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Research Article

**TO DETERMINE THE CYCLOSPORINE
NEUROPROTECTIVE EFFICIENCY AFTER TRAUMATIC
INJURY OF BRAIN IN RATS**

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Abstract:

Aim: To evaluate the possible neuroprotective effects of systemic administration of cyclosporin (cyclosporin A) after traumatic brain injury in rats.

Materials And Methods: The modified Feeney method was used as a model of trauma in male Sprague Dawley rats. After the injury, 20 mg / kg of cyclosporin was administered intraperitoneally to a group of rats (n = 15). Twenty-four hours after injury, the individuals were sacrificed and brain samples were collected. The brain edema level was assessed by lipid peroxidation rate, wet dry weight method and histological evaluation by transmission electron microscopy.

Results: The brain edema level and the rate of lipid peroxidation were significantly reduced in the rats receiving cyclosporin. Ultrastructural neuro-destruction was assessed, and comparison of the results between experimental groups revealed a significant neuroprotective effect of cyclosporine.

Conclusion: The results showed that systemic administration of cyclosporine resulted in a statistically significant reduction in both the level of brain edema and the rate of lipid peroxidation compared to the "untreated" state. It is also known that cyclosporine, which is regularly used as an immunosuppressant, prevents the opening of the pores of mitochondrial permeability by separating cyclophilin from the mitochondrial matrix. The transition pores regulation for the permeability of mitochondria by cyclosporine suggests that mitochondrial dysfunction following traumatic brain injury is an important event in progressive loss of neuronal tissue.

Keywords: cyclosporine, neuroprotection, transitional pores of mitochondrial permeability, traumatic brain injury, rats

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INTRODUCTION:

Traumatic Brain Injury (TBI) is a serious health problem with huge social and economic consequences. While the incidence of TBI is declining, TBI is expected to become one of the most common causes of death and disability in the near future¹⁻². The effects of the main effect are considered irreversible even if the effect is avoidable. A group of adverse events occurs as a cascade called secondary trauma that begins immediately after the injury and progresses over the days³⁻⁴. Experimental studies have revealed some mechanisms of secondary damage that lead to the main neuropathological symptoms seen after primary trauma, including changes in endogenous stimulatory aminoacids and the production of pro-inflammatory agents⁵⁻⁶. The factors responsible for the destructive cascade need to be understood so that they can be manipulated to protect the brain tissue and thus improve outcomes. Neuroprotection is an expanding area of research based primarily on pharmacological interventions aimed at preventing cascades of secondary injuries and thus optimizing outcomes. One of the promising drugs is cyclosporine (cyclosporin A [CsA])⁷⁻⁸. CsA is widely used as an immunosuppressant after organ transplantation. In addition to its known immunosuppressive activity, CsA has a neuroprotective effect by inhibiting calcineurin, blocking the opening of the transition pores of mitochondrial permeability (mPT) and the release of a neurotrophic factor, stabilizing the mitochondrial membrane⁹⁻¹⁰. Thus, CsA is a therapeutic agent to such an extent that it alleviates the effects of secondary trauma and improves outcomes. Our goal was to investigate the neuroprotective effects of CsA after injury. We assessed key parameters such as brain edema (wet dry weight method), rate of lipid peroxidation and histological results (using the ultrastructural neurodestruction classification system [UNGS] for ultrastructural outcomes).

METHODS AND MATERIALS:

Materials

CsA was acquired from Novartis International AG (Basel, Switzerland). CsA was prepared with polyethylene glycol (MilliporeSigma, St. Louis). Anesthesia is provided by a mixture of xylocaine (10 mg / kg) and ketamine HCl (90 mg / kg).

Animal model

The protocol of the experiment was approved by the Local Ethics Committee. We used male Sprague Dawley rats weighing 250 to 300 g each. The animals were anesthetized intramuscularly with the xylocaine / ketamine HCl mixture (10/90 mg / kg). Under stereotaxic conditions, patients were immobilized and a rectal probe and heating pad were

used to monitor body temperature. The rats were divided into groups A, B, C and D; each group consisted of 15 mice. All animals were craniectomized using a high-speed drill; This was the magnitude of the injury in the rats of group A. Traumatic injury was achieved by dropping sterile 24 g metal rods from a 9.3 cm tube onto the dura mater. Group B rats were untreated; The rats in group C were given only one carrier (polyethylene glycol) so that we could inspect its probable effect. Group D rats were administered CsA (20 mg / kg) intraperitoneally instantly after injury. The rats were then kept at 25 ° C for 24 hours. The rats were then sacrificed and the intact brain tissues removed 24 hours after injury. Samples were taken from the injured area and placed in liquid nitrogen until analysis. The experimental groups are described in Table I.

