



2013).

Pentylentetrazole (PTZ) is a gamma-aminobutyric acid (GABA) antagonist which is popularly used as a chemo-convulsant agent (Qu et al., 2005). However, the exact mechanism of PTZ-induced epileptogenesis is unknown. Mounting evidence has shown that PTZ has some effects on the astrocytes (Qu et al., 2005). Moreover, PTZ use in animal models has increased the markers of astrocyte damage (Meng et al., 2014). By considering the role of astrocyte dysfunction in the epileptogenesis, it is possible to assume that the astrocytes apoptosis may be involved in the epileptogenesis mechanisms of PTZ. Thus, an investigation of the mechanisms of PTZ-induced gliotoxicity may help to discover more targets for screening anti-epileptic drugs.

S100B is an astrocyte-derived cytokine with both beneficial and detrimental effects on neurons (Donato et al., 2009). Some studies have shown that there is an increase in S100B levels in the temporal lobe and the cerebrospinal fluid (CSF) of patients with epilepsy (Griffin et al., 1995; Steinhoff et al., 1999), which may result from the increased production or the passive release by the damaged astrocytes (Sen and Belli, 2007). Arundic acid is a glial modulating agent that decreases S100B synthesis and secretion from astrocytes (Fernandes and Ingle, 2013). Arundic acid has produced neuro- and glioprotective effects against several stressors (Kato et al., 2004; Keshavarz et al., 2019). Moreover, PTZ has increased S100B levels in the CSF and serum of animals (Meng et al., 2014). Thus, arundic acid is a suitable candidate to explore the role of S100B in the apoptotic action of PTZ.

Another glial-derived factor that may influence epileptogenesis is nitric oxide (NO), a gaseous neurotransmitter synthesized by the nitric oxide

synthase (NOS) enzyme (Garry et al., 2015). Some studies have documented that an enhanced activity of NOS isoforms has a role in activated glia in epileptogenesis. Animal models have also shown that NO mediates chemical-induced epileptogenesis (Banach et al., 2011). Moreover, NO is a mediator for the neurotoxic or gliotoxic effects of S100B in pathological conditions (Hu and Van Eldik, 1996; Hu et al., 1997). Nevertheless, the effects of NO and S100B on gliotoxicity of chemical convulsants such as PTZ is unknown. Therefore, we aimed to evaluate the effects of S100B and NO on PTZ gliotoxicity in the 1321NI1 astrocytic culture.

## Materials and methods

### Chemicals and reagents

The 1321NI astrocyte cells (the Pasteur Institute, Tehran, Iran) were used in this study. Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12), fetal bovine serum and penicillin-streptomycin were obtained from the Gibco® life technologies™ (New York, USA). We also obtained PTZ (a chemical convulsant and neural toxic agent), L-arginine (a NO donor), N-nitro-L-arginine methyl ester (L-NAME, a non-specific inhibitor of NOS enzyme isoforms), 7-nitroindazole (7-NI, a selective inhibitor for neuronal NOS), aminoguanidine (AG, a selective inducible NOS inhibitor), methylthiazolyldiphenyl-tetrazolium bromide (MTT), phosphate buffered solutions (PBS) and dimethyl sulfoxide (DMSO, the solvent for arundic acid) from the Sigma-Aldrich Chemie (St. Louis, USA). We purchased arundic acid (ONO-2506) from the Tocris Bioscience (USA). We dissolved PTZ, L-arginine, L-NAME, 7-NI, and AG in PBS and arundic acid in a solution contained PBS and DMSO (5% v/v).

**Table 1:** The organized groups to compare the cell viability and S100B protein level of 1321NI astrocyte cultures

Group								
1	control	PTZ	PTZ + arg100	PTZ + arg500	PTZ + AA	PTZ + AA + arg100	PTZ + AA + arg500	AA
2	control	PTZ	PTZ + NAME100	PTZ + NAME 500	PTZ + AA	PTZ + AA + NAME 100	PTZ + AA + NAME 500	AA
3	control	PTZ	PTZ + NI30	PTZ + NI100	PTZ + AA	PTZ + AA + NI30	PTZ + AA + NI100	AA
4	control	PTZ	PTZ + AG50	PTZ + AG1	PTZ + AA	PTZ + AA + AG50	PTZ + AA + AG100	AA

The cells were treatment with pentylentetrazole (PTZ: 40mM), arundic acid (AA: 50µM), L-arginine (arg: 100 or 500µM), N-nitroarginine methyl ester (L-NAME: 100 or 500µM), 7-nitroindazole (NI: 30 or 100µM) and/or aminoguanidine (AG: 50 or 100µM) for 24h.

