Clinical and Experimental Health Sciences

Naringenin Reduces Hepatic Inflammation and Apoptosis Induced by Vancomycin in Rats

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ABSTRACT

Objective: This investigation aimed to detect the possible protective impacts of naringenin (NAR) on vancomycin (VCM)-induced liver toxicity through measuring caspase-3, – 8 and – 9 activities as markers of apoptosis and the levels of tumor necrosis factor-alpha, cyclooxygenase-2 and vascular endothelial growth factor as inflammation markers and assessing the histopathological alterations in rats.

Methods: The rats were allocated into seven groups as, the control group (saline, intraperitoneally (i.p.)), VCM group (400 mg/kg/day, i.p.), carboxymethyl cellulose (CMC) group (0.5%, orally), NAR100 group (100 mg/kg/day, orally), VCM+NAR25 group (25 mg/kg/day, orally), VCM+NAR50 group (50 mg/kg/day, orally), VCM+NAR100 group (100 mg/kg/day, orally). The caspase enzyme activities and inflammation markers were measured using colorimetric methods and ELISA, respectively. Histopathological examinations were performed.

Results: The caspase activities and levels of inflammation markers were significantly higher in the VCM group as opposed to the other groups. The caspase activities were significantly ameliorated in the VCM+NAR25 group compared to the VCM+NAR50 and VCM+NAR100 groups, but the levels of inflammation markers were significantly reduced in the VCM+NAR50 group and, especially, the VCM+NAR100 group compared to the VCM+NAR25 group.

Conclusion: NAR has potential protective impact on liver injury caused by VCM, and the protective impacts of NAR at distinct doses may occur via different molecular mechanisms.

Keywords: Vancomycin, naringenin, liver, apoptosis, inflammation

1. INTRODUCTION

Drug-induced hepatotoxicity is the most frequent reason for acute liver failure. It constitutes approximately 10% of acute liver failure worldwide and approximately 40-50% of all cases of liver damage (1). Furthermore, antibiotic-induced hepatotoxicity accounts for 25-45% of drug-induced liver injuries (2). Vancomycin (VCM) is a glycopeptide antibiotic which is mostly employed to cure aerobic and anaerobic gram-positive bacteria, which include methicillin-resistant *Staphylococcus aureus* infections (3). Approximately 90% of VCM is eliminated with glomerular filtration (4), thus, its major side effect is nephrotoxicity (5). Also, VCM has been reported to have side effects on the liver that restrict its therapeutic use in patients with impaired liver function (6), and sufficient evidence has been found that potential VCM causes idiosyncratic hepatotoxicity and a type of hepatocellular lesion (7).

VCM–induced nephrotoxicity has been reported extensively, but its mechanism is still not exactly known (5). Lately, studies on animal model have revealed that apoptotic cell death, oxidative stress and inflammation can conduce to the pathogenesis of VCM-induced nephrotoxicity (3,5). Inflammation and oxidative stress are important factors that affect the progression of renal injury. These factors also play a significant part in the progression of liver injury. The liver is the most frequently targeted organ in drugs-induced damages (2) and is an organ that is majorly attacked by reactive oxygen species (ROS) (8). In the event of the excessive formation of ROS by various factors, redox homeostasis is impaired which leads to oxidative stress. Oxidative stress and inflammation in hepatocyte cause mitochondrial dysfunction and permeability and induce cell death via necrotic and/or apoptotic mechanisms, which in turn leads to cellular and tissue injuries (8,9). Little is known on the mechanism of VCM-induced hepatotoxicity, however, it is thought that these factors might play a role in liver injuries.

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Flavonoids, which are naturally occurring substances, have various therapeutic uses and pharmacological impacts and are significant sources of medicine worldwide. Some flavonoids owing to their phenolic structures have antioxidant activity and prevent processes related to free radical, and thus flavonoids, which are antioxidants, have the properties that can improve apoptosis (5). Naringenin (4',5,7-trihydroxyflavanone, NAR) is a flavonoid that is present in vegetables, flowers, leaves, seeds, fruits, plants bark etc. (10). NAR has biological properties such as pharmacologically potent antioxidant, anti-inflammatory, nephroprotective, antimutagenic, antinitrosative, antifibrogenic, neuroprotective, antiatherogenic, anticarcinogenic and antitumor activities (5,11). NAR has also very specific hepatoprotective properties (12). NAR has been reported to have protective effects against liver damage resulting from cadmium (5), ethanol (11), carbon tetrachloride (CCl_4) (13), arsenic (14), lead (15) in rats and acetaminophen (16) in mice. Previous studies have shown that NAR has a preventive effect against apoptosis, inflammation and oxidative stress caused by drugs or chemicals in liver tissues (9-11,13). Therefore, it can be stated that NAR may have potential protective effect on VCM-induced liver injuries. Nonetheless, as far as we know, there are no prior studies conducted on the protective impact of NAR on apoptosis and inflammation caused by VCM in the livers of rats.

