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The Impact of Salubrinal in Preventing Fetal Brain Damage in a Model of Chorioamnionitis Induced by LPS

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Abstract

Objective
Chorioamnionitis (CRY), with membrane rupture, preterm labor, prolonged labor, smoking, and bacterial or viral infection origin; is a condition that presents a risk for both maternal and neonatal sequelae. Our study aimed to investigate the effect of Salubrinal (SLB), an endoplasmic reticulum (ER) stress inhibitor, against damage to placental tissue and fetal brain in the Lipopolysaccharide (LPS) induced CRY model.

Material and Method
In this study, 24 Wistar Albino rats on the 17th gestational day; were divided into 4 groups; control, LPS (1 mg/kg intraperitoneal (ip)), LPS + SLB (1 mg/kg LPS ip and 1 mg/kg SLB ip) and SLB (1 mg/kg ip). After an experimental hysterectomy, the placenta and fetal brain tissues were taken into formaldehyde solution for histopathological analysis.

Results
According to the findings obtained; widespread congestion in the basal zone, degeneration of trophoblastic cells in the labyrinth zone, and inflammatory cell infiltrations in both basal and labyrinth zones were observed in the placental tissues of the LPS group. No pathology was detected in only the SLB group. While edema and congestion were detected in the ventricular and intermediate zones in the fetal brain tissues of the LPS group, a significant improvement was observed in these findings with SLB treatment.

Conclusion
As a result; ER stress is one of the mechanisms that play a role in placental tissue and fetal brain damage due to CRY, and SLB therapy might prevent this damage.

Keywords: Chorioamnionitis, endoplasmic reticulum stress, lipopolysaccharide, salubrinal

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Introduction

Preterm labor (PL) is defined as birth before 37 completed weeks of gestation and is the leading cause of neonatal mortality. Survivors of PL are likely to experience long-term neurological disorders (1,2).

PL usually occurs due to intrauterine infection. In pregnancies with signs of intrauterine infection, an increase in the levels of cytokines such as interleukin-1beta, interleukin-8, and tumor necrosis factor-α (TNF-α) has been observed and it has been revealed that these cytokines predispose to the formation of PL (3,4). In addition, inflammatory cytokines play a central role in the intrauterine infection process that triggers brain damage. There is a risk of white matter damage and thus, cerebral palsy in premature babies who are exposed to high concentrations of pro-inflammatory cytokines in the amniotic fluid or fetal blood (5,6). Intracerebral and systemic Lipopolysaccharide (LPS) injections induce inflammatory responses and significant white matter damage (7).

The endoplasmic reticulum (ER) is an organelle that ensures that secretory and membrane proteins fold correctly and travel to the target site after transcription (8). It acts as a quality control center for protein synthesis. When the ER cannot fulfill its function for various reasons, unfolded or misfolded proteins accumulate in the ER lumen, leading to ER stress. To cope with this stress, the ER activates intracellular signaling pathways called unfolded protein response (UPR) and tries to restore homeostasis (9).

Salubrinal (SLB) is a selective inhibitor of the de-phosphorylation of eukaryotic initiation factor 2α (eIF2α), which is critical for ER stress. Inhibition of dephosphorylation of eIF2α provides a cytoprotective effect against ER stress by reducing its workload (10,11).

This study aimed to investigate the effect of SLB, which inhibits the dephosphorylation of eIF2α in the LPS-induced chorioamnionitis model, on placental and fetal brain damage in rats.

Material and Method

Experimental Animals

The study was carried out at Süleyman Demirel University Experimental Animal Production and Experimental Research Laboratory. Ethical approval was obtained from the Animal Experiments Ethics Committee of Süleyman Demirel University with the decision of 17.10.2019/04.

Pregnant Wistar Albino female rats, 8-10 weeks old, were used in our study. The animals' environmental conditions are 12 hours of light and 12 hours of darkness, humidity (55-60%), and constant temperature (22±2 ºC) were provided in Euro type-4 cages. Standard care, nutrition, and housing conditions were provided for the experimental animals. Male and female rats were kept in the same housing environment. To detect vaginal plaque formation in rats, cotton swabs were wetted with physiological saline (SF), and a swab sample was taken from the vagina of female rats using these swabs and spread on a slide. In addition, swab samples taken to determine whether there was sperm in the vaginas of female rats were immediately diagnosed with a binocular light microscope. For the staining process, the swab samples showing sperm were kept in the open air to dry and then stained with crystal violet. 0.2 g of crystal violet was dissolved in 200 mg of distilled water for dyeing. The samples were kept in the dye solution for 1 minute and were washed with distilled water 2 times in succession for 1 minute each. After all these procedures, the samples were sealed using an aquose-mounte solution. Using the smear results, female rats were divided into 4 groups, each with 6 pregnant rats.

