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Ethanol exacerbates manganese – induced functional alterations along the hypothalamic-pituitary-gonadal axis of male rats

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ABSTRACT

Manganese (Mn) exposure has been reported to induce reproductive dysfunction in animal and humans. Studies have shown that a large percentage of adolescent and adult populations tend to consume alcohol in a binge pattern. However, there is no information on the influence of alcohol on Mn – induced functional alteration along the hypothalamic – pituitary – gonadal axis. This study aimed to evaluate the influence of ethanol (EtOH) on Mn – induced functional alteration along the hypothalamic – pituitary – gonadal axis. Rats were exposed to Mn alone at 30 mg/kg body weight or co-expose with EtOH at 1.25 and 5 g/kg body weight for 35 consecutive days. Results showed that EtOH exposure significantly ($p \leq 0.05$) exacerbated Mn – induced decrease in anti-oxidant enzymes activities, glutathione level and increased oxidative stress biomarkers in the hypothalamus, testes an epididymis of the exposed rats. Moreover, induction of inflammation was associated with disruption of histo-architecture of the hypothalamus, testes and epididymis of rats treated with Mn alone, EtOH alone or in combination. Furthermore, EtOH significantly exacerbated Mn – induced diminution in reproductive hormones and marker enzymes of testicular functions coupled with decreased sperm quantity and quality. Taken together, EtOH exacerbates Mn – induced functional alteration along the hypothalamic – pituitary – gonadal axis in rats *via* mechanisms involving induction of oxidative/nitrosative stress, lipid peroxidation and inflammation in rats.

1. Introduction

Manganese (Mn) is a well-known trace element and nutrient which plays an indispensable role in many biological processes involved in the maintenance and regulation of development, urea cycle, energy metabolism, anti-oxidative role, lipid metabolism, reproduction and brain function [1]. Mn is used in the production of nutritional supplements and multivitamin preparation [2]. Although diets remain one of the important routes of Mn exposure for humans, occupational and environmental exposure to dusts and fumes airborne in occupations has contributed immensely to Mn toxicity [2]. Mn intoxication has been reported to be linked to neurotoxic effects such as poorer memory and attention and hyperactive behavior [3]. For instance, epidemiological studies showed that occupational exposure to Mn containing dusts by men resulted in decreased sperm motility, sperm concentration, libido and impotency [3].

Previous studies have demonstrated that Mn toxicity caused significantly degeneration of the seminiferous epithelium, reduction in sperm motility and sperm count, depletion of spermatids number associated with absence of spermatocytes in the seminiferous tubules [4]. Also, Mn exposure has been implicated in induction of oxidative stress, DNA damage, apoptosis and alteration in ATP enzyme systems [5]. Moreover, Mn exposure inhibit steroidogenesis via reduction of p450 enzymes activities, StAR protein expression, 3β – HSD and apoptosis induction in rat Leydig cells [5,6]. Furthermore, Mn preferentially bio-accumulate in the mitochondria, where it generates reactive oxygen species (ROS) via oxidative phosphorylation disruption [6]. Generated intracellular ROS may induced oxidative stress and damage proteins, lipids, cell structures and nucleic acids [6], especially in membrane with high polyunsaturated fatty acids contents like the hypothalamus, testes and epididymis. ROS induced oxidative stress may cause reproductive dysfunction [7]. Therefore, we hypothesized that alcohol consumption may exacerbate Mn – induced functional alteration along the hypothalamus – pituitary – gonadal axis.

Alcohol has a long history of several uses worldwide. It is found in alcoholic beverages sold to adults, as fuel, and also has many scientific, industrial and medical uses. Alcoholic beverages contain about 3–40% alcohol by volume. Excessive consumption of alcohol has been linked to

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so many pathological conditions such as fetal injuries during pregnancy, liver disease, cancer, brain damage and reproductive dysfunction [8]. Consumption of alcohol in a binge pattern by both youth and adults has also been reported in many developed countries [9]. Furthermore, exposure of people to occupational and environmental Mn toxicity and consumption of alcohol is very possible. For instance, the populace in Southern Nigeria are exposed to Mn in seafood and groundwater [10] and this populace consume alcohol on a daily basis, especially farmers, fishermen and sand diggers. We report for the first time, the exacerbating effects of EtOH on Mn – induced toxicity along the hypothalamus – pituitary – gonadal axis via induction of oxidative/nitrosative stress, lipid peroxidation, inflammation, and disruption of hormonal balance, testicular function markers, spermatogenesis and steroidogenesis in rats.

2. Materials and methods

2.1. Chemicals

Manganese chloride ($MnCl_2.4H_2O$, ≥ 99.9%), epinephrine, 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide, 5′,5′- dithio-bis-2-nitrobenzoic acid (DNTB), trichloroacetic acid (TCA), glutathione and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acid phosphatase (ACP), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) assay kits were obtained from RANDOX laboratories (CrumLin, UK). All other reagents were of highest analytical grade and were purchased from the British Drug Houses (Poole, Dorset, UK).

