ORIGINAL ARTICLE

The effect of ethyl pyruvate administration on intestinal mucosal damage in neonatal rat models of necrotizing enterocolitis

Efecto de la administración de piruvato de etilo sobre el daño de la mucosa intestinal en modelos de enterocolitis necrosante en ratas neonatas

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Abstract

Objective: *The purpose of this study was to determine whether intraperitoneal administration of ethyl pyruvate (EP) affects intestinal mucosal damage in neonatal rats with a model of necrotizing enterocolitis (NEC).* **Methods:** *The study comprised 46 Sprague-Dawley newborn rats delivered on the 21st gestational day. The rats were divided into four groups: Sham Group (n = 10), EP Group (n = 10), NEC Group (n = 13), and NEC + EP Group (n = 13).* **Results:** *The mean body weights of rats in Sham and EP groups significantly increased (p < 0.05), whereas those in NEC groups significantly decreased (p < 0.05). The changes were similar between groups (p > 0.05). There was no significant difference in the clinical sickness score (CSS) between groups (p > 0.05). The CSS in the NEC groups showed an increase from day 1 to day 4. Based on the histopathological grading, there was a statistically significant difference (p < 0.001) observed between the Sham Group, the NEC Group, and the NEC + EP Group. The differences in biochemical values among the other groups were found to be statistically significant (p < 0.05) for all variables.* **Conclusions:** *The application of EP in high-mortality diseases such as NEC is expected to reduce surgical needs, establishing a basis for future studies on EP as an innovative anti-inflammatory agent.*

Keywords: *Necrotizing enterocolitis. Neonate. Ethyl pyruvate. Interleukin 6. Malondialdehyde. Sprague-Dawley rat.*

Resumen

Objetivo: *Determinar si la administración intraperitoneal de piruvato de etilo (PE) afecta al daño de la mucosa intestinal en ratas neonatas con un modelo de enterocolitis necrosante (ECN).* **Métodos:** *El estudio incluyó 46 ratas recién nacidas Sprague-Dawley entregadas en el día gestacional 21. Las ratas se dividieron en cuatro grupos: grupo simulado (n = 10), grupo PE (n = 10), grupo ECN (n = 13) y grupo ECN + PE (n = 13).* **Resultados:** *El peso corporal medio de las ratas en los grupos simulado y PE aumentó significativamente (p < 0.05), mientras que en los grupos ECN disminuyó significativamente (p < 0.05). Los cambios fueron similares entre grupos (p > 0.05). No hubo diferencia significativa en el puntuación clínica de enfermedad (PCE) entre grupos (p > 0.05). El CSS en los grupos ECN mostró un aumento del día 1 al día 4. Según la clasificación histopatológica, se observó una diferencia estadísticamente significativa (p < 0.001) entre el grupo simulado, el grupo ECN y el grupo ECN + PE. Las diferencias en los valores bioquímicos entre los otros grupos resultaron ser estadísticamente significativas (p < 0.05) para todas las variables.* **Conclusiones:** *La aplicación de PE en enfermedades de alta mortalidad como la ECN se espera que reduzca las necesidades quirúrgicas, estableciendo una base para futuros estudios sobre el PE como un innovador agente antiinflamatorio.*

Palabras clave: *Enterocolitis necrotizante. Neonato. Piruvato de etilo. Interleucina 6. Malondialdehído. Rata Sprague-Dawley.*

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Introduction

Necrotizing enterocolitis (NEC) is a pathological condition characterized by inflammation of the intestines that primarily affects neonates. It is sometimes observed in conjunction with sepsis, leading to the occurrence of intestinal perforation, peritonitis, and ultimately, mortality. The etiology of NEC encompasses various factors, including hypoxia, hyperosmolar enteral nutrition, intestinal immaturity, and intestinal dysbiosis.[1] Hypoxia and disruption of the intestinal epithelial barrier due to failure to provide enteral nutrition play an important role in premature infants.[2]

