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RESEARCH ARTICLE

Syzygium cumini (L.) Seed Extract Protects Embryofoetal Brains against Intrauterine Oxidative Toxicity in Rats during Hypoxia-reperfusion Injury

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ABSTRACT

Intrauterine hypoxia-reperfusion is an important cause of neonatal brain injury. Thus, we tested whether *S. cumini* seed extract (SE) protects the incidence of embryofoetal oxidative toxicity in rat fetal brains produced by intrauterine hypoxia-reperfusion injury. Hypoxia was induced by uteroplacental occlusion followed by reperfusion. Brain proinflammatory tumor necrosis factor alpha (TNF-α), the levels of lipid peroxides (LPO) and reduced glutathione (GSH), activities of catalase (CAT) and superoxide dismutase (SOD) and acetylcholine esterase (AChE) enzymes were assessed. Histology of the fetal brain slices was studied after hematoxylene/eosin staining. The hypoxia-reperfusion increased the levels of TNF-α, LPO and decreased the activities of CAT and SOD enzymes and levels of GSH in the fetal brains. The oral preadministration of the *S. cumini* (L.) seed extract to the mother rats, however, significantly inhibited the rises of the levels of TNF-α and the oxidative stress in the fetal brains and increased the activities of CAT and SOD enzymes and levels of GSH in the fetal brains concurrently. Histological studies revealed decreased structural disintegration and inflammatory necrosis in the fetal brains of *S. cumini* + hypoxic mothers. We, therefore, conclude that oral preadministration of *S. cumini* (L.) seed extract to pregnant rats might prevent reproductive and fetal development impairments caused by intrauterine proinflammatory and hypoxia-reperfusion injury.

KEYWORDS

Oxidative stress, TNF-α, embryofoetal brain, hypoxia, *Syzygium cumini* (L.), antioxidative enzymes

INTRODUCTION

Intrauterine hypoxia (also referred to as birth or perinatal asphyxia) is used to describe a physiological situation that results from inadequate supply of oxygen immediately prior to, during or just after delivery. The perinatal hypoxia causes functional motor and cognitive deficits[1,2] . Hypoxia followed by cerebral artery occlusion, also increases pro-inflammatory TNF-α protein in the neurons, astrocytes, microglia, choroid plexus, endothelial cells, and infiltrating polymorphonuclear leukocytes[3,4].

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Primary energy failure and oxidative stress are responsible for neuronal damage and tissue injury followed by excitotoxicity, inflammation, and apoptosis[5]. In the primary stage, free radicals affect lipids, proteins and carbohydrates, leading to lipid peroxidation and disintegration of cell membranes[6,7]. Impairments of protein synthesis act as an early indicator of cell death due to primary hypoxic event[8].

A second wave of neuronal cell death occurs during the "reperfusion" phase that causes the production and release of oxygen radicals, synthesis of nitric oxide (NO) and inflammatory

reactions[9,10]. These reports thus support that by inhibiting or preventing the oxidative stress/proinflammatory response antioxidants can protect the fetal brain from ischemic damage if these can be administered to pregnant mother during the gestation period.

Therefore, *Syzygium cumini* (L.) seed extract, one of our most investigated natural antioxidants, can be used as one of the oxidative stress-defenders. We have recently reported that the *S. cumini* (L.) seed extract improves learning-related memory deficits in old rats, hypoxia-induced oxidative stress after carotid artery occlusion and brain cerebral oxidative potentials of alcoholic rats[11,12]. Fenton-reagent-induced *in vitro* oxidative stresses in the brain and liver tissue homogenates also were inhibited after co-incubation with *S. cumini* (L.) seed extract[13].

These reports are of significant importance because of the fact that Bangladesh has a high maternal mortality ratio, with 320 deaths per 100,000 births[14]. Bangladesh is continually fighting, as one of the highest priority issues, for the reduction of her higher fetal mortality. Although the understanding of perinatal asphyxia-related pathophysiology is gradually increasing, limited therapeutic options are available to prevent or even mitigate the devastating process that unfolds after injury[15]. A potential solution might be in the protective use of antioxidants[16-18].

