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Ethanol via Regulation of NF-κB/p53 Signaling Pathway Increases Manganese-Induced Inflammation and Apoptosis in Hypothalamus of Rats

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Abstract

The diet is a major route of manganese (Mn) exposure for humans. Interestingly, several epidemiological data demonstrated an increase in the incidence of alcohol consumption globally. Chemical-chemical interaction subsequent to chemical mixtures exposure may result in a synergism or antagonism effects. The present study investigated the influence of co-exposure to ethanol (EtOH) and Mn on inflammation and apoptosis in the hypothalamus of rats. The study consisted of five groups of rats that were exposed to drinking water alone, EtOH alone at 5 g/kg, Mn alone at 30 mg/kg or co-expose with EtOH at 1.25 and 5 g/kg body weight by oral gavage for 35 consecutive days. The results indicated that the significant ($p < 0.05$) increases in pro–inflammatory cytokines, namely tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) as well as cyclooxygenase-2 (COX-2) and nuclear factor kappa B (NF-κB) activation in the hypothalamus following individual exposure to Mn and EtOH to rats were intensified in the co-exposure group. Moreover, immunohistochemistry analysis showed marked decrease in B cell lymphoma-2 (Bcl-2) protein expression as well as the increases in the apoptotic proteins, namely Bax and caspase-3 along with p53 in the hypothalamus of rats treated with Mn or EtOH alone were intensified in the co-exposure group. Taken together, these findings highlight that EtOH exacerbated the induction of inflammatory and apoptotic biomarkers via regulation of NF-κB/p53 signaling pathways in the hypothalamus of rats. These alterations may have profound disrupting effects on the hypothalamus functions such as impairment of it metabolic and autonomic nervous system functions.

Keywords Ethanol · Manganese · Inflammation · Apoptosis · NF-κB/p53 signaling pathways

Introduction

Manganese (Mn) is an essential metal required for the maintenance of various important physiological functions, such as energy metabolism, cell growth and development, wound healing, reproduction, urea cycle, digestion, immunity, blood clothing, and antioxidant capacity [1–4]. Mn is found in the environment as a constituent of dry batteries, fireworks, paints, fungicides, glazes, rubber, lacquers, and fertilizers [2]. Mn is also found in multivitamin preparations and nutritional supplements [5]. The central nervous system is the main target of Mn toxicity; the brain has the ability to retain Mn for a long period of time because of its inability in eliminating excess Mn [6]. Mn has the ability to bypass the blood–brain–barrier to the different parts of the brain to exert its toxicity via an axonal transport system [6]. Excessive accumulation of Mn in the brain causes neurodegenerative disorder called manganism [6, 7].

There are several reports on the adverse effects of Mn associated with either deficient or excess Mn levels. Animal models have been used to explore the diverse forms of Mn exposure which include nutritional, parenteral, occupational, and environmental exposure [8, 9]. Both in vivo and in vitro studies have greatly contributed to the description of uptake of Mn and it neurotoxicological mechanisms of action [9]. Zhoa et al. [10] reported that Mn-induced dopaminergic neurodegeneration and microglial activation in rat's substantia nigra. Moreover, Mn-

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induced oxidative stress and subsequent activation of intracellular signaling pathways involving mitogen-activated protein kinases, MAPKs [11–14]. Furthermore, Mn have been reported to induce mitochondrial damage thereby resulting in the inhibition of ATP production to cause the activation of apoptotic pathways and necrosis [15]. However, the influence of ethanol (EtOH) on Mn-induced inflammation and apoptosis in the hypothalamus is not yet known.

Alcohol abuse is prevalent in many developing and developed countries in the world today [16], especially in African. African Countries are ranked among the highest consumers of alcohol per capita [17]. A report conducted in the southern part of Nigeria shows that 52% of male and 40% of female respondents reported episodic drinking of alcohol in previous years with many consuming high amounts of alcohol regularly [17]. Alcohol abuse has been linked to many pathological conditions such as fetal alcohol syndrome, liver disease, cancer, and brain damage. Alcohol abuse results in massive stimulation of glial and neuronal apoptosis in rat's brain [18–20]. The hypothalamus is especially prone to alcohol toxicity [21]. EtOH induces neurodegeneration via necrosis and apoptosis via activation of caspases [22] and inhibition of the electron transport chain, causing mitochondrial dysfunction and mitochondrial membrane depolarization resulting in the activation apoptotic pathway [23, 24].

