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FABRÍCIA DE SOUZA PREDES

ASSOCIAÇÃO DE CÁDMIO E *GINKGO BILOBA* EM RATOS: AVALIAÇÃO DOS TESTÍCULOS QUANTO A MODIFICAÇÕES DE ESTRUTURA, ULTRA-ESTRUTURA E MORFOMETRIA

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

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ÍNDICE

1. RESUMO

O cádmio (Cd) é um dos poluentes ambientais importantes que causam danos a vários órgãos e tecidos, incluindo os testículos. O objetivo deste trabalho é investigar o efeito protetor e curativo do extrato de G. *biloba* (GbE) nos testículos de ratos adultos Wistar tratados com dose única de Cd. Trinta animais foram divididos aleatoriamente em 5 grupos que receberam: Grupo 1: água por 56 dias (controle); Grupo 2: GbE por 56 dias; Grupo 3: $CdCl_2$ (dose única) e água por 56 dias; Grupo 4: $CdCl_2$ (dose única) e GbE por 56 dias (Cd + GbE); Grupo 5: pré-tratamento com GbE por 30 dias e $CdCl_2$ no 31º dia (GbE→Cd). O GbE foi administrado diariamente na dose de 100 mg/Kg de peso corporal por gavagem. O cloreto de cádmio ($CdCl_2$) foi injetado intraperitonealmente em dose única de 3 μmol/Kg de corporal. Após a coleta de sangue, os ratos foram fixados por perfusão com Karnovsky modificado. Fragmentos de testículo incluídos em metacrilato foram secionados na espessura de 3μm e corados com azul de toluidina/ borato de sódio 1% para estudo morfométrico em microscopia óptica. Para observação em microscópio eletrônico de transmissão fragmentos de testículo foram refixados com tetróxido de ósmio 1% no mesmo tampão a 4ºC, desidratados em acetona e incluídos em Epoxy. Cortes ultrafinos foram feitos com navalha de diamante e contrastados com acetato do uranila 2% e citrato de chumbo 2%. Os níveis de testosterona plasmática obtida por RIA não foram alterados neste estudo. Não houve alteração do peso corporal e testicular, do epidídimo e das glândulas acessórias, vesícula seminal e próstata além do índice gonadossomático. A proporção volumétrica e o volume absoluto dos componentes testiculares não alteraram após os tratamentos. O grupo 3 que recebeu somente Cd teve redução significativa no volume individual das células de Leydig. Entretanto, animais que receberam GbE como pré-tratamento ou GbE após a dose de Cd não apresentaram esta redução. Isto sugere que GbE é eficaz em manter o volume das células de Leydig em ratos tratados com Cd. O número de células de Leydig não variou nos grupos estudados. Em microscopia eletrônica foram visíveis os efeitos do Cd nos testículos. Nas células de Sertoli foram observados vacuolização citoplasmática, alterações nas junções celulares e acúmulo de gotículas de lipídio e corpos residuais. Espaços intercelulares expandidos foram observados no epitélio

germinativo. Cromatina irregularmente condensada e acrossoma anormal foram encontradas em espermátides alongadas. Algumas células endoteliais sofreram alterações morfológicas no núcleo e perderam algumas junções. As células de Leydig mostraram o citoplasma denso com organelas, como retículo endoplasmático liso e mitocôndrias, mal definidas. A administração de GbE após a dose de Cd mostrou-se eficaz em manter a ultraestrutura normal dos testículos do rato. Diferentemente, o pré-tratamento com GbE não foi eficaz em proteger o epitélio seminífero da toxicidade do Cd. Entretanto, as células de Leydig apresentaram morfologia normal com o retículo endoplasmático liso bem definido e numerosas mitocôndrias. Conclui-se que a administração de GbE por 56 dias após dose única de 3 μmol de $CdCl_2$ é capaz de proteger todo o parênquima testicular dos efeitos tóxicos do Cd. Entretanto, o pré-tratamento com GbE não protegeu o epitélio seminífero, mas foi eficaz em proteger as células de Leydig dos efeitos tóxicos do cádmio.

2. ABSTRACT

Cadmium is one of the important environmental pollutants that affect various organs and tissues, including the testis. The aim of this study was to investigate the protective and curative effect of *G. biloba* extract (GbE) on cadmium-induced testis damage of adult Wistar rats. Thirty rats were randomly divided in five groups that consisted in :Group 1: water for 56 days (control); Group 2: GbE for 56 days; Group 3: $CdCl_2$ (single dose) and water for 56 days; Group 4: $CdCl_2$ (single dose) and GbE for 56 days (Cd + GbE); Group 5: pre-treatment with GbE for 30 days and $CdCl_2$ on the thirty-first day (GbE→Cd).The GbE was administered daily in a dose of 100 mg/Kg BW by gavage. The cadmium chloride ($CdCl_2$) was injected i.p. in a single dosage of 3 μmol/Kg BW. After blood collection, rats were fixed by whole-body perfusion in Karnovsky fixative. Methacrylate-embedded testis fragments were sectioned at 3μm thickness and stained with toluidine blue/sodium borate 1% for morphometric study in the light microscope. For electron microscopy transmission examination, testis fragments were post-fixed with 1% osmium tetroxide in the same buffer at 4ºC, dehydrated in acetone and embedded in epoxy resin. Ultrathin sections were cut with a diamond knife and stained with 2% uranyl acetate and 2% lead citrate prior to observation. Plasma testosterone values obtained by the RIA method were not modified in this study. No change occurred in the average of corporal and testicular weight, gonadosomatic index, epididymis and accessory glands weight. The volumetric proportion and absolute volume of testicular components did not change after the treatments. Group 3 had a significant reduction in Leydig cell individual volume. However, animals that received GbE as pre-treatment or GbE after a cadmium dose did not present this reduction of Leydig cell volume. This suggests that GbE is effective in maintaining Leydig cell volume in cadmium-damaged testis. The number of Leydig cells did not vary in any group studied. In electron microscopy observations, this Cd dose caused visible alterations in the testis. Cytoplasmic vacuolation, disruption of tight junctions, accumulation of lipid droplets and residual bodies were observed in Sertoli cells. Expanded intracellular spaces were observed between the tubule cells. Irregularly condensed chromatin and abnormal acrosomes were found in late spermatids. Endothelium was affected, showing some

disruption of tight junctions and morphologically altered nuclei. Leydig cells showed a dense cytoplasm with poorly defined organelles such as SER and mitochondria. The administration of GbE after the Cd dose is effective in maintaining almost normal rat testis ultrastructure. However, GbE pretreatment was not able to protect testis from Cd toxicity, since toxicity signals were observed in the seminiferous epithelium. However, Leydig cells showed the normal morphology with well-defined and abundant smooth endoplasmic reticulum and mitochondria. It can be concluded that, GbE administration for 56 days after a single dose of 3 μmol of $CdCl_2$ could protect the testicular parenchyma from Cd toxic effects. However, GbE pretreatment did not protect the seminiferous epithelium, although it was effective in protecting Leydig cells from Cd toxic effects.

3. INTRODUÇÃO

3.1. Cádmio

Durante as últimas décadas tem crescido a preocupação com os possíveis efeitos de contaminantes químicos aos quais o homem está constantemente exposto. Dentre as várias substâncias tóxicas, os metais pesados são particularmente graves em sua ação (Gupta et al., 2004a).

O cádmio (Cd) é um metal pesado que ocorre naturalmente no ambiente, sendo eliminado por plantas e como resultado de atividade vulcânica. Adicionalmente, fontes antropogênicas, relacionadas principalmente com a mineração, incineração de resíduos e combustão de carvão mineral e óleo, contribuem para a disseminação deste elemento (Robards e Worsfold, 1991). O Cd é encontrado naturalmente na maioria dos minérios e solos quase sempre associados ao zinco. É obtido como subproduto da refinação do zinco e de outros minérios como chumbo-zinco e cobre-chumbo-zinco (Salgado, 1996).

Constitui um importante poluente ambiental, pois é amplamente utilizado nas indústrias, estando presente em um grande número de produtos agrícolas. É encontrado em pigmentos, esmaltes e tinturas têxteis, baterias recarregáveis de níquel-cádmio, varetas para soldagem, tubos para televisores, plásticos, cigarros e alguns fertilizantes (Robards e Worsfold, 1991; Salgado, 1996). Sais de Cd são também usados como anti-helmínticos, anti-sépticos, acaricidas e nematicidas (Robards e Worsfold, 1991).

As principais rotas de exposição humana ao Cd podem ser identificadas como exposição aguda em ambientes de trabalho (inalação de fumaça e poeira) e exposição aguda e crônica a pequenas doses, na população em geral, via alimentação, ar e água (Robards e Worsfold, 1991; Hoyer, 2001).

A característica mais importante que distingue os metais pesados dos outros poluentes tóxicos é que eles não são biodegradáveis e, uma vez no ambiente, seu potencial de toxicidade é controlado pela sua forma físico-química. O Cd é, portanto, caracterizado por persistência ambiental e meia-vida biológica (10 a 30 anos) longa, o que explica sua bioacumulação em indivíduos (Robards e Worsfold, 1991).

A identificação do Cd como um elemento distinto ocorreu em 1817. Sua toxicidade aguda é facilmente reconhecida e seus sintomas, descritos por Marmé em 1867, incluem vertigem, vômito, síncope, dificuldade respiratória, perda da consciência e cãibra. Os primeiros casos registrados de intoxicação por Cd foram devido à exposição industrial, envolvendo a inalação de pó de Cd, embora o primeiro caso registrado em 1858 tenha sido de natureza doméstica (Robards e Worsfold, 1991).

Ao longo da história foram registrados vários casos de intoxicação por Cd. Na Suécia, um incidente de intoxicação oral envolveu crianças que consumiram suco de frutas armazenado em uma máquina que possuía o reservatório revestido por Cd (Robards e Worsfold, 1991). Em São Paulo, na cidade de Bauru, crianças, animais e produtos agrícolas foram contaminados por vários metais pesados, dentre eles o Cd, devido à emissão de poluentes pela Indústria de Acumuladores Ajax (Aceituno, 2002). O caso mais preocupante ocorreu no Japão e ficou conhecido como acidente de "Itai-Itai". A fonte de Cd foi arroz cultivado com irrigação por água contaminada proveniente de afluentes de mineração (Robards e Worsfold, 1991).

O Cd é considerado um dos metais de transição mais tóxicos, pois causa graves danos a uma variedade de tecidos e órgãos incluindo fígado, rins e testículos (Gupta et al., 2004; Shimada et al., 2004).

