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Research Article

# Vitamin D mitigates ethanol preference in rats via the DRD2-GDNF and antioxidant signaling in vivo

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## Abstract

Alcohol abuse is a widespread mental disorder with significant health complications. A relationship between vitamin D (VitD) and ethanol abuse has been established. VitD, as a vital element for the development and metabolism of dopaminergic neurons, affects dopamine and glial cell line-derived neurotrophic factor (GDNF) synthesis and may play a role in the pathogenesis of alcohol-related disorders. In this study, we aimed to examine the role of VitD in the attenuation of ethanol-induced conditioned place preference in rats via dopamine D2 receptor-GDNF and antioxidant signaling in vivo. Rats were randomly divided into control, ethanol (Eth), and three Eth+VitD groups for behavioral experiments in conditioned place preference (CPP) and an open field. The activities of different brain oxidants (MDA, NO, and TOS), as well as antioxidative parameters (CAT, GPx, SOD, and TAC), plus oxidative stress index (OSI) and plasma VitD levels, were measured. Finally, hippocampal NOX, GDNF, and DRD2 expression were examined by western blotting. VitD (500 IU) combined with the effective dose of Eth (1.25 g/kg) resulted in the maximum decrease in CPP with no effect on locomotion. VitD supplementation elevated plasma VitD levels post-conditioning, increased the activities of brain antioxidants, and overexpressed hippocampal NOX, GDNF, and DRD2 proteins. We demonstrate that VitD attenuates alcohol-induced conditioning in rats via DRD2-GDNF and antioxidant signaling in vivo. Hence, targeting GDNF signaling may be a novel approach for the development of new drugs to treat Eth-related disorders among VitD users.

*Keywords*: Alcohol conditioning; CPP; Dopamine; GDNF; in-vivo; Vitamin D.

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## 1. Introduction

Alcohol abuse is among the main reasons for the worldwide problem of substance abuse [1]. The syndromes related to alcohol abuse are the most widespread psychiatric dilemma, with detrimental outcomes on the body, mind, and social interactions [2].

Chronic alcohol abuse results in an imbalance of oxidative stress and antioxidative defense [3, 4]. The oxidative injury is a consequence of the accumulation of reactive oxygen species (ROS) and lipid peroxides. ROS may be produced by multiple enzymes, mainly NADPH oxidase [4]. Superoxide-generating NADPH oxidase (NOX) facilitates the formation of potentially harmful free radicals [5].

Long-term high-dose ethanol (Eth) intake is associated with increased bone fractures [6, 7], impaired cognitive function [6], and it has been shown that alcohol abusers have lower vitamin D (VitD) levels compared with their healthy counterparts [7]. A review of relevant literature shows a positive association between syndromes related to alcohol abuse and serum VitD [8], as well as an association of low serum VitD levels with alcohol abuse syndromes [1].VitD deficiency has been linked to substance or ethanol abuse [9], and it has been shown that VitD has the potential to modulate all aspects of the signaling and metabolism of dopamine [10-14].On the other hand, a large number of studies have revealed the relationship between VitD and addictive states. For instance, there is an increased frequency of VitD deficiency among established opioid abusers, along with a reverse association of VitD levels with self-reported opioid abuse [15]. VitD, as a vital element for the development and metabolism of dopaminergic neurons, affects dopamine and glial cell line-derived neurotrophic factor (GDNF) synthesis [16, 17]. GDNF is extensively expressed in multiple brain regions, including the hippocampus [16]. GDNF positively regulates dopaminergic transmission in the reward circuit [18, 19]. The concept that GDNF functions as a reverse regulator of alcohol intake suggests that it is responsive to voluntary alcohol consumption. Therefore, its dysregulation can result in alcohol abuse syndromes [18]. GDNF plays a role in the development and neurogenesis of the dopaminergic system and regulates the mechanisms of drugs of abuse through negative feedback [20].

A large body of evidence reveals that VitD intake causes the overexpression of dopamine receptor D2 (DRD2) in animals [10, 13, 14]. However, the mechanism of VitD administration on the expression of DRD2 in Eth conditioning has not yet been clearly identified. Recently, GDNF has been suggested as a therapeutic strategy for alcohol-related disorders in non-human primates [21]. Considering that all experiments reported in this regard are in vitro, the present study attempted for the first time to evaluate the role of VitD in the attenuation of ethanol-induced conditioned place preference in rats via D2R-GDNF and antioxidant signaling in vivo.

## 2. Materials and Methods

### *2.1. Animals*

Male Wistar rats, weighing 180-220 g, were obtained from the Animal Department of Kashan University of Medical Sciences (KAUMS) and used in all investigations. The animals were housed in an animal facility with a standard 12-hour light-dark cycle and had free access to food and water at all times. The temperature in the lab was maintained between 22 and 25 °C. Every behavioral test was administered between 8:00 a.m. and 2:00 p.m. The university's Ethics Committee registered and approved the study, and all experimental procedures were conducted in accordance with KAUMS' ethical regulations (code: IR.KAUMS.AEC.1401.007).

