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Vitamin D protects against hippocampal apoptosis related with seizures induced by kainic acid and pentylenetetrazol in rats

Sevim Şahin[a,*], Seren Gülşen Gürgen[b], Uğur Yazar[c], İmran İnce[d], Tülay Kamaşak[a], Elif Acar Arslan[a], Betül Diler Durgut[e], Beril Dilber[a], Ali Cansu[a]

[a] Department of Pediatric Neurology, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey
[b] Department of Histology and Embriyology, Faculty of Medicine, Celal Bayar University, Manisa, Turkey
[c] Department of Neurosurgery, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey
[d] Department of Medical Biochemistry, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey
[e] Department of Pediatric Neurology, Maternity and Children Diseases Education and Research Hospital, Giresun University, Giresun, Turkey

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ABSTRACT

Objectives: The hippocampus is susceptible to damage in patients with epilepsy and in animals with seizures caused by excitotoxic agents. The effect of vitamin D on hippocampal apoptosis related with seizures has not been reported. However, epileptic patients have an increased risk of hypovitaminosis D which is most likely due to the effects of antiepileptic drugs. Therefore, in this study, it was aimed to evaluate the effects of vitamin D on hippocampal apoptosis related with seizures by using pentylenetetrazol (PTZ) and kainic acid (KA) in rats.
Methods: Male Sprague Dawley rats, aged 5.5 weeks, were randomly divided into six groups: control, vitamin D, PTZ, KA, PTZ + vitamin D and KA + vitamin D groups. The groups that received vitamin D were given 500 IU/kg of vitamin D daily for two weeks in addition to a standard diet. At the end of this period, PTZ and KA were applied to trigger seizures in the rats in the seizure groups. 24 h after the administration of PTZ and KA, the rats were decapitated. In the hippocampal region, apoptosis was assessed by TUNEL and brain-derived neurotrophic factor (BDNF), Bax, caspase-3 and c-fos activation were evaluated by immunohistochemical method.
Results: BDNF level increased while c-fos, Bax and caspase-3 levels decreased ($p < 0.0001$, in all) in the hippocampal neurons of the groups that were pre-treated with vitamin D before the administration of PTZ and KA, in comparison with the PTZ and KA groups. Vitamin D significantly decreased the number of apoptotic cells in these rats in comparison with the PTZ and KA groups ($p < 0.0001$).
Conclusion: This study indicates that vitamin D has neuroprotective effects on hippocampal apoptosis induced by PTZ and KA in rats. With this study it is suggested that keeping vitamin D levels within normal limits may be beneficial for patients with epilepsy, especially children.

1. Introduction

In the last decade, the broad physiological effects of vitamin D have become increasingly understood (Deluca et al., 2013). Vitamin D is a fat-soluble vitamin, however it acts like a hormone (Sintzel et al., 2017). Vitamin D shows its biological effects by binding to the intracellular vitamin D receptor (VDR) (Sintzel et al., 2017; Yang et al., 2012). VDR and its ligand link to the vitamin D responsive elements located in the promoter region of the hundreds of target genes (Chabas et al., 2013). This relationship regulates the expression of more than 500 genes that carry out various physiological functions (Sintzel et al., 2017). The wide distribution of VDR suggests that vitamin D is regulatory in various physiological pathways such as brain development, inflammation, neurological functions, cell cycle control, immunomodulation and apoptosis (Yuan et al., 2017; Eyles et al., 2013). To our knowledge, the effect of vitamin D on hippocampal apoptosis related with seizures has not been studied. However, in an animal model of temporal lobe epilepsy, it was reported that epilepsy development was related with dysregulation in the vitamin D3 metabolism. The plasma vitamin D3 levels showed a depletion of in acute and latent phase of epileptogenesis (Heischmann et al., 2016). Moreover, patients with epilepsy, especially children, have an increased risk for vitamin D deficiency due to antiepileptic drug (AED) side-effects (Lee et al., 2015). Many AEDs cause an increase in vitamin D metabolism by the induction of hepatic CYP450, resulting in a decrease in the 25-hydroxy vitamin D levels (Lee et al., 2015; Nettekoven et al., 2008).

* Corresponding author at: Division of Pediatric Neurology, Department of Pediatrics, Faculty of Medicine, Karadeniz Technical University, 61080 Trabzon, Turkey.
E-mail address: sevimsahin1@yahoo.com (S. Şahin).

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The hippocampus has an important role in memory and learning functions (Coras et al., 2014). Postmortem studies indicated that acute neuronal loss was significant in the hippocampus of patients who died of convulsive status epilepticus (Walker, 2015; DeGiorgio et al., 1992). Information on the mechanisms related with hippocampal damage due to prolonged seizures was mostly obtained from studies conducted on animals (Walker, 2015). Kainic acid (KA) and pentylenetetrazol (PTZ), excitotoxic agents that lead to seizures, were used to evaluate the effects on animals (Korhonen et al., 2003; Naseer et al., 2013). KA is a glutamate analog, and its systemic administration leads to seizures, and the death of neurons in specific brain regions such as hippocampus, pyriform cortex, thalamus and amygdala (Lin et al., 2015; Ben-Ari and Cossart, 2000). PTZ interacts with the picrotoxin-barbiturate binding site which inhibits GABA-A receptors and blocks chlorine channels (Zhu et al., 2012; Qu et al., 2005). PTZ-induced seizures have been reported to increase the apoptotic activity of caspase-3 in the hippocampus of developing rats (Naseer et al., 2013). Apoptosis is a form of cell death and constitutes a part of the common mechanism for cell replacement, tissue remodeling and removal of damaged cells Thompson, 1995). Apoptotic cell death can be detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, which detects DNA fragmentation (Hwang et al., 2013). In this study, it was aimed to determine whether vitamin D plays a protective role in hippocampal apoptosis induced by KA and PTZ in rats. The TUNEL and immunohistochemical studies were performed for this purpose.

2. Methods

The present study was performed in accordance with the guidelines provided by the Experimental Animal Laboratory, and was approved by the Laboratory Animal Ethics and Use Committee (allowance number: 53488718-716, Karadeniz Technical University). This study conformed to the internationally accepted ethical standards (Guide for the Care and Use of Laboratory Animals, 8th edition, NIH Publication, 2011).

2.1. Animals

Male Sprague Dawley rats, aged 5.5 weeks, were included in the study. Their average weights were 120 g. All the rats were housed in smooth-bottomed plastic cages at 22 °C with a 12-h light / dark cycle and 60% humidity. Standard commercial laboratory food and water were available ad libitum.

2.2. Drugs

Daily vitamin D3 (Devit-3 oral drops, Deva, Turkey) was given orally at a dose of 500 U/ kg (Seif and Abdelwahed, 2014). Kainic acid (Cayman Chemical Company, USA) was administered intraperitoneally at a dose of 10 mg / kg after being dissolved in 1.8 ml / kg of isotonic sodium chlorure. Pentylenetetrazole (Santa Cruz Biotechnology, Inc. Canada) was dissolved in isotonic sodium chlorure, and was given at a dose of 40 mg / kg intraperitoneally in a volume which did not exceed 10 ml / kg. When necessary, the administration of PTZ was repeated at a dose of 10 mg / kg every 10 min for a maximum of two times (up to a total 60 mg / kg of PTZ) until stage-3 seizure occurred, according to the Racine scale.

