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Research Article / Araştırma Makalesi

Antioxidative Effects of Silymarin in A Neonatal Rat Model of Necrotizing Enterocolitis

Nekrotizan Enterokolitli Yenidoğan Rat Modelinde Silimarinin Antioksidan Etkileri

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Abstract

Necrotizing enterocolitis (NEC) is the most common gastrointestinal problem in premature infants. The aim of this study is to evaluate the protective and antioxidant effects of silymarin (SLY) in newborn rats with NEC model. Twenty-eight Sprague-Dawley rats were included in the study. The rats were randomized into four groups: control (C), C+SLY, NEC and NEC+SLY. NEC was induced by hyperosmolar enteral formula feeding, and the pups were exposed to hypoxia and cold stress. Macroscopic scoring of the intestinal tissue was evaluated and tissue samples were obtained for biochemical, histopathological examination. Superoxide dismutase (SOD), glutathione peroxidase (GPx), nitric oxide (NO), malondialdehyde (MDA), total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) levels were biochemically evaluated. Results: In the NEC + SLY group, there was a considerable rise in tissue TAS ($p = 0.007$), SOD ($p = 0.004$) and GPx levels, as well as a decrease in NO levels. Significantly higher tissue MDA, TOS ($p = 0.001$) and OSI ($p = 0.001$) values were detected in the NEC group. The intestinal tissue of rats in the NEC + SLY group had better histopathology than rats in the NEC group when evaluated. Silymarin has beneficial effects against NEC in neonatal rat. It appears that SLY reduces free radical levels and oxidative stress, increases antioxidant capacity, and ameliorates the severity of intestinal damage due to NEC

Keywords: Necrotizing enterocolitis; Silymarin; Neonatal rats; Oxidative stress

Özet

Nekrotizan enterokolit (NEK), prematüre bebeklerde en sık görülen gastrointestinal problemdir. Bu çalışmanın amacı, NEK modeli oluşturulan yenidoğan sıçanlarda silimarin (SLY)'nin koruyucu ve antioksidan etkilerini değerlendirmektir. Yirmi sekiz Sprague-Dawley sıçanı çalışmaya dahil edildi. Sıçanlar rastgele dört gruba ayrıldı: kontrol (C), C+SLY, NEK ve NEK+SLY. NEK, hiperosmolar enteral formül beslenmesi ile indüklendi, yavru sıçanlar hipoksi ve soğuk stresine maruz bırakıldı. Bağırsak dokusunun makroskopik skorlaması değerlendirildi ve biyokimyasal, histopatolojik inceleme için doku örnekleri alındı. Süperoksit dismutaz (SOD), glutatyon peroksidaz (GPx), nitrik oksit (NO), malondialdehit (MDA), total antioksidan status (TAS), total oksidan status (TOS) ve oksidatif stres indeksi (OSI) düzeyleri biyokimyasal olarak değerlendirildi. NEK+SLY grubunda doku TAS ($p = 0,007$), SOD ($p = 0,004$) ve GPx düzeylerinde önemli artış ve NO düzeylerinde azalma vardı. NEK grubunda doku MDA, TOS ($p = 0,001$) ve OSI ($p = 0,001$) değerleri anlamlı derecede yüksek saptandı. NEK + SLY grubundaki sıçanların bağırsak dokusu, değerlendirildiğinde NEC grubundaki sıçanlardan daha iyi histopatolojiye sahipti. Silimarin, neonatal sıçanlarda NEK'e karşı faydalı etkilere sahiptir. SLY'nin serbest radikal düzeylerini ve oksidatif stresi azalttığı, antioksidan kapasiteyi arttırdığı ve NEK'e bağlı bağırsak hasarının şiddetini iyileştirdiği görülmektedir.

Anahtar Kelimeler: Nekrotizan enterokolit; Silimarin; Neonatal rat; Oksidatif stres

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1. Introduction

Necrotizing enterocolitis (NEC) is a potentially fatal gastrointestinal disease that often affects premature newborns or extremely low birth weight infants (1). Malnutrition, bloating, reduced activity, blood in the stool, bile vomiting, multiple organ failure, and even death are all possible symptoms (2). Prematurity, immature intestinal barrier function, formula feeding, pathological bacterial colonization, intestinal ischemia-reperfusion injury due to hypoxia, free oxygen radicals formed in the environment as a result of infection and inflammation play an important role in the development of NEC (3,4). Silymarin (SLY) is derived from the milk thistle, silybum marianum, and has been used to treat liver illness for centuries, while it has also been researched for its therapeutic effects on cardioprotection, neuroprotection, metabolic disease progression, and cancer (5-9). Silymarin is reported to have potent antioxidant, anti-inflammatory, antiapoptotic, immunomodulatory, antifibrotic, anticancer, and antiviral effects (10,11). The effects and roles of SLY on NEC are unknown, although it is an established modality in the treatment of a variety of illnesses. As a result, the goal of our research was to see how SLY affected intestinal damage in a rat model of NEC.

