# The Protective Effect Of Allopurinol On Neural Tissue On Regional Cerebral Ischaemia: An Experimental Study On Rats.

IŞIK DİLEK, ALPER BAYSEFER, FERRUH GEZEN, ÖZCAN ÇIKLATEKERLİOĞLU, HAKAN KAYALI, SAİT ŞİRİN

Departments of Neurosurgery Gülhane Medical School 06018, Ankara - Türkiye

**Abstract :** In this experimental study the neuroprotective effect of XO inhibitor allopurinol on focal cerebral ischaemia created by permanent middle cerebral artery (MCAO) occlusion has been investigated. In the study made on 43 white rats, the size of the cerebral tissue injury caused by MCAO after allopurinol pretreatment was compared with the control groups after 4 and 24 hours. In the rats which had allopurinol pretreatment, the infarct area could not be seen 4 hours after MCAO. However it was observed to be reduced by 32% when compared with the control group at 24 hours. It has been concluded that allopurinol pretreatment protects the neural tissue in the early period after occlusion, and in the late period it prevents cerebral injury especially in the perifocal area by preventing the formation of free radicals with XO inhibition.

**Key Words :** Allopurinol, Brain Ischaemia, Rats.

## INTRODUCTION

The aerobic metabolism of the cell provided by mitochondrial oxidative phosphorilation is affected first in ischaemia. In this period, production of ATP slows down or ceases. The lack of ATP and consequently inadequate Na/K pump activity leads to acute cytotoxic oedema. All these processes are reversible. Besides, in the following period, irreversible cellular damage will occur, characterized by deteriorated cell membrane function because of membrane damage and irreversible mitochondrial disability despite reperfusion and oxygenation. Here the cell membrane damage developed by lipid peroxidation is believed to be the basic factor (28,33). It has been shown that the free radicals are effective on the lipid peroxidation and damage of the membrane proteins (20,24,32). There are many potential sources of free radicals, including xanthine oxidase system, mitochondrial oxidation, lactic acidose, and activated leucocytes (27,29,30). Mc Cord (21) asserted that xanthine oxidase (XO) was the primary free radical-generating enzyme. Betz et al (2) demonstrated the existence of XO in cerebral tissue. With medical treatment against free radicals in ischaemia models of the small intestine (31), heart (35) and kidney (1) remarkable tissue protection has been provided.

Allopurinol, one pharmacological agent used for neural tissue damage produced by the free radicals, is an analogue of hypoxanthine and inhibits biosynthesis of uric acid (7). Either allopurinol or oxypurineol inhibits XO. Cerebral tissue concentration of allopurinol which also diffuses into all body fluids is one third of the other tissues (7). The aim of our study was to investigate the effect of allopurinol in the protection of neural tissue damage caused by regional cerebral ischaemia.

## MATERIALS AND METHODS

Forty three albino-rats of either sex, weighting 300-400 gr. were used. All rats were anaesthetized with 20 mgr/kg (IM) ketamine hydrochloride (Ketalar, Eczacıbaşı, İstanbul) and 2 mgr/kg intraperitoneal (IP),

atropine sulfate (Atropin, İbrahim Ethem, İstanbul) four days before MCAO and cannulated for measuring arterial blood pressure by exploring the left common carotid artery. After measuring arterial blood pressure with a polygraph (San-Ei,Japan), body temperature with a rectal probe, and obtaining 1 cc of arterial blood from the CCA and 0.5 cc of venous blood sample from the left jugular vein, the left common carotid artery (CCA) was encircled with 10–0 monofilament nylon suture (Ethicon, England). In this way, arterial blood pressure, pH, haemoglobin, haematocrit, $PaO_2$, $PaCO_2$, blood glucose and uric acid levels were measured in all rats before MCA occlusion. This procedure was repeated before decapitation.

In this study, forty rats were divided into four groups, of ten in order to observe the protective effect of allopurinol in regional cerebral ischaemia 4 and 24 hours after MCAO.

Group I: 150 mg/kg 10% Allopurinol (Atabay, İstanbul) as a suspension in 2% carboxymethyl cellulose (CMC) (Merck, USA) was given intragastrically 1,24 and 48 hours before MCAO. Ischaemic changes were observed 4 hours after MCAO.

Group II: 2% CMC solution was given orally alone at the same times before occlusion of the MCA. Ischaemic changes were observed 4 hours after MCAO (control).

