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Efeito hepatoprotetor da silimarina (*Silybum marianum*) sobre a hepatotoxicidade induzida por paracetamol (APAP) em ratos espontaneamente hipertensos (SHR)

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências da Saúde.
Área de concentração: Saúde Humana

Orientador: Prof. Dr. Roberto Kenji Nakamura Cuman

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Dedico este trabalho a todos aqueles que contribuíram para sua realização.

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"A simplicidade tem magia e genialidade. Sustente o impulso com um grande sonho".

(Jorge Paulo Lemann)

Efeito hepatoprotetor da silimarina (*Silybum marianum*) sobre a hepatotoxicidade induzida por paracetamol (APAP) em ratos espontaneamente hipertensos (SHR)

RESUMO

Este estudo teve como objetivo investigar o efeito da silimarina sobre o estado hipertensivo e as alterações das funções hepáticas induzidas por paracetamol (APAP) em ratos espontaneamente hipertensos (SHR). Os animais foram divididos em 6 grupos experimentais (n=12 por grupo): (I), ratos normotensos da linhagem Wistar (N) utilizados como grupo controle, receberam solução salina (NaCl 0,9% , via oral); (II), SHR que receberam salina (NaCl 0,9 %, via oral); (III) e (IV) , ratos N e SHR tratados com APAP (3g/kg; via oral), respectivamente; (V) e (VI), N e SHR, respectivamente pré-tratados com silimarina (SLM) (200mg/kg , via oral), durante 7 dias antes da administração de APAP. Após doze horas da administração de APAP, todos os animais foram eutanaziados e a função hepática foi determinada pelos marcadores plasmáticos: alanina (ALT) e aspartato aminotransferase (AST), fosfatase alcalina (ALP), glicose (GLU) e gama-glutamil transferase (γ-GT). Amostras de tecido hepático foram utilizadas para determinar: a atividade da enzima mieloperoxidase (MPO), a produção de óxido nítrico (NO) e, posteriormente amostras foram seccionadas para análise histológica. Os resultados demonstram que a hepatotoxicidade está aumentada em animais SHR e N (grupos III e IV) e, que o tratamento com a SLM (grupos V e VI) reverteu as alterações observadas. Os dados foram expressos como a média ± SEM para cada grupo. Os resultados foram analisados estatisticamente por meio de análise de variância (ANOVA One-way) seguida pelo teste de Tukey ($p < 0,05$). Os resultados foram comparados com o grupo de animais N. Não houve diferença significativa entre animais SHR e N nos níveis de ALT indicando que o estado hipertensivo não interfere na função hepática basal. Porém, houve aumento significativo nos níveis de ALT pelo tratamento com APAP em ambos os grupos, o pré-tratamento com SLM reverteu esta alteração, **ALT**: (*I*) 51,6 ± 1,9 U/L; (*II*) 57,3 ± 3 U/L; (*III*) 181,5 ± 18,3* U/L **(72%)**; (*IV*) 196 ± 23,7* U/L **(71%)**; (*V*) 65 ± 4,7** U/L (**64%)**; (*VI*) 83,1 ± 7,9** U/L (**58%),** $p<0,0001$; houve diferença significativa nos níveis de AST entre os grupos de animais N e SHR, com o aumento significativo pelo tratamento com APAP. O pré-tratamento com SLM promoveu uma redução nos níveis de AST em animais N e SHR, **AST:** (*I*) 74,3 ± 4,2 U/L; (*II*) 103,5 ± 5 U/L; (*III*) 189,5 ± 16,2* U/L **(61%)**; (*IV*) 224,7 ± 23,8* U/L (54**%)**; (*V*) 113,6 ± 11,9** U/L **(40%)**; (*VI*) 131 ± 7,9** U/L **(42%)**,

p<0,0001; houve diferença significativa entre animais SHR e N e também, após o tratamento com APAP nos níveis de ALP. Entretanto, não houve diferença significativa pelo tratamento com SLM, **ALP:** (*I*) 198 ± 14,87 U/L; (*II*) 270,4 ± 4,71$^{\#}$ U/L; (*III*) 178,2 ± 6,49 U/L **(11%)**; (*IV*) 245,3 ± 17,62$^{\#\#}$ U/L **(10%)**; (*V*) 204,3 ± 33,92 U/L (13**%)**; (*VI*) 195,3 ± 7,05 U/L **(20%)**, p=0,0015; não houve diferença significativa nos níveis de GLU entre os animais SHR e N, e nos tratamentos com o APAP e SLM, **GLU:** (*I*) 150,4 ± 7,9 mg/dl; (*II*) 150,4 ± 6,7 mg/dl; (*III*) 156,4 ± 5,2 mg/dl **(4%)**; (*IV*) 143,4 ± 10,3 mg/dl **(5%)**; (*V*) 167,2 ± 3,4 mg/dl **(7%)**; (*VI*) 167,8 ± 4,84 mg/dl **(16%)**, p=0,1735. Não houve diferença significativa nos níveis de γ-GT entre os animais SHR e N. Apenas em SHR quando comparados estes tratados com APAP e SLM, **γ-GT:** (*I*) 1,94 ± 0,2 U/L; (*II*)1,85 ± 0,2 U/L; (*III*)1,55 ± 0,4 U/L **(25%)**; (*IV*) 4,12 ± 1 U/L* **(55%)**; (*V*) 1,85 ± 0,2 U/L **(19%)**; (*VI*) 1,66 ± 0,1**U/L **(61%)**, p=0,0102; a resposta inflamatória foi avaliada pela atividade da MPO e pela produção de NO no tecido hepático, e também por meio da histologia e infiltração leucocitária no tecido. A infiltração leucocitária pela atividade de MPO foi de maior intensidade após o tratamento com APAP e o tratamento com SLM diminuiu a migração de leucócitos, em ambos os grupos. Não houve diferença significativa para a atividade da MPO entre os animais SHR e N, **MPO:** (*I*) 0,11 ± 0,02U/L; (*II*) 0,19 ± 0,03U/L; (*III*) 0,36 ± 0,03*U/L **(69%)**; (*IV*) 0,54 ± 0,05*U/L **(65%)**; (*V*) 0,13 ± 0,04**U/L **(64%)**; (*VI*) 0,23 ± 0,04**U/L **(57%)**, p<0,0001; a produção de NO, um radical livre presente no processo inflamatório não foi diferente entre os grupos de animais SHR e N. Após o tratamento com APAP, houve aumento significativo na produção de NO para ambos os grupos. O tratamento com SLM diminuiu a produção deste radical livre, sugerindo atividades anti-radicais livres e anti-inflamatórias da SLM, **NO:** (*I*) 34,9 ± 4,7; (*II*) 38,5 ± 3,8; (*III*) 68 ± 9,6* **(49%)**; (*IV*) 84,5 ± 4,8* **(54%)**; (*V*) 38,9 ± 2** **(43%)**; (*VI*) 45,9 ± 3,9** **(46%),** p<0,0001. A lesão hepática foi avaliada através de estudos histológicos corados por hematoxilina e eosina. Nossos dados, em conjunto, indicam que o estado hipertensivo tem influência significativa na hepatotoxicidade induzida pelo APAP. Além disso, que a SLM por reduzir as alterações funcionais e histopatológicas induzidas pelo APAP reduz esta toxicidade, provavelmente devido aos efeitos desta substância sobre a produção de radicais livres pelo APAP, que poderiam induzir a lesão hepática.