The fruit *S. cumini* (L.), locally known as Jaam, is produced in huge amount in Bangladesh, however, its seeds are only limitedly or unknowingly used in traditional folk medicines and/or mostly discarded despite of the fact that it has strong antioxidative property. Thus one of the aims the present investigation was to widen our knowledge on the possible protective effects of *S. cumini* (L.) on embryonic brains.

The study was, therefore, undertaken to examine whether the *S. cumini* (L.) seed extract decreases proinflammatory TNF-α and protects against oxidative stress by setting of a rat model of embryofoetal brain hypoxic–reperfusion injury.

METHOD

Collection and Extraction of *S. cumini (L.)* Seeds

The *S. cumini* (L.) was purchased from local market. The plant material was identified and authenticated by the Department of Botany Jahangirnagar University, Savar, Dhaka and a voucher specimen for this collection has been deposited in the University Herbarium. The seeds were separated from the pulp and rinsed with water. Then seeds were sun dried and powdered by a mechanical grinder. The ethanolic extract was prepared by Soxhlet apparatus and concentrated with a rotary evaporator. The extract was stored at -20°C and used for the experiments.

Experimental Design

Thirty (15 male and 15 female) Wistar rats (3 months of age) were housed separately in plastic cages for the acclimatization to the laboratory conditions (12 h light/dark cycle) for 7 days. Then, the female rats were randomly divided into three groups: Control group (CON), Hypoxia group (HPX) and *S. cumini* (L.) seed Extract + Hypoxia group (SE+HPX). Each group was composed of 5 female rats. The *S. cumini* (L.) seed extract (400 mg/kgBW/day) was orally given through gastric gavage to the SE+HPX group female rats for 1 month. Equal volume of saline without *S. cumini* (L.) seed extract was administered to the CON and HPX group female rats. After 1 month, the rats were kept in pair (one male + one female) for 10 days in experimental case for courtship and to get pregnant the female rat. The oral administration of *S. cumini* (L.) and/or saline to the female rats were continued up to the end of the gestation period (~22 days at our laboratory condition). Hypoxia was performed under sterile condition in laminar air flow at the 19-20th day of gestation (*i.e.* ~2 days prior to delivery). The abdomen was incised and uteroplacental main blood circulation was blocked partially with the aid of metallic clips for ~1 hour. Afterwards, the clips were released for the reperfusion of placental blood for another half an hour. Embryofoetal brains were isolated under light

microscope for biochemical, enzymatic and histological analyses. The study was approved by the Biosafety, Biosecurity & Ethical Committee of Jahangirnagar University [Ref. No. BBECJU/M2013(1)], and accordingly, the rats were cared for and sacrificed during experimentation.

Embryofoetal Brain Tissue Preparation

Fetal brain tissues were homogenized (10 mg/ml) in phosphate buffer (50 mM, pH 7.4) and centrifuged at 500 rpm to remove unruptured tissues. The resulting supernatants were assigned as whole homogenates. Some parts of the whole homogenates were again centrifuged at 12000 rpm for 30 min to separate the supernatants (cytosolic fractions). Samples were immediately subjected to the assays and/or stored at −20 ºC.

ELISA for TNF-α

The multi-well plate was coated with cytosolic fraction in 0.1 M sodium bicarbonate, pH 9.6 at 4ºC overnight and then blocked with 1% BSA in Tris-buffered saline (TBS). The primary antibody anti-rabbit TNFα (Santa Cruz Biotechnology, CA, USA), at 1:1000 dilutions was incubated in the plate for overnight at 4ºC. Horseradish peroxidase-coupled anti-rabbit IgG (Biosource International, Inc., Camarillo, CA, USA) was used as the secondary antibody and incubated for 2h at room temperature before the addition of tetramethylbenidine (Invitrogen™, Life Technologies, USA) substrate to develop color. The reaction was stop by addition of 0.1N HCl after incubation for 30 min at room temperature. Wells coated with only 0.1M carbonate buffer, pH 9.6 was used as blank. The plates were analyzed with a multiwell plate reader (Erba Lisascan II, Mannheim, Germany) at 450 nm.

