Original Article

# Cobalamin modulate neurotoxic effects of trimethyltin chloride on hippocampus neural cells and cognitive function

Zeinab Hamidizad[1,3], Shima Ababzadeh[2,3], Fatemeh Heidari[2,3], Narges-al-Sadat Haeri[3], Mohsen Eslami Farsani[2,3], Mehdi Sadegh[4,5]* iD

1. Department of Physiology, School of Medicine, Qom University of Medical Sciences, Qom, Iran
2. Department of Anatomy, School of Medicine, Qom University of Medical Sciences, Qom, Iran
3. Cellular and Molecular Research Center, Qom University of Medical Sciences, Qom, Iran
4. Department of Physiology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran
5. Traditional and Complementary Medicine Research Center, Arak University of Medical Sciences, Arak, Iran

## Abstract

**Introduction:** Cobalamin (vitamin $B_{12}$) is essential for metabolism of the nervous system and its supplementation attenuate neuropathic and neuroinflammatory diseases. We designed to investigate the neuroprotective effects of cobalamin against the trimethyltin chloride (TMT) induced structural and functional damages in the hippocampus.

**Methods:** Adult male Wistar rats were divided into four groups: 1) control: received saline; 2) TMT: received a single dose of TMT (8mg/kg; ip) to induce hippocampal damages; 3) cobalamin: received cobalamin (18mg/kg; ip) for five consecutive days and 4) TMT+cobalamin: received single ip injection of TMT then were treated with cobalamin for five consecutive days. In day six of the experiments, behavioral effects of TMT and cobalamin were evaluated through shuttle box and novel object recognition task. After the behavioral tests, animals were perfused transcardially and Nissl staining was used on hippocampus to assess neural cell damages.

**Results:** Novel object exploring time was significantly decreased in TMT treated rats and treatment with cobalamin after TMT injection significantly recompensed this effect of TMT. In passive avoidance, TMT significantly decreased latency to enter the dark box, while cobalamin administration after the TMT injection significantly abolished this effect of TMT. Neural cell counted in the areas of hippocampus was significantly decreased in the TMT group and cobalamin treatment after the TMT injection significantly prevented neural cell loss.

**Conclusion:** These results indicate a neuroprotective role for cobalamin against the TMT induced memory impairment and hippocampal neuronal loss.

# Introduction

Neurotoxicity has been linked to a number of common drugs and chemical components (Roberts et al., 2015). Trimethyltin chloride (TMT) an organotin compound, previously had been used generally in industry and agriculture, but it has been limited

because of its dangers to human (Lee et al., 2016). Administration of TMT induces cognitive deficits in experimental models which are referred to severe hippocampal neuronal damage (Kaur et al., 2013). Therefore, it is a useful model for neurotoxicity to study the effects of chemical exposure on hippocampal function (Geloso et al., 2011). In rats, TMT first damages granule cells of the dentate gyrus (DG) and then pyramidal cells in the CA1 and CA3 subfields (Ogita et al., 2005). The precise mechanisms underlying TMT-induced neurotoxicity are not clear. But experimental studies supported the role of oxidative stress and neuroinflammation in TMT induced neurodegeneration (Kim, 2016). It has been proposed that TMT produces excess generation of cellular oxidative species, which leads to apoptotic cell death (Kim, 2016; Ebrahimpour et al., 2017). Also TMT leads to an elevated Bax/Bcl-2 ratio and enhanced the expression of caspase-3 and nuclear factor-kB (NF-kB) (Shuto et al., 2009; Silakhori et al., 2019).