Examination of the degree of cerebral edema

Wet-dry method

Each rat brain hemispheres were weighed immediately after killing the animal; the results were wet weights. The dry masses were obtained after drying the same hemispheres in an oven at 70 ° C for 36 hours. The percentage of water content in each hemisphere was calculated as follows:

$$\% \text{ Water content} = \left[\frac{(\text{wet-dry weight})}{\text{wet weight}} \right] \times 100$$

Determination of the amount of malondialdehyde

Free radicals, especially hydroxyl radicals, react with cell membranes to induce a destructive mechanism by which unsaturated fatty acids are replaced by lipid peroxidation. The degree of change is an indicator of the reaction of membrane phospholipids with free radicals. The lipid peroxidation reaction results in the formation of malondialdehyde, the amount of which can be quantified by reaction with thiobarbituric acid. Lipid peroxidation per gram of wet tissue in nanomoles is calculated by measuring the color produced by malondialdehyde by reaction with thiobarbituric acid at $\lambda = 532 \text{ nm}$ as shown by a spectrophotometer (Shimadzu UV 120-2).

Ultrastructural classification system for neurodestruction

Samples were taken from the cortical brain area in approximately 3 x 3 x 3 mm cubes. Cubes were fixed with glutaraldehyde solution for 24 hours immediately after tissue removal. Ultra-thin sections were obtained with an LKB-Nova ultramicrotome and stained with lead citrate and uranyl acetate. We use UNGS to measure ultra-structural findings; the results are summarized in Table II.

RESULTS:

Statistical analysis was performed using the Mann-Whitney U test at a significance level of 0.05. The

water content ratios for animals receiving 20 mg / kg intra-peritoneal CsA (group D) were significantly

lower ($p < 0.05$) compared to the control group (group B) receiving trauma but no treatment.

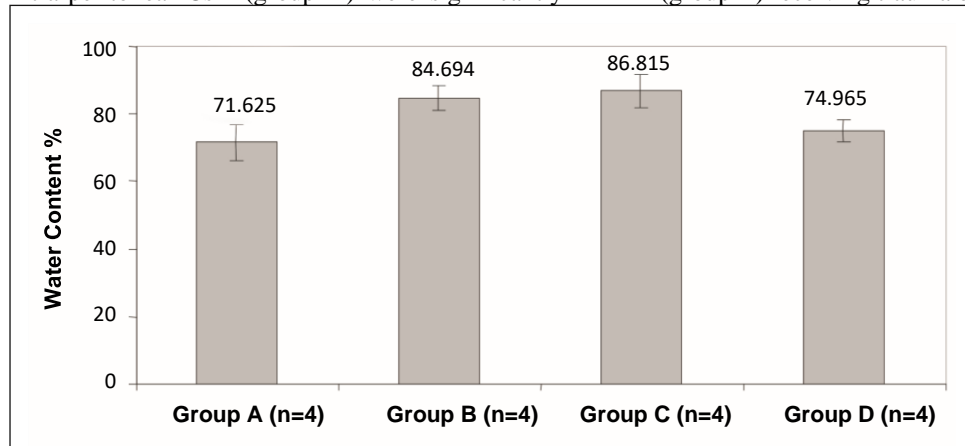


Figure 1 Water content ratios in the experimental groups (**Group A** only craniectomy; **Group B** traumatic injury after craniectomy but no treatment; **Group C** traumatic injury after craniectomy, followed by administration of the vehicle; **Group D** traumatic injury after craniectomy, followed by intraperitoneal administration of cyclosporine [Cs-A]).

In rats from group C (traumatic intraperitoneal polyethylene glycol), the weight of wet-dry tissues did not decrease significantly compared to the results of group B ($p < 0.05$). The results are summarized in Figure 1.

Table I: Experimental Groups

Group	Craniectomy	Trauma	Treatment
A	+	-	-
B	+	+	-
C	+	+	i.p. Polyethylene Glycol (PEG)
D	+	+	i.p. CsA (within PEG)

PEG: Polyethylene glycol, CsA: Cyclosporine A, i.p.: intraperitoneal.

