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Efeitos da Associação de Cloreto de Cádmio e Guaraná (*Paullinia cupana*) no Testículo de Ratos Wistar: Análise Morfométrica, Estrutural e Ultraestrutural.

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

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Lista de Abreviaturas

ABP – Proteína de Ligação a Andrógenos

Bm – Basal Membrane

BW – Body Weight

F – Fibrosis

FSH – Homônio Folículo Estimulante

Gc – Germ Cells

Is – Intertubular Space

L – Lipid

Lc – Leydig Cell

LH – Hormônio Luteinizante

M – Macrophage

Se – Sertoli Cell

Sg – Spermatogonia

Sp – Spermatocyte

St – Spermatid

ST – Seminiferous Tubule

Sz – Spermatozoa

V – Blood Vessel

Va – Vacuole

Sumário

Resumo

O guaraná (*Paullinia cupana* Mart. var. Sorbilis) é uma planta originária do Brasil encontrada na região da Bacia Amazônica. Estudos prévios vêm demonstrando a propriedade antioxidante do extrato de guaraná e sua capacidade neutralizar radicais livres. Essa planta é tradicionalmente usada com fins terapêuticos diversos e recentemente tem-se investigado sua capacidade de atuar no tratamento de patologias relacionadas ao desequilíbrio da atividade oxidante da célula. O cádmio é um metal pesado com propriedades tóxicas aos organismos e a contaminação por este elemento torna-se preocupante devido ao seu grande efeito cumulativo e meia-vida biológica longa. Esse metal tem sido considerado um importante poluente ambiental, sendo as suas principais fontes de exposição o despejo industrial, a poluição atmosférica e o tabaco. Sabe-se que a natureza tóxica do cádmio é, em parte, devida à sua ação oxidante no organismo, estimulando a produção de radicais livres que causam danos celulares. Os testículos são particularmente vulneráveis aos efeitos tóxicos desse metal, sendo que neste órgão a propriedade cumulativa do cádmio parece ser acentuada. Portanto, os testículos atuam como um importante modelo experimental para estudos referentes aos efeitos tóxicos do cádmio e à possíveis alternativas terapêuticas para os efeitos adversos deste metal. Baseando-se nestas informações, o presente estudo avaliou o potencial do guaraná em atenuar os danos causados pelo cádmio no testículo de ratos Wistar adultos. Foram desenvolvidos dois modelos experimentais diferentes: 1º- administração preventiva do guaraná durante 56 dias antes da exposição ao cádmio; 2º- administração do guaraná durante 56 dias após a exposição ao cádmio. Para análises comparativas, também foram montados um grupo tratado somente com cádmio e um grupo tratado somente com guaraná. As amostras de testículo foram coletadas e submetidas a análises histológicas, morfométricas e ultra-estruturais, e os resultados obtidos estão expostos em dois artigos científicos.

Abstract

Guaraná (*Paullinia cupana* Mart. var. Sorbilis) is a Brazilian plant, found in the Amazon basin. Previous studies have demonstrated the antioxidant properties of guaraná and its potential to neutralize free radicals. This plant is traditionally used for several therapeutic purposes and, recently, has been investigated to ascertain the potential of guaraná to counterbalance cellular oxidative disorders. Cadmium is a toxic heavy metal, and the contamination by this element is more harmful due to its long biological half-life, and therefore its accumulation in the organism. This metal is considered important for environmental pollution, in which exposition occurs through industrial wastes, atmospheric pollution and tobacco smoking. Cadmium is known to be toxic partly due to its oxidative effect in the organism, stimulating the production of free radicals that cause cellular damage. The testicles are particularly vulnerable to the toxic effects of this metal and this organ has higher cumulative properties. Therefore the testicles are an important experimental model for studies of the toxic effects of cadmium and the possible therapeutic alternatives for the adverse effects of this metal. Based on this information, the present study evaluated the potential of guaraná to attenuate the harm caused by cadmium in adult rat testicles. Two experimental models were developed: 1) Preventive administration of guaraná during 56 days before exposition to cadmium; 2) Administration of guaraná during 56 days after exposition to cadmium. For comparative analysis, a group was also treated only with cadmium or only with guaraná. Testicle samples were collected and submitted to histological, morphometrical and ultrastructural analysis and the results are presented in the form of two scientific articles. The results show that guaraná is beneficial in both experimental models, contributing to the maintenance of testicular morphology.

Introdução

Aspectos gerais do testículo

Os testículos são órgãos pares localizados no escroto, fora da cavidade abdominal. Cada testículo é envolvido por uma cápsula de tecido conjuntivo denso denominada albugínea testicular. Esta é compactada na superfície posterior do testículo e constitui o mediastino, do qual partem septos fibrosos que formam compartimentos denominados lóbulos do testículo. Cada lóbulo é ocupado por túbulos seminíferos que se alojam como novelos dentro de um tecido conjuntivo frouxo, o espaço intertubular, rico em vasos sanguíneos e linfáticos, nervos, células de Leydig, macrófagos e mastócitos (Russel et al., 1990; Junqueira e Carneiro, 2004).

Os túbulos seminíferos consistem de um lúmen central revestido por um epitélio seminífero especializado, contendo duas populações celulares distintas: (1) as células de Sertoli somáticas e (2) as células espermatogênicas ou germinativas (espermatogônias, espermatócitos e espermátides). O epitélio seminífero é envolto por uma membrana basal e uma parede formada por fibras colágenas, fibroblastos e células mióides contráteis (Kierszenbaum, 2004). Esta parede externa do túbulo seminífero é chamada de túnica própria (Stevens e Lowe, 1999).

As células espermatogênicas são uma população em constante processo de proliferação e diferenciação organizadas em camadas distintas no epitélio seminífero (Ross et al., 1993). As espermatogônias, células germinativas primitivas que iniciam a espermatogênese, são relativamente pequenas e encontradas próximas à membrana basal. As espermatogônias se diferenciam por mitose em espermatócitos, estes últimos localizados na região mediana do epitélio seminífero. Após a segunda divisão meiótica os espermatócitos originam as espermátides, localizadas próximas ao lúmen tubular. Estas últimas se diferenciam em espermatozóides através de um processo conhecido como espermiogênese (Junqueira e Carneiro, 2004).

As células de Sertoli constituem uma população celular não proliferativa que se localizam na região basal e formam a parede dos túbulos seminíferos. Análises ultra-estruturais mostram que esta célula possui morfologia típica, caracterizada por um citoplasma extenso, um núcleo exibindo indentações e numerosas mitocôndrias esféricas ou

alongadas com cristas tubulares (Bizarro et al., 2003). No miscroscópio de luz é facilmente reconhecida devido ao seu nucléolo evidente. O citoplasma se estende da região basal ao lúmen do túbulo seminífero, e as células de Sertoli adjacentes estão interconectadas por junções de oclusão. As junções de oclusão basolaterais subdividem o epitélio seminífero em um compartimento basal e um compartimento adluminal, e são componentes que determinam a chamada barreira hemato-testicular, que protege os espermatozóides em desenvolvimento e as espermátides das reações auto-imunes (Kierszenbaum, 2004). Basicamente as funções das células de Sertoli são: secretar as proteínas inibina e activina; secretar a proteína de ligação a andrógenos (ABP); sustentar, proteger e nutrir as células espermatogênicas em desenvolvimento; eliminar, por fagocitose, os corpos residuais liberados pelas espermátides ao final da espermiogênese; secretar um fluído rico em íons e proteínas no lúmen do túbulo seminífero (Russel e Griswold, 1993; Sharpe, 1994; Kierszenbaum, 2004).

Agregados de células de Leydig estão presentes no espaço intertubular, nas proximidades dos vasos sanguíneos e dos vasos linfáticos (Kierszenbaum, 2004). Estas células são as principais responsáveis pela síntese e secreção androgênica no organismo, correspondendo a aproximadamente 95% da testosterona encontrada no soro (Yang et al., 2003; Kierszenbaum, 2004). Como na maioria das células produtoras de esteróides, as células de Leydig contém gotículas lipídicas, mitocôndrias com cristas tubulares características e retículo endoplasmático bem desenvolvido (Stevens e Lowe, 1999; Kierszenbaum, 2004). Ao microscópio de luz são facilmente reconhecidas, não somente por serem as células predominantes do espaço intertubular, mas também por apresentarem morfologia arredondada ou poligonal com um núcleo central proeminente (Stevens e Lowe, 1999; Junqueira e Carneiro, 2004).

Hormônios são os fatores mais importantes no controle da espermatogênese. Esta depende da ação dos hormônios FSH e LH da hipófise nas células do testículo. O hormônio luteinizante (LH) age nas células de Leydig, estimulando a produção de testosterona necessária para o desenvolvimento normal das células espermatogênicas. O hormônio folículo estimulante (FSH) age nas células de Sertoli estimulando a síntese e a secreção da proteína de ligação a andrógenos (ABP). O complexo andrógeno-ABP é transportado ao

lúmen dos túbulos seminíferos e mantém altos os níveis de andrógenos nas proximidades das células germinativas. As proteínas inibina e activina secretadas pela célula de Sertoli exercem, respectivamente, um feedback negativo e positivo na liberação de FSH pelo hipotálamo e pela hipófise. A remoção experimental da hipófise gera a interrupção no processo espermatogênico. (Junqueira e Carneiro, 2004; Kierszenbaum, 2004).

Aspectos gerais do cádmio

Juntamente com o chumbo e o mercúrio, o cádmio é um dos três metais pesados mais venenosos encontrados como poluentes ambientais (Manahan, 1988). Este elemento apresenta número atômico 48, massa atômica relativa de 112.4 e raramente é encontrado em seu estado puro, estando geralmente associado a outros elementos como minério de cobre, chumbo e zinco (WHO, 1992).

O cádmio é amplamente distribuído na crosta terrestre, apresentando uma concentração de aproximadamente 0,1 mg/kg, e altos níveis deste metal estão naturalmente presentes em rochas sedimentares na proporção de 15mg/kg (Gesamp, 1984). Tempestades e erosão resultam no transporte fluvial de grande quantidade de cádmio aos oceanos, representando o maior fluxo global cíclico deste elemento, estimado em 15.000 toneladas anuais (Gesamp, 1987). A concentração estimada deste metal no mar é de 0,1 μg/litro (Korte, 1983), enquanto que em águas fluviais é de 1.1 a 13.5 ng/litro (Shiller e Boyle, 1987). A atividade vulcânica é a maior fonte natural de cádmio atmosférico, apresentando um fluxo anual estimado em 100 a 500 toneladas (Niagru, 1989).

Ao contrário de outros metais pesados, o cádmio só foi utilizado na indústria e em larga escala a partir de 1940 (Sherlock, 1984), e quantidades significantes e progressivas deste metal vêm sendo introduzidas no meio ambiente, seja a partir de fontes naturais como por processos antropogênicos (Bernard e Lauwerys, 1984; Hoels et al., 1999).

Até os anos 50 o cádmio não era reconhecido como uma ameaça em termos de contaminante ambiental (Bernard e Lauwerys, 1984), ou como sério contaminante em alimentos (Sherlock, 1984). Atualmente, este elemento torna-se um importante poluente ambiental, amplamente utilizado na produção industrial de fertilizantes fosfatados,

pigmentos, baterias, plásticos, estabilizadores, entre outros (Istomim et al., 1999; Kumar et al., 2000). O fumo também é uma importante fonte de cádmio, e a concentração deste metal

é em torno de duas vezes maior em fumantes quando comparados a não fumantes (Manahan, 1988; Milnerowicz et al., 2000).

A natureza tóxica do cádmio foi revelada por volta de 1900, quando trabalhadores americanos inalaram vapores do metal durante o processamento de minério de cobre. Estudos posteriores utilizando animais de laboratório revelaram alguns dos efeitos da sua exposição, que são: dano tubular renal, necroses placentárias e testiculares, osteomalácia, tumores testiculares, malformações, anemias, hipertensão, edema pulmonar, enfisema pulmonar crônico e agravamento de deficiências induzidas por ferro, cobre e zinco (Manahan, 1988). Há também referências de hemorragia cerebral, hemorragia central, redução de crescimento bem como interferências no desenvolvimento pulmonar (Ragan e Mast, 1990).

Os casos clínicos de intoxicação aguda e crônica por cádmio têm aumentado significativamente em humanos (Ragan e Mast, 1990), e o público em geral pode ser exposto, predominantemente, por água e alimentos contaminados, como também pela inalação de fumaça de cigarros, fumaça industrial e poluição atmosférica (Milnerowicz et al., 2000; Shimbo et al., 2000).

Pesquisas referentes à influência do tráfego de veículos automotores na poluição e intoxicação por cádmio vêm demonstrando que os níveis teciduais deste metal em humanos são mais elevados nas zonas urbanas quando comparadas à zona rural (Sanches, 2000), considerando que a queima de combustíveis fosseis consistem em uma importante fonte de cádmio ao ambiente (Who, 1992). Análises de águas naturais vêm demonstrando desequilíbrio na concentração normal de cádmio, sendo este fato devido às descargas de efluentes industriais, como na produção de pigmentos, soldas e materiais fotográficos. O padrão de potabilidade determinado pela portaria 1469 é fixado em 0,005 mg/l. Um aumento de 0,005mg/l provocado por uma mina de Zinco no Japão foi o responsável pela síndrome de ´´Itai-Itai``, na qual a população contaminada apresentou severas patologias como a osteomalácia e mau funcionamento dos rins (Cetesb, 2006).

Toxicidade Testicular ao Cádmio

Uma característica importante na toxicologia do cádmio é sua excepcional tendência de acumular-se no organismo, apresentando uma meia-vida biológica longa, de 10 a 30 anos (Robards e Worsfold, 1991). Esta tendência acumulativa é acentuada nos testículos (Oteiza, 1997), provavelmente devido ao fato de este órgão estar em constante processo de divisão e diferenciação, tornando-o mais suscetível a substâncias tóxicas (Yano e Dolder, 2002).

A dose mínima deste metal necessária para causar danos testiculares foi relatada por Gunn et. al. (1966), sendo de aproximadamente 0,44 mg/kg. Estudos utilizando doses maiores têm descrito diversas alterações morfológicas e bioquímicas nos testículos de mamíferos, tais como necrose testicular (Gunn et al., 1968), degeneração de células germinativas (Hew et al., 1993) e aumento na taxa de peroxidação de lipídios (Oteiza et al., 1997; Koyuturk et al., 2006). Estudos *in vitro* demonstraram que o cádmio também afeta a viabilidade de células de Leydig, reduzindo a produção de testosterona (Yang et al., 2003).

Um grande número de evidências indica que um dos mecanismos de ação tóxica deste metal é o aumento da produção de radicais livres, os quais geram danos oxidativos em DNA, lipídios e proteínas (El-Demerdash et al., 2004; Ilkes et al., 2004). O termo ´´radicais livres`` é utilizado para designar as espécies reativas de oxigênio, como por exemplo o radical hidroxila ($HO^{\cdot}$) que, uma vez formado, inicia o processo de oxidação de lipídeos e forma uma cadeia autocatalítica na produção de ainda mais radicais livres (Haslam, 1989).