Palavras-chave: Silimarina, hipertensão, paracetamol e hepatotoxicidade.

Hepatoprotective effect of silymarin (*Silybum marianum*) on hepatotoxicity induced by acetaminophen (APAP) in *Spontaneously Hypertensive Rats* (SHR)

ABSTRACT

The aim of this work was to investigate the effect of silymarin on a hypertensive state and the hepatic function alterations in an acetaminophen-induced model (APAP) of hepatotoxicity in Spontaneously Hypertensive Rats (SHR). The animals were divided into 6 experimental groups (n=12 each group): (I), normotensive Wistar rats (N) were used as a control group, received a saline solution (NaCl 0,9% , orally); (II), SHR that received a saline solution (NaCl 0,9% , orally); (III) and (IV) , N rats and SHR treated with APAP (3g/kg; orally), respectively; (V) and (VI), N and SHR, pretreated with silymarin (SLM) (200mg/kg, orally) respectively during 7 days before the APAP administration. Twelve hours after APAP administration, all animals were euthanized and the hepatic function was determined by plasmatic biomarkers: alanine (ALT) and aspartato aminotransferase (AST), alkaline phosphatase (ALP), glucose (GLU) and gamma glutamyl transferase (γ-GT). Samples of hepatic tissue were used to determine: the activity of the myeloperoxidase enzyme (MPO), the nitric oxide (NO) production. Indeed, tissue samples were selected for histological examination. The results showed that hepatotoxicity was increased in SHR and N animals (groups III and IV). Thus, SLM treatment reversed all changes observed (groups V and VI). Data were expressed as means ± SEM for each group. The results were statistically analysed by ANOVA (One-way), followed by Tukey's test ($p < 0, 05$). The results were compared with normotensive animals. None significant differences were observed between the animals SHR and N related to ALT levels, indicating that hypertensive state did not interfere in basal hepatic functions. Although there was a significant increase in the ALT levels for APAP treated animals in both groups, the SLM pre-treatment restored these alterations, **ALT**: (*I*) 51.6 ± 1.9 U/L; (*II*) 57.3 ± 3 U/L; (*III*) 181.5 ± 18.3* U/L **(72%)**; (*IV*) 196 ± 23.7* U/L **(71%)**; (*V*) 65 ± 4.7** U/L (**64%)**; (*VI*) 83.1 ± 7.9** U/L (**58%),** $p<0.0001$; a significant difference in the AST levels was observed between N and SHR groups, increased after APAP treatment. The pre-treatment with SLM reduced the AST levels in N and SHR animals, **AST:** (*I*) 74.3 ± 4.2 U/L; (*II*) 103.5 ± 5 U/L; (*III*) 189.5 ± 16.2* U/L **(61%)**; (*IV*) 224.7 ± 23.8* U/L (**54%)**; (*V*) 113.6 ± 11.9** U/L **(40%)**; (*VI*) 131 ± 7.9** U/L **(42%)**, $p<0.0001$. After APAP treatment a significant difference in the ALP levels was observed between the SHR and N, which was nor restored after SLM treatment, **ALP:** (*I*) 198 ± 14.9 U/L; (*II*)

270.4 ± 4.7$^{\#}$ U/L; (*III*) 178.2 ± 6.5 U/L **(11%)**; (*IV*) 245.3 ± 17.62$^{\#\#}$ U/L **(10%)**; (*V*) 204.3 ± 33.9 U/L (**13%)**; (*VI*) 195.3 ± 7.05 U/L **(20%)**, p=0.0015; there was no significant difference on GLU levels between SHR and N groups, in both treatments with APAP or SLM, **GLU:** (*I*) 150.4 ± 7.9 mg/dl; (*II*) 150.4 ± 6.7 mg/dl; (*III*) 156.4 ± 5.2 mg/dl **(4%)**; (*IV*) 143.4 ± 10.3 mg/dl **(5%)**; (*V*) 167.2 ± 3.4 mg/dl **(7%)**; (*VI*) 167.8 ± 4.8 mg/dl **(16%)**, p=0.17. In the γ-GT levels between SHR and N, none significant difference was verified. However it was observed only for SHR animals when compared to that treated with APAP or SLM **γ-GT:** (*I*) 1.94 ± 0.2 U/L; (*II*)1.85 ± 0.2 U/L; (*III*)1.55 ± 0.4 U/L **(25%)**; (*IV*) 4.12 ± 1 U/L* **(55%)**; (*V*) 1.85 ± 0.2 U/L **(19%)**; (*VI*) 1.66 ± 0.1**U/L **(61%)**, p=0.01; the inflammatory response was evaluated by the activity of MPO, by the production of NO on the hepatic tissue, by histological analysis and by leukocyte infiltration into hepatic tissue. The leukocyte infiltration and MPO activity were increased after APAP treatment whereas SLM reduced the leukocyte migration in both groups. The MPO activity was similar when compared SHR and N groups, **MPO:** (*I*) 0.11 ± 0.02U/L; (*II*) 0.19 ± 0.03U/L; (*III*) 0.36 ± 0.03*U/L **(69%)**; (*IV*) 0.54 ± 0.05*U/L **(65%)**; (*V*) 0.13 ± 0.04**U/L **(64%)**; (*VI*) 0.23 ± 0.04**U/L **(57%)**, p<0.0001; the NO production, a free radical involved in the inflammatory process, was similar between SHR and N groups. A significant increase in NO production was observed in these groups after APAP treatment. The treatment with SLM decreased NO contents, suggesting anti-free radical activities and anti-inflammatory of the SLM, **NO:** (*I*) 34.9 ± 4.7; (*II*) 38.5 ± 3.8; (*III*) 68 ± 9.6* **(49%)**; (*IV*) 84.5 ± 4.8* **(54%)**; (*V*) 38.9 ± 2** **(43%)**; (*VI*) 45.9 ± 3.9** **(46%),** p<0.0001. Liver injury was assessed using histological studies by hematoxylin and eosin staining. Together, our data indicated that hypertensive state affects significantly in the acetaminophen-induced hepatotoxicity. In additions, SLM by acts reducing the functional and histopathological alterations reduces the APAP-hepatotoxicity; probably due to their effects in the free radical production inducing hepatic injury.

Keywords: Silimarina, hypertension, acetaminophen and hepatotoxicity

LISTA DE ILUSTRAÇÕES

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SUMÁRIO

CAPÍTULO I

HEPATOTOXICIDADE

Nos Estados Unidos, mais de 50% dos casos de falência aguda hepática estão relacionados aos fármacos [1]. Diferentes enfermidades hepáticas podem ser desencadeadas pelo uso de xenobióticos [2, 3], incluindo hepatites agudas e crônicas, hepatite fulminante, cirrose hepática, doenças hepáticas colestáticas, esteatose hepática, distúrbios vasculares do fígado e tumores hepáticos [2, 3, 4, 5].

