



# Bardoxolone methyl attenuates acetaminophen-induced acute kidney injury by suppressing oxidative stress, inflammation and apoptosis

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## Abstract

**Aim:** Nuclear factor erythroid 2-related factor 2 (Nrf2) is important in ameliorating several diseases caused by oxidative stress and inflammation, including acute kidney injury (AKI). Bardoxolone methyl (BM) is powerful Nrf2-activating drug. This study evaluated the renoprotective effects of BM against acetaminophen (N-acetyl-para-aminophenol; APAP) induced AKI in rats.

**Materials and Methods:** Forty-two rats were evenly split into 6 groups; control (saline), vehicle (sesame oil), APAP, APAP+N-acetylcysteine (NAC) (160 mg/kg), APAP+BM (5 mg/kg), and APAP+BM (10 mg/kg). Kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels, total oxidant status (TOS) and total antioxidant status (TAS) were analysed in the kidney tissue. Histopathology was performed on glomerular and tubular structures. For apoptosis, caspase-3 was assessed by immunohistochemistry.

**Results:** APAP caused an increase in KIM-1, NGAL, TNF- $\alpha$ , oxidative stress and apoptosis and histopathological changes in the kidney. BM dose-dependently reduced APAP-induced AKI, including renal oxidative stress, histopathology and apoptosis. BM also decreased KIM-1, NGAL and TNF- $\alpha$  in the kidney.

**Conclusion:** BM has demonstrated therapeutic effects against APAP-induced AKI by enhancing the antioxidant system, modulating inflammatory cytokines and inhibiting apoptosis in rat kidney.



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## Introduction

Acute kidney injury (AKI) is a serious clinical condition characterised by a sudden and rapid decline in renal function. Drug-induced kidney injury, one of the causes of AKI, is common in clinical practice. Acetaminophen also known as N-acetyl-para-aminophenol (APAP) or paracetamol is a non-opioid analgesic and antipyretic agent. APAP, which is widely used worldwide, is a reliable drug in therapeutic doses. However, APAP causes dose-dependent hepatotoxicity and nephrotoxicity in both humans and animals [1].

Pharmacological doses of APAP are largely metabolised in the liver by conjugation with sulphate or glucuronic acid. APAP is also metabolized by cytochrome p450 (CYP450) enzymes to N-acetyl-p-benzoquinone imine (NAPQI) [2].

When APAP is taken at very high doses, NAPQI is produced in excess of its ability to bind to glutathione (GSH) and the formation of reactive oxygen and nitrogen species is initiated by this reactive molecule, leading to tissue apoptosis and necrosis, and ultimately dysfunction of organs [3, 4]. Renal NAPQI is mainly formed by the cortical CYP450 enzymes and medullary prostaglandin endoperoxide synthase [5]. Although the mechanism of APAP-induced AKI has not been fully elucidated, NAPQI-induced reactive oxygen species (ROS) have been implicated in renal injury [6, 7].

Bardoxolone methyl (BM) is a synthetic triterpenoid compound derived from oleanolic acid that exhibits potent antioxidant and anti-inflammatory effect by activating Nrf2 and inhibiting nuclear factor- $\kappa$ B (NF- $\kappa$ B) [8]. The renoprotective effect of Nrf2 in the kidneys has also been demonstrated in several experimental studies [9, 10]. In type 2 diabetic patients with chronic kidney disease, BM has been shown to improve renal function [11]. The BEA-

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CON trial validated beneficial effects of BM, but the trial was stopped because of heart failure and a high mortality rate [12]. However, recent studies suggest that BM may be safe in patients who are not at cardiovascular risk [13, 14]. The renoprotective effect of BM has also been shown in experimental studies [15, 16].

In view of the potent antioxidant effect of BM, we hypothesised that BM might have a protective effect on APAP-induced AKI. The aim of this study was to investigate the renoprotective effect of BM against high-dose APAP-induced AKI in rats.

## Materials and Methods

### Animals

42 male Wistar rats (230-290 g) were supplied by the Experimental Animal Breeding Research and Application Centre of Kütahya Health Sciences University (Kütahya, Türkiye). The animals were maintained at a temperature of  $25.0 \pm 1.0$  °C, relative humidity of  $60 \pm 5\%$  and under a 12/12 h light/dark cycle. Rats were given standard chow and water ad libitum. The Kütahya Health Sciences University Local Ethics Committee for Animal Experiments approved the experiment (Decision No: 2019.01.10). All animal procedures were performed in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Animals were randomly assigned to the 6 groups (n=7); (1) control, (2) vehicle (sesame oil), (3) APAP, (4) APAP + NAC, (5) APAP + BM-5 (5 mg/kg), and (6) APAP + BM-10 (10 mg/kg). Randomisation was carried out by writing animal numbers on the cards and then randomly selecting from the numbers. When one-way analysis of variance (ANOVA), was used to show the statistical difference between the 6 groups in terms of the data for the quantitative variables examined, a power analysis was performed with an effect size of 0.40 (large), 4 standard deviations, and a significance level of 0.05. When the number of samples in each group was 7, the minimum desired power value of 0.80 was achieved. A total sample size of 42 was studied, with the number of samples in each group being 7. G\*Power 3.1 program was used for the calculation.