Studies in the past have shown that ethyl pyruvate (EP) has anti-inflammatory benefits.[3,4] EP is a derivative of pyruvic acid and serves as an intermediary compound in the process of energy metabolism. Under anaerobic conditions, it facilitates the conversion to lactic acid through the process of dehydrogenation, thereby contributing to energy generation. Consequently, it exhibits antioxidant properties and diminishes the concentration of free radicals.[5] EP is a chemical compound that serves as a derivative of pyruvic acid. It functions as an intermediary molecule in the process of energy metabolism. In anaerobic conditions, it facilitates the conversion of pyruvate to lactic acid by dehydrogenation, thereby contributing to energy generation. Previous research has demonstrated that EP possesses therapeutic properties in the treatment of intra-abdominal sepsis and colitis by modulating intestinal permeability and fortifying the mucosal barrier systems. According to reports, EP has been found to possess a mitigating impact on intestinal inflammation through the inhibition of toll-like receptor 4 (TLR-4).[6] The experimental colitis model has demonstrated that EP exhibits beneficial effects by suppressing the "T Helper 1" immune response.[7] The study investigated the impact of *Escherichia coli* lipopolysaccharide-induced sepsis and septicemia on arterial hypotension in a shock model. The researchers found that the administration of EP solution during resuscitation in rats led to a reduction in arterial hypotension, resulting in prolonged survival. Furthermore, it was observed that the concentrations of interleukin-6 (IL-6) and nitrite/nitrate exhibited a decrease; however, the levels of IL-10 were found to be elevated.[8-10]

The applications of EP are currently being investigated in the experimental study phase, with administration occurring through various channels such as intravenous, intraperitoneal, and intrapulmonary methods.[9,11-13] There is no experimental research on the intraperitoneal administration of EP in NEC models in the literature.

The purpose of this study was to determine whether intraperitoneal administration of EP affects intestinal mucosal damage in neonatal rats with a model of NEC.

Methods

After the approval of the Acibadem University Experimental Animals Local Ethics Committee (January 08, 2020–2020/01), the study was approved by the Scientific Research Projects Unit in the University of Health Sciences, Turkey, which is in accordance with legislation for the protection of animals used for scientific purposes.

To create newborn rats, male and female Sprague-Dawley rats were housed together. If the veterinarian observed sperm in the vaginal smear between 08:30 and 09:30 am, this was considered the start of the 1st gestational day. The mother rats were provided with unlimited access to food at a standard room temperature of 24.5°C. They were kept in a controlled environment with a 12-h cycle of darkness and light.[10] On the 21st gestational day, a total of 46 Sprague-Dawley rats were born by cesarean section and were randomly divided into four groups. The study consisted of four groups: Sham Group (n = 10), NEC Group (n = 13), EP Group (n = 10), and NEC + EP Group (n = 13). The rats in the sham group were allowed to stay with their mothers and were given unlimited access to food. To eliminate the protective effect of breast milk against NEC, the rats designated for the NEC model were isolated from their mother. They were then placed in an incubator set at a temperature of 32°C with 60% humidity.[11]

Sham group

The newborn rats were immediately given to their mother after birth and began to be nourished with breast milk. A total of 0.02 mL of 0.9% NaCl (saline) was administered intraperitoneally into the left lower quadrant at 1:00 p.m. for the initial 4 days.

EP group

Newborn rats, who were being fed breast milk and staying close to their mother for the first 4 days after birth, were given 50 mg/kg of EP intraperitoneally. This was administered as a solution in 0.9% NaCl, with a dosage of 0.02 mL.

NEC group

The newborn rats were fed a hyperosmolar formula consisting of 15 g of formula mixed with 75 mL of puppy or canine milk. This mixture was administered every 4 h using a 24 Gauge orogastric catheter, starting from the 3rd h. The feeding schedule was maintained every 4 h, with an increase of 0.1 mL/day in the subsequent days. The rats used in the NEC model experiment were subjected to specific conditions. They were exposed to hypoxia for a duration of 90 s inside a box filled with nitrogen gas flowing at a rate of 10 L/min. Following this, they were exposed to cold temperatures of 4 °C for a period of 10 min. In addition, the rats were placed in an incubator twice a day, specifically at 9:00 a.m. and 5:00 p.m., for a total of 3 days. A total of 0.02 mL of 0.9% NaCl (saline) was administered intraperitoneally from the left lower quadrant at 1:00 pm for the initial 4 days.[10]

NEC + EP group

The initial steps were identical to those in Group C. During the initial 4 days, a dose of 50 mg/kg of EP, in the form of a solution in 0.9% NaCl, was administered intraperitoneally from the left lower quadrant at 1:00 pm.