O tecido hepático está envolvido na biotransformação de diversas substâncias endógenas, exógenas e de medicamentos. Doenças hepáticas são causadas principalmente por agentes químicos tóxicos (álcool, tetracloreto de carbono, hidrocarbonetos clorados e gases CO_2 e O_2), e medicamentos: anticancerígenos (azatioprina, doxorrubicina, cisplatina), imunossupressores (ciclosporina), analgésicos anti-inflamatórios (paracetamol, tioacetamida), drogas anti-tuberculose (isoniazida, rifampicina), biológicos (vacina Bacillus Calmette-Guerin-), radiações gama, metais pesados (cádmio, arsênico), micotoxinas (aflatoxinas), galactosamina, lipopolissacarídeos, etc... Vários são os fatores de risco para lesão hepática, dentre eles: idade, sexo, alcoolismo, nutrição e polimorfismos genéticos de enzimas do citocromo P450 [3, 4].

PARACETAMOL

O paracetamol (APAP) é denominado quimicamente por: N-acetil-p-aminofenol; 4-hidroxiacetanilida; 4-acetamidofenol ou N-(4-hidroxifenil) acetamida. É uma das drogas mais prescritas para o tratamento da dor e hipertermia sendo seguro em doses terapêuticas [6]. Entretanto, em doses elevadas é uma das principais causas de intoxicação levando a danos hepáticos [2 - 7] e podendo evoluir para insuficiência hepática [3].

No Brasil há poucos relatos da toxicidade pelo APAP, diferentemente do observado em alguns países europeus e nos Estados Unidos onde é a causa mais comum de falência aguda do fígado [3, 8].

A hepatotoxicidade induzida por APAP ocorre por uma reação de biotransformação via citocromo P-450 (complexo enzimático envolvendo oxidases), com formação do metabólito reativo N-acetil-p-benzoquinona-imina (NAPQI). O metabólito é normalmente conjugado com glutationa reduzida (GSH). No entanto, com altas doses de APAP, onde ocorre a hepatotoxicidade, os níveis de NAPQI estão elevados, enquanto os níveis de GSH reduzidos. Esta alteração leva a uma disfunção mitocondrial, gerando a formação de espécies reativas de oxigénio (ROS) e de peroxinitrito nas mitocôndrias [9, 10]. A produção elevada de ROS pode resultar em danos celulares em diferentes tecidos [11, 12]. Subsequentemente, os grupos

sulfidrilas de proteínas hepáticas reagem com o metabólito ativo, promovendo necrose hepática [13, 14]. As alterações estruturais, a degeneração e a necrose tecidual, associam-se à elevação de marcadores séricos hepáticos [12, 13] tais como a elevação da alanina (ALT) e aspartato aminotransferase (AST), fosfatase alcalina (ALP) e gama-glutamil transferase (γ-GT) que indicam a hepatotoxicidade. Há relatos na literatura de que a lesão celular induzida pelo APAP envolve a participação do óxido nítrico (NO) [12], marcador do stress oxidativo, e presente em diferentes patologias, incluindo doenças renais [11], inflamatórias, cardiovasculares [14, 15], dentre outras [16].

A lesão hepática induzida por APAP em ratos é um modelo experimental [6, 10, 12, 13] utilizado para a triagem de substâncias com potencial atividade hepatoprotetora.

HIPERTENSÃO

As doenças cardiovasculares, dentre elas, a hipertensão arterial (HA), promovem alterações funcionais e estruturais nos vasos sanguíneos e no coração. Estas doenças podem levar à hipertrofia do ventrículo esquerdo, acidente vascular cerebral, infarto do miocárdio, morte súbita, insuficiências renais e cardíacas, alterações visuais e isquemia de órgãos vitais [17-19]. Doenças associadas à HA, envolvendo elevação da glicemia e dislipidemias são frequentes na população e estão associados ao aumento da mortalidade por doenças cardiovasculares e das complicações microvasculares [20]. Além disso, a HA é caracterizada por provocar alteração de fluxo sanguíneo, podendo interferir na perfusão sanguínea de diferentes órgãos, tais como os rins, fígado e pulmão.

Para o estudo da HA, são propostos vários modelos experimentais, dentre eles, os ratos espontaneamente hipertensos (SHR), que se assemelha à hipertensão essencial humana [20].

PRODUTOS NATURAIS

Várias substâncias têm sido estudadas em protocolos experimentais e clínicos para reduzir ou prevenir a hepatotoxicidade induzida por APAP. Atualmente, os produtos naturais (derivados de plantas) têm recebido considerável atenção devido as suas diversificadas propriedades farmacológica e, efeitos hepatoprotetores [21 - 24].

O mercado de fitoterápicos vem crescendo aproximadamente 15% ao ano, girando em torno de 50 bilhões de dólares anualmente, sendo mais evidente em países europeus e asiáticos [12]. A população faz o uso destes recursos fitoterápicos, como suplementos alimentares/dietéticos e remédios naturais. Plantas medicinais são utilizadas como alternativa terapêutica para várias doenças e apresentam eficácia no tratamento e/ou prevenção de

diversas patologias [25, 26]. Sendo assim, recomenda-se a utilização dos fitoterápicos como forma de reduzir os custos dos programas de saúde pública, promovendo fácil acesso, principalmente em países de baixas condições socioeconômicas. Porém, há necessidade de realizar estudos para determinar a eficácia e segurança desses fitoterápicos [13].

A Silimarina (SLM), comercializada como fitoterápico no Brasil é utilizada para o tratamento e/ou prevenção de doenças hepatobiliares [27, 28], sendo referência em pesquisas enquanto droga-padrão hepatoprotetora [21]. É uma planta originária do sul da europa, norte da África e ásia menor e bem aclimatada nas américas do sul e do norte e sul da Austrália. Popularmente é conhecida no Brasil como "cardo-mariano", "cardo-leiteiro", "cardo-de-santa-maria", "cardo-branco", "cardo-de-nossa-senhora" ou "cardo-santo". O extrato de SLM é constituído por um complexo de flavolignanas (silibina, isosilibina, silicristina e silidianina), sendo a silibina o composto majoritário [29].

FÍGADO

O fígado é o principal órgão de biotransformação de fármacos, sendo responsável por frequentes efeitos adversos de drogas, devido às ações diretas agudas e crônicas sobre este órgão. As hepatopatias podem ser caracterizadas por lesão e necrose celular, resposta imunológica e regeneração nodular que comprometem a estrutura hepática e a capacidade funcional dos hepatócitos [30]. Nas hepatopatias, há uma elevação dos níveis séricos de marcadores bioquímicos, como ALT, AST, ALP e γ-GT em casos hepatotoxicidade e também na atividade da MPO e no conteúdo de NO, indicadores do processo inflamatório [31].

Portanto, medicamentos utilizados rotineiramente na prática clínica podem induzir a lesão hepática, o que poderá limitar o seu uso e os benefícios esperados.

