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Obestatin improve spatial memory impairment in a rat model of fetal alcohol spectrum disorders via inhibiting apoptosis and neuroinflammation

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ABSTRACT

Clinical and experimental evidence have demonstrated that, use of alcohol during pregnancy can interrupt brain development. Alcohol-induced neurocognitive deficits in offspring's are involved with activation of oxidative-inflammatory cascade joined with extensive apoptotic neurodegeneration in different brain regions such as hippocampus. Obestatin is a newly discovered peptide with anti-inflammatory, antioxidant, activities in different animal models. In this study, we aimed to evaluate the protective effects of obestatin on alcohol-induced neuronal apoptosis and neuroinflammation in rat pups with postnatal ethanol exposure.

Through intragastric intubation, ethanol (5/27 g/kg/day) was administered in male Wistar rat pups on postnatal days 2–10 (third trimester in humans). The animals received Obestatin (1 and 5 μg/kg, S.C.) on postnatal days 2–10. Thirty-six days after birth, the spatial memory test was performed using Morris water maze test, and then, antioxidant enzymes and TNF-α levels were measured by ELISA assay. The expression level of GFAP and caspase-3 proteins was determined via immunohistochemical staining after the behavioral test.

Obestatin significantly improved spatial memory deficits ($P < .01$), and obestatin treatment could significantly increase glutathione and total superoxide dismutase activity ($P < .05$), reduce level of malondialdehyde ($P < .05$) and TNF-α in comparison with the ethanol group ($P < .01$). It's also reduced caspase-3 level, and decreased GFAP-positive cells in the hippocampus of ethanol-exposed rat pups ($P < .01$). The result of this study shows the potential involvement of oxidative-inflammatory cascade-mediated apoptotic signaling in cognitive deficits due to postnatal ethanol exposure, the results also indicated the neuroprotective effects of Obestatin on alcohol-related behavioral, biochemical and molecular deficits.

1. Introduction

Maternal alcohol use during pregnancy can result in fetal alcohol spectrum disorders (FASD), which are associated with multiple neurocognitive and behavioral disorders, besides structural anomalies in newborns. Ethanol (ET) is described as a neuroteratogen, which can result in neuronal loss in the developing central nervous system (CNS) and produce many symptoms in children suffering from FASD (Riley and Mcgee, 2005).

Multiple long-term neurobehavioral disorders, including cognitive, physical, behavioral, and learning disabilities, are associated with prenatal alcohol exposure (Guerri et al., 2009). Patients with FASDs exhibit long-term disorders in behavioral flexibility and working memory (Rasmussen, 2005; Connor et al., 2000), which require simultaneous functioning of different brain regions. Many of these brain regions are being developed in the third trimester and show particular sensitivity to alcohol exposure (Patten et al., 2014). Rodents are the most commonly used for FASD research. The development period of the rodent brain is also divided into trimester equivalents; and therefore more closely look like brain development in human beings. In mice and rats, the first trimester comparable is from gestational day (GD) 1–10, the second trimester comparable corresponds to GD 10–20 (just prior to birth) and the third trimester equivalent and “brain growth spurt” occurs following birth from postnatal day (PND) 1 to 10, also developmental of prefrontal cortex, hippocampus cerebellum occurs in third trimester (Kelly et al., 2009). Thus, existing rodent models of FASD may involve exposure to alcohol during the prenatal period, postnatal period, or the combination of the two. But administration of ethanol in prenatal period has a limitation such as, alcohols is metabolized by placenta enzyme and alcohol blood levels in fetus is lower than mother. In prenatal alcohol administration maternal behavioral is abnormal and this can affect the development of the fetus, Also alcohol suppress

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lactation in mother (Giglia and Binns, 2006; Shankar et al., 2007).

A possible mechanism, contributing to ET-related neuronal damage, is oxidative stress, associated with the extreme formation of reactive oxygen species (ROS). ROS is generally formed during ET catabolism or mitochondrial damage as a result of ET exposure. ROS at low concentrations is recognized as a major regulating agent of cellular functions; however, excessive formation of ROS can lead to oxidative stress and consequently neurodegeneration (Tang et al., 2011).

According to various studies, necrosis and apoptosis are associated with ET-induced oxidative stress in neuronal cells (Goodlett and Horn, 2001). Moreover, antioxidant enzymes, including glutathione peroxidase and glutathione reductase, are suppressed by ET (Siler-Marsiglio et al., 2004). On the other hand, the developing brain, with lower antioxidant activities compared to the adult brain, may show greater vulnerability to oxidative stress and its neurotoxic effects (Wang and Michaelis, 2010).