Histopathological evaluation

Two double-blind observers evaluated the daily weight and clinical sickness scores (CSSs) of rats in all groups.[14] On the 4th day, following hypothermic sedation with an ice pack and 200 mg/kg phenobarbital, the surgical site was cleaned with povidone-iodine. A 3 cm segment of the terminal ileum was extracted through a 2 cm median incision above the umbilicus. Subsequently, all rats were euthanized.

Evaluation of the macroscopic appearance of the intestines was made by double-blind observers according to the "Macroscopic Gut Score"[12] (Table 1). Histopathological evaluation is based on the "NEC Grading Score" after 10% formaldehyde fixation and Hematoxylin and Eosin staining (Intact villus Grade 0, villi deletion 1, mild villus damage 2, complete villus necrosis 3, and transmural necrosis 4) performed by pathologists. Grades 2, 3, and 4 were accepted as NEC.[13]

Tissue samples of 3 cm in length, obtained from the terminal ileum, were subjected to storage at a temperature of −80°C. Subsequently, these samples were homogenized in a phosphate-buffered saline solution at a weight-to-volume ratio of 1/10. The tissue homogenates underwent centrifugation at a speed of 3000 revolutions/min (rpm) at a temperature of +4°C. Subsequently, the supernatants obtained were gathered and put into polypropylene tubes. The levels of IL-6 and Malondialdehyde (MDA) were quantified using the Rat-IL-6 Enzyme-Linked Immuno Sorbent Assay (ELISA) kit (Catalog Number E0135Ra) and the Rat-MDA ELISA kit (Catalog Number E0156Ra), respectively.[14,15]

Table 1. Scoring system for macroscopic gut assessment

	Macroscopic gut assessment		
Score	**Gut consistency**	**Gut colour**	**Gut dilatation**
0	Normal	Normal	Normal
1	Moderately Friable	Patchy discoloration	Patchy dilatation
2	Extremely Friable (jelly-like)	Extensive discoloration	Extensive dilatation

Macroscopic Gut assessment, from Zani et al.[12].

Statistical analysis

The data was documented using the Microsoft Office 2019 Pro-Excel software. The data were analyzed using the IBM Statistical Package for the Social Sciences 26.0 software (IBM, Armonk, New York, US). The data are presented as means and standard deviations, or as counts and percentages (n%). The Shapiro-Wilk test was used to assess the normality of the data distribution. To assess the distinctions between groups, analysis of variance tests and *post hoc* tests were employed. In order to examine temporal variance, the Friedman and Wilcoxon tests were utilized. The results were assessed using a 95% confidence interval and a statistical significance level of $p < 0.05$.

Results

Nine rats survived in both the sham group and the EP group. One rat from each group could not be found in the cage, leading to the assumption that the mother rat might have eaten the baby rat. Six of 13 rats in the NEC group were euthanized on day 3 due to deterioration in their general condition and increased clinical disease scores. One rat in the NEC + EP group was excluded from the study due to its unfortunate demise in the cage on the 2nd day. Three of them were sacrificed on the 3rd day and included in the study due to

Table 2. Clinical sickness score

Clinical sickness score				
Score	**Appearance**	**Natural activity**	**Response to touch**	**Body color**
0	Tonic and well hydrated	Moving normally in the cage	Alert (without stimulation)	Pink
1	Slimmer but still tonic and hydrated	Able to wriggle if put supine	Responding to mild stimulation	Pale (just at the extremities)
2	Skinny, floppy, and dehydrated	Not able to wriggle if put supine	Responding to vigorous stimulation	Pale (whole body)
3	Gasping and in agony	Not moving limbs and lying still	Unresponsive notwithstanding vigorous stimulation	Grey

deterioration in general condition and an increase in CSSs on the 3rd day (Table 2).

The mean body weights of rats in Sham and EP groups significantly increased ($p < 0.05$), while those in NEC groups significantly decreased ($p < 0.05$). The changes were similar between groups ($p > 0.05$) (Fig. 1).

There was no statistically significant difference between the groups in terms of the CSS values on the 1st day ($p > 0.05$). The CSS was consistent across all groups on day 1. The results showed that there was no significant difference between the groups on day 1 ($p > 0.05$), but there was a significant difference on days 2-4 ($p < 0.05$). There was no significant difference in the CSS between groups ($p > 0.05$). The CSS in the NEC groups showed an increase from day 1 to day 4 (Fig. 2).