Measurement of Catalase and Super Oxide Dismutase (SOD) Activity

Catalase activity was assayed according to the method of Claiborne (1985)[19]. The assay mixture consisted of phosphate buffer (pH 7.0), H_2O_2 and tissue homogenates in a final volume of 3 ml. Change in absorbance was recorded at 240 nm. Catalase activity was expressed as U/mg protein by using the molar absorption coefficient of H_2O_2 as 43.6 $M^{-1}cm^{-1}$. SOD activity was measured by the method of Jewett and Rocklin (1993)[20]. The reaction mixture contained 0.050 M $NaHCO_3$-Na_2CO_3 buffer (pH 10.2) with 0.0001M EDTA, tissue sample and epinephrine (0.3M in 0.020M HCL).

Measurements of Lipid Peroxide (LPO) and Reduced Glutathione (GSH)

LPO was determined by estimating the thiobarbituric acid reactive substances (TBARs) in the whole homogenate according to the method of Hashimoto et al. (2002)[20]. Briefly, brain tissue homogenates were added to 8.1% sodium dodecylsulphate, thiobarbituric acid in 20% acetic acid (pH 3.5) and distilled water. Each tube was tightly capped and heated at 95°C for 1 h. After cooling, n-butanol-pyridine (15:1, v/v) was added and shaken, centrifuged at 1200 rpm. The absorbance of the upper organic layer was measured at 532 nm. TEP (1,1,3,3-tetraethoxypropane) was utilized as standard. The GSH level was determined by the Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid), as previously described by Hashimoto et al (2002)[21]. The tissue homogenate was added with equal volume of 20% trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 2000 rpm. The supernatant was then transferred to a new set of test tubes and added to Ellman's reagent. After 15 min of incubation at room temperature, the absorbance was taken at 412 nm against the blank. The GSH standard curve was made by dissolving known amounts of GSH in 0.3M phosphate-citrate buffer and mixed with equivalent volume of the Ellman's reagent solution.

Measurement of Acetylcholine Esterase (AChE) Activity

Acetylcholine esterase activity was assessed, as previously described Hossain et al. (2004)[22]. The method is based on the hydrolysis of the substrate acetylthiocholine. The reaction mixture for the assay contained 100 mM

phosphate buffer (pH 8.0), 1 mM $MgCl_2$, 0.50 mM acetylthiocholine, 0.125 mM 5,5'-dithiobis-2-nitrobenzoic acid and tissue homogenate. The reaction was recorded at 412 nm and the rate was calculated as µmol of substrate hydrolyzed per min per mg protein. Acetylcholine esterase activity was expressed as U/mg of protein. Total protein concentration was determined by using Pierce, Rockford, IL, and assay kit with BSA as standard.

Histology

Formalin fixed fetal brains were processed with paraffin. Sections with a thickness of 10 µm were cut and stained with hematoxylin and eosin for examining the overall morphology. Sections were mounted on gelatin–chrome–alum coated slides. The microscopic observation was made by fluorescent microscope normal spectra in 40x.

Statistical Analyses

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed with one way analysis of variance (ANOVA), followed by Fisher's PLSD for post hoc comparisons. Software's used were GraphPadPrism, StatView4. A value of $P < 0.05$ was considered significance.

RESULTS

Levels of TNF-α in fetal brains

The levels of the TNF-α in the fetal brains of the HPX groups were significantly ($P<0.05$) higher (by 42%) than that of the fetal brains of CON groups. The levels of the TNF-α, however, were significantly reduced (28%) in the fetal brains of the SE+HPX groups, when compared to those of fetal brains of the hypoxic mothers (Fig. 1a).

Activity of the Catalase and SOD in Fetal Brains

Hypoxia caused a significant ($P< 0.05$) decrease (approximately by 50%) in the catalase activity of the embryofoetal brains, as compared to those of the controls. The catalase activity, however, was significantly higher in the fetal brain of the SE+HPX groups, when compared to those of the HPX groups (Fig. 1b). The fetal brain tissues of HPX groups showed a significant lower activity ($P< 0.05$) of SOD enzyme, as compared to those of the fetal brains of CON groups (Fig. 1c).

