ingestion of mate was stimulating enough to raise the production of this enzyme, apparently due to a higher metabolism that rises the LPO rate but does not damage the molecular machinery that produces the enzyme. Cd contamination was a strong stimulus but it probably reduced the efficiency of enzyme production, resulting in a somewhat lower production. The highest SOD level was observed for the MCd group where the initial stimulus of the tea was increased by that of the Cd contamination, but the enzyme production was not affected in the 24 hours before it was measured.

Determining the exact quantity of proteins in a solution is very often necessary in the biochemical practice. Estimation of protein concentration was necessary in this research to perform the catalase assay. The Biuret method, used in this research, is based on the complexation of Cu^{2+} to functional groups in the protein's peptide bonds. The formation of a Cu^{2+} protein complex requires two peptide bonds and produces a violet-colored chelate product which is measured by absorption spectroscopy at 540 nm [68]. Protein concentration was not significantly different between groups.

The hematopoietic system is the most sensitive one to reveal pathological conditions under the stress induced by toxicants in both humans and animals [69]. In this study, the following parameters: Hb, Htc and MCV decreased in Cd and CdM groups compared to control, indicating profound alterations of health conditions. Although leucocyte and platelet counts increased in these groups, they may be compared to control groups. A high white blood cell and platelet count does not indicate a specific disease, but can indicate a problem, such as infection, stress, inflammation, trauma and allergy.

Testis exposed to cadmium presented lower cadmium concentration in tissues. Low doses of Cd ranging from 1 to 2 mg/kg BW have been reported to cause

testicular damage without pathological changes to other organs after both acute and chronic exposures [32, 33]. The higher sensitivity of the testes to Cd exposure rather than other organs may be attributed to its unique vasculature of this organ [70]. Regardless of the primary site of toxic injury, most testicular toxicants cause germ cell degeneration and depletion to a greater or lesser extent. Although Sertoli cells are very sensitive to functional perturbation, they are remarkably resistant to cell death [71]. The alterations of the testis, such as the absence of a lumen and seminiferous tubules completely filled with degenerated germ cells, multinuclear giant cells, and few developed sperm, were verified after 7 days. The progressive degeneration resulted in lesion of tubules lined only by Sertoli cells after 56 days, indicating spermatogenesis impairment [30].

Daily sperm production and sperm production rate were significantly higher in M compared to the control group, while the MCd group showed a significant increase in the above parameters, in comparison to the control group. This suggests that cadmium affects DSP and sperm production rate over a period of time. On the other hand, DSP and sperm production rate significantly decreased in Cd and CdM groups. Higher DSP and sperm production could be due to a greater release of spermatozoa caused by the cadmium. Then the lower levels found for cadmium could be due to the depletion of spermatozoa after the large release. Lower M levels could be due to a somewhat greater release.

Reduction of integral membrane proteins, compromise the hemato-testicular barrier. The consequent disruption of cell adhesion, promotes germ cell loss, altering sperm production and their liberation after complete [72, 73]. This process could have contributed to reduced sperm count as well as to their abnormal morphology. Moreover, cadmium has a direct effect associated with inflammatory process induction, with an increase in pro inflammatory cytokine levels, contributing to epithelium disruption [56] and triggering a permanent spermatogenesis loss [74]. Pires et al. [75] described an extensive inflammatory process and epithelium damage caused by the same cadmium dosage used in the present research. According to Abd-Allah et al. [74] DNA damages can contribute to the development of this process.

CONCLUSION

In conclusion, our findings confirm that cadmium in a dose of 1.2mg/kg b.w. can severely destroy testicular tissues and affect spermatogenesis. Cadmium

stimulates the antioxidant activities probably due to the induction of an adaptive response, in the way of maintenance and/or increase of physiological activities under this concentration of Cd. With the analyses performed, we can report that *Ilex paraguariensis* infusion 15-days treatment is only partially efficient in the protection of the testicles from cadmium induced damages.

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APÊNDICE 2
TABLES

Table 1: Hematological analyses.

