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Original Article

The Effects and Mechanisms of Sucralfate on Intestinal Epithelial Cells in an *In Vitro* Model of Necrotizing Enterocolitis

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ABSTRACT

Aim: Necrotizing enterocolitis (NEC) is an important disease which particularly affects premature babies. Although its pathogenesis is not fully explained, it is thought that the mucosal barrier of the intestine is disrupted. Sucralfate is a cytoprotective drug which supports the mucosal barrier. This study aimed to investigate the effects and mechanisms of sucralfate on enterocytes in an *in vitro* NEC model.

Materials and Methods: Intestinal epithelial cells were cultured. NEC was created with lipopolysaccharide (LPS). Sucralfate dose and concentration (106 µL/cm^2 2:1 diluted) were determined according to the results of the cell viability test. Control group: This was the cell culture without treatment. Sham group: Only sucralfate was applied to the cell culture. NEC group (NG): Only LPS was applied to the cell culture. Treatment group (TG): the cell culture was first treated with LPS and then sucralfate. Prophylaxis group (PG): First sucralfate and then LPS were applied to the cell culture. Terminal deoxynucleotidyl transferase dUTP end-labeling (TUNEL), caspase 3-8-9, RIPK 1-3, MLKL, occludin, claudin, ICAM and MadCAM were investigated immunohistochemically. Differences between the groups were compared via the one-way ANOVA test.

Results: TNF-α and IL-8 levels were higher in the NG ($p<0.05$). TUNEL positive cells were 65.6±8.2% in the NG and 15.4±3.2% in the TG ($p<0.05$). Caspase-8,9 and RIPK1 were higher in the NG ($p<0.05$). The RIPK3 level was low in the NG ($p<0.05$). MLKL was high in the NG, low in the TG and PG ($p<0.05$). ICAM-1 was not significantly different between groups. MadCAM-1 was higher in the NG than in the TG and PG ($p<0.05$). Occludin expression was high and claudin expression was low in the TG ($p<0.05$).

Conclusion: *In the vitro* NEC model, apoptosis and necroptosis and the expression of cell adhesion molecules change. Sucralfate helps regulate apoptotic - necroptotic activity and cell adhesion molecules. The prophylactic administration of sucralfate does not appear to be as effective as therapeutic administration.

Keywords: Necrotizing enterocolitis, sucralfate, cell culture, apoptosis, necroptosis, cell adhesion molecules

Introduction

Necrotizing enterocolitis (NEC) primarily affects premature infants and it is associated with significant morbidity and mortality. While its pathogenesis is not fully understood, NEC is characterized by a disruption of the intestinal mucosal barrier and an invasion of the intestinal wall by Gram-negative bacteria (1). Despite ongoing research, no definitive solution has been found, though breast milk and some probiotics may be effective in preventing NEC.

Sucralfate (aluminum sucrose sulfate) is a cytoprotective agent which supports the mucosal barrier. Approved

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by the Food and Drug Administration for preventing or treating upper gastrointestinal ulcers, sucralfate adheres to inflamed epithelium and has shown effectiveness in epithelial wound healing, chemotherapy-induced mucositis, radiation proctitis, oral ulcers, and burn wound treatment (2,3). Given that NEC involves mucosal barrier disruption leading to inflammation and necrosis, sucralfate may be beneficial in treating or preventing NEC. Previous studies demonstrated that oral sucralfate partially prevented and treated NEC in a neonatal rat model and proved beneficial in preventing ischemia-reperfusion injury in another model (4,5). However, these studies did not clarify the extent of sucralfate's reach to the damaged bowel area or its underlying mechanism of action.

This study aimed to investigate the effects and mechanisms of sucralfate, which forms a protective layer on damaged intestinal epithelium, in an *in vitro* experimental NEC model.

Materials and Methods

This study's design was approved by the Health Sciences Ethics Committee of Manisa Celal Bayar University Faculty of Medicine (date: 02.06.2021, approval no.: 20.478.486/842) and it was funded by Manisa Celal Bayar University Scientific Research Projects Office (2021-073).