### *2.2. Experimental design*

Thirty-five rats were randomly divided into five groups (n = 7 per group): control, Eth, and three Eth + VitD groups. The control group received saline injections for eight days during the conditioning phase. The Eth group received eight days of alternating gavage administrations of either saline (days 3, 5, 7, and 9) or Eth (days 2, 4, 6, and 8) during the conditioning phase. Based on previous studies [22, 23], with some modifications and using different applied doses of Eth (1.25, 2.5, and 5 g/kg), a dose-response study was conducted, from which Eth (1.25 g/kg) was selected as the effective dose. The Eth+VitD groups were administered VitD (250, 500, and 1000 IU/kg) before receiving saline or the effective dose of Eth (1.25 g/kg via gavage) during the conditioning phase for eight days. After completing the CPP tests, the spontaneous locomotion of the animals was recorded using the open-field test. Following all behavioral studies, blood samples were taken by cardiac puncture to assess oxidant/antioxidant parameters and plasma VitD levels. The animals were then decapitated, and their brains and hippocampi were removed and preserved at -70 °C for biochemical and molecular assessments.

### *2.3. Drugs and treatments*

Eth was supplied by Sigma-Aldrich (England). Our pilot study served as the basis for selecting the initial dose-response. To prepare a new Eth solution for intragastric (i.g.) gavage, it was dissolved in 0.9% sterile saline. Compared to our other applied doses (1.25, 2.5, and 5 g/kg), the 1.25 g/kg dose of Eth efficiently developed conditioning in the CPP, and hence, it was selected as the effective dose. Eth was used to dilute VitD (Daroupakhsh Distribution Co., Iran), and it was freshly prepared to the designated doses.

### *2.4. Behavioral testing*

#### *2.4.1. Conditioned place preference*

According to our previous study [24], the conditioned place preference (CPP) model was applied using a CPP apparatus (MazeRouter, Tabriz, Iran). The apparatus consists of three chambers (designated A, B, and C) divided by guillotine doors. Chambers A and B, two sizable conditioning chambers, are identical in size but have distinct patterns and colors. While the walls and floor of chamber B were decorated horizontally with black and white patterns, those of chamber A were decorated vertically. A guillotine door connected chambers A and B. Chamber C was smaller than chambers A and B and was situated in the center. Two days prior to the start of the experiments, all animals were allowed to acclimate to the apparatus. Following this, on day 3, in the preconditioning phase, the animals were placed in the middle chamber for 15 minutes while the guillotine doors were raised to allow access to the other chambers. The time spent in each chamber was recorded for this phase. The CPP protocol was followed for 10 consecutive days, consisting of three distinct phases: preconditioning, conditioning, and post-conditioning tests [25]. Every rat was administered alternating gavages of Eth (1.25 g/kg/gavage) or saline (1 ml/kg, gavage) for eight days during the conditioning period (days 4–11); the rats were treated with Eth on days 4, 6, 8, and 10 and with saline on days 5, 7, 9, and 11. Using an "unbiased" method, the rats were placed in the saline chamber right after receiving a saline injection and in the Eth chamber for 45 minutes following Eth administration. A CPP test was administered on day 12 of the post-conditioning trial. Similar to day 3, the animals were given unrestricted access to the chambers for 15 minutes during this phase, which involved placing them in the central chamber and removing the guillotine doors. For this phase, the amount of time spent in each chamber was also noted. The difference (in seconds) between the time spent in the Eth-receiving chamber on day 12 and day 3 was used to calculate the preference change (**Figure 1**) [24, 26].

#### *2.4.2. Open-field test*

As previously reported [25], all rats underwent testing in an open-field apparatus to assess their spontaneous locomotor activity after the termination of CPP tests on day 13. The test was conducted over two days: the first (habituation) and the second (test) day. Accordingly, each rat was placed in a 40 × 40 × 30 cm black metal open-field apparatus and allowed to explore for five minutes to measure locomotor activity. A digital optical animal activity tracing system (Maze Router, Tabriz, Iran) and a computer-based CCD camera were used to measure activity in the open field. The total distance traveled (in cm) was recorded as locomotor activity.

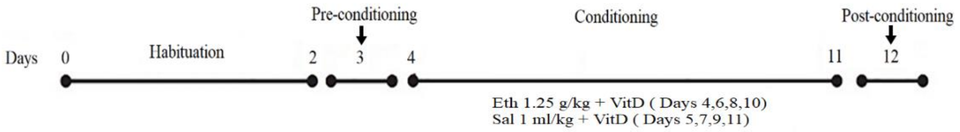

**Figure 1.** Experimental procedure.

### *2.5. Oxidative/antioxidative parameters*

The activities of brain oxidants (MDA, NO, and TOS), as well as antioxidative parameters (CAT, GPx, SOD, and TAC), were measured by ELISA according to the manufacturer's instructions (Kiazist Co., Iran). Moreover, the oxidative stress index (OSI) was calculated using the following formula: OSI (arbitrary units) = TOS / TAC [27].

### *2.6. Plasma VitD measurement*

ELISA measured the plasma VitD level according to the manufacturer's instructions (Kiazist Co., Iran).

