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ORIGINAL ARTICLE

Ethanol Consumption Promotes TNF-α Signaling Pathway in Rat Kidney: Rescue Effect of Curcumin

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KEYWORDS

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ABSTRACT: Tumor necrosis factor-α (TNFα) has several biological effects, including cell death, cell apoptosis and proliferation, and differentiation, as well as immune modulation. We characterized the alteration in TNF-α and the key receptors and molecular mediators related to TNF-α signaling pathway in the kidney after exposure to ethanol alone or in combination with curcumin (Cr). Accordingly, 24 male Wistar rats in 3 groups of control, ethanol, and Cr-treated -ethanolic groups were treated for six weeks. The ethanol group showed a significant elevation in TNF-α, nuclear factor-κB (NF-κB), and endothelin 1 (ET1) than the control group. TNF-α receptor 1 (TNFR1), TNF-α receptor 2 (TNFR2) and vascular endothelial growth factor receptor 2 (VEGFR2) were found with a significant down-regulation, and of TNF-receptor-associated factor 2 (TRAF2) and receptor-interacting protein-1 (RIP-1), and activator protein-1 (AP-1) were found with an up-regulation in the ethanol group than the control group. Cr and ethanol decreased the gene expression of TRAF-2, RIP-1 and AP-1, as well as increased the gene expression of TNFR1. Cr administration restored the increased levels of TNF-α, NF-κB and endothelin to these levels in the control group. Therefore, ethanol-related kidney injury addressed by our previous studies and others may in part be associated with the TNF-α signaling pathway, and such impacts can be rescued by Cr as an antioxidant and anti-inflammatory compound.

INTRODUCTION

Chronic ethanol exposure induces structural damages of the kidney[1], however, l the accurate molecular mechanisms of ethanol to damage to kidney is not clear. Recently, ethanol has been shown to cause tissue damage via inflammatory stress [2, 3]. Tumor necrosis factor-α (TNF-α) as a proinflammatory cytokine is mainly generated by immune cells, like macrophages and can stimulate the inflammatory mediators, like eicosanoids, interleukin-1 (IL-1), and platelet-activating factor [4]. TNF- α appears free in the plasma or is bound to circulating TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). It can attach to the type 1 and 2 transmembrane receptors originating from separate gene products[5]. During kidney inflammation, the

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expression of TNF-α and its receptors is observed in infiltrating leukocytes[3] as well as glomerular and tubular cells [6-8]. Most TNF receptor superfamily members and their signaling mediators, such as TNFR-associated factor 2 (TRAF2) and receptor-interacting protein kinases 1 (RIP-1), activate NF-κB and protein 1 (AP-1) in the TNF signaling pathway [9-11]. NF-κB can regulate the excretions of several genes associated with different biological processes, including inflammation and immune responses[12, 13]. NF-κB as a central mediator of mediator of signal transduction is activated via many inflammatory cytokines, including TNF-α and IL-1 [13]. In renal ischemia-reperfusion injury, TNF-α NF-κB-dependently is produced, and then TNF-α, attaches to its receptor for stimulating NF-κB activation, leading to a positive feedback for NF-κB regulation [14]. Overproduction of angiotensin II in renal injury activates NF-κB[15]. Therefore, NF-κB activation is crucial for inflammatory responses in renal injury[16]. Several plant-derived biologically active agents are effective in ethanol-related kidney tissue diseases[17, 18]. Of plant-derived active compounds, curcumin (Cr) is unique because of its anti-inflammatory effects [19, 20]. The inflammatory nature of the alcohol-induced abnormalities on the one hand, and the anti-inflammatory properties of Cr on the other hand, tempt us to investigate how the inflammatory pathway of TNF-α and its mediators changes in the kidney following alcohol consumption. We also assessed the possible inhibitory impacts of Cr on the inflammatory changes associated with this pathway.