2.3. Experimental design

A total of 48 rats were randomly divided into six groups: 1) control (n = 8), 2) vitamin D (n = 8), 3) PTZ (n = 8), 4) KA (n = 8), 5) PTZ + vitamin D (vD-PTZ) (n = 8) and 6) KA + vitamin D (vD-KA) (n = 8) groups. Half of the randomly selected rats were given vitamin D3 orally for two weeks. At the end of this period, after excluding the vitamin D and control groups, PTZ and KA were administered to the rats, when they weighed an average of 220 g. The rats in the control group were treated with the same dose of 0.9% saline. After the administration of PTZ and KA, seizure behavior was observed during the first hour, and the seizures were scored according to the Racine scale (Gleeson et al., 2010). According to the scale, the seizure patterns were assessed in five stages. Stage-1 involved the twitching of the face and whiskers, and staring and freezing. Stage-2 involved the swinging of the head, repetitive itching, spinning and shaking. Stage-3 involved the forelimb clonus, erect tail, and lordotic posture. Stage-4 involved the rearing up and the forelimb clonus. Stage-5 involved rearing accompanied with forelimb clonus, and falling due to generalized motor convulsion. The time of the seizure onset after the administration of excitotoxic agents, the stages of the seizures at onset and following period, and onset time of the seizures in each stage, and the total duration of the recurrent seizures were recorded. The effect of vitamin D on seizure threshold was evaluated by taking into account the latency period from PTZ and KA administration to the first seizure.

All rats were decapitated 24 h after the administration of KA and PTZ. Thereafter, brain tissues were dissected and immediately fixed with 10% formalin. The brain tissues were then embedded in paraffin and 5-μm sections were obtained with the microtome for immunohistochemical study.

2.4. TUNEL study

In situ apoptosis detection kit was used to determine DNA fragmentation and apoptotic cell death. Sections were held overnight at 37 °C and then incubated for one hour at 57 °C in order to facilitate the deparaffinization. The preparations were left active in xylol for 15 min twice in order to complete the deparaffinization. Thereafter, they were passed through 100%, 96% and 80% alcohol series, respectively, for 10 min each. The sections were incubated with 20 μg / ml proteinase K for 10 min after being washed twice with distilled water for five minutes in order to get rid of the alcohol. After being washed with Phosphate Buffer (PBS), the tissues were activated with 3% hydrogen peroxide (TA-015-HP, Lab Vision, USA) for 15 min, and endogenous peroxidase activity was blocked. The sections washed with PBS were incubated in equilibrated buffer for 10–15 minutes and incubated in TdT enzyme (77 μL Reaction Buffer + 33 μL TdT Enzyme) for 60 min at 37 °C in a humidified environment. They were then left at room temperature for 10 min in a preheated stop / wash buffer and incubated for 45 min in Anti-Digoxigenin. In each step, they were carefully washed with PBS. After the washing, DAB staining was performed to identify TUNEL positive cells. Methylene green was applied for five minutes for floor staining. The stained slides were dehydrated by passing through the increasing alcohol series and then kept in the xylol for 20 min to obtain transparency. Then, they were closed with entellan and lamellae, and were evaluated with a computer-equipped photo-light microscope. Approximately 100 cells were counted from five randomly selected areas in each slide. The brown colored apoptotic cells were identified as “TUNEL positive cells” and the percentage of the TUNEL positive cells were calculated.

2.5. Immunohistochemistry

The sections were deparaffinized and rehydrated by passage through ethanol series. To retrieve antigenic sites, the sections were left in 2% trypsin (Sigma-Adrich) in Tris buffer (150 mM NaCl and 50 mM Tris base dissolved in distilled water) for 15 min at 37 °C. These sections were incubated for 15 min in 3% H_2O_2 to inhibit endogenous peroxidase activity, thereafter they were left in a non-immune serum blocking solution (Invitrogen, CA, USA) for 10 min. They were then incubated in a humidified chamber for one hour with primer antibody counterparts: Brain-derived neurotrophic factor (BDNF) (Rabbit polyclonal, Biorbyt, USA), c-fos (Rabbit polyclonal, Genetex, CA, USA), Bax (Rabbit polyclonal, Bioss, USA), caspase-3 (Rabbit polyclonal, Bioss, USA). All antibodies were diluted 1: 100. Subsequently, biotinylated