2. Materials and Methods

Animals and Experimental Conditions The study was confirmed by the Ethics Committee for Animal Care and Usage at Dumlupınar University, Kütahya, Turkey. A total of 28 newborn Sprague-Dawley (SD) rats (5–6 g; ages 1–8 hours) were obtained from pregnant SD rats on day 21 of gestation. Rats were kept in a 12-hour light-dark cycle at 30°C and 60% humidity. Experimental Design; twenty eight SD neonatal rats were randomly divided into control (C), C+SLY, NEC, and NEC+SLY groups (n = 7). Three times a day, newborn rats were given 0.2 mL of a customized rodent formula (15g Similac60/40; Ross Pediatrics, Columbus, OH) mixed with 75 mL of puppy-canine milk replacement (Beaphar-Bogena, BV, Sedel, Netherlands). Experimental NEC was induced by hypoxia in an airtight chamber perfused with 100% CO_2 for 5 min. By the end of this period, the puppies were cyanotic and gasping. The rat pups were reoxygenated for 10 minutes with 100 percent O_2 and exposed to +4 C cold for 5 minutes twice daily for 4 days after hypoxia (12). The control rats were fed breast milk on a regular basis with no stimulation or intervention. SLY was given to the rats in the C+SLY and NEC+SLY groups at a dose of 100 mg/kg intraperitoneally once a day for four days. Tissue Preparation, on the fourth day of the experiment, all of the rats were sacrificed. Each rats abdomen was opened and the gastrointestinal tissues (3 cm intestinal resection, including the terminal ileum and cecum) were removed. After that, the samples were frozen in liquid nitrogen for biochemical examination and stored at -80°C until the results were available. A histopathological examination was also performed. The intestines were inspected for findings of consistency, color and dilatation related to NEC. Biochemical Analysis 0.1 g of intestinal tissue samples were weighed and 1 ml of 0.1 M pH: 7.4 phosphate buffer was added and homogenized using the Next Advance Bullet Blender Storm BBY24MCE (Next Advance, Inc., Averill Park, NY, USA) brand homogenizer and Next Advance brand zirconium oxide beads. Then the supernatant was separated by centrifuging the homogenates at 10,000 g for 15 min. and it was stored at -80°C until evaluation. Tissue protein was measured using a Protein Quantification Kit-Rapid (Sigma-Aldrich Chemie, Buchs/Switzerland). The results were given in µg/ml. Determination of SOD, MDA, NO and GPx activity. Tissue SOD, MDA and NO levels were measured using a superoxide dismutase, TBARS Assay Kit and Nitrate/Nitrite Colorimetric Measurement Kit (Cayman Chemical, Ann Arbor, Michigan, USA). The SOD, MDA and NO activity were calculated as U/mg protein, µmol/g protein and µmol/g protein, respectively. GPx measurement was performed with a glutathione peroxidase measurement kit (Sunred Biological Technology Co. Ltd., Shanghai, China). GPx activity were given in ng/mg protein. Absorbance levels were measured with a ChemWell® 2910 ELISA reader (AwarenessTechnology Inc. Martin Hwy. Palm City, USA). Determination of TAS, TOS and OSI Tissue TAS level was

measured using Rel Assay Diagnostics Total Antioxidant Status Assay Kit and tissue TOS level was measured using a Total Oxidant Status Assay Kit (Rel Assay Diagnostics, Mega Tıp San. ve Tic. Ltd. Sti., Sahinbey/Gaziantep/Turkey). TAS levels were calculated as mmol Trolox Equiv./g protein, and TOS levels as μmol H_2O_2 Equiv./g protein (13,14). Using the TAS and TOS data, the oxidative stress index (OSI=[(TOS/TAS)100]) was calculated. Histopathological Evaluation; For the macroscopic diagnosis of NEC, all tissues were examined for signs of discoloration, edema, deterioration of tissue integrity, ileal distension, bleeding, perforation and necrosis. 3 cm long ileum segment was taken from the ileocecal valve at a distance of 1 cm. It was washed with cold saline solution and fixed in a 10% formaldehyde solution at room temperature in a paraffin block. The 5 μm sections were stained with hematoxylin and eosin (H&E) and examined with a light microscope (Olympus BX51, Tokyo, Japan, 2000). The evaluation was carried out by a pathologist who did not know the characteristics of the groups to be objective. Changes in the intestines were graded on a scale of 0 to 4 in the histological evaluation: Grade 0 (normal): normal tissue structure, intact intestinal mucosa, Grade 1 (mild): little submucosa and lamina propria separation, Grade 2 (moderate): increased submucosa and lamina propria, submucosa and lamina propria separation, edema in the submucosa and muscle layers, Grade 3 (severe): severe submucosa and lamina propria separation, severe muscle and submucosa edema, local villi detachment, and Grade 4 (necrosis): villi structural loss and necrosis. A histological score of 2 or more was considered to be NEC.

