Pediatric RESEARCH

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BASIC SCIENCE ARTICLE

The effects of IGF-1 and erythropoietin on apoptosis and telomerase activity in necrotizing enterocolitis model

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BACKGROUND: Apoptosis that occurs after hypoxia/reoxygenation (H/R) has an important role in the pathogenesis of necrotizing enterocolitis (NEC). Telomerase activity, showing the regeneration capacity, may also be important in the recovery process. Therefore, we aimed to investigate the effects of insulin-like growth factor-1 (IGF-1) and erythropoietin (EPO) on apoptosis and telomerase activity in an H/R model.

METHODS: Young mice were divided into four groups each containing ten Balb/c mice. Group 1 (H/R) were exposed to H/R; group 2 and group 3 were pretreated with IGF-1 and EPO, respectively, for 7 days before H/R. Group 4 served as control. Intestinal injury was evaluated by histological scoring and assessment of apoptosis was performed by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) test. Proapoptotic and antiapoptotic gene expressions and telomerase activity were analyzed by real-time PCR.

RESULTS: IGF-1- and EPO-treated animals had decreased histological damage and apoptosis, confirmed by TUNEL test and caspase activity. Telomerase activity was increased in these animals in addition to increased expression of antiapoptotic genes. However, proapoptotic gene expressions were not statistically different.

CONCLUSIONS: The protective effects of IGF-1 and EPO in H/R damage may be through increased expression of antiapoptotic genes and increased telomerase activity, especially for IGF-1.

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IMPACT:

- This is a comprehensive study measuring various variables, namely IGF-1, EPO, apoptosis, apoptotic and antiapoptotic genes, and telomerase activity in the NEC model.
- The intestinal protective effects of IGF-1 and EPO in H/R damage may occur through increased expression of antiapoptotic genes and increased telomerase activity.
- To the best of our knowledge, telomerase activity has not been investigated in the NEC model before.
- Regarding our results, novel strategies may be implemented for the early definitive diagnosis, robust preventive measures, and effective treatment modalities for NEC.

INTRODUCTION

Necrotizing enterocolitis (NEC) is the second most common cause of morbidity and mortality in premature infants with an incidence of 1–5%.[1] Initial symptoms include feeding intolerance, abdominal distension, increased gastric residuals, and bloody stools. These symptoms may progress rapidly to acidosis, intestinal perforation, peritonitis, and systemic intravascular coagulation, which require intensive medical support.[2] The exact pathogenesis of NEC has not been elucidated yet, but it is well known that early diagnosis and interventions have a great role to prevent the worsening of the symptoms.[3] Numerous factors, namely gastrointestinal ischemia, immaturity, enteral feeding, perinatal asphyxia, bacterial colonization, and hypoxia, were considered in the etiopathogenesis.[4] Recent epidemiological studies showed that the primary causative factor for NEC is prematurity. About 65–95% of NEC is observed in premature infants. High gastric pH, reduced intestinal motility, poor absorption function, immature epithelial structure, insufficient immunoglobulin A secretion, reduced mucus, and regenerative capacity, were shown to be the predisposing factors in premature infants.[5,6] It has also been demonstrated that hypoxic–ischemic damage, which occurs due to perinatal asphyxia, is closely related to NEC. Continuous hypoxia leads to permanent damage in the intestinal mucosa.[7] Free oxygen radicals and mediators, particularly platelet-activating factor and tumor necrosis factor-α (TNFα), have been shown to increase the intestinal mucosal permeability causing mucosal damage. In addition, many of these mediators have also been reported in the intestinal epithelial cells to cause apoptosis, which also has an

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important role in the pathogenesis of NEC.[8–10] Proapoptotic and antiapoptotic proteins, such as Bcl-2, Bcl-xL, Bcl-w, BAX, BAK, and Bcl-xs, were known to mediate apoptosis. Moreover, caspase-3 has a main role in apoptosis; its level is used to screen apoptosis and it has been revealed that apoptosis may be prevented by using caspase-3 inhibitors.[11–14] Insulin-like growth factors (IGFs) are potent mitogenic factors and IGF-1 is the member of this family, which has a role in cell proliferation and differentiation. It also activates its receptor (IGFR) that works to inhibit the activation of caspases.[15] Antiapoptotic effect of IGF-1 receptors shows their effect by inhibiting proapoptotic Bax and Bcl-xs and activating antiapoptotic Bcl-xL and Bcl-2. It also has antiapoptotic effect by inhibiting phosphatidylinositol 3-kinase.[16] Telomerase activity, showing the regeneration capacity, may also be important in the recovery process. Although telomerase activity is present in immortal and malign cells, it has also been demonstrated in hematopoietic cells, intestinal crypt cells, endothelial cells, endometrial cells, and basal cells of the epidermis that are actively proliferating.[17]