3.2. Cádmio nos testículos e órgãos reprodutivos acessórios

Recentemente, algumas evidências apontam a perda da função testicular devido à redução da espermatogênese associada a fatores tais como agentes químicos, remédios e elementos tóxicos provenientes da poluição ambiental (Yano e Dolder, 2002).

O testículo é um importante alvo do Cd, tanto na toxicidade aguda como na crônica, o qual pode afetar o sistema reprodutivo masculino de homens e animais (Gupta et al., 2003; Zhou et al., 2004). Acredita-se que este órgão seja fortemente afetado por substâncias tóxicas por estar em constante divisão e diferenciação (Yano e Dolder, 2002).

Altas doses de Cd (maior que 10 μmol/ Kg) causam lesões testiculares extremamente graves como necrose hemorrágica (Gupta et al., 2004; Zhou et al., 2004), tumor de células intersticiais (Zhou et al., 2004), alterações vasculares (Aoki e Hoffer,

1978; Gouveia, 1988; Hew et al., 1993), alterações dos tecidos intersticiais (Aoki e Hoffer, 1978; Yano, 2001), diminuição dos níveis séricos de testosterona (Biswas et al., 2001; Gupta, 2004) e diminuição do peso do testículo e dos órgãos reprodutivos acessórios (Biswas et al., 2001).

Baixas doses de Cd (menor que 5 μmol/ Kg), que não causam danos evidentes no testículo, mas podem inibir a espermiogênese (Hew et al., 1993), causar apoptose em células espermatogênicas (Gupta et al., 2004; Zhou et al., 2004), além de reduzir o número de células que compõem o epitélio germinativo (Gupta et al., 2003). Segundo Zhou et al. (2004), baixas doses de Cd também causam significativas alterações na expressão gênica. Além disso, sabe-se que o Cd causa diminuição na produção de testosterona, mas os eventos celulares que explicam este efeito não estão bem estabelecidos (Lau et al., 1978; Sakasena et al., 1978; Yang et al., 2003).

Sabe-se que a administração de Cd em ratos machos resulta em danos à função testicular, embora as alterações do metabolismo celular que fundamentam os efeitos tóxicos ainda não tenham sido claramente estabelecidas (Gupta et al., 2004).

Há indicativos de que espécies reativas de oxigênio (ERO) estejam envolvidas em danos tissulares mediados por Cd. Tanto a exposição aguda quanto a crônica estão associadas com elevadas taxas de oxidação de lipídios nos pulmões, cérebro, rins, fígado, eritrócitos e testículos (Oteiza et al., 1999). Os testículos são particularmente vulneráveis à injúria com Cd, podendo apresentar significantes alterações (hemorragia, atrofia e calcificação) as quais podem ser atribuídas ao estresse oxidativo que inclui o aumento da oxidação de lipídios, o aumento da produção de peróxido de hidrogênio e a redução de glutationa (Oteiza et al., 1999; Gupta et al., 2004). Segundo Oteiza et al. (1999), um mecanismo que fundamenta a toxicidade celular do Cd é o dano oxidativo excessivo ao tecido.

3.3. Ginkgo biloba

O extrato de folhas de *Ginkgo biloba* é um dos mais antigos medicamentos vegetais que é ainda hoje utilizado como agente terapêutico na farmacologia moderna (Lugasi et al., 1999).

Extrato de *Ginkgo biloba* tem efeitos terapêuticos sobre uma variedade de processos patofisiológicos como trombose, inflamação, agregação plaquetária, infarto do miocárdio, hipóxia (Paick e Lee, 1996), Síndrome de Alzheimer, perda de memória (Yoshioka et al., 2004) e doenças vasculares periféricas (Sugiyama et al., 2004). É considerado neuroprotetor e vaso regulador (Defeudis, 2003), mostrando também ação contra radicais livres (Louajri et al., 2001; Ellnain-Wojtaszek et al., 2003; Parsad et al., 2003). Adicionalmente, o extrato de *Ginkgo biloba* tem efeito protetor contra danos causados por radicais livres em sistemas biológicos incluindo lesões causadas por isquemia-reperfusão no cérebro, coração e retina, peroxidação lipídica induzida por Ciclosporina A (Lugasi et al., 1999) e lesões no fígado causadas por tetracloreto de carbono (He et al., 2006).

A descoberta de substâncias com propriedades de recrutamento de radicais livres ou que estimulem a atividade de enzimas antioxidantes pode representar um campo na pesquisa terapêutica (Louajri et al., 2001) sendo valiosa na prevenção e tratamento de várias desordens relacionadas à doença induzida por radicais livres.

Uma vez reconhecido que o extrato de *Ginkgo biloba* tem efeito protetor contra radicais livres, nós acreditamos que este seja capaz de prevenir ou amenizar os danos causados pelo Cd nos testículos de ratos adultos.

3.4. Organização do Testículo

O testículo é recoberto por uma cápsula conjuntiva fibrosa resistente denominada albugínea testicular. Nos ratos e camundongos há uma pequena quantidade de tecido conjuntivo que se estende internamente, chamado de tecido conjuntivo intertubular (Russell et al., 1990). O testículo exibe dois compartimentos principais: o tubular, formado pelos túbulos seminíferos onde se dá o processo de espermatogênese e o intersticial, formado por tecido intertubular contendo tecido conjuntivo, vasos sanguíneos e linfáticos, nervos e células de Leydig.

3.4.1. Compartimento Tubular

Os túbulos seminíferos são alças contorcidas que possuem duas extremidades conectadas à *rete testis* pelo túbulo reto (*tubuli recti*). Embora numerosas convoluções estejam presentes em várias alças, existem porções retas que acompanham o eixo

longitudinal do testículo (Russell et al., 1990). Os túbulos seminíferos são delimitados por endotélio linfático, células mióides e elementos acelulares**.** As células mióides são contráteis e acredita-se que forneça a principal força para o movimento do fluido e propulsão dos espermatozóides no interior dos túbulos seminíferos. Juntamente com a lâmina basal, as células mióides fornecem um suporte estrutural no qual as células de Sertoli e as células do compartimento basal do epitélio seminífero se apóiam (Russell et al., 1990).

As células de Sertoli são altas (75-100 μm), simultaneamente de formato colunar e estrelado, com a base aderida à membrana basal. Sua região apical alcança o lúmen tubular, e numerosos prolongamentos laterais e apicais se estendem ao redor de todas as células germinativas. Seu núcleo é bem caracterizado pela forma alongada, com cromatina finamente dispersa e nucléolo bem distinto (Russell e Griswold, 1993). Desempenham papel crucial na espermatogênese (Monsees et al., 2000) devido às suas múltiplas funções desempenhadas simultaneamente. São responsáveis pela sustentação e manutenção das gerações de células germinativas em diferenciação localizadas no compartimento adluminal do epitélio seminífero (Russell e Griswold, 1993). São também responsáveis pela fagocitose e eliminação de corpos residuais citoplasmáticos liberados pelas espermátides alongadas no momento da espermiogênese, além da pinocitose ou endocitose de fase fluida no seu ápice (Russell e Griswold, 1993).

Células de Sertoli adjacentes se unem por junções oclusivas formando uma barreira para macromoléculas conhecida como barreira hemato-testicular (Monsees et al., 2000). Isto resulta na formação de dois compartimentos no epitélio seminífero: o basal, que contém espermatogônias e espermatócitos primários iniciais, e o adluminal, que contém espermatócitos mais tardios e espermátides em várias etapas da espermiogênese. Provavelmente, a presença desta barreira faz com que as células de Sertoli estejam envolvidas no transporte de substâncias, do compartimento basal para as células germinativas no compartimento adluminal (Russell e Griswold, 1993). Outro papel importante desta célula é a transposição dos espermatócitos primários em preleptóteno, do compartimento basal para o adluminal. Após transposição as células de Sertoli

restabelecem contatos e prendem-se umas às outras por novas junções (Russell et al., 1990; Russell e Griswold, 1993).

A liberação dos espermatozóides do epitélio seminífero ou espermiogênese tem ativo envolvimento da célula de Sertoli. Este fenômeno é complexo e se inicia com a expulsão das espermátides alongadas das criptas da célula de Sertoli em direção ao lúmen. Com a cabeça encapsulada individualmente por prolongamentos da célula de Sertoli, a espermátide fica retida na superfície do epitélio seminífero. Posteriormente, a espermátide se desliga, mas a célula de Sertoli retém e fagocita os corpos residuais liberados pelas espermátides (Russell et al., 1990; Russell e Griswold, 1993).

3.4.2. Compartimento intersticial

O compartimento intersticial do testículo consiste de células de Leydig, as quais são produtores de esteróides sexuais, macrófagos, vasos sanguíneos e linfáticos e nervos (Kim e Yang, 1999). Em ratos e camundongos, os vasos linfáticos representam canais irregulares ou sinusóides que estão limitados por células endoteliais e são denominados espaços linfáticos, os quais estendem entre as células do interstício e os túbulos seminíferos (Russell et al., 1990).

A principal função do compartimento intersticial, mais especificamente das células de Leydig, é a produção de hormônios esteróides, dos quais a testosterona é o principal (Kim e Yang, 1999). As células de Leydig possuem forma arredondada ou poligonal, núcleo central e citoplasma repleto de inúmeras gotículas de gordura. Apresentam características de células secretoras de esteróides, com retículo endoplasmático liso bem desenvolvido e numerosas mitocôndrias (Payne et al., 1996). A produção de esteróides andrógenos acontece sob controle do eixo hipotalâmico-hipofisário-testicular. O hormônio luteinizante (LH) é o fator primário necessário para manter a estrutura e função das células de Leydig. O volume e a função esteroidogênica das células de Leydig são dependentes do LH, mas o número de células de Leydig é regulado independentemente (Keeney e Ewing, 1990).

3.5. Espermatogênese

O processo espermatogênico é bastante complexo e acontece nos túbulos seminíferos. Pode ser dividido em três fases com base em considerações funcionais: (a) fase proliferativa (espermatogonial), na qual as células sofrem divisões mitóticas sucessivas rápidas; (b) fase meiótica (espermatócitos), na qual o material genético é recombinado e segregado; (c) fase espermiogênica, ou de diferenciação, na qual as espermátides se transformam em espermatozóides, células altamente especializadas e estruturalmente equipadas para alcançar e fertilizar o óvulo (Russell et al.,1990).