Células e tecidos normalmente possuem mecanismos endógenos de defesa antioxidante (eg. Superóxido desmutase) e mecanismos provenientes da dieta (eg. Vitamina C), que são especializados na remoção de radicais livres (Haslam, 1989). No entanto, alterações no sistema endógeno de defesa antioxidante foram observados após a exposição ao cádmio, inativando importantes enzimas antioxidantes como superóxido desmutase e glutationa peroxidase (Manca, 1991; Hussain, et al., 1987).

A propensão dos testículos aos efeitos acumulativos e tóxicos do cádmio torna este órgão um importante modelo experimental, possivelmente aplicado não somente a estudos referentes à fertilidade de mamíferos, como também ao organismo em geral. Uma vez que um dos mecanismos de atuação tóxica do cádmio é o estímulo a processos oxidativos no

organismo, estudos envolvendo o potencial antioxidante de extratos vegetais e outras substâncias são importantes na busca de alternativas terapêuticas para os danos causados por este metal, assim como para outras patologias relacionadas ao desequilíbrio da atividade oxidante celular.

Aspectos gerais do guaraná (***Paullinia cupana*** Mart var. Sorbilis)

O Guaraná é uma planta originária da Bacia Amazônica brasileira (Henman, 1982) que vem sendo usada há séculos como um estimulante pelos índios Saterê-Mauê, habitantes desta região (Henman, 1986). Diversas propriedades desta planta são cientificamente comprovadas, como proteção gástrica contra lesões causadas por etanol (Campos et al, 2003), perda de peso (Boozer et al., 2001) e aumento na capacidade de memorização (Espínola et al., 1997).

O guaraná é rico em cafeína, flavonóides e taninos, substâncias com propriedades estimulantes, efeito adaptógeno e ação contra diarréia (Brekhman, 1980; Carlson e Thompson, 1998). O termo adaptógeno é usado para classificar extratos vegetais ou outras substâncias que aumentam a resistência corporal de forma não específica, protegendo o organismo contra ações estressantes (Brekhman e Dardymov, 1969). Estas substâncias são usadas cronicamente para aumentar a resistência física e atenuar desordens resultantes do envelhecimento, como perda de memória, fraqueza e impotência sexual (Fulder, 1890; Carlini, 1991; Russo, 2001). Podem também ser usadas por pessoas saudáveis, não somente como profiláticos, mas também para aumentar as capacidades físicas e cognitivas (Wagner et al., 1994; Rege et al., 1999).

Estudos recentes vêm demonstrando a propriedade antioxidante do guaraná e sua possível utilização fitoterápica na prevenção e tratamento de doenças relacionadas ao desequilíbrio da atividade oxidante da célula. Mattei et al (1998), utilizando testes *in vitro* com cérebros de rato, demonstrou que o guaraná exerce um claro efeito antioxidante, inibindo o processo de peroxidação de lipídeos. Resultados concordantes foram obtidos por Basile et al (2005), nos quais o extrato de guaraná demonstrou reduzir significativamente a oxidação de lipídeos, resultando em 62,5% menos danos celulares causados por esse processo.

A propriedade antioxidante desta planta é provavelmente devido à sua alta concentrações de taninos, e é possível que grande parte dos efeitos revitalizantes relacionados ao guaraná sejam devidos à propriedade de redução dos processos oxidativos do organismo (Mattei, 1998). Os taninos são compostos fenólicos amplamente distribuídos entre os vegetais superiores (Haslam, 1989) e informações vêm sendo acumuladas ao longo dos últimos anos demonstrando sua capacidade de eliminar radicais livres (Haslam, 1996; Galato et al., 2001).

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Objetivos

O objetivo deste trabalho foi avaliar o potencial do extrato de *Paullinia cupana* em atenuar os danos testiculares em ratos Wistar adultos expostos ao cádmio. Dois modelos experimentais diferentes foram montados:

Modelo exp*erimental I* - administração preventiva do extrato de guaraná durante 56 dias antes da exposição ao cádmio.

Modelo Experimental II - administração do extrato de guaraná durante 56 dias após a exposição ao cádmio.

Resultados

Os resultados obtidos neste trabalho estão expostos em dois artigos científicos:

Modelo Experimental I: Protective Effect of Guaraná (*Paullinia cupana* Mart.) Supplementation on Cadmium-induced Damages in Adult Wistar Rat Testis.

Modelo Experimental II: Protective Effect of Guaraná (*Paullinia cupana* Mart.) relative to Cadmium-induced Damages in Testis of Adult Wistar Rats.

Protective Effect of Guaraná (*Paullinia cupana* Mart.) Supplementation on Cadmium-induced Damages in Adult Wistar Testis.

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Abstract

Guaraná (*Paullinia cupana* Mart var. Sorbilis) is a plant originally from Brazil, found in the Amazon Basin. Recent studies have been demonstrated the antioxidant potential of guaraná seed extract, probably due to the high concentration of polyphenols. On other hand, several reports indicated that one of the mechanisms underlying cadmium-induced cellular damage is free radical mediated, consequently increasing the oxidative processes in the organism. The present study was performed to investigate the potential of guaraná to attenuate the cadmium-induced damages in Wistar rats testis, since this organ is particularly vulnerable to the metal. Adult male Wistar rats were preventively treated with 2mg/kg bw of *P. cupana* during 56 days and then injected with a cadmium chloride in a dose of 1,15mg/Kg bw. The animals were sacrificed 48 hours after the cadmium exposition and the testis samples were evaluated by morphometry and transmission electron microscopy analyses. The cadmium only and guaraná plus cadmium treated groups presented evident morphological alterations relative to control animals, and the most sensitive animals presented massive cell death in the seminiferous epithelium as well as in the interstitial tissue components. However, the treatment with *P. cupana* during 56 days before cadmium exposition reduced the morphological changes in the Leydig cells, prevented vasoconstriction and reduced the inflammatory response in the intertubular space relative to the cadmium only treated animals. Also, this study showed that the animals treated with *P. cupana* only presented a significant increase in the plasma testosterone levels followed by a significant increase in the volumetric proportions of seminiferous tubules, suggesting that this extract stimulated the spermatogenic process.

Introduction

The therapeutic potential of natural products is a large research field that has grown in the last years, especially in countries with a large biodiversity, such as Brazil. *Paullinia cupana* var. Sorbiles Mart. (Sapindaceae), popularly called guaraná, is a plant found in the Amazon basin (Henman, 1982) that has been used for centuries as a stimulant by the Saterê-Mauê Indians, who live in the Amazon region (Henman, 1986). Some therapeutic properties of guaraná are scientifically proven, such as the increase in physical capacity (Espínola et al., 1997), prevention arteriosclerosis (Bydlowski et al., 1998) and gastric protection against lesions induced by ethanol (Campos et al., 2003). The chemical composition of guaraná features xanthic base such as caffeine, theophylline and theobromine, the later two found in guaraná bark, flowers and leaves, but are absent in seeds (Henman, 1986). There is also a high concentration of polyphenols such as tannins and flavonoids (Brekhman, 1980; Carlson and Thompson, 1998), substances that have proven antioxidant action (Galato et al., 2001). Therefore, it is possible that part of the revitalizing effects of guaraná may be due to the antioxidant action, attributed to the high concentration of tannins in the guaraná seeds (Mattei et al., 1998).

Cells and tissues normally possess antioxidant defense mechanisms to ensure the removal of reactive oxygen species that can be provided endogenously and by dietary contributions (Haslam, 1996). In conditions of oxidative stress, the intracellular concentration of free radicals is increased, presumably because cells either overproduce these substances or are deficient in their ability to destroy them (Cerutti, 1985). As a result, free radicals have been implicated in various human diseases including the process of aging, cancer, multiple sclerosis, Parkinson's disease and inflammation (Haslam, 1996).

A large number of reports indicated that one of the mechanisms underlying cadmium-induced cellular damage is free radical mediated (Sarkar et al., 1998; Mikhailova et al., 1997; Koizume and Li, 1992). Cadmium is widely used in industry, such as in plating, metal smelting and batteries (Friberg et al., 1986), and is one of the most toxic industrial and environmental heavy metal, and it has been known to damage the hepatic, respiratory and reproductive systems (WHO, 1992). It has long been recognized that

cadmium is extremely toxic to the testicular tissues of rats and mice, thus several morphological and biochemical changes in testis due to cadmium exposure have been described for mammals (Oteiza et al., 1997; Hew et al., 1993; Koyuturk et al., 2006). One important feature in cadmium toxicology is the exceptional trend towards testis accumulation, probably due to this organ undergoing in constant mitosis and meiosis (Yano and Dolder, 2002). Therefore, the aim of this study was to evaluate the potential of guaraná to prevent or attenuate the damages in Wistar rat testis caused by cadmium exposure.

Material and methods

Animals

Male Wistar (14 days of age), were obtained from the Multidisciplinary Center for Biological Investigation (State University of Campinas, SP, Brazil). Animals were housed two per cage under controlled conditions of temperature (24 ± 4^{o}C) with a 12h-dark/light cycle and provided with water and food *ad libitum*. After a maturation period, adult Wistar rats of 110 days of age were divided into 4 groups for the treatments (described below). The number of rodents per group was 5 for the control group, 5 for the guaraná only treated group, 9 for the cadmium only treated group and 9 for the guaraná plus cadmium treated group. This research project was approved by the institutional Committee for Ethics in Animal Research of this University (Protocol n^{o} 1202-1).

Paullinia cupana

Ground guaraná seeds were obtained from EMBRAPA Amazônia Occidental (Brazilian Enterprise for Agricultural and Cattle Raising Research of the Western Amazon) and were conserved in dry conditions at ± 4°C during the treatment. Guaraná (2mg/g BW) was freshly diluted in 1ml of water and administered by gavage once a day. The dose of the guaraná powder was chosen based on previously study, which demonstrated the best chemopreventive effects (Fukumasu et al., 2005).

Treatment protocol

Twenty eight adult male Wistar rats of 110 days of age were divided into four groups:
Group I – Control (5 animals)
Group II – Cadmium only (9 animals)
Group III – *P. cupana* plus Cadmium (9 animals)
Group IV – *P. cupana* only (5 animals)

The groups III and IV received the *P. cupana* extract by gavage during 56 days. The group II received water gavage during the same period. On the 56th day, the animals of groups II and III received an intraperitonial injection of cadmium chloride (1,15 mg/kg bw.) and were sacrificed 48 hours after the metal exposition. The group I received water gavage during 56 days and together with the group IV was sacrificed on the 57th day.

Fixation and Processing of the tissue

At the end of each experiment, animals were anesthetized with Xylazine and Ketamine (5 and 80 mg/kg BW, respectively). An incision was made in the scrotum and the left testicle was exposed and dissected free. The tunica albuguinea was gently fissured and the organ was immersed in a solution of glutaraldehyde 4% and paraformaldehyde 4% in 0.1 M phosphate buffer at pH 7.2. After ten hours of fixation small fragments was removed from the testis and immersed in a solution of glutaraldehyde 2,5% and paraformaldehyde 4 %, which is more appropriate for electron microscopy due to lower glutaraldehyde concentration. The testis fragments were fixed overnight and subsequently processed for both transmission electron microscopy and light microscopy. For histological analysis the testis fragments were embedded in hydroxyethyl methacrylate (Historesin ® Leica), sectioned at a thickness of 3 μm and toluidine blue stained. For ultrastructural analysis the testis fragments were embedded in epoxy resin and sectioned in ultra thin sections of 20-60 nm. Subsequently, this material was stained with 2% uranyl acetate (25 min.) and 2% lead citrate (7 min.) and documented in a transmission electron microscope (Zeiss, Leo 906).

Hormone Assay

Before removal of the testis, the abdomen and thoracic cavities were opened and a blood sample was collected from the vena cava with a heparinized syringe. Plasma was obtained after centrifuging the samples at 3000 rpm for 10 min. and subsequently frozen at -80° C until analyzed. Testosterone in plasma of rats was measured by enzyme-immunoassay.

Morphometrical analyses

Representative fields of testicular tissue were photographed with an Olympus Bx-40 microscope and than submitted to morphometrical analyses in image system Pro-Plus software. A grid mask system was used to measure volumetric proportions (%) of seminiferous tubules and intertubular space components. To measure the proportions between the seminiferous tubule and intertubular space, a grid mask with 475 points was used, superposed over 15 images, viewed with a 40x objective lens, giving a total of 7.125 points counted per animal. To measure the proportions of intertubular space components, a grid mask of 2028 points was used, superposed over 30 images, viewed with a 100x objective lens, measuring a total of 60.840 points counted per animal. In the intertubular space, the proportions of blood vessels, Leydig cells nuclei and inflammatory cells nuclei were measured. The percentage of these elements was obtained multiplying by 100x the number of points counted for each element and, subsequently, dividing this product by the number of points of the respective grid mask. The volume, expressed in ml, was obtained multiplying by 100x the volumetric proportions (%) of each testicular component and this product was divided by the total volume of the testis, which was considered identical to testis weight (Mori and Christensen, 1980). To obtain a more precise liquid testis volume, 6,5% relative to the tunica albuguinea was excluded from this organ weight (Russel and França, 1995).

Leydig Cell Morphometry

Representative areas of testicular tissue were photographed with an Olympus Bx-40 microscope and than submitted to morphometrical analyses in an Image Pro-Plus Program version 4.5 (Media Cybernetics). The proportion between nucleus and cytoplasm was assessed using a grid mask with 475 points superposed over each image viewed with a 100x objective lens. One thousand points on Leydig cells per animal were counted. The nuclear diameter of Leydig cells was obtained assessing 10 nuclei/animal. The nuclear volume was calculated using the $4/3\pi r^3$ formula, where ´´r`` was the mean nucleus radio obtained from half the diameter. The individual volume of Leydig cells was obtained from the nucleus volume and the proportion between nucleus and cytoplasm. The number of Leydig cells per testis was obtained dividing the total nuclear volume of these cells by the individual nuclear volume of each cell.

Statistical analysis

All data was presented as the mean ± (standard deviation) and analyzed via Anova (Tukey test) by using the system for statistical analyses (SAEG 9.0). The significance level was $p<0.01$ or $p<0.05$.

Results

Histological and Ultrastructural analyses

The testis sections of animals treated with cadmium only (4 of 9) and with guaraná plus cadmium (2 of 9) showed massive cell death in the seminiferous epithelium as well as among Leydig cells. Analyses with light microscopy showed a significant increase in the intertubular space, followed by evident edema and large proportion of dead cells (fig. 3). An increased fibrosis was also observed diffused in the intertubular space (Fig. 3C). The blood vessels were constricted and/or occluded by blood cells (Fig. 3A,C,D). Some seminiferous tubules, although surrounded by the basal membrane, were almost completely

empty of recognizable cells (Fig. 3B). There are also some foci of apoptotic germ cells, characterized by condensed chromatin and nucleus fragmentation (Fig. 3E,F). Analysis with transmission electron microscopy (fig. 5) showed that the seminiferous epithelium was largely substituted by cellular debris and a high proportion of lipids droplets (Fig. 5B,C). Almost no intact germ cell was observed. However, some Sertoli cells nuclei were observed in the basal compartment of the seminiferous tubule (Fig. 5A). Also, ultrastructural analysis showed the blood vessel was completely occluded by agglutinated erythrocytes, with very little plasma detected between the densely packed cells (Fig. 5D). Infiltrated erythrocytes were also present in the intertubular space, indicating a hemorrhagic process (Fig. 5A). The damages due to cadmium exposition were also observed in the Leydig cells, which presented a large increase in the cytoplasmic vacuolation (Fig. 5A).Since these animals presented a strongly sensitive response to cadmium, resulting in testis destruction, they were excluded from the morphometrical analysis.