### *2.7. Western blotting*

Hippocampal tissues from rats were lysed and homogenized in ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Triton X-100, containing 1% from 1X protease inhibitor cocktail (Melford). Samples were kept on ice for 30 min and then centrifuged at 13,000 rpm at 4 °C for 35 minutes. Supernatants were transferred to new microtubes, and the protein concentration was measured using the Bradford method [28]. For Western blot analysis, equal amounts of protein (50 µg/sample) were electrophoresed on a 12.5% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% BSA by incubation at room temperature for 1 hour, the membranes were incubated overnight with primary antibodies (1:1000) at 4 °C. The blots were washed with TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000) for 2 hours at room temperature. Protein bands were visualized using the Amersham ECL advanced kit and quantified using ImageJ software. Finally, the density of each band was normalized to the density of control β-actin.

### *2.8. Statistical analyses*

The findings are presented as mean ± SEM. After evaluating the normality of the data, the analyses of variance (ANOVA) and paired t-test, followed by Tukey's post hoc test, were used for statistical analysis. The Prism software (version 9) was used for analysis. The significance level was considered a p-value < 0.05.

## 3. Results and Discussion

### *3.1. The effect of different doses of Eth on the CPP change to determine the effective dose of Eth*

**Figure 2** reveals the effect of different doses of Eth (1.25, 2.5, and 5 g/kg) on CPP change (Sec). When compared to the control group, the one-way ANOVA showed a significant increase in CPP change only with the 1.25 g/kg dose of Eth (F3, 12 = 32.64, p < 0.0001). Thus, the effective dose of Eth in this study was determined to be 1.25 g/kg (p< 0.001).

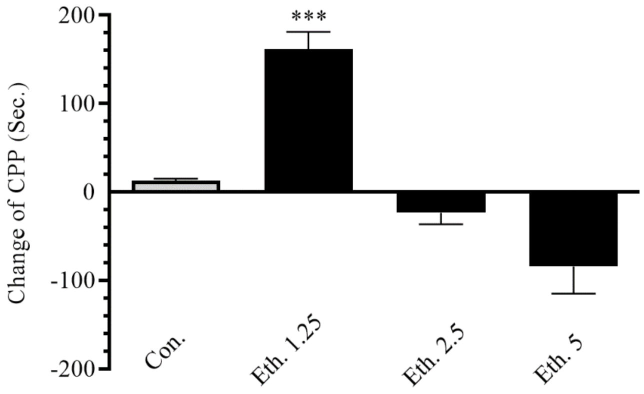

**Figure 2.** The effects of different doses of Eth on CPP for determining the effective dose of Eth. Eth, Ethanol; VitD, vitamin D; CPP, conditioned place preference. Data are expressed as mean±SEM ($n = 7$ in each group). $^{***}p < 0.001$ compared to the control group. Comparisons between different groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

### *3.2. The effect of the effective dose of Eth and Eth+ VitD on CPP change*

**Figure 3** reveals the effect of the effective dose of Eth (1.25 g/kg) per se and Eth + VitD (250, 500, and 1000 IU/i.p.) on CPP change (Sec). When compared to the control group, ANOVA revealed a significant difference between the groups (F (5, 29) = 12.48, p < 0.0001). Moreover, the post hoc analysis revealed a significant increase in CPP change in the Eth group compared to the control (p = 0.0001). While all concentrations of VitD in combination with the effective dose of Eth (1.25 g/kg) decreased CPP compared to the Eth group, only the 500 IU dose of VitD had the maximal decrease (p = 0.0001).

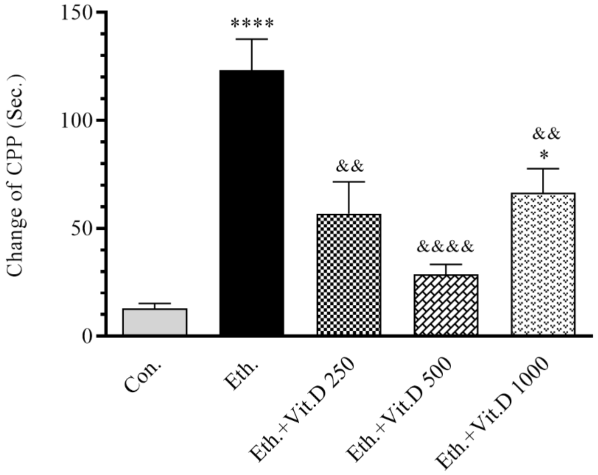

**Figure 3.** The effect of treatment with the effective dose of Eth (1.25 g/kg) per se and Eth. + VitD (250, 500 and 1000 IU/i.p.) on CPP change (Sec). Data are expressed as mean±SEM (*n* = 7 in each group). ). $^{*}p < 0.05$ and $^{****}p < 0.00001$ compared to control group. $^{\&\&}p < 0.01$ and $^{\&\&\&\&}p < 0.0001$ compared to Eth group. Comparisons between different groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

### 3.3. The effect of Eth. and Eth+ VitD on spontaneous locomotion in the open field

**Figure 4** reveals the effect of the effective dose of Eth (1.25 g/kg) and Eth + VitD (250, 500, and 1000 IU/i.p.) on locomotion in the open field. When compared to the control group, the ANOVA showed no significant difference between the groups (F (5, 34) = 0.3996, p = 0.8456).

