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FABRÍCIA DE SOUZA PREDES

"EFEITO ANTIOXIDANTE DA BARDANA (*Arctium lappa*) NO TESTÍCULO, EPIDÍDIMO E FÍGADO DE RATOS DANIFICADOS PELO CÁDMIO: BIOQUÍMICA, MORFOLOGIA, MORFOMETRIA E ULTRAESTRUTURA"

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1. ABSTRACT

Cadmium (Cd) is a heavy metal associated with severe damage in various organs, including male reproductive organs and liver. *Arctium lappa* is widely used in popular medicine for treating hypertension, gout, hepatitis and other inflammatory disorders. Pharmacological studies indicated that *A. lappa* roots have hepatoprotective, free radical scavenging and antiproliferative activities. The objectives of this work were evaluate: (1) histomorphometrically the threshold modifications of seminiferous tubules caused by single acute low doses of Cd, (2) antioxidant properties of different *A. lappa* extracts to choose the best one for "*in vivo*" study, (3) the ability of *A. lappa* to attenuate cadmium-induced damage to the testis, epididymis and liver of male rats. Dichloromethanic, ethanolic, aqueous extracts, hydroethanolic and total aqueous extract of *A. lappa* roots were investigated regarding radical scavenging activity and the total phenolic content. The extracts were tested for their antiproliferative activity *in vitro* using human cancer cell lines. The higher radical scavenging activity and yield were found in the hydroethanolic extract. Higher phenolic contents were found in the dichloromethanic and hydroethanolic extracts. Only the dichloromethanic extracts exhibited activity against cancer cell lines. High-resolution electrospray ionization mass spectroscopy for the hydroethanolic extract demonstrated the presence of: arctigenin, quercetin, chlorogenic acid and caffeic acid. The hydroalcoholic lyophilized extract was chosen for the experiments *in vivo*. To study the association of Cd and *A. lappa*, adult Wistar rats were assigned to four groups: control (C), *A.lappa* extract (*Al*), cadmium (Cd) and cadmium plus *A. lappa* (Cd*Al*). The animals

received water or *A. lappa* hydroethanolic extract (300 mg/Kg BW) by gavage and i.p. injections of saline or 1.2 mg/Kg BW of cadmium chloride. The animals were sacrificed after 7 and 56 days of treatment. The Cd caused reduction in testis, epididymis and seminal vesicle weights, seminiferous tubule (ST) proportion, total length of ST and ST diameter. Degenerated germ cells and multinucleated aggregates were observed in ST and progressive damage resulted in tubules lined only with Sertoli cells. There was a decreased in tubular proportion and increased epithelial height in epididymis after 56 days. Cd caused an increase in plasma GOT and GPT levels after 56 days, but *A. lappa* extract improved these levels. No relevant alteration was observed in liver transaminase levels. After 7 days, the Cd group showed decrease in hepatocyte proportion and increase sinusoid capillary proportion, also the nuclear density decrease. However, after 56 days, only the hepatocyte nuclear density decreased. In *Al* and Cd*Al* groups, after 7 days, the hepatocyte nuclear proportion increased and the cytoplasmic decreased, with no alteration in capillary sinusoid proportion. In *Al* and Cd*Al* groups, after 56 days, hepatocyte nuclear proportion increased and the sinusoid capillary proportion decreased. Also, an increase of hepatocyte nuclear density was observed in *Al* and Cd*Al* groups. This study revealed that low doses of Cd cause progressive morphological and morphometrical alterations on rat testis and that Cd has a subtle toxic threshold. The dichloromethanic extracts showed antiproliferative activity against K562, MCF-7 and 786-0 human cancer cell lines. The hydroethanolic extract showed higher free radical scavenger activity and higher phenolic content, and was chosen as the best extract for the "*in vivo*" studies. *A. lappa* hydroethanolic extract was ineffective

in protecting the testis and epididymis against Cd-induced damage, however effectively protected liver tissue.

2. RESUMO

Cádmio (Cd) é um metal pesado associado a graves danos em vários órgãos, incluindo órgãos reprodutivos e fígado. Estudos farmacológicos indicam que *Arctium lappa* possue atividade hepatoprotetora, sequestradora de radicais livres e antiproliferativa. O objetivo deste trabalho foi avaliar: (1) as modificações nos túbulos seminíferos causadas por pequenas doses de Cd, (2) as propriedades antioxidantes de diferentes extratos de *A. lappa*, (3) a capacidade de *A. lappa* em atenuar os danos induzidos por cádmio no testículo, epidídimo e fígado. A capacidade sequestradora de radicais livres e quantidade de compostos fenólicos totais foram analisados nos extratos da raiz de *A. lappa* para determinação da capacidade antioxidante. A propriedade antiproliferativa *in vitro* de cada extrato foi avaliada em células tumorais. O extrato hidroetanólico apresentou alto rendimento e capacidade sequestradora de radicais livres, e juntamente com os diclorometânicos, maior teor de compostos fenólicos. Somente os extratos diclorometânicos apresentaram atividade antiproliferativa contra células tumorais. A espectroscopia de massa revelou a presença dos compostos arctigenina, quercetina, ácido clorogênico e ácido caféico no extrato hidroetanólico. O extrato hidroetanólico liofilizado foi escolhido para os estudos *in vivo*. Para estudar os efeitos de Cd e *A. lappa*, ratos Wistar adultos foram distribuídos em quatro grupos: controle (C), extrato de *A. lappa* (*Al*), cádmio (Cd) e cádmio e extrato de *Al* (Cd*Al*). Os animais receberam água ou extrato de *Al* (300 mg/Kg BW) por gavagem e dose única i.p. de salina ou 1,2 mg/kg de cloreto de cádmio. Os animais foram sacrificados após 7 e 56 dias. O Cd causou redução do peso do testículo,

epidídimo e vesícula seminal, na proporção dos túbulos seminíferos (TS), no comprimento total dos TS e no diâmetro dos TS. Células germinativas degeneradas e células gigantes multinucleadas foram observadas nos TS e os danos progressivos resultaram em túbulos preenchidos apenas com células de Sertoli. Nos grupos Cd e *CdAl*, houve diminuição da proporção tubular e aumento da altura do epitélio no epidídimo após 56 dias. Cd causou aumento no nível plasmático de TGO e TGP após 56 dias, mas o extrato de *A. lappa* amenizou estes efeitos. Nenhuma alteração relevante foi observada para as transaminases hepáticas. No grupo Cd 7 dias, a proporção de hepatócitos diminuiu, a de sinusóides aumentou e o número de núcleo de hepatócito reduziu, entretanto após 56 apenas o número de núcleos de hepatócitos diminuíram. Após 7 dias, nos grupos *Al* e Cd*Al*, a proporção nuclear aumentou e a citoplasmática diminuiu, sem alteração dos capilares sinusóides. Após 56 dias, nos grupos *Al* e Cd*Al*, a proporção nuclear aumentou e a proporção dos capilares sinusóides reduziu. Houve aumento na densidade de núcleos nos grupos *Al* e Cd*Al*. Conclui-se que doses baixas de Cd causam alterações morfológicas e morfométricas no testículo e que o Cd tem sutil limiar tóxico. Os extratos diclorometânicos apresentaram atividade antiproliferativa contra as linhagens tumorais K562, MCF-7 e 786-0. O extrato hidroetanólico apresentou alto conteúdo fenólico e atividade antioxidante, portanto foi escolhido para os estudos *in vivo*. O extrato de *A. lappa* foi ineficaz na proteção do testículo e epidídimo contra danos induzidos pelo Cd, porém protegeu o tecido hepático.

3. INTRODUÇÃO

Os problemas ambientais têm aumentado exponencialmente nas últimas décadas, devido à poluição industrial e ao rápido crescimento da população humana. Os produtos químicos tóxicos, tais como íons de metais pesados lançados no ar, água e solo entram na cadeia alimentar provenientes do meio ambiente e perturbam os processos bioquímicos, levando a alterações da saúde e em alguns casos, a consequências fatais. Um desses metais pesados é o cádmio (Çavu o lu *et al.*, 2009).

O cádmio é um elemento natural presente na crosta terrestre e usualmente é encontrado como um mineral combinado com oxigênio, cloro e enxofre (Manna *et al.*, 2008) ou também associado aos minérios de zinco, cobre e chumbo (Wirth e Mijal, 2010). É liberado no ambiente por forças naturais ou atividades humanas (Wirth e Mijal, 2010). A atividade vulcânica, queima de combustíveis fósseis, incêndios florestais, e transporte de partículas do solo contaminado pelo vento constituem as principais atividades naturais responsáveis pela liberação de cádmio no meio ambiente. Este metal é frequentemente usado em galvanoplastia, pigmentos, tintas, solda e baterias Ni-Cd (Ji *et al.*, 2009).

Com o aumento da produção e utilização de cádmio, não apenas os trabalhadores industriais, mas também a população em geral está exposta aos efeitos tóxicos deste metal pesado (Ognjanovic *et al.*, 2009). A população está exposta ao cádmio através da água potável e dos alimentos (Ji *et al.*, 2009). Outra fonte importante de exposição humana ao cádmio é fumaça de cigarro, pois as plantas de tabaco seletivamente acumulam cádmio proveniente do solo (Joseph, 2009).

O cádmio pode causar numerosas lesões em diversos órgãos e sistemas tais como rim, fígado, sistema cardiovascular, respiratório, nervoso e reprodutor, tanto em animais silvestres e experimentais, quanto na população humana (Thompson e Bannigan, 2008; Ognjanovic *et al.*, 2009; Wirth e Mijal, 2010). É importante ressaltar que a toxicidade do cádmio em animais experimentais é influenciada por um grande número de fatores, tais como, via de administração, dose, duração da exposição e idade dos animais (Tzirogiannis *et al.*, 2003). Dentre os efeitos induzidos pelo cádmio, na grande variedade de tecidos que afeta, estão o aumento da peroxidação lipídica, diminuição da expressão e atividade das enzimas antioxidantes tais como superóxido desmutase e catalase (El-Sokkary *et al.*, 2009).

Exposições agudas ao cádmio resultam, primariamente, em acúmulo deste metal no fígado (Tzirogiannis *et al.*, 2003; Koyu *et al.*, 2006; El-Sokkary *et al.*, 2009) sendo os hepatócitos e as células endoteliais os principais alvos (Koyu *et al.*, 2006). Características histopatológicas da hepatotoxicidade do cádmio incluem necrose, infiltração gordurosa e inchaço dos hepatócitos. Fibrose, cirrose e inflamação também foram observadas, e os danos ao fígado são frequentemente acompanhados por infiltração de leucócitos (Tzirogiannis *et al.*, 2003).

Estudos anteriores mostraram que o cádmio reduz o peso testicular e dos órgãos sexuais acessórios (Manna *et al.*, 2008; Fouad *et al.*, 2009). Após a exposição aguda, danos testiculares induzidos pelo cádmio foramr encontrados tanto nos túbulos seminíferos quanto no interstício (Blanco *et al.*, 2007). A disfunção testicular pode resultar de distúrbios nas células de Sertoli, como rompimento da barreira hemato-testicular, ou nas células de Leydig, comprometendo a produção de andrógenos sob controle do eixo hipotálamo-

hipófise-testicular (Bizarro *et al.*, 2003; Manna *et al.*, 2008; Siu *et al.*, 2009). No epidídimo, o Cd pode causar atrofia, diminuição do lúmen do ducto, espessamento de seu epitélio (Herak-Kramberger *et al.*, 2000) e apoptose das células epiteliais (Huang *et al.*, 2005).

Nos últimos anos, a patogênese das lesões causadas pelo cádmio vem sendo atribuída ao estresse oxidativo (Sinha *et al.*, 2007; Borges *et al.*, 2008; Ola-Mudathir *et al.*, 2008). O estresse oxidativo é um estado de desequilíbrio na geração de espécies reativas de oxigênio (ERO) e radicais livres e no sistema de defesa antioxidante. As enzimas antioxidantes constituem o sistema de defesa mais importante que limita a toxicidade associada com os radicais livres e ERO (Sreepriya e Bali, 2006). Em baixas concentrações, as ERO estão envolvidas na regulação de vários processos fisiológicos tais como proliferação celular, diferenciação, apoptose e senescência. Em altas concentrações, as ERO são extremamente tóxicas para a célula (Gupta *et al.*, 2004). A produção de ERO tais como íon peróxido, radicais hidroxila e peróxido de hidrogênio, entre outros, levam ao aumento da peroxidação lipídica, danos ao DNA, degradação de proteínas e carboidratos, expressão gênica alterada e apoptose (Gupta *et al.*, 2003; Koyu *et al.*, 2006; Koyuturk *et al.*, 2006; Sinha *et al.*, 2007).

Estudos recentes mostram que sequestradores de radicais livres e antioxidantes são eficientes na proteção contra a toxicidade do Cd (Koyuturk *et al.*, 2006). Antioxidantes, tais como carotenóides (β-caroteno e licopeno), vitamina E (α-tocoferol) e vitamina C (ascorbato), desempenham papel importante na saúde animal, inativando os radicais livres prejudiciais produzidos pela atividade celular normal e por vários agentes estressores (El-

Missiry e Shalaby, 2000; El-Demerdash *et al.*, 2004; Gupta *et al.*, 2004; Sen Gupta *et al.*, 2004; Koyuturk *et al.*, 2006). As vitaminas E, C e/ou β-caroteno têm a capacidade de diminuir consideravelmente os efeitos prejudiciais do Cd, tais como alterações no peso corporal e de órgãos, na atividade enzimática, nos parâmetros hematológicos e bioquímicos, na qualidade dos espermatozóides, na produção de hormônios esteróides e na peroxidação lipídica (El-Missiry e Shalaby, 2000; Gupta *et al.*, 2003; El-Demerdash *et al.*, 2004; Gupta *et al.*, 2004; Koyuturk *et al.*, 2006; Yang *et al.*, 2006).

A exposição humana a contaminantes ambientais que afetam severamente a função reprodutiva masculina tem aumentado e se tornado uma grande preocupação de saúde pública (Ola-Mudathir *et al.*, 2008). Portanto, nos últimos anos, tem crescido o interesse na descoberta de plantas medicinais com compostos terapeuticamente ativos e atenção tem sido dada especialmente à propriedade antioxidante. Várias plantas e seus compostos têm sido testados quanto à sua capacidade de proteger diversos tecidos contra os danos causados pelo cádmio.

Estudos com o extrato aquoso de *Hibiscus sabdariffa* demonstraram efetividade na proteção contra os danos causados por cádmio no fígado, próstata e testículo, comprovados através de estudos bioquímicos. Neste estudo, ratos Wistar receberam doses intraperitoneais semanais de 3 mg/Kg de peso corporal de cloreto de cádmio por quatro semanas e o extrato da planta duas vezes por semana, oralmente (Asagba *et al.*, 2007). Pesquisadores estudaram o extrato vegetal Picroliv (fração isolada de raízes e rizomas de *Picrorhiza kurroa*) administrado preventivamente à intoxicação aguda por cádmio (3 mg/Kg de peso corporal) em ratos albinos Druckrey. A capacidade de minimizar os danos causados pelo cádmio foi

comprovada através do estudo do perfil sérico de transaminases e da análise histopatológica do fígado e testículo (Yadav *et al.*, 2005). Sinha e colaboradores isolaram uma proteína de 43 Kilodaltons da planta *Cajanus indicus* L., que diminui os danos induzidos pelo cádmio em hepatócitos *in vitro* isolados de camundongos Swiss. Tal proteína restabeleceu a atividade das enzimas antioxidantes, atenuou o aumento de peroxidação lipídica e aumentou a viabilidade celular (Sinha *et al.*, 2007).

A bardana (*Arctium lappa* Linne- Astaraceae) é uma planta originária da Ásia e aclimatada no Brasil. Por muitas décadas, a bardana vem sendo consumida em vários países, tanto na forma de alimento como bebida. Esta planta é amplamente utilizada na medicina popular em todo o mundo por suas conhecidas aplicações terapêuticas (Pereira *et al.*, 2005). Várias partes da planta possuem efeitos comprovados tais como o fruto (Matsumoto *et al.*, 2006), a semente (Ming *et al.*, 2004), a folha (Pereira *et al.*, 2005; Gentil *et al.*, 2006) e a raiz (Maruta *et al.*, 1995; Lin *et al.*, 1996; Lin *et al.*, 2000a; Lin *et al.*, 2002; Chen *et al.*, 2004; Leonard *et al.*, 2006; Wang *et al.*, 2007).

