Research Article

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Sildenafil for the treatment of necrotizing enterocolitis: an experimental study

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Abstract

Objectives: This study was conducted to evaluate whether sildenafil effectively treats necrotizing enterocolitis (NEC).

Methods: Thirty-eight rat pups were divided into 4 groups: control, sildenafil-control, NEC, and sildenafil-NEC (Sil-NEC). NEC was induced by hypoxia/reoxygenation and cold stress. The pups were treated by administering 1 mg/kg sildenafil by intraperitoneal injection once a day until the fourth postnatal day. The tissues were stained with haematoxylin/eosin staining and examined with the TUNEL test for apoptosis. The intestinal levels of malondialdehyde (MDA), interleukin β (IL-1β), inducible nitric oxide synthase (iNOS), caspase-3, and glutathione peroxidase (GSH-px) activity were quantified.

Results: TUNEL positivity (p=0.002) and intestinal damage grade (p<0.001) were found to be significantly lower in the Sil-NEC group. In addition, MDA, IL-1β, iNOS, caspase-3 levels, and GSH-px activity were also found to be significantly lower in the Sil-NEC group (p<0.001, p=0.004, p=0.011, p=0.026, p=0.002 respectively).

Conclusions: In this study, sildenafil has been shown to reduce intestinal damage and prevent the development of necrosis biochemically and histopathologically, with its antioxidant, anti-apoptotic, and anti-inflammatory effects, in the treatment of the experimental necrotizing enterocolitis model. This may suggest that sildenafil can be used to treat necrotizing enterocolitis, but further clinical studies are required.

Keywords: iNOS; necrotizing enterocolitis; neonate; sildenafil; TUNEL.

Introduction

Necrotizing enterocolitis (NEC) is a gastrointestinal emergency in neonates with high morbidity and mortality despite early diagnosis and aggressive treatment. Although it was first described in 1965, its pathogenesis is still not fully understood and an effective treatment has not been found, and so studies in this direction are ongoing [1].

Necrotizing enterocolitis is claimed to be multifactorial in many studies. More than 90% of affected infants are born before 32nd gestational week [2]. Exposure of the gastrointestinal tract to ischemia contributes to the development of NEC. Circulatory failure and intestinal ischemia are associated with NEC, especially in term infants. Reperfusion occurring in response to hypoxia after decreased intestinal blood flow has been thought to possibly contribute to intestinal damage. Increased basal intestinal vascular resistance after birth, an imbalance between mediators,
such as nitric oxide and endothelin, perinatal asphyxia, recurrent apnea, hypotension, left–right shunt heart disease, patent ductus arteriosus, indomethacin therapy, umbilical artery catheterization, polycythemia, anemia, and blood transfusion all reduce mesenteric blood flow and increase the risk of necrotizing enterocolitis [3, 4]. In the early stage of life, enteral feeding can lead to ischemia, and immature bowel fails to provide adequate hemodynamic responses after feeding, causing necrotizing enterocolitis to develop. Regulating the microcirculation of the immature intestine can prevent the development of necrotizing enterocolitis [5]. In a rat pup model of NEC, Downard and colleagues found that the main mechanical event in NEC is vasoconstriction in the mesenteric arteriole [6]. Agents that improve mesenteric blood flow and cardiovascular function, such as dobutamine, have been shown to be useful in the treatment of NEC [6, 7].

Sildenafil, a phosphodiesterase-5 (PDE-5) inhibitor, causes vasodilation of the vascular smooth muscle bed by increasing the cyclic guanosine monophosphate (cGMP) level. In addition, intracellular cGMP accumulation has been shown to reduce inflammation by decreasing proinflammatory cytokines and oxidative damage in experimental models [8]. In many experimental studies, it has been suggested that sildenafil may be beneficial in the treatment of various diseases by thanks to its anti-inflammatory, antioxidant and antiapoptotic properties [9–11]. In an experimental study of intestinal ischemia, intraperitoneal administration of sildenafil was shown to reduce intestinal mucosal damage [9]. Thus, in this study, sildenafil was investigated to test whether it would be effective in the treatment of experimentally induced necrotizing enterocolitis.

