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Research Article

THE EFFECTS OF AMNIOTIC FLUID IN EXPERIMENTAL NECROTIZING ENTEROCOLITIS MODEL

DENEYSEL NEKROTİZAN ENTEROKOLİT MODELİNDE AMNİYOTİK SIVININ ETKİLERİ

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ABSTRACT

Objective: Necrotizing enterocolitis (NEC) is a serious disease that affects the gastrointestinal tract of premature infants and newborns. In the fetal period, swallowed amniotic fluid has a possible preventive role, protecting the infant from NEC, and contributes to gastrointestinal tract development. The aim of this study was to investigate the effects of amniotic fluid on an experimental NEC model.

Method: Newborn rat pups were divided into five groups. Group 1 (G1) served as the control group. The remaining groups (G2–G5) were exposed to hypoxia and hypothermia to induce NEC and were then fed with breast milk (G2), amniotic fluid (G3), formula (G4), or formula prepared with amniotic fluid (G5). NEC development, intestinal perforation, and survival rates were evaluated.

Results: There were no signs of NEC development or perforation in the hypoxic-hypothermic stress–induced and breast-fed rat pups (G2). This was associated with the protective effect of breastfeeding. Symptoms of perforation and NEC developed in the other groups. The lifespan of the group fed amniotic fluid (G3) was shorter than that of the groups fed formula (G4). This short lifespan was connected to the low energy and protein content of amniotic fluid, although the intestinal perforation rate was lower than in G4. The NEC signs were fewer in number and less severe in G3 and G5 than in G4.

Conclusion: The results suggest that amniotic fluid, especially when combined with formula, may reduce the severity of NEC and intestinal perforation in newborn rats, indicating its potential protective role.

Keywords: Necrotizing enterocolitis, amniotic fluid, experimental model, newborn rat, breast milk

ÖZET

Amaç: Nekrotizan enterokolit (NEK), prematüre bebeklerin ve yenidoğanların gastrointestinal sistemini etkileyen ciddi bir hastalıktır. Fetal dönemde yutulan amniyotik sıvı, bebeği NEK'ten koruyacak olası bir koruyucu role sahiptir ve gastrointestinal sistem gelişimine katkıda bulunur. Bu çalışmanın amacı, amniyotik sıvının deneysel bir NEK modelindeki etkilerini araştırmaktır.

Yöntem: Yenidoğan sıçan yavruları beş gruba ayrıldı. Grup 1 (G1) kontrol grubuydu. Diğer gruplar (G2-G5) NEK oluşturmak için hipoksi ve hipotermiye maruz bırakıldı ve ardından anne sütü (G2), amniyotik sıvı (G3), mama (G4) veya amniyotik sıvı ile hazırlanmış mama (G5) ile beslendi. NEK gelişimi, bağırsak perforasyonu ve sağkalım değerlendirildi.

Bulgular: Hipoksik-hipotermik stres oluşturulan ve anne sütüyle beslenen sıçan yavrularında (G2) NEK gelişimi veya perforasyon bulgularına rastlanmadı. Bu durum emzirmenin koruyucu etkisiyle ilişkilendirildi. Diğer gruplarda perforasyon ve nekrotizan enterokolit (NEC) semptomları gelişti. Amniyotik sıvı ile beslenen grubun (G3) yaşam süresi, mama ile beslenen grupların (G4) yaşam süresinden daha kısa bulundu. Bu kısa yaşam süresi, amniyotik sıvının düşük enerji ve protein içeriğiyle ilişkilendirildi; ancak bağırsak perforasyon oranı G4'e göre daha düşüktü. G3 ve G5'te NEC bulguları G4'e göre daha az sıklıkta ve daha hafifti.

Sonuç: Bu çalışmanın sonuçları amniyotik sıvının, özellikle mama ile birlikte kullanıldığında, yenidoğan sıçanlarda nekrotizan enterokolit ve bağırsak delinmesinin şiddetini azaltabileceğini ve potansiyel bir koruyucu rol oynayabileceğini göstermiştir.

