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Running title: Cerebrolysin improved ethanol-induced memory impairments

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Abstract

The present study investigates the potential neuroprotective effect of cerebrolysin (CBL), a combination of neurotrophic factors, on the cognitive and biochemical alterations induced by chronic ethanol administration in rats. The animals were divided into 5 groups as follows: Control, ethanol (4 g/kg, for 30 days) plus normal saline (Ethanol + NS), ethanol plus CBL 1 ml/kg (Ethanol + CBL 1), ethanol plus CBL 2.5 ml/kg (Ethanol + CBL 2.5), and ethanol plus CBL 5 ml/kg (Ethanol + CBL 5). Morris water maze (MWM) test was performed to assess cognitive impairment. The status of the lipid peroxidation marker (MDA), antioxidant capacity as well as alterations of the apoptotic factors such as Bcl-2, BAX, and cleaved-caspase 9 and 3 were evaluated in the hippocampus. The results showed that CBL treatment not only normalized the increased MDA levels in the alcoholic rats and enhanced antioxidant defense but also reduced Bax/Bcl-2 ratio and cleaved-caspase 9 and 3 in the hippocampus. These results were parallel with improvement in the spatial memory performance in the MWM test. The findings of the present study provide evidence for the promising therapeutic effect of CBL in chronic ethanol consumption through counteracting oxidative stress and apoptosis markers.

Keywords: Ethanol; Cerebrolysin; Oxidative stress; Apoptosis; Spatial memory
Introduction

Alcohol abuse is one of the most common health problems worldwide resulting in millions of deaths each year. Ethanol has deleterious effects on many organs especially the heart, liver, and the central nervous system (CNS). Owing to dependence-producing properties of alcohol the global prevalence of alcohol abuse is increasing especially among adolescents (Casswell & Thamarangsi, 2009; Jørgensen, Pedersen, & Tønnesen, 2011; Orellana et al., 2017).

From the pharmacological point of view, ethanol, the active ingredient of alcoholic beverages, has a high solubility in fat and quickly is absorbed in the brain, particularly, due to high blood flow and percentage of fat (Geil et al., 2014). There is substantial evidence that acute or chronic alcohol abuse is associated with many deleterious effects on the brain function such as cognitive performance and neurological disorders, which are linked to neurodegeneration (Anstey, Mack, & Cherbuin, 2009; Dry, Burns, Nettelbeck, Farquharson, & White, 2012; McKinney, Coyle, & Verster, 2012; Mintzer, 2007; Russo et al., 2001; Wright et al., 2003). Indeed, chronic alcohol consumption leads to neurodegeneration in the various brain regions involved in cognitive processes such as the hippocampus (Haorah et al., 2008; Topiwala et al., 2017). Both human and animal studies have demonstrated that chronic alcohol consumption can lead to structural and functional damages to the hippocampus (Baydas, Yasar, & Tuzcu, 2005; White, Matthews, & Best, 2000). Several structural magnetic resonance imaging studies have shown a reduction of hippocampal volume in heavy alcohol drinkers (Beresford et al., 2006; Ozsoy, Durak, & Esel, 2013; Wilhelm et al., 2008). Other studies also showed that alcohol consumption is associated with decreased nerve growth factor (Sakai et al., 2005), reduction of hippocampal neurogenesis in the dentate gyrus (Golub et al., 2015; Morris, Eaves, Smith, & Nixon, 2010; Taffe et al., 2010), and inhibition of neuronal activity in the hippocampus (White & Best, 2000). In addition, alcohol affects the functions of multiple neurotransmitters involved in the learning and memory processes (Banerjee, 2014; Matthews & Morrow, 2000; Myhrer, 2003) and some of these side effects may persist even after the cessation of alcohol consumption (Santucci, Cortes, Bettica, & Cortes, 2008).
Among several proposed mechanisms, oxidative stress is one of the major mechanisms underlying alcohol-induced cognitive function impairments (Bailey, 2003; González-Reimers, Santolaria-Fernández, Martín-González, Fernández-Rodríguez, & Quintero-Platt, 2014; Reddy, Padmavathi, Kavitha, Saradamma, & Varadacharyulu, 2013; Russo et al., 2001). Alcohol metabolism results in the generation of reactive oxygen species (ROS) and oxidative stress (Das & Vasudevan, 2007; Mukherjee, 2014; Reddy et al., 2013) leading to neurotoxicity and cell death (Ceccatelli, Tamm, Zhang, & Chen, 2007; Hai Chen et al., 2011). Furthermore, it has been shown that chronic alcohol consumption decreases antioxidant enzyme activities (Ahmad, 1995; Saravanan, Rajendra Prasad, & Pugalendi, 2003).

