

Alcohol exposure in utero perturbs retinoid homeostasis in adult rats

Youn-Kyung Kim¹, Michael V. Zuccaro¹, Changqing Zhang², Dipak Sarkar², Loredana Quadro¹

¹Department of Food Science, Rutgers Center for Lipid Research, ²Department of Animal Sciences and Endocrine Research Program, Rutgers University, New Brunswick, NJ 08901, USA

Contributions: (I) Conception and design: L Quadro; (II) Administrative support: L Quadro; (III) Provision of study materials or patients: YK Kim, MV Zuccaro, D Sarkar; (IV) Collection and assembly of data: YK Kim, MV Zuccaro, C Zhang; (V) Data analysis and interpretation: YK Kim, MV Zuccaro, L Quadro; (VI) Manuscript writing: L Quadro; (VII) Final approval of manuscript: All authors.

Correspondence to: Loredana Quadro. Department of Food Science, Rutgers Center for Lipid Research, Rutgers University, New Brunswick, NJ 08901, USA. Email: quadro@aesop.rutgers.edu.

Background: Maternal alcohol exposure and adult alcohol intake have been shown to perturb the metabolism of various micro- and macro-nutrients, including vitamin A and its derivatives (retinoids). Therefore, it has been hypothesized that the well-known detrimental consequences of alcohol consumption may be due to deregulations of the metabolism of such nutrients rather than to a direct effect of alcohol. Alcohol exposure in utero also has long-term harmful consequences on the health of the offspring with mechanisms that have not been fully clarified. Disruption of tissue retinoid homeostasis has been linked not only to abnormal embryonic development, but also to various adult pathological conditions, including cancer, metabolic disorders and abnormal lung function. We hypothesized that prenatal alcohol exposure may permanently perturb tissue retinoid metabolism, predisposing the offspring to adult chronic diseases.

Methods: Serum and tissues (liver, lung and prostate from males; liver and lung from females) were collected from 60-75 day-old sprague dawley rats born from dams that were: (I) fed a liquid diet containing 6.7% alcohol between gestational day 7 and 21; or (II) pair-fed with isocaloric liquid diet during the same gestational window; or (III) fed ad libitum with regular rat chow diet throughout pregnancy. Serum and tissue retinoid levels were analyzed by reverse-phase high-performance liquid chromatography (HPLC). Serum retinol-binding protein (RBP) levels were measured by western blot analysis, and liver, lung and prostate mRNA levels of lecithin-retinol acyltransferase (LRAT) were measured by qPCR.

Results: Retinyl ester levels were significantly reduced in the lung of both males and females, as well as in the liver and ventral prostate of males born from alcohol-fed dams. Tissue *LRAT* mRNA levels remained unchanged upon maternal alcohol treatment.

Conclusions: Prenatal alcohol exposure in rats affects retinoid metabolism in adult life, in a tissue- and sex-dependent manner. We propose that the alcohol-induced perturbations of vitamin A metabolism may predispose to detrimental consequences on adult health.

Keywords: Alcohol; liver; lung; prostate; retinoids

Submitted Nov 14, 2014. Accepted for publication Dec 16, 2014.

doi: 10.3978/j.issn.2304-3881.2015.01.06

View this article at: <http://dx.doi.org/10.3978/j.issn.2304-3881.2015.01.06>

Introduction

Vitamin A is an essential nutrient responsible for regulating and maintaining mammalian vision, embryogenesis, immunity, proliferation and differentiation (1). Once obtained from the diet as retinol, retinyl esters, and

pro-vitamin A carotenoids (mainly β -carotene), vitamin A is predominantly stored in the liver as retinyl ester, synthesized by lecithin-retinol acyltransferase (LRAT). From the liver, retinol generated upon hydrolysis of retinyl esters is secreted into the circulation bound to retinol-binding protein (RBP) to reach the extrahepatic target tissues. Within the target

cells, retinol dehydrogenases (RDHs), and also alcohol dehydrogenases (ADHs), convert retinol into retinaldehyde, which is subsequently oxidized by retinaldehyde dehydrogenases (RALDHs) to generate retinoic acid, the active form of vitamin A that functions as a transcriptional regulator (2-7).