Cobalamin is one of the large and structurally complex, non-polymeric biomolecules and an evolutionarily ancient cofactor with carbon–metal bonds (Randaccio et al., 2010). In humans, cobalamin plays an important role in the methionine synthesis and succinyl coenzyme-A production (Randaccio et al., 2010). Cobalamin is essential nutrient for metabolic functions of the nervous system (Romano et al., 2014; Gunes et al., 2018; Arora et al., 2019). Its deficiency is associated with neurological disorders characterized by demyelination followed by axonal degeneration and irreversible neuronal damage (Stabler, 2013). Cobalamin supplementation reduces nociceptive, inflammatory, neuropathic pain and also is beneficial in treatment of many inflammatory and oxidative stress-associated diseases (Chan et al., 2018; Gunes et al., 2018). Cobalamin regulates the transcription factor NF-kB, which activate or suppress the inflammatory response and its resolution. Many studies suggest that cobalamin may provide a basis for more beneficial treatments of nervous disorders and promising approach to systemic inflammatory response syndrome (Hajihashemi et al., 2017; Guo et al., 2019). Taken together, studies who revealed indispensable role of cobalamin in the neural system metabolism, also high prevalence of low cobalamin intake, in the present study was designed to investigate neuroprotective effects of Cobalamin against TMT induced cognitive dysfunction and hippocampal neuronal loss.

# Materials and methods

### Animals
The study was conducted on 40 male Wistar rats (150-200g). The animals were kept in cages under similar controlled conditions in terms of light (12h light/dark intermittently), room temperature (23±2°C) and had free access to food and water. All research and animal care were approved by the Review Board and Ethics Committee of Qom University of Medical Sciences. All efforts were made to minimize the number of animals used and their suffering.

### Drugs and experimental groups
Animals were divided into four experimental groups (n=10 for each group): 1) control group: received normal saline; 2) TMT group: administered single intraperitoneal (ip) injection of TMT (Merck, Darmstadt, Germany) dissolved in normal saline (8mg/kg) (Shin et al., 2005; Kaur et al., 2013; Kim, 2016); 3) cobalamin group: received cobalamin (Merck, Darmstadt, Germany) (18mg/kg; ip) for five consecutive days (Kopruszinski et al., 2012) and 4) TMT+cobalamin group: administered single ip injection of TMT then after one hour were treated with cobalamin for five consecutive days. In day six of the experiments, five rats from each group were randomly selected and were used in behavioral tastes and other five rats were used for histological study.

### Novel object recognition task (NOR)
For the NOR test, rat were individually habituated to an open-field box consisted of a black-painted wood small chamber (60×50×50cm) for 3 days. During the training session, the animals were individually exposed for 10min to two of the same objects placed in the open-field box. The time of touching and sniffing of the objects was recorded as the time spent exploring each object. During the test session, the animals were individually placed back into the same box 24h after training, in which box one of the objects used during the training was replaced by a novel object and allowed to explore freely for 10min. Discrimination ratio for each rat was expressed by:

TN/(TN+TF) ratio [TF= time spent exploring familiar object; TN= time spent exploring the novel object]. The objects were cleaned with 10% ethanol solution between trials (Botton et al., 2010; Antunes and Biala, 2012).

### Passive avoidance task

Twenty-four hours after the NOR experiment, animals were used for passive avoidance task through a shuttle box which included identical light and dark Plexiglas square boxes (30×20×20cm) which were separated by a guillotine door. The light compartment contained a 50W bulb and its floor was composed of 2mm steel rods spaced 1cm apart. The floor of the non-illuminated, dark compartment also consisted of 2mm steel rods spaced 1cm apart. The animals underwent two separate trials: a training trial and a test trial 24h later. During the training trial each rat was placed in the lighted compartment, as soon as it entered the dark compartment, an inescapable electric shock was provided (0.5mA, 1s). In the testing trial, one day after the training trial, the rat was again placed in the lighted compartment and the time until it re-entered the dark compartment was measured (the step-through latency maximum testing limit was 300s) (Choi et al., 2012; Eagle et al., 2016).

### Histology

After the behavioral tests, the animals were perfused transcardially with normal saline solution, followed by 110ml of phosphate-buffered saline (0.1M), pH=7.4 and 4% paraformaldehyde. Then the brains were placed in the post-fix solution overnight. The samples were processed and embedded in the paraffin. Sagittal sections (5μ thickness) were prepared by a microtome rotary (LEICA RM 2235) and were stained with Nissl staining (cresyl violet acetate: 0.1%) (Kopruszinski et al., 2012). The pyramidal neurons in the CA1, CA3 and DG regions of the hippocampus were counted in 3 sections (which found between -2.8 to -3.1mm from bregma) for each animal.

