ORIGINAL ARTICLE

Chronic alcohol administration increases serum prolactin level and pituitary cell proliferation, and alters hypothalamus neurotransmitters in rat

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Abstract

OBJECTIVES: Alcohol induces hyperprolactinemia in both alcoholic men and women, but the mechanism is not fully established. The aim is to investigate the mechanism involved in elevation of serum prolactin level after chronic alcohol administration.

METHODS: In this study, healthy female SD rats were given alcohol for 8 weeks and checked for serum prolactin level by radioimmunoassay. Interior pituitary cell proliferation was determined by immunohistochemistry score of Ki-67, and hypothalamic neurotransmitters were detected by Coulomb HPLC electrochemical array.

RESULTS: We demonstrated that serum prolactin level and wet pituitary weight of alcohol-fed rats were significantly increased. Interior pituitary cell proliferation was significantly enhanced; hypothalamic dopamine, 5-HT and GABA levels were reduced while glutamate level was increased by chronic alcohol administration; hypothalamic noradrenalin level remained unchanged.

CONCLUSION: Our study suggested that chronic alcohol administration resulted in elevated serum prolactin level in normal SD rats probably through enhancing pituitary gland cell proliferation combined with altered hypothalamic neurotransmitters that regulate prolactin level.

INTRODUCTION

Hyperprolactinemia (HPRL) is a condition of elevated serum prolactin concentration (>25 ng/ml). The incidence is higher in females, about 0.4% in the general population and 9~17% for those with reproductive disorders (Li 2008). Physiological HPRL is a part of normal bodily changes during pregnancy and breastfeeding. Patients with HPRL often exhibit amenorrhea, galactorrhea, anovulatory or sterility. The major causes of pathological HRRL involve diseases affecting the hypothalamus and pituitary gland, such as prolactinomas, the tumor of pituitary lactotroph cells (Mancini *et al.* 2008).

Ethanol is an ancient and ubiquitous elixir. Chronic ingestion of ethanol leads to alcoholism, a psychiatric disease that brings various physiological, psychological and social consequences to the affected individual. The mechanisms relating to

ethanol addiction and reversal, its toxicity and intoxication have been studied for generations. However, its role in the reproductive system is not yet fully understood. The correlation of ethanol with reproductive disorder has been reported. For instance, long-term ethanol consumption can result in amenorrhea or sterility (Hadi *et al.* 1987), a common symptom observed in HPRL patients. Indeed, further studies found that alcohol-induced hyperprolactinemia in both alcoholic men and women (Seki *et al.* 1991; Ida *et al.* 1992); the serum alcohol levels were positively correlated with the progression of HPRL in the patient (Soyka *et al.* 1991). But the mechanism of how alcohol causes hyperprolactinemia is not fully established.

The pituitary consists of three distinct anatomical and functional parts: posterior, intermediate and anterior lobes. The posterior pituitary consists of axon terminals originating from neurons with somata in the hypothalamus. Cells in the intermediate pituitary release peptidergic hormones under the control of neurotransmitters released into the synaptic cleft from the innervating neurons. In contrast, secretory activity of anterior pituitary (AP) is controlled by blood-borne hypothalamic factors. AP releases hormones to control growth, development, reproduction, and respond to stress (Kreft & Zorec 2008). Prolactin is produced in the lactotroph cells of the anterior pituitary gland. The primary function of prolactin is to enhance breast development during pregnancy and to induce lactation. It is well documented that secretion of prolactin is under tonic inhibitory control from the hypothalamus.

Recent evidence demonstrated that neurotransmitters from hypothalamus interact with specific surface membrane receptors on pituitary lactotroph cells and subsequent activation of intracellular signaling mechanisms control exocytosis and secretory output from anterior pituitary cells (Kreft & Zorec 2008). For example, the tonic inhibitory control from hypothalamus is mediated by dopamine, which acts via D2-type receptors located on lactotrophs; other hypothalamic peptides and neurotransmitters also play important regulatory roles in prolactin production (Keith *et al.* 2005; Fitzgerald & Dinan 2008). Animal studies provided evidence for alcohol's effect on lactotropes proliferation *in vivo*, but not *in vitro* (De *et al.* 2002). Alcohol also enhances estradiol's mitogenic action on the lactotropes probably through alteration of the production of estrogen-regulated growth factors (Dipak *et al.* 2007).

