

Chronic ethanol ingestion induces glomerular filtration barrier proteins genes expression alteration and increases matrix metalloproteinases activity in the kidney of rats

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(Received: March 13, 2018; Revised manuscript received: April 16, 2018; Accepted: April 23, 2018)

Abstract: *Background:* Chronic alcohol ingestion-induced kidney structure and function alterations are very well known, but the precise underlying molecular mediators involved in ethanol-induced kidney abnormalities remain elusive. The aim of this study was to investigate the effect of chronic ethanol exposure on matrix metalloproteinase 2, 9 (MMP), glomerular filtration barrier proteins (nephrin and podocin), as well as vascular endothelial growth factor receptor 1, 2 (VEGFRs) isoforms gene expression in the kidney of rats. *Methods:* Sixteen male Wistar rats with an initial body weight of 220 ± 10 g were divided into the following two groups: (1) control and (2) ethanol (4.5 g/kg BW). *Results:* After 6 weeks of treatment, the results revealed a significant increase in isoforms VEGFR1 and VEGFR2 of VEGFR gene expression, significant increases of MMP2 and MMP9 activities, as well as significant decrease of nephrin and podocin gene expressions in the ethanol group, compared with that in the control group. *Conclusion:* These findings indicate that ethanol-induced kidney abnormalities may be in part associated with alteration in expressions of VEGFRs, nephrin, and podocin and in increasing activities of MMP2 and MMP9 as key molecular mediators in the kidney function.

Keywords: ethanol, kidney, VEGFR, nephrin, matrix metalloproteinase

Introduction

Our recent work indicated that chronic ethanol exposure led to impairing kidney function by significant enhancement of plasma creatinine, urea, and cystatin C levels. In addition, ethanol ingestion resulted in shrinkage of glomerular pores that were manifested by enhancement of cystatin C/plasma creatinine ratio as suggested by Shirpoor et al. [1] and Grubb et al. [2]. As an indicator of glomerular filtration rate, creatinine clearance also showed decreases followed by ethanol consumption [1].

In agreement with our previous work, several lines of studies revealed the deleterious effects of ethanol on kidney function and structure as well. Filtration defects, low blood concentrations of key electrolytes, severe alterations in the body's acid-base balance, and increased circulation of blood urea nitrogen (BUN) and creatinine following ethanol ingestion have also been reported by previous studies [3–5]. In addition, ethanol administration caused a wide range of kidney structural alterations such as moderate to severe cell proliferation in different parts of nephron tubules, kidney tissue fibrosis, swelling of

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tubular epithelial cell, dilation of tubular lumen, thickening of basement membrane of glomerulus, as well as interstitial edema and renal hypertrophy which have been demonstrated by previous studies [1, 6–8]. Although previous studies have identified some features of ethanol-induced kidney function and structure abnormalities, the stage intervening between the exposure of kidney to ethanol and initiation of the flow of responses inducing abnormalities in renal system and output are still far from understanding. On the other hand, studies have often postulated that mechanisms such as oxidative stress and inflammatory reactions explain the structural and functional alterations in the kidney following ethanol exposure [1, 3, 4, 9]; however, they have not provided precise information regarding the specific molecules that could influence kidney's function as a molecular mediator following ethanol exposure. Among dozens of molecular mediators related to the kidney function, nephrin and podocin are the two fundamental protein components of the podocyte slit diaphragm (SD) and glomerular filtration barrier [10, 11]. Previous studies have shown that decreasing of nephrin and podocin and alterations of their gene expression are associated with the development of proteinuria and progressive renal disease [10, 11]. Matrix metalloproteinases (MMPs) including collagenases, gelatinases, matrilins, and membrane-type MMPs are other important mediators playing a prominent role in normal physiology and abnormal pathology of the kidney functions with corresponding of cell proliferation, angiogenesis, and apoptosis [12]. Moreover, recently, the association between the expressions of vascular endothelial growth factor (VEGF) receptors (VEGFR) in the podocyte cells of glomerular filtration barrier and kidney function has attracted the researchers' attention. A study by Kitamoto et al. [13] confirmed that VEGF mediated the organogenesis of the kidney, particularly glomerulogenesis, as a message molecule between Bowman's capsule and capillary endothelia cell in paracrine way. However, VEGF is an endothelial mitogen-promoting angiogenic factor with a wide binding sites distribution in the rat, including the kidney [14]. Due to the fundamental role of the aforementioned observations, in this study, we focused on examining the effect of long-term ethanol consumption on nephrin, podocin, and VEGFRs expression transition in rats' kidney. It was also planned to find out whether chronic ethanol consumption can influence the activities of MMP2 and MMP9 in kidney tissue or not.