1- Control Group (n=6): Pregnant rats were injected with saline (0.5-1 ml volume, intraperitoneal [ip]) from their right inguinal region twice consecutively on the 17th day of the experiment.

2- LPS Group (n=6): Pregnant rats were injected with LPS (0.5-1 ml volume, 1 mg/kg, ip) in their right inguinal region on the 17th day of the experiment and then saline (0.5-1 ml volume, ip) was injected into the same region (12).

3- LPS+SLB Group (n=6): On the 17th day of the experiment, pregnant rats were given LPS (L2630, Sigma-Aldrich, MerckSa, Darmstadt, Germany) (0.5-1 ml volume, 1 mg/kg, ip) from their right inguinal region and then SLB (SML0951, Sigma-Aldrich, MerckSa, Darmstadt, Germany) (0.5-1 ml volume, 1 mg/kg, ip) were given from the same region (13).

4- SLB Group (n=6): On the 17th day of the experiment, pregnant rats were given saline (0.5-1 ml volume, 1 mg/kg, ip) from their right inguinal region and then SLB (0.5-1 ml volume, 1 mg/kg, ip) were given from the same region.

Preterm labor was performed via hysterotomy operation 6 hours after saline injection in the control group and 6 hours after LPS injection in the other

groups. Hysterotomy was performed under anesthesia by applying 10 mg/kg isoflurane to all experimental animals. Following the abdominal incision, placental tissues and fetuses were removed by clamping the artery feeding the placental tissues. Then, blood was taken from the inferior vena cava of the rats and surgical exsanguination was performed. The removed placental tissues, fetuses, and fetal brain tissues taken from fetuses were stored in formaldehyde.

Histopathological Evaluations

The collected tissues were kept in a 10% neutral formaldehyde solution for fixation for at least 48 hours. To perform the tissue tracking procedure, the tissues were washed with running water overnight after their fixation and were kept in alcohol-xylol solutions for specific periods. They were then embedded with paraffin. 5µm sections were taken from paraffin blocks with a Leica RM 2155 RT microtome (Leica Microsystem, Nussloch, Germany). After one day of drying, the slides were passed through the xylol and alcohol series. Then, tissues stained with hematoxylin-Eosin were evaluated under an imaging-assisted binocular light microscope (ECLIPSE Ni-U, Nikon, Japan), and photographs were obtained. Histopathological scoring was made according to the degree of the detected finding as (-): no finding, (+): low-level finding, (++): moderate finding, (+++): severe finding.

Results

Smear Results

To determine the animals to be included in the study, pregnancy status was checked by the presence of sperm in the vaginal canal. In crystal violet staining performed for this purpose, vaginal epithelial cells and sperm were observed in positive samples (Fig.1).

Histopathological Findings in Fetal Placenta Samples

In the histopathological evaluation of the tissues, no pathological findings were found in the fetal placenta samples of the rats in the control group. Significant histopathological changes (diffuse congestion in the basal zone, degeneration of trophoblastic cells in the labyrinth zone, and inflammatory cell infiltrates in both the basal and labyrinth zones) were observed in the fetal placenta samples of rats in the LPS group. A decrease in congestion in the basal zone and degeneration of trophoblastic cells in the labyrinth zone was detected in the fetal placenta samples of rats in the LPS+SLB group compared to those in the LPS group. In addition, inflammatory cell infiltrates in the basal and labyrinth zones seen in the LPS group had a near-normal histological appearance in the LPS+SLB group. A similar appearance was observed in the fetal placenta samples of SLB group rats as in the control group. Fetal placenta basal zone histologic images are presented in Figure 2, fetal placenta labyrinth zone histologic images are displayed in Figure 3, and the scoring table of histological evaluations is shown in Table 1.

