

Neuroprotective effect of astaxanthin (ATX) against cognitive impairment on PTZ-induced epileptic seizures in rats and against PTZ-induced neurotoxicity in SH-SY5Y human neuroblastoma cell culture

Astaksantin'in (ATX) ratlarda oluşturulan PTZ ile indüklenmiş epileptik nöbetlerde oluşan bilişsel bozukluğa karşı ve insan SH-SY5Y nöroblastoma hücre kültüründe PTZ ile indüklenen nörotoksisiteye karşı nöroprotektif etkisi

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Received/Accepted: March 05, 2019 / March 26, 2019

Conflict of interest: There is not a conflict of interest.

SUMMARY

Objective: Epilepsy is a common brain disorder that seizures could cause neuronal loss in the hippocampus. Oxidative stress has an important role in the pathology of this way. The aim of this study was to investigate the neuroprotective effect of astaxanthin (ATX), on pentylenetetrazole (PTZ) induced epileptic seizures in rats and in SH-SY5Y human neuroblastoma cell culture.

Method: In our study, we used 42 male 230-250 g Wistar Albino rats. Animals were divided into seven groups as control, saline (PTZ; 1 ml/kg serum physiologic), positive control (2,5 mg/kg diazepam), 10 mg/kg, 20 mg/kg, 40 mg/kg and 80 mg/kg ATX for seven days. Thirty min after the administration of the last drug at the indicated doses, PTZ was administered 45 mg/kg to induce an epileptic seizure. The animals were observed for 30 min. Seizure stages according to the Racine Scale (RC) and first myoclonic jerk times (FMJ). Twenty four hours after PTZ injection, passive avoidance test was performed, and then brain tissues were removed for biochemical and histopathological evaluation. The hippocampal Cornu Ammonis 1 (CA1), CA3 and dentate gyrus (DG) regions were evaluated histopathologically regarding neuronal damage. Besides, oxidative stress markers total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) were measured in brain tissues. Furthermore, ATX was performed in vitro SH-SY5Y human neuroblastoma cell culture to evaluate PTZ-induced neurotoxicity.

Results: When epileptic behaviors were evaluated, ATX did not affect RC and FMJ ($p > 0, 05$). However, ATX reduced both cognitive impairment in passive avoidance test and neuronal damage in the hippocampus ($p < 0, 05$). Moreover, ATX reduced both TOS levels and OSI in the brain ($p < 0, 05$). Besides of these in vitro studies, ATX increased neuronal viability in vitro.

Conclusions: Although ATX does not have antiepileptic properties directly, it has a protective effect on not only in vivo but also in vitro. These effects may occur by possible oxidative pathways.

Keywords: Astaxanthin, cognitive impairment, epileptic seizures, SH-SY5Y cell culture, neuroprotective effect.

ÖZET

Amaç: Epilepsi, nöbet sonrası hipokampusta nöronal kayba neden olduğu bilinen yaygın bir beyin hastalığıdır. Oksidatif stres bu patolojide önemli bir yere sahiptir. Bu çalışmanın amacı, astaksantin (ATX), ratlarda pentilenetrazol (PTZ) ile indüklenen epileptik nöbetlerde nöroprotektif etkisinin araştırılması ve ATX' in SH-SY5Y insan nöroblastoma hücre kültüründe PTZ ile indüklenen nörotoksisite üzerine etkisini araştırmaktır.

Yöntem: Çalışmamızda 42 adet 230-250 gr Wistar Albino cinsi erkek rat kullandık. Hayvanlar kontrol, salin (PTZ; 1 ml / kg serum fizyolojik), pozitif kontrol (2,5 mg / kg diazepam), 7 gün boyunca 10 mg / kg, 20 mg / kg, 40 mg / kg ve 80 mg ATX verilen olarak yedi gruba ayrıldı. Son ilacın belirtilen dozlarda uygulanmasından otuz dakika sonra, epileptik nöbeti indüklemek için PTZ 45 mg / kg uygulandı. Hayvanlar 30 dakika gözlemlendi. Racine Scale (RC) ve ilk miyoklonik jerk zamanlarına (FMJ) göre nöbet aşamaları kayıt edildi. Ratlara PTZ enjeksiyonundan yirmi dört saat sonra pasif kaçınma testi yapıldı ve daha sonra biyokimyasal ve histopatolojik değerlendirme için beyin dokuları çıkarıldı. Hipokampal Cornu Ammonis 1 (CA1), CA3 ve dentat girus (DG) bölgeleri, nöronal hasar açısından histopatolojik olarak değerlendirildi. Ayrıca, beyin dokularında oksidatif stres belirteçleri total antioksidan status (TAS), total oksidan status (TOS) ve oksidatif stres indeksi (OSI) ölçüldü. Ayrıca ATX' in PTZ'nin neden olduğu nörotoksisiteye olan etkisini değerlendirmek için in vitro SH-SY5Y insan nöroblastoma hücre kültürü gerçekleştirildi.