Studies show that several neurotrophins, such as Brain-Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) induce neuroprotection during nerve injury, as well as promote recovery process and neuronal regeneration after damage (Feng et al., 2017; Luo & Miller, 1998; Mohapel, Frielingsdorf, Hägglad, Zachrisson, & Brundin, 2005; Wong, Cheung, Yu, Sha, & Cho, 2014). Accordingly, the use of therapeutic strategies containing neurotrophic factors may have an impact on neurological impairments (Mahmoudi et al., 2018). In this regard, cerebrolysin (CBL) is a pharmaceutical composition isolated from porcine brain containing biologically active low molecular weight peptides and free amino acids (Huiping Chen et al., 2015). Several studies have shown that CBL has neurotrophic capabilities and induces neuronal sprouting and increases neuronal survival and neurogenesis, particularly in the dentate gyrus, and thus improves spatial memory (Huiping Chen et al., 2015; Juárez, González, Mena, & Flores, 2011; Schauer et al., 2006; Tatebayashi, Lee, Li, Iqbal, & Grundke-Iqbal, 2003; Windisch, Gschanes, & Hutter-Paier, 1998). In addition, CBL treatment reduces the free radical formation and cellular oxidative stress during cerebral ischemia (Amiri-Nikpour et al., 2014; Gonzalez, Francis, & Castellano, 1998).

To this date, there is no report about neuroprotective properties of CBL on alcohol-induced memory dysfunction. Therefore, this study was conducted to address whether CBL is able to improve spatial memory deficit and reduce hippocampal oxidative stress-induced cell death in alcohol-treated rats.
Materials and methods

Animals

Seventy adult male Wister rats, weighing 220-250 g, were purchased from the laboratory animal unit of Tabriz University of Medical Sciences. Rats were kept at 21.0 ±2 °C temperature and on a 12 h light/dark cycle with ad libitum access to food and water. This study was confirmed by the Ethical Committee of the Tabriz University of Medical Sciences (TBZMED.REC.1394.838) and all of the experimental procedures were performed in accordance with NIH guidelines.

Study design

Animals were randomly allocated to control (n=14) and ethanol groups (n=56). Animals in the control group received normal saline (5 ml/kg) for 60 consecutive days (For the first 30 days through intra-gastric route and for the second 30 days through intraperitoneal (i.p.) route). Rats in the ethanol group received daily ethanol dose of 4 g/kg for 30 consecutive days through intra-gastric route. At the end of this period and after ethanol withdrawal, alcoholic rats were randomly assigned into 4 subgroups (n=14 in each group) and received their respective treatments for 30 days: Ethanol + normal saline (NS), receiving i.p. injection of normal saline (5 ml/kg); Ethanol + CBL 1, receiving i.p. injection of 1 ml/kg CBL (EBewe Arzneimittel, Austria); Ethanol + CBL 2.5, receiving i.p. injection of 2.5 ml/kg CBL; and Ethanol + CBL 5, receiving i.p. injection of 5 ml/kg CBL. The study design is shown in Fig. 1.

Fig. 1. The timeline of the study.