Enterocyte Cell Culture

An Intestinal Epithelioid Cell line (IEC-6, CRL-1592, ATCC, USA) was purchased from ATTC. The cells were cultured with 90% fetal bovine serum, 1% penicillin-streptomycin and 10% α-MEM (Minimum Eagle's Medium) at 37 °C, 5% CO_2 until 80% confluency.

NEC Model

Lipopolysaccharide (LPS, L2630, Sigma-Aldrich, Germany, 200 µg/mL) was added onto IEC-6 cells (6,7) for 24 and 48 hours to model. After this, the levels of TNF-α and IL-8 in the culture media were determined by ELISA (DZE201120083 for TNF-α, and DZE SRB-T-83151 for IL-8, Sun Red Biotechnology Company) using the instructions of the kits' protocols. Samples were read in a microplate reader at 450 nm absorbance.

Cell Viability Test (MTT) at Different Sucralfate Doses and Concentrations

The amount of sucralfate (Antepsin 250 mL suspension, Bilim Pharmaceuticals, Turkey) to be applied to the cell culture wells was calculated as follows: Based on the adult body surface area and the maximum daily dose of sucralfate of 4 g, the maximum dose that could be applied per square centimeter was calculated to be 108 µL/cm^2. In determining the optimal nontoxic dose, values below (104 and 106 µL/cm^2) and above (112-114 µL/cm^2) the calculated dose were also tested.

Evaluation of the cytotoxicity of sucralfate on IEC-6 cells was performed using the colorimetric method, 3'-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) salt. Cells were seeded in 96-well culture (1x10^3 cells in each well) dishes with 100 µL of culture medium in each well and incubated for 24 hours. Doses of sucralfate were applied as 3 replicates and incubated for 24 or 48 hours. After incubation, 10 µL of MTT solution was added to each and incubated for 4 hours. The medium was removed and 50 µL of dimethyl sulfoxide was added and the absorbance was measured at 540 nm.

Experimental Groups

In the control group, the cells were cultured only in the culture medium. When the sucralfate was applied, the group was called Sham. In the NEC group, LPS was applied to the cell culture for 48 hours. After LPS administration, when the sucralfate was added for 48 hours, the group was called treatment. In the prophylaxis group, LPS administration was applied after sucralfate administration.

Terminal Deoxynucleotidyl Transferase dUTP End-labeling-TUNEL Method

Apoptotic cells were determined using the terminal deoxynucleotidyl transferase dUTP end-labeling (TUNEL) assay (S7101, Millipore, USA). All groups of cells were fixed with 4% paraformaldehyde for 30 minutes. After washing with phosphate buffer saline (PBS), they were permeabilized with 0.1% Triton X-100 for 10 minutes on ice and then washed with PBS. After incubation with Tdt enzyme at 37 °C for 1 hour, the samples were washed with equilibrium buffer solution and incubated with anti-POD peroxidase for 30 minutes. After washing with stop buffer, the samples were stained with diaminobenzidine (DAB) for 5 minutes and washed 3 times with PBS. For background staining, the samples were stained with Mayer's hematoxylin for 1 minute. After washing with distilled water, they were cover slipped with occlusion medium and examined by light microscopy. After staining, nuclei in 100 cells counted in 5 fields in each group were considered as TUNEL-positive cells, and the ratio was expressed as a percentage.

Immunocytochemical Staining

After the fixation of all of the groups of cells with 4% paraformaldehyde, 3% hydrogen peroxidase for 10

minutes was added and washed with PBS. Permeabilization was performed with 0.1% Triton X-100 for 10 minutes on ice. The blocking solution was added for 1-hour, then primary antibodies caspase-3 (BT-AP01199 Bioassay Technology Laboratory, China), caspase-8 (BS-0052R Bioss antibodies, USA), caspase-9 (BS-0049R Bioss antibodies, USA), RIPK1 (5805R Bioss, USA), RIPK3 (SC-374639 Santa Cruz Biotechnology, USA), MLKL (SC-293201 Santa Cruz Biotechnology, USA), occludin (SC-133256 Santa Cruz Biotechnology, USA), claudin (AB-203563 Abcam, UK), ICAM (SC-8439 Santa Cruz Biotechnology, USA), and MadCAM-1 (365934 Santa Cruz Biotechnology, USA) were added and incubated overnight at 4 °C. After washing with PBS, biotinylated secondary antibody and horseradish peroxidase-streptavidin were added to each of them for 30 minutes. The slides were stained with DAB for 5 minutes and washed with PBS and then with distilled water. After Mayer's hematoxylin staining for 1 minute, the slides were mounted with a mounting medium. The immunoreactivity density was scored as negative (-), mild (+), moderate (++), or severe (+++), and the H scores were calculated according to the immunohistochemical staining results. The formula ∑Pi (I+1) was used for the H-score (I: staining intensity, Pi represents the percentage of stained cells for each intensity).