In the present study, we looked at the effect of chronic alcohol administration on serum prolactin level and pituitary cell proliferation in normal female SD rats. Consequences of chronic alcohol administration in terms of hypothalamus monoamine neurotransmitters such as norepinephrine (NE), dopamine (DA), 5-hydroxytryptamine (5-HT), as well as inhibitory and excitatory amino acid neurotransmitters gamma-aminobutyric acid (GABA), glutamate (GLU) levels/expression were investigated.

MATERIALS AND METHODS

Animals

40 healthy female SD rats (8 week old) obtained from the SLAC Laboratory Animal Center (Shanghai, China) were housed in a controlled environment with temperature 24 °C, humidity 60%, natural circadian rhythm of light, and provided with certified rodent chow and water. Rats underwent one week of adaptive intragastric feeding (Hao *et al.* 2006) with water and were randomly divided into 2 groups (n=20 in each group). Experimental group was given 20% ethanol 2 g/kg twice daily by intragastric feeding for 8 weeks, while the control group was given equal volume of water for the same period of experimental time.

Serum preparation and prolactin measurement

At the end of experiment, rats were sacrificed by decapitation and blood samples (3 ml each) were collected. After natural solidified at room temperature for 20 minutes, serum was separated by centrifuge at 8 000 g for 10 minutes. All serum samples were saved as aliquots in –80 °C freezer. PRL analysis was performed by radioimmunoassay (Jiu Ding Medical Biological Company, Tianjing, China).

Immunohistochemical staining and score for pituitary cell proliferation

After rats were sacrificed, anterior pituitaries were removed, weighed, fixed in 10% neutral formalin solution and used for immunohistochemistry staining with Ki67 primary antibody (Abcam, USA).

Ten non-overlapping fields of view were examined per biopsy in a systematic random sampling pattern (magnification 400×). Staining was evaluated using an evaluation nomogram as previously reported (Ota & Igarashi 1993). Briefly, each section was graded according to the frequency of positive cells and intensity of staining. The frequency was defined as 1+, 2+ or 3+ when the number of positive cells in the pituitary in each section was <10%, 10–50% or >50% respectively. Intensity was defined as 3+ when staining of the cells was as strong as that observed in the positive controls, as 1+ when staining was weakly positive but distinct from the negative controls, and as 2+ when the staining was between 1+ and 3+. The sections were ranked from 1 to 5 according to the evaluation nomogram. Capture and score of images were done by two blinded-individuals.

HPLC analysis of hypothalamic neurotransmitters

Hypothalamus from alcohol-fed and control rats were excised immediately on ice, homogenized in PBS and centrifuged. Supernatants were used for detection of neurotransmitters (DA, NE, 5-HT, GABA and GLU) by Coulomb HPLC electrochemical array (Model 5600A Coulomb Array Detector-4, ESA, US).

