THERAPEUTIC POTENTIAL OF SILYMARIN IN ACETAMINOPHEN-INDUCED NEPHROTOXICITY IN RATS

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ABSTRACT

Acetaminophen (N-acetyl-p-aminophenol; APAP), a widely used analgesic and antipyretic drug, can cause life-threatening renal dysfunction. Factors which play a role in this toxicity are still ambiguous and no specific treatment was ascertained. The aim of this study was to investigate the potential protective role of silymarin to attenuate the nephrotoxicity induced by a single oral dose (3 g/kg) of APAP in rats. Four groups of Sprague-Dawley rats were used: control, silymarin, APAP and silymarin plus APAP-receiving animals. Interestingly, oral supplementation of silymarin (200 mg/kg/day) for nine days before APAP intoxication dramatically reduced APAP-induced nephrotoxicity as evidenced by measuring serum total protein, serum urea, creatinine clearance (Ccr) and urinary excretions of NAG (N-acetyl-β-D-glucosaminidase). Silymarin administration markedly prevented the generation of thiobarbituric acid reacting substances (TBARS) with substantial improvement in terms of reduced glutathione content (GSH) and activities of antioxidant enzymes in the kidney homogenates. Nitric oxide (NO) levels of urine and renal tissue were significantly inhibited in silymarin pre-treated animals. Furthermore, silymarin administration significantly inhibited the reduction of kidney content of adenosine triphosphate (ATP) parcels associated with this nephropathy. These results suggest that the protective role of silymarin in the prevention of APAP-induced nephrotoxicity in rats was associated with the decrease of oxidative and nitrosative stress in renal tissue as well as its capacity to improve the mitochondrial energy production. Thus, one could conclude that silymarin might be considered for the treatment of APAP-induced nephrotoxicity in rats. However, clinical studies are warranted to investigate such an effect in human subjects.

Keywords: Acetaminophen, Silymarin; Nephrotoxicity; Nitric oxide, Rat.

INTRODUCTION

Acetaminophen (N-acetyl-p-aminophenol; APAP), popularly called Paracetamol, is widely used in general medicine. It is an effective over-the-counter analgesic and antipyretic drug. Therapeutic dose of APAP is considered safe. Conversely, overdosing or chronic use can result in both hepatotoxicity and nephrotoxicity, although the later toxicity is less common and independent of APAP-induced hepatotoxicity (**Boutis and Shannon, 2001**). A little is known about the mechanism of APAP toxicity in the kidney and its prevention (**Stern et al., 2005**). Oxidative damage induced by reactive oxygen species (ROS) and lipid peroxidation products was frequently reported in the APAP-induced nephrotoxicity. APAP toxic metabolite, N-acetyl-para-amino-benzoquinone imine (NAPQI), is thought to be responsible for its nephrotoxicity. NAPQI is produced in excessive amounts through cytochrome P450 system in APAP overdose, and only part of it can be detoxified by conjugation with reduced glutathione (GSH) (**Adeneye and Benebo, 2008**). The remaining part of NAPQI binds to cellular proteins and induces oxidative stress, which initiates cell

death of renal tubular cells (**Abdel-Zaher** *et al.,* **2008; Cekmen** *et al.,* **2009; Das** *et al.,* **2010**). Besides, mitochondrial dysfunction observed after APAP overdose is due to covalent binding of NAPQI to mitochondrial proteins, which consequently inhibits mitochondrial respiration leading to adenosine triphosphate (ATP) depletion (**Qiu** *et al.,* **2001; Ahmad** *et al.,* **2012**).

Silymarin, a flavonoid extract derived from seeds of milk thistle (*Silybum marianum*), is composed of a mixture of four isomeric flavonolignans which are silibinin (the main active component), isosilibinin, silydianin and silychristin (**Crocenzi and Roma, 2006**). It is one of the popular complementary therapeutic agents, well tolerated, and almost without unfavourable effects (**Nencini** *et al.,* **2007**). It exhibits a potent anti-hepatotoxic effect (**Saller** *et al.,* **2001; Gazák** *et al.,* **2007**), and has also been proved to have antioxidant properties (**Halim** *et al.,* **1997; Galhardi** *et al.,* **2009**). Moreover, it is used as a standard agent for studies evaluating the hepatoprotective effects of different plant principles (**Dhiman and Chawla, 2005**). Nevertheless, silymarin is still a subject of various re-assessment studies concerning its renal protective effect owing to the comparative little information available.

