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The expressional disorder of the renal RAS mediates nephrotic syndrome of male rat offspring induced by prenatal ethanol exposure

Yanan Zhu[a,1], Na Zuo[a,1], Bin Li[a], Ying Xiong[a], Haiyun Chen[a], Hangyuan He[a], Zhaoxia Sun[a], Shuangshuang Hu[a], Hui Cheng[c], Ying Ao[a,b,*], Hui Wang[a,b,*]

[a] Department of Pharmacology, School of Basic Medical Science of Wuhan University, Wuhan, 430071, China
[b] Hubei Provincial Key Laboratory of Developmentally Originated Disorder, Wuhan, 430071, China
[c] Department of Nephrology, Renmin Hospital of Wuhan University, Wuhan, 430060, China

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ABSTRACT

This study aimed to prove that prenatal ethanol exposure (PEE) can induce nephrotic syndrome in male rat offspring and to explore the underlying intrauterine programming mechanisms. Pregnant Wistar rats were intragastrically administered ethanol (4 g/kg d) from gestational day (GD) 9 to GD 20, and the male fetuses were delivered by cesarean section at GD20 and the male adult offspring were euthanized at postnatal week (PW) 24. *In vitro*, the primary metanephric mesenchyme cells were treated with ethanol at concentrations of 15–60 mM. The results indicated that the kidneys of adult offspring in the PEE group exhibited glomerulosclerosis as well as interstitial fibrosis. The levels of serum creatinine and urine protein were elevated; the serum total cholesterol level was increased and the serum albumin concentration was reduced. In the fetal kidney, developmental retardation was presented in the PEE group via pathological examinations, accompanied by the expressional inhibition of the glial-cell-line-derived neurotrophic factor/c-ret tyrosine kinase receptor (GDNF/c-ret) signaling pathway. Although serum angiotensin II (Ang II) level and the gene expression of renal angiotensin-converting enzyme (ACE) were increased in the PEE group, the expression of renal angiotensin II type 2 receptor (AT_2R) was significantly inhibited, accompanied by a reduction in the H3K27ac level on the AT_2R gene promoter. In the non-classical renin-angiotensin system (RAS), the expression of renal angiotensin converting enzyme 2 (ACE2) and Mas receptor (MasR) were inhibited in the PEE group. The above changes of the classical and non-classical RAS all sustained from utero to adulthood. *In vitro*, ethanol elevated the gene expression of ACE and angiotensin II type 1a receptor ($AT_{1a}R$) whereas it reduced the expression of AT_2R, ACE2, and MasR, accompanied by a reduction in the H3K27ac level on AT_2R gene promoter. Taken together, these results suggested that PEE can induce fetal kidney developmental retardation and adult nephrotic syndrome, and direct regulation of ethanol to the renal RAS was involved in the mechanism of nephrotic syndrome induced by PEE.

1. Introduction

Intrauterine growth restriction (IUGR), as a common developmental toxicity, is diagnosed clinically when the fetal birth weight is two standard deviations less than the mean birth weight at the same gestational age (Huang et al., 2012). Epidemiological studies indicated that low birth weight predicted subsequent hypertension, insulin resistance, osteoporosis and coronary heart disease in later life (Godfrey et al., 2016; Reynolds et al., 2001). Experimental data also showed that IUGR increased the risk of metabolic syndrome in offspring animals (Malo et al., 2013; Sohi et al., 2011). These studies demonstrated that IUGR is associated with fetal original diseases of the offspring.

Prenatal exposure to toxicants is a definite cause of IUGR. Alcohol consumption is a very common phenomenon among pregnant women

