ORIGINAL ARTICLE

Glycine reduces liver lipid peroxidation in neonatal hypoxia/reoxygenation-induced necrotizing enterocolitis

A glicina reduz a peroxidação lipídica hepática na enterocolite necrotizante neonatal induzida pela hipóxia-reoxigenação

Karine Furtado Meyer[1], José Luiz Martins[2], Mauricio Macedo[3], Luiz Gonzaga de Freitas Filho[4], Maria Luiza Vilela Oliva[5], Francy Reis da Silva Patrício[6], Lina Wang[7], Ana Cristina Garcia Ferreira[8]

ABSTRACT

Objective: To assess the protective effect of glycine in liver of neonatal hypoxia/reoxygenation-induced necrotizing enterocolitis in rats. **Methods:** Forty-four (44) neonatal Wistar rats were distributed into three groups: G1 – normal control group (n=12); G2 Group (n=16) with animals that underwent hypoxia-reoxygenation; G3 Group (n=17) with animals submitted to hypoxia-reoxygenation following a 5% intraperitoneal glycine infusion. The groups were subdivided into A: euthanasia 12 h after hypoxia-reoxygenation and B: euthanasia 72 h after hypoxia-reoxygenation. The liver was removed for determination of tissue malondialdehyde. **Results:** Malondialdehyde values did not differ significantly in subgroups G1 and G3. The animals in G2A had mean malondialdehyde values significantly lower than those in G2B. Malondialdehyde values did not differ significantly for animals in subgroup A. In subgroup B, the malondialdehyde values did not differ significantly among the animals in G1 and G3. G2 animals had mean malondialdehyde values significantly higher than G3 animals. **Conclusion:** Glycine reduces liver lipid peroxidation in hypoxia/reoxygenation-induced necrotizing enterocolitis.

Keywords: Glycine; Enterocolitis, necrotizing; Ischemia; Liver/injuries

RESUMO

Objetivo: Avaliar o efeito protetor da glicina no fígado de ratos recém-nascidos com enterocolite necrotizante induzida por hipóxia-reoxigenação. **Métodos:** Quarenta e quatro ratos Wistar recém-nascidos foram distribuídos em três grupos: G1: grupo controle; G2: animais submetidos à hipóxia-reoxigenação e G3: animais submetidos à hipóxia-reoxigenação após a infusão intraperitoneal de glicina 5%. Dentro dos grupos, os animais foram subdivididos em: A: eutanásia 12 horas após hipóxia-reoxigenação e B: eutanásia 72 horas após hipóxia-reoxigenação. O fígado foi removido em bloco para dosagem de malondialdeído tecidual. **Resultados:** Os valores de malondialdeído não diferiram significantemente entre os subgrupos dos grupos G1 e G3. Os animais do grupo G2A apresentaram valores médios de malondialdeído significantemente menores que os animais do grupo G2B. Os valores de malondialdeído não diferiram significantemente entre os animais do subgrupo A. No subgrupo B, os valores de malondialdeído não diferiram significantemente entre os animais do grupo G1 e G3; o G2 apresentou valores médios de malondialdeído significantemente maiores que os dos animais do G3. **Conclusão:** A glicina reduz a peroxidação lipídica hepática em modelo experimental de enterocolite necrosante induzida pela hipóxia-reoxigenação.

Descritores: Glicina; Enterocolite necrosante; Fígado/lesões

INTRODUCTION

Despite significant advances in care provided to high-risk newborns, necrotizing enterocolitis (NEC) continues to be the most important cause of mortality and morbidity in low-birth-weight infants. NEC is the most common gastrointestinal emergency in neonatal

Study carried out at Department of Surgery, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP) Brazil.

[1] MD. Specialization course in Pediatric Surgery, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP), Brazil.

[2] Adjunct Professor of the Discipline of Pediatric Surgery; Post-doctorate degree, Department of Surgery, Universidade Federal de São Paulo – UNIFESP; Pediatric surgeon of Hospital Israelita Albert Einstein – HIAE, São Paulo (SP), Brazil.

[3] Graduate student in Surgery and Experimentation; Pediatric surgeon of the Hospital Israelita Albert Einstein – HIAE, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP), Brazil.

[4] Affiliate Professor of the Discipline of Pediatric Surgery, Department of Surgery, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP), Brazil.