The average macroscopic bowel assessment score for all groups of rats was 1.58. There was no significant difference in macroscopic bowel scoring between the Sham and the EP Group. However, there was a statistically significant difference between all other groups ($p < 0.05$). Figure 3 displays the macroscopic bowel appearances of the different groups.

During the histopathological examination, it was observed that 78% of the rats in the sham Group had grade 0 NEC, while the remaining 22% had grade 1 NEC. Within the NEC group, 7.7% of the rats exhibited grade 1, while another 7.7% displayed grade 2. The majority, 30.7%, had grade 3 NEC, and the highest proportion, 53.8%, exhibited grade 4 NEC. All rats in the EP Group exhibited no signs of NEC, as they were assigned a grade of 0. In the NEC+EP Group, the distribution was as follows: 1 in 8.4%, 2 in 33.3%, 3 in 33.3%, and 4 in 25%. The histopathological microscopic images are displayed in figure 4. Based on the histopathological grading, there was a statistically significant difference ($p < 0.001$) observed between the Sham Group, the NEC group, and the NEC + EP Group. There was a statistically significant difference ($p < 0.001$) between the NEC Group and the EP Group. A statistically significant difference ($p < 0.001$) was observed between the EP and NEC + EP Group. There was no statistically significant difference observed in the histopathological evaluation between the NEC group and the NEC + EP group. However, when considering the distribution, it was observed that the percentage of grade 3 and 4 rats in the treatment group was lower compared to the untreated NEC group. Table 3 presents the statistical results pertaining to the differences between groups.

Table 3. Histopathological changes

Group (n)	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Sham (9)	7 (77.8)	2 (22.2)	0	0	0
EP (9)	9 (100)	0	0	0	0
NEC (9)	0	1 (7.69)	1 (7.69)	4 (30.7)	7 (53.8)
NEC+EP (12)	0	1 (8.3)	4 (33.3)	4 (33.3)	3 (25)

Statistically significant differences were observed between Sham vs NEC, Sham vs NEC + EP, NEC vs EP, and EP vs NEC + EP groups ($p < 0.001$). No statistically significant difference was observed between NEC and NEC + EP groups.
NEC: necrotizing enterocolitis; EP: ethyl pyruvate.