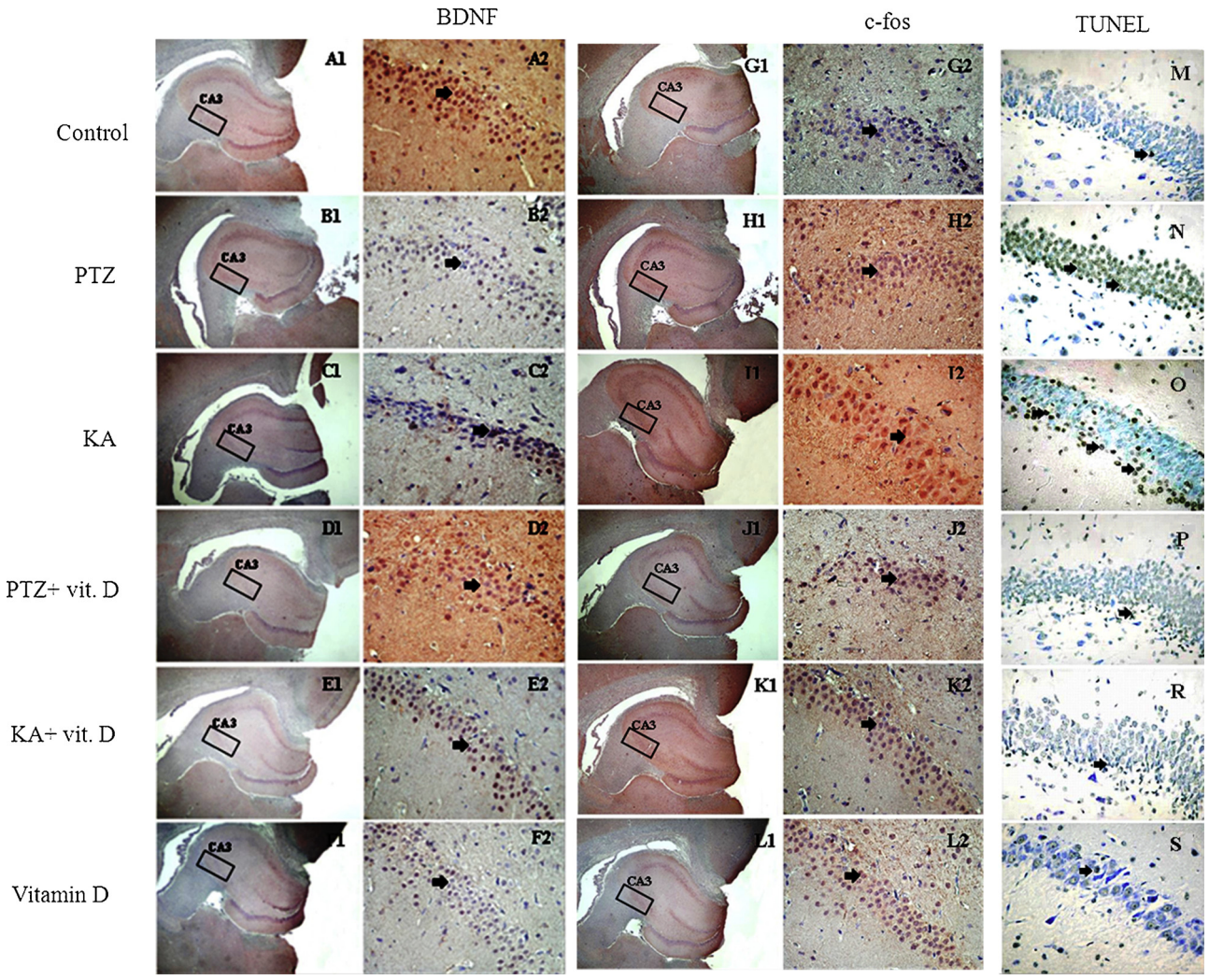

Fig. 1. BDNF (A–F), c-Fos (G–L) and TUNEL staining (M–S; X400 magnification) in the CA3 region of the hippocampus. BDNF expression was strong in the hippocampal neurons of the control group, whereas it was low in the PTZ and KA groups (A–C). In the groups pre-treated with vitamin D before the administration of PTZ, the BDNF reaction was strong similar to the control group. In the vitamin D and vD-KA groups, there was a moderate to strong BDNF staining (D–F). There was a weak c-fos staining in the vitamin D and control groups (G, L). In the PTZ and KA groups, c-fos reaction was strong, while it changed to weak or moderate when pre-treated with vitamin D (H–K). The numbers of TUNEL positive cells were significantly increased in the PTZ and KA groups (N, O), while they were only a few in the control and vitamin D groups (M, S). In the groups pre-treated with vitamin D, the numbers of TUNEL positive cells were decreased in comparison with the PTZ and KA groups (P, R). General hippocampus X40 (1), X400 (2); ➡: positive stained cells. BDNF: brain-derived neurotrophic factor, CA3: cornu ammonis area 3, KA: kainic acid, vD-KA: kainic acid + vitamin D group, PTZ: pentylenetetrazol.

secondary antibody and streptavidin conjugated horseradish peroxidase (85-9043, Invitrogen, CA, USA) were applied to the sections for 20 min. Lastly, 3-Amino-9-Ethylcarbazole (AEC, ASS-060, Spring, CA, USA) was applied for 3–5 minutes, the nuclei were stained with Mayer's hematoxylin for counterstaining and were closed with Mounting medium (DMM-060, Spring, CA, USA). All steps of the staining were performed with PBS, pH 7.4.

The slides were visualized, and images were obtained using a photo-light microscope (CX31 Olympus, Germany) attached to a digital camera (C-5060 Olympus, Germany). Immunohistochemical analysis was performed on brain cross-sections, using image-analyzing software (Leica Q Win V3 Plus Image). Two independent observers blind to the treatment regimen performed separate immunolabeling score evaluations. In each of the preparations, the intensities of involvement were evaluated by selecting five random areas at X400 magnification in each preparation. The intensities of involvement were scored semi-quantitatively as 0 (-, no involvement), 1 (+, weak immunoreactivity), 2 (+ +, moderate immunoreactivity) and 3 (+ + +, strong immunoreactivity). The staining was assessed with the histochemical score (HSCORE) as previously described (Chan et al., 2012), considering both the percentage and staining intensity of the positively labeled cells. For each field, HSCORE was calculated, using the equation HSCORE $= \Sigma Pi(i + 1)$, where i is the intensity of labeling (range: 0–3) and Pi is the percentage of labeled cells for each intensity (range: 0–100). The HSCORE ranges from 0 to 300. Total score was obtained by adding the scores of each field. Total scores were used for statistical comparison.

2.6. Statistical analysis

Data were presented as mean ± standard deviation (SD). Before determining the statistical significance of different data sets, the data distribution pattern and homogeneity of variance were evaluated by Kolmogorov-Smirnov and Levene tests, respectively. One way analysis of variance (ANOVA) was used in situations where the normal distribution was present in all groups (the BDNF, c-fos, Bax and TUNEL). For the post-Hoc analysis, the Tukey's multiple comparison test (the Bax and TUNEL) was used in the case of the homogeneity of variance, and

the Tamhane T2 multiple comparison test (BDNF and c-fos) was used in the other cases. The Kruskal Wallis method was used in the data set which did not show the normal distribution (the caspase-3), and Mann Whitney-U test was used in pairwise comparisons, considering statistically significant p-value as < 0.0033 (0.05 / the number of comparisons) with a Bonferroni correction.

Fisher's exact test was performed for the comparison of PTZ dosages in the PTZ and vD-PTZ groups and for the evaluation of the effects of vitamin D on seizure severity. Student T test as parametric test, and Mann Whitney-U test as nonparametric test were used according to data distribution in the comparison of apoptosis in PTZ subgroups formed by PTZ dosage; and were used in the evaluation of the effects of vitamin D on seizure latency and total seizures period after the administration of excitotoxic agents. The correlations of seizure latency and total seizure periods with apoptosis related biomarkers were evaluated with Spearman's correlation analysis.

In the comparison of apoptosis-related biomarkers in the groups, statistically significant p-value was accepted as < 0.01 (0.05 / 5), considering the Bonferroni correction procedure for multiple testing correction (Benjamini et al., 2001); and $p < 0.05$ was considered in other studies.

3. Results

3.1. General considerations

This study was initiated with eight rats in each of the six groups. However, two rats in the PTZ group died due to status epilepticus. For this reason, the study results included six rats in the PTZ group. Stage-3 seizure could not be obtained in two rats in the PTZ group, and in three rats in the vD-PTZ group. However, there was no significant difference between the PTZ and vD-PTZ groups in terms of seizure stages ($p > 0.05$, Fisher's exact test). Therefore, these rats were not excluded from the study.

3.2. Microscopic examination results

In the cornu ammonis area 3 (CA3) region of the brain hippocampus, the number of TUNEL positive cells were significantly high in the PTZ and KA groups. However, these numbers were low in the control and vitamin D groups. The number of TUNEL positive cells significantly decreased in the vD-PTZ and vD-KA groups in comparison with the PTZ and KA groups (Fig. 1).

In the control group, the CA3 region of the hippocampus showed strong expression of BDNF (Fig. 1) while c-fos (Fig. 1), Bax and caspase-3 (Fig. 2) were weak. In the vitamin D group, c-fos staining was weak and also Bax and caspase-3 expressions were markedly low, but BDNF expression was between moderate and strong (Figs. 1 and 2). In the PTZ and KA groups; c-fos, Bax, and caspase-3 reactions were strong, but BDNF expression was weak (Figs. 1 and 2). In the groups pre-treated with vitamin D before the administration of PTZ and KA, Bax and caspase-3 reactions were markedly low, and c-fos expression changed between weak and moderate (Figs. 1 and 2). The BDNF reaction in the vD-PTZ group was similar to that of the control group, whereas the staining changed between the moderate and strong in the vD-KA group (Fig. 1).

3.3. Statistical comparison of the apoptosis related biomarkers

As a result of the normal distribution of the data, ANOVA test was performed for the statistical comparison of Bax, BDNF, c-fos and TUNEL values between the groups. All of these values showed statistically significant differences. Therefore, the tests for post-hoc analysis were determined according to the homogeneity of variance in the Levene test. Thus, the Tamhane-T2 post-hoc multiple comparison test for BDNF and c-fos, and Tukey's post-hoc multiple comparison test for TUNEL and Bax were performed. Since the caspase-3 data did not show the normal distribution in the vD-PTZ group, Kruskal-Wallis variance analysis was used to compare the caspase-3 levels in the groups. For this data, subgroup comparisons were made by Mann-Whitney-U test, and the statistical significance was accepted as $p < 0.0033$, with a Bonferroni correction. The following results show the p-values obtained in the post-hoc analyzes.