O processo inicia-se com a célula germinativa primitiva, espermatogônia A, célula fonte localizada próxima à membrana basal. As espermatogônias se dividem por mitose e as células recém formadas podem seguir dois caminhos: (a) continua igual à célula mãe e sofrem novas mitoses, sendo, portanto uma fonte de espermatogônias ou (b) param de se dividir e crescem, tornando-se maiores que as espermatogônias, sendo então chamadas de espermatócitos primários ou espermatócitos I que posteriormente entram em meiose. Como a prófase da primeira divisão meiótica é muito demorada, passando pelas etapas de leptóteno, zigóteno, paquíteno e diplóteno, a maioria dos espermatócitos I vistos nas preparações histológicas está nessa fase. Os espermatócitos I são as maiores células da linhagem espermatogênica e se caracterizam pela presença de cromossomos em diferentes níveis de condensação. Desta primeira divisão meiótica resultam células menores, chamadas de espermatócitos secundários ou espermatócitos II. Estes ficam mais próximos do lúmen dos túbulos seminíferos, sendo difícil observá-los em cortes histológicos, pois entram rapidamente na segunda divisão meiótica, originando as espermátides. As espermátides caracterizam-se por apresentarem pequeno tamanho, núcleos com zona de cromatina condensada, e por se localizarem próximas do lúmen dos túbulos seminíferos. As espermátides não sofrem novas divisões celulares iniciando a espermiogênese, um processo de modificações complexas que levam à formação dos espermatozóides. Por isso, pode-se observar espermátides com as mais variadas morfologias, de acordo com a fase de espermiogênese em que se encontram (Junqueira e Carneiro, 2004). Assim, os espermatozóides formados são liberados no fluido do túbulo seminífero.

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5. OBJETIVOS

Geral: Avaliar a capacidade do extrato de *G. biloba* em proteger e/ou recuperar os testículos de ratos adultos após tratamento com dose única de cádmio.

Específicos: Analisar os efeitos do tratamento com cádmio e/ ou *Ginkgo biloba* sobre os aspectos morfológicos e funcionais de ratos adultos através da:

- Morfometria testicular: IGS, proporção volumétrica, volume dos componentes testiculares e número de células de Leydig.
- Variação no peso dos órgãos reprodutivos acessórios.
- Concentração plasmática de testosterona .
- Ultraestrutura testicular.

6. ARTIGOS CIENTÍFICOS QUE SERÃO SUBMETIDOS À PUBLICAÇÃO

6.1. *Ginkgo biloba* ameliorate cadmium-induced damage in rat testis: morphometric study

6.2. Rat testicular ultrastructure after cadmium and *Ginkgo biloba* treatment

6.1. *Ginkgo biloba* ameliorate cadmium-induced damage in rat testis: morphometric study

Running title: Testis submitted to cadmium/*Ginkgo biloba*

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ABSTRACT

Cadmium (Cd) is one of the important environmental pollutants affecting various organs and tissues, including the testis. The aim of this study is to investigate the association of Cd and *Ginkgo biloba,* which has antioxidant properties, on the testis and accessory glands of adult Wistar rats. Thirty rats were randomly divided into five groups that consisted in: Group 1: water for 56 days (control); Group 2: GbE for 56 days; Group 3: $CdCl_2$ (single dose) and water for 56 days; Group 4: $CdCl_2$ (single dose) and GbE for 56 days (Cd + GbE); Group 5: pre-treatment with GbE for 30 days and $CdCl_2$ on the thirty-first day (GbE→Cd). The GbE was administered daily in a dose of 100 mg/Kg BW by gavage. The cadmium chloride ($CdCl_2$) was injected i.p. in a single dose of 3 μmol/Kg BW (0,6 mg/Kg BW). After blood collection, rats were fixed by whole-body perfusion in Karnovsky fixative. The plasma testosterone value obtained by the RIA method showed no modifications in this study. No change in the average corporal and testicular weight, gonadosomatic index, epididymis and accessory glands weight. The volumetric proportion and absolute volume of testicular components did not change after the treatments. Group 3 had significant reduction in Leydig cells volume. However, animals that received GbE as a pre-treatment or GbE after a cadmium dose did not present this volume reduction. The number of Leydig cell did not vary in any of the groups studied. This suggests that GbE is effective in maintaining Leydig cell volume in cadmium-damaged testis.

Keywords: *Ginkgo biloba*, cadmium, testis, Leydig cell and morphometry

INTRODUCTION

Cadmium (Cd) is a wide spread environmental pollutant, characterized by its toxicity in various organs (El-Demerdash et al., 2004) of humans and animals. Low doses are more relevant to human exposure since the general population is exposed to Cd in contaminated food and cigarette smoke at relatively low, but constant levels, rather than to acute high levels (Zhou et al., 2004). According to World Health Organization the cadmium dose permitted for humans is 0.4- 0.5 mg/person/wk.

Cd accumulates in several tissues: liver, kidney, brain, lung and testis are target organs following Cd exposure. Spermatogenic cells are particularly sensitive to low doses of Cd exposure (Zhou et al, 2004). The severity of their intoxication depends on the route, dose, and duration of the exposure to the metal (El-Missiry and Shalaby, 2000).

In adult male rats, acute or chronic treatment with Cd induces a well-documented toxicity in the reproductive organs and production of androgens (Herak-Kramberger et al, 2000). Among the reported effects of Cd are reduction in the size and mass of testes and accessory sex organs, necrosis of seminiferous tubules, Leydig cell damage and decreased serum testosterone concentration (Herak-Kramberger et al, 2000; Gupta et al, 2004).

Several lines of evidence indicate that oxidative stress is involved in cadmium-induced testicular damage (Gupta et al, 2004a). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the body's natural antioxidant defense mechanisms, causing damage to macromolecules such as DNA, proteins and lipids (El-Missiry and Shalaby, 2000; El-Dermerdash et al, 2004; Gupta et al, 2004b). Many defense mechanisms within living organisms have developed to limit the level of ROS and the oxidative damage they induce. Among these defenses are the antioxidant enzymes and proteins and small molecular antioxidants (El-Missiry and Shalaby, 2000). Several studies have shown that free radical scavengers and antioxidants are useful in protecting against Cd toxicity such as β-carotene (El-Dermerdash et al, 2004; El-Missiry and Shalaby, 2000) vitamin C (Gupta et al, 2004a) and E (El-Dermerdash et al, 2004; Gupta et al, 2004a).

The *Ginkgo biloba* L. leaf extract (GbE) is one of the oldest herbal medicines that continue to be used as a therapeutic agent in modern pharmacology. GbE is mainly recommended for treatment of peripheral arterial disease and cerebral insufficiency in the elderly (Lugasi et al, 1999). Recently, the antioxidant properties of GbE have been intensively examined as a potential mechanism for its beneficial action. It has been reported that GbE scavenges several radical species *in vitro* and *in vivo* (Bridi et al, 2001; Huang et al, 2005; He et al, 2006). In addition, GbE show protective effects against free radical-mediated damage in biological system, including carbon tetrachloride-induced liver injury in rats (He et al, 2006), development of fibrosis in aged rats, (Huang et al, 2005) ischemia-reperfusion injury of the brain and heart and cyclosporin A induced lipid peroxidation (Bridi et al, 2001). This studies have demonstrated that the antioxidant activity of GbE contributes to the prevention and treatment of diseases associated with oxidative stress.

Since GbE is known to exert protective influences against the action of free radicals, we hypothesized that application of such an extract might prevent the cadmium-induced damage in adults rats. Therefore, the purpose of this study was investigating the protective and curative effect of GbE on cadmium-induced damage in testis of adult rats with a morphometric study.

MATERIAL AND METHODS

Animals

The study was carried out on 90-day-old adult male Wistar rats obtained from the Multidisciplinary Center for Biological Investigation (State University of Campinas, Campinas, SP, Brazil). The experiments were carried out according to the Guide for Care and Use of Laboratory Animals and were approved by the Committee for Ethics in Animal Experimentation of UNICAMP. Animals were housed 3 per cage. Food and water were provided *ad libitum.*

Treatment

Thirty rats were randomly divided in five groups that consisted of :

Group 1: water for 56 days (control);

Group 2: GbE for 56 days;

Group 3: $CdCl_2$ (single dose) and water for 56 days;

Group 4: $CdCl_2$ (single dose) and GbE for 56 days (Cd + GbE);

Group 5: pre-treatment with GbE for 30 days and $CdCl_2$ on the thirty-first day (GbE→Cd).

Ginkgo biloba extract (Bioflavin gotas – Herbarium Laboratório Botânico LTDA, Brazil) was administred daily by gavage in a dose of 100 mg/ kg BW (Bridi et al, 2001, Al-Yahya et al, 2006). The body weight was recorded weekly in order to calulate the dose. Cd was injected intraperitoneally with a single dose of 3 μmol/ BW of cadmium chloride ($CdCl_2$) (Yano, 2001). Group 5 was sacrificed 48 h after cadmium dose becase it is known that Cd causes damage in the testis a few hours after the injection (Aoki and Hoffer, 1978, Fiorini et al, 2004). Groups 1 and 3 received water by gavage to maintain the same conditions. Groups 1, 2, 3 and 4 were euthanasied after 56 days, because this interval represents the duration of spermatogenesis according to Russell (1990).

Hormone Measurement

Rats were intraperitoneally anesthetized with Ketamine (80 mg/BW) and Xylazine (5 mg/BW). Blood samples were obtained from cava caudal vein in heparinized syringe. The plasma was separated by centrifugation and stored at -72 ºC for subsequent hormone assays. Plasma levels of total testosterone were estimated by Testosterona Total RIA – Catálogo # DSL 4000. The assay sensitivity was 0.8 ng/ml.

Tissue Preparation

The animals, still under anesthetic, 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, with 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.

Biometry and Morphometry

The testicular albuginea was dissected out and weighed. The weight of testicular parenchyma was obtained subtracting the mass occupied by the albuginea from the testis total weight, thus providing the net weight of the organ's functional portion. The gonadosomatic index (GSI) was recorded and the testes weight expressed as a percentage of the total body weight, GSI = (testes weight/total body weight) x 100. The volumetric proportions of testicular tissue components were determined by light microscopy, by projecting a 432-intersection grid in Image Pro Plus software associated to an Olympus BX-40 microscope. Ten fields were chosen randomly (4320 points) over testicular parenchyma in each animal at 400X magnification. Points were scored and classified as one of the following testicular components: seminiferous tubule, Leydig cell, blood vessels, lymphatic space and connective tissue. The volume of each component of the testis was determined as the product of the volumetric proportion and parenchyma volume. For subsequent morphometric calculations, the specific gravity of testis tissue was considered to be 1.0 (Mori and Christensen, 1980). Individual volume of a Leydig cell was obtained from the nucleus volume and the proportion between nucleus and cytoplasm. For this purpose, Leydig cell nucleus diameter was obtained from the assessment of 10 cells/animal in Image Pro Plus software associated to an Olympus BX-40 microscope at 1000X magnification. Leydig cell nuclear volume was expressed in μm^3 and obtained by the formula $(4/3)\pi R^3$, where R = nuclear diameter/2. To calculate the proportion between nucleus and cytoplasm, a 432- intersection grid was projected in Image Pro Plus software associated to an Olympus BX-40 microscope at 1000X magnification. One thousand points over nuclei and cytoplasm of Leydig cells were counted for each animal. The cytoplasm volume was obtained by the formula: (% of cytoplasm X nuclear volume)/ % nucleus. The number of Leydig cells per testis and per gram of testis was estimated from the Leydig cell individual volume and the volume occupied by Leydig cells in the testis parenchyma.