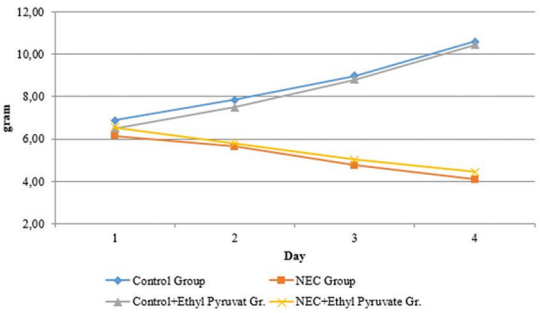

Figure 1. *Time change of mean body weights of the groups.*

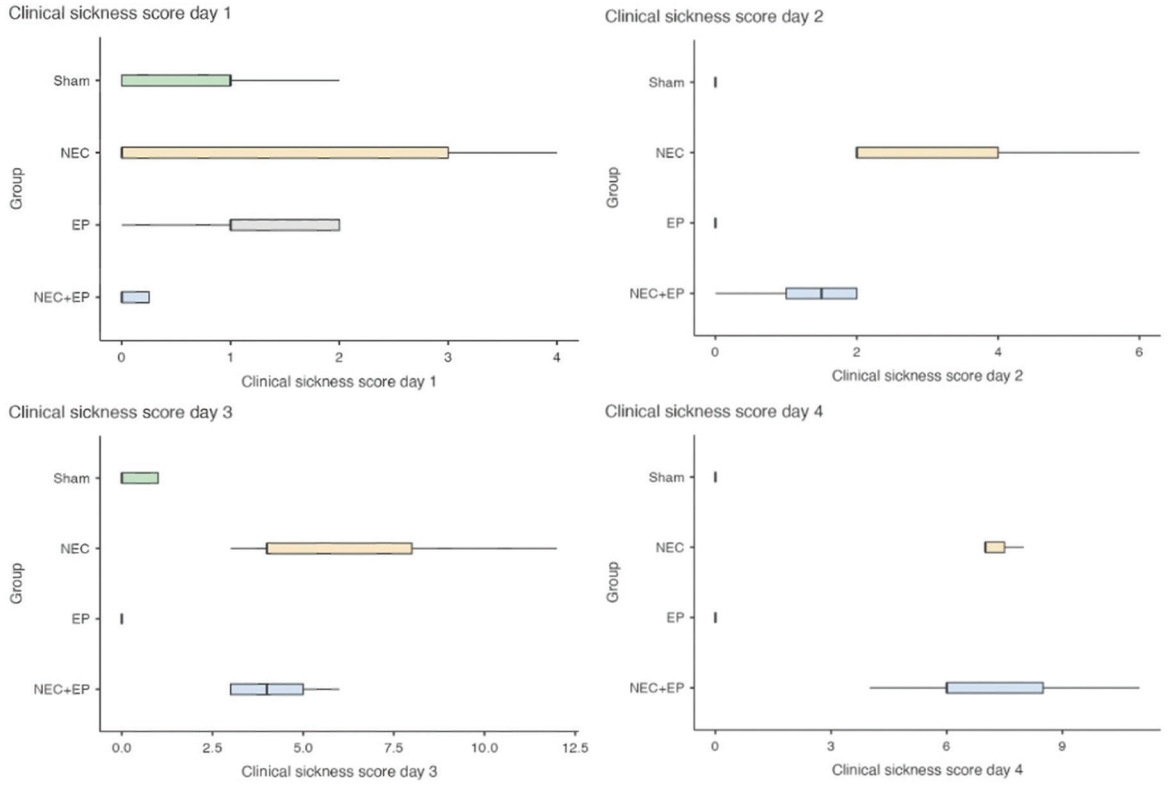

Figure 2. *Change of clinical sickness scores over time.*

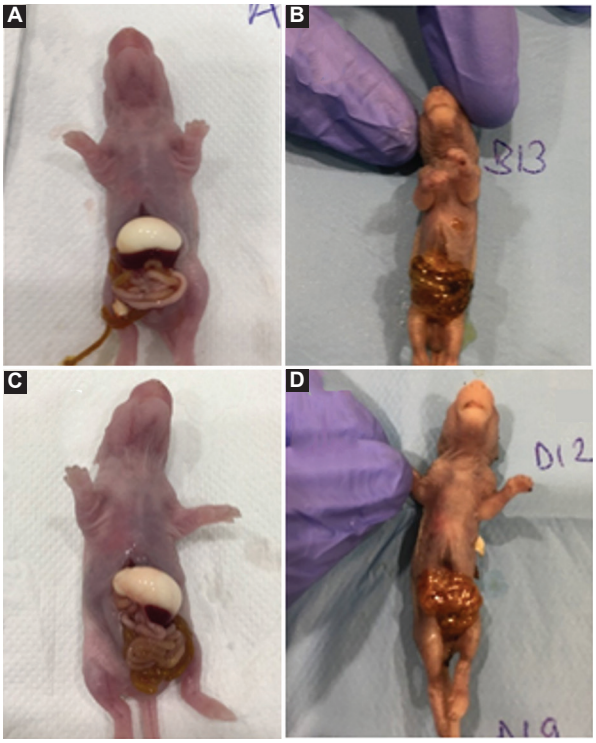

Figure 3. *Macroscopic bowel appearance. (**A**) Sham group, (**B**) necrotizing enterocolitis (NEC) group, (**C**) ethyl pyruvate (EP) group, (**D**) NEC + EP group.*

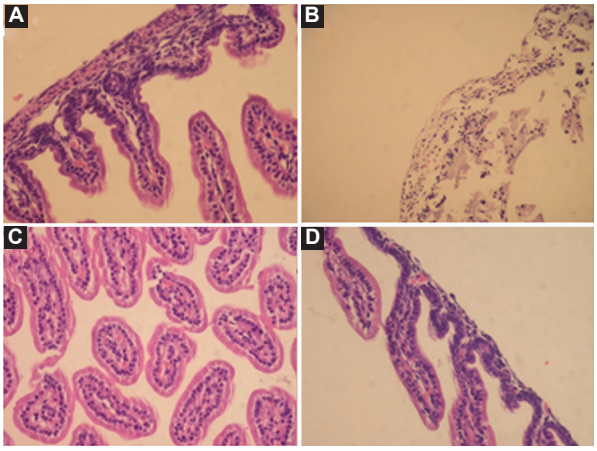

Figure 4. *Histopathological changes. (**A**) Sham (Grade 1), (**B**) necrotizing enterocolitis (NEC) (Grade 3), (**C**) ethyl pyruvate (EP) (Grade 0), (**D**) NEC + EP (Grade 1).*