In the PTZ (n = 6) and KA (n = 8) groups, the number of TUNEL positive cells was significantly higher than the control (n = 8) and vitamin D (n = 8) groups ($p < 0.0001$ in all pairwise comparisons; %, mean ± SD; 37,83 ± 3.31 in PTZ, 40.25 ± 3.01 in KA, 4.25 ± 1.9 in control, 4.37 ± 3.13 in vitamin D groups). The TUNEL positive cell counts of the groups pre-treated with vitamin D significantly decreased in comparison with the cell counts of the PTZ and KA groups ($p < 0.0001$ in all; %, mean ± SD; 10.12 ± 2.03 in vD-PTZ, 8.5 ± 2.45 in vD-KA groups). However, the number of apoptotic cells in the groups pre-treated with vitamin D were higher in comparison with the control group. That is, the number of TUNEL positive cells in the vD-PTZ group (n = 8) was higher than the control and vitamin D groups ($p < 0.0001$, $p = 0.001$, respectively; %, mean ± SD; 10.12 ± 2.03 in vD-PTZ, 4.25 ± 1.9 in control, 4.37 ± 3.13 in vitamin D groups). Additionally, the number of TUNEL positive cells in the vD-KA group (n = 8) was higher in comparison with the control and vitamin D groups ($p = 0.017$, $p = 0.022$, respectively; % mean ± SD, 8.5 ± 2.45 in vD-KA group, 4.25 ± 1.9 in control, 4.37 ± 3.13 in vitamin D groups) (Fig. 3).

In the PTZ and KA groups, in comparison with the control and vitamin D groups, there was a decrease in BDNF levels, and an increase in c-fos and Bax levels ($p < 0.0001$ in all), and also an increase in caspase-3 levels ($p < 0.0001$ in comparison of KA group with control and vitamin D groups, and $p = 0.001$ in comparison of PTZ group with control and vitamin D groups; score mean ± SD, **BDNF:** 99.3 ± 6.3 in PTZ, 100.7 ± 7.1 in KA, 304 ± 11.6 in vD-PTZ, 291.4 ± 3.2 in vD-KA, 305.7 ± 12.3 in control, 296 ± 12.7 in vitamin D groups; **c-fos:** 319.3 ± 5.4 in PTZ, 326.1 ± 6.2 in KA, 123.2 ± 5.1 in vD-PTZ, 116.6 ± 21.4 in vD-KA, 105.2 ± 6.3 in control, 100.1 ± 6.8 in vitamin D groups; **Bax:** 315.8 ± 3.8 in PTZ, 317.2 ± 5.8 in KA, 113.9 ± 5.7 in vD-PTZ, 108.6 ± 4.2 in vD-KA, 94 ± 7.5 in control, 98.6 ± 10.4 in vitamin D groups; **caspase-3:** 314.3 ± 6.6 in PTZ, 315.4 ± 5.2 in KA, 113.9 ± 5.7 in vD-PTZ, 108.6 ± 4.2 in vD-KA, 100.4 ± 7 in control, 97 ± 4.8 in vitamin D groups). Pre-treatment with vitamin D before the administration of PTZ and KA significantly increased the BDNF levels and decreased the c-fos and Bax levels ($p < 0.0001$ in all), and also decreased the caspase-3 levels ($p < 0.0001$ in comparison of KA group with the control and vitamin D groups, and $p = 0.001$ in comparison of PTZ group with control and vitamin D groups). Additionally, in the groups pre-treated with vitamin D, the BDNF and caspase-3 levels were similar to those of the control and vitamin D groups ($p > 0.05$). In the vD-KA group, Bax levels were higher in comparison with the control group ($p = 0.001$), whereas c-fos levels were similar ($p > 0.05$). In the vD-PTZ group, when compared with the control and vitamin D groups, both c-fos ($p < 0.0001$ in each comparison) and Bax levels ($p < 0.0001$ and $p = 0.001$, respectively) were higher (Fig. 4).

3.4. Was there any effect caused by the difference in the dosages of PTZ on apoptosis?

A single dose of PTZ was enough to achieve stage-3 seizures in three rats in the vD-PTZ group, although an additional dose of PTZ was required for all the rats in the PTZ group. However, the additional dose requirement of PTZ did not differ significantly between the two groups ($p > 0.05$, Fisher's exact test). In the vD-PTZ group, when the rats given PTZ at a dosage of 40 mg/kg or higher were compared, the BDNF, c-fos, Bax and caspase-3 levels were not different ($p > 0.05$ in all, Mann Whitney-U). However, considering the possibility of results