Although the neuropathological effects of alcohol on the fetus have been reported, simultaneous reactive changes in astroglia, which are often associated with these neuropathologies, have not been identified. Generally, astrogliosis is recognized as a common reaction to neuronal degeneration or CNS damage in the mature brain (Boschen et al., 2016; Goodfellow et al., 2018). After reactive astrocytes become hypertrophied, they produce thick fibrillary processes and lead to a significant increase in the content of glial fibrillary acidic protein (GFAP), which is known as the primary component of intermediate filaments of astrocytes (Hol and Pekny, 2015). Importantly, reactive gliosis can occur in neonatal rat brain in response to damage just as in the adult brain (Brolese et al., 2014). Obestatin, encoded by the ghrelin gene, is a peptide, comprised of 23 amino acids. Formation of this peptide is associated with the posttranslational processing of preproghrelin peptide. At first, Obestatin was considered an endogenous ligand of G-protein-coupled receptor 39 (GPR39). The expression and release of Obestatin in different peripheral tissues have been indicated; however, stomach and gastrointestinal tract produce Obestatin similar to ghrelin (Liu et al., 2011; Zhang et al., 2005).

Obestatin prevents apoptosis in pancreatic islets of both humans and rodents (Granata et al., 2010) It also plays a role in protection against myocardial infarction, preconditioning ischemia, and isolated ventricular myocytes (Alloatti et al., 2010). A previous study showed the anti-inflammatory and protective effects of peripheral injection of Obestatin on subarachnoid hemorrhage due to brain injury (Erşahin et al., 2013). Another study demonstrated that peripheral administration of Obestatin could reduce the severity of pentylenetetrazole-induced seizures, improve memory dysfunction, and reduce neuronal damage by limiting oxidative damage (Koyuncuoğlu et al., 2017). Therefore, considering the underlying mechanisms of ET induced neurotoxicity and the protective effect that was reported about obestatin, in the present study for the first time, we investigate the effects of obestatin administration on neuronal damage following ET induced neurotoxicity.

2. Materials and methods

The sixty rat pups were randomly allocated into five groups after birth. The groups (12 pups per group) were as follows: 1-control group, 2- milk solution + saline, 3- ET group, 4- ET + Obestatin group 1 μg/kg/SC, 5- ET + Obestatin group 5 μg/kg/SC. Treatments were initiated on postnatal (PD) day 2 and continued until PD 10, i.e., the third pregnancy trimester in humans. On PD 2–10, the ET group was administered ET via intragastric intubation. A dose of ET (5.27 g/kg) in a milk solution (27.8 ml/kg) was administered every day, producing maximum blood alcohol concentrations of 300–400 mg/dl For intubation, a PE10 intramedic tube, which was attached to a syringe and immersed in corn oil lubricant, was located down the esophagus; the solution was then directly injected into the stomach (Otero et al., 2012; Patten et al., 2014). Ethics code is IR.SHMU.REC.1397.158.

2.1. Morris water maze (MWM) task

To examine spatial learning and memory, the hippocampus-dependent MWM task was performed 36 days after birth. The MWM task is generally a common tool for evaluating cognitive deficits. The setting included an illuminated room with some external cues, which remained in the same location in the training and probe tests (Aski et al., 2018).

2.2. Spatial training test

Over four consecutive days, the animals were trained on how to find a hidden submerged platform in four trials (30-s intervals) from day 36 to day 39 after birth. There was a platform in the northwest quadrant of the tank, and the rats were randomly placed in water at one of the quadrants. The rats were given time to swim until finding and climbing the platform; they were trained to stay on the platform for 30 s. The animals were guided by the experimenter if they failed to find the escape platform in one minute. A video tracking system automatically recorded the total distance, velocity, and escape latency (time to reach the platform). The average of four daily trials was then measured.

2.3. Spatial probe test

One day after the final training trial (40 days after birth), the platform was removed from the tank in order to perform the 60 s spatial probe test for the previous location of the platform. The time spent in the target quadrant was also determined.

2.4. Biochemical analysis

Immediately after the memory test, six rats were anesthetized with ketamine (80 mg/kg/ip) and xylazine (10 mg/kg/ip) from each group. For the biochemical analysis, the animals' brains were removed. For this purpose, the hippocampal samples (six rats per group) were homogenized on ice in cold RIPA buffer with protease inhibitors. Centrifugation was carried out for 20 min at 3000*g* (4 °C). The supernatant was used to examine the enzymes (Mehrjerdi et al., 2018).