In view of this, an experimental study was designed to investigate whether oral supplementation of silymarin protects against APAP-induced nephrotoxicity, and if so, what is the mechanistic basis of such nephroprotective effects in this toxicity paradigm.

MATERIALS AND METHODS

Chemicals

Acetaminophen (APAP) and silymarin were purchased from Sigma-Aldrich Chemical Company. All other chemicals used were of the highest available commercial grade.

Animals

Thirty two male Sprague-Dawely rats, weighing 160-180 g, were obtained from the animal facility of the Faculty of Pharmacy at Al-Azhar University, Cairo, Egypt. The animals were fed a standard chow (El-Nasr Company, Abou-Zaabal, Cairo, Egypt) with free access to water, and kept in wire-floored cages under standard laboratory conditions at room temperature (25±2°C), and a 12-h light/12-h dark cycle. The animal experiments were conducted according to the guidelines for the care and use of laboratory animals stated by College of Pharmacy, Al-Azhar University, Cairo, Egypt.

Experimental design

Rats were randomized and divided into four groups of eight animals each. One group received silymarin, suspended in arabic gum 1%, by gavage at a dose of 200 mg/kg of body weight for 10 consecutive days. The second group received a vehicle (arabic gum 1% suspension) by the same route of administration for 9 days and on the tenth day, a single dose of APAP (3 g/kg) suspended in arabic gum 1% was orally administered. The third group received silymarin (200 mg/kg/day) for ten days and on the last day, they received the single dose of APAP (3 g/kg) 1 h before silymarin. The selected doses of APAP and silymarin were based on the results of previous studies (**Mansour** *et al.,* **2006; Nencini** *et al.,* **2007**). Last group of rats served as control and received only vehicle (arabic gum 1% suspension) for 10 days.

Twenty four hours following the administration of APAP, rats in all groups were housed for another 24 h in individual metabolic cages for urine collection. During urine collection animals had water *ad libitum*, whereas food was allowed for the first 12 h only. The volume of collected urine was measured, and then filtered to ascertain urine parameters

[creatinine, N-acetyl-β-D-glucosaminidase (NAG), and nitric oxide (NO)]. Blood samples from starved animals were taken from abdominal aorta under light ether anesthesia, and used for determination of total protein, urea, and creatinine levels. All animals were then killed by cervical dislocation and both kidneys from each rat were quickly removed, rinsed in ice-cooled physiological saline, blotted dry on filter paper, weighed, and then 10% (w/v) homogenate of the kidney was made in ice-cold 0.15 M KCl solution using a Potter-Elvehjem homogenizer. Aliquots of the homogenates were used for determination of tissue contents of thiobarbituric acid reacting substances (TBARS), GSH, ATP and NO. The enzyme activities of glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) were carried out in kidney homogenate. Total protein content of homogenates was also determined to ascertain tissue parameters.

Biochemical analysis

Serum urea and creatinine were measured according to the method of **Patton and Crouch (1977)** and **Bonsnes and Taussky (1945),** respectively using commercially available kits. Creatinine clearance (Ccr) was calculated after estimation of serum and urinary creatinine levels using the standard formula. NAG activity of the collected urine samples was measured using the methods of **Moore and Morris (1982)** which based on the enzymatic hydrolysis of p-nitrophenyl-N-acetyl-glucosaminide with subsequent assay of the liberated p-nitrophenol. Kidney ATP content was determined according to the method of **Adams (1963)** by assessing the oxidation of NADH+H$^+$ paired with dephosphorylation of 1,3 bisphosphoglycerate, which is produced from ATP and 3-phosphoglycerate reaction. Protein content of the serum, and homogenate samples was measured by the method of **Lowry *et al.* (1951)**.