Bulgular: Epileptik davranışlar değerlendirildiğinde, ATX, RC ve FMJ'yi etkilememiştir ($p > 0, 05$). Bununla birlikte, ATX hem pasif kaçınma testinde bilişsel bozulmayı hem de hipokampüsteki nöronal hasarı azaltmıştır ($p < 0, 05$). Ayrıca, ATX beyindeki hem TOS düzeylerini hem de OSI'yi düşürmüştür ($p < 0, 05$). İn vitro SH-SY5Y insan nöroblastoma hücre kültüründe ATX' in in vitro nöronal canlılığı artırdığı tesbit edildi.

Sonuç: Her ne kadar ATX doğrudan antiepileptik özelliklere sahip olmasa da, sadece in vivo olarak değil in vitro olarak da nöroprotektif bir etkiye sahiptir. Bu etkilerin olası oksidatif yollarla oluşabileceği düşünülmektedir.

Anahtar sözcükler: Astaksantin, bilişsel bozukluk, epileptik nöbet, SH-SY5Y hücre kültürü, nöroprotektif etki.

INTRODUCTION

Epilepsy, a neurological disorder that is a short-term paroxysmal disturbance of brain functions observed between sudden, abnormal and hyper-synchronization discharges and seizures of a group of neurons in the central nervous system¹. Seizures are known to damage the hippocampus, by inducing oxidative stress and neurodegenerative changes. Reactive oxygen species (ROS) are generated in the hippocampus after epileptic seizures. The acquired causes such as head injury or infection or exposure to toxic chemicals can initiate one or more seizures or status epilepticus. Patients with epilepsy are at significant risk for cognitive impairment and behavioral abnormalities. This is dramatically and catastrophically situation for patients affected with epileptic seizures².

Various antiepileptic drugs are (AED) widely used both long-term combined therapy and monotherapy in epilepsy. Drug-resistant epilepsy is defined as failure to achieve seizure control despite adequate trials of antiepileptic therapy, and approximately one-third of epileptic patients do not respond efficiently to present AED's. Many available AED's may also cause toxicity. Another excellent treatment modality is epilepsy surgery. As is known surgical resection of focal epileptic lesions may be cause altered cerebral glucose metabolism and postoperatively cognitive, behavioral and psychosocial changes³⁻⁶.

The brain tissue consumption high oxygen and the antioxidant system is weak, that means brain tissue could be exposed easily to oxidative damage than other tissue types. Oxidative stress enables the cell death by activation of the glutamate receptor gate, followed by affecting ionic homeostasis and neurotransmission, and able to entrance of elevated Ca^{2+} ⁷. There are many antioxidant agents have been given particular attention as protective agents against epileptic seizures in the literature^{8,9}.

One of them is astaxanthin (ATX) that is a red, lipid-soluble carotenoid pigment, and widely distributed in nature, such as in algae, crustaceans, salmonoids, and many other organisms. ATX has various activities, such as antioxidative, anti-inflammatory and anti-apoptotic and bio-activities including anti-cancer, anti-obesity; triglyceride and cholesterol activity, cardioprotective activity, anti-diabetic activity, hepatoprotective activity. ATX has multiple mechanisms of action, such as the scavenging of singlet oxygen via conjugated double bonds, suppression of lipid peroxidation, and inhibition of nuclear factor-kappa B (NF-kB)¹¹⁻¹³. ATX also can pass through the blood-brain barrier, and protects brain vessels from cerebrovascular diseases, such as cerebral ischemia and subarachnoid hemorrhage¹⁴. ATX was found to ameliorate oxidative stress, which is intimately involved in the exacerbation of various diseases due to the overproduction of reactive oxygen species (ROS), the superoxide radical, hydroxyl radical, and hydrogen peroxide (H_2O_2)¹⁵.

In this respect the investigation of more influential and safer treatment modalities and effective and innovative drug combinations for epilepsy and related cognitive impairment are necessary. Despite the potentially beneficial properties of ATX, the effects of this compound on the neuronal loss by PTZ-induced epileptic seizures have not been investigated. This study aimed to investigate the neuroprotective effect of astaxanthin on pentylenetetrazole-induced both epileptic seizures *in vivo* and neurotoxicity *in vitro*.

MATERIAL AND METHODS

Animals

All experimental protocols were performed following the guidelines for the local ethics committee on the care and use of animals. All mice were housed and bred 12 h light/12 h darkness in polypropylene cages and a temperature of 20-22°C, with free access to both food and water.