Table II: Scores Obtained with the Ultrastructural Neuro-Destruction Grading System

Mitochondria (M)	Score	
	0	: Normal
	1	: Significant crystal
	2	: Swelling
	3	: Amorphous accumulation
Myelinated Axons		
(High Dimension)	Score	
	0	: Normal
	1	: Separation in the myelin layers
	2	: Destruction in the myelin layers
	3	: Honeycomb appearance
Myelinated Axons (Medium Dimension)		
	Score	
	0	: Normal
	1	: Separation in the myelin layers
	2	: Destruction in the myelin layers
	3	: Honeycomb appearance

Determination of the amount of malondialdehyde

To validate the experimental methods, the amounts of malondialdehyde were determined as nanomoles per gram of wet tissue to measure the rate of lipid peroxidation. The results of the intact control group (Group A) were compared with the results of the injured control group (Group B) to confirm the experimental model of injuries and surgery. The results clearly showed that cerebral edema did not occur with the craniectomy alone ($p < 0.05$). Comparison of the results of group D with those of groups B and C revealed a significant difference in the rate of lipid peroxidation ($p < 0.05$, $n = 6$). The results are shown in Figure 2.

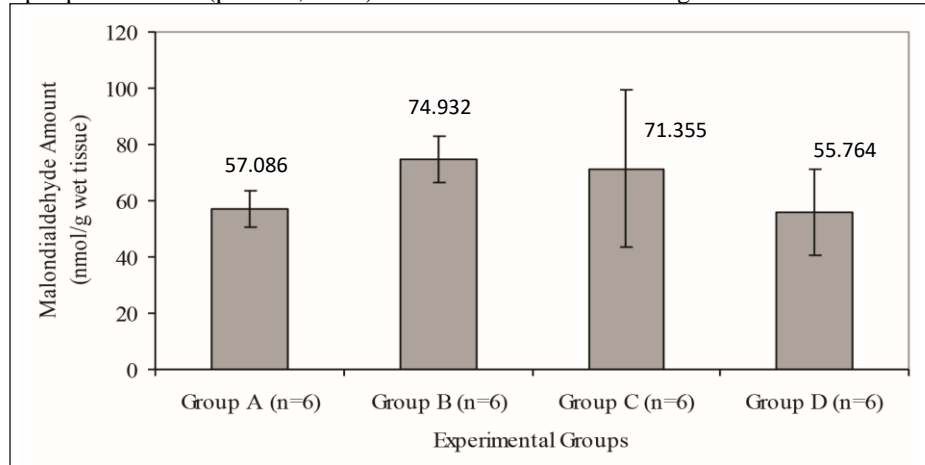


Figure 2 lipid peroxidation ratios, expressed in terms of malondialdehyde amounts (**Group A** only craniectomy; **Group B** traumatic injury after craniectomy but no treatment; **Group C** traumatic injury after craniectomy, followed by administration of the vehicle; **Group D** traumatic injury after craniectomy, followed by intraperitoneal administration of cyclosporine [Cs-A]).

