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The Consequence of Antioxidant Signaling Pathways on Acetaminophen Nephrotoxicity

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Abstract Renal injuries and death from renal diseases are one of major issues in medical field nowadays. Proper right dose of over-the-counter oral analgesic medication is important to be more controlled and decrease health risk. Acetaminophen is a commonly used over the counter oral analgesic and antipyretic, and it should be used at the appropriate therapeutic dose. Herein we shed the light on the potential impact of thymoquinone, turmeric and α-lipoic acid (either alone or in several combinations) on AP-induced renal injury and to explore the underlying mechanisms and signaling pathways of antioxidants under investigation. N-acetyl cysteine was used as a reference antidote drug. Acute AP on nephrotoxicity was elucidated in male Wister rats 24hr after the administration of an overdose of oral AP (1000 mg/kg). The investigated antioxidants were administered 3hr before that and carried on for 72hr after AP intoxication. The administration of AP enhanced the secretion of renal biomarkers including serum urea, creatinine, and uric acid. As well as the production of inflammatory biomarkers including C-reactive protein, toll like receptor-4, nuclear factor kappa-B (NF-κB) and ameliorated apoptotic biomarker P53. On the other hand, renal tissue showed a marked depletion in glutathione level in addition to the elevation of nitric oxide and malondialdehyde amount. Overexpression of fibrotic biomarkers, including transforming growth factor (TGF-β1) was detected. However, P53 was downregulated compared to the corresponding level in the control group. In conclusion, treatment with the antioxidants altered inflammatory and apoptotic biomarkers. Importantly, the combination regimen of LA and turmeric showed the most reno-protective impact via the upward of anti-inflammatory and antioxidant signaling.

Key Words acetaminophen, thymoquinone, lipoic acid, TGF-β1, CRP

1. Introduction

A growing body of evidence deduced that approximately 1-2% of patients who received acetaminophen (AP) overdose developed renal insufficiency [1]. Previous study demonstrated that the main etiology of renal toxicity in acetaminophen intoxication is the cytochrome P-450 mixed function in the kidney, whereas other known mechanisms include the activity of prostaglandin synthetase and N-deacetylase enzymes have been identified [2]. Contrarily, glutathione is thought to be a crucial component in the detoxification of AP and its metabolites, despite the fact that a creation of nephrotoxic substances was linked to the conjugates [2].

The hepato-renal syndrome, which can exacerbate hepatic failure, can be distinguished from AP-induced renal failure, which often manifests after hepatotoxicity. It's still unknown what role N-acetylcysteine therapy plays in cases of renal failure brought on by acetaminophen. In the context of acute acetaminophen intoxication, the pathogenesis, clinical characteristics, and therapy of renal insufficiency have previously been reported [3]. When given in therapeutic dose to adults, acetaminophen is metabolized nearly 63% via glucuronidation and 34% by sulfation. Water-soluble metabolites are eliminated by the kidney which are normally processed in the liver. The cytochrome P-450 enzyme system oxidizes 5% of APAP at therapeutic levels to the reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI) [4]. This electrophilic metabolite is then declined by glutathione during therapeutic treatment, and subsequently eliminated as a relatively harmless substance, mercapturic acid. Glutathione and sulfate reserves are decreased in the presence of high level of APAP. This produces more NAPQI reactive intermediates by diverting more acetaminophen to the CYP-450 mixed function oxidase system. When AP is taken in high dosages, glutathione is depleted more severely with the induction of high number of metabolites, which increases