### Statistical analysis

All data was expressed as a mean±SEM. Analyses were performed at GraphPad Prism 5.0. After the data were tested for normal distribution (D'Agostino-Pearson normality test), one-way ANOVA or two-way ANOVA and Tukey post-test were used to compare statistically significant differences. Significantly confidence were acknowledged when $P<0.05$.

# Results

### Novel object recognition analysis

Tow-way ANOVA and Tukey post-test statistical analysis showed no significant difference between experimental groups in time spent for exploring the identical objects (left and right) in the training session (Fig. 1A). In the test session, no significant difference were detected between groups in time spent for exploring the familiar object, while TMT treated rats significant explored the novel object lesser than control group ($P<0.05$). In addition, treatment with cobalamin after TMT injection significantly reduced the effect of TMT and TMT+cobalamin group explored the novel object significantly more than TMT group ($P<0.05$, Fig. 1B). Cobalamin administration without TMT had not significant effect on novel or familiar objects exploring times in compare with control group.

Exploratory preference scores for the novel object task significantly decreased in TMT group in compared with control group ($P<0.001$), while it showed significant increase in TMT+cobalamin group when compared with TMT group ($P<0.001$, Fig. 1C).

### Avoidance memory analysis

One-way ANOVA and tukey's post-test showed TMT treatment in TMT group significantly affects avoidance memory and decreased latency to enter the dark box in compared with control group ($P<0.001$). While cobalamin administration after the TMT injection in TMT+cobalamin group abolished the TMT-induced avoidance memory impairment and significantly increased latency to enter the dark box in compared with TMT group ($P<0.001$, Fig. 2).

### Histological analysis

Nissl stained sample section of hippocampal areas are demonstrated in Figure 3A. sample section of these areas are demonstrated in Figure 3B-E for experimental groups. One-way ANOVA and tukey's post-test revealed the number of neurons in the dentate gyrus, CA1 and CA3 of hippocampus in the TMT group was significantly fewer in compare with corresponding areas of control group ($P<0.05$ for CA1; $P<0.001$ for CA3 and dentate gyrus). In addition, cobalamin treatment in TMT+cobalamin
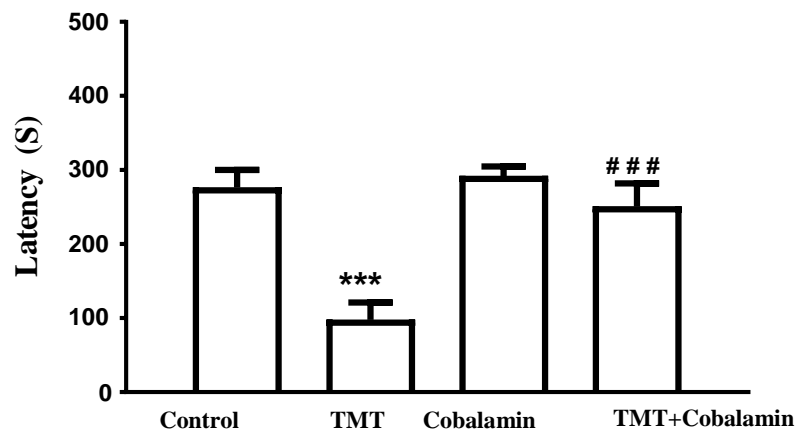
**Fig.2.** Cobalamin treatment following the trimethyltin chloride (TMT) compensates avoidance memory impairment due to TMT. TMT injection significantly increased latency in passive avoidance task when compared with control. Cobalamin administration in TMT+cobalamin group prevents this effect of TMT. Data are expressed as the mean±SEM; $^{***}P<0.001$ in compare with control; $^{###}P<0.001$ when compared with TMT group; n=5 for all groups.

group significantly improved counted number of neurons in all three area of hippocampus when compared with TMT group ($P<0.05$ for CA1; $P<0.001$ for CA3 and dentate gyrus, Fig. 4). Cobalamin administration without TMT had not significant effect on neural cells count in compare with control group.