This study aimed to clarify whether VCM induced apoptosis and inflammation in the livers of rats and to detect possible protective impact of NAR on VCM-induced liver toxicity through measuring caspase-3, – 8 and – 9 activities as markers of apoptosis and the levels of cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF-α) as inflammation markers and assessing histopathological alterations.

2. METHODS

2.1. Animals

Animals were purchased from the Laboratory of the Animal Production Unit of Mersin University, Mersin, Turkey. Forty-nine adult male Wistar albino rats that weighed between 180 and 250 g were employed for the experiment. The rats were maintained at standard laboratory conditions, under cycles of 12 hours of light and 12 hours of dark with relative humidity 55±8 % and temperature of 25±2 °C. The animals had a standard diet and access to drinking water ad libitum. The current investigation was endorsed by the Animal Experiments Local Ethics Committee of Mersin University, Turkey with the ethical approval number of 2016/HADYEK/E.98180, 2016/21.

2.2. Chemicals

NAR was supplied from Sigma-Aldrich Chemistry (St. Louis, MO, USA) and VCM was obtained from Kocak Farma (Istanbul, Turkey). All other chemicals were of analytical grade. The chemicals used in the experiment were prepared fresh daily.

2.3. Experimental Design

NAR and VCM doses were chosen according to previous studies (9,10,17-19). 49 rats were randomly assigned to one of the seven groups, each of the group consisted of seven rats. The experimental plan of the investigation is schematized in Figure 1 and illustrated in Table 1. The control and VCM groups were taken from our previous study (20).

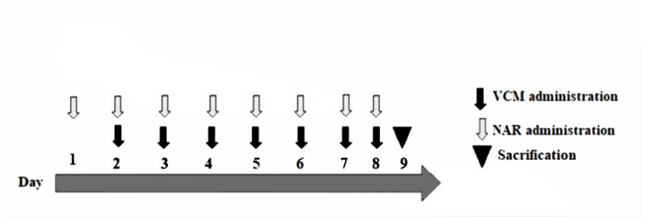

Figure 1. *Schematic presentation of the experimental plan of the study*

Table 1. *The experimental plan of the study*

Group	n	Treatment regimen
Control[a]	7	Saline administration was applied intraperitoneally (i.p.) once a day along 8 days
CMC	7	Carboxymethyl cellulose (0.5%) was administered via gavage once a day along 8 days
VCM[a]	7	VCM administration was applied at a dose of 400 mg/kg/day i.p. once a day at 24 hours intervals along 7 days
NAR100	7	NAR dispersed in CMC was applied orally at dose of 100 mg/kg once a day along 8-day
VCM+NAR25[b]	7	NAR25 administration was applied orally at a dose of 25 mg/kg once a day along 8-day. One day after the initial administration of NAR, administration of VCM (400 mg/kg/day) was initiated and proceeded along 7 days.
VCM+NAR50[b]	7	NAR50 administration was applied orally at a dose of 50 mg/kg once a day along 8-day. A day after the initial administration of NAR, VCM administration (400 mg/kg/day) was initiated and proceeded along 7 days.
VCM+NAR100[b]	7	NAR100 administration was applied orally at a dose of 100 mg/kg once a day along 8-day. A day after the initial administration of NAR, VCM administration (400 mg/kg/day) was initiated and proceeded along 7 days.

[a]*Control and VCM group were taken from our previous study (20);* [b]*VCM was administered 1 h after NAR administration; VCM: vancomycin; CMC: carboxymethyl cellulose; NAR: naringenin; VCM+NAR: VCM+NAR administered group.*

On the 9th day of the experiment and 24 hours after receiving the last dose, all of the rats were sacrificed under xylazine hydrochloride (10 mg/kg i.p.) and ketamine hydrochloride (30 mg/kg i.p.) anesthesia. The liver tissues were promptly excised and stored at –20 ^{0}C for the biochemical analyses and histopathological examinations.

