scientific reports

Check for updates

OPEN

Sildenafil pretreatment attenuates apoptosis and intestinal injury in a hypoxia–ischemia–induced NEC-like rat model

Şenay Kurtuluş[1], Özlem Öztopuz[2], İhsan Karaboğa[3] & Hakan Aylanç[4]✉

Necrotising enterocolitis (NEC) is a devastating gastrointestinal disease of premature infants, in which hypoxia-induced apoptosis plays a critical role in intestinal epithelial injury. The balance between pro-apoptotic and anti-apoptotic proteins, particularly Bax and Bcl-2, as well as the hypoxia-responsive transcription factor HIF-1α, are key determinant of epithelial survival. Sildenafil citrate, a phosphodiesterase-5 inhibitor, has been shown to improve microcirculation and exert anti-apoptotic effects through nitric oxide–cGMP signalling, but its role in NEC-related intestinal injury remains poorly understood. This study aimed to evaluate the protective effects of sildenafil on apoptosis in a hypoxia–ischemia–induced NEC-like intestinal injury model. A total of 75 Wistar rat pups were randomly assigned to six groups: control, NEC, vehicle (saline+DMSO), and sildenafil-treated groups (1, 5, and 10 mg/kg/day). Sildenafil was administered intraperitoneally for 4 days before NEC induction. NEC was induced by hypoxia (100% nitrogen for 60 s, twice daily for 48 h) and followed by cold stress (10 min at 4 °C). Intestinal tissues were collected for histopathological scoring, caspase-3 immunohistochemistry, and quantitative RT-PCR analysis of HIF-1α, Bax, and Bcl-2 expression. Data were analysed using ANOVA and Kruskal–Wallis tests, with $p \leq 0.05$ considered statistically significant. Pups subjected to hypoxia-induced intestinal ischemia exhibited severe villous damage, elevated histological injury scores, and marked upregulation of HIF-1α and Bax, along with downregulation of Bcl-2. Caspase-3 immunoreactivity strongly correlated with histological injury (Spearman's $\rho = 0.795$, $p < 0.001$). Sildenafil pretreatment reduced histological damage and apoptosis in a dose-dependent manner. The Sil_10 group showed the lowest injury score (1.06 ± 0.4, $p < 0.0001$ vs. NEC), significantly reduced Caspase-3 labelling, suppressed Bax and HIF-1α expression, and increased Bcl-2 levels, resulting in a markedly reduced Bax/Bcl-2 ratio compared with NEC ($p < 0.0001$). Sildenafil attenuates hypoxia-induced, ischemia-dominant NEC-like intestinal injury by modulating apoptotic and hypoxia-related pathways, lowering HIF-1α expression, reducing Bax/Bcl-2 ratio, and preserving epithelial integrity. These findings provide novel histological and molecular evidence for the anti-apoptotic effects of sildenafil in experimental NEC, highlighting HIF-1α modulation as a potential therapeutic strategy.

Keywords NEC-like intestinal injury, Apoptosis, HIF-1α, Bax/Bcl-2 ratio, Caspase-3, Sildenafil

Necrotising enterocolitis (NEC) is a serious gastrointestinal disease that primarily affects premature newborns. The overall incidence of NEC has been rising due to improved survival rates among premature infants, affecting approximately 7% of very low birth weight babies[1–3]. Multiple risk factors, including formula feedings, microvascular events that cause hypoxia, impaired intestinal barrier and immunity, and abnormal bacterial colonisation, have been shown to play a role in NEC development[4–6]. Hypoxia is a significant risk factor for NEC and is thought to be a critical initial injury that triggers multiple inflammatory reactions in the gut. Ischaemia-induced hypoxia is believed to be the first injury that triggers a variety of inflammatory reactions[7]. Baregamian et al. found significant tissue hypoxia in the intestinal mucosa in the NEC model[8]. Apoptosis is critical in NEC pathology, contributing to intestinal epithelial damage and tissue necrosis[9,10]. Hypoxia can trigger apoptosis by

[1]Department of Pediatric Surgery, Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye. [2]Department of Biophysics, Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye. [3]Department of Histology and Embryology, Faculty of Medicine, Kırklareli University, Kırklareli, Türkiye. [4]Department of Pediatrics, Division of Neonatology, Faculty of Medicine, Çanakkale Onsekiz Mart University, Çanakkale, Türkiye. ✉email: hakanaylanc@comu.edu.tr

activating specific pathways, and the balance between pro-apoptotic and anti-apoptotic proteins determines whether a cell undergoes apoptosis or survives[11,12].

Intestinal microcirculation is primarily regulated by nitric oxide (NO) produced through endothelial NO synthase (eNOS) activity[13,14]. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that regulates the cellular response to hypoxia and is essential for maintaining oxygen homeostasis.HIF-1 controls NO production for cellular adaptation to hypoxia[15]. HIF-1 is a heterodimer consisting of α and β subunits. Under normal oxygen levels, HIF-1α is rapidly degraded. However, when exposed to hypoxia, the HIF-1α is stabilized and forms a heterodimer with HIF-1β[16–18]. Additionally, upregulated HIF-1α expression suggested that hypoxia-induced intestinal cell damage and HIF-1α activation regulates critical cellular responses during NEC[8]. Zhang et al. observed that mice lacking HIF-1α in the intestinal epithelium exhibited an upregulation of the TLR4 signalling pathway in NEC[19]. These mice showed increased susceptibility to NEC-induced intestinal injury, characterized by elevated oxidative stress, increased apoptosis, and reduced cellular proliferation. These findings highlight the critical role of intestinal epithelial HIF-1α in modulating NEC pathology. Disturbances in redox chemistry have been reported to play a critical role in the pathogenesis of NEC, with hypoxia-inducible factor-1 alpha (HIF-1α) and inducible nitric oxide synthase (iNOS) contributing to oxidative stress in this process[20].

Apoptosis involves the activation of cysteine-dependent aspartate-specific proteases known as caspases, with caspase-3 acting as a key effector in the execution phase of programmed cell death. Once caspase-3 is activated, the apoptotic process becomes irreversible[21]. Hypoxia can trigger apoptosis by activating specific pathways, and the balance between proapoptotic and anti-apoptotic proteins determines whether a cell undergoes apoptosis or survives[11,12]. Apoptosis plays a central role in the pathogenesis of NEC, contributing to intestinal epithelial damage and tissue necrosis[9,10]. In a neonatal rat model, it has been shown that the increase in the rate of epithelial apoptosis not only precedes subsequent gross intestinal wall necrosis but is also an underlying cause[10]. Apoptosis is associated with the upregulation of proapoptotic proteins and the downregulation of antiapoptotic proteins. The balance of Bax and Bcl-2 plays an important role in the process of apoptosis; furthermore, the Bax/Bcl-2 ratio can increase the Caspase-3 expression[22].

Sildenafil citrate, a phosphodiesterase-5 (PDE-5) inhibitor, prevents the breakdown of 3′, 5′-cyclic guanosine monophosphate (cGMP), a key regulator of smooth muscle relaxation[23]. The accumulation of cGMP enhances nitric oxide (NO) production via the NO/cGMP pathway, thereby improving intestinal perfusion. Experimental studies have shown that sildenafil improves intestinal microcirculatory function and attenuates apoptosis by modulating NO synthesis and the NO–cGMP signaling pathway[24]. Moreover, investigations in various tissues and animal models indicate that sildenafil may confer direct protection against necrosis and apoptosis following ischemia–reperfusion injury through NO-dependent mechanisms[24–27]. In addition, sildenafil has been reported to exert protective effects in other experimental models of gastrointestinal injury; for instance, in a rat model of ethanol-induced gastric injury, it conferred protection through activation of the NO/cGMP/KATP pathway, an NO-dependent process abolished by pretreatment with the nonselective NOS inhibitor L-NAME[28]. Beyond NO/cGMP-mediated vasodilation, sildenafil has also demonstrated antioxidant and anti-inflammatory effects in pulmonary arterial hypertension—lowering serum 4-hydroxynonenal (4-HNE) and improving phospholipid fatty-acid profiles[29].