The well-known detrimental effects of alcohol on adult tissues include the impairment of retinoid (vitamin A and its derivatives) metabolism following both acute and chronic alcohol exposure (8). This effect has been ascribed to the biochemical similarity between ethanol and retinol metabolism, which also share enzymes such as ADH and cytochrome P450 2E1 (CYP2E1) (8). In humans, chronic consumption of alcohol progressively decreases plasma retinol levels and depletes hepatic retinoid stores, in both hepatocytes and stellate cells, affecting both retinol and retinyl ester levels (9-15), and overall, predisposing alcoholics to develop clinical signs of vitamin A deficiency (VAD) (8). Studies in various animal models, although predominantly in rodents, have confirmed the findings in humans with the exception of the decreased plasma retinol levels (8). Interestingly, chronic alcohol consumption also has an effect on extrahepatic retinoid homeostasis by increasing, rather than decreasing, retinoid concentrations in various brain regions, the colon, esophagus, kidney, lung, testes and trachea (16-22). The effects of alcohol consumption on tissue retinoic acid levels have not been unequivocally defined, due to discrepant data reported in the literature (23). Multiple mechanisms have been proposed to explain the effects of alcohol on retinoid metabolism, including the possibility that alcohol stimulates mobilization of hepatic retinoid stores towards extra-hepatic tissues and retinoid catabolism (8).

Retinoid homeostasis in the developing tissues is critical to normal embryonic development in mammals (24). Therefore, both deficiency and excess of vitamin A during gestation result in fetal death or in a spectrum of congenital defects (25). Alcohol exposure in utero has also been shown to perturb retinoid metabolism and signaling in developing tissues (26). Although, an exact mechanism of this disturbance has yet to be established, it has been associated with the development of fetal alcohol spectrum disorders (FASD) (27,28). This term comprises a broad array of diverse pathological conditions of children born from mothers consuming alcohol during pregnancy (29). The most severe of these conditions is called fetal alcohol syndrome (FAS), with central nervous system dysfunctions, impaired growth, facial abnormalities, and both structural and functional brain

damage (30). Many features of FAS mimic those of VAD or vitamin A excess, including poor fetal growth, eye and brain deficiency, heart defects, and impaired neural crest cell migration (27). The similarity between VAD and FAS has led to the hypothesis that alcohol-induced embryonic VAD contributes to FAS (27,28). Initially, it was proposed that ethanol competes with retinol for the enzyme ADH IV (ADH-IV), thus reducing the formation of retinoic acid from retinol (28,31). More recently, however, others have postulated that alcohol-induced vitamin A toxicity contributes to FAS, as hippocampal retinoic acid levels were elevated in alcohol-exposed fetuses (21).

In addition to inducing FASD, intrauterine alcohol exposure is also one of many factors known to predispose offspring to adult diseases by deregulating developmental and/or metabolic pathways heavily interconnected to one another. For example, maternal alcohol exposure can predispose progeny to cancer (32-34), circadian disruptions (35-40), and metabolic diseases in response to other stressors in adult life (41). Given that many adult pathological conditions have been attributed to alterations in vitamin A metabolism (42-45), to better understand the mechanisms whereby prenatal exposure to alcohol compromises health later in life, this study investigates the effects of maternal consumption of alcohol on retinoid metabolism of adult offspring in sprague dawley rats. We chose to analyze the liver, lung and prostate as diseases linked to pathological conditions of these organs have been associated with perturbation in retinoid metabolism (42-45). We show that prenatal alcohol exposure in rats affects retinoid metabolism in adult life, in a tissue- and sex-dependent manner.