Cr as an antioxidant and anti-inflammatory ingredient from *Curcuma longa* L, reveals many biological effects, like anticancer, anti-atherosclerotic, antimutagenic and immunomodulatory activities[21]. It was reported that, Cr ameliorates kidney injury against various nephrotoxic agents, such as gentamicin, cyclosporine, chloroquine, doxorubicin, and ischemia-reperfusion injury [22]. Huang et al reported that Cr could alleviate diabetic nephropathy through suppressing the SphK1-S1P signaling pathway activation of [23].

MATERIALS AND METHODS

Study design

The Principles of Laboratory Animal Care (NIH publication No. 85-23, revised, 1985) were followed and the experiments were confirmed by the Animal Care Committee, Urmia University of Medical Sciences. Male Wistar rats (n=24, 220±10 g) were assigned to the control, (2) ethanol, and (3) Cr-treated ethanolic group (n=8per group). The animals were kept under standard conditions with proper ventilation, temperature (22±2°C 1), under a 12:12 light-dark cycle. The ethanol group was treated with ethanol (4.5 g kg^{-1} body weight; Merck KGaA, Germany) diluted with tap water (20% w/v) daily through gavage for six weeks. The control group received tap water. The Cr -treated ethanolic group received Cr 40 mg kg^{-1} body weight via intragastric gavage plus their daily food routine and the same amount of ethanol. Six weeks later, anesthetization was done by2% xylazine (10 mg kg^{-1}, i.p.) and 10% ketamine (80 mg kg^{-1}, i.p.) and its depth was measured via pinching a hindpaw. The left kidney was excised and then washed with ice-cold saline for the removal of adventitial tissues, fat, and blood clots, and kept at -80 °C for gene expression tests.

Regarding total RNA isolation, kidney tissue (100 mg; from1/3 mid-part) was immersed in RiboxEX (1 ml; total RNA isolation solution) (GeneAll, South Korea) followed by homogenization in ice-cold extraction buffer (10% wt/vol), that included phosphate buffer (50 Mm; pH 7.4) with an Ultra Turrax homogenizer (T10B, Germany) and storing at −80°C until RNA isolation. For measuring TNF-α, endothelin, and NF-κB, different kidney parts were minced and were subjected to homogenization in ice-cold extraction buffer (10% wt/vol) that included phosphate buffer (50 mM; pH 7.4) through an Ultra Turrax homogenizer (T10B, Germany). Then, centrifugation of the homogenates was done (10,000×g; 4°C/ 20min (HERMLE, Germany). We employed the supernatant for TNF-α, endothelin, and NF-κB assessment.

TNF-α, endothelin and NF-κB measurement

Kidney TNF-a content was measured by a double-sandwich ELISA method and the ELISA kit (Bioassay Tech LAB, China**). In** Brief, monoclonal antibody (precoated antibody) and detecting antibody (polyclonal antibody) labeled as biotin were employed. Specimens and biotin labeling antibody were added to the wells of ELISA plate and washed using PBS. Next, ELISA wells were provided with Avidin- peroxidase conjugates in order. we applied TMB substrate for coloring following washing the reactant completely with PBS. The blue color of TMB was observed in peroxidase catalytic and changed into yellow affected by acid. There was a positive correlation between color depth and testing factors in specimens.

An enzyme-linked immunosorbent assay kit (Bioassay Tech LAB, China) was employed for measurinng the concentrations of endothelin (CAT NO.E0462Ra) and NF-κB (CAT NO.E0287Ra) in the kidney tissue. The ELISA procedure was carried out based on the manufacturer's instructions.