Histopathological Findings in Fetal Brain Samples

In histopathological evaluation, the fetal brain tissues of the control group animals had a normal histological appearance. Edema and congestion were observed in the ventricular and intermediate zones in the fetal brain tissues of LPS group animals. A significant decrease in edema and congestion in the ventricular and intermediate zones was observed in the fetal brain tissues of LPS+SLB group animals compared to the LPS group. No histopathological findings were detected in the fetal brain tissues of SLB group animals, like the control group. Histological images of

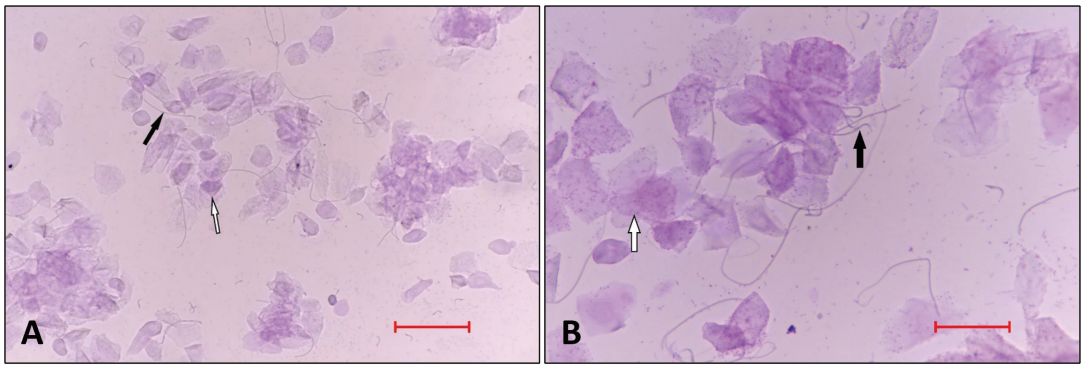

Figure 1
Positive vaginal smear.
Sperms (black arrow) and vaginal epithelial cells (white arrow).
Crystal violet, A: 20x, scale bar = 100µm, B: 40x, scale bar = 50µm.

Table 1 Histopathological evaluations of the basal zone and labyrinth zone in the placenta

Parameter/Group		Control	LPS	LPS+SLB	SLB
Basal zone findings	Congestion	-	+++	+	-
	Inflammatory cell infiltrates	-	+++	+	-
Labyrinth zone findings	Trophoblastic cell degeneration	-	+++	+	-
	Inflammatory cell infiltrates	-	++	-	-

LPS – Lipopolysaccharide; SLB – Salubrinal.

Table 2 Histopathological evaluations of the basal zone and labyrinth zone in the placenta

Parameter/Group	Control	LPS	LPS+SLB	SLB
Congestion	-	+++	+	-
Edema	-	+++	+	-

LPS – Lipopolysaccharide; SLB – Salubrinal.

fetal brains are shown in Figure 4, and the score table of histological evaluations is shown in Table 2.

Discussion

Despite the developments in treatment strategies in the last 20 years, systemic inflammation, which can develop for various reasons during pregnancy, can have a dramatic course for the mother and the fetus. Although the survival rates of these babies have increased day by day with improvements in treatment methods, severe pathological conditions, especially neurodevelopmental disorders, occur in surviving premature babies (14).

Damage mechanisms such as oxidative stress and apoptosis in systemic inflammation not only affect the vascular structures and cause damage to the endothelium, disrupting the blood supply of the tissue but also bind to receptors in the tissues through circulating cytokines and activate some intracellular pathways. With the activation of intracellular pathways, the synthesis and release of cytokines or damage markers occur. In this context, Karakuyu et al. suggested that exposure to LPS triggered cell death in lung cells by triggering ROS production (15). Bao et al. reported that after LPS administration, there was an increase in the levels of TNF-α, IL-6, and chemokine ligand 1 in the placentas of Sprague Dawley rats and that these cytokines disrupted the typical structure of the placenta and increased pathological damage (16). In our study, the labyrinth zone of the placenta facing the fetus, the basal zone facing the mother, and the brain tissue were examined separately. As a result of these histopathological examinations, degeneration of trophoblastic cells was observed in the labyrinth zone, and inflammatory cell infiltrates were observed in both the basal zone and the labyrinth zone. In the study by Yavuz et al., slight hyperemia and edema findings were obtained in the histopathological analysis of the rat cerebral cortex after 1 mg/kg intraperitoneal LPS administration. Considering the results obtained in our study, this proves the accuracy of the LPS dose and route of administration (17). Areas of congestion in the tissue should be evaluated as causing a tendency to bleed in the damaged area. The brain tissue results, which should be parallel to this placental inflammation, also support our hypothesis that inflammation first causes damage to the placenta and affects the brain tissue due to increased permeability. The fact that congestion and edema developing in the ventricular and intermediate zones are also

