Saccharomyces boulardii exerts anti-apoptosis and anti-necroptosis effects on neonatal mice necrotizing enterocolitis by increasing reactive oxygen species consumption

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Received March 2, 2019; Accepted May 10, 2019; Epub August 15, 2019; Published August 30, 2019

Abstract: Oxidative stress and exaggerated inflammatory response play crucial roles in necrotizing enterocolitis (NEC). Although Saccharomyces boulardii (S. boulardii) participates in many different biological activities, it remains unknown whether S. boulardii inhibits cell death induced by NEC. Furthermore, mechanisms against oxidative stress levels have not yet been elucidated. The present study established an experimental mouse model of NEC obtained after artificial feeding + hypoxia/re-oxygenation cooling stimulation + lipopolysaccharide for 3 days. Hematoxylin and eosin (HE) staining and morphologic studies were performed to define the model. After S. boulardii treatment, weights and survivor numbers were significantly increased, while intestinal tissue damage was reduced. Necroptosis and apoptosis were inhibited by S. boulardii. Protein and mRNA expression levels of caspase-3, caspase-8, and RIP3 further confirmed the protection of S. boulardii. Furthermore, S. boulardii increased super oxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) levels, while promoting clearing of reactive oxygen species (ROS) and malondialdehyde (MDA). The current study provides the first evidence that S. boulardii exerts anti-apoptosis and necroptosis effects in NEC intestinal tissues through increasing ROS consumption.

Keywords: Necrotizing enterocolitis, Saccharomyces boulardii, necroptosis, apoptosis, ROS consumption

Introduction

Necrotizing enterocolitis (NEC) is a neonatal inflammatory bowel disease. It is one of the most life-threatening disease in neonatal gastroenterology [1]. Although the first report of the disease appeared in 1964, causes and pathological mechanisms of the disease have not been clearly stated. A recent study demonstrated that exaggerated oxidative stress and inflammatory response, secondary to abnormal intestinal bacterial colonization in the immature gut, play crucial roles in NEC pathogenesis, although intestinal ischemia, hypoxia, enteral feeding, and prematurity were also included [2]. Large amounts of reactive oxygen species (ROS), pro-inflammatory cytokines, and nitric oxide have been observed in animal models [3, 4] and human NEC cases [5-7]. Initial stress in the immature gut induces the release of platelet activating factor and inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). This is followed by the migration of activated polymorph nuclear leukocytes, leading to excess ROS production [8-10]. In addition, malondialdehyde (MDA), an intermediate metabolite of lipid peroxidation, emerges after the initial insult, causing injuries of the intestine mucosal [11]. Together, these factors result in a series of events that eventually lead to destruction of the intestinal mucosa barrier and severe NEC [12].

Under normal conditions, there is a balance between ROS metabolism through the reduction of oxygen via redox metabolic reactions and effective removal by superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) [13]. Imbalances in this counterpoise caused by excess ROS generation or inadequate antioxidant capacity may lead to mucosal damage, apoptosis, and necroptosis,
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due to peroxidation of cellular membrane lipids and proteins [14]. Strengthening antioxidant capacity is pivotal in NEC infants. A shortage of antioxidant enzyme is an important factor that causes intestinal damage [12].

There are two types of cell death, programed cell death (apoptosis) and non-programed cell death (necrosis). A recent study revealed a new form of cell death, programmed necrosis (also known as necroptosis). This is a type of cell death that is driven as a result of deregulated activity of RIPK1 and RIPK3 [15]. Cellular apoptosis [16] and necroptosis [17, 18] are significantly related to ROS intervention. Many studies have shown that NEC can cause apoptosis of intestinal epithelial cells. However, whether NEC can induce cell necroptosis remains unclear.

Saccharomyces boulardii is a non-toxic yeast that is beneficial in many abnormal bacterial growth related gastrointestinal disorders [19]. S. boulardii is beneficial due to its probiotic effects [20] and the ability to produce polypeptides [21]. In addition, S. boulardii can prevent or manage traveler’s diarrhea and antibiotic-associated diarrhea, as it can resist most antibiotics [22]. However, the roles of S. boulardii concerning NEC prevention remain unclarified.