Statistical Analysis

In the analysis of the data obtained from all the parameters studied, the differences between the groups were examined using Graphpad Prism 9 and the non-parametric one-way ANOVA test. P values less than 0.05 were considered statistically significant.

Results

MTT Results

At the 48-hour of incubation mark, the cell viability rate of 106 µL/cm^2 dose (diluted 2:1) of sucralfate was 1.8 times higher than the control group. This showed that at 48 hours, sucralfate application had led to the loss of approximately 20% of the cells. However, cell loss was slightly higher at other doses and dilutions (Figure 1). Therefore, the 106 µL/cm^2 dosage was chosen in this study.

Model of NEC Established in IEC-6 Cells

The confirmation of the NEC model, TNF-α and IL-8 levels after 24 and 48 hours of application of LPS were evaluated. The highest level of TNF-α was obtained at 48 hours of LPS incubation (Figure 2).

TUNEL Staining

While TUNEL positive cells were 13.8±2.77% in the control group, they were 65.6±8.26% in the NEC Group. TUNEL positive cells were found to be lower in the treatment and prophylaxis groups with respect to the NEC group ($p<0.05$) (Figure 3).

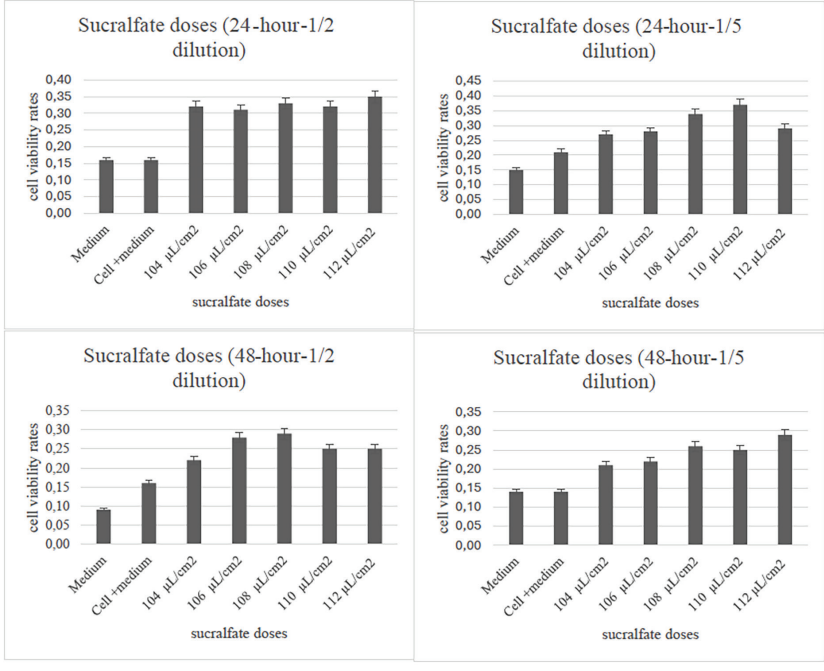

Figure 1. Effect of different dilutions and doses of sucralfate on cell viability rates (MTT test). At the 48th hour of incubation, the cell viability rate of 106 µL/cm^2 dose (diluted 2:1) of sucralfate was 1.8 times higher than the control group