Drug administration

ATX was dissolved in olive oil, and pentylenetetrazole was dissolved in physiological saline. The drugs and all chemicals were purchased from Sigma Aldrich Co., St Louis, MO, USA. All of the solutions were prepared freshly on experiment days.

Experimental design

42 male (230-250 g) Wistar Albino rats were used for this research. Animals were divided into seven groups as control, saline (PTZ; 1 ml/kg serum physiologic), positive control (2,5 mg/kg diazepam), 10 mg/kg, 20 mg/kg, 40 mg/kg and 80 mg/kg ATX for seven days. Thirty min. After the administration of the last drug at the indicated doses, PTZ was administered 45 mg/kg to induce an epileptic seizure. The animals were observed for 30 min. Seizure stages according to the Racine Scale(RC) and first myoclonic jerk times (FMJ). Twenty four hours after PTZ injection, passive avoidance test was performed, and then brain tissues were removed for biochemical and histopathological evaluation. For this purpose hippocampal CA1, CA2 and DG tissue samples from each group were homogenized within 10 volumes of the ice-cold homogenization buffer and centrifuged at $12,000 \times g$ for 10 min at 4 °C. The supernatant was collected, for protein concentration determination with a Bradford protein assay kit (Merck, Germany) and for TAS and TOS determination by using TAS, TOS kit (Rel Assay Diagnostics). The hippocampal Cornu

Ammonis (CA) 1, CA3 and dentate gyrus regions were evaluated histopathologically regarding neuronal damage. Besides, oxidative stress markers (total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI)) were measured in brain tissues. Furthermore, ATX was performed *in vitro* neuroblastoma cell culture to evaluate PTZ-induced neurotoxicity.

Histopathology

Formalin-fixed brain sections (5µm) were stained with toluidine blue stain to quantify the number of dark neurons. All sections were examined and photographed with Olympus C-5050 digital camera at Olympus BX51 microscope. In hippocampal CA1, CA3 (Cornu Ammonis) and DG (Dentate gyrus) regions, dark neurons and survival neurons were counted in six sections per studied animal (n=3 for each group) by an image analysis system (Image-Pro Express 1.4.5, Media Cybernetics, Inc. USA). The numbers of dark neurons were given as a percentage (toluidine blue stained neurons*100/survival neuron). The observers blinded to the study groups accomplished all histological assessments.

Cell lines Maintenance

The human SH-SY5Y cells were purchased from the ATCC (American Type Culture Collection, Rockville, MD, USA). Cell culture materials including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), sterile phosphate buffer saline (PBS), and Penicillin-Streptomycin were obtained from Sigma-Aldrich (St Louis, Missouri, USA). Astaxanthin and pentylenetetrazole were also obtained from Sigma-Aldrich (St. Louis, USA). XTT reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) was purchased from Roche Diagnostic.

Neuronal Cell Culture

The human SH-SY5Y cells were grown in DMEM media supplemented with 10 % fetal bovine serum, 100U/ml penicillin, and 100µg/ml streptomycin. The cells were seeded at a density of 10^5 cells/well in the 96-well plates for the XTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) experiments. The plates were maintained at 37°C in 95 % humidified atmosphere (air) with 5 % CO₂. After 24 hr of incubation, the cells were treated with PTZ and ATX.

Passive avoidance test

The passive avoidance (PA) learning test based on negative reinforcement was carried out. The apparatus had a grid floor and comprised two compartments: one dark and the other one lighted, with a small gate which connected these two parts. This test is performed with the knowledge that rats have a native preference to the dark environment. Before beginning the training session, the animals were familiarized with the apparatus for two successive days (300 seconds per day). On the ensuing day, they were placed in the lighted compartment and the time latency for entering the dark compartment was noted down. During the training phase, the animals were located in the lighted compartment while facing toward the walls and away from the gate and received an electric shock (1 mA, 5 s duration) when they were entered the dark part. The animals were then returned to their cages. In retention or test phase which was carried out at 1 h after the training sessions, the rats were placed in the light compartment, and time latency to enter the dark compartment was recorded.

Neuroprotective Assay

The effective neurotoxic concentration of pentylentetrazole was obtained through dose-response experiments and XTT assay. Cell viability of neuronal cells was determined by using (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (XTT) assay. We used four different groups of cells: (1) untreated SHSY5Y cells; ² SHSY5Y cells incubated with pentylentetrazole (30 μ M final concentration) for 24 hours; ³ SHSY5Y cells treated with astaxanthin (final concentration ranging from 3.125 to 100 μ M) for 24 hours; ⁴ SHSY5Y cells treated astaxanthin for 4 hours + pentylentetrazole for 24 hours. In brief, SH-SY5Y cells were seeded into a microtitre plate at a density of 10^5 cells/well and allowed to adhere overnight. 50 μ L of astaxanthin was added into the cells with the final concentration ranging from 3.125 to 100 μ M. The pretreated cells were incubated for four h followed by 30 μ M PTZ treatment for 24 h.