Materials and Methods

Animals and treatments

The authors of this manuscript have certified that they comply with the principles of ethical publishing in *Interventional Medicine & Applied Science*: Szél Á,

Merkely B, Hüttl K, Gál J, Nemes B, Komócsi A: Statement on ethical publishing and scientific authorship. *IMAS* 2, 101–102 (2010). Sixteen male Wistar rats initially weighing 220 ± 10 g were randomly divided into two groups: (1) control and (2) ethanol groups. Similar to our previous study, rats in the ethanol group received ethanol with a dose of 4.5 g/kg body weight (Merck KGaA, Darmstadt, Germany) saluted in tap water (20% w/v) intragastrically by gavage once a day, for 6 weeks [15]. The control group was treated with tap water only. After 6 weeks of treatment, the rats were anesthetized by 10% chloral hydrate (0.5 mL/100 g body weight, IP). The anesthesia depth was assessed by pinching a hind paw. At termination, the abdominal cavity was opened and the left kidney was excised. The excised kidney was freed from adventitial tissues, fat, and blood clots and was subsequently washed in ice-cold physiological saline. For total RNA isolation, 100 mg of kidney tissue was (from 1/3 mid-part) immersed in 1 ml RiboxEX (total RNA isolation solution) (GeneAll, Seoul, South Korea) and restored at -80 °C up to the time of RNA isolation. In order to measure kidneys' MMP2 (gelatinase A) and MMP9 (gelatinase B) activities, other parts of the kidneys were minced and then homogenized in ice-cold extraction buffer (10% wt/vol), containing a 50-mM phosphate buffer (pH 7.4) using Ultra Turrax homogenizer (T10B, IKA, Germany). Next, the homogenates were centrifuged at $10,000 \times g$ at 4 °C for 20 min (HERMLE, Z 360 K, Germany). Moreover, the supernatant sample was used for MMP2 (gelatinase A) and MMP9 (gelatinase B) assays.

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

Total RNA in frozen left kidney was extracted using a kit (GeneAll; Cat no. 305-101), in accordance with the manufacturer's instruction. RNA concentration was verified by spectrophotometric measurement of the absorbance at 260–280 nm and determined by mixture of Tris base, acetic acid, and EDTA (TAE) agarose gel electrophoresis.

Reverse transcriptase (RT) was measured using HyperScript™ Reverse Transcriptase (GeneAll). RT-PCR was performed using an amplification reagent kit (Ampliqon, Denmark) by the XP-Cycler instrument (TCXPD, Bioer, USA) with nephrin, podocin, VEGFR1, VEGFR2, and the rats' glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers. To amplify the cDNA, the 5' and 3' primer sequences (forward and reverse) of the nephrin, podocin, and VEGFRs genes designed by the Gene Bank (<http://blast.ncbi.nlm.gov/Blast.cgi>) revealed that the primers were gene-specific. Furthermore, Gene Runner software verified all the primers (Table I). Primers (forward and reverse) were also synthesized to amplify

Table 1 Sequences of primers used to evaluate expressions of GAPDH, nephrin, podocin, VEGFR1, and VEGFR2

Target gene	Primer sequence		Product size (bp)
GAPDH	Forward	5'-AGACAGCCGCATCTTCTTGT-3'	207
	Reverse	5'-CTTGCCGTGGGTAGAGTCAT-3'	
Nephrin	Forward	5'-ATCCACTTTAGGGGGTCATTA-3'	231
	Reverse	5'-CTTGTGCTTCTCCTCTCTCAG-3'	
Podocin	Forward	5'-GGCGAGTGGACAAGAGTAAT-3'	201
	Reverse	5'-TGAATGATGAGACGACCCAC-3'	
VEGFR1	Forward	5'-TTAGGACCAGGAAACAGCAC-3'	201
	Reverse	5'-AAGGAGCCAAAAGAGTGTCG-3'	
VEGFR2	Forward	5'-ACGGGGCAAGAGAAATGAAT-3'	159
	Reverse	5'-TTCTCCTCCACAAAACCTGA-3'	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; VEGFR: vascular endothelial growth factor receptor

the cDNA encoding GAPDH as a house-keeping gene; the sequences of related primers are also provided in *Table 1*.