JUSTIFICATIVA

As alterações de fluxo sanguíneo hepático, os processos de metabolização e de biotransformação de drogas, os efeitos adversos hepáticos e os níveis de marcadores bioquímicos decorrentes de uma hepatotoxicidade induzida por diversas drogas, têm sido estudados, tanto em humanos [10, 13] como em animais [9, 11, 12]. As alterações estruturais e funcionais hepáticas podem ser devidas ao aporte sanguíneo, e as alterações cardiovasculares, principalmente as vasculares podem agravar o quadro de hepatotoxicidade. Entretanto, há poucos relatos na literatura avaliando a associação entre HA e a hepatotoxicidade. Os resultados desta pesquisa auxiliarão a prescrição mais cuidadosa de fármacos em pacientes portadores de doenças hepáticas, bem como as prováveis interações medicamentosas em pacientes que utilizam a polifarmácia. O conhecimento da eficácia de drogas com atividade hepatoprotetora será útil para o tratamento desses pacientes evitando alguns dos efeitos adversos decorrentes da toxicidade medicamentosa.

Nesta pesquisa investigamos a associação entre a hipertensão e a hepatotoxicidade por meio da avaliação da função hepática, alterações estruturais e histopatológicas e o papel da SLM enquanto droga hepatoprotetora em modelo de hepatotoxicidade experimental induzida pelo APAP em ratos SHR.

OBJETIVOS

GERAL

Verificar a associação entre a hipertensão e a hepatotoxicidade por meio da avalição da função hepática, alterações estruturais e histopatológicas e o papel da SLM enquanto droga hepatoprotetora em modelo experimental de hepatotoxicidade induzida pelo APAP em ratos SHR e normotensos (N).

ESPECÍFICOS

Verificar a lesão hepática induzida pelo APAP em animais SHR e N;

Demonstrar as alterações funcionais (bioquímicas) e estruturais (histopatológicas) na intoxicação pelo APAP;

Investigar a participação do NO e a migração leucocitária (MPO) na intoxicação pelo APAP;

Avaliar o efeito do pré-tratamento com SLM sobre as alterações encontradas na intoxicação induzida pelo APAP.

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CAPÍTULO II

ARTIGO I: "Hepatoprotective effect of silymarin (*Silybum marianum*) on hepatotoxicity induced by acetaminophen (APAP) in *Spontaneously Hypertensive Rats* (SHR)"

Hepatoprotective effect of silymarin (*Silybum marianum*) on hepatotoxicity induced by acetaminophen (APAP) in *Spontaneously Hypertensive Rats* (SHR)

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ABSTRACT

This study was aimed to investigate the effect of Silymarin on the hypertension state and the liver function changes induced by acetaminophen (APAP) in *spontaneously hypertensive rat* (SHR). The animals were divided into 6 experimental groups (n=12 per group): (*I*) male Wistar rats were used as control normotensive group (N) that received saline (NaCl 0.9%, oral); (*II*) SHR that received saline (NaCl 0.9%, oral); (*III*) N and (*IV*) SHR treated with APAP (3 g/kg; oral); N (*V*) and SHR (*VI*), pre-treated orally with silymarin (200 mg/kg, oral) during 7 days, before APAP administration. Twelve hours after APAP administration, plasmatic levels of liver function markers: alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (GLU), gamma glutamyl transferase (γ-GT) and alkaline phosphatase (ALP) of all groups were determined. Liver injury was assessed using histological studies by hematoxylin and eosin staining. Samples of their livers were then used to determine the myeloperoxidade (MPO) activity, nitric oxide (NO) productin and were also sectioned for histological analysis. Data were expressed as the mean ± SEM for each group. Results were statistically analyzed by using one-way variance analysis (ANOVA) followed by Tukey's test ($p<0.05$). About hepatic markers, no differences were observed for ALT, γ-GT and GLU levels between SHR and normotensive rats groups. However, AST and ALP levels were increased in hypertensive animals. APAP treatment promoted an increase in ALT, AST in both SHR and N. However, only for SHR, γ-GT levels were increased. The inflammatory response evaluated by MPO activity and NO production showed that SHR were more susceptible to APAP effect, by increase leucocyte infiltration. Silymarin treatment (LEGALON®) restored the hepatocyte functional and histopatological alterations induced by APAP in normotensive and hypertensive animals.

Keywords: Silymarin, hypertension, acetaminophen and hepatotoxicity

1. Introduction

A growing number of patients require several drugs to treat multiple chronic disorders. The prescription of multiple drugs is related to increase risk of adverse drug-related events, among these, hepatic injury. The liver is of vital importance in intermediary metabolism and is continuously exposed to xenobiotics, environmental pollutants, and chemotherapeutic agents, since it is involved in detoxification and elimination of toxic substances (Xinsheng and Manautou, 2013). Hepatic damage is associated with altered metabolic functions and it is still a severe health problem, since conventional drugs used in the treatment of liver diseases serious adverse effects (Hayden and Sowers, 2008).

Cardiovascular diseases such as arterial hypertension (AH) promote functional and structural changes in blood vessels and myocardium, which lead to left ventricular hypertrophy, myocardial infarction, cerebral vascular accident, renal disease and complications of vital organs (Plante, 2002; Hayden and Sowers, 2008; Mambelli, 2011). Furthermore, AH promotes changes in blood flow, and can influence the blood perfusion of different organs, such as kidneys, lungs and liver. Thus, hypertensive patients could develop severe hepatic damage and several experimental hepatotoxicity models are used to investigate new pharmacologic treatment. However, there are few studies investigating the new strategic therapeutic in hypertensive animals with hepatotoxicity.

APAP is widely used as analgesic-antipyretic drug and is considered remarkably safe drug when used at usual therapeutic doses. APAP toxicity is due to the formation of toxic metabolites when a part of it is metabolized by cytochrome P450 (Joydeep *et. al.*, 2010). It is metabolized by sulfation and glucuronidation of the parahydroxyl group. APAP hepatotoxicity is caused by its reactive metabolite *N*-acetyl-p-benzoquinone imine (NAPQI), which causes oxidative stress and glutathione (GSH) depletion, a prerequisite for APAP-induced hepatotoxicity (Aubert *et. al.*, 2012; Zhang *et. al.,* 2013). In overdoses, it is a potent hepatotoxin, producing fulminant hepatic and renal tubular necrosis, which can be lethal in human and animal. Several studies about protection against hepatotoxicity has been investigated to ameliorate the livers disorders treatment. Many formulations containing herbal extracts are used for regeneration of hepatic cells and for protection of the liver against damage (Jaeschke *et. al.*, 2013). Silymarin (SLM) is a lipophilic extract isolated from the seeds and fruits of *Silybum marianum,* a herbaceous plant belonging to the family Compositae and native to a narrow area of the Mediterranean. SLM is composed of several flavonolignans isomers (65-80%) with small amounts of flavonoids and fatty acids (20-35%) and other polyphenolic compounds (Kim *et. al.,* 2003; Lee *et. al.*, 2006; Elmowafy *et. al.,* 2013). The main isolated and structural active component of SLM is silybin, which comprising about 33% of total SLM weight, and is clinically used (Pradhan and Girish, 2006; Féher