Determination of the extent of lipid peroxidation and GSH

Lipid peroxidation was assessed in kidney homogenate as TBARS according to the method of **Mihara and Uchiyama (1978)**. Colorimetric determination of TBARS is based on the reaction of malondialdehyde, the end product of lipid peroxidation, with thiobarbituric acid at low pH, and at a temperature of 95 °C for 30 min, to form TBARS. The absorbance of the resultant pink colour can be measured spectrophotometerically at 535 nm. GSH was determined in renal tissue according to the method described earlier by **Ellman (1959)**. The procedure is based on the reduction of Ellman's reagent [5,5-dithiobis(2-nitrobenzoic acid)] by GSH to produce an intense yellow colour which is measured spectrophotometrically at 412 nm.

Determination of NO

Urine and tissue contents of NO were measured as its stable metabolites, nitrate and nitrite. Nitrate was first reduced by nitrate reductase into nitrite. Nitrite is then detected by formation of red pink colour upon treatment with the Griess reagent and quantified spectrophotometrically at 540 nm **(Green *et al.,* 1982)**.

Determination of glutathione related enzymes

Glutathione related enzymes were determined in the kidney homogenate. GPx activity was measured by quantifying the rate of oxidation of glutathione by hydroperoxide as catalyzed by GPx present in the sample. Oxidized glutathione is then converted to its reduced form with a concomitant oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The disappearance of NADPH was assayed by measuring the decrease in absorbance at 340 nm **(Rotruck *et al.,* 1973).** GR activity was determined by measuring the rate of consumption of NADPH by the GSH regenerating reaction through monitoring of NADPH absorbance decline at 340 nm **(Pinto and Bartley, 1969)**. GST activity was assayed

by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH which is accompanied by an increase in absorbance at 340 nm **(Habig *et al.,* 1974).**

Statistical analysis

Data are expressed as mean ± S.D. for all groups. Data analysis was evaluated by one-way ANOVA followed by Tukey-Kramer post-test for multiple comparisons. A 0.05 level of probability was used as the criterion for significance.

RESULTS

APAP-induced nephrotoxicity

Injection of animal with APAP provoked an observed nephrotoxicity, as evidenced by significant decline in serum total protein (45%), and expressive elevation of serum urea (88%). Besides, serious decrease of Ccr (84%), and highly significant urinary excretion of NAG (107%) were observed when compared to the control values of vehicle receiving rats (Table 1). Kidneys of APAP-treated rats showed significant increase of TBARS levels (132%) and significant decrease of GSH content (51%) compared to the kidneys of vehicle-receiving animals (Table 2). Likewise, APAP treatment noticeably declined the activities of GPx (54%) (Figure 1), GR (45%) (Figure 2), and GST (52%) (Figure 3) in nephrotic kidneys when compared to the kidneys of control rats. ATP was measured in kidney homogenate as shown in Figure 4, APAP administration significantly affected the renal energy status as characterised by decreased level of kidney ATP (27%) compared to control animals. Moreover, APAP treatment significantly increased both urinary excretion of nitrite (107%), and nitrite concentration of kidney homogenate (74%) when compared to the control levels (Table 3).

Table 1: **Effect of silymarin administration on serum total protein, serum urea, creatinine clearance (Ccr) and urinary NAG, modified by APAP treatment in rats.** Values are expressed as mean ± S.D., *n* = 8. Multiple comparisons were achieved using one way ANOVA followed by Tukey-Kramer as post-ANOVA test. *a, b, c*: indicate significant change from control, silymarin or APAP-treated groups respectively, at p<0.05.

Parameters	Control	Silymarin	APAP	APAP + Silymarin
Serum total protein (g/dl)	5.6 ± 0.5	5.8 ± 0.45	3.1 ± 0.4 [a,b]	5.2 ± 0.5 [c]
Serum urea (mg/dl)	46 ± 3.8	44 ± 3.9	83 ± 8.8 [a,b]	51 ± 5.7 [c]
Creatinine clearance (Ccr) (ml/min)	0.44 ± 0.05	0.42 ± 0.06	0.·7 ± 0.·2 [a,b]	0.29 ± 0.·4 [a,b,c]
Urinary NAG (nmol/mg creatinine)	236 ± 24.5	242 ± 23	488 ± 37 [a,b]	307 ± 25 [a,b,c]

Table 2: **Effect of silymarin administration on renal TBARS and GSH modified by APAP treatment in rats.** Values are expressed as mean ± S.D., *n* = 8. Multiple comparisons were achieved using one way ANOVA followed by Tukey-Kramer as post-ANOVA test. *a, b, c*: indicate significant change from control, silymarin or APAP-treated groups respectively, at p<0.05.