Parte aérea da bardana (*Arctium lappa*)

Folhas

Raízes

Desde a década de 90, muitos pesquisadores têm estudado o efeito antioxidante e sequestrador de radicais livres atribuídos à *A. lappa*. Lin e colaboradores relataram que o extrato aquoso da raiz da bardana possui efeito antioxidante e sequestrante de radicais livres em fígado de ratos por inibir a hepatoxicidade induzida por tetracloreto de carbono (CCl_4). Além disso, esse mesmo extrato apresentou efeito antiinflamatório, reduzindo o edema de pata induzido por carragena (espessante alimentício) (LIN *et al.*, 1996). Lin e

colaboradores verificaram o mesmo efeito protetor no fígado de ratos tratados com acetaminofeno (paracetamol) e CCl_4 (Lin *et al.*, 2000a). Lin e colaboradores atribuíram a diminuição da hepatoxicidade induzida pelo etanol e potencializada pelo CCl_4, à ação antioxidante da bardana (Lin *et al.*, 2002). A eficácia de proteção dos extratos de *A. lappa* contra diferentes agentes oxidantes motivou a investigação da associação da planta ao agente estressor, cádmio.

Estudos anteriores, com administração de infusão de folhas de *A. lappa,* mostraram ausência de sinais de toxicidade em parâmetros bioquímicos plasmáticos e na morfologia hepática, além de ausência de alterações no peso dos órgãos sexuais acessórios e na proporção volumétrica dos compartimentos testicular e na morfometria das células de Leydig (Predes *et al*., 2007; Predes *et al.*, 2009).

É bem conhecido que a exposição ao cádmio tem efeitos danosos nos órgãos reprodutivos de ratos machos, o que está documentado através de muitos estudos bioquímicos de estresse oxidativo. Entretanto, poucos estudos investigaram e/ou quantificaram as alterações morfológicas causadas por este metal no testículo e epidídimo. O propósito deste trabalho foi avaliar histomorfometricamente as modificações causadas por uma dose aguda de cádmio e se a utilização do extrato hidroetanólico de raiz *A. lappa* é capaz de amenizar os efeitos danosos do cádmio.

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5. OBJETIVOS

- Determinar o limiar de ação do cádmio sobre o testículo

- Avaliar o potencial antioxidante e a atividade antiproliferativa *in vitro* de diferentes extratos de *A. lappa*

- Escolher o extrato mais eficiente para administração em ratos machos intoxicados com cádmio

- Analisar as alterações morfológicas, morfométricas/estereológicas e ultra-estruturais no testículo de ratos Wistar submetidos ao tratamento de Cd e/ou *A. lappa*.

- Analisar as alterações morfológicas, morfométricas/estereológicas no epidídimo de ratos Wistar submetidos ao tratamento de Cd e/ou *A. lappa*.

- Analisar as alterações morfológicas, estereológicas e bioquímicas no fígado de ratos Wistar submetidos ao tratamento de Cd e/ou *A. lappa*.

6. ARTIGO 1: TESTIS RESPONSE TO TWO LOW DOSES CADMIUM IN WISTAR RATS

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Abstract

Although it is well known that cadmium (Cd) causes adverse effects on male rat reproductive organs, few studies have quantified alterations caused by low doses. Quantification of these alterations, especially in the testis, was measured using morphometric techniques. A single dose of cadmium chloride (1 or 1.2 mg/Kg BW) was injected i.p. in adult rats, sacrificed after 7 or 56 days. The lower dose caused slight alterations as measured by morphometrical analysis. The higher dose caused significant reduction in testis and epididymis weight, gonadossomatic index and length of seminiferous tubule (ST) after 7 and 56 days. Cd significantly reduced the ST diameter after 56 days. Decreased volume density of ST, after 7 and 56 days, was accompanied by an increase in interstitium volume density. Observations with light microscopy, at the dose of 1.2 mg/Kg, showed progressive damage. After 7 days, the tubule lumens were filled with degenerated germ cells and multinucleated spermatid aggregates. Vacuolization of the seminiferous epithelium was also observed. After 56 days, increased damage resulted in vacuolated ST, often consisting only of Sertoli cells. SEM examination of this tissue showed in the group treated with 1.2 mg/Kg cadmium and sacrificed after 56 days, showed the interstitial tissue presenting a compact and fibrous appearance with absence of fenestrae. In the ST, the epithelium height diminished and the absence of spermatozoa in the lumen can be noted. The results show that a very small difference of Cd dose causes a sudden increase in testicular damage, apparently overpowering this tissue's natural defenses. **Keywords:** Testis, reproductive organs, cadmium, morphometry, light microscopy, scanning electron microscopy.

1. Introduction

Cadmium (Cd) is a heavy metal and a major environmental toxicant. The general population is exposed to Cd via contaminants found in drinking water and food, while occupational exposition to Cd usually takes place during mining or manufacturing of batteries and pigments that contain Cd. Industrial activities, such as smelting and refining of metals, and municipal waste incineration also release Cd into the atmosphere (Siu et al. 2009). Tobacco smoke is another important source of Cd exposure (Blanco et al. 2007). Cd toxicity is associated with severe damage in various organs, particularly the testes, in both humans and animals (Fouad et al. 2009). The adverse consequences of exposure to this heavy metal on the reproductive organs have been widely considered. After acute exposure, cadmium-induced damage can be found at interstitial and tubular levels. Permeability changes in the capillary endothelium, which cause edema, hemorrhage or necrosis, seem to be implicated in the histopathological mechanism of these lesions (Blanco et al. 2007). The severity of intoxication depends on the route, dose, and duration of exposure to the metal (El-Missiry et al. 2000). An important factor involved in cadmium toxicity is metallothionein (MT). MT is a family of cysteine-rich, metal-binding proteins which play a role in Cd detoxification. It has been shown that Cd is an effective inducer of MT synthesis, which in turn protects host tissue from Cd damage. However, it was clear that MT synthesis is induced with more difficulty in the testis than in the liver, for example, which may account for the higher susceptibility of the testis to Cd toxicity (Xu et al. 2005).

Although it is well known that Cd is associated to adverse effects on male reproductive organs, culminating in diminishing reproductive capacities, very few studies have

quantified the morphological alterations caused by low doses of Cd exposure. These low doses were preferred for this research because preliminary tests using 1.4 mg/Kg of $CdCl_2$ (results not shown) resulted in extensive testicular damage, making morphometrical studies impossible. The current study was undertaken to evaluate histomorphometrically the threshold modifications of seminiferous tubules caused by single acute low doses of Cd.

2. Material and methods

2.1. Animals

Male Wistar rats were obtained from the Animal Multi-Research Center for Biological Investigation (State University of Campinas, Campinas, SP, Brazil). The animals were housed three per cage, with a 12 h light–dark cycle. They were supplied with standard laboratory chow and water, *ad libitum*. The experimental protocol followed the Guide for Care and Use of Laboratory Animals and was approved by the Committee for Ethics in Animal Experimentation of UNICAMP (1232-1).

2.2. Experimental design

Wistar rats, randomly assigned to three groups, were injected with saline or cadmium chloride ($CdCl_2$) solution, in which the first group (n = 12) received a single i.p. injection of saline (vehicle of cadmium) and served as the control group. The animals of the second (n=12) group received a single dose of 1 mg/kg of body weight (BW) of $CdCl_2$ while the

third group (n =12) received a single dose of 1.2 mg/Kg BW of $CdCl_2$. Six animals of each group were sacrificed after either 7 or 56 days.

2.3. Tissue Preparation

The animals under Ketamine (80 mg/BW) and Xylazine (5 mg/BW) anesthesia were fixed by whole body perfusion. Briefly, after a saline wash to clear the vascular bed of the testis, they were perfused with glutaraldehyde 2.5% and paraformaldehyde 4% in sodium phosphate buffer 0.1 M, (pH 7.2) for 25-30 minutes. Testis, epididymis, prostate, seminal vesicles and coagulating gland were removed, post fixed in the same solution overnight and then weighed. Historesin®-embedded testis fragments were sectioned at 3 µm thickness and stained with toluidine blue/1% sodium borate.

2.4. Biometry and Morphometry

The weight of testicular parenchyma was obtained subtracting the mass occupied by the albuginea from the total testis weight, thus providing the net weight of the organ's functional portion. The gonadosomatic index (GSI) was expressed as percentage of the total body weight in relation to the testis weight, GSI = (testes weight/total body weight) X100. The volume density of testicular tissue components were determined with light microscopy using Image Pro Plus software associated to an Olympus BX-40 microscope at 400X magnification. The total area occupied by seminiferous tubules and interstitial tissue was determined to calculate the volume density of each component. The volume of each testis

component was determined as the product of the volume density and parenchyma volume. For morphometric calculations, the specific gravity of testis tissue was considered to be 1.0 (Mori and Christensen, 1980). The tubular diameter of seminiferous tubules was measured at 100x magnification with the Image Pro Plus program associated to an Olympus BX-40 microscope. Thirty tubular profiles that were round or nearly round were chosen randomly and measured for each animal. The total length of the seminiferous tubule, expressed in meters, was obtained by dividing seminiferous tubule volume by the squared radius of the tubule multiplied by the pi value. These values were expressed per testis and per gram of testis (Russell et al. 1990).

2.5. Scanning electron microscopy (SEM)

After whole body perfusion fixation, the specimen fragments were fixed in Karnovsky´s fixative and rinsed three times with 0.1 M sodium phosphate buffer, pH 7.2, then in increasing saccharose solutions (0.5, 1.5, 3 %). The tissue was frozen in liquid nitrogen, fractured, post fixed in 1% osmium tetroxide, rinsed, dehydrated in an ascending ethanol series prior to critical point drying. The specimens were mounted on stubs, sputter-coated with gold and examined with the scanning electron microscope (Geol -JMS 560).

2.6. Statistical Analysis

Comparison of the values of control and treated groups was done by the variance analysis, Statistical (ANOVA), followed by Tukey's test. The results were considered

significant for $p < 0.05$. For all values, the means ± standard error mean (SEM) was calculated.

3. Results

3.1. Biometry and morphometry

The biometric and morphometric data are presented in Tables 1 and 2. No significant difference was found between the body weight of control and cadmium-treated rats in all of the groups studied. No difference was observed in liver weight in all treated groups, compared with the control. After 7 days, the higher dose of Cd caused a significant reduction in kidney weight. However, no alteration was observed for other treatments. At the Cd dose of 1.0 mg/Kg, no alteration was observed in testicular weights, however at the 1.2 mg/Kg dose, significant reduction occurred after 7 and 56 days. The epididymis weights in the low dose of cadmium were not different in any of the groups studied but at the higher dose a significant reduction was observed after 7 and 56 days. There was only a significant decrease for the higher dose group in the seminal vesicle, after 7 days. However, the ventral prostate, seminal vesicle and coagulating gland weights were not altered in all the groups studied. At the Cd dose of 1.0 mg/Kg, the GSI did not alter in all groups compared with the control. At the higher dose, a significant reduction was observed after 7 and 56 days. Cd significantly reduced the seminiferous tubule (ST) diameter in the higher dose, after 56 days, however in the other groups no alteration was detected. At the lower dose of Cd, no alteration was detected in the total length of ST per testis and per gram of testis in relation

to the control. At the higher dose, the total length of ST per testis diminished significantly after 7 and 56 days, however the value per gram of testis was not altered. The volume density of ST in the group submitted to the lower Cd dose increased, while the volume density of the interstitium decreased, both significantly, after 56 days. However, in the animals submitted to the higher dose of Cd after 7 days, the volume density of ST decreased and the volume density of interstitium increased significantly, and after 56 days this alteration was more prominent. The volume occupied by ST and the interstitium did not alter in the lower dose Cd-treated animals, compared with the control. In the higher dose, the volume density of ST diminished significantly but the volume of the interstitium was not changed, resulting in testicle shrinking.

Table 1: Body and organ weights of adult rats treated with two low doses of cadmium (mean ± S.E.M.).

Parameters	Control		Cd 1mg/Kg		Cd 1.2 mg/Kg	
	7 days	56 days	7 days	56 days	7 days	56 days
Body	387.83 ± 14.83	435.83 ± 15.93	383.17 ± 10.08	436.33 ± 10.09	366.83 ± 11.26	448.83 ± 21.67
Kidney	1.58 ± 0.07	1.64 ± 0.05	1.44 ± 0.05	1.67 ± 0.06	1.31 ± 0.05*	1.62 ± 0.06
Liver	14.58 ± 0.3	14.49 ± 1.04	13.66 ± 0.56	14,84 ± 1.16	13.83 ± 0.74	16.80 ± 1.05
Epididymis	0.49 ± 0.02	0.51 ± 0.02	0.45 ± 0.02	0.53 ± 0.01	0.40 ± 0.01*	0.35 ± 0.04*
Seminal vesicle	0.97 ± 0.05	1.04 ±0.06	0.96 ± 0.04	1.23 ± 0.08	0.63 ± 0.09*	1.18 ± 0.08
Coagulating gland	0.20 ± 0.02	0.22 ± 0.01	0.19 ± 0.05	0.25 ± 0.02	0.14 ± 0.02	0.22 ± 0.02
Ventral prostate	0.35 ± 0.02	0.48 ± 0.03	0.33 ± 0.01	0.57 ± 0.03	0.29 ± 0.03	0.43 ± 0.02

* Statistically significant differences, $p<0.05$.

Table 2: Testis weight and testicular morphometric parameters of adult rats treated with two low doses of cadmium (mean ± S.E.M.).

Parameters	Control		Cd 1mg/Kg		Cd 1.2 mg/Kg	
	7 days	56 days	7 days	56 days	7 days	56 days
Testis	1.68 ± 0.04	1.60 ± 0.10	1.59 ± 0.07	1.69 ± 0.07	0.86 ± 0.08*	0.91 ± 0.16*
GSI	0.88 ± 0.04	0.73 ± 0.03	0.83 ± 0.04	0.78 ± 0.02	0.48 ± 0.05*	0.40 ± 0.06*
Tubular diameter	292.10 ± 6.30	298.22 ± 6.87	285.71 ± 2.83	295.90 ± 4.01	262.93 ± 13.24	250.92 ± 16.88*
CT/ testis	20.70 ± 0.70	19.33 ± 1.95	19.96 ± 1.11	20.70 ± 0.87	10.94 ± 1.86*	10.56 ± 0.91*
CT/ g of testis	12.31 ± 0.51	11.94 ± 0.58	12.56 ± 0.25	12.23 ± 0.34	12.40 ± 1.06	13.52 ± 1.66
Seminiferous tubule	85.39 ± 0.73	85.97 ± 0.55	84.15 ± 0.80	87.53 ± 0.36*	71.98 ± 1.24*	65.19 ± 1.61*
Interstitial tissue	14.61 ± 0.73	14.03 ± 0.55	15.85 ± 0.80	12.47 ± 0.36*	28.02 ± 1.24*	34.81 ± 1.61*

* Statistically significant differences, $p<0.05$.

3.2. *Light and scanning electron microscopy*

Observations with light microscopy (Figure1) showed no marked changes in testicular tissue of animals submitted to the dose of 1 mg/Kg in relation to controls, as demonstrated by morphometry. Thus, normal spermatogenesis was seen with the usual interaction of germ cells and Sertoli cells. Also, in the interstitium and Leydig cells no alterations were observed. However, at the dose of 1.2 mg/Kg, progressive damage was observed after 7 and 56 days. After 7 days, the tubule lumens were filled with degenerated germ cells and multinucleated spermatid aggregates with apoptotic nuclei. Vacuolization of the seminiferous epithelium was also observed. After 56 days, increased damage resulted in vacuolated seminiferous tubules, often consisting only of Sertoli cells. SEM examination of this tissue (Figure 2) showed the interstitial tissue of control animals as having an intricate three-dimensional network. The interstitial tissue forms a delicate lattice with large fenestrae and well spaced cells, surrounding each seminiferous tubule. The seminiferous epithelium was intact and the seminiferous tubules showed the lumen filled with sperm flagella. A distinct space for fluid was observed between the interstitial tissue and the seminiferous tubules. In the group treated with 1.2 mg/Kg cadmium and sacrificed after 56 days, the interstitial tissue presents a compact, fibrous appearance with absence of fenestrae but with a larger number of cells. In the seminiferous tubule, the epithelium height diminished and the absence of spermatozoa in the lumen can be noted. Between the interstitium and the tubules the fluid space was diminished or not clearly observed.