### Astrocyte cell culture and treatment

In this study, 1321N1 astrocytes were maintained under the standard condition at 37°C in 95% humidified atmosphere contains 5% CO<sub>2</sub> until became confluent (Keshavarz et al., 2019). According to the previous studies, we used a dose range for each agent to obtain the best concentration. The dose range was as follow: PTZ (1-100mM) (Zhu et al., 2012), arundic acid (1-100µM) (Keshavarz et al., 2019), L-arginine (100-1000µM) (Quintas et al., 2014), L-NAME (100-1000µM) (Quintas et al., 2014), 7-NI and AG (1-100µM) (Efremova et al., 2015; Gupta et al., 2015). The confluent culture was exposed to the PTZ (40mM), arundic acid (50µM) or both of them for 24 or 48h. In addition, L-arginine (100 or 500µM), L-NAME (100 or 500µM), 7-NI (30 or 100µM) and AG (50 or 100µM) were added to PTZ, arundic acid or both of them in separate groups and incubated for 24 or 48h. The studied groups were summarized in table 1. Cell viability was measured by the MTT reagent using a microplate reader (Synergy HT, Biotek®, Winooski, VT, USA) at 570nm.

### S100B level measurement by an ELISA test

The intracellular S100B levels were measured using a commercially available ELISA kit (Mybiosource Inc., USA). The procedure was performed according to the manufacturer instruction. In brief, we poured 100µl of standard or sample into each well and incubated for 90min at 37°C. We removed the supernatant and added 100µl of biotinylated detection antibody to each well and it was incubated for 1h at 37°C. After washing them for three times, 100µl of horseradish peroxidase conjugate was added to each well and was incubated for 30min at 37°C. After washing for five times, 90µl of substrate reagent was poured into the wells and it was incubated for 15min at 37°C. Finally, 50µl of the stop solution was added to each well. The S100B protein level was determined using a microplate reader (Synergy HT, Biotek®, Winooski, VT, USA) at 450nm. The concentration of S100B protein was estimated by a standard curve.

### Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey's test were used to analyze of the data in each group. *P*-value of <0.05 was considered statistically significant. SPSS software version 23

(SPSS Inc, Chicago, IL) was used for the statistical analysis of the data.