Group III: 150 mg/kg 10% allopurinol as a suspension in 2% CMC was given orally at the same times before the MCAO. Ischaemic changes were observed 24 hours after MCAO.

Group IV: 2% CMC solution was given orally alone at the same times before MCAO. Ischaemic changes were observed 24 hours after MCAO(control). CMC was used to stabilize the allopurinol solution. The left CCA's of the remaining three rats were cannulated without treatment and MCA and physiological parameters were observed.

MCA occlusion: Rats in all four groups were anaesthetized with 50 mg/kg IM ketamine and 10 mg/kg IM xylazine (Rompun 2%, Bayer, Germany). After a vertical incision was made between the left eye and ear, the temporalis muscle was dissected from the temporoparietal connections using bipolar cautery. The zygoma was detached from the squamous portion of the temporalis bone. Subtemporal craniectomy was performed using a dental drill. The dura mater was incised and the MCA was occluded from a point where the lateral striate artery leaves the MCA (median edge of the olfactory tract) to the inferior cerebral vein using bipolar cautery. Later, the temporal fascia and skin were closed. All the surgical procedures were carried out under an operating microscope (Zeis Opmi-6, Germany).

Four hours after MCAO, the rats in groups I-II and 24 hours after MCAO, those in groups III-IV were decapitated following arterial blood pressure determination and obtaining 1.5 cc of blood. The brains were rapidly removed and chilled for ten minutes at -20°C. Then 2 mm - thick coronal slices were taken using a microtome blade (Disposable blade, Shandon, England). Sectioning was started 2 mm from the anterior pole. Sections were placed in a phosphate buffer solution (pH :7.4) containing 1% TTC (Triphenyltetrazolium chloride, Sigma, USA) and incubated at 37°C for forty minutes. After staining, the sections were photographed and the infarcted tissue volumes were measured stereo-photogrometrically (Planicomp Analytic Equipment C-140, Zeiss, Germany). Photogrometry is the technique of obtaining data about the form and structure of the object photographed by a special camera situated in a distinct place. This technique includes calculation and measurement to determine the form and size of the objects. In photogrometry, assessment is usually done by two photographs and three dimensional images of the objects are obtained. This is called stereophotogrometry.

Glycaemia was determined by the enzymatic calorimetric method and uric acid by the Folin-Denis calorimetric method. For this RA-1000 auto analyzer equipment (RA-1000 auto analyzer, Techniqueon, France) was used. Haemoglobin, haematocrit, pH, $PaO_2$ and $PaCO_2$ measurements were made by ABL-510 (ABL-510 Radiometer, Denmark) blood gas analyzer.

## RESULTS

Before and after MCAO, the body temperature of all animals varied between 35°C and 36°C. Physiological parameters are shown in tables I and II. There was no significant difference in the analysis of observed parameters except for, uric acid level.

Treated with allopurinol before MCAO, Groups I and II rats' plasma uric acid levels were remarkably low.

**Table I. Physiological parameters**

| Parameter | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| MAP | BS 102 ± 8.71 | 101 ± 8.06 | 99 ± 10.86 | 102 ± 6.42 |
| | AS 100 ± 8.89 | 98 ± 5.74 | 97 ± 12.16 | 100 ± 6.48 |
| pH | BS 7.35 ± 0.01 | 7.35 ± 0.01 | 7.35 ± 0.01 | 7.37 ± 0.01 |
| | AS 7.35 ± 0.01 | 7.34 ± 0.01 | 7.32 ± 0.01 | 7.34 ± 0.01 |
| $PaO_2$ | BS 77 ± 6.48 | 79 ± 7.81 | 86 ± 3.74 | 86 ± 4.79 |
| | AS 76 ± 7.28 | 79 ± 7.74 | 85 ± 4.00 | 85 ± 3.46 |
| $PaCO_2$ | BS 40 ± 3.16 | 41 ± 8.12 | 36 ± 5.29 | 38 ± 4.24 |
| | AS 42 ± 4.35 | 42 ± 7.87 | 39 ± 5.91 | 39 ± 3.16 |
| Hb | BS 13.6 ± 3.00 | 13.9 ± 1.33 | 14.7 ± 1.18 | 14.4 ± 0.74 |
| | AS 12.9 ± 2.00 | 13.5 ± 1.41 | 14.5 ± 0.58 | 14.2 ± 0.54 |
| Htc | BS 42 ± 7.14 | 44 ± 3.28 | 44 ± 2.26 | 43 ± 3.66 |
| | AS 38 ± 4.89 | 42 ± 4.34 | 43 ± 2.84 | 41 ± 3.19 |

Values are mean ± SD. BS: Before Surgery, AS: After Surgery.