and Lengyel, 2012) as hepatoprotector to treat liver injuries (Hau *et. al.*, 2010). Besides, other biological activies for SLM, such as hepatoprotective/hepatoregenerador, immunomodulator, anti-inflammatory, antioxidant and antifibrotic activities were described (Feher and Langyel, 2008; Das *et. al.*, 2011; Elmowafy *et. al.*, 2013). There is evidence that SLM is effective in hepatic disease induced by different drugs (Hau *et. al.*, 2010; Sherif and All-Gayyar*,* 2013). Currently, SLM is used as a reference drug in the screening of new drugs hepatoprotective (Cordero-Pérez *et. al.*, 2013; Elmowafy *et. al.*, 2013; Raj and Gothandam, 2014). This study aimed to investigate the effect of silymarin on changes in the liver function after APAP administration in SHR, because cardiovascular diseases (including AH) which promotes structural and functional changes in blood vessels and myocardium (Houshyar *et. al.,* 2012), may interfere in the blood perfusion of different organs, such as kidney, lungs, heart and also liver.

2. Methods

2.1 Animals

Normotensive Wistar male rats (N, systolic = 124 ± 5 mmHg, diastolic = 95 ± 2 mmHg) and SHR (*spontaneously hypertensive rat*, systolic = 231 ± 1 mmHg, diastolic = 191 ± 2 mmHg), (Salsoso *et. al*., 2014) aged 14 - 16 weeks, weighing 250 - 330 g were provided by the Central Animal House of the State University of Maringá. The animals were housed at 23 ± 2ºC under a 12/12 h light/dark cycle water and ration (Nuvilab®) *ad libitum*. Prior the experiments, the animals were fasted overnight 12 h and water *ad libitum*. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (144/2012 CEAE/UEM).

2.2 Hepatotoxicity induced by APAP and treatment of animals with Silymarin (SLM)

The animals were divided into 6 experimental groups of 12 animals each-one. Group *I* and *II*: N and SHR received orally APAP vehicle (saline containing 2% Tween 80), respectively; *III* and *IV*, N and SHR received APAP-treatment (3g/kg; orally) respectively, as described (Fakurazi *et. al.,* 2008); *V* and *VI*, N and SHR, pre-treated orally with the standard drug, SLM (200mg/kg, orally) respectively, during 7 days, before APAP-induced hepatotoxicity as described (Quan *et. al.,* 2011).

2.3 Determination of serum AST, ALT, GLU, γ-GT and ALP levels

After 12 hours of hepatotoxicity induced by APAP, all rats were anesthetized with halothane 3% and blood was collected from inferior vena cava for determination of plasmatic ALT (alanine aminotransferase), AST (aspartate aminotransferase), GLU (glucose), γ-GT

(Gamma glutamyl transferase) and ALP (alkaline phosphatase) using the Analyze ® Gold Kits.

2.4 Determination of MPO (myeloperoxidase) activity

The MPO enzyme activity was measured in the supernatant of homogenate of liver tissue sections. Briefly, the liver sections were put in phosphate buffered saline (PBS) in a Potter homogenizer and the homogenate was stirred in a vortex and centrifuged. Ten microliters of the supernatant was added to each well in triplicate in a 96-well microplate. The PBS solution (200 µl) that contained 4.21 mg o-dianisidine dihydrochloride (Sigma), 22.5 ml double-distilled water, 2.5 ml potassium phosphate buffer (pH=6), and 10 µl of 1% H_2O_2 was added. The enzyme reaction was stopped by 30 µl the addition of sodium acetate (2.23 g in 20 ml of double-distilled water). Enzyme activity was determined by the absorbance measured at 450 nm using a microplate spectrophotometer (Asys Expert Plus®).

2.5 Determination of NO (oxid nitric) production

The NO production was determined by the Griess method in the supernatant of liver tissue sections, which determines the nitrite production [Saleh *et. al.,* 1999]. Two hundred microliters of the supernatant was added to each well in triplicate in a 96-well microplate. Sequentially, solution (50 µl) was added to Griess (1g sulfanilamide in 2.5ml fosforic acid and 0.1g dihydrochloride of N-1-naftiletilonodiamina milli-Q water) at room temperature. The reading was taken using an ELISA plate reader at a wavelength of 550 nm, as described [35]. ON production were calculated from a standard curve of sodium nitrite. The results were expressed as µM.

2.6 Liver Index and Histopathological Analysis

The livers of rats were collected and inspected macroscopically. The liver index was calculated as liver weight divided by body weight. The largest right lobe of each liver was excised and fixed in a 10% formalin solution for histopathologic analyses of all animals. Subsequently, the livers were dehydrated in increasing concentrations of alcohol (80 - 100%, v/v) and embedded in paraffin blocks which were sectioned in 6 µm thickness on a Leica Rotary Microtome (Leica Microsystems, Gladesville, NSW, Australia). The organ sections were stained with hematoxylin/eosin (H&E) for evaluation of tissue morphology using light microscopy. The changes in tissue morphology were assessed for nuclear variations, cytoplasmic eosinophilia, swelling and vacuolation in both periportal and central areas.

2.6 Statistical analysis

Data were expressed as the mean ± SEM for each group. Results were statistically analyzed by using one-way variance analysis (ANOVA) followed by Tukey's test. Differences were considered significant when $p<0.05$. Statistical analyzes were performed using GraphPad Prism® (Version 5.0 GraphPad Software, Inc). Results are expressed and representative in separate experiments.

3. Results

The SLM effects on liver weight in rats with APAP-induced hepatotoxicity are presented in Table 1. Significant differences in the liver weight were observed after hepatotoxicity induced by APAP or pretreatment with the SLM, in both groups. The liver index was increased after APAP treatment, but restored to normal values after SLM pretreatment (Table 1).

The effects of SLM on plasmatic ALT, AST, ALP, γ-GT and GLU, were investigated after APAP treated rats and were represented in Figure 1. Data not showed a significant difference in plasmatic ALT levels between N and SHR animals. However, a significant increase (72% and 71%, respectively) in ALT levels after APAP treatment, in both groups, was verified. The SLM treatment restored (64% and 58%) to normal levels (Fig. 1-A).

Data showed a significant difference in plasmatic AST levels between N and SHR animals. However, a significant increase (61% and 54%, respectively) in AST levels after APAP treatment in both groups, restored by SLM (40% and 42%) (Fig. 1-B). In the same manner, significative difference in ALP when compared to SHR with N groups was found APAP and SLM administration in both groups did not alter ALP levels (Figure 1-C).

The levels of γ-GT (figure 1-D) were increased in SHR+APAP treatment (55%) when compared to that of SHR, which was restored by SLM treatment (61%). Therefore, significant differences not found for APAP treatment and SLM pretreatment in both groups to GLU levels (Fig. 1-E).

The effects of SLM on MPO activity and NO production in liver tissues, were investigated after APAP treated rats and were represented in Figure 2. The MPO activity in SLM pretreated rats was significantly decreased in N (64%) and SHR (57%), when compared with that of APAP group (69 increased of% in N and 65% in SHR) (figure 2-A).