Therefore, the objectives of the current study were: (1) To investigate whether S. boulardii treatment can protect against neonatal mouse NEC; (2) To determine whether the protective effects of S. boulardii against neonatal mice NEC are achieved via inhibition of apoptosis and/or necroptosis; and (3) To verify whether S. boulardii protects neonatal mice NEC by inhibiting ROS production.

Materials and methods

Reagents

Anti-caspase-3 (1:1,000 dilution, #9662), caspase-8 (1:1,000 dilution, #8592), and β-actin (1:1,0000 dilution, #3700) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-RIP3 (1:1,000 dilution, ab56164) was purchased from Abcam (Cambridge, UK). LPS (E. coli 055:B5) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). An in situ cell death detection kit (cat. no. 11684795910) was purchased from Roche Diagnostics (Indianapolis, IN, USA). Dabco4 and 6-diamidino-2-phenylindole (DAPI; cat. no. C1005) was purchased from Beyotime Institute of Biotechnology (Haimen, China). Superoxide dismutase (SOD; #A001-1), malonaldialdehyde (MDA; #A002-1), and catalase (CAT; #A007-1) detection kits were purchased from JIANCHENG Bioengineering Institute (Nanjing City, P.R China). Cellular glutathione peroxidase (GPx; #S0056) assay kit was purchased from Beyotime Institute of Biotechnology (Nantong City, P.R China). The TIANamp Genomic DNA kit was purchased from Tiangen Biotech Co., Ltd. (Beijing, China).

Experimental design

C57BL/6 mice (1.4-1.6 g) were purchased from the Laboratory Animal Center of Xuzhou Medical University (Jiangsu, China). Immediately after birth, the neonates were weighed and randomized into four groups. Group A was breast-fed. Group B was housed in an incubator (Ohio Medical Products, Madison, WI) and garaged with a special rodent formula three times daily. Neonatal mice were fed Esbilac puppy formula (~200 kcal/kg/day) every 3 hours via an orogastric feeding catheter. The feeding volume began at 0.1 cc every 3 hours and increased incrementally to account for somatic growth [23]. Newborn mice in Group C were treated with artificial feeding + hypoxia/re-oxygenation cooling stimulation + lipopolysaccharide (10 mg/kg). Hypoxia/re-oxygenation cooling stimulation: The oximeter was zeroed. The oximeter probe was connected to the top of the closed anoxic chamber and the tape is closed to prevent outside air from entering. The inlet of the closed anoxic tank was filled with pure nitrogen gas and the flow rate of nitrogen was controlled to 15 L/min. Neonatal mice were placed in an anoxic chamber and were removed from the box after 90 seconds of suffocation. They were then placed in an oxygen chamber with 100% O₂ for 10 minutes of reoxygenation stimulation. Afterward, the neonatal mice were removed from the oxygen chamber and immediately stimulated for 10 minutes in a refrigerator at 4°C. After hypoxia + reoxygenation + cold stimulation. Newborn mice were immediately returned to the incubator. LPS administration: The lipopolysaccharide was diluted with 0.1 mL of sterilized water and the concentration was 10 mg/kg after dilution. The neonatal rats were orally perfused through the oral cannula with a demineralized indwelling needle once a day for 3 days. Group D was treated in a similar way to
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Group C. However, they were subjected to S. boulardii before feeding. The newborn mice were orally administered with S. boulardii once a day at a dose of 800 mg/kg/d (the dose of 800 mg/kg is most effective in 200, 400, 600, 800, 1000, and 1200). At the end of the gavage, the newborn rats were returned to the incubator. The neonatal mice were killed on day 3 and harvested the last 2 cm of terminal ileum. All experiments were conducted in accordance with the guidelines of the Animal Care Committee of Xuzhou medical University. The number of mice in each of the 9 groups of average growth weight studies and in the control group, artificial feed group, NEC group, and NEC+SB group of body weight measurements was 3. The number of animals in the survival quantity study was 22-24 per group. Animal numbers and protocols were approved by the Xuzhou medical University Animal Care Committee. Effort was made to minimize suffering. Any animals showing evidence of pain, distress, tachypnea, hypotension, or sepsis were immediately euthanized.