Sildenafil remains an alternative treatment option for persistent pulmonary hypertension, particularly in settings of iNO non-response or limited access to the newborn. Given its mechanism of action, which lowers pulmonary arterial pressure, it concerns that sildenafil may cause severe systemic hypotension and severe hemodynamic instability have not been proven. While there is debate about whether sildenafil affects the normal development of visual function in premature infants through its effect on PDE6 receptors in the eye, there is no proven evidence of a direct link between sildenafil treatment and ocular complications[30–32].

In this context, the present study aimed to investigate the protective effects of sildenafil pretreatment on intestinal apoptosis in a rat model of hypoxia-induced intestinal ischemic injury. Specifically, we sought to evaluate its anti-apoptotic potential in preserving intestinal epithelial integrity, a key pathogenic component of necrotizing enterocolitis.

Methods

Animals

All animal procedures were approved by the Çanakkale Onsekiz Mart University Animal Experiments Local Ethics Committee (Approval No: 2022/01–05) and conformed to the standards of the Office of Laboratory Animal Welfare regarding the housing and medical care of Wistar rats used in research. This study was conducted in compliance with relevant guidelines and regulations and is reported in accordance with the ARRIVE guidelines. Fourteen-day-old Wistar rat pups were obtained from the Çanakkale Onsekiz Mart University Experimental Research Application and Research Centre (ÇOMUDAM). The experimental pups were separated from their mothers and placed in cages under optimal laboratory conditions (temperature: 20 ± 2 °C; humidity: 50%; light/dark cycle: 12/12 hours). All pups were individually numbered and randomly assigned to 6 groups without regard to gender.

Drug administration

Sildenafil citrate salt (Sigma-Aldrich, CAS No. 171599-83-0) was used as an active compound. The powder was weighed on a precision balance under sterile conditions and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/mL to ensure complete dissolution, as sildenafil is poorly soluble in aqueous isotonic solutions (e.g., phosphate-buffered saline). Intraperitoneal (IP) administration was selected to ensure reliable and reproducible systemic exposure[33].

The pups were randomly assigned to six groups: control ($n = 7$, no intervention), Intestinal ischemia ($n = 8$, hypoxia–hypothermia–induced NEC-like intestinal injury without sildenafil), Vehicle control (saline+DMSO)

($n = 15$, 10 µL DMSO in saline IP), Sil_1mg ($n = 15$, 1 mg/kg/day sildenafil in 10 µL DMSO IP), Sil_5mg ($n = 15$, 5 mg/kg/day sildenafil in 10 µL DMSO IP), and Sil_10mg ($n = 15$, 10 mg/kg/day sildenafil in 10 µL DMSO IP). All administrations were performed once daily for 4 days.

Experimental design-Intestinal Ischemia Induction and tissue collection

An experimental **intestinal ischemia model**, predominantly driven by hypoxia-induced ischemic injury, was employed. The induction protocol was adapted from previously published models[34,35]. At 18 days of age, rat pups in the experimental groups were subjected to twice-daily hypoxia (100% nitrogen exposure for 60 s), immediately followed by cold stress at 4 °C for 10 min. This regime continued for 48 h. Unlike classical neonatal NEC models that incorporate enteral feeding and bacterial exposure, the present protocol focused on **hypoxia-and hypothermia-induced intestinal ischemic injury** to allow controlled evaluation of epithelial damage and apoptosis. This model was specifically selected to investigate **ischemia-driven epithelial injury and apoptotic pathways**, which represent a central pathogenic mechanism in NEC. The experimental design and animal allocation are summarised in Fig. 1.

After the experimental period, pups were anesthetized with an intramuscular injection of ketamine (70 mg/kg) and xylazine (7 mg/kg). A midline laparotomy was then performed, the abdomen was explored, and the ileum was carefully dissected and removed. At the end of the protocol, all pups were euthanized by exsanguination via transdiaphragmatic cardiac puncture under deep ketamine/xylazine anesthesia, in accordance with institutional animal care guidelines.

The intestinal lumen was flushed with cold phosphate-buffered saline (PBS; pH 7.4). Each ileal sample was divided into two portions: one portion was snap-frozen in liquid nitrogen and stored at − 80 °C for subsequent gene expression analysis; the other portion was fixed in 10% neutral-buffered formalin for histopathological and immunohistochemical evaluation.

Histopathological examination

Intestinal tissue samples were fixed with 4% formalin, embedded in a paraffin dispenser (Leica, HistocoreArcadia), and 4–5 µm thick sections were obtained using a semi-motorised microtome (Leica, RM2245) were stained with Hematoxylin-Eosin (H&E) (MerckMilipore) and Masson trichrome (MT) (TK, EpiFarma, Biooptica). The prepared sections were used for histochemical and immunohistochemical labelling of Caspase-3. Two blind observers scored morphological changes in the intestinal epithelium according to the modified scoring system used by Caplan et al.[36]. The definition of each histological grade was as follows: 0-normal intestine, 1-mild

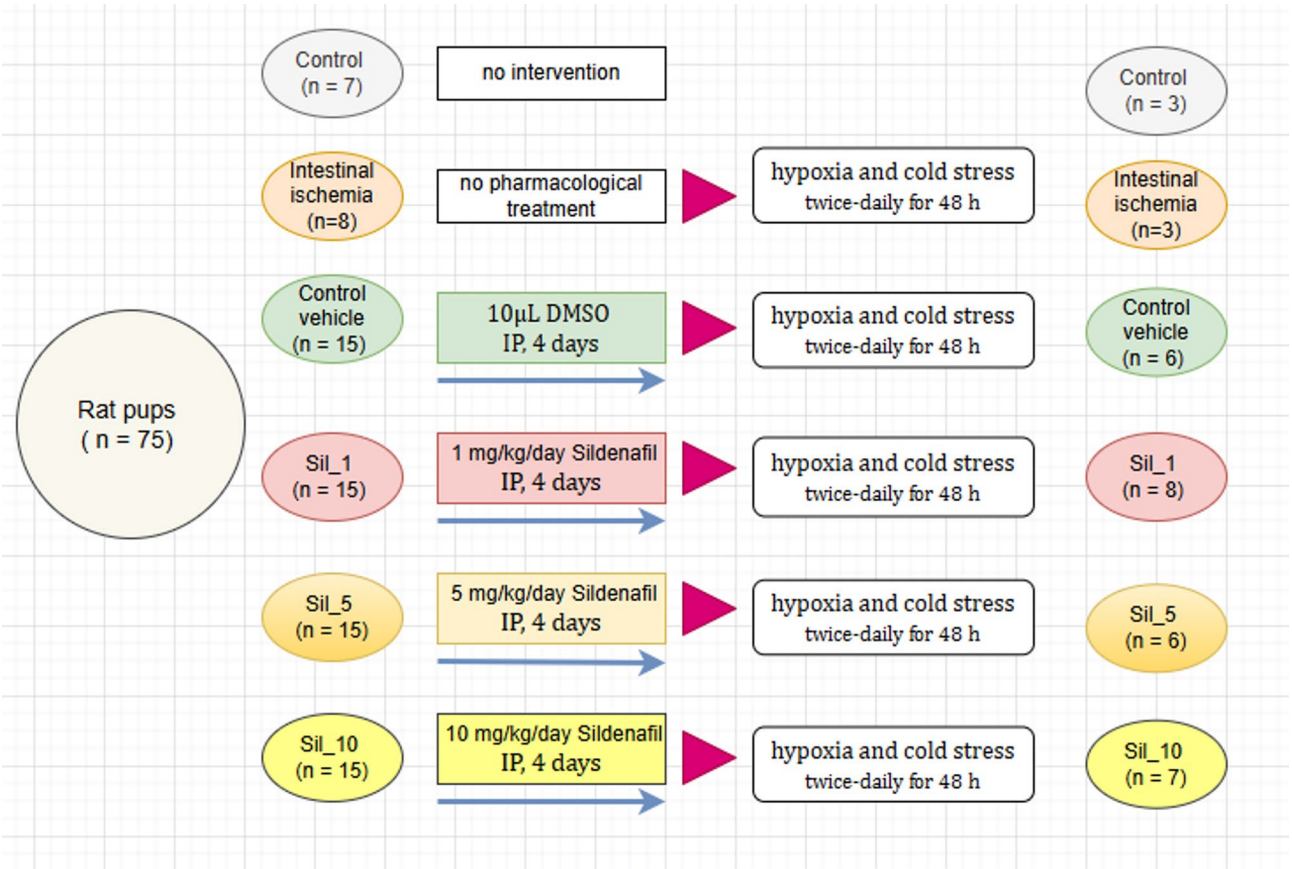

Fig. 1. Experimental design and group allocation for the intestinal ischemia model induced by hypoxia and cold stress.

epithelial shedding in the intestinal mucosa, 2-moderate villus damage, 3-severe villus damage, and 4-transmural necrosis.