Parameters/ Groups	C	M	Cd	MCd	CdM
Hemoglobin content (Hb)	17.68 ± 0.96	16.48 ± 0.54 ***	14.46 ± 0.31 *	17.4 ± 0.66	15.67 ± 1.7 **
Hematocrit (HCt)	64.28 ± 3.9	59.46 ± 2,9 ***	51.86 ± 2.05 *	64.24 ± 2.6	57.33 ± 5.0
Mean corpuscular vol. (MCV)	70.64 ± 1.33	72.18 ± 2.55	68.2 ± 1.08[a]	73.33 ± 0.51	69.5 ± 1.8[b]
Mean corpuscular vol. conc. (MCHC)	27.64 ± 0.53	27.74 ± 1.05	27.92 ± 1.05	27.12 ± 0.72	27.43 ± 0.93
Leucocyte (Leu)	3620 ± 1248	3560 ± 931	6725 ± 950	7900 ± 1299	13133 ± 5962[c]
Platelet Count (Pl)	578.6 ± 113.5	643.4 ± 49.5	880 ± 42.5[e]	595 ± 61.4	1397 ± 284[d]

Values are mean ± SD. C: control group; M: mate group; Cd: cadmium group; MCd: mate and cadmium single dose after 15 days treatment; CdM: cadmium single dose and and mate treatment. (n=5). A significant difference was found for the following: *p *< 0.0003 with C and MCd; **p=0,04 with C; ***p=0,01 with Cd; [a]p<0,02 with M and MCd; [b]p=0,04 with MCd; [c]p<0,001 with C, M and Cd; [d]p<0,0001 with C, M and MCd; [e]p<0,04 with C and MCd.*

Table 2: Spermatid counts in the testis and in the epididymis (head/body and cauda epididymis), daily sperm production and sperm transit time.

Parameters/ Groups	C	M	Cd	MCd	CdM
DSP (x10^6/ testis/day)	13.04±1.53^a	21.33±5,19^b	6.48±2.88	18.66±3.07^c	4.18±2.49
Sperm production rate (x10^6)					
Per testis	79.54±9.35^a	130.1±31.68^b	39.52±17.56	113.9±18.76^c	25.48±15.2
Per gram of testis	56.31±8.66^a	85.5±26.54^b	52.82±8.14	92.36±6.63^c	34.05±16.22
Epididymal sperm (x10^6)					
Head/ body	95.25±18.49	68.16±2.2^d	21.76±7.63^e	75.72±13.24^f	0.81±0.2^g
Cauda	216.6±15.69^h	150.6±12.55^i	39.19±16.39	126.9±12.23	18.28±20^j
Sperm transit time (epididymis in days)					
Head/ body	4.39±0,87	2.31±0,35	0.76±0.55	8.69±4.49^k	0.18±0.21
Cauda	9.98±0.72	5.07±0.58	1.41±0.98	22.67±11.09$^{l;m}$	2.94±2.65

Values are mean ± SD. C: control group; M: mate group; Cd: cadmium group; MCd: mate and cadmium single dose after 15 days treatment; CdM: cadmium single dose followed by mate treatment. A significant difference was found for the following: ^{a}p*<0.02 with M;*^{b}*p<0.0009 with Cd, and CdM;* ^{c}*p<0.006 withCd and CdM;* ^{d}*p=0.018 with C;* ^{e}*p<0,0003 with C and M;* ^{f}*p<0.0001 with Cd and CdM;* ^{g}*p<0.0001 with C and M;* ^{h}*p<0.0001 with M, Cd, MCd and CdM;* ^{i}*p<0.0001 with Cd and CdM;* ^{j}*p<0.0001 with C, M, Cd and MCd;* ^{k}*p<0.001 with M, Cd and CdM;* ^{l}*p<0.001 with Cd and CdM;* ^{m}*p<0.008 with A and M.*

Table 3: Sperm morphology – proportion of normal and abnormal sperm (%).