Materials and methods

Animals

The pregnant Wistar albino rats used in this study were supplied by Kahramanmaraş Sütçü İmam University Medical Sciences Experimental Research Center. A total of 38 male rat pups were divided into 4 groups: control (n=10, control rat pups fed exclusively with breast milk ad libitum); Sil-control, control + sildenafil (n=10, control rat pups fed with breast milk ad libitum and administered sildenafil); NEC, necrotizing enterocolitis (n=8, necrotizing enterocolitis procedure performed and administered saline); and Sil-NEC, necrotizing enterocolitis + sildenafil (n=10, necrotizing enterocolitis procedure performed and administered sildenafil). The male rat pups in the NEC and Sil-NEC groups were weaned from their mothers immediately after birth and held in a moisturized incubator at 37 °C to ensure avoidance of the protective effects of breast milk. They were fed with 0.2 mL of specific rodent formula six times a day [12, 13].

Necrotizing enterocolitis procedure

Experimental necrotizing enterocolitis was established using the hypoxia/reoxygenation and cold injury method according to the procedure described by Guven et al. [14–16]. The pups were exposed to 100% CO₂ in an airtight plexiglass box for 10 min and maintained in a hypoxic environment. At the end of this process, the pups were breathless and cyanotic. Necrotizing enterocolitis was induced by subjecting the animals to an environment with a temperature of 4 °C for 5 min followed by the administration of 97% O₂ for 10 min two times per day. This process was performed for three days for each rat pup. The pups’ daily weights were measured and recorded.

Sildenafil administration

The rat pups in the Sil-NEC were administered an intraperitoneal injection of 1 mg/kg sildenafil once a day from birth until the postnatal fourth day [9]. The necrotizing enterocolitis group administered an intraperitoneal physiological saline alone.

Histopathological analysis

An experienced pathologist blinded to the study protocol performed histopathological tissue examinations. Intestinal specimens were fixed with 10% neutral-buffered formalin solution for light microscopy. The blocks of paraffin-embedded samples were cut into 4-μm-thick slices and stained with hematoxylin and eosin (H&E). Histopathological examination was graded as follows: grade 0 indicates normal tissue; grade 1 indicates mild disease with separation of the villous nucleus without other abnormalities; grade 2 indicates moderate disease with villous nucleus detachment, submucosal edema, and epithelial peeling; grade 3 indicates severe disease with villous, full-thickness necrosis, or epithelial peeling with loss of perforation; grade 4 indicates total intestinal injury, complete destruction of the mucosa, transmural necrosis, and pneumatosis intestinalis [17, 18]. If the intestinal damage scoring was ≥2, the rat pup was considered to have necrotizing enterocolitis.

TUNEL method

After mounting 4- to 6-μm-thick sections of paraffin blocks on polyglyzed slides, an ApopTag Plus Peroxidase in situ Apoptosis Detection Kit (Chemicon, USA) (Cat No: S7101) was used to detect apoptotic cells. The sections, deparaffinized with xylene, were passed through graded alcohol series and washed with phosphate-buffered saline (PBS). Tissues were incubated with 0.05% proteinase K for 15 min, and then incubated with 3% hydrogen peroxide for 10 min to prevent endogenous peroxidase activity. After washing the tissues with PBS, they were incubated with equilibration buffer for 6 min and left to incubate for 60 min with the working solution at 37 °C in a humid environment. The samples were then retained in stop/wash buffer for 10 min. Afterward, the apoptotic cells were observed by dropping diaminobenzidine
substrate on tissues treated with anti-digoxigenin-peroxidase for 40 min. Tissue contrasted with Harris hematoxylin, were covered with entellan and examined under light microscope and photographed. An average 500 normal and apoptotic average 500 cells were counted in sections at a magnification of 10×. The apoptotic index (AI) was calculated by proportioning apoptotic cells to total (normal-apoptotic) cells.

