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Chronic ethanol intake leads to structural and molecular alterations in the rat endometrium

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ABSTRACT

We described the effects of low- and high-dose ethanol intake on the structure and apoptosis signaling of the uterine endometrium of UChA and UChB rats (animals with voluntary ethanol consumption). Thirty adult female rats, 90 days old, were divided into three groups ($n = 10$/group): UChA rats fed with 10% (v/v) ethanol *ad libitum* (free choice for water or ethanol) drinking < 1.9 g/kg/day; UChB rats fed with 10% (v/v) ethanol *ad libitum* (free choice for water or ethanol) drinking from 2 to 5 g/kg/day; control rats without ethanol (only water). After 120 days of treatment, rats displaying estrus were euthanized. Uterine epithelial cells of the UCh rats showed dilated cisterns of the rough endoplasmic reticulum, presence of lipid droplets, altered nuclear chromatin, and disrupted mitochondria. The UCh rats exhibited intense atrophied epithelial cells with smaller areas and perimeters of cytoplasm and nuclei. The endometrium of UChA rats showed higher levels of caspase-3 while Xiap and Bcl2 varied from moderate to weak. Both UChA and UChB rats exhibited a stronger immunoreaction to Ki-67 and IGFR-1 on epithelial and stromal cells. Chronic ethanol intake leads to structural and molecular alterations in the uterine endometrium of UCh rats, regardless of low- or high-dose consumption, promoting reproductive disorders.

Introduction

Ethanol ingestion has increased in recent decades with social consequences and has become one of the most serious problems of global public health (World Health Organization, 2011). It is a toxic agent that disturbs the integrity of biochemical and physiological functions and the development of organic structures involved in reproduction, causing severe damage to the signaling of hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) axes (Wallock-Montelius et al., 2007). Ethanol may affect the pregnancy rate with higher risk of spontaneous abortions; prolonged consumption can lead to male and female infertility (Clave, Joya, Salat-Batlle, Garcia-Algar, & Vall, 2014; Kesmodel, Wisborg, Olsen, Henriksen, & Secher, 2002). Furthermore, chronic ethanol exposure is associated with malformations in fetal development (Ornoy & Ergaz, 2010) and some cancers (Surtel, Klepacz, & Wysokińska-Miszczuk, 2014).

The prevalence of ethanol abuse and dependence is higher among men. However, recent studies have shown that consumption among women is growing, while male consumption has remained stable (Day, Helsel, Sonon, & Goldschmidt, 2013; Lyngsø et al., 2014). Researchers showed that women initiate ethanol consumption later than men, but seek treatment at the same age, when they develop an alcohol-associated pathology, which can be associated with more rapid progression of alcoholism in women with morbidity varying from 1.5 to 2 times higher compared to men with a similar pattern of intake (Fernandez-Sola, Nicolas, Estruch, & Urbano-Marquez, 2005; Hochgraf, Zilberman, & Andrade, 1995). Only a few consequences of excessive ethanol ingestion on uterine tissue have been reported in the literature, such as atrophy of the uterine horn and endometrial lining thickness, and morphometrical and ultrastructural alterations to the epithelial and glandular cells

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(Buthet et al., 2013; Martinez et al., 1999, 2001). Recent findings have focused on the effects of chronic ethanol consumption and the relationship between circulating sex hormones and their receptors in female reproduction (Chuffa et al., 2013). Specifically to uterine tissue, long-term ethanol consumption was negatively correlated to the levels of androgen receptor (AR), progesterone receptor (PR), and estrogen receptor (subunit ER-α).

According to Buthet et al. (2013), the rat uterine horn has different metabolic pathways able to generate acetaldehyde and free radicals from ethanol. This long-term accumulation may cause cell injury, and promote cancer and related pathologies. We have previously described that high ethanol consumption by rats is associated with induction of apoptosis in different tissues (Oliveira, Chuffa, et al., 2014; Oliveria, Fontanelli, et al., 2014), which may occur due to oxidative stress (Chu, Tong, & Monte, 2007). While most studies have reported that tissue damage arises as a result of prenatal alcohol exposure, we proposed to evaluate the function of endometrial lining during the estrus phase, focusing on apoptotic and anti-apoptotic signaling. To date, no study has addressed the effects of high- and low-ethanol intake on uterine apoptosis.

The UCh ethanol-preferring rat model (Mardones & Segovia-Riquelme, 1983) is derived from original Wistar rats and represents a special model for understanding the basis of alcoholism-linked characteristics such as those described in alcohol-related human diseases. Therefore, the present study aimed to investigate the effects of low- and high-dose ethanol consumption on the structure and apoptotic signaling of the uterine endometrium in two ethanol-preferring rat models.