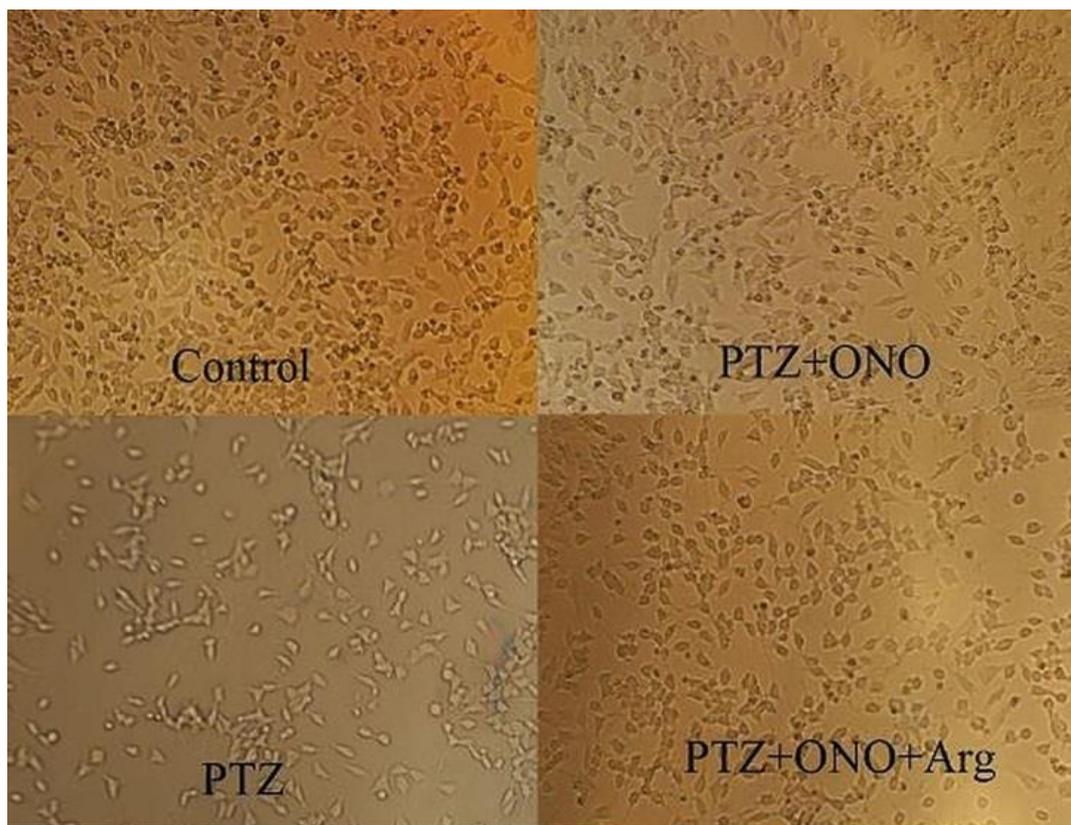
## Results

### The effects of different treatments on astrocyte viability

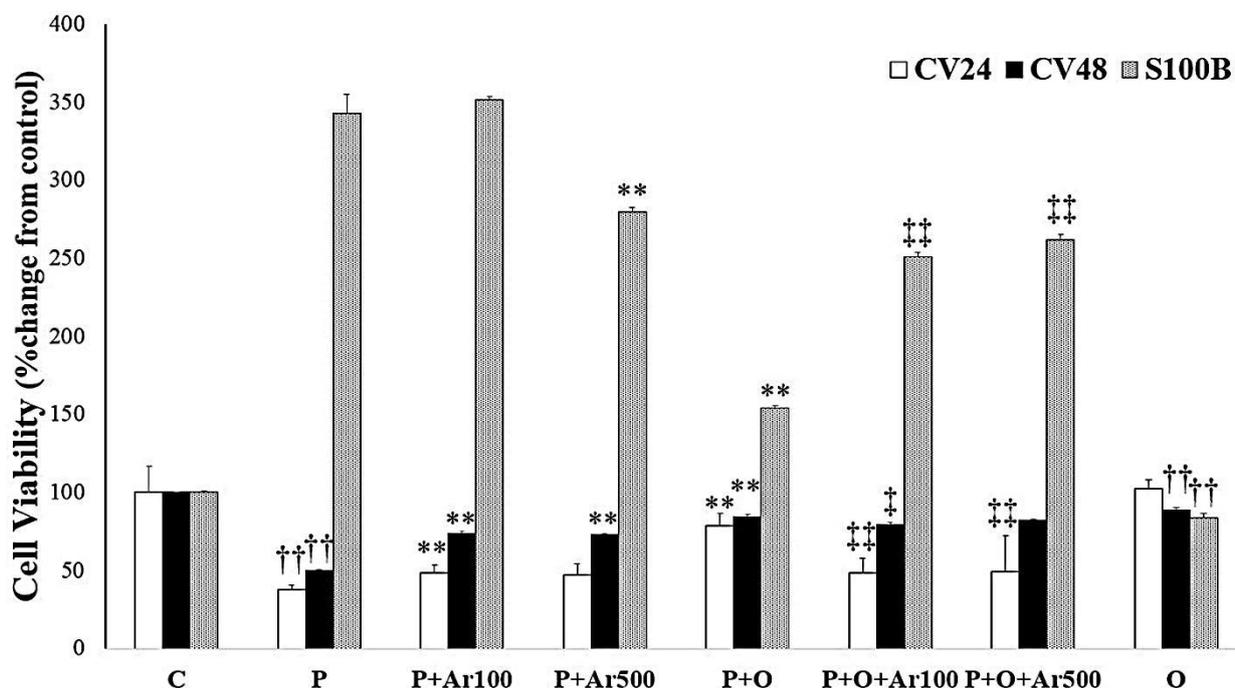
PTZ (40mM) significantly decreased the glial cell viability after 24 and 48h treatment in comparison with the control group (*P*<0.001, Figs. 1 and 2). However, arundic acid at a concentration of 50µM reversed the PTZ toxic effects on the glial viability (Figs.1 and 2).