2.4. Biochemical Studies

2.4.1. Tissue homogenization

The homogenization of rat livers was performed in an ice-cold lysis buffer. The centrifugation of homogenates was made for 10 minutes at 14 000 g at 4 °C, and the resulting supernatants were used to study the selected biochemical parameters.

2.4.2. Determination of the activities of the caspase-3, – 8, and – 9 enzymes

The activities of caspase-3, – 8 and – 9 enzymes were assessed using Colorimetric Test Kits (BioVision Research Product, Mountain View, CA, USA) in line with manufacturer's directions. The protein levels were detected by Lowry test (21). Accordingly, the appropriate volume of dilution buffer was added to 50 µg of protein and the protein was diluted to 50 µL. Caspase – 3, – 8 and – 9 assay kits were used for colorimetric analysis to determine chromophore *p*-nitroanilide (*p*NA) after separation from the labeled substrate. The caspase – 3, – 8 and – 9 enzymes' substrates were Asp-Glu-Val-Asp (DEVD)-*p*NA, Ile–Glu–Thr–Asp (IETD)-*p*NA and Leu–Glu–His–Asp (LEHD)-*p*NA, respectively. A microplate reader at 405 nm was used to measure pNA light emission.

2.4.3. Determination of hepatic TNF-α, COX-2 and VEGF levels

Measurement of COX-2, TNF-α and VEGF levels were done using enzyme-linked immunosorbent assay kits (ELISA kits, Sunred Biological Technology, Shanghai, China) in line with the manufacturer's directions. The intensity of the color of the samples was determined for TNF-α, COX-2, and VEGF at 450 nm with the ELISA plate reader. The values were demonstrated as ng/mg protein.

2.5. Histopathological Study

Liver tissues from each rat were removed and weighted. The tissues were cut into 1 cm^3 pieces and fixed in a 10% neutral buffered formalin solution for 48 hrs. Upon fixation, the samples were employed routinely, embedded in paraffin and cut at 5 µm on a rotary microtome. The cut sections were then mounted on glass slides, stained with hematoxylin and eosin (H&E) and Masson's Trichrome, and visualized under the light microscope (Olympus BX50, Olympus GmBH Tokyo, JAPAN) for histopathological evaluations. The histopathological studies were conducted by two histopathologists and the grading was done according to a semiquantitative scale: (–) no significant histopathological injury, (+) mild degree of injury, (++) moderate degree of injury and (+++) severe degree of injury. The gradings were done according to the following parameters: nuclear pleomorphism, inflammation, pyknosis, capsule thickening, fibrosis, necrosis, vacuolization, and sinusoidal dilation (22,23).

2.6. Statistical Analysis

Statistical analyses were done using SPSS Version 25.0 statistical software package. The values were represented as means ± standard deviation (SD). Comparisons of the caspase activities and the levels of inflammation markers among the groups were made using One-way analysis of variance (ANOVA) with Tukey's *post hoc* test and Mann Whitney U test or Kruskal Wallis H test, respectively. Statistically significant was considered when a *p* value was below 0.05.

3. RESULTS

3.1. Caspase Enzyme Activities

The alterations in the hepatic caspase enzymes activities were shown in Figure 2. The caspase-3 and – 9 enzyme activities were detected to be significantly more elevated in the NAR100, VCM, VCM+NAR (50 and 100) groups as opposed the control group ($p<0.05$). The caspase-3 and – 9 activities in the CMC and VCM+NAR25 groups were detected to be significantly more reduced than those in the VCM group ($p<0.05$). Furthermore, statistically reduced caspase-3 and – 9 activities were detected in the VCM+NAR25 group when compared with the NAR100 and VCM+NAR (50 and 100) groups ($p<0.05$). Caspase-8 activity was significantly more elevated in the VCM group as opposed to the control, CMC, NAR100, VCM+NAR25 groups ($p<0.05$). Caspase-3, – 8 and – 9 activities were significantly more reduced in the CMC group versus the VCM and VCM+NAR (50 and 100) groups. These findings revealed that the hepatic activities of caspase-3, – 8, and – 9 caused by VCM were significantly reduced by NAR25 administration.