Caspase-3 immunolabeling

Immunohistochemical detection of apoptosis-related protein, Caspase-3, was carried out on 4–5-mm-thick deparaffinized sections. Sections were hydrated in graded ethanol and washed with phosphate-buffered saline (PBS). For antigen retrieval, sections were placed in citrate buffer (0.01 M, pH 6.0; Thermo Scientific) and boiled in a microwave oven at 700 W for 5 min. After cooling to room temperature, endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide for 5 min. A hydrophobic barrier pen was then used to create a barrier around the tissue. Non-specific binding was blocked with normal serum, and sections were incubated with primary antibody (Elabscience, E-AB-63510) in a humid chamber at 37 °C for 1 h. Subsequently, sections were incubated with a biotinylated secondary antibody, followed by streptavidin, for 30 min at room temperature. 3-Amino-9-ethylcarbazole (AEC) was used as the chromogen, and Mayer's hematoxylin was applied as the counterstain.

The obtained preparations were evaluated using an Olympus BX40 microscope (Olympus, Japan) equipped with a camera attachment and an image analysis program. Caspase-3 immunoreactivity was evaluated as previously described[37]. Staining distribution was scored as 0.1 (≤25%), 0.4 (26–50%), 0.6 (51–75%), and 0.9 (76–100%). Staining intensity was scored as 0 (no staining), 0.5 (very weak), 1 (weak), 2 (moderate), and 3 (very strong). The final immunohistochemical score was calculated as distribution × intensity.

Determination of HIF1-α, Bcl-2, Bax gene expressions, and Bax/Bcl-2 ratio

RNA isolation and cDNA synthesis

Total RNA isolation from the samples was performed using a combination of TRIzol™ Reagent and Purelink RNA mini kit (Thermo Scientific, USA). The purity of the collected RNAs was determined by 260/280 nm (1.8–2.0.8.0 absorption ratio) using an Optizen NanoQ microvolume spectrophotometer (Mecasys, South Korea). Complementary DNA synthesis was performed to enable the RNAs obtained after the synchronisation process to be amplified by PCR. A High-Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) was used at this stage. The cDNAs obtained were stored at – 20 °C for ongoing studies.

RT-PCR to detect mRNA expression cDNAs were obtained from RNAs isolated and amplified in qRT-PCR by the Power Sybeer Green qPCR MasterMix (Thermo, USA) protocol and were amplified using the Applied Biosystems QuantStudio 5 Real-Time PCR device. Ct values of the peaks obtained during amplification were used to determine gene expression. Several reports indicate that GAPDH and beta-actin (ACTB) expression varies across tissues, during cell proliferation, throughout development, and during hypoxia/serum/growth factor stimulation. Therefore, we considered the simultaneous use of the two housekeeping genes to be appropriate. With endogenous control, expression levels of HIF1-α, Bcl-2, and Bax were computed using automatic output by RT-PCR (Thermo Fisher Scientific). The primers used to investigate the changes in the expression of these genes were given in order 5′–3′ (Table 1). For the qRT-PCR reaction to take place, step 1: Enzyme activation: 95 °C–10 min; step 2: Denaturation: 95 °C–15 s; Primer Annealing-Chain extension: 60 °C–1 min; step 3: Melting curve: 95 °C–15 s, 60 °C–1 min, and 95 °C–15 s. The Ct values of the peaks obtained during the amplification process were used to determine gene expression, and gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The normality of the data distribution was assessed using the Kolmogorov–Smirnov and Shapiro–Wilk tests. Parametric tests were applied for quantitative gene expression data obtained by the $2^{-\Delta\Delta Ct}$ method in RT-PCR assays. Differences between group means were analyzed using one-way ANOVA followed by Dunnett's or Tukey's post hoc tests, as appropriate. For non-normally distributed data, including histopathological injury scores, the Kruskal–Wallis's test was used, followed by pairwise comparisons with Bonferroni correction. Caspase-3 immunohistochemical scores were similarly analyzed using non-parametric tests. Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA), and graphs were generated with GraphPad

Gene	Primer sequence (5′–3′)
HIF1-α	F: 5′-CCACCGCAACTGCCACCACT-3′
	R: 5′-AGGGGCACGGTCACCTGGTT-3′
Bcl-2	F: 5′-GAGTACCTGAACCGGCATCT-3′
	R: 5′-GAAATCAAACAGAGGTCGCA-3′
Bax	F: 5′-TTGCTACAGGGTTTCATCCA-3′
	R: 5′-GAGTACCTGAACCGGCATCT-3′
B-Actin	F: 5′-CCCGCGAGTACAACCTTCT-3′
	R: 5′-CGTCATCCATGGCGAACT-3′
GAPDH	F: 5′-GGTCACCAGGGCTGCTTTT-3′
	R: 5′-ATCTCGCTCCTGGAAGATGGT-3′

Table 1. Primer sequences of genes.

Prism version 8 (GraphPad Software, San Diego, CA, USA). A p-value ≤ 0.05 was considered statistically significant.

Sample size calculation was performed using G*Power version 3.1. For one-way analysis of variance (ANOVA) and repeated ANOVA comparison (number of repetitions = 3), the effect size ("f") was determined to be 0.25, the Type I error limit was 0.05, and the Type II error limit was 0.8537[38]. A total sample size of 35 subjects was required to achieve a statistical power of 0.90. Thus, the number of surviving experimental animals at the end of the experiment was 33, and the statistical power was 0.90, close to the confidence interval, and within acceptable limits.

Correlation between histological injury scores and caspase-3 immunoreactivity was evaluated using Spearman's rank correlation, selected as the primary method due to non-normal data distribution. For completeness, Pearson's correlation coefficients were also calculated. All analyses were two-tailed with $\alpha = 0.05$ and performed using SPSS v20.0 (IBM Corp., Armonk, NY, USA).

Results

Sildenafil attenuates intestinal injury

Histopathological changes in intestinal tissue were determined by hematoxylin-eosin and Masson trichrome staining. Administration of a phosphodiesterase-5 inhibitor reduced the incidence and severity of hypoxia-induced intestinal ischemic injury. Histopathological examination showed significant villous damage, sometimes severe in the intestinal ischemia group, whereas moderate villous damage and epithelial shedding were observed in the Sil_1 group. The incidence of intestinal injury (defined as an injury score ≥ 2) was significantly higher in the intestinal ischemia group compared to the control group (2.89 ± 0.5 and 0.18 ± 0.4, respectively) ($p < 0.0001$). The Sil_1 group demonstrated partial protection, with a mean score of 2.46 ± 0.6, while the Sil_5 and Sil_10 groups showed further reduction in injury (1.68 ± 0.6 and 1.06 ± 0.4, respectively). The Sil_10 group had the lowest injury score among the experimental groups, which was significantly lower than that of the intestinal ischemia group (1.06 ± 0.4) ($p < 0.0001$). Masson's trichrome staining further confirmed that the connective tissue structure of the mucosa and submucosa layers was preserved to a greater extent in the Sil_10 group compared to the intestinal ischemia group (Fig. 2).

Sildenafil reduces Caspase-3 immunoreactivity

Immunohistochemical labelling of Caspase-3, a specific marker of apoptosis, was performed to evaluate apoptotic changes in the ileum of rat pups in all experimental groups (Fig. 3). Caspase-3 immunoreactivity was predominantly localized in villus enterocytes and, to a lesser extent, in the superficial epithelium of the intestinal crypts. The NEC group exhibited significantly higher Caspase-3 positivity compared to the control group (1.38 ± 0.40, 0.001 ± 0.003, respectively) ($p < 0.0001$). Sildenafil treatment reduced apoptotic activity in a dose-dependent manner: mean immunoreactivity scores were 0.75 ± 0.382 in the Sil_1 group, 0.504 ± 0.329 in the Sil_5 group, and 0.216 ± 0.145 in the Sil_10 group. Among sildenafil treated groups, Sil_10 exhibited the lowest Caspase-3 activity, with values approaching those of the control group ($p < 0.0001$ vs. NEC) (Fig. 3).