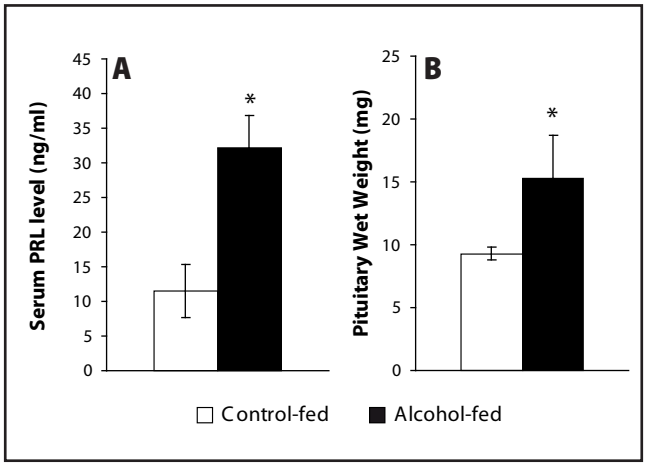

Fig. 1. Chronic alcohol administration increases serum PRL level and pituitary wet weight. (A) Serum PRL level was measured by radioimmunoassay in alcohol-fed and control-fed female SD rats (n=20 in each group). (B) Pituitary wet weights were obtained after 8 weeks of alcohol or control feeding. Data are means ± SD of 20 animals in each group. *p<0.05, significantly different between the two groups.

Statistics

All experimental data were expressed as mean ± standard deviation (± SD). Statistical analysis was performed using the Statistical Package for the Social Sciences Version 17.0. Difference between the two groups was determined by independent sample t test. p<0.05 was considered significant.

RESULTS

Effects of alcohol on serum PRL and pituitary wet weight

8 weeks of alcohol administration resulted in significant increase of the mean serum PRL concentration (32.1 ± 4.7 ng/ml, n=20, p<0.05) compared to the serum PRL in control-fed group (11.5 ± 3.8 ng/ml, n=20) (Figure 1A). Pituitary wet weights were also significantly higher in alcohol-fed rats (15.26 ± 3.45 mg, n=20, p<0.05) than that of control-fed rats (9.30 ± 0.48 mg, n=20) (Figure 1B).