In the figure 2-B the N and SHR animals group treated with an overdose of APAP developed significant hepatic damage, which was observed by a substantial increase in the NO production. Administration of SLM after APAP treatment resulted in a significant reduction (43% in N and 46% in SHR) in NO production in APAP groups (49% and 54%, respectively) and appears to be protective in reducing the injurious effect of APAP.

The livers untreated of SHR and W groups not showed histopatological alteration (Figure 3-A and 3-B), whereas the treatment with APAP showed severe injury characterized by features typical of inflammatory hepatic tissues, including the presence of moderate infiltration of neutrophils and inflammation, characteristics of hepatic damage as indicated by biochemical and enzymatic assays (Figure 3-C and 3-D). The histopathological analysis of the livers obtained from the SLM-pretreated group SHR and N (200 mg/kg) no showed pathological hallmark after APAP treatment (Figure 3-E and 3-F).

4 Discussion

Chronic patients often are treated with multiple drugs. During hypertensive state many structural and functional alterations occur, such as: increase in the blood flux, organs blood perfusion and burst of the oxidative stress, releasing ROS, should affect vital organs. Thus, it is plausible to assume that hypertensive state could enhance the hepatic damage and others injuries in various organs.

The liver is involved in detoxification and elimination of drugs and a liver injury could affects pharmacokinetics parameters of drugs, and increases the risk of adverse drug-effects.

Liver injuries induced by APAP is one of the best characterized system of xenobiotic-induced hepatotoxicity. It is commonly used as a model for the screening of hepatoprotective activities of drugs, where free radicals and oxidative processes play an important role in hepatotoxicity (Jaeschke *et. al.*, 2013).

A decrease in total serum protein after APAP treatment could be associated with the decrease in the number of hepatocytes, which in turn may result in the decreased hepatic capacity to synthesize protein and, consequently, decrease liver weight (Bhadauria, 2010; Oyaqbemi and Odetola, 2010). Our results showed that livers weight of hypertensive and normotensive rats did not differ. However, changes are observed in the liver index that was increased after APAP treatment, but restored to normal values after SLM pretreatment.

Hepatotoxic drugs, such as APAP, are known to cause marked elevation in serum level of enzymes, such as ALT, AST, ALP, and bilirubin, indicating significant hepatocellular injury (Green *et. al.,* 2010). When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver injury (Alkiyumi *et. al.*, 2012; Bell *et. al.*, 2012). Our data showed that the liver activity functional is altered, since ALT, AST, γ-GT and ALP levels are increase in SHR, but not for glucose levels (Binda *et. al.*, 2001). Generally, there is a raised activity of serum transaminases in intoxicated rats (Dremza *et. al.,* 2010), as observed in the present study. However, ALT and AST levels was increased but this is enough to be attributed to the damaged structural integrity of the liver because transaminases are cytoplasmic enzymes in nature and are released into the circulation after cellular damage (Kelava *et. al.*, 2013). Interesting, in our work, γ-GT levels were increased after APAP

treatment, but not in normotensive animals. γ-GT is a common biomarker of liver injury and alcohol consumption (Nakanishi *et. al.*, 2004). However, recent epidemiologic and clinical studies have also found a close association between γ-GT level and the risk of cardiovascular disease, diabetes, and metabolic syndrome, demonstrating an association between hypertension and hepatic injury (Houchyar *et. al.*, 2012; Ryu *et. al.*, 2011; Lee *et. al.*, 2007), as demonstrated in by our results.

Silymarin has hepatoprotective properties and is used in treatment of various liver diseases (Elmowafy *et. al.*, 2013). Various studies indicate that silymarin exhibits strong antioxidant activity (Simeonova *et. al.*, 2013) and shows protective effects against hepatic toxicity induced by a wide variety of agents by inhibiting lipid peroxidation (Binda *et. al.*, 2001; Bosisio *et. al.*, 1992). Higher total phenolic content has been known to contribute to the antioxidant activity of extracts (Guo *et. al.*, 2003)**,** while antioxidant activity has also been linked to the hepatoprotective effect of some extracts (Dursun *et. al.*, 2009)**.** These findings corroborate with our results on the ability of SLM to exert a hepatoprotective activity.

In this study, SLM treatment restore to normal values the levels of ALT, AST in all animals treated with APAP, γ-GT levels in SHR. The reduced concentrations of these enzymes as a result of SLM administration might probably be, in part, due to the presence of chemical constituents in the extract (Cordero-Pérez *et. al.*, 2013). The plasmatic reduction of these marker enzymes, to return to near normally values would be owing to the anti-hepatotoxic effect of SLM.

Histopathologic studies supported the evidence of biochemical parameters analised in this study. Histological analyses of rat liver treated with APAP showed significant hepatotoxicity, characterized by inflammatory hepatic tissues, including the presence of moderate infiltration of neutrophils. There was extensive infiltration of inflammatory cells around the central vein and loss of cellular boundaries in all groups, after hepatotoxicity induced by APAP. High activity of MPO and NO production was used to demonstrate an acute inflammatory process as observed after the liver injury induced by APAP. The inflammatory response evaluated by MPO activity and NO production showed that SHR more susceptible to APAP effect, by increasing the leucocyte infiltration. The NO production, a free radical present in the inflammatory process did not differ in SHR and N groups. After treatment with APAP there was a significant increase in NO production in both groups of animals. Pretreatment with SLM decreased NO production, suggesting activities anti-inflammatory and anti-free radicals release of SLM. SLM treatment reduced the severity of hepatic damage, when compared to that observed after APAP treatment, decreasing neutrophil infiltration in the hepatic tissue which further indicated its significant hepatoprotective effect.

6 Conclusion

In light of our study, here we demonstrated an important association between hypertension and hepatic damage. SLM was effective to protect the liver of damage induced by APAP. The use of natural products as SLM to treated hepatotoxicity could be important to patients receiving several medicines, such as observed for hypertensive individuals.

7 Conflict of interest

The authors declare that there are no conflicts of interest.