Ethanol administration schedule

The protocol for yielding peak blood ethanol concentration (99.80 mg/dl) was adopted from Abadi et al. (Abadi, Vaghef, Babri, Mahmood-Alilo, & Beirami, 2013). In this method, the initial 5% (v/v)
concentration of ethanol was followed by addition of 5% ethanol every two days to achieve a final concentration of 20% (v/v). This concentration maintained for the remaining 22 days of study.

**Morris Water Maze (MWM)**

One day after the last intervention, spatial learning and memory were assessed using Morris Water Maze (MWM) task in all study groups. A black circular water pool (with a diameter of 136 cm and a depth of 80 cm) was filled with water at 23±1°C temperature and positioned in a room with visual cues on the walls. Then, a small, black escape platform (10 cm×10 cm) was placed in the center of a predetermined quadrant of the tank submerged 1.5 cm under the water surface (to make it invisible to the rats). The pool was conceptually divided into four equal quadrants with four points designed as starting positions (N, S, W, and E) (Pereira, Strapasson, Nabinger, Achaval, & Netto, 2008). The behaviors of the animals were tracked by a ceiling-mounted video camera placed directly above the pool and then analyzed using Noldus tracking software (Ethovision XT, Noldus Information Technology, Wageningen, Netherlands).

*Visible platform*

All animals were submitted to a visible platform task to test eyesight and swimming ability. The escape platform was placed over the water level (1 cm) in one quadrant of the pool. The animals were initially placed on the platform for 30 s to familiarize them with the cues. The experiment was included four trials in which animals were allowed to search the platform for a period of 60 s. The starting position remained constant throughout the visible testing, but the platform location was randomized among trials to overcome any residual preference for the previous location of the platform.

*Acquisition*

The following day after the visible platform task, rats were trained on the hidden platform task to assess spatial acquisition. In this task, the platform was submerged 1.5 cm below the water surface and remained in the same position during all trials. The animals were subjected to four trials a day for three successive days. In each trial, rats were placed in the water, facing the wall of the pool, in one of the four starting locations (N, S, W, and E) and permitted them to swim up to 60 s to find the
escape platform. At the end of 60 s, if the animals failed to find the platform within the allowed time, the animal was gently guided to the platform and allowed to remain there for 15 s. The next trial started immediately after their removal from the platform. After completion of the fourth trial of the block, rats were removed from the pool and placed them in a temporary holding cage under a heat lamp.

**Probe test**

Spatial memory retention was evaluated using a 60 s-probe trial which was carried out 24 h after the last acquisition trial. The platform was removed from the pool, and rats were placed in the water in either the adjacent right or the adjacent left quadrant according to the training quadrant. The time spent in each quadrant and escape latency time were recorded.

**Oxidative Stress Parameters**

**Tissue Preparation**

At the end of the behavioral assessment, animals were sacrificed by cervical dislocation under ketamine and xylazine (200 mg/kg and 10 mg/kg, i.p, respectively) anesthesia. Oxidative stress was assessed in the homogenized hippocampal tissues of the animals. The hippocampus was rapidly dissected on an ice-cold surface, and frozen in liquid nitrogen and subsequently stored at −80 °C until biochemical analysis. The frozen hippocampal tissues were homogenized in cold phosphate buffer (0.1 M, pH 7.4), containing 140 mM KCl and 1mM EDTA. Subsequently, the homogenates were centrifuged at 1000 rpm for 10 min at 4°C and used the resulting supernatant in the experiments.

**Superoxide Dismutase Activity**

Superoxide dismutase (SOD) activity was determined using a RANSOD kit (Randox Labs., Crumlin UK) according to the Delmas-Beauvieux et al. method (Delmas-Beauvieux et al., 1995). Briefly, xanthine and xanthine oxidase were employed to generate superoxide radicals which react with 2-(4-iodophenyl)-3 (4-nitropheno)-5-phenyl tetrazolium chloride (ITN) to form a red formazan dye. The SOD activity was measured by the degree of inhibition of this reaction at 505
nm using a spectrophotometer. The activities were expressed as units per milligram of protein (U/mg of protein).

Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity was assessed according to the Paglia and Valentine method (Paglia & Valentine, 1967) using a RANSEL kit (Randox labs. Crumlin UK Randox).

Malondialdehyde levels

Malondialdehyde (MDA) concentration was determined as an indicator of lipid peroxidation according to the Draper and Hadley method (Draper & Hadley, 1990). Briefly, the content of MDA as thiobarbituric acid reactive substances (TBARS) was evaluated using a spectrophotometer at 532 nm. The results were expressed as nmol/mg of protein.

Total Anti-oxidant Capacity (TAC)

The assessment of total antioxidant capacity provides an integrated parameter to determine all the antioxidants present in the biologic samples (Ghisselli, Serafini, Natella, & Scaccini, 2000). Using TAC kit, this parameter was measured and expressed as nmol/mg of protein.

Western blotting

Western blotting was carried out using the previously described method with 12.5% polyacrylamide gel (Salehpour et al., 2017). Primary antibodies (Santa Cruz Biotechnology, U.S.A.) including anti-caspase-9 (1:500, sc-81663), anti-caspase-3 (1:500, sc-136219), anti-Bcl-2 (1:500, sc-492), anti-Bax (1:500, sc-493) antibodies were used. Anti β-actin (1:500, sc-130656) antibody was applied as an internal control of the procedure. Each band signal intensity was quantified using Image J 1.62 software (National Institutes of Health, Maryland, U.S.A.) and normalized to the matching internal control.

Statistical Analyses

The data are expressed as mean ± standard error of mean (SEM). All analyses were performed using Graph Pad Prism 6.01 (Graph Pad Software Inc., La Jolla, CA, USA). The significance of differences in the escape latency time was determined using two-way ANOVA. For all other
variables one-way ANOVA followed by post hoc Tukey test was performed for comparison of the differences between groups. In all comparisons, $p<0.05$ was considered statistically significant.

**Results**

**Body Weight**

Our results showed no significant effect of the treatments on the body weight of rats during the study (data are not shown).

**MWM**

*Visible Platform Task of MWM*

The results of the visible platform showed no significant differences in swimming velocity, total pathlength, and escape latency (Table 1) among different groups indicating that all the animals had no vision, sensation, or motor dysfunction.

**Table 1** Pathlength, escape latency, and swimming speed in the visible platform task of Morris water maze (MWM) in different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pathlength (cm)</th>
<th>Escape latency (s)</th>
<th>Swimming speed (cm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>854.12±39.27</td>
<td>41.11±2.08</td>
<td>23.12±1.38</td>
</tr>
<tr>
<td>Ethanol + NS</td>
<td>869.76±40.12</td>
<td>42.40±3.17</td>
<td>21.33±1.21</td>
</tr>
<tr>
<td>Ethanol + CBL1</td>
<td>857.15±47.23</td>
<td>39.03±2.17</td>
<td>22.5±1.09</td>
</tr>
<tr>
<td>Ethanol + CBL2.5</td>
<td>873.23±41.03</td>
<td>42.14±3.25</td>
<td>22.16±1.17</td>
</tr>
<tr>
<td>Ethanol + CBL5</td>
<td>860.65±33.45</td>
<td>40.26±3.01</td>
<td>24.01±0.97</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM for 12 animals in each group. [NS: normal saline; CBL: cerebrolysin].

