Protective Role of the Combination of Resveratrol and N-Acetylcysteine in Preventing Acute Paracetamol-Induced Nephropathy in Rats

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ABSTRACT

As an overdose of Paracetamol (PCM) is nephrotoxic, the present study aimed to investigate and compare the individual therapeutic effects of Resveratrol (RES), N-acetyl cysteine (NAC), and their combination against PCM induced nephrotoxicity in rats in addition to illustrate possible mechanisms of action. Fifty adult Wistar rats were divided into 5 groups (n=10/group): 1) control group, 2) PCM group (1000 mg/kg b.wt), 3) PCM post-treated with RES (25 mg/kg b.wt), 4) PCM post-treated with NAC (1.2 g/kg b.wt), and 5) PCM post-treated with concomitant RES and NAC. PCM was administered during the first 2 days and all other treatments were administered intraperitoneally daily for the post PCM induction five days. At the end of the study, serum electrolytes, renal levels of oxidative stress marker, mRNA expression levels of ɣ-glutamyl cysteine ligase (ɣ-GCL), and Na^+/K^+ ATPase were measured. PCM intoxication caused oxidative stress-induced renal damage as evident by the inhibition of mRNA expression of ɣ-GCL and glutathione (GSH) levels, and by the inhibition of the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST). PCM induced renal damage was mediated by a parallel decrease in activity and expression levels of Na^+/K^+ ATPase. However, while delayed NAC administration to post PCM intoxicated rat poorly improved these parameters and changes in kidney structure, the effect of RES was also partially but more profound. On the other hand, concomitant administration of both NAC and RES completely reversed the alterations of serum electrolytes, renal architectures histological parameters, serum electrolytes, oxidative stress parameters, renal architectures histological parameters, and resulted in normal renal mRNA expression of both ɣ-GCL and Na^+/K^+ ATPase. These data suggest that NAC and RES act in a synergistic manner and completely ameliorate PCM induced nephrotoxicity.

Key Words: N-acetylcysteine; Nephrotoxicity; Oxidative stress.

INTRODUCTION

Drug-induced nephrotoxicity (DIN) is considered as a major cause of acute kidney injury and increased mortality and morbidity (Dolin and Himmelfarb, 2008). It is well reported that Paracetamol (PCM) overdose is considered highly nephrotoxic in both humans and experimental animals (Guo and Nzerue, 2002).

Metabolism of ingested PCM is achieved by three pathways including conjugation with sulfate and glucuronide (95%) and through cytochrome p450 oxidase enzyme system (5%) (Gamel El-din *et al.*, 2003; Slitt *et al.*, 2005, Presscott, 2005). The metabolism of PCM by cytochrome p450 enzyme system produces the metabolite N-acetyl-p-benzoquinone imine (NAPQI) which is normally toxic to liver and kidney tissues (Gulnaz *et al.*, 2010). In this regards, under therapeutic dose, this low levels of NAPQI rendered ineffective by conjugation with cellular reduced glutathione (GSH) and the resulted complex is excreted by kidneys (Melo *et al.*, 2006).

However, in PCM overdose exaggerates NAPQI production which in turns causes a huge depletion in both mitochondrial and cytosolic pools of GSH, diminishes cellular capability to make new glutathione, and reduces the antioxidant capacity of the cells (Elhabib *et al.*, 2007; Sandhu *et al.*, 2010). This generates both reactive oxygen (ROS) and nitrogen species (RNS) (Nakae *et al.*, 1990; Michael *et al.*, 1999; Knight *et al.*, 2001). Moreover, high levels of NAPQI allow it to bind covalently with intracellular proteins causing mitochondrial dysfunction, and further generation of both superoxide and peroxynitrite radicals (Saito *et al.*, 2010). Oxidative stress is well reported to cause oxidation of protein, lipids, and DNA leading to kidney necrosis (Michael *et al.*, 1999. Knight *et al.*, 2001). Given the important

role of Na^+/K^+ ATPase in the renal tissue, both ROS and NAPQI have been reported to directly inhibit Na^+/K^+ ATPase activity and expression after PCM intoxication. This pathway was considered as one of the most important pathways of PCM induced renal injury (Helms *et al.*, 2015).

Therapeutic options for prevention and/or treatment of PCM nephrotoxicity are limited (James *et al.*, 2003). Thus, the search for a golden therapeutic agent becomes imperative. Logically, an excellent therapeutic drug against PCM nephrotoxicity should be able to enhance intracellular GSH levels and has potent antioxidant potentials. Although many biological compounds with antioxidant properties such as melatonin, vitamin E, and N-acetyl-cysteine (NAC) were reported to be effective against PCM induced nephrotoxicity when used individually, no complete cure was achieved (Bessems and Vermeulen, 2001). Hence, the use of antioxidants therapy in combination has been suggested to be superior to individual effects as reported in several different animal models of oxidative stress and renal dysfunction (Petronilho *et al.*, 2008; Puica *et al.*, 2008).

NAC is a synthetic small molecule with a cysteinyl thiol group that is freely filterable and accessible to intracellular compartments (Yamada et al., 2013). NAC is able to reduce the incidence and afford partial protection against various nephrotoxicity conditions such as gentamicin (Patel Manali *et al.*, 2011), ifosfamide (Chen *et al.*, 2008), and contrast-induced nephropathies (Al-Ghonaim and Pannu, 2006). In addition, unlike clinical evidence, more recent accumulative data confirm the effectiveness of NAC against PCM induced nephrotoxicity in rats (Kandiş *et al.,* 2011; Cermik *et al.,* 2013; Canayakin *et al.,* 2016). The mechanism of NAC-related organ protection is primarily attributed to scavenging ROS either directly by reduced thiol (SH) group or indirectly through increasing intracellular GSH concentrations by acting as a GSH precursor and activator of glutathione S-transferase (GST) enzyme

(Tylicki *et al.*, 2003).

On the other hand, Resveratrol (RES) is a polyphenolic compound found in various plants, including grapes, berries, and peanuts (Morales *et al.* 2002). RES has been reported to ameliorate several types of renal injury in animal models, including diabetic, hyperuricemic, aldosterone-induced injuries, ischemia-reperfusion, and sepsis-related injuries well as gentamicin -induced injury (Morales *et al.* 2002; Chander *et al.* 2005; Albertoni and Schor, 2015). RES has been reported to be a potent antioxidant agent that acts as a reactive oxygen species (ROS) scavenger and iron chelator (Kitada and Koya, 2013). Furthermore, RES is a potent activator of the NAD^+-dependent deacetylase, silent mating type information regulation 2 homolog sirtuin 1 (SIRT1) (Kim *et al.*, 2011), a regulatory protein that enhances adaptations to cellular stress and cell survival as well as mitochondrial biogenesis and is able to reduce inflammation.