Total RNA extraction and quantitative real-time polymerase chain reaction (real-time PCR)

Gene expression was measured using real-time PCR. Through a kit (GeneAll; Cat no. 305-101), we extracted total RNA in the frozen kidney homogenate based on the produre's guidelines. RNA level was confirmed using spectrophotometric assessment of the absorbance at 260–280 nm and calculated with the combined Tris base, acetic acid, and EDTA (TAE) agarose gel electrophoresis. Reverse transcriptase (RT) was assessed by Hyperscript™ Reverse Transcriptase (GeneAll). RT-PCR was done by the amplification reagent kit (Ampliqon, Denmark) using the TCXPD XP-Cycler (USA) and TNFR1, TRAF2, RIP1, and AP-1 as well as the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. For amplifying the cDNA, the forward and reverse 5 ′ and 3′ primers of the TNFR1, TRAF2, RIP1, and AP-1 genes designated through the Gene Bank (http:// blast. ncbi.nlm.gov/Blast.cgi) indicated gene-specific primers. Also, the primers were approved by the Gene Runner software (Table 1). For amplifying cDNA-encoding GAPDH (a house-keeping gene) Primers were generated. The Real-Time PCR Master Mix Green kit (Ampliqon) was used for Real-time quantification of the targeted genes as instructed. In addition, genes expression was assessed by the iQ5 real-time PCR detection system (Bio-Rad, USA). We recorded a melting curve for verifying the amplification specificity. The samples were replicated three times and the threshold cycle (Ct) value was similar to the corresponding average. Using the $2^{-\Delta\Delta Ct}$ method, the mRNA relative fold expression was determined ($-\Delta\Delta Ct = \Delta Ct$ test sample $- \Delta Ct$ calibrator sample [24].

Statistical analysis

The Kolmogorov–Smirnov tested the normal distribution of data using SPSS 16.0. The one-way analysis of variance (ANOVA) and Tukey's post-hoc test were used to assess statistical differences between the groups and the findings are expressed as mean ± SD, and $p<0.05$ was regarded as statistically significant.

Table 1. Sequences of primers employed for evaluating the GAPDH, TNFR1, TRAF2, RIP1, and AP-1 expressions.

Target gene	Primer sequence	Product size, bp
GAPDH	Forward 5′-GTTGTGGATCTGACATGCCG-3′ Reverse 5′-GGTGGAAGAATGGGAGTTGC-3′	159
TNFR1	Forward5′-GAAACAGTCTGCGAGGTGTG-3′ Reverse5′-TTCTTCTTGCAGCCACACAC-3′	158
TNFR2	Forward 5′- GCAGGGAACACAGTCAACTC-3′ Reverse 5′- GCCTGGCTTCTACAATGAGG-3′	187
TRAF2	Forward5′-CATTCCTGCTGACTGAGTGC-3′ Reverse5′-CCTCGTAGTGTAGCTCCAGG-3′	153
RIP1	Forward5′-TGGTCTGCATAAACTCTGGGA-3′ Reverse5′-ACTTGCCACATCCTCTTCTACA-3′	197
AP-1	Forward5′-TCGTGAAGGACTGTGAGGAC-3′ Reverse5′-GTTGATGTCGTGGAGCTTGG-3	185
VEGFR2	Forward 5′- ACGGGGCAAGAGAAATGAAT-3′ Reverse 5′-TTCTCCTCCACAAAACCTGA-3′	159

TNFR1: Tumor necrosis factor receptor 1;GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; TRAF2: TNF receptor-associated factor 2; RIP1: Receptor-interacting protein 1; AP-1: Activator protein 1