2.5. Malondialdehyde (MDA) measurement

To indicate the lipid peroxidation products, MDA levels were determined in the hippocampal samples using the MDA assay kit (ZellBio GmbH, Germany). The collected tissues were homogenized in ice-cold 1.15% KCl for two minutes. The MDA levels were measured based on the thiobarbituric acid reactive substance (TBARS) formation method. After mixing cold trichloroacetic acid 10% (1 ml) and thiobarbituric acid 10% (2 ml) with homogenized tissues, they were heated at 100 °C for one hour. The precipitate was removed after cooling down the solution at room temperature and centrifugation; afterwards, the pink-colour supernatant was moved to the microplate. The reaction mixture absorbance was measured at 535 nm using a microplate reader (ELx800, BioTek, USA). The MDA level (μM) was calculated according to the standard curve (Shafahi et al., 2018).

2.6. Glutathione (GSH) and total superoxide dismutase activity (SOD) measurements

According to GSH and SOD kit instructions (ZellBio GmbH, Germany), the GSH and SOD contents were determined. After adding a certain amount of PBS (100 mM, pH 7.4), the hippocampal samples were homogenized and centrifuged. The supernatants were carefully collected. After SOD and GSH interacted with the chromogen reagent, absorbance was read with a microplate reader (ELx800, BioTek, USA) at 420 and 412 nm to measure total SOD activity (U/mg protein) and GSH (U/mg protein), respectively, based on the defined formulae (Erfani et al., 2018b).

2.7. Tumor necrosis factor-α (TNF-α) measurements

The instructions of TNF-α kit (Diaclone), which is a solid-phase sandwich ELISA assay, were followed to determine the level of TNF-α. The wells of microtiter strips were coated with TNF-α-specific monoclonal antibodies. Then, samples, including unknown and known concentrations of TNF-α, were pipetted in the wells. The TNF-α antigen and biotinylated monoclonal antibody for rat TNF-α were incubated concurrently. After washing, the streptavidin-peroxidase enzyme was added. Following incubation and complete removal of unbound enzymes, a substrate solution acting on the bound enzyme was added, forming a colored reaction product. The product intensity was relative to the level of TNF-α in the samples.

2.8. Tissue preparation

Six of the animals from each group were anesthetized immediately after the memory test. Then, transcardiac perfusion was carried out using 0.9% saline and then 4% paraformaldehyde in phosphate buffer (0.1 M; pH, 7.4). The animals' brains were embedded in paraffin after being removed and postfixed for three days. Then, 7-μm coronal sections were cut based on the Paxinos Atlas (from 3.3 to 4.2 mm posterior to the bregma), using a microtome for different staining methods (Khaksari et al., 2017).

2.9. Immunohistochemical staining

For determining astrocyte activation and apoptosis, immunohistochemical staining was carried out to determine caspase-3 and GFAP in 7-μm tissue sections. Following paraffin removal through incubation at 60 °C for 30 min, the sections were cleared with xylene, and subtractive series of alcohols was used for rehydration. In order to prevent endogenous peroxidase activity, 10% H_2O_2 was used in methanol for treatment during 10 min. Afterwards, a tris-buffer preparation was used to wash the sections. Antigen was retrieved after 11 min of autoclaving in citrate buffer.

After washing in PBS, fetal bovine serum (1% FBS) in 0.3% triton X-100 was used to fix the sections. Following overnight incubation at 4 °C, a primary antibody (Abcam, UK) was used. The dilution ratio was 1:100. For detection of the antigen, a goat polyclonal secondary antibody (Abcam, UK) was left for 30 min in the room, and then, DAB (Sigma, USA) was added. For microscopic visualization, hematoxylin (Sigma) counterstaining was carried out. The GFAP-positive cells were counted in the right CA1 region of the hippocampus in each slide at 400× magnification. The person in charge of counting procedures was blind to the study objectives. The primary antibody was not included in the negative control slides (Erfani et al., 2018a).

2.10. Data analysis

The normal distribution of variables was indicated by Kolmogorov-Smirnov test. Data are presented as mean ± SEM. ANOVA was applied to compare the groups. After adjusting the level of significance, Scheffe post hoc test or Dunnett T3 test was used to evaluate the differences. If the variances were homogenous, Scheffe test was used, and if not, Dunnett's T3 post hoc test was employed. $P \leq .05$ was considered as the level of significance. For data analysis, SPSS version 16 was used.