### Acetaminophen nephrotoxicity protocol and treatments

All rats were fasted for 24 h prior to the administration of APAP, but were allowed to drink water ad libitum. APAP (Sandoz, Türkiye) was given at a single dose of 2g/kg in normal saline suspension by oral gavage. The plasma half-life ( $t_{1/2}$ ) of APAP following a therapeutic dose is approximately 2 hours, but  $t_{1/2}$  is prolonged after overdose due to liver damage and nephrotoxicity [17]. For this reason and also to avoid affecting the absorption of APAP, the animals were given sufficient water and food four hours after the administration of APAP [18]. The chosen dose of APAP was based on the following previous studies [19].

Group 1 rats were given a single dose of 0.5 ml saline and served as the control. The rats in group 2 were given a single dose of 0.5 ml of sesame oil (BM vehicle). Group 3 rats were treated with APAP plus 0.5 ml sesame oil. Group 4 rats received APAP plus 0.5 ml of 160 mg/kg NAC (Bilim İlaç, Türkiye) [20]. Groups 5 and 6 rats received APAP +

BM (Sigma Chemicals, USA) at doses of 5 mg/kg and 10 mg/kg, respectively. BM was dissolved in 0.5 ml of sesame oil. The BM dose was determined according to previous studies [21]. Saline, NAC and BM were administered a single dose by oral gavage 1 h before APAP administration.

### Preparation of tissue homogenate

After 24h of APAP administration, all rats were anesthetized with ketamine (70 mg/kg ip, Ketalar, Pfizer) and xylazine (10 mg/kg ip, Alfazyne, Atafen). For histopathological and biochemical analysis, the rats were euthanised by collecting blood from the heart and both kidneys were removed. Left kidney tissue samples including cortex, medulla and glomeruli were fixed in 4% buffered formaldehyde. The right kidney was homogenized in phosphate buffer (50 mmol/L, pH 7.40) (SpeedMill PLUS, Analytik Jena, Germany) for biochemical analysis. The homogenates were centrifuged and the supernatants were stored at  $-80^{\circ}\text{C}$  [22]. A nanodrop spectrophotometer was used to measure protein concentrations in the homogenates. (MaestroNano Spectrophotometer, Taiwan).

### Determination of renal dysfunction and renal cytokine

Kidney injury molecule-1 (KIM-1) [22], neutrophil gelatinase-associated lipocalin (NGAL) [23] and Tumor Necrotizing Factor- $\alpha$  (TNF- $\alpha$ ) [24] levels in the kidney the homogenates were determined using rat ELISA kits (Elabscience Biotechnology, PRC).

### Determination of renal oxidative stress

Renal total antioxidant status (TAS) and total oxidant status (TOS) were analysed by spectrophotometry (Rel Assay Diagnostic Kit, Türkiye) [25]. The oxidative stress index (OSI) was calculated according to the following formula:  $\text{OSI (arbitrary unit)} = [(\text{TOS}, \mu\text{mol H}_2\text{O}_2 \text{ equivalent/l}) / (\text{TAS}, \mu\text{mol Trolox equivalent/l}) \times 100]$ .

### Histopathology of the kidney

Buffered 4% neutral formalin was used to fix the kidney. Paraffin-embedded specimens were sectioned on slides, stained with hematoxylin and eosin (H&E) and examined by light microscope to assess tubular and glomerular changes. Tubulointerstitial fibrosis was also evaluated using Masson's trichrome [26].

Kidney injury in the cortex and medulla was determined by examination ten randomly selected sections. Tubular dilatation and injury, necrosis, atrophy, interstitial inflammatory cell infiltration, exfoliation of tubular epithelium, vacuolization, cast formation, edema, and hemorrhage were evaluated. The injury scores were as follows [27]. 0: normal, 1: mild, less than 25% involvement of injury in the cortex, 2: moderate, 25-50% involvement of injury in the cortex, 3: severe, 50-75% involvement of injury in the cortex, 4: widespread, 75-100% involvement of injury in the cortex.

### Determination of renal apoptosis

Caspase-3 was stained immunohistochemically to evaluate apoptosis in the kidneys. Sections were incubated with

anti-caspase-3 antibody (Abcam, USA) and viewed under the light microscopy. Apoptotic nuclei were counted in areas stained positive for caspase-3 in the glomeruli, tubules and interstitial cells. Results were expressed as the percentage of immunopositive cells compared to the total number of cells [28].

#### Statistical analysis

Results are presented as mean  $\pm$  SEM. The IBM SPSS 20 package (SPSS, Chicago, IL) was used for statistical analysis. GraphPad Prism 10 (GraphPad Software, USA) was used for graphical presentation. The Shapiro-Wilk test was used to assess the normality of the data distribution. One-way analysis of variance (ANOVA), a parametric hypothesis test, was used to examine differences between groups (post-hoc Tukey's test). The arithmetic mean, standard error and associated confidence interval of the normally distributed data are presented in the tables in the results section. Data for the tubular injury scores were compared using the chi-square test, and the results are presented in a table. All analyses were assessed at 95% level of confidence ( $p < 0.05$ ).