Parameters	Control	Silymarin	APAP	APAP + Silymarin
TBARS (nmol/gm wet tissues)	88 ± 11.2	81 ± 10.3	204 ± 16.9 [a,b]	133 ± 14.6 [a,b,c]
GSH (μmol/gm wet tissues)	3.5 ± 0.27	3.7 ± 0.28	1.7 ± 0.12 [a,b]	3.2 ± 0.23 [b,c]

Table 3: **Effect of silymarin administration on urinary excretion and renal tissue NO modified by APAP treatment in rats.** Values are expressed as mean ± S.D., *n* = 8. Multiple comparisons were achieved using one way ANOVA followed by Tukey-Kramer as post-ANOVA test. *a, b, c*: indicate significant change from control, silymarin or APAP-treated groups respectively, at p<0.05.

Parameters	Control	Silymarin	APAP	APAP + Silymarin
Urinary NO (μmol/mg creatinine)	1.5 ± 0.06	1.4 ± 0.05	3.1 ± 0.15 [a,b]	2.2 ± 0.09 [a,b,c]
Renal tissue NO (nmol/mg protein)	512 ± 22	501 ± 23	889 ± 45 [a,b]	606 ± 24 [a,b,c]

Effect of silymarin on APAP-induced nephrotoxicity

Daily administration of silymarin significantly decreased APAP-induced high serum urea (39%), and high urinary excretion of NAG (37%), whereas serum protein and Ccr were significantly increased (68 and 314%, respectively) compared to APAP-treated rats (Table 1). Silymarin, at a dose of 200 mg/kg body weight for consequent ten days, resulted in a realistic reversal of APAP-induced alterations of the levels of TBARS and GSH content in renal tissue. Silymarin significantly decreased renal TBARS level (35 %) and increased renal GSH content (88 %) (Table 2). Silymarin