*Hidden Platform Task of MWM*

A two-way ANOVA of escape latency time (Fig. 2A) and mean path length (Fig. 2B) in the hidden platform task using group and day as factors showed a main effect of group ($F_{(4, 165)} = 9.748$, $p<0.001$), day ($F_{(2, 165)} = 66.78$, $p<0.01$), and group × day interaction ($F_{(8, 165)} = 2.861$, $p<0.01$) for escape latency and main effect of group ($F_{(4, 165)} = 22.22$, $p<0.001$), day ($F_{(2, 165)} = 77.67$, $p<0.001$), and group × day interaction ($F_{(8, 165)} = 1.145$, $p>0.05$) for path length. Intergroup analysis indicated
that chronic administration of ethanol increased escape latency time on the 2\textsuperscript{nd} and 3\textsuperscript{rd} days of hidden platform session of MWM compared to the control group ($p<0.01$ for both days). In addition, there was a significant difference between control animals and Ethanol+NS group in the mean path length on days 2 and 3 ($p<0.001$ for both days). Nevertheless, chronic CBL treatment at the dose of 5 ml/kg decreased escape latency time ($p<0.05$ for both days) as well as mean path length ($p<0.01$ for both days) on the 2\textsuperscript{nd} and 3\textsuperscript{rd} days compared to Ethanol + NS received rats. However, there was no significant difference in the swimming speed among different groups (Table. 2).

\textit{Time Spent in the Target Quadrant in Probe Trials}

The result of one-way ANOVA showed a significant difference in the time spent in the target quadrant of the probe test among different groups ($F_{(4, 30)} = 5.82, p<0.001$). Post-hoc analysis revealed that ethanol administration for 30 days decreases the time spent in the target quadrant in the Ethanol + NS and Ethanol + CBL1 ($p<0.05$ for both) groups as compared to the control group in rats. On the other hand, treatment with CBL at 5 ml/kg dose increased the time spent in the target quadrant and returned it values to those of the healthy control group (Fig. 2C)
Fig. 2. A) Escape latency time and B) Mean path length during training days on the hidden platform task of MWM in different groups. C) Time spent in the target quadrant in probe trials of MWM task in different groups. Each bar represents the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001
compared with the control, and *p*<0.05 and **p*<0.01 compared with the Ethanol + NS group. NS: normal saline; CBL: cerebrolysin; MWM: Morris water maze.

**Table. 2** Mean swim speed (cm/min) in the hidden platform task.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.12±0.97</td>
<td>22.09±1.03</td>
<td>21.18±1.39</td>
</tr>
<tr>
<td>Ethanol+NS</td>
<td>23.76±1.02</td>
<td>20.22±1.23</td>
<td>23.36±0.96</td>
</tr>
<tr>
<td>Ethanol+CBL1</td>
<td>22.15±1.23</td>
<td>19.23±0.87</td>
<td>25.21±0.96</td>
</tr>
<tr>
<td>Ethanol+CBL2.5</td>
<td>19.23±0.93</td>
<td>22.34±0.93</td>
<td>18.09±1.58</td>
</tr>
<tr>
<td>Ethanol+CBL5</td>
<td>20.65±1.45</td>
<td>23.33±1.22</td>
<td>22.12±1.04</td>
</tr>
</tbody>
</table>

Data are shown as mean±SEM (n=12). There was no significant difference between different groups.

**Biochemical Analysis**

*Lipid peroxidation levels*

The result of one-way ANOVA demonstrated a significant difference in the hippocampal MDA levels among different groups (F (4, 25) = 5.65, *p*<0.001). Post-hoc analysis revealed that chronic ethanol administration significantly increased MDA levels in the hippocampus of the Ethanol + NS received rats compared to the control group (*p*<0.01). Also, the results revealed that CBL administration at 5 ml/kg reduced MDA levels in alcoholic rats and MDA values was approached to those of the healthy control group (Fig. 3).
Fig. 3. The impacts of alcohol and treatments on the MDA levels in the hippocampus of rats (mean ± SEM). **$p<0.01$ compared to control group. NS: normal saline; CBL: cerebrolysin; MDA: malondialdehyde.

SOD Activity

One-way ANOVA analysis also showed a significant difference in the hippocampal SOD activity among different groups ($F_{(4, 25)} = 7.12, p<0.001$). Post-hoc analysis revealed that prolonged alcohol consumption decreases SOD activity in the hippocampus of Ethanol + NS ($p<0.01$), Ethanol + CBL1 ($p<0.01$), and Ethanol + CBL2.5 ($p<0.05$) groups compared to the control group. Further analysis revealed that CBL administration at 5 ml/kg dose improved SOD activity in the hippocampus of Ethanol + NS rats and approached its values to those of the healthy control group (Fig. 4).