Statistical Analysis

Comparison of the values between control and treated groups was done by analysis of variance Statistical (ANOVA) followed by Tukey's test. The results were considered significant for $P< 0.05$. All values arepresent the means ± standart error mean (SEM).

RESULTS

Hormone Measurement

The plasma testosterone levels are shown in Figure 1. Plasma testosterone levels did not change significantly in any treated group compared with the control.

Light microscopy

The seminiferous epithelium of both control and GbE treated groups showed the typical association of germ cells types and Sertoli cells in the different stages of the seminiferous cycle. The majority of the cross sections showed normal seminiferous tubules with the characteristic morphological organization of cell types. Typical myoid cells were observed surrounding the seminiferous tubule. The normal interstitial tissue contained vascular elements, connective tissue, Leydig cells and macrophages. Leydig cells were morphologically normal and occurred in clusters of polymorphic cells, and that were usually associated with small blood vessels. Macrophages were usually detected close to or adherent to the Leydig cells (Figure 2a and 2b).

In the cadmium treated group, interstitial components were for the most part similar to those in the control animals. However the majority of the Leydig cells were smaller in size and this was confirmed by the morphometric results (Table 5). In some seminiferous tubules, vacuolization of Sertoli cell cytoplasm was observed (Figure 2c).

The treatment with GbE associated to cadmium minimized the damage to the seminiferous epithelium, such as vacuolization of Sertoli cell cytoplasm. Leydig cells were morphologically normal (Figure 2d).

However, the pretreatment with GbE was ineffective in protecting the seminiferous epithelium, since vacuolization of Sertoli cell cytoplasm was observed in most of the seminiferous tubules. The Leydig cells exhibited normal morphology (Figure 2e).

Biometry

No statistically significant differences were observed between the control and treated groups in body weight gain and the weight of testis, testicular parenchyma, albuginea and GSI (Table 1).

The weight of epididymis, prostate and seminal vesicle did not show statistically significant differences among all experimental groups. Statistical evaluation showed the

coagulating gland weight of groups 4 and 5 to be significantly different from the control group (Table 2).

Volumetric proportion (%) of testicular parenchyma components

The volumetric proportion of testicular parenchyma components are shown in Table 3. No significant changes were observed in all groups studied. The absolute volumes of the parenchyma components were not significantly different for all the experimental groups (Table 4).

Leydig cell volume and number

A statistically significant reduction of nuclear diameter, nuclear volume, cytoplasmatic volume and individual Leydig cell volume was observed in the cadmium treated group when compared with control groups. In GbE treated group were observed a significant reduction of Leydig cell cytoplasm. The number of Leydig cells per testis and per gram of testis were not different for the groups studied (Table 5) and (Figure 3).

DISCUSSION

The functional diversity of the male reproductive system and the complexity of its hormonal regulation provide a vast number of potential sites for chemical disturbance (Creasy, 2001). The testis is known to serve as one of the important targets of cadmium. Several mechanisms of cadmium-induced testicular toxicity have been proposed, but this is still controversial.

In this study, the initial and final body weights were very different among the groups studied so we compared the body weight gain. The body weight gain was not statistically significant. Beek et al (1998) affirms that in long-term toxicity studies in rat (27 weeks) GbE did not induce any significant toxic effect up to the dose of 500 mg/ Kg daily. According to Biswas et al (2001), animals that received 0.45 mg/Kg BW of $CdCl_2$ injected subcutaneously did not change their body weight. This result is similar to that reported by Zielinska-Psuja (1976) although the dose has been bigger (0.025 mM/Kg BW). The present investigation showed no change in the testis weight, which compares favorably with previously published results using cadmium (Laskey et al, 1983) and GbE (Beek et al,

1998). By contrast, in several studies of cadmium treatment, decreased testicular weight was found (Zielinska-Psuja, 1976; Biswas et al, 2001; Gupta et al, 2003; Zeng et al, 2003), although, these studies used different doses, exposure routes and rat strains, making them difficult to compare.

The treatments used in this study did not alter the weight of epididymis, prostate and seminal vesicle. The weight of the coagulating gland was higher in the group that received GbE and cadmium (group 4) and lower in the group that received GbE as pre-treatment (Group 5). Except for coagulating gland, these results are similar to those reported by Castro et al (2005) and Al-Yahya et al (2006) in GbE studies. However, according to these authors, cadmium has been reported to have a pronounced effect on sex organ weight, which is the primary indicator of possible alteration in androgen status (Biswas et al, 2001; Creasy, 2001; Gupta et al, 2003). The hormone assay showed that testosterone plasma level remained unchanged after 48 hours or 56 days after a single dose of cadmium, indicating that the dose of cadmium was unable to alter testosterone plasma level and, as a consequence, the sex organs weight.

In the present study we have also obtained quantitative morphometric data. The volumetric proportion and absolute volume of testicular compartments and interstitial components did not present any significant variation between the groups, showing that the treatments did not alter these parameters. No other study was found reporting changes in morphometric parameters of cadmium and/or GbE treated rats. However, similar information was found in the studies of Mori and Christensen (1980), Russell and França (1995) and Yang and Kim (1999) for volumetric proportion and absolute volume of testis components for untreated rats.

The major function of the Leydig cells is the production of steroid hormones, including testosterone (Creasy, 2001; Yang et al, 2003; Gupta et al, 2004b). According to Yang et al (2003) and Gupta et al (2004a) cadmium could be directly toxic to Leydig cells. However, in the present study we believe that the cadmium dose used was insufficient to alter the plasma testosterone levels, since we did not find significant alteration in accessory sex organs weight, which are the primary indicators of possible alteration in androgen status. Their secretory function is androgen dependent and vary sensitive to circulating concentrations of testosterone (Creasy, 2001).

The morphometric analysis of Leydig cells did not show alterations in GbE treated group compared to the control group. In spite of the variability of morphometric analysis, these data were comparable with Kim and Yang (1999), Russell and França (1995) e Mori and Christensen (1980) studies. In contrast, the cadmium treated group showed statistically significant reduction of Leydig nucleus diameter accompanied by the decrease in absolute volume of individual Leydig cell, as well as of the cytoplasmatic and nuclear volume. Kim et al (2002) reported that deprivation of endogenous LH caused Leydig cell hypotrophy and reduced steroidogenic capacity. According to Gunnarsson et al (2003), it is possible that Cd interferes, desensitizing the LH receptor.

However, the association of GbE and cadmium is effective in maintaining, or recuperating, the volume parameters of Leydig cells, similar to the control group. The number of Leydig cells per testis and per gram of testis did not change for any group. As was observed, the volume of Leydig cells decreased, however the number of Leydig cells remained unaffected. This finding was consistent with Keeney and Ewing (1990) and Kim et al (2002), which reported that the luteinizing hormone (LH) is the only pituitary hormone required to maintain Leydig cell volume, but it is not required to maintain the Leydig cell number in testes of adult rats. In spite of the increase in Leydig cell number, this number was not statistically different among all experimental groups. Keeney and Ewing (1990) affirm that Leydig cells have the capacity to regenerate following destruction of the mature population by cadmium treatment. Ichihara et al (2001) suggests that the increase in number of this cell may be due to the differentiation of immature precursor cells, without mitosis.

The analysis of the results, show that rats exposed to a single i.p. dose of 3 μmol $CdCl_2$/ Kg BW did not present severe symptoms of cadmium toxicity in the testis and accessory sex organs. However this study suggests that the cadmium dose could alter LH levels, as suggested by the decrease in volume of individual Leydig cells in cadmium treated rats. Considering that groups, which received cadmium and GbE did not present volume reduction similar to that found for the group that received only cadmium, we suggest that GbE furnishes a protection from cadmium damage in Leydig cells.

In summary, our results indicated that cadmium damages Leydig cells and that both pre-treatment and treatment after cadmium dose with GbE demonstrates the efficacy of this

plant in lowering Leydig cell damage induced by cadmium.

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TABLES

Table 1- Basic data (g) and GSI of adult rats treated with GbE and/or cadmium (mean ± SEM).

Parameters	Control	GbE	$CdCl_2$	$CdCl_2$ + GbE	GbE→ $CdCl_2$
Body weight gain	93.83 ± 16.84	44.40 ± 8.78	70.50 ± 1.65	80.00 ± 17.23	54.00 ± 8.07
Final body weight	415.33 ± 23,85	387.00 ± 16.68	442 ±8,40	415.67 ± 8,74	402 ± 8,36
Testis weight	1.61 ± 0.03	1.5 ± 0.12	1.63 ± 0.04	1.59 ± 0.08	1.44 ± 0.07
Testicular parenchyma	1.53 ± 0.03	1.47 ± 0.12	1.56 ± 0.03	1.51 ± 0.08	1.36 ± 0.07
Albuginea (mg)	71.50 ± 3.64	71.00 ± 2.39	74.50 ± 4.62	76.33 ± 4.34	75 ± 4.84
GSI	0.79 ± 0.05	0.79 ± 0.05	0.74 ± 0.02	0.77 ± 0.04	0.72 ± 0.04

n = 6 for each group.

Table 2- Organs weight (mg) of adult rats treated with GbE and/or cadmium (mean ± SEM).

Parameters	Control	GbE	$CdCl_2$	$CdCl_2$ + GbE	GbE→ $CdCl_2$
Epididymis	535.83 ± 19.29	543.80 ± 33.96	544 ± 12.73	538 ± 23.24	507.67 ± 15.10
Ventral prostate	443 ± 40.31	430.40 ± 37.67	532.67 ± 33.17	444.33 ± 28.94	488.17 ± 26.43
Dorsolateral prostate	349.83 ± 31.26	373.80 ± 15.13	414.83 ± 31.64	365 ± 24.70	339.83 ± 25.58
Coagulating gland	207.67 ± 22.09	235.40 ± 13.14	214.33 ± 6	254.83 ± 9.73*	192 ± 10.10*
Seminal vesicle (g)	1.16 ± 0.14	0.95 ± 0.06	0.99 ± 0.04	1.08 ± 0.07	0.92 ± 0.02

n = 6 for each group. * Indicates significant differences ($P < 0.05$) between control and treated groups.