# Discussion

In the present study our results confirmed neuroprotective effect of cobalamin against the cognitive impairment and neuronal loss resulted from the TMT exposure. It had been shown that neurodegenerative effect of TMT appear through the pyramidal cell loss in the hippocampus and limbic cortex which leading to various behavioral and histological deficits (Gasparova et al., 2014). In agreement with previous studies, our data showed that single injection of TMT induced neuronal cell loss and significantly decreased neural cells in the hippocampal areas. Also, disrupt recognition and memory performances in novel object and passive avoidance experiments. Pathogenesis of TMT induced neurotoxicity and neural cell death includes multiple processes such as neuroinflammation, oxidative stress, glutamate excitotoxicity, necrosis and apoptosis (Corvino et al., 2013; Jianhai et al., 2019). TMT induced up-regulation of tumor necrosis factor (TNF)-α, interleukin-1 and NF-kB which might be responsible for initiating the neuronal cells death (Figiel and Dzwonek, 2007; Corvino et al., 2013). TMT increased expression of inducible nitric oxide

synthase (iNOS) through the activating of NF-kB and nitric oxide generation increase gene expression of Bax and induces mitochondrial mediated apoptosis and cell death (Röhl and Sievers, 2005; Kim et al., 2019).

Our results indicating chronic cobalamin treatment prevent TMT induced neuronal cell death in the hippocampus areas. In behavior level, also decreased recognition and memory dysfunction induced by TMT. In agreement these, previous studies had been shown considerable effects of cobalamin on inflammation through the regulation of NF-kB. According to the important effect of NF-kB on gene expression of pro and anti-inflammatory factors, preventing effects of cobalamin on neuronal cell loss might be explained (Wheatley, 2006). Studies also had shown significant inverse linear relationship between cobalamin and TNF-α levels which is important during the neuroinflammation induced apoptosis (Hajihashemi et al., 2017).