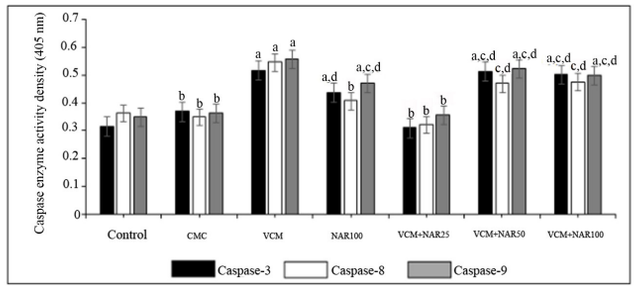

Figure 2. *Impact of NAR against hepatic activities of caspase-3, – 8, and – 9 caused by VCM. Values are mean ± SD; [a]p< 0.05 vs. control group; [b] p< 0.05 vs. VCM group;, [c]p< 0.05 vs. CMC group; [d]p<0.05 vs. VCM+NAR25 group; NAR: naringenin; VCM: vancomycin; SD: standard deviation; CMC: carboxymethyl cellulose.*

3.2. Hepatic TNF-α, COX-2 and VEGF Levels

As seen in Figure 3, the hepatic COX-2, TNF-α and VEGF levels were significantly more elevated in the VCM group as opposed to the control group (p<0.05). The hepatic levels of TNF-α were significantly more elevated in the VCM group than in the CMC, NAR100, VCM+NAR (25, 50 and 100) groups (p<0.05). However, no significant difference was noted among the VCM+NAR groups (p>0.05). Also, the COX-2 levels in the

VCM group were also significantly more elevated compared to the CMC, NAR100 and VCM+NAR (50 and 100) groups ($p<0.05$). The levels of COX-2 were significantly lower in the VCM+NAR100 group as opposed to the VCM and VCM+NAR25 groups ($p<0.05$). Interestingly, no significant difference was noted between the VCM+NAR50 and both the VCM+NAR25 and VCM+NAR100 groups in terms of COX-2 levels. The hepatic VEGF levels were significantly higher in the VCM group as opposed to the CMC, NAR100 and VCM+NAR100 groups ($p<0.05$). Among the VCM+NAR groups, the VEGF levels in the VCM+NAR100 were significantly lower in comparison to the VCM+NAR25 and VCM+NAR50 groups ($p<0.05$). No important difference was noted between the VCM+NAR25 and VCM+NAR50 groups in terms of VEGF levels ($p>0.05$). These results indicated that increased hepatic TNF-α, COX-2 and VEGF levels caused by VCM were significantly decreased by NAR treatments, particularly NAR100 mg.

3.3. Histopathological Examination

The liver tissues were evaluated according to the determined histopathological parameters, namely vacuolization, nuclear pleomorphism, sinusoidal dilatation, pyknosis, inflammation, necrosis, capsule thickening and fibrosis (22,23). As shown in Table 2 and Figures 4-6, vacuolization, pyknosis, nuclear pleomorphism, and sinusoidal dilatation were not observed in the study groups. Furthermore, no histopathological changes were determined in the control and CMC groups. However, fibrosis was mildly observed in the VCM, VCM+NAR (25, 50, and 100) and NAR100 groups. Necrosis and capsule thickening were mildly detected in the VCM, VCM+NAR50 and VCM+NAR100 groups, while mild inflammation was detected in the VCM and VCM+NAR50 groups. These findings showed that VCM caused histopathological damages, even if they were not severe in rat liver, and that the damages were ameliorated by the administration of NAR25 mg.

Table 2. *Scores of histopathological alterations observed in study groups*

Histopathological parameters	Mean Score						
	Control	CMC	VCM	NAR100	VCM+NAR25	VCM+NAR50	VCM+NAR100
Vacuolization	-	-	-	-	-	-	-
Nuclear pleomorphism	-	-	-	-	-	-	-
Sinusoidal dilatation	-	-	-	-	-	-	-
Pyknosis	-	-	-	-	-	-	-
Inflammation	-	-	+	-	-	+	-
Necrosis	-	-	+	-	-	+	+
Capsule thickening	-	-	+	-	-	+	+
Fibrosis	-	-	+	+	+	+	+

+++: severe; ++: moderate; +: mild; – : none. VCM: vancomycin, CMC: carboxymethyl cellulose, NAR: naringenin, VCM+NAR: VCM+NAR administered group.