RESULTS

Kidney tissue TNF-α, NF-κB and endothelin protein concentrations were measured using the ELISA technique (Figure 1A, B, and C). Based on Figure1A. ethanol exposure at 4.5 g kg^{-1} body weight for 42 consecutive days significantly increased TNF-α concentration in the kidney tissue compared with control group rats (658.9±14 vs. 240.25±5.1, $p<0.05$). Ethanolic rats treated with Cr significantly reversed the increases in the kidney TNF-α content (359.69±8.14 vs. 658.9±14, $p<0.05$). The NF-κB protein concentration in the renal tissue of controls was 193.56±7.6 ng ml^{-1}, while it was 263.59±6.5 ng ml^{-1} in the ethanol-exposed animals. The amount of NF-κB protein in the kidney tissue of ethanol-fed animals was found with a significant increase in comparison with the control tissues ($p<0.05$, Figure1B). Also, the ethanol-fed rats indicated a significant increase in endothelin concentration (38.75±3.2 ng ml^{-1}) in comparison with the control group (27.51±0.84 ng ml^{-1}) ($p<0.05$, Figure 1C). Treatment of ethanol-fed animals with Cr significantly decreased kidney tissue NF-κB ($p<0.05$) and endothelin ($p<0.05$) content. The real-time RT-PCR was performed for quantification of TNFR1, TNFR2, TRAF2, VEGFR2, RIP1, and AIP1 mRNA expression a (Figs.2). The mean TNFR1 and TNFR2 relative expression was 1.1±0.15 and 1.17±0.08 vs. 0.64±0.1 and 0.35±0.03-fold in the control and ethanol-fed animals, respectively. Based on Figure 2A and B, treatment with ethanol for 42 days caused a significant decrease in the TNFR1 and TNFR2 mRNA expression than the control animals ($p<0.05$). However, Cr treatment significantly increased the TNFR1 and TNFR2 mRNA expression than the ethanol-exposed animals ($p<0.05$). The real-time RT-PCR results of AP1, RIP1, TRAF2, and mRNA levels indicated that the mRNA expressions of these markers showed a significant increase in ethanol-fed animal kidney tissue compared with the control animals ($p<0.05$). These

results also illustrated that there was a significant decrease in gene expression of TRAF2, RIP1, and AIP1 in the group treated with Cr than the ethanol group ($p<0.05$, Figure 2 C,D,E and F respectively). According to Figure 2.F, the kidney expression of VEGFR2 significantly decreased in ethanol-fed animals, and no significant difference was found ($p<0.05$) in VEGFR2 between the ethanol group and the Cr -treated animals.