Abbreviations: PEE, prenatal ethanol exposure; GD, gestational day; PW, postnatal week; PD, postnatal day; GDNF, glial-cell-line-derived neurotrophic factor; c-ret, c-ret tyrosine kinase receptor; Ang II, angiotensin II; ACE, angiotensin-converting enzyme; AT_2R, angiotensin II type 2 receptor; $AT_{1a}R$, angiotensin II type 1a receptor; RAS, renin-angiotensin system; ACE2, angiotensin-converting enzyme 2; MasR, Mas receptor; IUGR, intrauterine growth restriction; Scr, Serum creatinine; BUN, Blood urea nitrogen; TC, total cholesterol; SPF, Specific pathogen free; HE, hematoxylin and eosin; PAS, periodic acid-Schiff; Masson, Masson's trichrome; MOD, mean optical density; Cdh11, Cadherin11; PI3K, phosphoinositide-3-kinase; Pax2, paired Box 2; GAPDH, glyceraldehyde 3- phosphate dehydrogenase; H3K9ac, histone 3 Lysine 9 acetylation; H3K14ac, histone 3 Lysine 14 acetylation; H3K27ac, histone 3 Lysine 27 acetylation; Agt, angiotensinogen; TGF-β, transforming growth factor-β; Ang(1–7), angiotensin 1–7; ND, nephric duct; UB, ureteric bud

* Corresponding authors at: Department of Pharmacology, School of Basic Medical Science of Wuhan University, Wuhan, 430071, China.
E-mail addresses: yingao@whu.edu.cn (Y. Ao), wanghui19@whu.edu.cn (H. Wang).
[1] These authors contributed equally to this work.

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in many countries (Zimatkin et al., 2006). In the United States, England and Canada, 20%–32% of the pregnant women drink, and in some European countries, the rate is even higher, exceeding 50% (May et al., 2005). It has been proven that prenatal ethanol exposure (PEE) impairs fetal development and causes IUGR (Jones and Smith, 1973; O'Leary, 2004; Valero De Bernabe et al., 2004). Our previous studies also demonstrated that PEE resulted in IUGR and increased the susceptibility to non-alcoholic fatty liver disease and osteoarthritis of the offspring (Pan et al., 2016; Shen et al., 2014).

Nephrotic syndrome is a common renal disorder characterized by intense proteinuria, hypoalbuminemia, generalized edema, and hyperlipidemia (Pereira et al., 2015). In many cases, nephrotic syndrome leads to end-stage renal disease, requiring renal replacement therapy (Pereira et al., 2015). The animal experiments and clinical data demonstrated that IUGR leads to renal development retardation and the reduction in the number of nephrons *in utero*, and the consequent glomerular over-filtration after birth resulted in kidney damage in adulthood (Baum, 2010; Brenner et al., 1988). The recent retrospective analysis also indicated that low birth weight is closely associated with the development of nephrotic syndrome (Plank et al., 2007; Zidar et al., 1998), indicating that IUGR might be the fetal developmental origin of nephrotic syndrome.

The classical renin-angiotensin system (RAS) plays an important role in fetal kidney development and regulating kidney function (Ao et al., 2015; Sun et al., 2015). Prenatal adverse environment, such as hypoxia, low-protein maternal diet, or lipopolysaccharide exposure inhibited the expression of the renal RAS and kidney development and increased the susceptibility of kidney diseases in adulthood (Gonzalez-Rodriguez et al., 2013; Hao et al., 2010; Woods et al., 2001). Moreover, Ang II-ACE2-Ang-(1–7)-MasR axis is a newly recognized non-classical RAS, that is also proven to be involved in fetal development (Zhang et al., 2001), and the deficiency of angiotensin-converting enzyme 2 (ACE2) resulted in the developmental retardation of the offspring (Bharadwaj et al., 2011). However, whether PEE leads to nephrotic syndrome through intrauterine programming of the classical or non-classical RAS pathway is still unknown.