The Levels of LPO and GSH in Embryofoetal Brain Tissues

The levels of lipid peroxide (LPO) significantly ($P< 0.05$) increased in the fetal brain tissues of the HPX groups, when compared to those of the CON group. The levels of LPO, however, significantly ($P< 0.05$) decreased in the fetal brains of the SE+HPX rats (Fig. 1d). The level of reduced glutathione (GSH) in the fetal brain tissues of the hypoxic rat mother decreased, by 29 %, as compared to that of the fetal brains of CON groups. The value, however, did not reach significance ($P < 0.03$) (Fig. 1e).

Acetylcholine Esterase Activity of Fetal Brain Tissue

Acetylcholine esterase activity of fetal brain tissues of HPX groups decreased significantly, as compared to that of the fetal brains of CON groups ($P< 0.05$). The activity of the enzyme, however, significantly increased ($P< 0.05$) in the fetal brain of the SE+HPX groups in comparison with those of the hypoxic rats (Fig. 1f).

Histological Feature Analyses

The gross hippocampus structure was abolished in the embryofoetal brains of HPX groups, while it was partially abolished in the fetal brain of the SE+HPX groups when compared to the relatively intact gross hippocampus structure in the embryofoetal brains of CON groups. Control embryofoetal brains showed a normal integrity of pyramidal cell layers in the hippocampus. Pyramidal cell layer was dispersed with a substantial number of inflamed pyramidal cells in fetal brains of the hypoxic rats. But the pyramidal cell layer was relatively integrated in SE+HPX fetal brains with a few inflamed pyramidal cells and infiltrated neutrophils. The cellular feature of S1BF (barrel field primary somatosensory cortex) region of brain cortex showed inflamed neural cells as well as

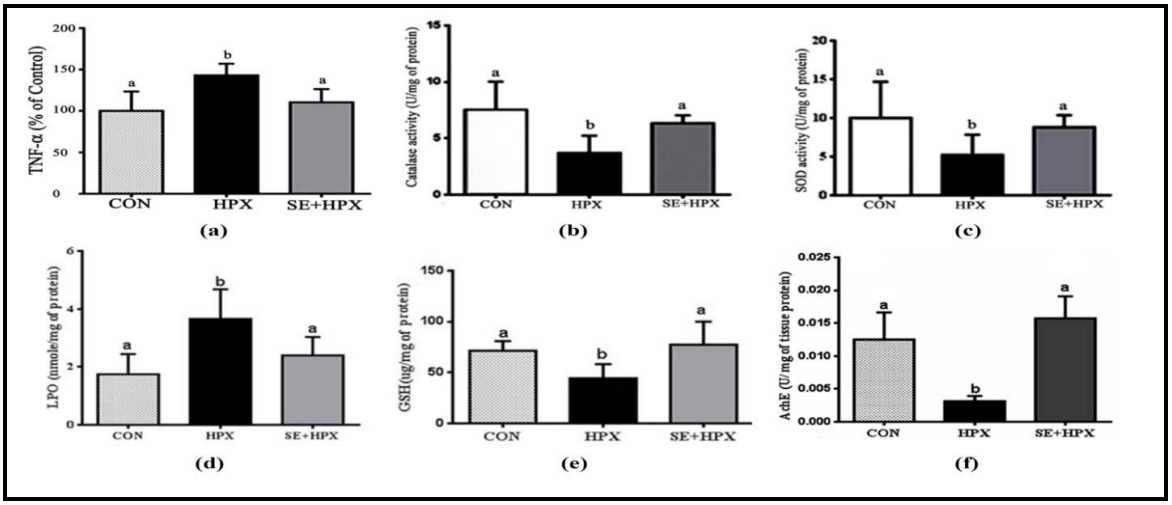

Figure 1**:** Effects of oral preadministration of *S. cumini* seed extract on fetal brain **a** Tumor necrosis factor (TNF-α) **b** Catalase **c** Superoxide dismutase (SOD) **d** Lipid peroxide (LPO) **e** Reduced glutathione (GSH) **f** Acetylcholine esterase (AChE) activity. Results are mean ± SEM (n=5) for triplicate determinations. Here, CON = Control; HPX= Hypoxic; and SE+HPX = *S. cumini* seed extract preadministered hypoxic rats. Bars with different alphabets are significantly different at P<0.05. Data were analyzed by one-way ANOVA followed by Fisher's PLSD for post hoc comparisons.