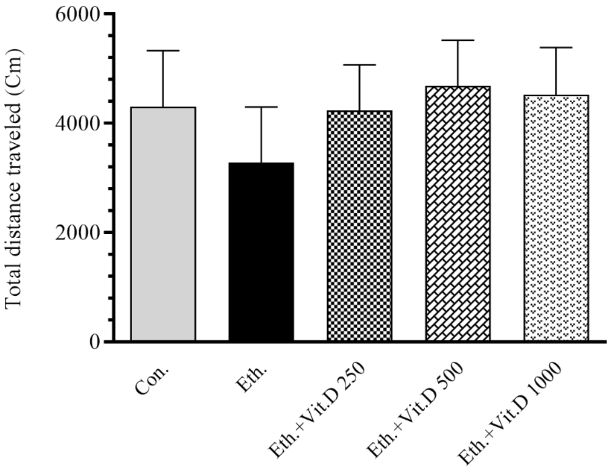

**Figure 4.** The effect of treatment with the effective dose of Eth (1.25 g/kg) per se and Eth. + VitD (250, 500, and 1000 IU/i.p.) on locomotion. Eth, Ethanol; VitD, vitamin D; CPP, conditioned place preference. Data are expressed as mean±SEM (*n* = 7 in each group). Comparisons between different groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

### 3.4. The effect of Eth and Eth+ VitD on plasma VitD level

**Figure 5** reveals the effect of different doses of Eth (1.25, 0.5, and 5 g/kg) and Eth + VitD (250, 500, and 1000 IU/i.p.) on plasma VitD level. The analysis of a paired t-test showed no significant difference between the pre- and post-conditioning in the control (P = 0.4083) and Eth (P = 0.23) groups. However, the paired t-test analysis also revealed a significant and dramatic increase in plasma VitD level in all Eth + VitD combination groups for pre- and post-conditioning (P = 0.0001).

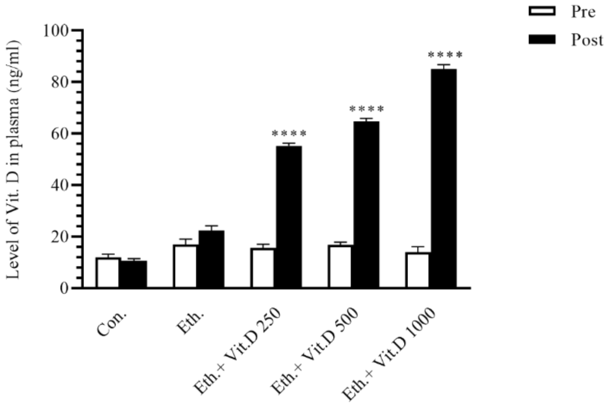

**Figure 5.** The plasma VitD level (ng/ml) of the pretreatment administration of different doses of VitD (250, 500, and 1000 IU/kg, i.p.) plus Eth before (Pre) and after (Post) the conditioning phase in all groups. Eth, Ethanol; VitD, vitamin D; CPP, conditioned place preference. Data are expressed as mean±SEM (*n* = 7 in each group). $^{***}p < 0.0001$ compared to pre (no VitD treatment) group. Comparisons between different groups were made using paired t-test analysis.

### 3.5. Effect of Eth and Eth + VitD on oxidative/antioxidative parameters

**Figure 6** reveals the effect of Eth and Eth + VitD on oxidant/antioxidant parameters in the brain homogenates of rats. The administration of different doses of VitD combined with the effective dose of Eth caused a significant decrease in the activities of brain oxidants, including MDA [F (5, 23) = 40.56, p < 0.0001], NO [F (5, 24) = 8.804, p < 0.0001], and TOS [F (5, 23) = 6.612, p = 0.0006], compared to the Eth group. In all combination groups, the highest decrease in oxidant parameters (MDA [p < 0.0001], NO [p < 0.01], and TOS [p < 0.01]) was observed in the Eth + VitD (500 IU/i.p.) group. Nonetheless, the administration of Eth (1.25 g/kg) significantly increased oxidative parameters by enhancing the activities of MDA (p < 0.0001), NO (p < 0.001), and TOS (p < 0.05) in the Eth group, compared to the control group.