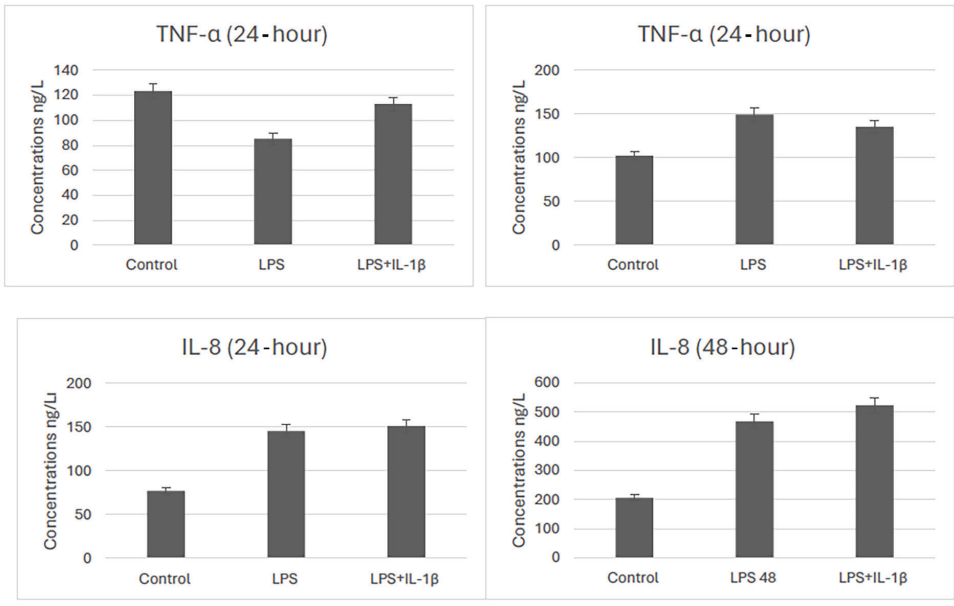

Figure 2. TNF-α and IL-8 levels triggered by lipopolysaccharide (LPS) and LPS +IL-1β (Interleukin 1β) added to enterocyte cell culture for 24 and 48 hours (TNF-α and IL-8 levels, which reached approximately 2-fold compared to the control group, were obtained only at the 48th hour of LPS application)

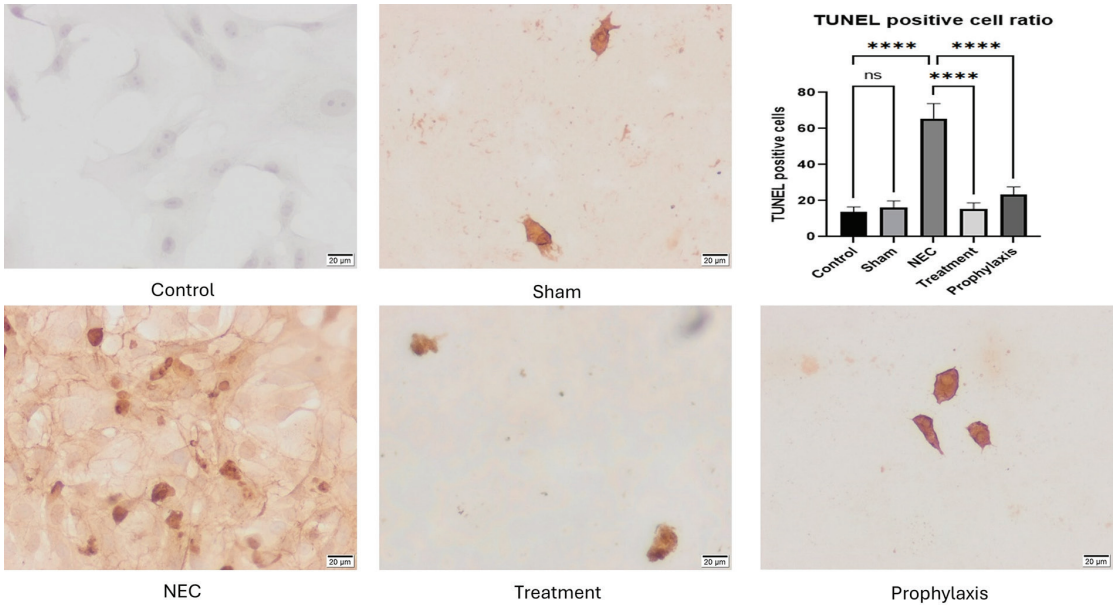

Figure 3. TUNEL (+) cell rates of the experimental groups are seen in the graph. TUNEL (+) cell rate was significantly lower in the treatment and prophylaxis groups than in the NEC group (NEC: Necrotizing enterocolitis, TUNEL: Terminal deoxynucleotidyl transferase dUTP end-labeling, ****p<0.0001, ns: non-significant). In immunohistochemical staining, TUNEL (+) cells were very dense in the NEC group compared to the control group, while the density was less in the treatment and prophylaxis groups (x20 μ)

