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Neuroprotective Effect of Magnesium Sulfate and Dexamethasone on Intrauterine Ischemia in the Fetal Rat Brain: Ultrastructural Evaluation

Fetal Rat Beyninde İntrauterin İskemide Magnezyum Sülfat ve Deksametazonun Nöroprotektif Etkisi: Ultrastrüktürel Değerlendirme

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ABSTRACT

AIM: The aim of this study was to investigate the neuroprotective effect of magnesium sulfate and dexamethasone on oxidative damage in intrauterine ischemia.

MATERIAL and METHODS: In this study, 19-day pregnant rats were divided into five groups. Fetal brain ischemia was achieved in the ischemia/reperfusion (I/R) group by bilaterally closing the utero-ovarian artery with aneurysm clips for 30 min and subsequently removing the aneurysm clips for 60 min for reperfusion. Mg (600 mg/kg) and dexamethasone (0.25 mg/kg) were administered 20 min before the I/R insult. The lipid peroxidation in the brain tissue was determined by the concentration of thiobarbituric acid reactive substances (TBARS). The mitochondrial score was calculated after an evaluation with electron microscopy.

RESULTS: Both the electron microscope and TBARS data showed a significant difference between the control and I/R groups. The Mg and dexamethasone treatment groups exhibited significantly lower TBARS values compared to the IR group. Similarly, the mitochondrial scores in the Mg and dexamethasone treatment groups were significantly lower than those in the I/R group.

CONCLUSION: Result showed that magnesium sulfate and dexamethasone prevent lipid peroxidation and reduce mitochondrial injury thus suggests neuroprotective effects in fetal rat brain in intrauterine ischemia-reperfusion (I/R) injury.

KEYWORDS: Dexamethasone, Fetal brain, Injury, Intrauterine, Ischemia, Lipid peroxidation, Magnesium

ÖZ

AMAÇ: Bu çalışmada amaç, intrauterin iskemide oluşan oksidatif beyin hasarında magnezum sülfat ve deksametazonun nöroprotektif etkisinin araştırılmasıdır.

YÖNTEM ve GEREÇLER: Bu çalışmada 19 günlük gebe sıçanlar beş gruba ayrıldı. Fetal beyin iskemisi, utero-ovaryan arterin bilateral olarak 20 dakika anevrizma klipleri ile kapatılması ve kliplerin çıkarılmasında 60 dakika sonra reperfüzyon ile elde edilmiştir. İskemi-Reperfüzyon (İ/R) hasarından 30 dakika önce intraperitoneal 600 mg/kg tek doz magnezyum sülfat ve 0.25 mg/kg dexametazon uygulanmıştır. Beyin dokusundaki lipid peroksidasyonu thiobarbituric acid reaktif madde (TBARS) konsantrasyonu olarak belirlenmiştir. Elektron mikroskopisi ile mitokondri incelemesi yapıldıktan sonra mitokondriyal skor hesaplandı.

BULGULAR: Hem electron mikroskobu ve hem de TBARS verileri kontrol ve iskemi grupları arasında anlamlı farklılık gösterdi. Mg ve deksametazon tedavi grupları iskemi gubuyla karşılaştırıldığında anlamlı derecede daha düşük TBARS değerleri gösterdi. Benzer şekilde Mg ve deksametazon tedavi gruplarında mitokondriyal skorlar iskemi gruplarından anlamlı düzeyde daha düşüktü.

SONUÇ: Sonuçlar magnesyum sülfat ve deksametazonun lipid peroxidasyonunu önlediğini ve mitokondriyal hasarı azalttığını, böylece fetal rat beyninde intrauterin iskemi-reperfüzyon (IR) hasarında nöroprotektif etkisi olduğunu destekledi.