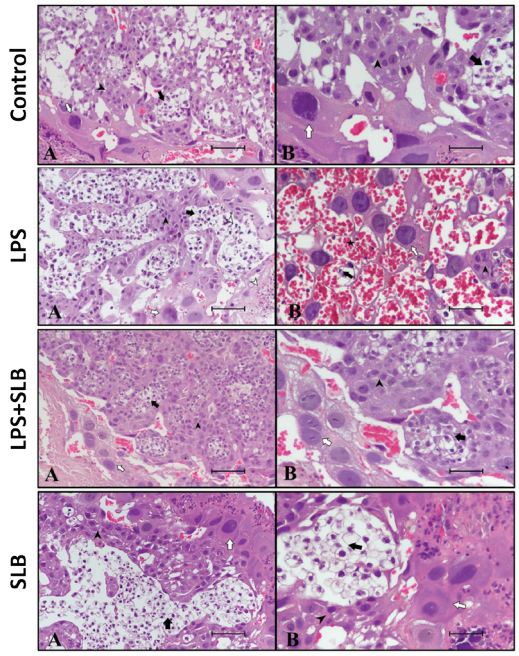

Figure 2

Fetal placenta basal zone histological images. (A) Trophoblastic giant cells (white arrow), glycogen cells (black arrow), spongiotrophoblasts (black arrowhead), inflammatory cell infiltrates (white arrowhead). H-E, 20x, scale bar; 100 µm. (B) Trophoblastic giant cells (white arrow), glycogen cells (black arrow), spongiotrophoblasts (black arrowhead), diffuse areas of congestion (black star). H-E, 40x, scale bar; 50 µm.

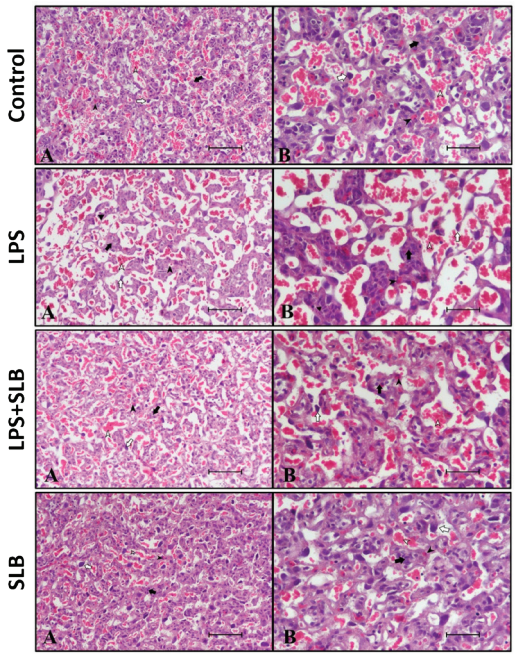

Figure 3

Fetal placenta labyrinth zone histological images. (A) Cytotrophoblasts (white arrow), syncytiotrophoblasts (black arrow), maternal vessels (white arrowhead), fetal vessels (black arrowhead), inflammatory cell infiltrates (black triangle). H-E, 20x, scale bar; 100 µm. (B) Cytotrophoblasts (white arrow), syncytiotrophoblasts (black arrow), maternal vessels (white arrowhead), fetal vessels (black arrowhead), inflammatory cell infiltrates (black triangle). H-E, 40x, scale bar; 50 µm.. LPS – Lipopolysaccharide; SLB – Salubrinal.