Anahtar Kelimeler: Nekrotizan enterokolit, amnion sıvısı, deneysel model, yenidoğan sıçan, anne sütü

Introduction

Necrotizing enterocolitis (NEC) is a life-threatening disease in the neonatal period, which is characterized by different degrees of transmural or mucosal necrosis of the gastrointestinal system, especially the bowels. While the exact etiology is unknown, it is thought to be multifactorial (1). NEC development has been generally attributed to gastrointestinal immaturity, high osmotic nourishment with infant formula, bacterial invasion, and hypoxic-ischemic injury (2). As there are many factors which can lead to stress and ischemia in the intrauterine period (low systemic oxygen saturation, hydrops fetalis, serious tachyarrhythmia and bradyarrhythmia, intensity of hypotensive situations of mother, etc.), NEC symptoms may also emerge in the fetal intrauterine period.

When oral nourishment is considered dangerous for newborns because of critical illness or prematurity, it has been suggested that the negative changes in the gastrointestinal system caused by deficient nutrition can be reduced with the enteral administration of artificial amniotic fluid (AF) (3). However, there have been few experimental and clinical studies examining the effects of the direct or indirect addition of AF to the nutrition program on NEC.

The hypothesis of this study was that, in addition to other factors, AF and the protective agents it contains may play a role in preventing the development of NEC in the neonatal period. The aim of the study was to evaluate the clinical, histological, and pathological effects of orally administered AF and AF-supported nourishment methods on NEC induced in an experimental rat model.

Materials and Methods

Sprague Dawley rats were obtained and mated for the use of newborns in the experiment. The pregnant dams were fed normally, and healthy newborn offspring were separated into five groups for the experiment (Table 1).

Table 1. The characteristics of groups in terms of nourishment contents

G1	Breastfed control group which has never been exposed to any stress
G2	Control group where NEC protocol is applied and then feeding with breast milk is continued
G3	NEC protocol applied group fed with human AF
G4	NEC protocol applied group which has been fed with only formula
G5	NEC protocol applied group fed with formula prepared with human AF

A total of 47 rat pups were obtained from five dams. The distribution of the offspring was 7 from the 1st mother (G1), 9 from the 2nd mother (G2), 11 from the 3rd mother (G3), 10 from the 4th mother (G4) and 10 from the 5th mother (G5).
The G1 rat pups were kept together with their mother and continued to be fed with breast milk. The rat pups in the other groups (G2, G3, G4 and G5) were fed as stated in Table 1. The NEC protocol was applied to all the subjects in all the groups. The method described in the literature for the NEC model was used, according to which the subjects were exposed to planned

hypoxia and hypothermia (4). Immediately after birth, the newborn rats were taken from their mothers for feeding and were then exposed to hypothermia in a refrigerator at +4°C for 10 minutes. The rat pups were then removed and placed in a glass jar, which was hermetically sealed with 3 openings. The air in the jar was aspirated from the first opening with a surgical aspirator device, then 100% nitrogen gas was added to provide equal media pressure, and the rat pups were exposed to hypoxia for 1 minute. The pups were observed to become cyanosed during the procedure. This NEC protocol was applied to all the rats for a period of 96 hours at 12-hour intervals.

The nourishment of the rat pups in the G3, G4, and G5 groups was administered using silicon taps of 24Gx19mm branulas and 1 ml insulin syringes. The branula taps were placed in the stomachs of the newborn rats by hyperextension of the mouth. Nourishment was administered for 96 hours. The rat pups were kept separate from their mothers in cages at an appropriate temperature and humidity. The newborn rat pups in G3 were fed with human AF, the G4 group was fed with formula, and the G5 group was fed with a formula prepared with 30 cc human AF and 1 measure of formula. The nourishment was administered in a 0.1 ml volume at 4-hour intervals.

Permission to use human AF in study was granted by the Clinical Research Ethics Committee. In the selection of pregnant amniotic fluid (AF) donors, the factors of chorioamnionitis, preeclampsia, premature membrane rupture, gestational diabetes mellitus, and hypertension were taken into consideration. Attention was paid to the donors having a smooth, problem-free pregnancy with good follow-up. AF was obtained under sterile conditions during the birth from women with a first pregnancy, where birth was cesarean delivery. It was ensured that the AF obtained was clean and transparent without meconium or blood. To confirm that the AF was not infected, samples were sent for biochemical analysis, cell count, and C-Reactive protein investigation.

The general condition, color, activity level, and abdominal tension of the newborn rats was recorded before and after each administration of nourishment. Weight was recorded daily. During this process, any animals with serious distension, hypokinesis and respiratory distress, or sepsis symptom development in the abdomen were sacrificed.