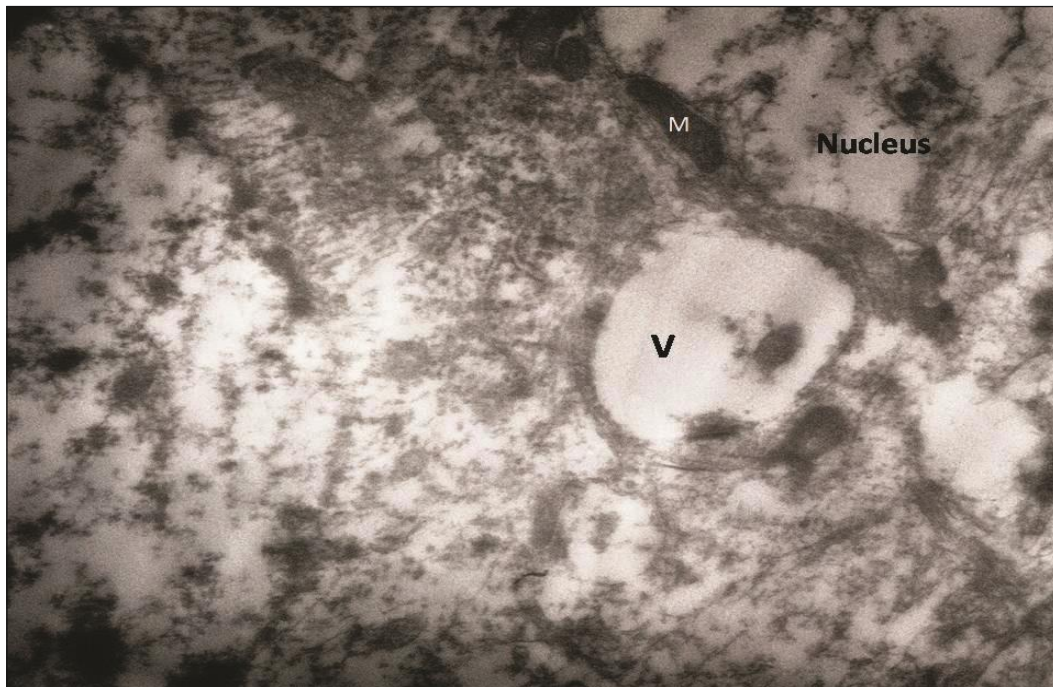
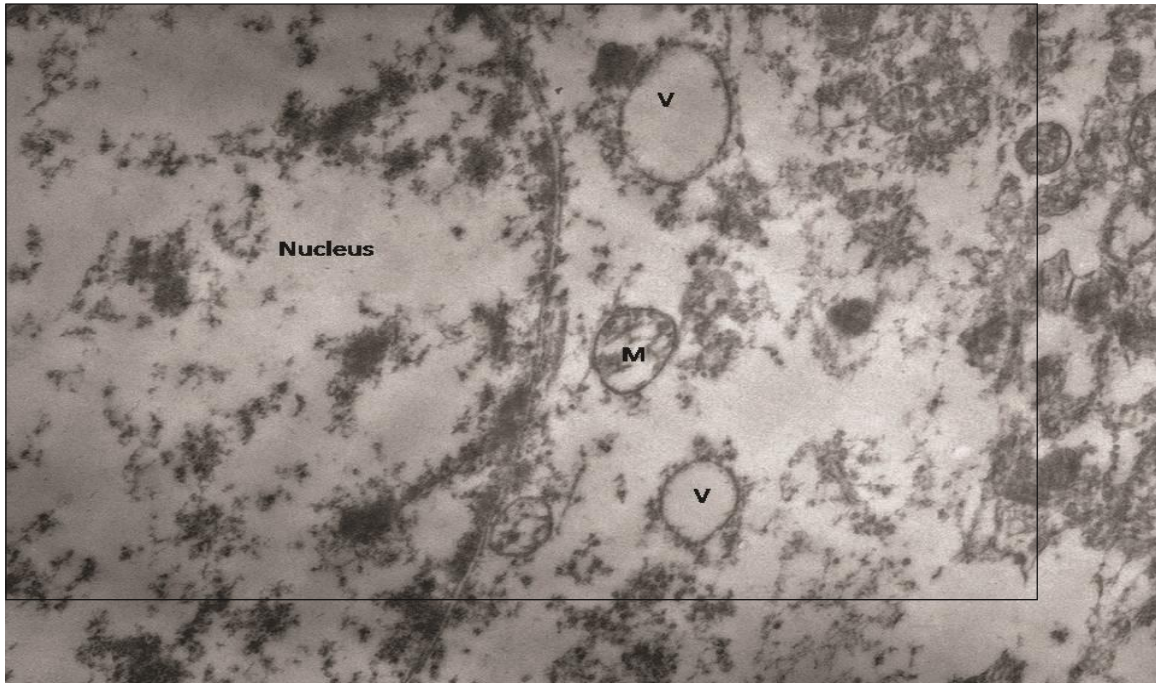
Ultrastructural classification system for neurodestruction

The UNGS scoring system was used to measure the data obtained from the microscopic assessments. The parameters and overall results tested for all groups are summarized in Table III.

Table III: Investigational Parameters and Scores of the Experimental Groups

Parameter	Group			
	A	B	C	D
Mitochondria 1	12	34	28	24
Mitochondria 2	09	28	26	22
Large Sized Myelinated Axons 1	07	43	35	36
Large Sized Myelinated Axons 2	5	36	33	32
Medium Sized Myelinated Axons 1	2	26	21	13
Medium Sized Myelinated Axons 2	4	20	25	12

The Mann-Whitney U test was used to compare all UNGS results for each of the tested parameters. The results of the research showed that the results of group D significantly differed from the results of group B ($p < 0.05$, $n = 2$). Ultrastructural electro-microscopic examples of groups B and D are shown in Figures 3 and 4.