## Results

#### Survival

There were no deaths observed in any of the groups during the study.

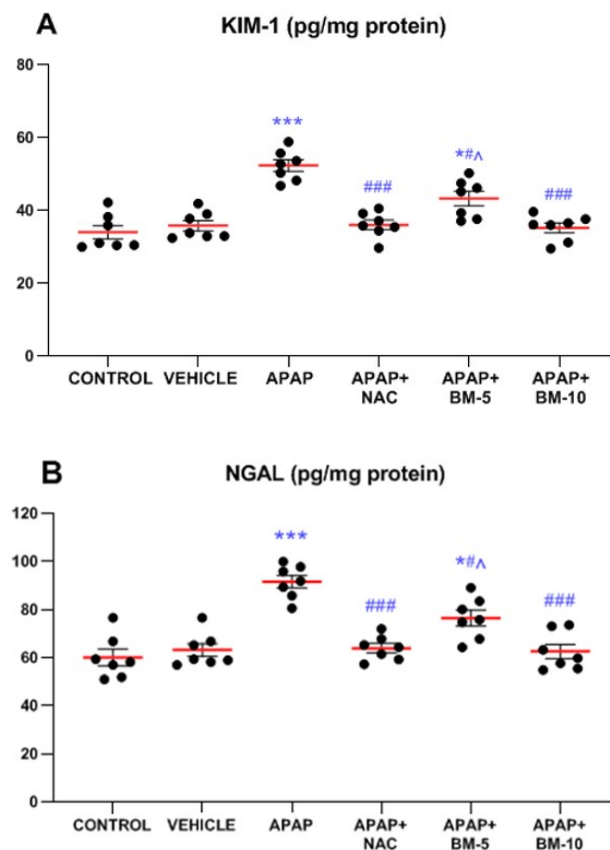
#### Effects of BM on renal KIM-1 and NGAL levels

Renal injury was assessed by measuring KIM-1 and NGAL levels. KIM-1 was significantly elevated in the APAP group ( $52.31 \pm 1.60$  pg/mg protein,  $p < 0.001$ ) compared to the vehicle ( $34.02 \pm 1.82$  pg/mg protein), indicating the induction of nephrotoxicity (Figure 1A, Table 1). Treatment with the NAC showed significant ( $36.01 \pm 1.33$  pg/mg protein,  $p < 0.001$ ) decrease in levels of renal KIM-1 levels compared to the APAP. BM reduced the KIM-1 concentration at a 5 mg dose compared to the APAP group ( $p < 0.05$ ). The greatest effect was seen in at a 10 mg/kg BM dose compared to the BM-5 group ( $p < 0.05$ ). At a dose of 10 mg/kg BM was also found to be as effective as NAC.

There was a significant increase in renal NGAL levels in the APAP group ( $91.56 \pm 2.62$  pg/mg protein,  $p < 0.001$ ) compared with the vehicle ( $63.12 \pm 2.64$  pg/mg protein). BM was found to be more effective in reducing NGAL at a dose of 10 mg/kg than at a dose of 5 mg/kg ( $p < 0.05$ ) (Figure 1B, Table 1). BM at a dose of 10 mg/kg was also found to reduce NGAL levels similarly to NAC.

#### Effects of BM on renal TNF- $\alpha$ levels

TNF- $\alpha$  was measured in the renal homogenates to assess the inflammatory status and renal injury caused by APAP. TNF- $\alpha$  levels in the APAP group ( $113.83 \pm 3.86$  pg/mg protein,  $p < 0.001$ ) were significantly higher than in the vehicle group ( $71.20 \pm 5.21$  pg/mg protein). NAC treatment showed a significant ( $71.96 \pm 3.43$  pg/mg protein,  $p < 0.001$ ) decrease in renal TNF- $\alpha$  levels compared to APAP. BM treatment decreased renal TNF- $\alpha$  levels in



**Figure 1.** Effects of BM treatment on KIM-1 (A) and NGAL (B) levels in rat kidney homogenates. Each column represents the mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  vs. vehicle group, #  $p < 0.05$ , ###  $p < 0.001$  vs. APAP group, ^  $p < 0.05$  vs. BM-10 group ANOVA ( $n=7$ ). APAP acetaminophen, NAC N-acetylcysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg, KIM-1 Kidney Injury Molecule-1, NGAL Neutrophil Gelatinase-associated Lipocalin.

a dose-dependent manner. BM was found to be more effective at a dose of 10 mg/kg than at a dose of 5 mg/kg (Figure 2, Table 1).

