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# The Role of RAAS Inhibition by Aliskiren on Paracetamol-Induced Hepatotoxicity Model in Rats

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## ABSTRACT

Paracetamol is one of the most popular and widely used analgesic and antipyretic agents, but an overdose can cause hepatotoxicity and lead to acute liver failure. Aliskiren directly inhibits renin which downregulates the renin–angiotensin–aldosterone system (RAAS). Recent findings suggest that RAAS system takes part in the pathogenesis of liver fibrosis. We aimed to reveal the relationship between hepatotoxicity and the RAAS by examining paracetamol induced hepatotoxicity. Rats were separated into five groups as follows: control, 100 mg/kg aliskiren (p.o.), 2 g/kg paracetamol (per os (p.o.)), 2 g/kg paracetamol + 50mg/kg aliskiren (p.o.), and 2 g/kg paracetamol + 100 mg/kg aliskiren(p.o.). Samples were analyzed at the biochemical, molecular, and histopathological levels. Paracetamol toxicity increased alanine aminotransferases (ALT), aspartate aminotransferases (AST), renin, and angiotensin II levels in the serum samples. In addition, the SOD activity and glutathione (GSH) levels decreased while Lipid Peroxidation (MDA) levels increased in the livers of the rats treated with paracetamol. Paracetamol toxicity caused a significant increase in TNF-α and TGF-β. Both aliskiren doses showed an improvement in ALT, AST, oxidative parameters, angiotensin II, and inflammatory cytokines. Only renin levels increased in aliskiren treatment groups due to its pharmacological effect. A histopathological examination of the liver showed that aliskiren administration ameliorated the paracetamol-induced liver damage. In immunohistochemical staining, the expression of TNF-α in the cytoplasm of the hepatocytes was increased in the paracetamol group but not in other treatment groups when compared to the control group. In light of these observations, we suggest that the therapeutic administration of aliskiren prevented oxidative stress and cytokine changes and also protected liver tissues during paracetamol toxicity by inhibiting the RAAS. J. Cell. Biochem. 117: 638–646, 2016. 

**KEY WORDS:** ALISKIREN; HEPATOTOXICITY; PARACETAMOL; RAT; TGF-β

Paracetamol is the most widely used drug and the most popular "over-the-counter" non-narcotic analgesic agent for the treatment of pain and fever that is safe when used in therapeutic doses. However, hepatotoxicity caused by overdose (usually doses greater than 10 g) is a worldwide problem [Gyamlani and Parikh, 2002]. Paracetamol toxicity is responsible for the vast majority of the world's acute liver failure [Larson et al., 2005]. It is known that using paracetamol at high doses or for suicidal purposes leads to hepatic and renal damage in humans, as well as in experimental animal models. Paracetamol is mainly metabolized in the liver, via conjugation with glucuronic acid and sulphate, into two main nontoxic metabolites which are excreted via the urine. However, a small amount of paracetamol (less than 5%) is metabolized by the cytochrome P450 (CYP) enzyme system (mainly CYP2E1) forming a highly reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI), which causes toxicity via an oxidative pathway [Bessems and Vermeulen, 2001]. NAPQI is a highly reactive electrophilic molecule and leads to damage by binding covalently to intracellular proteins. At therapeutic doses, it reacts with reduced glutathione (GSH) to form a nontoxic conjugate that will be excreted [Jaeschke et al., 2012]. However, taking paracetamol at high doses leads to saturation of the conjugation pathway and exhaustion of GSH; this

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results in an increase in the formation of toxic reactive metabolites which in turn cause mitochondrial dysfunction and oxidative stress [Hinson et al., 2010]. GSH is one of the most important cellular defense molecules against reactive toxic compounds and oxidative stress [Prescott, 1983]. Reduced GSH levels leads to the suppression of mitochondrial respiration, adenosine triphosphate (ATP) consumption, and mitochondrial oxidative stress. Consequently, cellular oncotic necrosis occurs in hepatocytes and sinusoidal endothelial cells [Mari et al., 2009].

The renin–angiotensin–aldosterone (RAAS) has been known to regulate systemic circulatory homoeostasis, and a growing body of evidence indicates that it takes part in the pathogenesis of liver fibrosis [Bataller et al., 2003, 2005; Friedman, 2008]. It is known that Angiotensin II (Ang II), the most important mediator of the RAAS, is an important molecule in liver fibrosis pathogenesis [Bataller and Brenner, 2005; Lubel et al., 2008]. Hepatic stellate cells (HSCs) are the cellular cites of Ang II′s main profibrotic action in the liver [Bataller et al., 2000]. In the process of chronic liver injury, increased Ang II promotes hepatic inflammation and fibrosis. Additionally, Ang II releases free radical precursor enzymes, such as nicotinamide adenine dinucleotide phosphate oxidase and xanthine oxidase, into vascular structures and leads to increased free radicals in the tissue [Mollnau et al., 2002; Landmesser et al., 2007]. Ang II increases hepatic oxidative stress [Bataller et al., 2003, 2005].

Aliskiren is a drug used in the treatment of hypertension; it directly inhibits renin, which is the first-rate limiting enzyme in the synthesis of Ang II and which produces Ang I from angiotensinogen. Inhibition of renin activity downregulates the RAAS [Gradman and Kad, 2008] and may be beneficial by decreasing oxidative stress and proinflammatory cytokine production in paracetamol induced hepatotoxicity. There are many studies showing that aliskiren decreases oxidative stress by inhibiting Ang II formation [Rashikh et al., 2012; Wang et al., 2013]. Aliskiren is found to have beneficial effects on carbon tetrachloride-induced liver damage [Lee et al., 2012].