Previous investigations on the interactive effects of Mn and EtOH on some chemical constituents of the liver and serum of rats have been reported [25, 26]. Mn and EtOH in combination resulted in extremely lethargic and in poor physical condition of the rats and also had a synergistic effect in altering the enzymes activity of adenylpyrophosphatase, manganese superoxide dismutase, saccharopine dehydrogenase, adenosine deaminase, and alpha-amylase along with elevation of calcium content and lipid peroxidation in the liver of rats. These alterations indicate that the toxic effects of Mn are enhanced when the metal and EtOH interact in the biological system. Deng et al. [27] also reported that occupational exposure to Mn can lead to a dose-dependent increase of liver enzyme concentrations, and interact with alcohol drinking to potentially aggravate the liver damage.

Nutritional co-exposure to Mn and EtOH is quite possible. For instance, seafood and drinking water in the Southern part of Nigeria are polluted with Mn [28–30] and majority of the population in this region consume alcohol on a daily basis, especially fishermen and farmers. Recent study in the region indicates that fishermen had elevated levels of Mn in their toenails when compared with permissible limits and typical communities in developed countries [31]. Hence, the present study investigated, for the first time, the influence of co-exposure to (EtOH and Mn on inflammation and apoptosis markers in the hypothalamus of rats by assessing the involvement of NF-κB/p53 signaling pathways.

Materials and Method

Chemicals

Manganese chloride (as $MnCl_2{\cdot}4H_2O$, ≥ 99.9%) and ethanol (99.9%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and British Drug Houses (Poole, Dorset, UK), respectively. ELISA kits for measuring TNF-α, IL-1β, and NF-κB were obtained from ABCAM (UK). While rabbit polyclonal primary anti-COX-2, anti-p53, anti-Bax, anti-Bcl-2, anti-caspase-3, and anti-rabbit secondary antibody were also obtained from ABCAM (UK) and Elabscience (China). All other reagents were of highest analytical grade and were purchased from the British Drug Houses (Poole, Dorset, UK).

Animal Care

Fifty matured male Wistar rats (8 weeks old; weighing between 140 and 160 g) obtained from the Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan were used for this investigation. The experimental animals were housed in standard polypropylene cages with a 12-h light/dark cycle in a well-ventilated rat house and acclimatized for a period of 2 weeks before the commencement of the experiment. The animals fed on rat pellets and had access to water ad libitum. Animal care and experimental protocols were executed according to the approved guidelines set by the University of Ibadan Ethical Committee, which is in compliance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science (NAS) and published by the National Institute of Health.

Experimental Design

The rats were randomly divided to five groups of ten 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 at 30 mg/kg body weight and ethanol at 1.25 g/kg body weight.
Group V (Mn + EtOH 2): Rats were orally co-treated with manganese at 30 mg/kg body weight 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*) used in the present study were chosen based on the results of the level of Mn in seafood commonly consumed in

the Southern part (Niger Delta region) of Nigeria [27] and from the pilot study in our laboratory and previously published data [28]. This design seeks to evaluate the effect of simultaneous dietary exposure to Mn and EtOH common in Ogoniland [27–31].

Tissues Sampling

The animals were sacrificed 24 h after the last administration. Three rats brain were fixed in 4% paraformaldehyde solution for immunohistochemistry analysis whereas the hypothalamus from seven rats were dissected and stored at − 20 °C before separate homogenization in eight volumes of 50 mM Tris–HCl buffer (pH 7.4) containing 1.15% potassium chloride. Subsequently, the resulting homogenate was centrifuged at 12,000×*g* for 15 min at 4 °C and the supernatant obtained was used for the assessment of the transcription factor and pro-inflammatory cytokines using enzyme linked immunosorbent assay (ELISA) technique.

Assessment of Tumor Necrosis Factor Alpha, Interleukin 1 Beta, and Nuclear Factor Kappa B Levels

The concentration of nuclear factor kappa B (NF-κB), tumor necrosis factor alpha (TNF-α), and interleukin 1 beta (IL-1β) were assayed in the hypothalamus homogenates using commercially available ELISA kits with the aid of NM 9602 Microplate Reader (ABCAM, UK) according to the recommendations of the manufacturer. The optical density was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm.

