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Allopurinol's effect on caspase-3 and caspase-8 activity in hypoxic-ischemic newborn rats

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Summary

Aim: During reperfusion period of hypoxia-ischemia, cyclooxygenase and xanthine oxidase pathways are induced. A xanthine oxidase inhibitor, allopurinol has been shown to be neuroprotective in hypoxic- ischemic encephalopathy. Caspase-8 and caspase-3 have a key role in neuronal apoptosis. We aimed to test repeated doses of allopurinol's effect on caspase-3 and caspase-8 activities in newborn rats with hypoxic-ischemic encephalopathy.

Material and Method: Seven days old newborn rats were taken and there were 10 rats in each group. After Ethical Committee was approved (TIBDAM-25), rats were subjected to left carotid artery ligation and hypoxia (8% oxygen and 92% nitrogen) for two and half hours. Hypoxic ischemic rats treated with 24 mg/kg allopurinol 30 minutes and 12 hours (AL48 group), and 30 minutes, 12 and 24 hours (AL72 group) after hypoxic- ischemic insult. Twenty four hours after last dose, rats were decapitated. The others groups were sham and saline- treated hypoxic- ischemic (H-I) group. Caspase- 3 and caspase- 8 activities were measured in both hemispheres.

Results: There was no difference in caspase-3 and caspase-8 activities between right and left brain hemispheres in each group ($p>0.05$). Caspase-3 and caspase-8 activities were significantly lower in sham group when compared to H-I group, AL48 and AL72 groups (all of it, $p=0.0001$). Even though there were no difference activities of caspase- 3 and caspase- 8 between H-I group and AL48 group ($p>0.05$), activities of caspase- 3 and caspase- 8 in AL72 group were significantly lower than H-I group and AL48 group (respectively $p= 0.0001$, $p=0.001$).

Conclusions: Decreased activities of caspase- 3 and caspase- 8 in AL72 group may suggest that totally dosage of 72 mg/kg allopurinol may be effective for reducing neuronal apoptosis in newborn rats with hypoxic- ischemic insult. *(Turk Arch Ped 2013; 48: 48-52)*

Key words: Allopurinol, caspase-3, caspase-8, hypoxic-ischemia, newborn rats

Introduction

Hypoxic ischemic encephalopathy is one of the most important causes of mortality in the newborn period and of neurological deficits in the long-term (1,2). In hypoxic ischemia, central nervous system tissues which are rich in unsaturated fatty acids are exposed to inflammation and oxidative stress (3). Increase in production of xantine oxidase, nitric oxide sythtase and prostaglandin especially in the reperfusion period of hypoxic ischemia leads to release of free oxygen radicals. When free oxygen radicals exceed the capacity of antioxidant defense systems, they cause to change in DNA and protein structures by lipid peroxidation and to apoptosis and cell death by leading to activation of caspases (4,5).

Formation of free radicals during hypoxic ischemia is a complex process. One of the most important metabolic pathways in formation of free radicals in the process of hypoxic ishemia is the xantine oxidase/xantine dehydrogenase enzyme system (6). Xantine dehydrogenase is transformed to xantine oxidase by a protease independent of calcium (7). Throughout the reperfusion period, xantine and hypoxantine

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increase with activation of xantine oxidase and this process leads to production of free radicals in presence of oxygen (8). In experimental hypoxic ischemic animal models, allopurinol which is a xantine oxidase inhibitor has been shown to decrease hypoxic ischemic brain damage (6,9,10,11). However, there is no consensus on the theraputic dose, treatment period and time of onset of allopurinol yet (12,13).

Neuronal apoptosis has the main role in hypoxic ischemic brain damage and contributes to neural tissue loss after acute hypoxic ischemic encephalopathy. Neuronal apoptosis starts with activation of caspase cascade. Caspase cascade is activated by intrinsic and extrinsic pathway. In the extrinsic pathway, caspase-8 is activated with stimulation of death receptors on the plasma membrane. Caspase-8 activates caspase-3 which has a key role in neuronal apoptosis (14,15,16,17). Studies have shown that caspase inhibition has neuroprotective effect (16).

