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Original Article

Maternal Ethanol Exposure Impairs the Kidney of Offspring by Alteration of Podocyte Proteins Genes Expression and Fibrosis

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Abstract

Background: This study examined the effect of prenatal and early postnatal ethanol exposure on the structural, functional, and molecular alterations of rat's offspring kidney on postnatal days 21 and 90.

Methods: Pregnant rats on gestation day 7 were divided into the two groups, namely control and ethanol groups. Rats in the ethanol group received ethanol (4.5 g/kg B.W) from gestation day 7 throughout lactation. Nephrin, podocin, vascular endothelial growth factor receptors (VEGFRs) 1 and 2 gene expression were measured by RT-PCR technique. The MMP2 and MMP9 levels in the kidney tissue and plasma cystatin C level were measured by ELISA method.

Results: The results revealed a significant alteration in mRNA expression of nephrin, podocin, and VEGFR, as well as MMPs amounts in the kidneys of the offspring. Cystatin C level, the ratio of cystatin C/serum creatinine, serum creatinine, and urine urea showed a significant increase, but urine creatinine and GFR showed a significant decrease in the offsprings of the ethanol group compared to the control group. Histopathological changes such as fibrosis, kidney cells proliferation, leukocytes infiltration, and vacuolization have also seen in the kidney of the offsprings after 21 and 90 days from birth.

Conclusion: Taken together, these results provide evidence that pre and early postnatal ethanol exposure renal toxicity is in part associated with alteration of nephrin, podocin, and VEGFRs genes expression, as well as MMPs amount changes. Furthermore, it was found that these molecular alterations were triggered by inflammatory reactions manifested by fibrosis, proliferation, and polymorphonuclear(PMN) leukocytes infiltration.

Keywords: Cystatin C, Ethanol, kidney, Matrix Metalloproteins, Nephrin

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Introduction

Current evidence available in the literature reveals that maternal ingestion of ethanol in high doses during pregnancy can lead to a wide range of growth and mental deficiencies, collectively termed fetal alcohol spectrum disorders (FASDs). The hazardous effects of FASD on the developing brain have received a great deal of attention over the last decades (1,2), but little is known about the effects of maternal ethanol exposure on the developing kidneys. Previous studies have reported significant changes in the function and structure of the kidney of offspring with maternal exposure to high levels of ethanol in the gestation period (3,4). Studies conducted on children diagnosed with FASD and animals prenatally exposed to ethanol have revealed structural changes such as small and misshapen kidneys, decreased renal protein and DNA content, as well as a reduced number of nephrons in kidneys of the offspring (5-7). Another study reported a mild-to-moderate swelling and rounding of mitochondria and cytoplasmic vacuoles of the epithelial cells in 90-day old rats prenatally exposed to ethanol (8). From a functional perspective, renal tubular dysfunction, including impaired urinary concentration, hyperzincuria, and impaired acidifying capacity has been reported in offspring from those mothers receiving ethanol in the gestation period (7,4). Although some functional and structural kidney alterations due to the maternal ethanol consumption have been identified in earlier and recent studies, the precise mediating steps between maternal exposure to ethanol and initiation of the cascade of responses leading to kidney abnormality in the offspring have not yet been completely clarified and the number

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of studies investigating the mechanism through which ethanol affects the developing kidneys is limited. Studies have often suggested mechanisms such as reduced ureteric branching morphogenesis, dysregulation of rennin-angiotensin system pathways, and inhibition of renal angiotensin II type 2 receptors as possible involved mechanisms through which prenatal ethanol exposure exerts its effects on kidneys of the offspring (3,4). According to the findings of some other studies and also the present study, prenatal ethanol exposure leads to oxidative stress and inflammatory changes in the testis, heart, and brain of the offspring (2,9-11).

Considering the limited understanding of the effect of FASD on the development of the renal system, in the current study, we evaluated the possible adverse effects of maternal pre- and early postnatal days of ethanol ingestion on the kidney of male offspring, in terms of functional, histological and molecular endpoints, 21 and 90 days after the birth. In the current study, proliferating cell nuclear antibody (PCNA) and Masson trichrome staining were applied in evaluating the histopathological alterations. In addition, creatinine clearance, cystatin C, and cystatin C/plasma creatinine ratios, as well as serum and urine creatinine and urea levels were measured in order to evaluate functional alterations of kidneys caused by exposure to ethanol during development. Due to the fundamental role of nephrin, podocin and vascular endothelial growth factor receptors (VEGFRs) 1 and 2 in both physiological and pathological status of the kidney, we also proposed that maternal ethanol consumption resulting in kidney abnormalities is mediated in part by nephrin, podocin, and VEGFR 1 and 2 related genes expression changes in the kidney tissue. The effect of maternal ethanol consumption on the amounts of matrix metalloproteinases 2 and 9 in the kidneys of offspring was also evaluated. Due to the importance of sex differences in response to ethanol, only male pups were used in the present study.

Materials and Methods

Experimental design

The animal care and research protocols were in accordance with the National Health and Medical research Care published by the National Institutes of Health (NIH publication, no.85–23, revised 1985), and the protocol was approved by the Urmia University of Medical Sciences Animal Care Committee (Ethical code: ir.umsu.iec.1396.238). Sixteen adult female Wistar rats weighing 220 ± 20 g were mated with males previously tested as being fertile. The presence of vaginal plugs in the next morning indicated successful mating, and that day was designated as gestation day (GD)1. Beginning on GD7, pregnant rats were singly housed and randomly divided into the two groups (8 in each group), namely control and ethanol-treated groups. Rats in the ethanol group received ethanol at a dose of 4.5 g/kg body weight (Merck KGaA, Darmstadt, Germany) diluted in tap water (20% w/v) intragastrically by gavage once a day from GD7 through postnatal (PN) 21. The mean body weight was measured from the beginning to the end of the study on weekly basis and was used to recalculate the dosage of ethanol. On PN1, pups were culled to three or four male pups/mother to reduce possible nutrition deficiencies due to competition among the litters and were then returned to mothers' cages. On PN21 and PN90, the offspring from each group (n = 8 offspring from each group of ethanol-treated and control on PN21 and PN90) were anesthetized with ketamine (10%, 80 mg/kg B.W, IP) and xylazine (2%, 10 mg/kg B.W IP). After weighing, the abdominal cavity was opened and blood samples were taken directly from the portal vein, collected in EDTA tubes, and centrifuged at 4000× g for 20 minutes within 30 minutes of collection. The plasma was stored at -80°C without repeated freeze-thaw cycles. Then, the right kidney was isolated for analysis as follows: a part of the kidney was fixed immediately in 10% formalin embedded in paraffin and sectioned at 5 µm for histological examinations. To perform biochemical analysis, a part of the right kidney was washed with ice-cold physiological saline and then dried on filter paper. Subsequently, an ice-cold extraction buffer (10% wt/vol) containing a 50mM phosphate buffer (pH 7.4) was added and then homogenized using Ultra-Turrax (T10B, IKA, Germany). Next, the homogenates were centrifuged at 10000×g at 4°C for 20 minutes. and at the end, the supernatant sample was obtained and then stored at -80°C up to the time of analysis. To extract total RNA, 100 mg of the right kidney tissue was placed into 1 mL RiboEX (total RNA isolation solution) (Gene All, Seoul, Korea) and stored at -80°C, up to the time of RNA isolation. To collect urine samples (only at PN90), 24 hours before anesthetizing, offspring were kept in metabolic cages individually, and their urine was collected and immediately centrifuged at -20°C to remove debris before storage.