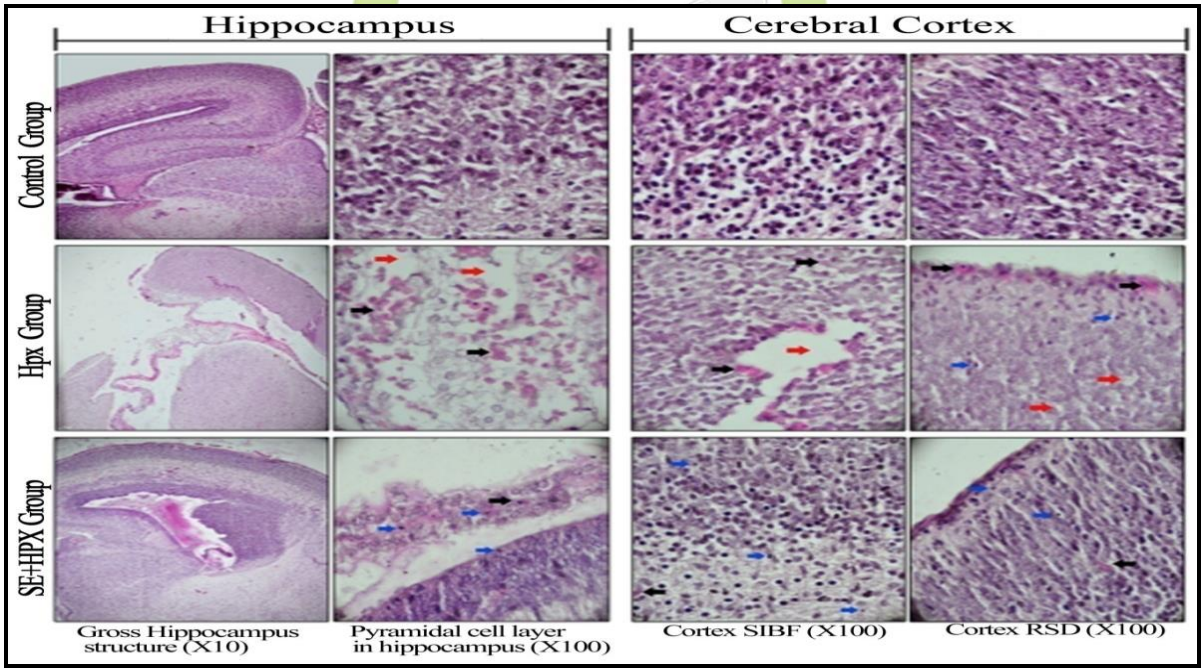

Figure 2: Hematoxylene and eosin dye stained representative brain slices of hippocampus and cortex of embryofoetal brain. Control (CON), hypoxic (HPX) and hypoxic + *S. cumini* seed extract preadministered (SE+HPX) rats. Black arrow shows the inflammatory neural cells with marked swelling. Red arrow shows vacuolation due to liquefaction or colliquative necrosis. Blue arrow shows infiltration of inflammatory cells like neutrophils. S1BF= Barrel Field Primary Somatosensory Cortex; RSD = Retrospel Dysgram.

vacuolation due to colliquative necrosis. But vacuolation was absent in SE+HPX fetal brains with increased neutrophils infiltration. Histological features of RSD (Retrospel Dysgram) region of cortex exhibited both the presence of vacuolation due to colliquative necrosis and inflamed neural cell in addition to infiltrated neutrophils. In the SE+HPX fetal brains vacuole was absent with relatively less number of inflamed neural cells and infiltrated neutrophils (Fig. 2).