3. Results

3.1. Effects of obestatin on memory and spatial learning impairment due to ET exposure

According to the MWM test, the groups were significantly different in terms of escape latency time and total distance travelled for reaching the platform on trial days. However, the groups showed no significant difference in terms of velocity to reach the platform. On experimental days, the ET group spent more time and travelled a longer distance to find the hidden platform in comparison with the control group ($P \leq .001$). Also, in the ET group receiving obestatin, the distance and time spent to reach the hidden platform reduced, compared to the ET group on experimental days ($P < .01$, Fig. 1).

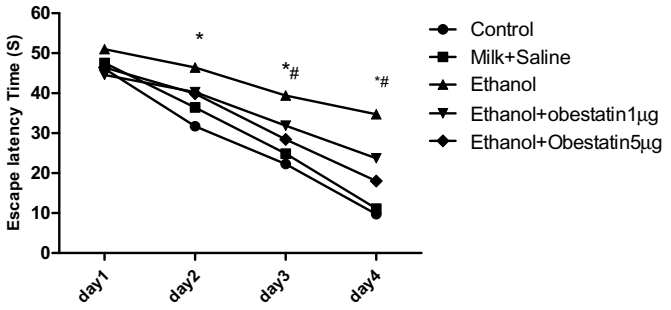

Fig. 1. Escape latency time, total distance moved and velocity to reach the platform during trial days in the Morris water maze task for different groups.
Significantly different between ET group with control and saline groups ($P < .001$).
* Significantly different between obestatin treated group with ethanol group ($P < .01$).

According to the probe trial (Fig. 2), the ET group spent less time in the target quadrant, compared to the control group ($P < .01$). The spent time in the target quadrant significantly increased in the obestatin groups ($P < .01$) (Fig. 3).

3.2. Obestatin increased the levels of total SOD activity and GSH after ET-induced neurotoxicity

A significant decline was observed in total SOD activity in the ET group, compared to the control group ($P < .01$). In obestatin treatment groups, total SOD activity increased in comparison with ET rats ($P < .05$). The GSH level decreased in the ET group versus the control group ($P < .01$). In obestatin (5 μg/kg) treatment group, the GSH level increased, compared with ET group ($P < .05$) (Fig. 4).

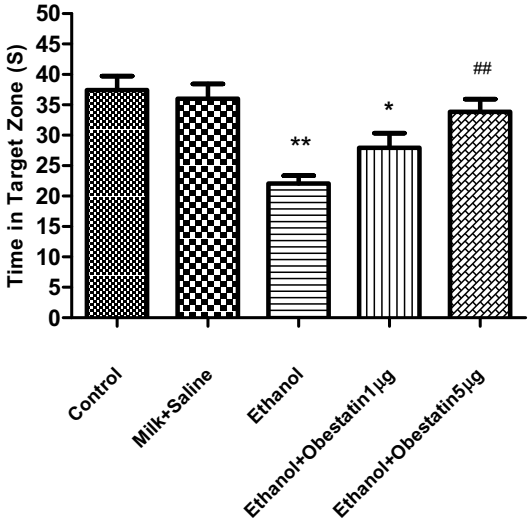

Fig. 2. Time spent in the target zone.
* Significantly different compared with ET group ($P < .05$).
** Significantly different compared with control group ($P < .01$).
Significantly different compared with ET group ($P < .01$).