was also able to attenuate APAP-induced decrease of antioxidant enzymes, as it increased renal GPx (91%) (Figure 1), renal GR (73%) (Figure 2), and renal GST (70%) (Figure 3) when compared to intoxicated animals. Similarly, silymarin supplementation almost normalized APAP-induced decrease of kidney ATP (Figure 4). Furthermore, the increase of urinary excretion and renal tissue contents of nitrite induced by APAP were significantly minimized following silymarin supplementation (Table 3). The individual administration of silyamrin had insignificant changes of the levels of all parameters measured, compared to the control animals.
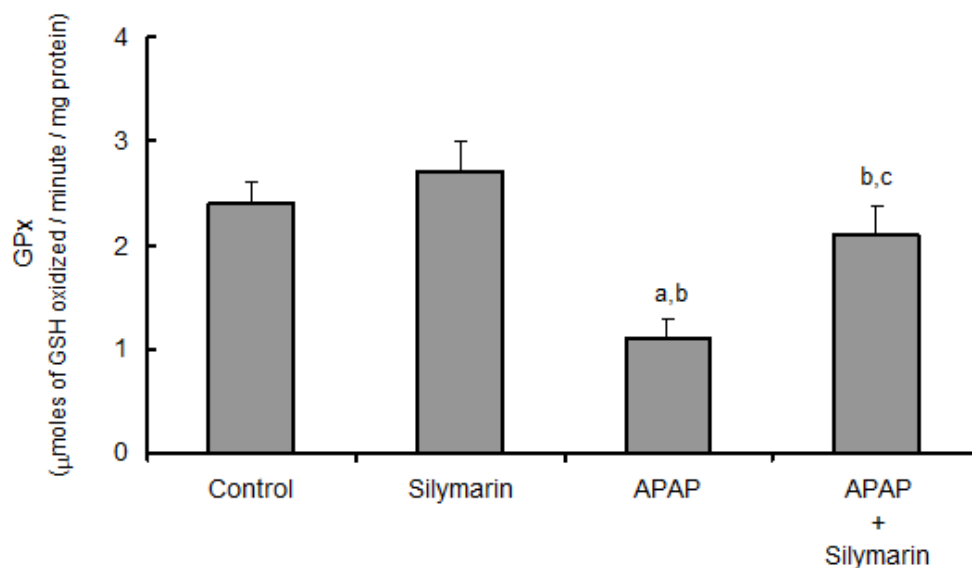
Figure 1: **Effect of silymarin administration on renal GPx activity modified by APAP treatment in rats.** Values are expressed as mean ± S.D., *n* = 8. Multiple comparisons were achieved using one way ANOVA followed by Tukey-Kramer as post-ANOVA test. *a, b, c*: indicate significant change from control, silymarin or APAP-treated groups respectively, at p<0.05.
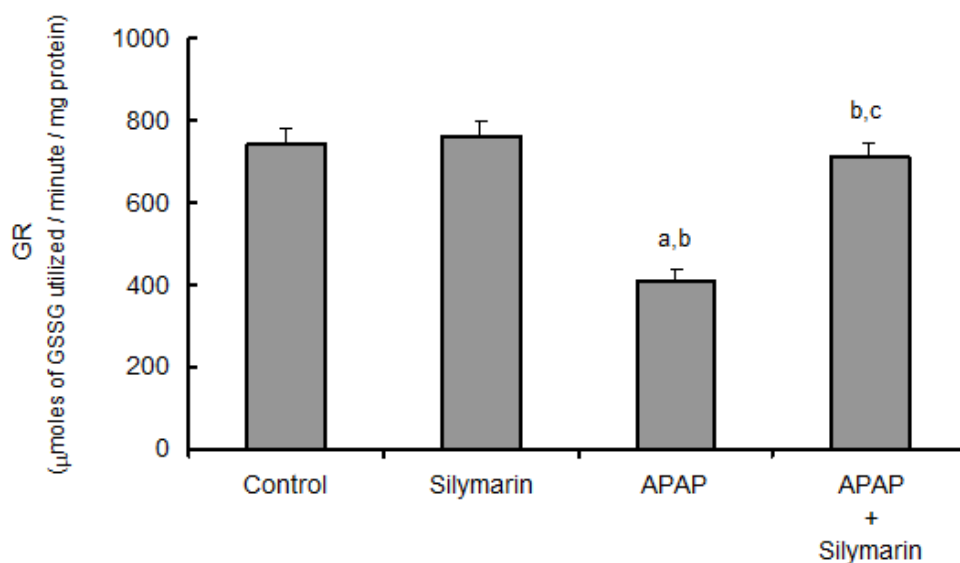

Figure 2: **Effect of silymarin administration on renal GR activity modified by APAP treatment in rats.** Values are expressed as mean ± S.D., *n* = 8. Multiple comparisons were achieved using one way ANOVA followed by Tukey-Kramer as post-ANOVA test. *a, b, c*: indicate significant change from control, silymarin or APAP-treated groups respectively, at p<0.05.