A previous clinical study has been reported low concentrations of vitamin B12 is associated with poor memory performance in amnestic patients and MRI study showed that is mediated by microstructural damage of the hippocampus in DG subfield ( Köbe et al., 2016; Smith, 2016). As DG is the major target of the input to the hippocampus via the perforant path, which carries information reaching the entorhinal cortex from many cortical regions, the memory deficit could be explained. In addition, considering to these fact that the DG is one of the few regions of the adult brain where neurogenesis occurs and vitamin B12 is
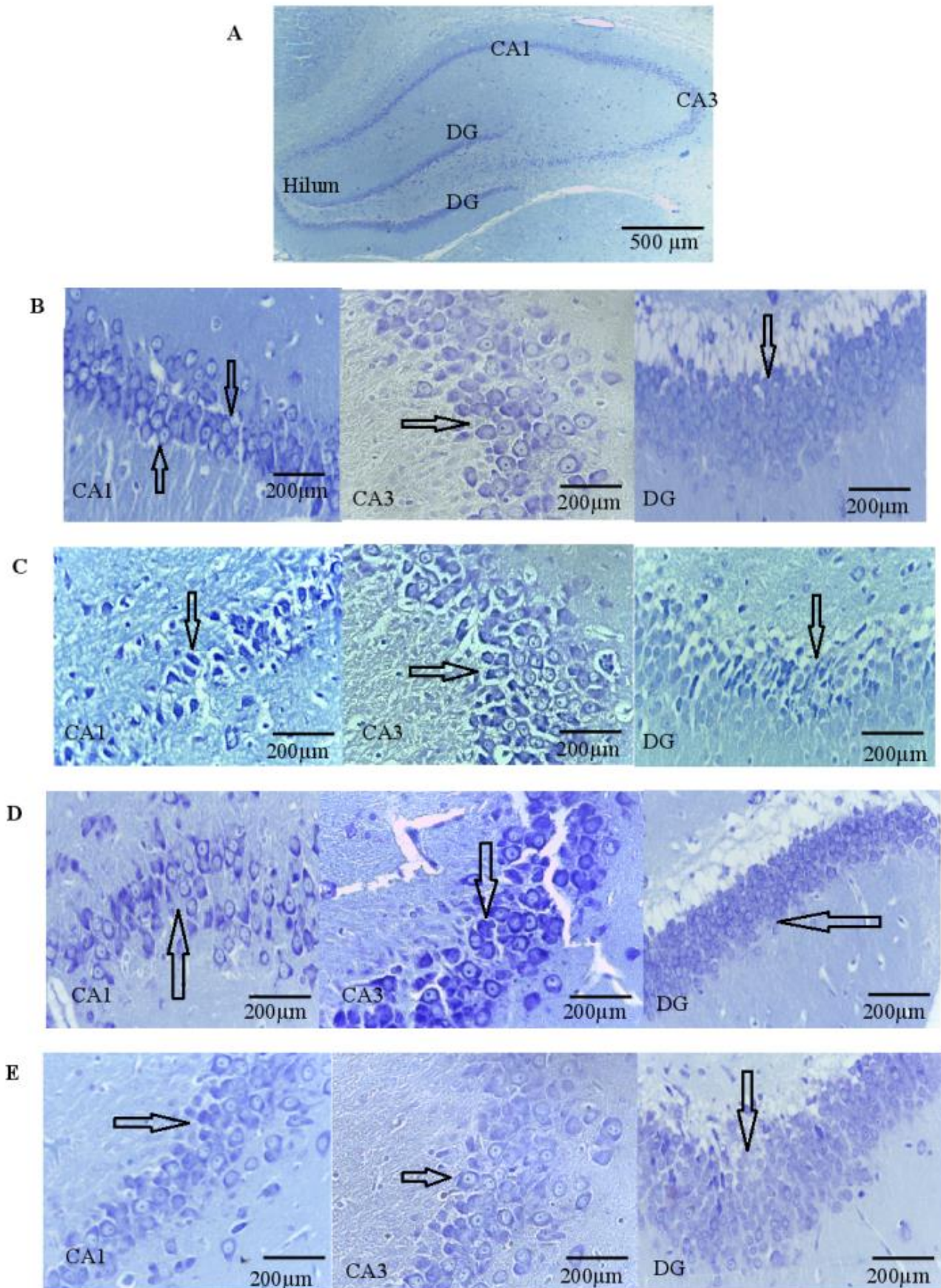
**Fig.3.** Sample section of the hippocampal areas with Nissl staining: 40X (A). Neural cells in the CA1, CA3 and dentate gyrus areas of hippocampus are demonstrated as sample of different experimental groups (400X); Control (B); trimethyltin chloride (TMT) (C), TMT+cobalamin (D); Cobalamin (E); arrows pointed neural cells and their alterations in experimental groups.
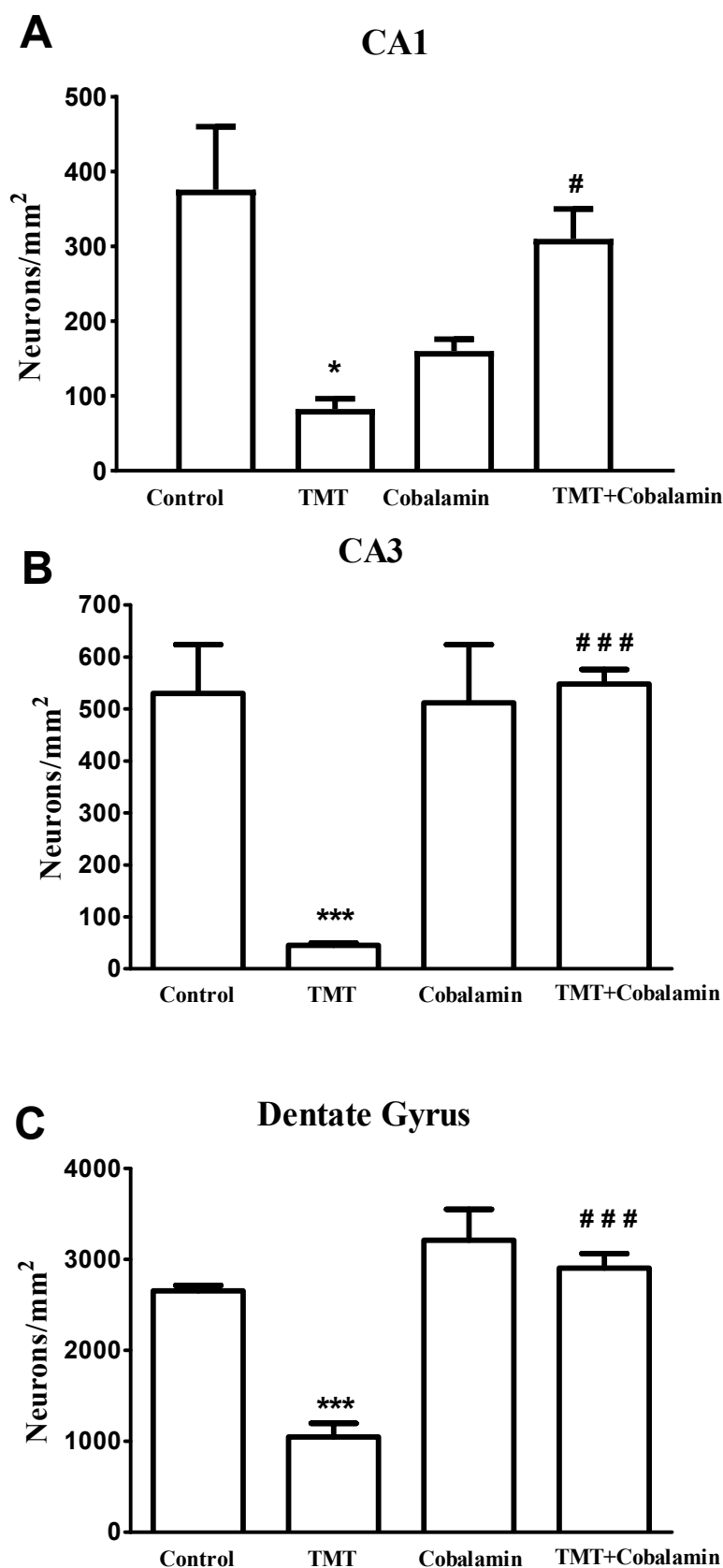
**Fig.4.** Cobalamin treatment following the trimethyltin chloride (TMT) reduced hippocampal neural cells loss due to TMT. Single injection of TMT significantly decreased the neuronal cell counted in all three areas of hippocampus in comparison with control. Cobalamin administrations after the TMT for five days prevent this effect of TMT and significantly caused an increase in the number of counted cells in all three areas of hippocampus when compared with TMT group. Data are expressed as the mean±SEM; $^*P<0.05$ and $^{***}P<0.001$ in compare with control; $^#P<0.05$ and $^{###}P<0.001$ when compared with TMT group; n=5 for all groups.

