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Is caffeine a predisposing risk factor for developing intestinal injury in newborn rats?

Kafein sıçan yavrularında bağırsak hasarı gelişiminde predispozan bir risk faktörü mü?

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Abstract

Purpose: To investigate of histopathologic and biochemical effects of caffeine citrate in newborn rats with hypoxia/reoxygenation (H/R)-induced intestinal injury.

Materials and methods: One-day-old, 32 Wistar albino newborn rats (n=8) were randomly divided into four groups: control group (group1, n=8), caffeine group (group2, caffeine citrate administered subcutaneously, n=8), H/R group (group3, exposed to H/R, n=8), and caffeine + H/R group (group4, caffeine citrate administered and exposed to H/R, n=8). Caffeine citrate was initiated at a loading dose of 20 mg/kg, followed by a maintenance dose of 5 mg/kg/day, subcutaneously. On day 4th, all animals except for groups 1 and 2 were exposed to H/R and sacrificed 6 hours after H/R procedure. Histopathological injury scores (HISs), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and oxidative stress index (OSI: total oxidant status "TOS"/total antioxidant status "TAS") levels were measured in intestinal samples.

Results: As histopathological, the most severe damage was observed in H/R-induced groups (p<0.01). Although not statistically significant, the mean HISs of caffeine group was higher than the control group and lower than the H/R group. The levels of TNF-α, IL-6 and OSI in the groups 2, 3 and 4 were significantly higher than the control group (p<0.05). However, these biochemical parameters in the caffeine group were significantly lower than those of the H/R-induced groups (p<0.01).

Conclusion: This study showed that caffeine citrate significantly increased the intestinal tissue levels of TNF-α, IL-6, and OSI. As a result, caffeine may be a predisposing risk factor for developing intestinal injury.

Key words: Caffeine, intestinal injury, newborn.

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Öz

Amaç: Hipoksi/reoksijenizasyon (H/R) ile intestinal hasarlanma oluşturulan sıçan yavrularında kafein sitratın histopatolojik ve biyokimyasal etkilerini araştırmak.

Gereç ve yöntem: Bir günlük 32 Wistar albino sıçan yavrusu rastgele dört gruba ayrıldı: kontrol grubu (grup1, n=8), kafein grubu (grup2, subkutan kafein sitrat uygulanan, n=8), H/R grubu (grup3, H/R uygulanan, n=8) ve kafein+H/R grubu (grup4, kafein sitrat verilen ve H/R uygulanan, n=8). Kafein sitrat, 20 mg/kg'lık bir yükleme dozunda başlatıldı, ardından subkutan 5 mg/kg/günlük idame dozu takip edildi. Dördüncü günde, grup 1 ve 2 dışındaki tüm hayvanlar H/R'ye maruz bırakıldı ve H/R prosedüründen 6 saat sonra öldürüldü.

Histopatolojik hasarlanma skorları (HIS), interlökin-6 (IL-6), tümör nekroz faktörü-alfa (TNF-α) ve oksidatif stres indeksi (OSI: toplam oksidan durum "TOS"/toplam antioksidan durum "TAS") seviyeleri bağırsak örneklerinde değerlendirildi.

Bulgular: Histopatolojik olarak en ciddi hasar H/R uygulanan gruplarda görüldü (p<0,01). İstatistiksel olarak anlamlı olmasa da kafein grubunun ortalama HIS'leri kontrol grubundan daha yüksek ve H/R grubundan daha düşüktü. Grup 2, 3 ve 4'teki TNF-α, IL-6 ve OSI düzeyleri kontrol grubundan anlamlı olarak yüksekti (p<0,05). Bununla birlikte, kafein grubundaki bu biyokimyasal parametreler, H/R uygulanan gruplardan anlamlı olarak daha düşüktü (p<0,01).

Sonuç: Bu çalışma, kafein sitratın TNF-α, IL-6 ve OSI'nin bağırsak doku düzeylerini önemli ölçüde arttırdığını gösterdi. Sonuç olarak, kafein bağırsak hasarı gelişiminde predispozan bir risk faktörü olabilir.

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Anahtar kelimeler: Kafein, intestinal hasarlanma, yenidoğan.