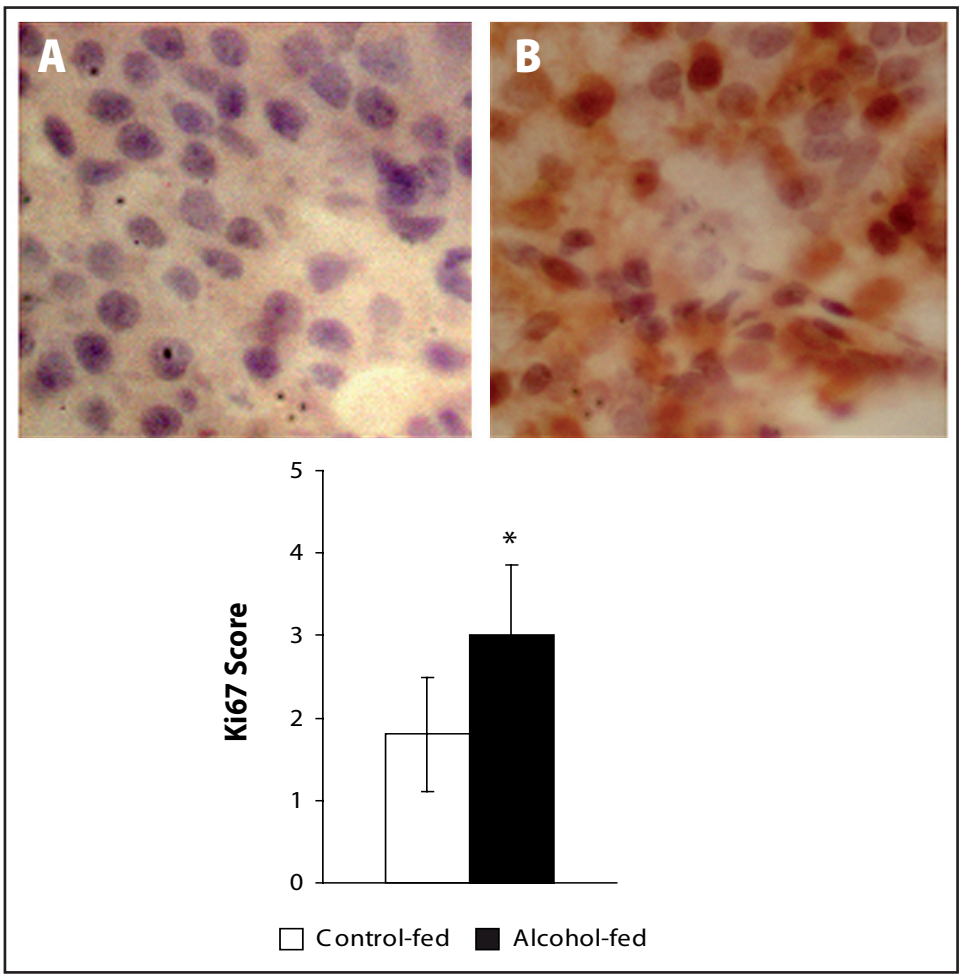

Fig. 2. Chronic alcohol administration enhances pituitary cell proliferation. Pituitary cell proliferation was determined by immunohistochemistry staining of Ki-67 in formalin fixed anterior pituitary sections. Representative images from alcohol-fed rats (A) or control animals (B) were shown. Images were further analyzed by evaluation monogram and the scores for positive staining were shown in figure (C). *p<0.05, significantly different between the two groups.

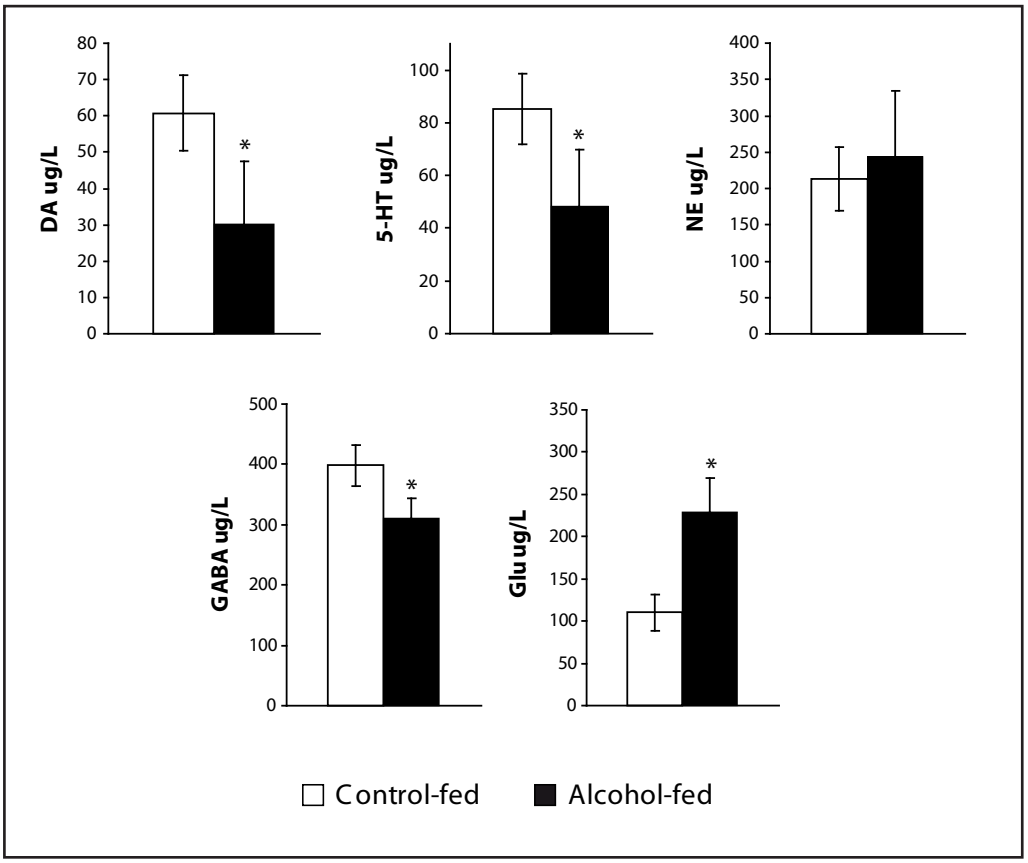

Fig. 3. Effects of alcohol on hypothalamic neurotransmitters. Hypothalamus homogenates were used for detection of neurotransmitters by Coulomb HPLC electrochemical array analysis. Effects of alcohol on hypothalamic dopamine (A), norepinephrine (B), 5 - hydroxytryptamine (C), gamma-aminobutyric acid (D) and glutamate (E) levels were compared between alcohol-fed and control-fed rats. *p<0.05, significantly different between the two groups.