Materials and methods

Animals and diet

Pregnant Sprague Dawley rats were purchased from Charles River (Wilmington, MA, USA) and individually housed in a controlled environment with a 12-hour light/dark cycle with the period of darkness between the hours of 19:00 and 07:00. Dams were acclimated to the environment for 2 days and, then, fed a liquid diet containing ethanol (alcohol-fed; F1258SP; Bio-Serv, Frenchtown, NJ, USA) or an isocaloric liquid diet (pair-fed; F1259SP; Bio-Serv, Frenchtown, NJ, USA) or an ad libitum rat regular chow (chow-fed; Purina, St Louis, MO, USA; LabDiet 5012). The liquid diets contained 6 IU of vitamin A/mL, and calories from protein, carbohydrates and fat were 15%, 49% and 36%,

respectively, giving rise to an estimated calorie intake of about 75 kcal/day and to an estimated vitamin A intake of 450 IU vitamin A/day, based on a consumption of 75 mL diet/day (46). The regular rat chow diet contained 12 IU vitamin A/g and 1.9 ppm of β -carotene/g. Calories from protein, carbohydrates, and fat were 27%, 60% and 13%, respectively, giving rise to an estimated calorie intake of about 70 kcal/day and to an estimated vitamin A intake of 264 IU vitamin A/day, based on a consumption of 20-25 g diet/day (47).

Experimental scheme

Dams were acclimated to the alcohol diet from day 7 to 9 of gestation by feeding a liquid diet containing 2.2% ethanol on day 7 and 4.4% ethanol on day 8. Once acclimated, dams were fed the liquid diet containing 6.7% ethanol from days 9 to 21. This concentration of ethanol represented 35% of total calories/day (32). Dams drinking 6.7% alcohol are expected to present with blood alcohol levels between 100 to 150 mg/dL (48,49), which translates to approximately three to five drinks in 2 hours in women (50). At birth, male and female pups were cross-fostered to dams fed ad libitum with the regular rat chow diet and litters were normalized to 8 pups per dam. Pups were weaned at 21 days of age and fed the regular rat chow diet ad libitum until 60-70 days of age (n=9-13 offspring pups per treatment group) when they were sacrificed by rapid decapitation. Serum, liver, lung and prostate were collected, frozen and stored at -80°C until further analyses. Prior to freezing, the three lobes of the prostate (dorsal, lateral and ventral) were quickly dissected and stored in separate tubes. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rutgers University Institutional Committee on Animal Care.

High-performance liquid chromatography (HPLC)

Measurements of retinol and retinyl ester in serum, and tissue (liver, lung and prostate) were performed by reverse-phase HPLC, as previously described (51,52). Retinol and retinyl esters (retinyl oleate, linoleate, palmitate and stearate) were identified by comparing retention times and spectral data of experimental compounds with those of authentic standards. Retinyl acetate (Sigma, MO, USA) was added as internal standard.

Western blot analysis

Analysis was performed as previously described (4) by using a rabbit polyclonal anti-rat RBP antiserum for immunodetection. Albumin, detected upon treating the membranes with either Coomassie Blue or Ponceau S stain, and detected by a rabbit polyclonal anti-albumin antibody (Abcam, Cambridge, MA, USA), was used as a loading control. The molecular weight of each detected protein is as follow: RBP, 21 kDa and albumin, 65 kDa. The quantification of the membranes was completed by densitometry analysis with Quantity One Program (Bio-Rad, CA, USA).

RNA extraction, cDNA synthesis, and qPCR

RNA extraction, cDNA synthesis, and qPCR were performed as previously described (53,54). To determine changes in gene expression of *Lrat*, the $\Delta\Delta\text{Ct}$ method was used. Gene expression changes were expressed as fold of the control group (PF). List of primers and amplicon size is as follows: LRAT forward primer 5'-AACCGTGTCTCGCCCATCTAAT-3', LRAT reverse primer 5'-TTCTGAGTGCGTTCCTTGTC-3', expected amplicon size is 68 bp; 18S reverse primer 5'-GCTGGAATTACCGCGGCT-3', 18S forward primer 5'-CGGCTACCACATCCAAGGAA-3', expected amplicon size is 187 bp.