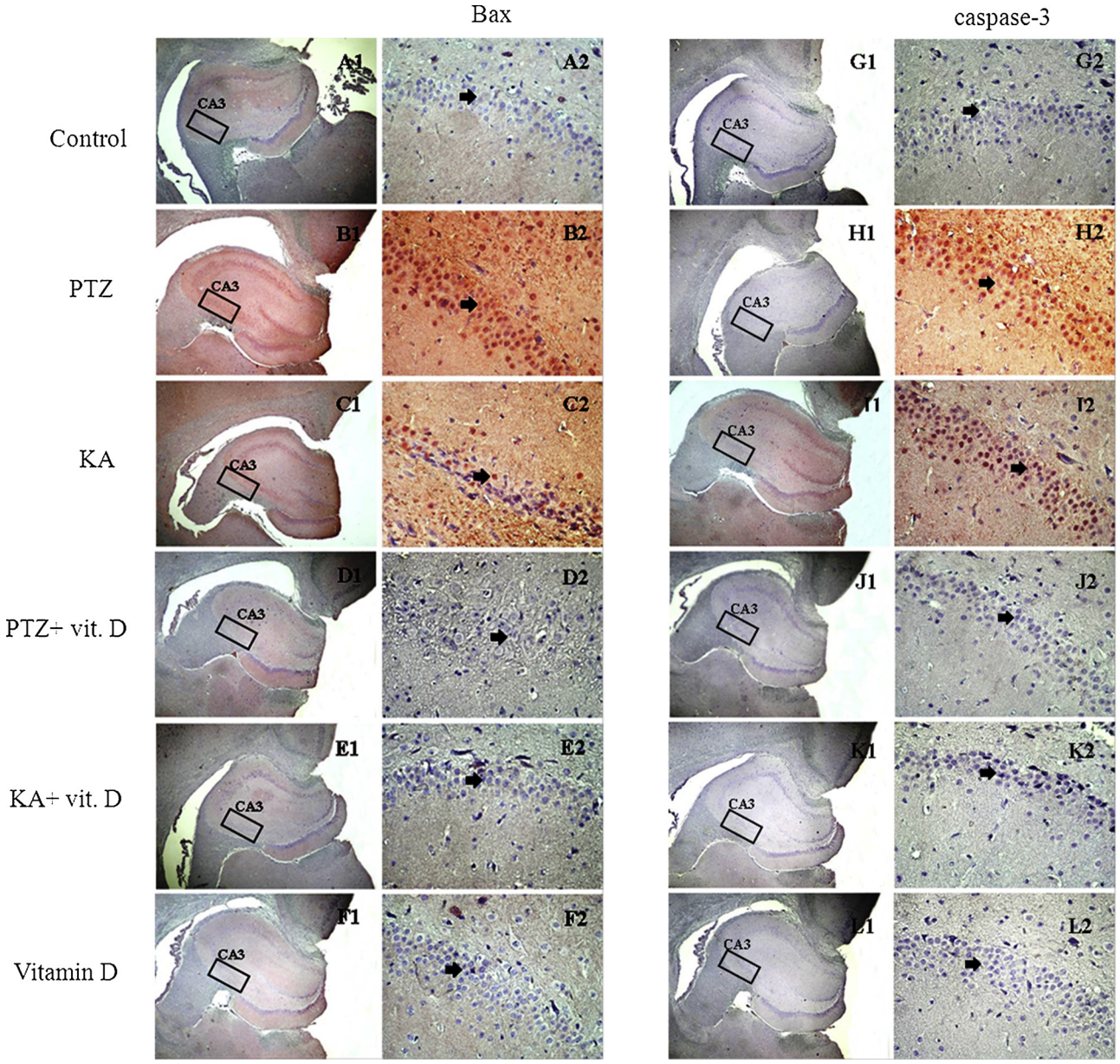

Fig. 2. The Bax (A–F) and Caspase-3 (G–L) immunohistochemical staining in the CA3 region of the hippocampus. The expressions of Bax and caspase-3 were increased in the PTZ and KA groups (B–C and H–I) while the staining was weak in the control and vitamin D groups (A, F and G, L). In the groups pre-treated with vitamin D, the Bax and caspase-3 reactionswere significantly decreased (D, E and J, K) in comparison with the PTZ and KA groups. General hippocampus X40 (1), X400 (2); ➡: positive stained cells. CA3: cornu ammonis area 3, KA: kainic acid, PTZ: pentylenetetrazol.

influencing due to PTZ dosage, animals with low dose of PTZ (40 mg/kg) were excluded and the statistical comparison was repeated. Similar to the previous results, in rats receiving vitamin D prior to PTZ, the BDNF levels were significantly higher and TUNEL, Bax and c-fos levels were significantly lower ($p < 0.0001$ in all, Student T). Caspase-3 levels were also significantly lower ($p = 0.004$, Mann Whitney-U).

3.5. The effect of vitamin D on seizures

In half of the rats in the KA group, stage-3 or greater seizures occurred, while seizures were below stage-3 in all the rats in the vD-KA group. That is, vitamin D significantly reduced the severity of seizures due to KA ($p = 0.038$, Fisher's exact test). The first seizures were stage-1 seizures seen in 3/8 of the rats in the PTZ group, while this ratio was 4/8 in the vD-PTZ group ($p > 0.05$, Fisher's exact test). The stage of the last seizure was stage-5 seen in 5/8 of the rats in the PTZ group, and this ratio was 2/8 in the vD-PTZ group. However, the difference was not statistically significant ($p > 0.05$, Fisher's exact test).

The effect of vitamin D on seizure threshold was evaluated by taking into account the latency period from the administration of PTZ and KA to the first seizure. Vitamin D effect on the seizure latency period was not statistically significant in both KA and PTZ induced seizures ($p > 0.05$, Student T; latency period, minutes, mean ± SD: 1.7 ± 0.8 in PTZ, 2.2 ± 1.2 in vD-PTZ, 2.2 ± 0.8 in KA, 3.1 ± 1.2 in vD-KA groups) (Fig. 5). In the rats receiving a total dose of 40 mg/kg PTZ in the vD-PTZ group, the seizure latency period was significantly shorter than the rats receiving the higher PTZ dosages ($p = 0.025$, Mann Whitney-U test, seizure latency period, minutes, mean ± SD: 0.9 ± 0.3 in 40 mg/ kg of PTZ, 3 ± 0.7 in > 40 mg/kg of PTZ). However, dosage of the PTZ did not cause significant differences in the TUNEL, Bax, BDNF, c-fos and caspase-3 levels ($p > 0.05$, Mann Whitney U test).

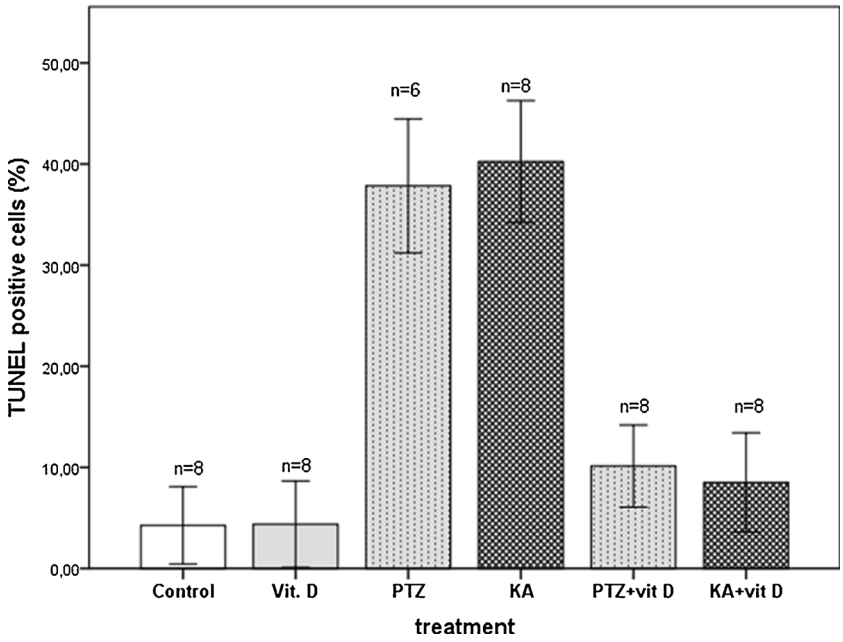

Fig. 3. Comparison of the percentages of TUNEL positive cells in the groups (n = 6 in PTZ group, n = 8 in other groups; $p < 0.0001$, ANOVA, Tukey's post-hoc multiple comparison test). Error bars indicate mean ± SD. KA: kainic acid, PTZ: pentylenetetrazol, SD: standard deviation.

In the rats treated with vitamin D prior to PTZ, stage-3 seizures, or stage-2 seizures in those who did not achieve stage-3 seizures were seen significantly earlier than in the PTZ group ($p = 0.034$, Student T; minutes, mean ± SD; 22.9 ± 15.7 in PTZ group, 7.6 ± 8.2 in vD-PTZ group). The total period of the seizure recurrences was significantly shorter in the vD-PTZ group ($p = 0.04$, Student T; minutes, mean ± SD; 22.8 ± 17.4 in PTZ, 6.7 ± 8.1 in vD-PTZ groups) (Fig. 5). However, vitamin D did not show a significant effect on the time of stage-2/-3 seizure in the rats treated with KA ($p > 0.05$, Student T; stage 2/3 seizure time, minutes, mean ± SD: 7.8 ± 2.8 in KA, 10 ± 9.2 in vD-KA groups). Additionally, total seizure period was not different between the two groups treated with KA ($p > 0.05$, Mann Whitney U; minutes, mean ± SD; 13.7 ± 18.2 in KA, 6.8 ± 9.3 in vD-KA groups) (Fig. 5).