Given their diverse individual roles as potent antioxidants and their various mechanistic roles within the cell, this study aimed to investigate if combined administration of both NAC and RES at their therapeutic doses could offer a golden therapy against PCM induced oxidative renal damage and impairment of renal Na^+/K^+ ATPase induced nephropathy.

MATERIALS AND METHODS
1. Drugs preparation and doses:
Paracetamol (PCM) as high analytical grade powder was obtained from the department of Pharmacy at King Khalid University, Abha, Saudi Arabia and freshly dissolved in 0.9% normal saline to a final volume of 100 mg/ml. The most active animal use form of Resveratrol (RES), (trans-Resveratrol), was purchased as powder from Sigma-Aldrich (St. Louis, MO, USA) and freshly dissolved in saline solution (0.9% NaCl) of 20% hydroxypropyl cyclodextrin (American Maize-Products Co., Hammond, IN, USA) to final concentration of 100mg/ml. N-acetyl cysteine (NAC, >99% purity)

were purchased as powder from Sigma Chemical Co. (St. Louis, USA, Cat No.) then dissolved in distilled water with heating to a final concentration of 100 mg/ml.

2. Animals:

Adult male Wistar rats (n=50), weighing 190-200g, were obtained from the animal house at King Khalid University, Abha, Saudi Arabia and kept under a photoperiod of 12h light: 12h darkness with automatically regulated temperature (22–23ᵒC). Each 10 animals were housed in stainless cage and received standard rat chow and water *ad libitum*. All experimental procedures in this study were approved by the Research Ethical Committee of the College of Science at King Khalid University.

3. Experimental design:

After 2 weeks of acclimatization, the rats were divided into 5 groups (n=10), designed as one control and four experimental groups. The control group was injected with 1 ml of 0.9% NaCl for 7 consecutive days. The other 4 groups received i.p. a total dose of PCM (1000 mg/Kg b.wt) given over the first two days as 500 mg/kg/day (Gulnaz *et al.*, 2010) and then treated as follows over the remaining five days, on daily basis: 1) PCM intoxicated rats: received 1 ml of 0.9% NaCl. 2) NAC post-treated rats: post administered NAC (1.2 g/Kg b.wt). 3) RES post-treated rats: post administered RES (25 mg/Kg b.wt), and 4) RES+NAC post treated rats: post administered a concomitant dose of both RES (25 mg/Kg b.wt) and NAC (1.2 g/Kg b.wt). In all experimental groups, all treatments were administered intraperitoneally. NAC dose was selected based on the study of Chen *et al.* (2008) who showed a protective effect of NAC against ifosfamide-induced nephrotoxicity in rats whereas RES dose was selected based on the study of Do-Amaral *et al.* (2008) who reported protective effect of RES at this dose against cisplatin-induced nephrotoxicity.

4. Samples' collection and tissue homogenization:

Twelve hours after the last treatment of day seven, all animals were anesthetized with sodium pentobarbital (70 mg/kg, i.p) and killed by cervical dislocation. Blood samples into plain tubes were collected separated after centrifugation at 5000 rpm for 10 min and stored at -20 ᵒC for future measurements. The animals were dissected then the kidneys were quickly removed and decapsulated. One kidney was stored at -80 ᵒC for RT-PCR experiments while the other one was homogenized in 0.01 M ice-cold sodium potassium phosphate buffer (10 % w/v, pH 7.4) containing 1.15 % KCl. Supernatants collected from these homogenates (10,000 rpm, 20 min.) was stored at -80 ᵒC and used later for the biochemical analysis.

5. Measurements of the biochemical parameters in the serum:

Serum Creatinine (Cr) concentration was determined by the commercial colorimetric assay kit (Cat., no. 700460) purchased from Cayman Company (Ann Arbor, MI, USA). Blood urea was determined by a kinetic reagent (Diagnostic Chemicals Limited, Cat., no. 283-30). Na^+ and K^+ levels were determined using an Olympus Autoanalyser (Olympus Instruments, Tokyo, Japan). All analyses were performed in accordance with the manuals provided by the manufacturers.

6. Measurements of biochemical parameters in renal homogenates:

The activity of Na^+/K^+ ATPase in the renal homogenates of all rats was determined as previously run in the labs of our institution (Bashir *et al*., 2014). In brief, the total enzyme activity was calculated by measuring the amount of inorganic phosphate (Pi) liberated from ATP in a premixed buffer (100 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA, 40 mM Tris/HCl (pH 7.4) and 3 mM Na 2ATP). Na^+/K^+ ATPase activity was calculated by considering the difference between the activities assayed in the absence and presence of 2 mM ouabain. Quabain

sensitive H^+/K^+ ATPase activity was blocked by the addition of Sch 28080 (0.2 mM). The amount of Pi liberated (micromolar) per 1 mg of protein during 1 h (micromolar per hour per milligram of protein) was used to express Na^+/K^+ ATPase activity.

Malondialdehyde (MDA) levels as Lipid peroxidation marker were measured in the homogenates as levels of thiobarbituric acid reactive substances (TBARS) using a commercial assay kit (Cat No. NWK-MDA01, NWLSS, USA). Assay kits for determination of activities of superoxide Dismutase (SOD, Cat. NO.706002), CAT (CAT, Cat. NO.703102) and Glutathione S-transferase (GST, Cat. NO.703302) and levels of reduced glutathione (GSH, Cat. No. 703002) were purchased from Cayman Chemical, Ann Arbor, MI, USA). All measurements were performed in accordance with manufacturer's instructions. MDA levels were expressed as nmol/mg protein. SOD CAT and GST activities were expressed as nmol/min/ml/mg protein. Calculated GSH and GSSG levels were expressed as μmol/mg protein and their ratios were expressed as %.

a. RNA Extraction and RT-PCR:

RT-PCR reaction was carried out at the stem cell research unit at the college of Medicine at King Khalid University, Abha, Saudi Arabia according to the established method there (Bashir *et al*., 2014). Primers used to study the mRNA expression of renal Na^+/K^+ ATPase, glutamylcysteine ligase (ɣ-GCL) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in rat's kidney are shown in table 1.

Table1: Primers sequences used in the RT-PCR.