Therefore, in this study, we aimed to:

- Investigate the roles of apoptosis and telomerase activity in the etiopathogenesis of NEC
- Reveal the possible protective effects of IGF-1 and erythropoietin (EPO) in an H/R mouse model

MATERIALS AND METHODS

Study groups

Young Balb/c mice (20–25 days old) weighing ~15 g were used in the study. All animals were housed in a rack-mounted cage, in a normothermic environment, each containing ten mice. The mice were divided into four groups ($n = 10$/group): group 1 (H/R) were exposed to H/R; group 2 (H/R + IGF-1) and group 3 (H/R + EPO) were pretreated with IGF-1 and EPO, respectively, for 7 days before H/R. Group 4 served as a control group. The study was approved by Ege University Animal Care, Use and Research Ethics Committee.

Administration of IGF-1 and EPO

Group 1 mice were given 1 ml of 0.9% NaCl intraperitoneally for 1 week. Group 2 and group 3 mice were administered 1 µg/kg of IGF-1 and 0.75 IU of EPO intraperitoneally for 1 week.[1,18] Group 4 mice did not receive any medication.

Generation of hypoxia and reoxygenation

After the administration of relevant medication, hypoxia was induced by placing young mice in a plexiglass chamber consisting of 10% oxygen for 60 min (Minolta Oxygen Monitor, Japan). After hypoxia, the mice were reoxygenated for 20 min with 100% oxygen. Group 4 mice were not exposed to hypoxia and reoxygenation. They were carried to their cages and were sacrificed 4 h after H/R.

Evaluation of apoptosis

Intestinal injury was evaluated by dissecting the abdominal wall and taking biopsies from injured areas. Then, the biopsies were collected and fixed with 10% formalin solution. The intestinal tissue was washed in cold phosphate-buffered saline (PBS) solution and stored in a liquid nitrogen tank at −80 °C until further study.

TUNEL test. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed to identify double-stranded DNA fragmentation and the characteristics of DNA degradation by apoptosis. Briefly, the samples were fixed with 10% formalin. Histological slides were prepared and stained with hematoxylin and eosin. The slides were scored under light microscope ranging from 0 (no change) to 4 (full intestinal necrosis). After deparaffinization, the slides were treated with 0.1 M sodium citrate buffer, rinsed with PBS, incubated with 0.03% H_2O_2 and methanol to block the endogenous peroxidase activity, and rinsed with PBS. The slides were TUNEL stained using an in situ cell death detection POD Kit (Roche, Germany). In two slides, the terminal transferase was omitted as a negative control and two slides were incubated with DNaseI for 10 min as a positive control. All slides were counterstained with hematoxylin. Apoptotic index was calculated by dividing the number of apoptotic cells to total cell number, multiplied by 100.

Caspase-3/CPP32 colorimetric assay. Caspase-3 activity was measured by using Caspase-3/CPP32 Colorimetric Assay Kit (Biovision, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. Briefly, 150 µg of protein was added to 50 µl of 2× reaction buffer and 5 µl of DEVD-pNA substrate. After incubation (2 h at 37 °C), DEVD-pNA cleavage was monitored by detecting enzyme-catalyzed release of pNA at 405 nm using a microplate reader.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from the tissue samples with TriPure isolation reagent according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The quality and quantity of RNA were determined with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). One microgram of total RNA was reverse transcribed using the Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany).

Quantitative analysis of hTERT mRNA

Quantitation of hTERT mRNA was performed with Telo TAGGG hTERT Quantification Kit (Roche Diagnostics GmbH, Mannheim, Germany). The amplification signals were detected in real time. hTERT mRNA was reverse transcribed, and a 198-bp fragment of the generated cDNA was amplified with specific primers during 40 cycles. The level of hTERT mRNA was determined as the relative ratio, which was calculated by dividing the level of hTERT mRNA by the level of the PBGD housekeeping gene (hTERT/PBGD).