DISCUSSION:

TBI is a medical and social problem characterized by high mortality and morbidity in adults, and accounts for approximately 30% of deaths from direct injury¹¹⁻¹². There are two main categories of head injury: primary and secondary. Primary trauma occurs immediately after the injury, and secondary trauma is caused by a series of adverse events that begin immediately after the injury¹³⁻¹⁴. Therefore, the primary trauma is exacerbated by the secondary damage that causes neurological dysfunction. Secondary damage appears to be associated with post-traumatic neurochemical changes that may exert a direct pathogenic effect on brain metabolism and ion homeostasis, and have a neurotoxic effect. These cellular and molecular changes can include changes in the synthesis and release of neuroprotective, self-destructive, or neurotoxic cascades¹⁵. Following TBI, decreased levels of cellular adenosine triphosphate (ATP) and increased oxidative stress are believed to be the main causes of cell death. In addition, an increase in glutamate levels causes an over-activation of N-methyl-d-aspartate (NMDA) receptors, resulting in a disproportionate influx of calcium (Ca²⁺) into the

cell matrix, which is detrimental, and it turned out to result in a neural outcome¹⁶. The increase in intracellular Ca²⁺ can be reduced by activation of ion pumps in the cell membrane as well as by cellular organelles such as the endoplasmic reticulum and mainly by the mitochondria. Mitochondria are considered essential organelles in the maintenance and management of intracellular Ca²⁺, especially through Ca²⁺ storage through various mechanisms. The marked increase in Ca²⁺ in the cell after glutamate exposure induces several cell damage reactions, such as an increase in free radical production and changes in the bioenergy capacity of the mitochondria. The overproduction of oxygen free radicals is associated with an increase in the Ca²⁺ level in the mitochondria; however, Ca²⁺ can cause mitochondrial dysfunction in cell death following traumatic injury. Destruction of mitochondria by Ca²⁺ leads to an imbalance in mitochondrial homeostasis; Disruption of the mitochondrial membrane potential causes an increase in free radical formation and a decrease in ATP levels¹⁷. Destruction of the mitochondria manifests itself in swelling and build-up of amorphous deposits, causing the neurons to cease to function. It has been suggested that the pore opening of MPT is an important response in the progressive neuropathological crisis following TBI. CsA is used extensively in immunosuppression, but has also shown neuroprotection after TBI. It can be used to inhibit neurotoxicity induced by overproduction of

NMDA, but it also significantly reduces cytoskeleton alterations and Ca²⁺ induced axon loss. Recently, it has been found that neuroprotection occurs in a variety of ways, including inhibiting calcineurin, closing the pores of the mPT, or restricting the pore opening of the mPT by inhibiting the binding of cyclophilin to the mitochondrial matrix. release of pores and neurotrophic factors¹⁸. The interaction of CsA with the matrix cyclophilin results in inhibition of the pore opening of the mPT. CsA also increases mitochondrial membrane homeostasis, which provides membrane potential and is essential for life. It increases the viability of neurons by inhibiting MTP pores, thereby providing mitochondrial membrane potential and restoring ATP levels to cellular activity. The studies cited in the previous paragraph showed that the neuroprotection achieved by CsA was dose-dependent and that the therapeutic window after injury was up to 24 hours. Sullivan and colleagues concluded that 20 mg / kg of CsA administered intraperitoneally is the most neuroprotective treatment, while 10 mg / kg intravenous CsA is more effective in the studies by Okonkwo and colleagues. In our study, we used 20 mg / kg. To analyze the effect of CsA on the occurrence of brain edema after TBI, we used the wet dry weight method as described above. The statistical interpretation of the results was assessed at a significance level of less than 0.05 using the Mann-Whitney U test¹⁹. In the animals receiving CsA, the water content was significantly lower than in the control groups (p <0.05). Free radicals react with membrane phospholipids after TBI to produce the final product, malondialdehyde. It is known that malondialdehyde is responsible for destabilizing the cell membrane and causes cell death. In our study, we used the malondialdehyde reaction to evaluate the rate of lipid peroxidation, which explains the degree of brain swelling²⁰. The Mann-Whitney U test helps to assess the difference between the experimental groups. The results of animals receiving systemic CsA treatment (group D) were compared with the results of both control groups (groups A and B). The difference between group D and group B results was statistically significant (p <0.05). Increased calcium levels following traumatic brain injury causes mitochondrial degeneration²¹. In quantitative analysis, both mitochondrial deformation and myelin axon deformity were observed in more than 100 electron microscopic mitochondrial studies. The UNGS is used to score these studies and allowed us to assess the statistical difference between the group that received CsA after TBI (Group D) and the group that did not receive treatment after the injury (Group B). By using UNGS for evaluation, electron microscopy studies can provide new insight into

post-traumatic mitochondrial dysfunction such as mPT pore opening due to increased intracellular Ca²⁺ and the destruction of mitochondrial homeostasis²².

CONCLUSION:

As TBI is one of the most important public health problems globally, new pharmacological agents should be developed to provide neuroprotective protection after TBI. In our study, post-trauma CsA proved to be a promising neuroprotective drug, maintaining calcium homeostasis and restoring energy necessary for cell viability. More research is needed to evaluate the clinical use of CsA.

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