Gene	Primers	Product Size
ATPase α1	F:5'GAAGCTCATCATCAGGCGACG3' R:5'CCAGGGTAGAGTTCCGAGCTC3'	159 bp
ɣ-GCL	F: 5'TTGGCAGCCTT CCTGATTTC3' R: 5'AACTTCTCCACAACCCTCTG3'	78 bp
GAPDH	F:5'AGAAGGCTGGGGCTCACT3' R:5'GGCATTGCTGACAATCTTGA3'	134bp

Thirty mg of renal tissue was used to prepare total RNA using RNeasy Mini Kit (Qiagen Pty. Ltd., Victoria, Australia). Absorbance 260/280 was used to test RNA purity. For Reverse transcription (RT), 1 μg of DNase I pretreated total RNA samples were incubated with 30 μL of reverse transcription mixture at 40°C for 70 min and then heat inactivation at 95°C for 3 min. Then, RT-PCR reaction (20 μL) was performed in Biorad thermal cycler using the following mixture: 2 μL RT products, 0.01 U/ml Taq polymerase, 100mM dNTPs, 1.5 mM MgCl2 and 50 mM Tris-HCl buffer. PCR reactions consisted of a first denaturing cycle at 97°C for 5 min, followed by 35 cycles of amplification as 1) denaturation at 96°C for 30 sec, 2) annealing at 60°C for Na^+/K^+ ATPase and GAPDH and 50°C for ɣ-GCL, for 30 sec and 3) extension at 72°C for 1 min. A final extension cycle of 72°C for 15 min was included. Reverse transcriptase was omitted from one sample on each run as a negative control. All RT-PCR product (10 μL) were run on 1.5% agarose gels containing 100 ng/ml ethidium bromides and photographed with a Polaroid camera under ultraviolet illumination. Images were analyzed using ImageJ (USA) and data of each gene were presented as mean ±SD of 10 samples after being normalized to GAPDH.

b. Histopathological studies:

Parts of the kidney were rapidly fixed in 10% neutral buffered formalin, dehydrated in ascending concentrations of ethyl alcohol

(70-100%) and then prepared using standard procedures for Hematoxylin and Eosin staining.

c. Statistical analysis:

Statistical analyses were performed by using GraphPad prism statistical software package (version 6).The data are represented as the mean ± SD. All comparisons were analyzed by one-way ANOVA followed by post hoc Tukey's t- test and accepted as significant at $P< 0.05$.

RESULTS

1. Serum parameters:

Table 2 shows the changes in the levels of urea, creatinine (Cr), Na^+ and K^+ in the serum of the control and the other experimental groups. After seven days of PCM intoxication, significant increases in the serum levels of urea and Cr with parallel decreases in the serum levels of Na^+ and K^+ were seen in the intoxicated group. Post-administration of NAC, RES and their combination resulted in significant decreases in the levels of serum urea and Cr and significantly elevated Na^+ and K^+ levels. The amelioration in the levels of all these parameters was significantly more profound in RES post-treated group as compared to NAC post-treated group. However, levels of all these measured parameters in RES post-treated group remained slightly different form their corresponding levels seen in the control group. Remarkably, normal levels of urea, Cr, Na^+, and K^+ have measured in the serum of rats received post treatment of combined RES+NAC.

Table 2: Biochemical parameters measured in the serum.

animal group \ parameter	Control	PCM	PCM then NAC	PCM then RES	PCM then RES+NAC
Urea (mg/dl)	32.4±4.6	84.5±6.7[a]	72.3±6.5[ab]	48.5±4.9[abc]	36.5±6.3[bcd]
Creatinine(mg/dl)	0.59±0.11	1.73±0.12[a]	1.34±0.19[ab]	0.89±0.15[abc]	0.64±0.13[bcd]
Na^+(mmol/l)	124±12.4	54.65±8.9[a]	56.7±7.3[ab]	98.7±11.6[abc]	124.6±9.4[bcd]
K^+(mmol/l)	5.4±0.12	1.3±0.08[a]	2.2±0.13[ab]	4.2±0.32[abc]	5.3±0.21[bcd]

Values are expressed as Mean ± SD for 10 rats/group. Values were considered significantly different at $P< 0.05$. [a] Significantly different when compared to control group. [b] Significantly different when compared to PCM intoxicated group. [c] Significantly different when compared to PCM then NAC. [d] Significantly different when compared to PCM then RES.