Statistical analyses

Normality of variables was assessed by the Shapiro-Wilk test. When the data were not normally distributed, values were logarithmically transformed prior to statistical analysis (female serum retinyl esters and hepatic retinol; male liver, lung, ventral and lateral prostate retinyl esters). Normally distributed values were statistically analyzed by one-way ANOVA for the comparison. Not normally distributed values were statistically tested by Kruskal-Wallis followed by Mann-Whitney test for the comparison. A P value of <0.05 was used to establish statistical significance. Analyses were performed by means of SPSS (SPSS Inc., Chicago, IL, USA).

Results

Serum retinoid and RBP levels

Serum retinol and retinyl ester concentrations were measured by reverse phase HPLC in male and female

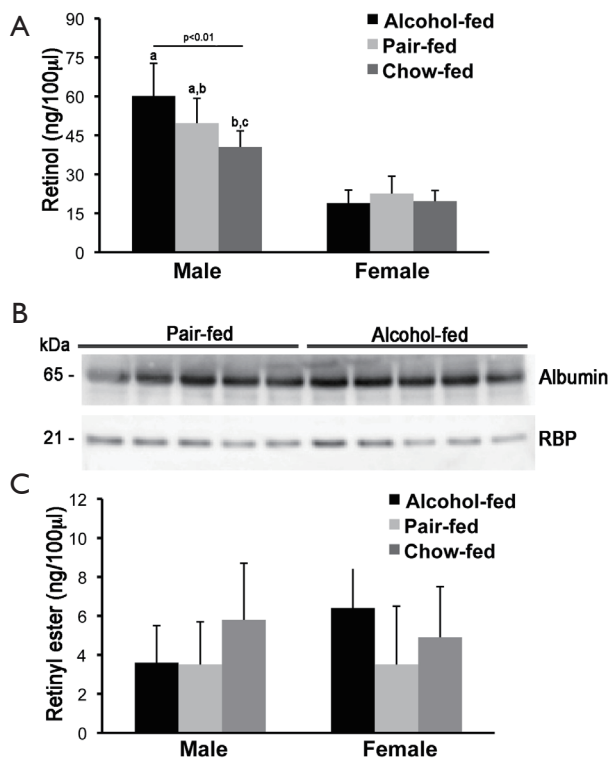


Figure 1 Serum retinol, retinyl ester and RBP levels. (A) Serum retinol and (C) retinyl ester levels were measured by HPLC analysis. Retinyl ester concentrations are the sum of retinyl oleate, linoleate, palmitate and stearate levels. Results are expressed as mean \pm SD; $n=6-7$ animals/group; (B) serum RBP levels of male rats were measured by western blot by using albumin as loading control. Molecular mass (kDa) of detected proteins is indicated on the left side of the panels. Statistical analysis as described in the materials and methods. RBP, retinol-binding protein; HPLC, high-performance liquid chromatography.

offspring rats from dams that were alcohol-fed, pair-fed or chow-fed during gestation. As previously reported, serum retinol levels were higher in males than in females rats (55-57). Whereas serum retinol levels were slightly but significantly elevated in the alcohol-fed compared to the chow-fed male group, females rat did not show significant differences in serum retinol concentrations, regardless of the maternal treatment (Figure 1A). In agreement with these data, serum Western blot analysis of RBP and the sole specific retinol carrier in the bloodstream (4), showed similar levels of the protein in both males (Figure 1B) and females (data not shown) when the alcohol-fed and the pair-fed groups were compared. Serum retinyl ester levels were not different among offspring from the three different maternal

treatment groups, regardless of the sex (Figure 1C).