the toxicity and releases more unbound reactive species. These electrophilic intermediates then combine with the glutathione and sulfhydryl moieties on cellular proteins to produce adducts [5]. Homeostasis is upset by this process, which then triggers the lysosomal and caspase enzymes that start apoptosis, or programmed cell death. Animal models have shown this in both liver and kidney tissue. The ensuing cell death causes tissue necrosis, which in turn causes organ malfunction. Based on data from both animal and human studies, there are various probable pathways for the etiology of kidney damage. The cytochrome P-450 pathway, prostaglandin synthetase, and N-deacetylase enzymes are examples of potential processes [2]. Both the liver and kidney contain the CYP-450 microsomal enzymes necessary for this function, however they differ somewhat in each organ. The CYP-450 inhibitor piperonyl butoxide can dramatically lessen the amount of reactive adducts in tissues and the severity of kidney injury. Additionally, it has been shown that circumstances linked to elevated CYP-450 system activity promote acetaminophen toxicity. The primary CYP-450 isoenzyme implicated in the biotransformation in the kidney is CYP 2E1, which testosterone can induce [6]. The kidney maintains the body fluid volume and composition as well as the acid base balance. Nevertheless, certain drugs could merit renal dysfunctions [1]. PA is a commonly utilized pain killer over-the-counter drug worldwide [2]. PA induced nephrotoxicity could evoke multisystem organ damage [3]. N-Acetylcysteine (NAC) is well known as the specific antidote of PA hepatotoxicity and has also been proven effective against PA nephrotoxicity in various experimental models of renal injury. NAC plays a reno-protective role, via an anti-apoptotic pathway [7].

Natural products have attracted the attention of numerous researchers in this field and turmeric (TR) is a phenolic phytochemical with a marked antioxidant, anti-inflammatory, antibacterial, immune-modulatory, and anticancer activities. It modulates the signaling pathways of growth factors, malondialdehyde (MDA)/antioxidant levels and scavenger free radicals in rats treated with PA increased its bioavailability and solubility [8]. Thymoquinone (TQ), is a well-documented powerful antioxidant agent, with protective impact against hepato-renal damage. The mechanisms of such protection included amelioration of oxidative and nitrosative stress, down regulation of the tumor necrosis factor (TNF)-α/NF-κB/cyclo-oxygenase (COX)-2 inflammatory pathway, and inhibition of apoptosis.

α-lipoic acid (LA) is a natural dithiol compound and a cofactor for many mitochondrial enzymes involved in energy metabolism. LA scavenger's free radicals and chelates metal ions [9].

Herein the current work the protective effects of NAC alone as a reference antidote drug as well as TQ, TR and LA either alone or in combinations against PA induce nephrotoxicity was investigated. Moreover, monitoring the various mechanisms underlying the actions of these antioxidants.

2. Materials and Methods

A. Chemicals

High analytical grade of chemicals was purchased from Sigma-Aldrich Chemical Corp (St. Louis, MO, USA). PA, NAC, LA, turmeric and TQ. The commercial kits for the assay of urea, uric acid and creatinine, were purchased from Randox Co. ELISA kits for the determination of CRP level was purchased from R & D Co. Primary antibodies against TGF-β1, Smad-2, NFκB, TLR-4 and P53 were purchased from Santa Cruz Biotechnology (Santa Cruz CA, USA). The secondary antibodies were purchased from Sigma-Aldrich Chemical Corp.

B. Experimental Animals

The Experimental Animal Care Center, King Fahad Medical Research Center, King Abdulaziz University provided the animals for this study. Seventy-two male Wister albino rats (170-190 g) were housed in controlled maintained environment (12-hour light/dark cycle, a temperature of $20 - 22^oC$, humidity of 55%), and standard rat pellet chow were fed. All animal care and treatment procedures followed the ethical guidelines approved by the Animal Care Committee of King Saud University (KSU-SE-20-32) following the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (NIH).

C. Experimental design

Animals were randomly divided into nine groups of 8 rats each after week of acclimatization, and were treated according to the following schedule:

- G1: Control administered carboxy methyl cellulose.
- G2: administered single oral toxic dose of acetaminophen 1000 mg/kg [10].
- Groups (3-9) were treated with oral doses of the natural compounds 3 h before and for 72h post acetaminophen administration.
- G3: administered 20 mg/kg of N-acetyl cysteine (NAC) [11].
- G4: administered 15 mg/kg Thymoquinone (TQ) [12].
- G5: administered 15 mg/kg turmeric (TR) [13].
- G6: administered 15 mg/kg α-amino lipoic acid (LA) [14].
- G7: administered of TQ (15 mg/kg) and TR (15 mg/kg).
- G8: administered TQ and LA.
- G9: administered TR and LA.