Cell Viability Assay

Twenty-four hours after treatments, the treatment medium was removed, and wells were washed with 100 μ L phosphate buffered saline (PBS). At the end of these periods, for determination of living cells, 100 μ L DMEM without phenol red and 50 μ L XTT labeling mixture were added to each well and then the plates were incubated for four h. The absorbance of XTT-formazan was

measured using a microplate (ELISA) reader at 450 nm against the control. All experiments were performed in three independent experiments, and the cell viability was clarified in % related to control index of neuronal viability.

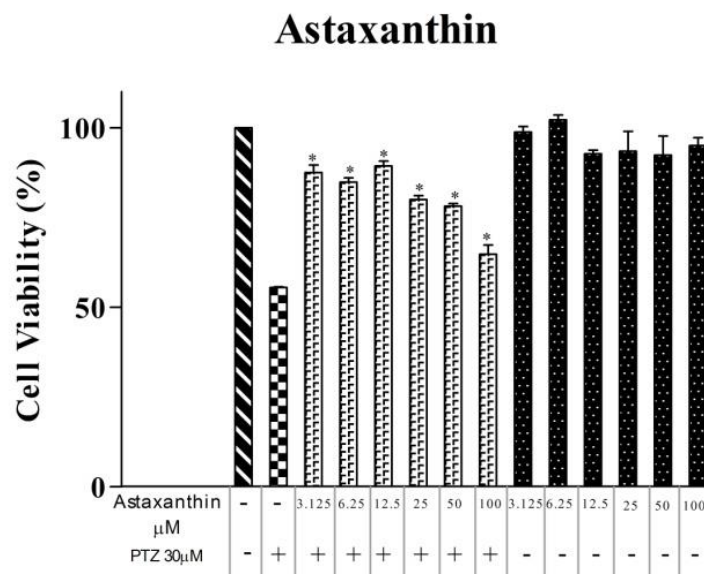
Statistical analysis

The results were expressed as a mean \pm standard error of the mean (SEM). The data analyses were performed with SPSS Version 21.0 for Windows. The RCS score, FMJ time and dark neurons were evaluated using a one-way analysis of variance (ANOVA). A posthoc Tukey test was utilized to identify the differences between the experimental groups, and a value of $p < 0.05$ was accepted as statistically significant.

RESULTS

Cell viability

XTT cell proliferation assay was used to evaluate the antiproliferative effects of pentylentetrazole and astaxanthin on SH-SY5Y cell line. As shown in Figure 1, Significantly decreased levels of cell viability were found in pentylentetrazole treated cells ($p < 0.05$) relative to untreated cells. Cell viability levels were unchanged in cells treated with astaxanthin relative to untreated cells. On the other hand, significantly increased cell viability levels were found in cells treated with PTZ + astaxanthin ($p < 0.05$) relative to PTZ incubated cells. Our cell viability results suggested that astaxanthin increases neuronal cell viability in the presence of PTZ.



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Figure 1. Neuronal Cell Viability Assay parameters in control human neuroblastoma (SHSY5Y) cells, in pentylenetetrazole (PTZ), incubated SHSY5Y cells, in SHSY5Y cells treated with ATX, and in SHSY5Y cells incubated with PTZ and then treated with astaxanthin. We performed statistical analysis using one-way analysis of variance (ANOVA) following the Tukey correction for cell viability.

The antiepileptic effect of ATX

A PTZ-induced epileptic rat model has been used to test if astaxanthin has antiepileptic effect in biological systems. Table 1 shows Racine Scale Scores and latencies to the 1st myoclonic seizures in both controls, saline, positive control, and ATX experimental groups. While diazepam reduced both Racine Scale Scores and latencies to

myoclonic seizures significantly (5.50 ± 0.22 vs. 2.50 ± 0.2 and 57.00 ± 2.01 vs. none, respectively) ($p < 0.05$), on the other hand, none of the ATX doses created a statistically significant effect on neither Racine Scale nor latencies which indicate the absence of antiepileptic effect of ATX in PTZ-induced epilepsy model ($p < 0.05$).

Table 1. Effect of ATX on seizures threshold (latency) in PTZ-induced seizures in the rat.

Group	Racine Scale	Latency to the 1st myoclonic seizures (sec)
Control	None	None
Saline (PTZ 45 mg/kg, i.p.)	$5,50 \pm 0,22$	$57,00 \pm 2,01$
Positive Control (Diazepam 2.5 mg/kg, i.p.)	$2,50 \pm 0,22^{ab}$	None
ATX (10 mg/kg i.p.)	$5,33 \pm 0,21$	$59,33 \pm 1,60$
ATX (20 mg/kg.i.p.)	$4,83 \pm 0,30$	$59,66 \pm 2,15$
ATX (40 mg/kg.i.p.)	$5,16 \pm 0,30$	$57,66 \pm 2,02$
ATX (80 mg/kg.i.p.)	$5,16 \pm 0,40$	$57,50 \pm 1,97$

Values are presented as mean \pm SEM. ^a $p < 0,01$ compared to PTZ group, ^b $p < 0,01$ compared to ATX groups.