Immunohistochemistry

Immunohistochemistry for COX-2, p53, Bax, Bcl-2, and Caspase-3 were performed on a 4% paraformaldehyde-fixed paraffin-embedded brain sections using rabbit polyclonal antibody. The immunohistochemistry analysis was performed in a two-step procedures. In the first step, the embedded paraffin sections were deparaffininized in xylene, rehydrated in graded series of alcohol before incubated with rabbit anti-COX – 2, p53, Bax, Bcl-2, and Caspase-3 (1/200 dilution) at 23 °C in a moist chamber for 1 h. In the second step, slides were incubated with horse-radish peroxidase labeled anti-rabbit polyclonal secondary immunoglobulins for 30 min. Lastly, 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen molecule for the immunological detection. The generated images were analyzed and processed with an open source Fiji-ImageJ Software (v1.51a NIH). Three micrographs from each of the representative groups were analyzed with ImageJ. These images were deconvolved to remove or filter microscope-associated noise while the color threshold was adjusted to a positive DAB stain region. The resultant images generated were further evaluated to obtain the mean intensity of immunoreactivity after removing the background intensity representing the total intensity of the image. The data were expressed as percentage (%) of high positive DAB intensity.

Statistical Analysis

Statistical analyses were carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Bonferroni's post hoc test using GRAPHPAD PRISM 5 software (Version 4; GraphPad Software, La Jolla, California, USA). Values of $p < 0.05$ were considered significant.

Results

Influence of EtOH Administration on TNF-α and IL-1β Levels in the Hypothalamus of Mn-Treated Rats

Figure 1(A, B) shows the effects of EtOH on pro-inflammatory cytokines, namely TNF-α and IL-1β in the hypothalamus of Mn-treated rats. The rats treated with EtOH alone and Mn alone show significant ($p < 0.05$) increases in TNF-α and IL-1β levels in the hypothalamus when compared with the control. However, co-exposure to Mn and EtOH significantly ($p < 0.05$) exacerbates Mn-induced increase in pro-inflammatory cytokines in the rat hypothalamus when compared with the control, EtOH alone, and Mn alone

Influence of EtOH Administration on NF-κB and COX-2 in the Hypothalamus of Mn-Treated Rats

Influence of separate administration of EtOH, Mn and their co-treatment on NF-κB are presented in Fig. 1(C). The experimental rats treated with EtOH alone and Mn alone show significant ($p < 0.05$) increase in NF-κB level in the hypothalamus when compared with the control. However, co-treatment with EtOH (i.e., Mn + EtOH 1 and Mn + EtOH 2) significantly exacerbates Mn-induced increase in NF-κB level in the treated rats when compared with the control, EtOH alone, and Mn alone. Similarly, experimental rats treated with EtOH alone and Mn alone significantly increased COX-2 protein expression in the hypothalamus when compared with the control. However, co-treatment with EtOH (i.e., Mn + EtOH 1 and Mn + EtOH 2) significantly ($p < 0.05$) exacerbated Mn-induced upregulation of COX-2 protein expression in the hypothalamus when compared with the control, EtOH alone, and Mn alone as shown in Fig. 2.