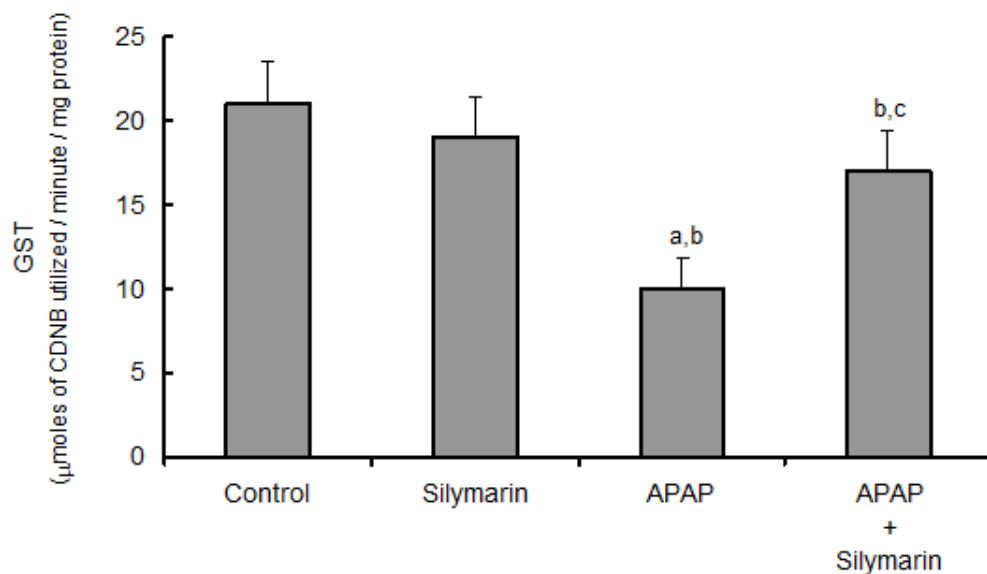**Figure 3**: **Effect of silymarin administration on renal GST activity modified by APAP treatment in rats.** Values are expressed as mean ± S.D., $n = 8$. Multiple comparisons were achieved using one way ANOVA followed by Tukey-Kramer as post-ANOVA test. *a, b, c*: indicate significant change from control, silymarin or APAP-treated groups respectively, at $p < 0.05$.
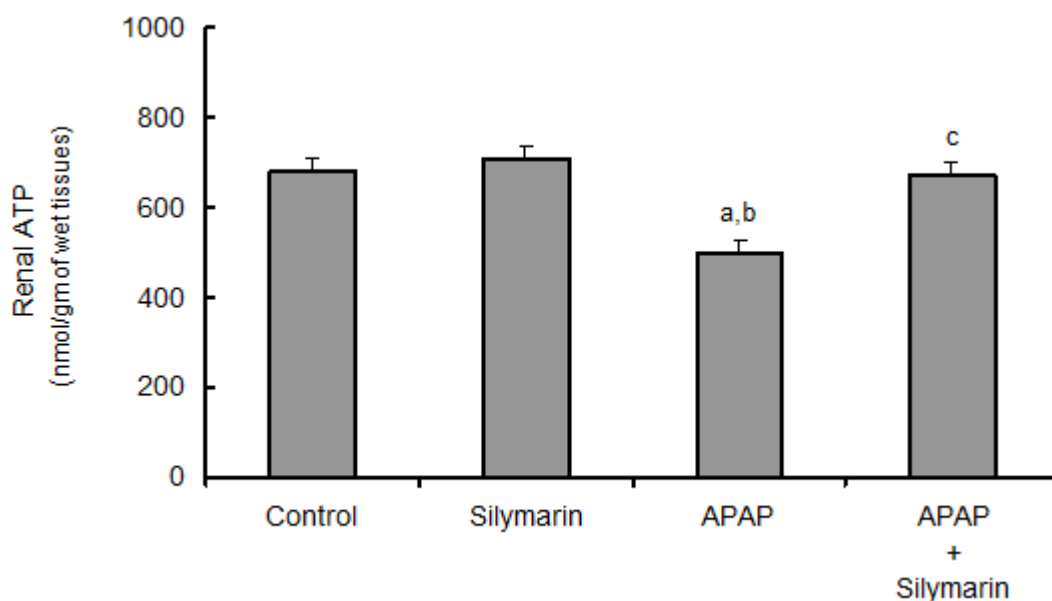

Figure 4: **Effect of silymarin administration on renal ATP modified by APAP treatment in rats.** Values are expressed as mean ± S.D., $n = 8$. Multiple comparisons were achieved using one way ANOVA followed by Tukey-Kramer as post-ANOVA test. *a, b, c*: indicate significant change from control, silymarin or APAP-treated groups respectively, at $p < 0.05$.