Fig. 4. The effects of alcohol and treatments on the SOD levels in the hippocampus of rats (mean ± SEM). *$p<0.05$, **$p<0.01$ compared to control group. NS: normal saline; CBL: cerebrolysin; SOD: superoxide dismutase.

GPx Activity

The result of one-way ANOVA analysis also demonstrated a significant difference in the hippocampal GPx activity among different groups ($F_{(4, 25)} = 5.63, p<0.001$). Additionally, the
Tukey post-hoc analysis revealed that GPx activity decreased in the hippocampus of the Ethanol + NS and Ethanol + CBL1 rats compared to the control group ($p<0.05$ for both). Further analysis showed that CBL administration at 5 ml/kg dose increased GPx activity in the hippocampus of alcoholic rats (Fig. 5).

Fig. 5. The impacts of alcohol and treatments on the GPx levels in the hippocampus of rats (mean ± SEM). *$p<0.05$ compared to control group. NS: normal saline; CBL: cerebrolysin; GPx: glutathione peroxidase.

Total antioxidant capacity (TAC)

Our results showed a significant difference in the hippocampal TAC levels among different groups ($F(4, 25) = 8.43$, $p<0.001$). The results of Tukey post-hoc analysis demonstrated that chronic alcohol administration significantly decreases TAC in the hippocampus of Ethanol + NS ($p<0.001$), Ethanol + CBL1 ($p<0.01$) as well as Ethanol + CBL2.5 ($p<0.01$) rats compared to the control group. Also, CBL administration at 5 ml/kg dose increased TAC in the hippocampus of Ethanol + NS rats and approached its values to those of the healthy control group (Fig. 6).
Fig. 6. The impacts of alcohol and treatments on TAC in the hippocampus of rats (mean ± SEM). **p<0.01, ***p<0.001 compared to control group. NS: normal saline; CBL: cerebrolysin; TAC: total antioxidant capacity.

Apoptosis biomarkers

Cleaved Caspase 9 and 3

One-way ANOVA analysis confirmed a significant difference in the cleaved caspase 9 (F (4, 15) = 152.4, p<0.001) and cleaved caspase 3 (F (4, 15) = 53.74, p<0.001) levels in the hippocampus of different groups. Post-hoc analysis revealed that prolonged ethanol administration significantly (for both p<0.001) increased cleaved caspase-9 as well as cleaved caspase 3 levels compared to the control group in the hippocampus of Ethanol + NS and Ethanol + CBL1 rats. Nevertheless, chronic CBL treatment at dose of 5 ml/kg significantly (Fig. 7B) decreased cleaved-caspase 9/pro-caspase 9 ratio in the ethanol-received rats. Furthermore, CBL administration at 2.5 and 5 ml/kg doses, but not at dose 1 ml/kg, decreased cleaved caspase-3 levels in the hippocampus of Ethanol + NS rats and approached its values to those of the healthy control group (Fig. 7C).

BAX/Bcl-2 ratio

One-way ANOVA analysis also showed a significant difference in BAX/Bcl-2 ratio (F (4, 15) = 12.53, p<0.001) in the hippocampus of different groups. Post-hoc analysis revealed that chronic ethanol administration significantly increased BAX/Bcl-2 ratio compared to the control group in the
hippocampus of Ethanol + NS (p<0.01) and Ethanol + CBL1 (p<0.05) groups. However, CBL administration at 5 ml/kg dose decreased BAX/Bcl-2 ratio in the hippocampus of alcohol-received rats (Fig. 7D).