2.2. Animals model

Sixty-five adult male Wistar rats between 140–160 g were obtained from the Central Animal House, College of Medicine, University of Ibadan, Ibadan. The animals were housed in standard plastic cages situated in a well-ventilated vivarium under 12-hour light/dark cycle. The rats were fed and given drinking water *ad libitum*. The rats were acclimatized for two weeks under standard laboratory conditions. The ethical approval of the University of Ibadan Ethical Committee involving the use of rats for this study was obtained. Animal care and experimental protocol were conducted in accordance with the Guide for the Care and Use of Laboratory Animals' as stipulated by the National Academy of Science (NAS) and reported by the National Institute of Health.

2.3. Experimental design

The rats were randomly divided to five groups of thirteen rats each and were treated for 35 consecutive days as follows:

Group I (Control): Rats received normal drinking water alone for 35 consecutive days.

Group II (EtOH alone): Rats were orally treated with ethanol alone at 5 g/kg body weight.

Group III (Mn alone): Rats were orally treated with manganese alone at a dose of 30 mg/kg body weight.

Group IV (Mn + EtOH 1): Rats were orally co-treated with manganese and ethanol at 1.25 g/kg body weight.

Group V (Mn + EtOH 2): Rats were orally co-treated with manganese and ethanol at 5 g/kg body weight.

The doses of Mn (30 mg/kg) and EtOH (1.25 and 5 g/kg – 40% v/v) adopted in the present study were chosen based on the results from the pilot study in our laboratory and previously published data [9].

Twenty-four hours after the last treatment, blood were collected via the retro-orbital venous plexus with the aid of heparin containing tubes prior to the animal sacrifice by cervical dislocation. The collected blood samples were centrifuged at 3000 *g* for 10 min to obtain the plasma, which were thereafter stored frozen at −20 °C before the hormone assays were carried out using ELISA strip reader (Robonik India Private Limited, Mumbai, India). The cranium was opened and the brain were cautiously removed and thereafter, the hypothalamus was excised from the brain on ice. Subsequently, the hypothalamus, testes and epididymis tissues were weighed and processed for different biochemical and histological analyses after being washed with ice-cold phosphate-buffered saline. Moreover, the organo-somatic-indices (OSI) of the hypothalamus, testes and epididymis were evaluated using the formula, OSI = 100 × organ weight (g)/body weight (g).

2.4. Evaluation of reproductive indices

Evaluation of sperm progressive motility was done according to the method described by Zemjanis [11]. Evaluation of epididymal sperm count was done according to an established method of the WHO [12]. The evaluation of sperm morphological abnormalities and viability was done according to the established method of Wells and Awa [13]. Determination of testicular sperm number (TSN) and daily sperm production (DSP) were evaluated using frozen testes according to the method of Blazak et al [14].

The circulatory levels of the pituitary and testicular hormones were assayed using available commercial enzymes immunoassay kits that is specific for rats such as FSH (RPN 2560, Amersham, United Kingdom), LH (RPN 2562, Amersham, United Kingdom) and testosterone (EIA – 5179, DRG Diagnostic GmbH, Marburg, Germany) according to the instructions of the manufacturer.

2.5. Tissues sampling and biochemical assays

The hypothalamus, testes and epididymis of the rats were homogenized separately in 50 mM Tris − HCl buffer (pH 7.4) containing 1.15% potassium chloride and resulting homogenates were centrifuged at 12,000 *g* for 15 min at 4 °C to obtain the post-mitochondrial fraction which was used for the biochemical estimations. Protein concentration was assayed at 595 nm according to the method of Bradford [15].

2.6. Determination of antioxidant and oxidative stress indices in the hypothalamus, testes and epididymis

Superoxide dismutase (SOD) was assayed according to the method of Misra and Fridovich [16]. Catalase (CAT) activity was assayed using hydrogen peroxide as substrate according to the method of Clairborne [17]. Glutathione –S- transferase (GST) activity was assayed according to the method of Habig et al [18]. Reduced glutathione (GSH) level was determined according to the method of Beutler et al [19]. Hydrogen peroxide (H_2O_2) generation was assayed according to the method of Wolff [10] while lipid peroxidation (LPO) was determined according to the method described by Farombi et al [20] with slight modification using a 752S UV-VIS Spectrophotometer (Ningbo, China).

2.7. Assessment of pro-inflammatory biomarkers

Nitric oxide (NO) level was assayed by measuring the nitrites content (i.e. the stable end product of nitric oxide (NO) according to the method of Green et al [21]. Myeloperoxidase (MPO) activity was determined according to the method described by Granell et al [22] with little modification.

2.8. Estimation of marker enzymes of testicular function

Marker enzymes of testicular function namely acid phosphatase (ACP), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were assayed in the supernatant of the testes. ACP and ALP activities were determined by the method of Tietz [23]. Determination of testicular LDH activity was based on the method of Vassault [24].