Cell viability in cells treated with PTZ+ arundic acid+ arginine (100µM) for 24h (*P*=0.025) and 48h (*P*=0.003) was higher compared with the PTZ+ arundic acid group (Fig. 2). The PTZ+ arundic acid+ arginine (500µM) group only after 24h treatment had a lower cell viability compared with the PTZ+ arundic acid group (*P*=0.026, Fig. 2). Treatment with PTZ+ L-NAME (100µM) for 24 or 48h and PTZ+ L-NAME (500µM) for 24h decreased the cell death compared to the PTZ-treated group (*P*<0.001, Fig. 3). The cells treated with PTZ+ arundic acid+ L-NAME (100µM) for 24h (*P*=0.003) or 48h (*P*<0.001) had higher cell viability than the PTZ+ arundic acid group (Fig. 3). The 7-NI treatment for 24 or 48h at the concentrations of 30 (*P*<0.001) and 100µM (*P*=0.029 and *P*<0.001, respectively) reversed the effects of PTZ on the cell viability (Fig. 4). Cell viability in the cells treated with PTZ+ arundic acid+ 7-NI (30µM) for 24h (*P*=0.010) was significantly higher than the PTZ+ arundic acid group (Fig. 4). In addition, the PTZ+ arundic acid+ 7-NI (100µM) group after 48h had a lower cell viability compared with the PTZ+ arundic acid group (*P*<0.001, Fig. 4). Treatment with PTZ+ AG (50 or 100µM) for 24 or 48h decreased cell death in comparison with the PTZ-treated group (*P*<0.001, Fig. 5). Moreover, the cell viability in the PTZ+ arundic acid+ AG (50µM or 100µM) groups after 24 or 48h was significantly lower than the PTZ+ arundic acid group (*P*<0.001, Fig. 5).

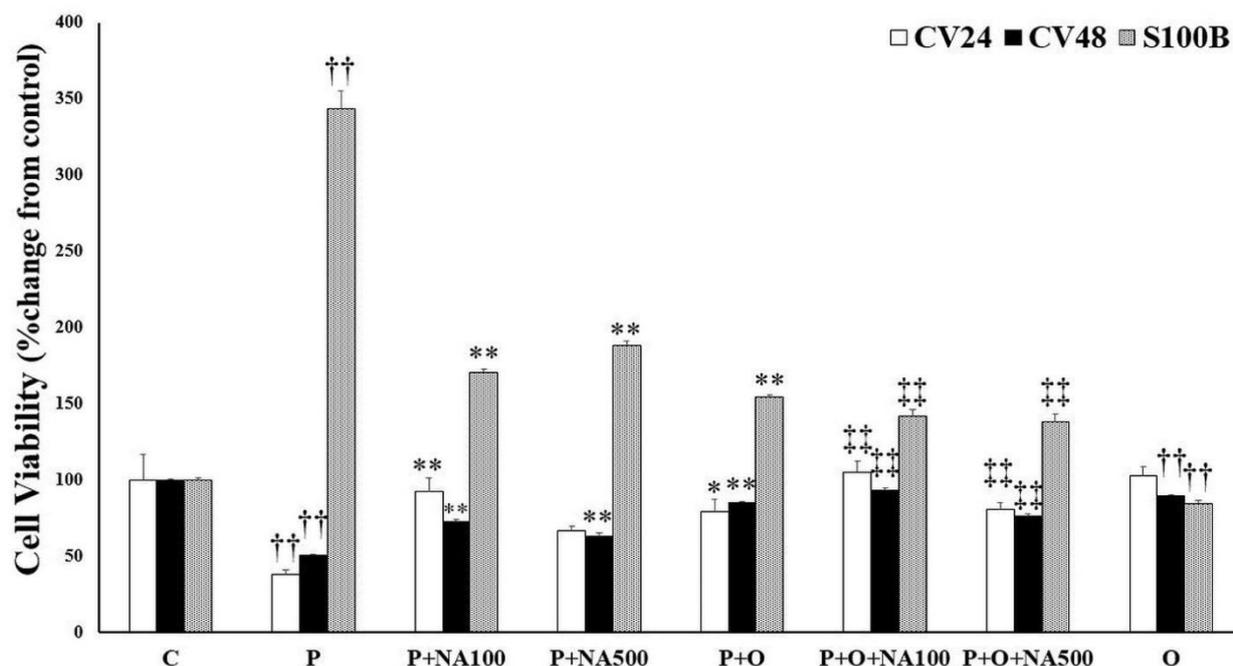
Addition of arginine (100 or 500µM) decreased the protective effects of arundic acid on the PTZ-induced cytotoxicity (*P*=0.024 and 0.025, respectively), but the addition of L-NAME (100 or 500µM), NI (30 or 100µM) and AG (50 or 100µM) did not change it (*P*>0.05). Addition of L-NAME (100µM or 500µM, *P*<0.001 and *P*=0.001, respectively), NI (30µM or