NAC, TQ, LA and TR were dissolved in carboxymethyl cellulose and 72hr after acetaminophen intoxication the rats were euthanized via carbon dioxide. Serum obtained by centrifugation at 2555 g for 20 min which was applied for all collected blood samples. Then the kidney tissue was collected, washed with saline solution, and homogenized in phosphate buffer for 20 min at 3000 rpm to obtain 20% homogenate. The supernatants and sera were stored at -80°C until they were biochemically analyzed. Four kidneys were rapidly frozen under a liquid nitrogen stream and stored at -80°C for western blotting.

D. Serum biochemical analysis

1) Determination of serum creatinine, urea, and uric acid

Serum creatinine, uric acid and urea were estimated calorimetrically using assay kits from Randox Co. [15], [16].

2) Determination of renal lipid peroxidation (MDA) level

From renal tissues, lipid peroxidation was assessed using Mohideen et al., [17] method to measure thiobarbituric acid reactive substances (TBARS).

3) Determination of renal total nitrate/nitrite concentration

Nitrate/nitrite concentrations in renal tissue were measured as an indirect indicator of NO synthesis using the method of Nrisinha, et al., [18]. Additionally, acid medium was used for Griess reagent, Sulfanilamide, and N- 1-naphthylnaphthydrochloride.

4) Determination of renal glutathione (GSH) activity

The reduced glutathione (GSH) was determined in the renal tissues according to the method of Kadry and Abdel Megeed, [19] based on the reaction of GSH with 5, 5'-dithiobis (2-nitrobenzoic acid) which yields the yellow chromophore, 5-thio-2-nitrobenzoic acid.

5) Determination of renal CRP level

CRP concentration level was assessed in renal tissue using an enzyme-linked immunosorbent assay kit (R&D systems, MN, USA), according to the manufacturer's instructions. Before the assay, certain antibodies applied for microplate pre-coated purposes. Next, the fixed antibody that attached to CRP was introduced, and the wells were enhanced with the enzyme-linked secondary antibody that targets CRP. The measurement of absorbance was conducted at a wavelength of 450 nm. The intensity of the color was measured at 450 nm (Agient BioTek Microplate reader, Neo2) [20], [21].

6) Western blot analysis for TGF-β1, Smad-3, NF-κB, TLR-4 and P53 levels

Western blots analyses were performed to determine the protein expression of TGF-β1, Smad-3, NFκB, TLR-4 and P53. The protein bands were visualized using enhanced chemiluminescence (ECL)-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. For quantitative estimation of protein expression, positive immunoreactive bands were scanned and quantified with β-actin as an internal control [22].

E. Statistical analysis

The data are expressed as means $\pm$ SEM for quantitative measures. The statistical comparison between the different groups was performed using a one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was set at P<0.05.

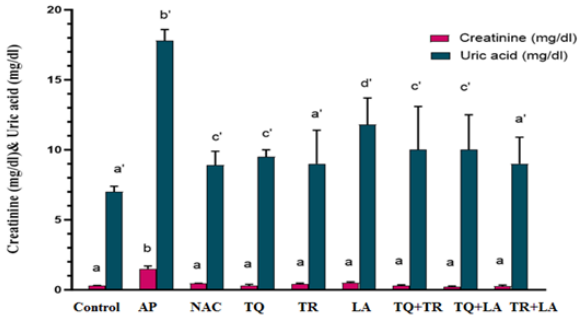

Figure 1: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on serum creatinine and uric acid post AP intoxication. Data are expressed as mean ± S.E.M (n = 8). P $\leq$ 0.05 value is considered significant. . Different letters are significantly different from each other while similar letters aren't significantly different using ANOVA, followed by Bonferroni as post ANOVA test