Aliskiren has a positive effect on inflammatory cytokines [Aihara et al., 2013; Ziypak et al., 2015] and oxidant/antioxidant balance [Ziypak et al., 2015]; as a result, it is thought to be used in the treatment of many diseases experimentally. Aliskiren may lower the production of Ang II during the process of chronic liver injury and concomitantly ameliorate inflammation and fibrosis. In this study, we aimed to reveal the relationship between hepatotoxicity and the RAAS by examining paracetamol induced hepatotoxicity in rats on a biochemical, molecular and h istopathological level. This was done by applying different doses of aliskiren to inhibit the RAAS.

## MATERIALS AND METHOD

### ANIMALS

In this study, we used 30 male albino Wistar rats, obtained from the Atatürk University Medical Experimental Research Center (ATADEM). The animals weighed between 210 and 220 g and were fed under normal temperature conditions (22°C) in separate groups before the experiment. The animal care and experimental protocols were approved by the Experimental Animal Ethics Committee, Atatürk University, Erzurum, Turkey (31.05.2013/6).

### CHEMICALS

Paracetamol was purchased from Doğa Ilaç Hammaddeleri Tic. Ltd Sti. (Istanbul, Turkey). We obtained thiopental sodium from IE Ulagay A.S. (Istanbul, Turkey) and aliskiren (Rasilez, 300 mg tb) from the Novartis Drug Company.

### TREATMENT

Animals fasted overnight and were divided into five equal groups (n = 6). Experiment groups and details are shown below. Aliskiren was administered one hour after acetaminophen in all treatment groups that received both drugs. After administration of the drugs, the rats were allowed food for the following 24 h until they were sacrificed.

Group 1: Control

Group 2: 100 mg/kg aliskiren, (2 ml) (p.o.)

Group 3: 2 g/kg paracetamol (suspended in 1% CMC in 1X PBS) 2 ml per rat, (p.o.)

Group 4: 2 g/kg paracetamol (suspended in 1% CMC in 1X PBS) 2 ml per rat, (p.o.) + 50 mg/kg aliskiren, (2ml) (p.o.)

Group 5: 2 g/kg paracetamol (suspended in 1% CMC in 1X PBS) 2 ml per rat, (p.o.) + 100 mg/kg aliskiren, (2 ml) (p.o.)

Twenty-four hours after the induction of paracetamol toxicity, the rats were anesthetized using a 50 mg/kg lethal dose of thiopental. Blood samples were collected into bottles using a heart puncture. The liver was removed immediately after sacrifice.

### BIOCHEMICAL INVESTIGATIONS

**Serum ALT and AST measurement.** In separated serum samples, alanine aminotransferases (ALT) and aspartate aminotransferases (AST) from each serum sample were measured, in duplicate, with ALT (Reference number: AL021) and AST (Reference number: AS071) kits obtained from Ben S.r.l. (Milano, ITALY) according to the manufacturer′s instructions with an auto analyzer (ChemWell 2910 - ELISA & Biochemistry analyzer, USA).

**Serum renin and angiotensin II determination.** Commercially available enzyme-linked immunosorbent assay kits were used to determine the serum concentrations of renin (No. 201-11-0549) and angiotensin II (No. 201-11-0656, SunRed, Shanghai, People′s Republic of China). All analyses were performed in accordance with the manufacturer′s instructions.

**Biochemical investigation of liver tissues (SOD, GSH, MDA, GR).** After the surgical procedures, approximately 100 mg of ground liver tissue was homogenized in 1 ml of phosphate-buffered saline (PBS) homogenate buffer in an eppendorf tube using the TissueLyser II and then centrifuged. SOD [Sun et al., 1988], GSH [Sedlak and Lindsay, 1968] and MDA [Ohkawa et al., 1979] levels from each sample′s supernatants and standards were measured at room temperature, in duplicate, according to the modified methods of the ELISA reader as previously described [Akpinar et al., 2014]. GR activity was determined spectrophotometrically by measuring the rate of NADPH oxidation at 340 nm [Carlberg and Mannervik, 1985]. GR and SOD results are expressed as U/mg protein; GSH and MDA results are expressed as nmol/mg protein.

### MOLECULAR INVESTIGATIONS

**Total RNA extraction and cDNA synthesis.** Tissues were stabilized in RNA Stabilization Reagent (RNAlater, Qiagen) and then disrupted using the TissueLyser II (1 × 5 min for liver; Qiagen). Total RNA was purified using RNeasy Mini Kit Qiagen according to the manufacturer's instructions in Qiaqube (Qiagen). The RNA samples were reverse-transcribed into complementary DNA by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). A total of 10 μl RNA was treated with 2 μl 10 X RT Buffer, 0.8 μl 25 X dNTPs mix, 2 μl 10X RT Random Primers, 1 μl MultiScribe Reverse Transcriptase, and 4.2 μl DEPC-$H_2O$. Reverse transcription was carried out at 25°C for 10 min, followed by 37°C for120 min, and finally, 85°C for 5 min using a Veriti 96-Well Thermal Cycler (Applied Biosystem). The cDNA concentration and quality was assessed and quantified by the Epoch Spectrophotometer System and Take3 Plate (Biotek) [Akpinar et al., 2014].