Immunohistochemistry Staining

Apoptosis

Although the distribution of caspase 3 was lower in the treatment group than in the NEC group, it was not statistically significant (p>0.05). Caspase-3 immunoreactivity in the prophylaxis group was similar to the NEC Group (p>0.05). While the intensity of caspase 8 was higher in the NEC group than in the control group (p<0.05), in the treatment and prophylaxis group, this immunoreactivity was lower than in the NEC group but statistically not significant (p>0.05). The intensity of caspase 9 was significantly different in the

NEC group than in the control group (p<0.05), although it was slightly lower but not significantly so in prophylaxis or treatment groups (Figure 4).

Necroptosis

RIPK1 immunoreactivity was higher in the NEC group in comparison to the control group (p<0.05). The intensity of RIPK1 in the both the treatment and prophylaxis groups was similar to the NEC group. However, the immunoreactivity of RIPK3 in the both treatment and prophylaxis groups was significantly less than that in the NEC group (p<0.05) (Figure 5).

MLKL immunoreactivity was increased in the NEC group compared to the control group (p<0.05). Sucralfate application decreased MLKL intensity in both the treatment and prophylaxis groups (p<0.05) (Figure 5).

Cell Adhesion Molecules

LPS application did not change ICAM-1 distribution in the NEC group. When both the treatment and prophylaxis groups were compared with the NEC group, there was no significant difference in ICAM-1 immunoreactivity. MadCAM-1 intensity was significantly higher in the NEC group than in the (p<0.05). Sucralfate administration decreased MadCAM-1 immunoreactivity in both the treatment and prophylaxis groups (p<0.05). Occludin intensity was higher in the NEC and treatment groups in comparison to the control group (p<0.05). The immunoreactivity of occludin was slightly less in the prophylaxis group (p<0.05) (Figure 6).

While claudin intensity was similar in the control and the NEC group, however, it was decreased significantly in the treatment group (p<0.05) (Figure 6).