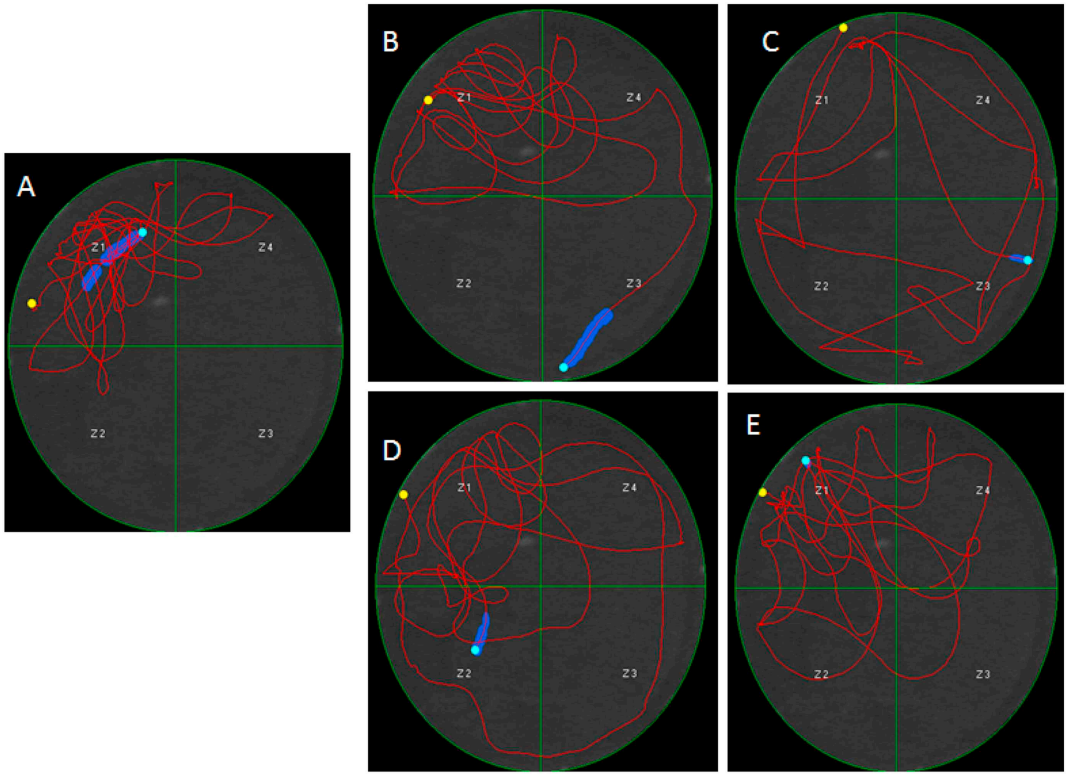

Fig. 3. Illustrative swim paths in probe trial day (5th day) in the Morris water maze task for different groups. A: control, B: milk solution + saline, C: ET group, D: ET + obestatin group 1 μg/kg/SC and E: ET + obestatin group 5 μg/kg/SC groups

3.3. Obestatin reduced the level of MDA and TNF-α after ET-induced neurotoxicity

According to the biochemical analysis, the hippocampus MDA level in the ET group was higher than that of the control group ($P < .01$). The MDA level decreased in obestatin groups versus the ET group ($P < .05$).

The hippocampus TNF-α level in the ET group was higher than that of the control group ($P < .01$). Obestatin treatment (5 μg/kg) decreased TNF-α level, compared to the ET group ($P < .01$; Fig. 5).

3.4. Obestatin decreased active caspase-3 activation in ET-induced neurotoxicity

The number of active caspase-3-positive cells in the ET group was significantly higher than that of the control group ($P < .001$). On the other hand, the percentage of active caspase-3 positive cell in the obestatin group was significantly lower than that of the ET group ($P < .01$) (Fig. 7).