Quantitative real-time PCR and RT-PCR

Total RNA was extracted using an extraction kit (Gene All, South Korea, Cat no 305-101) according to the manufacturer's protocol. The cDNA was synthesized using an amplification reagent kit (Ampliqon, Denmark) by the XP-Cycler instrument (TCXPD, Bioer, USA). The real-time quantification of the target genes was carried out as described previously in our earlier study (12). The 2-(ΔΔCt) method was used to determine relative quantitative levels of target genes and normalize them to GAPDH as a housekeeping gene. The results were expressed as the fold difference to the housekeeping gene.

Biochemical examinations

The MMP2 and MMP9 levels in the kidney tissue and the

amount of plasma cystatin C were measured conducting the quantitative sandwich enzyme immunoassay method using a commercial rat MMP2, MMP9, and cystatin C kit (Elisa kit Bioassay Technology Laboratory, China) in accordance with the protocol provided by the manufacturer. Plasma and urine creatinine and urea levels were measured using urea and a creatinine commercial kit (Pars Azemoon, Karaj, Iran). Glomerular filtration rate (GFR) was determined by calculating creatinine clearance (GFR = [UCr × V]/SCr), using plasma and urine creatinine concentrations and the urine flow rate or volume.

Histopathological examinations

The fixed kidney specimens were handled according to Bancroft and Stevens (13). Briefly, 5-micron kidney tissue sections embedded in serial paraffin were cut and stained with hematoxylin and eosin (H&E) and examined under a light microscope. The immunohistochemical method for PCNA staining was carried out as previously detailed to identify proliferating cells (12). To determine the percentage of PCNA-positive cells, four microscopic fields of view per section from two to three sections per animal were randomly selected and analyzed. The number of positively stained cells (brown nuclear) were counted and then reported as the percentage of the total number of counted cells for each field of view. The criteria applied to scoring the PCNA positive indices were classified as follows: 1) normal (less than 5%), 2) mild (5-25%), 3) mild to moderate (25-50%), 4) moderate to severe (50%-75%), 5) severe (75%-100%). To evaluate the kidney tissue fibrosis, sections were stained using Masson trichrome, following the manufacturer's instructions (Asiapajohesh, Amol, Iran). The intensity of tissue fibrosis was scored using a semi-quantitative method described by Ashcroft et al and our published protocol (14,15). The criteria applied in scoring the kidney fibrosis were as follows: 0 = normal kidney, 1 = minimal fibrosis thickening of kidney tissue, 2 and 3 = moderate thickening of kidney tissue without obvious damage to the structure of kidney tissue, 4 and 5 = increased fibrosis with definite damage to the architecture of the kidney and formation of small fibrosis masses, 6 and 7 = severe distortion of the structure and large fibrosis areas, and 8 = total fibrotic involvement (15). For evaluating the general histological alteration of kidney tissue stained by H&E, the following semi-quantitative scoring method were applied as follows: no (-), weak (+), moderate (+ +), and severe (+ + +).

Statistical analyses

Normal distributions of data within each group were verified by applying the Kolmogorov-Smirnov test. Statistical differences between the groups were tested by conducting an independent samples *t* test. In each test, the data were expressed as the mean ± standard error (SE), and $P < 0.05$ was considered to be statistically significant.

Results

Kidney and body weight changes

Separate analyses of the offspring body weight, kidney weight, and kidney weight/body weight ratio on PN21 and PN90 are shown in Table 1. The bodyweights of pups from the ethanol-treated rats were significantly lower than those of the control group on PN21 and PN90 ($P < 0.05$). Similarly, right and left kidney weights were significantly lower in the ethanol group than those in the control group ($P < 0.05$). The kidney weight/body weight ratio of the ethanol rats (maternal ethanol consumption during pregnancy and lactation period) was significantly higher on PN21 compared with the control rats ($P < 0.05$). On PN90, the kidney weight/body weight ratio showed no significant difference between the ethanol and the control offspring.

Gene expression alteration in different groups

Figure 1 shows the results of treatment of mothers with ethanol on the gene expression alteration of nephrin, podocin, VEGFR1, and 2 involved in the kidney function and structure. Ethanol administration significantly decreased the expression of kidney nephrin, podocin, VEGFR1and 2 mRNA levels on PN21 ($P < 0.05$). In the kidneys obtained from PN90 offspring, podocin, and VEGFR2 mRNA levels were significantly lower than the expression of the same genes in the control offspring; however, Nephrin and VEGFR1 gene expression increased in the ethanol-treated group when compared with the control group ($P < 0.05$).

Biochemical changes

Changes in the kidney tissue MMP2 and MMP9 concentrations, serum and urine creatinine and urea, and serum cystatin C levels, as well as GFR in the offspring of the experimental groups have been shown in Figure 2. Serum levels of cystatin C on PN21 and PN90 were significantly higher in the ethanol group offspring,

Table 1. Mean body and kidney weight of offspring on PN21 and PN90

	Control 21	Ethanol 21	Control 90	Ethanol 90
B.W (g)	50.28 ± 3.7	32.87 ± 2.8*	251.29 ± 5.04	211.149±4.7*
R. K. W (g)	0.23 ± 0.01	0.18 ± 0.01*	0.82 ± 0.01	0.64 ± 0.05*
L.K.W (g)	0.23 ± 0.01	0.17 ± 0.01*	0.83 ± 0.02	0.65 ± 0.05*
T.K.W (g)	0.463 ± 0.02	0.361 ± 0.022*	1.66 ± 0.034	1.29 ± 0.11*
R.K.W(mg)/B.W (g)	4.54 ± 0.24	5.37 ± 0.05*	3.28 ± 0.09	3.03 ± 0.26
L.K.W(mg)/B.W (g)	4.78 ± 0.11	5.2 ± 0.17*	3.34 ± 0.11	3.7 ± 0.24
T.K.W(mg)/B.W (g)	9.3 ± 0.042	10.5 ± 0.021*	3.3 ± 0.23	3.04 ± 0.69

Values expressed as mean ± SEM.
*$P < 0.05$: significant differences compared to the control.
B.W: Body weight, R.K.W: Right kidney weight, L.K.W: Left kidney weight, T.K.W: Total (right +left) kidney weight.