[5] Adjunct Professor of the Discipline of Molecular Biology, Department of Biochemistry, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP), Brazil.

[6] Adjunct Professor of the Discipline of Medical Pathology, Department of Pathology, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP), Brazil.

[7] Resident of the Pediatric Surgery Service , Hospital do Servidor Público Estadual – HSPE, São Paulo (SP), Brazil.

[8] Medical student, Universidade Federal de São Paulo – UNIFESP, São Paulo (SP), Brazil.

Corresponding author: Karine Furtado Meyer – R. Rio Grande, 551 – Ap 162 A – Vila Mariana – CEP 04018-001 – São Paulo (SP), Brazil – Tel.: 11-5084.5342 – e-mail: karine_meyer@uol.com.br

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intensive care units; approximately 5% of low-birth-weight infants develop this condition[1].

Intestinal lesion in NEC causes damage to the organ affected, and can result in dysfunction and failure of multiple organs, by triggering the release of inflammatory mediators into the blood stream. This failure is the most common cause of morbidity and mortality in necrotizing enterocolitis patients[2]. The liver is located in a position that makes it face the toxic mediators released by the intestine[3].

Different hypotheses have been formulated to explain the origin of NEC; however, it remains a disease of unknown etiology and uncertain pathogenesis. Management is still based on empirical observations and no prevention method showed to be fully successful[1]. The combination of gastrointestinal ischemia, which results from a redistribution of splanchnic blood flow to vital organs, such as the heart and the brain, enteral nutrition that possibly predisposes to lesion of the mucosal layer, and translocation of pathogenic bacteria, associated with not mature immune mechanisms of the bowel, would be involved in the development of the disease[1].

Many animal models have been used to assess hypoxia-reoxygenation (HR) effects as the origin of NEC and shown that reduced intestinal perfusion as well as tissue lesion would be caused by an HR mechanism[4-9].

Glycine is a non-essential simple amino acid formed by a carbon molecule bound to an amino group and to a carboxyl group. On the plasma membrane, it activates a chlorine channel that either stabilizes or hyperpolarizes the membrane potential. As a result, it blocks the additional intracellular calcium triggering the cytokine formation cascade[10]. Thus, glycine has an anti-inflammatory, immunomodulating and cytoprotective effect. It protects against the shock caused by both hemorrhage and endotoxins, prevents ischemia-reperfusion-associated injury in several organs and tissues, such as liver, kidney, heart, bowel and skeletal muscle, and reduces renal and liver injury caused by drugs[10].

We previously demonstrated that glycine prevents lipid peroxidation of intestinal tissue in the same NEC model[11]. The group undergoing hypoxia-reoxygenation (HR) had mean malondialdehyde (MDA) values significantly higher than those in the group undergoing HR but who were previously protected by using glycine ($p = 0.021$). The absence of difference between the control group and the group that used glycine ($p = 0.992$) showed that the level of protection provided by glycine was so important that it provided a peroxidation level similar to that of normal control rats[11].

OBJECTIVE

To assess the capacity of glycine to prevent liver lipid peroxidation caused by hypoxia-reoxygenation in a previously described experimental model of NEC[8] was assessed.

METHODS

Experimental Design. The animal experiment was approved by the Research Bioethics Committee of the Universidade Federal de São Paulo - UNIFESP-EPM, and registered under number 0560/04.

Forty-four (44) neonatal OUT B EPM-1 Wistar rats (*Rattus norvegicus albinus*, *Rodentia mamalia*), from a litter of six female rats and weighing 4 to 6 grams, were used. The animals were randomized into three groups: normal control G1 group (n = 12); G2 group (n = 15) with animals undergoing HR; G3 group (n = 17) with animals undergoing HR following a glycine intraperitoneal infusion. The animals underwent hypoxia in a rodent CO_2 kill chamber where they received an air flow containing 100% CO_2, for 5 minutes. Following hypoxia the animals were resuscitated with an air flow containing 100% O_2 for 5 minutes and then kept close to their respective mothers in a normothermic environment[9]. All animals were given breast milk before and after the procedure. In Group 3, the animals received 0.2 ml of 5% glycine solution in saline solution[12-14]. The glycine injection was administered 30 minutes prior to hypoxia-reoxygenation and it was maintained once a day until animal euthanasia. The animals were submitted to euthanasia by decapitation. The groups were subdivided to demonstrate that liver injury was not caused by HR alone. In subgroup A, euthanasia occurred 12 hours after HR and in subgroup B, 72 hours after HR.

