Reproductive Toxicology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Reproductive Toxicology

journal homepage: www.elsevier.com/locate/reprotox

Alcohol intake stimulates epithelial proliferation in an authentic model of the human breast

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ARTICLE INFO

Article history:
Received 23 June 2014
Received in revised form
19 September 2014
Accepted 24 October 2014
Available online xxx

Keywords:
Alcohol
Mammary gland
Terminal ductal lobular unit

ABSTRACT

The voluntary consumption of alcohol by humans is a modifiable lifestyle factor that has been consistently linked to a woman's risk of developing breast cancer. We have used an animal model that closely recapitulates breast development in humans to study the effect of alcohol intake on breast growth and morphology. Pubertal female pigs were fed alcohol for 4–5 weeks at 19–21% of total caloric intake, which led to average blood alcohol concentrations of 115–130 mg/dL. Alongside increased liver mass, alcohol intake promoted the formation of distended ductules within lobular units in association with increased epithelial proliferation. Alcohol consumption also increased phosphorylation of the transcription factor STAT5 in the mammary epithelium, but did not lead to any evidence of precocious lactogenesis. In conclusion, feeding alcohol to female pigs having a similar physiology and mammary gland morphology to humans during a reproductive state equivalent to human adolescence leads to increased mammary gland proliferation and development of atypical lobular structures. These changes may phenocopy how alcohol intake increases the risk for developing breast cancer in humans.

1. Introduction

An association between alcohol intake and neoplasia of the breast was first reported in 1977 [1], and has since been reinforced in a number of epidemiological studies [2–4] including in a meta-analysis of 98 studies [5]. The relationship between alcohol intake and breast, oral or digestive tract cancers is specific in that there is no increased risk for other cancers including ovarian, endometrial, thyroid or renal cancers [2]. Indeed, an estimated 4% of the breast cancers diagnosed in developed countries can be attributed to alcohol intake [4].

The risk for developing breast cancer is increased even by moderate alcohol intake (10 g/day) [6], and increases by 7–12% for every additional drink imbibed per day [2,4,5,7]. At least 50% of women between 18 and 44 years of age regularly consume alcohol, and of these, 17% binge drink, which is defined as consuming alcohol to a BAC in excess of 80 mg/dL [8]. For women, binge drinking typically involves consuming 4 or more drinks within approximately 2 h [8]. In younger women, 43% of high school girls report alcohol use and, of these, 58% binge-drink [9]. A recent prospective study revealed that teenage girls who consumed one drink per day had a 1.5-fold increased risk for developing benign breast disease compared to those who drank less than once/week, while girls who consumed alcohol 6–7 days/week had a 5.5-fold higher risk for developing benign breast disease [3]. Along these same lines, adults who reported alcohol consumption between ages 18 and 22 had higher rates of developing proliferative benign breast disease [10]. Given that the breast undergoes substantial development during adolescence in response to a changing endocrine environment [11], any increase in epithelial proliferation or exposure to transforming events during this window may increase the risk for developing breast cancer later in life [12,13].

Abbreviations: DAB, 3,3′-diaminobenzidine tetrahydrochloride; PH3, phosphorylated histone H3; P-Stat5, phosphorylated signal transducer and activator of transcription-5; MG, mammary gland; TDLU, terminal ductal lobular unit; BAC, blood alcohol concentration; IGF-I, insulin-like growth factor-I; IGFBP3, insulin-like growth factor binding protein-3; PR, progesterone receptor.

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http://dx.doi.org/10.1016/j.reprotox.2014.10.020
0890-6238/

The mammary glands (MG) of pigs represent an excellent model for the human breast given similarities in the histological and morphological features of the epithelium and stroma [14,15]. At the same time, pigs are genetically more similar to humans than mice [16] and have a similar physiology to humans [17]. Moreover, pigs voluntarily consume alcohol unlike many laboratory species [18], making them an ideal model to investigate the consequences of alcohol exposure on MG development.

Herein we examined the effect of alcohol intake on various parameters of MG histology, morphology and proliferation in pigs, where we hypothesized that alcohol consumption would stimulate precocious MG development consistent with a mechanism for increasing breast cancer risk in women. Our data reveal that voluntary alcohol intake increased the proliferation of mammary epithelial cells alongside increased phosphorylation of signal transducer and activator 5 (STAT5) and disruption of epithelial histomorphogenesis.

2. Methods

2.1. Animals and tissues

Experiments were performed in accordance with the NIH Guide for The Care and Use of Laboratory Animals, as approved by the UC Davis Institutional Animal Care and Use Committee. Hampshire × Yorkshire (F1) nulliparous pubertal 6-months old female pigs were housed under standard management conditions at the University of California, Davis with free access to water, and were fed a corn and soybean mash (14.4% protein, 0.78% w/w lysine, 3174.6 kcal/kg metabolizable energy). Animals were fed twice-daily, at 8 am and 8 pm.