**Table II. Plasma glycaemia and uric acid measurements**

| Parameter | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|
| GLYCAEMIA | BS 148 ± 21.7 | 154 ± 19.3 | 156 ± 16.8 | 152 ± 18.6 |
| | AS 146 ± 16.8 | 151 ± 20.2 | 154 ± 11.5 | 151 ± 16.7 |
| URIC ACID | BS 2.9 ± 0.59 | 3.2 ± 0.52 | 3.2 ± 0.60 | 3.3 ± 0.33 |
| | AS 1.3 ± 0.26 | 3.3 ± 0.68 | 1.6 ± 0.30 | 3.8 ± 0.88 |

Values are mean ± SD. BS: Before Surgery, AS: After Surgery.

We did not confirm any TTC vital stained area of infarction in the allopurinol administrated Group I rats decapitated four hours after MCAO (figure 1). But all sections of the untreated Group II rats decapitated four hours after MCAO were stained and the areas of infarction were slightly limited (figure 2).

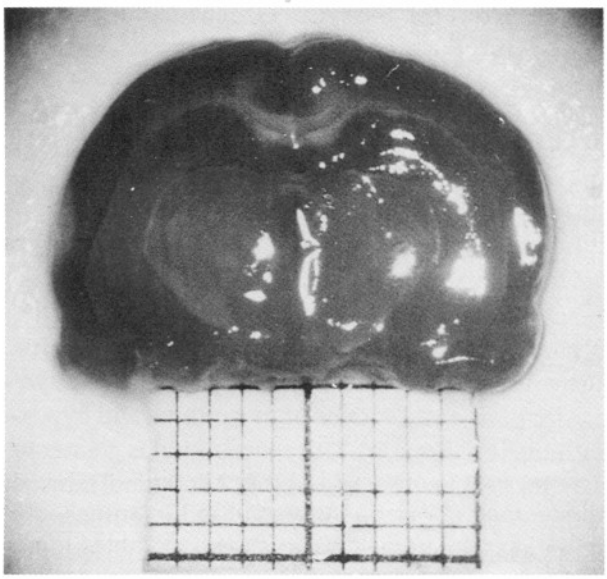

*Fig. 1 : No infarct area was observed at the 4th hour after occlusion with allopurinol pretreatment.*

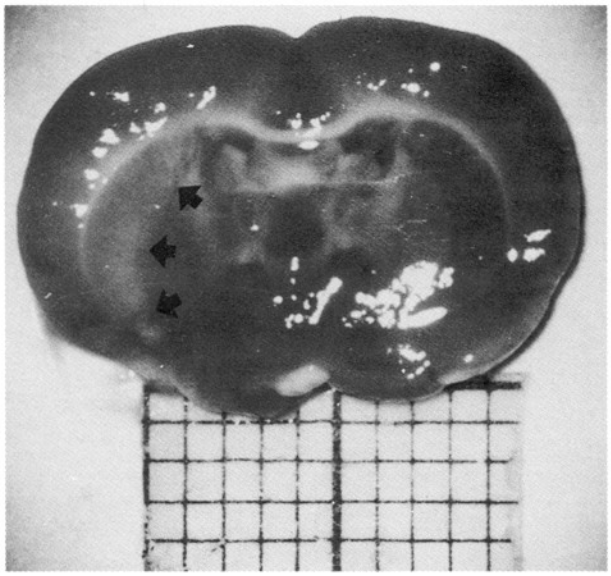

*Fig. 2 : The infarct area at the 4th hour after occlusion in the non-treated group (arrows).*

We observed definite infarction in the sections taken from Group III and IV rats 24 hours after MCAO. The volumes of infarcted tissue measured by stereo-photogrometry are shown in table III. Together with the volumes of the MCA occluded hemisphere and opposite hemisphere. The infarction areas of all the sections in Groups III and IV were compared and were all small at the cortex. There was no significant difference in the areas of infarction in the basal ganglia region. A 32% reduction was seen in the infarction volumes of Group III Allopurinol pretreated rats (figure 3), compared with the untreated Group IV rats (figure 4). This was statistically significant ($p<0.05$).