Real-time RT-PCR

LightCycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany) was used for real-time reverse transcription–polymerase chain reaction (RT-PCR) reactions. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-actin* genes were selected as housekeeping genes. The expression levels of *TERT*, *GAPDH*, *P53*, *STAT3*, *TNFa*, *FAS*, *BCL-2*, *BCL-XL*, *BCL-W*, *ACTB*, *BAX*, *NFKB*, and *CASPASE3* genes were studied according to the manufacturer's instructions.

RESULTS

Histological findings

Macroscopic changes were observed in all animals exposed to H/R. Histopathological damage was most severe in the H/R group followed by the H/R + EPO group, and manifested as edema and inflammatory cells in lamina propria, degeneration of villi, and Paneth cell hypertrophy. The H/R + IGF-1 group had less damage compared to the H/R and H/R + EPO groups (Fig. 1). No change was observed in regard to mitotic activity among study groups.

Evaluation of apoptosis

The number of apoptotic cells was found to be increased in all study groups compared to the control group, confirmed by TUNEL test (Fig. 2). In parallel to this, fluorescence microscopy revealed that apoptosis was decreased in IGF-1- and EPO-treated animals compared to the H/R group (Fig. 3).

Spectrophotometric findings

Caspase-3 activity was significantly increased in study groups compared to the control group. However, IGF-1- and EPO-treated

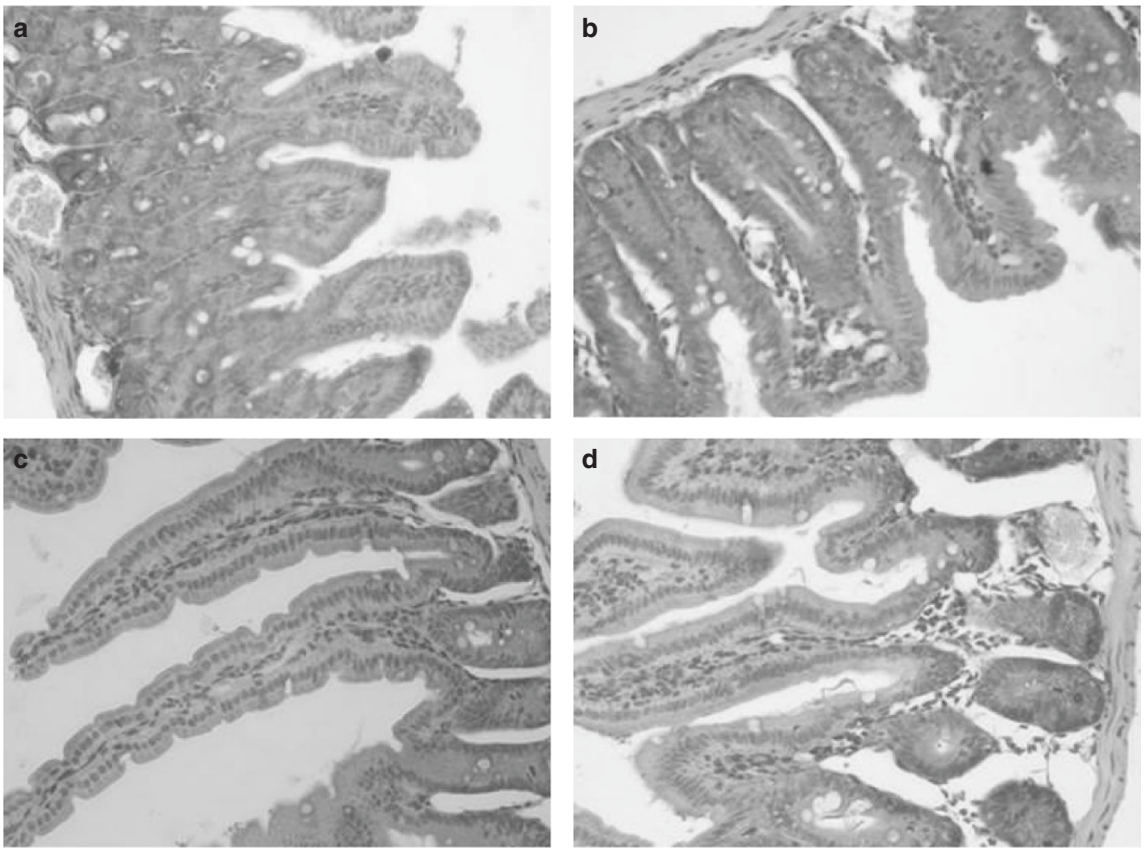

Fig. 1 Hematoxylin and eosin staining of the intestinal tissue of study groups (microscopic view, ×40). a Control group, **b** H/R group, **c** H/R + IGF-1 group, **d** H/R + EPO group. H/R hypoxia/reoxygenation, IGF-1 insulin-like growth factor-1, EPO erythropoietin.