Figure 2 shows passive avoidance latencies in both training and test trial. After a successful training trial period, as shown in the control group, animals successfully learned to avoid the harmful stimulus which has been proven by increased latency durations. Nevertheless, passive avoidance latencies dropped dramatically in

animals with PTZ-induced seizures ($p < 0.05$). In the presence of ATX, latencies were recovered and went back to control levels ($p > 0.05$). However, there was no significant difference between different doses of astaxanthin regarding restoring passive avoidance latencies ($p > 0.05$).

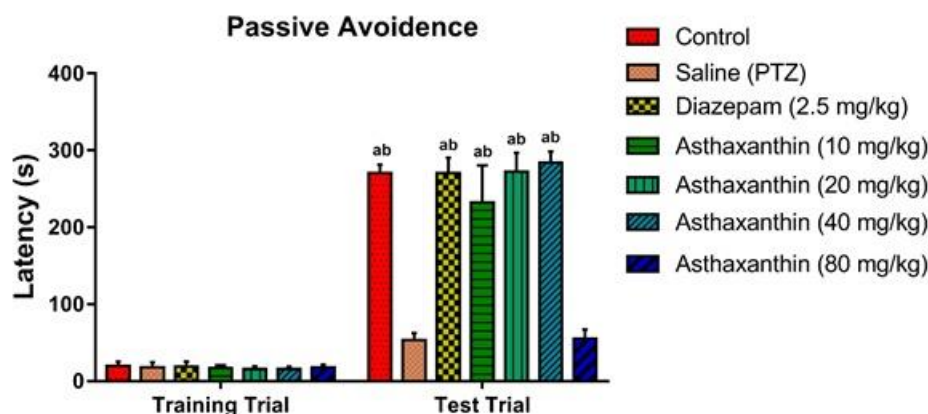


Figure 2. Passive Avoidance Latencies in both Training and Test Trial (s).

Antioxidant effect of ATX

After determining the neuroprotective effect of ATX in histological examinations, TAS, TOS, and OSI levels in rat brain after PTZ-induced seizures were evaluated to show the role of oxidative stress in the mechanism of action. Table 2 shows the Effect of ATX on TAS, TOS, and OSI in rat brain after PTZ-induced seizures. There was no significant change in TAS levels after

neither PTZ induction nor ATX administration ($p < 0.05$). On the other hand, induction of seizures via PTZ increased TOS levels in the saline group significantly (0.56 ± 0.00 vs. 0.70 ± 0.02) ($p < 0.05$). In the positive control group (Diazepam 2.5 mg/kg, i.p.), TOS levels dropped back to control group levels as in astaxanthin groups ($p > 0.05$). There was no significant difference between 10, 20, 40 and 80 mg/kg astaxanthin doses ($p < 0.05$).

Table 2: Effect of ATX on TAS, TOS, and OSI in rat brain after PTZ-induced seizures.

Group	TAS ($\mu\text{mol/mg protein}$)	TOS ($\mu\text{mol/mg protein}$)	OSI
Control	$0,14 \pm 0,04$	$0,56 \pm 0,00^a$	$393,35 \pm 17,36^a$
Saline (PTZ 45 mg/kg, i.p.)	$0,13 \pm 0,00$	$0,70 \pm 0,02$	$507,49 \pm 12,30$
Positive Control (Diazepam 2.5 mg/kg, i.p.)	$0,13 \pm 0,00$	$0,57 \pm 0,04^a$	$443,38 \pm 8,80^a$
ATX (10 mg/kg, i.p.)	$0,12 \pm 0,00$	$0,57 \pm 0,00^a$	$459,74 \pm 7,86$
ATX (20 mg/kg, i.p.)	$0,13 \pm 0,00$	$0,57 \pm 0,00^a$	$431,82 \pm 11,81^a$
ATX(40 mg/kg, i.p.)	$0,13 \pm 0,00$	$0,49 \pm 0,01^a$	$372,61 \pm 5,42^{ab}$
ATX (80 mg/kg, i.p.)	$0,13 \pm 0,00$	$0,58 \pm 0,00^a$	$448,20 \pm 14,13$

Values are presented as mean \pm SEM. ^a $p < 0,01$ compared to PTZ group, ^b $p < 0,01$ compared to ATX (10, 20 and 80 mg/kg) and Diazepam groups.