#### Effects of BM on renal TAS and TOS levels

To evaluate oxidant and antioxidant status due to APAP toxicity, TAS and TOS were measured in kidney homogenates. In the APAP group, TAS levels decreased ( $p < 0.01$ ) and TOS levels increased ( $p < 0.001$ ). OSI was increased in the APAP group compared to the vehicle group ( $p < 0.001$ ). This result indicated that APAP increased renal oxidative stress. BM treatment dose-dependently increased TAS levels and reduced TOS and OSI levels in the kidney compared to the APAP group. BM was also as effective as NAC at a dose of 10 mg/kg (Table 2).

#### Effects of BM on renal histopathology findings

Histopathological examination of kidneys in the control and vehicle groups showed normal appearance of glomeruli

**Table 1.** Effects of APAP-induced acute kidney injury and BM treatment on renal KIM-1, NGAL (B) and TNF- $\alpha$  levels.

Groups	KIM-1 (pg/mg protein) (95% confidence interval for mean)	NGAL (pg/mg protein) (95% confidence interval for mean)	TNF- $\alpha$ (pg/mg protein) (95% confidence interval for mean)
Control (saline)	34.02 $\pm$ 1.82 (29.57-38.46)	60.07 $\pm$ 3.38 (51.79-68.35)	68.26 $\pm$ 3.63 (59.37-77.15)
Vehicle (sesame oil)	35.78 $\pm$ 1.40 (32.34-39.23)	63.12 $\pm$ 2.64 (56.67-69.57)	71.20 $\pm$ 5.21 (57.43-84.97)
APAP	52.31 $\pm$ 1.60 (48.40-56.22) ***	91.56 $\pm$ 2.62 (85.16- 97.96) ***	113.83 $\pm$ 3.86 (103.62-124.04) ***
APAP+NAC	36.01 $\pm$ 1.33 (32.75-39.26) ###	63.89 $\pm$ 1.92 (59.21-68.58) ###	71.96 $\pm$ 3.43 (62.91-81.01) ###
APAP+BM-5	43.28 $\pm$ 1.98 (38.45-48.11) *#^	76.44 $\pm$ 3.27 (68.44-84.45) *#^	95.01 $\pm$ 2.06 (89.57-100.44) ***^
APAP+BM-10	35.21 $\pm$ 1.36 (31.89-38.53) ###	62.50 $\pm$ 2.98 (55.21-69.80) ###	77.37 $\pm$ 4.22 (66.22-88.52) ###

Data are presented as mean  $\pm$  SEM and 95% confidence interval for mean. \* p< 0.05, \*\* p< 0.01, \*\*\* p< 0.001 vs. vehicle group, # p< 0.05, ### p< 0.001 vs. APAP group, ^ p< 0.05 vs. BM-10 group ANOVA (n=7). APAP acetaminophen, NAC N-acetylcysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg. KIM-1 Kidney Injury Molecule-1, NGAL Neutrophil Gelatinase-associated Lipocalin, TNF- $\alpha$  Tumor Necrosis Factor- $\alpha$ .

**Table 2.** Effects of APAP-induced acute kidney injury and BM treatment on renal TAS, TOS and OSI levels in rats.

Groups	TAS (TroloxEq/mg protein) (95% confidence interval for mean)	TOS (H <sub>2</sub> O <sub>2</sub> Eq/mg protein) (95% confidence interval for mean)	OSI (arbitrary unit) (95% confidence interval for mean)
Control (saline)	2.25 $\pm$ 0.17 (1.76-2.65)	8.36 $\pm$ 0.12 (7.98-8.65)	0.38 $\pm$ 0.02 (0.33-0.45)
Vehicle (sesame oil)	2.19 $\pm$ 0.19 (1.66-2.60)	7.98 $\pm$ 0.19 (7.51-8.46)	0.39 $\pm$ 0.04 (0.30-0.49)
APAP	1.26 $\pm$ 0.09 (1.02-1.50) **	15.39 $\pm$ 0.12 (15.07-15.71) ***	1.27 $\pm$ 0.11 (1.00-1.54) ***
APAP+NAC	1.97 $\pm$ 0.14 (1.64-2.30) #	8.75 $\pm$ 0.18 (8.30-9.18) ##	0.46 $\pm$ 0.04 (0.36-0.55) ##
APAP+BM-5	1.69 $\pm$ 0.17 (1.17-2.06) *	9.10 $\pm$ 0.20 (8.60-9.59) *	0.58 $\pm$ 0.07 (0.45-0.79) *
APAP+BM-10	1.91 $\pm$ 0.11 (1.62-2.20) #	8.82 $\pm$ 0.19 (8.37-9.33) ##	0.47 $\pm$ 0.02 (0.42-0.53) ##

Data are presented as mean  $\pm$  SEM and 95% confidence interval for mean. \* p< 0.05, \*\* p<0.01, \*\*\* p< 0.001 vs. the vehicle group # p< 0.05, ## p< 0.01 vs. the APAP group ANOVA (n=7). APAP acetaminophen, NAC N-acetyl cysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg. Total antioxidant status TAS; Total oxidant status, OSI Oxidative stress index.