Alcohol enhanced pituitary gland cell proliferation

Chronic alcohol fed significantly enhanced Ki-67 expression in pituitary cells (Figure 2 A, B). The average immunohistochemistry score of Ki-67 in alcohol-fed rats was 3.00 ± 0.86 (n=20, p<0.05), significantly higher than the score in control-fed rats (1.80 ± 0.70, n=20) (Figure 2C).

Effects of alcohol on hypothalamic neurotransmitters

At the end of experiment, the average hypothalamic dopamine in alcohol-fed and control-fed group (30.31 ± 17.16 μg/L, n=20, p<0.05)) was significantly reduced compared to the control-fed group (60.71 ± 10.38 μg/L, n=20). Similarly, significant decrease of the average concentration of hypothalamic 5-HT was found in alcohol-fed rats (48.21 ± 21.54 μg/L, p<0.05) in compare with the value of control-fed rats (85.19 ± 13.38 μg/L) (Figure 3 A, B). But the average concentration of hypothalamic NE remained comparable between the two groups (243.40 ± 91.13 μg/L in alcohol-fed group and 212.73 ± 43.71 μg/L in control-fed group, p>0.05) (Figure 3 C).

We also looked at the effect of alcohol on hypothalamic inhibitory amino acid neurotransmitters GABA and the excitatory amino acid neurotransmitters GLU using the same approach. The average hypothalamic GABA was significantly reduced by 22% in alcohol-fed group (p<0.05) (Figure 3D); while the average concentration of hypothalamic GLU was significantly increased by 52% in response to alcohol administration (p<0.05) (Figure 3E).

DISCUSSION

Alcohol abuse has become a global medical and social problem. The influence of alcohol on human health and its mechanisms are receiving more attention than ever before in medical studies. Clinical reports showed elevated plasma prolactin in both alcoholic women and men. Alcohol induced hyperprolactinemia has also been demonstrated in animal studies of nonhuman primates, which suggest that ethanol consumption is a positive risk factor for hyperprolactinemia (Sarkar 2010). Our present study with rats further confirmed

the positive correlation between alcohol and serum prolactin level.

How ethanol increases blood prolactin is not well understood. One report using bromodeoxyuridine (Brdu) incorporation assay showed alcohol causes hyperprolactinemia by increasing the number of lactotropes in the anterior pituitary gland (De *et al.* 2002). In the present study, we examined the expression of Ki-67, a cellular proliferation and mitotic activity marker to determine the effect of ethanol on cell proliferation. We found with immunohistochemistry that the expression of Ki-67 in the pituitary gland was significantly higher in female rats given alcohol than control rats. Our results are consistent with the previous study done by Sarkar's group (De *et al.* 2002) which suggested that enhanced pituitary cell proliferation is one of the causes of alcohol-induced hyperprolactinemia.

In the current study, changes in different neurotransmitters in the hypothalamus were compared between alcohol-fed rats and control rats. We found significantly reduced dopamine and serotonin levels, but not NE levels in the hypothalamus after chronic alcohol administration. This suggests that dopamine and serotonin were involved in alcohol's effect. Besides the change of monoamine neurotransmitters by alcohol, we found that GABA, an inhibitory amino acid neurotransmitter widely distributed in the central nervous system including the hypothalamus, also decreased after ethanol exposure. There is evidence showing GABA exhibits an inhibitory effect on prolactin secretion (Lee and Pan 2001; Racagni *et al.* 1979). Glutamate is known as one of the excitatory amino acid neurotransmitters. Its role in regulation of prolactin secretion has not been relevantly reported. In this study, we found that glutamate content in hypothalamus was elevated in alcohol-fed rats, thus glutamate may function as a prolactin release factor (PRF) in hypothalamus, which is under the regulation by ethanol.