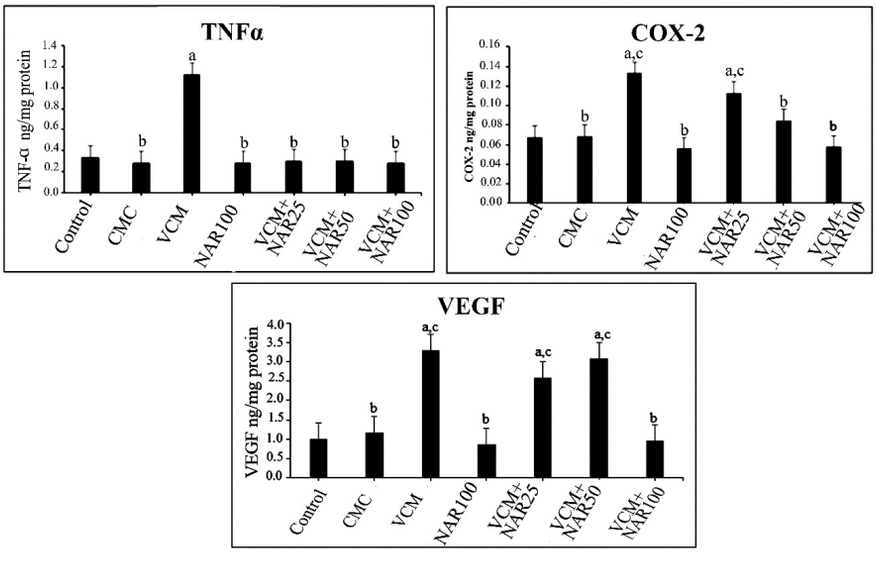

Figure 3. *Impact of NAR against hepatic TNF-α, COX-2 and VEGF levels induced by VCM. Values are mean ± SD; [a]p<0.05 vs. control group; [b]p<0.05 vs. VCM group; [c]p<0.05 vs. VCM+NAR100 group; NAR: naringenin; TNF-α: tumor necrosis factor-alpha; COX-2: cyclooxygenase-2; VEGF: vascular endothelial growth factor; VCM: vancomycin; SD: standard deviation.*

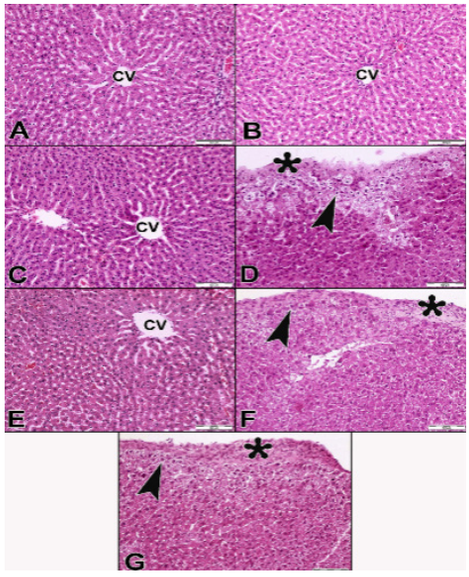

Figure 4. *Representative photomicrographs of H&E-stained liver sections. Control group (A), CMC group (B), NAR100 group (C), VCM group (D), VCM+NAR25 group (E), VCM+NAR50 group (F), VCM+NAR100 group (G); central vein (CV), necrotic cells with cytoplasmic vacuoles (black arrowhead), capsule thickening (black asterisk) (X100, scale bar 100 µm); CMC: carboxymethyl cellulose; NAR: naringenin; VCM: vancomycin.*