Tissue retinol and retinyl ester levels

Retinol and retinyl ester levels were measured by reverse phase HPLC in liver, lung and prostate from the same groups of rats described above. As previously reported, liver retinol and retinyl ester levels were generally higher in female than in male rats (Figure 2A,B) (55-58). Hepatic female retinol levels were significantly lower in the chow-fed group compared to both alcohol-fed and pair-fed female offspring (Figure 2A). In contrast, hepatic retinyl ester levels were not significantly different among female rats from the three different maternal treatment groups (Figure 2B). In the case of the male offspring, whereas hepatic retinol concentrations were similar among the three experimental groups (Figure 2A), hepatic retinyl ester levels were lower in males alcohol-fed *vs.* pair-fed and chow-fed groups (Figure 2B).

Lung retinol and retinyl ester levels were generally similar in female compared to male rats (55,58). In the male offspring, lung retinol levels were not different among the three experimental groups (Figure 2C), whereas in the females, lung retinol levels were lower in the chow-fed group compared to the pair-fed animals of the same sex (Figure 2C). Retinyl ester levels were significantly lower in alcohol-fed *vs.* pair-fed, regardless of the gender (Figure 2D). Moreover, lung retinyl ester concentrations were significantly greater in the female pair-fed *vs.* the chow-fed group of the same sex (Figure 2D).

Finally, in the case of the ventral prostate, whereas retinol levels were unchanged among the various groups (Figure 2E), retinyl ester (Figure 2F) levels were significantly decreased in animals of the alcohol-fed *vs.* pair-fed group. Retinol and retinyl ester levels were not different in the dorsal and lateral prostate, regardless of the maternal treatment (Figure 2E,F). Note that alterations of the acyl composition of the retinyl ester stores of the offspring from alcohol-fed dams were not observed in any of the tissues analyzed.

Overall, these data indicate that maternal alcohol intake decreased retinyl ester levels in the adult offspring, in a tissue- and sex-dependent manner.

Transcriptional levels of lecithin-retinol acyltransferase (LRAT)

To establish whether the changes in retinyl ester concentrations observed in liver, lung and prostate of the