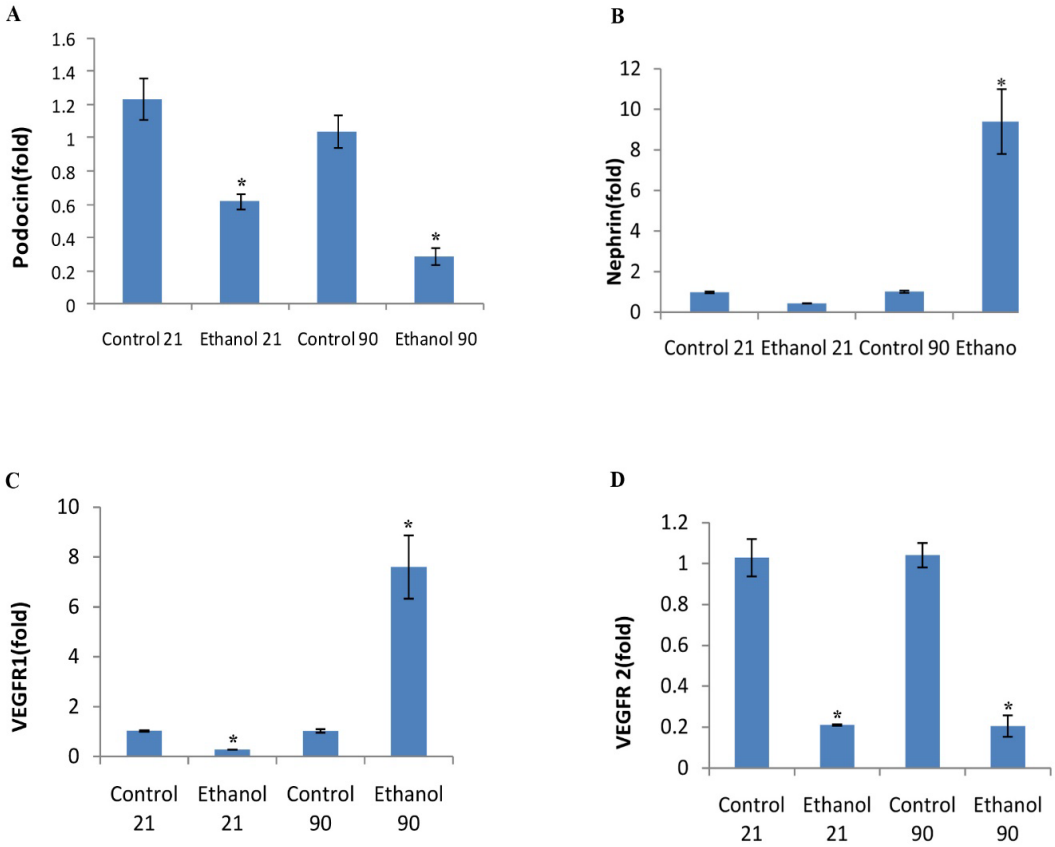

Figure 1. Maternal ethanol exposure during gestation and lactation -induced alteration of nephrin, podocin, and VEGFs gene expression in the kidney tissue of male offspring on PN21 and PN90. *$P<0.05$: significant differences compared to the control.

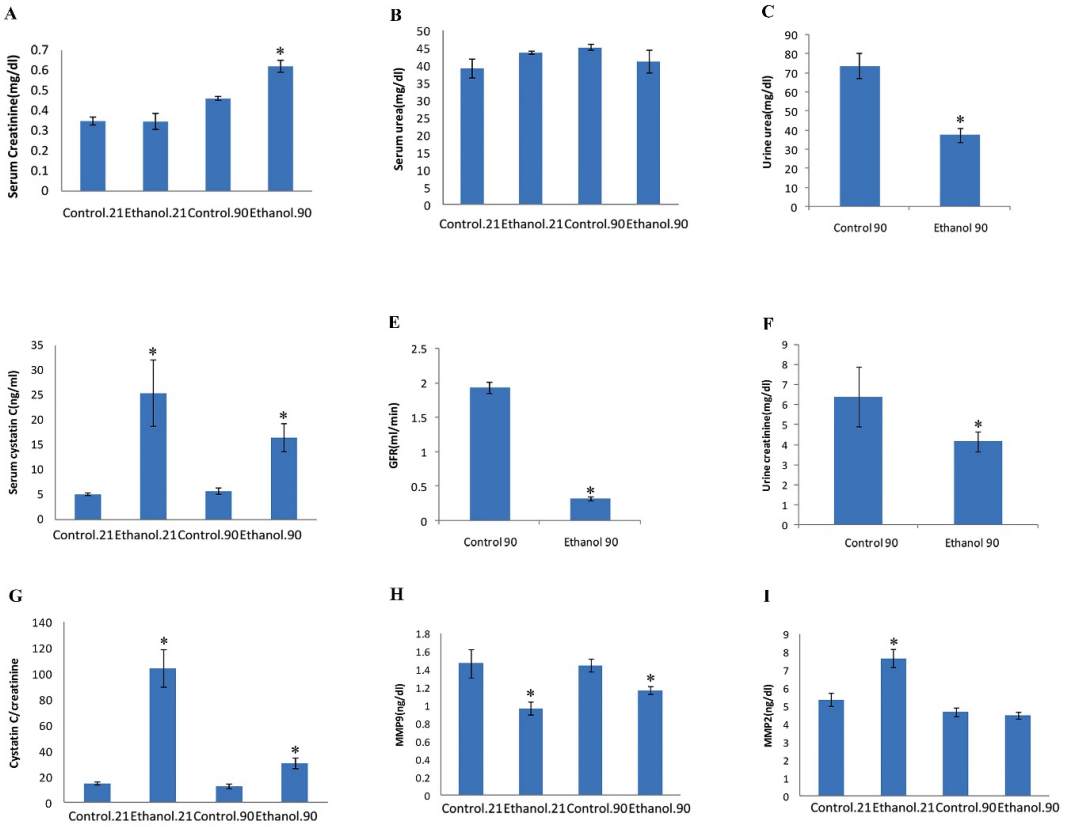

Figure 2. Maternal ethanol exposure-induced changes in kidney, urine and plasma levels of creatinine, urea, cystatin C, creatinine clearance, Cys C/ Creatinine ratio and MMPs in male offspring on 21PN and 90PN. *$P<0.05$: significant differences compared to the control.

compared with those in the control group offspring. In comparison with the control group, the levels of serum creatinine and urea showed no significant difference in the ethanol-treated offspring on PN21. On PN90, the serum creatinine level showed a significant increase in the ethanol rats, compared to the control rats. Serum urea level, however, was not significantly different in the ethanol and control groups. Urine creatinine and urea levels showed significant decreases in ethanol-treated group on PN90 compared to the control group. On PN90, the creatinine clearance, as an indicator of GFR, was significantly lower in the ethanol group than that in the control group. Moreover, the cystatin C/serum creatinine ratio was significantly higher in the ethanol- treated group, compared with that in the control group on PN21 and PN90 ($P < 0.05$). The kidney tissue MMP9 content on both PN21 and PN90 was significantly lower in ethanol group compared with the control group. However, the MMP2 levels in the ethanol group showed a significant increase on PN21 and remained unchanged on PN90, compared to the control group.