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Introduction

Apnea of prematurity is a developmental disorder due to immaturity of respiratory control system in premature infants. Classically, it is defined as the cessation of breathing for greater than 15 to 20 seconds. Shorter events (<15 seconds) may also be identified as apnea if along with oxygen desaturation and bradycardia (1). Methylxanthines, such as theophylline and caffeine, are the most commonly used pharmacologic agents for the treatment of apnea of prematurity. It is known methylxanthines (ex. aminophylline) have acute adverse effects such as tachycardia, cardiac dysrhythmias, feeding intolerance, and seizures. However, these adverse effects are rarely seen with the use of caffeine at therapeutic doses [1]. First 3 days of life caffeine usage has beneficial effects on neonatal outcomes, including mortality, bronchopulmonary dysplasia, periventricular leukomalacia, retinopathy and patent ductus arteriosus [2, 3]. Caffeine therapy is recommended to use for all premature neonates with high risk of needing mechanical ventilation at a loading dose of 20 mg/kg, followed by a maintenance dose of 5-10 mg/kg daily [4]. Although, the meta-analysis reported by Park et al. [3] suggested that early caffeine usage in very low birth weight (VLBW) infants did not increase the risk of necrotizing enterocolitis (NEC), recently published studies showed that there was a potential association between caffeine usage and the development of NEC in premature infants [5, 6]. Moreover, a recent experimental study showed that caffeine administration to newborn rats impaired lower esophageal sphincter (LES) and gastrointestinal motor function [7].

Necrotizing enterocolitis is the most common gastrointestinal disease of premature infants. This disease has a multifactorial etiology, however; hypoxia and ischemia appear to play an important role in the pathogenesis of NEC [8-11]. The effects of hypoxia have been studied using several animal models [9-11]. Okur et al. [9] reported that histopathologic lesions in newborn rats with hypoxia/reoxygenation (H/R)-induced intestinal injury were similar to those found in early NEC. Therefore, we used the method described by Okur et al. for H/R in this study.

There is controversy and limited information about the usage of caffeine and its impact on the development of NEC [3, 5, 6]. We aimed to investigate the effects of subcutaneously caffeine citrate at loading dose of 20 mg/kg followed by a maintenance dose of 5 mg/kg on H/R-induced intestinal injury in newborn rats.

Materials and methods

Animals

This study was approved by the Pamukkale University Animal Research Committee and was performed on newborn (1-4 days old) Wistar albino rats.

Experimental design

Thirty-two, one day old Wistar albino newborn rats were randomly divided into four groups. Grup 1; Control group, untreated and not exposed to H/R, n=8. Grup 2; Caffeine group, treated with caffeine but not exposed to H/R, n=8. Grup 3; H/R group, exposed to H/R but not treated, n=8. Grup 4; Caffeine + H/R group, treated with caffeine and exposed to H/R, n=8. All newborn rats were kept in a normothermic environment (at 22-23 C), and breast-fed. Caffeine citrate (Peyona 20 mg/ml flacon, Chiesi, İstanbul) was initiated at a loading dose of 20 mg/kg at first postnatal day, followed by a maintenance dose of 5 mg/kg per day, subcutaneously during 3 days.

On postnatal fourth day, newborn rats in groups 3 and 4 were exposed to the H/R procedure described by Okur et al. [9]. Hypoxia was accomplished by placing the rats in an airtight Plexiglas chamber that was perfused with 100% CO_2 for five minutes. At the end of the procedure, the rats were afflicted with cyanosis and hyperventilated. After hypoxia, the newborn rats were reoxygenated for 5 minutes with 100% oxygen. After sixth hours of H/R procedure, all newborn rats in all groups were sacrificed on the 4th day of their life [10]. Distal ileum

segments of all newborn rats were extracted for histopathologic and biochemical assessment. Histopathologic injury score (HIS), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and oxidative stress index (OSI: total oxidant status "TOS" / total antioxidant status "TAS") were measured on intestinal tissue samples. Intestinal tissue samples were stored in -80°C for biochemical analysis of these parameters, and all biochemical assessment was performed at 4°C.

Histopathological assessment

A section of distal ileum from each newborn rats was removed, fixed in 10% buffered formalin, placed in paraffin blocks, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for histologic evaluation. Histopathologic changes in intestinal architecture were scored by a pathologist in a blinded fashion and graded as follows: grade I, normal histology; grade II (minimal) hydropic degeneration and/or surface epithelial disruption from lamina propria; grade III (mild) limited epithelial cell necrosis in tips of villi; grade IV (moderate) total villi necrosis; and grade V (severe) transmural necrosis [11].