Group/ Parameter	C	M	Cd	MCd	CdM
NORMAL SPERM	105,4 ±34,8	83,8±16,3	42,6±14,1*	68,8±6,1	54±29,4*
Bent tail	49,8±24,8	70,6±16,2	79,4±22,6	75,2±22,9	59,8±10,5
Coiled tail	24,2±5,7	36±18,1	11,4±10,5	21,4±13,4	0,6±0,9*
Short tail	6,4±2,9	3,4±3,6	0,4±0,6*	3,6±3,3	1,8±2,2
Amorphous head	6,4±4	0,4±0,6*	28,6±8,4	14±9,9	23±8,5
Head absent	6,6±7,1	5,8±6,5	37,4±35,5*	16,4±8,6	47,6±64,7*

Values are mean ± SD. C: control group; M: mate group; Cd: cadmium group; MCd: mate and cadmium single dose after 15 days treatment; CdM: cadmium single dose and and mate treatment. (n=5). A significant difference was found for the following: *p*< 0.05 with C.*

APÊNDICE 3
FIGURES

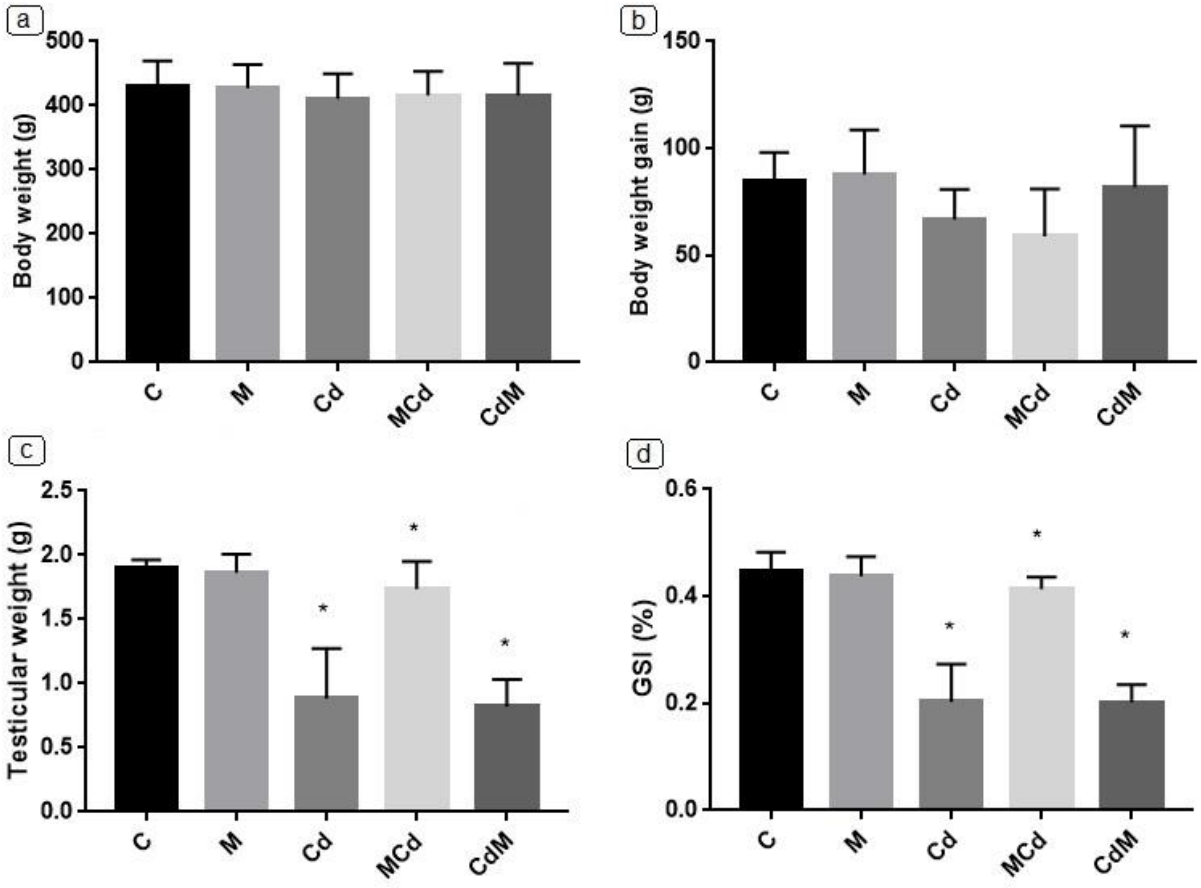

Figure 1: Biometric data. (a) body weight; (b) body weight gain; (c) testicular weight; (d) GSI, gonadossomatic index. C: control group; M: mate group; Cd: cadmium group; MCd: mate and cadmium single dose after 15 days treatment; CdM: cadmium single dose and and mate treatment. (n=5). A significant difference was found for the following: * p<0,001 with C and M.