Morphologic studies

Hematoxylin and eosin (H&E) slides were prepared, according to standard protocol [24]. Morphological changes were graded as normal, mild, moderate, or severe in the intestinal epithelium. The definition for each histologic grade was as follows: (1) Mild, separation of the villus core, without other abnormalities; (2) Moderate, villus core separation, submucosal edema, and epithelial sloughing; and (3) Severe, denudation of epithelium with loss of villi, full thickness necrosis, or perforation. For data analysis, only specimens that displayed moderate or severe histologic abnormalities were considered to have experimental necrotizing enterocolitis [25].

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA of intestinal specimens was extracted using TRIzol Reagent, according to manufacturer protocol (Tiangen Biotech, Beijing, China). Complementary DNA (cDNA) was synthesized from 1 mg total RNA using the ImProm-II™ Reverse Transcription System (Promega Corp., Madison, WI, USA). PCR amplification of cDNA and quantification was performed using SYBR Green (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). Relative product levels were quantified using the 2(ΔΔC(T)) method [26]. β-actin serving as an internal standard was arbitrarily assigned a value of 1.0. Sequence-specific primers used for qPCR were as follows:

Caspase-8 sense: 5'-CATGTCCTGATTTTGATG-G-3' and antisense: 5'-AGCCAGATGGCTCCA-TAC-3'; caspase-3 sense: 5'-CTGAGCTGCGTA-TTGAGAC-3' and antisense: 5'-CCGGTGCGG-TAGGTAAGC-3'; RIP3 sense: 5'-GAGTTGCAA-CGAACCATC-3' and antisense: 5'-CTTTGGAAGGCTGCCACCTACC-3'; β-actin (control) sense: 5'-GGTCAAGAGGTCTATG-3' and antisense: 5'-GGTCTCAACATGATCTGGG-3'.

Western blotting analysis

For Western blotting analysis, tissues were solubilized in a lysis buffer (10 mmol/l Tris-HCl, 20% glycerine, 200 mmol/l dithiothreitol and protease inhibitors; pH 6.8). Protein concentrations from the supernatant were determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). A total of 50 ng protein per lane was separated by 12% SDS-PAGE (Bio-Rad, USA) and transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk for 2-3 hours at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. Caspase-3, caspase-8, and RIP3 rabbit polyclonal and rac1 antibodies were used at a dilution of 1:1,000. β-actin antibody was used at a dilution of 1:10,000. Next, fluorescence-labeled secondary antibodies were added for 1 hour at 37°C: Anti-rabbit IgG H+L DyLight™ 800 4X PEG (1:30,000; cat. no. 5151) and anti -mouse IgG (H+L DyLight™ 680, 1:15,000; cat. no. 5470) (both from Cell Signaling Technology, Inc.) and membranes were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). ImageJ software (v1.50; NIH, Bethesda, MD, USA) was used for quantification.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for apoptosis and RIP3 for necroptosis in vivo

Samples from the terminal ileum were embedded in an optimum cutting temperature compound tissue medium (Sakura Finetek Europe B. V., Flemingweg, the Netherlands), snap-frozen on dry ice, and stored at -80°C. Apoptosis in vivo was determined by double-labeling TUNEL immunofluorescence staining. This was performed with an in situ cell death detection
kit. Necroptosis was identified using RIP3 antibodies. Specimens were blocked with 5% bovine serum albumin (Vicmed Life Sciences, Xuzhou, China) for 30 minutes at room temperature and incubated with anti-RIP3 overnight at 4°C. They were then washed three times in PBS. Specimens were incubated with goat anti-mouse immunoglobulin G (IgG; H+L) cross-absorbed secondary antibody (Alexa Fluor®488/green; 1:200; A11001; Thermo Fisher Scientific, Inc.). According to manufacturer protocol, TUNEL staining was performed using a cell death detection kit (Roche, USA). Before TUNEL staining, the solution was mixed with an enzyme-to-label ratio of 1:9. The samples were incubated and contained at 37°C for 1 hour. They were then washed three times in PBS. DAPI staining was conducted at room temperature for 15 minutes. It was used to count the total number of nuclei. The percentage of apoptotic and necroptosis cells was calculated as the ratio of the number of TUNEL-positive cells and the RIP3-positive cells to the total number of cells. These were counted in three different random fields of view.