Table 3 – Volumetric proportion (%) of testicular parenchyma components of adult rats treated with GbE and/or cadmium (mean ± SEM).

Parameters	Control	GbE	$CdCl_2$	$CdCl_2$ + GbE	GbE→ $CdCl_2$
Seminiferous tubule	75.77 ± 4.98	79.58 ± 1.44	76.42 ± 2.86	77.60 ± 1.79	73.44 ± 4.69
Interstitium	24.23 ± 4.98	20.42 ± 1.44	23.58 ± 2.86	22.40 ± 1.79	26.56 ± 4.69
Lymphatic space	14.67 ± 3.84	11.96 ± 1.31	14.25 ± 1.96	14.35 ± 1.65	16.55 ± 4.27
Blood vessels	3.38 ± 0.5	2.81 ± 0.30	3.37 ± 0.69	2.39 ± 0.35	3.01 ± 0.81
Connective tissue	0.93 ± 0.41	1.07 ± 0.13	1 ± 0.23	0.63 ± 0.12	0.56 ± 0.09
Leydig cells	5.25 ± 0.76	4.55 ± 0.35	4.95 ± 0.65	5.03 ± 0.30	6.44 ± 0.67

n = 6 for each group.

Table 4 – Absolute volume (mL) of testicular parenchyma components of adult rats treated with GbE and/or cadmium (mean ± SEM).

Parameters	Control	GbE	$CdCl_2$	$CdCl_2$ + GbE	GbE→ $CdCl_2$
Seminiferous tubule	1.166 ± 0.087	1.161 ± 0.095	1.189 ± 0.051	1.174 ± 0.068	0.998 ± 0.077
Interstitium	0.368 ± 0.070	0.305 ± 0.030	0.366 ± 0.043	0.358 ± 0.031	0.366 ± 0.070
Lymphatic space	0.222 ± 0.054	0.180 ± 0.023	0.222 ± 0.03	0.217 ± 0.028	0.227 ± 0.061
Blood vessels	0.052 ± 0.008	0.041 ± 0.005	0.052 ± 0.01	0.036 ± 0.005	0.043 ± 0.013
Connective tissue	0.015 ± 0.006	0.016 ± 0.002	0.016 ± 0.004	0.009 ± 0.002	0.008 ± 0.002
Leydig cells	0.080 ± 0.010	0.067 ± 0.007	0.077 ± 0.01	0.075 ± 0.005	0.089 ± 0.012

n = 6 for each group.

Table 5 – Nuclear diameter (μm), individual Leydig cell volume (μm^3) and number of Leydig cells in testis of adult rats treated with GbE and/or cadmium (mean ± SEM).

Parameters	Control	GbE	$CdCl_2$	$CdCl_2$ + GbE	GbE→ $CdCl_2$
Nuclear diameter	6.32 ± 0.07	6.11 ± 0.13	5.30 ± 0.07*	6.15 ± 0.08	6.10 ± 0.16
Nuclear volume	132.37 ± 4.27	120.11 ± 7.39	78.27 ± 3.28*	122.03 ± 4.67	120.21 ± 8.93
Cytoplasmic volume	325.08 ± 28.77	270.70 ± 17.41*	183.88 ± 8.38*	318.27 ± 17.61	309.41 ± 38.50
Leydig Cell volume	457.45 ± 32.94	390.81 ± 23.04	262.15 ± 11.07*	440.30 ± 19.63	429.62 ± 41.58
Nº per testis (10^6)	186.7 ± 41.2	179.1 ± 30.4	294 ± 36.3	174.3 ± 15.6	210.7 ± 26.7
Nº per g of testis (10^6)	128.5 ± 29.8	119.4 ± 11.9	189.7 ± 24.5	115.6 ± 8.8	154.3 ± 17.0

n = 6 for each group. * Indicates significant differences ($P < 0.05$) between control and treated groups.

FIGURE LEGENDS

Figure 1- Mean plasma testosterone levels of adult rats treated with GbE and/or cadmium. The results are means ± SEM (standard error mean).

Figure 2. Light micrographs of general aspect of testicular interstitium of rats treated with GbE and/or cadmium stained with toluidine blue. (a) Control; (b) GbE; (c) $CdCl_2$; (d) $CdCl_2$ + GbE; (e) GbE→ $CdCl_2$. ST = seminiferous tubule; LY = lymphatic space; LC = Leydig cell; B = blood vessels. Scale bar = 0,03 mm.

FIGURES

Figure 1

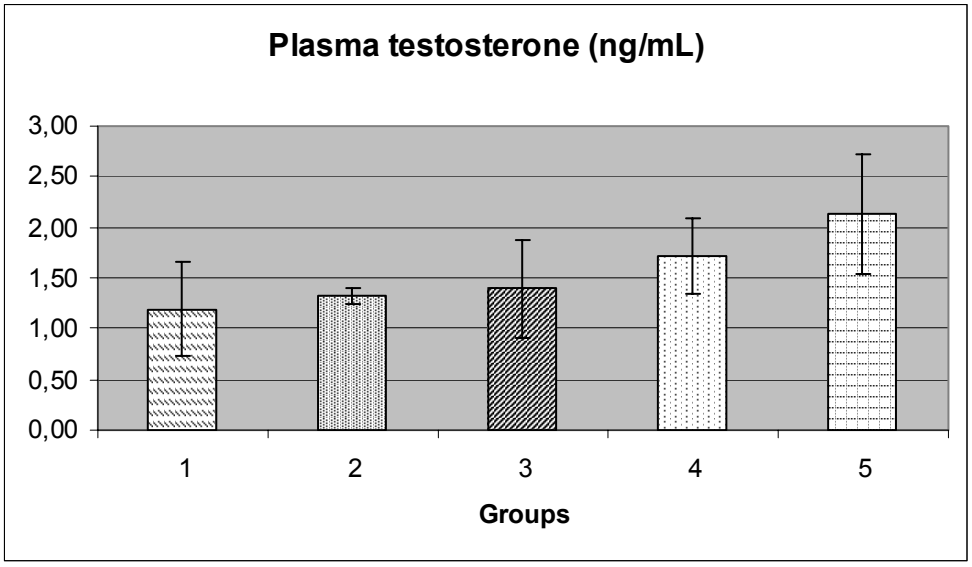
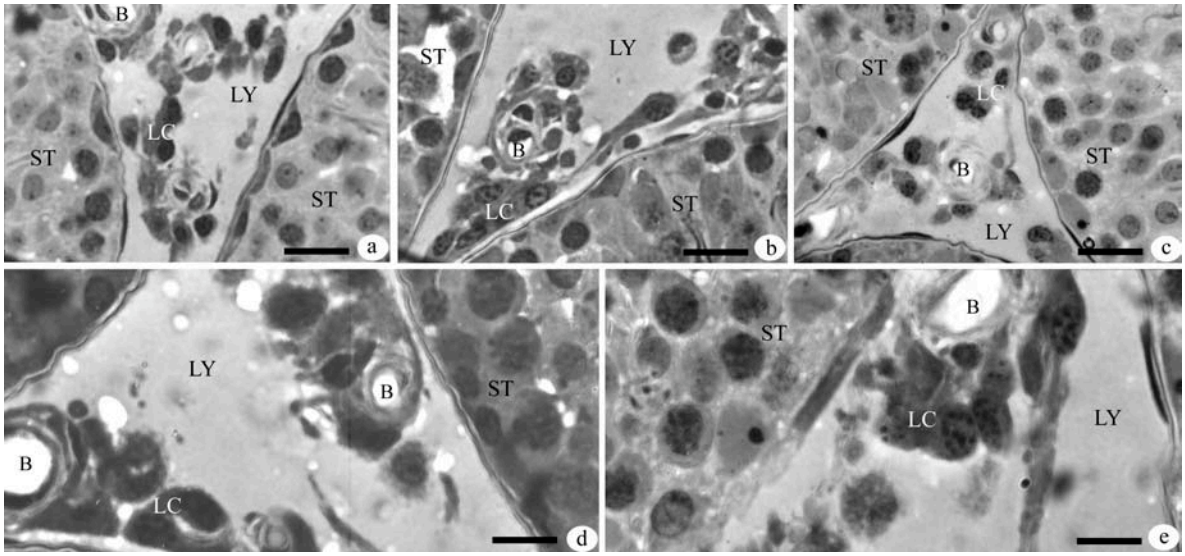


Figure 2



6.2. Rat testicular ultrastructure after cadmium and *Ginkgo biloba* treatment

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ABSTRACT

This study investigated the effects of cadmium (Cd) and *Ginkgo biloba* extract (GbE) on rat testis by electron microscopic observation. Thirty rats were randomly divided in five groups that consisted in: Group 1: water for 56 days (control); Group 2: GbE for 56 days; Group 3: $CdCl_2$ (single dose) and water for 56 days; Group 4: $CdCl_2$ (single dose) and GbE for 56 days (Cd + GbE); Group 5: pre-treatment with GbE for 30 days and $CdCl_2$ on the thirty-first day (GbE→Cd). The GbE was administered daily in a dose of 100 mg/Kg BW by gavage. The cadmium chloride ($CdCl_2$) was injected i.p. with a single dose of 3 μmol/Kg BW. This Cd dose has caused visible alterations in the testis. In Sertoli cells, various alterations were observed, including cytoplasmic vacuolation, disruption of tight junctions, accumulation of lipid droplets and residual bodies. Expanded intercellular spaces were observed among the cells. Irregularly condensed chromatin and abnormal acrosomes were found in late spermatids. Blood vessels were also affected. Leydig cells showed dense cytoplasm with poorly defined organelles, such as smooth endoplasmic reticulum and mitochondria. The administration of GbE after the Cd dose was effective in maintaining the normal morphology of rat testis. However, GbE pretreatment (group 5) was not able to protect the testis from Cd toxicity since the typical cadmium alterations could be identified in the seminiferous epithelium. However, Leydig cells showed the normal morphology with well-defined and abundant smooth endoplasmic reticulum and mitochondria. Summarising, GbE administration for 56 days after a single dose of 3 μmol of $CdCl_2$ could protect the testicular parenchyma and the Leydig cells from Cd toxic effects. However, GbE pretreatment did not protect seminiferous epithelium, but is effective in protecting Leydig cell from toxic effects caused by cadmium.