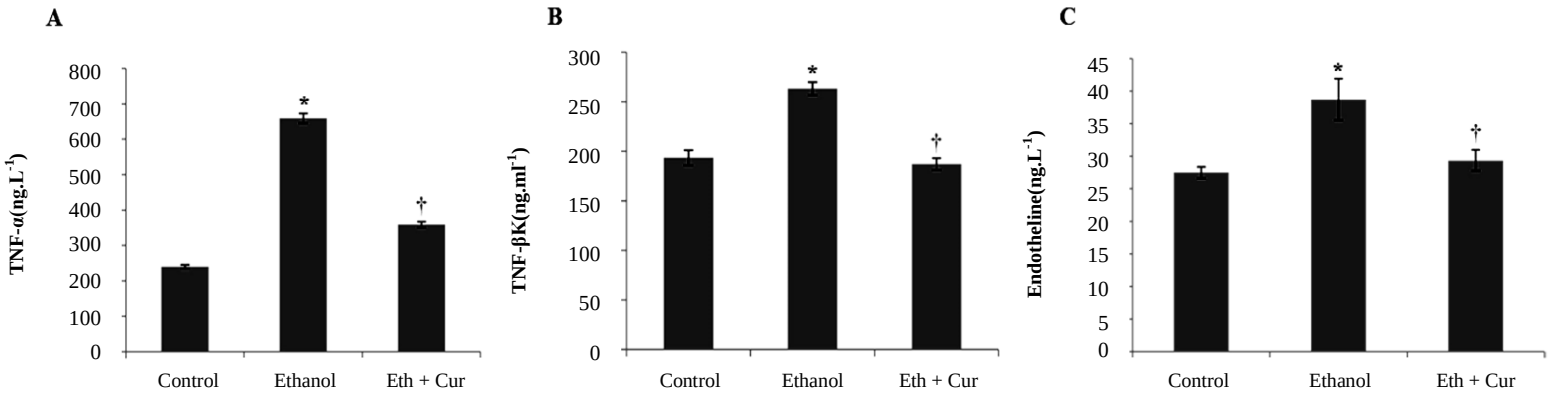

Figure 1. Effect of ethanol exposure and curcumin treatment on A) tumor necrosis factor-alpha receptor (TNF-α), B) NF-kappaB (NF-kB), and C) endothelin levels in the kidney tissue of rats. Data are given as Mean±SD. Values are significantly different (*in comparison with controland † in comparison with ethanol group; $p<0.05$). Eth: Ethanol; Cur: Curcumin