**Table 3.** BM treatment on tubular injury score.

Groups	Tubular injury n (%)					Total
	0	1	2	3	4	
Control (saline)	5 (71.4)	2 (28.6)	0 (0)	0 (0)	0 (0)	7(100)
Vehicle (sesame oil)	5 (71.4)	2 (28.6)	0 (0)	0 (0)	0 (0)	7(100)
APAP	0 (0)	0 (0)	0 (0)	2 (28.6)	5 (71.4)	7(100)
APAP+NAC	2 (28.6)	5 (71.4)	0 (0)	0 (0)	0 (0)	7(100)
APAP+BM-5	1 (14.3)	6 (85.7)	0 (0)	0 (0)	0 (0)	7(100)
APAP+BM-10	4 (57.1)	3 (42.9)	0 (0)	0 (0)	0 (0)	7(100)
Total	17 (40.5)	18 (42.9)	0 (0)	2 (4.8)	5 (11.9)	42(100)

$$X^2=51.059; p<0.001$$

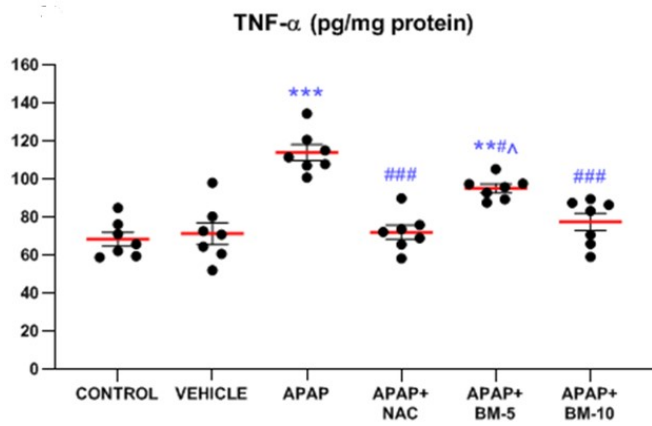
Chi-square test, p<0.001 0: Normal, 1: Mild, 2: Moderate, 3: Severe, 4: Widespread, injury in the cortex. APAP acetaminophen, NAC N-acetyl cysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg.

and tubules (Figure 3A). Significant histopathological deterioration was observed in the APAP group, including exfoliation of tubular epithelium, vacuolization, multiple haemorrhages in the intertubular space, and edema in the cortex and medulla. Tubular degeneration and necrosis were also observed in the medulla (Figure 3B). Deterioration in glomeruli and tubules was improved by NAC treatment. (Figure 3C). BM treatment showed a dose-dependent protective effect on the kidney, as evidenced by the reduced tubular and glomerular damage, with a better protective effect observed in the 10 mg/kg BM group (Figure 3E). In the APAP group, the tubular injury score was significantly higher than in the vehicle group (Table 3). The tubular injury score was also significantly decreased

dose-dependently in the BM treatment groups (Table 3). Masson's trichrome staining showed normal histology in the control group (Figure 4A). Reduced tubulointerstitial fibrosis was observed in the treatment groups, particularly in the 10 mg/kg BM group (Figure 4E).

#### Effects of BM on renal apoptosis

Kidneys from rats in the APAP-treated group showed extensive caspase-3 positive staining compared to the vehicle group (p<0.001). In the NAC-treated group, caspase-3 immunopositive cells were decreased. BM treatment dose-dependently decreased the percentage of caspase-3 immunopositive cells (Figure 4D, 4E and 5, Table 4).



**Figure 2.** Effects of BM treatment on TNF- $\alpha$  levels in rat kidney homogenates. Each column represents the mean  $\pm$  SEM. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. vehicle group, #  $p < 0.05$ , ###  $p < 0.001$  vs. APAP group, ^  $p < 0.05$  vs. BM-10 group ANOVA ( $n=7$ ). APAP acetaminophen, NAC N-acetylcysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg, TNF- $\alpha$  Tumor Necrosis Factor- $\alpha$ .

