Research Article

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The effect of Paracetamol exposure on hepatic and renal tissues during statin usage

Statin kullanımı sırasında parasetamol maruziyetinin hepatik ve renal dokular üzerine etkisi

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Abstract

Objective: Statins and Paracetamol have widespread use in clinic and both drugs possess similar side effects; therefore, we investigated if drug-interaction occurs when the combination of these two drugs is used during therapy.
Materials and methods: A total of 32 (12–15 months old) grown-up male rats were divided into four groups: Control group, RSV group (10 mg/kg Rosuvastatin/daily), APAP group (50 mg/kg Paracetamol/5 days/weekly), RSV + APAP (10 mg/kg Rosuvastatin/daily + 50 mg/kg Paracetamol/5 days/weekly). At the end of 8 weeks of chronic treatment, the blood and tissue samples were taken under the Ketamine and Xylasine anesthesia (50 mg/kg and 5 mg/kg, respectively).
Results: In the liver, sinusoidal dilatations, pyknotic nuclei and hemorrhagic foci are more frequently seen in the group receiving combination therapy; although serum liver functions among groups were not significantly different. Kidney histopathologic alterations in APAP and RSV + APAP groups were found more distinct than in RSV alone group. Inducible nitric oxide synthase activity was highly increased with combination therapy in liver and kidney tissues.
Conclusion: RSV-Paracetamol interaction may occur as an important drug interaction histopathologically even before it is manifested biochemically in the clinic.

Keywords: Acetaminophen; Hepatotoxicity; Nephrotoxicity; Rational drug use; Statin.

Öz

Amaç: Statinler ve Parasetamol klinikte yaygın olarak kullanılmaktadır ve her iki ilaç da benzer yan etkilere sahiptir; Bu nedenle, tedavi sırasında bu iki ilacın kombinasyonu kullanıldığında ilaç etkileşimi oluşup oluşmadığını araştırdık.
GereçveYöntem: Toplam 32 (12–15 aylık) yetişkin erkek sıçan 4 gruba ayrıldı: Kontrol grubu, RSV grubu (10 mg/kg Rosuvastatin/günlük), APAP grubu (50 mg/kg Parasetamol/5 gün/haftalık), RSV + APAP (10 mg/kg Rosuvastatin/günlük + 50 mg/kg Parasetamol/5 gün/haftalık). 8 haftalık kronik tedavinin sonunda, kan ve doku örnekleri Ketamin ve Ksilazin anestezisi altında (sırasıyla 50 mg/kg ve 5 mg/kg) alındı.
Tartışma: Serum karaciğer fonksiyonları gruplar arasında anlamlı farklılık göstermemesine ragmen, karaciğerde, sinusoidal dilatasyonlar, piknotik nükleuslar ve hemorajik odaklar, kombinasyon tedavisi alan grupta daha sık görüldü. Böbrek histopatolojik değişiklikleri, APAP ve RSV + APAP gruplarında tek başına RSV alan gruptan

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daha belirgin bulundu. Karaciğer ve böbrek dokularında, kombinasyon tedavisi ile indüklenebilir nitrik oksit sentaz aktivitesi yüksek oranda artmıştır.
Sonuç: RSV-Parasetamol etkileşimi, klinikte biyokimyasal olarak ortaya çıkmadan önce bile histopatolojik olarak önemli bir ilaç etkileşimi olarak görülebilir.

Anahtar Kelimeler: Asetaminofen; Hepatotoksisite; Nefrotoksisite; Rasyonel İlaç Kullanımı; Statin.

Introduction

Statins, HMGCoA reductase inhibitors, are among the medications that are prescribed by a physician according to blood cholesterol level and are recommended to be used under observation and are considered safe. Utilization of these drugs in order to reduce blood lipid has increased recently. This group of drugs is metabolized through the liver CYP enzyme systems and especially during prolonged high doses usage, hepatic and renal toxicity is reported as a significant side effect. Within this group, after Lovastatin (the first drug approved by FDA) other compounds have come into use and Rosuvastatin (RSV), a completely synthetic compound, was approved in 2003 [1].