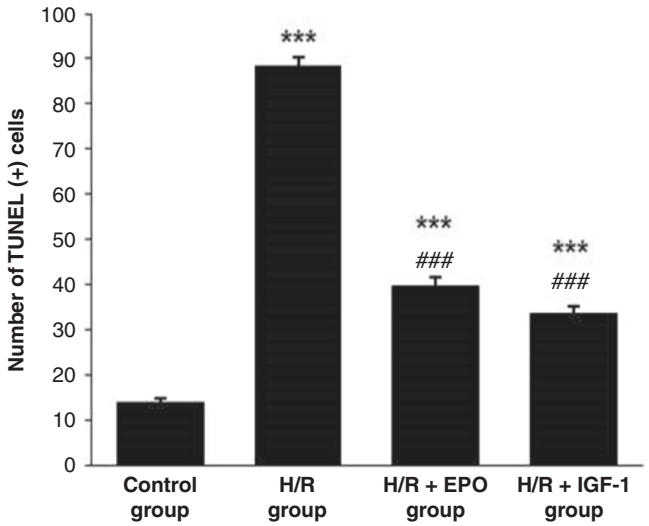

Fig. 2 The number of apoptotic cells found in study groups. $^{***}P < 0.001$ compared to the control group and $^{\#\#\#}p < 0.001$ compared to the H/R group. H/R hypoxia/reoxygenation, IGF-1 insulin-like growth factor-1, EPO erythropoietin.

animals had decreased caspase-3 activity compared to animals without treatment (H/R group) (Fig. 4).

Real-time RT-PCR results

Expression of antiapoptotic gene levels showed variability among study groups. *BCL-XL* and *BCL-2* were increased in all H/R groups compared to controls (Fig. 5). In addition to this, the H/R + IGF-1 group of animals had a decreased ratio of *BAX/BCL-W* compared to the H/R group. Expression of other genes, such as *NFKB1*, *p53*, *STAT*, and *TNFa*, was increased in the H/R group compared to control; however, this trend did not reach statistical significance. Only FAS expression was increased significantly in the H/R group compared to controls.

Telomerase activity was evaluated by telomerase reverse transcriptase (TERT) RT-PCR. TERT expression was similar in the H/R and H/R + EPO groups, but increased significantly in the IGF-1-treated animals compared to the control group ($p < 0.05$) (Fig. 6).

DISCUSSION

NEC continues to be a common and devastating disease in premature neonates and the exact etiopathogenesis remains a challenge. This study revealed the decreased intestinal injury in H/R-induced mice, which were treated with IGF-1 and EPO. At the same time, antiapoptotic gene expressions were increased in addition to increased telomerase activity in IGF-1 treated mice.

There are several best clinical practices achieved to reduce the risk of NEC, namely, breastfeeding, probiotic supplementation, standardized feeding protocols, and limiting prophylactic antibiotic use. But there is no effective treatment protocol that has yet been established.[19] It is well known that, in breast milk, there are many growth factors, such as IGF-1, EPO, epidermal growth factor, transforming growth factor, and taurin, that can slow down the occurrence of NEC.[20] IGF-1 is a kind of pleiotropic cytokine known to have roles in growth, development, cell differentiation, and in various metabolic pathways. It has unique physiological functions, such as promoting growth, development, and healing of the intestinal tissue; however, the effects of IGF-1 on NEC still remains unclear at present.[21] In one study, it was shown that the enteral

