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The role of nitric oxide in an experimental necrotising enterocolitis model

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ABSTRACT

Background: Necrotising enterocolitis (NEC) causes a significant life-threatening gastrointestinal system (GIS) disease with severe mortality and morbidity, particularly in premature infants. Nitric oxide (NO) has many functions in the GIS. Therefore, in the present study, we evaluated the effects of NO in experimentally induced NEC of newborn 1-day-old rats following hypoxia/reoxygenation (HR). **Materials and Methods:** Thirty Wistar albino rats (weight, 5–8 g) were randomly divided into three groups: group 1 (HR), group 2 (HR + nitroglycerine), and group 3 (control). HR was achieved by placing the rat in carbon dioxide (CO2) for five minutes at 22°C, which was followed by five minutes of 100% oxygen. After HR, nitroglycerine was administered for three days at 50 μg/Kg/day. On day 4, the rats were decapitated and the intestines between the duodenum and sigmoid colon were resected and histopathologically examined. **Results:** The histopathological findings of groups 1 and 2 were characteristic of NEC. Intestinal injury in group 1 was significantly more prevalent than that in group 2 ($\chi^2 = 21.55$, $P = 0.000$). The intestinal injury score in group 3 was significantly lower than that in the other groups ($P < 0.05$). **Conclusions:** NO treatment was effective for treating experimentally induced NEC.

Key words: Hypoxia reoxygenation, necrotising enterocolitis, nitric oxide, premature, rat, treatment

INTRODUCTION

Necrotising enterocolitis (NEC) is believed by some to have been first described by Siebold in 1825. However, the first case of NEC was published by Paltau in 1888.[1] Although the etiopathogenesis has not been fully explained, hypoxia and ischaemia are the most significant effects of NEC.[1-6] Hypoxia and ischaemia have physiological and pathological effects on both the integrity of the intestinal epithelial barrier (IEB) and hemostasis, leading to intestinal epithelial damage.[7,8] Translocation of intraluminal bacteria and IEB products results in the expression of inflammatory mediators and epithelial damage, ultimately leading to intestinal tissue necrosis.[9,10]

Approximately 90% of neonates with a gastrointestinal system (GIS) perforation have perinatal complications such as low Apgar score, cyanosis, and respiratory distress syndrome, which cause hypoxia.[11,12]

Nitric oxide (NO) is found in GIS veins and the myenteric nerves of the endothelium. NO has been shown to increase cyclic guanosine monophosphate, which causes relaxation of the nerves and membrane hyperpolarization, ultimately resulting in a reduced GIS intrinsic neural reflex and intestinal motility.[13] In addition, NO cleanses free oxygen radicals from the water-electrolyte transport system of the GIS, inhibits inflammation, and increases blood flow to the mucous membranes, which increases permeability to protect the GIS from damage.[2,10,14,15] NO not only protects the epithelium directly from trauma, but also plays a role in epithelial renewal.[16]

Inducible NO synthase (NOS) activity increases approximately three hours after the onset of NEC.[15] In this study, we evaluated the therapeutic effects of NO [hypoxia/reoxygenation (HR)] in newborn rats in which NEC was experimentally induced with hypoxia.

MATERIALS AND METHODS

This experimental study protocol was approved by the Experimental Animal Ethics Committee of the Şişli Etfal Training and Research Hospital. This study was

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performed at the Istanbul University Experimental Medical Research and Application Institute. We used newborn Wistar albino rats housed at 20±1°C under a 12 hour light: 12 hour dark cycle. This study conformed to the Guidelines for the Care and Use of Laboratory Animals (U.S. National Institutes of Health Publication no. 85-23, revised 1996).

Thirty one-day-old Wistar Albino rats (weight: 5–8 g) were randomly allocated into three groups of 10; group 1 (HR + no treatment), group 2 [HR + nitroglycerin (perlinganit)], group 3 (control, no application). A 0.1 ml aliquot of 0.9% sodium chloride (NaCl) was administered intraperitoneally (i. p.) to groups 1 and 3 as a control injection. The experimental model used in this study has been described previously.[5] The drug was prepared at 0.05 mg/10 mL and 0.1 mL was injected i.p., as the effective perlinganit dose for humans is 10–100 μg/kg/min.

Normothermic (22–23°C) rats were placed in a vacuum glass bell jar at room temperature (Vacunit70, Eschmann, England). The air in the vacuum apparatus was emptied with an aspirator from one aperture and 100% carbon dioxide (CO_2) was provided through the other aperture to generate hypoxic conditions. After approximately five minutes, the rats exposed to hypoxia became motionless and cyanotic from lack of air. The rats were then removed from the bell jar and exposed to 100% oxygen (O_2).

Approximately five minutes later, the rats regained a pink color, began to move, had regular respiration, and were returned to their mothers. Animals in the treatment group (group 2) were given a single dose of i.p. nitroglycerine (Perlinganit, ADEKA, Istanbul, Turkey) at 24 hours after HR and daily for three days thereafter. The control group did not undergo the HR procedure. After the procedures, the rats were placed in cages with their mothers and provided unlimited food. The rats were decapitated four days after the HR procedure and examined histologically.