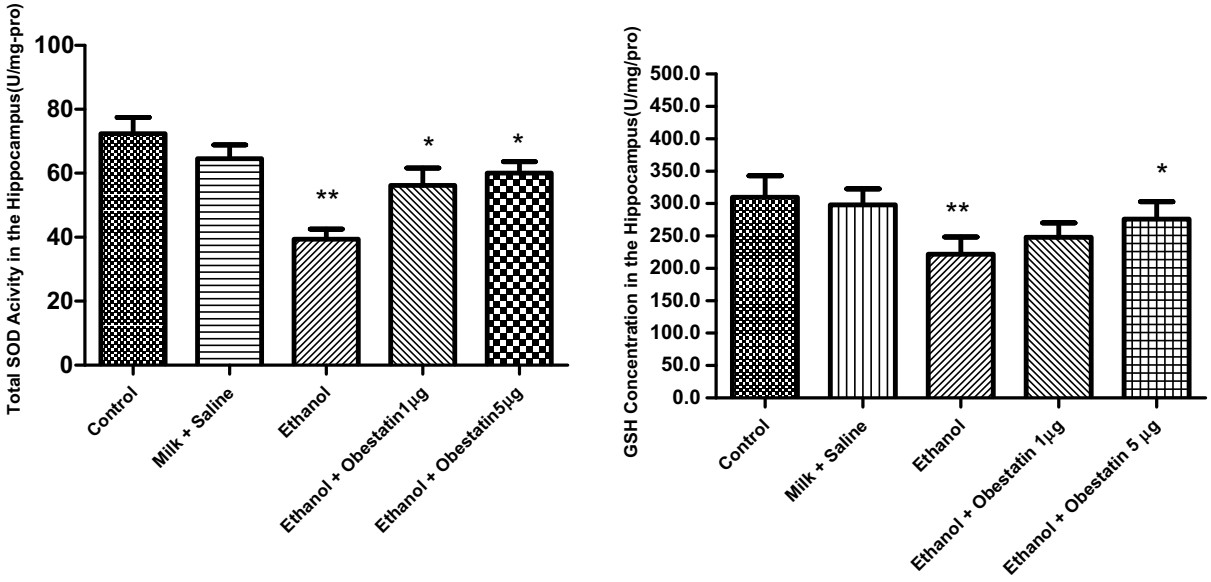

Fig. 4. Effect of treatment with obestatin on total SOD activity and GSH concentration in the hippocampus following ET induced neurotoxicity.
* Significantly different compared with ET group ($P < .05$).
** Significantly different compared with control group ($P < .01$).

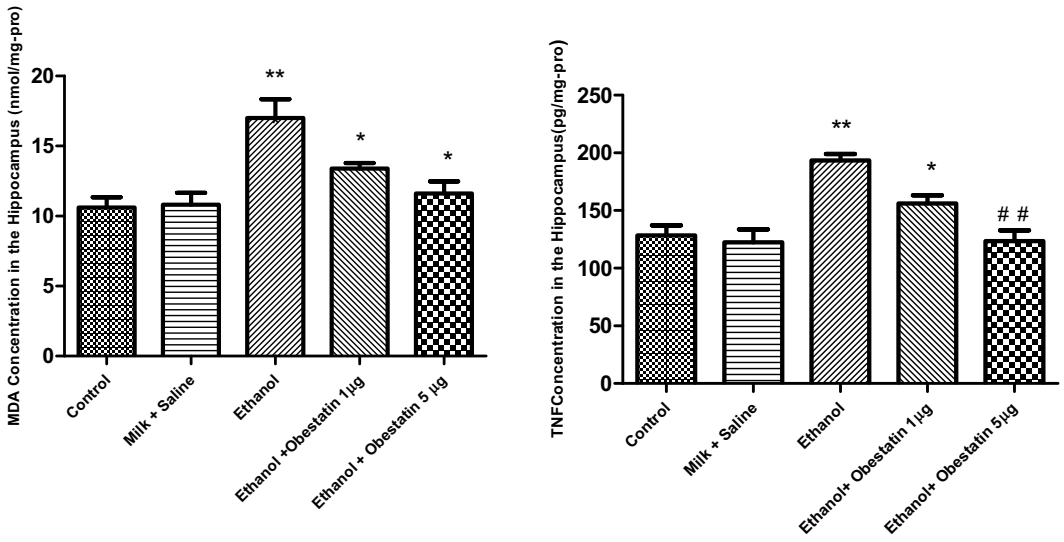

Fig. 5. Effect of treatment with obestatin on MDA and TNF-α concentration in the hippocampus following ET induced neurotoxicity.
* Significantly different compared with ET group ($P < .05$).
** Significantly different compared with control group ($P < .01$).
Significantly different compared with ET group ($P < .01$).

3.5. Obestatin decreased the level of GFAP after ET-induced neurotoxicity

GFAP expression was poor in the sham group. Also, the number of GFAP-positive cells in the ET group was higher than that of the control group ($P < .001$). In the obestatin group, GFAP-positive cell percentage was lower than the ET group ($P < .01$), (Fig. 6).

4. Discussion

The present study showed that administration of obestatin as a novel peptide could significantly improve memory impairment following ET induced neurotoxicity. It also decreased the active caspase-3 positive cell count in the pyramidal cells of CA1 region and reduced the level of GFAP as a neuroinflammation factor. Moreover, obestatin could prevent lipid peroxidation and significantly increase the capacity of antioxidant enzymes in ET neurotoxicity. Also, this study showed that obestatin attenuated TNF-α production induced by ET neurotoxicity.

Postnatal ET exposure causes neuroinflammation mostly in the hippocampus among other brain regions; this may be particularly harmful during early development due to the low levels of antioxidants in the brain (Shah et al., 2015). Increased neuroinflammation and decreased antioxidant properties may interfere with the normal processes of neural development, including neurogenesis and synaptic plasticity. This damage is thought to contribute to impairments in executive function, learning, and memory in FASD patients and rodent models (Rasmussen, 2005; Swart et al., 2017).