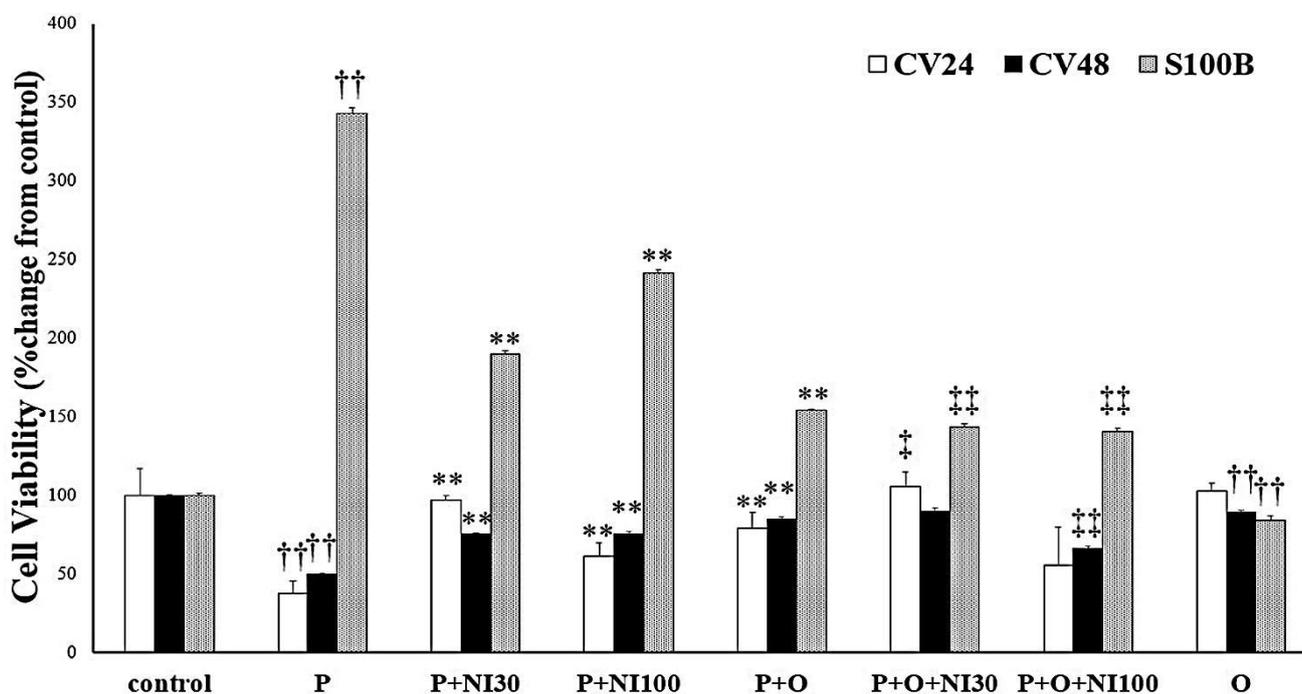
**Fig.1.** The light microscope photos of control, pentylenetetrazole (PTZ, 40mM), pentylenetetrazole plus arundic acid (ONO) and pentylenetetrazole plus arundic acid (ONO) plus arginine (Arg, 100µM) after 24h treatment with these agents.