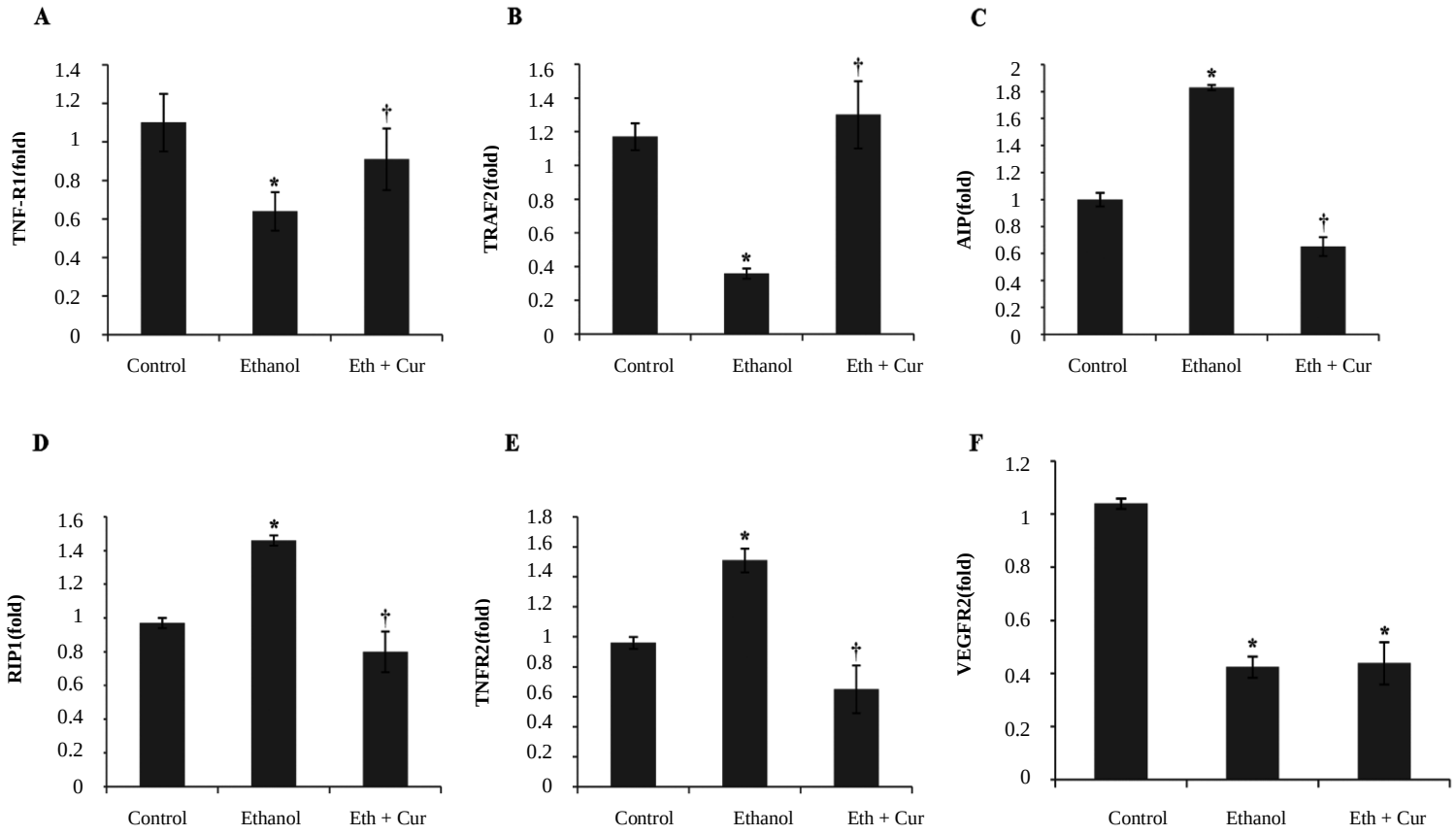

Figure 2. Impact of ethanol exposure and curcumin administration on TNFR1, TNFR2, TRAF2, VEGFR2, RIP1, and AIP1 mRNA expression in the kidney tissue of animals. Data are given as Mean±SD. Values are significantly different (*compared to controland † in comparison with ethanol group; $p<0.05$). Eth: Ethanol; Cur: Curcumin

DISCUSSION

The TNF-α tissue concentration and TNF-α receptors expression and its downstream adapter mediators were investigated in the kidney of heavy ethanol-consuming animals. The heavy ethanol exposure caused significant changes in the kidney level of TNF-α, NFkB, endothelin, as well as the expression of TNF-α signaling pathway mediator, including TNFR1, TNFR2, TRAF-2, RIP-1, and AP-1. Cr administration restored ethanol-induced changes in the above-mentioned mediators in the kidney.

Little is known about ethanol damage to the kidney, and the accurate effect of TNF-α as well as its adapter protein to cause ethanol-induced kidney inflammation is unclear. Heavy ethanol exposure induced a significant elevation in the TNF-α concentration in the kidney and that Cr treatment prevents ethanol-induced TNF-α elevation. Our data are in line with those of other investigations reporting that Cr can ameliorate autoimmune disorders through the regulation of inflammatory cytokines, like IL-1β, IL-6, TNF-α, and IFN-γ, JAK-STAT, AP-1, and NF-κB signaling pathways associated with immune cells and renal ischemia-reperfusion injury [25-27]. TNF-α is a strong mediator of inflammation, and the up-regulated renal