The remaining animals of cadmium only treated group (5 of 9) (fig, 2C,D) and guaraná plus cadmium treated group (7 of 9) (fig. 2A,B) presented a less drastic response to cadmium exposure, with discernible testicular tissue structures in light microscopy. Histological analysis of these animals showed tenuous alterations in the seminiferous epithelium relative to control animals. However, there were evident alterations of the spacing between germ cells. Analysis in transmission electron microscopy confirmed this result, showing increased intercellular spaces between germ cells (fig. 4C) and a large proportion of lipid droplets (Fig. 4A). The ultrastructural analysis also showed some foci of shrunken cells, which probably represent apoptosis (Fig. 4D). Furthermore, cytoplasmic fragmentation was observed in some Sertoli cells as well as germ cells, which are probably in early stages of necrosis (Fig. 4B).

Histological analyses of the animals treated with *P. cupana* only (Fig 1C,D) presented morphological features very similar to control animals (fig. 1A,B). However, the increase in the volumetric proportions of the seminiferous tubule was evident, followed by a reduction in the intertubular space. Also, these animals presented a visible increase in the blood vessel lumen. These observations were confirmed by the morphometrical analyses, as described below.

The results of transmission electron microscopy are not conclusive to permit the comparison of the cadmium only treated group with the *P. cupana* plus cadmium treated group, since the response to cadmium was very heterogeneous, even within some testis samples. On other hand, histological analysis demonstrated evident morphological differences between these groups, as described below in the morphometrical analyses.

Body and testis weight

The results of body and testis weight statistical analyses can be found in table 1. There is no significant difference in the body and testis weight between the control and the treated groups. However, the animals treated with *P. cupana* only presented a moderate reduction in the body weight; even so this result is not corroborated by statistical analyses.

Morphometrical analyses

The morphometry statistical analyses of volume (ml) and volumetric proportion (%) can be found in Tables 2 and 3 respectively. There is no significant alteration in the measures of the seminiferous tubule and intertubular space between control animals and both groups exposed to cadmium. On other hand, the *P. cupana* only treated animals presented a significant increase in the volumetric proportion (%) of the seminiferous tubule, followed by a significant decrease in the volumetric proportions of the intertubular space compared with the control animals.

The cadmium only and guaraná plus cadmium treated animals presented a moderate increase in the volume and volumetric proportions of Leydig cells nuclei. However, this result appears to be due to the increase in the individual nucleus volume presented by these animals exposed to cadmium, as described below in the Leydig cell morphometry.

The cadmium only treated animals presented a significant increase in the volume and volumetric proportion of inflammatory cells relative to control animals. The *P. cupana* plus cadmium and the *P. cupana* only treated animals presented no significant alteration in the proportions of inflammatory cells.

A moderate reduction in the volumetric proportions and volume (ml) of blood vessel lumen was evidenced in the cadmium only treated animals; however this result is not statistically significant. On other hand, the guaraná plus cadmium treated animals presented a significant increase in the volumetric proportions and volume (ml) of blood vessel lumen. The *P. cupana* only treated animals presented a moderate increase in the volumetric proportion and volume (ml) of blood vessel lumen, even so this result is not statistically significant.

Leydig cell morphometry and plasma testosterone levels

The statistical analysis of plasma testosterone levels and individual Leydig cell morphometry can be found in Tables 1 and 4, respectively. The cadmium only treated group presented evident alterations in the Leydig cell morphology relative to control animals. A significant increase was verified in the nuclear diameter as well as in the nuclear volume. The cytoplasmic volume is also significantly larger than the results found in the control animals. Therefore, the total volume of Leydig cell is significantly larger than control. These animals also presented a moderate decrease in the number of Leydig cell per testis; however this result is not statistically confirmed. The plasma testosterone level is not significantly altered in these animals relative to the control.

The *P. cupana* plus cadmium treated animals also presented a moderate increase in the nuclear diameter and nuclear volume. However, this alteration is not statistically significant relative to the control animals. The cytoplasmic volume as well as the total volume of Leydig cell also did not presented significant alterations relative to control animals. Besides, the number of Leydig cell per testis is similar to the results found to control. However, the plasma testosterone level is lower in these animals when compared to control animals.

The *P. cupana* only treated animals presented Leydig cell morphological proportions that are very similar to the results found for control animals. Besides, the number of Leydig cell per testis is practically the same counted for the control animals. Also, these animals presented a significant increase in the plasma testosterone levels relative to control.

Discussion

Several morphological changes in testis due to cadmium have been described for mammals (Aoki and Hoffer, 1978; Hew et al., 1993, Koyuturk et al., 2006), since it is well known that this organ is very sensitive to acute cadmium contamination. Hew et al. (1993) demonstrated that rats injected with cadmium chloride in a single dose of 1mg/kg body wt. did not show any significant change in the seminiferous tubules as well as blood vessels under the light microscope 48 hours after metal exposition. Slightly higher doses, 1.3 mg/kg of body weight resulted in hemorrhagic testicular necrosis with subsequent degeneration (Gunn et al., 1968).

The cadmium dose used in the present study is between the doses reported above, since it was expected that the animals would present evident but not dramatic morphological changes. While the testis from most animals exposed to cadmium showed discernible testicular tissue structures, testis sections from some cadmium-treated rats (4 of 9) and a few of the guaraná plus cadmium treated group (2 of 9) showed massive cell death in the seminiferous epithelium as well as among Leydig cells and blood vessels. This heterogeneous response to cadmium exposure is in agreement with previous study (Hew, et al., 1993), and suggests that some animals are more sensitive to cadmium. Studies of cadmium-induced testicular damages have shown that strain and species differences in sensitivity may be related to variations in the ability of the cells to accumulate cadmium (King et al., 1999; Dalton et al., 2005).

Previous studies demonstrated that the vascular endothelium is an important target of cadmium toxicity, which causes specific changes in the ultrastructure of the adhering junctional complexes that mediate adhesion between the capillary endothelial cells (Niewenhuis, 1997), resulting in increased vascular permeability (Sacerdote and Cavicchia, 1983). The mechanisms underlying this effect are not well known, but some reports suggest that this alteration in endothelium permeability involves the ability of cadmium in disrupt cadherin-dependent cell-cell junctions (Prozialeck, 2000), resulting in leakage of fluid and blood cells from the capillaries into the interstitial space (Aoki and Hoffer, 1978).

Our study clearly showed that the most sensitive cadmium only treated animals (4 of 9) presented a significant increase in the intertubular space, followed by evident edema

and infiltration of blood cells into the intertubular space. Electron microscopy analyses of these animals showed blood vessels completely occluded by agglutinated erythrocytes, with very little plasma detected between the densely packed cells. Also, infiltrated erythrocytes were present in the intertubular space, indicating hemorrhagic process. This elevation in blood viscosity as well as hemorrhagic infiltration could be explained due to increased vascular permeability, resulting in loss of plasma and hence, in concentration of erythrocytes (Aoki and Hoffer, 1978).

The remaining cadmium only treated animals (5 of 9), which could be evaluated by morphometric measurements, showed no significant increase in the intertubular space relative to the control group, even so, moderate increase of edema was observed. These animals presented evident reduction of blood vessel lumens relative to control animals. Although this vascular alteration is not confirmed by statistical analyses, our observations with light microscopy as well as the analysis of volumetric proportions are pertinent for this affirmation. This vasoconstriction due to cadmium toxicity was also described in preceding literature (Skoczynska and Martynowicz, 2005; Tzotzes et al., 2007), and could be explained, at least in part, by the observations that this cation induces intracellular oxidative stress, resulting in decreased nitric oxide synthesis (Martynowicz et al., 2004; Skoczynska and Martynowicz, 2005) and consequent reduction in vasodilatation. Cadmium also tends to accumulate in the smooth muscle cells of blood vessels (Kaji et al. 1996), affecting the contraction response of these cells. Furthermore, the increased vascular permeability leads to infiltration of fluids from the blood vessels into the intertubular space, resulting in agglutination of erythrocytes, as demonstrated by our ultrastructural analysis. Therefore, it is possible that the decreased blood plasma in the affected vessels, associated with others vascular damages described above, have resulted in the reduction of blood vessel lumens.

One of the mechanisms underlying cadmium induced cell toxicity is the increased free radical production, resulting in oxidative damages of DNA, lipids and proteins (El-Demerdash et al., 2004; Ilkes et al., 2004). The term free radical is used to designate the reactive oxygen species (ROS), which once formed, can then initiate the process of oxidative stress. Cells and tissues normally possess antioxidant defense mechanisms to ensure the removal of reactive oxygen species, such as endogenous control (e.g,. superoxide dismutase) and that provided by dietary sources (e.g., vitamin C and E)

(Haslam, 1996). However, with cadmium exposure the balance between oxidant/antioxidant status is disturbed, not only by free radical increase, but also because the cellular antioxidant defense is suppressed (Hussain et al., 1987; Manca, 1991).

Several reports describe the protective effects of antioxidants against cadmium induced damage to testis, such as vitamin E and carotenoids, which play a role as free radical scavengers (El-Demerdash et al., 2004; Yang et al., 2006; Koyuturk et al., 2006). Ground *Paullinia cupana* seeds posses a high concentration of polyphenols such as tannins and flavonoids (Brekhman, 1980; Carlson and Thompson, 1998), substances that have proven antioxidant action (Galato et al., 2001). It has been documented that red wine polyphenols and *Paullinia pinnata* polyphenols induce endothelium-dependent vasodilatation, not only by increasing nitric oxide (NO) activity but also by protecting NO degradation through their antioxidant properties (Zamble et al. 2006; Zenebe et al. 2006).

The animals that received only *P.cupana* during 56 days presented a moderate increase in blood vessel lumens relative to control animals. Although this morphological change was not statistically significant, histological evaluations showed clearly altered blood vessels. The possibility of guaraná having stimulated the processes of new blood vessel growth was suggested. However, it has been demonstrated that several polyphenols extracted from various plants have been found to be potent inhibitors of angiogenesis (Cao et al. 2002), acting as suppressants of cell proliferation (Fotsis et al., 1997; Brakenhielm et al., 2001) and inhibiting endothelial cell migration (Fotsis et al., 1997). Thus, it is likely that this vascular alteration is due to the capacity of polyphenols to induce endothelium vasodilation and not angiogenesis.

The animals preventively treated with *P. cupana* during 56 days before cadmium exposition presented no reduction in blood vessel lumen, contrasting with the cadmium only group, which showed vasoconstriction. On other hand, these animals treated with *P.cupana* followed by cadmium showed evident vasodilation, presenting a blood vessel lumen significantly larger than control animals. As described above, vasodilation was also observed in the animals treated with *P. cupana* only, consequently suggesting that the administration of guaraná extract affected the contractile process of endothelial cells in both the *P. cupana* only and the *P. cupana* plus cadmium treated animals. This result is supported by previous studies, which reported the potential of polyphenols to prevent injury

induced by an oxidative process to human cultured endothelial cells (Vieira et al., 1998). Therefore, it is possible that *P.cupana* protected the endothelial cells against an oxidative stress, consequently providing suitable conditions for nitric oxide synthesis. Also, Barrouillet et al. (1999) demonstrated a pretreatment of cultured mesangial cells (a smooth muscle-like structure) with polyphenols resulted in a complete attenuation of the myocontracturant response caused by cadmium exposition. On other hand, the animals exposed to cadmium only presented a significant cellular contraction relative to control animals. In analogy with this study, we could suggest that the preventive treatment with *P. cupana* was efficient in counterbalancing the toxic effects of cadmium in the endothelial smooth muscles cells, consequently preventing the vasoconstriction in these animals, and probably preventing anoxia.

Typical cadmium related pathological changes were seen in the testis of both the cadmium only and the guaraná plus cadmium treated animals with transmission electron microscopy. In the seminiferous tubule it was evident the increased spacing between the germ cells, occasionally surrounding the deserted spaces which result from cell death. We also observed some foci of Sertoli cell fragmentation, probably due to a necrotic process. Sertoli cells possess extensive tight junctions that form a physiological barrier in the seminiferous tubule, providing intercellular contact between Sertoli cells and Sertoli cells and germ cells. Previous study demonstrated that the exposition to cadmium affects this permeability barrier (Fiorine et al., 2004). So, it is likely that the spacing between the seminiferous epithelium cells is due not only to Sertoli cells death, but also due to disruption of cell-cell junctions.

Results of both in vivo and in vitro studies have demonstrated that the letal effects of cadmium in cells involve two distinct pathways, necrosis (Yang et al., 2007) and apoptosis (Xu et al., 1996). Our evaluations are not conclusive about the mechanisms underlying cadmium-induced cell death, although we suggest that both necrosis and apoptosis occurred in the animals exposed to this cation.

Our analyses with electron and light microscopy showed the typical process of necrosis in the cadmium only and guaraná plus cadmium treated animals, in the form of plasma membrane breakdown and increased cytoplasmatic vacuolation (Majno and Joris, 1995). Furthermore, morphometrical analysis demonstrated significant increase in the

inflammatory response, probably due to the releasing of necrotic cell content into the extracellular space (Majno and Joris, 1995). On the other hand we also observed features typical of apoptosis in these animals, especially with light microscopy, such as condensed chromatin and fragmented nuclei of basal germ cells as well as Leydig cells (Hengatner, 2000).

Our morphometrical analysis showed that the Leydig cells of the cadmium only group presented a significant increase in their cytoplasmic and nuclear volume, resulting in a cellular volume larger than that observed for Leydig cells of control animals. The cellular swelling is one of the morphological changes that characterize necrosis, resulting in subsequent breaks in the plasma membrane. Since our histological and ultrastructural analyses showed typical necrotic processes in the testicular tissue, it is very likely that the abnormal increase in Leydig cell volume resulted from cadmium-induced necrosis. Furthermore, observations with the electron microscope showed an evident increase in cytoplasmic lipids droplets in the Leydig cell, probably due to a reduction in the androgen synthesis.

Our analyses are not conclusive in relation to the influence of this cation on the number of Leydig cell per testis, since the statistical analyses showed no significant difference between the animals treated only with cadmium in relation to control animals. However, our observations with light microscopy as well as the results of morphometrical mean suggest that a moderate decrease of these cells occurred in the animals exposed to cadmium. These results could explain, at least in part, the elevated number of inflammatory cells, which also suggests that the cell death content is released into the intertubular space.

On other hand, the animals preventively treated with *P. cupana* before cadmium exposure presented a Leydig cell volume larger than control animals, but very lower than the results found for cadmium only treated animals. Besides, the estimated number of Leydig cells per testis in these animals was very near normal, suggesting that *P.cupana* was efficient in reducing death of this cellular population. These results could be supported by the fact that these animals presented reduced inflammatory response, probably due to decreased cell content released into the intertubular space.