ANAHTAR SÖZCÜKLER: Deksametazon, Fetal beyin, Travma, İntrauterine, İskemi, Lipid peroksidasyonu, Magnezyum

INTRODUCTION

Brain injury due to intrauterine ischemia is one of the main causes of perinatal death and neurological injury (2, 19, 26). The reperfusion period that begins following ischemia is the most critical period when severe injury occurs. Treatments given before this period may be able to prevent or reduce hypoxic brain injury (1, 9, 24). Many interrelated mechanisms, such as the excessive stimulation of excitatory amino acid receptors, accumulation of intracellular calcium, lipid peroxidation, and free radical production play a role in pathophysiology of hypoxic brain injury (1, 2, 24). Animal studies have made important contributions toward explaining these mechanisms and identifying new treatment approaches (2, 9, 26).

The neuroprotective effects of magnesium sulfate and dexamethasone in ischemic brain injury are still being investigated. Their neuroprotective effects are explained by increased regional blood flow in the brain tissue, the nonspecific antagonism of voltage-dependent calcium channels, the noncompetitive inhibition of glutamate-dependent N-methyl-D-aspartate (NMDA) receptors, the inhibition of glutamate release, the reorganization of the ischemia-induced disruption of cellular metabolism, and the balancing of calcium levels in the mitochondria (2, 8, 26). The aim of the present study was to investigate the neuroprotective effects of magnesium sulfate and dexamethasone in intrauterine ischemic brain injury by brain tissue lipid peroxidation levels and brain tissue ultrastructural findings.

METHODS

This study was approved by the Institutional Animal Care and Use Committee of the Ankara Training Hospital in Ankara. The experiments were performed on 19-day-old pregnant Sprague Dawley rats (animal laboratory of Ankara Training Hospital in Ankara). The rats were housed separately at room temperature with a 12h dark-light cycle and were allowed free access to water and food. The rats were randomly divided into five groups, with six animals in each group. First group was the control group. Brain tissue was taken from the control group immediately after laparotomy (**Group 1**: Control). Brain tissue was taken from the sham group 90 min after laparotomy (**Group 2**: Sham). Fetal brain ischemia was achieved in the ischemia-reperfusion (I/R) group by bilaterally closing the utero-ovarian artery with aneurysm clips for 30 min and then removing the aneurysm clips for 60 min for reperfusion (**Group 3**: Ischemia-reperfusion). The magnesium sulfate treatment group received a single 600 mg/kg intraperitoneal dose of magnesium sulfate 20 min before the I/R injury (**Group 4**: magnesium sulfate). The dexamethasone treatment group received a 0.25 mg/kg IP dose of dexamethasone 20 min before the I/R injury (**Group 5**: dexamethasone). Tissue samples were taken immediately after I/R injury in Groups 3,4,5.

The surgical procedure was performed under general anesthesia with 10-mg/kg intramuscular injection of xylazine (Xylazine, Bayern, Istanbul, Turkey) and 60-mg/kg injection of ketamine hydrochloride (Ketalar, Pfizer, Warner Lambert, Istanbul, Turkey). After adequate anesthesia, the pregnant rats were placed in a supine position for the laparotomy procedure. Fetal ischemia was induced by bilaterally closing the utero-ovarian artery with aneurysm clips for 30 min. A surgical microscope was used to assure the complete blockage and restoration of blood flow in the utero-ovarian arteries. Reperfusion was achieved by removing the clips from the arteries and restoring circulation for 60 min. Whole fetal rat brains were removed at the end of the reperfusion period for tissue sampling. A very small portion of the brain tissue (≈1mm^3) from left motor cortex was preserved in glutaraldehyde for the electron microscopic analysis. The rest of the whole brains were stored in liquid nitrogen (-196^{0}C) for lipid peroxidation.

Determination of Lipid Peroxidation

The frozen tissue samples were weighed and homogenized in 1:10 (w:v) potassium phosphate buffer (50 mM, pH 7.4) with a Dounce homogenizer. The TBARS were measured as an index of lipid peroxidation using the method of Uchiyama and Mihara (22), and calculated as nano moles per gram of wet tissue.