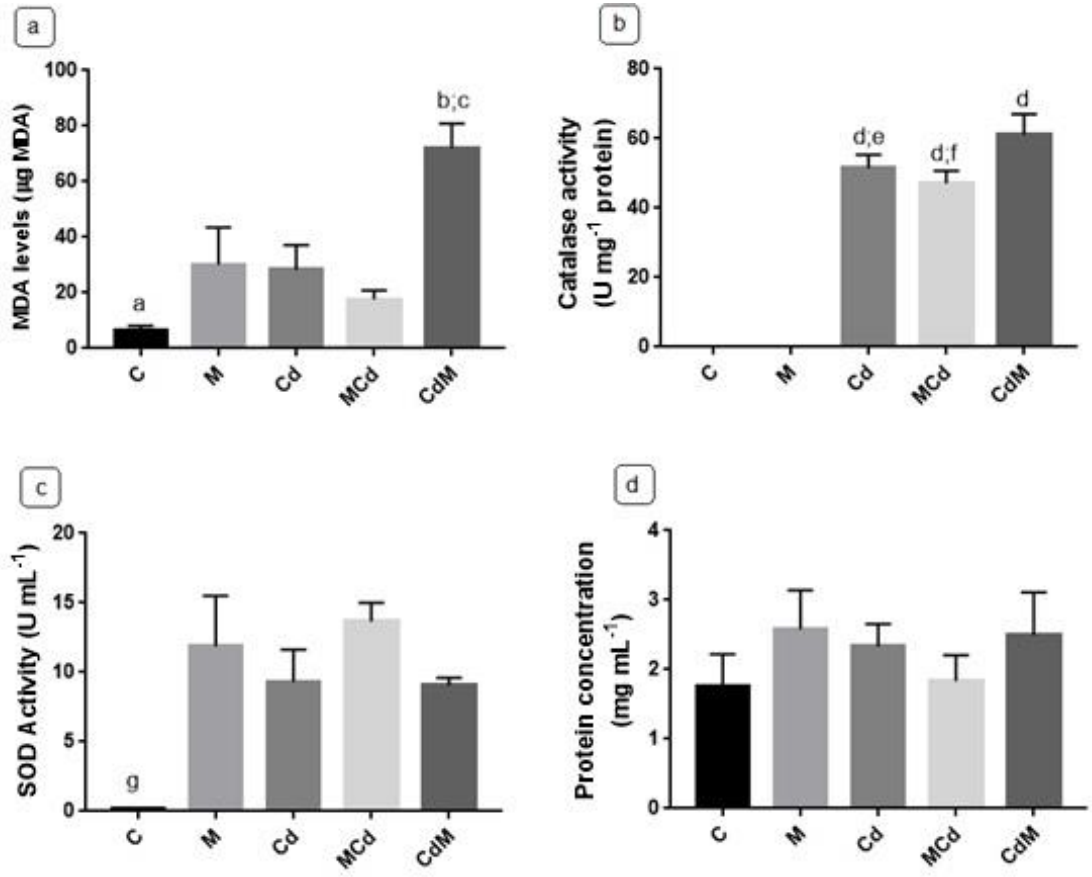

Figure 2: Enzymatic and non-enzymatic assays, and protein concentrations. (a) MDA levels; (b) Catalase activity; (c) Superoxide dismutase – SOD activity; (d) Protein concentrations. C: control group; M: mate group; Cd: cadmium group; MCd: cadmium single dose after 15 days mate treatment; CdM: cadmium single dose followed by mate treatment. (n=5). A significant difference was found for the following: *[a]p<0.03 with Cd and M; [b]p<0.0001 with C, M, and MCd; [c]p=0.0002 with Cd; [d]p<0.0001 with C and M; [e]p<0.0226 with CdM; [f]p<0.0015 with CdM; [g]p<0.002 with M, Cd, MCd and CdM.*