DISCUSSION

Our observation in the present study emphasizes that intrauterine hypoxia-reperfusion has induced significant increases in the levels of proinflammatory cytokine TNF-α and oxidative stress indicator LPO, concurrently with decreases in the levels of reduced glutathione GSH and activities of antioxidative enzymes, namely catalase and SOD. The activities of cholinergic neurotransmission-related enzymes AChE also were significantly reduced after hypoxia-reperfusion injury. Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that increases and exacerbates ischemic injury in the brain after injury. The oral administration of *S. cumini* (L.) significantly decreased the levels of TNF-α in the fetal brains of the SE + HPX rats, thus demonstrating that the *S, cumini* (L.), by somehow, ameliorated the proinflammatory response in the fetal brains. Antioxidant enzymes play an important role in the removal of ROS and therefore can have significant effects in reducing the oxidative damage during re-oxygenation. Catalase, a common antioxidative enzyme found in nearly all living organisms exposed to oxygen, catalyzes the decomposition of hydrogen peroxide to water and oxygen and protects the cell from ROS. Due to overflow of oxygen during re-oxygenation in the brain tissues, the catalase might have been coped with over-production of ROS, and so doing, the overall catalase activity reduced in the embryofoetal brains of the hypoxic rats. This explanation is consistent with the decreased activity of catalase in the hypoxic fetal brain than that of the controls. SOD is involved in the protection against ischemia/reperfusion injury[23]. Likewise, the SOD also decreased in the hypoxic rats. These results indicate that hypoxia/reperfusion (re-oxygenation)-induced oxidative stress can be ascribed to the declines in the activities of antioxidant enzymes, including catalase and SOD.

The hypoxia/reperfusion was also accompanied by the elevated levels of LPO in the fetal brains. The decreased level of GSH in the hypoxic brains is thus again consistent with increases of oxidative stress in the hypoxic brains. The increased oxidative stress, as reflected by the decreased levels of catalase, SOD, GSH and increased levels of LPO, in the present hypoxic model also is consistent with other studies[24,25] reporting that the hypoxia/ ischemia cascade leads to formation of free radicals. However, the deteriorations of the oxidative defense in the fetal brains were reversed in the embryofoetal brains of the *S. cumini* (L.)- pretreated mothers, suggesting the *S. cumini* (L.) seed extract protected the oxidative free radical-induced toxic effects. The intrauterine hypoxia caused an inhibition of acetylcholine esterase activity in the fetal rat brain. AChE is a postsynaptic membrane-bound enzyme that splits ACh neurotransmitter and plays an important role for the wellbeing of the cognitive functions. This might relate to the impairments of cognitive functions in the prenatally hypoxia-induced rats. In the present study, the preadministration of *S. cumini* (L.) to the mother rats increased the AChE activity, concurrently with the protection against oxidative toxicity in the hypoxic fetal brains. Thus, it could be hypothesized that the neuroprotective activity of *S. cumini* (L.) might be also due to its influence on cholinergic neurotransmission thereby improving the cognitive functions of the rats[11]. Furthermore, the beneficial effects of *S. cumini* (L.) were evident in the histological examinations. Thus, *S. cumini* (L.) seed extract may potentially be useful in the treatment of neurodegenerative conditions that may involve free radical production, such as perinatal hypoxia-reperfusion. The results of the present investigation are indicative of a prospective use of *S. cumini* (L.) seed as a potential protectant

and a supportive therapeutic agent in pregnancies with high risk of pre-eclampsia, perinatal asphyxia or pre-term delivery, in which oxidative injury may play a crucial role. Further experiments with animal models are needed, so more accurate and precise insights into the mechanisms of hypoxia- reperfusion induced brain injury could be successfully treated and/or at least managed on the bearable level.

CONCLUSION

The aim of our investigation was to find out whether the outcomes of the study could be exploited to combat, at least partially, the hypoxia/reperfusion-related toxicity seen in the fetal brains. The therapeutic opportunities are very limited for prenatal hypoxia related complications, and though the exact injury mechanisms remain to be determined in human hypoxic cases, which suggests a more complex cellular mechanism than that anticipated from experimental animal models, still our animal model study demonstrates that the detrimental effects of hypoxia/reperfusion can, at least partially, be run down by the preadministration of *S. cumini* (L.) to the pregnant mothers.

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Statement of Conflict of Interest

None of the contributing authors has any conflict of interest to declare with regards to the described research or the publication of the result.

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