3.6. Correlation study results

Considering all the rats treated with PTZ and KA (n = 32), there was a statistically significant positive correlation between duration of seizures and caspase-3 levels ($r = 0.507$, $p = 0.004$). The latency period for the first seizure did not show statistically significant correlation with biomarkers related with apoptosis; but although not statistically significant, it showed a negative correlation with c-fos levels ($r = -290$, $p > 0.05$) (Fig. 6).

In the rats that were not treated with vitamin D prior to the administration of excitotoxic agents (n = 16), the positive correlation between seizure period and caspase-3 levels ($r = 0.551$, $p = 0.041$), and the negative correlation between seizure latency period and c-fos levels ($r = -377$, $p > 0.05$) were stronger than in the other rats (Fig. 6).

In the rats treated with vitamin D prior to seizure induction (n = 16), the positive correlation between seizure duration and caspase-3 levels was not statistically significant ($r = 0.315$, $p > 0.05$). The seizure latency period did not show a correlation with c-fos levels ($r = -0.085$, $p > 0.05$) (Fig. 6).

4. Discussion

The present study suggests that vitamin D has a neuroprotective effect against hippocampal apoptosis induced by KA and PTZ in rats. The increase in TUNEL-positive and caspase-3-positive cell counts in the hippocampus in comparison with the control group indicates an increase in apoptotic neuronal cell death in both the PTZ and KA groups (Hwang et al., 2013). Vitamin D caused a significant reduction in these apoptotic effects ($p < 0.0001$). In some invitro studies, vitamin D has been reported to exhibit neuroprotective effects in the hippocampal (Brewer et al., 2001; Kajta et al., 2009), dopaminergic neurons (Orme et al., 2013) and neocortical neurons (Kajta et al., 2009). In this study, vitamin D provided a markedly increase in hippocampal BDNF levels and a markedly decrease in hippocampal c-fos, Bax and caspase-3 levels, thus preventing hippocampal apoptosis due to seizures induced by PTZ and KA in rats. It was reported that the genes related with synaptic transmission, cell communication and G protein function were up-regulated after the administration of a high dose of vitamin D in rats (Latimer et al., 2014). In the present study, vitamin D did not cause any changes in the levels of apoptosis related biomarkers in rats without seizure induction. This result suggests that vitamin D might have an inhibitory effect on a step that occurs as a response to the seizures and induces apoptosis.

The neuroprotective effects of BDNF have been demonstrated in previous studies, as in the present study (Hwang et al., 2013). BDNF is a neurotrophic factor produced by astrocytes (Zhu et al., 2012). In addition to promoting the proliferation and differentiation of neurons, it also affects the shape and number of dendritic spines affecting the morphological development of neurons (Cohen-Cory et al., 2010; Zhu et al., 2012). BDNF was found at higher concentrations in the hippocampus (Hwang et al., 2013). Increased BDNF expression in the hippocampus improves both short-term and long-term memory, as well as contributing to neuronal survival and differentiation (Hwang et al., 2013; Suzuki et al., 2011). Additionally, BDNF has been reported to play a role in the amelioration of KA-induced neurotoxicity (Gleeson et al., 2010).

In this study, vitamin D suppressed Bax activity in the hippocampus, as well as caspase-3 activity. The intrinsic pathway of apoptosis is characterized by permeabilization of mitochondrial outer membrane, death-inducing signaling complex formation, DNA fragmentation and caspase-3 activation (Wu et al., 2012; Yuan et al., 2017). Caspase-3 is the major caspase that actively causes neuronal cell death, among various subtypes of caspases (Tamatani et al., 1998; Yuan et al., 2017).