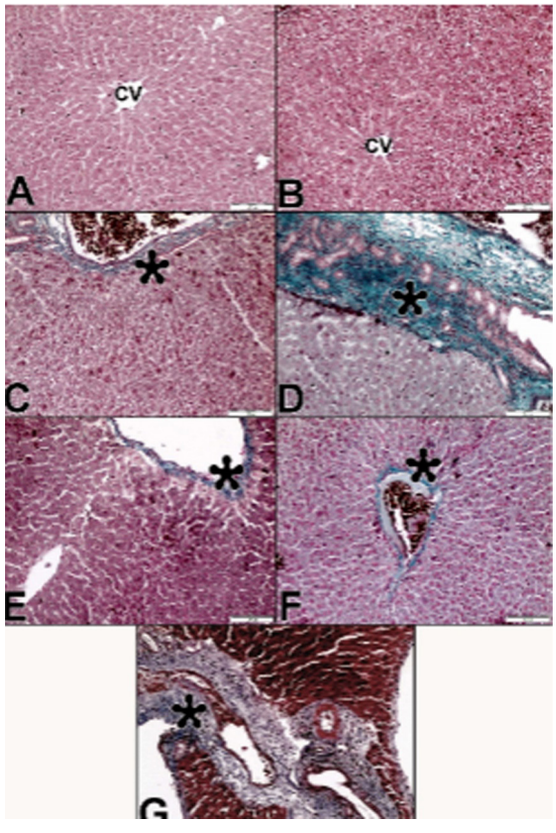

Figure 5. *Representative photomicrographs of Masson's trichrome-stained liver sections. Control group (A), CMC group (B), NAR100 group (C), VCM group (D), VCM+NAR25 group (E), VCM+NAR50 group (F), VCM+NAR100 group (G); central vein (CV), fibrosis (asterisk) (X100, scale bar 100 µm); CMC: carboxymethyl cellulose; NAR: naringenin; VCM: vancomycin.*

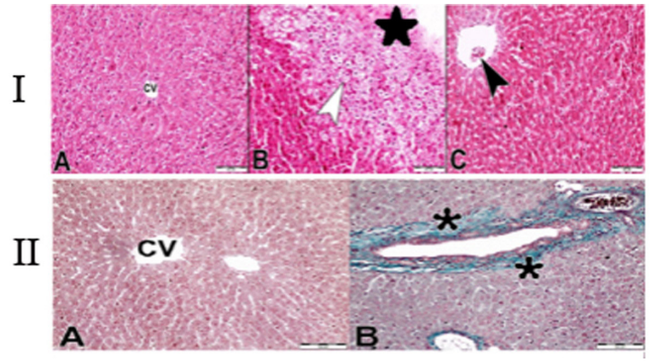

Figure 6. *H&E (I) and Masson's trichrome (II) staining in control and VCM groups. Control group (A), VCM group (B and C); central vein (CV), necrotic cells with cytoplasmic vacuoles (white arrowhead), capsule thickening (black star), cell debris spilling into vein lumen (black arrowhead), fibrosis (asterisk). A, B and C X100; VCM: vancomycin.*

4. DISCUSSION

The possible protective impacts of NAR were surveyed by measuring caspase-3, – 8 and – 9 activities as markers of apoptosis and levels of COX-2, TNF-α and VEGF as inflammation markers, and by examining the histopathological alterations. In this study, it was determined that the protective impact of NAR on apoptosis and inflammation occurred at its different doses. This investigation is the first study to show the potential protective impacts of NAR on VCM-induced apoptosis and inflammation in the livers of rats. This study is important as it provided valuable data on the protective mechanism of NAR.

A variety of inflammatory, toxic and metabolic insults lead to liver injury and disease through the activation of apoptotic and/or necrotic cell death (24). Apoptosis is a primary factor in numerous liver diseases and injuries (24) and is induced via two pathways, which are the extrinsic (death receptor pathway) and the intrinsic (mitochondrial pathway) pathways, and the increased activities of caspase-8 and – 9, which are initiator caspases, stimulate the extrinsic and intrinsic pathways of apoptosis, respectively and activate caspase-3 that is a primary executioner caspase in apoptosis (5). Very little is known about the mechanism of VCM-induced liver injuries and caspase-dependent apoptosis caused by VCM in the liver tissue. In this study, it was determined that the hepatic activities of caspase-3, – 8, and-9 were significantly more elevated in the VCM group as opposed to the control group ($p<0.05$). Additionally, the activities of caspase-3, – 8 and – 9 enzymes in the VCM+NAR25 group were significantly lower compared to the VCM group ($p<0.05$). Therefore, it can be concluded that NAR25 significantly inhibits the activities of caspase-3, – 8 and – 9 enzymes induced by VCM.

Flavonoids are known to indicate both pro-oxidant and antioxidant activity based on their high concentrations, polyphenolic structure and type of cell and exhibit cytotoxic activities at comparatively high doses in the micromolar concentration range (25). Flavonoids at high concentrations

have been shown to able to be produced ROS via auto-oxidation. ROS are the main signaling molecules that modulate cell death. The findings of the current study revealed that a 25 mg dose of NAR had more potential inhibitory impacts on VCM-induced caspase activity in liver tissue compared to 50 and 100 mg doses of NAR. This might be due to NAR at high concentration displaying cytotoxic activity and the generation of ROS as a result of their pro-oxidant activity. NAR25 significantly reduced the increase in caspase activities in the liver tissues.