The hippocampus is particularly susceptible to inflammatory stress. This region of the brain in involved in spatial memory and navigation

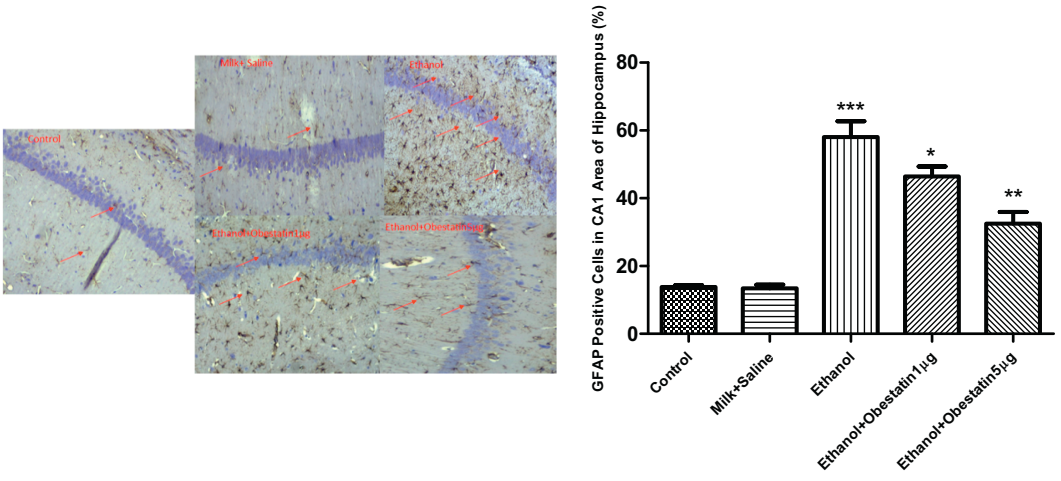

Fig. 6. left figures photomicrographs of immunohistochemical staining of active caspase −3 in the CA1 area of right hippocampus after ethanol induced neurotoxicity. (Magnifications ×400). Red arrows in the photomicrographs shows active caspase-3 expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Right figure effects of obestatin treatment on the active caspase −3 levels in the hippocampal CA1 area following the ethanol induced neurotoxicity. Treatment with obestatin significantly attenuated the ethanol -induced increase in active caspase-3 levels.
* Significantly different compared with ethanol group ($P < .05$).
** Significantly different compared with ethanol group ($P < .01$).
*** Significantly different compared with control group ($P < .001$).

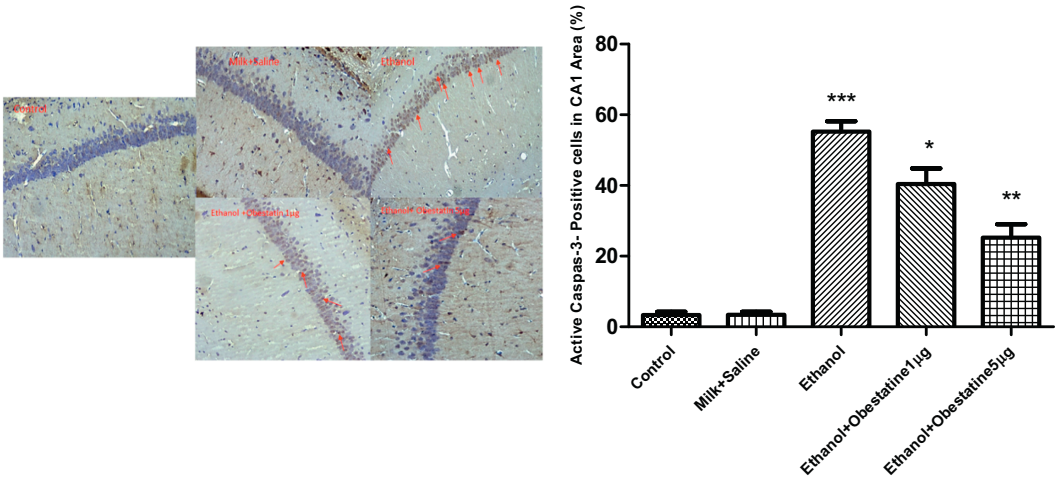

Fig. 7. left figures photomicrographs of immunohistochemical staining of GFAP in the CA1 area of right hippocampus after ethanol induced neurotoxicity. (Magnifications × 400). Red arrows in the photomicrographs shows GFAP expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Right figure effects of obestatin treatment on the GFAP levels in the hippocampal CA1 area following the ethanol induced neurotoxicity. Treatment with obestatin significantly attenuated the ethanol -induced increase in caspase-3 levels.