Fig. 7. The effect of alcohol and treatments on A) Representative image of Pro and Cleaved-caspase-9, Pro and Cleaved caspase-3, Bax, Bcl-2, and β-actin identified by Western blotting. B) Cleaved caspase-9/Pro-caspase 9 ratio, C) Cleaved caspase-3/Pro-caspase 3 ratio, and D) BAX/Bcl-2 ratio in the hippocampus of rats. β-actin was used as an internal loading control. Data are shown as mean ± SEM (n=4). *p<0.05, **p<0.01, ***p<0.01 compared to control group. NS: normal saline; CBL: cerebrolysin; Casp: caspase.
**Discussion**

The results of the present study demonstrated that alcoholic rats had longer escape latency and shorter time spent in the target quadrant than control rat indicating memory impairment. However, CBL treatment improved memory function in the MWM test. Moreover, CBL reduced ethanol-induced oxidative stress and enhanced enzymatic antioxidant activities in the hippocampus. In addition, hippocampal BAX/Bcl-2 ratio and cleaved caspase-3 protein expression level were decreased by CBL treatment.

The hippocampus is a critical structure for learning and memory formation (Deng, Aimone, & Gage, 2010), particularly for spatial memory and navigation (Eichenbaum, 2014). Previous studies reported that the hippocampus is particularly vulnerable to the deleterious effects of alcohol consumption, and damage to the hippocampus results in spatial memory deficits (Farajdokht et al., 2017; Morris et al., 2010; Tagliaferro, Vega, Evrard, Ramos, & Brusco, 2002; Topiwala et al., 2017). In the current study, MWM task was performed to assess the spatial learning and reference memories which are correlated with hippocampal function (D’Hooge & De Deyn, 2001). In agreement with the previous reports (Cippitelli et al., 2010; Mintzer, 2007; Wright et al., 2003), our study showed that chronic ethanol exposure resulted in spatial memory impairment as indicated by a significant increase in escape latency time, while CBL administration decreased this parameter indicating improved acquisition. Moreover, ethanol significantly decreased the time spent in the target quadrant in the probe test which was considerably reversed by CBL (5 ml/kg), representing that CBL treatment improved memory function. Several clinical and experimental studies have documented that CBL, a combination of active neuropeptides fragments, improves memory performance through preserving the integrity of the neuronal circuits and stimulating the generation of new functional synapses (Flores & Atzori, 2014; Masliah & Diez-Tejedor, 2012; Zhang et al., 2015). In addition, CBL has been proposed as a treatment strategy for cognitive impairment in ischemic stroke and neurodegenerative disorders (Pourmemar et al., 2017; Ubhi et al., 2013; Zhang et al., 2015).
Heavy and regular alcohol consumption is associated with smaller brain volumes and substantial cortical thinning possibly due to promoting neurotoxicity (Monnig, Tonigan, Yeo, Thoma, & McCrady, 2013; Squeglia, Jacobus, & Tapert, 2014). According to the previous studies, prolonged ethanol exposure is associated with enhanced ROS production and lipid peroxidation resulting in oxidative damage to dynamic components including DNA, proteins, and lipids. Moreover, ethanol is capable to reduce the endogenous antioxidants capacity (González-Reimers et al., 2014; McDonough, 2003; Mukherjee, 2014; Patil, Tawari, Mundhada, & Nadeem, 2015). Similarly, we found that ethanol increased hippocampal MDA levels and diminished the activity of enzymatic antioxidant defense, SOD and GPx activities, and TAC levels in the hippocampus. According to previous studies, overproduction of ROS results in a depletion of intracellular antioxidant enzymes, abnormalities of the cell structure and metabolism, and eventually neuronal degeneration (Blokhina, Virolainen, & Fagerstedt, 2003; Lo, Moskowitz, & Jacobs, 2005).