Histological and immunohistochemical analyses

The histopathological changes in the kidney sections of both the control and ethanol groups are shown in Figures 3 to 4, and Table 2. Ethanol treatment at the dose of 4.5 g/kg body weight during gestation and lactation induced several histopathological changes in kidney sections including vacuolization, lymphocyte aggregation in the interstitium, and perivascular chronic inflammatory cell (mostly lymphocyte) infiltration, and aggregation on both PN21 and PN90 (Figure 3). The semi-quantitative scoring for histopathological changes were as follows: no (-), weak (+), moderate (+ +), and severe (+ + +). The results have been shown in Table 2. Masson trichrome staining results revealed that there were no lesion scores in the bowman capsule, proximal tubule, distal tubule, and medulla in the kidney of control groups on PN21 and PN90 (grade = 0). On PN21, the microscopic lesion scores in the bowman's capsule, proximal tubule, distal tubule, and medulla were 0, 4 and 5, 0, 2, and 3 respectively in the ethanol group, which indicate increased damage to the architecture of proximal and moderate thickening of medulla tissue without obvious damage to the structure of kidney tissue (Figure 4). On PN90, the microscopic lesion scores in the bowman's capsule, proximal tubule, distal tubule, and medulla were 2 and 3, 4 and 5, 2 and 3, and 1, respectively, which indicate a moderate thickening

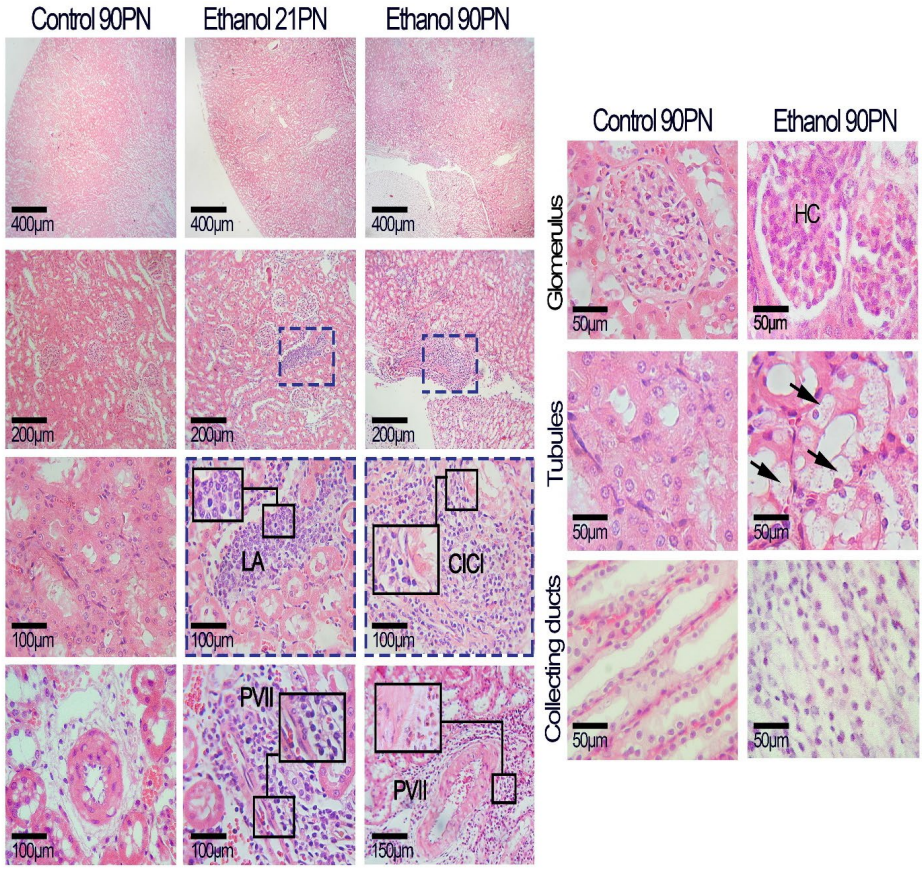

Figure 3. Photomicrographs of hematoxylin and eosin stained sections of kidney tissues from each group are shown. Ethanol treatment at dose of 4.5 g/kg body weight during gestation and lactation induced several histopathological changes in kidney sections as follows: Mild mesangial hypercellularity (HC), vacuolization (→), lymphoid aggregate of the interstitium (LA), perivascular chronic inflammatory cell (mostly lymphocyte) infiltration (PVII), and Chronic inflammatory cell infiltration (CICI) of the interstitium. Note higher magnifications of glomerulus, tubules, and collecting ducts from control and ethanol-received groups that are representing normal histological characteristics in the control sections and pathological changes in the glomerulus, tubules (vacuolization of tubular cells), and nonspecific pathologic changes in the collecting ducts.