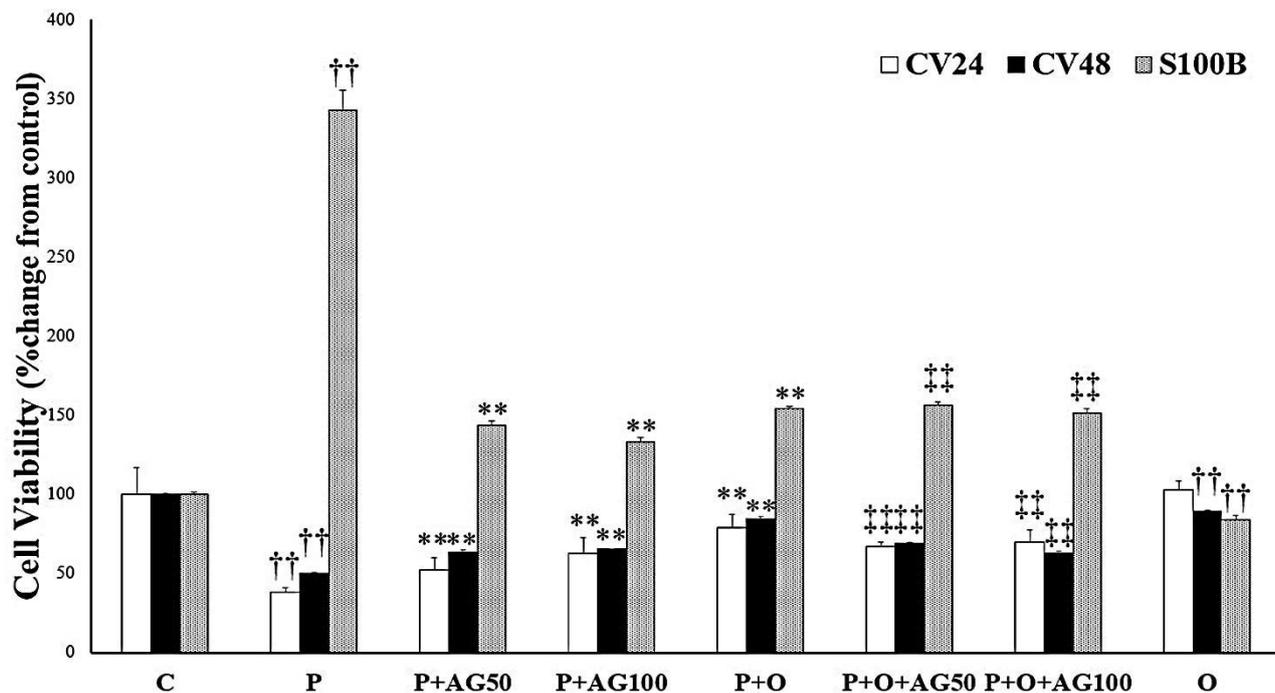
**Fig.2.** The effects of 24 or 48h treatment with pentylenetetrazole, arundic acid and/or arginine on the cell viability and the intracellular S100B protein in the 1321N1 astrocyte cell line (n=4). Cell viability was measured by methylthiazolyldiphenyltetrazolium bromide (MTT) method and S100B using an ELISA test. One-way analysis of variance (ANOVA) followed by the Tukey's test was used to analyze the variables of each group. \*\* $P < 0.001$  compared to the pentylenetetrazole-treated group; †† $P < 0.001$  compared to the control group; ††† $P < 0.001$  compared to the pentylenetetrazole+ arundic acid group. C: control, P: pentylenetetrazole (40mM), Ar100: arginine (100µM), Ar500: arginine (500µM) and O: arundic acid (50µM).



**Fig.3.** The effects of 24 or 48h treatment with pentylenetetrazole, arundic acid and/or N-nitroarginine methyl ester on the cell viability and the intracellular S100B protein in the 1321N1 astrocyte cell line (n=4). Cell viability was measured by methylthiazolyldiphenyl-tetrazolium bromide (MTT) method and S100B using an ELISA test. One-way analysis of variance (ANOVA) followed by the Tukey's test was used to analyze the variables of each group. \* $P < 0.05$  and \*\* $P < 0.001$  compared to the pentylenetetrazole-treated group; †† $P < 0.001$  compared to the control group; ††† $P < 0.001$  compared to the pentylenetetrazole+ arundic acid group. C: control, P: pentylenetetrazole (40mM), NA100: N-Nitroarginine methyl ester (100 $\mu$ M), NA500: N-Nitroarginine methyl ester (500 $\mu$ M) and O: arundic acid (50 $\mu$ M).



**Fig.4.** The effects of 24 or 48h treatment with pentylenetetrazole, arundic acid and/or 7-nitroindazole on the cell viability and the intracellular S100B protein in the 1321N1 astrocyte cell line (N=4). Cell viability was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) method and S100B using an ELISA test. One-way analysis of variance (ANOVA) followed by the Tukey's test was used to analyze the variables of each group. \* $P < 0.05$  and \*\* $P < 0.001$  compared to the pentylenetetrazole-treated group; †† $P < 0.001$  compared to the control group; ††† $P < 0.001$  compared to the pentylenetetrazole+ arundic acid group. P: pentylenetetrazole (40mM), NI30: 7-nitroindazole (30 $\mu$ M), NI100: 7-nitroindazole (100 $\mu$ M) and O: arundic acid (50 $\mu$ M).



**Fig. 5.** The effects of 24 and 48h treatment with pentylenetetrazole, arundic acid and/or aminoguanidine on the cell viability and the intracellular S100B protein in the 1321N1 astrocyte cell line (n=4). Cell viability was measured by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) method and S100B using an ELISA test. One-way analysis of variance (ANOVA) followed by the Tukey's test was used to analyze the variables of each group. \*\* $P < 0.001$  compared to the pentylenetetrazole-treated group; †† $P < 0.001$  compared to the control group; ††† $P < 0.001$  compared to the pentylenetetrazole+ arundic acid group. C: control, P: pentylenetetrazole (40mM), AG50: aminoguanidine (50 $\mu$ M), AG100: aminoguanidine (100 $\mu$ M) and O: arundic acid (50 $\mu$ M).