Transmission Electron Microscopic Examination

The tissue samples were fixed in 2.5% glutaraldehyde for 6 hours, washed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) for 2 hours, and dehydrated in increasing concentrations of alcohol. Next, the tissue samples were washed with propylene oxide and embedded in epoxy-resin embedding media. Semi-thin sections measuring approximately 2 Gm in thickness and ultra-thin sections of approximately 60 nm in thickness were cut with a glass knife on an LKB-Nova (LKB-Produkter AB, Bromma, Sweden) ultramicrotome. The semi-thin sections were stained with methylene blue and examined using a Nikon Optiphot (Nikon Corporation, Tokyo, Japan) light microscope. The tissue blocks were trimmed after this examination, and ultra-thin sections were cut using the same ultramicrotome and stained with uranyl acetate and lead citrate. Following staining, all of the ultra-thin sections were examined with a Jeol JEM 1200 EX (Jeol Ltd., Tokyo, Japan) transmission electron microscope. The electron micrographs were taken by the same electron microscope. An investigator blinded to the study protocol examined the tissues. We used a grading system to perform a quantitative evaluation. The system was based on the method similar to that used for evaluating spinal cord tissue (10, 11). The mitochondria scoring parameters were as follows; 0: normal mitochondrion, 1: mitochondrion with prominent crista, 2: mitochondrion with cloudy swelling, 3: amorphous material collection inside the mitochondrion.

Statistical Analysis

The data were analyzed using a non-parametric Kruskal-Wallis test. The Mann-Whitney U test was used as a post-hoc analysis for the pairwise comparison of groups within any particular

assay. The data were expressed as the mean value ± SE. A p-value <0.05 was considered to be statistically significant.

RESULTS

Lipid Peroxidation

There were no significant differences between the control and sham groups. TBARS data exhibited a significant difference between the control and I/R groups (p<0.001 each), suggesting that the intrauterine I/R model generated significant rise in lipid peroxidation in the intrauterine brain injury. Both magnesium sulphate and dexamethasone treatment groups showed significantly lower TBARS values compared to the I/R group (p<0.01 and p<0.001, respectively). There was no difference between the TBARS results for the magnesium sulphate or dexamethasone treatment groups and the control groups. This finding suggests that pre-treatment with either magnesium sulphate or dexamethasone was sufficient to fully prevent to increase in lipid peroxidation in the brain tissue after I/R injury (Figure 1). When compared to each other magnesium sulphate and dexamethasone had no statistical difference in their effects on TBARS values or mitochondrial scores.

EM Findings

The transmission electron microscopic examination of the tissue samples from the control group revealed normal neuron, nucleus and mitochondria. There were no significant differences between the control and sham groups. Thus abdominal operation itself did not cause intrauterine ischemia. Statistical data revealed that there was significant difference between the control and I/R groups (p<0.001 each). Ischemia-reperfusion group revealed worse ultrastructural findings. Nearly all of the mitochondria in this group showed ultrastructural pathological changes and most of the mitochondria were swollen. Large vacuoles and intercellular edema were seen in the cytoplasm. No additional ultrastructural pathology was detected in nuclei, cell membranes or other organelles of the neurons. This result suggests that present intrauterine-I/R model generated significant mitochondrial damage and lipid peroxidation in the brain tissue exposed to I/R injury. Magnesium sulphate and dexamethasone treatment groups revealed less intercellular edema and better mitochondrial protection than ischemia-reperfusion group (Figure 2A-E). The mitochondrial scores in the magnesium sulphate or dexamethasone-treatment groups were significantly lower than those in the I/R injury group (p<0.001). However, there was also a significant difference between the drug-treated and control groups (p<0.001) (Figure 3), suggesting that pre-treatment with either of these drugs could decrease the mitochondrial damage, but not to the pre-injury levels.

When compared to each other magnesium sulphate and dexamethasone had no statistical difference in their effects on mitochondrial scores.