**Table 4.** BM treatment on caspase-3 immunopositive cells (%).

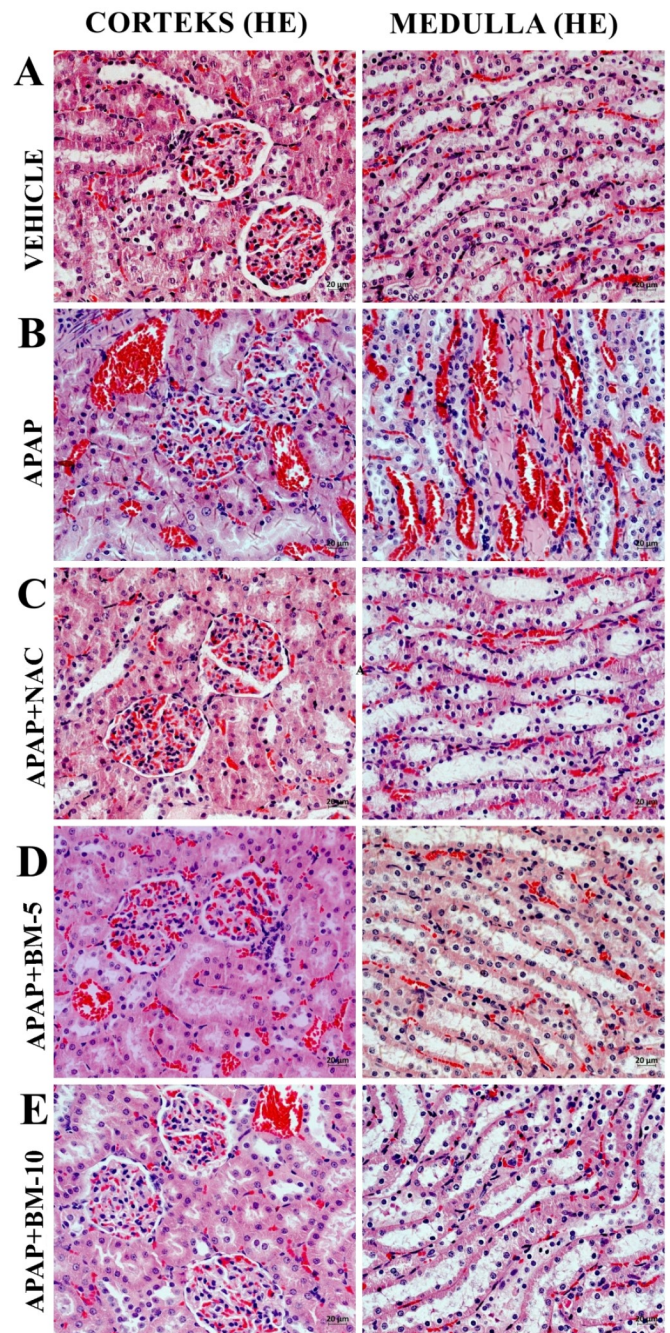
Groups	Caspase-3 immunopositive cell (%) (95% confidence interval for mean)
Control (saline)	8.45 $\pm$ 1.11 (5.71-11.19)
Vehicle (sesame oil)	7.64 $\pm$ 1.13 (4.88-10.40)
APAP	73.54 $\pm$ 2.45 (68.69-78.38) ***
APAP+NAC	37.91 $\pm$ 2.49 (31.82-43.99) **###
APAP+BM-5	38.86 $\pm$ 1.64 (34.82-42.89) **###
APAP+BM-10	10.91 $\pm$ 1.06 (8.30-13.51) ###

Data are presented as mean  $\pm$  SEM and 95% confidence interval for mean. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs. vehicle group, ##  $p < 0.01$  and ###  $p < 0.001$  vs. APAP group, ANOVA ( $n=7$ ). APAP acetaminophen, NAC N-acetylcysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg.