Due to its analgesic and antipyretic effect, Paracetamol (Acetaminophen, APAP) can be consumed freely without any prescription obligation in many countries. These drugs are reported to have the greatest side effects on liver. They are metabolized in the liver by three ways; conjugation with sulfate, glucuronidation and oxidation. The ways of metabolism described above come short on repetitive APAP doses and the drug transforms into toxic metabolite called N-acetyl-p-benzoquinone (NAPQI) through 1A2 and 3A4 as well as especially CYP2E1 [from microsomal liver enzymes (cytochrome P450)] [2]. This metabolite causes damage in organs such as liver and kidney by creating oxidative stress [3]. Generally metabolite becomes harmless by interacting with Glutathione (an endogenous antioxidant). However, an increase occurs in the amount of metabolite after APAP is taken prolonged time or at higher doses.

Available Glutathione cannot bind excess amount of NAPQI molecule that has come off. The liberated NAPQI, since it could not be bounded by Glutathione, leads to damage in the hepatic tissue by binding to other larger molecules in liver covalently. The Glutathione stores consumed away in liver due to reasons such as nutritional deficiency and alcohol dependence facilitates poisoning [4, 5].

APAP has nephrotoxic effects as well as side effects in liver [6]. Although APAP's hepatotoxicity risk is much higher than of nephrotoxicity, it may cause acute renal inefficiency at a rate of 1–2% and may become fatal at higher doses [7].

A patient being treated with Statins, may use APAP concomitantly due to their analgesic characteristics. Statin-APAP interaction may highly important for rational drug use in clinical practice and physicians should consider their adverse effects. Although there are many studies carried out related to APAP, no controlled study was found in literature demonstrating the hepatic and renal damage which may occur when it is used with statins. Therefore, in this study we evaluated possible drug interactions between RSV and APAP biochemically and immuno-histopathologically.

Materials and methods

A total of 32 (12–15 months old) grown-up male rats were randomized into four groups (eight in each group):

Control group; healthy, no drug was applied.

RSV group; was given drinking water and 10 mg/kg Rosuvastatin/daily for 8 weeks.

APAP group; received Paracetamol of 50 mg/kg through intraperitonal injection, 5 days a week for 8 weeks.

RSV + APAP group; was applied Paracetamol of 50 mg/kg through intraperitonal injection, 5 days a week for 8 weeks, as well as drinking water and RSV of 10 mg/kg.

The aim of project, number of animals to be used, the types of drugs to be applied to animals, drug doses and length and way of application, consistency with posology and ethical rules were scrutinized and approved by Adnan Menderes University Animal Experiments Ethical Committee (HADYEK 64583101/2014/035). Consistent with the ages when statins are used more frequently, our study was planned paying attention to 12–15 months old rats (their life span is approximately 26–28 months). We weighted the rats and adjusted the drug doses every Monday.

At the end of 8 weeks, after chronic drug application, intracardiac blood samples were taken and decapitation was performed under ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia. Hepatic and renal tissues were removed and placed in neutral formalin of 10% for histological follow-up. Serum samples obtained from centrifuged blood were kept at –80°C until study day. We could not find any evidence concerning hemolysis that will effect interpretation of test results in blood samples.

In all groups, in order to assess the effects of medications on hepatic and renal functions, we examined the serum aspartate transaminase (AST), serum alanine transaminase (ALT) (the indicators of liver damage) and

serum albumin levels. Also, to evaluate renal functions the urea, cystatin-C and creatinine levels in serum by spectrophotometric measurement method using automatic analyzer systems were studied (Architect i2000, Abbott, USA). In addition, as an indication of renal functions, we studied Lipocalin (NGAL) manually by enzyme immunoassay method (RayBio rat Lipocalin-2 Elisa). Results were obtained through Biotech Eliza Plate Reader and a standard curve was drawn using K-C Junior Software and Lipocalin standard and calculated as pg/mL.

We studied Nitrate and Nitrite levels manually through Nitrate/Nitrite colorimetric working kit (Cayman, USA) using serum and the results were obtained as optic density using Biotech Eliza Plate Reader (USA) device. A standard curve was drawn using nitrate standard and all results were calculated using Gene 5 software program and evaluated as μmol/mL. We obtained 5 μm thick cross-sections from the paraffin blocks prepared for histopathologic study by using Leica type slide-microtom and dyeing was performed using hematoxylin-eosin (H-E). During visualization we used Olympus BX-50 type binocular research microscope.