Statistical Analysis

The SPSS® Statistics 16 pocket application was used for statistical analysis (Chicago, IL, USA). All of the results were presented as mean ± standard error. Multiple groups were compared using the Kruskal-Wallis test. To compare two groups, the Mann-Whitney U test was utilized. $p \leq 0.05$ were accepted as statistically significant.

3. Results

Silymarin increases SOD and GPx activity levels in the intestinal tissues Tissue SOD levels were found to be statistically significantly different among C (3.46 ± 0.32 U/mg protein), C+SLY (3.71 ± 0.48 U/mg protein), NEC (2.49 ± 0.17 U/mg protein) and NEC+SLY (3.49 ± 0.22 U/mg protein) ($p = 0.011$). The NEC group had a significantly lower tissue SOD levels than the C, C+SLY, and NEC+SLY groups ($p = 0.009$, $p = 0.013$, and $p = 0.004$, respectively) (Figure 1). The differences in the GPx levels in the intestinal tissue among the C (34.0 ± 2.68 ng/mg protein), C+SLY (37.0 ± 6.26 ng/mg protein), NEC (22.7 ± 4.31 ng/mg protein) and NEC+SLY (45.3 ± 4.01 ng/mg protein) groups were significant ($p = 0.016$). The NEC+SLY group had significantly greater GPx levels than the C and NEC groups, which were $p = 0.025$ and $p = 0.004$, respectively (Figure 1). Silymarin arranges NO levels in the intestinal tissues Tissue NO levels differed considerably among the groups of C (1.81 ± 0.68 μmol/g protein), C+SLY (0.72 ± 0.27 μmol/g protein), NEC (2.55 ± 0.96 μmol/g protein) and NEC+SLY (0.70 ± 0.26 μmol/g protein), ($p = 0.05$). The NEC group had considerably greater tissue NO levels than the C + SLY and NEC+SLY groups ($p = 0.025$, and $p = 0.003$ respectively) (Figure 1). Silymarin decreases MDA activity levels in the intestinal tissues There were notable differences in the intestinal tissue MDA levels among C (3.08 ± 0.21 μmol/g protein), C+SLY (2.21 ± 0.22 μmol/g protein), NEC (4.45 ± 1.64 μmol/g protein) and NEC+SLY (2.09 ± 0.16 μmol/g protein), ($p = 0.023$). MDA levels were substantially lower in the C + SLY group than in the C group, which was $p = 0.025$. In addition, when compared to the C and NEC groups, MDA levels in the NEC+SLY group were dramatically reduced, $p = 0.013$ and $p = 0.035$ (Figure 1). Silymarin increases TAS level and decreases TOS and OSI in the intestinal tissues Tissue TAS levels showed statistically significant variations among the groups of C, C+SLY, NEC, and NEC+SLY ($p = 0.007$). Tissue TAS levels in the NEC group were found to be considerably lower than in the C and C+SLY groups ($p =$

0.025 and p = 0.002). Furthermore, when comparing the NEC+SLY group to the NEC group, the TAS levels were found to be significantly higher in the NEC + SLY group, p = 0.030 (Table 1). The levels of tissue TOS and OSI showed significant disparities among the groups of C, C+SLY, NEC, and NEC+SLY (p = 0.001 and p = 0.001). The NEC group showed a considerable increase in tissue TOS and OSI levels as compared to the C, C+SLY, and NEC+SLY groups (p = 0.002, respectively) (Table 1). Silymarin improves inflammatory conditions of intestinal tissues H&E staining was used to grade the inflammatory states of each group's intestinal tissues. The NEC group had epithelial cell swelling, fragility intestinal edema, discolouration, and weakness of tissue integrity, loss of villi, and a decrease in goblet cells, according to histological evaluation. Figure 2 shows the damage scores and comparisons between the groups. When comparing the NEC+SLY group to the NEC group, the results showed that the NEC+SLY group had less intestinal inflammation.