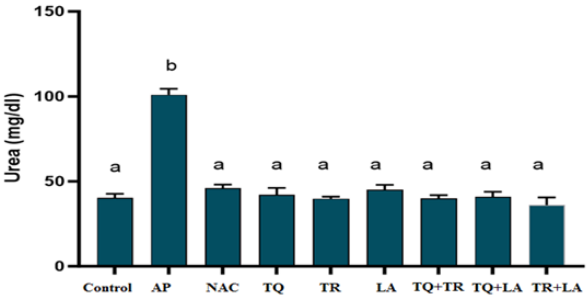

Figure 2: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on serum urea post AP intoxication. Data are expressed as mean ± S.E.M (n = 8). P $\leq$ 0.05 value is considered significant. Different letters are significantly different from each other while similar letters aren't significantly different using ANOVA, followed by Bonferroni as post ANOVA test

3. Results

A. Modulation of renal function

AP administration significantly elevated renal function biomarkers (creatinine, uric acid, and urea) compared to those of the control group (P$\leq$ 0.05, Figures 1 & 2 respectively). However, treatment with NAC alone as a reference antidote drug and TQ, TR, and LA (either alone or in different combinations), significantly decreased these elevated values. The combination of TR and LA showed the most marked reduction in creatinine level compared to NAC treated group.

B. Modulation of oxidative stress

Oxidative stress biomarkers, including MDA and NO were significantly elevated in renal tissue following AP intoxication. However, the GSH level was reduced compared with the control group (p$\leq$ 0.05). On the other hand, treatment with

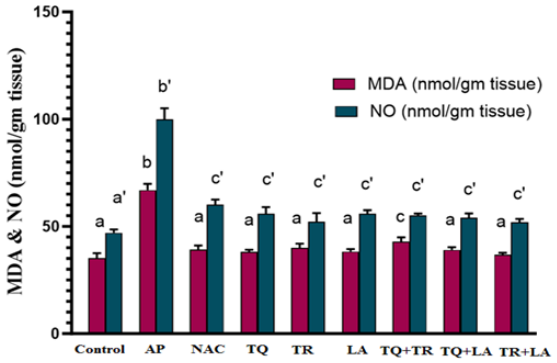

Figure 3: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on renal oxidative stress markers (MDA and NO) post AP intoxication. Data are expressed as mean ± S.E.M (n = 8). P $\leq$ 0.05 value is considered significant. Different letters are significantly different from each other while similar letters aren't significantly different using ANOVA, followed by Bonferroni as post ANOVA test

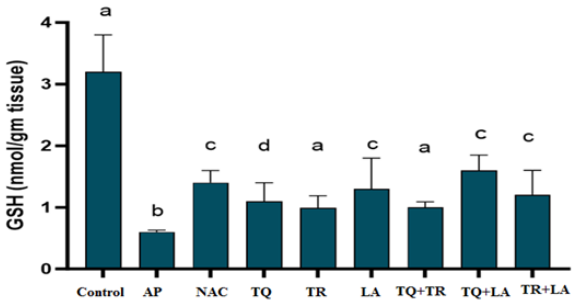

Figure 4: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on renal glutathione (GSH) post AP intoxication. Data are expressed as mean ± S.E.M (n = 8). P $\leq$ 0.05 value is considered significant. Different letters are significantly different from each other while similar letters aren't significantly different using ANOVA, followed by Bonferroni as post ANOVA test

TQ, TR and LA either alone or in different combinations, significantly attenuated oxidative stress in response to AP intoxication. Furthermore, combined treatment with TR and LA was the most effective in modulating the levels of MDA, NO and GSH compared to the reference antidote drug (NAC) (Figures 3 & 4).

C. Modulation of inflammation

Inflammatory biomarkers (NFκB, TLR-4 and CRP) as well as fibrotic markers (TGF-β1 and Smad-3) were markedly increased in renal tissue post AP intoxication. Treatment of AP intoxicated rats with NAC alone or with, TQ, TR, and LA (either alone or in different combinations) significantly decreased the elevated protein expression levels for both inflammatory markers (Figures 5 and 8), and fibrotic markers