DISCUSSION

APAP-induced nephrotoxicity is a well established experimental model of drug-induced renal injury. APAP nephrotoxicity results from its highly reactive and toxic metabolite NAPQI. NAPQI is detoxified by conjugation with GSH. Once GSH is depleted, NAPQI covalently binds to proteins of the proximal tubule, causing alterations in

intracellular homeostasis and eventually lead to cell necrosis of renal tubules (**Emeigh Hart et al., 1994; Tarloff and Kinter, 1997; Jamshidzadeh et al., 2008**).

In the present study, we investigated whether the acute nephrotoxic effect of APAP could be prevented or alleviated by silymarin. Current experiment showed that single dose of APAP (3 g/kg body weight) revealed a severe nephropathy 24 h after APAP administration as reflected by high level of serum urea with substantial hypoproteinemia. This nephrotic toxicity was also associated with obvious decrease of Ccr and apparent renal tubular injury, as indicated by the increased urinary excretion of NAG. This distinctive figure of APAP-induced nephrotoxicity is comparable to those reported by other research groups (**Abdel-Zaher et al., 2008; Adeneye and Benebo, 2008; Das et al., 2010**). Silymarin administration in a dose of 200 mg/kg of body weight for 10 consecutive days significantly reduced APAP-induced renal injury, which led us to investigate the underlying mechanisms of this distinguishing nephroprotective effect.

The formation of free radicals and consequent oxidative stress in the induction of APAP-induced liver damage is widely accepted throughout several studies, but in comparison less information is available about the extrahepatic effects of APAP. Nevertheless, ROS generation and oxidative stress have been proposed as a mechanism by which many chemicals can induce nephrotoxicity (**Devipriya and Shamala, 1999; Somani et al., 2000; Renugadevi and Prabu, 2009**). Results of the present study revealed a significant increase in TBARS generation accompanied by a significant decrease in the level of total GSH content in renal tissues of APAP-treated rats. Besides, activities of antioxidant enzymes GPx, GR and GST were significantly reduced. The apparent inhibition of GSH related enzymes and associated GSH depletion make the cells more susceptible to damage by ROS. This ability of APAP-induced nephrotoxicity to modulate kidney oxidative status proves the role of oxidative stress in this pathogenesis. However, silymarin administration prevented the increase of TBARS level and the depletion of GSH content. Furthermore, silymarin restored the reduced activities of GSH-related enzymatic antioxidants. Similar mechanisms have been proposed to explain the protective effects of silymarin against adriamycin-induced nephrotoxicity (**El-Shitany et al., 2008**). Silymarin, in addition to its well known hepatoprotective effect, is recognized to have a scavenging activity of free radicals, and ability to stimulate antioxidant regeneration via increase of gene expression of antioxidant enzymes (**Sonnenbichler and Zetl, 1986; Soto et al., 2010**).

Moreover, we have observed a marked increase of NO production in the renal tissue and urine of APAP-treated rats. Physiological levels of NO chiefly contributed in the conservation of renal haemodynamics, mainly due to its vasodilator and antithrombogenic properties. However, higher level of NO production is associated with glomerulonephritis (**Cattel, 1995**). **Beckman et al. (1990)** highlighted an important issue, as they reported that NO may interact with superoxide anion and generate peroxynitrite. On the contrary, NO and superoxide anion, peroxynitrite is a potent oxidant that can induce lipid peroxidation and cytotoxicity. Similar studies reported that overproduction of NO is correlated with APAP-induced nephrotoxicity and hepatotoxicity in rats (**Gardner et al., 1998; Abdel-Zaher, 2008; Cekmen et al., 2009**). Results of this study revealed that pretreatment of rats with silymarin, restrained NO overproduction induced by acute toxic dose of APAP. It has been reported that silymarin is able to decrease the synthesis of NO by either inhibiting the expression of endothelial nitric oxide synthase enzyme (eNOS) or reducing the induction of its inducible form (iNOS) (**Cho et al., 2009; Shaarawy et al., 2009**).