Further study showed that cadmium is directly toxic to primary cultured Leydig cells, resulting in decreased cell viability and reducing the testosterone secretion (Yang et

al., 2003). In spite of the morphological changes observed in the Leydig cells of cadmium only treated group, these animals presented no significant alteration in the testosterone concentration relative to control animals. This result is also compatible with preceding literature, which showed that a single subcutaneous injection of cadmium chloride (1 mg) did not alter significantly the testosterone levels in the male rats 7 days after metal exposition. However, the same study reported a significant reduction in testosterone levels 15 days after cadmium administration, suggesting that the influence of cadmium in testosterone synthesis is time-dependent (Saksena et al., 1977). In the present study the dose of cadmium used (approximately 0,415 mg per animal) is considerably lower than the dose utilized by the above cited author and the animals were sacrificed 48 hours later. Therefore, it is understandable that no alteration in testosterone levels were found in these animals.

On other hand, the *P. cupana* only group showed a significant rise in the testosterone concentration relative to control animals. Sönmez et al. (2005) demonstrated that the administration of ascorbic acid reduced the oxidative stress and increased the plasma testosterone levels in Wistar rats. Since *P. cupana* possess antioxidant properties, it is possible that this plant extract stimulated suitable conditions for testosterone synthesis. Furthermore, these animals presented a significant increase in the volumetric proportions of the seminiferous tubule, followed by a reduction in the volumetric proportions of the intertubular space. According Sinha-Hikim et al. (1989), the tubular diameter possesses a positive correlation to spermatogenic activity. As a result, we could suggest that the increased testosterone production provided by *P. cupana* administration stimulated the spermatogenic process and hence, enlarged the volumetric proportions of seminiferous tubule.

The animals preventively treated with *P. cupana* before cadmium exposition presented an evident reduction in the testosterone concentration relative to control animals. The significance of this result is not clear, since the cadmium only treated group presented normal testosterone levels, and the *P. cupana* only treated animals showed a high concentration of testosterone. Besides, the morphometrical analyses of these animals demonstrated that the Leydig cells presented morphological features similar to control

animals, contrasting with the results found for the cadmium only group. Additional research should be undertaken to resolve the inconsistency for this group.

The present work showed that the cadmium only and the *P. cupana* plus cadmium treated animals presented evident histopathological alteration in the testis tissue. These results are in agreement with a vast preceding literature. However, the animals preventively treated with *P. cupana* before cadmium exposition presented some testicular components with characteristics similar to the results found to control animals. Therefore, it is possible that a chemopreventive effect could be attributed to the antioxidant properties of polyphenols found in this extract. On other hand, some results observed in the animals preventively treated with *P. cupana* before cadmium exposition are not conclusive, and would be necessary additional research to investigate these alterations. Furthermore, the present study also showed that the *P. cupana* only treated animals presented a significant increase in the plasma testosterone level, followed by a significant increase in the volumetric proportions of seminiferous tubule. We have not found preceding literature reporting this property for guaraná.

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Tables

Table 1: Biometric values and plasma testosterone levels in adult male Wistar rats treated with *P. cupana* and/or cadmium.

Parameters	Group I	Group II	Group III	Group IV
Initial body weight	362.600 ± 48.22	360.000 ± 21.66	379.000 ± 18.54	385.40 ± 19.74
Final body weight	452.800 ± 75.21	472.600 ± 30.14	444.833 ± 33.90	405.40 ± 26.90
Testis weight	1.782 ± 0.22	1.756 ± 0.07	1.838 ± 0.25	1.73 ± 0.15
Testosterone(ng/ml)	1.266 ± 1.23^{b}	1.307 ± 0.89 ^{b}	0.530 ± 0.35 ^{b}	5.044 ± 4.26 ^{a}

Group I = Control (n = 5); Group II = Cadmium only (n = 5); Group III = *P. cupana* plus Cadmium (n = 7); Group IV: *P. cupana* only (n = 5)
$^{a-b}$In each row, values with different superscripts are significantly different ($p < 0.05$).

Table 2: Volume (mL) of the testis components in adult Wistar rats treated with *Paullinia cupana* and/or exposed to cadmium.

Parameters	Group I	Group II	Group III	Group IV
Seminiferous Tubule	1.414 ± 0.21	1.398 ± 0.10	1.465 ± 0.17	1.502 ± 0.16
Intertubular Space	0.330 ± 0.04^{ab}	0.351 ± 0.05^{a*}	0.367 ± 0.09^{a}	0.228 ± 0.02^{b*}
Inflammatory Cells	0.001 ± 0.00^{b}	0.009 ± 0.00^{a}	0.003 ± 0.00^{b}	0.002 ± 0.00^{b}
Blood Vessel Lumen	0.015 ± 0.00^{b*}	0.010 ± 0.00^{b}	0.033 ± 0.01^{a*}	0.021 ± 0.00^{ab}
Leydig Cell Nucleus	0.030 ± 0.00	0.039 ± 0.00	0.041 ± 0.00	0.032 ± 0.00

Group I = Control (n = 5); Group II = Cadmium only (n = 5); Group III = *P. cupana* plus Cadmium (n = 7); Group IV: *P. cupana* only (n = 5)
$^{a-b}$In each row, values with different superscripts are significantly different ($p < 0.01$) and *($p < 0.05$).

Table 3: Volumetric Proportions (%) of the components of the intertubular space in adult Wistar rats treated with *Paullinia cupana* and/or exposed to cadmium.

Parameters	Group I	Group II	Group III	Group IV
Seminiferous Tubule	80.92 ± 0.07^{b*}	79.76 ± 3.26^{b}	80.12 ± 3.10^{b}	86.46 ± 2.07^{a*}
Intertubular Space	19.07 ± 0.07^{b*}	20.23 ± 3.26^{b}	19.86 ± 3.10^{b}	13.52 ± 2.07^{a*}
Inflammatory Cells	0.09 ± 0.02^{b}	0.55 ± 0.27^{a}	0.20 ± 0.08^{b}	0.15 ± 0.08^{b}
Blood Vessel Lumen	0.94 ± 0.20^{b*}	0.59 ± 0.27^{b}	1.76 ± 0.59^{a*}	1.24 ± 0.22^{ab}
Leydig Cell Nucleus	1.75 ± 0.30	2.27 ± 0.45	2.28 ± 0.47	1.87 ± 0.47

Group I = Control (n = 5); Group II = Cadmium only (n = 5); Group III = *P. cupana* plus Cadmium (n = 7); Group IV: *P. cupana* only (n = 5)
$^{a-b}$In each row, values with different superscripts are significantly different ($p < 0.01$) and *($p < 0.05$).

Table 4: Leydig Cell morphometry in adult Wistar rats treated with *Paullinia cupana* and/or exposed to cadmium.

Parameters	Group I	Group II	Group III	Group IV
Nucleus Diameter LC (µm)	5.72 ± 0.26^{b}	$6.90 \pm 0.34^{a*}$	6.25 ± 0.71^{ab}	$5.88 \pm 0.10^{b*}$
Volume of a LC (µm³)	659.41 ± 82.02^{b}	$1375.79 \pm 215,45^{a}$	806.92 ± 324.70^{b}	784.69 ± 97.63^{b}
Nuclear Volume (µm³)	97.57 ± 12.12^{b}	$172.98 \pm 25.53^{a*}$	131.97 ± 46.96^{ab}	$106.31 \pm 5.86^{b*}$
Cytoplasmic Volume (µm³)	561.84 ± 71.87^{b}	1202.88 ± 191.17^{a}	674.94 ± 279.97^{b}	678.38 ± 94.82^{b}
Number LC/testis (10^6)	304.80 ± 62.05	228.20 ± 42.74	328.00 ± 74.81	303.20 ± 77.89
Number LC/g testis (10^6)	178.16 ± 13.92	130.82 ± 28.53	186.25 ± 32.36	175.79 ± 52.35

Group I = Control (n = 5); Group II = Cadmium only (n = 5); Group III = *P. cupana* plus Cadmium (n = 7); Group IV: *P. cupana* only (n = 5)

[a-b]In each row, values with different superscripts are significantly different ($p < 0.01$) and *($p < 0.05$).

Figure Legends

Figure 1 - Light microscopy of the testicular tissue of Wistar rats: A and B – Representative areas of control animals showing a well organized seminiferous epithelium with normal blood vessels and clusters of Leydig cells; C and D – Representatives areas of *P. cupana* only treated animals with a morphology similar to the control, but with dilated blood vessels. ST = seminiferous tubule; Ls = Lymphatic space; Lc = Leydig cell; V = blood vessels; (arrow) Sertoli cell. Toluidine blue staining.

Figure 2 - Light microscopy of the testicular tissue of Wistar rats: A and B – Images from *P. cupana* plus cadmium treated animals showing very dilated blood vessels, seminiferous epithelium and Leydig cells morphologically similar to control. C and D – Images from cadmium only treated animals showing a constricted blood vessel and increased proportion of macrophages. The Leydig cell nuclei appear to be turgid and the cytoplasm is weakly stained with toluidine blue. ST = seminiferous tubule; Ls = Lymphatic space; Lc = Leydig cell; V = blood vessels; M = macrophage; (arrow) Sertoli cell. Toluidine blue staining.

Figure 3 - Light microscopy of the testicular tissue of Wistar rats: Representative areas of the most sensitive animals from the cadmium only(4 of 9) (fig.A,C,E) and the *P. cupana* plus cadmium (2 of 9) (fig.B,D,F) groups. A and B - General view of the testicular tissue showing extensive damages in the seminiferous epithelium; C - Increased fibrosis in the intertubular space; D – Occluded blood vessel; E – Intertubular space with Leydig cells apparently undergoing apoptosis; F – Germ cells appear to be apoptotic. ST = seminiferous tubule; Ls = Lymphatic space; Lc = Leydig cell; V = blood vessels; M = macrophage; (arrow) Apoptosis; F = Fibrosis; Bm = basal membrane. Toluidine blue staining.

Figure 4 – Electron micrographs of the less sensitive animals from the cadmium only (5 of 9) (fig.A,C) and *P. cupana* plus cadmium animals (7 of 9) (fig.B,D). A – General view of the seminiferous epithelium showing Sertoli cells (Se), spermatogonia (Sg), abnormal spermatid (St), a few vacuoles probably due to cell death (Va) and large lipids droplets (L); B – Seminiferous epithelium showing fragmentation (arrow) of Sertoli cell (Se) and

spermatogonia (Sg) plasmic membrane, abnormal disposition of spermatids (St) near to the basal compartment and small lipid droplets (L); C – Seminiferous epithelium showing an evident separation between spermatocytes (Sp); D - Seminiferous epithelium showing a shrunken cell (*), probably an apoptotic body and basal membrane (Bm) with abnormal concentration of lipids droplets (L).

Figure 5 – Electron microscopy micrographs of the most sensitive animals from cadmium only (4 of 9) (fig.A,B) and *P. cupana* plus cadmium animals (2 of 9) (fig.C,D)). A - General view of the seminiferous epithelium showing a large proportion of vacuoles probably due to cell death (Va), abnormal lipids droplets (L), and intact Sertoli cell nucleus (Se). Leydig cell (Lc) with extensive cytoplasmic vacuolation (Va) can be observed. B – Seminiferous epithelium showing a large proportion of cellular debris (seta) diffused in the tubular compartment between germ cells (Gc) and abnormal spermatids (St). C – Micrograph of the adluminal compartment of the seminiferous epithelium showing large lipids droplets (L), abnormal spermatids (St) and some spermatozoa (Sz). D – Micrograph of the intertubular space (Is) showing an occluded blood vessel, with very little open space normally occupied by plasma, between erythrocytes (V).

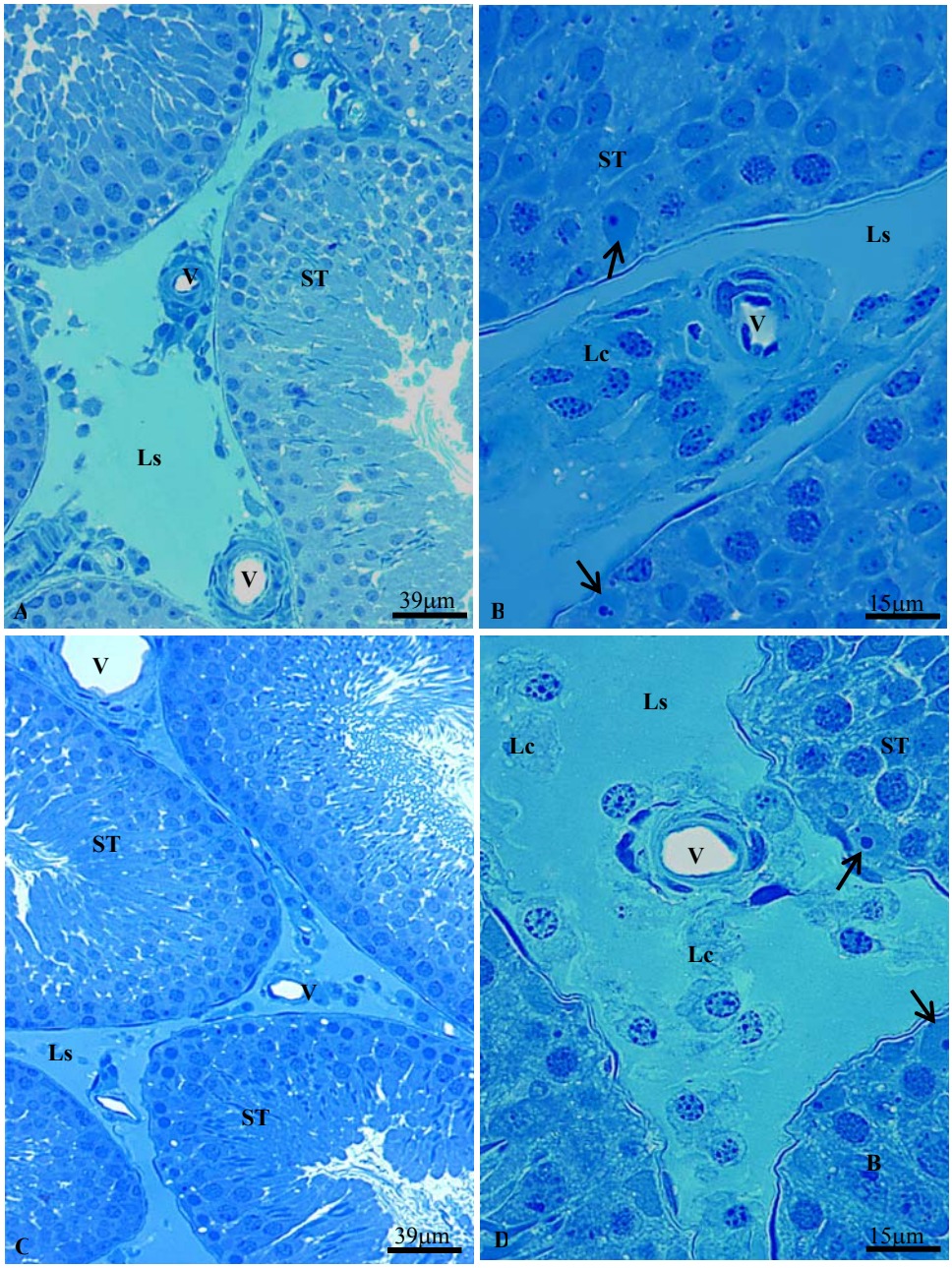
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Figure 1