Real-time quantification of the target genes was performed by using a Real-Time PCR Master Mix Green kit (Ampliqon) in a total volume of 25 μ l and in accordance with the manufacturer's instructions. Furthermore, the mentioned genes expressions were analyzed using an iQ5 real-time PCR detection system (Bio-Rad, CA, USA). The reactions were then prepared for 10 min at 95 °C in a 96-well optical plate followed by 40 cycles of 20 s each at 59 °C. In order to confirm the specificity of the amplification reactions, a melting curve was recorded. Each sample was replicated three times; the value of the threshold cycle (Ct) was the same as that of the corresponding mean. The relative fold expression of each mRNA was calculated by conducting the $2^{-\Delta\Delta C_t}$ method ($-\Delta\Delta C_t = \Delta C_t$ test sample $- \Delta C_t$ calibrator sample), with Ct being the threshold cycle. Next, the calculated levels were normalized to GAPDH and were then analyzed for statistical significance performing an independent samples *t*-test.

Biochemical assay

Kidneys' MMP2 (gelatinase A) and MMP9 (gelatinase B) activities were measured by adopting the quantitative sandwich enzyme immunoassay method and using a commercial rat MMP2 (gelatinase A) and MMP9 (gelatinase B) ELISA kit; Bioassay Technology Laboratory, China).

Statistical analysis

Normal distribution of data within each group was verified by carrying out a Kolmogorov–Smirnov test. The statistical differences between the groups were tested by

conducting an independent samples *t*-test. The data obtained from each test are presented as the mean \pm SE, and $p < 0.05$ is considered as statistically significant.

Results

For quantification of nephrin and podocin mRNA expression, we applied real-time RT-PCR, and the relative expression results are shown in *Fig. 1*. The results showed that the means for relative expression of podocin were 1.18 ± 0.11 and 0.36 ± 0.036 fold in the control- and ethanol-treated groups, respectively. As shown in *Fig. 1*, 42 days administration of ethanol significantly decreased the podocin mRNA expression compared to the control group ($p < 0.05$). In the same way, the ethanol exposure significantly decreased the nephrin mRNA expression (0.33 ± 0.09) as compared to that in the control group (1.19 ± 0.12 fold) ($p < 0.05$). VEGFR1 and VEGFR2 isoforms mRNA levels in the kidney tissue were detected by real-time RT-PCR and the relative expression results are shown in *Fig. 2*. The VEGFR1 mRNA expression

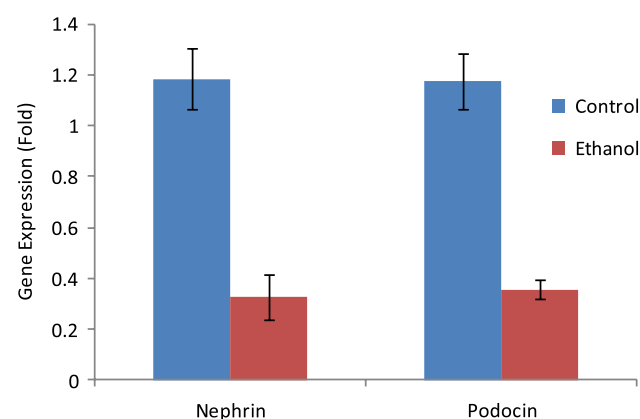


Fig. 1. Ethanol consumption significantly decreased nephrin and podocin genes expression compared with the control group

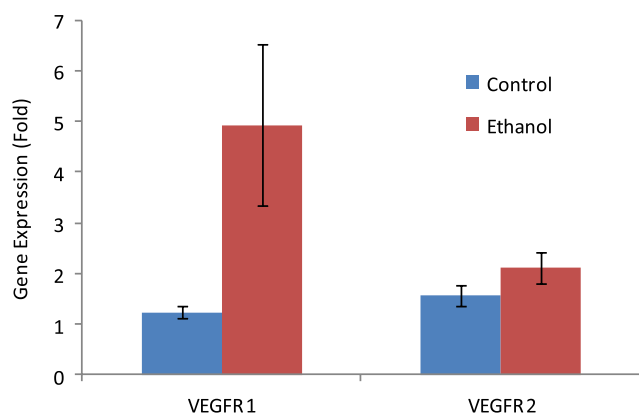


Fig. 2. Ethanol consumption significantly increased VEGFR1 and VEGFR2 genes expression compared with the control group

ratio (amount of VEGFR1 mRNA/amount of GAPDH mRNA) was 4.95 ± 1.6 fold in the ethanol-treated kidney tissue, whereas the corresponding value in the kidney tissue harvested from control animals was 1.23 ± 0.12 fold. VEGFR1 mRNA expression in ethanol-treated kidney tissue was significantly higher than that in the control tissue ($p < 0.05$). Similarly, VEGFR2 mRNA expression in the ethanol-treated kidney tissue (2.12 ± 0.42) was also significantly higher than that in the kidney tissue of control (1.57 ± 0.2) group ($p < 0.05$).