Biochemical analysis

Whole tissue specimens were washed with saline solution and stored at −80 °C for further analysis. The tissues weighed before analysis were homogenized with 50 mM (pH 7.4) phosphate buffer at 14,000 rpm for 30 min. Then, the supernatants were separated by centrifugation at 10,000 g for 30 min at +4 °C. The lipid peroxidation level was measured by the Ohkawa method which is based on the spectrophotometric measurement of the color formed during the reaction of thiobarbituric acid with malondialdehyde (MDA) at 535 nm [19]. The MDA levels were expressed in mM/g protein. The Paglia and Valentine method was used to determine glutathione peroxidase (GSH-px) activity [20]. In this method, GSH-px catalyzes the oxidation of glutathione in the presence of hydrogen peroxide, and while converting oxidized glutathione to a reduced form, the GSH-px activity is determined by measuring the absorbance change that occurs by oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. The GSH-px activity was expressed in U/g protein for tissue. Protein was measured according to the method outlined by Lowry [21].

The rat interleukin (IL)-β levels were quantified by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Rel Assay Diagnostics, Gaziantep, Turkey cat no:RLDR0281). Its sensitivity was 0.022 g/mL, and the detection range was <10%. The results were expressed in ng/mL. An automatic ELISA reader (Thermo Scientific) was used for ELISA studies. The intra-assay CV was <8%, and inter-assay CV was <10%. The results were quantified by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Rel Assay Diagnostics, Gaziantep, Turkey cat no:RLDR0281). Its sensitivity was 0.23 g/mL, and the detection range was <10%. The results were expressed in ng/mL. An automatic ELISA reader (Thermo Scientific) was used for ELISA studies. The intra-assay CV was <8%, and inter-assay CV was <10%. The results were expressed in ng/mL. An automatic ELISA reader (Thermo Scientific) was used for ELISA studies.

Statistical analysis

Statistical analyses were performed using SPSS, version 25.0 (IBM Corp., NY, USA). Normality of distribution was checked using the Shapiro–Wilk test. Normally distributed data were expressed as mean ± standard deviation and non-normally distributed data as median (min–max). Parametric variables were compared by means of the T-test and one-way ANOVA with post-hoc Scheffe-test. A p-value <0.05 was considered statistically significant.

Results

All the rat pups survived until the postnatal fourth day. Although no difference in weight was found between the groups at the beginning of the study, the weights of rat pups were significantly lower in the NEC group than in the other groups at the end of the study (p<0.001, One-way ANOVA test with post hoc Scheffe test) (Table 1, Figure 1).

Histopathological findings

Intestinal damage was shown with microscopic examination after H&E staining. The H&E images of the tissue samples under light microscope are shown in Figure 2. Intestinal histological injury scores of each group are shown on Table 2 and Figure 3. Histopathological examination group could not be performed on two samples from the Sil-control group because they showed signs of were autolysis. All other tissue samples were examined. Mucosal integrity and histological appearance were labeled as normal in the control group (Figure 2A). In the Sil-control group, except for submucosal edema in 1 pup (intestinal injury score=1) and focal necrosis in 2 pups (intestinal injury score=1), the other specimens had normal mucosal integrity and histological appearance (Figure 2B). Hemorrhage, submucosal edema, ulceration and focal necrosis were observed in the NEC group (Figure 2C). In the NEC

Table 1: Comparison of weights and biochemical assessments of experimental rat pups.