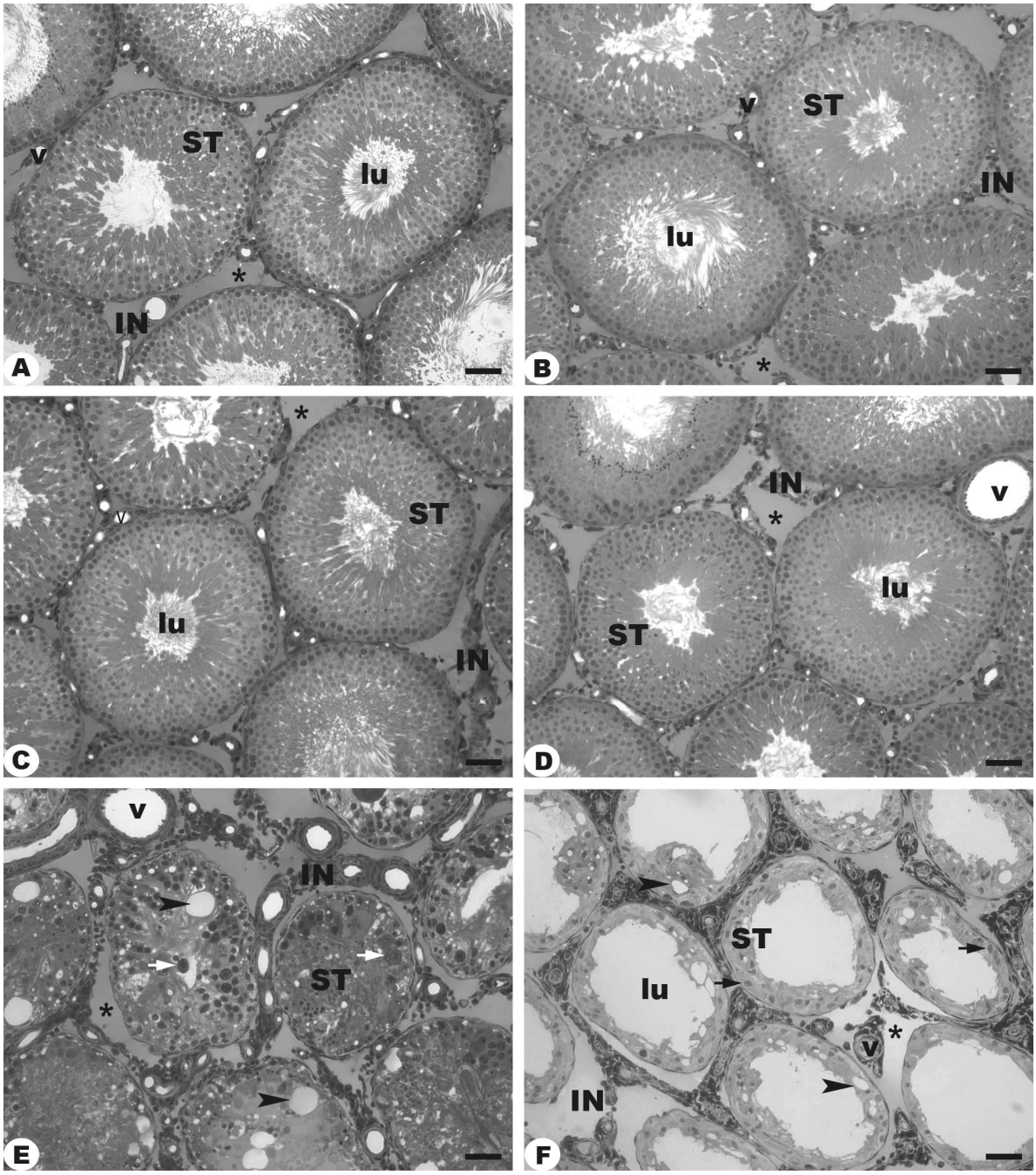

Figure 1- Light microscopy of a control rat testis and under the effect of cadmium. The seminiferous tubules (ST) are well preserved in controls (A: 7 days and B: 56 days) and in rats treated with cadmium 1 mg/Kg BW (C: 7 days and D: 56 days). Rats treated with cadmium 1.2 mg/Kg BW (E) exhibit degenerated seminiferous tubules with multinucleated spermatid aggregates with apoptotic nuclei, after 7 days. Note the reduced tubular diameter, absence of lumen (lu) and the thick, fibrous interstitium (IN). After 56 days (F), the progressive damage resulted in seminiferous

tubules lined only by Sertoli cells. Multinucleated spermatid aggregates with apoptotic nuclei and Sertoli cells are indicated by white and black arrows respectively. Blood vessels (v). Fluid space (Star). Vacuolization (Arrow head). Scale bars: 50 µm.

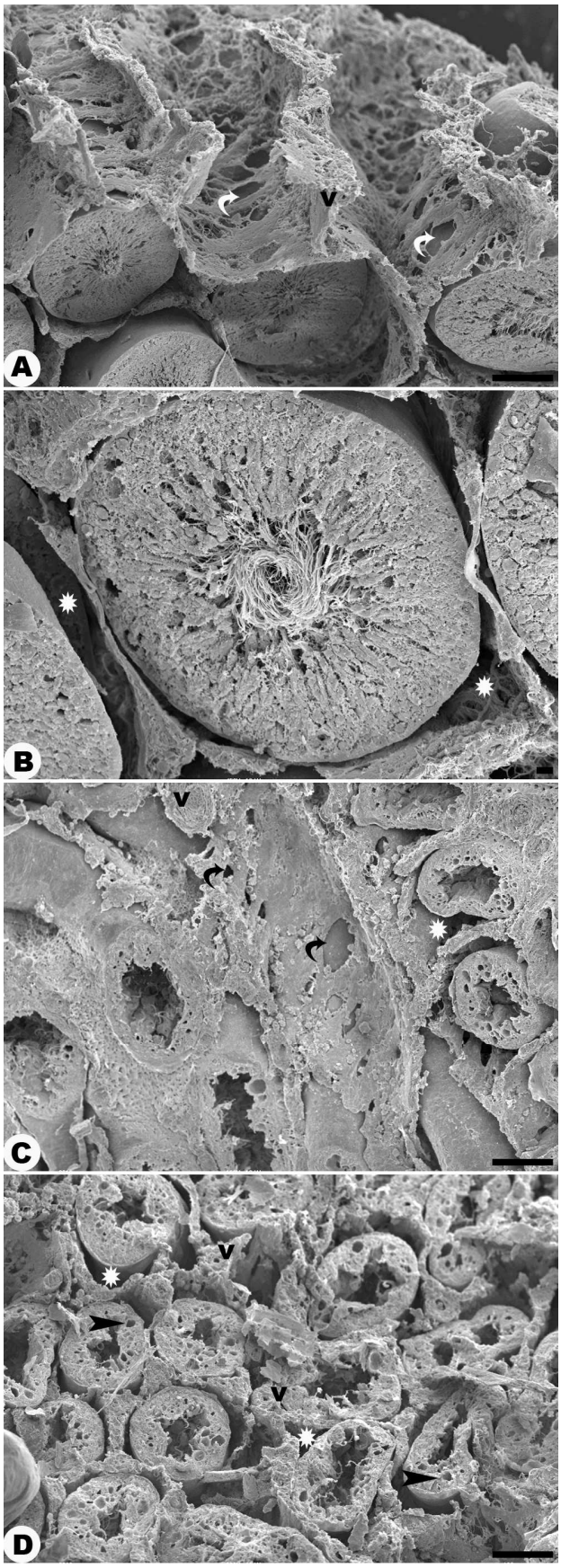

Figure 2- Scanning electron microscopy of control rat testis and under the effect of cadmium after 56 days. (A) Testicular parenchyma shows a delicate lattice with large fenestrae of interstitial tissue. The seminiferous tubules are well preserved in the control group. A large space for fluid was observed between the interstitial tissue and the seminiferous tubules. (B) Detail of the seminiferous tubule with intact epithelium and the presence of spermatozoa in the lumen. (C) In cadmium (1.2 mg/Kg BW) treated group, the interstitium shows a dense fibrous aspect with few fenestrae and reduced fluid space (white star). (D) The reduced epithelium height and vacuolization is observed in the seminiferous tubules. Note the absence of spermatozoa in the lumen. The fluid space was diminished or not clearly observed. Blood vessels (v) have thicker walls. Curved arrows: fenestrae. Scale bars: A, C, D: 100 µm; B: 10 µm

4. Discussion

Cadmium is an environmental and industrial pollutant that adversely affects the male reproductive system in humans and animals (El-Demerdash et al. 2004; Amara et al. 2008). Rodent testes are especially sensitive to the toxic effects of Cd exposure. Cd impairs reproductive capacity by causing severe testicular degeneration, seminiferous tubule damage and necrosis in rats (Burukoğlu & Bayçu, 2008). The present results showed that the toxic effects of Cd in the male reproductive system were dose-dependent. The difference between the lower and higher doses is very small but the morphological and morphometrical results are clearly very different. This suggests that the defenses of the testis against Cd contamination are efficient up to a very precise level, possibly depending on the level of metallothioenin (MT) present. It is widely accepted that the Cd bound to MT is nontoxic, thus MT protects tissue against Cd toxicity in this way. The balance between Cd-MT and free Cd in the tissue has been shown to be crucially important for toxicity (Xu et al. 2005). Moreover, Siu et al. (2009) and Xu et al. (2005) affirm that Cd treatment induces metallothionein production, but this appears to be limited to a certain level in the testis. Toxic effects probably result as the amount of MT becomes insufficient to bind with the Cd present, resulting in oxidative stress and disruption of spermatogenesis (Xu et al. 2005). The level of defense appears to vary for each animal, since the effects were not uniform for the two groups. Previous studies (Gupta et al. 2003; El-Dermerdash et al. 2004) have reported reductions of accessory sex organs after Cd administration. In the present research, after the higher dose of cadmium, the seminal vesicle reduced in weight, when measured on the seventh day, but after 56 days the usual weight was restored. However,

prostate and coagulating gland showed unaltered weight, with no alterations after either dose. Studies of the consequences of Cd contamination have demonstrated that the testis is more sensitive to Cd than other important organs, and that low doses, with no detectable effects on general health, can interfere with testis function (Blanco et al. 2007). These observations agree with our results showing that the weight and morphology of the testes were clearly affected only by the higher dose of Cd, whereas the physical appearance, body, kidney and liver weights of the animals remained unaltered. Previous studies of acute Cd exposure have reported diminished testicular weight in relation to the Cd dosage and attributed this effect to the necrotic and degenerative cadmium-induced changes (Blanco et al. 2007). With histological analysis, the absence of tubular lumen, germ cell loss and presence of multinuclear giant cells were observed after short term. These findings for the higher dose of Cd were in accordance with previous studies (Biswas et al. 2001; Yang et al. 2006; Blanco et al. 2007). When massive cellular loss from seminiferous epithelium occurs, a sharp decline in testicular morphometric parameters can be verified (França & Russell, 1998). Indeed, this decline has also been observed in the current work. A positive relationship usually exists between the tubular diameter and the spermatogenic activity of the testis (França & Russell, 1998; Sinha Hikim et al. 1989). Our data showed a marked reduction of seminiferous tubular diameter after the higher dose of Cd, together with the conspicuous decrease of the tubular volume density, which means that Cd caused a significant reduction in the relative seminiferous tubule length. Taking into account the fact that the weight of the testis was also reduced, it can be deduced that the total length of seminiferous tubule clearly diminished as a consequence of cadmium exposure. Supporting

these results, França & Russell (1998) state that the total seminiferous tubule length is related to three structural parameters: testis size, tubular diameter and seminiferous tubule volume density. An increase in testicular interstitial tissue and lymphatic space volume density occurred after Cd treatment; probably due to lymphatic and/or interstitial edema. According to Lirdi et al. (2008), this edema is a direct consequence of disruption of the endothelial layer, allowing fluids from the blood to flow into the interstitium. This study demonstrates the importance of morphometrical methods, as very sensitive instruments to evaluate threshold changes which could not be confirmed merely by the observation of testis morphology. The sensitivity of this method permitted the detection of differences such as the fact that few of the small modifications verified for the higher dose were found for the lower dose, and that there were differences in the tissue alterations according to a shorter or longer time lapse when the effect on spermatogonia can be felt. Clark (1976) describes the three dimensional organization of normal Sprague-Dawley testicular interstitial tissue. All parts of the interstitium are directly continuous and all seminiferous tubules are totally surrounded by interstitial tissue. However, the interstitium and the tubules are completely separated without cellular connections. Thus, a fluid space is formed and the fenestrae in the interstitium allow the fluid around adjacent tubules to communicate freely. No attention has been paid to the configuration of the tissue as a whole in cadmium treated animals. The 3-dimentional SEM observation in this study, showed cadmium-induced alterations in the interstitial tissue architecture that are not observed with light microscopy and morphometry analysis. In the present study, the interstitial organization was similar to that in control animals, which corroborates the data of Clark (1976). After

cadmium treatment, the interstitium had a thick, compact, fibrous aspect with few fenestrae. The few fenestrae could affect the fluid movement and the communication between the tubules. An increase in the cellular component, possibly fibroblasts, was observed, as well as thicker walled capillaries. The presence of vacuoles in Sertoli cells and the almost complete absence of spermatozoa demonstrate the impairment of spermatogenesis. According to Creasy (2001), vacuolization is the most common morphological response of Sertoli cells to various injuries. Subsequent to vacuolization, germ cell degeneration, disorganization, or exfoliation is generally seen, as observed in this experiment. Injury to the Sertoli cell has potentially serious consequences because of its pivotal role in supporting spermatogenesis. Although cell death rarely occurs, the metabolic and regulatory pathways of Sertoli cells are easily disturbed (Creasy, 2001). In conclusion, our findings showed the progressive evolution of morphological and morphometrical alterations caused by low doses of Cd due to their direct effect on rat testis. Our results highlight the direct relationship of dose and time lapse to morphological alterations. The results show the progressive damage that cadmium causes to the testis, suggesting that the eventual recuperation of the testis over longer periods is not to be expected.

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7. ARTIGO 2: ANTIOXIDATIVE AND IN VITRO ANTIPROLIFERATIVE ACTIVITY OF *Arctium lappa* ROOT EXTRACTS

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Abstract

Background: *Arctium lappa*, known as burdock, is widely used in popular medicine for hypertension, gout, hepatitis and other inflammatory disorders. Pharmacological studies indicated that burdock roots have hepatoprotective, anti-inflammatory, free radical scavenging and antiproliferative activities. The aim of this study was to evaluate total phenolic content, radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and *in vitro* antiproliferative activity in human cancer cell lines of different *A. lappa* root extracts. **Results:** Hot and room temperature dichloromethanic, ethanolic and aqueous extracts; hydroethanolic and total aqueous extract of *A. lappa* roots were investigated regarding radical scavenging activity by DPPH, total phenolic content by Folin–Ciocalteau method and antiproliferative *in vitro* activity was evaluated in human cancer cell lines. The hydroethanolic extract analyzed by high-resolution electrospray ionization mass spectroscopy demonstrated the presence of arctigenin, quercetin, chlorogenic acid and caffeic acid compounds, which were identified by comparison with previous data. Higher radical scavenging activity was found for the hydroethanolic extract. The higher phenolic contents were found for the dichloromethane, obtained both by Soxhlet and maceration extraction and hydroethanolic extracts. The dichloromethane extracts were the only extracts that exhibited activity against cancer cell lines, especially for K562, MCF-7 and 786-0 cell lines. **Conclusions:** The hydroethanolic extracts exhibited the strongest free radical scavenging activity, while the highest phenolic content was observed in Soxhlet extraction. Moreover, the dichloromethanic extracts showed selective antiproliferative activity against K562, MCF-7 and 786-0 human cancer cell lines.