DISCUSSION

Intrauterine ischemic brain injury and its associated complications are serious problems in both developed and developing countries. The lack of adequate effective treatments for the late period after intrauterine ischemic brain injury caused researchers to investigate treatments that are designed to prevent intrauterine ischemic brain injury and reduce the damage that occurs in the early period (1, 10, 26). Although intrauterine ischemic brain injury causes complications, such as cerebral palsy, hearing and vision loss, attacks, and learning difficulties, it may be possible to treat it at the cellular level by reducing necrosis and apoptosis (8, 9, 26). In the early period, necrotic cell death occurs as a result of hypoxia and ischemia (primary injury) (1, 10, 11). After hours and days, neuronal death occurs during the late period, as a result of a complicated series of biochemical and molecular events that result in apoptosis (secondary injury) (1, 8, 11, 24). The chain of cellular events following ischemia and reperfusion period causes the excessive release of the excitatory amino acid glutamate, the stimulation of NMDA and other receptors, an increase in the calcium levels within

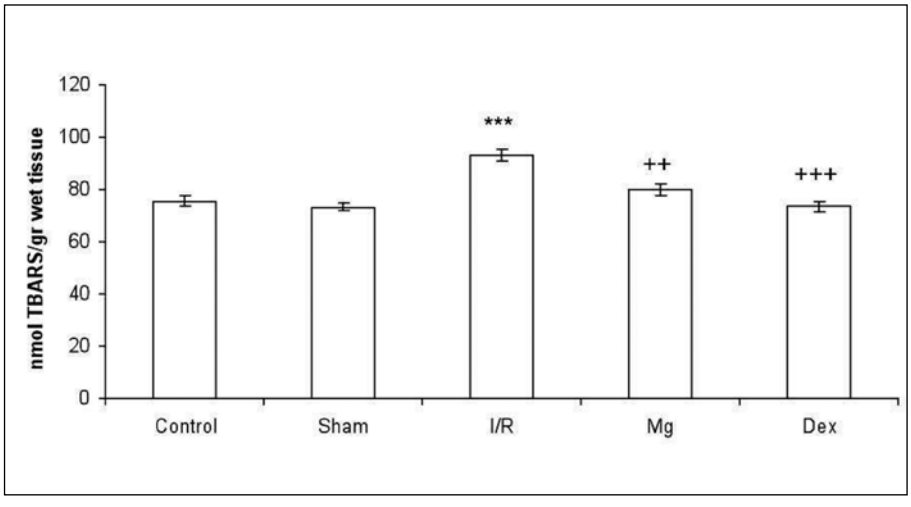

Figure 1: The levels of thiobarbituric acid reactive substances (TBARS) in fetal rat brain in control, sham, I/R and drug-treated groups. There is no significant difference in the levels between the control and sham groups. The lipid peroxidation products increased after ischemia-reperfusion (I/R) injury, but treating mothers with either magnesium sulfate (Mg) or dexamethasone significantly decreased the TBARS values compared to the I/R group. Asterisk represents comparison relative to the control and plus sign represents comparison relative to the I/R injury group (*** or +++ for p<0.001, ++ for p<0.01).