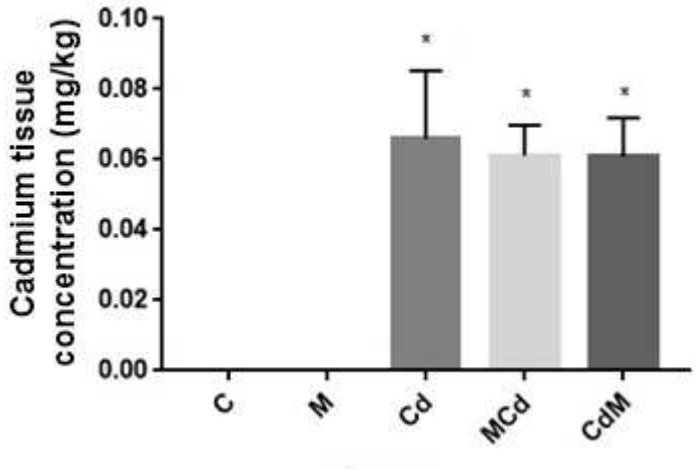

Figure 3: Cadmium testis concentration. C: control group; M: mate group; Cd: cadmium group; MCd: mate and cadmium single dose after 15 days treatment; CdM: cadmium single dose followed by mate treatment. A significant difference was found for the following: ** p<0.001 with C and M,* but not in the comparison among these three groups.