Materials and methods

Animals and experimental design

Thirty adult female rats (*Rattus norvegicus albinus*), 90 days old, were obtained from the Department of Anatomy, Bioscience Institute/Campus of Botucatu (IBB/UNESP) – Univ Estadual Paulista. The rats were divided into three groups ($n = 10$/group): UChA rats fed with 1:10 (v/v) ethanol *ad libitum* (free choice for water or ethanol) drinking from 0 to 1.9 g/kg/day; UChB rats fed with 1:10 (v/v) ethanol *ad libitum* (free choice for water or ethanol) drinking from 2.0 to 6.0 g/kg/day; control rats without access to ethanol (only water). When the animals reached 60 days of age they were given, during a period of 20 days, a choice between two bottles containing either water *ad libitum* (1) or 10% (v/v) ethanol solution (2). Afterward, 10 animals per group displaying ethanol consumption within the expected average were selected according to the criteria of Mardones and Segovia-Riquelme (1983). In this study, the preference ratio associated with ethanol-seeking behavior was approximately 60%. To ensure higher efficiency and to maintain constant consumption throughout the experiment, rats were kept under observation for 10 days. During all the treatments, estrus cycles were monitored daily at 9:00 AM using a vaginal-swab technique. All rats were housed in polypropylene cages (43 × 30 × 15 cm) with laboratory-grade pine shavings as bedding and maintained under controlled temperature (23 ± 1 °C) and lighting conditions (12 h light/12 h dark photoperiod, lights switched off at 7:00 AM). Experimental protocols followed the Ethical Principles in Animal Research of the Brazilian College of Animal Experimentation (no65/07-CEEA). After 120 days of treatment, females in estrus phase were anesthetized and euthanized by decapitation for further analysis.

Body, genital organs weight, and tissue processing

The rats were weighed in an Owalabor analytical balance and submitted to abdominal-pelvic laparotomy in order to collect genital organs (uterine horn, ovaries, and uterine tubes), which were weighed together. The uterine horn, ovaries, and uterine tubes were dissected under a surgical microscope (D. F. Vasconcelos), and the uterus fixed in Bouin solution during 6 h. After sequential washes, the uterus was conditioned in 70% (v/v) ethanol. Dehydration steps were made using 80%, 90%, 95%, and 100% alcohol for 4 h, following diaphanization and paraplast embedding (Oxford Labware, St. Louis, MO, USA).

Morphometry, light microscopy, and immunohistochemistry

The blocks were sectioned at 4 μm thickness using a LEICA 2145 microtome and stained with hematoxylin and eosin (H&E). The slides were imaged by digital photomicroscope (Axiophot II Zeiss). For histological analysis and counting measures, slides with H&E were used, and the values were obtained in five slides per rat in three areas per section with 20× magnification (interval between each section was 50 μm).

Rabbit primary antibodies (1:100 Santa Cruz Biotechnology, CA, USA) were used for IGFR-1, Ki-67, Bcl2, and Xiap analysis, and rabbit primary antibodies (1:100 Biocare, USA) were used for caspace-3 analysis, according to the manufacturers' procedures ($n = 5$/group). After immunoreactions, the slides were washed in TBS-T buffer and incubated with secondary antibody (anti-mouse IgG or anti-rabbit IgG; DAKO® CYT; Glostrup, Denmark) for 1 h. Then, slides were reacted with diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) and counterstained with hematoxylin. Five slides of each uterine horn containing four sections were stained for morphometric analysis. The immunohistochemistry results were analyzed by a pathologist using a Zeiss Axiophot II microscope (Carl Zeiss, Oberkochen, Germany), based on the levels of staining intensity, which were scored as absent (0), weak (+), moderate (++), or strong (+++/++++) immunoreactivity.

Transmission Electron Microscopy (TEM)

For TEM analysis, five rats per group were decapitated, and the uterine horn was collected and immersed in modified Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.2). The 0.5 mm uterine horn fragments remained in the fixative solution for 3 h at 4 °C. Afterward, they were post-fixed in 1% osmium tetroxide (OsO_4), sodium phosphate buffer, and then embedded in Araldite® plastic resin. After embedding, sections 0.5 μm thick were obtained by using an LKB 8800 ultramicrotome, *Ultratome III,* with a glass blade, and stained with toluidine blue and azur II. After selecting specific areas, the blocks were trimmed and ultrathin sections were obtained, being contrasted by uranyl acetate and lead citrate. All the analysis and documentation were performed using a Philips CM 100 transmission electron microscope (Electron Microscopy Center of IBB/UNESP).

Scanning Electron Microscopy (SEM)

The uterine horns were fixed with Karnovsky's solution for 2 h and post-fixed with 1% OsO_4 for 2 h. They were immersed in 2% tannic acid for 2 h, dehydrated in graded ethanol, which was replaced with isoamyl acetate, and dried to a critical point with CO_2 (Balzers CPD-010). The specimens were coated with gold (Balzers MED-010) and examined in a Philips FEM 515 scanning electron microscope.

Hormone levels

After decapitation, trunk blood samples were obtained from rats ($n = 10$/group) to verify LH, FSH, estradiol, and progesterone, and