For immuno-histochemical study, we placed cross-sections on slides with lysine and incubated tissues in 1/50 dilution at the room temperature for 1 h using primary antibody inducible nitric oxide synthase (iNOS) (Epitopa Specific Rabbit Antibody, Labvision Fremont CA 94539, USA). Secondary antibody was treated with Biotinylated Goat Anti-Polyvalent-Labvision TP-125-BN for 20 min. To determine iNOS intensity in cross-sections where reverse-staining was performed with hematoxylin, we used semi-qualitative assessment method in order to interpret staining as (–): No staining; (+): Very slight staining; (++): Little staining; (+++): Medium level staining; (++++): Dense staining. In statistical evaluation of immune-histochemical staining we used chi-square (χ^2) analysis by Fisher exact test. The normality of the numeric values were evaluated by using Shapiro Wilk test. AST, ALT and Cystatin-C have shown non–parametric distribution and evaluated by Kruskal-Wallis test; the other biochemical tests have shown normal distribution and evaluated by one way ANOVA. Values with $p < 0.05$ was considered statistically significant.

Results

Biochemical findings

We could not find any statistically significant difference between groups in terms of ALT and AST levels. We established that serum albumin levels in the group where only APAP was used, tended to decrease compared to control group; however, this decline was statistically significant in the group where APAP was used together with RSV ($p = 0.006$). Serum urea values were found higher in RSV group compared to other groups ($p < 0.001$), but any significant difference could not be found between groups in terms of creatinine and NGAL levels that are other indicators of renal functions. Although it is not statistically significant, we detected an increase in serum NO levels in all drug groups than in control group (Table 1).

Similarly, iNOS staining become evident in tissues. During liver examination, iNOS level was found higher in statin group; when compared to other groups, it was also higher in kidneys of the group where two drugs were used together.

Liver tissue was with normal appearance in control group with H-E staining. In the liver where APAP therapy was applied hemorrhagic foci accompany to sinusoidal dilatations. A histopathologic appearance progressing to liver necrosis is available. In the hepatic tissues where RSV therapy was applied sinusoidal dilatations are less frequently seen compared to APAP group; however, sporadic pyknotic nuclei and alteration progressing to

Table 1: Biochemical results in all groups.

	Control group	RSV group	APAP group	RSV + APAP group
Aspartate transaminase (AST) (U/L)	140.25 ± 76.78	157.63 ± 61.17	96.43 ± 17.14	120.63 ± 21.77
Alanine transaminase (ALT) (U/L)	68.25 ± 22.74	64.0 ± 18.24	54.0 ± 8.49	66.8 ± 12.23
Albumine (g/dL)	2.83 ± 0.07	2.85 ± 0.26	2.71 ± 0.19	2.61 ± 0.17[a]
Urea (mg/dL)	16.25 ± 1.67	23.13 ± 3.14[b]	16.43 ± 1.27	16.38 ± 2.39
Creatinine (mg/dL)	0.53 ± 0.05	0.57 ± 0.06	0.51 ± 0.03	0.50 ± 0.02
Cystatin C (mg/L)	0.16 ± 0.04	0.16 ± 0.04	0.17 ± 0.06	0.13 ± 0.04
N Gal (pg/mL)	310.40 ± 124.2	214.13 ± 94.0	167.25 ± 80.2	227.40 ± 100.0
Nitric oxide (μmol/mL)	3.11 ± 1.52	5.06 ± 2.99	4.18 ± 2.16	4.50 ± 2.37

Mean ± SEM, [a]$p < 0.01$, [b]$p < 0.001$.

necrosis are observed in hepatic cell structure. In the group that received RSV + APAP combination therapy, there are hemorrhagic foci, sinusoidal dilatations and sporadic necrotic areas as well as granular and vacuolar degeneration.

In the livers of APAP group, there are sinusoidal dilatations and hemorrhagic foci and an appearance progressing to necrosis is available with H-E staining. In the group receiving RSV therapy, even though there are alterations, pyknotic nuclei and necrotic findings in the hepatic cell structure, the rate of sinusoidal dilatations is smaller compared to APAP group. Dilatations, pyknotic nuclei and hemorrhagic foci are more frequently seen in the group receiving combination therapy.