100 $\mu$ M,  $P < 0.001$  and  $P = 0.008$ , respectively) and AG (100 $\mu$ M,  $P = 0.004$ ) decreased the cytotoxic effects of PTZ.

#### Effects of different treatments on the intracellular S100B level

Our study also showed that the cell viability in the astrocytes had a negative correlation with the intracellular ( $r = -0.397$ ,  $P < 0.001$ ) S100B levels. The post-hoc comparison showed that the cells received PTZ ( $P < 0.001$ ), PTZ+ arginine (100 $\mu$ M,  $P < 0.001$ ) and PTZ+ arginine (500 $\mu$ M,  $P < 0.001$ ) had higher intracellular S100B levels compared to the control group (Fig. 2). The cells treated with arundic acid had a lower intracellular S100B compared to the control group ( $P = 0.003$ , Fig. 2). Addition of arundic acid to the PTZ decreased the PTZ effects on the intracellular S100B level (Fig. 2). In contrast, the cells treated with PTZ+ arundic acid+ arginine (100 $\mu$ M,  $P < 0.001$ ) and PTZ+ arundic acid+ arginine (500 $\mu$ M,  $P < 0.001$ ) had higher intracellular S100B levels than the PTZ+ arundic acid- treated group (Fig. 2).

The intracellular S100B levels in the cells treated with

PTZ+ L-NAME (100 $\mu$ M,  $P < 0.001$ ) and PTZ+ L-NAME (500 $\mu$ M,  $P < 0.001$ ) were significantly lower than those treated with PTZ (Fig. 3). Moreover, the intracellular S100B levels in the cells received PTZ+ arundic acid+ L-NAME (100 $\mu$ M,  $P = 0.049$ ) and PTZ+ arundic acid+ L-NAME (500 $\mu$ M,  $P = 0.006$ ) were lower than the PTZ+ arundic acid-treated group (Fig. 3). The intracellular S100B levels in the cells treated with PTZ+ 7-NI (30 $\mu$ M,  $P < 0.001$ ) and PTZ+ 7-NI (100 $\mu$ M,  $P < 0.001$ ) were significantly lower than the PTZ-treated group (Fig. 4). Moreover, the cells treated with PTZ+ arundic acid+ NI (100 $\mu$ M) had a lower intracellular S100B level compared to the PTZ+ arundic acid-treated group (Fig. 4). The addition of AG (50 or 100 $\mu$ M) to PTZ diminished the PTZ effects on the intracellular S100B level ( $P < 0.001$ , Fig. 5). The addition of arginine (100 or 500 $\mu$ M) increased while adding L-NAME (100 or 500 $\mu$ M) and 7-NI (100 $\mu$ M) decreased the effects of PTZ+ arundic acid on the intracellular S100B level. The addition of arginine (500 $\mu$ M) increased the effects of PTZ on the intracellular S100B level, but the addition of L-NAME (100 or 500 $\mu$ M), NI (30 or 100 $\mu$ M) and AG (50 or

100 $\mu$ M) decreased it.

## Discussion

In this study, our finding showed that PTZ decreased the astrocyte viability, while arundic acid, L-NAME (a non-specific NOS inhibitor), 7-NI (a selective neuronal NOS inhibitor) and AG (a selective inducible NOS inhibitor) reversed the toxic effects of PTZ on the astrocytes. Moreover, arginine as a NO donor, decreased the protective effects of arundic acid against PTZ-induced cell toxicity. In addition, the cells which received PTZ or PTZ+ arginine, had higher levels of S100B than the control group. However, arundic acid, L-NAME, 7-NI and AG suppressed the effects of PTZ on the intracellular S100B protein.

PTZ is a convulsant agent frequently used in the screening of new anti-epileptic drugs (Löscher, 1999). The antagonistic effects on the GABA<sub>A</sub> receptors may be responsible for its pro-convulsant effect (Macdonald and Barker, 1978), but its exact mechanism of action is relatively unknown (Eloqayli et al., 2003). In our study, the findings showed that the administration of PTZ induced astrocyte death. In accord with our experiment, Zhu et al. (2012) showed that PTZ (20 and 40mM) increased cell death in rat primary astrocyte culture. Moreover, some *in vitro* and *in vivo* experiments in the literature have shown that PTZ reduces astrocytic glucose uptake (Qu et al., 2005) and metabolism (Eloqayli et al., 2004). The impaired glial functions or astrocyte death may be the cause or promoter of epileptogenesis (Kang et al., 2006; Wetherington et al., 2008). Thus, PTZ-induced glial death may contribute to the convulsive mechanism of this agent.