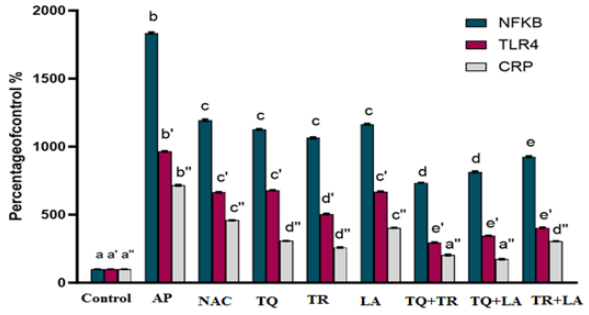

Figure 5: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on renal nuclear factor kappa-B (NFKB), Toll like receptor-4 (TLR4) and C-reactive protein (CRP) protein expression post AP intoxication. Data are expressed as mean ± S.E.M (n = 8). P $\leq$ 0.05 value is considered significant. Different letters are significantly different from each other while similar letters aren't significantly different using ANOVA, followed by Bonferroni as post ANOVA test

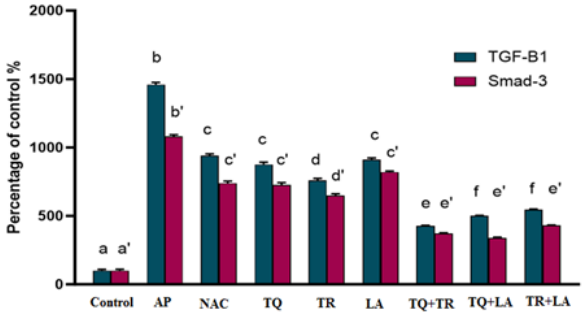

Figure 6: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on renal transforming growth factor (TGF-β1) and Smad-3 protein expression post AP intoxication. Data are expressed as mean ± S.E.M (n = 8). P $\leq$ 0.05 value is considered significant. Different letters are significantly different from each other while similar letters aren't significantly different using ANOVA, followed by Bonferroni as post ANOVA test

(Figures 6 and 8). Treatment with the different combinations (TQ+ TR, TQ+ LA and TR+ LA) was the most effective in alleviating the elevated protein expression levels of inflammatory and fibrotic biomarkers.

D. Modulation of apoptosis

AP induced a significant reduction in the apoptotic biomarker P53 protein expression (Figures 7 & 8). Treatment with the afore-mentioned antioxidants (NAC, TQ , TR, and LA) mitigated the altered biomarker level. The combinations (TQ+ TR, TQ+ LA and TR+ LA) showed the most significant impact. Figure 9 represents a heat map for different protein expressions.

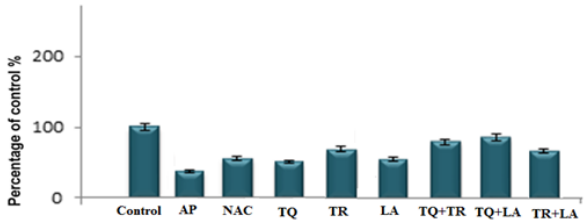

Figure 7: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on renal P53 protein expression post AP intoxication. Data are expressed as mean ± S.E.M (n = 8). $P \leq 0.05$ value is considered significant. Different letters are significantly different from each other while similar letters aren't significantly different using ANOVA, followed by Bonferroni as post ANOVA test

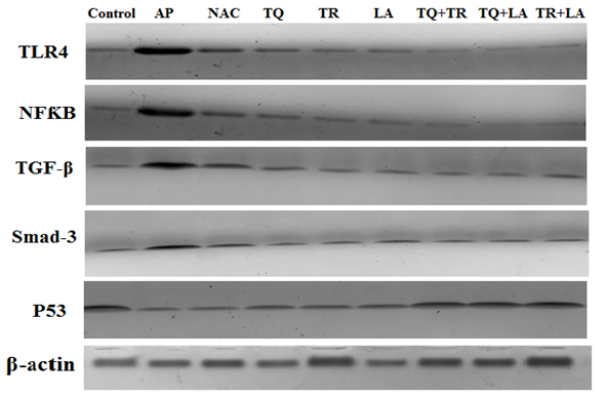

Figure 8: Impact of NAC (used alone as a reference drug) and TQ / TR/ LA (alone and in different combinations) on renal western blot analysis for TLR-4, NFκB, TGF-β, Smad-3 and p53 in acetaminophen induced toxicity