**Quantitative PCR analysis.** Relative TNF-α and TGF-β mRNA expression analyses were performed with StepOne Plus Real-Time PCR System (Applied Biosystem) using cDNA synthesized from rat liver RNAs. qPCR was performed by using hydrolysis probes for rat TNF-α Rn00562055_m1, TGF-β Rn00572010_m1and β-actin Rn00667869_m1 (Applied Biosystem). Results were expressed as relative-fold and compared to control groups. Expression data of β-actin in each cell group were used as reference genes. For each cell group, triplicate determinations were performed for both targets in a 96-well optical plate using 9 μl of cDNA (100 ng), 1 μl of Primer Perfect Probe mix, and 10 μl of QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany) in each 20 μl reaction. The plates were heated for 2 min at 50°C, 10 min at 95°C, and a subsequent 40 cycles of 15 s at 94°C and 60 s at 60°C. All data were expressed as fold-change in expression compared to animal groups using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001].

**Immunohistochemical study.** Tissue sections were deparaffinized in xylene and rehydrated in ethanol followed by water and phosphate-buffered saline. Subsequently, the sections were washed in distilled water and immersed in 3% hydrogen peroxide for 15 mi. After several washes in PBS, the sections were immersed in an equilibration buffer at room temperature for 20 min. The tissue sections were then incubated with TNF-α solution (Abcam, Cambridge, Anti-TNF alpha antibody (ab6671)) for 1 h at room temperature. Sections were incubated with PBS containing normal goat serum without a primary antibody. Reactions were performed on an automated Ventana BenchMark ULTRA immunostainer, using an UltraView Universal DAB Detection Kit (Ventana, reference760–500, Mannheim, Germany). The sections were counterstained with Mayer's hematoxylin before being examined and photographed under a light microscope (Olympus BH-40).

### STATISTICAL ANALYSES

For the molecular analyses, we used GraphPad Prism, version 5.0, and the results are presented as the means ± standard deviation (SD). Comparisons between the groups were performed using the one-way analysis of variance (ANOVA) and Tukey's multiple comparison test; significance was accepted at $P < 0.05$. For the biochemical analyses, we used IBM's SPSS Program, version 20, and the results are presented as the means ± SD. Group comparisons were performed using the one-way ANOVA and Duncan's multiple comparison test; significance was accepted at $P < 0.05$.

## RESULTS

### BIOCHEMICAL RESULTS

**Effects of aliskiren on serum ALT and AST levels.** The effects of aliskiren on the serum enzymes ALT and AST are shown in Table I. There were statistically significant ($P < 0.05$) increases in AST and ALT levels in the group treated with paracetamol as compared with the control. Aliskiren administration with paracetamol significantly decreased the serum enzyme level when compared with the group treated with paracetamol alone ($P < 0.05$).

**Serum renin and Ang II Levels.** In the present study, serum renin concentration significantly increased in the paracetamol treated group compared to control group ($P < 0.05$, Fig. 1A). Both doses of aliskiren (50 and 100 mg/kg) further increased serum renin levels when administered to paracetamol treated rats. In addition while paracetamol treatment significantly increased the serum Ang II concentration compared to the control group, each aliskiren dose significantly reduced Ang II levels ($P < 0.05$) (Fig. 1B).

**Effects of aliskiren on liver oxidant and antioxidant levels.** The activity of SOD, GR and the level of GSH, as well as MDA levels, were evaluated in all rat livers. The results are shown in Table II. The hepatic SOD activity and GSH level are markedly reduced in the group treated with paracetamol ($P < 0.05$), whereas the MDA level and GR activity increased as compared to the control group. In contrast with the group treated with paracetamol, the GSH level and SOD activity increased, and MDA level and GR activity decreased, in both aliskiren treatment groups.

### MOLECULAR PARAMETERS

As shown in Figure 2A, the TNF-α gene expression was increased 4.26-fold in the group treated with paracetamol compared to the control groups ($P < 0.01$). 50 mg/kg and 100 mg/kg aliskiren administration with paracetamol decreased TNF-α mRNA expression to 2.99- and 2.11-fold. Similarly, TGF-β mRNA gene expression significantly increased (Fig. 2B) in the group treated with paracetamol compared to the control group (11.48- fold) ($P < 0.001$). With both doses of aliskiren administration with paracetamol, TGF-β mRNA gene expression decreased 3.44- and 1.58-fold compared to the control group. For these parameters,

TABLE I. Effect of Aliskiren on Changes in Enzymatic Activities of ALT and AST Levels

| Groups | ALT (U/L) | AST (U/L) |
|---|---|---|
| Control | 33.34 ± 7.84[a] | 56.44 ± 10.64[a] |
| Aliskiren 100 mg/kg | 31.45 ± 8.93[a] | 61.89 ± 21.63[a] |
| Paracetamol 2 g/kg | 127.15 ± 29.39[b] | 179.33 ± 45.22[b] |
| Paracetamol + Aliskiren 50 mg/kg | 84.34 ± 22.88[c] | 104.88 ± 23.09[c] |
| Paracetamol + Aliskiren 100 mg/kg | 70.33 ± 21.22[c] | 133.65 ± 15.29[d] |

Means in the same column by the same letter are not significantly different according to the One Way ANOVA-Duncan test ($P = 0.05$). Results are means ± SD of two measurements.