The histopathological examination was performed by a pathologist blinded to the study group. A 1 cm length of intestinal tissue was taken from the distal jejunum, the terminal ileum, and the proximal colon of each animal and fixed in 10% formalin. Tissues sections (8–10 μm) in paraffin blocks were stained with hematoxylin and eosin and examined under light microscopy. Intestinal changes were scored from 0 to 4 according to the classification system described.[17] The following classification procedure was applied to each rat separately: Grade 0 (normal histology), healthy villi; grade 1 (minimal), villus necrosis and villus crypts (mild epithelial disintegration); grade 2 (mild-moderate), villus and crypt necrosis, mucosal and submucosal damage (moderate level villus damage); grade 3 (moderate), necrosis extending to the muscle layer (total villus damage); and grade 4 (severe), transmural necrosis. These scores were applied to the three intestinal segments for each animal and were analyzed statistically.

Statistical evaluation

A one-way analysis of variance (ANOVA) was conducted. The Kruskal–Wallis test was used to detect specific differences between the histopathological groups. When a difference was determined based on the Kruskal–Wallis test, Dunn's multiple comparison test was used to identify the difference. A P value <0.005 was considered to be statistically significant.

RESULTS

The experimental animals tolerated HR well, and no mortalities were observed. The mean birth weight was similar among the three groups at the beginning of the study. Weight loss from the pups in group 1 was significantly higher than that in the groups 2 and 3 ($P<0.05$). Birth weights in groups 1, 2, and 3 were, 6.9±0.6, 6.7±0.5, 6.6±0.8 g, respectively. At the end of the experiment, weights in groups 1, 2, and 3 were 6.2±0.9, 7.4±0.7, and 8.2±0.3 g, respectively.

No microscopic or macroscopic effects were observed in the intestines of group 3, whereas microscopic and macroscopic effects were observed in all animals of the other two groups. Specifically, a colour difference, oedema, and microscopic effects in the villi and mucous were observed [Figure 1]. In group 1, the intestinal mucous was affected in one case, whereas grade 2 damage was observed in the intestinal samples of the remaining nine cases, particularly in the distal ileal segment, but also in the jejunum ($n=4$) and colon ($n=6$). These results were considered moderate-level villus and crypt necrosis with submucosal damage.

In group 2, the intestinal mucous was normal in one case, whereas four cases revealed grade 1 villus necrosis and villus crypts. In all group 2 animals, the ileum was affected. In addition, the colon was affected in two animals. Grade 2 effects were observed in four animals in group 2, with one in the jejunum and three in the colon [Table 1].

All intestinal specimens in group 3 were normal

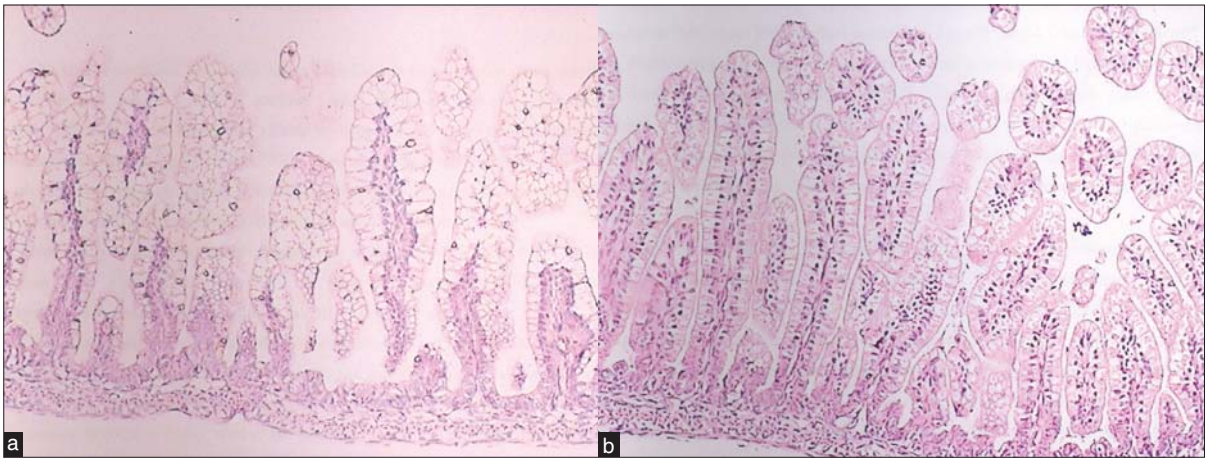


Figure 1: (a) Microscopic image of the intestines of newborn rats with created necrotising enterocolitis, (b) Microscopic image of the intestines of newborn rats with normal intestine

Table 1: Distribution of the damage to the intestine sections of the groups by HR model

Clinical features	Nonaffected intestine	Jejunum	Ileum	Colon
Group 1 (n = 10)	1	4	9	6
Group 2 (n = 10)	1	1	9	3
Group 3 (n = 10)	10			

histopathologically, whereas mucosal oedema and congestion of the muscularis propria in the epithelium cells covering the villi were observed in the other two groups. Although the villi were moderately affected, significant damage had occurred in the distal ileum. All specimens with histopathological changes had significant tissue damage compared to that in group 3 ($P<0.005$). A significant difference was observed between groups 3 and 2 with respect to the histopathological changes ($P<0.000$).