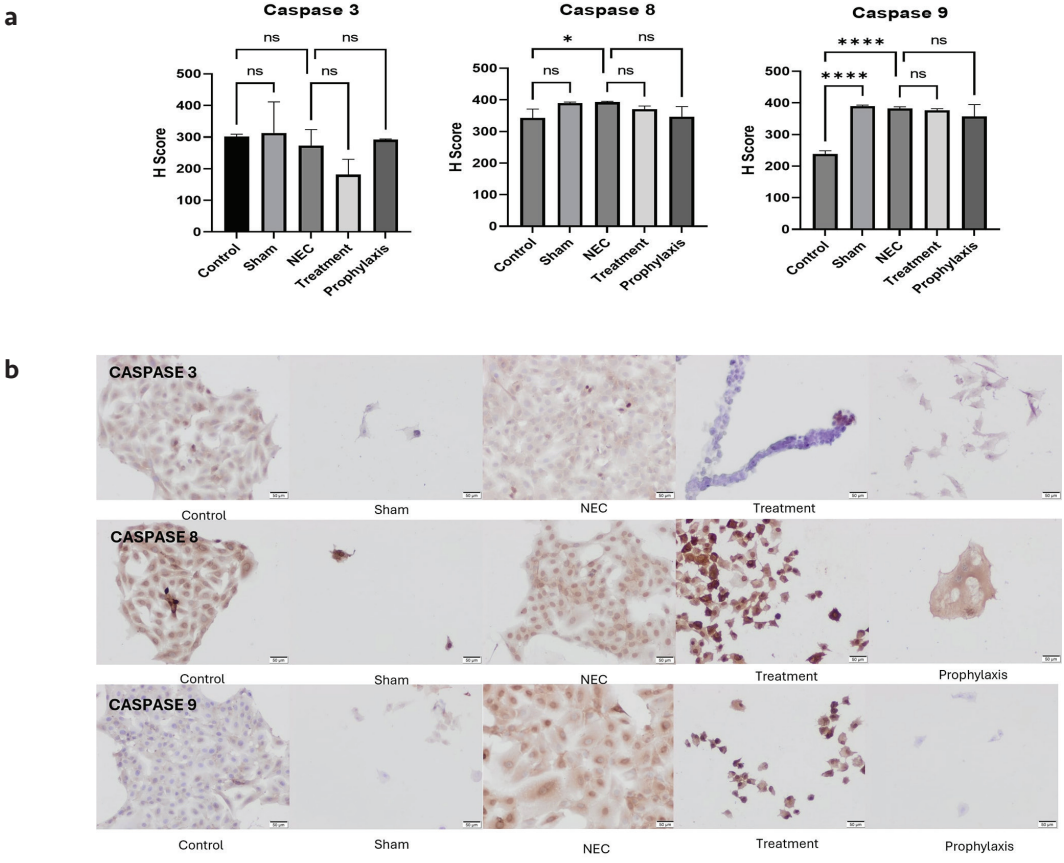

Figure 4. a) The H scores of caspase 3, 8 and 9 are seen in the graph. Especially when the treatment and prophylaxis groups were compared with the NEC group, although the H score of Caspase 3 was lower in the treatment group, no significant difference was found between the groups. When the NEC group was compared with the control group, the H scores of caspase 8 and 9 were significantly higher in the NEC group (*p<0.05, ****p<0.0001, ns: non-significant). b) Immunohistochemical staining of caspase 3,8,9 can be seen at the bottom. Here, especially in the NEC group, caspase 8 and 9 immunoreactivities are seen to be stained more intensely. Sham groups showed lower caspase 3, 8 and 9 immunoreactivities and cell density compared to control groups (x50 µm)

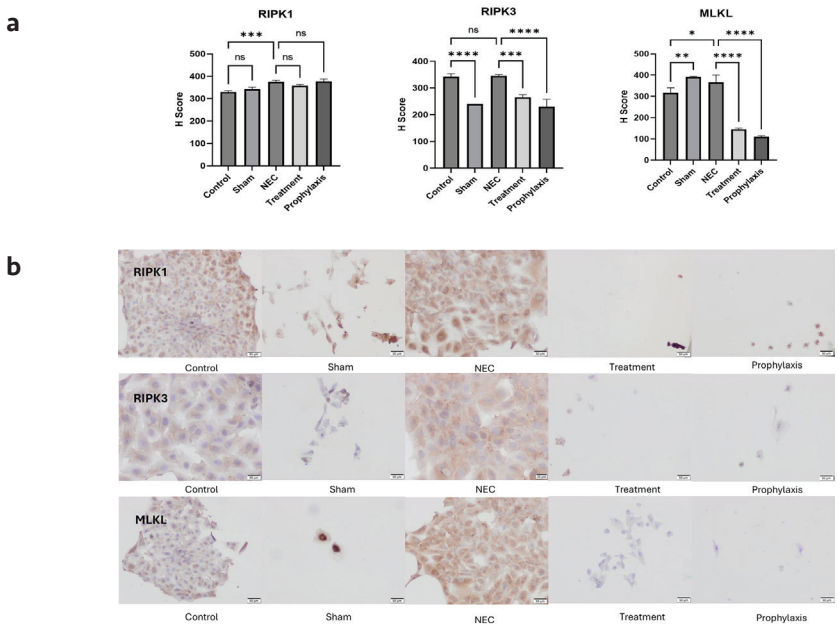

Figure 5. a) Evaluation of the necroptotic pathway. The H score results of RIPK1, RIPK3 and MLKL are shown in the graph above. When the treatment and prophylaxis groups were compared with the NEC groups, the H scores of RIPK3 and MLKL were found to be significantly lower. When the NEC groups were compared with the control groups, the H scores of RIPK1 and MLKL were significantly higher. (*p <0.05, **p<0.01, ns: non-significant). b) The immunohistochemical staining results are shown below (x50 µm). While RIPK1 and MLKL immunoactivity density was higher in the NEC group, RIPK3 and MLKL density was found to be less intense in the treatment and prophylaxis groups. Sham groups showed lower RPIK1, RIPK3 and MLKL immunoreactivities and cell density compared to control groups (x50 µm)