2. Renal oxidative stress and lipid peroxidation:

PCM intoxicated rats showed a significant increase in renal lipid peroxidation as measured by MDA level with concomitant significant decreases in the activities of SOD, CAT, and GST as compared with the control group. Post-treatment with NAC and RES produced significant decreases in MDA levels and significant increases in the activities of SOD, CAT, and GST as compared to control rats. In these groups, MDA level remained significantly higher and SOD, CAT and GST activities remained significantly lower as compared to control rats with more significant improvements was noticed in RES post-treated rats. However, in spite of these improvements, the levels of these parameters remained significantly different as compared to their corresponding levels seen in the control group. On the other hand, normal levels of MDA and activities of SOD, CAT, and GST were seen in the group of rats post-treated with RES and NAC combination (Fig. 1).
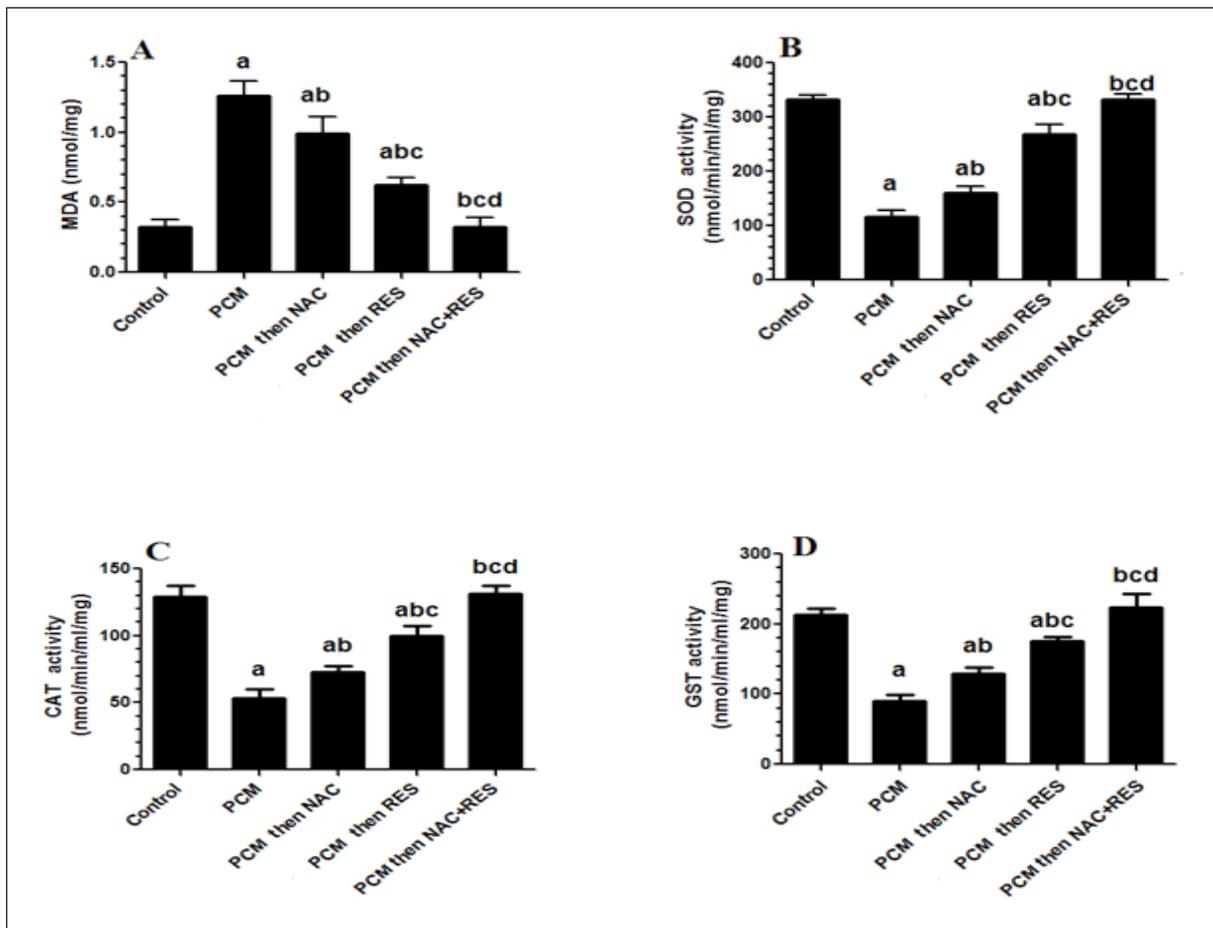
Fig. 1: Levels of malondialdehyde (MDA) (A); and activities of superoxide dismutase (SOD), (B), Catalase (CAT), (C) and glutathione S-transferase (GST), (D) in renal homogenates of all experimental groups of rats. Values are expressed as Mean ± SD for 10 rats/group. Values were considered significantly different at $P< 0.05$. [a] Significantly different when compared to control group. [b] Significantly different when compared to PCM intoxicated group. [c] Significantly different when compared PCM then NAC. [d]significantly different when compared to PCM then RES.

3. GSH and ɣ-GCL mRNA levels:

Figure 2 shows renal levels of reduced glutathione (GSH, **A**) and expression levels of ɣ-glutamylc ysteine ligase (ɣ-GCL) in the kidneys obtained from the control and all other experimental groups of rats. mRNA levels of ɣ-GCL and GAPDH (internal control) were within expected value. A thick mRNA band of ɣ-GCL was seen in the control group (Fig. 2, lane 1). Significant decreases in the levels of GSH and relative mRNA levels ɣ-GCL were detected in the kidneys of PCM intoxicated rats as compared to control group. (Fig. 2, Lane 2) A gradual increase in the levels of GSH and relative mRNA expression of ɣ-GCL were seen in the renal tissues of rats post-treated with NAC, RES and their combination, respectively (Fig. 2, Lanes 3-5). Data showed that RES post- administration to PCM intoxicated rats resulted in more significant increases in the of GSH and ɣ-GCL mRNA levels as compared to intoxicated rats received NAC as a post treatment, However, complete normalization in the levels of GSH and ɣ-GCL mRNA were achieved in the PCM intoxicated rats post-treated with RES and NAC.
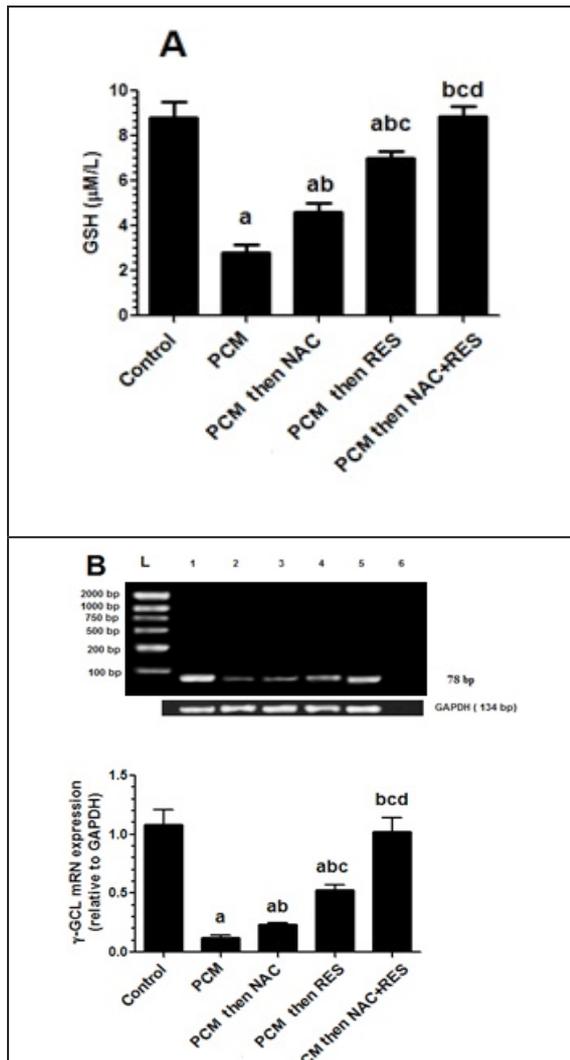
Fig. 2: Levels of reduced glutathione(GSH), (A) and mRNA expression levels of ɣ-glutamylcystine ligase (ɣ-GCL),(B), in the renal homogenates and tissues, respectively, of all experimental groups of rats. Values are expressed as Mean ± SD for 10 rats/group. Values were considered significantly different at P< 0.05. [a] Significantly different when compared to control group (Lane 1). [b] Significantly different when compared to PCM intoxicated group (Lane 2). [c] Significantly different when compared PCM then NAC (Lane 3). [d] significantly different when compared to PCM then RES (Lane 4). Lane 5: PCM then NAC+RES. Lane 6: negative control.