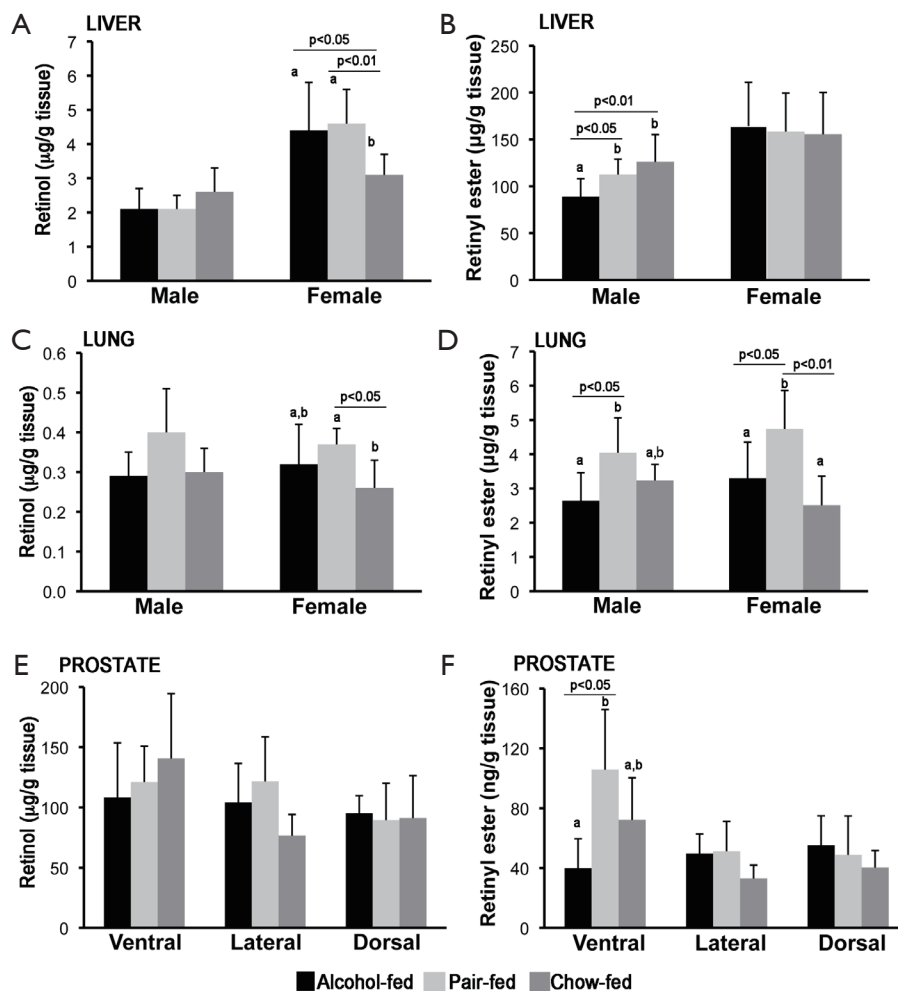


Figure 2 Tissue retinoid levels. Hepatic retinol (A) and retinyl ester (B) levels; lung retinol (C) and retinyl ester (D) levels; and prostate retinol (E) and retinyl ester (F) levels were measured by HPLC analysis. Retinyl ester concentrations are the sum of retinyl oleate, linoleate, palmitate and stearate levels. Results are expressed as mean \pm SD; $n=5-6$ animals/group. Statistical analysis as described in the materials and methods. HPLC, high-performance liquid chromatography.

offspring from dams alcohol-fed during pregnancy was correlated to an attenuated transcription of *Lrat*, the main enzyme that synthesizes retinyl ester in mammalian tissues (2,59), we measured *Lrat* mRNA levels by qPCR. No differences were observed in *Lrat* mRNA levels between alcohol-fed and pair-fed groups (males: liver, lung and prostate; females: liver and lung; Figure 3), suggesting that alcohol exposure in utero does not affect *Lrat* transcription in these organs.

Discussion

Over the past decade, it has become clear that the

intrauterine environment plays a critical role in determining the susceptibility to develop chronic pathological conditions, such as diabetes, cardiovascular diseases and cancer, later in life (60). Among the factors that have been shown to influence fetal programming, maternal diet is an important determinant (61).

Alcohol consumption during pregnancy occurs at surprisingly high rates. Roughly 40% of women drink during pregnancy, with about 10% of them drinking heavily (62). Although ideally this condition would be prevented by total sobriety during pregnancy, the prevalence of alcohol use among women of child-bearing age is over 50% (62). Drinking alcohol at any level during pregnancy has been