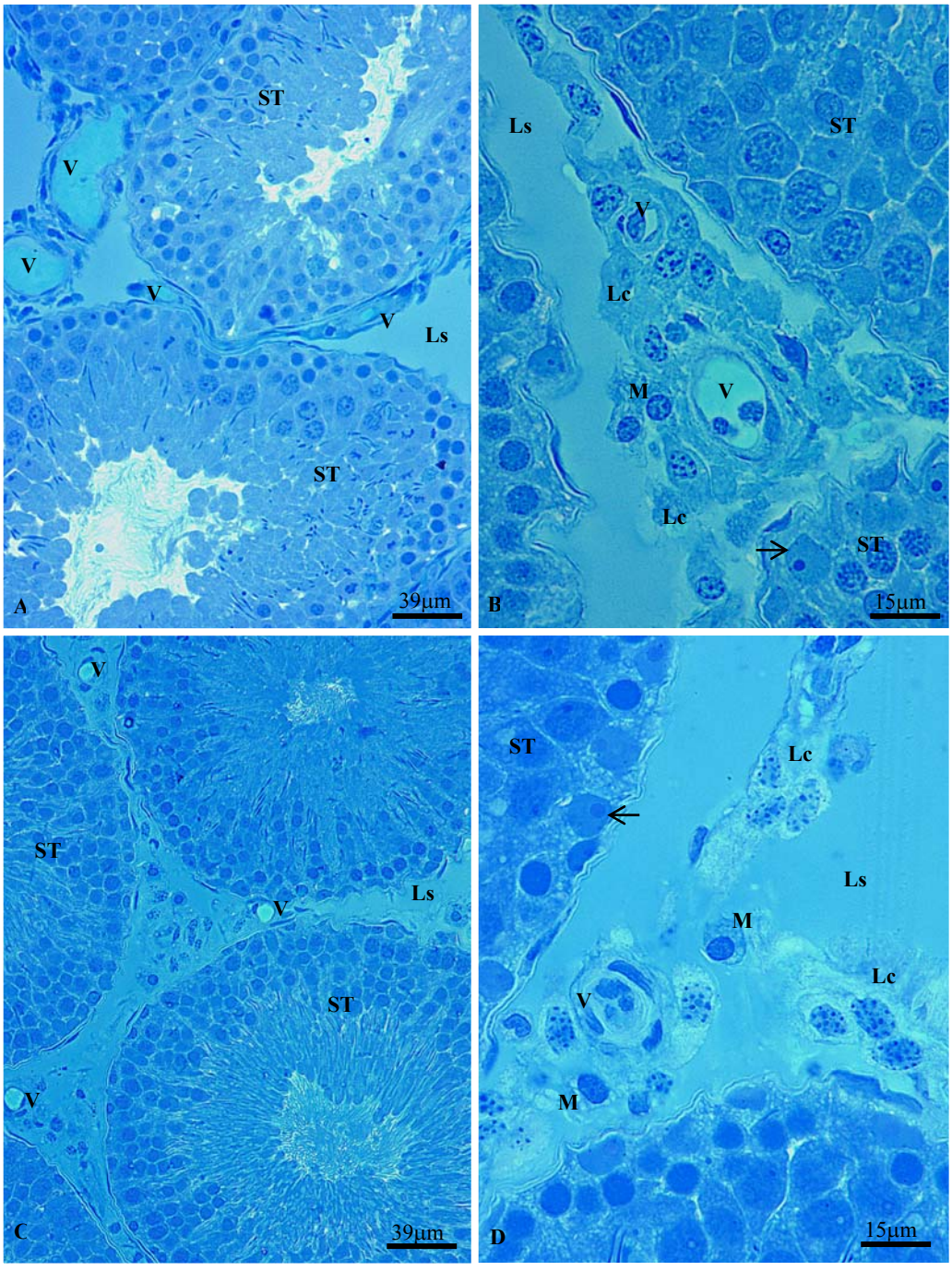
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Figure 2

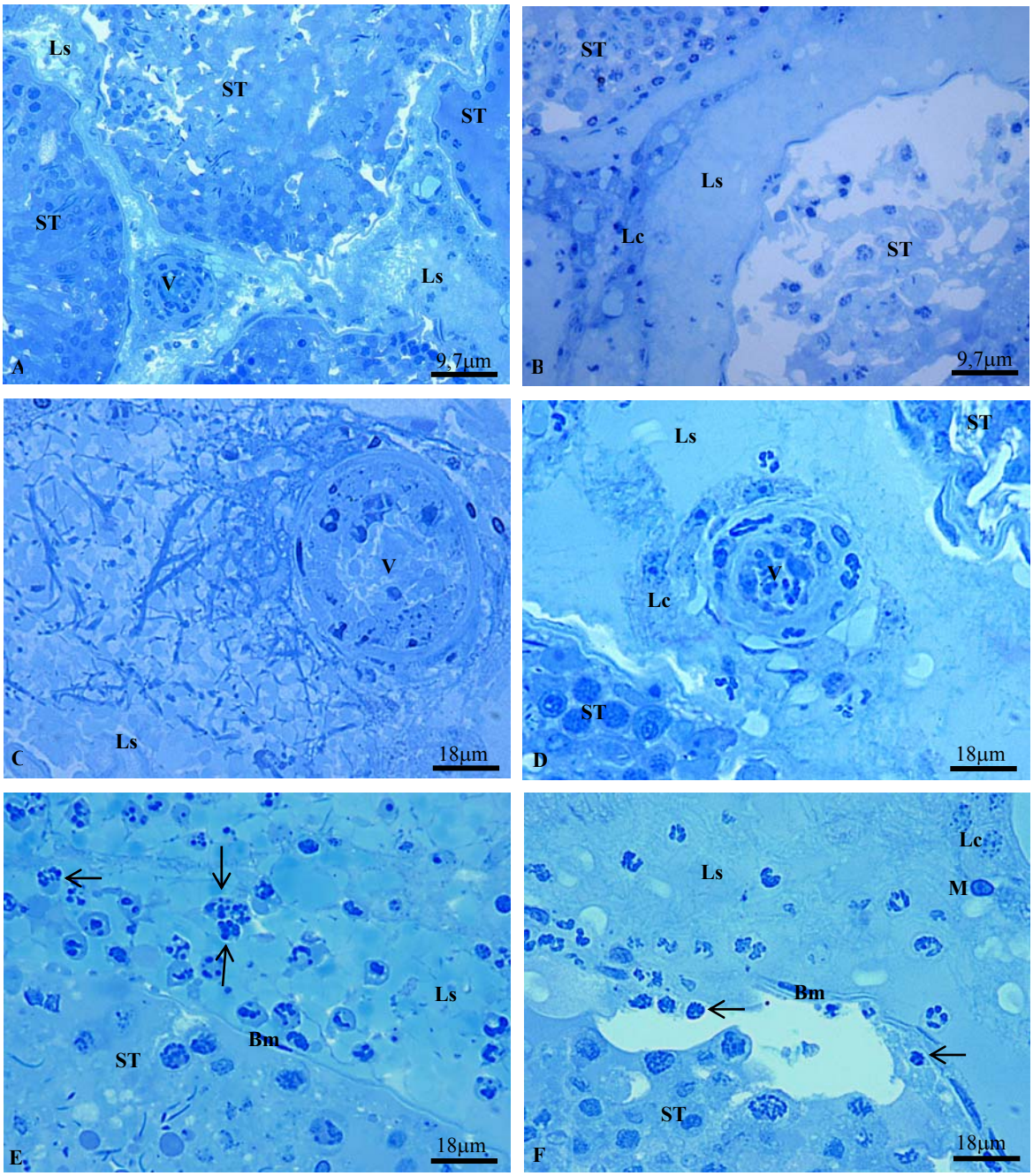
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Figure 3

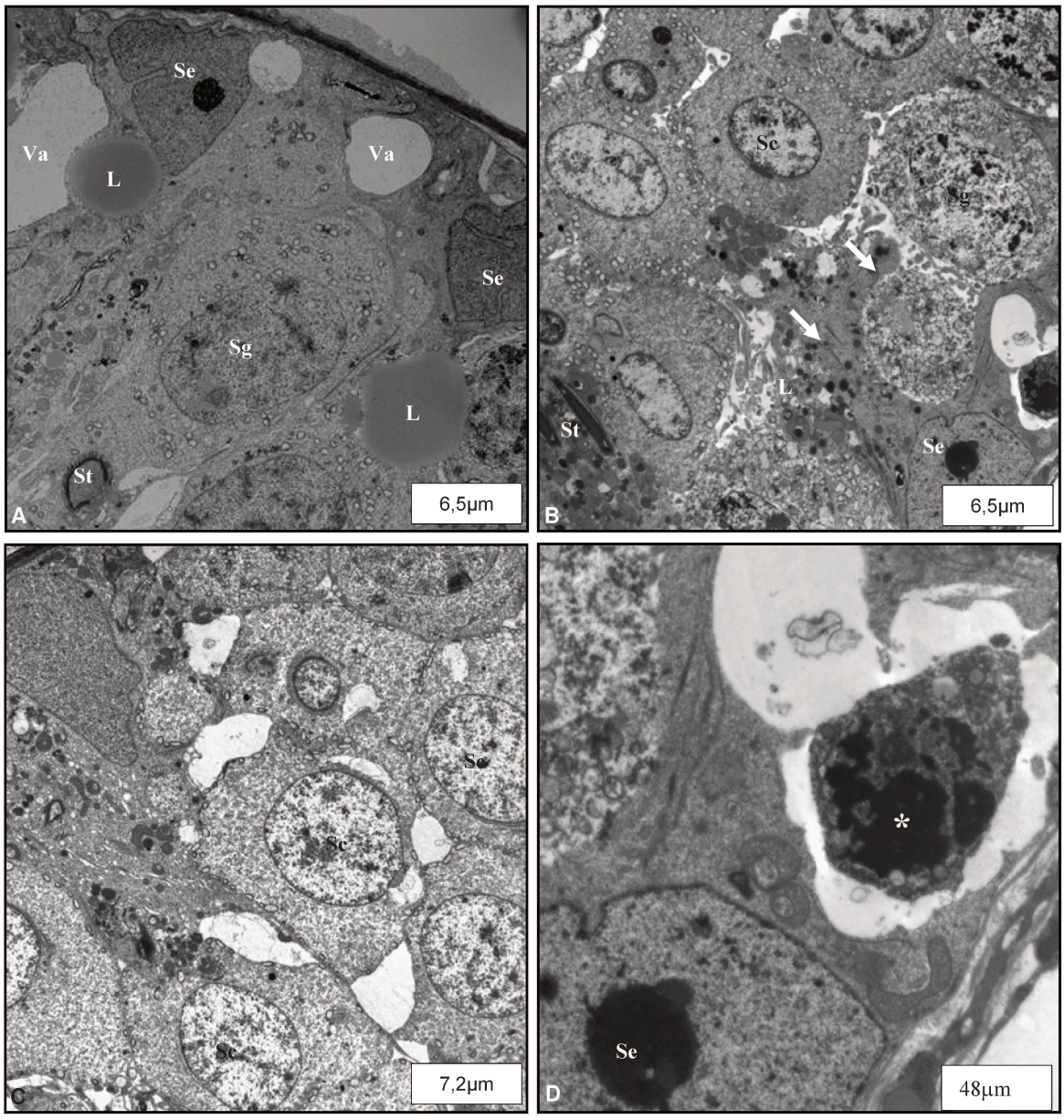
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Figure 4

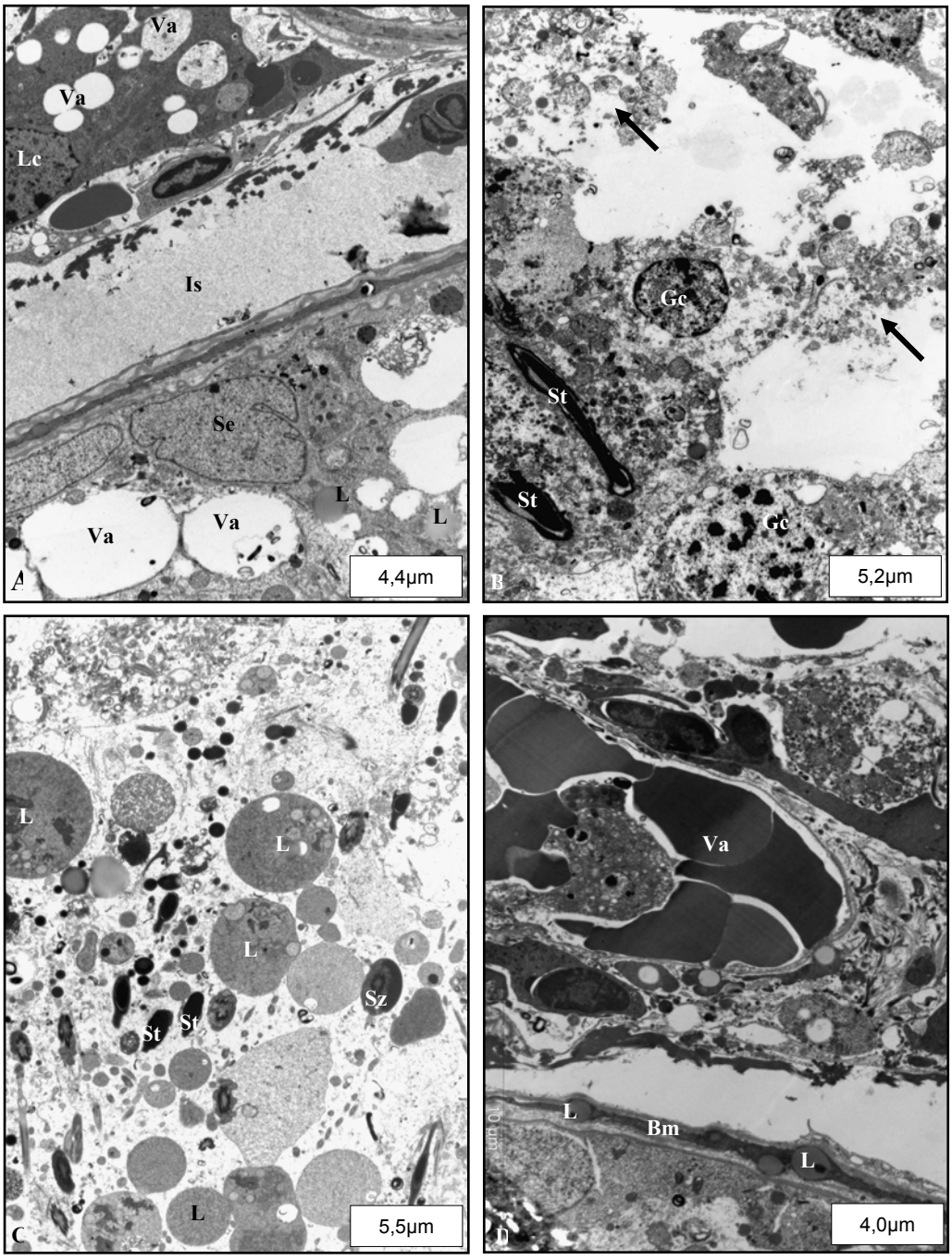
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Figure 5

Advantage of Guaraná (*Paullinia cupana* Mart.) Supplementation relative to Cadmium-induced Damages in Testis of Adult Wistar rats.