4. Activities and mRNA expression levels of Na⁺/K⁺ ATPase in the renal tissues:

Figure 3 shows the transcriptional changes in the levels of Na^+/K^+ ATPase α-1 subunit and the total activity of the enzyme in the rats' kidneys of all groups of rats.
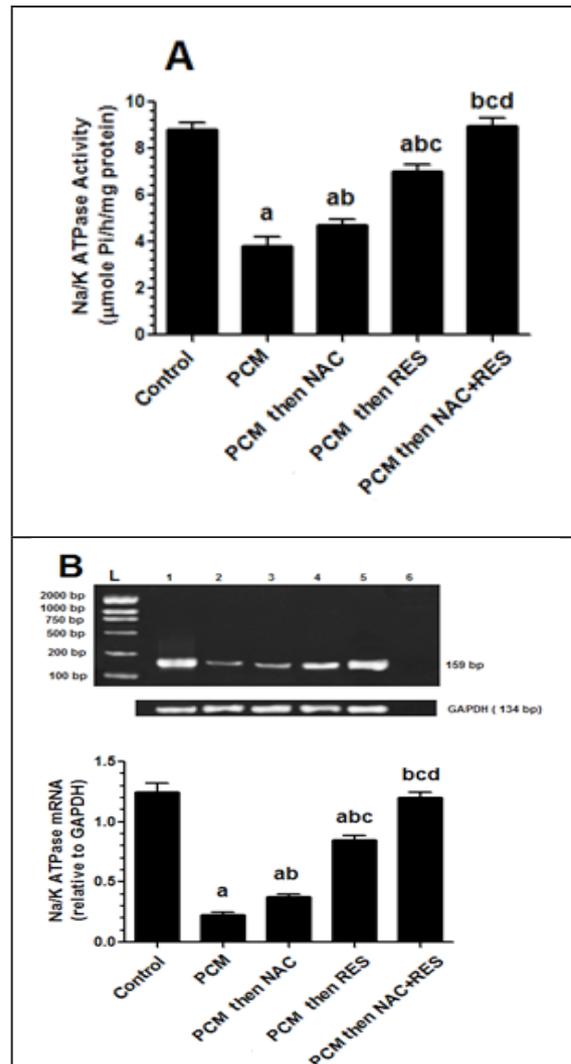
Fig. 3: Activities (A) and mRNA expression levels (B) of Na^+/K^+ ATPase in the renal homogenates and tissues, respectively, of all experimental groups of rats. Values are expressed as Mean ± SD for 10 rats/group. Values were considered significantly different at P< 0.05. [a] Significantly different when compared to control group (Lane 1). [b] Significantly different when compared to PCM intoxicated group (Lane 2). [c] Significantly different when compared to PCM then NAC (Lane 3). [d] significantly different when compared to PCM then RES (Lane 4). Lane 5: PCM then NAC+RES. Lane 6: negative control.

GAPDH mRNA expression from the same sample was used as internal control. All tested transcripts were detected and RT-PCR resulted in fragments similar in size to those expected values A thick prominent band for Na^+/K^+ATPase α-1 mRNA was seen in the control (Fig. 3, lane 1). The

activity of Na$^+$/K$^+$ ATPase and the relative mRNA expression of its α-1 subunit were significantly decreased in the kidney of PCM intoxicated rats and a very thin band was detected (Fig. 3 B Lane 2). On the other hand, a small significant enhancement in Na$^+$, K$^+$ ATPase activity and relative mRNA expression of the enzyme α-1 subunit were seen in PCM intoxicated rats post-treated with NAC (Fig. 3, lane 3). As compared to NAC post-treated group, a more significant increase in the activity of this enzyme and its relative mRNA expression were seen in PCM intoxicated rats post-treated with RES (Fig. 3, Lane 4). However, normal activity and mRNA expression were seen in the kidneys of PCM intoxicated rat post-treated with the combined dose of NAC+RES (Fig. 3, Lane 5).

Histopathological Findings

Histological examination of renal tissue in Control groups showed normal renal structures. All parts of kidney showed normal appearance. Dense rounded renal corpuscles comprising the glomeruli appeared and they were surrounded by Bowman's capsules which were lined by the squamous epithelial cells. The cortical tubules were found in a bulk and were both proximal, distal convoluted tubules as well as collecting tubules were seen. prominent brush border lined the proximal convoluted tubules which were lined by simple cuboidal epithelium. Distal convoluted tubules were identified clearly by having defined lumen and closely packed nuclei per section (Fig. 4, A). Severe glomerolus damage as evident by glomerular degeneration, bleeding, and shrinkage as well as sever rapture of capsule endothelia was seen in rats intoxicated with PCM. Sever damage and dilation were seen in both proximal and distal convoluted tubes of PCM intoxicated rats. Loss of brush border of proximal convoluted