In this experimental study, caspase-3 and caspase-8 activities were measured following different doses of allopurinol which is an unselective xantine oxidase inhibitor in newborn rats with hypoxic ischemic encephalopathy and it was aimed to evaluate the potential effects of caspase-3 and caspase-8 on neuronal apoptosis.

Material and Method

This study was conducted in newborn Wistar-albino rat models of hypoxia-ischemia. Approval for the study was obtained from the ethics committee established in Çukurova University, Medical Faculty, Medical Experiencies Research and Application Center.

Ten rats were included in each group randomly. The groups were divided as the control group (S) in which surgical procedure was performed and no treatment was given, the hypoxia-ischemia group (H-I) in which serum saline was given, the AL48 treatment group in which 24 mg/kg allopurinol was given at the 30th minute of ischemia and at the 12th hour of ischemia (a total of 48 mg/kg) and the AL72 treatment group in which 24 mg/kg allopurinol was given at the at the 30th minute, 12th hour and 24th hour of ischemia (a total of 72 mg/kg).

For rat model of hypoxia-ischemia the method which was described by Levin (18) was used (18). Rat youngsters who were with their mothers and were breastfed were included in the study when they were 7 day old. The left arteria carotis communis was found with a 0.5 cm cut just left to the trachea under ether anesthesia. The artery was tied bilaterally with 4/0 silk suture and cut in the middle. The rats who were given to their mother until the effect of anesthesia disappeared were kept in a cage containing 8% oxygen and 92% nitrogen in quinary groups for 2.5 hours. Afterwards they were left to recover in room air for half an hour and allopurinol and serum saline were administered intraperiotenally. Allopurinol (Allopurinol vial 5 g Sıgma-Aldrich-USA) was given at a total dose of 48 mg/kg in two doses at the 30th minute and 12th hour after hypoxia-ischemia to the AL48 group and at a total dose of 72 mg/kg in three doses at the 30th minute,and 24th hour 12th hour after hypoxia-ischemia to the AL72 group. In the hypoxia-ischemia group, only serum saline was administered at the 30th minute after hypoxia-ischemia as a single dose. 24 hours after the application all rats in all groups were decapitated under ether anesthesia. The brain was removed rapidly and the right and left hemispheres were separated and placed in tupes kept in dry ice. They were stored at -20 ºC until the study day.

Caspase-3 was measured with Caspase-3/CPP32 Colorimetric Assay Kit (Oxford BiomedicalResearch, Oxford, MI), Caspase-8 was measured with FLICE/Caspase-8 Colorimetric Protease Assay Kit (Oxford Biomedical Research, Oxford, MI) in accordance with the instructions contained in the kit. The method is based on spectrophotometric measurement of chromophor p-nitroaniline (pNA) which is manifested by breaking of the labeled substrate. The amount of pNA was determined by measuring the absorption value at 405 nm using a microtiter plaque reading device (ELX 800, Biotek, Turkey).

Statistical analysis

It was checked if the variables were distributed normally and variable tests were used in the analyses, since the variables showed normal distribution. The data were entered into the SPSS 15 package program and analysed. The graphics were prepared in STATISTICA 6.1. A p value of <0.05 was considered to be statistically significant.

Results

The mean weight of the newborn rats was found to be 11.3 ± 2.3 (8.2-16) g. There was no statistically significant difference between the groups in terms of gender and weight ($p>0.05$).

Caspase-3 and caspase-8 activities were studied in the right and left brain hemispheres obtained from the rats in the study group. No statistically significant difference was found inside the groups in terms of caspasae-3 and caspase-8 activities of the right and left brain hemispheres ($p>0.05$) (Figure 1 and Figure 2).

The caspase-3 and caspase-8 activities in the left and right brain hemispheres of the rats in the control group were found to be significantly lower compared to the caspase-3 and caspase-8 activities of the rats in the H-I group, AL48 group and AL72 group (Table 1 and Table 2) for all $p=0.0001$).