Epidemiological investigations and animal experiments showed that the male rats were more sensitive to renal damage than the female rats, shown as increased glomerular filtration rate, reduced nephron number, proteinuria (Cattran et al., 2008; Doublier et al., 2011; Gray et al., 2010). Therefore, the male offspring were selected as research subjects in this study. The present study aimed to explore whether PEE can cause nephrotic syndrome in the male offspring kidney and discover the RAS-related programming mechanism. This study will be beneficial for thoroughly understanding the recent and long-term toxicity of adverse utero environment to offspring kidney and clarifying the fetal-originated mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol was obtained from Zhen Xin Corp. (Shanghai, China) and Kemiou Chemical Reagent Corp. (Tianjin, China). Isoflurane was purchased from Baxter Healthcare Corp. (Deerfield, IL, USA). Serum creatinine (Scr) and blood urea nitrogen (BUN) assay kits were bought from Jiancheng Bioengineering Institute (Nanjing, China). Serum total cholesterol (TC) assay kits were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Angiotensin II (Ang II) radioimmunoassay kits were obtained from the North Institute of Biological Technology (Beijing, China). Protein G Sepharose (No. 17061802) was obtained from General Electric Company (Millipore, USA). Primary antibodies such as rabbit IgG (No. ab172730) and rabbit anti-Ki67 (No. ab15580) were purchased from Abcam Inc. (Cambridge, UK), rabbit anti-AT_2R (No.bs-0438R) was obtained from Biosynthesis Biotechnology Inc. (Beijing, China). Chromatin immunoprecipitation antibodies for anti-H3K9 (No. A7255), anti-H3K14 (No. A7254), and anti-H3K27 (No. A7253) were purchased from Abclonal Co., Ltd. (Wuhan, China). The RNA-Solv reagent and HiBind™ PCR DNA extraction kit were provided by Omega Bio-Tek Inc. (Norcross, GA, USA). Reverse transcript and quantitative PCR (RT-qPCR) kits were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). All rat oligonucleotide primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). DMEM/F12 (1:1) and fetal bovine serum (FBS) were provided by Gibco Inc. (St Louis, MO, USA). Streptomycin and penicillin were purchased from Genom Co., Ltd. (Hangzhou, China). All of the chemicals and reagents were of analytical grade.

2.2. Animals and treatment

Animal experiments were performed at the Center for Animal Experiments of Wuhan University (Wuhan, China), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All animal experimental procedures were approved by and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee and the International Council on Research Animal Care.

Specific pathogen-free (SPF) Wistar rats (with weights of 200–240 g for females and 260–300 g for males) were obtained from the Experimental Center of Hubei Medical Scientific Academy (Wuhan, China). The animals were grouped and treated as previously described (Shangguan et al., 2017). Briefly, after one week of accommodation, 2 females mated with 1 male every night. Gestational day (GD) 0 was confirmed by the appearance of sperm in a vaginal smear. The pregnant females were transferred to individual cages and randomly divided into two groups: a control group and a PEE group (n = 8–10 for each group). The PEE group was administered ethanol 4 g/kg once daily by gavage from (GD9) until term delivery (GD20), while the control group was given the same volume of distilled water. The pregnant rats were kept until normal delivery (GD21), and on postnatal day 1 (PD1) the numbers of pups were normalized to 8 pups per litter to assure adequate and standardized nutrition until weaning (postnatal week 4, PW4). At PW24, the male offspring rats were anesthetized with isoflurane and decapitated (Huang et al., 2015). Serum was prepared and stored at −80 °C. The kidneys were dissected and weighed. The right kidneys were split longitudinally and fixed in 4% paraformaldehyde solution for histological examination, and the left kidneys were immediately frozen and stored at −80 °C.

In the experiment on the fetus, the pregnant rats were euthanized under isoflurane anesthesia at GD20 about 1 h after the last dose of ethanol. The pregnant rats with litter sizes of 10 to 14 were considered qualified. IUGR was diagnosed according to the reference (Dong et al., 2017). Three to five fetal kidneys from 3 dams (one kidney per litter) from each group was randomly selected and routinely fixed for histological and ultra-structural examination, and the rest of the fetal kidneys from each group were immediately frozen and stored at −80 °C for gene expression analyses.

2.3. Blood sample analysis

Scr, BUN, and serum TC were detected using assay kits following the manufacturer's protocol.

2.4. Histological and immunohistochemistry analysis

The adult right kidney tissue was fixed for 24 h in 4% paraformaldehyde solution and embedded in paraffin, sectioned into 4-μm-thick slices, and stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) reagent, and Masson's trichrome (Masson). The sections were observed and photographed using an Olympus AH-2 light microscope (Olympus, Tokyo, Japan).