**Table III : Comparison of right and left hemispheres, and volumes of infarct in groups III and IV.**

| Group | Left Hemisphere volume | Right Hemisphere volume | Infarct volume | % |
|---|---|---|---|---|
| Group III | 557 ± 40.38 | 569 ± 33.56 | 141 ± 19.44 | 25 ± 2.87 |
| Group IV | 553 ± 32.27 | 611 ± 31.25 | 229 ± 21.36 | 37.5 ± 2.72 |

Values are mean ( SD.11

Three control rats had no remarkable difference either in physiological parameters or cerebral tissue sections.

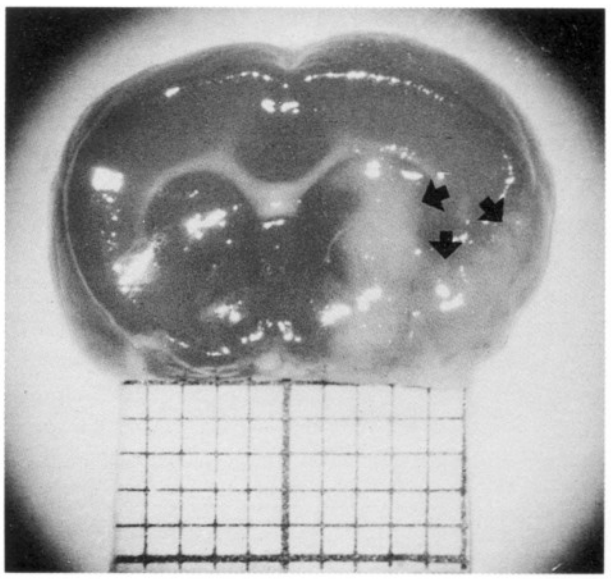

*Fig. 3 : Reduction in the infarct area was observed at the 24th hour after occlusion in the group pretreated with allopurinol (arrows).*

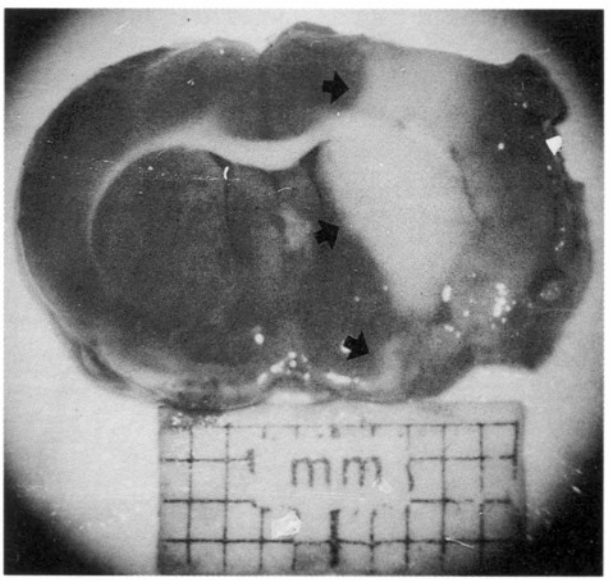

*Fig. 4 : The infarct area at the 24th hour after occlusion in the nontreated group (arrows).*

## DISCUSSION

In normal cerebral tissue , 92.4 % percent of XO is in the form of XD and while hypoxia, in the first 30 minutes 43.7% of XD transforms to XO (6, 8). It has been demonstrated that this transformation is probably proteolitic (3). There is evidence in many studies that XO is the primary free-radical generating system (11,12,19,23,24). Besides, Martz et al. (11) in their study with Dymethylthiourea (DMTU) have shown that especially the hydroxyl radical, one of the free radicals, is the primary mediator. However some authors believe that XO is not the primary source of free radicals (3,19,24,25).

In the ischaemic period, XO catabolizes hypoxanthine to xanthine and xanthine to uric acid which are the metabolites in purine nucleotide degradation (1). It has been shown that although purine nucleotide degradation produces uric acid, plasma level does not increase, uric acid accumulates 6–8 times higher than plasma level in the ischaemic cerebral areas. The same studies proved that the deposition is protected by allopurinol (13,17,29).

Uric acid blood levels varied between 1.6-4.1 mg/ml in the 43 rats in our study. Since allopurinol inhibits the biosynthesis of uric acid (When 1.6 mg/ml is accepted as the physiological lower limit) uric acid level reduction to the lower limit in the treatment group was observed (7,15). No increase in the plasma uric acid value was experienced in any of the rats after MCAO. Kanemetsu et al.(13) showed that there was no increase in plasma uric acid value after MCAO but a decrease with allopurinol treatment.