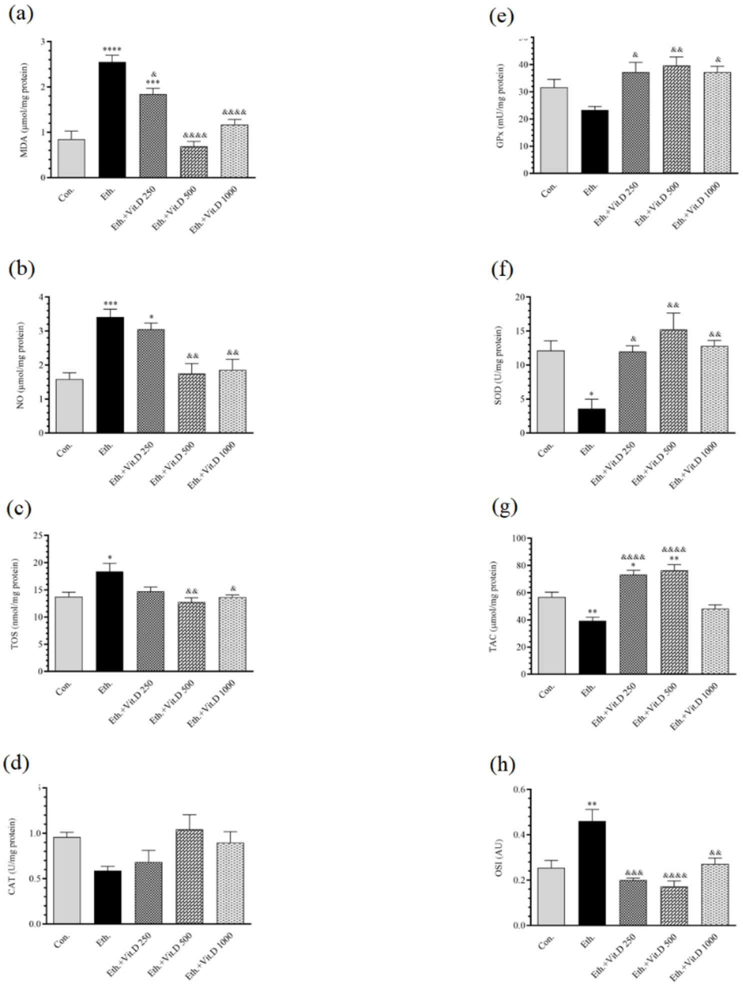

**Figure 6**. The activity of oxidative parameters: MDA (nmol/mg protein) (a), NO (nmol/mg protein) (b), and (c) TOS in brain tissue after VitD treatment in all groups. The activity of antioxidative parameters: CAT ($\mu$M/mg protein) (d), GPx (mU/mg protein) (e), SOD (U/M) (f), and TAC (µ mol/mg protein) in brain tissue after VitD treatment in all groups. The OSI values (AU) (h) of all groups after the treatment with different doses of VitD (250, 500, and 1000 IU/kg, i.p.) in all groups. VitD, vitamin D; MDA, malondialdehyde; NO, nitric oxide; TOS, total oxidative status. Eth, Ethanol; CAT, catalase; SOD, superoxide dismutase; TAC, total antioxidant capacity; OSI, oxidative stress index. $^{*}p < 0.05$, $^{***}p < 0.001$ and $^{****}p < 0.0001$ compared to control group. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ and $^{\&\&\&\&}p < 0.0001$ compared to Eth group. Comparisons between different groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

The administration of different doses of VitD combined with the effective dose of Eth caused a significant increase in the activities of brain antioxidants, including GPx [F (5, 24) = 9.915, p < 0.0001], SOD [F (5, 16) = 8.452, p = 0.0004], and TAC [F (5, 29) = 28.99, p < 0.0001], as compared to the Eth group. In all combination groups, the highest increase in antioxidant parameters (GPx [p < 0.01], SOD [p < 0.01], and TAC [p < 0.0001]) was observed in the Eth + VitD (500 IU/i.p.) groups. Nonetheless, the administration of Eth (1.25 g/kg) significantly attenuated antioxidative parameters by decreasing the activities of SOD (p < 0.05) and TAC (p < 0.01) in the Eth group, as compared to the control group. Moreover, while the lowest OSI value was determined in the Eth + VitD (500 IU/i.p.) group, the highest OSI value was found in the Eth group (p < 0.01).

### 3.6. *Effect of Eth and Eth + ViD on the hippocampal NOX expression*

**Figure 7** (upper panel) reveals the relative NOX/β-actin expression (%) in the hippocampus following the administration of Eth and VitD using western blotting.

The densitometric analysis showed dose-related notable variations in the hippocampal expression of NOX. The lower panel reveals the dose-dependent underexpression of NOX in the hippocampi of rats administered Eth + VitD compared to the Eth group (F(5, 12) = 12058 , p < 0.0001). Nonetheless, the administration of Eth revealed the overexpression of NOX in the Eth groups (p = 0.0001), compared to the control group.

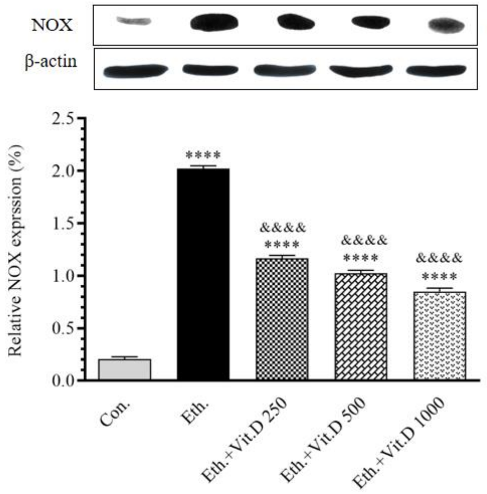

**Figure 7.** The effect of VitD (250, 500, and 1000 IU/kg, i.p.) on NOX expression (%). All animals received pretreatment saline (1ml/kg)/different doses of VitD (250, 500, and 1000 IU/kg, i.p.) before the effective dose of Eth (1.25 g/kg, i.p.). The evaluation of NOX expression level was performed in five groups: Control (group 1, no treatment); Eth (1.25 g/kg) (group 2); and Eth + VitD groups (groups 3, 4, and 5), followed by CPP testing and then protein expression using western blotting (upper panel shows the immunoblotting profile). (lower panel)The brain expression levels of NOX in CPP. The analysis indicated that NOX was significantly increased in the Eth group and decreased in the Eth + VitD groups compared to the control group. $^{****}p < 0.0001$ compared to the control group. $^{\&\&\&\&}p < 0.0001$ compared to the Eth group. Comparisons between different groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Eth, ethanol; VitD, vitamin D.