<table>
<thead>
<tr>
<th></th>
<th>Control n=10, Mean ± SD</th>
<th>Sil-control n=9, Mean ± SD</th>
<th>NEC n=8, Mean ± SD</th>
<th>Sil-NEC n=9, Mean ± SD</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>5.13 ± 0.478</td>
<td>5.1 ± 0.298</td>
<td>5.31 ± 0.488</td>
<td>5.16 ± 0.522</td>
<td>0.773</td>
</tr>
<tr>
<td>Last weight, g</td>
<td>11.47 ± 0.751</td>
<td>11.14 ± 1.077</td>
<td>5.91 ± 0.814</td>
<td>9.24 ± 1.440</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MDA, nmol/mg protein</td>
<td>0.95 ± 0.281</td>
<td>1.63 ± 0.812</td>
<td>6.08 ± 4.720</td>
<td>1.75 ± 1.405</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GSH-px, U/mg protein</td>
<td>1.35 ± 0.757</td>
<td>4.72 ± 1.426</td>
<td>18.15 ± 15.600</td>
<td>8.31 ± 11.494</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-1β, pg/μg protein</td>
<td>4.99 ± 3.197</td>
<td>4.08 ± 1.420</td>
<td>20.63 ± 19.677</td>
<td>5.75 ± 6.683</td>
<td>0.004</td>
</tr>
<tr>
<td>iNOS, ng/μg protein</td>
<td>0.16 ± 0.099</td>
<td>0.21 ± 0.166</td>
<td>0.70 ± 0.750</td>
<td>0.17 ± 0.121</td>
<td>0.011</td>
</tr>
</tbody>
</table>

Data are shown as statistics: *one-way ANOVA test, post hoc Scheffe. GSH-px, glutathione peroxidase; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; NEC, necrotizing enterocolitis; Sil, sildenafil.
group, 1 pup exhibited grade 1 NEC, 3 pups exhibited grade 2 NEC, and 4 pups exhibited grade 3 NEC. Grade 4 NEC was not seen in any offspring. In the Sil-NEC group, intestinal damage was not observed in 4 pups (Figure 2D). Grade 1 NEC was seen in 5 pups and grade 2 NEC in 1 pup. There was a significant improvement in histopathological findings in the Sil-NEC group compared with the NEC group \(p<0.001\), one-way ANOVA, post hoc Scheffe test).

### Biochemical analysis

Biochemical analysis of all tissue samples was performed except for an autolyzed sample from the Sil-NEC group. The levels of MDA, GSH-px activity, IL-1\(\beta\), inducible nitric oxide synthase (iNOS), and caspase-3 are shown on Table 1. The MDA level (nmol/mg protein) was 6.08 ± 4.72 in the
The NEC group, 0.95 ± 0.28 in the control group, 1.63 ± 0.81 in the Sil-control group, and 1.75 ± 1.41 in the Sil-NEC group (p<0.001). The GSH-px activity (U/mg protein) was determined to be 18.15 ± 15.800 in the NEC group, 1.35 ± 0.76 in the control group, 4.72 ± 1.43 in the Sil-control group, and 8.31 ± 1.43 in the Sil-NEC group (p=0.002). The IL-1β level (pg/μg protein) was 20.63 ± 19.68 in the NEC group, 4.99 ± 3.197 in the control group, 4.08 ± 1.42 in the Sil-control group, and 5.75 ± 6.48 in the Sil-NEC group (p=0.004). The iNOS level (ng/μg protein) was 0.70 ± 0.75 in the NEC group, 0.16 ± 0.10 in the control group, 0.21 ± 0.17 in the Sil-control group, and 0.17 ± 0.12 in the Sil-NEC group (p=0.011). The caspase-3 level (ng/μg protein) was 19.97 ± 12.50 in the NEC group, 13.28 ± 6.48 in the control group, 7.64 ± 3.79 in the Sil-control group and 13.05 ± 9.50 in the Sil-NEC group (p=0.026) (one-way ANOVA test, post hoc Scheffe test).

**Findings of the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling method**

The results of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) test was performed to determine apoptotic cells with light microscopy, and TUNEL positivity revealed that the results were similar in the control group (Figure 4A) and the Sil-control group (Figure 4B) (p=0.589). TUNEL positivity was significantly higher in the NEC group (Figure 4C) compared with the control group (p=0.002) and significantly higher than in the Sil-control group (p=0.002). NEC, necrotizing enterocolitis; Sil, sildenafil.