There was no statistically significant difference in the biochemical values of the Sham Group and the EP Group when comparing the results of IL-6 level and tissue MDA level. The differences in biochemical values among the other groups were found to be

Table 4. Cytokine levels of groups

Cytokines	Sham (n = 9)	EP (n = 9)	NEC (n = 9)	NEC+EP (n = 12)	p
IL-6 (pg/mg tissue)	0.21 ± 0.02	0.21 ± 0.02	0.43 ± 0.06[a]	0.28 ± 0.05[a]	0.0001
MDA (pg/mg tissue)	0.06 ± 0.01	0.05 ± 0.01	0.12 ± 0.02[a]	0.08 ± 0.02[a]	0.0001

[a]Statistically significant difference between group NEC versus NEC + EP p = 0.001.
NEC: necrotizing enterocolitis; EP: ethyl pyruvate; IL-6: interleukin-6; MDA: malondialdehyde.

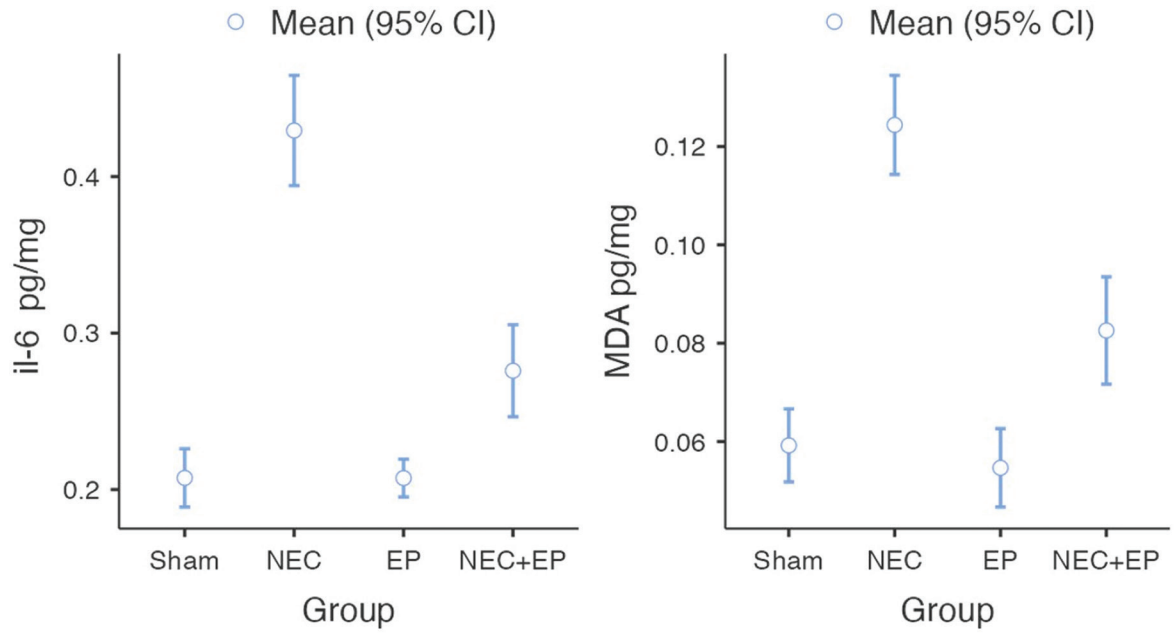

Figure 5. *Comparison of the cytokine levels.*

statistically significant ($p < 0.05$) for all variables (Table 4 and Fig. 5).

Discussion

The advancement of neonatal intensive care units has highlighted NEC as a major concern due to its high rates of illness and death in premature infants. In preventing NEC, administering EP intraperitoneally may play a key role in lessening intestinal harm by reducing intestinal necrosis. This study is the first to examine the impact of EP on neonates with an NEC model. Our research aimed to shed light on NEC's causes and development, as well as on strategies for its prevention. The insights gained from this study lay the groundwork for further research in this field.