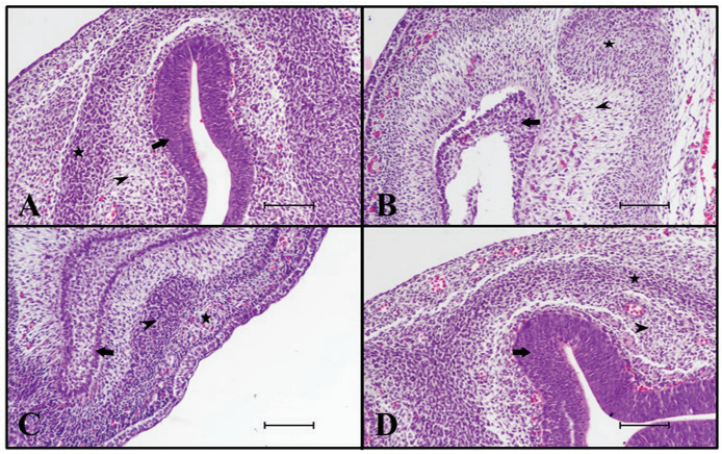

Figure 4

Histopathological images of fetal brain tissues (A) Control group; ventricular zone (arrow), intermediate zone (arrowhead), and cortical plate (star) have normal histological appearance. (B) LPS group; Edema and congestion in the ventricular zone (arrow) and intermediate zone (arrowhead), normal histological appearance in the cortical plate (asterisk). (C) LPS+SLB group; Decrease in histopathological findings in the ventricular zone (arrow), intermediate zone (arrowhead), and cortical plate (asterisk). (D) SLB group; ventricular zone (arrow), intermediate zone (arrowhead), and cortical plate (star) have a normal histological appearance. H-E, 20x, scale bar = 100 µm.

observed in the fetoplacental region is evidence that increased permeability may also damage cerebral tissue. According to the study conducted by Moradi Vastegani et al., it was found that the permeability of the blood-brain barrier (BBB) was increased in LPS-treated rats compared to the control group (18). This confirms that the increase in permeability of the BBB in the baby, through cytokines that cause systemic inflammation, plays a significant role in the progression of the damage.

Our findings concluded that SLB treatment prevented LPS-induced placental and fetal brain damage and did not show any signs of damage in the SLB alone group. SLB has an essential role in ER stress as a blocker of the dephosphorylation of eIF2α. According to the results obtained from our histopathological analysis, the regression of the damages shows that ER stress plays a role in the pathogenesis of the mentioned damage.

SLB has been described as a neuroprotective agent in different nervous system pathologies, demonstrating the importance of reducing ER stress as a therapeutic target to alleviate neural damage (13). Therefore, it has been suggested that treatment with SLB provides cytoprotection related to protein kinase RNA-like endoplasmic reticulum kinase (PERK)- Eukaryotic Initiation Factor 2 alpha (eIF2α) signaling in spinal cord injury (19). It has been demonstrated that in traumatic brain injury, SLB prevents neuronal death in the cortex of mice exposed to trauma by alleviating ER stress (20).

The neuroprotective effect of SLB on ischemic injury has been reported in the middle cerebral artery occlusion and global cerebral ischemia (GCI) model. The neuroprotective effect of SLB in GCI was demonstrated as a reduction in neuron loss seven days after damage to the cornu ammonis 1 (21). This study also hypothesizes that the UPR in cornu ammonis1 cannot overcome ER stress after ischemia and that SLB contributes more to overcoming ER stress.

One of the study's limitations is that the effect of SLB in alleviating the damage to the placenta and fetal brain tissues secondary to experimental CRY was revealed only by histopathological analysis. Cellular pathways need to be shown with more studies. In addition, within the scope of our study model, we believe that a single dose of SLB after injury will not be sufficient to generalize the current results. In this context, studies evaluating the post-treatment effects of SLB for more extended treatment periods and longer follow-up periods are needed.

In line with the findings obtained in this study, we concluded that SLB alleviates LPS-induced placental and fetal brain damage. In this context, the mechanisms of placental and fetal brain damage secondary to systemic inflammation should be investigated more, ER stress-related mechanisms should be detailed, target genes should be increased, and new treatment modalities should be tried. We think the results of this study will shed light on future research on this subject.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
The study was carried out at Süleyman Demirel University Experimental Animal Production and Experimental Research Laboratory. Ethical approval was obtained from the Animal Experiments Ethics Committee of Süleyman Demirel University with the decision of 17.10.2019/04.

The animals' environmental conditions are 12 hours of light and 12 hours of darkness, humidity (55-60%), and constant temperature (22±2 ºC) were provided in Euro type-4 cages. Standard care, nutrition, and housing conditions were provided for the experimental animals.

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Availability of Data and Materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors Contributions
PI: Conceptualization; Data curation; Investigation; Methodology; Validation; Visualization; Writing-original draft.

SO: Conceptualization; Data curation; Investigation; Methodology; Validation; Visualization; Writing-original draft.

MS: Conceptualization; Data curation; Investigation; Methodology; Validation; Visualization; Writing-review & editing.

KG: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing- review & editing.

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