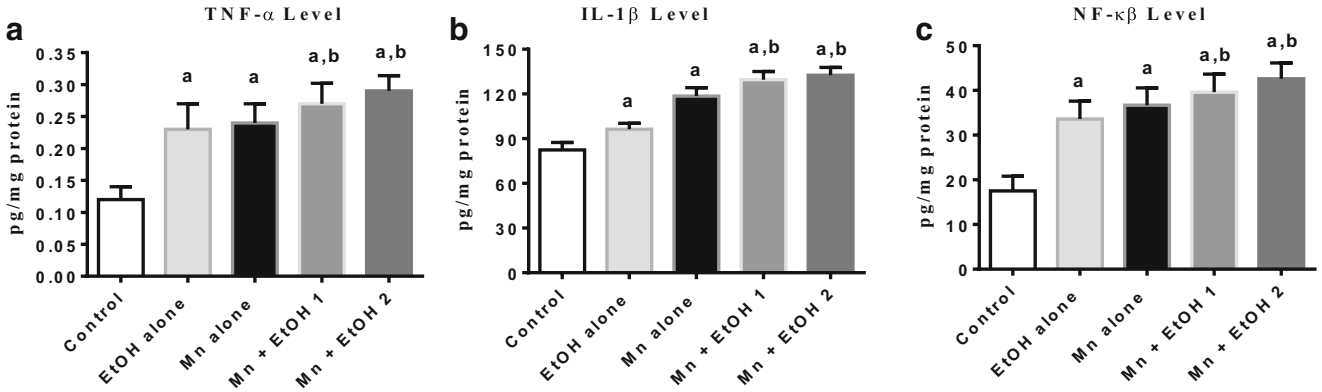

Fig. 1 The influence of EtOH on Mn-induced activation of NF – α (**a**), IL - 1β (**b**) and NF- κB (**c**) in the hypothalamus of rats. Mn, manganese; EtOH, ethanol. Mn (30 mg/kg body weight); EtOH 1, (1.25 g/kg body weight); EtOH 2, (5 g/kg body weight). The data are expressed as mean ± S.D. for 7 rats per group. (a) Values differ significantly from control ($p \leq 0.05$). (b) Values differ significantly from EtOH and Mn alone at $p \leq 0.05$.

Influence of EtOH on Apoptotic Proteins Expression in the Hypothalamus of Mn-Treated Rats

Figures 3 and 4 show the influence of EtOH on apoptotic proteins (p53, Bax, Bcl-2, and Caspase-3) in the hypothalamus of Mn-treated rats. The immunoreactivity for p53, Bax, and Caspase-3 were weak in the control group when compared with the treated groups. The protein expression of p53, Bax, and Caspase-3 in the hypothalamus of rats exposed to EtOH alone and Mn alone were significantly upregulated whereas the protein expression of anti-apoptotic marker, Bcl-2, was markedly downregulated following separate administration of EtOH and Mn to the rats. However, rats co-treated with EtOH (i.e., Mn + EtOH 1 and Mn + EtOH 2) exhibited significantly exacerbation in the upregulation of p53, Bax, and Caspase-3 as well as in the downregulation of Bax protein expression in the hypothalamus of the treated rats when compared with the control, EtOH alone, and Mn alone.

Discussion

The hypothalamus plays an important role in metabolic processes and other autonomic nervous system activities. It synthesizes and secretes neurohormones such as hypothalamic hormones (releasing hormones) responsible for the stimulation or inhibition of pituitary hormones secretion [28]. It also controls hunger, sleep, body temperature, fatigue, circadian rhythms [32, 33]. Any alteration of the hypothalamus function would impair it metabolic and autonomic nervous system