Different animal models have been developed for NEC research, but they vary in mechanism and pathophysiology. The human and animal immune systems differ; for example, humans get immunoglobulins via the placenta, while pigs get them through the gastrointestinal tract. Barlow and Santulli's 1974 NEC model, the first successful one, focuses on altered intestinal bacteria and the lack of breast milk. Its creation involved formula feeding, hypoxia, and cold exposure.[16]

Numerous agents have undergone investigation in experimental animal models to explore their potential for preventing and treating NEC.[17-19] EP is a pyruvic acid derivative and has been reported to provide improvement in conditions such as ischemia-reperfusion injury, organ dysfunctions, hemorrhagic shock, sepsis, acute respiratory distress syndrome, acute pancreatitis, and skin burns.[9] EP has the ability to reduce the action of peritoneal macrophages by decreasing the secretion of HMGB1 protein.[4] Previous studies have demonstrated that EP possesses therapeutic effects in the treatment of intra-abdominal sepsis and colitis by targeting the intestinal permeability

and mucosal barrier systems.[6,7] In the colitis model induced by 2,4,6-trinitrobenzene sulfonic acid, it has been demonstrated that intraperitoneal administration of EP at a dose of 50 mg/kg for 1 week improves pathological damage and reduces T-helper-17 cells in the local tissue.[7]

Yurttutan et al.'s study showed newborn rats with an NEC model gained more weight with etanercept, a tumor necrosis factor alpha (TNF-α) blocker.[17] Another study found increased weight in NEC rats fed growth factor-enriched formula.[15] Our study observed normal growth in breastfed control groups and significant weight loss in NEC groups, with no difference in weight gain from intraperitoneal EP application.

The CSS was used to assess the clinical condition of newborn rats with NEC.[14] Korkut et al.'s study showed lower scores in rats treated with obestatin.[11] Another study found improvement with mesenchymal stem cell therapy.[18] Our research observed worsening conditions and increased scores in the NEC and NEC + EP groups, with significant deterioration by the 4th day. No difference in scores was noted between these groups, aligning with high morbidity and mortality rates in NEC models.

The macroscopic gut assessment method evaluates bowel appearance in the NEC model newborn rats.[14] A study found that cytidine 50-diphosphocholine significantly reduced macroscopic bowel scores in treated rats.[20] Our study, using double-blind observers, showed that EP treatment in NEC rats led to improved bowel appearance and lower scores, indicating potential reduction in intestinal damage.

A grading system has been established to determine the histopathological diagnosis of NEC. The histopathological grading is assessed using the NEC grading score. This scoring system assigns a value of 0 for intact villi, 1 for villi deletion, 2 for mild villus damage, 3 for complete villus necrosis, and 4 for transmural necrosis.[13] In a study performed by giving pentoxifylline to newborn rats, for which the NEC model was created, it was shown that histopathological NEC findings were milder.[21] Previous studies similar to ours have reported a morbidity rate of 75% in the experimental NEC model.[10] A study was conducted to investigate the effects of pioglitazone, a peroxisome proliferator-activated receptor-γ agonist, on an NEC model. The study compared a control group with a treatment group. The results showed that the treated group had a lower macroscopic bowel score and exhibited milder intestinal damage in histopathological examinations. The study found that there was no difference between the two groups in terms of the amount of IL-6 and TNF-α messenger RNA (mRNA) in the tissue.[17] In our study, we observed histopathological findings in newborn rats. Specifically, we found that 53% of rats in the NEC Group had grade 4 NEC, whereas 30% had Grade 3 NEC. In the group treated with NEC+EP, the distribution of rat grades was as follows: 33% were Grade 2, 33% were Grade 3, and 25% were Grade 4 NEC. There was no statistically significant difference between the two groups; distributionally, there was a decrease in the severity of NEC in the NEC + EP Group. In grades 3 and 4, NEC was detected in 83% of the rats in the NEC group. There was a significant difference between the control groups and the NEC groups in relation to the increase in histopathological grading. There was no statistically significant difference observed in histopathological grading between the NEC group and the NEC + EP group. However, the percentage of rats in the treatment group with Grade 3 and 4 distributions was lower compared to the untreated NEC group. In order to achieve statistical significance, it may be necessary to increase the sample size. The results indicate that the therapeutic effect of EP on NEC can be observed through intraperitoneal administration, as seen in the histopathological findings.