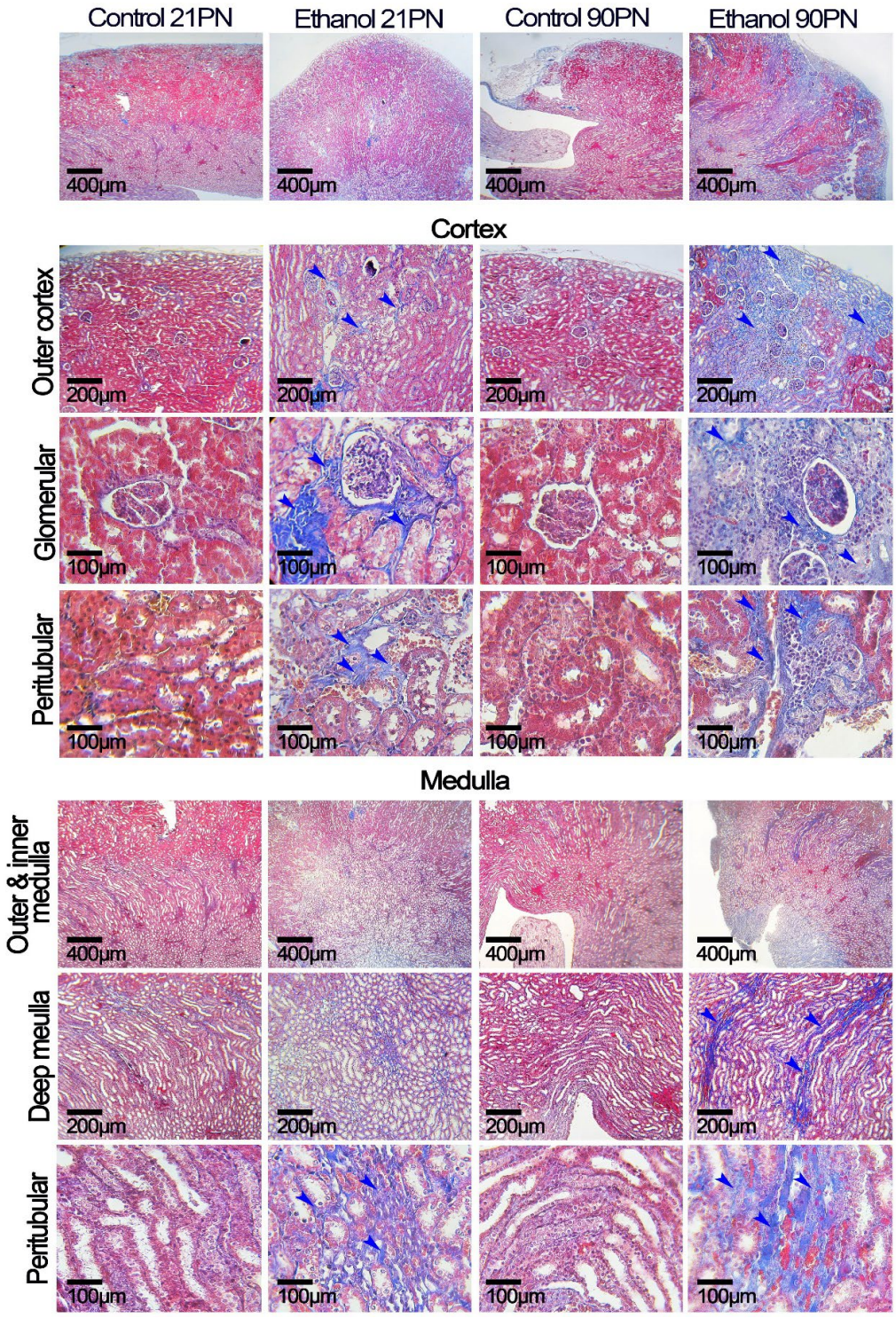

Figure 4. Photomicrographs of kidney tissue staining by Masson's trichrome from control and ethanol offspring on 21 and 90 PN, (Original magnification × 200), fibrosis band (→).

Table 2. The severity of histopathological alterations in the kidney in ethanol and control groups on PN21 and PN90

Histological changes	Control 21	Ethanol 21	Control 90	Ethanol 90
Vacuolization	-	+++	-	+++
Minimal mesangial hypercellularity	-	++	-	++
Lymphocyte aggregation in interstitium	-	++	-	+++
Perivascular inflammatory cells aggregates	-	++	-	+++

Grade: no (-), weak (+), moderate (+ +), severe (+ + +).

of bowman's capsule and distal tubules without obvious damage to the structure of kidney tissue in the ethanol group, increased damage to the architecture of proximal, as well as minimal fibrotic thickening of the kidney tissue in the medulla (Figure 4). The percentage of the glomerulus, proximal tubule, distal tubule, and medulla proliferative cells that are considered as PCNA positive indices in four main parts of the nephron in both control and ethanol groups on PN21and PN90 have been shown in Table 3. On PN21 the percent of proliferating cells were mild in the control group, and moderate to severe in the glomerulus, proximal tubule, and medulla, and mild to moderate in the distal tubule of ethanol-treated group. Ethanol-exposed offspring examined at PN90 showed mild to moderate cell proliferation in the glomerulus and all parts of the nephron tubule.

Discussion

Maternal proper nutritional status is important to maintain the optimal environment for fetal development and producing healthy offspring (16). According to the literature, alcohol consumption during pregnancy disrupts the intake of nutrient and impair the quality and quantity of proper nutrients (17). It is reported that alcohol consumption during pregnancy interferes with the uptake of essential amino acids and vitamins, particularly B_1 (thiamine), B_2 (riboflavin), B_6 (pyridoxine), vitamin A and C, and folic acid (19). When the maternal nutritional status is compromised by alcohol, the supply of essential nutrients is not available for correct growth and developing fetus that results in suboptimal health outcomes like intrauterine growth restriction or FASDs (16,18). The current study's results showed growth restriction in offspring of the ethanol group by significantly low body weight and kidney weight in the offspring of ethanol-treated group compared to the offspring of control mothers. Real-time PCR identified changes in gene expression of proteins and enzymes involved in the glomerular filtration barrier, normal physiology, and abnormal pathology of the kidney functions with corresponding cell proliferation, and fibrosis. In the present study, we showed that maternal ethanol exposure caused a significant decrease in podocyte podocin mRNA expression over 90 days after ethanol treatment. Nephrin mRNA showed a significant decrease in PN21 and a significant increase in PN90. New aspects of kidney disease pathogeneses and glomerular filter and filtrate quality have been identified by the recent description of gene defects of the podocytes including nephrin and podocin in congenital nephrotic syndrome (19,20). Based on size and charge, ultrafiltration of plasma takes place through the glomerular filtration barrier, which is composed of three specialized layers including epithelial cells called podocytes, a fenestrated capillary endothelium, and an intervening glomerular basement membrane (21). Recently, it has been evidenced that several specific proteins such as podocin, and nephrin are present in podocytes and interaction among these maintains the integrity of the slit diaphragm as an interdigitation of adjacent podocyte foot (22,23). The stoichiometric ratio between these filtration complex proteins is important to maintain normal ultrafiltration (22). Podocin interacted with nephrin in the slit diaphragm (24) is believed to increase the efficiency of nephrin signaling (25), and connects with nephrin to preserve the normal structures and functions of the glomerular filtration barrier (26). Interestingly, the results of our study showed a significant increase in the expression of nephrin mRNA and a decrease in the expression of podocin mRNA parallel with kidney impairment in the ethanol-treated group compared to the control group on PN90. Recently, the study by Schaefer et al indicated that upregulation of nephrin expression with downregulation of podocin expression in anti-Thy1.1-induced glomerulonephritis was highly manifested as a dysregulated filtration barrier and increased proteinuria (24). In addition, deficiency of nephrin, as it is found in congenital nephrotic syndrome, results in severe proteinuria and renal damage (27). Similar findings have also been reported suggesting that the knockout of the nephrin gene in slit diaphragm of mice results in podocyte foot process effacement and congenital nephritic syndrome (28,29). Although the mechanism through which ethanol leads to nephrin and podocin gene expression alteration is not fully understood, it may be the result of proinflammatory cytokines and inflammatory reactions. Recent findings in human embryonic kidney podocyte cells have discovered that proinflammatory cytokines such as interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) induce upregulation of nephrin mRNA levels (30,31). In addition, it has been previously reported that placental and fetal membrane nephrin and Neph 1 gene