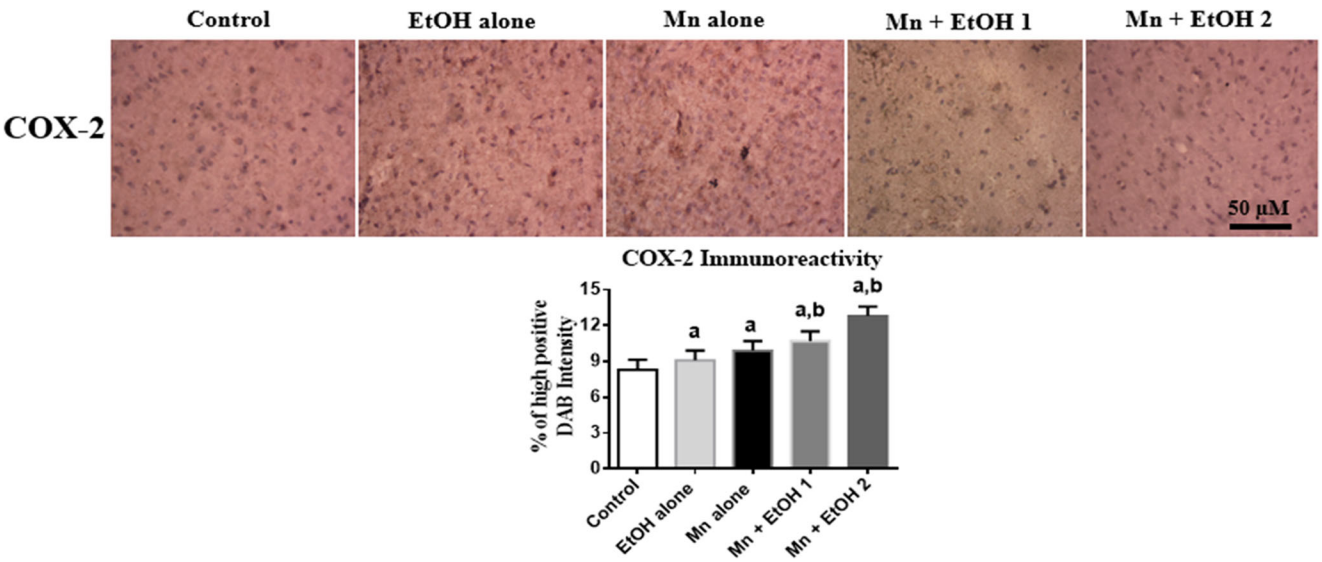

Fig. 2 Immunohistochemical staining showing the influence of EtOH on Mn-induced protein expression of COX-2 in the hypothalamus of rats' brain. Mn, manganese; EtOH, ethanol. Mn (30 mg/kg body weight); EtOH 1, (1.25 g/kg body weight); EtOH 2, (5 g/kg body weight). A = quantification of COX-2 protein expression in rat hypothalamus. The data are expressed as mean ± S.D. for 3 rats per group. (a) Values differ significantly from control ($p \leq 0.05$). (b) Values differ significantly from EtOH and Mn alone at $p \leq 0.05$

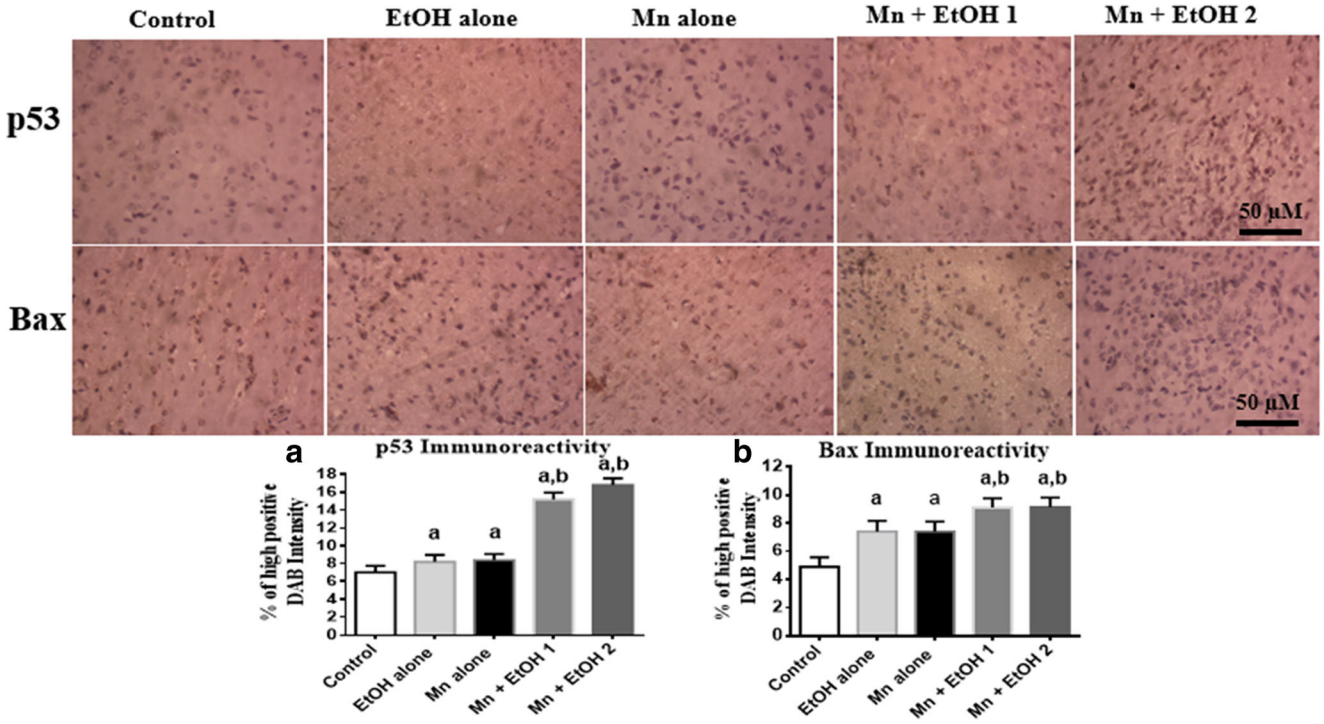

Fig. 3 Immunohistochemical staining showing the influence of EtOH on Mn-induced protein expressions of p53 and Bax in the hypothalamus of rats' brain. Mn, manganese; EtOH, ethanol. Mn (30 mg/kg body weight); EtOH 1, (1.25 g/kg body weight); EtOH 2, (5 g/kg body weight). (A, B) = quantification of p53 and Bax protein expression in rat hypothalamus, respectively. The data are expressed as mean ± S.D. for 3 rats per group. (a) Values differ significantly from control ($p \leq 0.05$). (b) Values differ significantly from EtOH and Mn alone at $p \leq 0.05$