Inflammation plays a significant part in the progression of liver injury. There is little information about how VCM causes inflammation in the liver tissue. TNF-α is an extremely pleiotropic cytokine that induces diverse biological effects, such as necrotic and/or apoptotic cell death, inflammatory responses, metabolic activation and cell proliferation (26). It plays a significant part in toxic liver damage. In this study, it was revealed that TNF-α levels were significantly more elevated in the VCM group versus the control group, while TNF-α levels in the VCM+NAR (25, 50 and 100) groups were significantly lower compared to the VCM group. No statistically significant difference was noted between the VCM+NAR25, VCM+NAR50 and VCM+NAR100 groups. Depending on the results, it can be concluded that NAR can reduce the levels of TNF-α. Similar to the results of this study, several studies have also reported that NAR can decrease proinflammatory cytokines like IL-6, IL-1β and TNF-α with the suppression of NF-κB, which is a signal transduction pathway promoting the transcription of gene coding for proinflammatory proteins (27,28).

COX-2 is another significant inflammatory mediator due to its rate-limiting synthesis of the precursors of thromboxanes and prostaglandins (29). Increased COX-2 levels cause eicosanoid production in high concentrations by initiating the COX-prostanoid pathway leading to necrosis and cellular inflammation (29). The COX-2 gene, which is mostly expressed in Kupffer cells, is expressed in response to various cytokines and proinflammatory agents. Also, COX-2 has a relationship with liver pathogenesis that includes cancer and fibrosis (30). In this study, it was revealed that the levels of COX-2 were significantly more elevated in the VCM group as opposed to the control group (p<0.05). COX-2 levels were significantly more reduced in the VCM+NAR50 and VCM+NAR100 groups as opposed to the VCM group (p<0.05). However, in terms of COX-2 levels, there was no significant distinction between the VCM+NAR50 group (36.1% reduction) and VCM+NAR100 (56.4% reduction) or VCM+NAR25 (15% reduction), but a significant distinction was between the VCM+NAR100 and VCM+NAR25 groups. Therefore, it can be concluded that dosages of 50 mg and, especially, 100 mg NAR effectively reduce the levels of COX-2 induced by VCM (Figure 3). In the study by Jayaraman *et al.* (31), by virtue of its anti-inflammatory effects, NAR (50 mg/kg) was reported to inhibit several inflammatory mediators such as COX-2, TNF-α and NF-κB, thereby contributing to the treatment of liver damage caused by exposure to ethanol. Esmaeili and Alilou (13) also reported that NAR (50 mg/kg) attenuated liver inflammation by downregulating the CCl_4-induced activation of nitric oxide synthase (iNOS), TNF-α and COX-2 at protein and mRNA levels in rats. Besides, Dong *et al.* (32) also administered 30, 60 and 120 mg/kg of naringin, the aglycone of which is naringenin, doses to CCI4-treated mice and revealed that NAR at a dose of 120 mg/kg sharply downregulated the expressions of TNF-α, COX-2, NF-κB, IL-6, IL-1β, HMGB-1, AP-1, iNOS and TLR4. These previous results are in agreement with the findings of this study.

VEGF, which is a signal protein stimulating angiogenesis, acts as a proinflammatory cytokine by improving endothelial permeability in vivo at nanomolar concentrations, inducing the expression of endothelial cell adhesion molecules and by acting as a monocyte chemoattractant (33). Liver VEGF is mostly present in hepatocytes and endothelial cells with the VEGF receptors (34) and the production of VEGF can also be induced by COX-2. The findings of this report revealed that the levels of VEGF were significantly higher in the VCM group as opposed to the control group (p<0.05). However, the VEGF levels are significantly lower in the VCM+NAR100 group versus the VCM group and also the VCM+NAR (25, 50 and 100) groups (p<0.05). Therefore, it can be concluded that a dose of 100 mg of NAR was more effective in increasing the levels of VEGF caused by VCM than lower doses (25, 50 mg).

In this study, it was determined that a dose of 25 mg of NAR was more effective for caspase-dependent apoptosis than higher doses (50 and 100 mg) while 50 mg and, especially, 100 mg of NAR was more effective for inflammation than lower dose (25 mg). The preventive effects of NAR on apoptosis and inflammation occurred at different doses. This is an extremely interesting result, and it may suggest that caspase-dependent apoptosis and inflammation are caused by the different mechanisms in distinct doses of NAR.