In liver IHC staining, control group seemed normal; however, the highest oxidative damage was seen in the RSV group followed by APAP group. We found a statistically significant difference between groups in terms of liver tissue rating ($p < 0.001$). The most obvious damage in immune-histochemical liver staining was observed in the group where RSV was applied alone (Figure 1).

In kidney H-E staining, the proximal and distal tubular renal particles are observed normal in control group. In APAP group there is granular degeneration in proximal convoluted tubule and also there are luminal dilatation and sporadic hemorrhagic foci in distal tubules. In RSV group, distal convoluted tubule and luminal dilatation is less than in APAP group; in addition, there are hemorrhagic foci in this group. In RSV + APAP group we observed that granular degeneration has continued in proximal and distal convoluted tubules; and there was increased luminal dilatation and presence of sporadic hemorrhagic foci. When compared with control group, hypertrophy was seen in renal particles of the groups receiving APAP and RSV + APAP. It's possible reasons are increase in volume due to increase in pressure, blood flow passing through glomeruli and hypertrophy developed in podocytes. Histopathologic alterations in APAP and RSV + APAP groups were found more distinct than in RSV alone group.

Even though, control group had normal appearance in IHC staining; the highest oxidative damage was

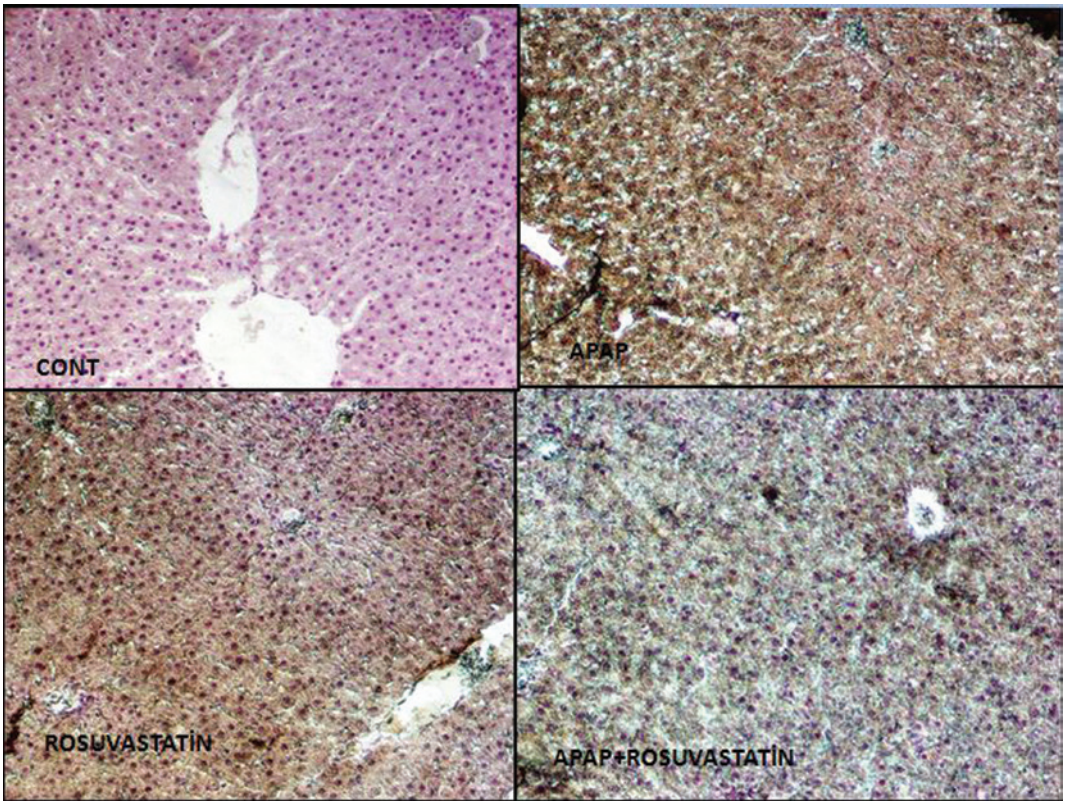

Figure 1: The Results of liver dyeing with immunohistochemistry.
In control group and in the groups where RSV, APAP and RSV + APAP therapy was applied, assessment of hepatic tissue by immunohistochemical method (magnification: 20×). We rated oxidative damage induced by our primary antibody iNOS. Rating was performed by assigning (+) to the lowest damage and (++++) to the highest damage and statistical analysis was carried out using chi-square (χ^2) method.