Conversely, CBL at dose of 5 ml/kg effectively attenuated MDA levels and enhanced SOD and GPx activities and TAC. Our results are consistent with previous reports demonstrating antioxidant activity of CBL in different organs. It has been reported that cerebrolysin attenuates the oxidative stress and neuronal damage induced by peripheral lipopolysaccharide administration in rats (Abdel-Salam et al., 2013). Boshra et al. have also shown that CBL attenuates oxidative stress-induced apoptosis in the rat model of myocardial ischemia (Boshra & Atwa, 2016). Recently, it has been demonstrated that CBL is capable of decreasing lipid peroxidation in diabetes induced by streptozotocin in rats (Sherif, 2017). Noor et al. also reported that CBL normalized the increased MDA and restored the glutathione levels of the midbrain in the animal model of Parkinson’s disease (Noor, Mohammed, Mourad, Khadrawy, & Ezz, 2016). Patočková et al. also demonstrated that a single injection of CBL attenuated insulin hypoglycemia induced lipid peroxidation in the brain of mice (Patočková, Kršiak, Marhol, & Tůmová, 2003). It seems that ethanol-induced memory deficit is possibly a consequence of hippocampal impairment secondary to oxidative stress damage. Hence, CBL may have the potential to protect ethanol-induced memory dysfunction through decreasing oxidative stress.
Moreover, the imbalance of oxidation and intracellular antioxidant defenses can stimulate apoptotic pathways (Kannan & Jain, 2000). Apoptosis is an active programmed cell death which is initiated by different signaling pathways or organelle damage (Elmore, 2007; Gronbeck et al., 2016). Bcl-2 family include anti-apoptotic and pro-apoptotic proteins (Bcl-2 and BAX, respectively) which either inhibit or promote apoptosis by regulating the permeability of the mitochondrial outer membrane (Sharifi, Mohajjel Nayebi, Farajnia, & Haddadi, 2015). Bcl-2 is a strong anti-apoptotic protein and its overexpression leads to resistance of the cells to oxidative stress-induced apoptosis (Brunelle & Letai, 2009). On the other hand, overexpression of Bax accelerates apoptosis and high Bax to Bcl-2 ratio is associated with apoptotic cell death (Elmore, 2007; Shamas-Din, Kale, Leber, & Andrews, 2013). Indeed, ROS overproduction may exceed the anti-oxidative capacity to initiate mitochondrial-dependent apoptotic signaling pathways and activate pro-apoptotic members of the Bcl-2 protein family such as BAX (X. Wang, 2001). Activated BAX induces the release of cytochrome c from the mitochondria. Consequently, cytochrome c generates apoptosomes and triggers the initiator caspase-9, which in turns, activates effector caspases such as pro-caspase 3 through cleavage (Orrenius, Gogvadze, & Zhivotovsky, 2007; Portt, Norman, Clapp, Greenwood, & Greenwood, 2011).

Our results also revealed that chronic alcohol consumption up-regulated pro-apoptotic proteins, BAX and cleaved-caspase 9 and 3, and down-regulated Bcl-2 expression in the hippocampal tissue. Similar to our results, previous reports have shown that 4 weeks exposure to ethanol induces hippocampal apoptosis through mitochondrial pathway associated with the up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins (Ku et al., 2007; Naseer et al., 2014; P. Wang et al., 2017). Therefore, it seems that the oxidative stress and apoptotic cell death may contribute to ethanol-induced memory impairment. Nevertheless, CBL treatment reversed these changes under ethanol stimulation and attenuated oxidative stress-induced apoptosis. Anti-apoptotic effect of CBL has also been reported in the myocardium and brain (Boshra & Atwa, 2016; Schauer et al., 2006; Xing et al., 2014).

Conclusion
To sum up, the findings of the present study demonstrated that CBL attenuated ethanol-induced memory dysfunction, at least in part, through inhibiting oxidative damage and apoptosis cell death in the hippocampus and may have beneficiary effect in the treatment of cognitive impairment associated with chronic alcoholism.

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Highlights

- CBL improves ethanol-induced spatial memory impairments.
- CBL decreases ethanol-induced oxidative stress in the hippocampus.
- CBL attenuates Bax/Bcl-2 ratio and cleaved-caspase 9 and 3 in the hippocampus of alcoholic rats.