The extrinsic pathway of apoptosis is activated by the binding of the death ligand to death receptors on the plasma membrane. Death ligands belong to the TNF superfamily, involving TNF-related apoptosis that induce ligand (TRAIL), TNF-α and Fas ligand (FasL) (35) and the most well-known death receptors are TNFR-1 and Fas (CD95), which are abundant in the liver. For the extrinsic pathway of NAR, it can be stated that Fas is activated by FasL, which subsequently binds to Fas-associated protein with death domain (FADD), and that the Fas-FADD complex activates procaspase 8, which subsequently activates other caspases, causing apoptosis (12). The Fas/FasL interaction is already known to be a significant initiator of apoptosis via the extrinsic pathway, which can trigger the caspase cascades in liver damage (32). Therefore, in this study, although a dose of 25 mg of NAR had potential prevention effects on caspase activities, it might be suggested that higher NAR doses (50 and 100 mg) induce the extrinsic pathway of apoptosis via FasL instead of TNF-α.

In the intrinsic pathway of apoptosis, the administration of VCM leads to ROS generation, and higher doses of NAR can act as pro-oxidants. This may also increase cells' oxidative status. ROS are the main signal molecules modulating cell death and increased ROS can induce apoptosis. The

antioxidant and/or pro-oxidant effects of NAR may vary depending on the flavonoid concentration, the model used and the radical formed (12). For the reason, higher doses of NAR, unlike lower dose, may have induced apoptosis.

However, higher doses of NAR may have inhibited inflammation by different mechanisms. NAR is known to suppress diverse NF-kB-regulated gene products involving COX-2, VEGF, matrix metalloproteinase-2 and – 9 (36). Hernández-Aquino *et al.* (37) examined the molecular mechanisms involved in the hepatoprotective impacts of NAR (100 mg/kg body weight, p.o. per day) on liver fibrosis induced by CCl_4. They reported that CCl_4-treated rats performed elevated IL-1, IL-10 and NF-κB protein levels, yet concomitant administration of NAR and CCl_4 precluded these increases. NAR suppresses NF-κB through the downregulation of toll-like receptor 2 (TLR2) and TLR4 protein and mRNA levels and the reduced translocation and DNA binding of NF-κB. This causes the suppression of the expression of NF-κB dependent interleukins, like IL-1 and IL-10, and accordingly, prevents necrosis. In a study carried out by Yilma *et al.* (38), NAR was demonstrated to suppress TLR2 and 4 signaling, giving rise to the attenuation of neuroinflammation induced by pathogen.

The activated TLRs are known to trigger various liver cells and lead to the release of cytokines facilitating the progression of liver disease (39). Polyphenols have been reported to decrease inflammation by TLR4 signaling pathway modulation (40). NAR is a polyphenol, and it could be stated that NAR at higher doses can reduce inflammation by inhibiting the signaling pathway of TLRs.

Histopathological examinations revealed mild necrosis, inflammation, capsule thickening, and fibrosis were observed in the VCM group. In the study by Bruniera *et al.* (4), the effect of distinct VCM dilutions on liver, kidney and endothelial damage was investigated by histopathological analysis and biochemical parameters. They reported changes in alanine aminotransferase (ALT) that featured hepatotoxicity and reported that the animal groups that were treated for 3 days (infusion of VCM – 10mg/kg/day – in dilutions of saline 5 mg/ml and 10 mg/ml) demonstrated minor changes in the histopathological examinations, which are similar to the results of this study. Also, they suggested that morphological changes might be observed in some cases, and that but the commonly used and proposed markers did not aid in the monitoring of toxic effects of VCM. The main elimination pathway of VCM is the kidney, on which VCM has a major side effect as it tends to accumulate there (41). Therefore, biochemical and histopathological damages caused by VCM can easily be observed in renal tissues. However, approximately 5 to 8.5% of VCM clearance is extra-renal, possibly through hepatic conjugation, leading to VCM crystalline degeneration products (42). Accordingly, the biochemical effects may be clearly observed rather than histopathological effects of VCM in liver tissue. Thus, routine continuous monitoring of hepatic events among patients receiving VCM is recommended (43).

5. CONCLUSION

This study performed that the administration of VCM was able to cause caspase-dependent apoptosis by increasing the activities of caspase – 3, – 8 and – 9 and inflammation by inducing TNF-a, COX-2 and VEGF. Furthermore, the results demonstrated that the administration of NAR at different doses attenuated the liver injuries induced by VCM by decreasing the activities of caspases and the levels of inflammation markers. Consequently, NAR has potential protective impacts on liver injury caused by VCM, and the protective impacts of NAR at distinct doses may occur via different molecular mechanisms.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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