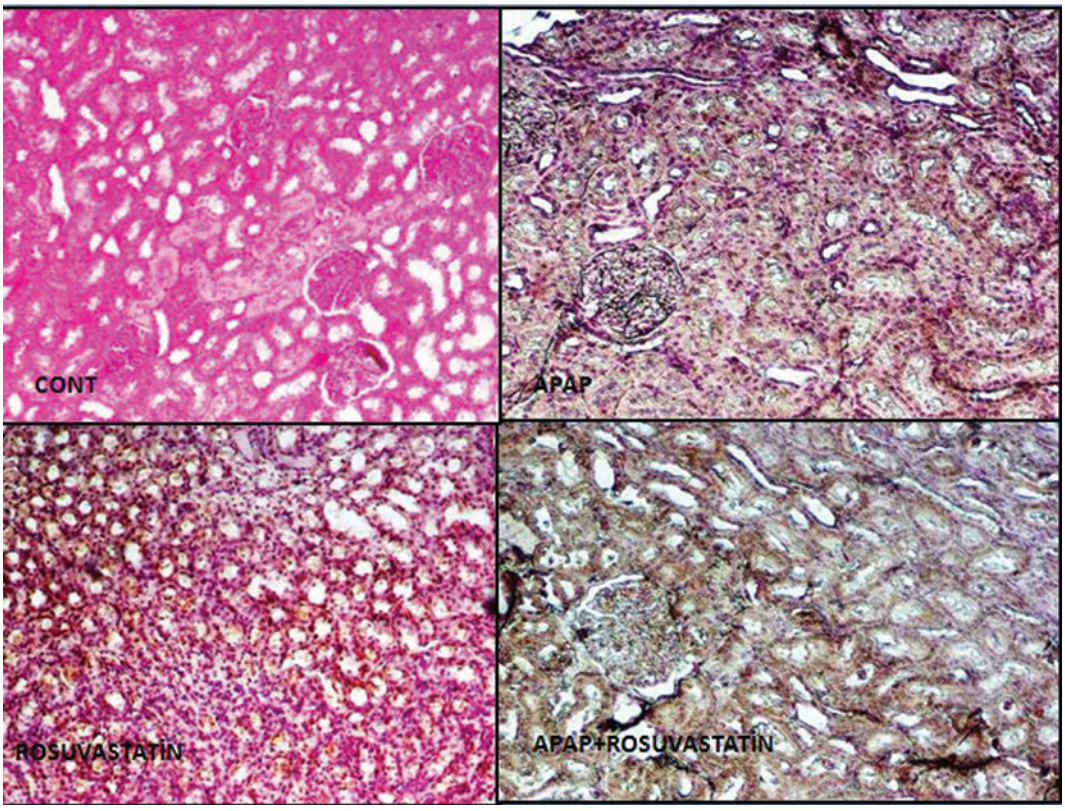

Figure 2: The Results of renal tissue dyeing with immunohistochemistry.
In control group and in the groups where RSV, APAP and RSV + APAP therapy was applied, assessment of renal tissue with immunohistochemical method (magnification: 20×). We evaluated oxidative stress induced by our primary antibody iNOS. Rating was performed by assigning (+) to the lowest damage and (+++) to the highest damage and statistical analysis was carried out using chi-square (χ^2) method.

observed in the combined therapy. Among the groups that received drug therapy, the least damage was seen in RSV group. There was a statistically significant difference between drug groups in terms of renal rating ($p < 0.001$). Immuno-histochemically, the most intensive histopathological alterations in kidneys were observed in the group that received APAP and RSV + APAP (Figure 2).