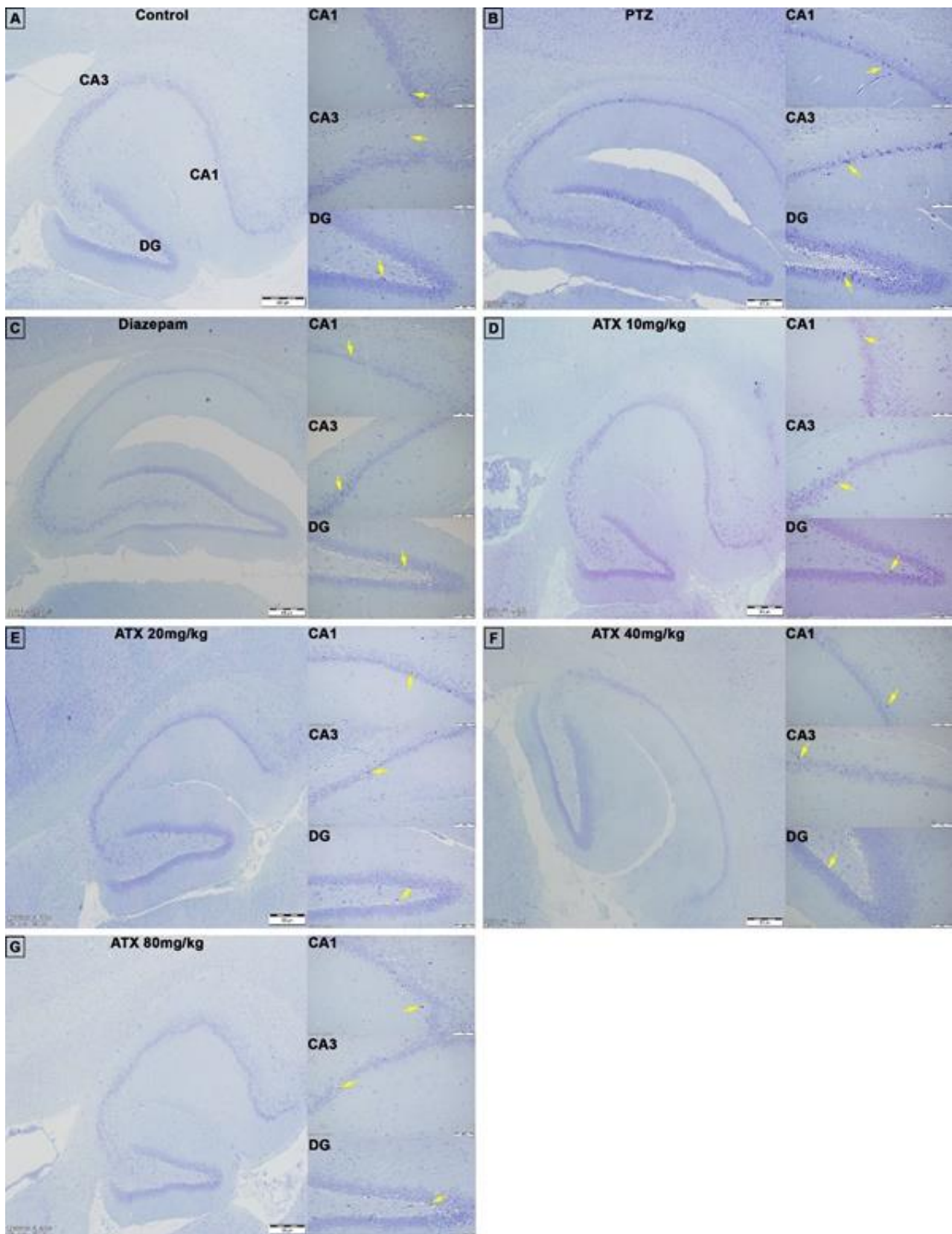


Figure 3. Histological imaging of dark neurons at hippocampus, DG, CA1 and CA3 regions in control, PTZ, Diazepam, ATX 10, ATX 20, ATX 40 and ATX 80 mg/kg groups.

Evaluation of groups in terms of dark neurons

In the present study, the identification of the neuroprotective effect ATX was made by histological evaluation of dark neurons which were identified by the neuronal shrinkage, cytoplasmic eosinophilia, nuclear pyknosis, and surrounding spongiosis in the total hippocampal formation, CA1, CA3, and DG hippocampal regions' formation. Administration of diazepam at the dose of 2.5 mg/kg significantly prevented the production of dark neurons due to PTZ induced

seizures in CA1, CA3 and DG regions of the hippocampus ($p < 0.05$) (Figures 3,4). Similarly, ASX 10, 20 and 40 mg/kg also created statistically significant neuroprotective effect regarding prevention of dark neurons induced by PTZ ($p < 0.05$). Interestingly, although 80 mg/kg dose of ATX also managed to produce significant neuroprotection when compared to the PTZ group, it was significantly less when compared to other doses.