Keywords: *Arctium lappa*, antioxidative and antiproliferative activity, male rats

Background

Arctium lappa L. (Asteraceae) is a Japanese plant and introduced in Brazil, which is widely used in popular medicine worldwide, as a diuretic and antipyretic tea as well as for hypertension, gout, hepatitis and other inflammatory disorders (Pereira *et al.*, 2005; Predes *et al.*, 2009b). The root has long been cultivated as a popular vegetable for dietary use and folk medicine (Lin *et al.*, 2002; Chen *et al.*, 2004). *A. lappa* tea has become a promising and important beverage, because of ample therapeutic activity (Lin *et al.*, 2002). In the literature, many health benefits have been reported due to different classes of bioactive secondary metabolites. These classes include, among others, flavonoids and lignans, for which *A. lappa* is an important natural source (Ferracane *et al.*). Pharmacological studies and clinical trials indicated that burdock roots have hepatoprotective (Lin *et al.*, 2000a; Lin *et al.*, 2002), anti-inflammatory (LIN *et al.*, 1996) and free radical scavenging activities (LIN *et al.*, 1996; Duh, 1998) attributed to the presence of caffeoylquinic acid derivatives (Maruta *et al.*, 1995). Recently, antiproliferative and apoptotic effects of lignans from *A. lappa* were described for leukemic cells (Awale *et al.*, 2006) as well as antitumor effects of arctigenin on pancreatic cancer cell lines (Matsumoto *et al.*, 2006). Consumption of dietary antioxidants from plant materials has been associated with lower incidence of diseases due to reduction of oxidative stress. Thus the aim of this study was to determine the total phenolic content by the Folin-Ciocalteau method, to evaluate the the antiradicalar

properties based on their ability to quench the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and *in vitro* antiproliferative activity in human cancer cell lines of eight different *A. lappa* root extracts.

Material and method

Plant material

The roots of *A. lappa* (Asteraceae) were collected at CPQBA, University of Campinas (UNICAMP), experimental field (Paulínia, Brazil) in August 2007. Dr. Glyn Mara Figueira was responsible for identification of the plant species. A voucher specimen was deposited at UNICAMP Herbarium under number 146021.

Extraction 1

Fresh milled roots (770 g) were extracted successively in a Soxhlet apparatus with dichloromethane, 95% ethanol and water (2:1 solvent/plant ratio), for 6 hours each solvent. The extracts were concentrated under vacuum (Buchi RE 215) until complete elimination of the organic solvent and subsequently freeze-dried for water elimination, providing dichloromethane (DHE), ethanolic (EHE) and aqueous hot (AHE) extracts.

Extraction 2

Fresh milled roots (276 g) were successively extracted by dynamic maceration with dichloromethane, 95% ethanol and water (1:5 plant/solvent ratio, 3 times each solvent), at room temperature, in an oscillating agitator (FANEM). The extracts were concentrated under vacuum (Buchi RE 215) until complete elimination of the organic solvent and subsequently freeze-dried for water elimination, providing dichloromethane (DE), ethanolic (EE) and aqueous (AE) extracts.

Extraction 3

Fresh milled roots (100 g) were extracted three times consecutively in Soxhlet extractor with water (1:5 plant/solvent ratio). The aqueous extract was freeze-dried, providing the total aqueous extract (TAE).

Extraction 4

Fresh milled roots (594 g) were extracted three times with 70% ethanol (1:5 plant/solvent ratio) under reflux, for 6 hours. The filtrates obtained were combined and concentrated under vacuum. The remaining water was freeze-dried resulting in the hydroethanolic extract (HE).

High-resolution eletrospray ionization mass spectroscopy (HRESI-MS) of hydroethanolic extract

HRESI-MS was recorded on a Q-Tof Mass Spectrometer (Micromass – U.K.) using direct infusion of a 10 µL.min-1 MeOH + 0.1 % formic acid solution and ionization by electrospray in the positive ion mode. Major operation conditions were as follows: capillary voltage of 3.5 kV, source temperature of 100 °C, desolvation temperature of 100 °C and cone voltage of 35 V.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Microplate DPPH assay was performed as described by Brand-Williams et al. (Brand-Williams *et al.*, 1995), modified by Brem et al. (Brem *et al.*, 2004). Briefly, in a 96-well plate, successive sample dilutions (100 µL/well, 0.25, 2.5, 25 and 250 µg/ mL), tested in triplicate, received DPPH solution (40 µM in methanol, 100 µL/well) and absorbance was measured at 550 nm with a microplate reader (VERSA Max, Molecular Devices). Results were determined every 5 min up to 150 min in order to evaluate the kinetic behavior of the reaction. The percentage of remaining DPPH was calculated as follows: % DPPH rem = 100 × ([DPPH] sample / [DPPH] blank). A calibrated Trolox standard curve was also made. The percentage of remaining DPPH against the standard concentration was then plotted in an exponential regression, to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (EC_{50}). The time needed to reach the

steady state for EC_{50} is defined as TEC_{50}. The antiradical efficiency (Jiménez-Escrig *et al.*, 2000), was calculated as follows: $AE = 1/(EC_{50} \times TEC_{50})$.

Total phenolic content

The total phenolic content was performed as described by Prior et al. (Prior *et al.*, 2005), with small modifications in order to use a microplate reader. Briefly, an aliquot (10 µL) of the sample (1 mg/mL) was diluted in distilled water (600 µL). Then, this solution was applied in a 96-well plate (150 µL per well), in triplicate, and received Folin-Ciocalteau solution (12.5 µL), sodium carbonate (37.5 µL, 1 M) and water (50 µL). After incubation at 37º C for 2 h, absorbance was measured at 725 nm with a microplate reader (VERSA Max, Molecular Devices). A calibrated Gallic acid standard curve was made and results were expressed as mg equivalents in Gallic acid per gram of sample.

***In vitro* antiproliferative activity assay**

Human tumor cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian expressing phenotype multiple drug resistance), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon), K562 (leukemia) were kindly provided by Frederick Cancer Research & Development Center – National Cancer Institute – Frederick, MA, USA. Stock cultures were grown in 5 mL of RPMI 1640 (GIBCO BRL, Life Technologies) supplemented with 5% fetal bovine serum. Penicilin: streptomycin

(1000 µg/mL:1000 UI/mL, 1mL/L) were added to the experimental cultures. Cells in 96-well plates (100 µL cells/well) were exposed to each extract in DMSO (0.25, 2.5, 25 and 250 µg/mL) at 37º C, 5% of CO_2 for 48 h. The final concentration of DMSO did not affect the cell viability. Then, a 50% trichloroacetic acid solution was added and after incubation (30 min at 4º C), washing and drying, cell proliferation was determined by spectrophotometric quantification (540 nm) of cellular protein content using sulforhodamine B assay. Using the concentration-response curve for each cell line the total growth inhibition (TGI), which is the concentration that produces total growth inhibition or a cytostatic effect, were determined through non-linear regression analysis using the software ORIGIN 7.5 (OriginLab Corporation) and corresponded to the test extract concentration necessary to inhibit proliferation of the cells.

Results and Discussion

The yields of the different extraction for *A. lappa* are listed in Table 1. The extraction efficiency of the solvents in the successive extractions increased in the order: ethanol > water > dichloromethane. The aqueous and hydroethanolic extraction exhibited the greatest yields.

The phenolic compounds are ubiquitous phytochemicals present in plant foods with various biological activities including antioxidant properties. They exert properties such as free radical scavenging and inhibiting the generation of reactive species (Gülçin, 2006; Zhang *et al.*, 2008). Phenolic compounds constitute a group of secondary metabolites that

are quite widespread in nature with several therapeutical properties(Gülçin, 2006; Gilioli A, 2007). Their antioxidant activity is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, free radical scavengers, singlet oxygen quenchers and metal chelators(Gilioli A, 2007).

Total phenolic content of all extracts are shown in Figure 1. The present study showed that the highest phenolic compound concentrations were obtained for Soxhlet extraction with dichloromethane (79.45 mg Gallic acid/g extract) and ethanol (77.26 mg Gallic acid/g extract) rather than extraction at room temperature. Whereas, the hydroethanolic extract (HE) showed a considerable phenolic content (72.61 mg Gallic acid/g extract). A previous study with *A. lappa* roots reported that the extraction with a chloroform and ethanol (1:1) mixture resulted in higher concentration to phenolic compounds (85.15 ± 0.55 mg Gallic acid/g dry extract), besides a great quantity of flavonoid (12.57 ± 0.05 mg quercetin/ g extract) in the chloroformic extract; moreover, they reported a phenolic content (65.92 ± 0.36 mg gallic acid/g extract) (Gilioli A, 2007) for the ethanol extract which is similar to that described herein. Also, researchers (Erdemoglu *et al.*, 2009) described that *Arctium minus* ssp *minus* leaves aqueous extract exhibited a total phenolic content of 58.93 ± 2.72 mg gallic acid/g of extract, while the ethanolic extract gave 48.29 ± 0.21 mg gallic acid/g of extract.

Many authors have reported a direct relationship between total phenolic content and antioxidant activity in various seeds, fruits and vegetables (Carvalho *et al.*; Gülçin, 2005; Gulcin *et al.*, 2008). Antioxidant properties, especially radical scavenging activities, are

very important due to the deleterious role of free radicals in foods and in biological systems [22]. The DPPH radical has been widely accepted as a tool for estimating free radical scavenging activities of various compounds and plant extracts (Gulcin *et al.*, 2008; Erdemoglu *et al.*, 2009). Although the present study evaluated the scavenging activity of all extracts, only hydroethanolic extract showed strong antiradicalar activity compared to the commercial standards used, licopene and trolox. Table 2 shows the scavenging effect of extracts and reference substances.

The hydroethanolic extract of *A. lappa* was then analyzed by high-resolution electrospray ionization mass spectroscopy and the presence of quercetin, arctigenin, chlorogenic acid and caffeic acid was demonstrated. These substances were identified by comparison with previous data (Ferracane *et al.*; Chen *et al.*, 2004; Matsumoto *et al.*, 2006). Phenolic compounds such as chlorogenic acid, caffeic acid (Chen *et al.*, 2004), and caffeoylquinic acid derivatives (Maruta *et al.*, 1995) were isolated from *A. lappa* roots. Also, flavonoids such as quercetin and rutin were isolated from leaves (Erdemoglu *et al.*, 2009) and roots (Scorzoni L, 2007) of *A. lappa*. Therefore, antioxidant properties of this plant could be attributed to these compounds. Moreover, Erdemoglu et al (Erdemoglu *et al.*, 2009) reported that *A. minus* leaves aqueous extract had antioxidant activity attributed to flavonoid thus corroborating our results for *A. lappa.*

The antiproliferative properties of the eight extracts of *A. lappa* roots were assessed by using nine human cancer cell lines, and the chemotherapeutic drug, doxorubicin, as a positive control. Among all extracts evaluated, dichloromethane extracts were the only ones

with antiproliferative activity. The most active extract (DE) presented a moderate activity for all cell lines with selectivity for K562 (TGI = 3.6µg/mL) and MCF-7 (TGI = 41.1µg/mL) (Figure 3) while DHE extract displayed the lowest activity with selectivity for K562 (TGI = 17.0µg/mL) and 786-0 (TGI = 155.7µg/mL) (Figure 3). The difference in the antiproliferative effects between hot and room temperature may have resulted from the different bioactive substances contained in the extracts due to the sensitivity to heat treatment. An antiproliferative activity study, using prostate cancer cells (LNCaP), attributed the inhibitory activity of *A. lappa* seeds hydromethanolic extract to the presence of compounds lappaol A, C and F (Ming *et al.*, 2004). A study performed with *A. lappa* showed that dichloromethane seed extract inhibits cancer cell viability under nutrient-deprived conditions, as observed in pancreatic cancer and hepatoma cell lines at 50 µg/ml concentration. The authors also reported the isolation of arctigenin which exhibits cytotoxicity by inducing necrosis in cancer cells (Awale *et al.*, 2006). Researchers (Matsumoto *et al.*, 2006)also reported that hydromethanolic extract of *A. lappa* fruits shows potent antiproliferative activity against B cell hybridoma cells (MH60) attributed to the presence of arctigenin. Ferracane et al (Ferracane *et al.*) recently isolated arctiin from *A. lappa* root, which demonstrated, according to other research groups, a strong cytotoxic effect on human hepatoma cell line (HepG2) (Moritani *et al.*, 1996), human lung cancer (A549), human ovarian cancer (K-OV-3), human skin cancer (SK-MEL-2); human CNS cancer (XF498) and human colon cancer (HCT15) (Ryu *et al.*, 1995).

A. lappa is plant popularly used in the diet as a vegetable and in alternative medicine because it has ample therapeutic action. Moreover, this plant is a component of

Flor-Essence® and Essiac®, which is two of the most widely used herbal products by cancer patients (Tamayo *et al.*, 2000; Tai *et al.*, 2004; Leonard *et al.*, 2006). Several experimental studies have shown evidence of biological activity of *A. lappa* extracts or active compounds including antioxidant, anti-inflammatory, free radical-scavenging, antibacterial and hepatoprotective actions (Predes *et al.*, 2009b). Thus the current study contributes to the growing literature which demonstrates that *A. lappa* show antioxidant and human tumor cell antiproliferative activities *in vitro*. Although, several studies demonstrated biological properties of *A. lappa in vitro*, further research is needed to elucidate the *in vivo* activities.

Conclusions

Our results demonstrated that hydroethanolic extracts exhibited the strongest free radical scavenger activity while the highest phenolic content was observed by Soxhlet extraction with dichloromethane, ethanol and hydroethanolic mixture. Moreover, the dichloromethanic extracts are the most important for this research in that they showed selective antiproliferative activity against K562, MCF-7 and 786-0 human cancer cell lines. On the other hand, the hydroethanolic extract had one of the greatest yield and shows free radical scavenger activity and high phenolic content, making this extract the best suitible for future studies.

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Authors' contributions

FSP: Was the person responsible for conception and design, acquisition of data, analysis and interpretation of data and drafted the manuscript.

MAF: made substantial contribution to conception and design, interpretation of data and revised it critically for important intellectual content

JEC: made substantial contribution to conception and design of the antiproliferative assay, interpretation of data and revised it critically for important intellectual content

ALTGR: made substantial contribution to conception and design of the antiproliferative, DPPH and Total phenolic content assay, interpretation of data and revised it critically for important intellectual content

Tables

Table 1: Yield of the different solvent extractions of *A. lappa* root

Extract	Yield
Dichloromethane hot extract	0.12 %
Ethanolic hot extract	6.39 %
Aqueous hot extract	2.87 %
Dichloromethanic extract	0.10 %
Ethanolic extract	4.45 %
Aqueous extract	3.51 %
Total aqueous extract	10.56 %
Hydroethanolic extract	10.25 %

Table 2: DPPH radical scavenging of *A. lappa* extract (mean ± SEM)

Sample	**EC_{50} (µg/ml)[a]**	**TEC_{50} (min)[b]**	**AE[c]**
Hydroethanolic extract	4.79 ± 0.15	5	0.0418 ± 0.001
Lycopene	21.28 ± 0.11	0.1	0.47 ± 0.002
Trolox	1.13 ± 0.1	0.1	8.98 ± 0.84

[a] Time needed to reach the steady state to EC_{50} concentration
[b] Antiradical efficiency = $1/EC_{50} \cdot TEC_{50}$
[c] Antiradical efficiency

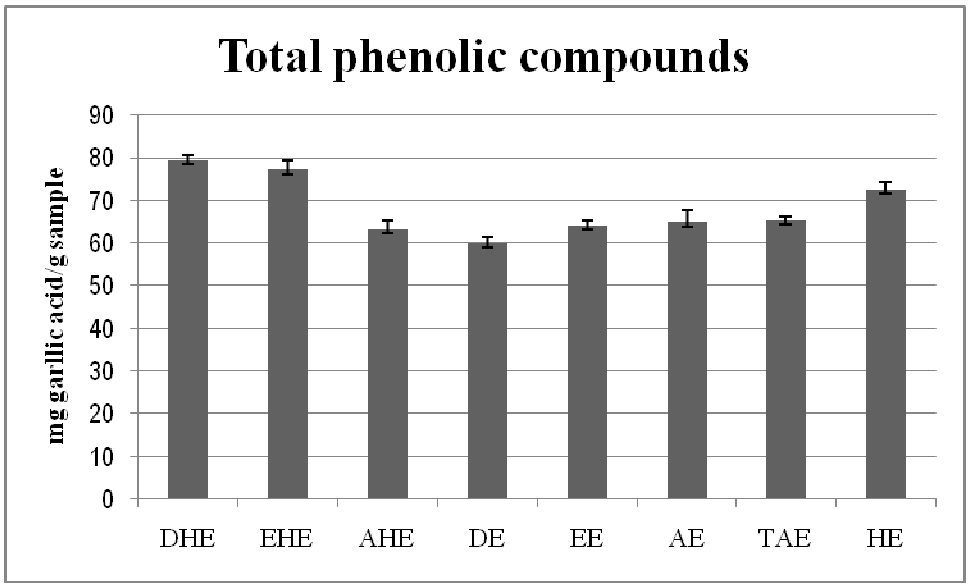
Table 3: Tumor growth inhibition (TGI) (µg/mL) induced by *A. lappa* extracts

	U	**M**	**A**	**7**	**4**	**P**	**O**	**H**	**K**
Doxo	3,22	0,16	16,79	0,20	0,05	0,34	>25	1,51	0,03
DHE	>250	>250	>250	155,79	>250	>250	>250	>250	17,06
DE	>250	41,12	>250	60,32	50,47	62,28	81,99	61,43	3,62

Legend: U = UACC-62 (melanoma), M = MCF-7 (breast), A = NCI-ADR/RES (expressing multiple drug resistance phenotype), 7 = 786-0 (renal), 4 = NCI-H460 (lung, non-small cells), P = PC-3 (prostate), O = OVCAR-3 (ovarian), H = HT-29 (colon), K = K562 (leukemia). Doxo= doxorubicin, DHE= dichloromethane hot extract, DE= dichloromethane extract.