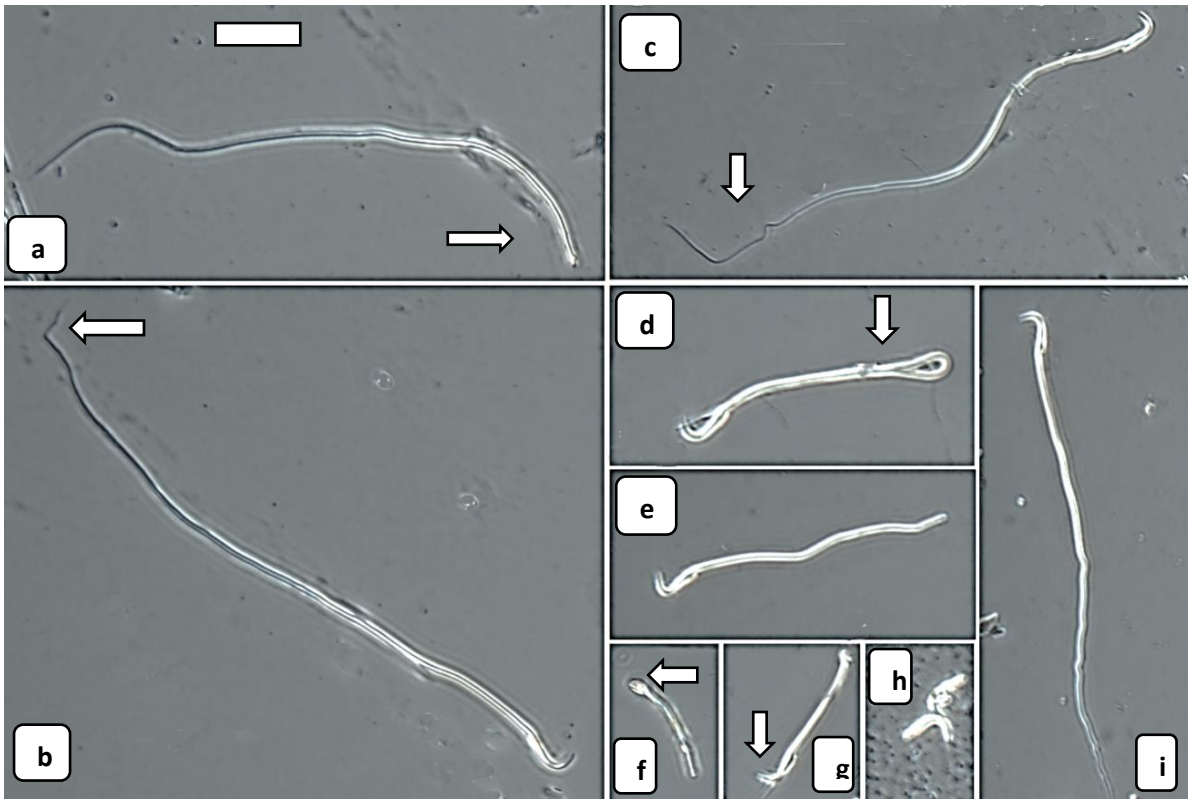

Figure 4: Sperm morphology alterations observed after 15 days treatment; (a) head absent; (b) and (c) bent tail; (d) coiled tail; (e) short tail; (f) and (g) amorphous head; (h) tail absent; (i) normal. Scale bar = 10,5µm.

5. CONSIDERAÇÕES FINAIS GLOBAIS

- A infusão de Ilex paraguariensis administrada durante 15 e 56 dias não interfere na morfologia testicular, podendo estimular a produção espermática após 15 dias de tratamento;
- O presente estudo confirma os danos testiculares causados pelo cádmio na dose de 1,2 mg/kg de peso, como degeneração dos túbulos seminíferos e redução da espermatogênese;
- Após um tratamento de 15 dias a erva-mate não protegeu os testículos dos danos causados pelo cádmio;
- Os testículos foram parcialmente protegidos dos efeitos causados pelo cádmio após tratamento durante 56 dias com infusão de erva mate;
- O cádmio estimulou a atividade antioxidante a fim de de induzir uma resposta adaptativa, com a finalidade de manter ou aumentar as atividades fisiológicas após exposição;
- A erva mate causa aumento da peroxidação lipídica e estimula a produção de enzimas antioxidantes, possivelmente por conter compostos que estimulam o metabolismo, porém estudos precisam ser realizados a fim de confirmar.