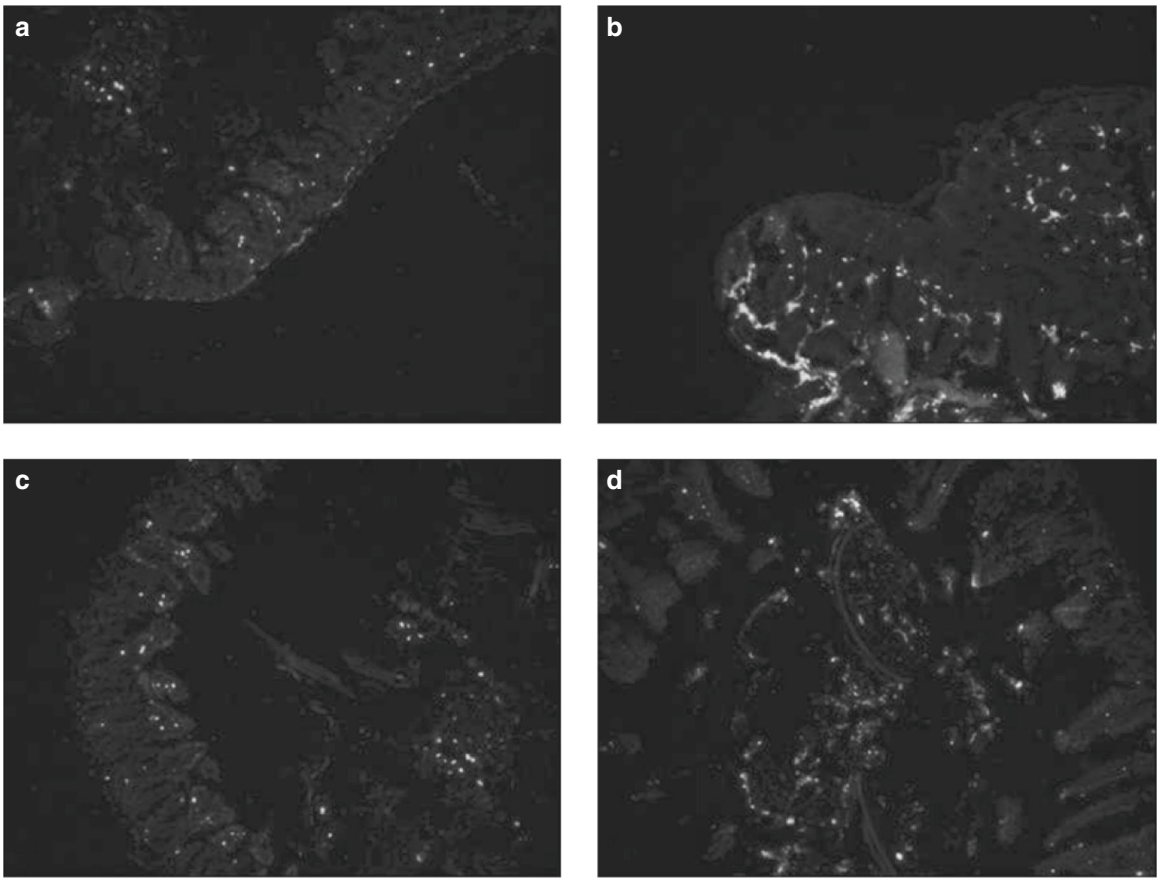

Fig. 3 Fluorescence microscopic view of TUNEL assay results (microscopic view, ×40). **a** Control group, **b** H/R group, **c** H/R + IGF-1 group, **d** H/R + EPO group. H/R hypoxia/reoxygenation, IGF-1 insulin-like growth factor-1, EPO erythropoietin.

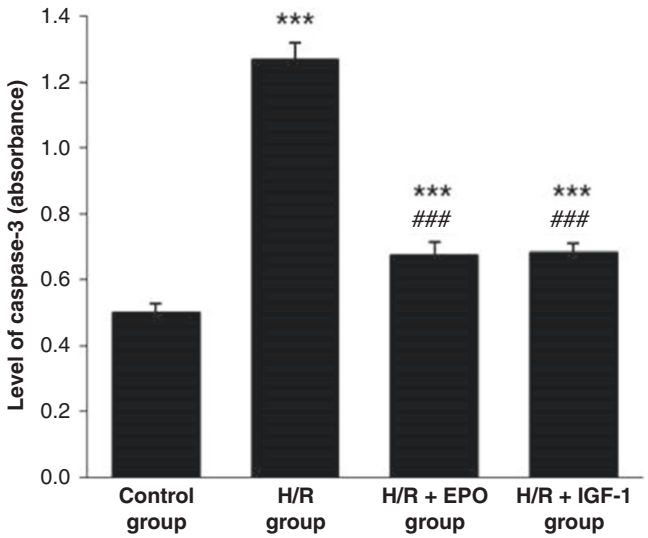

Fig. 4 Caspase-3 levels detected in study groups. $^{***}P < 0.001$ compared to control group and $^{###}p < 0.001$ compared to the H/R group. H/R hypoxia/reoxygenation, IGF-1 insulin-like growth factor-1, EPO erythropoietin.