Experiments were conducted in two blocks. Block 1 was a pilot experiment using two animals ($n = 1$ control and alcohol-fed; average bodyweight 91 ± 6 kg), where eight animals were used in block 2 ($n = 4$ per treatment; 124 ± 2 kg). In block one, females underwent a one-week adaptation period during which feed and water volume were increased each day. Ethanol (3.99 kcal/g metabolizable energy, the same as soluble carbohydrate) [19,20] was introduced on day 4 at 140 mL/day. Feed was increased for both animals on day 5 to 2.27 kg/day with 1.6 L water (±ethanol) which was then maintained for the duration of the experiment. Ethanol was increased daily to a maximum allocation on day 8 (550 mL/day), at which point the alcohol-fed females were assigned to receive 19% of their total caloric intake as ethanol. Pigs were weighed on days 1 and 36 of the study. For females in block 2, feed supplied was increased daily during the adaptation period to 2.27 kg/day with water content increasing to 1.6 L on day 4. Control-fed females were maintained on this level of feed for the duration of the study. Ethanol was first introduced on day 4 (50 mL/day) then increased daily to a maximum allocation on day 10 (600 mL ethanol/day). On day 12 alcohol-fed pigs had their feed reduced to 1.8 kg/day mash with 19–21% of their total caloric intake as ethanol (460–500 mL/day) in a final volume of 1.6 L/day. Females were weighed weekly. Stage of estrous was checked using a boar twice during the treatment period and confirmed that all females underwent regular estrous cyclicity. Pigs were euthanized on day 36 (block 1), or day 46 or 47 (block 2) in a USDA-inspected facility by electrocution followed by exsanguination. Liver, kidneys, heart, uterus and MG were collected and weighed at necropsy, samples were snap frozen in liquid nitrogen and stored at −80 °C or fixed in 4% paraformaldehyde (block 1) or 10% formalin (block 2).

2.2. Blood alcohol concentration (BAC)

Blood for BAC measurement was collected from pigs by jugular venipuncture into Vacutainer tubes containing sodium fluoride and potassium oxalate twice (days 17 and 35) during block 1, and every 2–7 days immediately following the morning feeding during block 2. Plasma was separated by centrifugation and stored in tightly closed tubes on ice prior to BAC analysis at the UC Davis Medical Center Clinical Laboratory (Sacramento, CA) using an enzymatic rate method on a Beckman Coulter D × C automated chemistry analyzer.

2.3. Mammary gland assessments

Fixed tissues obtained from three MG, distributed across the entire mammary chain in each pig, were prepared as whole mounts, without acetone defatting, as described [14,21]. Carmine alum stained semi-thick sections ($n = 3$ per MG) were mounted in Permount (Thermo Fisher Scientific, Hampton, New Hampshire, USA) and imaged with a dissecting microscope (Olympus SZX16, Shinjuku, Tokyo, Japan).

Sections (4–5 μm) of paraffin-embedded tissue were stained with either Gomori's trichrome, or hematoxylin and eosin. Sections stained with hematoxylin and eosin were scored for the presence of distended ductule lumina within terminal ductal lobular unit (TDLU) structures using a subjective scale by an investigator blinded to treatment. The scoring scale reflected the number and size of ductules within TDLU that were enlarged beyond those recorded in a TDLU2 that had minimal luminal expansion. The scoring scale ranged from 1 (no expansion in any ductules) to 10 (all ductules atypically engorged). Five fields were scored for each MG, with three MG scored per pig (besides one pig that had two MG scored), excluding sections that did not contain adequate epithelial tissue (3 sections among 29).

Morphological development of epithelial TDLU structures within the pig MG was determined as we previously described and validated [14], using criteria described for the human breast [22]. Briefly, a TDLU1 in the human breast is a simple collection of approximately 11 ductules, a TDLU2 contains an average of 47 ductules, and the largest and most complex TDLU3 structures contain approximately 81 ductules [22]. We classified an average of $n = 12$ TDLU (range $n = 3$–18) in each of three whole mounts that were prepared from different regions within each of three MG.

2.4. Immunohistochemistry

Immunohistochemistry for phosphorylated histone H3 (PH3), phosphorylated STAT5 (P-STAT5) and progesterone receptor (PR) was performed using procedures outlined by VanKlompenberg et al. [23]. Sections of MG2, 3 and 5 (cranial to caudal) from each female were analyzed for PH3 and P-STAT5, while one section from MG3 was analyzed for PR. Each immunohistochemistry run included one section from every MG in a particular position plus a positive control section and a no primary antibody negative-control section. Blocking for PR immunohistochemistry was performed with 5% horse serum in PBS + 0.05% Tween-20 for 30 min at room temperature. Immunoreactive PH3 was detected with rabbit anti-PH3 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) incubated at RT for 1 h, while PR was detected using a rabbit polyclonal anti-human PR (1:100, Dako North America Inc., Carpenteria, CA) and p-STAT5 was detected using a rabbit monoclonal anti-P-STAT5 (Tyr694) antibody (1:50; Cell Signaling Technology, Danvers, MA), both overnight at 4 °C. Immunocomplexes were visualized using NovaRED chromagen (PR; Vector Laboratories, Burlingame, CA) or 3,3′-diaminobenzidine tetrahydrochloride (DAB; PH3 and P-STAT5; Life Technologies, Carlsbad, CA). The number of PH3, P-STAT5 or PR-positive cells and the total number of epithelial cells in each field was counted using the tag feature in Image Pro Express 6.3 (PH3; Media Cybernetics, Rockville, MD), Aperio ImageScope v10.2.2.2319 (P-STAT5; Leica Biosystems, Buffalo Grove, IL) or

Please cite this article in press as: Schennink A, et al. Alcohol intake stimulates epithelial proliferation in an authentic model of the human breast. Reprod Toxicol (2014), http://dx.doi.org/10.1016/j.reprotox.2014.10.020