Kidney tissue MMP2 and MMP9 proteins levels were detected by ELISA method and the relative results are shown in *Fig. 3*. The MMP2 protein amount was 23.52 ± 2.5 ng/ml in the ethanol-treated kidney tissue, whereas the corresponding value in the kidney tissue harvested from control animals was 53.36 ± 1.76 ng/ml. MMP2 level in ethanol-treated kidney tissue was significantly higher than that in the control tissue ($p < 0.05$). Similarly, MMP9 amount in the ethanol-treated kidney tissue (2.1 ± 0.3 ng/ml) was also significantly higher than that in the kidney tissue of control (1.36 ± 0.03 ng/ml) group ($p < 0.05$).

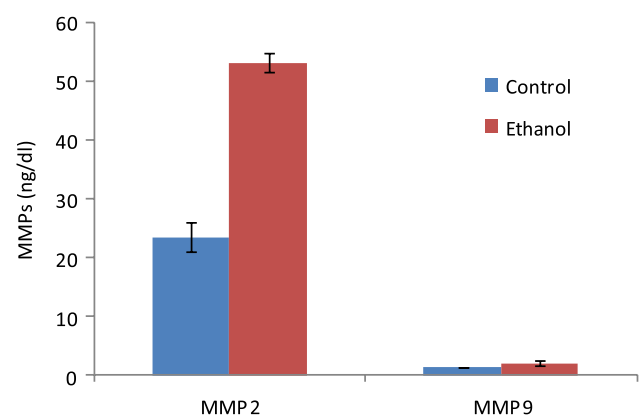


Fig. 3. Ethanol exposure significantly increased the activities of MMP2 and MMP9 in kidney tissue compared with control group

Discussion

Several studies have reported that chronic ethanol consumption affects the kidney filtration quality and renal glomerulus, the site of plasma ultrafiltration, and make up the majority of kidney diseases leading to acute kidney injury and sudden loss of kidney filtration with retention of nitrogenous waste in blood [1, 16, 17]. Disappointingly, however, the molecular mechanisms involved in the development of glomerular disease and kidney injury through ethanol exposure are poorly understood. Therefore, the study of the precise molecular mediating steps between exposure of kidney to ethanol and initiation of the cascade of responses leading to glomerular disease and kidney injury has become of greatest interest. The filtration property of the filtration barrier layers, including fenestrated endothelium, glomerular basement membrane (GBM), and podocytes, forms a specialized filter that rigorously restricts the transcapillary passage of large-molecular weight proteins, while permitting the permeation of smaller molecules, electrolytes, and water from the circulating blood into the primary urine for excretion. Several line of molecular mediators, cell signaling pathways, and proteins play role in the filtration barrier layers for maintained normal kidney function and glomerular permeability. Among several proteins that are known to be critical for podocyte phenotype and function, nephrin and podocin are two key molecules in the glomerular SD, an ultrathin zipper-like structure, which is a podocyte-specific slit between interdigitating podocytes foot processes implicated in the formation of a size barrier to protein leakage [18]. According to literature, mutations in expression of genes encoding nephrin and podocin were shown to play a major role in the alteration of glomerular permeability and early onset of nephritic syndrome in humans [19]. Mechanistically, nephrin and podocin exert its effect on the organization and preservation of the specialized cell-cell junction through several ways. Nephrin interacts with the cytoskeletal through several proteins such as podocin, CD2AP, alpha-actinin-4 (ACTN4), as well as other proteins, which still need to be identified, to build the filtration complex at SD in the glomerular podocyte [20]. Regarding the special structural and signaling functions of every protein in filtration complex, the stoichiometric ratio between filtration complex proteins is essential for a normal functioning of ultrafiltration [21]. Alteration in genes expression associated with each element in the filtration complex leads to a dysfunction of the barrier, flattening of foot process, and uncontrolled permeability of the glomerular filtration barrier [22]. In addition, besides the important role of nephrin in scaffolding of SD, nephrin triggers signal transduction by transmitting extracellular domain from the SD to the intracellular actin cytoskeleton, and this signal transduction augments by another podocyte protein called podocin [20, 23]. There is growing evidence