### *3.7. Effect of Eth and Eth + VitD on the hippocampal GDNF expression*

Figure 8 (upper panel) reveals the relative GDNF/β-actin expression (%) in the hippocampus following the administration of Eth and VitD using western blotting. The densitometric analysis showed dose-related notable variations in the hippocampal expression of GDNF. The lower panel shows the overexpression of GDNF in the hippocampus of rats administered Eth + VitD compared to the Eth group (F(5, 12) = 3370, p < 0.0001). Nonetheless, the administration of Eth revealed the under-expression of GDNF in the Eth group (p < 0.0001) as compared to the control group.

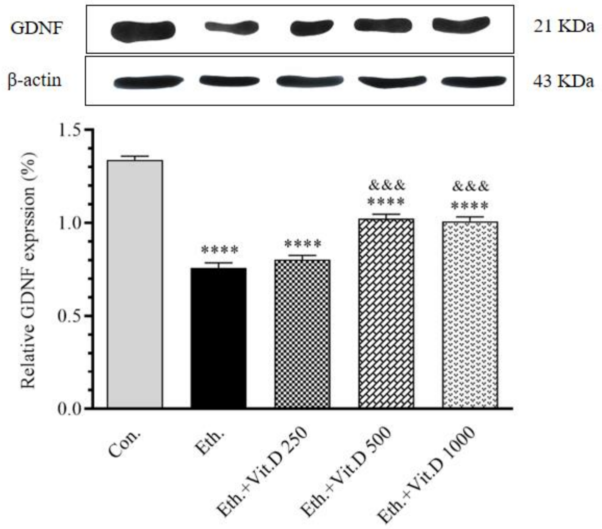

**Figure 8.** The effect of VitD (250, 500, and 1000 IU/kg, i.p.) on GDNF expression (%). All animals received pretreatment saline (1ml/kg)/different doses of VitD (250, 500, and 1000 IU/kg, i.p.) before the effective dose of Eth (1.25 g/kg, i.p.). The evaluation of GDNF expression level was measured in five groups: Control (group 1, no treatment); Eth (1.25 g/kg) (group 2); and Eth + VitD groups (groups 3, 4, and 5), followed by CPP testing and then protein expression using western blotting (upper panel shows the immunoblotting profile). (lower panel) The brain expression levels of GDNF in CPP. The analysis indicated that GDNF was significantly decreased ($p < 0.0001$) in the Eth group and increased in the Eth + VitD groups compared to the control group. $^{****}p < 0.0001$ compared to the control group. $^{\&\&\&\&}p < 0.0001$ compared to the Eth group. Each value represents the mean±SEM ($n = 7$ in each group). Comparisons between different groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Eth, ethanol; VitD, vitamin D.

### *3.8. Effect of Eth and Eth+ VitD on the hippocampal DRD2 expression*

Figure 9 (upper panel) reveals the relative DRD2/β-actin expression (%) in the hippocampus following the administration of Eth and VitD using western blotting. The densitometric analysis showed dose-related notable variations in the hippocampal expression of DRD2. The lower panel illustrates the overexpression of DRD2 in the hippocampus of rats administered Eth + VitD compared to the Eth group (F(5, 12) = 164000, p < 0.0001). Nonetheless, the administration of Eth revealed under-expression of DRD2 in the Eth group (p < 0.0001) compared to the Con group.