Table 1. The total antioxidant-oxidant status and oxidative stress index values of the tissues

Groups	C (n=7)	C + SLY (n=7)	NEC (n=7)	NEC + SLY (n=7)	p
TAS	0.94 ±0.17[a]	1.07 ± 0.16[b]	0.44 ±0.03[abc]	0.72 ± 0.03[c]	0.007
TOS	1.67 ± 0.07[a]	1.64 ±0.10[b]	3.04 ±0.37[abc]	1.48 ± 0.07[c]	0.001
OSI	0.22 ± 0.04[a]	0.17 ± 0.03[b]	0.69 ± 0.06[abc]	0.25 ± 0.03[c]	0.001

p: Shows the differences between all groups (Kruskal Wallis test).
a,b,c; In each line, the difference between the means with same letters are significant, p≤0.05 (Mann-Whitney U test).
C – control; C - SLY – control -silymarin; NEC – necrotizing enterocolitis; NEC - SLY – necrotizing enterocolitis -silymarin; TAS – total antioxidant status; TOS – total oxidant status; OSI – oxidative stress index

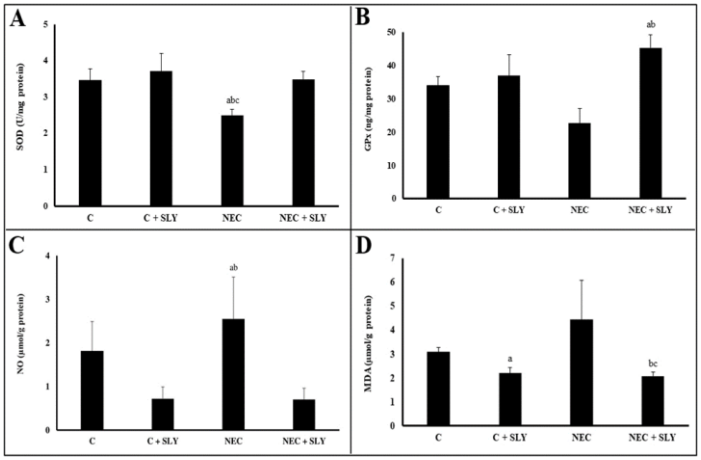
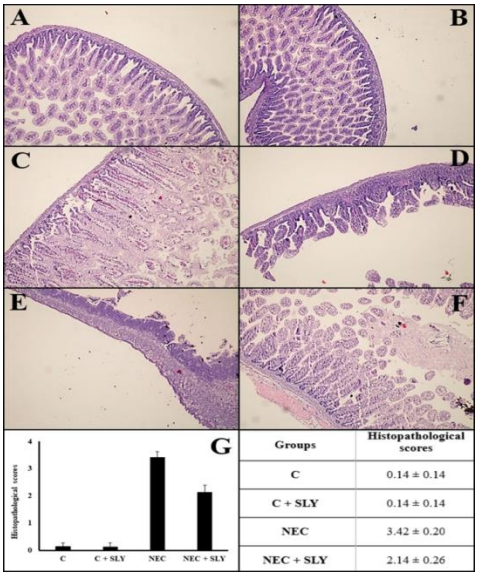


Figure 1. SOD, GPx, NO, MDA activity levels in the intestinal tissue of rats in Control (C), control + silymarin (C + SLY), necrotizing enterocolitis (NEC), necrotizing enterocolitis + silymarin (NEC + SLY) groups.

(A): [a]; Shows significance between C and NEC groups, [b]; Shows significance between C + SLY and NEC groups,
[c]; Shows significance between NEC and NEC + SLY groups (p≤0.05) (Mann Whitney U test).
(B): [a]; Shows significance between C and NEC + SLY groups, [b]; Shows significance between NEC and NEC + SLY groups (p≤0.05) (Mann Whitney U test).
(C): [a;] Shows significance between C + SLY and NEC groups (p≤0.05) (Mann Whitney U test), [b;] Shows significance between NEC and NEC + SLY groups (p≤0.05) (Mann Whitney U test).
(D): [a]; Shows significance between C and C + SLY groups, [b]; Shows significance between C and NEC + SLY groups, [c]; Shows significance between NEC and NEC + SLY groups (p≤0.05) (Mann Whitney U test).
Superoxide dismutase (SOD), Glutathion Peroxidase (GPx), Nitric Oxide (NO), Malondialdehyde (MDA)



Groups	Histopathological scores
C	0.14 ± 0.14
C + SLY	0.14 ± 0.14
NEC	3.42 ± 0.20
NEC + SLY	2.14 ± 0.26

Figure 2. Hematoxylin and eosin stained sections of intestinal tissue harvested from in Control (C), control + silymarin (C + SLY), necrotizing enterocolitis (NEC), necrotizing enterocolitis + silymarin (NEC + SLY) groups.
(A): C group, and (B): C + SLY group; Normal tissue morphology, intact intestinal mucosa and no muscular edema detected (H&E x 100). (C) (D) (E): NEC group; epithelial cell swelling, intestinal edema, discoloration, fragility and weakness of tissue integrity, loss of villi and decrease in goblet cells were detected (H&E x 100).
(F): NEC + SLY group; cell epithelium villus loss, cellular swelling, goblet cell loss were not detected (H&E x 100). (G): The histological scores of groups was statistically analyzed.