Biochemical analysis

Measurements of the TNF-α and IL-6

The terminal ileum tissues were placed into eppendorf tubes. These tissues were stored at -80°C until assaying. Intestinal tissues were homogenized at 4 °C in 50 mM phosphate buffer solution (pH: 7.4, 1/10 g/mL) containing 0.2 μM phenylmethanesulfonyl fluoride, 1 mM EDTA, and 1 μM leupeptide. Homogenates were centrifuged at 10.000 g for 5 minutes. Clear upper supernatant fluid was obtained and assayed for biochemical analyses including TNF-α and IL-6. The levels of these parameters were determined by enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Thermo Fisher, Camirillo, CA, USA). The levels of TNF-αand IL-6 were expressed as picograms per ml of wet tissue, pg/ml.

Measurement of the TAS

Serum TAS levels were determined using a novel automated measurement method, developed by Erel kits (Reel Assay Diagnostics kit; Mega Tıp, Gaziantep, Turkey). In this method, the antioxidative effect of the sample against the potent free radical reactions, which is initiated by the produced hydroxyl radical, was measured. The results were expressed as mmol Trolox Eq/L [12].

Measurement of the TOS

Serum TOS values were determined using a novel automated measurement method, such as TAS, developed by Erel (Reel Assay Diagnostics kit; Mega Tıp, Gaziantep, Turkey). The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide (H_2O_2) and the results were expressed in terms of micro molar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/L) [13].

Calculation of OSI

The OSI was defined as the ratio of the TOS level to TAS level (OSI (arbitrary unit) = TOS (μmol H_2O_2 Eq/L)/TAS (μmol Trolox Eq/L) [13].

Statistical analysis

For statistical analysis, the results were subjected to nonparametric tests (Kruskal-Wallis test, mann-Whitney *U* test) using Statistical Packages for Social Sciences for Windows (Version 22.0; SPSS Inc, Chicago, Illinois, USA), as appropriate. All values are expressed as median, minimum-maximum. *P* values of less than.05 were considered significant.

Results

This experimental model was well tolerated by the animals and none of them died during the experimental procedure. There was no significant difference in weight of the newborn rats among the groups ($p>0.05$, Table 1). On histopathological evaluation, the control group had grade 1 HIS, normal histology (Fig.1). Caffeine group had grade 2 HIS, hydropic degeneration from lamina propria (Fig. 2). In contrast, median HIS was grade 3 in the H/R-induced groups and it was also significantly higher than that in the control group (Fig. 3 and 4, $p<0.01$, Table 2). The highest levels of TNF-α and IL-6 were detected in the H/R-induced groups and were statistically higher than those in the control and caffeine groups ($p<0.05$). Moreover, these cytokines were statistically higher in the caffeine group compared to control group ($p<0.01$). The highest mean value of OSI was also observed in the H/R-induced groups and was statistically higher than that in

Table 1. The weight (in grams) of the rat-pups in the groups at the first, second, and third day of life

Groups*	1st day Median (min-max)	2nd day Median (min-max)	3rd day Median (min-max)
Group 1 (control, n=8)	7.25 (7-8)	8.25 (8-9)	9.38 (9-10)
Group 2 (caffeine, n=8)	7.75 (7-9)	8.75 (8-10)	9.88 (9-11)
Group 3 (H/R, n=8)	7.63 (7-8)	8.63 (8-9)	9.63 (9-10)
Group 4 (caffeine+H/R, n=8)	8.0 (7-9)	9.0 (8-10)	10.25 (9-10)

H/R: hypoxia/reoxygenation-induced intestinal injury

* There was no significant difference in weight of the rat-pups among the groups (p>0.05)

Table 2. Histopathologic injury score (HIS) of intestinal tissue samples in the groups

Groups	HIS median Grade (min-max)
Group 1 (control, n=8)	1.0 (1-1)
Group 2 (caffeine, n=8)	2.0 (1-2)
Group 3 (H/R, n=8)	3.0 (2-3)*
Group 4 (caffeine+H/R, n=8)	3.0 (2-4)**

H/R: hypoxia/reoxygenation-induced intestinal injury
* Group 3 > group 1 (p<0.01)
** Group 4 > groups 2 and 1 (p<0.01)

the control and caffeine groups (p<0.01). Also, its value was statistically higher in the caffeine group compared to control group (p<0.01, Table 3).