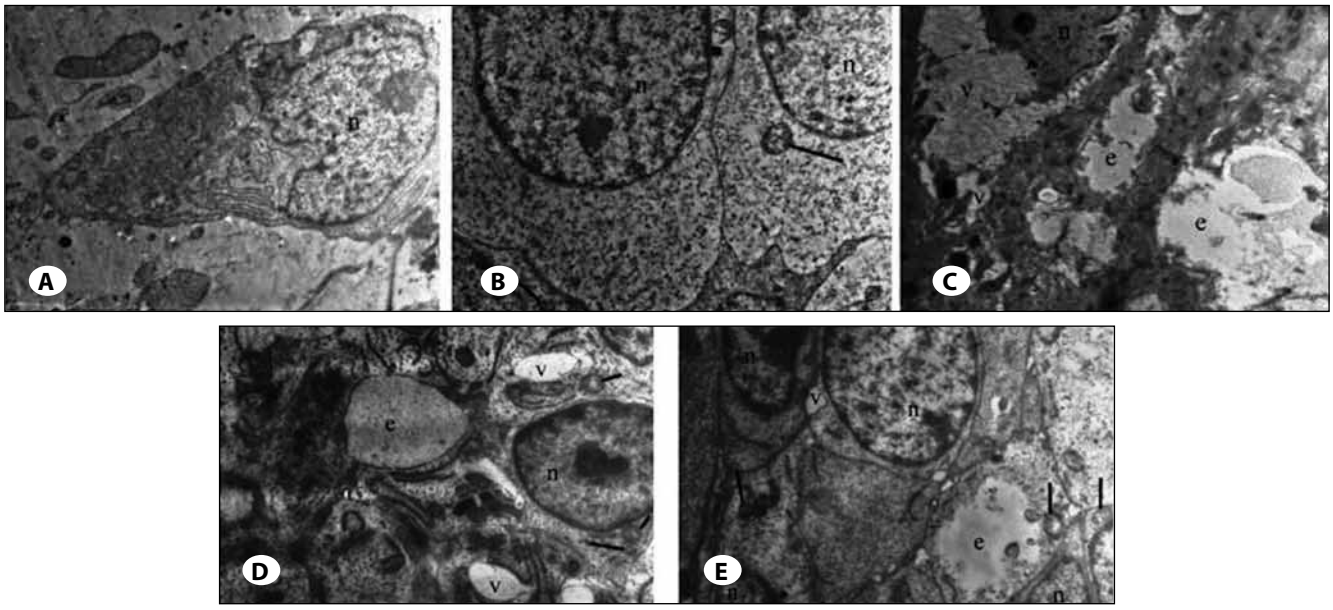

Figure 2: Electron microscopic evaluation. **(A)** A normal neuronal structure is observed in the control group. **(B)** Normal neurons and mitochondria are observed in the sham group (arrow). **(C)** Severe injury is observed in the ischemia-reperfusion (I/R) group. Intercellular edema is observed among the neurons and intracytoplasmic organelles attached to the large vacuoles. **(D)** Intercellular edema, intracytoplasmic vacuoles and mitochondria, and prominent crista are observed in the magnesium sulfate (Mg)-treatment group (arrows). **(E)** Intercellular edema, intracytoplasmic vacuoles and mitochondria, and prominent crista are seen in the dexamethasone-treatment group (arrows). Magnification: 6000X. Abbreviations: **n:** neuron nucleus, **e:** edema, **v:** vacuole.

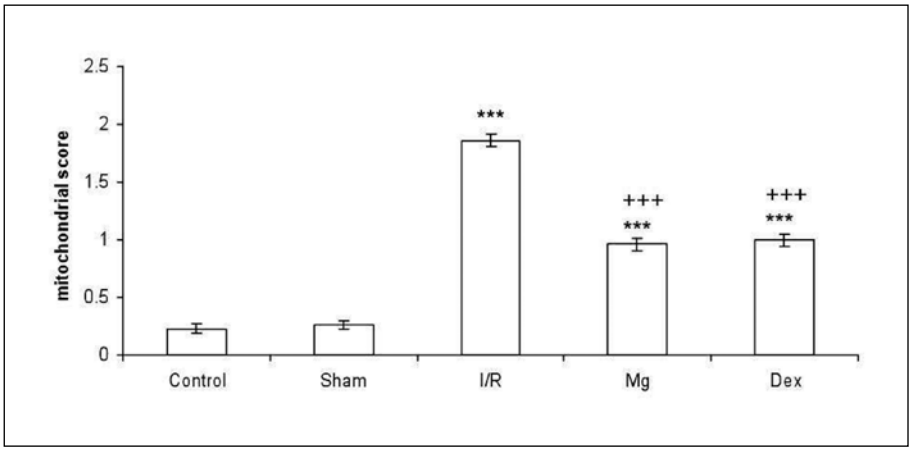

Figure 3: Mitochondrial scores of fetal rat brain in control, sham, I/R and drug-treated groups. The ultrastructural evaluation shows that mitochondrial damage occurred in the ischemia-reperfusion (I/R) group, but treating mothers with either magnesium sulfate (Mg) or dexamethasone significantly decreased the mitochondrial score compared to that of the I/R group. Asterisk represents comparison relative to the control and plus sign represents comparison relative to the I/R injury group (*** or +++ for $p<0.001$).