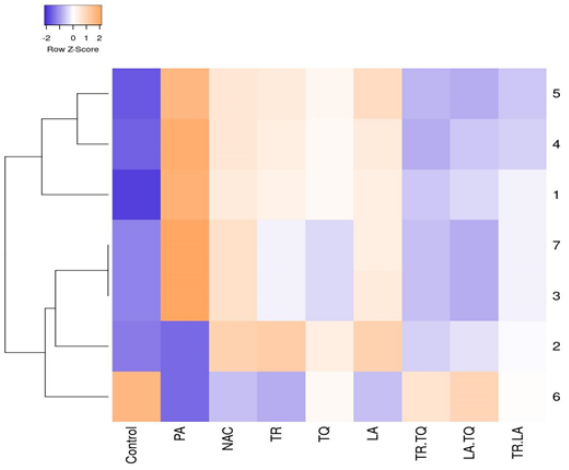

Figure 9: Heat map for different protein expression. Blue represents low score meanwhile orange represents high score

4. Discussion

A growing body of evidence deduced that renal diseases remain one of the most serious causes of mortality worldwide. Renal dysfunction due to ingestion or inhalation of reno-toxin has a widespread index [23], [24]. Acetaminophen (AP) is a commonly used as analgesic and antipyretic when administered in the appropriate dose, but excessive usage can cause fatal kidney damage. The metabolism of AP in the liver generates the toxic by product NAPQI contributing to reno-toxicity [25].

The study found a notable increase in serum urea levels, and uric acid in AP intoxicated rats, indicated to the deterioration in the kidney function due to the toxic impact of the drug. Moreover, a marked depletion in the GSH activity with a concomitant increase in MDA and NO levels in renal tissue was observed following AP intoxication compared to the control value. These results are in accordance with those of Ismail and Salem, [26] who reported that a portion of PA can be metabolized into a toxic and extremely active metabolite by the microsomal P-450 enzyme system into N-acetyl-p-benzoquinone-imine (NAPQI). Intracellular GSH can bind NAPQI, resulting in mercapturic acid conjugate, that is eliminated through the renal tubules thus performs a vital role in PA detoxification. Nevertheless, in PA overdose, the portion of active NAPQI exceeds the binding capacity of GSH, leading to the accumulation of NAPQI. Thus, active NAPQI binds the intracellular macromolecules contributing to tissue damage. Additionally, subsequent triggering of lysosomal enzymes promotes tissue necrosis and lastly organ dysfunction. PA toxicity targets proximal tubules due to their active secretory and absorptive actions thus PA can trigger life-threatening renal necrosis and lesions. NO could play an important role in the pathogenesis of AP-induced renal damage by combining with superoxide ion (O2) forming peroxynitrite which initiates lipid peroxidation thus promoting apoptosis [27], [28]. AP-induced nephropathy is highly correlated with increased lipid peroxidation and decreased GSH activity as well as the activity of other antioxidant enzymes in the kidney [6].

It was previously reported that AP induced renal injury reflected by an increase in blood urea as well as serum creatinine and damage in renal tubules [29]. Indeed, several biological compounds with antioxidant properties have attracted the attention of many researchers by their potential renal protection versus the deleterious impact of AP overdose [30]. N-acetyl cysteine (NAC) is a widely used standard drug and protects against renal dysfunction, via its antioxidant, anti-inflammatory and anti-apoptotic activities [7].

After a toxic dose of AP, the total renal GSH is depleted to a crucially low level. The protective impact of TQ was previously reported in doxorubicin, carbon tetrachloride, cisplatin, ethanol, and aflatoxin induced oxidative damage. Furthermore, anti-inflammatory, antitumor, antimicrobial, antihistaminic and immune-modulatory impact of TQ has been previously reported. TQ was highly useful in treating AP-induced hepatotoxicity and renal toxicity in rats [3]. TQ

treatment has a marked therapeutic and antioxidant impact in AP induced nephrotoxicity in rat model [7]. Tumeric (TR) is an antioxidant which displayed effective protection against acetaminophen overdose induced nephrotoxicity [3]. It possesses a wide index of biological activities including antioxidant [31], anticarcinogenic and anti-inflammatory activities [32]. Previous results clarified the powerful protective impact of TR on AP induced renal toxicity [33].