TNF-α expression activates local inflammation as well as tissue destruction [28]. The majority of the members in the TNF-α receptor superfamily and their several inflammatory signaling mediators, including TRAF2 and RIP-1, can transfer inflammatory signaling through activating NF-κB and AP-1 that enter the nucleus and activate the transcription of pro-inflammatory gene targets[9-11]. Regarding the TNF-α pathophysiological effect on kidney, our results are in line with other studies in which TNF-α showed a high expression level in the kidney of subjects with lupus glomerulonephritis, diabetic nephropathy, and other kidney diseases [29-31]. Therefore, ethanol exerts some of its deleterious effects on the kidney tissue via TNF-α alteration. Some studies have reported that kidney injury in animals receiving ethanol is linked to the leukocyte use and activation, and these activated leukocytes initiate the acute phase of inflammation[3, 32]. Other studies have suggested that alcohol metabolism and hypoxia, resulting from alcohol metabolism, directly produce reactive oxygen species (ROS) as well as activating NF-κB[33]. Hypoxia induces the inflammatory reaction[34]. Overall, limited data is available regarding these inflammation inducers and the role they play in alcohol-induced inflammation. TNF-α affects TNFR1 and TNFR2 [35]. Our results revealed that TNFR1 and TNFR2 expression decreased in ethanol-fed rats. Down-regulation of these receptors as a regulatory mechanism may be due to chronic elevation of TNF-α in the kidney. However, the up-regulation of RIP and TRAF-2 and also NF-κB and AP-1 in renal tissues from heavy ethanol-fed animals indicates that the proinflammatory activity mediated by TNF-α was facilitated in the local tissue. It is clear that the TNFR1 and TNFR2 effect on the kidney is much more complicated. TNFR1 and TNFR2 may be found to activate independent and distinct signal transduction pathways. Animal model studies suggest that inhibition of individual TNFRs, TNFR1, or TNFR2 can balance the pro-inflammatory and immunomodulatory effects of TNF in kidney disease[36]. In plasma, TNF-α seems as free or bound to circulating TNFR1 and TNFR2[5]. Increased levels of sTNFR1 and sTNFR2 show a strong association with kidney failure in diabetes[37]. The generalization of this finding to other kidney diseases, including ethanol-induced kidney inflammation, should be done with caution. As previously mentioned, the results of our study demonstrate kidney NF-κB elevation following heavy ethanol administration. The mechanism of NFkB activation is not completely described, according to previous reports, NF-kB activation is not applicable for distinguishing TNFR1 from TNFR2 signaling because they can induce NF-kB activation[38]. NF-κB is essential for normal immune reactions, is a pivotal mediator of TNF-α signal transduction, and thus participates in the effector phase of inflammation[13]. Deregulated NF-κB activation can cause various inflammatory diseases[16]. The role of alcohol has been confirmed in the generation of ROS and activation of NF-κB. Assuming that a strong antioxidant and anti-inflammatory agent could reduce alcohol-induced renal damages, Cr was used in the present study. Cr administration significantly restored ethanol-induced changes in the TNF-α inflammatory pathway mediator in the kidney. Cr also inhibited ethanol-induced NF-κB elevation in the kidney tissue. Consistent with our results, recent studies have suggested that the anti-neuroinflammatory effect of Cr mainly happens by the inhibition of NF-kB and MAPKs signaling[39, 40]. Tianfu Wu et al. indicated that curcumin treatment reduces the activation of the NF-kB, MAPK, AKT, and pBAD pathways systemically or within the inflamed kidneys [25-27]. Activation of endothelial/epithelial protein tyrosine kinase (Etk) is another signaling pathway downstream of TNFR2. TNFR2-mediated Etk activation can partly activate vascular endothelial growth factor receptor 2 (VEGFR2), leading to the activation of Akt and/or protein kinase B[41].VEGFR2 expression markedly decreases in ethanol-fed rats. Our results demonstrate that Cr has no significant on VEGFR2 expression in the kidney of heavy ethanol-fed rats. However, it is

reported that Cr blocks the VEGF-VEGFR-2 signaling pathways in human umbilical vein endothelial cells (HUVECs) through inhibition of the phosphorylation of VEGFR-2 caused by VEGF[42]. Hypoxia is also known for its induction of inflammatory responses. The role of hypoxia to induce inflammatory responses has been shown[32]. Hypoxia-inducible factor 1 (HIF-1α), a vital mediator of hypoxia signaling, controls the production of several proangiogenic factors, like ET-1, and VEGF. The results of our study revealed that ET-1 significantly increases in the kidney of ethanol-fed animals, and the administration of Cr significantly decreases ET-1. According to the previous reports, ET-1 induces VEGF generation by increasing the level of HIF-1α [43].

CONCLUSIONS

It will be of great interest to identify precise downstream events in the TNF-α signaling pathway in heavy ethanol exposure-induced kidney injury. our findings showed that ethanol-induced kidney injury, addressed by our previous studies and those of others, may be in part associated with the TNF-α signaling pathway, and that these impacts can be reduced using Cr as an antioxidant and anti-inflammatory compound.

Author contributions

Amin Abdollahzade Fard and Alireza Shirpoor. Contributed to conception, design, data analyses, and drafting the manuscript, Mahrokh samadi conceived and designed the experiments, and drafting the manuscript, Yousef Rasmi. Critically revised the manuscript.

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Conflict of interests

The authors have each completed and submitted an International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. There is no conflict of interest

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