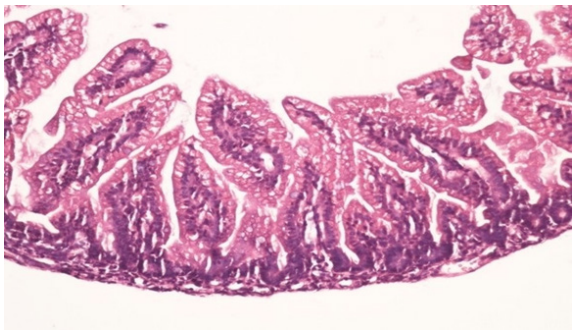

Figure 1. Grade I injury: normal histology (H&E, x200)

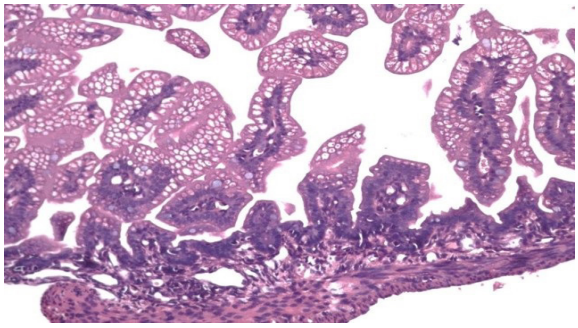

Figure 2. Grade II injury: hydropic degeneration from lamina propria (H&E, x200

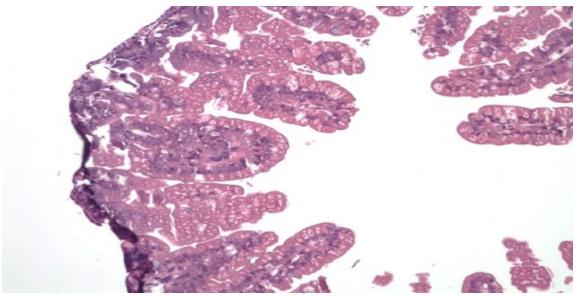

Figure 3. Grade III injury: limited epithelial cell necrosis in tips of villi (H&E, x200)

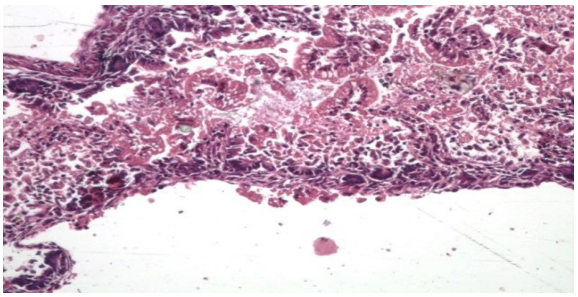

Figure 4. Grade IV injury: total villi necrosis (H&E, x200)

Table 3. Biochemical evaluation of TNF-α, IL-6 levels, and OSI in intestinal tissue samples of the groups

Groups	TNF-α Pg/ml wet tissue Median (min-max)	IL-6 Pg/ml wet tissue Median (min-max)	OSI Median (min-max)
Group 1 (control, n=8)	10.71 (3.11-15.03)	59.63 (45.94-70.93)	20.08 (8.02-28.86)
Group 2 (caffeine, n=8)	30.20 (21.67-37.33)*	89.77 (47.14-138.2)*	32.90 (20.68-52.11)*
Group 3 (H/R, n=8)	43.63 (32.83-66.44)**	133.67(93.14-191.55)**	63.94(24.51-103.18)**
Group 4 (caffeine+H/R, n=8)	38.98 (29.95-62.71)**	129.62 (87.42-235.84)**	76.81 (36.37-148.7)**

TNF-α: tumor necrosis factor-alpha; IL-6: interleukin-6; OSI: oxidative stress index;
H/R: hypoxia/reoxygenation-induced intestinal injury
* Group 2 > group 1 (p<0.01)
** Groups 3 and 4 > groups 1 and 2 (p<0.001 and p<0.05, respectively)

Discussion

Although the specific pathogenesis of NEC is not clearly understood, it is known that this disease is related to prematurity, enteral feeding, intestinal ischemia/asphyxia, and bacterial colonization [8]. Caffeine therapy is also used in premature neonates, especially with VLBW infants, to treat apnea of prematurity and prevention of bronchopulmonary dysplasia [1-3]. A 1- to 3-day-old rat would correspond to a human fetus at about 22/24 to 28/32 weeks [14, 15]. Therefore, this study was designed to investigate the effects of caffeine citrate on the biochemical and histopathologic alterations on 1- to 4-day-old newborn rats exposed to hypoxia/reoxygenation-induced intestinal injury.