Figure

Figure 1- Total phenolic compounds of *A. lappa* extracts



DHE: dichloromethane hot extract; EHE: ethanolic hot extract; AHE: aqueous hot extract; DE: dichloromethane extract; EE: ethanolic extract; AE: aqueous extract; TAE: total aqueous extract; HE: hydroethanolic extract. The black bars represent standard deviation.

8. ARTIGO 3: INVESTIGATION OF *Arctium lappa* ROOT EXTRACT EFFECT ON CADMIUM DAMAGED SEMINIFEROUS TUBULE AND EPIDIDYMIS OF ADULT WISTAR RATS

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ABSTRACT

Acute cadmium (Cd) exposure causes testicular and epididymal damage. This study has evaluated the potential protective effect of *A. lappa* (*Al*) root hydroethanolic extract. Male rats received a single i.p. dose of $CdCl_2$ (1.2 mg/Kg BW) with or without co-administration of *Al* extract daily by gavage (300 mg/kg BW) for 7 or 56 days. The cadmium caused significant reduction in the testis, epididymis and seminal vesicle weights, in the seminiferous tubule (ST) proportion, in total length of ST and in ST diameter. In the epididymis, this metal caused diminished tubular proportion and increased epithelium height. Degenerated germ cells and multinucleated aggregates with apoptotic nuclei were observed and the progressive damage resulted in tubules lined only with Sertoli cells. We concluded that *A. lappa* extract administration was inefficient to protect testis and epididymis against Cd toxicity.

Keywords: cadmium, *A. lappa*, testis, epididymis,

INTRODUCTION

Environmental problems have recently increased exponentially because of industrial pollution and the rapid growth of the human population. Toxic chemicals such as heavy metal ions discharged into the air, water and soil are introduced in the food chain. By entering the biological systems, they disturb biochemical processes, leading to health abnormalities and in some cases to fatal consequences. One of these heavy metals is cadmium (Cd) (Çavu o lu *et al.*, 2009). Cd toxicity is associated with severe damage in various reproductive organs in both humans and animals (Fouad *et al.*, 2009). After acute exposure, cadmium-induced testicular damage can be found at interstitial and tubular levels (Blanco *et al.*, 2007). The testicular dysfunction may arise from disturbances in Sertoli cells, which support spermatogenesis, or Leydig cells, that are responsible for androgen production under the regulation of the hypothalamic-pituitary-testicular axis (Bizarro *et al.*, 2003). In the epididymis, Cd causes atrophy, diminished diameter of the duct lumen, thickening of duct epithelium (Herak-Kramberger *et al.*, 2000) and apoptosis of epithelial cells (Huang *et al.*, 2005).

Many investigators proposed that a possible mechanism of Cd toxicity is the disturbance of pro-oxidant and antioxidant balance by generation of reactive oxygen species (El-Shahat *et al.*, 2009). Therefore, natural antioxidants and free radical scavengers have been used as a promising therapeutic approach toward diminishing Cd-induced damage. Recently, it was reported that *Hibiscus sabdariffa* (Asagba *et al.*, 2007), *Pluchea lanceolata* (Jahangir *et al.*, 2005), *Allium cepa* Linn, *Allium sativum* Linn (Ola-Mudathir *et al.*, 2008) extracts and *Camellia sinensis* tea (El-Shahat *et al.*, 2009) have the ability to

prevent or attenuate cadmium toxicity in various organs in rats and mice. *Arctium lappa*, a well-known traditional plant of Asiatic origin, has proven hepatoprotective action against several hepatotoxicants, such as carbon tetrachloride, acetaminophen, carrageenan and chronic ethanol consumption. Its property is attributed to the antioxidant and free radical scavenging activities (Lin *et al.*, 1996; Lin *et al.*, 2000; Lin *et al.*, 2002).

The quest for natural compounds and encouraging data on the efficacy of *A. lappa* against hepatotoxicants, prompted us to evaluate its therapeutic efficacy in cadmium-induced damage to male rat reproductive organs, especially in the architecture of testicular and epididymal tissue.

MATERIAL AND METHODS

Extract preparation

Fresh milled roots of *A. lappa* (Asteraceae) were extracted three times with 70% ethanol (1:5 plant/solvent ratio) under reflux, for 6 hours. The filtrates obtained were combined and concentrated under vacuum. The remaining water was freeze-dried, resulting in the hydroethanolic extract. This extract was diluted in water to treat the animals. The dose of 300 mg / kg body weight was chosen according to Lin *et al*. (1996) and Lin *et al*. (2002).

Animals

Male Wistar rats (90 days old) were obtained from the Animal Multi-Research Center for Biological Investigation (Campinas State University, Campinas, SP, Brazil). The animals were housed three per cage, with a 12 h light–dark cycle. They were supplied with standard laboratory chow and water, *ad libitum*. The experimental protocol followed the Guide for

Care and Use of Laboratory Animals and was approved by the Committee for Ethics in Animal Experimentation of UNICAMP (1232-1).

Experimental design

Adult Wistar rats were randomly assigned to four groups of 12 animals. The control group (C) received water by gavage. The cadmium group (Cd) received a single i.p. dose of 1.2 mg/Kg BW of cadmium chloride ($CdCl_2$) solution. *A. lappa* group (*Al*) received *A. lappa* extract (300 mg/Kg/BW) by gavage. The cadmium and *A. lappa* (Cd*Al*) group received the single dose of cadmium and the *A. lappa* extract by gavage as described for the groups Cd and *Al*. The C and Cd groups received water by gavage and the C and *Al* were injected with saline solution to simulate the same stress as the other groups. The animals were sacrificed after 7 and 56 days of treatment. Since cadmium is considered to be genotoxic (Giaginis *et al.*, 2006)**,** a 56 day interval was chosen considering the period necessary to complete a spermatogenic cycle (Russell *et al.*, 1990). The 7 day interval was chosen to observe short-term modifications caused by the cadmium (Predes *et al.*, 2010) associated with the proven antioxidant activity of the plant in this period (Lin *et al*., 1996; Lin *et al*., 2002).

Tissue Preparation

The animals under Ketamine (80 mg/BW) and Xylazine (5 mg/BW) anesthesia were fixed by whole body perfusion. After a brief saline wash to clear the vascular bed of the testis, they were perfused with glutaraldehyde 2.5% and paraformaldehyde 4% in sodium phosphate buffer 0.1 M, (pH 7.2) for 25-30 minutes. Testis, epididymis, prostate, seminal vesicles and coagulating glands were removed, post-fixed overnight in the same solution

and then weighed. Glycol methacrylate-embedded testis and epididymis fragments were sectioned at 3 µm thickness and stained with toluidine blue/1% sodium borate. For transmission electron microscopy, tissue samples were post-fixed in 1% osmium tetroxide, dehydrated in acetone, and embedded in epoxy resin. Ultrathin sections were cut using diamond knives and stained with 2% uranyl acetate and 2% lead citrate prior to observation with a transmission electron microscope (Zeiss, Leo 906).

Biometry, morphometry and stereology

The weight of testicular parenchyma was obtained subtracting the weight of albuginea from total testis weight, thus providing the net weight of the organ's functional portion. The gonadosomatic index (GSI) was expressed as a percentage of the total body weight in relation to the testis weight, GSI = (testes weight/total body weight) X 100. The morphometry and stereology was performed using Image Pro Plus software associated to an Olympus BX-40 microscope. The area of testicular tissue components was determined measuring the area occupied by seminiferous tubules and interstitium in fifteen fields per animal, at 400x magnification. The total area was used to obtain the volumetric proportion of each component. The tubular diameter of seminiferous tubules was measured at 100x magnification. Thirty tubular profiles that were round or nearly round were chosen randomly and measured for each animal. The total length of the seminiferous tubule, expressed in meters, was obtained by dividing seminiferous tubule volume by the squared radius of the tubule multiplied by the *pi* value. These values were expressed per gram of testis (Russell *et al.*, 1990). The area of epididymal tissue components was determined measuring the area occupied by epididymal tubules and interstitium in ten fields per animal

at 200x magnification. The total area was used to obtain the proportion of each component in caput and cauda epididymis. The caput and cauda epididymis epithelium height was measured in ten tubules per animal at 200 X magnification.

Statistical Analysis

Comparison of the values of control and treated groups was done by the variance analysis, Statistica (ANOVA), followed by Tukey's test. The results were considered significant for $p < 0.05$. For all values, the means ± standard error mean (SEM) was calculated.

RESULTS

Biometric data

Biometric data is in Table 1. After 7 days, no statistical change was observed in body weight. Over 56 days, all the animals gained weight and the final body weight was not statistically different among the groups. The epididymis weight was significantly reduced in Cd (7 days) and Cd and Cd*Al* (56 days) groups. The seminal vesicle and coagulating gland weights diminished significantly only in the Cd treated rats after 7 days. The testis and testicular parenchyma weight were decreased after 7 and 56 days in the Cd and Cd*Al* groups. The albuginea weight did not change in any of the groups (data not shown).

Table 1: Body and organ weights (g) of adult rats treated with hydroethanolic extract of *A. lappa* root and/or cadmium (mean ± S.E.M.).

Parameters	**7 days**				**56 days**			
	C	Cd	*Al*	Cd*Al*	C	Cd	*Al*	Cd*Al*
Body weight	387.83 ± 14.83	366.83 ± 11.26	371.50 ± 12.98	380.17 ± 13.37	435.83 ± 15.93	448.83 ± 21.67	437.67 ± 10.32	453.33 ± 18.42
Testis	1.68 ± 0.04	0.86 ± 0.08*	1.54 ± 0.09	1.2 ± 0.13*	1.60 ± 0.10	0.91 ± 0.16*	1.71 ± 0.05	0.87 ± 0.12*
Epididymis	0.49 ± 0.02	0.40 ± 0.01*	0.46 ± 0.02	0.47 ± 0.02	0.51 ± 0.02	0.35 ± 0.04*	0.55 ± 0.01	0.32 ± 0.03*
Seminal vesicle	0.97 ± 0.05	0.63 ± 0.09*	0.91 ± 0.01	0.82 ± 0.1	1.04 ± 0.06	1.18 ± 0.08	1.12 ± 0.03	1.07 ± 0.07
Coagulating gland	0.20 ± 0.02	0.14 ± 0.02*	0.20 ± 0.005	0.18 ± 0.02	0.22 ± 0.01	0.22 ± 0.02	0.24 ± 0.007	0.19 ± 0.01
Ventral prostate	0.35 ± 0.02	0.29 ± 0.03	0.33 ± 0.02	0.28 ± 0.02	0.48 ± 0.03	0.43 ± 0.02	0.50 ± 0.04	0.44 ± 0.05

* Values significantly different ($P < 0.05$).

Morphometry and stereology

A significant reduction was observed in the GSI of Cd and Cd*Al* after 7 and 56 days. The proportion of seminiferous tubule (ST) decreased significantly in the Cd (7 days) and in the Cd and Cd*Al* groups (56 days), consequently, an increase occurred in the interstitium proportion in these groups. The tubular diameter decreased only after 56 days in the groups Cd and Cd*Al*. The total length of ST had a significant decrease in the groups Cd and Cd*Al* at both 7 and 56 days (Table 2).

The proportion of epididymal tubule and interstitium of the caput and cauda of the epididymis did not change after 7 days in all treatments. However, after 56 days, percentage of tubules of the caput was reduced only in the Cd group. In the epididymis cauda, a decrease of tubular proportion in Cd and Cd*Al* groups was also observed, as well as an increase in the interstitial proportion. The epithelium height increased significantly in epididymis caput and cauda after 7 days in Cd and Cd*Al* groups. However, after 56 days, this increase was observed only in the epididymis cauda in both Cd and Cd*Al* groups (Table 3).

Table 2: Testicular morphometry and stereology parameters of adult rats treated with hydroethanolic extract of *A. lappa* root and/or cadmium (mean ± S.E.M.).

Parameters	7 days				56 days			
	Control	Cd	*Al*	Cd*Al*	Control	Cd	*Al*	Cd*Al*
GSI	0.88 ± 0.04	0.48 ± 0.05*	0.83 ± 0.05	0.63 ± 0.05*	0.73 ± 0.03	0.40 ± 0.06*	0.78 ± 0.02	0.38 ± 0.04*
Tubular diameter (µm)	292.10 ± 6.30	262.93 ± 13.24	298.10 ± 6.3	269.31 ± 16.03	298.22 ± 6.87	250.92 ± 16.88*	315.76 ± 11.95	241.26 ± 5.00*
Seminiferous tubule (%)	85.39 ± 0.73	71.98 ± 1.24*	85.54 ± 0.56	81.05 ± 3.13	85.97 ± 0.55	65.19 ± 1.61*	87.56 ± 0.36	63.86 ± 5.81*
Interstitial tissue (%)	14.61 ± 0.73	28.02 ± 1.24*	14.46 ± 0.56	18.95 ± 3.13	14.03 ± 0.55	34.81 ± 1.61*	12.44 ± 0.36	36.14 ± 5.81*
TL/ testis (m)	20.70 ± 0.73	10.94 ± 1.86*	18.23 ± 1.06	15.54 ± 0.80*	19.33 ± 1.95	11.52 ± 1.48*	18.64 ± 1.21	10.80 ±1.63*
TL/ g of testis (m)	12.31 ± 0.51	12.40 ± 1.06	11.87 ± 0.51	13.54 ± 1.16	11.94 ± 0.58	13.52 ± 1.66	10.35 ± 1.20	12.53 ± 1.17

* Values significantly different (P < 0.05).

Table 3: Epididymis morphometry and stereology parameters of adult rats treated with hydroethanolic extract of *A. lappa* root and/or cadmium (mean ± S.E.M.).