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Abstract

Cadmium is known to be toxic for several organs, including the testis. One of the mechanisms underlying cadmium-induced cellular damage is free radical mediated, resulting in cellular oxidative damages. The aim of this study was to evaluate if *Paullinia cupana*, a Brazilian plant with antioxidant properties, popularly called guaraná, could protect the Wistar rat testis against cadmium-induced damages. Adult male Wistar rats with 110 days of age received an intraperitonial injection of cadmium chloride in a dose of 1,15 mg/kg body weight and were subsequently treated with *P. cupana* during 56 days. Furthermore, groups receiving only *P. cupana* or only cadmium were also tested. After the treatment period, the testis samples were submitted to histological and morphometrical analyses. Drastic effects of cadmium were observed in the both cadmium only and cadmium plus *P. cupana* treated groups after 56 days of exposition to this cation. However, the animals treated with guaraná after cadmium exposition presented a significant reduction in the seminiferous epithelium damages. Also, this study showed that no significant alterations were observed in the Leydig cell morphology in the both groups exposed to cadmium, indicating that there is a high potential for testis recovery of functional Leydig cells after cadmium damages. This result is corroborated by hormonal analyses, since these animals presented no significant alteration in the testosterone plasma levels in relation to control animals. It was also shown that, despite the extensive damages in the seminiferous epithelium, these animals presented a basal layer of Sertoli cells in the seminiferous tubule.

Introduction

Paullinia cupana var. sorbilis Mart. (Sapindaceae), commonly called guaraná, is a plant found in the Amazon Basin in Brazil (Henman, 1982). Guaraná has been used for centuries as a stimulant by the Saterê-Mauê Indians who live in the Amazon region (Henman, 1986), and is popularly used for a variety of therapeutic purposes, including as a tonic, to treat chronic diarrhea and hypertension. Furthermore, some therapeutic properties of guaraná have been scientifically assessed, such as a chemopreventive of mouse hepatocarcinogenesis (Fukumasu et al., 2005), protection against gastric lesions induced by ethanol (Campos et al., 2003) and weight loss (Boozer et al., 2001). Among its chemical constituents the plant contains xanthic bases such as caffeine, theophylline and theobromine; the last two have been found in guaraná bark, flowers and leaves, but are absent in seeds (Henman, 1986). The xanthic base of guaraná, however, does not explain all the therapeutic action attributed to this plant (Benowitz, 1990). Polyphenols such as tannins and flavonoids are also found in the guaraná seeds (Brekhman, 1980; Carlson and Thompson, 1998), substances that have proven antioxidant action (Galato et al., 2001). In vitro studies demonstrated that the antioxidant effects of *P. cupana* are related to the inhibition of spontaneous oxidative processes, probably due to a high concentration of tannins in guaraná seeds (Mattei et al. 1998). Therefore, it is possible that part of the revitalizing effects of guaraná may be due to the antioxidant properties presented by this plant.

Cadmium is a heavy metal of continuing environmental and occupational concern, characterized by a wide variety of adverse effects. This metal is widely used in industry, such as in plating, metal smelting and batteries (Friberg et al., 1986). In the environment, cadmium is dangerous because humans consume both plants and animals that absorb this cation efficiently and concentrate it within their tissues (Moore and Ramamoorthy, 1984). A vast preceding literature indicates that one of the mechanisms underlying cadmium-induced cellular damage is free radical mediated (Koizume and Li, 1992; Mikhailova et al., 1997; Sarkar et al., 1998). Free radical lead to oxidative deterioration of lipids, proteins and DNA, hence initiating various pathological conditions in men and animals (Manca, 1991;

Sarkar, 1997). Therefore, estimation of free radical generation and antioxidant defense has become an important aspect of investigations in mammals. One important feature in cadmium toxicology is the exceptional trend toward testis accumulation of this metal, probably due to the fact that this organ is in constant mitosis and meiosis (Yano and Dolder, 2002). Given the observations described above, in the present study we investigated the potential of *P. cupana* to attenuate testicular damages induced by cadmium.

Material and methods

Animals

Male Wistar rats (14 days of age) were obtained from the Multidisciplinary Center for Biological Investigation (State University of Campinas, SP, Brazil). Animals were housed two per cage under controlled conditions of temperature (24 ± 4o.C) with a 12h-dark/light cycle and provided with water and food *ad libitum*. After a maturation period, adult rats with 110 days of age were divided into 4 groups for the treatment. The number of rodents per group was 5 for the control group, 5 for the guaraná only group, 6 for the group that received cadmium only and 6 for the group treated with cadmium plus guaraná. This research project was approved by the institutional Committee for Ethics in Animal Research of this University (Protocol n^{o} 1202-1).

Paullinia cupana

Powdered guaraná seeds were obtained from EMBRAPA Amazônia Occidental (Brazilian Enterprise for Agricultural and Cattle Raising Research of Western Amazon) and was conserved in dry conditions at ± 4ºC during the treatment. Guaraná (2mg/g BW) was freshly diluted in 1ml of water and administered by gavage once a day. The dose of the guaraná powder was chosen based on previous study, which demonstrated better chemo-preventive effects for this dose (Fukumasu et al., 2005).

Treatment protocol

Twenty two adult male Wistar rats of 110 days of age were divided into four groups:
Group I – Control (5 animals)
Group II – Cadmium only (6 animals)
Group III – Cadmium plus *P. cupana* (6 animals)
Group IV – *P. cupana* only (5 animals)

The groups II and III received an intraperitonial injection of $CdCl_2$ (1,15 mg/kg bw) dissolved in water in the first day of the experiment. After this procedure, the cadmium only treated animals (group II) received water gavage once a day during 56 days and the cadmium plus *P. cupana* (group III) received the plant extract during the same period. The animals treated only with *P. cupana* (group IV) received the plant extract during 56 days and the animals of the control group received water gavage during the same period. These groups were sacrificed on the 57^{th} day after begin the treatment. The period of 56 days of treatment was chosen since this interval represents the duration of spermatogenesis in rats (Russel et al., 1990)

Fixation and Processing of the tissue

At the end of each experiment, animals were anesthetized with Xylazine and Ketamine (5 and 80 mg/kg BW, respectively). An incision was made in the scrotum and the left testicle was exposed and dissected free. The tunica albuguinea was gently fissured and the organ was immersed in solution of glutaraldehyde 4% and paraformaldehyde 4% in 0.1 M phosphate buffer at pH 7.2. The testis fragments were fixed overnight and subsequently processed for light microscopy using routine techniques. For histological analysis the testis fragments were embedded in hydroxyethyl methacrylate (Historesin R, Leica), sectioned at a thickness of 3 μm and toluidine blue stained.

Hormone Assay

Before removing the testis, the abdomen and thoracic cavity were opened and a blood sample was collected from the vena cava with an heparinized syringe. Plasma was obtained after centrifuging the samples at 3000 rpm for 10 min. and subsequently frozen at -80° C until analyzed. Testosterone in plasma of rats was measured by enzyme-immunoassay.

Morphometrical analyses

Representative areas of testicular tissue were photographed with an Olympus Bx-40 microscope, than submitted to morphometrical analyses in an image system Pro-Plus software. A grid mask system was used to measure the volumetric proportion (%) of seminiferous tubules, intertubular space and intertubular components. To measure the proportions between the seminiferous tubule and intertubular space, a grid mask with 475 points was used, superposed over 15 images viewed with a 40x objective lens, consisting in a total of 7.125 points counted per animal. To measure the proportions of intertubular space components, a grid mask of 2028 points was used superposed over 15 images viewed with a 100x objective lens, making a total of 30.420 points counted per animal. In the intertubular space, the proportions of blood vessels, Leydig cells nucleus and inflammatory cells nucleus were measured. The percentage of the elements measured was obtained by multiplying by 100x the number of points counted for each element and, subsequently, dividing this product by the number of points of the respective grid mask. The volume, expressed in ml, was obtained multiplying by 100x the volumetric proportions (%) of each testicular component with this product being divided by the total volume of the testis, which was considered identical to the testis weight (Mori and Christensen, 1980). To obtain a more precise liquid testis volume, 6,5 % relative to the tunica albuguinea was excluded from this organ's weight (Russel and França, 1995).

Leydig cell morphometry

Representative areas of testicular tissue were photographed with an Olympus Bx-40 microscope and than submitted to morphometrical analysis in an image system Pro-Plus. The proportion between nucleus and cytoplasm was assessed using a grid mask with 475 points superposed over each image viewed with a 100x objective lens. One thousand points were counted over the Leydig cells per animal. The nuclear diameter of Leydig cells was obtained assessing 10 nuclei/animal. The nuclear volume was calculated using the $4/3\pi r^3$ formula, where ´´r`` indicates the mean nucleus radius obtained from half the diameter. The individual volume of the Leydig cell was obtained from the nucleus volume and the proportion between nucleus and cytoplasm. The number of Leydig cells per testis was obtained from the individual nucleus volume and the total nuclear volume of these cells in the intertubular space.

Statistical analyses

All data was presented as the mean ± (standard deviation) and analyzed via Anova (Tukey test) by using the system for statistical analyses (SAEG 9.0). The significance level was $p<0.01$ or $p<0.05$.

Results

Body and testis weight

The results of body and testis weight statistical analysis can be found in the Table 1. The animals treated with cadmium only and cadmium plus guaraná presented a significant reduction in the testis weight relative to control animals. However, there was no difference in the testis weight between these two groups exposed to cadmium. The animals treated only with *P. cupana* showed a testis weight very similar to the results found for control animals. Despite the drastic reduction in the testis weight observed in all animals exposed to cadmium, there was no significant alteration in their body weight compared to the

control group. On other hand, the animals treated with *P. cupana* only presented a moderate reduction in body weight; even so, this result is not confirmed by statistical analysis.

Histological and Morphometrical evaluations

The morphometric statistical analyses of volume (ml) and volumetric proportion (%) can be found in the Tables 2 and 3, respectively. Morphometrical analyses of the cadmium only and cadmium plus guaraná treated animals showed an evident alteration in the testicular tissue relative to control animals. The cadmium only treated animals (Fig. 2) presented a significant reduction in the volumetric proportions and volume of seminiferous tubules. This alteration was followed by a significant increase in the intertubular space. Furthermore, histological analysis demonstrated that, although the tubular basement membranes of seminiferous tubules were identified with an irregular morphology, all the animals presented massive degeneration of germ cells. On other hand, the cadmium plus guaraná treated animals (Fig. 3) presented no significant alteration in the volumetric proportions of the seminiferous tubule and intertubular space. The testis sections of some of these animals (3 out of 6) showed a relatively well-preserved seminiferous epithelium, with distinctive somatic and germ cell populations. The remaining animals (3 out of 6) presented extensive cell death in the tubular compartment, with no discernible differences in the seminiferous epithelium relative to the cadmium only treated animals. However, despite the extensive damages observed in the germ cells, these animals presented a large number of seminiferous tubules with a circular shape and no identifiable material content. The animals treated with cadmium only and the most damaged animals of the cadmium plus guaraná group (3 out of 6) presented the basal membrane of the seminiferous tubule having a thin basal lining of Sertoli cells. On other hand, animals treated only with *P. cupana* (Fig.1) presented an evident increase in the volumetric proportions of seminiferous tubule followed by a reduction in the volumetric proportions of the intertubular space; even so, this result is not corroborated by statistical analyses. Histological evaluations of these animals showed a well-preserved seminiferous epithelium and intertubular space components, with characteristics similar to the control animals.

Morphometrical evaluation showed a significant increase in the volumetric proportions of inflammatory cells and Leydig cell nuclei in both groups exposed to cadmium. However, no alteration was encountered in the total volume (ml) of these cells relative to control animals.

A moderate reduction in the volumetric proportions of blood vessel lumen was observed in both groups exposed to cadmium; however this result is not statistically significant. On other hand, the volume (ml) of blood vessel lumen is significantly lower in these animals when compared with the control animals. The animals treated with *P. cupana* only presented a moderate increase in the volumetric proportion of blood vessel lumen relative to the control animals; even so, this result is not confirmed by statistical analysis. However, the increase in blood vessel lumen found in animals treated with *P. cupana* is statistically larger than the results found for both groups exposed to cadmium.

Leydig cell morphometry and plasma testosterone levels

The statistical analyses of plasma testosterone levels and Leydig cell morphometry can be found in the table 1 and 4 respectively. There is no significant alteration in the Leydig cell morphology between control and the treated groups. However, the cadmium only treated animals presented a moderate reduction in the number of Leydig cells per testis; even so, this result is not statistically significant. The plasma testosterone level is also not altered in both groups exposed to cadmium relative to the control animals. Even though, the *P. cupana* only treated animals presented a high increase in the plasma testosterone levels relative to the control; however, this result is not statistically significant.

Discussion

Several studies have described the morphological and biochemical changes in mammal testis exposed to cadmium. The minimal dose of this metal required to cause testis damage was related by Gunn et. al. (1966), and it is approximately 0,44 mg/kg body weight. Hew, et. al. (1993) demonstrated that a dose of 1 mg/kg of body weight caused failure in the spermatogenic process 48 hours after the metal contamination, characterized

by abnormal cellular organization, reduced spermatid population and increased number of immature spermatids. Although all spermatogenic stages were observed in the most of the animals, the more sensitive individuals presented massive degeneration of the seminiferous epithelium.

In the present study the animals were exposed to cadmium in a dose of 1,15 mg/kg body wt. and sacrificed 56 days after administration of the metal. Our results showed extensive testicular damage in the animals exposed to cadmium in the both the cadmium only and cadmium plus guaraná treated groups, especially in the seminiferous epithelium. Histological evaluations demonstrate that in the group treated with cadmium only, although the tubular basement membranes of seminiferous tubule were identified, all the animals presented massive degeneration of germ cells, in agreement with previous literature (Yang et al., 2006 Koyuturk et al., 2006). In these animals, the tubular compartment presented an irregular morphology, probably due to the empty spaces caused by germ cells death. Morphometrical analyses confirmed these results, demonstrating that cadmium only treated animals presented a significant reduction in the volumetric proportions of seminiferous tubules relative to control animals.

On other hand, the animals treated with *P. cupana* during 56 days after cadmium exposition showed a reduced proportion of germ cell damage. Testis sections of these animals (3 out of 6) showed relatively well-preserved seminiferous epithelium, with distinctive somatic and germ cell populations. The remaining animals (3 out of 6) presented extensive cell death in the tubular compartment, with no discernible differences in the seminiferous epithelium relative to animals treated only with cadmium. However, despite the extensive damages observed in the germ cells, these animals presented a large number of seminiferous tubules with a circular shape, contrasting with the irregular morphology found in all the cadmium only treated animals. These tubules contained no identifiable material, which probably represents cellular debris resulting from cell death. It is possible that guaraná protected the myoid cells that form a continuous peritubular layer surrounding the seminiferous tubule, hence aiding in the maintenance of the circular shape. These results are corroborated by morphometrical analyses, since the cadmium plus guaraná treated group presented volumetric proportions of the seminiferous tubule very similar to the results found for control animals.