Allopurinol enters the brain as a result of its affinity for the transporter in the brain capillaries. (2,16). However the cerebral tissue concentration compared with other tissue levels is only 1/3. It has been determined that a single dose of allopurinol maintains its effect for 24 hours (14). The treatment was started in the early period in the allopurinol treatment (11,13,20,24,30). Because oxypurinol, the metabolite of allopurinol oxidation, is a more potent XO inhibitor (15). Hegstad et al (10) have shown that allopurinol given after hypoxia has no protective effect.

Tissue hypoxia results in a progressive depletion of tissue ATP proportional to the duration of the ischaemic injury. When uric acid forms from purine nucleotide it is irreversible. But it is possible to synthesize purine nucleotides from xanthine and hypoxanthine (reutilization). This reutilization is greater in the CNS than in other tissues (34). Allopurinol inhibits de Novo purine biosynthesis either by reutilization of hypoxanthine and xanthine or by ribonucleotides which are tissue metabolites of allopurinol and oxypurinol (15).

In our study, it was thought that the significant volume difference between the two hemisphere sections taken 24 hours after MCAO depended on cerebral oedema and/or swelling. The vasogenic component of cerebral oedema is the result of capillary endothelial damage that lets macromolecules such as albumin and water pass the blood–brain barrier (30). There is four times more XO in the brain capillary system than the brain tissue in rats (2). That is why, the most harmful effect of the free radicals produced by XO is on the capillary endothelial. Endothelial cells are demonstrated to be the potential source of cytotoxic superoxide and hydroxyl radicals (30).

Itoh et al.(12) have demonstrated in hypertensive rats with ischaemia/reperfusion model that treatment with allopurinol reduces cortical $Na^+$ and $H_2O$ content. Chan et al (5) reported that intracerebral injection of free-radical system to rats leads to neural damage, increased permeability in the blood-brain barrier and arise in water content of the brain.

In our study there was no significant difference between the hemispheric areas and volumes in the sections taken 24 hours after MCAO in group III treated with allopurinol. This result did not contradict previous studies and showed the protection of allopurinol against cerebral oedema. There was no significant difference between the hemispheric areas and volumes of the sections from each allopurinol treated and control group after four hours. We explain this by insufficient cerebral oedema and/or swelling formation in the fourth hour. Lin et al (18) reported that remarkable oedema could develop 6 hours after MCAO.

Bolonder (4) ,and Hakim (9) examined cerebral blood flow after MCAO. In Hakim's study, the ipsilateral CCA was encircled in addition to MCAO. In both studies, while blood flow was reduced at the side of MCAO, that at the opposite side decreased below 50-60% in the fifth and twentieth minutes. Here we can talk about reperfusion damage, but cerebral damage was not observed in the hemisphere opposite the MCAO. Reperfusion damage can not be achieved in rats by clipping the CCA bilaterally and allowing blood flow some time later, because in this period, cerebral blood flow reduces only by 16-41 % (30).

In our study, reduction of cortical infarction in the sections taken at 24 hours from allopurinol treated rats was considered to be a result of protection against free radicals generated in the cortex, as the perifocal region, by increased of blood flow with XO inhibition and particularly protection of capillary endothelials. Moorhouse et al. (26) put forward that allopurinol and particularly oxipurineol were hydroxyl radical scavengers, and considered that the cortex was protected in that way. Martz et al. (20) observed infarction in the control and allopurinol treated groups at three hours, but in some studies, it is reported that infarction can be determined only at six hours (18,22). We demonstrated infarction in the control group at four hours. But no development of infarction in the allopurinol treated group at four hours made us consider that especially the caudaputamen was protected by a mechanism differing from the protection against $O_2$ dependent free radicals generated by XO because of low blood flow. In our study, tissue protection 4 hours after MCAO can be explained with the last mentioned effect of allopurinol.

## CONCLUSION

Allopurinol is effective in tissue protection or in decreasing damage against experimentally induced ischaemic cerebral damage in rats. But to observe this useful effect, Allopurinol treatment must be started prior to ischaemia.

So far, experimental studies have presented no evidence for clinical usage of allopurinol. For this reason, more detailed investigations should be made. Besides it may be used in future in patients at risk of transient or permanent disarrangement of arterial circulation during surgery or in patients with transient ischaemic attack in order to decrease or protect cerebral damage in the next serious attack.

**Correspondence** : Alper Baysefer
GATA Lojmanları Rieder Paşa Apt. No: 32
Etlik, Ankara - Türkiye

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