* Significantly different compared with ethanol group ($P < .05$).

** Significantly different compared with ethanol group ($P < .01$).

*** Significantly different compared with control group ($P < .001$).

(Boitard et al., 2014; Shamsaei et al., 2015). Therefore, common memory disorders following ET neurotoxicity are normally associated with reduced hippocampal functioning (Guerri and Pascual, 2010). Predisposition to alcohol at moderate to high levels during the process of development can reduce the count of pyramidal cells in the CA1 and CA3 regions (Mcgoey et al., 2003). Moderate to high levels of alcohol exposure during gestation trigger marked cytoarchitecture changes in pyramidal cell dendrites and impair long-term potentiation (LTP) in the pyramidal cells of CA1 region (Byrnes et al., 2004).

Recent studies have focused on ET-related damages to brain structures by activating the neuroinflammatory system (Drew and Kane, 2014). Early overactivation of inflammatory responses due to ET exposure can damage the developing hippocampus and result in cognitive deficits later in life (Terasaki and Schwarz, 2016). Moreover, based on different studies, reactive astrocytes following FASD play a pivotal role in inflammation regulation from a primary source of chemokines and proinflammatory cytokines. The major proinflammatory products include interleukin (IL) 1β and TNF-α (Komada et al., 2017).

In addition, the fluidity of mitochondrial membranes changes due to ET exposure, producing acetaldehyde and oxidative species (Henderson et al., 1999), such as hydrogen peroxide, hydroxyl radicals, and free radicals, which rapidly activate Nuclear factor-κB (NFκB) (Crews et al., 2006). According to a study by Crews et al. (2006), microglial activation, NFκB activation, and improved COX2 immunoreactivity occur as a result of alcohol-induced neurodegeneration, leading to the release of proinflammatory cytokines, including IL-1β and TNF-α (Crews et al., 2006; Alfonso-Loeches et al., 2014).

TNF-α stimulates the production of endothelial adhesions and release of inflammatory mediators from neutrophils and microglial cells. TNF-α leads to direct neuronal toxicity by forming reactive oxygen species through ceramide and results in neuronal apoptosis (Andrieu-Abadie et al., 2001). In line with our findings, recent research indicates that obestatin reduces TNF-α, IL-1β, and IL-6 levels after subarachnoid hemorrhage (Erşahin et al., 2013). Also, obestatin improves ischemia/reperfusion-induced renal injury via antiinflammatory mechanisms (Koç et al., 2014).

Substantial evidence suggests the role of oxidative stress in FASD, as indicated by ROS production in both in vitro and animal models. Increased production of ROS and reactive nitrogen species by oxidizing proteins affects neuronal death by damaging DNA and increasing lipid peroxidation in the cell membrane (Koç et al., 2014). Obestatin increases the activity of SOD and GSH-Px; therefore, the level of DNA damage also decreases. MDA cytotoxic compound, produced by lipid peroxidation, is a biomarker of oxidative stress, indicating free radical production and tissue damage (Kadiiska et al., 2005). Antioxidant enzymes, such as GSH and SOD, show protective effects against oxidative damage. These enzymes survive aerobic organisms through a defense mechanism.

The present results indicated that SOD and GSH levels significantly reduced the plasma antioxidant activity following ET induced neurotoxicity, although the level of MDA significantly increased, consistent with previous findings (Smith et al., 2005). However, plasma SOD and GSH levels significantly increased following obestatin treatment, although the level of MDA significantly reduced in rats. Recent studies have also shown that obestatin ameliorates renal ischemia/reperfusion-injury and protects cardiomyocytes against ischemia/reperfusion by antioxidative properties (Koç et al., 2014; Alloatti et al., 2010).

The present study showed that obestatin prevents neuronal apoptosis and inflammation induced by ET neurotoxicity. The beneficial effects of obestatin may be attributed to changes in the expression of apoptosis-related proteins and endogenous inflammatory mediators, leading to alterations in the number of apoptotic neuronal cells. The hippocampus is generally involved in spatial memory and navigation. Therefore, common memory disorders following cerebral ischemia are normally associated with hippocampal dysfunction.

The present study showed that obestatin treatment significantly reduced CA1 cell death and improved spatial memory following ET-related neuronal damage in the rat hippocampus. Considering the effects of obestatin on pathological diseases, it can be considered as an efficient therapeutic option for diseases such as FASD; however, further research is necessary in the future.

Conflict of interests

The authors declare no conflict of interests related to this work.

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