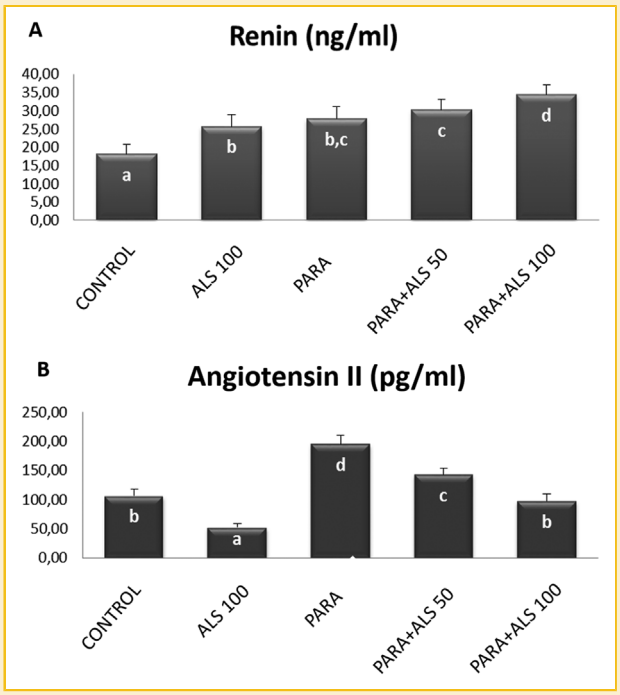


**Fig. 1.** Serum renin (A) and angiotensin II (B) concentration in treatment groups. Means in the same column by the same letter are not significantly different according to the Duncan test ($P = 0.05$). Results are means ± SD.

both doses of aliskiren were effective when compared to the group treated with paracetamol.

### HISTOPATHOLOGICAL RESULTS

Regular normal liver histology was seen in the control group (Fig. 3A). In the livers of the group treated with paracetamol, thin severe inflammatory cell infiltration and gross necrosis of the entire centrilobular areas were obvious. In the hepatocytes of this group, hydropic degeneration was found; perinuclear cytoplasm of these hepatocytes was dense or cloudy, and the nucleus was abnormally shaped and showed basophilic staining. Furthermore, central vein and sinusoidal congestion and hemorrhage were found in this paracetamol treated group (Fig. 3B). The findings for the hepatocytes of the aliskiren 100 mg/kg group were similar to the control group, showing normal liver histology (Fig. 3C). The Paracetamol + aliskiren 50 mg/kg (Fig. 3D) and paracetamol + aliskiren 100 mg/kg (Fig. 3E) groups also presented similar findings. Their hepatocytes and sinusoids had a normal appearance. Their parenchyma structures were regular with typical Kupffer cells.

### IMMUNOHISTOCHEMICAL RESULTS

Control (Fig. 4A) and aliskiren 100 mg/kg (Fig. 4C) groups showed a negative expression of TNF-α in the cytoplasm of the hepatocytes. In the group treated with paracetamol, the specimen showed expression of TNF-α in the cytoplasm of a hepatocyte (Fig. 4B). Positive cells were located predominantly around the central vein areas after paracetamol treatment. The paracetamol + aliskiren 50 mg/kg (Fig. 4D) and paracetamol + aliskiren 100 mg/kg (Fig. 4E) groups showed negative expression of TNF-α in the cytoplasm of the hepatocytes.

## DISCUSSION

In this study, we investigate the effect of RAAS inhibition via the new drug aliskiren in paracetamol-induced hepatotoxicity in rats. Paracetamol is a commonly used analgesic and antipyretic agent. However, overdoses is known to be hepatotoxic in humans and experimental animal models [Uzkeser et al., 2012; Karakus et al., 2013]. At therapeutic doses, paracetamol is metabolized into nontoxic metabolite sulfate and glucuronide conjugates and is eliminated via urine. Only a small amount of the paracetamol is metabolized via the CYP 450 (predominately CYP2E1 isoenzyme) enzyme system into the highly reactive metabolite NAPQI. At therapeutic concentrations, this toxic reactive metabolite is detoxified by GSH. However, at overdoses, GSH, one of the most important molecules in the cellular defense pool of the liver, is depleted, and the toxic metabolite NAPQI increases and causes damage to liver tissues [Corcoran et al., 1980]. Since paracetamol is mainly metabolized with CYP 2E1 isoenzyme to its toxic metabolite, drug-drug interactions may lead to increase paracetamol toxicity. Aliskiren is mainly eliminated as unchanged drug and in a lesser amount oxidative metabolism by CYP 3A4 isoenzyme. Aliskiren showed no clinically significant interactions during coadministration with a wide range of potential concominant medications except p-glycoprotein inhibitors. Aliskiren does not inhibit or induce CYP isoenzymes activity [Vaidyanathan et al., 2008]. Therefore, aliskiren does not influence the bioactivation of paracetamol.

Among the available biochemical markers of hepatotoxicity, serum ALT and AST levels are the first to change and should be evaluated directly following an overdose of paracetamol. These are sensitive enzymes used in the diagnosis of liver diseases [Karakus et al., 2013]. Yayla et al. investigated the role of bosentan in paracetamol-induced hepatotoxicity; elevated serum ALT and AST levels in the paracetamol-induced hepatotoxicity group were reduced by treatment with bosentan [Yayla et al., 2014]. Jiang et al.'s study also found that these enzyme levels increased with paracetamol toxicity and decreased with a pretreatment of the major six bioactive lignans of *Fructus Schisandrae* [Jiang et al., 2015]. Similarly, in our study, serum ALT and AST levels were elevated in the group treated with paracetamol; aliskiren treatment decreased these enzyme levels compared to the group treated with paracetamol alone. Aliskiren treatment were increased AST levels in high dose compared to low dose but AST levels were already lower than paracetamol treatment group.