Keywords: *Ginkgo biloba*, cadmium, ultrastructure, Sertoli cell and Leydig cell

INTRODUCTION

Cadmium (Cd) is a wide spread environmental pollutant, characterized by its toxicity in various organs [1] of humans and animals. It accumulates in several tissues; liver, kidney, brain, lung and testis are the target organs following Cd exposure. The severity of the intoxication depends on the route, dose, and duration of the exposure to the metal [2].

In adult male rats, acute or chronic treatment with Cd induces a well-documented toxic reaction in the reproductive organs and, particularly the testes are extremely susceptible to Cd [3, 4]. Metal-induced testicular dysfunction may arise from disturbances in Sertoli cells, that support spermatogenesis, or Leydig cells, that are responsible for androgen production under control of the hypothalamic-pituitary-testicular axis [5]. Within the testis tissue, Sertoli cells appear to be highly sensitive to Cd toxicity [4].

Several lines of evidence indicate that oxidative stress is involved in cadmium-induced testicular damage [6]. Many defense mechanisms within living organisms have evolved to limit the level of reactive oxygen species (ROS) and the oxidative damage they induced. Among this defenses are the antioxidant enzymes and proteins and small molecular antioxidants [2]. Several studies have shown that free radical scavengers and antioxidants are useful in protecting against Cd toxicity.

Recently, the antioxidant properties of *Ginkgo biloba* extract (GbE) have been intensively examined as a potential mechanism for its beneficial action. It has been reported that GbE scavenges several free radical species *in vitro* and *in vivo* [7-9]. In addition, GbE shows protective effects against free radical-mediated damage in biological systems, including carbon tetrachloride-induced liver injury in rats [8], development of fibrosis in aged rats [9], ischemia-reperfusion injury of the brain and heart and cyclosporin A induced lipid peroxidation [7]. Since GbE is known to exert protective influences against the action of free radicals, we hypothesized that application of such an extract might prevent the cadmium-induced damage in testis of adult rats since this tissue is pivotal for reproduction. Ultrastructural modifications in the testis were investigated, since this tissue is in constant division and cell differentiation, which makes it more vulnerable to toxic substances.

MATERIAL AND METHODS

Animals

The study was carried out on 90-day-old adult male Wistar rats obtained from the Multidisciplinary Center for Biological Investigation (State University of Campinas, Campinas, SP, Brazil). The experiments were carried out according to the Guide for Care and Use of Laboratory Animals and were approved by the Committee for Ethics in Animal Experimentation of UNICAMP. Animals were housed three per cage. Food and water were provided *ad libitum.*

Treatment

Thirty rats were randomly divided in five groups that consisted in:

Group 1: water for 56 days (control);

Group 2: GbE for 56 days;

Group 3: $CdCl_2$ (single dose) and water for 56 days;

Group 4: $CdCl_2$ (single dose) and GbE for 56 days (Cd + GbE);

Group 5: pre-treatment with GbE for 30 days and $CdCl_2$ on the thirty-first day (GbE→Cd).

Ginkgo biloba extract (Bioflavin gotas – Herbarium Laboratório Botânico LTDA, Brazil) was administred daily by gavage in a dose of 100 mg/ kg BW [7, 10]. Cd was injected intraperitoneally using a single dose of 3 μmol/ BW of cadmium chloride ($CdCl_2$) [11]. Group 5 was sacrificed 48 h after the cadmium dose. The groups 1 and 3 received water by gavage to maintain the same conditions. Groups 1, 2, 3 and 4 were euthanasied after 56 days, since this interval represents the duration of spermatogenesis according to Russell [12].

Tissue Preparation for Transmission Electron Microscopy

Rats were anesthetized with Ketamine (80 mg/BW) and Xylazine (5 mg/BW). The animals 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% 0.1 M sodium phosphate buffer, with pH 7.2 for 25-30 minutes and then post fixed in the same solution for 24 hours, at 4ºC. The tissues were post-fixed with 1% osmium tetroxide in the same buffer at 4ºC, dehydrated in acetone and embedded in epoxy resin. Ultrathin sections (20-60 nm) were cut with diamond knives and stained with 2% uranyl acetate (25 min) and 2% lead citrate (10 min) prior to observation with a transmission electron microscope (Zeiss, Leo 906).

RESULTS

In the control and GbE treated groups, the seminiferous tubule consisted of normal Sertoli and germ cells. The Sertoli cells and spermatogonia occur just above the basal lamina, while the spermatocytes, round and elongated spermatids were closely associated with Sertoli cell prolongations (Figs. 1a and 1b). Sertoli cells displayed an irregular nucleus with condensed patches of chromatin and a well-defined nucleolus (Fig. 1b). The Sertoli cell cytoplasm contained smooth endoplasmic reticulum (SER), numerous mitochondria and variable amounts of lipid inclusions, as well as ribosomes, lysosomes and Golgi membranes that were not altered. Several residual bodies retained from the excess spermatid cytoplasm were also apparent (Fig. 1c). Specialized intercellular tight junctions between adjacent Sertoli cells and Sertoli cell-germ cell were present. The adluminal compartment contained the other types of germ cells: late spermatocytes, round and elongated spermatids and spermatozoa with normal associations. Round spermatids were characterized by a well-defined nucleus with a distinct nuclear membrane and chromatin network and the cytoplasm predominantly occupied by mitochondria. Acrosome formation, marked by an acrosomal cap, appeared normal (Figs. 1c and 1d). The tubule lumen was filled with typical spermatozoa. Leydig cells showed a large irregular nucleus, which usually possessed a proeminent nucleolus (Figs. 1e and 1f). The cytoplasm contained Golgi complexes, lysosome-like structures, small patches of rough endoplasmic reticulum (RER), well-defined, abundant SER and numerous mitochondria containing tubular and lamellar cristae surrounding the nucleus. The blood vessels follow the usual structure, with elongated endothelial nucleus.

In the cadmium treated group, Sertoli cell cytoplasm showed vacuolation and loss of cytoplasmic organelles and contained large, lysosome-like vacuoles, with polymorphous interiors e electron lucent lipid droplets (Fig. 2a). The structures of Sertoli-Sertoli cell junctions and the Sertoli cell-germ cell junctions were affected. There was an increase in the intracellular space due to disorganization of germ cells in some tubules. This disorganization showed cells isolated at the basal compartment, namely, Sertoli cells, spermatogonia and preleptotene spermatocytes. The adluminal compartment was observed to have lost germ cell attachment, resulting in expanded intercellular spaces between spermatocytes, spermatids where Sertoli cell prolongations are observed (Fig. 2b). The elongated spermatids exhibited heterogeneous and granular chromatin. Structural alterations in some acrosomes was observed (Figs. 2b and 2d). Blood vessels were affected. Occasional open endothelial junction could be detected and the nucleus of endothelial cells was irregular in shape (Fig. 2a). The Leydig cells showed a nuclear envelope with many deep indentations and dense cytoplasm, and poorly defined organelles as SER and mitochondria.

The administration of GbE after a single dose of $CdCl_2$ is effective in maintaining partially normal ultrastructure of rat testis (Fig. 3). However, the pretreatment with GbE was not effective. In Sertoli cells, in this group, cytoplasmic vacuolation and the accumulation of lysosome-like structures with polymorphous interiors, as well as a few electron lucent lipid droplets occurred frequently (Figs. 4a and 4b). The blood vessels and the nucleus of endothelial cells were irregular in shape. Irregularly condensed chromatin was found in late spermatids (Fig. 4b). They were random orientated in the adluminal compartment and were surrounded by abundant cytoplasm. Leydig cells showed a large irregular nucleus, which usually possessed a proeminent nucleolus (Fig. 4c). The cytoplasm contained Golgi complexes, lysosome-like structures, RER, well-defined, abundant SER and numerous mitochondria.

DISCUSSION

Spermatogenesis is a complex process in which the interaction of diverse cells such as Sertoli, Leydig and germinal cells is tightly regulated by hormonal signal and receptor

stimulation [5]. If pollutants damage one specific cell type, then the whole process could be disrupted leading to reproductive failure, infertility or sterility [5, 13].

Metal-induced testicular dysfunction may arise from disturbance in Sertoli cells that support spermatogenesis or Leydig cell responsible for androgenic production under control of hypothalamic-pituitary-testicular axis [5].

It is known that Cd causes damage in the testis a few hours after the injection [14, 15], so the animals of group 5 were euthanasied 48 hours after this. Therefore, in the present study, the group that had received Cd after treatment with GbE showed the common effects of the Cd in the tubular compartment. These results suggest that GbE was ineffective in preventing the early effects of Cd in this compartment. However, the group that had received the single dose of Cd and GbE for 56 days did not show the severe toxic effects of Cd, especially in Sertoli cells. This result is indicative that GbE was able to protect or maintain the seminiferous epithelium or that this tissue could recover its morphology after a complete spermatogenic cycle.

The accumulation of lysosome-like structure with polymorphous interiors and residual bodies, cytoplasmic vacuolization in Sertoli cells and irregularly condensed chromatin in late spermatids, as observed in the present study, may be the consequences of cadmium toxicity.

The Sertoli cells are one of the most common target cells for toxicity, in testicular tissue, as a consequence of its role in supporting spermatogenesis [4, 16-18]. Sertoli cells are supportive in the seminiferous epithelium, and orchestrate spermatogenesis by providing structural and nutritional support for germ cells. Any functional compromise of this cell will have rapid secondary effects on the survival of the dependent germ cells [17].

One of the most common morphological responses of Sertoli cell to injury is vacuolization [17]. Probably, the cytoplasmic vacuolization of Sertoli cells results from the swelling and coalescence of cytoplasmic membrane-bounded organelles, such as endoplasmic reticulum and vesicles [17,19].

In addition to vacuolization, increase in lipid droplets and residual bodies was observed. Some authors suggest that lipid accumulation is due to degenerating germ cells, which have been phagocytized by Sertoli cells [20, 21]. Here, the numerous residual bodies

observed in Sertoli cell cytoplasm render support this hypothesis. Subsequent to the vacuolization, germ cell degeneration, disorganization, or sloughing is generally seen [17] and was observed also in this study.

Many of the sloughing germ cells appear morphologically normal suggesting that the primary cadmium effect is on the cell-to-cell junctions between Sertoli and germ cells. The occurrence of a stem cell population without alterations in this study indicates a resistance of these cells, either because of their long cell cycles or their low mitotic activity, according to Gopalkrishnan [20].