tubules was prominent. Intraluminal cell debris, karyorrhexis, vacuolization and dark pink cytoplasm were observed as indicators of the cell death were also observed section (Fig. 4, B and C). Improvements in kidney histology were seen after individual NAC or RES post-administration to PCM intoxicated groups. Significant improvement in the glomerulus structure as evident by its round capsule and the presence of endothelia were seen in NAC post-treated group. However, in this group of rats, the presence of some glomerulus capillaries degenerations and moderate tubular degenerations, dilation and vacuolization were prominent loss of brush boarders of the proximal tubules and the intraluminal cell debris's were evident. More clear improvements in all the mentioned renal structures with less tubular degenerations, vacuolization and dilation as well as improvements in proximal tubules brush boarders were seen in RES post-treated group. On the other hand, the maximum improvement in kidney architectures were seen in the group of rats post-treated with the concomitant dose of RES+NAC. This group of rats showed very little tubular degeneration and complete absence of intraluminal cell debris's. However, some glomerulus capillary degeneration persist (Fig. 4, B and C).
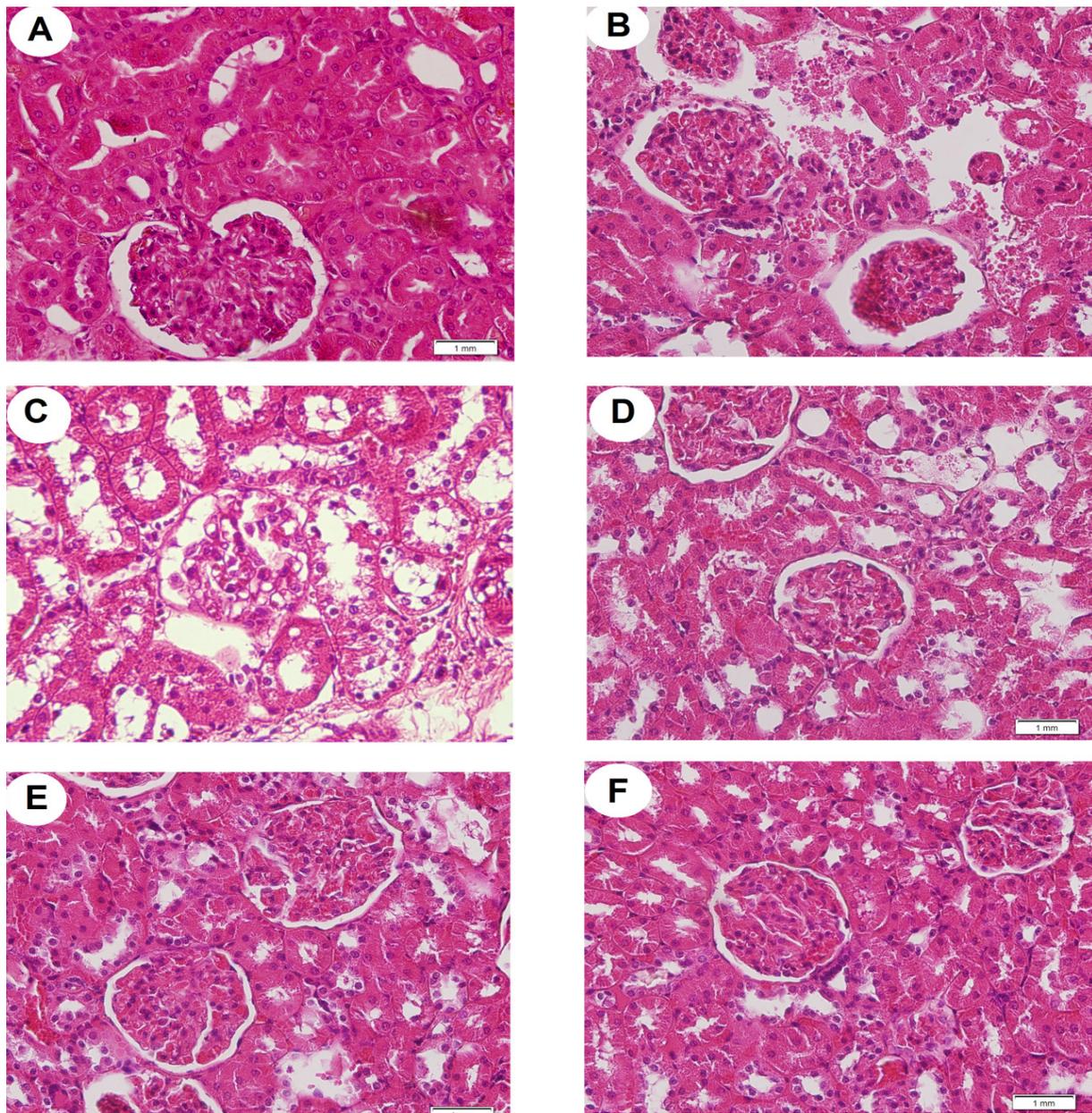
Fig. 4: Histopathological findings in all groups of rats. A: a section taken from control rat showing normal kidney architectures including dense rounded renal corpuscles comprising the glomeruli surrounded by Bowman's capsules. Both proximal lined with brush border and normal distal convoluted tubules well preserved and both line with simple cuboidal epithelium. B and C: sections taken from PCM intoxicated rats showing sever glomerular degeneration as evident by bleeding, shrinkage, and rapture of capsule endothelia. Increased vacuolization and dilation were seen in both proximal and distal convoluted tubules with loss of brush border. Intraluminal cell debris, karyorrhexis and dark pink cytoplasm were observed as indicators of the cell death. D: a section taken from PCM intoxicated rats post treated with NAC showing an improvement in the glomerulus structure as evident by its round capsule and the presence of endothelia. The presence of some glomerulus capillaries degenerations and shrinkage, moderate tubular degenerations, dilation and vacuolization and loss of brush boarders were prominent. E: a section taken from PCM intoxicated rats post-treated with RES showing clear improvements in all the mentioned renal structures with less tubular degenerations, vacuolization and dilation as well as improvements in proximal tubules brush boarders. F: a section taken from PCM intoxicated rats post treated with a concomitant dose of NAC+RES showing a maximum improvement in kidney architectures, very little tubular degeneration and complete absence of intraluminal cell debris's. Some glomerulus capillary degeneration persists.

DISCUSSION

The present study is the first to describe that combination therapy of NAC and RES provides an excellent preventative therapy rather than their individual usage against PCM induced nephrotoxicity in rats. the major exclusive findings clearly shows that such combination of these two drugs act synergistically to restore renal architecture and attenuate changes in serum electrolytes, urea and creatinine in mechanisms related to antioxidant potentials and amelioration of levels and activity of renal Na^+/K^+ATPase. It is well documented that high serum urea and creatinine levels are considered the major nephrotoxic markers (Adelman et al., 1981). Higher levels of urea and creatinine were seen in the serum of PCM intoxicated rats that are in accordance to those observed by Isik et al. (2006) in rats after 1 g/kg body weight of PCM administration which is mainly due to enhanced rate of production rather than clearance resulting in their accumulation (Mayne, 1994). Also, it was previously reported the significant elevation of urea was strongly correlated to PCM induced oxidative stress (Karadeniz et al., 2008). In support, PCM intoxicated rats had lower intracellular GSH levels and antioxidant enzymes activities including superoxide dismutase (SDO), catalase (CAT) and Glutathione S-transferase GST as well enhanced levels of MDA, a common lipid peroxidation marker (Helms et al., 2015). In this regards, PCM induced tissue toxicity is mainly a result of the excessive generation of a highly reactive intermediate metabolite, N-acetyl-para-benzoquinone-imine (NAPQI). In the kidney, NAPQI enhances the generations of ROS and initiates cell necrosis by direct depletion in GSH levels and mitochondrial dysfunction (Bessems and Vermeulen, 2001; James et al., 2003; Knight et al., 2001) which explains the GSH levels and lower antioxidants enzymes activities that lead to renal damage. These findings were confirmed by the histological findings, which showed reduced glomerular size, raptured bowman's capsules, enhanced vacuolation, dilation, and degeneration in the proximal and distal convoluted tubules of PCM intoxicated rats. Such results were previously described and are in accordance to those previously reported by Abdel-Zaher et al (2007), and Khorsandi and Orazizadeh (2008).