Table 3. Ethanol exposure during gestation and feeding induced cells proliferation in the kidney of offspring on PN21 and PN90

	Control-21	Ethanol-21	Control-90	Ethanol-90
Glomerulus%	22 ± 3.16	71.8 ± 1.5*	4.75 ± 0.69	48 ± 3.8*
Proximal%	24.8 ± 1.2	59.58 ± 3.5*	4.6 ± 0.4	50.8 ± 2.3*
Distal%	15.43 ± 1.6	47.7 ± 2.5*	4 ± 1.2	40 ± 4.3*
Medulla %	12.6 ± 3.5	54 ± 4.7*	5 ± 1.4	48.2 ± 2*

Values expressed as mean ± SEM.
*$P < 0.05$; significant differences compared to the control.

expressions upregulate in response to inflammation (32). Our previous work has indicated that exposure of rats to ethanol either in pregnant female rats or male adult rats leads to the enhancement of pro-inflammatory markers such as IL-6 and TNF-α, as well as heart tissue fibrosis and proliferation (10,33). Recently, our study and the study conducted by Zhu et al have demonstrated that prenatal ethanol exposure leads to heart abnormality via inflammation and oxidative stress, renal fetal development retardation, and congenital nephritic syndrome in adult offspring rats through expressional disorders of genes related to the renin-angiotensin system (RAS) pathway (4,10). In the present study, the expression of renal VEGFR2 in the ethanol group reduced from fetus time to maturity at 90 days. Furthermore, VEGFR1 expression was reduced on PN21 but increased on PN90. The observed contrary results related to VEGFR mRNA expression on PN90 may be explained by distinct actions mediated by different VEGFR receptors. VEGFR2 mainly mediated pro-survival signals, but VEGFR1 interceded pro-inflammatory and fibrogenic effects. Moreover, downregulation of VEGFR2 (34,35) VEGF and VEGF receptors have an important role in the normal physiology and pathophysiology of the kidney, and their alterations are associated with defects in the glomerular podocyte architecture and kidney pathology (36). Previous studies have shown that blocking of VEGF in the mice kidney leads to proteinuria and glomerulosclerosis suggesting defects in the fenestrations of glomerular capillaries (36,37). Another novel finding of the current study was that gestational and early postnatal ethanol exposure induced alteration of MMPs amount accompanied by glomerular and tubule-interstitial fibrosis in the kidney of offspring. It has been well established that early damage at critical stages of embryo development in the intrauterine programming processes leads to permanent structural and/or functional changes in tissue (38). Remodeling of extracellular matrix (ECM) changes during the development, is one of the most important mechanisms mediating the intrauterine programming and fibrosis (39). Presently, it is widely accepted that MMPs enzymes are essential regulators in maintaining constant remodeling of ECM including invasion and branching of the ureteric bud in the metanephrinc mesenchyme during renal development (39,40). Gelatinase enzymes including MMP2 and MMP9 preserve the balance between production and degradation of extracellular matrix. Therefore, alterations in MMP2 and MMP9 activities may directly change ECM turnover, glomerular scarring, and kidney dysfunction (39,41).

Our study results also showed a significant increase in plasma cystatin C and creatinine levels, cystatin C/ creatinine ratio, and a significant decrease in creatinine clearance as an indicator of GFR in the ethanol offspring compared to the control. In contrast, other studies have suggested that intrauterine ethanol exposure does not significantly change GFR and/or increase GFR in the male offspring (42,43). The explanation for this inconsistency between the results of our study and those of the others might be due to ethanol exposure duration (our ethanol exposure duration was much longer), offspring age, or doses of given ethanol. Although cystatin C is an important marker for prediction of the kidney function, the Achilles heel of this marker as a predictor of the kidney function is that its amount is affected by some non-renal factors such as body weight, higher white blood cells count, and increased markers of inflammation (44-46). To overcome this issue, Grubb et al considered the ratio of cystatin C/ creatinine to be an indicator for detecting the severity of glomerular filtration barrier deficiency, especially glomerular pores shrinkage as follows: the shrinking of glomerular pores in a condition named “shrunken pore syndrome” leads to the enhancement of the ratio of serum cystatin C/ creatinine, and this event manipulates the composition of glomerular filtrate quality (47). A low decrease in pore size causes an increase in serum concentrations of large molecules such as cystatin C. More shrinkage of pores, however, leads to retention of smaller molecules such as urea and creatinine, which results in their accumulation in the blood. Therefore, the ratio of cystatin C/creatinine increases when a lower degree of pore shrinkage occurs and cystatin C is retained more than creatinine. This is because the cystatin C molecule is 100 times larger than creatinine (45,47). Interestingly, the results of the current study revealed a significant increase in the cystatin C/ creatinine ratio in the kidney of offspring exposed to ethanol in the development period. In the current study, inflammatory damage and inflammatory reaction were also evidenced by fibrosis, tubular cells proliferation, local hemorrhage, and causing severe congestion in peritubular and glomerular capillary and focal ploymorphonuclear leukocytes (PMN) infiltration in the kidneys obtained from the ethanol treated groups, compared to those obtained from the control group.

In general, the measurement of secondary products of inflammation and oxidative stress has been accepted as a trustworthy way of measuring the ongoing oxidative and inflammatory stress and damage. In agreement with the findings of the present study, a recent study conducted by Zhu et al reported that prenatal ethanol exposure led to fibrosis and tubular cells proliferation (4). The series of our previous works have also confirmed that ethanol exposure of mother in gestation and early postnatal days leads to apoptosis, fibrosis, proliferation, as well as oxidative and inflammatory stress in hippocampus, testis, and heart of the offspring (2,9,10). The mechanism through which ethanol induces cell proliferation and fibrosis is not fully understood; even though, it may have resulted from oxidative stress and inflammatory

reactions, as well as infiltrated PMN. The study by Sasaki et al clarified that infiltrated leukocytes and their resultant PMNs induced fibrosis and proliferation in the skin tissue cultured cells (48).

Conclusion

In conclusion, the results of this study have demonstrated that, in the long term, maternal ethanol consumption during gestation and sucking periods affects renal function and structure. At the molecular level, ethanol treatment promotes or inhibits the expression of genes involved in glomerular filtration barrier normal physiology and abnormal function. Moreover, it changes the amount of MMPs involved in kidney ECM turnover. The alteration in protein expression related to the glomerular filtration barrier resulted in renal function failure of pups. Besides, permanent functional alteration, structural changes with inflammatory nature including fibrosis and proliferation in the kidney of ethanol group, led us, with acknowledging previous works, to assume that ethanol induces its deleterious effects on kidney development via inflammatory reaction. These results provide the foundation for further research and clarifying the molecular mechanism underlying the developmental toxicity of gestational ethanol exposure through which ethanol disturbs kidney function and structure in developing periods.