The liver was removed in a block and immediately frozen at -80°C for subsequent homogenization and measurement of tissue MDA.

Determination of MDA. MDA is a final product of lipid peroxidation and a well-established parameter to determine the increase of free radicals in intestinal tissue[8]. To determine MDA levels, the thiobarbituric acid (TBA) reaction proposed by Kohn and Liversedge[15] was used and the levels were expressed in nmol MDA/mg of protein. The protein content of the homogenate was determined by the coomassie brilliant blue (CBB) procedure. Tissue samples were defrosted, weighed, and a volume equivalent to five times the weight of TRIS 0.01M/pH 7.4 buffer solution was then added. Tissue samples were homogenized in ice bath four times, for 30 seconds each, and subsequently centrifuged for 5 minutes at 10000 rpm, at 4°C.

Protein measurement. The CBB reactant interacted with protein enabling its quantification by using a standard albumin curve with known concentrations.

Preparation of the CBB reactant. One hundred milligrams of CBB 250G was dissolved into 50 ml of 95% ethanol. Later, 100 ml of 85% phosphoric acid was added and stirred constantly. Distilled water for a final one liter volume was added. The reactant was let to rest for 24 hours and then it was filtered and kept in a dark vial. By using a bovine serum albumin (BSA) 10 mg/ml stock solution, we prepared 200 ìl of the following solutions: 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml and 1 mg/ml (bovine serum albumin/ml of water) to have the bovine serum albumin standard curve (standard curve for protein measurement). The homogenate was collected, 25 ìl and 50 ìl, and then diluted in five times a TRIS 0.01M/pH 7.4 buffer; 2.5 ml of the CBB was added and readings were taken at 595 nm, 10 minutes after the CBB reactant was added.

MDA Measurement. Four hundred microliters of the centrifuged homogenate supernatant were collected and the following was added to: 1 ml of 20% trichloroacetic acid and 400 ìl of 1.6% thiobarbituric acid. The mixture was incubated for 30 minutes at 95°C. Lipids were extracted by adding n-butanol (1.6 ml) and stirring vigorously. The sample was again centrifuged for 10 minutes at 3000 rpm. Absorbance of the organic layer was determined through reading at 510, 532, and 560 nm. The following equation proposed to minimize the interference of both heme pigments and hemoglobin in the measurement of MDA[16] was used:

$$MDA_{532} = 1{,}22[(A_{532})-(0{,}56)(A_{510})+(0{,}44)(A_{560})]$$

The calibration curve was drawn with 1,3,3 tetramethoxypropane (malondialdehyde bis) (acetyldimethyl).

Statistical Analysis. The quantitative variables were represented by mean, standard error (SE), minimum and maximum, and the qualitative variables were represented by absolute (n) and relative (%) frequencies. The Kolmogorov-Smirnov test was applied to test the normal distribution of parameters. The one way analysis of variance (ANOVA) was performed to compare the study groups as nmol/mg of protein and nmol/mg of tissue. The differences were found using the Tukey multiple comparison test. The Kruskal-Wallis test was used to analyze the extent of tissue injury in three sites assessed. The differences were found using the Dunn multiple comparison test. The significance level established was $p < 0.05$ for all tests and was represented by an asterisk (*).

RESULTS

MDA levels of the groups are shown in table 1. MDA values did not differ significantly among the subgroups in group G1 (p = 0.901) and group G3 (p = 0.094). The animals in group G2 subgroup A had mean MDA values significantly lower than those in subgroup B (p = 0.020).

Table 1. Tissue MDA (nmol/mg of protein)

		nmolMDA/mg protein		
Group	Subgroup	Mean	SE	N
G1	A	0.8310	0.1848	6
	B	0.8043	0.0946	6
G2	A	0.7508	0.1135	7
	B	1.9328	0.3924	8
G3	A	1.0662	0.1178	7
	B	0.6891	0.1552	10

MDA values did not differ significantly in subgroup A (p = 0.256). In subgroup B, MDA values did not differ significantly in groups G1 and G3 (p = 0.949). The animals in group G2 had mean MDA values significantly higher than those in G1 (p = 0.023). Group G2 had mean MDA values significantly higher than G3 (p = .004).