administration of IGF-1 decreased the clinical symptoms and occurrence rate in neonatal NEC rat models. They proposed that IGF-1 down-regulates the TLR4 mRNA expression to inhibit the production of inflammatory mediators to protect the mechanical and immunobarrier functions of the intestinal mucous.[22] IGF-1 was also shown to exert an antiapoptotic effect by phosphatidylinositol 3-kinase activation and protect intestinal mucosa.[23] On the other hand, EPO is a glycoprotein cytokine that has roles in the proliferation, differentiation, and maturation of erythroid progenitor cells. EPO and its receptors are widely distributed in different tissues also found in the intestines of infants, which indicate that EPO may play a role in the development of the gastrointestinal tract. Previous studies suggested that the recombinant EPO was able to reduce the inflammatory response, decrease autophagy and apoptosis, and limit the intestinal mucosal necrosis, thus improving NEC injury.[18,24–27] In our study, we were clearly able to see the protective effect of IGF-1 and EPO in the intestinal mucosa.

Caspases (cysteine-aspartic proteases) are a family of protease enzymes playing essential roles in apoptosis and inflammation. There are more than 10 confirmed caspases carrying out a variety of cellular functions. Caspase-3 is known to be activated in the apoptotic cell both by extrinsic and intrinsic pathways. TUNEL assay is also used to detect apoptotic DNA fragmentation.[28] In a study, it was shown that EPO treatment protects intestinal epithelium from excessive autophagy and apoptosis in experimental NEC.[27] In our study, the number of apoptotic cells and caspase-3 activity was found to be increased in all study groups compared to the control group. However, apoptosis and caspase activity was decreased in IGF-1 and EPO-treated animals compared to the H/R group. H/R-induced intestinal injury resulting in apoptosis is accompanied by increased expressions of both antiapoptotic (*BCL-2*, *BCL-XL*) and proapoptotic (*FAS*) genes. This dual response could be interpreted as an attempt of the organism to limit the existing damage. In the H/R-induced intestinal injury model, both EPO and IGF-1 pretreatments provide protection seen

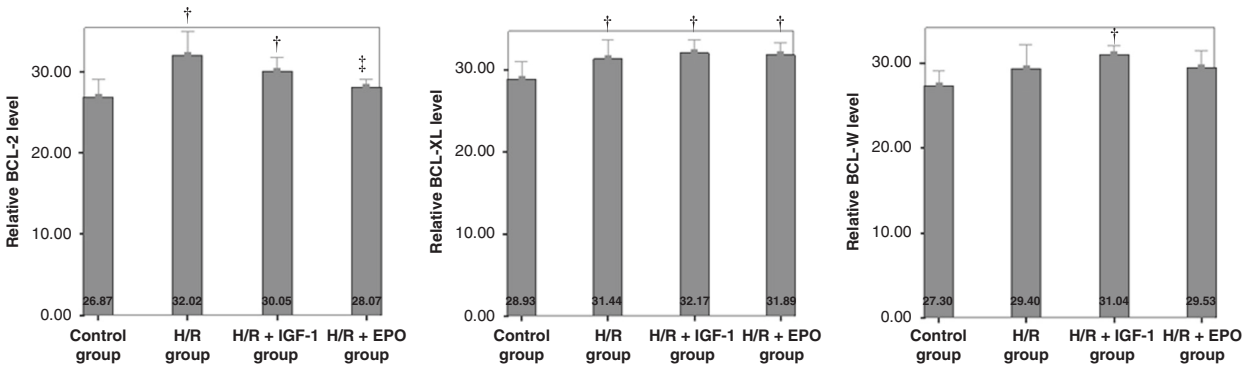

Fig. 5 Relative antiapoptotic gene levels among study groups detected by real-time RT-PCR. $^{\dagger}P < 0.05$ compared to the control group and $^{\ddagger}p < 0.05$ compared to the H/R group. H/R hypoxia/reoxygenation, IGF-1 insulin-like growth factor-1, EPO erythropoietin.