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Figures/Table Legend

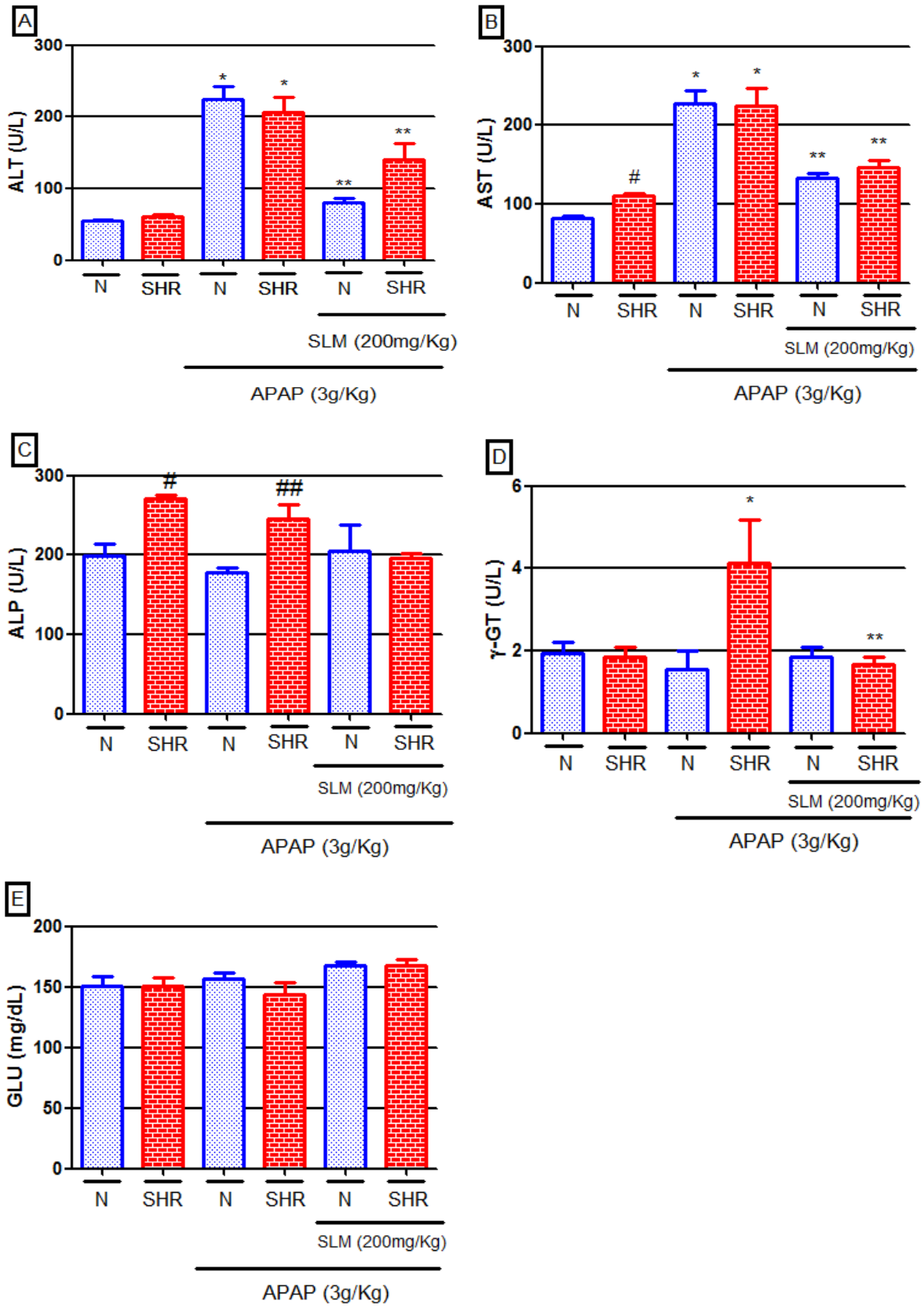
Figure 1. Serum parameter of rats of all groups untreated, treated with APAP (3 g/kg, orally) and pretreated with SLM (200 mg/kg), of ALT (A), AST (B), ALP (C), γ-GT (D) and GLU (E) were determined 12 h after APAP intoxication. Results represent mean ± SEM of 12 rats per group. * p<0.05 N+APAP versus N, **p<0.01 N+SLM+APAP versus N+APAP, *p<0,05 SHR + APAP versus SHR, **p<0,05 SHR+SLM+APAP versus SHR+APAP, $^{\#}$p<0,05 N versus SHR, $^{\#\#}$p<0,05 N+APAP versus SHR+APAP.

Figure 2. Serum parameter of rats of all groups untreated, treated with APAP (3 g/kg, orally) and pretreated with SLM (200 mg/kg), of MPO activity (A) and NO production (B) were determined 12 h after APAP intoxication. Results represent mean ± SEM of 12 rats per group. * p<0.05 N+APAP versus N, **p<0.05 N+SLM+APAP versus N+APAP, *p<0,05 SHR + APAP versus SHR, **p<0,05 SHR+SLM+APAP versus SHR+APAP.

Figure 3. Histopathology of livers 12 hours after APAP injection. The livers were collected 12 hours from all groups after APAP administration (3 g/kg). Panel A and B: N and SHR group that received only vehicle, respectively, C and D: N and SHR group that received APAP, respectively. E and F: N and SHR group pre-treated with standard drug (SLM, 200mg/kg per 7 days). Sections were stained with H&E (magnification, x 40).

Table 1. Body weight (g), liver weight (g) and liver index (%) in all groups of rats 12 hours after APAP administration (3 g/kg) and pretreated with SLM (200 mg/kg). Média ± SEM, n=12 por grupo. * p<0.05 N+APAP versus N,* p<0.01 N+SLM+APAP versus N+APAP, **p<0.05 SHR + APAP versus SHR, **p<0.05 SHR+SLM+APAP versus SHR+APAP, $^{\#}$p<0.05 N versus SHR, $^{\#\#}$p<0.05 N+APAP versus SHR+APAP, $^{\#\#\#}$p<0.05 N+SLM+APAP versus SHR+SLM+APAP.

Figure 1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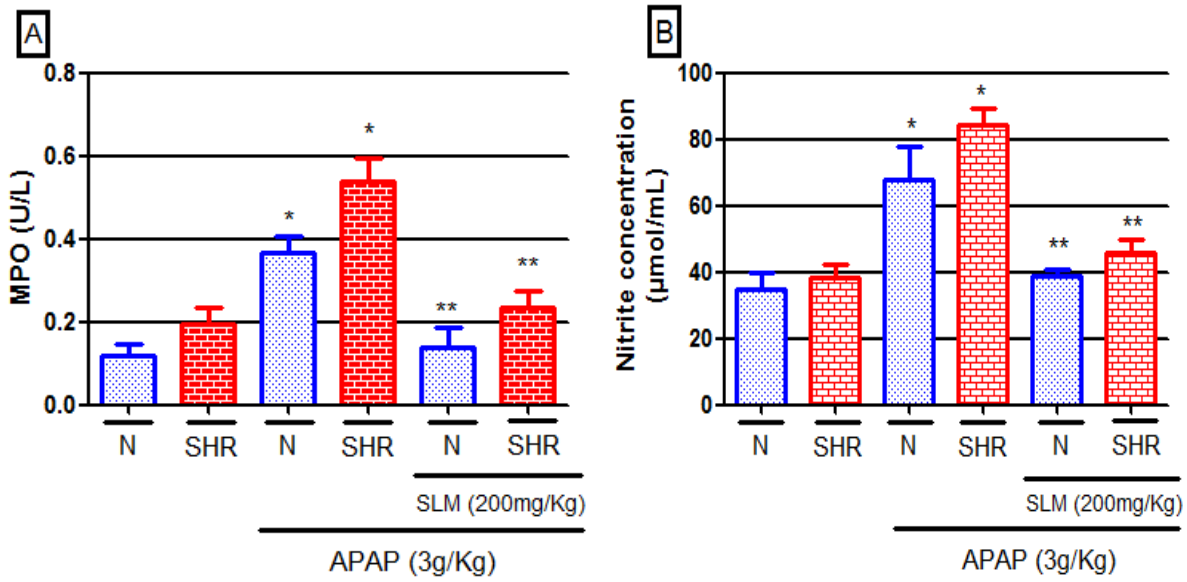
SLM (200mg/Kg)
APAP (3g/Kg)