In contrast to our study, after a month of aliskiren treatment with antiepileptic drugs in a 61-year-old woman, asymptomatic acute liver cirrhosis had progressed, with ALT and AST levels dramatically increased [Crepin et al., 2014]. In another study, aliskiren was found to be effective in carbon tetrachloride-induced hepatotoxicity in mice [Lee et al., 2012]. These studies show that while long term treatment of aliskiren leads to hepatic damage, acute treatment does not, and aliskiren even improves hepatic damage enzymatically by decreasing AST and ALT levels.

TABLE II. Effect of Aliskiren Treatment on Superoxide Dismutase (SOD) and Glutathione Reductase (GR) Activity, Lipid Peroxidation (MDA), and Total Glutathione (GSH) Levels in the Liver Tissues of Rats

| Groups | SOD (U/mg protein) | GSH (nmol/mg protein) | MDA (nmol/mg protein) | GR (U/mg protein) |
|---|---|---|---|---|
| Control | $35.59 \pm 7.92^{c}$ | $4.87 \pm 0.51^{c}$ | $1.68 \pm 0.37^{a}$ | $24.27 \pm 5.37^{a}$ |
| Aliskiren 100 mg/kg | $30.74 \pm 3.78^{b,c}$ | $4.25 \pm 1.01^{b,c}$ | $1.95 \pm 0.78^{a,b}$ | $24.37 \pm 4.67^{a}$ |
| Paracetamol 2 g/kg | $20.18 \pm 8.74^{a}$ | $2.65 \pm 1.04^{a}$ | $4.24 \pm 1.08^{c}$ | $29.89 \pm 4.31^{b}$ |
| Paracetamol + Aliskiren 50 mg/kg | $28.55 \pm 2.85^{b}$ | $4.25 \pm 0.86^{b,c}$ | $2.46 \pm 1.37^{a,b}$ | $26.8 \pm 8.76^{a,b}$ |
| Paracetamol + Aliskiren 100 mg/kg | $23.86 \pm 7.35^{a,b}$ | $3.91 \pm 0.81^{b}$ | $3.00 \pm 1.26^{b}$ | $23.55 \pm 8.6^{a}$ |

Means in the same column by the same letter are not significantly different according to the One Way ANOVA-Duncan test ($P = 0.05$). Results are means ± SD of three measurements.

In previous studies, it was seen that antioxidant enzyme activities, inflammatory cytokines, histopathological examination and oxidant levels provide other markers of liver failure due to paracetamol toxicity [Uzkeser et al., 2012; Ferah et al., 2013]. It has been demonstrated that increased circulatory Ang II induces hepatic oxidative stress [Bataller et al., 2003, 2005]; oxidative stress induces hepatocyte damage and activates HSCs and Kupffer cells that result in liver fibrosis [Dooley and ten Dijke, 2012]. The physiological function of SOD is to protect cells against the harmful effects of superoxide radicals. In previous studies it was shown that SOD activity decreased in paracetamol toxicity-induced hepatic damage, and with treatment strategies, SOD activity increased [Ferah et al., 2013; Karakus et al., 2013]. Our results also support these findings. Furthermore, aliskiren treatment increases the SOD activity of kidneys in renal ischemia/reperfusion injury showing high antioxidant properties [Ziypak et al., 2015]. Similar to this study, aliskiren treatment increased SOD activity in low dose. High dose of aliskiren treatment also increased SOD activity but not as high as low dose. In the mitochondrial matrix SOD converts superoxide anion into hydrogen peroxide. Accumulated hydrogen peroxide can diffuse to the cytosol and generate more reactive free radicals. For this reason, mitochondria must provide a balance between the activity of SOD and the GSH redox cycle to dispose hydrogen peroxide. Lack of catalase in the mitochondria, the metabolism of hydrogen peroxide is accomplished by GSH (mainly) with the participation of GSH peroxidase and conversion of GSSG back into GSH by GR [Mari et al., 2009]. In our study, GR activity increased in paracetamol treated groups and both doses of aliskiren administration leads to decrease these levels. Paracetamol toxicity leads to decrease GSH levels and GR activity increased to convert GSSG to GSH to eliminate toxic metabolite NAPQI.

It is known that NAPQI, as an oxidative stress mediator, leads to a decrease in GSH levels; due to decreased GSH levels, lipid peroxidation increased in acetaminophen overdoses [Corcoran et al., 1980]. In a Karakus et al. study, GSH levels were decreased in the paracetamol-induced hepatotoxicity group and treatment with agomelatin decreased GSH levels [Karakus et al., 2013]. Further aliskiren increased GSH levels in a study performed on kidney tissue [Ziypak et al., 2015]. Similarly, both doses of aliskiren treatment increased the GSH levels that had been compromised by the paracetamol toxicity.