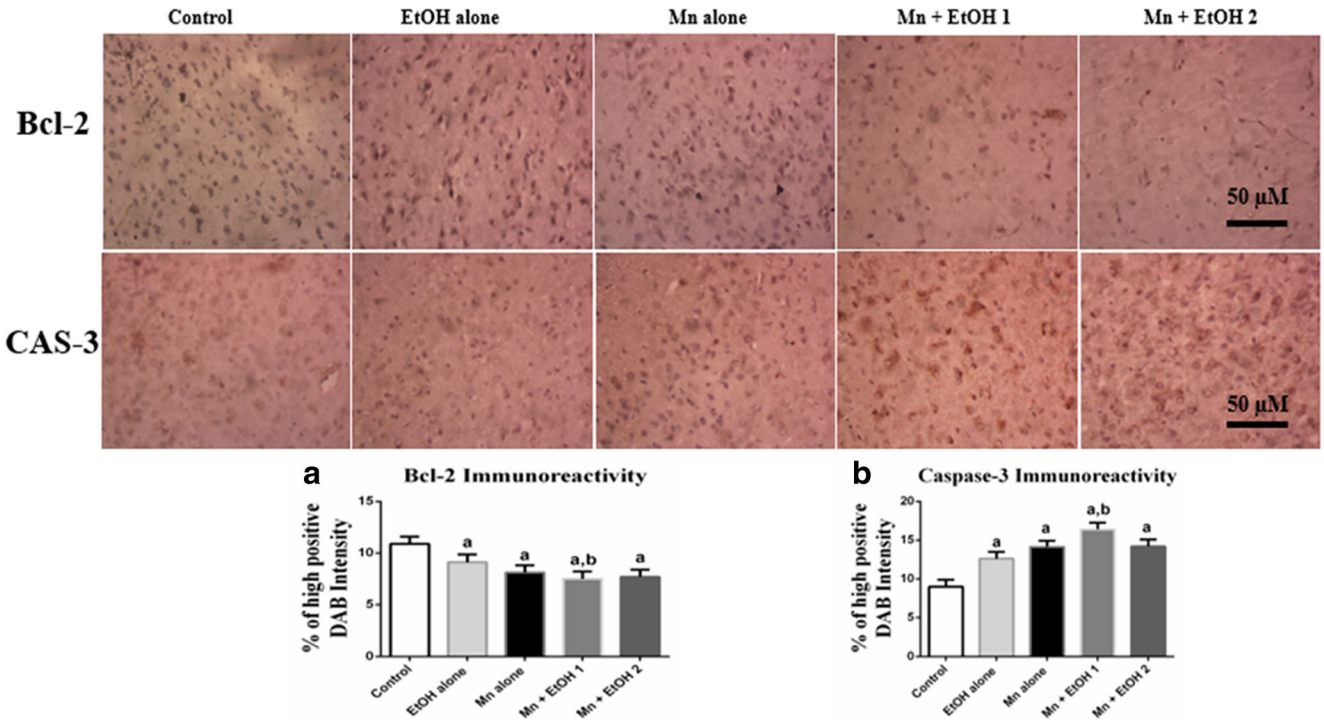

Fig. 4 Immunohistochemical staining showing the influence of EtOH on Mn-induced protein expressions of Bcl-2, and caspase-3 in the hypothalamus of rats' brain. Mn, manganese; , EtOH, ethanol; CAS3, caspase-3. Mn (30 mg/kg body weight); EtOH 1, (1.25 g/kg body weight); EtOH 2, (5 g/kg body weight). (A, B) = quantification of Bcl-2 and caspase-3 protein expression in rat hypothalamus, respectively. The data are expressed as mean ± S.D. for 3 rats per group. (a) Values differ significantly from control ($p \leq 0.05$). (b) Values differ significantly from EtOH and Mn alone at $p \leq 0.05$

function. The present study aimed at characterizing the effects of co-exposure to EtOH and Mn on inflammation and apoptosis in the hypothalamus rats.