Detection of intracellular ROS generation

DHE was used to stain for intracellular ROS, as previously described [27]. DHE, an oxidant-sensitive probe, has been widely used for detection of ROS. Tissues were incubated with 10 µM DHE for 30 minutes at 37°C, according to manufacturer protocol. They were then washed with DMEM without FBS three times. Fluorescence was observed under a fluorescence microscope. Fluorescence was calculated by viewing in four randomly selected fields for each group. Image-Pro Plus software (v6.0; Media Cybernetics, Inc.) was used for quantification.

Measurement of T-SOD, MDA, CAT, and GPx

T-SOD, MDA, GPx, and CAT activities were examined using the xanthine oxidase method, TBA method, ultraviolet spectrophotometric method, and colorimetric method. Data was observed using a multi-mode microplate reader (Synergy 2, Bio-Tek, USA).

Statistical analysis

Results are expressed as the mean ± standard error of the mean. All statistical analyses were conducted with SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA). Results were analyzed using two-tailed Student’s t-tests or repeated-measures one-way analysis of variance, followed by Fisher’s least significant difference tests. P < 0.05 indicates statistically significant differences.

Results

Effects of Saccharomyces boulardii on weight gain and survivor numbers

Although S. boulardii had been shown to be effective in the prevention and management of diarrhea, the effects of S. boulardii on newborn NEC prevention remain unclarified. To investigate the most optimal concentration of S. boulardii, NEC mice were treated with S. boulardii at concentrations ranging from 200 to 1200 mg/kg. The average growth weights of neonatal mice are presented in Figure 1A, indicating that S. boulardii at a concentration of 200 mg/kg did not increase the average growth weight up to a concentration of 800 mg/kg. S. boulardii increased the average growth weight by 0.6 g, compared with the NEC group. In contrast, treatment with S. boulardii at a concentration of 1,200 mg/kg significantly reduced the average growth weight, compared with 800 mg/kg. The concentration of 1,000 mg/kg was not significantly increased, compared with that of the 800 mg/kg. This biphasic dose-response phenomenon indicated that the best concentration of S. boulardii was 800 mg/kg, in agreement with a previous study. It was hypothesized that S. boulardii could protect the NEC mice. To test this hypothesis, weights and survivor numbers were tested. As shown in Figure 1B and 1C, NEC decreased the newborn mice weights and survivor numbers. However, S. boulardii significantly increased these values. Current results demonstrate that S. boulardii significantly protected the NEC mice. Present data also indicates that high doses of S. boulardii decreased the weights of the NEC mice.

S. boulardii decreases severity of intestinal injuries in neonatal mice intestines

At baseline, the control group exhibited similar intestinal appearances, with a median histologic score of 0 (IQR = 0-1). The artificial group and NEC group exhibited significantly worse intestinal injuries, compared to the control group. The artificial group had a median score of 2 (IQR = 1-3, Figure 2A). The NEC group had a higher incidence of NEC development, with a median histologic injury score of 3.25 (IQR = 2.25-3.625). As seen in Figure 2B, hematoxylin...
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S. boulardii conferred anti-apoptotic and anti-necroptosis effects in the NEC mice. As seen in Figure 3B and 3C, S. boulardii suppressed the necroptosis ratio more than the apoptosis ratio. Results suggest that S. boulardii inhibited both apoptosis and necroptosis. More importantly, the effects of S. boulardii on necroptosis were significant.

S. boulardii inhibited apoptosis and necroptosis associated factor expression

To further assess the anti-apoptosis and anti-necroptosis effects of S. boulardii, caspase-8, caspase-3, and RIP3 protein expression and mRNA levels were tested. As presented in Figure 4A, caspase-8 and caspase-3 proteins increased after artificial feeding, while no significant effects were observed after treatment with RIP3. Expression of these three proteins significantly increased in the NEC group, whereas S. boulardii decreased expression, especially for RIP3. Moreover, mRNA levels of caspase-8, caspase-3, and RIP3 exhibited the same trend (Figure 4B-D). Present results indicate that S. boulardii inhibited both apoptosis and necroptosis. More importantly, the effects of S. boulardii on necroptosis were more significant, similar to results shown in Figure 3.