3. Discussion

NEC is particularly prevalent in premature infants. In NEC, apoptosis and necrosis form in the tissue mainly due to intestinal motility loss, mucosal integrity impairment and inflammation (15). Many agents used to treat NEC have unproven efficiency and their use is controversial. Thus, research is still required to find safe and efficient NEC-preventive treatment methods (16, 17). The goal of this study was to see if SLY has an influence on the symptoms of necrotizing enterocolitis rats. The effects of SLY in a rat model of NEC are being investigated for the first time, according to our findings. It's probable that oxidative stress contributes to the onset of NEC. The antioxidant enzyme activity of tissue SOD, GPx, and TAS was greatly reduced, whereas tissue MDA, NO, TOS and OSI concentrations with oxidative effect were significantly raised in the NEC. According to Guven et al. MDA and NO levels in the intestinal tissue of rats with NEC were higher, although SOD and GPx levels were lower (12). NO metabolism is increased in some animal models of NEC (18, 19). Enterocyte apoptosis is caused by nitric oxide, which is created by inducible NO synthase (iNOS) and reactive NO oxidation, and inhibits enterocyte proliferation and migration resulting in the intestinal barrier being ruptured (20, 21). Aydemir et al. reported that TOS levels increased, but TAS levels did not change (22). In another study, Yazıcı et al. they found no difference in TAS levels between NEC and control groups (23). In another study by Tayman et al. shown that, the TAS has been reported as decreased (24). The TOS and OSI levels were significantly higher in the NEC group, whereas the TAS level was significantly lower, according to Akduman et al. these findings were in line with prior studies had shown that the development and etiology of NEC are linked to oxidative stress (25). Silymarin has shown anti-inflammatory, antioxidative and immunomodulatory effects against diseases in various animal models (26, 27). The antioxidant effect of silymarin is thought to be due to directly removing the formed free radicals from the environment and inhibiting

specific enzymes responsible for free radical production. SLY has been tested in several experimental models and found to protect cells from reactive oxygen radical damage and strengthen cellular antioxidant systems (28, 29). Some studies have shown the antioxidant characteristic of SLY to be responsible for its protective effects on tissue (30, 31). We discovered that SLY reduced oxidative stress in NEC pups intestines by raising TAS, SOD and Gpx levels while decreasing TOS, OSI, MDA and NO levels. Although the effects of silymarin on different diseases have been shown in various studies, its effect on NEC is unknown. Therefore, we are not able to compare our results. In an animal study, Jouhari et al. reported that silymarin as an antioxidant agent, significantly reduced the size and histopathological scores of endometriotic lesions and increased serum TAS levels (32). Ghaznavi et al. demonstrated that SLY and melatonin significantly lowered the elevated renal reactive oxygen species and MDA levels, and enhanced renal glutathione level and SOD activity in rats with gentamicin-induced nephrotoxicity (33). Mazhari et al. found that SLY increased in testicular TAS, SOD and GPx levels and decreased in cyclo-oxygenase-2 (COX2) expression NO and MDA contents in experimental varicocele induced pathogenesis (34). We concluded that the antioxidant effect of SLY in newborn rats who developed NEC due to hypoxia and hypothermia may be effective in decreasing TOS, OSI, MDA, NO levels and increasing TAS, SOD, GPx levels in the intestinal tissue.

4. Conclusion

In conclusion it was determined that SLY could be beneficial in the treatment of NEC. This effect was thought to be associated with reduced intestinal tissue damage in rats with NEC, as demonstrated in both histopathological and biochemical parameters. SLY could be considered a new candidate for treatment of intestinal injury due to including increased antioxidant enzyme activities, decreased oxidative stress. SLY should clearly be considered as an alternative option for NEC treatment. However, more studies are needed to evaluate its positively affect

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Ethics

Ethics Committee Approval: The study was approved by Animal Care and Usage at Dumlupınar University Kutahya Ethical Committee (Number: 2020.11.03, Date: 26.11.2020).

Informed Consent: None

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