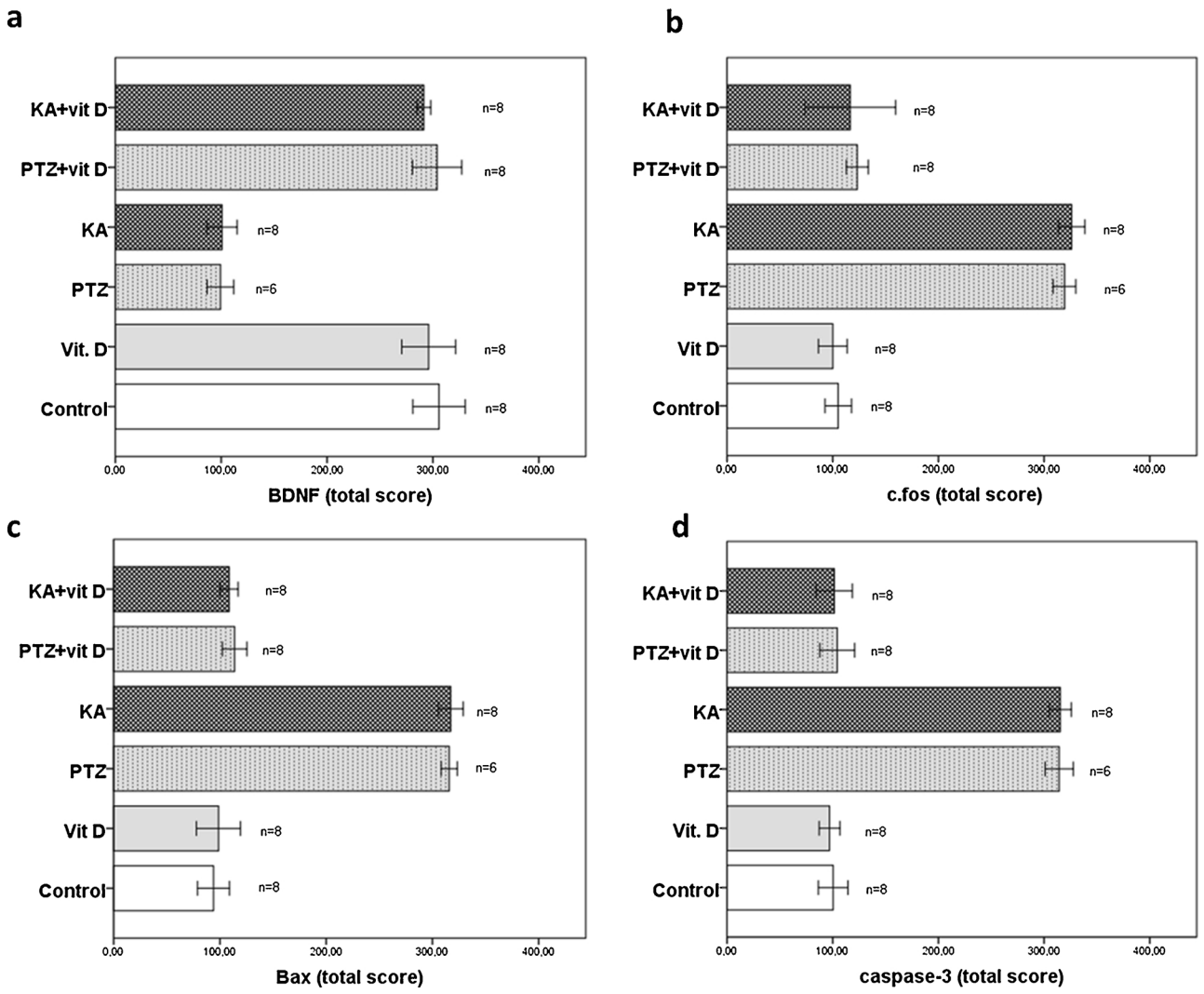

Fig. 4. Comparison of the BDNF **(a)**, c-fos **(b)**, Bax **(c)** and caspase-3 **(d)** activity between the groups (n = 6 in PTZ group, n = 8 in other groups). Significance and statistical tests are indicated as follows: **a)** $p < 0.0001$, ANOVA, post-hoc Tamhane T2 multiple comparison test; **b)** $p < 0.0001$, ANOVA, post-hoc Tamhane T2 multiple comparison test; **c)** $p < 0.0001$, ANOVA, post-hoc Tukey's multiple comparison test; **d)** $p < 0.0001$, Kruskal Wallis test. The total score was obtained by adding each HSCORE of five randomly selected areas in the hippocampus. HSCORE was defined by the following equation: HSCORE $= \Sigma Pi\ (i + 1)$ as previously described (i, intensity of labeling ranged from 0 to 3; Pi, percentage of the stained cells for each intensity, ranged from 0 to 100). Error bars indicate mean ± SD. BDNF: brain-derived neurotrophic factor, KA: kainic acid, PTZ: pentylenetetrazol, SD: standard deviation.

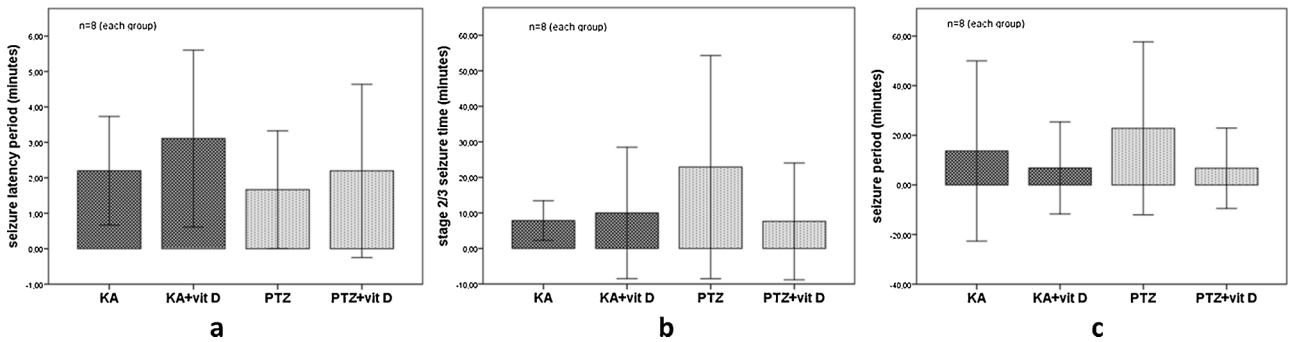

Fig. 5. Vitamin D effects on the seizure latency period **(a)**, onset time of the stage-3 or stage-2 seizures in those who did not achieve stage-3 seizures **(b)**, and total period of seizure recurrences **(c)** (n = 8 in all groups). **a)** Vitamin D did not show a statistically significant effect on the seizure latency period ($p > 0.05$, Student T test). **b)** The stage 2/3 seizures occurred earlier in the rats treated with vitamin D prior to the administration of PTZ, as compared to the PTZ group ($p = 0.034$, Student T test). This was not statistically significant in KA-induced seizures ($p > 0.05$, Student T). **c)** Vitamin D significantly shortened total seizure period in PTZ-induced seizures ($p = 0.04$, Student T). This was not observed in KA-induced seizures ($p > 0.05$, Mann Whitney U). Error bars indicate mean ± SD. KA: kainic acid, PTZ: pentylenetetrazol, SD: standard deviation.

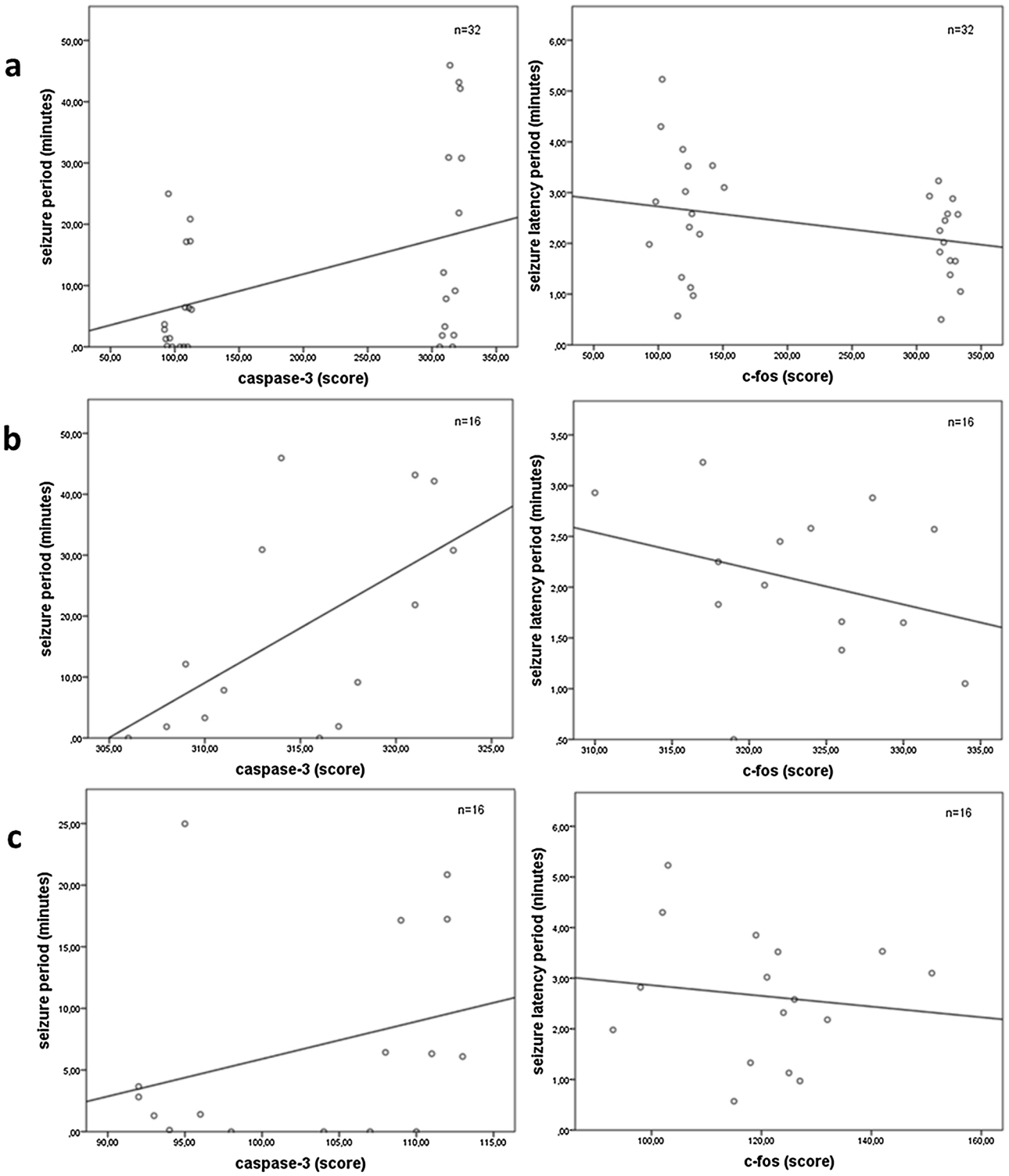

Fig. 6. Correlation of the apoptosis-related biomarkers with seizure latency period and total period of all seizures. **a)** Taking into account all the rats with seizure induction (n = 32), the duration of seizures was statistically significantly correlated with the caspase-3 levels (r = 0.507, p = 0.004). The seizure latency period was negatively correlated the most with the c-fos levels, but without a statistical significance (r= -290, p > 0.05). **b)** In the rats that were not treated with vitamin D prior to seizure induction, the correlation of the seizure period with caspase-3 levels (r = 0.551, p = 0.041) and the correlation of seizure latency period with c-fos levels (n= -377 p > 0.05), were stronger, as compared to the above results. **c)** In the rats treated with vitamin D before the seizure induction, the correlation between caspase-3 levels and seizure period was not statistically significant (r = 0.315, p > 0.05). The correlation between the seizure latency period and c-fos levels was not detected (r= -0.085, p > 0.05) (Spearman's correlation analysis).