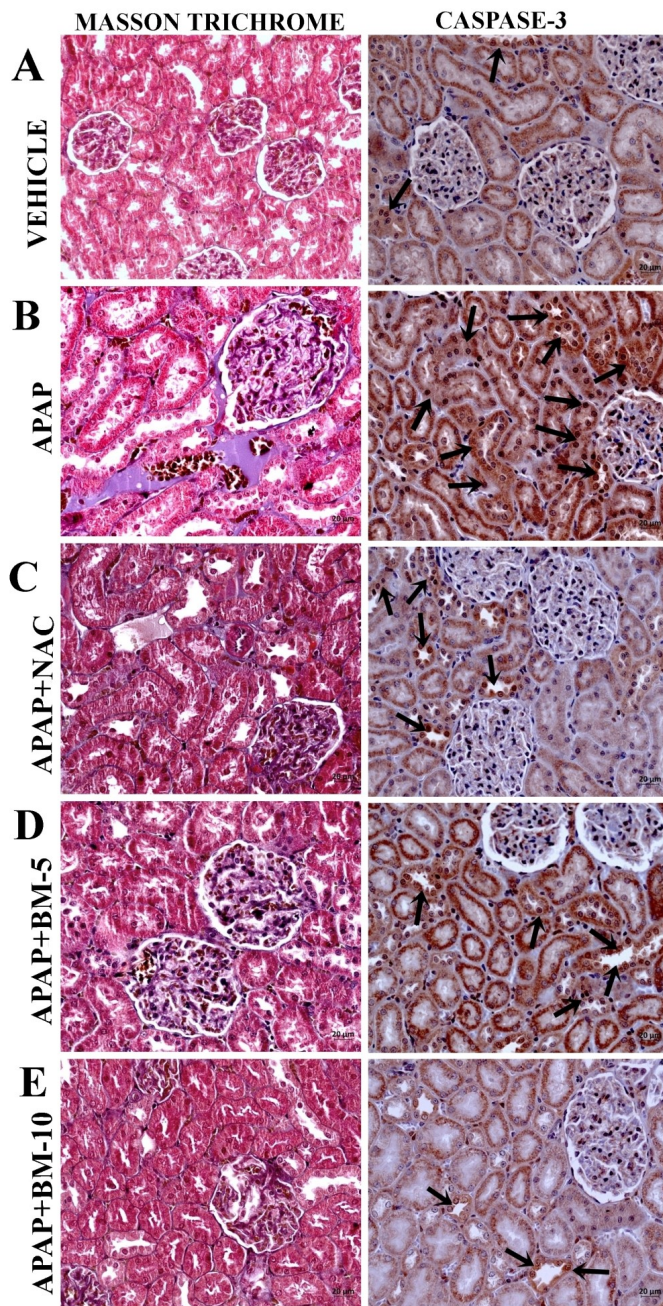
## Discussion

In this study, we found that BM had a renoprotective effect in APAP-induced AKI. We can say that BM dose-dependently reduces APAP-induced AKI based on the following evidence; (1) decreased KIM-1 and NGAL levels, (2) decreased TNF- $\alpha$  production, (3) reduced oxidative stress, (4) improved renal histological changes and (5) reduced apoptosis.

It is well known that APAP is toxic to the liver and kidney at high doses. KIM-1 and NGAL which are more specific biomarkers of renal injury, were evaluated in our study. KIM-1, a type I transmembrane glycoprotein, is a specific and sensitive biomarker of acute proximal tubular injury [29]. NGAL, or lipocalin 2, is another biomarker used to detect kidney damage, and its expression increases during kidney injury [29]. In our study, we found that APAP significantly elevated kidney levels of KIM-1 and NGAL. Hua et al. reported that APAP increased mRNA levels of NGAL and KIM-1 in the kidney of mice [30]. Shin

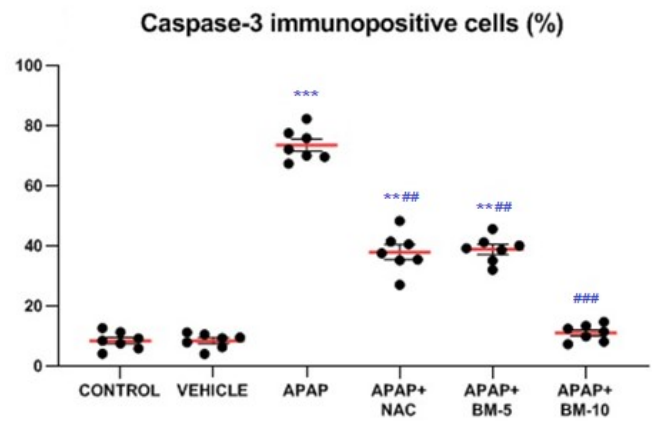


**Figure 3.** Photomicrographs of rat renal cortex (left panel) and medulla (right panel) (H&E, 100 $\times$ ) from: (A) vehicle (control) group showing normal renal architecture; (B) APAP group showing significant interstitial inflammatory cell infiltration, tubular degeneration, tubular dilatation, necrosis, exfoliation of tubular epithelium, vacuolization, multiple hemorrhages and casts formation; (C) APAP+NAC group showing well preserved architecture than APAP group (D) APAP+BM-5 group showing improvement in the histological picture (E) APAP+BM-10 group showing marked improvement in the histological picture which is comparable to that of the APAP group. APAP acetaminophen, NAC N-acetylcysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg.



**Figure 4.** Masson trichrome (left panel) and immunohistochemically staining for caspase-3 (right panel, brown nuclear staining) in rat kidney (100 $\times$ ). Masson trichrome staining revealed normal histological findings in the control group (A). Tubulointerstitial fibrosis was reduced in the 10 mg/kg BM group (E). In the APAP group, the rat kidney tissues were strongly positive for caspase-3 expression (B); NAC treatment was significantly reduced the expression of caspase-3 (C); BM treatment reduced the expression of caspase-3 in a dose-dependent manner (D and E). APAP acetaminophen, NAC N-acetylcysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg.

et al. also found that APAP increased the expression of KIM-1 and NGAL in the kidney of rat [31]. The results of our experiment are supported by these studies. The metabolite NAPQI has been reported to play a key role



**Figure 5.** BM treatment on caspase-3 immunopositive cells (%). Data are given as mean  $\pm$  SEM. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs. vehicle group, ##  $p < 0.01$  and ###  $p < 0.001$  vs. APAP group, ANOVA ( $n=7$ ). APAP acetaminophen, NAC N-acetylcysteine, BM-5 bardoxolone methyl 5 mg/kg, BM-10 bardoxolone methyl 10 mg/kg.

in APAP-induced nephrotoxicity. NAPQI leads to renal tubular cell death by causing arylation of proteins [32]. On the other hand, accumulation of NAPQI in the kidney leads to in mitochondrial dysfunction, GSH depletion, lipid peroxidation and increased ROS and cytokine production. As a result, apoptosis is initiated in the kidney cells and necrosis develops [33]. The histopathological findings in our study support nephrotoxic effect of APAP. Exfoliation of the tubular epithelium, vacuolization, multiple hemorrhages in the intertubular space, and edema in the medulla and cortex were observed in the APAP group in our study. Tubular degeneration and necrosis were also seen in the medulla. APAP also induced apoptosis as evidenced by a significant increase in caspase-3 immunoreactive cells. These results are consistent with previous studies [33, 34].