In the current study, 7-NI and AG suppressed the cytotoxic effects of PTZ on astrocytes. Abundant evidence shows that AG (100 $\mu$ M) selectively inhibits iNOS in astrocyte culture (Hewett et al., 1994). Therefore, iNO and eNO might mediate the cytotoxic effects of PTZ. NO is a neuro-modulatory agent with potential cytotoxic effects at higher concentrations (Hu and Van Eldik, 1996; Lipton, 1999). S100B-induced NO elevation causes astrocytic death (Hu et al., 1997). Our study showed that arundic acid prevented astrocytic death and arginine, a NO donor, reversed anti-apoptotic effects of arundic acid against PTZ. Thus, the reduction of S100B by arundic acid may cause NO suppression and prevent PTZ-

induced toxicity. Moreover, the inhibition of iNOS suppressed PTZ effects on S100B elevation. Some reports have shown that iNOS activation causes astrocyte apoptosis (Quintas et al., 2014). Therefore, the protection of astrocytes by iNOS inhibition may reduce passive release of S100B from astrocytes. Furthermore, iNOS may mediate the PTZ effects on active S100B secretion from astrocytes. Extracellular S100B protein, at higher concentrations, activates iNOS and causes astrocytes apoptosis (Hu et al., 1996). The iNOS inhibitors may suppress PTZ effects on S100B secretion and function. Accordingly, iNOS inhibitors may protect astrocyte from apoptotic pathways. In addition, some studies have reported that the NO elevation produces pro-convulsive effects in the PTZ-induced seizure model (Watanabe et al., 2013). Astrocytic dysfunction or death may affect epileptogenesis (Kang et al., 2006). Consequently, the PTZ-induced NO elevation may damage the astrocytes and trigger epileptogenesis.

In our study, PTZ and arginine increased the S100B level and cell death. It is noteworthy to mention that the group with higher concentrations of arginine (500 $\mu$ M) plus PTZ had lower S100B level compared to the PTZ group. This effect may arise from the NOS inhibition with higher concentrations of arginine or NO. In contrast, arundic acid and NOS inhibitors reversed the PTZ effects on the intracellular S100B level, and the astrocyte death. Furthermore, arginine reversed the arundic acid effects on the astrocyte viability and the intracellular S100B level. These results may imply that NO is involved in the synthesis and release of S100B. moreover, higher concentrations of S100B can increase the expression of NO and induce astrocyte cell death (Hu and Van Eldik, 1996). Our study also showed that the effects of PTZ on NO may elevate S100B level and induce astrocytic cell death. Therefore, NO may mediate PTZ effects on the astrocyte apoptosis and the elevation of S100B protein.

Glioprotective effects of arundic acid may have potential implications in the neuroprotective effects of this agent. Accordingly, the protection of astrocytes by arundic acid may prevent either the active or the passive release of S100B into the extracellular space and may suppress the glial activation and neuronal death. The increased level of S100B can be detrimental to neurons and it can affect the CNS homeostasis in several ways (Mrak and Griffinb,

2001). Thus, the arundic acid neuroprotection may arise, at least in part, from the glioprotective effects and the inhibition of S100B release from the dead astrocytes.

In the present study, 7-NI and AG reversed the effects of PTZ on the intracellular S100B and decreased the intracellular S100B, which may show that the NO produced from iNO and eNO may mediate the PTZ effects on S100B in the astrocytes. Some studies have previously shown that S100B protein enhances the expression of inducible NOS (Hu et al., 1996). Our study added the notion that the inhibition of different NOS isoforms activity in astrocytes decreased the intracellular S100B as a marker of astrocyte damage. These findings suggest that NOS function is very important in the astrocyte viability in the presence of a stressor such as PTZ.

## Conclusion

Nitric oxide and S100B may have a role in the gliotoxic effects of PTZ in the cell culture. Arundic acid suppressed PTZ-induced S100B elevation and gliotoxicity possibly by modulating the NO pathway. The neuroprotective effects of arundic acid may arise from its glioprotective effects.

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## Conflict of interest

All the authors confirm that there were no conflicts of interest.

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