On the other hand, it has been suggested that impairment of renal Na^+/K^+ ATPase expression and activity play an essential role in the pathology and progress of PCM induced nephropathy (Helms et al., 2015). Indeed, both NAPQI or oxidative stress directly inhibit renal Na^+/K^+ ATPase (Corcoran et al., 1987; Nicotera et al.; 1990; Tukel, 1995; Helms et al., 2015).

In the current study, severe inhibition of Na^+/K^+ ATPase was noticed in the renal homogenates of PCM intoxicated rats and these changes were associated with disturbances in the electrolytes, which are manifested by significant decreases in Na^+ and K^+ levels in the serum. Hence, these results confirm the involvement of this enzyme in the pathophysiology of PCM induced nephropathy. Since ATP levels were not measured in the renal homogenates, it cannot be identified if Na^+/K^+ ATPase inhibition is due to disruption of the cell cytoskeleton or due to other biochemical events as previously suggested (Nelson et al., 1990; Canfield et al. 1991).

In addition, significant decreases in the renal mRNA levels of Na^+/K^+ ATPase were seen after PCM intoxication. Elevation of intracellular Ca^{+2} has been reported to highly up-regulate the transcription rate of the α1 and β1 subunit mRNAs (Rayson, 1991). During early stages of PCM-induced hepatotoxicity, a rise in liver Ca^{+2} content has been reported in in vivo (Corcoran et al., 1987) as well as in vitro studies (Moore et al., 1985; Shen et al., 1991). Although intracellular Ca^{+2} mediated processes are beyond the scope of the present study, the participation of Ca^{+2}, as well as other second messengers as intracellular mediators in PCM induced alterations in Na^+/K^+ ATPase abundance, will be a matter of further

investigations. In PCM intoxication, NAC is one of the best antidotes of choice (Corcoran and Wong, 1986; Gregory and Kelly, 1998). NAC can stimulate GSH synthesis, enhance GST activity, promote detoxification, and act directly on reactive oxidant radicals (Tylicki et al., 2003). Thus, by enhancing GSH levels, NAC preserves membrane fluidity and of the activities of CAT, mitochondrial SOD and the different forms of glutathione peroxidases (GPx) (Tylicki et al., 2003).

In the current study, i.p. administration of NAC 24 h after induction of nephrotoxic dose of PCM significantly but partially improved renal GSH levels and activities of SOD, CAT, and GST and slightly upregulated the expression of Glutamate–cysteine ligase (ɣ-GCL). Also, renal tissue histopathology was partially improved with abundant necrotic tubular and glomerulus degenerations. Even more, Na^+/K^+ ATPase activity and expression were poorly but significantly improved with relatively high levels of serum Na^+ and K^+. These data indicates that delay in NAC administration after PCM intoxication is partially beneficial and not much useful.

Although many clinical studies have suggested that NAC is not effective in treating PCM induced toxicity in rats, Currently accumulative evidence contradict this in animal studies. This has been explained by the fact that such judgment was derived primarily from retrospective studies and case reports and there have not been any large-scale randomized clinical trials (Mazer et al., 2008). This is the reason behind the fact that no difference in peak Cr levels between nephropathy patients treated with NAC and those who were not treated (Eguia et al., 1997; Mour et al., 2005). This further reinforces the concept that discrete and different mechanisms of PCM toxicity exist between the liver and the kidney.

However, our findings are in accordance with Smilkstein et al (1988) who showed that within eight hours of acetaminophen ingestion, NAC is protective regardless of the initial plasma PCM concentration;

however, protection efficacy decreased when treatment was further delayed. In the same line, NAC administration to PCM intoxicated rats improved histological architectures and enhanced levels of SOD and GSH and significantly lowered serum levels of Cr and MDA levels in the renal tissues (Canayakin et al., 2016). In addition, NAC administration was more effective than erdosteine treatment in protection against PCM induced nephrotoxicity in rats (Kandiş et al., 2011). Furthermore, one given as a sole treatment or in combination with hyperbaric oxygen, NAC significantly attenuated PCM induced nephrotoxicity in rats by reducing inflammatory mediators and improved kidney structure (Cermik et al., 2013).

On the other hand, accumulative evidence has shown that RES could offer an excellent option to protect and treat various forms of drug-induced nephrotoxicity including cisplatin, glycerol, gentamicin, and cyclosporine (Chander et al., 2005, Do-Amaral et al. 2008). In the same line, all the biochemical parameters histological alterations seen in PCM intoxicated rats were highly significantly attenuated by RES and the attenuation in the levels of any of these parameters was always higher than that measured in measure as compared o NAC post-treated group. Interesting, beside the higher amelioration in GSH levels and activities of SOD, CAT and GST, RES solely administration resulted in a significant higher increase in the renal activities and expression levels of Na^+/K^+ ATPase, as compared to NAC post treated rats. However, no complete cure was achieved with RES and proximal and glomerular degeneration persisted but at a lower level.

The delayed beneficial effect of RES could be explained by the diverse mechanism of RES. Resveratrol is a natural antioxidant that can directly scavenge various forms of ROS, (Holthoff et al., 2010). It is able to modulate the expressions and activities of antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), through