In our study we also evaluated another parameter that showed oxidative stress MDA level which is an end product of lipid peroxidation. Lipid peroxidation due to free oxygen radicals is an important mechanism of paracetamol-induced liver damage. In previous studies, increased MDA levels due to paracetamol-induced liver damage were decreased with treatment [Ferah et al., 2013; Yayla et al., 2014] and it has been shown that aliskiren decreases MDA levels [Hassanin and Malek, 2014]. Our results show that the increased MDA in paracetamol toxicity was decreased by both doses of aliskiren treatment. Oxidative stress is involved in the physiopathology of

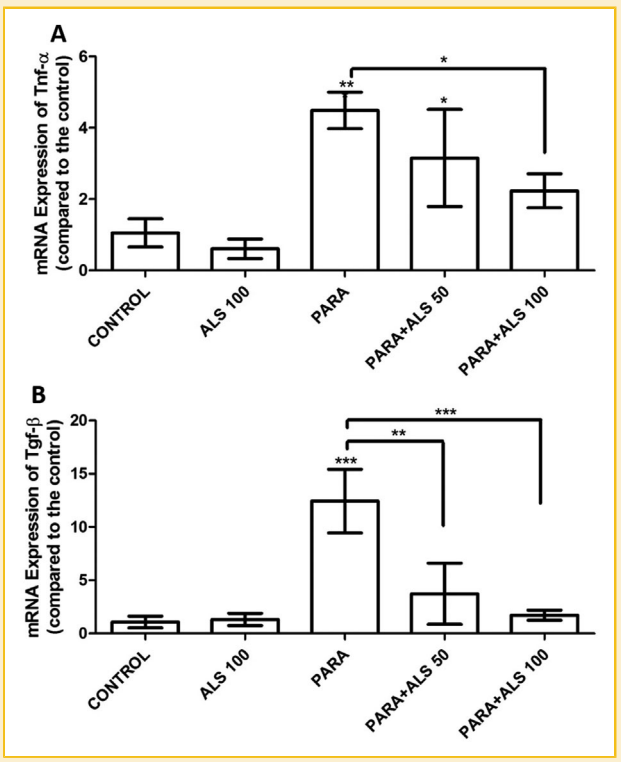


Fig. 2. Relative mRNA expression levels of TNF-α (A) and TGF-β (B) in liver tissue of experimental rat groups. The expression of mRNAs was detected using quantitative qPCR analysis. β-Actin was used as the reference gene. Results are expressed as relative fold compared with control animals. Gene-specific probes were used as outlined under "Materials and methods" section. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Statistical comparisons were made using one-way ANOVA followed by Tukey's test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Results are means ± SD.

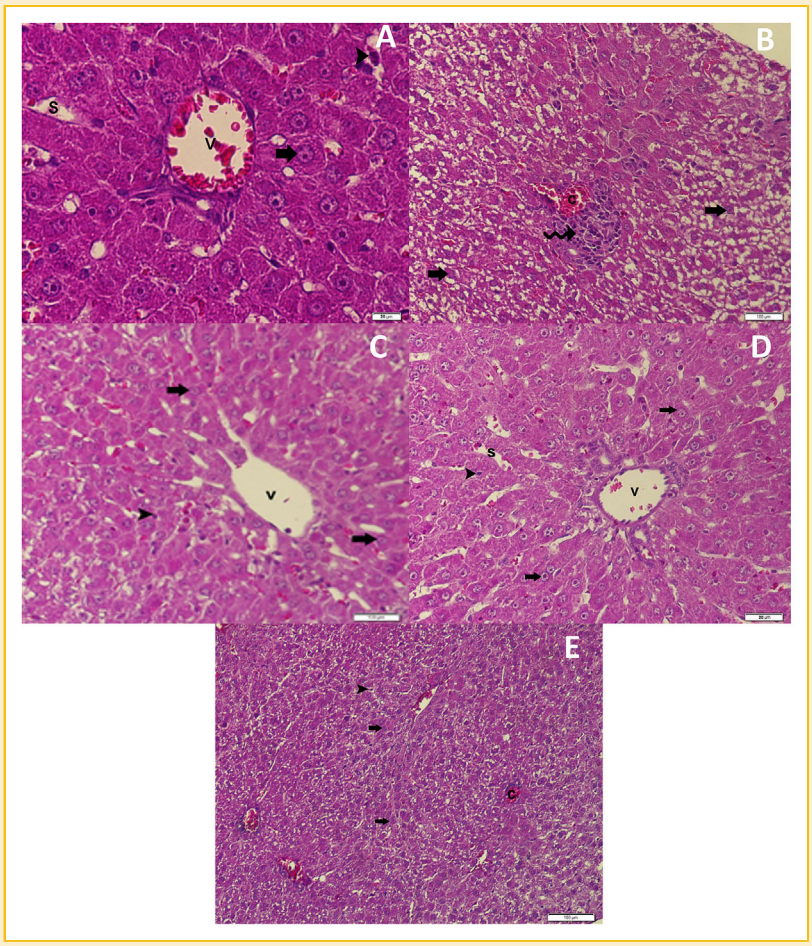


**Fig. 3.** Haematoxylin and eosin results in rats' liver tissues; Magnification 100X. Control group (A), PARA group (B), ALS 100 group (C), PARA + ALS 50 group (D), PARA + ALS 100 group (E). Fig 3A: Hepatocytes (arrow) and sinusoids (s) with normal appearance in control group. Fig 3B: Severe inflammatory cell infiltration (spirally arrow) and gross necrosis of the entire centrilobular areas were obvious. Hepatocytes with hydropic degeneration (arrow). Central vein (v) and sinusoidal congestion and hemorrhage are seen. Fig 3C: Typically hepatocytes (arrow). Sinusoids (s) normal appearance with radial organized structure, Kupffer cell (arrow head) and central ven (v) are seen. Fig 3D–E: Hepatocytes (arrow) and sinusoids (s) with normal appearance are seen. Regular parenchyma with radial structure were shown. Kupffer cell (arrow head) is seen. PARA, Paracetamol; ALS, Aliskiren.