Despite of the massive germ cell death found in the animals treated with cadmium only and the most damaged animals of the cadmium plus guaraná group (3 out of 6), a thin line of Sertoli cells was detected internally lining the basal membrane of seminiferous tubules. In previous studies, administration of cadmium destroyed various types of testicular cells, including both germ cells and Sertoli cells (Parizek, 1960). However, according to (Hascheck and Rousseaux, 1998), in most cases of testicular toxicity, germ cell death or depletion is followed either by regeneration of the germ cell population or by the persistence of shrunken tubules lined only with Sertoli cells.

Previous studies described the protective effects of antioxidants against damages induced by cadmium in the testis, such as vitamin E and carotenoids, which play a role as free radical scavengers (Koyuturk et al., 2006Yang et al., 2006). Ground seeds of *Paullinia cupana* reportedly present antioxidant properties, which probably is due to the high concentration of polyphenols, as tannins (Mattei, 1998). To our knowledge there is no preceding literature investigating the therapeutic potential of *P. cupana* against cadmium-induced damages, although the protective effects of this plant have been related to several kinds of pathological disorders involving oxidative processes. Therefore, we could suggest that *P. cupana* protected the seminiferous tubules in the animals exposed to cadmium, consequently reducing the proportion of epithelial cell death and preserving the volumetric proportions of the tubular compartments in these animals.

The vascular endothelium is an important target of cadmium toxicity, and has been related to earlier events of histological damages caused by this cation. The exposition to cadmium causes specific ultrastructural changes in the capillary endothelial cell-cell junctions (Niewenhuis et al., 1997), resulting in increased vascular permeability (Sacerdote and Cavicchia, 1983) and hence, increased leakage of fluid and blood cells from the capillaries into the interstitial space (Aoki and Hoffer, 1978). Previous experiments accomplished in our laboratory (Leite, R. et al., 2007, to be submitted for publication) showed that the animals exposed to cadmium in a dose of (1,15 mg/kg bw) presented a moderate reduction in blood vessel lumen volumetric proportions followed by increased interstitial edema 48 hours after the metal administration. In this present study, reduction in the blood vessel lumen volumetric proportion was also evident for both cadmium only and cadmium plus *P. cupana* treated animals, 56 days after the metal administration. Although

this vascular alteration is not statistically significant, the analysis of morphometrical means (Table 4) as well as the similarity with the results found in ours prior experiments (Leite, R. et al., 2007, to be submitted for publication) are pertinent to this affirmation. Furthermore, the volume (ml) of blood vessels in these animals is significantly lower than the measurements made for control animals, probably due to the reduction in the volumetric proportions of blood vessel lumen. This vasoconstriction in animals exposed to cadmium was also described in preceding literature (Skoczynska and Martynowicz, 2005; Tzotzes et al., 2007), and could be explained, at least in part, by the observation that this cation induces intracellular oxidative stress, resulting in decreased nitric oxide synthesis (Martynowicz et al., 2004; Skoczynska and Martynowicz, 2005) and consequently reduced vasodilation. Besides, cadmium also tends to accumulate in the smooth muscle cells (Kaji et al., 1996) and probably contributes to the contractile response of endothelium.

On other hand, the animals treated only with *P. cupana* presented a moderate increase in the blood vessel lumen relative to control animals. It was shown that extracts from red wine polyphenolic compounds can induce vasorelaxant effects that could be explained, at least in part, by nitric oxide (NO) production (Púzserová et al., 2006).

The animals of both groups exposed to cadmium presented a significant increase in the volumetric proportion of Leydig cell nuclei and inflammatory cells relative to the control animals. However, this result appears to be due to reduced testis volume in these animals, consequently agglomerating these cellular populations. This hypothesis is confirmed by the measurement of volume (ml), since these animals showed a total volume of Leydig cell nuclei and inflammatory cells very similar to the results found for control animals. Thus, these animals did not present a real increase in the inflammatory response and in the number of Leydig cells per testis.

We observed in previous experiments (Leite, R. et al., 2007, to be submitted for publication) that exposition to this cation reduced the number of Leydig cells per testis 48 hours after the metal administration. In the present study, our morphometrical analysis also showed a moderate reduction in the number of Leydig cells per testis, 56 days after cadmium exposition. The estimated number of cells in these animals ($228{,}2 \times 10^6$) is very similar to the results found in our previous study ($229{,}6 \times 10^6$), in which the counting of Leydig cells was accomplished 48 hours after cadmium injection. These comparisons are

relative to control animals, which presented a number of Leydig cells per testis of $304{,}8x10^6$. Although these results are not confirmed by statistical analyses, the consistent repetition of the numerical means described above suggests this histological alteration.

On other hand, the animals treated with *P. cupana* during 56 days after cadmium exposition presented a number of Leydig cells per testis that is very similar to the proportions found in the control animals. This result is in agreement with our previous study (Leite, R. et al., 2007, to be submitted for publication), in which the animals preventively treated with *P. cupana* during 56 days before cadmium exposition presented a nearly normal number of Leydig cells per testis. The coincidence of these results strongly suggests that the administration of guaraná is efficient in protecting the Leydig cells against cadmium damages, maintaining the numerical proportions of theses cells.

Results of in vitro studies demonstrated that cadmium is directly toxic to primary cultured Leydig cells, resulting in decreased cell viability and reducing the testosterone secretion (Yang, et al., 2003). The present study showed that cadmium only and cadmium plus guaraná treated animals presented no significant alteration in the testosterone production relative to control animals, 56 days after cadmium injection. Furthermore, despite the extensive damages observed in the seminiferous epithelium of these animals, morphometrical analysis showed no significant alterations in the Leydig cell morphology. This result is also compatible with previous study, which suggested that the cadmium-damaged testis recovered or regained some functional Leydig cells that contribute to testosterone production (Saksena et al. 1977).

On other hand, the animals treated only with *P. cupana* presented a large increase in the testosterone production relative to control animals; however this result is not statistically significant. Furthermore, these animals also presented an evident increase in the volumetric proportions of the seminiferous tubule, suggesting an increase in the spermatogenic process. According to Sinha-Hikin et al. (1989) the tubule diameter has a positive correlation with the spermatogenic activity. Based on this information, it is very likely that the increase in testosterone production in the animals treated with *P. cupana* only resulted in an increase in the spermatogenic process and hence, in the increase in the seminiferous tubule volumetric proportions.

Castro et al. (2002) observed a significant correlation between the number of Leydig cells per testis and both plasma and testicular testosterone levels in rabbit. The author suggested that the increase in testosterone might occur as a consequence of the increase in the number of Leydig cells per testis. We observed that despite the high levels of testosterone observed in the animals treated with *P. cupana* only, the number of Leydig cells found in these animals is very similar to the results found for control animals. Furthermore, although we showed a reduction in this cellular population in the cadmium only treated group, these animals presented no reduction in the testosterone plasma levels. Thus, it is likely that the increase in the testosterone production observed in the animals treated with *P. cupana* only be related to increased Leydig cell synthetic activity.

The present research showed that the cadmium only and the cadmium plus *P. cupana* treated animals presented evident histopathological alterations in the testis tissue, especially in the seminiferous epithelium. However, the animals treated with *P. cupana* during 56 days after cadmium injection presented a significant reduction in the proportion of the seminiferous tubules damages. The morphometrical analyses showed a few alterations in the intertubular components of both groups exposed to cadmium. The number of Leydig cells in these animals is very similar to the results found for control animals, indicating the capacity of the testis to recover or regain some functional Leydig cells. This result is attested by the results of hormonal analyses, since these animals did not present significant alteration in the concentration of testosterone relative to control animals. On other hand, the blood vessel lumens appeared to be very constricted in the animals exposed to cadmium, in agreement with previous literature.

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Tables

Table 1: Biometric values and plasma testosterone levels in adult male Wistar treated with *P. cupana* and/or cadmium.

Parameters	Group I	Group II	Group III	Group IV
Initial body weight	362.600 ± 48.22	365.66 ± 21.88	347.66 ± 32.53	385.40 ± 19.74
Final body weight	452.80 ± 75.21	432.66 ± 6.74	451.83 ± 44.21	405.40 ± 26.90
Testis weight	1.782 ± 0.22 [b]	0.81 ± 0.24 [a]	0.98 ± 0.46 [a]	1.73 ± 0.15 [b]
Testosterone(ng/ml)	1.266 ± 1.23	1.400 ± 1.40	1.400 ± 1.50	5.044 ± 4.26

Group I = Control (n = 5); Group II = Cadmium only (n = 6); Group III = Cadmium plus *P. cupana* (n = 6); Group IV: *P. cupana* only (n = 5)
[a-b]In each row, values with different superscripts are significantly different ($p < 0.01$)

Table 2: Volume (ml) of the testis components in adult Wistar treated with *Paullinia cupana* and/or exposed to cadmium

Parameters	Group I	Group II	Group III	Group IV
Seminiferous Tubule	1.414 ± 0.21[b]	0,400 ± 0,08[a]	0,732 ± 0,41[a]	1.502 ± 0.16[b]
Intertubular Space	0.330 ± 0.04	0,413 ± 0,18	0,247 ± 0,08	0.228 ± 0.02
Inflammatory Cells	0.001 ± 0.00	0,005 ± 0,00	0,002 ± 0,00	0.002 ± 0.00
Blood Vessel Lumen	0.015 ± 0.00[b]	0,006 ± 0,00[a]	0,006 ± 0,00[a]	0.021 ± 0.00[b]
Leydig Cell Nucleus	0.030 ± 0.00	0,031 ± 0,00	0,037 ± 0,00	0.032 ± 0.00

Group I = Control (n = 5); Group II = Cadmium only (n = 6); Group III = Cadmium plus *P. cupana* (n = 6); Group IV: *P. cupana* only(n = 5)
[a-b]In each row, values with different superscripts are significantly different ($p < 0.01$)

Table 3: Volumetric Proportions (%) of the testis components in adult Wistar treated with *Paullinia cupana* and/or exposed to cadmium

Parameters	Group I	Group II	Group III	Group IV
Seminiferous Tubule	80.92 ± 0.07[bc]	50,60 ± 10,60[a]	72,40 ± 9,68[c*]	86.46 ± 2.07[b*]
Intertubular Space	19.07 ± 0.07[bc]	49.37 ± 10.60[a]	27.58 ± 9.67[c*]	13.52 ± 2.07[b*]
Inflammatory Cells	0.09 ± 0.02[b*]	0.27 ± 0.08[a*]	0.29 ± 0.12[a]	0.15 ± 0.08[ab]
Blood Vessel Lumen	0.94 ± 0.20[ab]	0.77 ± 0.23[b]	0.64 ± 0.15[b]	1.24 ± 0.22[a]
Leydig Cell Nucleus	1.75 ± 0.30[b]	3.98 ± 1.12[a]	4.08 ± 0.78[a]	1.87 ± 0.47[b]

Group I = Control (n = 5); Group II = Cadmium only (n = 6); Group III = Cadmium plus *P. cupana* (n = 6); Group IV: *P. cupana* only (n = 5)
[a-b-c]In each row, values with different superscripts are significantly different ($p < 0.01$) and *($p < 0.05$).

Table 4: Leydig Cell morphometry in adult Wistar rats treated with *Paullinia cupana* and/or exposed to cadmium

Parameters	Group I	Group II	GroupIII	Group IV
Nucleus Diameter LC (µm)	5.72 ± 0.26	6.39 ± 0.33	6.37 ± 0.80	5.88 ± 0.10
Volume of a LC (µm³)	659.41 ± 82.02	857.90 ± 136.06	834.33 ± 270.00	784.69 ± 97.63
Nucleus Volume (µm³)	97.57 ± 12.12	136.65 ± 20.13	140.00 ± 47.90	106.31 ± 5.86
Cytoplasm Volume (µm³)	561.84 ± 71.87	721.24 ± 117.25	694.33 ± 222.54	678.38 ± 94.82
Number LC/testis (10^6)	304.80 ± 62.05	229.66 ± 61.39	293.16 ± 134.97	303.20 ± 77.89
Number LC/g testis (10^6)	178.16 ± 13.92	290.85 ± 86.56	333.288 ± 166.16	175.79 ± 52.35

Group I = Control (n = 5); Group II = Cadmium only (n = 6); Group III = Cadmium plus *P. cupana* (n = 6); Group IV: *P. cupana* only (n = 5)

Figure Legends

Figure 1 - Light microscopy of the testicular tissue of Wistar rats: A and B – Representative areas of control animals showing a well organized seminiferous epithelium with normal blood vessels and clusters of Leydig cells; C and D – Representative areas of *P. cupana* only treated animals with structures similar to the control, exempting the blood vessels, which appear to be dilated. ST = seminiferous tubule; Ls = Lymphatic space; Lc = Leydig cell; V = blood vessels; (arrow) Sertoli cell. Toluidine blue stained.

Figure 2 - Light microscopy of the testicular tissue of Wistar rats: Representative areas of the cadmium only treated animals. A and B – Images showing extensive damages in the seminiferous epithelium and shrunken seminiferous tubules. The intertubular space is largely amplified and contains many Leydig cell clusters. C - Dense clusters of Leydig cells occur in the intertubular space surrounding blood vessels that appears to be constricted. D - Image showing the absence of germs cells in the seminiferous epithelium, which presents only a basal thin line of Sertoli cells. The intertubular space presents Leydig cell clusters and a blood vessel with normal morphology. ST = seminiferous tubule; Ls = Lymphatic space; Lc = Leydig cell; V = blood vessels; M = macrophage; (arrow) Sertoli cell. Toluidine blue stained.

Figure 3 - Light microscopy of the Wistar rat testicular tissue: Representative areas of the cadmium plus *P. cupana* treated animals. A – Image showing seminiferous tubules and intertubular space with morphology similar to that found for control animals. B – Image representing the heterogeneous response to cadmium in the testicular tissue: seminiferous tubules with normal morphology are adjacent to a seminiferous tubule with drastic damages in the seminiferous epithelium. C - Image showing the absence of germs cells in the shrunken seminiferous tubule and the intertubular space with dense clusters of Leydig cells. D - Seminiferous tubule with circular shape, containing no identifiable material. The intertubular space presents dense Leydig cell clusters. E – Image showing the agglomeration of Leydig cells in the intertubular space. F – Image showing the absence of

germs cells in the seminiferous tubule, which presents only a thin line of Sertoli cells. The intertubular space presents dense Leydig cell clusters surrounding a blood vessel that appears constricted. . ST = seminiferous tubule; Ls = Lymphatic space; Lc = Leydig cell; V = blood vessels; M = macrophage; Bm = basal membrane; (arrow) Sertoli cell. Toluidine blue stained.