Histological injury scores showed a strong positive correlation with caspase-3 immunoreactivity (Spearman's $\rho = 0.795$, $p < 0.001$; Pearson's $r = 0.756$, 95% CI: 0.65–0.83; $N = 87$), indicating that increasing mucosal injury was closely associated with enhanced apoptotic labelling. These findings indicate that increased histological injury was associated with higher caspase-3 expression, whereas sildenafil administration attenuated this apoptotic activity and preserved epithelial integrity.

Hypoxia-induced intestinal ischemia upregulates HIF-1α expression

It was found that HIF1-α gene expression was regulated at the highest level in the intestinal ischemia group, while it was regulated at the lowest level in the Sil_10 group, similar to the control group. This difference was statistically significant ($p < 0.0001$). The HIF1-α gene expression level in the experimental group was approximately 7 times higher than in the Sil_1 group and 13 times higher than in the Sil_5 group. ($p < 0.05$ and $p \leq 0.0001$, respectively) (Fig. 4). Marked upregulation of HIF-1α during intestinal ischemia supports a central role for hypoxia-driven HIF-1α activation in modulating cellular pathways involved in epithelial injury and apoptosis.

Effect of Sildenafil on the expression of pro-apoptotic and anti-apoptotic gene mRNA

To further assess apoptosis in the ileum subjected to hypoxia-induced intestinal ischemic injury, real-time PCR analysis was performed to quantify the expression levels of the pro-apoptotic gene Bax and the anti-apoptotic gene Bcl-2 (Fig. 5). Bax mRNA expression was highest in the intestinal ischemia group and was significantly downregulated in the sildenafil-treated groups, particularly in the Sil_5 and Sil_10 groups ($p < 0.0001$). Conversely, Bcl-2 mRNA expression was significantly increased in the Sil_10 group compared with the intestinal ischemia group ($p < 0.0001$). Analysis of the Bax/Bcl-2 ratio revealed a marked increase in the intestinal ischemia group, whereas this ratio was significantly reduced in all sildenafil-treated groups, with the most pronounced reduction observed in the Sil_10 group ($p < 0.0001$) (Fig. 5).

When we evaluated the Bax/Bcl-2 ratio to assess shifts in the balance of apoptotic genes that promote cell survival, we found a significant increase in the Bax/Bcl-2 ratio within the intestinal ischemia group. In contrast, this ratio was significantly lower in all sildenafil-treated groups, particularly in the Sil_10 group ($p < 0.0001$) (Fig. 5). Additionally, the Sil_10 group exhibited a substantially lower Bax/Bcl-2 ratio than the Sil_1 and Sil_5 groups ($p < 0.0005$ and $p < 0.044$, respectively). These results suggest that sildenafil citrate, a PDE-5 inhibitor, shifts the balance of apoptotic proteins toward enhanced cell survival, indicating its potential anti-apoptotic effects. This increase in Bcl-2 was demonstrated in our study as a result of Sildenafil's anti-apoptotic effect.

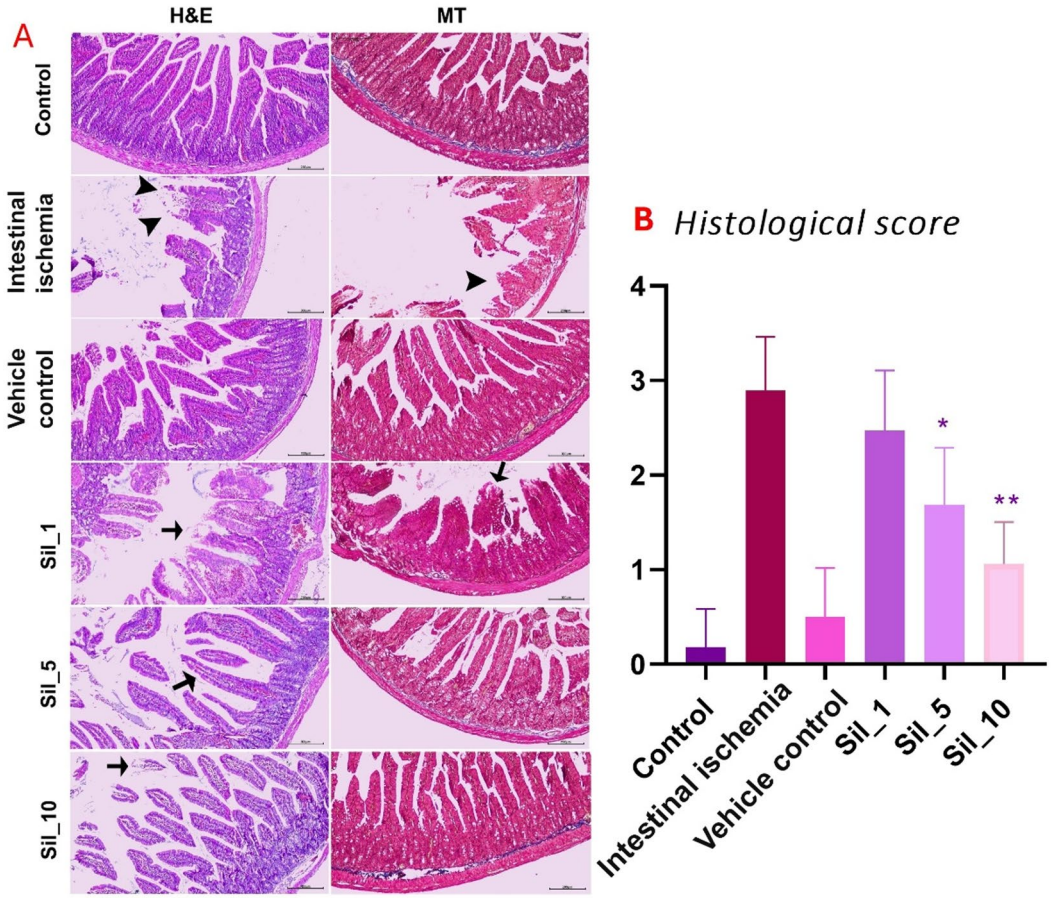

Fig. 2. Intestinal H&E and MT staining findings and histopathological injury scores. (**A**) Normal intestinal architecture was observed in the control group. The intestinal ischemia group exhibited severe villous damage. The Sil_1 group showed moderate villous damage with epithelial shedding. In the Sil_5 and Sil_10 groups, epithelial disruption was limited, and the intestinal structure in the Sil_10 group closely resembled that of the control group. MT staining demonstrated preserved connective tissue architecture in both the mucosal and submucosal layers, particularly in the Sil_10 group. (**B**) The incidence of intestinal injury (injury score ≥ 2) was significantly higher in the intestinal ischemia group compared with the control group ($p < 0.0001$), whereas the Sil_10 group exhibited the lowest injury score ($p < 0.0001$). (Black arrowhead: villous damage; black arrow: epithelial shedding). H&E: hematoxylin–eosin; MT: Masson's trichrome.

Discussion

NEC remains a significant cause of morbidity and mortality in premature infants; however, its precise etiology remains incompletely understood, and both prevention and management continue to present challenges. Histopathologically, NEC is characterized by a spectrum of intestinal injury ranging from mucosal damage to full-thickness necrosis and perforation[39]. Although NEC is a multifactorial disease involving feeding patterns, bacterial colonization, and hypoxic stress, it is widely accepted that intestinal ischemia represents a central initiating mechanism.

Accordingly, the present study employed a hypoxia–hypothermia–based experimental model to specifically investigate ischemia-driven epithelial injury and apoptosis and is therefore best described as an NEC-like intestinal injury model. Consistent with previous experimental studies, our histopathological evaluation demonstrated marked villous damage and epithelial disruption in the NEC group, reflecting characteristic features of NEC-associated intestinal injury.