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Authors' Contribution

FNM and AS contributed to conception, design, data analyses, and drafting the manuscript, ATA contributed to data analysis, and drafting the manuscript, FK, RN and MP conceived and designed the experiments and YR critically revised the manuscript.

Conflict of Interests

The authors report no conflict of interest.

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References

1. Mukherjee RA, Hollins S, Turk J. Fetal alcohol spectrum disorder: an overview. J R Soc Med. 2006;99(6):298-302. doi: 10.1258/jrsm.99.6.298.
2. Shirpoor A, Salami S, Khadem-Ansari MH, Minassian S, Yegiazarian M. Protective effect of vitamin E against ethanol-induced hyperhomocysteinemia, DNA damage, and atrophy in the developing male rat brain. Alcohol Clin Exp Res. 2009;33(7):1181-6. doi: 10.1111/j.1530-0277.2009.00941.x.
3. Gray SP, Kenna K, Bertram JF, Hoy WE, Yan EB, Bocking AD, et al. Repeated ethanol exposure during late gestation decreases nephron endowment in fetal sheep. Am J Physiol Regul Integr Comp Physiol. 2008;295(2):R568-74. doi: 10.1152/ajpregu.90316.2008.
4. Zhu Y, Zuo N, Li B, Xiong Y, Chen H, He H, et al. The expressional disorder of the renal RAS mediates nephrotic syndrome of male rat offspring induced by prenatal ethanol exposure. Toxicology. 2018;400-401:9-19. doi: 10.1016/j.tox.2018.03.004 .
5. Taylor CL, Jones KL, Jones MC, Kaplan GW. Incidence of renal anomalies in children prenatally exposed to ethanol. Pediatrics. 1994;94(2 Pt 1):209-12.
6. Gray SP, Cullen-McEwen LA, Bertram JF, Moritz KM. Mechanism of alcohol-induced impairment in renal development: could it be reduced by retinoic acid? Clin Exp Pharmacol Physiol. 2012;39(9):807-13. doi: 10.1111/j.1440-1681.2011.05597.x.
7. He H, Xiong Y, Li B, Zhu Y, Chen H, Ao Y, et al. Intrauterine programming of the glucocorticoid-insulin-like growth factor 1 (GC-IGF1) axis mediates glomerulosclerosis in female adult offspring rats induced by prenatal ethanol exposure. Toxicol Lett. 2019;311:17-26. doi: 10.1016/j.toxlet.2019.04.022.
8. Assadi FK, Zajac CS. Ultrastructural changes in the rat kidney following fetal exposure to ethanol. Alcohol. 1992;9(6):509-12. doi: 10.1016/0741-8329(92)90088-r.
9. Shirpoor A, Norouzi L, Khadem-Ansari MH, Ilkhanizadeh B, Karimipour M. The protective effect of vitamin E on morphological and biochemical alteration induced by pre and postnatal ethanol administration in the testis of male rat offspring: a three months follow-up study. J Reprod Infertil. 2014;15(3):134-41.
10. Shirpoor A, Nemati S, Khadem-Ansari MH, Ilkhanizadeh B. The protective effect of vitamin E against prenatal and early postnatal ethanol treatment-induced heart abnormality in rats: a 3-month follow-up study. Int Immunopharmacol. 2015;26(1):72-9. doi: 10.1016/j.intimp.2015.03.008.
11. Brocardo PS, Gil-Mohapel J, Christie BR. The role of oxidative stress in fetal alcohol spectrum disorders. Brain Res Rev. 2011;67(1-2):209-25. doi: 10.1016/j.brainresrev.2011.02.001.
12. Tofighi A, Ahmadi S, Seyyedi SM, Shirpoor A, Kheradmand F, Gharalari FH. Nandrolone administration with or without strenuous exercise promotes overexpression of nephrin and podocin genes and induces structural and functional alterations in the kidneys of rats. Toxicol Lett. 2018;282:147-53. doi: 10.1016/j.toxlet.2017.10.015.
13. Bancroft JD, Stevens A. Theory and Practice of Histological Techniques. Edinburgh: Churchill Livingstone; 1990.
14. Ashcroft T, Simpson JM, Timbrell V. Simple method of estimating severity of pulmonary fibrosis on a numerical scale. J Clin Pathol. 1988;41(4):467-70. doi: 10.1136/jcp.41.4.467.
15. Shirpoor A, Rezaei F, Abdollahzade Fard A, Taghizadeh Afshari A, Hosseini Gharalari F, Rasmi Y. Ginger extract protects rat's kidneys against oxidative damage after chronic ethanol administration. Biomed Pharmacother. 2016;84:698-704. doi: 10.1016/j.biopha.2016.09.097.
16. Young JK, Giesbrecht HE, Eskin MN, Aliani M, Suh M. Nutrition implications for fetal alcohol spectrum disorder. Adv Nutr. 2014;5(6):675-92. doi: 10.3945/an.113.004846.
17. Lieber CS. Relationships between nutrition, alcohol use, and liver disease. Alcohol Res Health. 2003;27(3):220-31.
18. Sebastiani G, Borrás-Novell C, Casanova MA, Pascual Tutusaus M, Ferrero Martínez S, Gómez Roig MD, et al. The effects of alcohol and drugs of abuse on maternal nutritional profile during pregnancy. Nutrients. 2018;10(8):1008. doi: 10.3390/nu10081008.
19. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. J Clin Invest. 2001;108(11):1583-7. doi: 10.1172/jci14629.
20. Antignac C. Genetic models: clues for understanding the pathogenesis of idiopathic nephrotic syndrome. J Clin Invest. 2002;109(4):447-9. doi: 10.1172/jci15094.
21. Zuo Y, Wang C, Sun X, Hu C, Liu J, Hong X, et al. Identification of matrix metalloproteinase-10 as a key mediator of podocyte injury and proteinuria. Kidney Int. 2021;100(4):837-49. doi:

10.1016/j.kint.2021.05.035.
22. Benzing T. Signaling at the slit diaphragm. J Am Soc Nephrol. 2004;15(6):1382-91. doi: 10.1097/01.asn.0000130167.30769.55.
23. Greka A, Mundel P. Cell biology and pathology of podocytes. Annu Rev Physiol. 2012;74:299-323. doi: 10.1146/annurev-physiol-020911-153238.
24. Schaefer L, Ren S, Schaefer RM, Mihalik D, Babelova A, Huwiler A, et al. Nephrin expression is increased in anti-Thy1.1-induced glomerulonephritis in rats. Biochem Biophys Res Commun. 2004;324(1):247-54. doi: 10.1016/j.bbrc.2004.09.042.
25. Huber TB, Kottgen M, Schilling B, Walz G, Benzing T. Interaction with podocin facilitates nephrin signaling. J Biol Chem. 2001;276(45):41543-6. doi: 10.1074/jbc.C100452200.
26. Chen S, Chen H, Liu Q, Ma Q. Effect of simvastatin on the expression of nephrin, podocin, and vascular endothelial growth factor (VEGF) in podocytes of diabetic rat. Int J Clin Exp Med. 2015;8(10):18225-34.
27. Putaala H, Soininen R, Kilpeläinen P, Wartiovaara J, Tryggvason K. The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. Hum Mol Genet. 2001;10(1):1-8. doi: 10.1093/hmg/10.1.1.
28. Gerke P, Huber TB, Sellin L, Benzing T, Walz G. Homodimerization and heterodimerization of the glomerular podocyte proteins nephrin and NEPH1. J Am Soc Nephrol. 2003;14(4):918-26. doi: 10.1097/01.asn.0000057853.05686.89.
29. Donoviel DB, Freed DD, Vogel H, Potter DG, Hawkins E, Barrish JP, et al. Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. Mol Cell Biol. 2001;21(14):4829-36. doi: 10.1128/mcb.21.14.4829-4836.2001.
30. Huwiler A, Ren S, Holthöfer H, Pavenstädt H, Pfeilschifter J. Inflammatory cytokines upregulate nephrin expression in human embryonic kidney epithelial cells and podocytes. Biochem Biophys Res Commun. 2003;305(1):136-42. doi: 10.1016/s0006-291x(03)00687-9.
31. Ren S, Xin C, Beck KF, Saleem MA, Mathieson P, Pavenstädt H, et al. PPARalpha activation upregulates nephrin expression in human embryonic kidney epithelial cells and podocytes by a dual mechanism. Biochem Biophys Res Commun. 2005;338(4):1818-24. doi: 10.1016/j.bbrc.2005.10.158.
32. Beall MH, Amidi F, Gayle DA, Wang S, Beloosesky R, Ross MG. Placental and fetal membrane nephrin and Neph1 gene expression: response to inflammation. J Soc Gynecol Investig. 2005;12(5):298-302. doi: 10.1016/j.jsgi.2005.02.009.
33. Shirpoor A, Norouzi L, Khadem-Ansari MH, Ilkhanizadeh B, Gharaaghaji R. Vasoprotective effect of vitamin E: rescue of ethanol-induced atherosclerosis and inflammatory stress in rat vascular wall. Int Immunopharmacol. 2013;16(4):498-504. doi: 10.1016/j.intimp.2013.04.024.
34. Zhang Z, Neiva KG, Lingen MW, Ellis LM, Nör JE. VEGF-dependent tumor angiogenesis requires inverse and reciprocal regulation of VEGFR1 and VEGFR2. Cell Death Differ. 2010;17(3):499-512. doi: 10.1038/cdd.2009.152.
35. Huang Y, Chen X, Dikov MM, Novitskiy SV, Mosse CA, Yang L, et al. Distinct roles of VEGFR-1 and VEGFR-2 in the aberrant hematopoiesis associated with elevated levels of VEGF. Blood. 2007;110(2):624-31. doi: 10.1182/blood-2007-01-065714.
36. Sugimoto H, Hamano Y, Charytan D, Cosgrove D, Kieran M, Sudhakar A, et al. Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem. 2003;278(15):12605-8. doi: 10.1074/jbc.C300012200.
37. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003;111(5):649-58. doi: 10.1172/jci17189.
38. Fowden AL, Giussani DA, Forhead AJ. Intrauterine programming of physiological systems: causes and consequences. Physiology (Bethesda). 2006;21:29-37. doi: 10.1152/physiol.00050.2005.
39. Lenz O, Elliot SJ, Stetler-Stevenson WG. Matrix metalloproteinases in renal development and disease. J Am Soc Nephrol. 2000;11(3):574-81. doi: 10.1681/asn.v113574.
40. Brenner CA, Adler RR, Rappolee DA, Pedersen RA, Werb Z. Genes for extracellular-matrix-degrading metalloproteinases and their inhibitor, TIMP, are expressed during early mammalian development. Genes Dev. 1989;3(6):848-59. doi: 10.1101/gad.3.6.848.
41. Lelongt B, Legallicier B, Piedagnel R, Ronco PM. Do matrix metalloproteinases MMP-2 and MMP-9 (gelatinases) play a role in renal development, physiology and glomerular diseases? Curr Opin Nephrol Hypertens. 2001;10(1):7-12. doi: 10.1097/00041552-200101000-00002.
42. Assadi FK, Manaligod JR, Fleischmann LE, Zajac CS. Effects of prenatal ethanol exposure on postnatal renal function and structure in the rat. Alcohol. 1991;8(4):259-63. doi: 10.1016/0741-8329(91)90321-m.
43. Gray SP, Denton KM, Cullen-McEwen L, Bertram JF, Moritz KM. Prenatal exposure to alcohol reduces nephron number and raises blood pressure in progeny. J Am Soc Nephrol. 2010;21(11):1891-902. doi: 10.1681/asn.2010040368.
44. Stevens LA, Schmid CH, Greene T, Li L, Beck GJ, Joffe MM, et al. Factors other than glomerular filtration rate affect serum cystatin C levels. Kidney Int. 2009;75(6):652-60. doi: 10.1038/ki.2008.638.
45. Bardallo Cruzado L, Pérez González E, Martínez Martos Z, Bermudo Guitarte C, Granero Asencio M, Luna Lagares S, et al. Serum cystatin C levels in preterm newborns in our setting: correlation with serum creatinine and preterm pathologies. Nefrologia. 2015;35(3):296-303. doi: 10.1016/j.nefro.2015.05.004.
46. Tangri N, Stevens LA, Schmid CH, Zhang YL, Beck GJ, Greene T, et al. Changes in dietary protein intake has no effect on serum cystatin C levels independent of the glomerular filtration rate. Kidney Int. 2011;79(4):471-7. doi: 10.1038/ki.2010.431.
47. Grubb A, Lindström V, Jonsson M, Bäck SE, Åhlund T, Rippe B, et al. Reduction in glomerular pore size is not restricted to pregnant women. Evidence for a new syndrome: 'shrunken pore syndrome'. Scand J Clin Lab Invest. 2015;75(4):333-40. doi: 10.3109/00365513.2015.1025427.
48. Sasaki T, Hori H, Arai K, Hattori S, Nagai Y. Effects of a factor derived from polymorphonuclear leukocytes on the growth and collagen metabolism in normal and scleroderma skin fibroblast cultures. J Dermatol Sci. 1996;11(1):10-8. doi: 10.1016/0923-1811(95)00410-6.