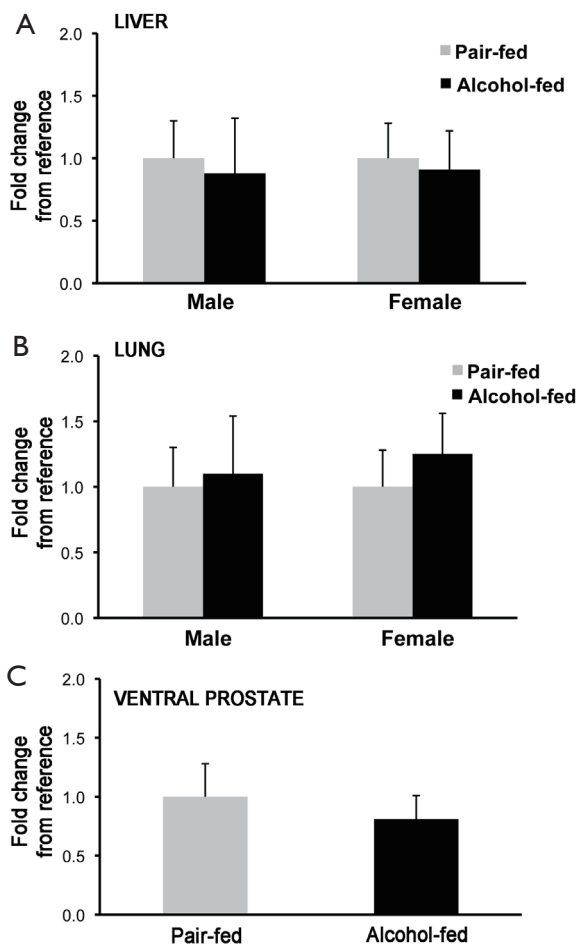


Figure 3 qPCR analysis of *Lrat* mRNA levels in various tissues. (A) Analysis performed in liver; and (B) lung of male and female rats; and (C) in ventral prostate. Values are expressed as mean \pm SD; $n=3-4$ /group. Statistical analysis as described in the materials and methods.

known to cause various pathological conditions of the offspring, depending upon the frequency and dose of alcohol intake, stage of gestation, maternal racial/ethnic population, and nutritional status and/or intake (63-65). In addition to the variety of neurobehavioral and physical abnormalities generically referred to as FASD, intrauterine alcohol exposure has also been linked to a variety of adult diseases of the offspring, likely resulting from the deregulation of developmental and/or metabolic pathways heavily interconnected to one another (66). For example, maternal alcohol consumption has been associated with increased susceptibility to carcinogen-induced mammary (32,33) and prostate (34) tumors in rodents and increased risk of acute

myeloid leukemia in children (67), circadian disruption (35-40) and inhibition of insulin signaling in certain tissues (68,69). The mechanisms of such effects are not fully understood. However, it is noteworthy that alcohol has been reported to interfere with the metabolism of various micro- and macro-nutrients such as lipids, vitamin A, folate and choline, for instance. Hence, the hypothesis that some of the effects of maternal (and adult) alcohol intake may be due to dysregulations of the metabolism of such nutrients rather than to a direct effect of alcohol (27,70).

In mammals, retinoids support numerous crucial biological functions (1), and disruption of retinoid homeostasis in tissues has been linked not only to abnormal embryonic development (25), but also to various adult pathological conditions, including proliferative diseases like cancer and skin disease (71-73), metabolic disorders such as obesity, diabetes and dyslipidemia (42-44) and even abnormal lung function (45). Given the crucial role of vitamin A in maintaining the health of the body throughout life, the current study was conducted to investigate whether alcohol exposure in utero may perturb retinoid metabolism in the adult offspring. By using a well-established rat model of prenatal alcohol exposure (34), we observed that maternal alcohol intake affects tissue retinoid concentrations of the adult offspring, in a tissue- and sex-dependent manner. Specifically, the levels of retinyl ester, the storage form of vitamin A, were significantly decreased in the lung of both males and females and in the liver and ventral prostate of males born from dams administered with alcohol during pregnancy. As discussed earlier, the well-known effects of alcohol consumption on both adult and embryonic retinoid metabolism have been linked to the similarity between alcohol and retinol metabolism, even though the molecular details of such interaction still need to be fully clarified (8). Our results add a novel layer of complexity to the relationship between alcohol and vitamin A metabolism suggesting, for the first time, a long-term impact of alcohol on tissue retinoid homeostasis.

Among the multiple mechanisms that have been proposed for the actions of alcohol, it has been shown that alcohol influences gene transcription by inducing various type of epigenetic modifications (74). We therefore hypothesized that the prenatal alcohol exposure in our rat model could have induced epigenetic modifications leading to attenuated transcription of *Lrat* and, thus, decreased synthesis of retinyl esters. However, the lack of differences in *Lrat* mRNA levels in the liver, lung and prostate between alcohol-fed and pair-fed groups does not

favor this possibility. We cannot exclude, at the moment, that post-transcriptional modifications of the LRAT protein might have possibly occurred resulting in the attenuation of its enzymatic activity, and ultimately in the reduced tissue retinyl ester levels. Alternatively, it could be considered that in utero alcohol exposure might have epigenetically modified genes controlling other key metabolic pathways that maintain tissue retinoid homeostasis, such as hydrolysis of the retinoid stores via retinyl ester hydrolases (75); retinaldehyde oxidation via RALDH2 (6); retinoic acid oxidation into non-active metabolites via CYP26A1 (76); and possibly cellular efflux of retinol mediated by the plasma membrane receptor STRA6 (77-79). All these hypotheses need to be further investigated.