necessary for DNA replication, so cobalamin supply could prevent impairment of neurogenesis which were induced by TMT. Another clinical study had shown that vitamin B supply was reduced atrophy of specific brain regions that are a key component of the Alzheimer disease including the medial temporal lobe (Douaud et al., 2013; Guo et al., 2019).

In addition, cobalamin treatment was effective in preventing the impairment of memory by TMT for novel object recognition. In addition, cobalamin significantly increased exploring the novel object and improved learning performance. Cobalamin acts as a good free radicals scavenger and is suggested to work as neuroprotectant (Hajihashemi et al., 2017). Cobalamin passes through the blood brain barrier (Obeid et al., 2007), so it might protect against neurodegenerative conditions. Cobalamin is responsible for the synthesis or availability of the NOS substrates and cofactors. That it decreased NO production, other reactive oxygen species and reactive nitric oxide species (Wheatley, 2007; van de Lagemaat et al., 2019). Therefore, cobalamin might improve associative learning and memory processes as assessed by passive avoidance task.

# Conclusion

In this study, cobalamin treatment immediately after the TMT injection prevents TMT effects on hippocampus neuronal loss and memory impairment induced by TMT. These results suggest neuroprotective potential for cobalamin against the neurodegenerative situation, if applied early after that situation.

## Acknowledgments

## Conflict of interest

There are no conflicts of interests for the authors.

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