DISCUSSION

NEC is still the most commonly encountered GI disease in neonatal intensive care units.[2,10] The incidence of NEC is approximately 1 in 1,000 live births, of which 1 in 7 are lost to the disease.[3,4,10,18,19] NEC is generally seen at 2–3 days postnatally in full-term infants and at 10–15 days postnatally in premature infants. An increase in the survival rates of premature infants has occurred due to technological developments and improved care provided by neonatal intensive care facilities. However, the incidence of NEC is also increasing.

In a previous study, a one-day-old rat was considered equivalent to a 22–24 week foetus and a three-day-old rat was equivalent to a 28–32 week foetus.[20] Therefore, we used one-day-old rats in the current study.

In a study by Barlow *et al.*, NEC developed in rats exposed to hypoxia and cold stress.[21] Development of NEC was observed in 60% of those exposed once to cold stress and in all of those exposed twice or more.[21] In the same study, NEC continued to develop in 20% of those with one hypoxic attack, in 60% of those with two attacks, in 80% of those with three attacks, and in 100% of those with four attacks.[2]

Hypoxia leads to ischaemia in the intestine through a reduction in mesenteric blood flow (diving reflex).[16] This reduction in mucosal blood flow causes a delay in the activation of the ischaemia-reperfusion process, which is responsible for GI damage.[5] In a study by Kazez *et al.*, tissue damage that developed in association with HR in a neonate rat model conformed to that of early NEC,[5] and up to grade 3 intestinal damage was seen.[5] However, in the current study, grades 1 and 2 intestinal damage were found. This may be explained by our inability to achieve an environment with 100% CO_2 or by changes in the duration and temperature. Hypoxia was a significant risk factor for the development of NEC. Previous studies have shown that the intestinal mucous is sensitive to ischaemia.[4,19]

In the current study, the ileum was most affected by NEC in the HR model, which is consistent with previous studies.[5,9,22] In previous studies, the coagulative or ischaemic lesions associated with NEC were more frequently observed in the ileocolic area.[22,23] NO is effective in alleviating intestinal damage associated with NEC.

Infusion of nitroglycerine, which is an NO donor, significantly reduces intestinal damage in the NEC model.[2,9,10,13,14] In these previous studies, NO, which

preserves the mucous, helped to correct permeability alterations and acted as a nonadrenergic-noncholinergic mediator of intestinal relaxation, thus correcting vascular tone and protecting the intestine from toxins.[8,10] In the present study, 50 μg/kg nitroglycerine was administered i.p. once a day for three days.

NO is a critical mediator expressed by inflammatory cells in response to inflammation during NEC pathogenesis.[17] Several inflammatory mediators, such as tumour necrosis factor-α (TNF-α), interleukin (IL)-1, 6, 8, and 10, platelet-activating factor (PAF), and NO, play important roles in NEC pathogenesis.[19] NO has a paradoxical role in intestinal physiology. By affecting various molecular pathways, NO and superoxide, together with peroxynitrite, which is a potent oxidant, prevent enterocyte apoptosis or necrosis enterocyte proliferation-migration and epithelial regeneration.[6,10,19]

The ratio of arginine to arginine asymmetric dimethylarginine (inhibited by NOS) in the blood of patients with NEC is low as compared with that of a control group.[6] Previous studies have reported success from intravenous, direct, and oral administration. The results of the current study were evaluated histopathologically for the effects of NO on NEC. Our findings support those of previous studies.

The intestinal mucous barrier includes the epithelium, intestinal lymphoid system, and mucous immune system, and it is not fully developed at birth. Slow peristaltic movements increase with age[20] and motility and vascular resistance in the premature intestine are low. A premature GIS has relatively insufficient levels of the NOS enzyme, which plays a role in the formation of NO.[11,22,23] Therefore, the incidence of NEC is increasing because of intestinal NOS insufficiency, particularly in premature infants. This is especially true when there is a trigger mechanism such as ischaemia.[11,17] As demonstrated in a study by Potoka *et al*., intestinal barrier insufficiency is caused by bacterial translocation.[15] By protecting the intestine from damage, preventing apoptosis and necrosis, and performing gap junction roles, endogenous NO preserves the epithelium entirely and reduces mortality rates.[4,6,10]

The present study had several limitations. First, we only conducted a histopathologic evaluation of the intestine. Second, our sample size was small and may not have been large enough for sufficient statistical power to detect differences. Third, i.p. drug administration can be harmful or a predisposing factor for intestinal injury; so the drug should have been administered subcutaneously. Thus, it is difficult to conclude that NO or its analogues may prevent or treat NEC.

In conclusion, hypoxia is a significant factor in the aetiology of NEC. Several studies have shown that NO and its analogues may be a good choice for protecting against and treating NEC. Future clinical studies should investigate the routine administration of NO to newborns, particularly premature infants, to treat and/or prevent NEC.

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