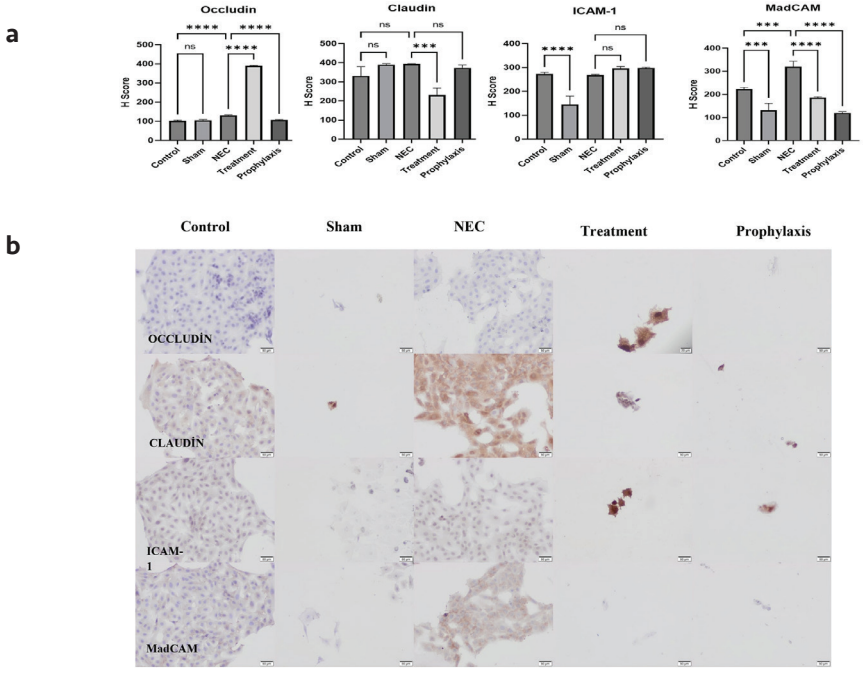

Figure 6. a) The graph (top) shows the H scores of cell adhesion molecules (Occludin, claudin, MadCAM-1, ICAM-1) in the experimental groups. H scores of the occludin were significantly higher in the treatment and prophylaxis groups than in the NEC group, while they were lower in the prophylaxis group. When the NEC group was compared with the control group, H scores of the occludin were higher in the NEC group. Claudin H scores were significantly lower only in the treatment group. ICAM-1 H scores did not differ significantly between the treatment and prophylaxis groups (***p<0.001, ****p<0.0001, ns: non-significant). b) In the lower part, immunohistochemical staining of cell adhesion molecules can be seen (x50µm). The treatment group showed a more intense occludin immunoreactivity compared to the NEC group, while claudin density was lower. The density of MadCAM was also lower in both the treatment and prophylaxis groups compared to the NEC group

Discussion

NEC predominantly affects premature infants and it is thought to result from a disruption of the intestinal mucosal barrier and bacterial colonization, leading to high morbidity and mortality (1,7). NEC appears to be the final common pathway of various pathologies causing inflammatory bowel disease in newborns (8-10).

Although many agents have been tested in NEC experimental models, none have entered clinical practice. Some studies suggest breast milk reduces NEC incidence, and agents that support the intestinal barrier may be effective treatments. Sucralfate, which protects the intestinal mucosal barrier, may have therapeutic or prophylactic effects in NEC.

Enterocytes can express proinflammatory cytokines such as IL-6, IL-8, IL-1β, TNF-α, δ-INF, and GM-CSF under different conditions (6,7). IL-1β and LPS (endotoxins) disrupt the intestinal barrier, reduce intestinal villus crypt formation, and increase permeability (7,11,12). We evaluated inflammatory response (TNF-α and IL-8 levels) by applying LPS and LPS+IL-1β to intestinal epithelial cell cultures for 24 and 48 hours. Higher levels of TNF-α and IL-8 were observed with 48-hour LPS administration compared to LPS+IL-1β, leading us to perform the NEC model with 48-hour LPS incubation alone.