**Table 3: Apoptotic index (%) of each group.**

<table>
<thead>
<tr>
<th></th>
<th>Apoptotic index (%), Mean (min–max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.83 (1.00–3.00)</td>
</tr>
<tr>
<td>Sil-control</td>
<td>1.50 (1.00–3.00)</td>
</tr>
<tr>
<td>NEC</td>
<td>17.50 (13.00–22.00)</td>
</tr>
<tr>
<td>Sil-NEC</td>
<td>2.83 (2.00–6.00)</td>
</tr>
</tbody>
</table>

Data are shown as median (min–max) or mean (min–max). aCompared to the Sil-control group (p=0.002). bCompared to the NEC group (p=0.002). NEC, necrotizing enterocolitis; Sil, sildenafil.
lower in the Sil-NEC group compared with the NEC group (Figure 4D) (p=0.002). AI (%) is shown on Table 3 and in Figure 5.

**Discussion**

In this study, the therapeutic effect of sildenafil was investigated in an experimental model of necrotizing enterocolitis in rat pups. Previously, experimental studies have shown that apoptosis increased at the onset of necrotizing enterocolitis [18, 22]. TUNEL positivity a marker of showing apoptosis was significantly lower in the Sil-NEC group compared with the NEC group. Also, intestinal damage was significantly lower in the Sil-NEC group in histopathological examination.

Mesenteric ischemia may cause necrotizing enterocolitis [23]. Free oxygen radicals, which are intensively generated by hypoxia/reoxygenation, harm intestinal tissue through tissue damage and apoptosis. Antioxidant systems exert a protective effect against this event by removing free oxygen radicals. Sildenafil has been found to prevent oxidative damage in other organs and tissues [24]. MDA is the end product of lipid peroxidation. It occurs when free oxygen radicals oxidize membrane lipids and is an indicator of oxidative stress [25]. GSH-px is an important factor of an antioxidant system [26]. In an experimental burn study conducted by Bostanci et al., it was shown that GSH-px activity and MDA levels were reduced in rats receiving sildenafil treatment after burn; thus, the authors of the study suggested a positive effect of sildenafil on wound healing in burns. In an experimental burn study Bostanci et al. demonstrated that sildenafil has a positive effect on wound healing in burns [27]. In another study, Marganis et al. reported that sildenafil decreased the level of colonic MDA in an experimentally created colitis model [28].

In this study, the levels of GSH-peroxidase and MDA the indicators of oxidative stress, were lower in the Sil-NEC group compared with the NEC group. This decrease in GSH-peroxidase and MDA levels in the Sil-NEC group is consistent with previous studies, suggesting that sildenafil may be beneficial in NEC thanks to its antioxidant effect.

Sildenafil prevents the breakdown of cGMP by inhibiting the PDE-5 enzyme. The PDE-5 receptors are in vascular smooth muscle, intestines, heart, platelets, placenta, and chondrocytes [29]. cGMP accumulating in vascular smooth muscle causes vasodilation. In addition, intracellular cGMP accumulation has been shown to reduce inflammation by reducing the amount of proinflammatory cytokines and mitigating oxidative damage in several experimental models [8]. Yazji et al. showed that when animals with necrotizing enterocolitis were treated with sildenafil, left in hypoxia for four days and fed with formula gavage, the severity of necrotizing enterocolitis was reduced [30]. Soydan et al. demonstrated the protective effect of sildenafil against intestinal damage after intestinal ischemia–reperfusion [31]. Moore et al. created ischemia–reperfusion damage in 12-week-old male mice and administered sildenafil intraperitoneally at various concentrations at follow-up. Consequently, they suggested that administering sildenafil following intestinal ischemia may limit intestinal mucosal damage [9]. In this study, sildenafil was found to be useful in treating necrotizing enterocolitis in the hypoxia/reoxygenation and cold model.