NAPQI-induced renal toxicity results in increased production of ROS, decreased levels of antioxidant enzymes, inflammation and upregulation of cytokines [35]. In our study, we found that APAP reduced TAS levels and elevated TOS levels in rat kidneys. These results indicated that APAP increases ROS formation and triggers inflammatory cytokine release in the kidney. Karthivashan et al. reported that APAP increased the levels of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the mouse kidney [32]. The increased production of pro-inflammatory cytokines such as TNF- $\alpha$  in kidney tissue indicates that inflammation is also involved in the pathogenesis of APAP-induced nephrotoxicity.

Nrf2 is the principal redox-sensitive transcription factor that is essential for the maintenance of renal function. Under stress conditions, Nrf2 translocates into the nucleus to increase the expression of the antioxidant response elements (AREs). AREs encode antioxidant defence enzymes, and attenuate cellular oxidative stress [36]. BM is a synthetic triterpenoid compound derived from oleanolic acid that is a potent inducer of the Keap1-Nrf2 pathway. Experimental studies have shown that BM and its analogs have beneficial effects in animal models of renal disease,

including amelioration of ischemic acute kidney injury in mice [37], protection against aldosterone and salt-induced kidney injury in rats [38], amelioration of angiotensin II-induced decreased glomerular filtration rate in rats [39], improvement of Nrf2 and NF- $\kappa$ B expression and kidney function in rats with 5/6 nephrectomy model [40], and prevention of the adverse effects of adriamycin-induced podocyte injury [41]. Our results are in alignment with these studies as we demonstrate renoprotective effect of BM in APAP-induced AKI. Our study showed that BM dose-dependently reduced APAP-induced increases of renal KIM-1 and NGAL. In addition, our histopathological examinations also showed that BM improved APAP-induced AKI.

The antioxidant and/or free radical scavenging activities of BM may mediate the renoprotective mechanism of BM against APAP-induced AKI. Considering the excessive ROS production associated with NAPQI, it can be suggested that BM acts as a Nrf2 activator with antioxidant activity. We found that BM dose-dependently elevated renal TAS and reduced TOS concentration in the APAP-treated rats. Wu et al. reported that BM ameliorated aristolochic acid-induced AKI via the Nrf2, and the antioxidant activity of BM played an important role in the renoprotective effect [42]. Aleksunes et al. also found that CDDO-IM, a compound similar to BM, reduced cisplatin-induced AKI by increasing the expression of Nrf2 [43]. On the other hand, the beneficial effects of BM on the expression of inflammatory cytokines may play a role in its renoprotective effect in APAP-induced AKI. In our study, BM significantly reduced renal TNF- $\alpha$  levels in APAP-treated rats. BM has been reported to be as an immunomodulator and inhibitor of NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 [37, 44].

Caspase-3 is a key protein required for apoptosis or programmed cell death. In this study, we observed that BM significantly downregulated the expression of caspase-3 in rats with APAP-induced AKI. According to these results, the inhibition of apoptosis may be an essential factor in the renoprotective effect of BM. BM has been reported to prevent apoptosis not only in acute or chronic kidney injury, but also in cardiomyocytes after myocardial ischaemia/reperfusion [45] and in chondrocytes after experimental osteoarthritis in rats [46].

NAC is known around the world as the antidote for APAP intoxication. NAC increases the synthesis and storage of GSH in the liver. Several studies have shown that NAC is protective against APAP-induced kidney injury [47, 48]. In our study, we observed that NAC provided both biochemical and histological improvement in APAP-induced AKI and BM showed a renoprotective effect similar to that of NAC.

## Conclusion

In conclusion, our study showed that BM has dose-dependent therapeutic effects against APAP-induced AKI by enhancing the antioxidant system, inhibiting inflammatory cytokine TNF- $\alpha$  and inhibiting apoptosis in rat kidney. BM can be used in acute and chronic kidney diseases, such as drug-induced AKI and diabetic kidney disease.

## Declaration of conflict of interest

The authors declare no conflict of interest.

## Consent to participate

Not applicable.

## Ethical approval

Animal experiments were carried out at Kütahya Health Sciences University (KSBU) Experimental Animal Breeding Research and Application Centre with the approval of the KSBU Animal Experiments Local Ethics Committee (Ethics Committee Decision No: 2019.01.10, Decision date: 15.02.2019). All animal experiments were carried out in accordance with the "Guidelines for the Care and Use of Laboratory Animals" issued by the National Institutes of Health (NIH).

## Author contribution

All authors participated in the conception and design of the study. YT designed the animal experiments, carried out the experiments on the animals, performed the ELISA measurements, analyzed the data, wrote the manuscript. EK carried out the experiments on the animals, analyzed the data, wrote the manuscript. FYÖ carried out the experiments on the animals, performed the ELISA measurement and analyzed the data. OÖ performed histologic and immunohistochemical experiments, analysis, and interpretation. The final version was read and approved for publication by all authors.

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