several diseases. As a result, overdose of paracetamol induced oxidative stress in liver tissues and aliskiren treatment ameliorate the oxidative stress in these tissues. RAAS activation and enhanced Ang II activity exacerbates oxidative stress [Bataller et al., 2003, 2005; Landmesser et al., 2007; Mollnau et al., 2002]. Inhibition of the RAAS by aliskiren together with decreased Ang II levels reduce the oxidative stress that causes tissue damage [Rashikh et al., 2012]. Therefore, the suppression of the RAAS with aliskiren in the paracetamol-induced hepatotoxicity group could reduce the oxidative stress-induced damage. Earlier studies performed with captopril and aliskiren showed that inhibition of Ang II reduced the production of free oxygen radicals [He et al., 2007; Ziypak et al., 2015]. Our previous studies showed that aliskiren protects against oxidative stress [Akpinar et al., 2014; Ziypak et al., 2015], which is consistent with our results on paracetamol toxicity.

We also evaluated the proinflammatory cytokine TNF-α expression levels, which has an important role in immunity, inflammation and apoptosis [Baud and Karin, 2001]. TNF-α and Angiotensin II are potent inducers of oxidative stress in a number of cell types. The inflammatory mediators are capable of upregulating various RAAS components in a variety of mammalian tissues [Mariappan et al., 2012]. TNF-α is released by activated Kupffer cells [Jaeschke et al., 2002], due to the acetaminophen overdose [Laskin et al., 1986] and has mediated innate immunity [Abbas and Poper, 1994]. The hepatoprotective role of Kupffer cells in paracetamol toxicity can be attributed to the TNF-α production and release [Chiu et al., 2003]. Blazka et al. suggested that TNF-α contributes to hepatotoxicity in paracetamol toxicity [Blazka et al., 1996]. In parallel with this study, inflammatory cytokines such as TNF-α, IL-1α and IL-6 have been associated with symptoms of liver diseases like cirrhosis and inflammation [Blazka et al., 1996; Chiu et al., 2003]. Blazka et al. indicated that increased TNF-α production leads to hepatic damage secondarily in addition to hepatic damage of metabolites [Blazka et al., 1995]. In addition, increased Ang II levels

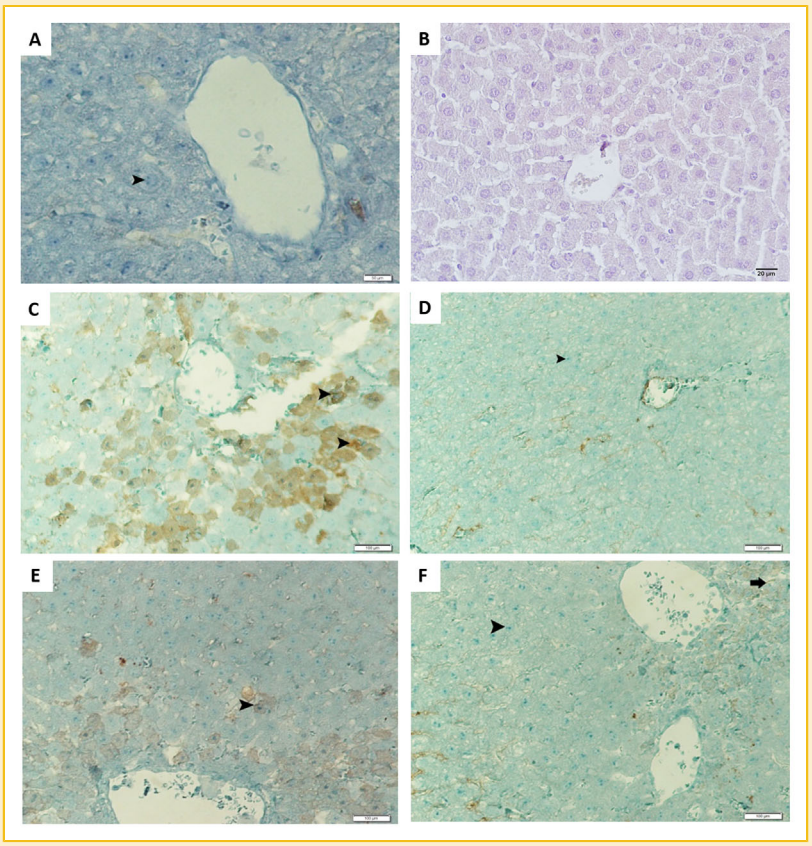


**Fig. 4.** Aliskiren pretreatment prevents hepatocyte apoptosis induced by paracetamol. A: Control group. We show regular parenchyma. Hepatocytes (arrow) (original magnification: × 400); B: Negative control (Haematoxylin) C: Liver samples were collected from PARA group. TNF-α positive hepatocytes (arrow head). D: ALS 100 mg/kg group. We show typical parenchyma. Hepatocytes (arrow). E: PARA + ALS 50 mg/kg group. We show normally parenchyma. Hepatocytes (arrow). F: PARA+ALS 100 mg/kg group. PARA, Paracetamol; ALS, Aliskiren.

were shown to aggravate oxidative stress and inflammation in previous studies [Kim et al., 2012; Ziypak et al., 2015]. In our study, the TNF-α levels that were increased in paracetamol toxicity and decreased in the aliskiren treatment group may be due to the inhibition of Ang II production, or the previously shown antioxidant or anti-inflammatory effects of aliskiren.