Inflammation plays a significant role in the development of necrotizing enterocolitis development. A previous study suggested that sildenafil caused vasodilation by
decreasing cGMP degradation and by increasing the sensitivity of vascular smooth muscles to endogenous and exogenous NO [32]. NO also has a regulatory role in immune response and inflammation [33]. Proinflammatory cytokines such as IL-1β, IL-1α, IL-18, IL-6, TNF-α which are released in the early stages of inflammation have all been shown to increase in NEC [34, 35]. In an experimental study, Araujo et al. showed that sildenafil-treated group’s IL-1β level decreased; the authors credited the anti-inflammatory effect of sildenafil [36]. In this study, IL-1β was higher in the NEC group but it was significantly lower in the sildenafil-treated NEC group.

Phosphodiesterase and iNOS enzymes are expressed in macrophages, dendritic cells, T cells, and neutrophils. iNOS is a type of nitric oxide synthetase that increases in inflammatory states [37]. Kosutova et al., in an experimental study on acute lung injury, detected lower levels of iNOS and stated that inflammation was less severe in the sildenafil-administered group [33]. In the present study, iNOS had a lower level in the Sil-NEC group than in the NEC group. This indicates that sildenafil administration mitigated inflammation in the experimental NEC study with rat pups. The caspase-3 is known to be one of the markers of apoptosis. Jia et al., in their study on neonatal rats, found a decrease in the caspase-3 level with the administration of sildenafil in hypoxic myocardial cells [38]. In the present study, the caspase-3 level was lower in the Sil-NEC group compared with the NEC group. This is a significant finding in that - it indicates the antiapoptotic effect of sildenafil in the treatment of NEC.

Sildenafil has been widely used in treating pulmonary hypertension in neonates and has been shown to be effective and safe in meta-analyses. A retrospective study by Hornik et al., involving 232 infants who were born at 24–41 weeks of age and who received sildenafil treatment, showed no relationship between systemic hypotension and sildenafil use [39]. Cohen et al. reported that low-dose sildenafil was well tolerated in a retrospective study of pediatric patients with pulmonary hypertension [40]. The fact that sildenafil was well tolerated and safe in pediatric patients in these two studies made us think that it can be used as an option in the treatment of necrotizing enterocolitis, a highly fatal disease.

An important limitation of our study was that we could not show the development of grade 4 NEC in rat pups. Despite this, various degrees of intestinal damage were demonstrated histopathologically in pups who underwent NEC induction. In addition, in the biochemical analysis, MDA, GSH-px, IL-1β, iNOS, caspase-3 were higher in the animals that developed NEC but significantly lower in the NEC group treated with sildenafil. This showed the therapeutic effect of sildenafil in necrotizing enterocolitis in our study.

This study was involved a small number of animals so as to meet the limitation imposed by the ethics committee. Another important limitation of this study was that sildenafil was administered simultaneously with the induction of necrotizing enterocolitis. Administering sildenafil after the development of NEC may be more helpful. Also, if we had carried out the steps of NEC induction separately, we could have shown exactly at which step sildenafil was more effective. These are the main limitations of our study. Therefore, clinical studies involving larger numbers of animals or humans are needed.

Conclusions

This study was conducted to test whether sildenafil, which has been proved to be safe in the aforementioned studies, may also be used for treating necrotizing enterocolitis. The results of the present study showed that sildenafil reduced intestinal damage induced by hypoxia/reoxygenation and cold injury with its antioxidant, anti-apoptotic and anti-inflammatory effects in an animal model of necrotizing enterocolitis.

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Competing interests: Authors state no conflict of interest.

Informed consent: Not applicable.

Ethical approval: Ethical approval for this study was obtained from the ethics committee of Kahramanmaras Sütçü Imam University Medical Faculty (2018/09-01).

References