Previous studies have demonstrated that oral sucralfate reduced the severity of intestinal damage in a neonatal rat NEC model and suppressed apoptosis in a rat intestinal ischemia-reperfusion model (4,5). However, these studies did not clarify how much of the sucralfate dose reached the damaged intestine, and effects beyond apoptosis were not evaluated. In this study, both apoptosis and necroptosis were observed in enterocytes in an *in vitro* NEC model.

In our study, TUNEL (+) cells were significantly reduced by sucralfate treatment and prophylaxis, indicating effectiveness in both groups. However, TUNEL positivity may also reflect necroptotic cells (13). Caspase 8 and 9 concentrations were significantly higher in the NEC group, suggesting apoptosis had begun but not been completed, as caspase 3 levels remained unchanged. Sucralfate did not significantly affect caspase 3, 8, or 9 levels.

RIPK1, a key molecule in necroptosis, was significantly increased in the NEC group, most likely due to TNF-α activation of cell death receptors. Sucralfate reduced RIPK1 levels in the treatment group, suggesting partial effectiveness, possibly by forming a protective layer. Despite this, persistent inflammation could lead to RIPK3 activation and necroptosis. Activated RIPK3 and MLKL form the necrosome complex. Phosphorylation occurs and the cell goes into necroptosis (14,15). The increase in MLKL in the NEC group indicated necroptosis, while sucralfate reduced both RIPK3 and MLKL levels, supporting its cytoprotective effect.

Tight junction proteins like occludin and claudin play crucial roles in maintaining intestinal mucosal barrier integrity (16,17). Occludin expression, reduced in NEC, was significantly increased by sucralfate, suggesting its benefit in preserving cell integrity. In the literature, it has been shown that occludin expression is reduced in NEC (18-20). Claudin regulates fluid ion diffusion between cells and, together with occludin, closes the gap between adjacent cells. Claudins are also associated with the actin cytoskeleton (20,21). Claudin density was higher in the NEC group but not statistically significant. Sucralfate's cytoprotective effect was observed in the treatment group but not in the prophylaxis group. Ares et al. (22) it was suggested that claudin-2 traffic to the cytoskeleton increased due to increased cell permeability in NEC, and therefore claudin-2 expression increased in NEC. The increase in claudin in the NEC group may have occurred for a similar reason.

ICAM-1, an important regulator in pathological conditions (23,24), showed no significant difference between the NEC and control groups, nor between the treatment and prophylaxis groups, suggesting LPS stimulation does not affect ICAM-1 in IEC-6 cells. Sumagin et al. (25) reported that TNF and LPS induced ICAM-1 expression in endothelial cells but not in IEC-6 cells.

MadCAM-1, an adhesion molecule induced in inflammatory diseases (23), was significantly higher in the NEC group. Sucralfate significantly reduced MadCAM-1 expression, suggesting it helps maintain cell integrity in the NEC model.

Study Limitations

Our study had certain limitations. Incubation with sucralfate for 48 hours reduced cell numbers in some experimental groups, possibly due to the high viscosity of the sucralfate coating the cells and reducing contact with the culture medium. Additionally, a difference was observed in the H scores of some cell adhesion molecules in the sham group compared to the control group. Although we could not fully explain this situation, we thought it might be related to the viscosity of sucralfate.

Conclusion

In conclusion, our study showed changes in apoptosis, necroptosis, and cell adhesion molecules in an *in vitro* NEC model. Sucralfate appears to protect intestinal epithelial

cells by regulating apoptotic-necroptotic activity and cell adhesion molecules. However, its prophylactic effect is less pronounced than its therapeutic effect. Further studies in other NEC models are needed to confirm sucralfate's effects. The involvement of apoptosis and necroptosis in NEC pathogenesis suggests potential future treatments using inhibitors of these pathways.

Ethics

Ethics Committee Approval: This study's design was approved by the Health Sciences Ethics Committee of Manisa Celal Bayar University Faculty of Medicine (date: 02.06.2021, approval no.: 20.478.486/842).

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Footnotes

Authorship Contributions

Surgical and Medical Practices: A.Ş., S.V., Concept: A.Ş., Design: A.Ş., S.V., Data Collection or Processing: A.Ş., S.V., Analysis or Interpretation: A.Ş., S.V., Literature Search: A.Ş., Writing: A.Ş.

Conflict of Interest: No conflict of interest was declared by the authors.

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