Parameters	7 days				56 days			
	Control	Cd	*Al*	Cd*Al*	Control	Cd	*Al*	Cd*Al*
Epididymis Caput								
Tubule (%)	88.12 ± 1.28	81.18 ± 3.03	85.74 ± 1.33	82.22 ± 2.92	86.80 ± 1.71	65.03 ± 3.05*	86.15 ± 1.54	80.04 ± 3.22
Interstitium (%)	11.88 ± 1.28	18.82 ± 3.03	14.26 ± 1.33	17.78 ± 2.92	13.20 ± 1.71	34.97 ± 3.05*	13.85 ± 1.54	19.96 ± 3.22
Epithelium height (µm)	28.56 ± 0.75	34.11 ± 0.67*	28.22 ± 0.99	34.06 ± 0.65*	28.05 ± 0.84	28.08 ± 0.60	29.30 ± 0.97	26.41 ± 0.85
Epididymis cauda								
Tubule (%)	89.28 ± 1.06	87.12 ± 1.07	85.25 ± 1.38	82.70 ± 3.56	90.20 ± 1.23	78.18 ± 3.20*	87.45 ± 0.45	80.42 ± 2.04*
Interstitium (%)	10.72 ± 1.06	12.88 ± 1.07	14.75 ± 1.38	17.30 ± 3.56	9.80 ± 1.23	21.82 ± 3.20*	12.55 ± 0.45	19.58 ± 2.04*
Epithelium height (µm)	16.31 ± 0.37	20.08 ± 1.30*	18.94 ± 0.97	24.12 ± 1.30*	17.70 ± 0.87	24.32 ± 0.84*	19.15 ± 0.81	24.60 ± 1.16*

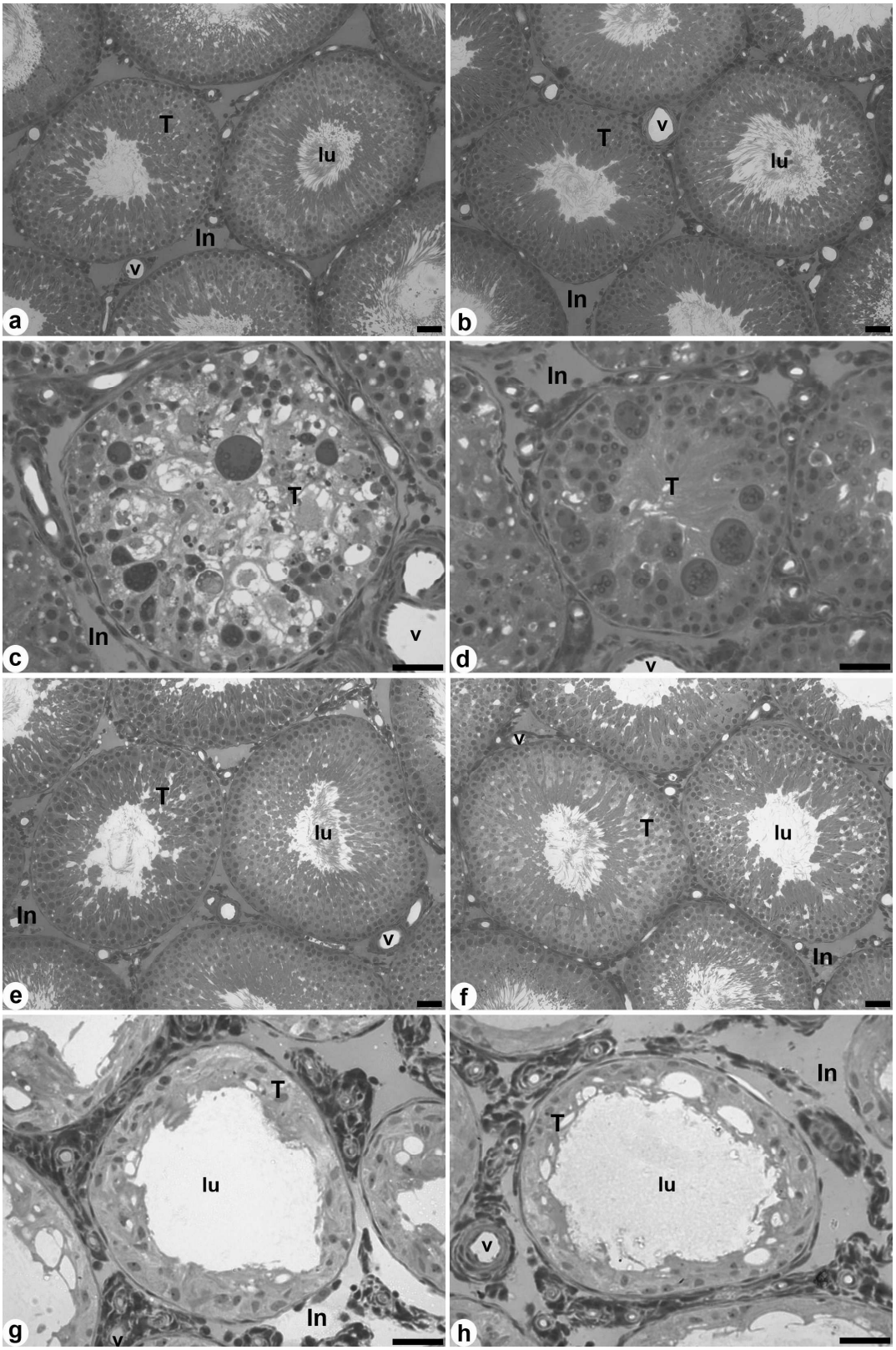
* Values significantly different (P < 0.05).

Testis morphology

Testis morphology was presented in Figure 1, 2 and 3. Light and transmission electron microscopy observations showed no changes in testicular tissue of *Al* groups compared to control group, as demonstrated by morphometry. Thus, normal spermatogenesis was seen with the usual association of germ cells and Sertoli cells.

Cd caused a severe damage as observed after 7 and 56 days both in Cd and Cd*Al* groups. After 7 days, the lumen was absent and the tubules were filled with degenerated germ cells and multinucleated aggregates containing apoptotic nuclei. Sertoli cells have numerous lipofuscin granules, residual bodies, lipid droplets and electron-lucid cytoplasmic vacuoles. Most cells of the seminiferous epithelium were degenerated, occupied the entire lumen of the tubule. Moreover, multinucleated giant cells were formed by clusters of spermatocytes and spermatids undergoing apoptosis, characterized by condensation of nuclear chromatin along the nuclear boundary. After 56 days, the testis of Cd and Cd*Al* groups showed increased damage, resulting in vacuolated seminiferous tubules consisting only of Sertoli cells. These cells presented numerous cytoplasmic vacuoles of irregular contour and are located at different levels of the cytoplasm from the basal area to the lumen. There was large amount of lipid droplets.

Figure 1: Testis morphology of adult rats treated with hydroethanolic extract of *A. lappa* root and/or cadmium. **a**-**d**: 7 days of treatment. **e**-**h**: 56 days of treatment. (**a and e**) Control groups. (**b and f**) *Al* groups. (e) After 7 days, groups Cd (**c**) and Cd*Al* (**d**) exhibit degenerated seminiferous tubules and multinucleated spermatid aggregates. Notice the reduced tubular diameter, absence of lumen (**lu**) and the thick and fibrous interstitium (**In**). After 56 days, the progressive damage resulted in seminiferous tubules lined only by Sertoli cells in the Cd (**g**) and Cd*Al* (**h**) groups. Scale bar: 15 (a,b,e,f) and 200 μm (c,d,g,h).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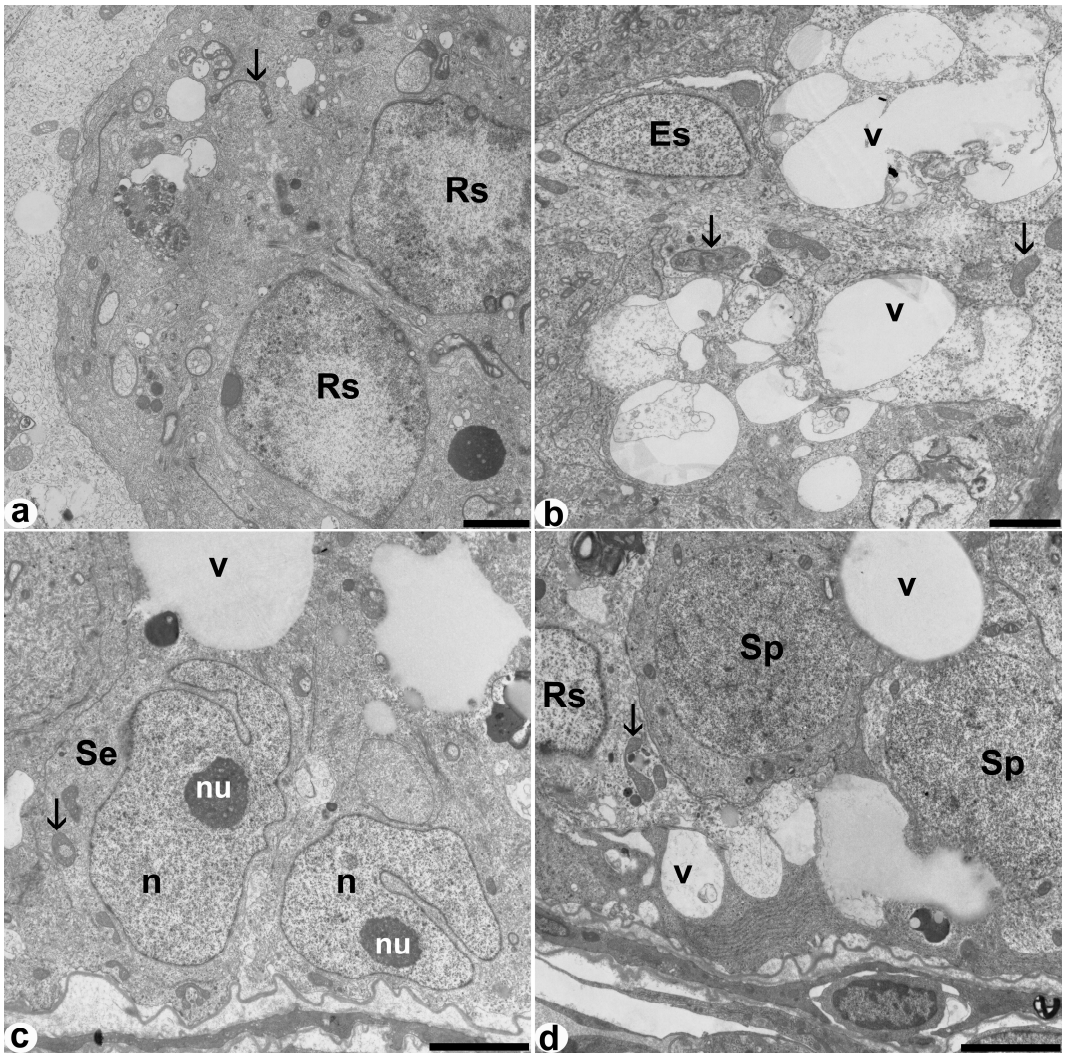


Figure 2- Testis ultrastructure of adult rats treated with cadmium and *A. lappa* root extract and cadmium after 7 days. **a** and **c**: Cd group. **b** and **d**: Cd*Al*. **a**: Multinucleated spermatid aggregates with apoptotic nuclei. **b**: Sertoli cell vacuolated cytoplasm. **c**: Sertoli cell irregular nuclei and vacuolated cytoplasm. **d:** Sertoli cell cytoplasm vacuolated. Rs: round spermatid. Es: elongated spermatid. v: vacuoles. Se: Sertoli cell. n: nucleus. nu: nucleolus. Sp: spermatocytes. Arrow: mitochondria. Scale bars: **a**: 2 µm. **b**: 3 µm. **c** and **d**: 4 µm.

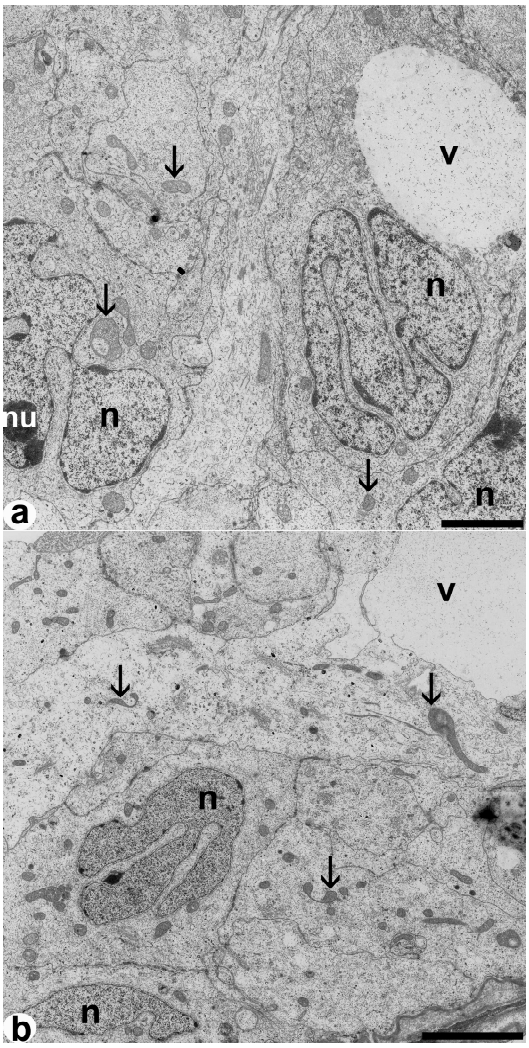
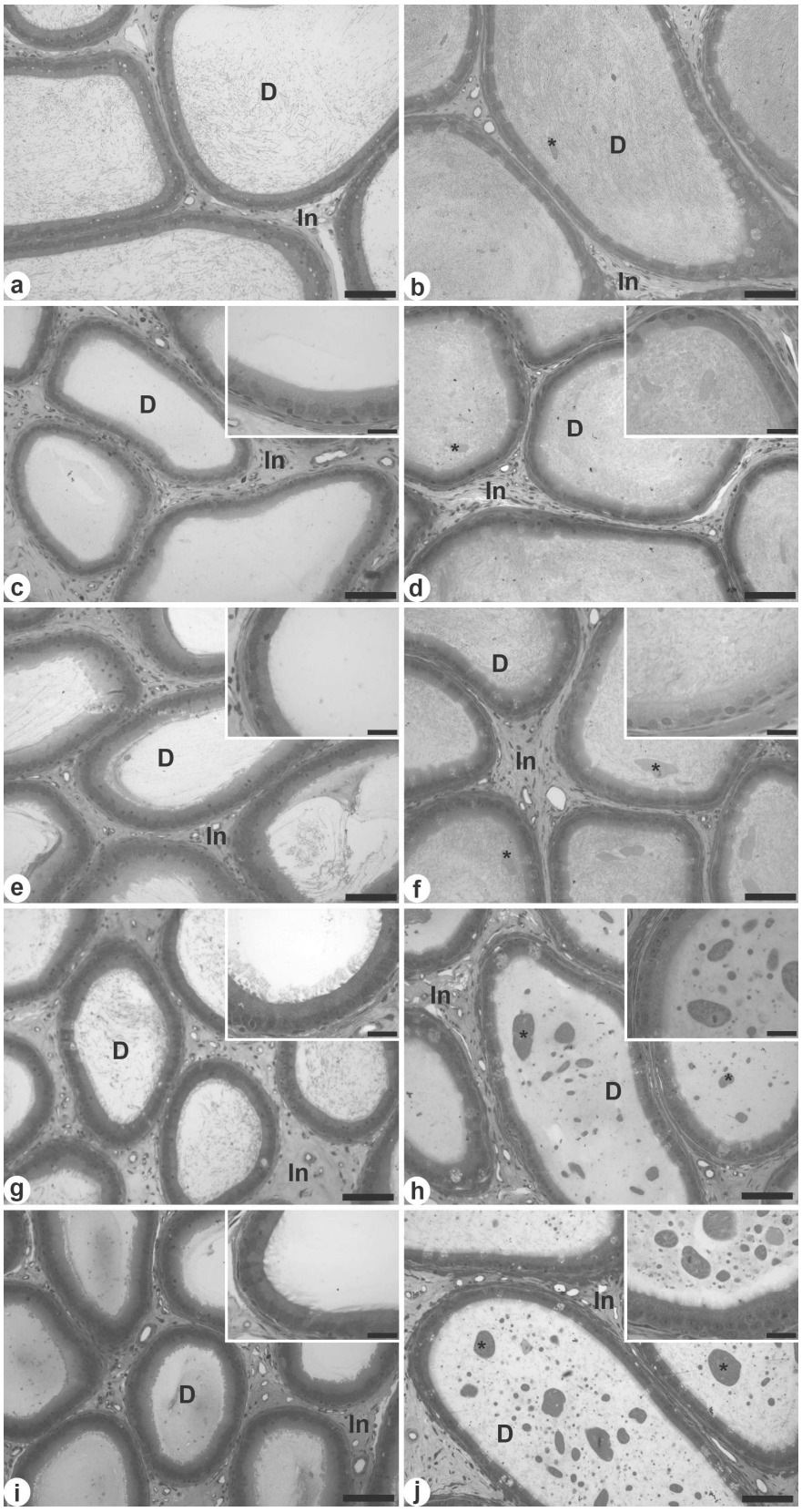


Figure 3- Testis ultrastructure of adult rats treated only with cadmium or *A. lappa* root extract and cadmium after 56 days. **a**: Cd group. **b**: Cd*Al*. **a** and **b**: Seminiferous tubule lined only by vacuolated Sertoli cells. v: vacuoles. n: nucleus. nu: nucleolus. Arrow: mitochondria. Scale bars: **a**: 2.5 µm. **b**: 5 µm.