that the reduction in podocyte-specific proteins, such as nephrin and podocin, resulted in glomerular hyperpermeability and possibly other renal diseases characterized by heavy proteinuria [24, 25]. Here, we demonstrated that the ethanol exposure induced a decrease in the expression of nephrin and podocin in the kidney tissue of rats. The underlying molecular mechanism through which the ethanol exerts its reduced expressive effects on nephrin and podocin genes expression is still not very well understood. Recently, the study conducted by Yang et al. [26] indicated that advanced oxidation protein products decrease expression of nephrin and podocin in podocytes through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species generation that activated p38 mitogen-activated protein kinase pathway. Interestingly, previous studies had demonstrated that ethanol exposure led to increase the level of NADPH oxidase in the kidney tissue, as well as in the cardiac tissue concurrent alteration of fetal gene expression [15, 27, 28]. Based on the results of this study and the previous ones, we suggest that ethanol induces NADPH oxidase to mediate oxidative stress, may trigger decreasing expression of nephrin and podocin in podocytes, and contribute, at least in part, to glomerular disease and kidney injury. Another novel finding of this study was that ethanol exposure induced overexpression of VEGF 1 and 2 receptors mRNA in the kidney tissue of animals. Although the role of VEGF in glomerular physiology and pathology is not very well known, a recent study by Eremina et al. [29] suggested that a balance of VEGF expression may be necessary for normal glomerular function and alteration of VEGF expression leads to distinct congenital and acquired renal disease. Various studies have also shown the association between increased VEGFRs expression of podocytes and development of nephropathy with symptoms, such as hyperpermeability, GBM thickness, and mesangial expansion in experimental diabetes [29–31]. In addition, the nephropathic role of VEGFs and its related receptors' overexpression was approved by data, which revealed that the administration of anti-VEGF antibodies improved diabetic nephropathy by reducing hyperpermeability, GBM thickness, and mesangial expansion in experimental diabetes [32]. Our previous study and others indicated that ethanol administration causes nephropathy with significant kidney structural and functional alteration, including hyperpermeability, GBM thickness, mesangial expansion, BUN and creatinine enhancement in the circulation, and dozens of other kidney dysfunction and structural changes [1, 33, 34]. Accordingly, the established nephropathic effect of ethanol consumption on one hand and the nephropathy resulted due to overexpression of VEGFRs as mentioned above on the other hand tempt us to speculate the theory as follows: besides reduced expression of nephrin and podocin, overexpression of VEGFR1 and VEGFR2 are

another molecular mediators that ethanol could induce its deleterious effects on kidney tissue.

Other important result of this study was that ethanol consumption leads to significant increases of MMP2 and MMP9 activities in rat's kidney tissue. It has been supposed that the gelatinase enzymes preserve balance between the production and degradation of extracellular matrix (ECM) [35]. Therefore, altered MMP2 and MMP9 activities may directly change ECM turnover, glomerular scarring, and kidney dysfunction [36]. In addition, MMPs' dysregulation activity leads to diabetic retinopathy, chronic non-healing diabetic ulcer and could harmfully affect podocyte integrity and GBM penetrability [37, 38]. For evidence, the study by Tveita et al. [39] demonstrated that the enhancement of MMP2 and MMP9 activities and their related genes expression within the glomerular mesangial space is along with ECM expansion and development of proteinuria in lupus-prone B/W mice. Moreover, increases of MMPs activities induce inflammatory cell influx [40]. Furthermore, other studies have implicated that reduced activity of gelatinases resulted in accumulation of ECM components [41]. Therefore, ECM accumulation and degradation persistently and ultimately cause chronic kidney disease and extensive fibrosis in kidney [42]. Combined with our recent work and this study's results, we conclude that ethanol ingestion induces kidney structural and functional abnormality [1] is mediated in part by enhancement of gelatinases (MMP2 and MMP9) activity and eventually inordinately degradation of ECM lead to imbalance in production and degradation of ECM that participate in renal disease.

In conclusion, this is the first study to suggest that alteration in expression of VEGFRs and podocyte-specific proteins, such as nephrin and podocin, as well as increase of gelatinase enzymes activities are likely to contribute to the pathology of ethanol-induced nephropathy. It would be of interest to extend this study in a large number of alteration of molecular mediators related to normal kidney function and various renal disorders associated with kidney dysfunction, such as ethanolic nephropathy. Furthermore, whether ethanol exposure induces kidney failure via over or downexpression of VEGFRs and podocyte-specific proteins genes needs to be discovered by studies using knockout of these genes expression analysis to elucidate the underlying molecular mechanism of the subjects.

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Funding sources: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contribution: MS contributed to data collection and/or processing. ATA contributed in concept preparation. AS performed supervision and provided critical review. FK performed real-time analysis and biochemical examination. YR and MS contributed to examination and analysis. All authors had full access to all data in the study

and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgements: This work was derived from a Master of Science thesis in Physiology by Urmia University of Medical Sciences, Urmia, Iran.

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