Although the role of sildenafil in NEC has not been extensively explored, its protective effects against ischemia–reperfusion (I/R) injury and its capacity to preserve intestinal epithelial integrity have been well documented in experimental settings[40–42]. While Moreira et al. reported that sildenafil could not prevent intestinal injuries in a classical experimental NEC model[43], our findings demonstrate that sildenafil pretreatment markedly attenuated ischemia-induced villous damage and epithelial shedding. These results suggest that sildenafil may preserve intestinal structural integrity by protecting epithelial and connective tissue components under ischemic conditions. Hunter et al. reported that sildenafil alleviated intestinal damage in experimental NEC through eNOS-independent mechanisms, and İpek et al. demonstrated significant anti-apoptotic and anti-inflammatory effects of sildenafil in rat-induced NEC[44,45].

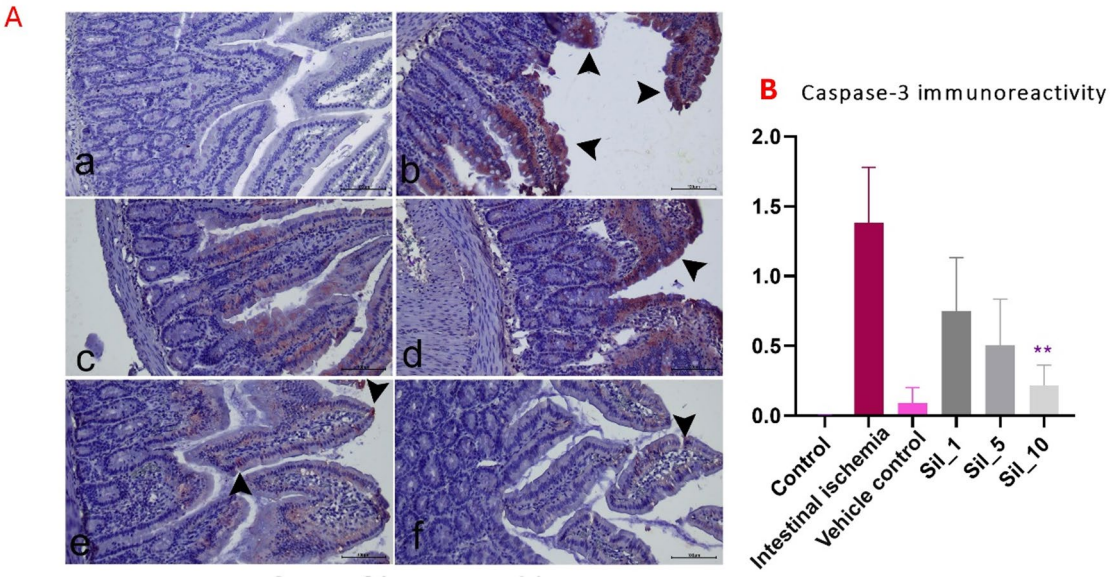

Fig. 3. Caspase-3 IHC labelling findings. (**B**) Marked caspase-3 immunoreactivity was observed in the intestinal ischemia group, particularly in enterocytes lining the intestinal villi. Reduced immunoreactivity was observed in the sildenafil-pretreated groups. (**A**) Caspase-3 immunoreactivity scores were significantly increased in the intestinal ischemia group ($p < 0.0001$) and significantly decreased in the Sil_10 group ($p = 0.0021$). Representative IHC images of caspase-3 staining in the (a) control, (b) intestinal ischemia, (c) vehicle (DMSO), (d) Sil_1, (e) Sil_5, and (f) Sil_10 groups (arrow: positively stained enterocytes). Mayer's hematoxylin counterstaining; original magnification ×200. Group comparisons: *a*, $p < 0.05$ vs. control; *b*, $p < 0.005$ vs. intestinal ischemia group. IHC: Immunohistochemistry.

Mitochondria are important sources of damage-associated molecular patterns (DAMPs). When mitophagy, which maintains cellular homeostasis by selectively removing dysfunctional mitochondria, is disrupted, excessive inflammation and pathological damage can be triggered by the accumulation of mitochondrial DAMPs. Increasing evidence links defective mitophagy to the pathogenesis of NEC, and the role of mitophagy-related genes in NEC is being actively investigated[46,47].

HIF-1α is a central regulator of cellular adaptation to hypoxic stress, playing a crucial role in hypoxia-induced intestinal epithelial injury[11,48,49]. Increased HIF-1α expression under hypoxic conditions has been shown to contribute to the maintenance of intestinal epithelial homeostasis by initiating mitophagy through activation of BCL2/adenovirus E1B 19-kDa protein-interacting protein 3-like (BNIP3L) signaling[50]. In addition, HIF-1α has been identified as a differentially expressed gene associated with mitophagy, and its expression has been significantly increased in hypoxia-induced epithelial damage models[50].

Zhang et al. demonstrated that deficiency of epithelial HIF-1α increases susceptibility to apoptosis through enhanced TLR4 signaling, underscoring a protective role for epithelial HIF-1α during intestinal ischemic stress[19]. However, the biological effects of HIF-1α are highly context-dependent and may vary according to cellular conditions and post-translational modifications. Notably, dephosphorylated HIF-1α has been shown to promote apoptosis by binding to and stabilizing p53, indicating a dual and context-specific role in epithelial injury and survival[51]. In line with this complexity, Baregamian et al. reported that suppression of intestinal HIF-1α expression can confer protection against ischemic intestinal injury[8].

In the present study, HIF-1α expression was markedly upregulated in the intestinal ischemia group compared with controls, supporting the role of hypoxia-induced ischemic stress in activating HIF-1α–mediated cellular responses. We found that sildenafil administered at 5 and 10 mg/kg/day significantly inhibited HIF-1α expression (Fig. 3). These findings suggest that sildenafil pretreatment may play a role in attenuating HIF-1α upregulation, with HIF-1α potentially contributing to its anti-apoptotic effects and the regulation of hypoxia-induced intestinal injury.

Our study demonstrated a significant increase in Caspase-3 immunoreactivity in enterocytes from the intestinal ischemia group, indicating heightened apoptotic activity within the intestinal epithelium. This finding is consistent with the established pathophysiology of ischemia-associated intestinal injury, which involves extensive epithelial cell death, contributing to mucosal injury and barrier dysfunction[8,52]. Caspase-3, a central executioner of apoptosis, plays a pivotal role in the terminal phase of programmed cell death; once apoptotic signaling reaches the level of caspase-3 activation, cellular recovery is no longer possible[21]. In the present study, sildenafil administration significantly reduced Caspase-3 immunoreactivity in enterocytes, These findings suggest that sildenafil exerts a protective effect by attenuating apoptosis, in line with previous reports showing that phosphodiesterase-5 inhibitors suppress apoptotic signaling through enhancement of nitric oxide-mediated pathways[24,25].

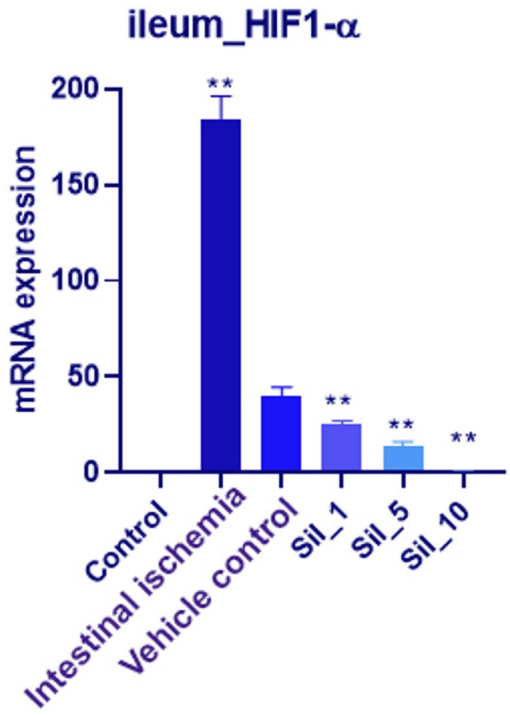

Fig. 4. Relative mRNA expression levels of HIF-1α in ileal tissues. Relative fold-change values of HIF-1α mRNA expression in the control, intestinal ischemia, and Sil_1, Sil_5, and Sil_10 groups. Data were normalized to β-actin and GAPDH using the multiple reference gene method. HIF-1α expression was significantly upregulated in the intestinal ischemia group compared with the control group. Sildenafil pretreatment, particularly at doses of 5 and 10 mg/kg/day, significantly downregulated HIF-1α expression. Symbols represent individual samples, and bars indicate mean ± SE. $p < 0.05$; $p \leq 0.0001$ ($n = 6$).