It was reported in different experimental studies that intestinal ischemia/hypoxia was associated with decreased intestinal perfusion and mucosal ischemic changes. These studies with H/R model showed that the histopathologic lesions ranged from normal histology to transmural necrosis and that the prominent microscopic lesions were located in distal small intestine [9-11, 16]. When we evaluated the histopathological changes in distal intestinal samples of the groups, while the control group had normal histology, the most severe damage was seen in H/R-induced groups. Hence, our experimental study confirmed findings in previously studies that hypoxia was an important risk factor for intestinal injury. Many investigators reported that increased pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, had been found in cases with NEC, and that these cytokines might be played a role in mediating intestinal injury [8, 17-19]. Moreover, it was reported that the metabolites of oxidative stress produced during reperfusion had also been proposed to play a critical role in the pathophysiology of NEC [10, 16, 19-22]. In the presented study, we also showed that the highest levels of TNF-α, IL-6, and OSI were detected in H/R-induced groups when compared to control group.

Park and co-workers reported that there was not an association between NEC development and early caffeine use in VLBW infants [3]. Also, in another retrospective cohort study conducted in preterm neonates (< 31 weeks' gestation) who received caffeine therapy and divided into two groups (as early=first 2 days of life and late=after 3 days of life), authors reported that there was no difference between the groups for NEC [23]. On the contrary, Cox et al. [5] reported that there was a potential association between the administration of caffeine and the development of medical and surgical NEC in premature infants. As histopathologic results of the presented study, all the newborn rats in the control group had normal histology, caffeine group had grade 2 HIS, and H/R-induced groups had grade 3 HIS. The mean HISs of the H/R group was significantly higher than the control group. Although it was not found statistically significant, we observed that the mean HISs of caffeine group were higher than the control group, but lower than the H/R group. However, when we evaluated the levels of TNF-α, IL-6,

and OSI among the groups, we observed that while these cytokines and OSI were statistically higher in the caffeine group compared to control group, the same parameters were statistically lower in the caffeine group compared to H/R-induced groups. All of these results suggest that caffeine may be contributed to the development of intestinal injury.

In a recent experimental study which investigated the effects of caffeine (administered 10 mg/kg intra peritoneal) on gastrointestinal function, investigators documented that caffeine significantly reduced lower esophageal sphincter, gastric, and bowel smooth muscle tone, and induced delayed gastric emptying in newborn rats [7]. Also, same investigators reported that the mechanism responsible for the caffeine-induced gastrointestinal muscle tone was mediated via smooth muscle cell ryanodine receptors [7]. There are a few studies evaluated the effects of caffeine on intestinal blood flow in preterm neonates in the literature [6, 24-26]. Hoecker et al. [24] reported that there was a significant reduction in superior mesenteric artery (SMA) blood flow velocity (BFV) of 30% at one and two hours following an oral loading dose of 25 mg/kg caffeine. In another study reported by the same investigator, when a loading dose of 25 mg/kg caffeine administered in two equal doses of 12.5 mg/kg four hours apart through a nasogastric tube to preterm neonates (< 34 weeks' gestation), it was reported that BFV in the coeliac artery and SMA had not changed significantly one hour after the second dose of caffeine [25]. Soraisham et al. [26] showed that a single 10 mg/kg intravenous loading dose of caffeine in preterm neonates (≤ 32 weeks' gestation) was not significantly reduced BFV in SMA. The last published study reported by Abdel Wahed et al. [6] investigated the effects of intravenous infusion of caffeine citrate at a loading dose of 20 mg/kg followed by a maintenance dose of 5-10 mg/kg/day on the SMA BFV in preterm infants (28-33^{+6} weeks' gestation). The authors showed that blood flow in SMA was significantly reduced after caffeine infusion at a loading dose of 20 mg/kg, and that this effect was continued for at least two hours and improved after six hours [6]. Soraisham et al. [26] and Abdel Wahed et al. [6] suggested that the effect of caffeine on intestinal blood flow might be dose-dependent. In the lights of these information, the effect of caffeine on intestinal system may be changed with dose-dependent and the way of giving. We used the standard dose for caffeine citrate; 20 mg/kg loading dose followed by 5 mg/kg per day subcutaneously as maintenance dose in this experimental study, and pro-inflammatory cytokines such as TNF-α, IL-6, and OSI were found to be significantly higher in caffeine group compared to control group. To the best of our knowledge, this experimental study is the first report evaluating the effect of caffeine on H/R-induced intestinal injury.

The limitations of this study are that caffeine serum levels are not measured in newborn rats and it could not compare different doses of caffeine between groups.

In conclusion, our study revealed that caffeine administration may be associated with the development of intestinal injury by increasing the pro-inflammatory cytokines and OSI. Therefore, further experimental and clinical studies are needed to determine the association between caffeine therapy and the development of intestinal injury.

Conflict of interest: The authors declare that there is no conflict of interest.

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