LA suppressed nitric oxide (NO) and lipid peroxidation. Meanwhile, it reduced GPx and elevated GSH activity in AP-intoxicated renal tissues [34]. The present study clarified that TQ, TR and LA, improved renal injury induced via AP reflected by the decline in blood urea, serum creatinine and uric acid. Moreover, they induced a marked increase in GSH activity with a concomitant decrease in both MDA and NO levels in renal tissue. Previously, Saeedi and his group clarified that AP caused a focal kidney necrosis inflammation, and fatty degeneration as well. Moreover, IL-6, TNF-α and CRP levels were elevated post AP nephrotoxicity [35]. The results of the present study showed an agreement finding with previous published work who reported that treatment with AP increased serum levels of the pro-inflammatory cytokines including VEGF and CRP [35].

TGF-β stimulates the gene expression of the death-associated protein kinase mediator of apoptosis which leads to the activation of TGF-β -dependent apoptosis by binding Smads to mitochondrial pro-apoptotic factors leading to apoptosis [36]. Herein, the protein expression of NFκB, TLR-4, TGF-β1 and Smad-3 was over expressed upon intoxication with AP while P53 level was down regulated.

The most significant impact on the induction of these proteins was demonstrated by TQ, TR and LA administration either alone or in combination with the combination treatment. This is in agreement with Fouad and Jresat, [37] study, they reported that AP induced a significant over expression in inflammatory biomarker nuclear factor-κB and a significant down regulation in p53 level in kidney tissue in rat model reflecting renal toxicity and apoptosis. It was reported that Benzyl alcohol attenuates AP-induced acute liver injury via down regulating Toll-like receptor-4 in mice [38]. Moreover, it has been reported that AP increase NF-κB, which controls the transcription of inflammatory and immune-modulatory biomarkers including TGF- β [39], IL-1, IL -6 and TNF- α [40]. Meanwhile, treatment with the aforementioned antioxidants markedly down regulated inflammatory and apoptotic biomarkers with the combination regimens showing the most significant impact reflecting their potent antioxidant, anti-inflammatory and anti-apoptotic impact.

In harmony, it was previously reported that TQ and TR reversed the methotrexate-induced inflammatory and apoptotic markers including (NFκB, TNF-α, COX-2 and caspase-3) [41], [42]. Moreover, LA was reported for its antioxidant, metal chelating and reducing the oxidized form of other antioxidant agents such as vitamin C and E and glutathione and modulating the signaling transduction of NFκB, TGF-β1, Smad-2 and apoptotic pathways in nephropathy of diabetic patients [43]. In addition, TR acts as a NO scavenger and inhibits COX-2 and NFκB [8]. Finally, NAC is the specific antidote of AP hepatotoxicity and effective in various experimental renal injury models. NAC plays a reno-protective role via its anti-apoptotic pathway [7], [44]. The burden of kidney disorders on the world's health has grown throughout time. Numerous hybrids or antioxidants have been used in conjunction to increase treatment effectiveness while minimizing negative effects. Numerous fields have effectively used these medicinal compounds to achieve and increase people health condition [45].

5. Conclusion

The combination of TQ, TR and LA obtain a promising candidate that able to suppress AP induced nephrotoxicity. Therefore, the combination of the presented elements (TQ, TR and LA) could be an excellent strategy to decease AP-induced renal injury incident. Finally, different pathways mediated the action of these antioxidant factors provide protection mechanisms for nephrotoxicity including antioxidant, anti-inflammatory and anti-apoptotic pathways.

Acknowledgments

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Conflict of Interest

Author declares no conflict of interests. Author read and approved final version of the paper.

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