This study demonstrated that separate exposure to EtOH and Mn resulted in a significant increase in inflammatory markers such as pro-inflammatory cytokines (TNF-α and IL-1β), COX-2 and NF-κB in the hypothalamus. Furthermore, EtOH exacerbates the Mn-induced increases in the inflammatory markers in the hypothalamus. The increases in these inflammatory markers may stimulate the innate immune cell in the brain (astrocytes) to activate pro-inflammatory cytokines as a result of ROS production via NF-κB mediated mechanisms to cause neuro-inflammation in the hypothalamus [34]. NF- κB is responsible for the regulation of gene expression involved in cell growth, inflammation and cell death via over-expression of pro-inflammatory cytokines and apoptosis induction [35–38]. Normally, NF-κB is bound to it inhibitory protein IκBα in the cytosol. However, the activation of IκBα leads to the phosphorylation and degradation of NF-κB [39]. This subsequently results in the phosphorylation and translocation of NF-κB into the nucleus. Activated NF-κB in the nucleus activates the transcription of TNF-α, IL-1β, and IL-6, in its target genes [39]. The present investigation demonstrated that EtOH exacerbates Mn-induced hypothalamic inflammation via the regulation of NF-κB signaling pathway involving the activation of TNF-α, IL-1β, and IL-6 levels in the hypothalamus of rats.

Moreover, rats administered with EtOH alone, Mn alone or in combination showed high immunoreactivity for COX-2 when compared with the control. However, Mn + EtOH 1 and Mn + EtOH 2 showed intense immunoreactivity when compared with rat hypothalamus exposed to EtOH alone and Mn alone. COX-2, an arachidonic acid converting enzyme is unexpressed under normal conditions in most cells, especially in the brain but elevated level is found in response to inflammatory stimuli in many inflammatory diseases [40, 41]. Also, the expression of COX-2 is highly transcriptionally upregulated by NF-κB activation [42] via reactive oxygen species/reactive nitrogen species (ROS/RNS)-mediated neuronal damages. This further confirmed that EtOH exacerbates Mn-induced hypothalamic inflammation.

The upregulation of protein expression of apoptotic markers, namely p53, Bax, and Caspase-3 and downregulation of the protein expression of the anti-apoptotic protein (Bcl-2) in rat hypothalamus treated with Mn alone, EtOH alone and in combination clearly indicates DNA damages as a result of apoptosis induction [43]. Upregulation of Bax initiate p53-mediated apoptosis via dependent transcription pathway involving the inhibition of Bcl-2 by Bax upregulation. Moreover, upregulation of p53 and Bax activate the cleavage of executional pro-apoptotic protein caspase-3 [44]. Furthermore, upregulation of caspase-3 cleaved it substrate; a protein/DNA repair enzyme (i.e., PARP-1) and degrade DNA via DNases proteolytic activation [45], thereby resulting in apoptosis via p53 dependent mechanism. Although p53 is known to be a general inhibitor of inflammation due to its antagonism of NF-κB [46]; however, excessive activation or abnormal regulation of p53 or NF-κB causes inflammation and apoptosis [47]. Thus, for both NF-κB and p53, normal physiological responses can lead to lethal consequences such as inflammation and apoptosis if activated to excessive levels [47]. The authors acknowledged the limitations to the present study which may be addressed in future investigations; for instance, the use of western blot and PCR analyses to complement the results of the immunohistochemistry data on the exacerbating effect of EtOH on Mn-induced hypothalamic toxicity. Our data demonstrated that EtOH alone and in combination with Mn lead to excessive activation of NF-κB/p53 signaling pathways, thereby exacerbating manganese-induced inflammation and apoptosis in rat hypothalamus. The observation may have profound effect on the hypothalamus function such as impairment of it metabolic and autonomic nervous system functions and consequently lead to the downstream alterations in the stimulation of pituitary hormones secretion, hunger, sleep, body temperature, fatigue, and circadian rhythms.

Conclusion

Taken together, our present study demonstrated that EtOH exacerbates Mn-induced neuro-inflammation and apoptosis in the hypothalamus of rats via regulation of NF-κB/p53 signaling pathway. The data presented herein are novel and have significant consequences on human health complications especially if extrapolated to individuals who are simultaneously exposed to both Mn and EtOH.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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