transcriptional regulation via nuclear factor E2-related factor 2 (Nrf2), activator proteins (AP)-1, forkhead box O (FOXO), and SP-1 or through enzymatic modification (Mokni *et al.*, 2007; Robb *et al.*, 2008; Pervaiz and Holme, 2009). Indeed, RES enhanced the expression levels of Nrf2 and its downstream enzyme, including ɣ-GCL, m-GST, and hemoxygenase-1 (HO-1). This could explain the enhanced activities of antioxidant enzymes and GSH levels as well as higher expression of ɣ-GCL in treated rats (Palsamy and Subramanian, 2011). Moreover, RES can directly inhibit CYP2E1, CYP3A11, and CYP1A2 activities, and significantly inhibited the bio-activation of PCM into toxic metabolite NAPQI (Wang *et al.*, 2015). Additionally, RES treatment significantly induced SIRT1 and then negatively regulated p53 signaling to induce cell proliferation-associated proteins including cyclin D1, CDK4, and PCNA to promote proliferation (Wang *et al.*, 2015). However, the data of the current study revealed complete recovery of kidney architecture and function when RES and NAC were added together and given as a delayed dose after PCM intoxication. Complete recovery of GSH levels, preservation of antioxidant enzymes and normal levels of lipid peroxidation, as well as electrolytes levels, were seen in RES+NAC post-treated group. This can be explained by the synergistic partial antioxidant potential of each drug and by the ability of RES to enhance the expression and activities of Na^+/K^+ ATPase. In fact, recent studies have recommended the use of antioxidants in combination that is superior to its individual usage as to treat oxidative stress-induced renal dysfunction in various animal models (Petronilho *et al.*, 2008; Puica *et al.*, 2008). Indeed, in many experiments, adding other antioxidants to NAC enhanced its efficiency. An example is the combination therapy of NAC and cimetidine (an H_2-receptor-antagonist drug and an inhibitor of hepatic microsomal oxidative enzymes) produced a more pronounced effect in the treatment of acetaminophen overdose than the use of NAC or cimetidine alone. In mice, the concomitant administration of NAC and cimetidine produced a 100 percent survival rate, reduced plasma GOT and GPT activities to within the normal range, and significantly raised hepatic GSH concentrations to values close to those measured in saline-treated control animals (Al-Mustafa *et al.*, 1997). The combination between NAC and Mg^{+2} produced a significant decrease of the lipid peroxidation production and an increase of the antioxidant enzymes in gentamicin-induced nephrotoxicity rat model (Abdel-malek, 2013). Moreover, combined treatment with NAC and folic acid significantly restored kidney functions, modulate hematological parameters, reduced lipid peroxidation, and enhanced reduced glutathione level in aspartame-induced nephrotoxicity rat model (Saleh, 2015).

However, in spite of these interesting findings and the concomitant protective effect of NAC and RES, this study still have some limitations. Among these, even individual uses of NAC or RES or their combination gradually and significantly improved serum Cr levels. Unfortunately, we could not adjust the dose to the renal function, i.e., estimating Cr clearance, as we could not metabolically collect the urine in these rats. In addition, even we could not find any change in the final body weight of rats between various treated (data not shown), a further investigation on NAC-RES drugs interactions were not investigated in this study as it is not a primary object in the current study. Hence, further pharmacological analysis on this should be considered taking into account absorption rates, liver metabolism, and peak serum and renal levels of RES as well as measuring renal NAC adducts. In addition, since drugs' interaction is affected by age and sex, Further analysis using different ages and female rats should be considered.

In conclusion, our novel data are the first to show that concomitant administration of NAC and RES, completely reverses nephrotoxicity induced by PCM. While delayed NAC

administration poorly improved renal GSH levels, antioxidant enzymes levels and activity and mRNA expression levels of Na^+/K^+ ATPase, RES effect was on these parameters was more profound. Overall, adding RES to NAC enhances its efficiency to act as a therapeutic agent against PCM induced nephrotoxicity and vice versa.

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CONFLICTS OF INTEREST

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الدور الوقائي لمزيج من الريسفيراترول و ن-أسيتيل سيستين لمنع اعتلال الكلى الحاد الناتج عن الباراسيتامول في الجرذان

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الملخص

بما أن التعرض لجرعة زائدة من الباراسيتامول يسبب التسمم الكلوي، فقد هدفت الدراسة الحالية إلى بحث التأثير العلاجي لمزيج من مركبي الريسفيراترول و ن- أسيتيل سيستين ضد التسمم الكلوي في الجرذان.

تم استخدام 50 جرذا بالغا، قسمت عشوائيا إلى 5 مجموعات بكل مجموعة 10 جرذان: 1) المجموعة الضابطة، 2) مجموعة الباراسيتامول (بجرعة مقدارها 1000 ملغ/ كغ من وزن الحيوان)، 3) مجموعة الباراسيتامول والريسفيراترول (بجرعة مقدارها 25 ملغ/ كغ من وزن الحيوان)، 4) مجموعة الباراسيتامول و ن-أسيتيل سيستين (بجرعة مقدارها 1.2 غرام/ كغ من وزن الحيوان)، 5) مجموعة الباراسيتامول والريسفيراترول + ن-أسيتيل سيستين. تم حقن الحيوانات داخل الصفاق بشكل يومي. تم فحص وظائف الكلى والتغير النسيجي في الكلية إضافة إلى قياس معدل أكسدة الدهون وأنشطة الإنزيمات المضادة للأكسدة، ومستويات إنزيم الجلوتاثيون المختزل، وكذلك مستويات التعبير الجيني لإنزيم ألفا غلوتاميل سيستين والإنزيم المنظم لتركيزات الصوديوم والبوتاسيوم في أنسجة الكلى لجميع الجرذان في التجربة.

أشارت النتائج إلى أن الحقن بالباراسيتامول أدى إلى تلف أنسجة الكلى؛ حيث كان هناك انخفاض في مستويات الجلوتاثيون المختزل وفي نشاط الإنزيمات المضادة للأكسدة (سوبر أوكسيد الديزميوتاز والكاتاليز وجلوتاثيون اس-ترانسفيراز). كان هناك أيضا انخفاض ملحوظ في النشاط والتعبير الجيني لإنزيم ألفا غلوتاميل سيستين والإنزيم المنظم لتركيزات الصوديوم والبوتاسيوم.

لقد أدى حقن الحيوانات المعرضة للباراسيتامول بمركبي ن-أسيتيل سيستين والريسفيراترول كل على حدة إلى تحسن طفيف في أنسجة الكلى والاختلالات في الإنزيمات التي تم قياسها، إلا أنه – ولأول مرة – اكتشفت هذه الدراسة أن المعالجة بمزيج الريسفيراترول و ن-أسيتيل سيستين استطاعت أن تؤدي إلى تحسن شبه كامل لأنسجة الكلى والاختلالات في الإنزيمات التي تم قياسها. وبذلك يستنتج من الدراسة أن مزيج الريسفيراترول و ن-أسيتيل سيستين يجعلها يعملان بشكل متآزر لمنع اعتلال الكلى الحاد الناتج عن استخدام الباراسيتامول.

الكلمات المفتاحية: السمية الكلوية، الضغط التأكسدي، ن-أسيتيل سيستين.