The animals that survived after 96 hours were euthanized using high-dose ketamine. The intestines were removed, and color change, edema, stenosis or necrosis development was noted, then they were sent for histopathological investigation. Histological preparates were prepared with Hematoxylon-Eosin staining, and the degree of microscopic injury was evaluated with a score of 0 - 4 according to the Musemeche et al. classification (5) (Table 2).

Table 2. Histopathological staging

•	0=No histopathological change
•	1=Partial mucosal necrosis
•	2=Whole layer mucosal necrosis
•	3=Partial muscularis necrosis
•	4=Necrosis in all bowel walls

Ethical Approval

This study was approved by the Experimental Animal Research Ethics Committee of the Faculty of Medicine, Kahramanmaraş Sütçü İmam University (Approval No: 2412/03-8). Additionally, permission for the use of human amniotic fluid was obtained from the Clinical Research Ethics Committee.

Statistical analysis

All statistical analyses were carried out using SPSS 22.0 software. Values were stated as mean ± standard deviation (SD), median (range), or number and percentage. The differences between groups were investigated using analysis of variance (ANOVA) and the post-hoc Tukey HSD test was applied to differences between the groups. A value of $p<0.05$ was considered statistically significant. A post-hoc power analysis was conducted using G*Power 3.1 to evaluate the study's ability to detect the observed effect size. The analysis was based on an effect size of Cohen's $d=1.2$, a significance level of $\alpha=0.05$, and sample sizes of $n1=11$ and $n2=10$. The achieved power ($1-\beta1$) was calculated to be 84.17%.

Results

The birthweight and daily weight of the rat pups were recorded daily throughout the study. The data related to birthweight, daily weight, life duration and presence of intestinal perforation are given in Table 3.

Table 3. Average body weights, life durations and intestinal perforation frequencies of objects by groups

Group	Body weights (gram)					Life duration (hour)	Intestinal Perforation%
	Birth	24th h	48th h	72nd h	96th h		
G1 (n:7)	5.64±0.55	6.53±0.59	7.24±0.59	8.06±0.57	8.89±0.57	96±0	0%
G2 (n:9)	5.57±0.41	5.96±0.34	6.46±0.37	6.90±0.32	7.54±0.41	96±0	0%
G3 (n:11)	5.53±0.29	5.80±0.27	5.50±0.28	.	.	41.5±6.22	36.4%
G4 (n:10)	5.69±0.27	5.97±0.25	5.98±0.19	.	.	45.2±6.39	60%
G5 (n:10)	5.79±0.29	6.11±0.39	6.27±0.47	.	.	51.3±10.47	30%

No difference was found between the groups in terms of average birth weight ($p > 0.05$). At the end of the first 24 hours, the G1 group had gained more weight than the G2 ($p = 0.027$), G3 ($p = 0.002$), and G4 ($p = 0.027$) groups. No significant difference was observed between the G1 and G5 groups regarding weight gain in the first 24 hours ($p = 0.159$). Due to a decrease in the number of rat pups, these weight comparisons could not be made at 48 hours. At 72 and 96 hours, the G1 rat pups had gained more weight than the G2 group ($p = 0.001$ for both).

In the macroscopic examinations of the subjects, their activity, abdominal distension, and color changes were evaluated (Figure 1).

Fig. 1. Distension and color change in the abdomen of the rat subjects on the left due to necrotising enterocolitis are visible.

During abdominal exploration, intestinal perforation was detected in 4 of the G3 pups (36.4%), 6 of the G4 pups (60%), and 3 of the G5 pups (30%). No perforations were observed in the G1 and G2 groups. During the macroscopic evaluation of gastrointestinal systems in pathology, the intestines of the control group rats were pink, intact and of normal appearance. The characteristics of the intestines removed from the rat pups fed with breast milk were similar. There was no pathological change in 27.3% of G3 pups, but changes related to different stages of NEC were detected in the rest of the G3 group and in all the subjects in G4 and G5.

The histopathological samples of the intestines were classified according to the Musemeche et al. method (4). The histopathological staging of the groups according to this method is shown in Table 4.