S. boulardii reduced the promotion of consumption of ROS to decrease apoptosis and necroptosis

As shown in a previous study, ROS plays a key role in NEC [28]. ROS levels were increased in...
Figure 2. S. boulardii decreases severity of intestinal injuries in neonatal mice intestines. A. Histologic injury scores were significantly worse in both artificial groups, compared to the control group. The NEC group had more severe injuries than the control group. **P < 0.01 vs. control group. B. Representative histologic sections of each group (H&E stained, 20 × magnification) demonstrate villus disarrangement and villous sloughing in the artificial groups, with significant villous core separation noted, specifically in the NEC group, bar = 500 μm. Data were compared using two-tailed Student’s t-tests.
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However, S. boulardii decreased ROS and MDA levels, which were increased in the NEC group (Figure 5A, 5B). In contrast, the activities of T-SOD, CAT, and GPx showed an opposite trend (Figure 5C-E). These results suggest that S. boulardii upregulated the activities of SOD, GPx, and CAT, promoting the clearing of ROS and MDA.
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Discussion

The current study first demonstrated that *S. boulardii* protects against neonatal mouse necrotizing enterocolitis by inhibiting apoptosis and necroptosis and increasing ROS consumption. *S. boulardii* with a concentration of 800 or 1,000 mg/kg showed significant positive effects on average growth weights, newborn weights, and survivor numbers in NEC newborn mice. Furthermore, there was a lower rate of intestinal injury in NEC newborn mice treated with 800 mg/kg of *S. boulardii*. Current results suggest that *S. boulardii* exerts protective effects on NEC newborn mice.

In addition, NEC not only induced intestinal epithelial cell apoptosis, but also caused cell necroptosis. RIP3 protein levels were significantly increased in the NEC+SB group, compared with the NEC group. Surprisingly, *S. boulardii* mitigated both cellular apoptosis and necroptosis in NEC newborn mice. Inhibition of necroptosis was more pronounced than inhibition of apoptosis. Tumor necrosis factor (TNF) signaling plays an important role in cellular necroptosis. TNF is a pleiotropic cytokine that regulates inflammation steered by tissue injuries and infections. TNF signaling induces gene expression that regulates inflammation. However, TNF is also a potent regulator of cell death under certain circumstances [29]. Although some early studies suggested that TNF promotes caspase independent cell via RIP1, most studies have demonstrated that TNF-induced cell death is associated with cellular apoptosis [30]. The confirmation of RIP1-targeted necroptosis inhibitors offers proof that TNF-induced cellular necrosis is a kinase-regulated process, named necroptosis [31]. Therefore, it was hypothesized that the anti-necroptosis effects of *S. boulardii* on NEC newborn mice may be related to TNF signaling pathways. However, additional studies and investigations are necessary.

RIP3 induced necroptosis involves mitochondrial fission and ROS production and consumption [32]. Thus, the current study explored the effects of *S. boulardii* on ROS levels and ROS consumption in NEC mice. It is noteworthy that ROS and MDA levels in NEC+SB group were significantly decreased, compared with the NEC group. Furthermore, *S. boulardii* increased the anti-oxidative enzymatic activity of SOD, CAT, and GPx in NEC newborn mice. Current results...
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suggest that S. boulardii protects NEC newborn mice by inhibiting ROS production and consumption. Moreover, S. boulardii significantly increased CAT activity, compared with SOD and GPx activity.

There were several limitations to the current study, including the inability to study the effects of S. boulardii on ROS consumption rather than both ROS production and consumption in NEC newborn mice. Although the current study investigated the protective effects of S. boulardii on weights and survivor numbers in newborn mice subjected to artificial feeding + hypoxia/re-oxygenation cooling stimulation + lipopolysaccharide for 3 days, it did not investigate results at longer time points.

In conclusion, the current study revealed that S. boulardii protects NEC newborn mice via its anti-apoptosis and anti-necroptosis effects and inhibition of ROS production and consumption. Present results offer a new mechanism in which S. boulardii inhibits NEC injuries in newborn intestine. Targeting of S. boulardii NEC pathways in neonatal gastroenterology may provide therapeutic benefits. Therefore, this new strategy may assist in alleviating intestinal injuries and fighting neonatal inflammatory bowel disease.

Acknowledgements

This work was supported by the Nanjing Military Innovation Project (15MS042).

Disclosure of conflict of interest

None.

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