It's well known that prolactin production and secretion is controlled by hypothalamic neurotransmitters. These neurotransmitters may regulate prolactin secretion through direct projection to the anterior pituitary from median eminence, such as the GABAergic neurons (Kreft & Zorec 2008), or projections to the posterior pituitary and then use neurotransmitters to access the anterior pituitary via the short portal vessels, such as glutamate (Caruso, *et al.* 2004). Importantly, a variety of neurotransmitter receptors have been found on the surface of lactotroph cells. For example, the glutamate NMDA receptor subunit 1 was found in all of the cell types in the anterior pituitary (Bhat *et al.* 1995), whereas the subunit GluR2 and GluR3 of AMPA receptors were only localized in lactotropes (Login 1990; Lindstrom & Ohlsson 1992). A widespread expression of 5-HT receptors was found in the rat anterior pituitary, and several of which are up-regulated by estrogen to mediate acute prolactin release (Papageorgiou & Denef 2007). The anterior pituitary cells express ionotropic and metabotropic GABA receptors and GABA stimulates the secretion of hormonal output from these cells (Anderson & Mitchell 1986; Nakayama *et al.* 2006; Zemkova *et al.* 2008). In addition, the β-adrenergic agonists and norepinephrine can stimulate prolactin release from rat anterior pituitary through receptors on the surface of lactotroph cells (Baes & Denef 1984). However, the most thoroughly studied prolactin inhibitory factor is dopamine, which acts via D2 receptor located on lactotroph cells (Keith *et al.* 2005; Fitzgerald & Dinan 2008). The wide distribution of neurotransmitter receptors in the anterior pituitary cells supports the significance of our finding that elevation of excitatory neurotransmitter (glutamate) and decreased inhibitory neurotransmitters (serotonin and GABA) in the hypothalamus are responsible for the upregulation of serum prolactin levels. Due to the inhibitory effect of activated D2 receptor, the decreased dopamine levels in the hypothalamus reduced the tonic inhibitory control of prolactin secretion. Indeed, the clinical application of bromocriptine and other dopamine receptor agonists on hyperprolactinemia has been shown effective (Crosignani 2006; Lee *et al.* 2010). But how alcohol affects the neurotransmitters in hypothalamus need further studies.

The current study demonstrated that ethanol-induced hyperprolactinemia was established by changes at least in two levels: ethanol altered hypothalamic neurotransmitters levels and stimulated anterior pituitary cell proliferation. The proliferation of lactotrophs will increase the basic prolactin level in cells and increased hypothalamic glutamate will activate cell signaling responsible for exocytosis and secretory output of prolactin from lactotrophs. It is currently unclear how hypothalamic glutamate and dopamine as well as other inhibitory neurotransmitters balance the basic serum prolactin level. GABA was found to stimulate the secretion of prolactin through GABA receptors in lactotrophs (Anderson & Mitchell 1986; Nakayama *et al.* 2006; Zemkova *et al.* 2008); conversely, we found that reduced GABA level in the hypothalamus correlated with elevated serum prolactin level. We propose that reduced GABA levels decreased its inhibition on GABA neurons located in median eminence (Kreft & Zorec 2008), subsequently increased GABA release from projection GABA neuron in the anterior pituitary and increased secretion of prolactin. The decreased dopamine and 5-HT levels will reduce their stimulation on D2 receptor and 5-HT receptors, respectively, which subsequently reduce the tonic inhibitory control of prolactin secretion.

In conclusion, we have shown that chronic alcohol administration causes elevated serum prolactin level in healthy female rats. Promoting effect on pituitary cell proliferation and disrupted selective inhibitory neurotransmitters along with enhanced glutamate as a prolactin releasing factor may be the mechanisms of hyperprolactinemia followed by alcohol administration.

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