We also evaluated the other cytokine TGF-β levels. TGF-β plays a key role in the regulation of chronic liver disease with contributing to all stages of disease progression from initial liver injury through inflammation and fibrosis to cirrhosis and hepatocellular carcinoma [Dooley and ten Dijke, 2012]. In the liver, a large proportion of TGF-β is synthesized by endothelial and Kupffer cells [Nakatsukasa et al., 1990]. TGF-β is the cytokine that regulates cell growth, differentiation and matrix production [Dooley and ten Dijke, 2012]. TGF-β is also known to have antiproliferative, proapoptotic and anti-inflammatory effects [Siegel and Massague, 2003; Li et al., 2006]. The increase in TGF-β activity plays an important role in fibrotic diseases characterized by excessive matrix material between tissues of the lung, kidney, liver and other organs [Border and Ruoslahti, 1992]. TGF-β levels increased in hepatotoxicity and studies showed that treatment strategies decreased these elevated levels [Lee et al., 2012]. In response to Ang II, Kupffer cells secrete TGF-β and TNF-α. Ang II can mediate and exacerbate liver fibrosis through HSC activation and by stimulating TGF-β via the angiotensin II receptor, type 1 ($AT_1$) receptor. Also, Ang I activates HSCs to secrete TGF-β. Aliskiren treatment normalized TGF-β levels in a previous study [Aihara et al., 2013]. Our findings are similar to previous studies; TGF-β levels were increased in paracetamol toxicity groups and aliskiren treatment showed a statistically significant decrease in these levels. Aliskiren may down-regulate the activation of Kupffer cells and HSCs via decreasing Ang II levels.

When looking at the serum renin level, a significant increase was observed in paracetamol treated groups compared to the control group. In our study, serum renin concentration levels increased under paracetamol toxicity and treatment with aliskiren increased renin levels. Aliskiren increases blood renin concentration as a defense mechanism to prevent the negative feedback mechanism of the RAAS [Azizi et al., 2006]. In addition, aliskiren treatment was found to cause a more intense increase in renin secretion as a result of decreasing serum Ang II levels. And also because of the aliskiren inhibits the active enzymatic part of the renin, renin production does not stop [Ziypak et al., 2015]. Our other result that Ang II levels substantially increased in paracetamol treated groups compared to the control group while aliskiren administration blocked paracetamol induced

increases in Ang II levels support our hypothesis. Increased renin production with paracetamol treatment causing an increase in the Ang II concentration. Decreases in Ang II levels suggest that aliskiren administration blocked the paracetamol toxicity induced increase. Therefore, decreased Ang II levels can be responsible for protective effects of aliskiren during paracetamol toxicity. In previous study, Lee et al demonstrated that Ang II levels were significantly high in carbon tetrachloride induce hepatotoxicity and aliskiren treatment normalized the Ang II levels [Lee et al., 2012]. In our study aliskiren potentially exerted protective effects on Ang II concentrations, the end product of the RAAS and, therefore, on paracetamol toxicity by decreasing inflammatory cytokines and oxidative stress. When we looking at biochemical and oxidative stress parameters, aliskiren has significant effects on paracetamol toxicity. Thus, it seems that using RAS inhibitors for paracetamol toxicity may be strong enough to stop paracetamol toxicity induced cellular damage by many mechanisms.

Paracetamol toxicity-induced liver necrosis not only causes enzymatic changes but also leads to histopathological changes in the liver. Overdoses of paracetamol result in centrolobular necrosis [Mitchell et al., 1973]. In our study, we observed severe necrotic foci in the liver of the paracetamol-induced hepatotoxicity group, supporting the findings of earlier experimental studies [Ferah et al., 2013; Karakus et al., 2013]. Both doses of aliskiren inhibit the paracetamol-induced toxicity in liver histopathologically. In aliskiren-treated paracetamol groups, the sequence of hepatocyte cords extending from the vena centralis in the parenchyma were quite different from the group treated with paracetamol but similar to those of the control group. Findings by Lawson et al. showed that TUNEL positive cells were located predominantly around the central vein areas after AAP treatment [Lawson et al., 1999]. Our findings support the findings of Lawson et al. When looking at all these results together, aliskiren has very important protective effects, and treatment with aliskiren provided protection against paracetamol-induced hepatic injury in rats, enzymatically and histopathologically.

In conclusion, we demonstrated that aliskiren might have a protective role against hepatic damage due to the overdose of paracetamol. Paracetamol-induced hepatotoxicity was ameliorated by aliskiren treatment, which was supported by progressively decreased serum levels of ALT and AST, its positive impact on the oxidant/antioxidant balance with decreased MDA levels and increased levels of antioxidants like SOD and GSH as well as positive regulation of the expression of TNF-α and TGF-β. Taken together, these results show the importance of the RAAS and suggest that the renin inhibitor aliskiren is a promising treatment for paracetamol-induced hepatotoxicity in the future.

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