Apart from caspases, Bcl-2 family proteins also play an important role in the regulation of apoptosis. This family includes pro-apoptotic proteins, including Bax, as well as anti-apoptotic proteins (Hwang et al., 2013; Sugawara et al., 2004). Bax reassembling into the mitochondrial membrane increases membrane permeability and causes cytochrome c to be released into the mitochondrial cytoplasm. Thus, cytochrome c causes apoptosis by activating caspase-9 which activates executioner caspase-3 and caspase-7 (Seif and Abdelwahed, 2014; Shuh et al.,

2013).

In this study it was found that vitamin D significantly suppressed c-fos activation induced by PTZ and KA, in the hippocampus, even such that c-fos expression was normalized completely in the KA group. It has been previously reported that c-fos was expressed at a high-level in the hippocampus after KA administration (Lin et al., 2015) c-Fos is an inducible transcription factor of the immediate-early gene family (Lin et al., 2015). Immediate-early genes play an important role in the regulation of neurological development and function, and are involved in the apoptosis regulation (Pérez-Cadahía et al., 2011; Yun et al., 2010). Increased c-fos reactivity after hypoxia-ischemia in the immature brain has been suggested to promote neuronal death (Nozaki and Beal, 1992). The results of this study suggest that c-fos suppression is involved in the anti-apoptotic mechanism of vitamin D.

Although the neuroprotective effects of vitamin D have been demonstrated in a variety of studies, little is known about its anti-apoptotic effects (Kajta et al., 2009). Moreover, the literature on this particular subject is inconsistent. Especially in neoplastic cell studies, pro-apoptotic effects of calcitriol have been demonstrated (Kajta et al., 2009). In neuronal tissues, vitamin D3 administered at low doses has been reported to exhibit an anti-apoptotic effect. In the rat hippocampal cell culture, it was demonstrated that dexamethasone-induced apoptosis decreased by calcitriol treatment (Kajta et al., 2009; Obradovic et al., 2006). In addition, systemically administered vitamin D3 protected the substantia nigra of the rat brain, against zinc-induced apoptosis (Kajta et al., 2009; Lin and White, 2004).

The protective effects of vitamin D against excitotoxicity may indicate that vitamin D may also be protective against seizures. However, in this study, vitamin D did not affect seizure threshold, but seizure severity induced by KA and the total period of seizure recurrences induced by PTZ were significantly decreased (Fig. 5). A few reports indicated anticonvulsant effects of vitamin D (Kalueff et al., 2005, 2006). In one of these reports, it was stated that the duration and frequency of seizures decreased in mice pre-treated with vitamin D prior to the administration of PTZ, as in the present study (Kalueff et al., 2005). In another report, it was indicated that, in VDR knockout mice, the onset of PTZ-induced seizures was earlier, the seizure severity and the mortality rate were higher (Kalueff et al., 2006). In addition, calcitriol has been reported to increase electroconvulsive seizure threshold and decrease seizure severity (Siegel et al.,1984).

In the present study, the shortened seizure period in rats treated with vitamin D prior to the administration of PTZ might have a contribution to the anti-apoptotic effects of vitamin D. Moreover, a strong correlation between seizure period and caspase-3 levels was found (Fig. 6). In epileptic rats, caspase-3 activation in hippocampal neurons has been reported to be closely associated with mitochondrial damage (Feng et al., 2018). Seizures induce mitochondrial oxidative damage, leading to an increase in permeability, which causes to the release of mitochondrial cytochrome c, thus resulting with mitochondrial dysfunction and apoptosis (Ali et al., 2018; Khurana et al., 2013). However, in this study, vitamin D treatment prior to the administration of PTZ and KA weakened the correlation between seizure period and caspase-3 levels (Fig. 6). This suggests that other mechanisms may also be involved in the vitamin D effects on apoptosis. At this point, the inhibitory effect of vitamin D on c-Jun N-terminal kinase (JNK) is noteworthy. Vitamin D has been suggested to inhibit caspase activation via inhibition of stress-induced p38 mitogen-activated protein kinase (MAPK) and JNK in keratinocytes, thus providing protection in keratinocytes (Diker-Cohen et al., 2006). JNK activation, with other putative apoptotic effectors, has been considered a part of the neuronal stress response (Herdegen et al., 1998). The JNK is considered a central mediator of excitotoxicity in neurodegeneration caused by KA-induced seizures (Zhao et al., 2012). A temporary increase in JNK activity after the administration of PTZ in rats was also reported (Herdegen et al., 1998).

It was thought that the neuroprotective effects of vitamin D were partially due to its antioxidative and immunomodulatory properties, and its trophic properties in neuronal maturation and differentiation (Buell and Dawson-Hughes, 2008; Ekici et al., 2009; Pilz et al., 2011). Rapid mechanisms are responsible in the effects of neurosteroids on neuronal survival (Kajta et al., 2009; Leśkiewicz et al., 2006). Vitamin D stimulates a variety of intracellular signaling pathways by providing rapid formation of secondary messengers, such as cAMP, and by activating various protein kinases (Kajta et al., 2009; Regulska et al., 2006). Calcitriol has been reported to induce activation of the extracellular signal-regulated kinase 1/2 pathways, thus suppressing neuronal apoptosis (Yuan et al., 2017).

Another remarkable point in the present study was that vitamin D showed a large inhibitory effect on hippocampal apoptosis, although vitamin D deficiency was not established in the rats or their mothers. This finding may also indicate the importance of vitamin D as a therapeutic agent. It has been thought that vitamin D and its analogs have promising therapeutic potential particularly in various skin diseases, cancer development, diabetes and multiple sclerosis (Sintov et al., 2014).

5. Conclusion

In conclusion, this study demonstrates that vitamin D has a protective effect against hippocampal apoptosis related with KA and PTZ induced seizures in rats. It is suggested that this finding indicates to the importance of keeping serum vitamin D levels within normal limits in patients with epilepsy. Monitoring vitamin D levels can be important especially for children with epilepsy because of the widespread presentation of vitamin D deficiency (Lee et al., 2015). In addition, AEDs increase the risk of vitamin D deficiency (Lee et al., 2015; Nettekoven et al., 2008). Some authors suggest that vitamin D supplementation is a simple, safe and inexpensive treatment option to reduce the disease burden in several health problems, considering its neuroprotective effects (Harms et al., 2011).

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Conflicts of interest

None of the authors has any conflict of interest to disclose.

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