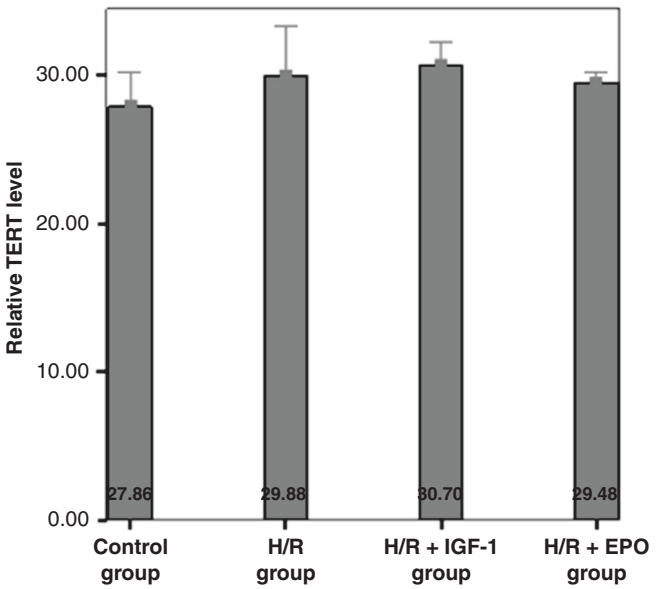

Fig. 6 Relative TERT expression levels among study groups. TERT telomerase reverse transcriptase, H/R group, H/R hypoxia/reoxygenation, IGF-1 insulin-like growth factor-1, EPO erythropoietin.

as decreased histopathological injury through inhibition of caspase-3 and subsequent apoptosis.

TERT is a catalytic subunit of telomerase, which, together with the telomerase RNA component, constitutes the telomerase complex.[29] Best to our knowledge, TERT has not been studied previously in NEC yet. A published study showed that telomerase activity may be derived from intestinal stem cells and this activity may be sufficient to provide extended proliferative capacity for the renewal tissues.[30] In addition, Jaskelioff et al.[31] revealed that telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. As an indicator of tissue regeneration, TERT activity was found to be increased in the IGF-1 group compared to the control group, which may indicate that IGF-1 also has further protective effects as increased regeneration capacity of the intestinal cells via telomere activation. Therefore, telomerase activity has likely to have promising regenerative effects on the intestinal injury, but further studies are needed to fully understand its impact on NEC.

Several animal models have been used and they are of significant value to better understand the pathogenesis of NEC and to develop more effective treatment strategies, such as growth factors, probiotics, stem cells, and breast milk. Although there is no standardized animal model for the study of NEC, they should at least replicate most of the histopathological features that are seen in human NEC.[32] As expected, there are certain advantages and limitations for each animal model. The most useful advantage of the mouse model is the ability to monitor and manipulate genetic variability and the use of transgenic animals.[33] Although models with small animals such as mice and rats have the advantages of being relatively low cost and readily available in large numbers, they lack the clinical robustness that is obvious in larger animal models such as piglets.[32] One of the major limitations of animal models is that humans have a naturally different pattern of gene expression and therefore a distinct immune response to NEC. In addition to this limitation, mice are also different due to their size compared with newborn rats or piglets.[33] However, considering these limitations, mouse models have produced greater insights into the significance of prematurity in animals that develop NEC.

Despite novel advances in the etiology and pathophysiology of NEC, clinicians are still seeking for early definitive diagnosis, robust preventive measures, and highly effective treatment modalities. We believe that molecular and histopathological results obtained from our study will accelerate the implementation of novel early diagnosis and treatment strategies in NEC. To measure the responses to IGF-1 and EPO treatment, antiapoptotic and proapoptotic gene expression levels may be examined rapidly by RT-PCR. By using these markers, this experience could be implemented to the clinic practice using peripheral blood or urine samples, without the need for intestinal biopsy for the early diagnosis. On the other hand, IGF-1 and EPO could be used for the therapy, and agents increasing the telomerase activity in the intestines could be administered to the patient for the treatment of the intestinal injury. However, in order to further evaluate the effects of IGF-1, EPO, and telomerase activity, more high-quality advanced clinical trials with a larger number of subjects are needed to verify the validity and long-term outcomes.

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AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: M.A., B.D., O.A.K., S.U., and U.A. Drafting the article or revising it critically for important intellectual content: M.Y., H.A., and O.C. Final approval of the version to be published: M.A., B.D., M.B., F.O., and N.K.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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