Epididymis morphology

Light microscopy observations showed no changes in epididymal histology of *Al* groups, after 7 and 56 days, compared with their respective controls, corroborating the stereology. After 7 days no spermatozoa were observed in the caput region of both Cd and Cd*Al* groups. The duct lumen in caput and cauda was narrow and the epithelial cells were transformed into tall columnar cells. However, in the cauda, spermatozoa were present along with cell debris from the testis. After 56 days, spermatozoa were absent both in the caput and in the cauda, and the quantity of cell debris was higher in Cd and Cd*Al* groups (Figure 4).

Figure 4: Epididymis morphology of adult rats treated with hydroethanolic extract of *A. lappa root* and/or cadmium. **a**- Control caput (7 days); **b**- *Al* cauda (56 days); **c**-**f**: (7 days) **c**- Cd caput; **d**- Cd cauda; **e**- Cd*Al* caput; **f**- CdAl cauda; **g**- **j**: 56 days; **g**- Cd caput; **h**- Cd cauda; **i**- Cd*Al* caput; **j**- Cd*Al* cauda. T- Tubule; In- interstitium *Cell debris. Scale bar: 80 μm. Inset: 60 μm.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DISCUSSION

Although a great variety of biochemical assays have been performed to study the consequences of cadmium injury in rat testis, histopathology is rarely used to demonstrate the adverse effects. This study emphasizes the importance of the use of multiple endpoints to elucidate the damage caused by toxicants. The molecular mechanisms of cadmium testicular toxicity have not been clearly elucidated but it is possible to identify the probable target cell of its toxicity by a careful examination of the earliest morphological changes. In the present study, we investigated the effects of administration of *A. lappa* extract in Cd-injuried in testicular tubules and epididymal tissue, and the possible involvement of this plant in protection against Cd-induced damage in male rats.

In adult male rats, acute and chronic treatment with cadmium presents a well-documented toxic effect on reproductive organs and the production of androgens (Herak-Kramberger *et al.*, 2000). An indicator of reduced plasma testosterone level is the regression of seminal vesicles and prostate. Their secretory function is androgen dependent and very sensitive to circulating concentration of testosterone (Creasy, 2001). However, in the current study, the reduction of seminal vesicle and coagulating gland weights only 7 days after Cd administration did not allow to affirm the action of cadmium on Leydig cell or in hypothalamic-pituitary-testicular axis as reported previously by these authors (Manna *et al.*, 2008). This discordant result may be due to the administration of only a single low dose of cadmium.

It has been reported that doses of Cd as low as 1 - 2 mg kg/ body weight can cause testicular damage without pathological changes to other organs (Prozialeck *et al.*, 2006). In this study the dose administered did not cause apparent sign of impairment of

the general health of the animals as observed by unaltered body, liver and kidney weights (data not shown), behavior and physical appearance. However, it severely affected the rat testis and epididymis as observed by their weight loss. Chronic oral treatment of mice with cadmium also showed the same pattern response observed in our study after a single i.p. dose of cadmium, in which testicular damage occurred while general health was unchanged (Blanco *et al.*, 2007; Monsefi *et al.*, 2009). It was postulated that the mammalian testis is more sensitive than other organs because of its unique vasculature (Prozialeck *et al.*, 2008; Siu *et al.*, 2009).

The testis weight or size generally establishes the normalcy of the testis, enabling the assessment of experimentally induced changes in testicular size and potential spermatozoa production (França and Russell, 1998). Previous study of acute Cd exposure reported testicular weight loss due to the necrotic and degenerative cadmium-induced changes (Blanco *et al.*, 2007). Regardless of the primary site of toxic injury, most testicular toxicants will cause germ cell degeneration and depletion to a greater or lesser extent. If the insult is severe and prolonged, an end-stage lesion comprising tubules lined only with Sertoli cells will result. Although Sertoli cells are very sensitive to functional perturbation, they are remarkably resistant to cell death (Creasy, 2001).The alterations of the testis, such as the absence of a lumen, seminiferous tubules completely filled with degenerated germ cell, multinuclear giant cells and few developed sperm were verified after 7 days. Progressive degeneration resulting in tubules lined only of Sertoli cells after 56 days also are clear signs of spermatogenesis impairment. Electron microscopy evaluation of this tissue revealed that the multinuclear giant cells corresponded in the round spermatid stage, involved in

apoptosis, which is confirmed by the fact that after 56 days the tubule were lined only by Sertoli cells.

França & Russell (1998) reported that massive cell loss from seminiferous epithelium causes a sharp decline in testicular morphometric parameters. Indeed, the severe damage in the testicular epithelium observed clearly caused the decline of the quantitative parameters evaluated in the testis of the cadmium treated rats.

The quantitative information obtained in the present study corroborates to the morphological findings observed in rat testes after cadmium-induced testicular damage. Earlier studies affirm that a positive relationship usually exists between the spermatogenic activity of the testis and tubular diameter (Sinha Hikim *et al.*, 1988; França and Russell, 1998). Our study corroborates this affirmative, since the spermatogenic activity was progressively compromised, measured at intervals of 7 and 56 days, while the diameter of the seminiferous tubules proportionally decreased. This reduction in tubular diameter was the principle factor affecting reduction of tubule volume, since there was no variation in total tubular length.

The epididymis is frequently overlooked as a potential site for toxicity (Creasy, 2001). This can be seen by comparing the number of studies that investigate the effect of cadmium in the testis and epididymis. The first report (Sacerdote and Cavicchia, 1983) of early epididymal damage by Cd (3 µmol $CdCl_2$/100 g body weight) in Holtzman rats showed striking alterations in the ultrastructure of their blood vessels, such as disorganized endothelial cell junctions. These alterations range from slight separation of leaflets to wide gaps which communicated freely with the peri-capillary tissue. These authors also affirm that there was no lesion in the epididymal epithelium or in any other peritubular tissue. A recent study (Huang *et al.*, 2005), which

investigated the protective effects of vitamin E against Cd (injected subcutaneously with 2 mg/kg/time of $CdCl_2$ twice a week for three months in mice) showed degenerated and necrotic and apoptotic epithelial cells in epididymal ducts caused by Cd and the protective effect of vitamin E. In the present study, a decrease of tubular proportion was observed accompanied by the thickening of epithelium height and decrease of duct lumen. According to these authors (Herak-Kramberger *et al.*, 2000), these morphological alterations may be related to the level of testosterone in the animals. Moreover, the transformation of the epithelium into a uniformly tall columnar shape might represent dedifferentiation of the cells into a morphological and functional state that is characteristic of prepubertal or castrated rats. Since the organs used as indicators of the hormonal status did not show relevant change in androgen level, we suggest that changes in epididymal tissue were due to the toxic effect of the cadmium.

The administration of *A. lappa* alone in the rat did not cause any alteration in testis and epididymis morphology, morphometry and stereology. This data is in accordance with previous study that showed non-toxicity of the aqueous extract of *A. lappa* leaves. In this case, unaltered body weight was verified, indicating normal functioning of vital organs and reproductive organs such as testis, epididymis and accessory reproductive glands (Predes *et al.*, 2009). In this study, unaltered data of testis and epididymis showed that *A. lappa* did not cause spermatogenesis impairment.

Many researchers (Lin *et al.*, 1996; Lin *et al.*, 2000; Lin *et al.*, 2002) have demonstrated the antioxidant and free radical scavenger properties of *A. lappa* and also showed the hepatoprotective effects of *A. lappa* against toxicants such as carrageenan, carbon tetrachloride, acetaminophen and ethanol. However, when *A. lappa* extract was administered in the Cd-treated animals, the same morphological, morphometric and

stereological parameters alterations were observed as in the animals treated only with the metal. So it can be concluded that the dose, route and period of administration of the extract used in this study were ineffective in avoiding testis and epididymis cadmium injury.

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9. ARTIGO 4: PROTECTIVE EFFECT OF *Arctium lappa* ROOT EXTRACT IN CADMIUM DAMAGED LIVER OF ADULT WISTAR RATS

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ABSTRACT

The present study was performed to determine the protective effects of *Arctium lappa (Al)* root extract against cadmium-induced oxidative damage in rat liver. Male rats received single i.p. dose of $CdCl_2$ (1.2 mg/Kg BW) with or without *Al* extract administered daily by gavage (300 mg/kg BW) for 7 or 56 days. The relative liver weight did not vary in all groups studied both after 7 and 56 days of treatment. After 7 days, *Al* caused plasma glutamic oxalacetic (GOT) and glutamic pyruvic (GPT) transaminases levels to decrease and Cd*Al* treatment caused reduction of GPT levels. After 56 days, GOT and GPT levels were higher in the Cd group. No alteration in plasma levels of creatinine, total bilirubin and total protein was verified in all treatments was verified, both at 7 and 56 days. In liver transaminase assay, only GOT level was increased in the Cd group and no alteration was observed in the other groups studied. After 7 days, in the Cd group, hepatocyte proportion decreased and sinusoid capillary proportion increased. In *Al* and Cd*Al* groups, the nuclear proportion increased and cytoplasmic proportion decreased with no change in capillary sinusoid proportion. The hepatocyte nuclei density reduced in the Cd and increased in the *Al* group. After 56 days, there was no alteration in hepatic lobular parenchyma proportion in the Cd group.

However, in *Al* and Cd*Al* groups, the nuclear proportion increased without cytoplasmic proportion variation, but sinusoid capillary proportion was reduced. The hepatocyte nuclei density decreased in the Cd group and increased in *Al* and Cd*Al* groups. In conclusion, *A. lappa* effectively protected liver tissue from cadmium-induced acute hepatotoxicity.

Keywords: cadmium, *A. lappa*, liver, transaminases

INTRODUCTION

Cadmium (Cd) is a common environmental pollutant and Cd emissions to the atmospheric, aquatic and terrestrial environment have increased dramatically during the twentieth century (Vicente-Sanchez *et al.*, 2008). Most human exposure comes from food, water as well as cigarette smoke and air contaminations (Jihen *et al.*, 2008). The wide environmental distribution of Cd led to greater interest in its toxicity and biological effects (Vicente-Sanchez *et al.*, 2008). Several studies revealed that Cd accumulates preferentially in hepatic and renal tissues. In the liver, Cd can, even in low concentration, cause distinct pathological changes (Jihen *et al.*, 2008). Cd is known to disturb the oxidative balance of the tissues, thus oxidative stress is involved in the mechanism by which this metal affects health (El-Sokkary *et al.*, 2009). Many studies have reported the efficiency of natural antioxidants and free radical scavengers, such as isolated substances or plant extracts, in reducing damage caused by Cd in various organs. *Arctium lappa*, a well-known traditional plant of Asiatic origin, has proven hepatoprotective action against several hepatotoxicants, such as carbon tetrachloride, acetaminophen, carrageenan and chronic ethanol consumption. Its property is attributed

to the antioxidant and free radical scavenging activity (Lin *et al.*, 1996; Lin *et al.*, 2000a; Lin *et al.*, 2002). The quest for natural compounds and encouraging data on the efficacy of *A. lappa* against hepatotoxicants, prompted us to evaluate its therapeutic effects in acute Cd intoxication of the liver.

MATERIAL AND METHODS

Extract preparation

Freshly milled roots of *A. lappa* (Asteraceae) were extracted three times with 70% ethanol (1:5 plant/solvent ratios) under reflux, for 6 hours. The filtrates obtained were combined and concentrated under vacuum. The remaining water was freeze-dried resulting in the dry hydroethanolic extract. This extract was diluted in water to treat the animals. The dose of 300 mg / kg body weight was chosen according to Lin *et al*. (1996) and Lin *et al*. (2002).

Animals

Male Wistar rats (90 days old) were obtained from the Animal Multi-Research Center for Biological Investigation (State University of Campinas, Campinas, SP, Brazil). The animals were housed three per cage, with a 12 h light–dark cycle. They were supplied with standard laboratory chow and water, *ad libitum*. The experimental protocol followed the Guide for Care and Use of Laboratory Animals and was approved by the Committee for Ethics in Animal Experimentation of UNICAMP (1232-1).

Experimental design

Adult Wistar rats were randomly assigned to four groups of twenty four animals. The control group (C) was i.p. injected with saline. The cadmium group (Cd) received a single dose of 1.2 mg/Kg BW of cadmium chloride ($CdCl_2$) solution. *A. lappa* group (*Al*) was injected once with saline and received *A. lappa* extract by gavage. The Cd and *A. lappa* (Cd*Al*) group received the single dose of cadmium and the *A. lappa* extract by gavage. The C and Cd group received water by gavage to simulate the same stress as the other groups. Twelve animals of each group were sacrificed after either 7 or 56 days of treatment. The 7 days interval was chosen because of the proven antioxidant activity of the plant in this period (Lin *et al*., 1996; Lin *et al*., 2002). The 56 day interval was chosen to study the long-term effect of the plant.

Tissue Preparation

Rats were anesthetized with Ketamine (80 mg/BW) and Xylazine (5 mg/BW). Blood samples were collected by cardiac puncture in a heparinized syringe. The fresh liver was collected in six animals of each group and kept at - 80º C for biochemical analysis. Six animals of each group were fixed by whole body perfusion with glutaraldehyde 2.5% and paraformaldehyde 4% in sodium phosphate buffer 0.1 M, (pH 7.2) for 25-30 minutes. The liver was removed, weighed and post fixed in the same solution overnight and then weighed. Paraffin-embedded liver fragments were sectioned at 5 µm thickness and stained with hematoxylin and eosin.

Biochemical analyses

Blood was centrifuged at 10000 rpm for 5 minutes. The plasma was removed to be assayed for glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), creatinine, bilirubin and total protein. The liver was homogenized

in cold potassium phosphate buffer (0.05 M, pH 7.4), centrifuged at 5000 rpm for 10 min at 4 ºC. The resulting supernatant was used for determination GOT and GPT activities by colorimetric assay kits according to the recommendations of the manufacturer (Bioclin, Brazil) using a spectrophotometer at 505 nm.

Stereology

The stereology was performed using Image Pro Plus software associated to Olympus BX-40 microscope. The proportion of hepatic parenchyma components (hepatocyte cytoplasm or nucleus, sinusoid capillary and Kupffer cell) was determined using a grid with 120 intersections in ten fields per animal at 200 X magnification. Two thousand points were counted for each animal. The number of hepatocyte nuclei were counted in a total tissue area of approximately 3.7 mm^2 (10 fields per animal) and the nuclei density was calculated as number of nuclei/mm^2.

Statistical Analysis

Comparison of the values obtained from control and treated groups was done by the variance analysis, Statistica (ANOVA), followed by Tukey's test. The results were considered significant for $p < 0.05$. For all values, the means ± standard error mean (SEM) was calculated.

RESULTS

Biometry

After 7 days, no statistically change was observed in body weight. Over the 56 days, all the animals gained weight and the final body weight was not statistically significant for all groups. The relative liver weight did not vary in all groups studied after both 7 and 56 days of treatment. The relative kidney weight in animals treated with cadmium decreased after 7 days, however the other treatments did not induce changes in this parameter even after 56 days.

Table 1: Body and organ weights (g) of adult rats treated with *A. lappa* root extract and/or cadmium (mean ± S.E.M.).

Parameters	**7 days**				**56 days**			
	C	Cd	*Al*	Cd*Al*	C	Cd	*Al*	Cd*Al*
Body weight	387.83 ± 14.83	366.83 ± 11.26	371.50 ± 12.98	380.17 ± 13.37	435.83 ± 15.93	448.83 ± 21.67	437.67 ± 10.32	453.33 ± 18.42
Liver relative weight	3.78 ± 0.10	3.77 ± 0.18	3.40 ± 0.16	3.98 ± 0.13	3.38 ± 0.35	3.75 ± 0.17	3.30 ± 0.12	3.53 ± 0.16
Kidney relative weight	0.41 ±0.02	0.36 ±0.01*	0.37 ±0.02	0.40 ±0.01	0.38 ±0.01	0.36 ±0.01	0.35 ±0.01	0.36 ±0.01

* Level of significance $p < 0.05$.