Figure 2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SHR
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SHR
SLM (200mg/Kg)
APAP (3g/Kg)
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APAP (3g/Kg)

Figure 3

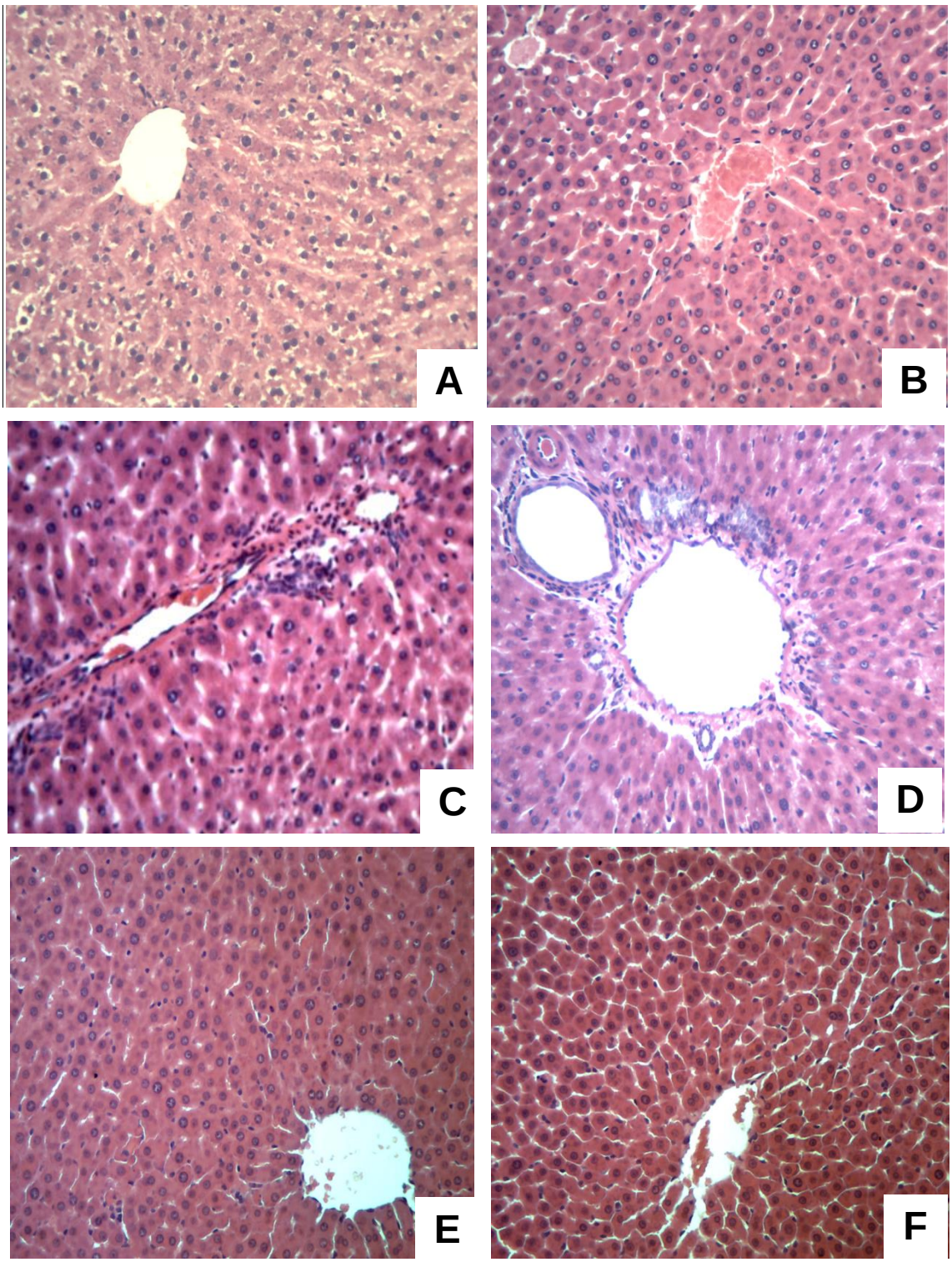

Table 1*:* Body weight (g), liver weight (g) and liver index (%) in rats 12 h after administration of APAP (3 g/Kg) alone or in pretreatment per 7 days with the SLM.

Treatment	*Body weight (g)*	*Liver weight (g)*	*Liver Index* (%)
N	309 ± 11	11.63 ± 0.42	3.4 ± 0.04
SHR	267 ± 9$^{\#}$	11.10 ± 0.15	3.8 ± 0.04
N + APAP	321 ± 6	11.39 ± 0.33	3.7 ± 0.1*
SHR + APAP	248 ± 10$^{\#\#}$	10.12 ± 0.36	4.4 ± 0.1**
N + SLM + APAP	317 ± 8	11.02 ± 0.37	3.5 ± 0.07*
SHR + SLM + APAP	233 ± 4$^{\#\#\#}$	8.89 ± 0.34	3.4 ± 0.1**

Média ± SEM, n=12 por grupo. * p<0.05 N+APAP versus N,* p<0.01 N+SLM+APAP versus N+APAP, **p<0.05 SHR + APAP versus SHR, **p<0.05 SHR+SLM+APAP versus SHR+APAP, $^{\#}$p<0.05 N versus SHR, $^{\#\#}$p<0.05 N+APAP versus SHR+APAP, $^{\#\#\#}$p<0.05 N+SLM+APAP versus SHR+SLM+APAP.

CAPÍTULO III

CONCLUSÓES

Neste estudo evidenciamos a associação entre as doenças crônico-degenerativas: a hipertensão e a insuficiência hepática (hepatopatia).

A injúria hepática, induzida por uma “overdose” de APAP, altera a atividade funcional do fígado, comprovada por alterações histopatológicas.

A Silimarina é um produto natural e eficaz para proteger o tecido hepático de lesões induzidas por medicamentos, tal como ocorre com o uso do APAP.

A Silimarina pode ser importante para pacientes hipertensos que utilizam a polifarmácia.

PERSPECTIVAS FUTURAS

Os resultados desta pesquisa auxiliarão a prescrição mais cuidadosa de fármacos em pacientes portadores de doenças hepáticas, bem como o efeito das prováveis interações medicamentosas em pacientes que utilizam a polifarmácia. Além disso, o conhecimento da eficácia de drogas com atividade hepatoprotetora, como a Silimarina, será útil para o tratamento destes pacientes portadores de doenças crônicas associadas.

Sugere-se que, sejam feitas novas pesquisas utilizando os compostos isolados da Silimarina, para que identifique os compostos responsáveis por esta atividade farmacológica.

ANEXO

Instructions For Authors

Scope of the Pakistan Journal of Pharmaceutical Sciences:

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