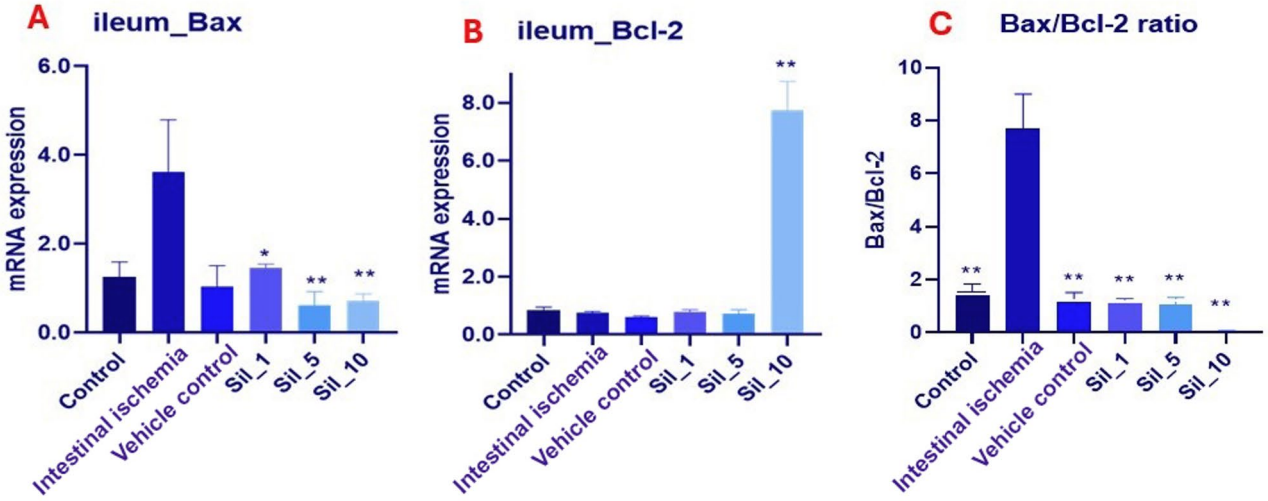

Fig. 5. Bax, Bcl-2 gene expressions and Bax/Bcl-2 ratio. Relative fold-change values of Bax and Bcl-2 mRNA expression in the control, intestinal ischemia, and Sil_1, Sil_5, and Sil_10 groups. Data were normalized to β-actin and GAPDH. (**A**) Pro-apoptotic Bax expression was highest in the intestinal ischemia group and was significantly reduced in the sildenafil-treated groups, particularly in the Sil_5 and Sil_10 groups ($p < 0.0001$). (**B**) Anti-apoptotic Bcl-2 expression was markedly increased in the Sil_10 group compared with the intestinal ischemia group ($p < 0.0001$). (**C**) The Bax/Bcl-2 ratio was significantly lower in the Sil_10 group ($p < 0.0001$). Symbols represent individual samples, and bars indicate mean ± SE. $p < 0.05$; $p \leq 0.0001$ ($n = 6$).

The balance between pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins is a critical determinant of apoptosis. Overexpression of Bcl-2 suppresses cell death, whereas an increased Bax/Bcl-2 ratio enhances susceptibility to apoptosis and promotes caspase-3 activation[21,22]. Evidence from human neonates suggests that an imbalance between pro-apoptotic Bax expression and anti-apoptotic Bcl-2 expression may contribute to ischemia-associated intestinal epithelial injury[53]. In the present study, rat pups subjected to intestinal ischemia exhibited markedly increased Bax mRNA expression. In contrast, sildenafil administration suppressed Bax expression while significantly upregulating Bcl-2 expression, particularly in the Sil_10 group, resulting in a substantially reduced Bax/Bcl-2 ratio. These findings indicate that sildenafil shifts the apoptotic balance toward enhanced cell survival, consistent with its observed anti-apoptotic effects in ischemic intestinal tissue.

Experimental studies in different ischemic injury models have demonstrated that sildenafil pretreatment exerts significant anti-apoptotic effects. In a rat model of renal ischemia–reperfusion injury, sildenafil pretreatment reduced apoptosis by increasing inducible and endothelial nitric oxide synthase (iNOS and eNOS) expression and decreasing the Bax/Bcl-2 ratio[54]. Similarly, Zhang et al. reported that sildenafil protected against post-resuscitation myocardial dysfunction by attenuating apoptosis, as evidenced by increased iNOS and eNOS expression together with reduced caspase-3 activity and a lower Bax/Bcl-2 ratio[24]. The marked reduction in the Bax/Bcl-2 ratio induced by sildenafil is therefore considered a key mechanism underlying its anti-apoptotic effects.

Das et al. further provided compelling evidence for the role of NO signalling in sildenafil's protective effects, showing that the absence of Bcl-2 upregulation in iNOS knockout mouse myocytes strongly supports a direct anti-necrotic and anti-apoptotic action of sildenafil via the NO pathway[55]. This mechanism, well-documented in cardiomyocytes, may similarly contribute to the protective effects of sildenafil observed in the intestinal epithelium in the intestine under ischemic conditions. Consistent with these findings, our results demonstrate that sildenafil significantly attenuates apoptosis in the intestinal tissue of rat pups subjected to hypoxia-induced intestinal ischemia. In contrast to the pronounced pro-apoptotic effects of ischemic stress, sildenafil pretreatment rendered the intestinal epithelium more resistant to apoptosis, further supporting its protective role in ischemia-driven intestinal injury.

At enrollment, rat pups weighed 24.54 ± 3.50 g (range, 16.4–33.4 g). Of the 75 randomized pups, 33 survived to the experimental endpoint (overall mortality 56%): control ($n=3$), intestinal ischemia ($n=3$), vehicle control ($n=6$), Sil_1 ($n=8$), Sil_5 ($n=6$), and Sil_10 ($n=7$). This mortality exceeds rates reported in some studies employing comparable hypoxia-based intestinal injury protocols[42]. Mortality occurred predominantly during the hypoxia challenge. Exposure to 100% N_2 triggered bradycardia within 20 s in many pups; although transient resuscitation was often successful, a substantial proportion succumbed at this stage. Consequently, pups could not be consistently exposed to 100% nitrogen hypoxia for the full 60–75 s specified in previously described protocols[18,19]. Although very small pups (1–3 g) are frequently used in neonatal experimental models, our experience indicates that consistent gavage feeding at these weights is technically challenging and may compromise both procedural consistency and animal welfare[43].

A limitation of the present study is that the experimental model does not fully recapitulate the classical neonatal NEC model, which involves enteral feeding, bacterial exposure, and hypoxia. Instead, our model primarily reflects hypoxia- and hypothermia-induced intestinal ischemic injury, representing an ischemia-dominant, NEC-like condition. While this approach allows controlled investigation of apoptosis and epithelial injury, future studies incorporating enteral feeding and bacterial exposure will be necessary to enhance translational relevance.

A further limitation of our study is that Masson's trichrome staining could only be evaluated qualitatively, as our current image analysis software did not allow quantitative collagen density measurements. Nevertheless, the representative images clearly demonstrated preserved connective tissue architecture in the sildenafil groups. In addition, Kaplan–Meier survival analysis was not performed, as the experimental design was not primarily intended to assess survival outcomes. Future studies incorporating survival analysis may provide further insight into the potential protective effects of sildenafil under ischemic intestinal injury.

Although this simplified model enabled reproducible induction of hypoxia-driven intestinal ischemic injury, it does not fully capture the multifactorial pathogenesis of necrotizing enterocolitis observed in human neonates. Despite this limitation, the model reliably produced intestinal injury, allowing controlled evaluation of apoptosis-related mechanisms and the protective effects of sildenafil.

Another limitation is the absence of Western blot or other quantitative protein assays to validate apoptotic marker expression at the protein level. Instead, apoptosis was assessed using immunohistochemical caspase-3 labeling in conjunction with gene expression analyses of Bax, Bcl-2, and HIF-1α, which consistently demonstrated the anti-apoptotic effects of sildenafil. Future studies with dedicated funding support will be required to confirm these findings using quantitative protein-based approaches.