While there was no difference between the caspase-3 and caspase-8 activities in the left and right brain hemispheres of the rats in the H-I group and the caspase-3 and caspase-8 activities of the rats in the AL48 group, the caspase-3 and

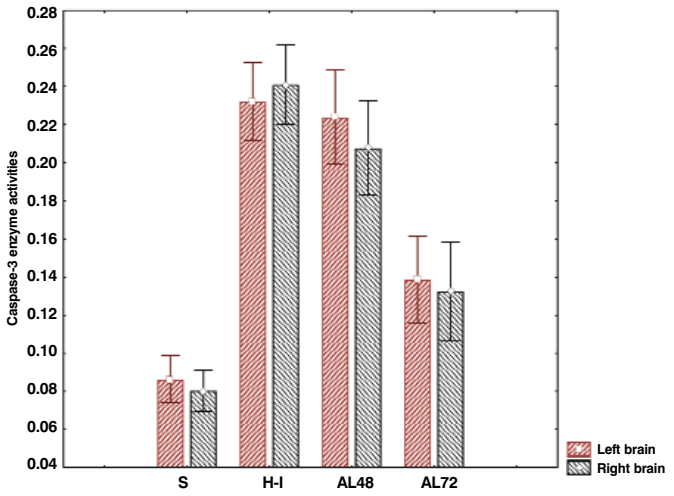

Figure 1. Caspase-3 activities of the left and right brain hemispheres in the rats in the study group

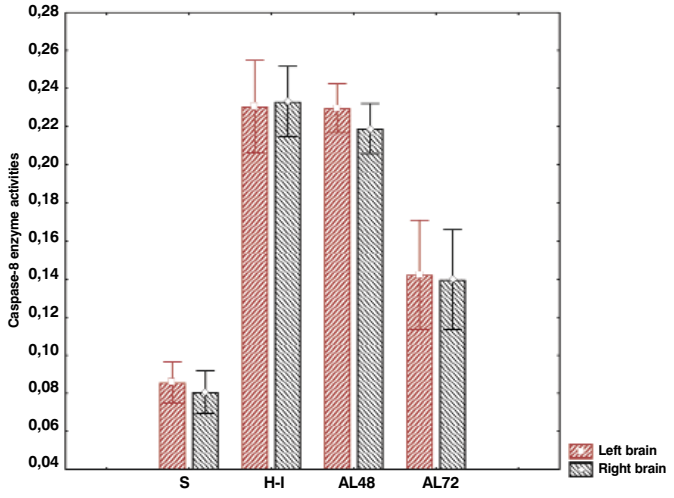

Figure 2. Caspase-8 activities of the left and right brain hemispheres in the rats in the study group

caspase-8 activities of the rats in the AL72 group were found to be significantly lower (Table 1 and Table 2) (p>0.05, p=0.0001).

When the rats in the AL48 group were compared with the rats in the AL72 group, the caspase-3 and caspase-8 activities in the left and right brain hemispheres were found to be lower compared to the rats in the AL48 group (p=0.0001).

Discussion

Hypoxic ischemic brain damage is a process which starts at the time of hypoxia-ischemia and continues during the recovery stage following recuscitation. The tissue damage which is observed as selective neuronal necrosis or infarct continues with destruction of the cellular structure of the neurons, glial cells and endothelial cells. Necrosis or programmed cell death (apoptosis) develop in the neurons which are located in the nearest area to the infarct area (2).

It is known that xantine oxidase is an important source for free radicals. Allopurinol which is an inhibitor of xantine oxidase enzymes inhibits the synthesis of xantine from hypoxantine and prevents formation of free radical superoxide (8,19).

A few studies have investigated the protective and healing effects of allopurinol administered immediately after brain ischemia (20,21,22). Recently, Güneş T et al. (22) conducted a study in asphyctic newborn babies and reported that serum NO level was significantly higher in asphyctic babies compared to the control group and NO level in the cerebrospinal fluid was compatible with the degree of encephalopathy. In addition, they found that serum NO levels decreased and better neurologic outcomes were obtained in the asphyctic newborn treated with allopurinol.