Table 4. Pathological staging of NEC in terms of groups

Group	Stage 0	Stage 1	Stage 2	Stage 3
G1 (n:7)	7 (100%)	0	0	0
G2 (n:9)	9 (100%)	0	0	0
G3 (n:11)	3 (27.3%)	5 (45.5%)	3 (27.3%)	0
G4 (n:10)	0	4 (40%)	5 (50%)	1 (10%)
G5 (n:10)	0	8 (80%)	2 (20%)	0

Discussion

In various previous studies and experimental NEC animal models, the effects of probiotics, immunoglobulins, antibiotics, essential amino acids, lactoferrin, and different dietary regimens on the development and progression of the disease have been investigated. In the current study, the protective and therapeutic role of AF in NEC was examined. The study was based on the consideration that AF swallowed by the fetus in utero may contribute to the protection and maturation of the intestines. The notion that NEC may have a protective aspect has also been suggested in the literature (6)

Various studies have shown that mediators present in the content of AF stimulate intestinal development (7,8). Trahair et al. (9) asserted that esophageal infusion of "gastrin-releasing peptide," which is found in high concentrations in biological fluids such as AF and colostrum, contributes to the development

of fetal organs and the immune system. It has been reported that the concentrations of arginine, ornithine, and polyamines rapidly increase in sheep from the early gestational period and remain at high levels throughout pregnancy; these AF-derived and swallowed polyamines contribute to the proliferation and differentiation of epithelial cells (10).

In the current study, the weight of the G3 rat pups at the end of the 1st and 2nd days was found to be lower than that of the G1 and G2 groups ($p < 0.05$). This lower weight gain was thought to be due to the lower energy content of AF, as a previous study of human breast milk reported 7.93 g/dl lactose and 0.99 g/dl protein, whereas the AF used in this study contained 21 mg/dl glucose and 0.7 g/dl total protein. Low osmolarity is recommended in feeding models during premature and neonatal periods. Although the osmolarity of the AF used in the study was low, its protein and energy content were deficient.

No statistically significant difference was found between the weights of the G3 and G4 groups at the end of the 1st and 2nd days ($p > 0.05$). The only difference during the follow-up of these stress-exposed groups was the form of nourishment. The calorie and protein content of the nutrition given to G3 was lower than that given to G4, and the higher solute load in the formula fed to G4, compared to the AF given to G3, was an important risk factor for the G4 subjects. High solute load is known to be a significant risk factor for NEC. The osmolarity of the AF used in this study was calculated as 252 mOsm/L. In a previous study investigating the solute loads of breast milk and other infant feeding products, the osmolarity of maternal breast milk was found to be 300 mOsm/L, while that of formula was 400 mOsm/L (11). Therefore, it is known that the G4 group in the current study was fed a higher solute load. The formula feed given to the G4 group is thought to have contributed to progression to necrosis and disruption of intestinal perfusion by damaging the intestinal mucosa structure, thereby affecting peristalsis and lengthening bowel transit time. Some agents in AF may be effective in promoting remodeling in the damaged intestine.

During this study, perforation was observed in 60% of the G4 cases, compared to 36.4% of the G3 cases. When comparing NEC changes in the intestines related to perforation, no NEC symptoms were observed in one-third of the G3 cases, while Phase 1 NEC was present in 55% and Phase 2 NEC in 27% of the group. In contrast, 100% of the G4 cases exhibited NEC symptoms, with 40% at Phase 1, 50% at Phase 2, and 4.8% at Phase 3 ($p < 0.05$). These results indicate that AF leads to fewer NEC cases than formula feeding. This lower rate may be attributed to the low viscosity, low solute load, and anti-inflammatory properties of AF.

In comparisons between G4 and G5, the development of NEC symptoms was found to decrease with the addition of AF to the formula feed, as perforation occurred in 60% of G4 cases but only in 30% of the AF-supplemented G5 group. Furthermore, while pathological staging was Phase 2 or higher in 60% of G4 cases, this proportion decreased to 20% in G5. These findings demonstrate that the addition of AF to the feed has a protective effect against tissue damage.

Limitations

In this study, human amniotic fluid was used to feed newborn rats. Although the inflammatory and histopathological processes are similar, it may be better to use AF from the same species because of possible molecular differences. Therefore, there is a need for furthermore extensive studies using larger subjects rather than rats to be able to obtain their own AF.

Conclusion

This study, examining the effects of amniotic fluid in an experimental NEC model, demonstrated the protective effects of amniotic fluid supplementation against the incidence and severity of NEC. In amniotic fluid supplementation therapy, both caloric needs and the osmotic load of the nutritional content should be considered. This beneficial effect needs to be further defined through larger experimental studies and, potentially, well-designed human trials. Such research may lead to new protective and therapeutic approaches against NEC.

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