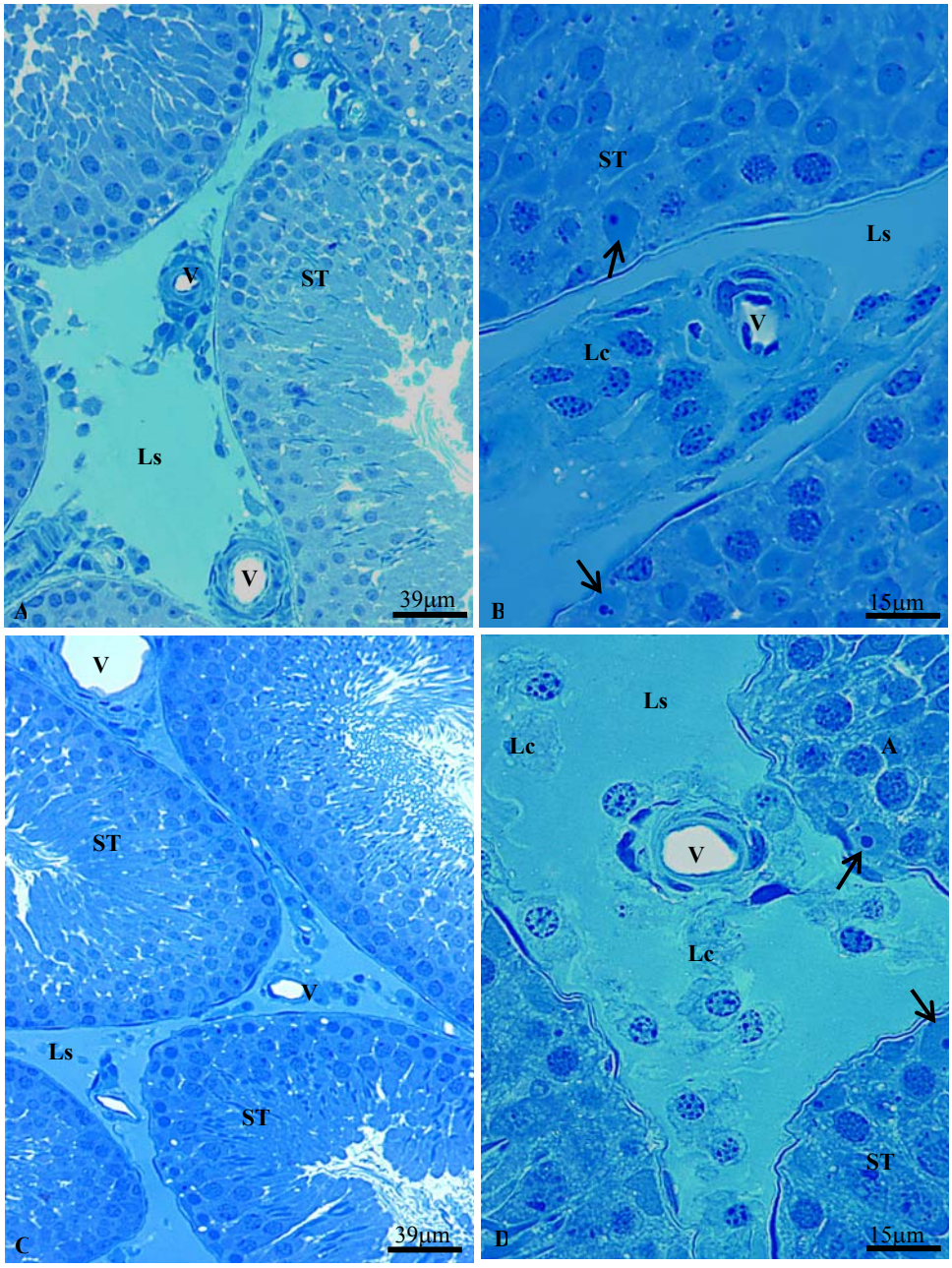
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Figure 1

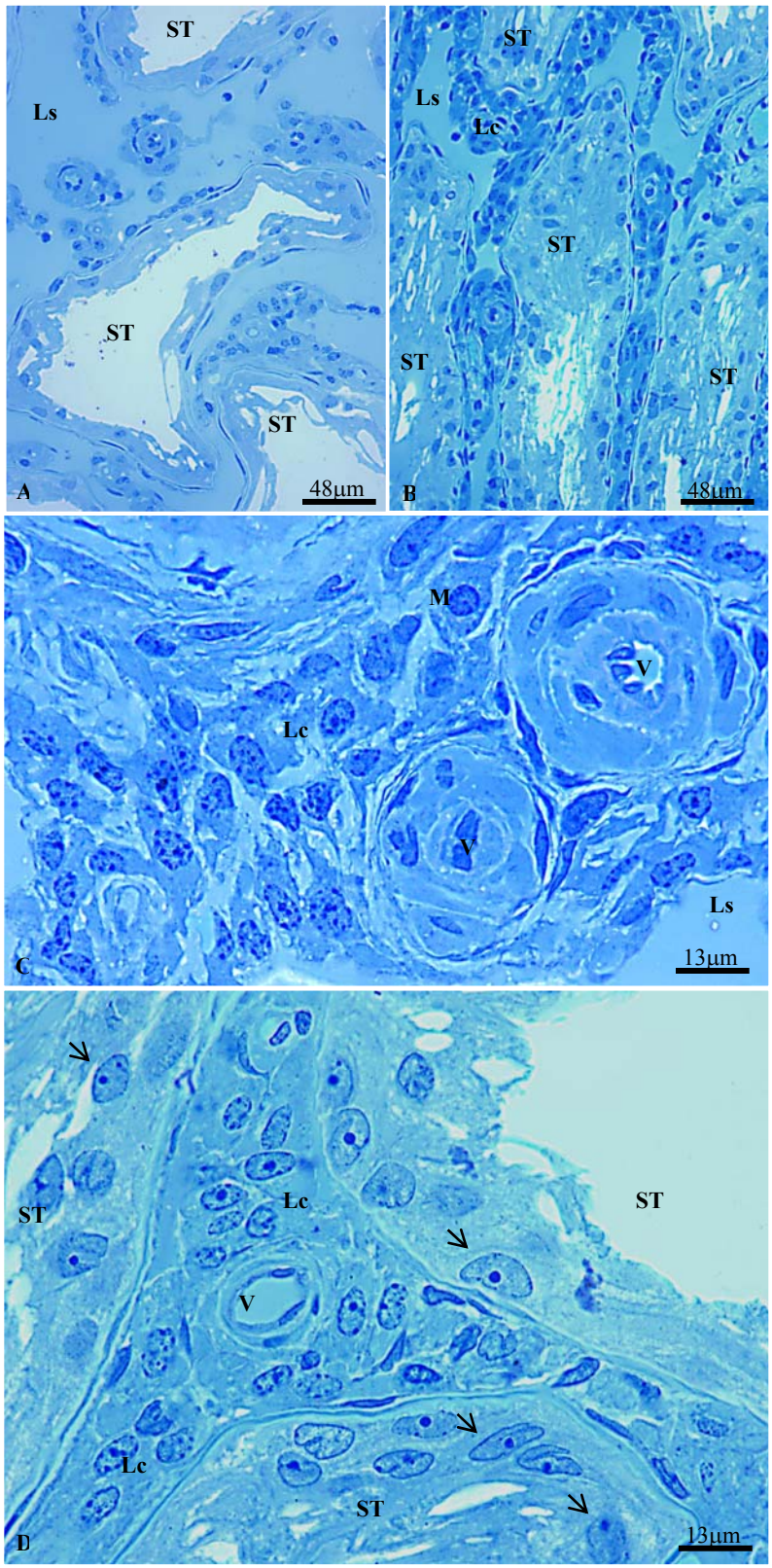
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Figure 2

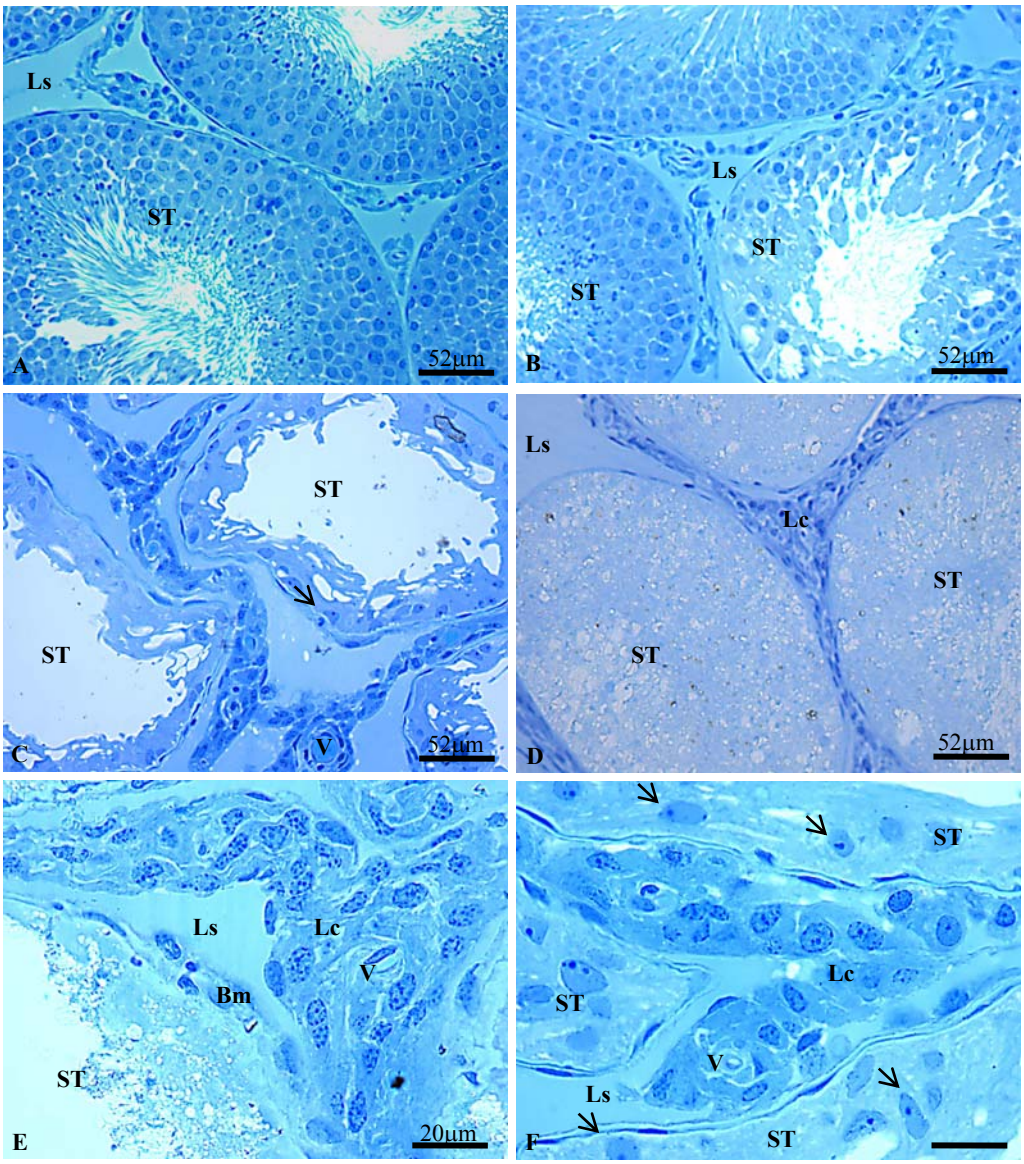
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Figure 3

Conclusões

Modelo Experimental I:

1 - A dose de cádmio administrada neste estudo é suficiente para gerar morte celular generalizada no epitélio seminífero assim como de células de Leydig nos animais mais sensíveis ao metal.

2 - O processo de degeneração do tecido testicular devido aos efeitos tóxicos do cádmio envolveu dois mecanismos, necrose e apoptose.

3 - Animais de mesma linhagem apresentam diferentes níveis de sensibilidade ao cádmio.

4 - A administração preventiva do guaraná durante 56 dias antes da exposição ao cádmio foi eficiente em prevenir o processo de vasoconstrição gerado por este metal.

5 - O tratamento preventivo com guaraná durante 56 antes da exposição ao cádmio amenizou as alterações morfológicas em células de Leydig e contribuiu na manutenção da proporção numérica desta população celular.

6 - A dose de cádmio utilizada não foi suficiente para alterar a concentração plasmática de testosterona medida 48 horas após a administração do metal. Por outro lado, o tratamento conjunto de cádmio e guaraná afetou negativamente a produção deste hormônio.

Modelo Experimental II:

1 - A administração do guaraná durante 56 dias após a exposição ao cádmio foi efetiva em reduzir parcialmente a proporção de túbulos seminíferos danificados.

2 - Ambos os grupos expostos ao cádmio apresentaram redução significativa no lúmen de vasos sanguíneos. Portanto, a administração do guaraná após a exposição ao cádmio não foi eficiente em evitar o processo de vasoconstrição gerado por este metal.

3 - Ambos os grupos expostos ao cádmio apresentaram células de Leydig com características morfológicas muito similares aos animais controle. Concluímos que após a exposição ao cádmio o testículo possui a capacidade de recuperar uma população de células de Leydig morfologicamente normal.

4 - Os animais expostos somente ao cádmio apresentaram células de Leydig, ainda que morfologicamente semelhante ao grupo controle, numericamente menor do que os animais controle. Os animais tratados com cádmio e guaraná simultaneamente apresentaram um número similar ao encontrado para os animais controle. Portanto, o guaraná contribuiu na manutenção da proporção numérica das células de Leydig.

5 - Ambos os grupos expostos ao cádmio não apresentaram alterações nas concentrações plasmáticas de testosterona. Concluímos que, após a exposição ao cádmio, o testículo possui a capacidade de recuperar uma população de células de Leydig com atividade normal de síntese.

6 - Ambos os grupos expostos ao cádmio apresentaram uma camada basal de células de Sertoli nos túbulos seminíferos destituído de epitélio. Este resultado sugere que as células de Sertoli são menos sensíveis ao cádmio e portanto permanecem nos túbulos seminíferos destituídos de células germinativas.

Influência do guaraná na morfologia testicular e na secreção de testosterona

1 - Os animais tratados somente com guaraná apresentaram um evidente aumento no lúmen de vaso sanguíneo. Concluímos que este fato se deve a propriedade vasodilatadora dos polifenóis, substâncias abundantes na semente do guaraná.

2 - A administração deste extrato gerou um aumento significativo na produção de testosterona assim como um aumento significativo na proporção volumétrica dos túbulos seminíferos. O significado deste resultado não é bem compreendido, ainda que podemos

supor que o aumento na síntese hormonal tenha estimulado a atividade das células germinativas.

3 – Apesar do aumento significativo na produção de testosterona, os animais tratados com guaraná não apresentaram aumento no número de células de Leydig. Portanto, concluímos que o efeito anabólico desta planta está relacionado ao aumento na atividade de síntese das células de Leydig e não ao aumento na proporção numérica destas células.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Mestrado** intitulada **Efeitos da Associação de Cloreto de Cádmio e Guaraná (*Paullinia cupana*) no Testículo de Ratos Wistar: Análise Morfométrica, Estrutural e Ultraestrutural.**

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

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

(X) tem autorização da Comissão de Ética em Experimentação Animal (Protocolo nº 1202-1).

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

Rodrigo Paula Leite
Aluno(a): (Rodrigo Paula leite)

Orientador(a): (Mary Anne Heidi Dolder)

Para uso da Comissão ou Comitê pertinente:

(X) Deferido ( ) Indeferido

Ana Maria Aparecida Guaraldo
Nome:
Função:

Profa. Dra. ANA MARIA A. GUARALDO
Presidente
Comissão de Ética na Experimentação Animal
CEEA/IB - UNICAMP