Palmer et al. (19) showed that young mice in whom allopurinol was administered to the brain 15 minutes after hypoxia-ischemia had lesser brain damage. In the study performed by Peeters-Scholte et al. (8) in pigs in whom experimental hypoxia was applied, no statistically significant differrence was found in caspase-3 levels between the group

Table 1. Brain caspase-3 activities in rats in the study group (optic density 405 nm)

Study groups	n	Caspase-3 left brain hemisphere Mean±SD	Caspase-3 right brain hemisphere Mean±SD	p
S	10	0.09±0.02*	0.08±0.02*	p>0.05
H-I	10	0.23±0.03*#Ω	0.24±0.03*#Ω	p>0.05
AL48	10	0.22±0.04*&Ω	0.21±0.04*&Ω	p>0.05
AL72	10	0.14±0.03*#&	0.13±0.04*#&	p>0.05

* A statistically significant difference was found between caspase-3 activities in the left and right brain hemispheres in S and H-I, S and AL48, S and AL72 group rats (p= 0.0001).
# A statistically significant difference was found between caspase-3 activities in the left and right brain hemispheres in H-I and AL72 group rats (p= 0.0001).
& A statistically significant difference was found between caspase-3 activities in the left and right brain hemispheres in AL48 and AL72 group rats (p= 0.0001).
Ω No statistically significant difference was found between caspase-3 activities in the left and right brain hemispheres in H-I and AL48 group rats (p>0.05).

Table 2. Brain caspase-8 activities in rats in the study group (optic density 405 nm)

Study groups	n	Caspase-8 left brain hemisphere Mean±SD	Caspase-8 right brain hemisphere Mean±SD	p
S	10	0.09±0.02*	0.08±0.02*	p>0.05
H-I	10	0.23±0.03*#Ω	0.23±0.03*#Ω	p>0.05
AL48	10	0.23±0.02*&Ω	0.22±0.02*&Ω	p>0.05
AL72	10	0.14±0.04*#&	0.14±0.04*#&	p>0.05

* A statistically significant difference was found between caspase-8 activities in the left and right brain hemispheres in S and H-I, S and AL48, S and AL72 group rats (p= 0.0001).
# A statistically significant difference was found between caspase-8 activities in the left and right brain hemispheres in H-I and AL72 group rats (p= 0.0001).
& A statistically significant difference was found between caspase-8 activities in the left and right brain hemispheres in AL48 and AL72 group rats (p= 0.0001).
Ω No statistically significant difference was found between caspase-8 activities in the left and right brain hemispheres in H-I and AL48 group rats (p>0.05)

who was given allopurinol, the group who was given desferroxamine and the control group. Allopurinol and oxypurinol levels ranged between 5 and 20 mg/L in the study and these values are known to inhibit xantine oxidase level. Safe use of allopurinol in newborns is still contradictory and mostly mutiple therapies have been experimented (12). In addition, there is no consensus on the therapeutical dose, time and intitiation time of allopurinol. In our study, allopurinol alone was given to newborn rats at a dose of 48mg/kg in the AL48 group and at a dose of 72 mg/kg in the AL72 group. When caspase-3 and caspase-8 levels of the right and left brain hemisphers were compared with the hypoxia-ischemia group, it was found that these levels were significantly lower in the AL72 group.

In this study we conducted, the fact that caspase-3 and caspase-8 levels in the right and left brain hemispheres were significantly lower in the AL72 group who was given 72 mg/kg allopurinol compared to H-I and AL48 groups suggested that allopurinol had a protective effect in hypoxic-ischemic encephalopathy throughout ischemia.

Conclusively, this study suggests that allopurinol might be effective in prevention of hypoxic ischemic brain damage by decreasing caspase-3 and caspase-8 levels. It may be thought that this action is due to decreased apoptosis, but further studies are needed to better understand the roles of caspase-3 and caspase-8 in hypoxic ischemic encephalopathy.

Conflict of interest: None declared.

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