APAP-treated rats showed a marked reduction of renal ATP content. This is similar to the previous findings of **Nagi et al. (2010)** who observed a significant decline of liver energy

parcels in APAP-treated mice. Protein complexes of mitochondrial respiratory chain, involved in oxidative phosphorylation and ATP production, are extremely sensitive to ROS **(Boveris and Chance, 1973; Shertzer *et al.,* 2006)** which might be the reason for their decreased activities and reduced ATP production. Interestingly, the present study exhibited reduced levels of kidney GSH and GPx activities which are especially important for mitochondrial function. GSH functions as an efficient scavenger and it also reduces H_2O_2 to water via GPx. Energy depletion in APAP-induced nephrotoxicity was completely restored following silymarin supplementation that could be due to its antioxidant properties and thereby maintained mitochondrial function and enhanced ATP production **(Pradeep *et al.,* 2007)**. Under our experimental conditions, silymarin single treatment did not significantly affect any of the studied parameters, compared to the control group.

In conclusion, data of the present study provided additional evidence that APAP-induced nephrotoxicity in rats was associated with elevated oxidative and nitrosative stress in renal tissue, alongside with reduced mitochondrial energy production. These findings also demonstrated that administration of silymarin exerts a renoprotective effect in APAP-induced nephrotoxicity possibly through reversing oxidant–antioxidant imbalance and regulation of NO bioavailability in the kidney. Additional study on human subjects is needed to investigate the possibility to make use of silymarin as a part of renoprotective strategies during APAP therapy or whenever cellular damage is a consequence of oxidative and/or nitrosative stress.

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الإمكانية العلاجية للسليمارين فى الجرذان المصابة بالتسمم الكلوى المحدث بالأسيتامينوفين.

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إن الأسيتامينوفين، و الشائع إستخدامه كمسكن و خافض للحرارة، من الممكن أن يسبب إعتلال كلوى خطيرولا تزال العوامل التى تلعب دورا فى هذه السمية غيرواضحة و بلا علاج محدد. تهدف هذه الدراسة الى بحث الدور الوقائى المحتمل للسليمارين فى التخفيف من التسمم الكلوى المحدث بجرعة واحدة من الأسيتامينوفين (٣جم/كجم) فى الجرذان. تم إستخدام اربع مجموعات رئيسية من جرذان سبراغ داولى ومعالجتهم كالتالى: صمغ عربى للمجموعة الأولى (الضابطة)، سليمارين للمجموعة الثانية، أسيتامينوفين للمجموعة الثالثة، أسيتامينوفين مع سليمارين للمجموعة الرابعة. ومما يدعو للإهتمام، إن التغذية بالسليمارين عن طريق الفم (٢٠٠مجم / كجم) يوميا لمدة تسعة أيام قبل إعطاء الأسيتامينوفين قد خفضت بشكل كبير الإعتلال الكلوى المحدث ويتضح ذلك من خلال قياس مصل البروتين الكلى و اليوريا فى الدم و تصفية الكرياتينين و الإفرازات البولية لإنزيم ن-أسيتيل بيتا-دى-جلوكوزامينيداز. إن إعطاء السليمارين قد قلل تكوين المواد المتفاعلة مع حمض الثيوباربيتيوريك مع تحسن كبير من حيث إنخفاض محتوى الجلوتاثيون و أنشطة الإنزيمات المضادة للأكسدة فى الخليط المتجانس لنسيج الكلى. كما نقص بشكل ملحوظ مستوى أكسيد النيتريك فى البول و أنسجة الكلى فى الحيوانات المعالجة مسبقا بالسليمارين. بالإضافة الى أن إعطاء السليمارين قد منع تقليل محتوى الأدينوسين ثلاثى الفوسفات فى أنسجة الكلى المصاحب لهذا الإعتلال. تشير هذه النتائج الى أن الدور الوقائى للسليمارين فى تقليل التسمم الكلوى المحدث بالأسيتامينوفين فى الجرذان كان مرتبط بالإنخفاض فى مستوى الإجهاد التأكسدى و النيترى فى أنسجة الكلى، فضلا عن قدرتها على تحسين إنتاج الطاقة من الميتوكوندريا. وهكذا نستنتج أنه يمكن أن يكون للسليمارين دور فى علاج حالات التسمم الكلوى المحدث بواسطة الأسيتامينوفين. ومع ذلك، لاتزال الإختبارات الإكلينيكية مطلوبة لبحث تأثيره الوقائى فى هذه السمية عند البشر.