As described by various authors [5, 13, 16, 22], Cd induces loss of intercellular junctions between Sertoli and germ cells. Hew [22, 23] reported that cadmium's effects on Sertoli cell tight junctions is due to its action on the actin filaments associated with the junctions and the disruption of microfilaments was related with failure of spermiation. Thus, the presence of elongated spermatids without chromatin condensation, random orientation and surrounded by residual cytoplasm confirms the claim that cadmium induces failed spermiation. Even a single low dose of cadmium can cause failure of spermiation, and this metal probably affects sperm release by acting on Sertoli cell microfilaments [19, 23].

The alteration in vascular endothelium, according to Prozialeck [24], occurred due to the breakdown of the junctions between endothelial cells of capillaries and venules, resulting in an increase in vascular permeability, followed by edema. This may occur because of Cd ability to disrupt cadherin-dependent cell-cell junctions [21]. Due to the small dose of Cd employed, the vascular endothelium did not entirely loose its barrier integrity, so the edema was not accentuated.

Leydig cell function is susceptible to any agent that interferes with the steroidogenic pathway or with circulating levels or receptor binding of its regulatory hormones, luteinizing hormone (LH) and prolactin [17]. It is known that cadmium is directly toxic to Leydig cells inhibiting testosterone production [3, 25-27]. The ultrastructural analysis showed decreased the amount of developed SER, only in the cadmium treated group. The decreased amount of SER suggests reduced testosterone production since this organelle is

essential for the production of this hormone [28, 29]. On the other hand, the pretreatment with GbE protected the Leydig cells from the toxic effects of Cd.

In conclusion, exposure to a single dose of 3 μmol of $CdCl_2$ results in rat testis damage. The administration of *Ginkgo biloba* extract for 56 days could ameliorate the testicular parenchyma and Leydig cells from Cd toxic effects, however, the pretreatment with GbE was not effective in shielding the epithelium seminiferous, but is effective in protecting Leydig cell. However, further studies are required to elucidate if protection is really linked to *Ginkgo biloba* antioxidant property [30,31].

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FIGURE LEGENDS

FIG. 1- Testis of control rats (a, c and e) and rat treated with GbE (b, d and f). (a, b) Seminiferous epithelium showing Sertoli cell (SC) and spermatogonia (G) in the basal compartment and round spermatids (RS) in the adluminal compartment. Interstitium showing lymphatic space (LY) and blood vessels (B). (c,d) Adluminal compartment showing round spermatids (RS) in different stages of differentiation and residual bodies (RB). (e, f) Leydig cells (LC) showing large irregular nucleus and cytoplasm rich in mitochondria (m), endoplasmatic reticulum (r) and Golgi complex (gc). Scale bars = 5 μm.

FIG. 2- Testis of rats treated with cadmium. (a) Seminiferous epithelium showing Sertoli cell (SC) with large vacuoles (V) and lipid droplet (L) and normal spermatogonia (G) in the basal compartment. S= spermatocyte I. Interstitium showing damaged blood vessel (B) and the lymphatic space (LY). Expanded intercellular space (*). (b) Presence of deformed elongated spermatid (ES) without condensed chromatin and altered acrosome (A) enclosed in fragmented Sertoli cell (SC) cytoplasm. Expanded intercellular space (*). (c) Leydig cell (LC) showing large irregular nucleus with proeminent nucleolus. Presence of dense cytoplasm with endoplasmatic reticulum (r) and few other cytoplasmic organelles. (d) Adluminal compartment with elongated spermatids (ES) with condensed chromatin and altered acrosomes (A) and round spermatids (RS) near vacuolated Sertoli cell (SC). Scale bars = 5 μm.

FIG. 3- Testis of rats treated with $CdCl_2$ and GbE on the first day and only GbE on the following days. (a) Seminiferous tubule showing basal compartment with Sertoli cell (SC) and spermatogonia (G) and adluminal compartment with round spermatids (RS). (b) Adluminal compartment with normal round spermatids (RS) and elongated spermatids (ES) without condensed chromatin enclosed in Sertoli cell (SC) cytoplasm. (c) Leydig cell (LC) showing large irregular nucleus and cytoplasm rich in mitochondria (m) and endoplasmatic reticulum (r). (d) Normal blood vessel with elongated endothelial cell nucleus. Scale bars = 5 μm.

FIG. 4- Ultrathin sections of testis of rats treated with GbE for 30 days and $CdCl_2$ on the thirty-first day. (a) Seminiferous epithelium showing Sertoli cell (SC) with some vacuoles (V) in the basal compartment. Interstitium showing damaged blood vessel (B) with irregular endothelial cell nucleus (E) and the lymphatic space (LY) with some Leydig cells (LC). (b) Presence of some elongated spermatid (ES) without condensed chromatin and others with condensed chromatin and random orientation, enclosed in fragmented Sertoli cell (SC) cytoplasm. Expanded intercellular space (*) and residual bodies (RB). (c) Leydig cell (LC) showing large irregular nucleus and cytoplasm rich in mitochondria (m), endoplasmatic reticulum (r) and Golgi complex (gc). Scale bars = 5 μm.