the cells, lipid peroxidation and the formation of arachidonic acid metabolites, the production of free oxygen radicals, and the activation of proteases, which result in irreversible cell death (2, 18, 19). To date, various pharmacological agents and methods have been used to prevent injury in intrauterine ischemic brain injury models of the newborn (2, 9, 24).

Magnesium sulphate is the main element in normal cellular functions, such as membrane integrity, cellular respiration, the establishment of normal sodium-potassium levels, and the regulation of calcium transport and accumulation (6, 7, 18, 21). It is used to limit secondary neuronal injury and ameliorate neurological consequences in some animal models of brain and spinal injury (6, 7, 12). Magnesium sulphate was shown to have a direct effect on cellular metabolism during injury development after traumatic brain injury, in addition to playing a critical role in the regulation of other secondary injury factors (7, 12, 21). Mg prevents Ca^{+2} entry into the cell by blocking NMDA receptors, thereby reducing intrauterine ischemic brain injury (14, 17, 18). It has been shown that, magnesium sulphate treatment was a safe and effective method for preventing neurological complications in obstetrics patients (6, 17, 18). Mg treated rats that were subjected to middle cerebral artery occlusion and reperfusion showed reduced brain infarction by accelerating electrophysiological and neurological healing (16, 17, 23).

Researchers have shown that magnesium sulphate treatment reduced the delayed cerebral ischemia that occurred in patients with aneurysmal subarachnoid hemorrhage (SAH). A clinical study compared the effects of magnesium sulphate and nimodipine on the prevention of a delayed ischemic neurological deficit and determined that the utilization of magnesium reduced morbidity and hospitalization time (14, 16, 23). We found that magnesium sulphate decreased both the TBARS values and mitochondrial scores when compared to ischemic brain injury group, however the effect of magnesium sulphate on mitochondrial scores was not as strong as its effect on TBARS. Our findings support that magnesium sulphate has neuroprotective effect by preventing lipid peroxidation and reducing mitochondrial damage and this is in accordance with the findings of other related studies (7, 17, 18).

Dexamethasone is a synthetic corticosteroid that was used in brain edema and spinal cord injuries for a long time. Dexamethasone has demonstrated a beneficial inhibition of lipid peroxidation and neuroprotective effects (4, 5, 15). The dexamethasone exerts a series of effects on injured neural tissue (13, 20). These effects are the regulation of energy metabolism, the prevention of progressive posttraumatic ischemia, the prevention of neurofilament degradation and the inhibition of membrane lipid hydrolysis (3, 4, 25). Thus, it has been long believed that, with dexamethasone treatment, the structural and functional integrity of biological membranes can be preserved (3, 15). The primary mechanism of the neuroprotective effect of dexamethasone is the inhibition of lipid peroxidation (4, 5, 15). In our work, dexamethasone decreased the TBARS values back to control levels and decreased the mitochondrial damage when compared to ischemic brain injury group. The neuroprotective effect of dexamethasone is well correlated with previous studies (4, 5, 13, 15). When compared to each other magnesium sulphate and dexamethasone had no statistical difference in their effects on TBARS values or mitochondrial scores suggesting that both drugs were equally neuroprotective in our study.

In summary, the neuroprotective effects of magnesium sulphate and dexamethasone on the central nervous system have been shown in previous experiments. In our work we evaluated the neuroprotective effects of magnesium sulphate and dexamethasone on fetal rat brain in an intrauterine ischemia-reperfusion model. According to our lipid peroxidation values and ultrastructural findings magnesium sulphate and dexamethasone may have a protective effect on intrauterine ischemic brain injury. The exact neuroprotective mechanisms of the treatments need to be elucidated in future studies.

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