DISCUSSION

NEC is the most frequent and lethal disease affecting the gastrointestinal tract of preterm infants. Although the etiology of NEC has not been well defined yet, hypoxia definitely plays an important role in its pathogenesis[4-9]. Several animal models were proposed to show the relevance of hypoxia in the development of NEC-associated lesions[4-9].

One of the reasons accepted to explain hypoxia-associated lesions is that neonatal asphyxia would lead to a redistribution of blood flow by triggering splanchnic vasoconstriction, diverting the flow to vital organs such as the heart and brain and causing intestinal ischemia[5]. Several mechanisms are involved in the onset and progression of this ischemia-associated lesion, such as increased production of hyperreacting peroxides, increased synthesis of adhesion molecules with neutrophilic infiltration, increased lipid peroxidation and increased production of inflammatory mediators such as cytokines[17].

Lipid peroxidation is a complex process that can occur in biological membranes composed of molecular oxygen-reactant polyunsaturated fatty acids, leading to production of lipid hydroperoxides and their metabolites. Most cases involving lipid peroxidation start from a chain reaction that spreads out, mediated by the presence of free radicals. Lipid hydroperoxides accumulate in the membrane, inactivating its receptors

and enzymes, affecting its functions, making it unstable and permeable to ions. A simple method of high sensitivity, very much used as a lipid peroxidation marker, involves thiobarbituric acid-reactive substances, such as lipid hydroperoxide derivatives. Hence, MDA is an adequate indicator of lipid peroxidation caused by free radicals[8].

Intestinal lesion in NEC causes damage to the affected organ, and, by triggering the release of inflammatory mediators into the blood stream, it can lead to dysfunction and failure of multiple organs, which is the most common cause of morbidity and mortality in necrotizing enterocolitis patients[2]. The liver is positioned to first encounter these toxic mediators released from the intestine[3].

Glycine is a non-essential amino acid that protects the gut against lesions caused by the ischemia-reperfusion phenomenon[12-14]. It is considered an anti-inflammatory and immune-modulating agent that has a direct cytoprotective function[10]. Lee et al.[12], in a model of intestinal ischemia and reperfusion, showed that local 20% glycine mesenteric intravenous infusion increased mucosal protein and DNA content, reduced the intestinal myeloperoxidase activity and maintained glutaminase activity in the mucosa. Two other studies[13-14], also in an intestinal HR model in rats, showed the protective effect of glycine used in systemic intravenous infusion, by reducing the apoptosis cascade[13] and preserving the integrity and contractility of the intestinal wall[14]. MDA intestinal levels, in our previous study using the NEC model proposed by Okur et al.[8], showed glycine as able to prevent lipid peroxidation. The group undergoing HR had mean MDA intestinal values significantly higher than those in the group undergoing HR and previously protected by using glycine ($p = 0.021$)[11].

Several techniques were used in an attempt to prevent multisystem organ failure due to intestinal ischemia-reperfusion injury. Vejchapipat et al.[18] showed that moderate hypothermia ameliorated liver energy failure after intestinal HR using magnetic resonance spectroscopy. Ferrer et al.[19)] demonstrated that somatostatine and N-acetylcisteine might improve prognosis and survival of patients with multiple organ failure mediated by oxidative stress after intestinal ischemia. Horie et al.[20] suggested that low-dose ethanol attenuates the gut ischemia-reperfusion hepatic microvascular dysfunction and sequential liver injury by increasing sinusoidal NO levels.

The group undergoing HR after 72 hours had mean MDA hepatic values significantly higher than those in the group undergoing HR and previously protected by the use of glycine ($p = 0.004$). The absence of difference between the control group and the group that used glycine ($p = 0.949$) showed that the level of protection provided by glycine in liver was so important that it provided a peroxidation level similar to that of normal control rats.

In the group submitted to HR after 12 hours, the MDA values did not differ significantly in the control group, suggesting that the liver peroxidation injury was not caused only by HR (the presence of intestinal lesion is required).

CONCLUSION

Our findings support the hypothesis that glycine reduces blood release of lipid peroxidation products from gut; thus, it prevents the increase of lipid peroxidation of liver.

It remains to be known, maybe in a not a very distant future, to what extent such findings can actually benefit infants with NEC. Changing the history of this disease that still claims many lives among low-birth-weight infants is essential.

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