Discussion

Our research was planned to determine if combined use of two drugs (Paracetamol and Rosuvastatin; these are prescribed in large numbers and used arbitrarily out of physician's knowledge) is safe and to make a significant contribution to the issue of "rational drug therapy" and to human health at this stage.

In line with The Commission of Adult Treatment Panel (ATP) III's strict control targets in reducing blood lipids and as a result of identification of high sensitive C reactive protein (h-CRP) as a cardiac risk factor, there is an increase in prescribing statins [8]. Vasudevan et al. described four clinic conditions for the liver damage caused by statins (acute liver failure, hepatitis, cholestasis and increase in asymptomatic transaminases) and emphasized that advanced age leads to an important predisposition [9, 10]. Rangnekar et al. reported that, despite their widespread usage, statins are not very effective in this type of damage; moreover, according to latest studies, statins are therapeutic and safe in chronic hyperlipidemic liver injury [11]. Besides, the studies that defined hepatic toxicity and myopathy [1, 9], their influence on kidneys have continued to be reported. Dormuth et al. suggested in a study that when RSV is used, acute renal failure may develop in patients within first 120 days [12]. In our study, we also evaluated the effect of Rosuvastatin (RSV; a statin that has a strong effect) on liver, as well as renal histopathology. Similar to other studies [13–15] in our experimental model the dose of chronic RSV exposure was created by adding RSV of 10/kg/daily to drinking water for a period of 8 weeks. In our study we have seen, granular and vacuolar

degeneration, sporadic picnotic nuclei, sinusoidal dilatations and hemorrhagic foci in the livers of RSV group. Also, iNOS expression has increased. Despite, presence of tissue damage that we demonstrated histopathologically, this condition was not reflected in blood parameters yet. American Food and Drug Administration (FDA) elucidated that serious hepatic injuries related to all statins are very rare; additionally they cannot be established in advance, therefore, routing follow-up of liver enzymes is not possible [16].

The normal serum hepatic function tests in our study are consistent with these data. In other words, existing tissue injury does not show up clinically, it lies snug for a certain period of time since liver functions do not deteriorate immediately.

In kidneys where RSV therapy was applied, there are luminal dilatation in distal convoluted tubes, hemorrhage foci and iNOS expression. In statin group, the slight increase in creatinine values as well as in urea was similar to the results of Nasri et al. who studied three groups by applying atorvastatin of 10, 50 and 150 mg for 7 days long. In their study, creatinine values were reported as 0.5, 0.53 and 0.55 mg/dL, they stated that they did not find any statistically significant difference biochemically. However, similar to the findings in our study they reported that atorvastatin given at a level of 150 mg has caused vacuolization, degeneration and dilatation in renal tubules [17]. In our study, creatinine levels were 0.53 mg/dL in control group and 0.57 mg/dL in RSV group and any significant difference could not be found. In clinical study carried out with RSV (1800-person JUPITER study), although it is not statistically significant, risk for acute renal failure at a rate of 19% was identified in 20 mg RSV users [12]. These explanations support our present study; we demonstrated in our study that renal structure is injured by RSV histologically.

APAP (over-the-counter; OTC) is an analgesic drug which has widespread usage and may be fatal at overdoses [5, 18]. Its toxic effects are primarily on liver and kidney [5, 19, 20]. It is known that in case of exhaustion of glutathione stores (a member of antioxidant system), excessively produced NAPQI (a metabolite of APAP) leads to hepatic necrosis, oxidative stress and lipid peroxidation by binding intracellular proteins [19]. In our study, consistent with these data, sinusoidal dilatations, hemorrhagic foci and a structure progressing to necrosis were observed in the livers of APAP group by the help of H-E staining. At the doses given, an increase was seen in iNOS expression of APAP. When renal tissues were examined, granular degeneration in proximal convoluted tubule; luminal dilatations in distal tubules; hypertrophy and obviously increasing iNOS expression in renal particles were established. In our study, similar to other studies carried out by Carroll, Kumar, Blecharz, subcutaneous APAP of 50 mg/kg, 5 days a week for 8 weeks long was applied as a "chronic Paracetamol application model" [21–24].