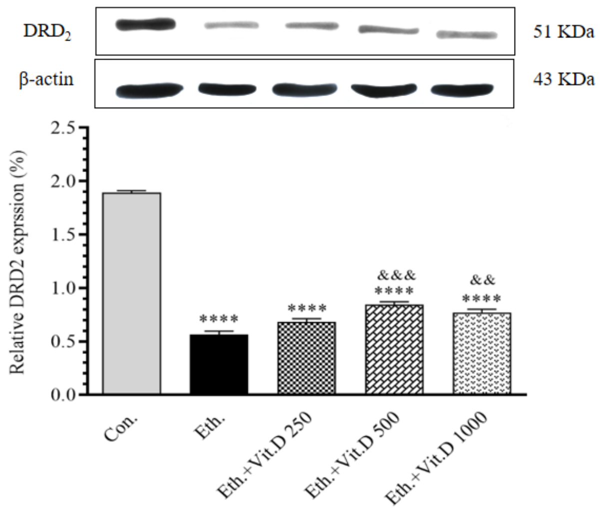

**Figure 9.** The effect of VitD (250, 500, and 1000 IU/kg, i.p.) on DRD2 expression (%). All animals received pretreatment saline (1ml/kg)/different doses of VitD (250, 500, and 1000 IU/kg, i.p.) before the effective dose of Eth (1.25 g/kg, i.p.). The evaluation of DRD2 expression level was performed in five groups: Control (group 1, no treatment); Eth (1.25 g/kg) (group 2); and Eth + VitD groups (groups 3, 4, and 5), followed by CPP testing and then protein expression using western blotting (upper panel shows the immunoblotting profile). (lower panel) The brain expression levels of DRD2 in CPP. The analysis indicated that DRD2 was significantly decreased ($p < 0.0001$) in the Eth group and increased in the Eth + VitD groups compared to the control group. $^{****}p < 0.0001$ compared to the control group. $^{\&\&\&\&}p < 0.0001$ compared to the Eth group. Comparisons between different groups were made using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Eth, ethanol; VitD, vitamin D.

The objective of our present study was to investigate the attenuation of Eth-induced conditioned place preference in rats by vitamin D via the DRD2-GDNF and antioxidant pathway in vivo. To achieve this objective, the results of our study, which examined the effect of different applied doses of Eth (1.25, 2.5, and 5 g/kg), revealed that only the dose of 1.25 g/kg developed a place preference for the ethanol-paired chamber. However, our other applied doses (2.5 and 5 g/kg) resulted in a place aversion to the Eth-paired chamber. In agreement with the approximate applied dose of our study (1.25 g/kg), some researchers have also reported the induction of Eth-conditioning [22, 23]. Therefore, it seems that the induction of conditioning by Eth is a dose-dependent process [29]. Specifically, Eth at doses below 1 g/kg fails to induce conditioning; doses higher than 1 g/kg induce conditioning, and doses above 2 g/kg induce aversion [30].

The combined effect of VitD with the effective dose of Eth on CPP change revealed that only the 500 IU dose of VitD had a maximal decrease in CPP change. This finding is consistent with the results of our previous study illustrating the effect of VitD on morphine preference in rats [25]. The possible mechanism may be explained by VitD's ability to inhibit dopaminergic neuronal loss [31]. Moreover, VitD also replenishes the dopamine transporter in rats [32]. Additionally, the hypothesis postulated by Eserian, suggesting the application of VitD as an effective therapeutic approach for drug abuse and dependence, supports these findings [33].

Furthermore, VitD supports neurotrophic factors, neurogenesis, and development via GDNF [34]. It also provides in vitro neuroprotection for dopaminergic neurons and enhances their responsiveness through the over-expression of GDNF [35].

The sole and combined effects of VitD on the open field revealed no change in locomotion. Consistent with our findings, Yunusoğlu et al. reported no change in locomotion at a dose of 2 g/kg Eth [36]. However, while Novier's study reported that low to moderate doses of Eth increase locomotor activity in adolescent and adult rats [37], Eaton's study reported decreased locomotor activity [30]. The 8-day conditioned quail with 2 g/kg Eth exhibited decreased locomotion [30]. Together, it seems that different age groups of rats show varying sensitivities to locomotion [38].

The effect of VitD on plasma VitD levels resulted in a significant and dramatic increase. While a relationship between serum VitD and its supplementation has been reported among alcoholics with memory disorders [39], Tardelli et al. in their systematic review reported that in some cases, there may be an association between ordinary alcohol use and serum VitD levels, or a positive association (or no association) between alcohol intake and VitD levels. They concluded that VitD signaling might play a role in the pathophysiology of addiction [8].

The effect of VitD on oxidative/antioxidative parameters, relative NOX expression (%), and OSI resulted in a significant dose-dependent under-expression of NOX, as well as a decrease in the activities of brain oxidants (MDA, NO, and TOS), and OSI. The administration of different doses of VitD combined with the effective dose of Eth caused a significant increase in the activities of brain antioxidants, including CAT, GPx, SOD, and TAC. In clear agreement, Nickovic, Miric et al. reported increased MDA and NO metabolites and decreased antioxidative parameters in alcoholics [3]. Moreover, it has been demonstrated that Eth elevates MDA, TOS, and OSI [40-43]. Additionally, regarding the OSI, the general trend observed for each of the oxidant parameters is similar to the OSI changes. However, the OSI ratio in combination groups (Eth + VitD) was independent of the VitD dose, and the lowest OSI was seen for VitD 500. A possible explanation for this apparent discrepancy may be that, as the OSI is the ratio of all oxidants to antioxidants, it may differ from the separate alterations of each parameter.

Alcohol suppresses ribosomal biogenesis and induces nucleolar stress in the nervous system, causing growth arrest and apoptosis [44]. Reportedly, VitD protects dopaminergic neurons against oxidative stress-induced neurotoxicity [45] by augmenting the glutathione system and preventing the formation of inducible NO-synthase [46], as well as by suppressing NADPH oxidase activity [47]. The NADPH oxidase enzyme is the main source of ROS in Eth metabolism [48]. Moreover, VitD signaling is involved in the modulation of mitochondrial function during stress [49].