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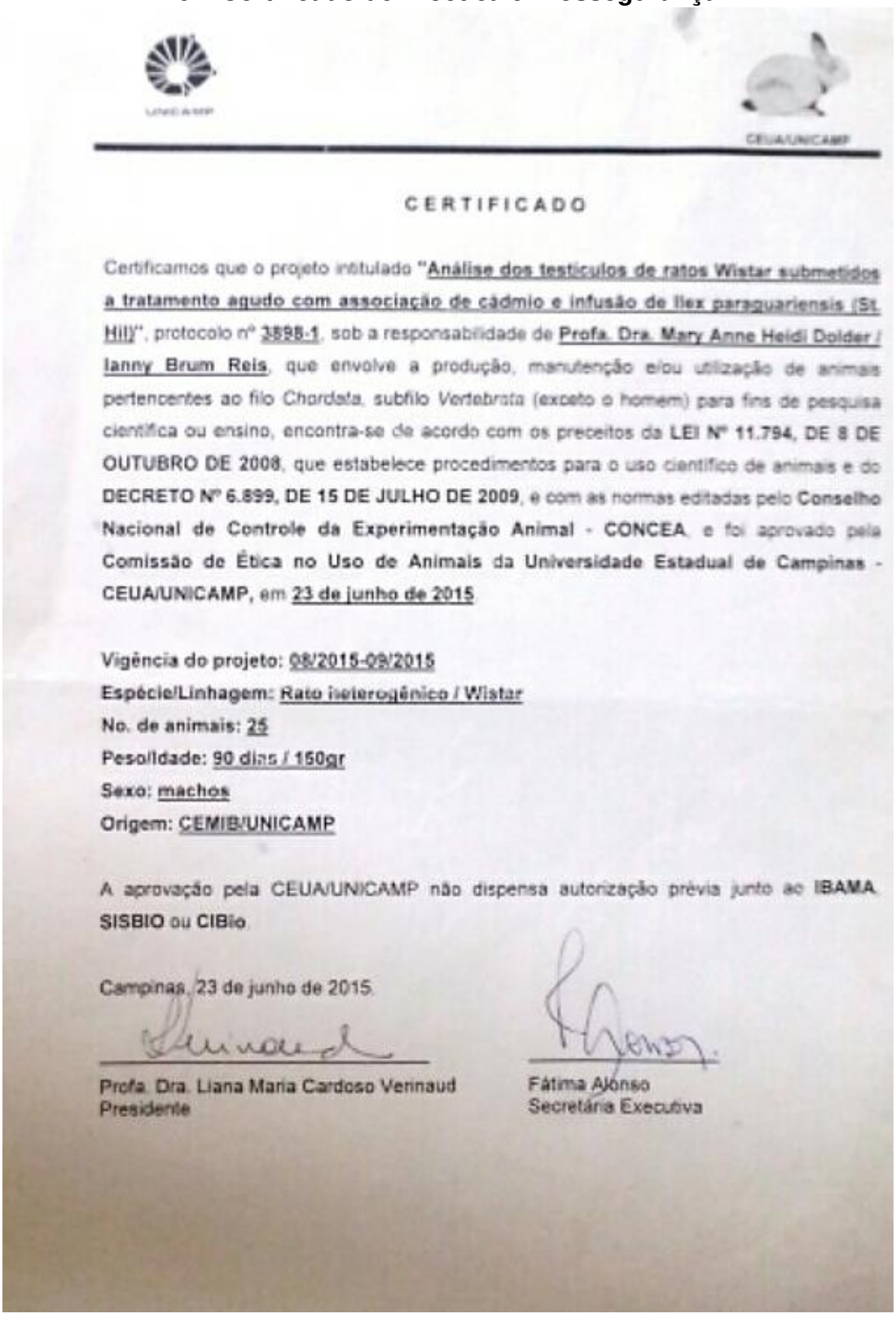
6. ANEXOS

6.1 Certificado de Bioética e Biossegurança



CEUA/UNICAMP

CERTIFICADO

Certificamos que o projeto intitulado "**Análise dos testículos de ratos Wistar submetidos a tratamento agudo com associação de cádmio e infusão de Ilex paraguariensis (St Hil)**", protocolo nº **3898-1**, sob a responsabilidade de **Profa. Dra. Mary Anne Heidi Dolder / Ianny Brum Reis**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica ou ensino, encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais e do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal - CONCEA**, e foi aprovado pela **Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP**, em **23 de junho de 2015**

Vigência do projeto: 08/2015-09/2015
Espécie/Linhagem: Rato iselerogênico / Wistar
No. de animais: 25
Peso/Idade: 90 dias / 150gr
Sexo: machos
Origem: CEMIB/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

Campinas, 23 de junho de 2015.

Profa. Dra. Liana Maria Cardoso Vennaud
Presidente

Fátima Ajonso
Secretária Executiva

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **Análise dos testículos de ratos Wistar submetidos a tratamento agudo com associação de cádmio e infusão de Ilex paraguariensis (St. Hil)** (protocolo CEUA/UNICAMP nº 3898-1), de responsabilidade da **Profa. Dra. Mary Anne Heidi Dolder** e **Ianny Brum Reis**, teve o título alterado para **Avaliação dos efeitos de *Ilex paraguariensis* (St. Hil) associada ao cádmio em diversos órgãos de ratos Wistar**.

Este documento é válido apenas se apresentado junto com os certificados emitidos originalmente pela CEUA/UNICAMP, sendo emitido em 23/06/2015.

Campinas, 29 de março de 2017.

Profa. Dra. Liana M. C. Verinaud
Presidente

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6.2 Declaração: dissertação não infringe dispositivos da lei nº9610/98

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **"Effect of *Ilex paraguariensis* (St Hil) on Cadmium Induced Damage to Wistar Rats Teticles"** , não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 08 de maio de 2017.

Assinatura : _____
Nome do(a) autor(a): **IANNY BRUM REI**
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