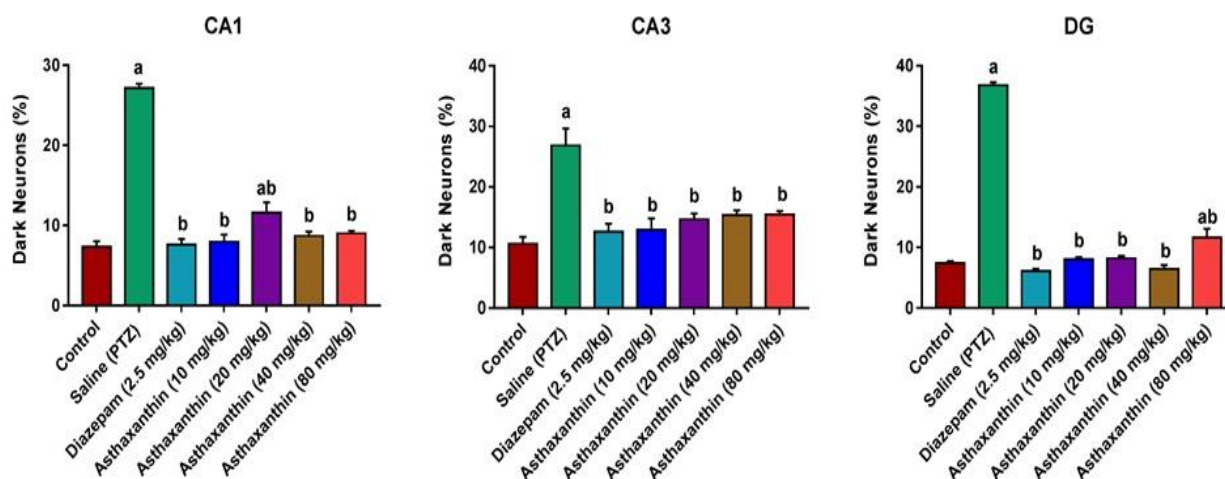


Figure 4. Dark Neuron percentage at A) CA1, B) CA3 and C) DG regions in control, PTZ, Diazepam, ASX 10, ASX 20, ASX 40 and ASX 80 mg/kg groups.

DISCUSSION

The seizures are generated due to abnormal hypersynchronous paroxysmal cerebral discharges from the neurons which eventually results in irreversible damage to them and their surroundings. There is promising evidence focusing on the contribution of oxidative stress and mitochondrial dysfunction in causing epileptic seizures. Various animal studies show the role of free radical production and oxidative damage to cellular proteins in epileptic seizures. Many studies have suggested the role of antioxidant therapeutic agents in the protection of brain against seizures, and improved seizure induce cognitive disorders. Thus, there is increasing attention in the nutritional supplements with antioxidant properties may be possible candidates for the prevention of oxidative neuronal damage and memory deficits associated with epilepsy^{7,8,16}.

Emerging evidence confirms that oxidative stress manifests as a consequence of the first seizure insult, which turns out later to become the cause

of epileptogenesis. The recurrent seizures can also result in overproduction of mitochondrial superoxide radicals in rodent models that can be converted to hydroxyl radicals. The hydroxyl radicals readily oxidize proteins, lipids, and DNA resulting in altered protein function, membrane permeability, and gene expression, respectively⁵.

ATX is a red carotenoid pigment, which is widely distributed in nature can pass through the blood-brain barrier and exhibits neuroprotective effects in animal models. Accordingly, with the literature, recent studies have demonstrated that ATX attenuated oxidative damage in experimental subarachnoid hemorrhage and global ischemia rat models^{14,17}.

In this study PTZ-induced epileptic rat model has been used to test if astaxanthin has antiepileptic and neuroprotective effects in biological systems. RC scores and latencies to the FMJ in both controls, saline, positive control, and given ATX experimental groups were recorded. While diazepam reduced both RC and latencies to FMJ, none of the astaxanthin doses created a statistically significant effect on neither RC nor

FMJ latencies which indicate the absence of antiepileptic effect of astaxanthin in PTZ-induced epilepsy model. In the present study, we detected that ATX does not have antiepileptic properties. Previously Chang et al. detected in their study that astaxanthin pretreatment increased the seizure latency and reduced the seizure score¹⁶. This result is consistent with a recent study from Lu et al., which showed that astaxanthin 75 mg/kg exerts anticonvulsant effects in an amygdala kindling animal model¹⁸. We believe that the difference in the results obtained in our study may be related to the method of epilepsy applied in other methods.