FIG. 1

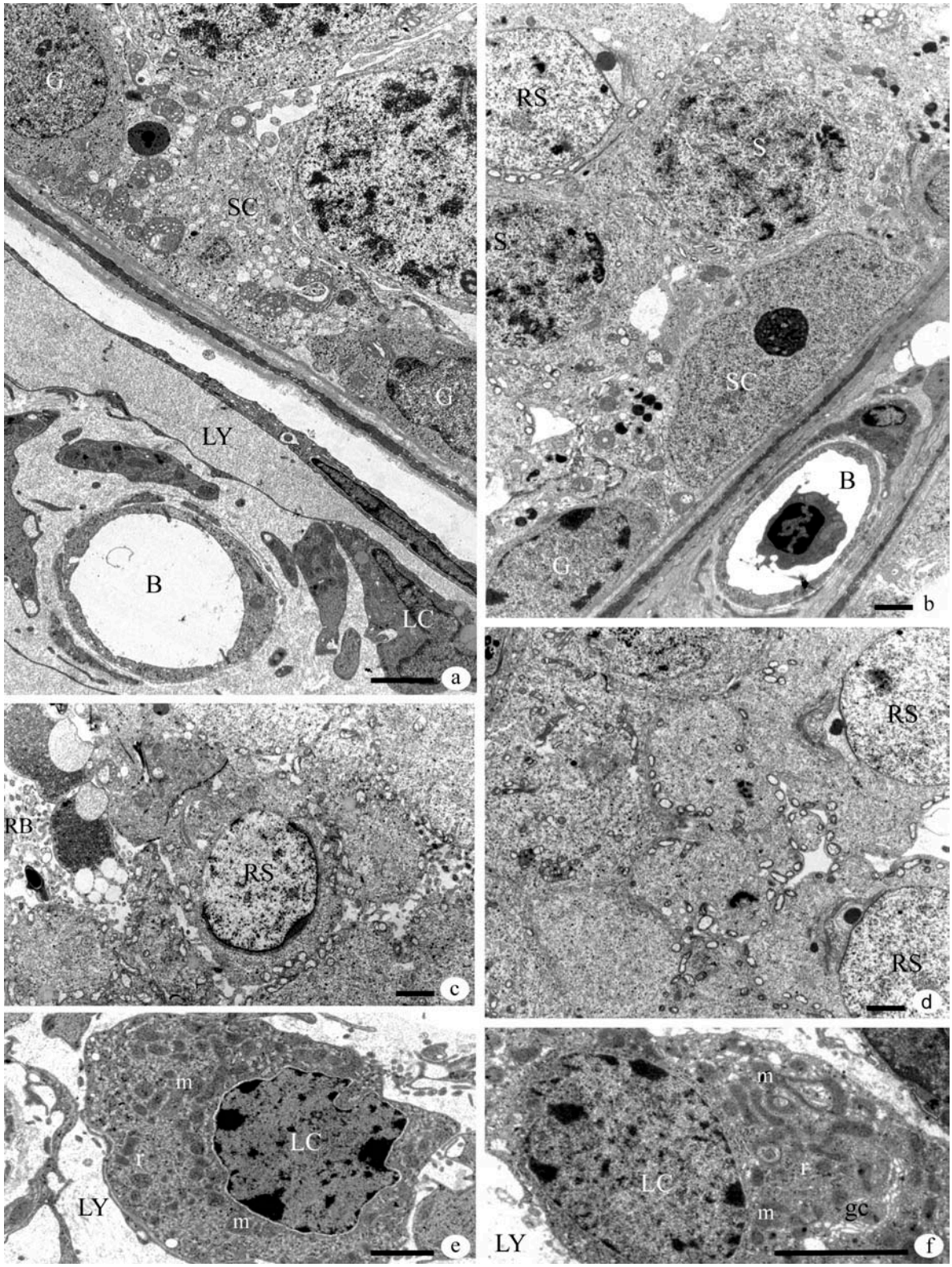


FIG. 2

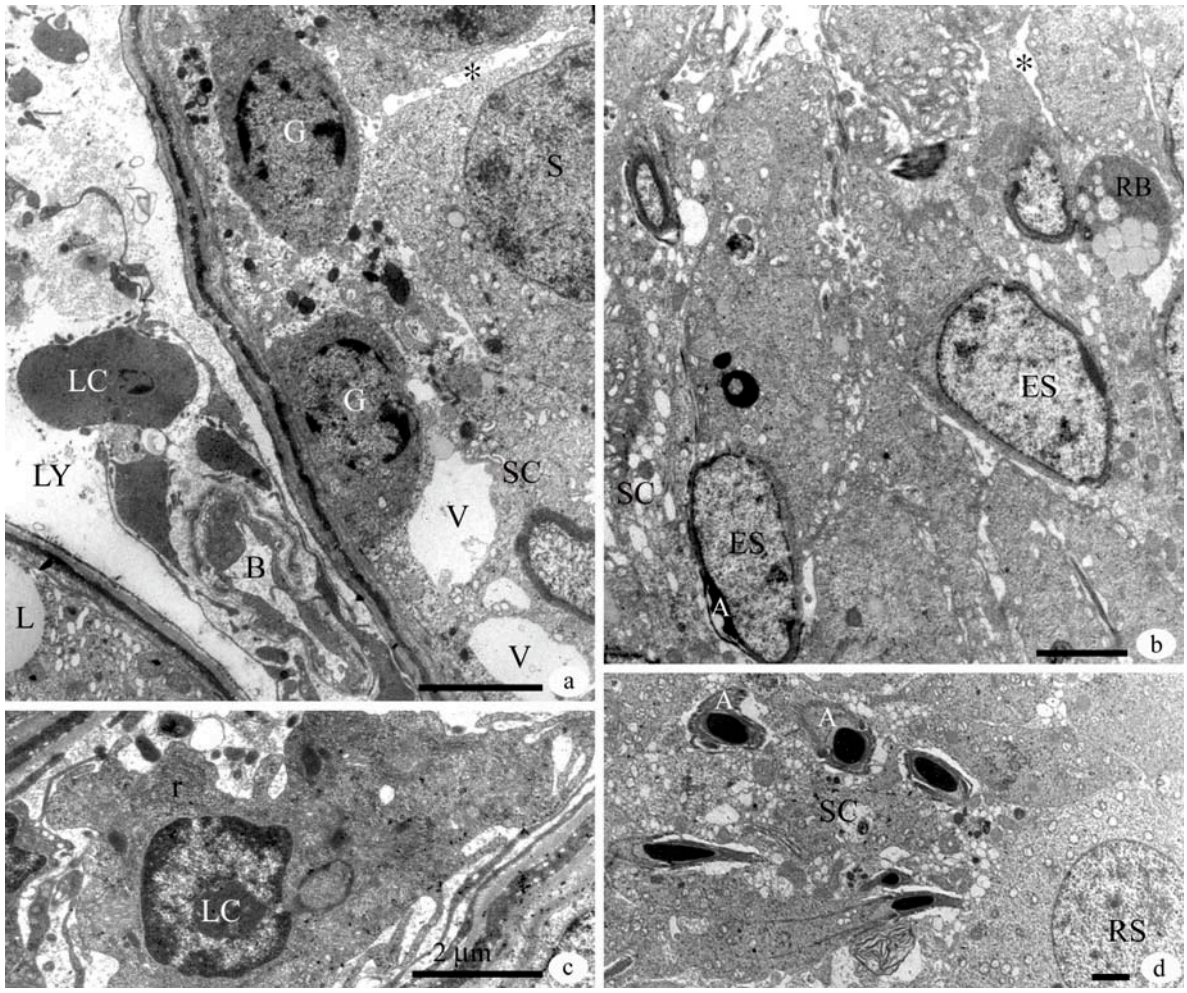


FIG. 3

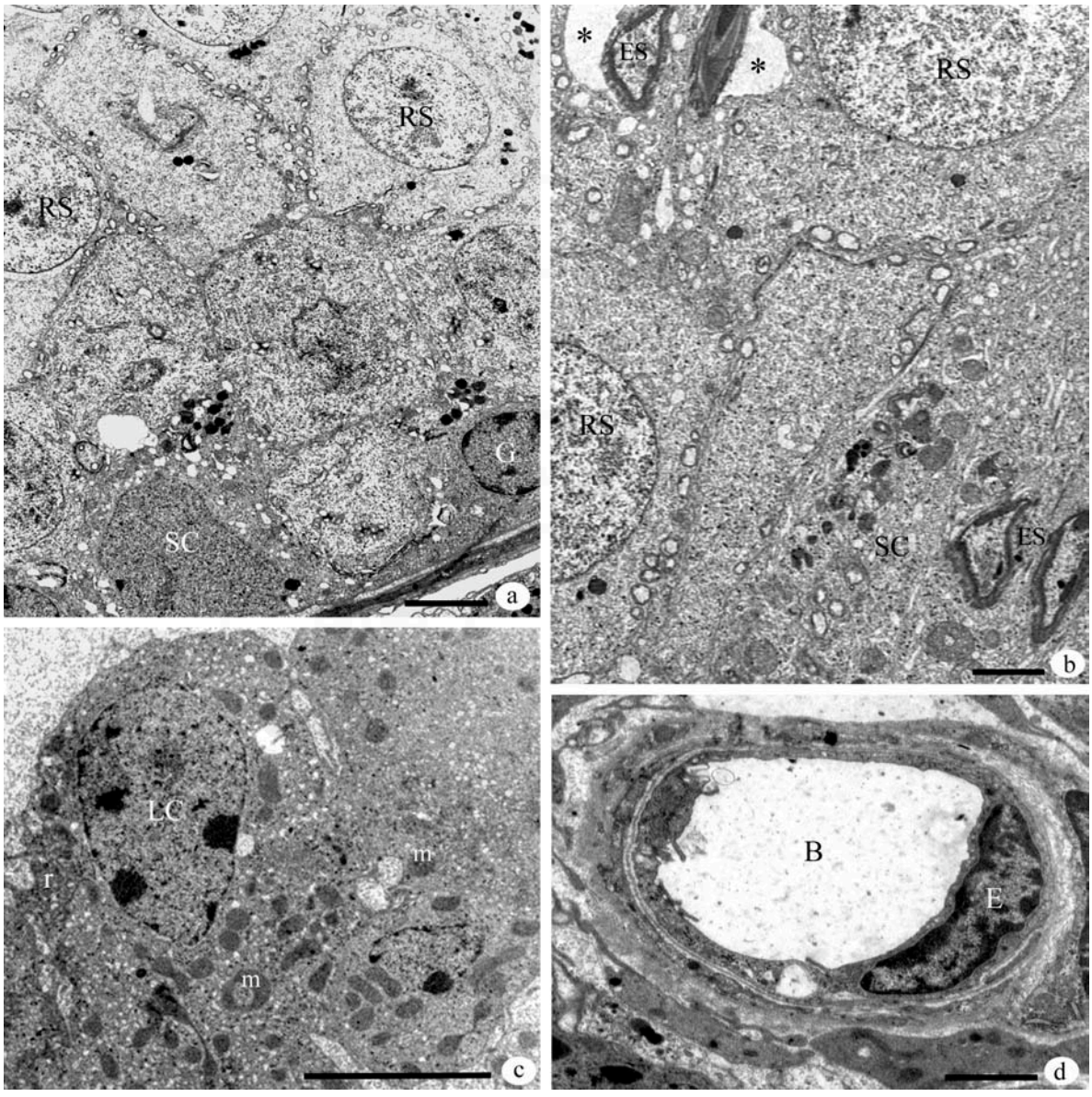
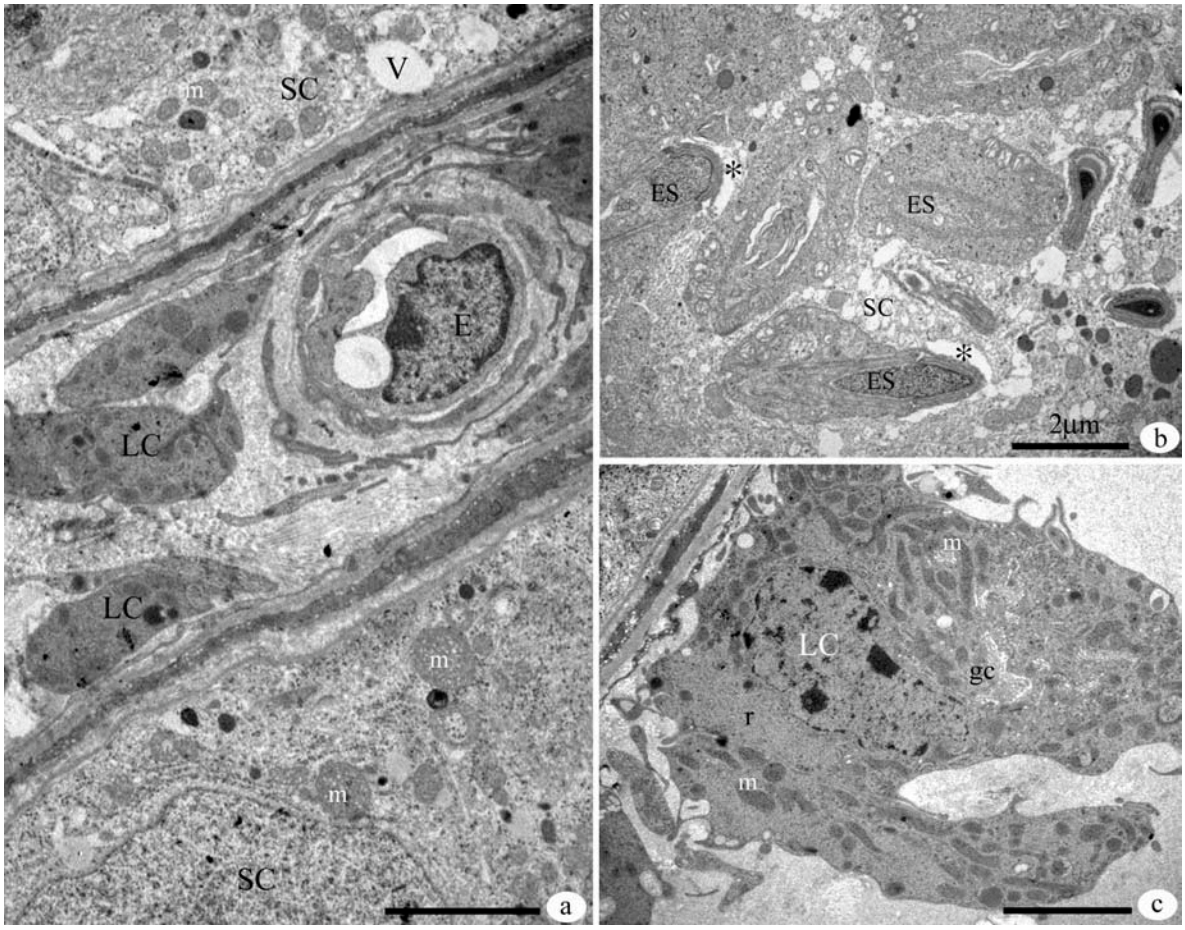


FIG. 4



7. CONCLUSÕES FINAIS

Após o tratamento com cloreto de cádmio e extrato de *Ginkgo biloba*, as avaliações morfológicas e morfométricas realizadas em microscopia de luz possibilitaram somente a detecção dos efeitos do cádmio nas células de Leydig. Estas células apresentaram redução de seu volume individual, mas observou-se que o extrato de *Ginkgo biloba* foi capaz de manter ou recuperar o volume destas células. Acreditamos que a dificuldade em observar os efeitos do cádmio em todo parênquima testicular se deu devido à pequena dose utilizada. Entretanto, a análise ultra-estrutural, em microscopia eletrônica de transmissão, permitiu a observação dos graves efeitos causados pelo cádmio no epitélio seminífero e confirmou os efeitos deste metal nas células de Leydig. Portanto, concluímos que a administração do extrato de *G. biloba* seqüencialmente à dose de cádmio (3 μmol/Kg BW) é eficaz em proteger todo parênquima testicular enquanto o pré-tratamento só previne danos causados às células de Leydig.