High mortality in this model may have limited statistical power and introduced survivor bias, thereby constraining generalizability. Although hypoxia exposure was curtailed when physiological instability occurred, mortality remained substantial, and Kaplan–Meier survival analysis was not prespecified in the experimental design. Future studies should prospectively incorporate survival endpoints and graded-severity injury protocols to improve reproducibility and enhance translational relevance.

Although sildenafil has not yet been clinically applied for the prevention or treatment of NEC, our findings provide experimental evidence supporting its potential role in modulating key pathogenic mechanisms. These results underscore the need for further preclinical validation and carefully designed clinical studies to evaluate the safety and efficacy of sildenafil in this context.

Conclusion

In conclusion, this study demonstrates that sildenafil attenuates hypoxia-induced, ischemia-dominant intestinal injury by reducing apoptotic signaling, as evidenced by a decreased Bax/Bcl-2 ratio and suppression of HIF-1α expression. These molecular effects were accompanied by preservation of intestinal morphology and reduced epithelial apoptosis. Collectively, our findings provide novel histological and molecular evidence supporting the potential protective role of sildenafil in ischemia-driven, NEC-like intestinal injury and suggest that modulation of hypoxia- and apoptosis-related pathways may represent a promising therapeutic strategy warranting further investigation.

Data availability

The data sets used and/or analyzed during the current study can be obtained from the corresponding author upon request.

Received: 27 October 2025; Accepted: 27 March 2026

References

1. Dominguez, K. M. M. R. Necrotizing enterocolitis. *Clin. Perinatol.* **39**(2), 387–401. https://doi.org/10.1016/j.clp201204011 (2012).
2. Fitzgibbons, S. C. et al. Mortality of necrotizing enterocolitis expressed by birth weight categories. *J Pediatr Surg* **44**(6), 1072–5 (2009).
3. Horbar, J. D. et al. Mortality and neonatal morbidity among infants 501 to 1500 grams from 2000 to 2009. *Pediatrics* **129**, 1019–26 (2012).
4. Neu, J. & Walker, W. A. Necrotizing Enterocolitis. *N. Engl. J. Med.* **364**(3), 255–264. https://doi.org/10.1056/NEJMRA1005408 (2011).
5. Hackam, D. & Caplan, M. Necrotizing enterocolitis: Pathophysiology from a historical context. *Semin Pediatr Surg* **27**, 11–18. https://doi.org/10.1053/j.sempedsurg201711003 (2018).
6. Egan, C. E. et al. Toll-like receptor 4-mediated lymphocyte influx induces neonatal necrotizing enterocolitis. *J. Clin. Invest.* **126**(2), 495–508. https://doi.org/10.1172/JCI83356 (2016).
7. Chen, Y. et al. The role of ischemia in necrotizing enterocolitis. *J. Pediatr. Surg.* **51**(8), 1255–1261. https://doi.org/10.1016/j.jpedsurg201512015 (2016).
8. Baregamian, N., Rychahou PG, Hawkins HK & Evers BM CD. Phosphatidylinositol 3-kinase pathway regulates hypoxia-inducible factor-1 to protect from intestinal injury during necrotizing enterocolitis. *Surgery* **142** (2), 295–302. https://doi.org/10.1016/j.surg200704018 (2007).
9. Ford, H. et al. The role of inflammatory cytokines and nitric oxide in the pathogenesis of necrotizing enterocolitis. *J Pediatr Surg* **32**(2), 275–282 (1997).
10. Jilling, T. et al. Intestinal epithelial apoptosis initiates gross bowel necrosis in an experimental rat model of neonatal necrotizing enterocolitis. *Pediatr. Res.* **55**, 622–629. https://doi.org/10.1203/01PDR00001134637043574 (2004).
11. van der Greijer, A. E. WE. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. J Clin Pathol ;57(10)1009-14. https://doi.org/10.1136/jcp2003015032. PMID: 15452150; PMCID PMC1770458. (2004).
12. McClintock, D. S. et al. Bcl-2 family members and functional electron transport chain regulate oxygen deprivation-induced cell death. *Mol Cell Biol* **22**, 94–104 (2002).
13. Ford, H. R. & Sorrells, D. L. K. A. Inflammatory cytokines, nitirc oxide, and necrotizing enterocolitis. *Semin Pediatr. Surg.* 5155–5159, (1996).
14. MA, M. Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268** (17), 12231–12234 (1993).
15. Wenger, R. H. & Stiehl, D. P. C. G. Integration of oxygen signaling at the consensus HRE. Sci STKE ;2005(306)re12 Publ 2005 Oct 18. https://doi.org/10.1126/stke3062005re12. (2005)
16. Huang, L. E., Gu, J. & Schau, M. B. H. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway.*Proc Natl Acad Sci.* https://doi.org/10.1073/pnas95147987 (1998).
17. Schumacker, P. T. P. Hypoxia-inducible factor-1 (HIF-1). *Crit Care Med* **33**(12), S423–S425. https://doi.org/10.1097/01CCM00001 9171638566E0 (2005).
18. GL S,. Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis. *J Lab Clin Med* **131**(3), 207–214. https://doi.org/10.1016/s0022-2143(98)90091-9 (1998).
19. Zhang, Y., Yan, M. & Yue, Y. C. Y. Hypoxia-Inducible Factor-1α Modulates the Toll-Like Receptor 4/Nuclear Factor Kappa B Signaling Pathway in Experimental Necrotizing Enterocolitis. Mediat Inflamm; 20244811500. https://doi.org/10.1155/mi/4811500. PMID 39719983; PMCID PMC11668547. (2024)
20. Gershner, G. H. and CJH. Redox Chemistry: Implications for Necrotizing Enterocolitis. *Int. J. Mol. Sci. vol* **25**,15 8416 1 Aug 2024, https://doi.org/10.3390/ijms25158416 (2024)
21. Parrish, A. B., FC., & SK. Cellular mechanisms controlling caspase activation and function. *Cold Spring Harb Perspect Biol* **5**, pii a008672 (2013).
22. Salakou, S. Kardamakis D, Tsamandas A. C, Zolota V, Apostolakis E, Tzelepi V et al. Increased Bax/Bcl-2 ratio up-regulates caspase-3 and increases apoptosis in the thymus of patients with myasthenia gravis. *Vivo*; 21123–21132. (2007).
23. Boolell, M., Allen MJ, Ballard SA, Gepi-Attee S, Muirhead GJ, Naylor AM et al. Sildenafil: an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. *Int. J. Impot. Res.*; 847–852. (1996).
24. Zhang, Q., Wang, G, Yuan, W., Wu J, Wang M. LC,. The effects of phosphodiesterase-5 inhibitor sildenafil against post-resuscitation myocardial and intestinal microcirculatory dysfunction by attenuating apoptosis and regulating microRNAs expression: essential role of nitric oxide syntheses signaling. *J Transl Med* **13**, 177. https://doi.org/10.1186/s12967-015-0550-9 (2015).
25. Salloum, F., Yin, C., Xi, L. & R, K. Sildenafil induces delayed preconditioning through inducible nitric oxide synthase-dependent pathway in mouse heart. *Circ Res.* **92**(6), 595–597. https://doi.org/10.1161/01RES000006685309821 98 (2003).
26. Das, S., Maulik, N., Das, D. Research PK and clinical, 2002 undefined. Cardioprotection with sildenafil, a selective inhibitor of cyclic 3', 5'-monophosphate-specific phosphodiesterase 5. Accessed March 22. https://europepmc.org/article/med/12776574 (2023).
27. Choi, D. E., Jeong, J. Y., Lim, B. J., et al. Pretreatment of sildenafil attenuates ischemia-reperfusion renal injury in rats. *Am. J. Physiol. Ren. Physiol.* **297** (2), F362–F370. https://doi.org/10.1152/ajprenal906092008 (2009).
28. Medeiros, J. V. R. et al. Role of the NO/cGMP/K(ATP) pathway in the protective effects of sildenafil against ethanol-induced gastric damage in rats. *Br J Pharmacol* **153**(4), 721–7. https://doi.org/10.1038/sj.bjp0707605 (2008).
29. Semen, K. et al. Sildenafil reduces signs of oxidative stress in pulmonary arterial hypertension: Evaluation by fatty acid composition, level of hydroxynonenal and heart rate variability. *Redox Biology* **7**, 48–57. https://doi.org/10.1016/j.redox201511009 (2016).
30. Simonca, L. T. R. Sildenafil in Infants and Children. *Child* **4**(7)60. https//doi.org/10.3390/children4070060