In the present study, the neuroprotective effect of ATX was evaluated against cognitive impairment induced by seizures in the PTZ kindled rats. The effects of epilepsy on cognition have been reviewed in several articles, suggesting the presence of at least a mild decline in intellectual performance in children and adults with epilepsy^{19,20}.

Recently reported that seizure activity had caused an increased level of free radicals and reduced activity of antioxidant defense mechanisms^{18,21}. In our study, we detected that ATX has a neuroprotective effect against oxidative stress and accordingly cognitive impairment in rats. We evaluated the effect of ATX on TAS, TOS, and OSI in rat brain after PTZ-induced seizures. There was no significant change in TAS levels after neither PTZ induction nor ATX administration. On the other hand, induction of seizures via PTZ increased TOS levels in the saline group significantly. In the positive control group (Diazepam 2.5 mg/kg, i.p), TOS levels dropped back to control group levels as in ATX groups. There was no significant difference between 10, 20, 40 and 80 mg/kg ATX doses. This imbalance between oxidant and antioxidant defense mechanism in the body may result in seizures and cognitive deficit.

In this study, PTZ caused a significant decrease in retention latency in the passive avoidance task which indicates impairment of memory of rats. In the groups that were administered ATX, the rats exhibited significantly increased retention latencies in the passive avoidance paradigm as compared to the animals administered PTZ alone. In the presence of ATX, latencies were recovered and went back to control levels. Previously accordingly with our study Patil et al. demonstrated that α -tocopherol decrease in retention latency in the passive avoidance these

improvements in cognition of kindled rats with antioxidant mechanisms¹⁰.

Previous studies showed that ATX, by attenuating oxidative damage, lipid peroxidation, and inhibiting the mitochondrial-related apoptotic pathway, protects hippocampal neurons against epilepsy-induced cellular loss²². Additionally, the previous studies reported that ATX prevents inflammation injury and improves cognition in diabetic mice²³. To investigate the possible neuroprotective role of ATX against the antiproliferative and toxic effects of PTZ we used XTT cell proliferation assay on SH-SY5Y cell line. Significantly decreased levels of cell viability were found in PTZ treated cells relative to untreated cells. Cell viability levels were unchanged in cells treated with ATX relative to untreated cells. On the other hand, significantly increased cell viability levels were found in cells treated with PTZ + ATX relative to PTZ incubated cells. Our cell viability results suggested that ATX increases neuronal cell viability in the presence of PTZ. Similarly, Lee et al. demonstrated that ATX showed neuroprotective effects in global cerebral ischemia model in rats and also ATX significantly inhibited production of nitric oxide synthase and suppressed oxidative stress process on SH-SY5Y cell line²⁴. Lobos et al. demonstrated in their work, the possible neuroprotective effects of ATX on some of the deleterious effects induced by amyloid- β peptide oligomers which cause neuronal damage and death and induce the cognitive deficits that characterize Alzheimer's disease, on primary hippocampal cultures. They found that these protective effects may result from the reduction of intracellular ROS promoted by ATX²⁵.

In our study, we evaluated the neuroprotective effect of ATX by histological evaluation of dark neurons which were identified by the neuronal damage and surrounding spongiosis in total hippocampal formation CA1, CA3 and DG regions. Diazepam at the dose of 2.5 mg/kg significantly prevented the production of dark neurons due to PTZ induced seizures of the hippocampus. Similarly, ATX 10, 20 and 40 mg/kg also created statistically significant neuroprotective effect regarding prevention of dark neurons. Although 80 mg/kg dose of ATX also managed to produce significant neuroprotection when compared to the PTZ group, it was significantly less when compared to other doses. ROS-mediated oxidative damage can damage neurons, induce neural apoptosis or/and necrosis, and inhibition of oxidative damage has been accepted as an effective strategy.

Importantly, it is reported that ATX could easily cross the brain-blood barrier (BBB) and show novel neuroprotective effects against neural damage involving anti-oxidation, anti-inflammation, and antiapoptosis²⁶. In the previous studies investigated the in vitro neuroprotective effects and mechanism of ATX against experimental -induced neural toxicity, and the results suggested that ATX has the potential to reverse experimental induced neurotoxicity and apoptosis by inhibiting mitochondrial dysfunction, ROS-mediated oxidative damage²⁷. Shen et al. reported in their experimental ischemic brain injury, apoptosis occurs following ischemia/reperfusion and can result from free radical damage and excessive glutamate release¹⁷. In their study they found that pretreatment with ATX reduced apoptosis and TUNEL labeling, suggesting that ATX inhibits DNA fragmentation in stroke brain.

CONCLUSION

Nevertheless ATX has no direct antiepileptic effects, it has strong neuroprotective effect in PTZ-induced epileptic model which may suggest that ATX may be a good candidate agent for preventing neuronal loss and cognitive function after an epileptic seizure in patient with the high risk of seizure.

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