Detailed information in literature related to utilization of APAP along with statin is not available; except a study carried out with rats in 1990 [13] and a case report [4]. In the subject rat study, Atorvastatin of 30 mg/kg was given three times a week (i.e. 90 mg/kg/weekly) and APAP of 0.75% was given 13 weeks long [13]. This is the first and only animal study that demonstrates the statin-APAP interaction. The case reported in Lithuania, a person had taken APAP in addition to Simvastatin, Ramipril, Metoprolol and Aspirin and referred to hospital with the complaints of increase in hepatic function tests and jaundice. Individual recovered after Simvastatin and APAP discontinued and it was suggested that the interaction between two drugs was effective in clinic of patient [4].

Like Leite et al., in our study, we did not select absolutely toxic doses of RSV; we just wanted to reveal the effect caused by selected dose of 10 mg/kg during application [25]. Similarly, we avoided to create an APAP poisoning model by applying APAP of 50 mg/kg 5 days a week and taking a break for 2 days. In APAP-statin combination therapy, liver granular vacuolar degeneration, sinusoidal dilatation, hemorrhagic focus were more prominent than the drugs given alone and iNOS expression were similar. In kidneys, we determined the presence of granular degeneration in proximal and distal convoluted tubules, an increase in the luminal width, hemorrhagic foci and extremely increased inflammation (that can be identified by hypertrophy and iNOS) in renal particles.

In our study group, we could not find a distinct difference either in groups where RSV and APAP were used alone or where RSV + APAP were used together, in terms of ALT and AST, marker of live cellular damage, compared to control group. Therefore, it was thought in our study that these doses of drugs could not cause hepatic injury to affect liver function tests in serum. The decline seen in serum albumin levels by combination of two drugs demonstrated that the synthesizing function of the liver has decreased (Table 1). Additionally, histopathologic alterations were observed in the livers of all study groups; slight histopathologic alterations in RSV group and more distinctive histopathologic alterations in RSV + APAP group. In case these two drugs, known to be metabolized through liver, are used together, even though there are no functional changes reflecting on biochemical tests; injury initiates histopathologically, hepatic damage will show

up on laboratory results in the late term. So, even though periodic routine biochemical examinations are normal, particularly statins may have side effects, we must avoid using them together with an additional medication such as APAP.

Nitric oxide (NO) is a compound that possesses high reactive oxidant capacity and can be produced by nitric oxide synthase (NOS) enzyme which can be induced from L-arginine amino acid in parenchymal cells and other non-parenchymal cells in liver. Excessive amount of NO produced in liver is thought to be a significant role in other models related to inflammatory reaction and damage in liver and endotoxic shock. In this study it was demonstrated through immune histochemical staining that APAP and RSV increase iNOS activity which is the indicator of oxidative stress in liver and kidney. In the group where APAP and RSV are used together, iNOS activity was more apparent. In a way that supports this, we scrutinized the nitrate and nitrite levels (end products of nitric oxide) in serum and found that it was tend to increase in drug groups. NO levels were 5.06 ± 2.99 μmol/mL in RSV group, 4.18 ± 2.16 μmol/mL in APAP group, 4.50 ± 2.37 μmol/mL in RSV + APAP group; they were higher than control group (3.11 ± 1.52). Since APAP and statin groups are frequently used by patients in daily life with or without prescription, our main objective was to investigate the harmful effects of these drugs on liver and kidney when used together. Some studies suggest that statins are molecules that have antioxidant features on different tissues [8, 26, 27], however, these defined characteristics do not prevent statins' side effects to occur. Moreover, considering that the most important treatment regime for APAP poisoning is antioxidant therapy [19] this characteristic suggested for statins has not been sufficient in preventing toxicity of APAP.

It is well known that, these medicines are the metabolites of different CYP enzyme systems and assuming that at some point, this molecular mechanism might protect the liver from serious drug interactions. But, focusing on (anti)oxidant and cytokine pathways rather than CPY enzyme analyses in this study has revealed that the injury of liver and kidney are inevitable; the effects in combination treatment were the most.

Conclusion

APAP and statin groups are frequently used by patients in daily life with or without prescription. Our study is presented some evidence that Statin-Paracetamol interaction may occur actually as an important drug interactions in clinic; therefore further monitoring of the patients and avoiding of polypharmacy should be the main precautions for rational drug therapy.

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