Currently, we do not understand the reasons for the tissue- and sex-specificity of the effect of alcohol on retinoid homeostasis. However, it is interesting that a compromised metabolism of vitamin A in the liver, lung and prostate has been associated with various adult pathological states. Although the cause-effect relationship has not been clarified in all instances, depleted hepatic retinoid stores have been correlated with fatty liver, hepatitis, and cirrhosis in patients with alcoholic liver disease (8). More generally, retinoids control a number of crucial pathways of lipid metabolism that, when compromised, can also lead to non-alcoholic fatty liver disease (NAFLD), which could be considered as the hepatic manifestation of the metabolic syndrome (80). In addition, since retinoids are key regulators of cell proliferation and differentiation (81), a compromised tissue vitamin A homeostasis has been linked to cancer onset and progression at various body sites, including prostate (82) and lung (83). Retinoid metabolism and susceptibility to carcinogenesis have been shown to be different in the various murine prostate lobes (82). Our data confirm that retinoid concentrations seems to be lobe-specific also in the rat prostate, but call for further studies to understand the functional differences that resulted in the retinoid stores being reduced only in the ventral prostate of the offspring from dams alcohol-fed.

Overall, it is intriguing to speculate that adverse influences during development, such as the impairment of tissue retinoid stores induced by maternal alcohol consumption, may cause permanent changes in various organs, leading to increased risk of diseases in adulthood. In our study, we did not measure retinoid levels in fetuses or offspring at birth. Therefore, we cannot establish with certainty that the decline in retinoid stores began in utero. However, it is intriguing that disruption of retinoic acid

signaling during embryonic development has been shown to have a negative impact on postnatal lung function in the offspring (45) and that reduced lung function at birth has been associated with an increased risk of airway diseases, such as asthma, later in life (84).

Finally, we would like to point out the sex- and tissue-specific differences in the retinoid concentrations of liver, lung and ventral prostate in the offspring of the dams fed the regular chow diet ad libitum compared to the offspring of the pair-fed dams. At the moment, we do not understand the reason for these differences. It is interesting, however, that the vitamin A intake of the chow-fed and the pair-fed dams was different (264 *vs.* 450 IU vitamin A/day, respectively), raising the possibility that the intrauterine vitamin A availability per se may also influence the ability to maintain retinoid homeostasis in certain organs later in life, in a sex- and tissue dependent manner.

Conclusions

In summary, this study is the first to report that prenatal alcohol exposure in rats affects retinoid metabolism in adult life, in a tissue- and sex-dependent manner. Given the crucial role of vitamin A in maintaining the health of the body throughout life, these data warrant further investigations to identify the molecular mechanism(s) through which alcohol exposure during embryonic development compromises retinoid metabolism of adult organs such as liver, lung and prostate, likely predisposing to detrimental consequences on health. It is desirable to perform future studies in genetically modified mouse models of altered retinoid metabolism to ease the identification of the targets of the alcohol action.

Acknowledgements

Funding: This work was supported by The Charles and Johanna Busch Memorial Fund and the NJ Agricultural Experiment Station at Rutgers and partially by the National Institute of Health (NIH) grants R01HD057493, R01HD057493-02S1 and R01HD057493-05S1 to Loredana Quadro; and partially by the NIH grant R37AA08757 to Dipak Sarkar.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rutgers University Institutional Committee on Animal Care.

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Cite this article as: Kim YK, Zuccaro MV, Zhang C, Sarkar D, Quadro L. Alcohol exposure in utero perturbs retinoid homeostasis in adult rats. *HepatoBiliary Surg Nutr* 2015;4(4):268-277. doi: 10.3978/j.issn.2304-3881.2015.01.06