31. Wardle, A. J. & Tulloh, R. M. Paediatric pulmonary hypertension and sildenafil: Current practice and controversies. *Arch. Dis. Child.* **98**, 141–147 (2013).
32. Samiee-Zafarghandy, S. et al. Sildenafil and retinopathy of prematurity risk in very low birth weight infants. *J. Perinatol.* **36**, 137–140 (2016).
33. Oya, Y. et al. The pharmacokinetics of sildenafil may be affected by intestinal absorption rate in children admitted to the intensive care unit. *Biol. Pharm. Bull.* **43**, 1917–1923. https://doi.org/10.1248/bpb.b20-00614 (2020).
34. Caplan, M. S., Hedlund, E., Adler, L. & Hsueh, W. Role of asphyxia and feeding in a neonatal rat model of necrotizing enterocolitis. *Pediatr. Pathol.* **14**, 1017–1028. https://doi.org/10.3109/15513819409037698 (1994).
35. Barlow, B., Santulli, T. V., Heird, W. C., Pitt, J. & Blanc, W. A. An experimental study of acute neonatal enterocolitis: the importance of breast milk. *J. Pediatr. Surg.* **9**, 587–595. https://doi.org/10.1016/0022-3468(74)90093-1 (1974).
36. Caplan, M. S., Miller-Catchpole R., Kaup S. et al. Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. *Gastroenterol* **117** (3), 577–583. https://doi.org/10.1016/s0016-5085(99)70450-6. (1999)
37. Carter, J. H. et al. Pak-1 expression increases with progression of colorectal carcinomas to metastasis. *Clin. Cancer Res.* **10**(10), 3448–3456. https://doi.org/10.1158/1078-0432CCR-03-0210 (2004).
38. Faul, F., Erdfelder, E. & Lang, A. G. B. A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods*. **39** (2), 175–191. https://doi.org/10.3758/bf03193146. PMID 17695343. (2007)
39. Rausch, L. A., Hanna, D. N. & Patel, A. B. M. Review of necrotizing enterocolitis and spontaneous intestinal perforation clinical presentation, treatment, and outcomes. *Clin. Perinatol.* **49**(4), 955–964. https://doi.org/10.1016/j.clp202207005 (2022).
40. Moore, H. M., Drucker, N. A., Hosfield, B. D. & Shelley, W. C. M. T. Sildenafil as a Rescue Agent Following Intestinal Ischemia and Reperfusion Injury. J Surg Res. 246512-518. https://doi.org/10.1016/j.jss201909037. (2020).
41. Inan, M. Uz, Y.H., Kizilay, G. et al. Protective effect of sildenafil on liver injury induced by intestinal ischemia/reperfusion. *J Pediatr Surg* **48**, 1707–15. https://doi.org/10.1016/j.jpedsurg201212054 (2013).
42. Soydan, G., Sökmensüer, C., Kilinç, K., et al. The effects of sildenafil on the functional and structural changes of ileum induced by intestinal ischemiareperfusion in rats. *Eur. J. Pharmacol.* 61087–61092. https://doi.org/10.1016/j.ejphar200903038. (2009)
43. Moreira, G. A., Dias, A. I. B. D. S., Cadena, S. M. S. C., Corrêa-Ferreira, M. L. & Ioshii, S. O. F. C. Use of sildenafil and L-arginine in an experimental rat model for the prevention of neonatal necrotizing enterocolitis. Rep.; 12(1)6206 Publ 2022 Apr 13. https://doi.org/10.1038/s41598-022-10323-8. (2022).
44. Hunter, C. E. et al. Sildenafil attenuates intestinal injury in necrotizing enterocolitis independently of endothelial nitric oxide synthase. *J. Pediatr. Surg.* **57**(12), 967–973. https://doi.org/10.1016/j.jpedsurg202206001 (2022).
45. İpek, M. S., Gürel, D., Güngör, T. & Yılmaz, G. A. I. Protective effect of sildenafil on experimental necrotizing enterocolitis in newborn rats: Anti-apoptotic and anti-inflammatory mechanisms. *Turk. J. Biochem.* **47**(4), 369–377. https://doi.org/10.1515/tjb-2021-0234 (2022).
46. Zhang, Q. Raoof, M., Chen, Y. et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nat; 464104-7. https://doi.org/10.1038/nature08780. (2010)
47. Dang, D. et al. Heme induces intestinal epithelial cell ferroptosis via mitochondrial dysfunction in transfusion-associated necrotizing enterocolitis. *FASEB J* **36**, e22649. https://doi.org/10.1096/fj202200853RRR (2022).
48. Blanco Pampin, J., Garcia Rivero, S.A., Otero Cepeda, X. L., et al. Immunohistochemical expression of HIF-1alpha in response to early myocardial ischemia. *J. Forensic Sci.* **51**, 120–124 (2006).
49. Bai, M., Lu, C., An L., et al. SIRT1 relieves Necrotizing Enterocolitis through inactivation of Hypoxia-inducible factor (HIF)-1a. *Cell Cycle* **19**, 2018–27. https://doi.org/10.1080/1538410120201788251 (2020).
50. Vincent, G. et al. Nix-mediated mitophagy modulates mitochondrial damage during intestinal inflammation. *Antioxid. Redox Signal.* **33**, 1–19. https://doi.org/10.1089/ars20187702 (2020).
51. Suzuki H, Tomida A TT. Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. Oncogene; 20(41):5779–5788. https://doi.org/10.1038/sj.onc1204742. (2001)
52. Yi, X., Chang, X., Wang, J., Yan, C. & Zhang, B. Intestinal trefoil factor increased the Bcl-2 level in a necrotizingenterocolitis neonate rat model. *TURKISH JOURNAL OF MEDICAL SCIENCES* **46**, 1–1. https://doi.org/10.3906/sag-1501-65 (2016).
53. Ates, U., Gollu, G., Kucuk, G., Billur, D., Bingol-Kologlu, M., Yılmaz, Y., Ozkan-Ulu, H., Bayram, P., Bagriacik, E. DH. Increase in pro-apoptotic Bax expression and decrease in anti-apoptotic Bcl-2 expression in newborns with necrotizing enterocolitis. Arch Argent Pediatr. ;114(3)243-7 English, Spanish. https://doi.org/10.5546/aap2016.eng243. Epub 2016 Jun 1. PMID 27164337. (2016).
54. Choi, D. E. Jeong, J. Y., Lim, B. J., Chung, S., Chang, Y. K., Lee, S. J., Na, K. R., Kim, S. Y., Shin, Y. T. L K. Pretreatment of sildenafil attenuates ischemia-reperfusion renal injury in rats. Am J Physiol Ren Physiol. 297(2)F362-70. https://doi.org/10.1152/ajprenal906092008. Epub 2009 May 27. PMID 19474186. (2009).
55. Das, A. & Xi, L. K. R. Phosphodiesterase-5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. *J. Biol. Chem.* **280**(13), 12944–12955. https://doi.org/10.1074/jbcM404706200 (2005).

Acknowledgements

We would like to thank Sait Elmas, the responsible veterinarian at the Çanakkale Onsekiz Mart University Experimental Research Application and Research Center (COMUDAM), and her colleagues for their valuable support throughout the experimental procedures.

Author contributions

Study concept and design (SK, ÖÖ, IK, HA); performed the animal experiment and data collection (SK, ÖÖ, IK, HA); prepared samples analyzed the performed histology analysis and interpretation of data (SK, ÖÖ, IK, HA); writing the manuscript (SK, ÖÖ); critical revision of the manuscript (SK, ÖÖ, IK, HA); statistical expertise (SK, ÖÖ); supervision (SK, HA).

Funding

Çanakkale Onsekiz Mart University Scientific Research Projects Coordination Unit supported the study (Project No: TSA-2022-4028). Sildenafil citrate salt (Sigma-Aldrich, CAS Number: 171599-83-0) was supplied by Kale.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to H.A.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

© The Author(s) 2026, modified publication 2026