Plasma biochemical analysis

After 7 days, the plasma levels of GOT and GPT were significantly reduced in the *Al* group and the GPT level was also reduced in the Cd*Al* group. After 56 days of treatment, the level of GOT and GPT were significantly increased in the Cd treated group. In the *CdAl* group, GOT and GPT levels were similar to those found in the control group. There was no change in plasma levels of creatinine, total bilirubin and total protein in all treatments, after both at 7 to 56 days (Table 2).

Liver transaminases analysis

The liver transaminase assay, after 7 days, showed no alteration in all other groups. After 56 days, despite the significant increase in concentration of GOT in Cd*Al* group, no more relevant results were found for GOT and GPT levels in the other groups (Table 2).

Table 2: Plasma and liver component levels of male rats treated with hydroalcoholic root of *A. lappa* and / or cadmium for 7 and 56 days.

Parameters	7 days				56 days			
	Control	Cd	*Al*	Cd*Al*	Control	Cd	*Al*	Cd*Al*
Total bilirrubin	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.00
Creatinin	0.57 ± 0.03	0.5 ± 0.00	0.5 ± 0.04	0.48 ± 0.04	0.52 ± 0.04	0.59 ±0.01	0.55 ± 0.03	0.57 ± 0.02
Total proteins	6.14 ± 0.05	6.10 ± 0.04	6.14 ± 0.03	6.05 ± 0.10	6.40 ± 0.07	6.43 ± 0.08	6.64 ± 0.08	6.33 ± 0.09
Plasma GOT	166.83 ± 13.97	192.67 ± 19.36	106.92 ± 9.13*	161.92± 2.60	169.73 ± 13.56	250.12 ± 9.60*	153.32 ± 13.39	143.67 ± 17.42
Plasma GPT	54.71 ± 2.06	55.86 ± 2.64	45.17 ± 3.12*	45.25 ± 1.68*	55.10 ± 2.72	64.67 ± 2.49*	50.83 ± 2.54	58.83 ±3.89
Liver GOT	255.71 ± 18.36	350.60 ± 32.68	292.38 ± 16.14	352.38 ± 43.70	111.88 ± 9.85	118.82 ± 5.51	126.24 ± 11.17	138.82 ± 3.75*
Liver GPT	242.27 ± 15.52	267.73 ± 23.90	250.45 ± 18.53	282.42 ± 32.66	210.76 ± 21.53	226.19 ± 5.56	232.10 ± 6.90	240.76 ± 5.84

* Level of significance $p < 0.05$. GOT: glutamic oxalacetic transaminase. GPT: glutamic pyruvic transaminase

Stereology and morphology

7 days

In the Cd group, the proportion of hepatocyte nuclei, cytoplasm, and consequently the hepatocyte proportion decreased significantly associated to the significant increase of sinusoid capillary proportion. In the *Al* and Cd*Al* groups, the nuclear proportion increased significantly accompanied with the decrease of cytoplasmic proportion, maintaining the hepatocyte proportion constant. Moreover, the capillary sinusoid proportion also did not change with the treatments. The Kupffer cell proportion did not alter in all groups studied. The density of hepatocyte nuclei was reduced in Cd group and increased in *Al* group. The administration of *A. lappa* after Cd treatment maintained the hepatocyte nuclei density similar to the control group. These stereological data confirm the histopathological examination. Light microscopic examination indicated the normal liver structure in the controls. Exposure to Cd induced hepatocyte cytoplasmic vacuolization and condensed nuclear chromatin, and sinusoidal widening. The administration of the extract after acute Cd dose alleviated the effects of the metal in the liver (Figure 1 and Table 3).

56 days

In the Cd group, no alteration was observed in the components of hepatic lobular parenchyma. However, the treatment with *Al* alone or after Cd administration, caused a significant increase in nuclear proportion associated with the greater hepatocyte proportion, without variation in cytoplasmic proportion. Consequently, the proportion of sinusoid capillaries was reduced. Kupffer cell proportion increased slightly in Cd*Al* group. The

hepatocyte nuclei density was decreased in the Cd group and increased in *Al* and Cd*Al* groups. Despite stereological Cd data showing no damage caused by Cd, morphological examination showed hepatocyte cytoplasmic vacuolization and condensed nuclear chromatin, and sinusoidal widening. The protective effects of the extract on liver histology are seen also after long term (Figure 1 and Table 3).

Figure 1 – Liver morphology of adult rats treated with *A. lappa* root extract and/or cadmium. **a**, **c**, **g**, **e**: 7 days of treatment. **b**, **d**, **f**, **h**: 56 days of treatment. **a** e **b**: Control groups. **c** e **d**: Cd groups. **e** e **f**: *Al* groups. **g** e **h**: Cd*Al*. Thin arrow: Kupffer cells. Thick arrow: hepatocyte nucleus. Star: sinusoid capillaries. Scale bar for all figures: 300 μm.

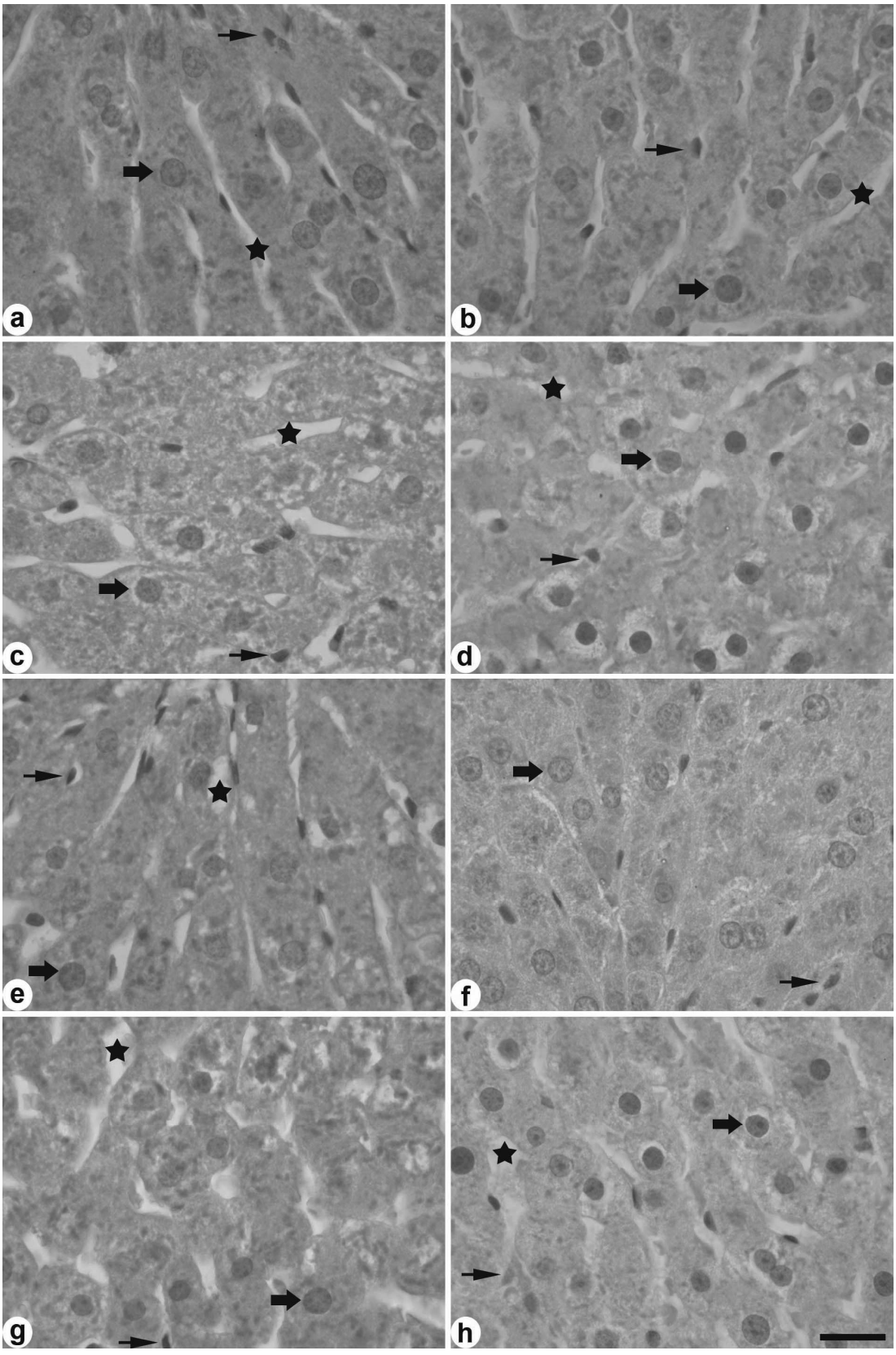
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Table 3: Liver components of male rats treated with *A. lappa* root extract and / or cadmium for 7 and 56 days.

Parameters	7 days				56 days			
	Control	Cd	*Al*	Cd*Al*	Control	Cd	*Al*	Cd*Al*
Hepatocyte nucleus (%)	9.91 ± 0.48	8.59 ± 0.27*	11.70 ± 0.42*	11.97 ± 0.40*	10.78 ± 0.24	10.64 ± 0.40	12.88 ± 0.40*	11.88 ± 0.46*
Hepatocyte cytoplasm (%)	76.26 ± 0.61	72.75 ± 0.69*	73.93 ± 0.30*	72.70 ± 0.97*	73.69 ± 0.65	73.17 ± 1.12	75.13 ± 0.46	74.71 ± 0.52
Sinusoid capillary (%)	12.18 ± 0.75	16.68 ± 0.67*	12.75 ± 0.41	13.46 ± 0.52	14.06 ± 0.61	14.71 ± 0.87	10.33 ± 0.34*	11.71 ± 0.44*
Kupffer cell (%)	1.65 ± 0.12	1.98 ± 0.17	1.63 ± 0.09	1.87 ± 0.25	1.46 ± 0.05	1.49 ± 0.12	1.66 ± 0.05	1.71 ± 0.05*
Hepatocyte nucleus number/mm^2	40.77 ±1.91	32.30 ±1.53*	45.86 ±0.81*	42.30 ±1.67	45.81 ±1.68	41.03 ±1.2*	58.51 ±1.1*	56.89 ±1.9*

* Level of significance $p < 0.05$

DISCUSSION

Natural products which possess free radical scavenger activity have become a central focus for research designed to prevent or diminish tissue injury. In the present study, the therapeutic efficiency of *A. lappa* was tested in preventing liver injury caused by cadmium.

In this study, the dose of Cd injected did not cause apparent signs of impairment of the animal's general health, as observed by unaltered body and liver weights and the behavior and physical appearance. The slight reduction in kidney weight observed only after 7 days could be attributed to the direct effect of acute Cd dose. However, after 56 days, kidney weight was restored, suggesting that this was a transient change. However, this dose of Cd causes severe weight loss in testis and epididymis after 56 days (Predes *et al*, 2009). The administration of *A. lappa* extract alone or after Cd intoxication did not cause any of the alterations of the parameters discussed above.

The biochemical indices monitored in the serum or plasma such as the electrolytes and other secretory substances of the liver and kidney can be used as 'markers' for assessing the functional capacities of the organs (Ashafa *et al.*, 2009). Some substances commonly used as indicators of damage are creatinine, the major catabolic product of muscle activity and bilirubin, a metabolic product of breakdown of heme group, which is derived from senescent red blood cells. These parameters of organ function, if altered, will demonstrate the impairment of the normal functioning of the organ (Ashafa *et al.*, 2009). The absence of effect on the plasma concentrations of total protein, bilirubin and creatinine

suggests that the secretory ability and normal functioning of these tissues were not altered in relation to these parameters for all treatments.

Most drugs and toxic chemicals are metabolized in the liver and these processes may cause liver injuries. The methods for monitoring hepatotoxicity include histopathological evidences as well as blood and liver indices that measure glutamate oxaloacetate and glutamate pyruvate transaminase levels (Mani *et al.*, 2007). In this study, Cd injection caused GOT and GPT plasma levels to increase after 56 days. Similar results were obtained when Cd was administered orally and intravenously (Nemmiche *et al.*, 2007). The elevation of transaminase levels can be attributed to the damaged structural integrity of the liver, because they are normally located in the cytoplasmic in location and are released to the circulation after cell damage (Mani *et al.*, 2007). Seven days of administration of *A. lappa* extract reduced the plasma GOT and GPT levels, however, after 56 of *A. lappa* administration, these levels returned to normal levels. The liver transaminase levels were not altered after all treatments except in Cd*Al* group after 56 days. However, the importance of this finding is not well established since the other groups did not change and it could be attributed to an individual variation of the animals.

In agreement with a large number of studies (Koyu *et al.*, 2006; Jihen *et al.*, 2008; El-Sokkary *et al.*, 2009), the dose of Cd injected caused already known the toxic signs of Cd damage in liver, such as hepatocyte cytoplasmic vacuolization, condensed nuclear chromatin and sinusoidal widening. According to previous report (Rikans and Yamano, 2000), the hepatocellular injury was produced *in vivo* as a result of ischemia caused by damage in endothelial cells. Secondary injury was thought to occur from the activation of

Kupffer cells and the cascade of events involving several types of liver cells and a large number of inflammatory and cytotoxic events. However, it was reported that acute Cd-induced hepatic trauma in Wistar rats does not involve inflammation; the lesion must be the result of direct effect of the metal and/or ischemia due to endothelial cell injury (Tzirogiannis *et al.*, 2003). Our results agree with the latter author, since we did not observe inflammatory infiltrates.

The administration of *A. lappa* extract alone showed that this plant has beneficial therapeutic effects since it reduced transaminase levels in the blood stream, and also alleviated the harmful effects of cadmium. In addition, *A. lappa* promoted an increase in number of hepatocytes nuclei when used alone or after Cd acute dose. This fact suggests the regeneration of this tissue, since a great number of binuclear hepatocytes were observed. In agreement with this observation, Lin and colleagues reported that the administration of *A. lappa* to Wistar rats after ethanol plus carbon tetrachloride liver injuries was capable to regenerate the liver tissue, as evidenced by binuclear hepatocytes (Lin *et al.*, 2002).

The results of the present study indicate that *A. lappa* helped to protect liver tissue against acute cadmium-induced hepatotoxicity. This can be concluded by associating the biochemical parameters and liver histology. Therefore, *A. lappa* could represent a potential therapeutic option to alleviate liver tissue damage resulting from acute cadmium intoxication, although more studies should be made to confirm its action.

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10. CONCLUSÕES FINAIS

1. O cádmio, mesmo em baixas doses, causa progressivas alterações morfológicas e morfométricas sobre os testículos de ratos, devido ao seu efeito direto sobre os mesmos. Pode-se observar que existe um limiar sutil de ação tóxica do cádmio no testículo. Nossos resultados destacam a relação direta da dose e tempo decorrido com as alterações morfológicas observadas decorrentes da ação do cádmio.

2. O extrato hidroetanólico apresentou relevante atividade limpadora de radical livre e alto teor de compostos fenólicos. Foi identificada a presença dos compostos quercetina, arctigenina, ácido clorogênico e ácido caféico. Além disso, os extratos diclorometânicos apresentaram atividade antiproliferativa seletiva contra as linhagens K562, MCF-7 e 786-0 de células tumorais humanas.

3. O cádmio, na dosagem de 1,2 mg/ kg causou severas alterações morfológicas e morfométricas tanto no testículo quanto no epidídimo. A administração do extrato de *A. lappa* pela via (gavagem), dose (300 mg/kg) e período utilizado (7 e 56 dias) foi ineficaz contra os danos causados pelo cádmio nestes órgãos.

4. O cádmio, na dosagem de 1,2 mg/ kg causou alterações bioquímicas plasmáticas, morfológicas e morfométricas no parênquima hepático. A eficiência do extrato *A. lappa* foi demonstrada pela melhora significativa nos parâmetros bioquímicos e na morfologia hepática alterados.

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1232-1**, sobre **"Efeito antioxidante da bardana (*Arctium lappa L.*) em testículos e epidídimos de ratos danificados pelo cádmio: bioquímica, morfologia, morfometria e ultraestrutura"**, sob a responsabilidade de **Profa. Dra. Mary Anne Heidi Dolder / Fabrícia de Souza Predes**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de 28 de março de 2007.

CERTIFICATE

We certify that the protocol nº **1232-1**, entitled **"Antioxidant effect of burdock (*Arctium lappa L.*) cadmium-induced damage in rat testis and epididymis: biochemistry, morphology, morphometry and ultrastructure"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on March 28, 2007.

Campinas, 28 de março de 2007.

Profa. Dra. Ana Maria A. Guaraldo
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