NEC is linked with increased levels of IL-6, a proinflammatory cytokine, which correlates with NEC severity. IL-6 stimulates C-reactive protein release and is regulated by nuclear factor kappa-light-chain-enhancer of activated B cells.[22] Studies in rats show that interventions can impact IL-6 levels.[23,24] Fecal microbiota transplantation normalized systemic IL-6, IL-1β, and TNF-α in a Wistar albino rat NEC model. Another study using an NEC model induced by formula feeding, lipopolysaccharide injection, and hypoxia found that subcutaneously administered glucagon-like peptide-2 significantly lowered TNF-α and IL-6 levels.[25] Ulinastatin treatment also significantly decreased tissue IL-6 levels.[26] N-Acetyl Cysteine given antenatally and postnatally reduced IL-6 and its mRNA expression in intestinal tissue.[27,28] Our study focused on the effect of EP on IL-6 levels in NEC rats. While control groups showed no significant IL-6 difference, NEC groups had higher IL-6, which was significantly reduced by EP treatment, suggesting EP's effectiveness in reducing NEC severity by lowering IL-6 in intestinal tissue.

EP has been found to be an effective agent in combating free oxygen radicals during instances of oxidative stress. Previous studies have reported that the

administration of EP reduces the levels of MDA, a free oxygen radical, in conditions such as hemorrhagic shock and ischemia-reperfusion injury.[29] In the case of NEC, oxidative stress leads to the formation of free oxygen radicals, which in turn causes an increase in tissue MDA levels.[23] A study was conducted to investigate the effects of quercetin, a flavonoid derivative known for its antioxidant properties, on newborn rats with NEC. After creating the NEC model, quercetin was administered to one group of rats while another group did not receive any treatment. The results revealed a decrease in oxidative stress parameters, specifically MDA levels, in the tissues of the treated group compared to the non-administered group.[30] A study conducted by Guven et al. demonstrated that the rats with the NEC model who were administered melatonin exhibited a decrease in the oxidative stress parameter, MDA, compared to the group that did not receive melatonin.[31] In our study, we did not find any significant difference in MDA levels in intestinal tissues between the two control groups. However, we did observe a significantly higher level of MDA in the NEC groups compared to the control groups. In the NEC group that received EP, it was observed that the levels of tissue MDA decreased significantly compared to the NEC group that did not receive EP.

This study is the first to examine the impact of EP on neonates with an NEC model. The study demonstrated that when EP was administered intraperitoneally to newborn rats with an NEC model, it did not have any impact on the clinical status or body weight of the rats. However, it did lead to an improvement in the macroscopic appearance of the bowel. Although the application of EP did not have a statistically significant effect on the histopathological improvement in the NEC group, it was observed that it improved the overall histopathological condition. It has been found that administering EP intraperitoneally to rats with an NEC model resulted in a reduction in the severity of NEC. This was achieved by decreasing the level of IL-6 in the intestinal tissue and reducing oxidative stress by lowering the MDA level.

This is a pioneering study to investigate the effects of EP on the NF-kB activation pathway and the TLR-4 inhibition pathway in the pathogenesis of NEC. Due to financial difficulties, this research could not be carried out. It may be necessary to enlarge the sample sizes to obtain more statistically significant results. These situations are the limitations of the study.

Conclusions

It is predicted that the application of EP in diseases with high mortality, such as NEC, can decrease the necessity for surgery. This finding will serve as the foundation for future phase studies exploring the use of EP as a new-generation anti-inflammatory agent.

Funding

This study was financed by the University of Health Sciences Scientific Research Projects Unit at the stage of procurement of materials, procurement of experimental animals, and procurement of laboratory services.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical considerations

Protection of human subjects and animals. The authors declare that the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the World Medical Association and the Declaration of Helsinki. The procedures were authorized by the Institutional Ethics Committee.

Confidentiality, informed consent, and ethical approval. This study did not involve human participants. All experimental animal procedures were approved by the Acibadem University Experimental Animals Local Ethics Committee (Approval date: January 08, 2020; Approval number: 2020/01) and were conducted in accordance with institutional and applicable ethical guidelines. The ethics committee approval document is attached for editorial records."

Declaration on the use of artificial intelligence. The authors declare that no generative artificial intelligence was used in the writing or creation of the content of this manuscript.

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