Altogether, the results from the effects of VitD on CPP and oxidative stress in the brain are not linearly dose-responsive, and the 500 IU dose of VitD showed the optimum effect. The findings are consistent with the findings of our previous study [25]. It may be hypothesized that the lack of a classic linear dose-response, which can be seen in supra-physiological doses of VitD, can sometimes result in paradoxical effects due to receptor downregulation, alternative metabolic pathways, or cross-talk signaling pathways. Another postulation for the discrepancy seen for the 500 IU dose against the other doses of VitD may be described by the notion that the effective dose of VitD may optimally activate VitD receptor-mediated signaling pathways, such as the upregulation of antioxidant enzymes and neurotrophic factors, without inducing compensatory mechanisms that may blunt its efficacy at higher concentrations. While, our data demonstrates the 500 IU dose of VitD as the most effective dose among the other applied doses, it underscores the importance of identifying an optimal therapeutic window for VitD supplementation rather than assuming that "more is better."

The effect of VitD on hippocampal GDNF expression revealed overexpression of GDNF in the hippocampus of rats administered with VitD. In agreement, Sönmez, Görgülü et al. reported fluctuations in BDNF and GDNF serum levels in individuals with alcohol dependence [50]. Moreover, Sanchez et al. reported changes in accumbal expression of GDNF following alcohol-taking behaviors in mice [31]. GDNF is involved in alcohol abuse and dependence behaviors in animals, and its alteration is due to changes in components of the reward signaling [51]. Endogenous overexpression of GDNF following alcohol intake occurs in response to the activation of the reward circuit [52]. GDNF functions as an Ethyl-Responsive gene, whereby different levels of gene expression can be regulated according to varying ethyl doses [18]. Particularly, human studies have described increased and decreased serum GDNF levels in acute and chronic cases, respectively [53, 54]. Given this approach, GDNF gene therapy in male non-human primates using therapeutic modulation of involved circuits is suggested as a means for treating human Eth abusers [21].

To the best of the authors’ knowledge, while the exact pathophysiology of these events is unknown, it has been proposed that the neuroprotective effect of VitD via the GDNF modulatory effect on dopaminergic neurons may play a role [33]. Alternatively, it may be explained by a neuronal mechanism that indicates that VitD regulates the firing of glutamatergic collaterals on dopaminergic neurons and, therefore, can regulate dopamine activity through dopamine transporters, enzymes, and/or receptors [8]. In addition to the in vitro inducing effect of VitD on endogenous GDNF, it also induces the overexpression of tyrosine hydroxylase [55].

In the next step, the effect of VitD on hippocampal DRD2 expression revealed the overexpression of DRD2 in the hippocampus of rats administered with Eth. In this regard, a relationship between endogenous dopamine levels and alcohol abuse in animals has been reported, which signifies the presence of different dopamine tones for different doses of alcohol [56]. Moreover, regarding the relationship between dopamine and GDNF, it has been reported that, in addition to the regulatory function of GDNF on dopaminergic neurons, dopaminergic signals also regulate GDNF [18]. Apart from the fact that a large corpus of studies indicates a relationship between DRD2 and GDNF in diverse physiological processes [57-60], the role of DRD2 in the developmental and plastic properties of human cell line-derived midbrain dopaminergic neurons has been identified [61]. In addition, findings from other isolated tissues suggest a mutual regulatory function between GDNF and dopaminergic activity. The application of a DRD2 agonist to midbrain cell lines results in a temporary overexpression of the DNA-binding transcription factor zinc-finger protein 268, followed by an upregulation of GDNF expression [62]. Moreover, chronic GDNF overexpression decreased dopamine transporters with no tyrosine hydroxylase expression in the rat mesostriatal system [63]. The fact that VitD results in the overexpression of tyrosine hydroxylase indicates that VitD could modulate dopaminergic functions [33]. Remarkably, all the aforementioned studies on the role of DRD2 and its involvement in alcohol studies have been conducted in isolated tissues and cell lines (in vitro). To our knowledge, our study is the first to integrate findings from D2R-GDNF signaling in vivo and address this question.

### *3.9. Limitations*

One issue that may be regarded as a limitation of the present study is the measurement of plasma alcohol levels. Another issue is that our method for studying NO was indirect. Therefore, we suggest applying some direct and more precise methods for NO assay in future studies (e.g., electron paramagnetic resonance spectroscopy) [64].

## 4. Conclusions

Taken together, our study shows that VitD mitigates alcohol-induced conditioning in rats via the DRD2-GDNF and antioxidant signaling in vivo. Hence, targeting GDNF signaling may be a novel approach for the development of new drugs to treat Eth-related disorders among VitD users.

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## Conflict of interest

The authors state that they have no conflicts of interest to disclose.

## Data availability

The corresponding author will provide the data upon request.

## Authors Contributions

Sajjad Sadri: Methodology, Data Curation. Behrang Alani: Data Curation. Ali Arjmand: Data Curation. Mohammad Esmaeil Shahaboddin: investigation, Data Curation. Sahar Ahmadi Asouri: Data Curation. Gholamreza Ghavipanjeh: Conceptualization, Methodology, Supervision, Formal analysis and investigation. Abolfazl Ardjmand: Methodology, Supervision, Writing - review and editing.

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## Using artificial intelligence chatbots

There was no use of artificial intelligence in the making of this article.

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