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Potential of high dose caffeine citrate to cause intracranial hemorrhage in a premature rat model

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ABSTRACT

Aim: To determine whether caffeine citrate, used in the treatment of apnea of prematurity, poses a risk of intracranial hemorrhage (ICH) when used at high doses (100 mg/kg) in premature neonates.

Methods: Wistar albino rats are divided into 3 groups. Each group consists of 8 rats. The first group constituted the control group and was not given any medication. The second group was determined as the group given normal dose (20 mg/kg) caffeine citrate, and the third group was determined as the group given high dose (100 mg/kg) caffeine citrate. Brain tomography was performed on the baby rats immediately after birth to rule out congenital ICH. Normal dose (20 mg/kg) and high dose (100 mg/kg) caffeine citrate were given to the 2nd and 3rd groups, respectively, with the help of orogastric tube. At the end of the 2nd hour after the first brain tomography, all rats in the 3 groups were subjected to a control brain tomography, and xylazine (1mg/kg) and high dose ketamine (20 mg/kg) were administered intraabdominally and sacrificed. The rats were sacrificed due to intracranial hemorrhage that was too small to be detected on brain tomography imaging, and then their brain tissue was evaluated histopathologically. Bleeding at the microscopic level was investigated by staining with hematoxylin-eosin dye.

Results: There was no intracranial hemorrhage detected in both brain tomography and brain tissue pathology of all the baby rats which was determined as the control group, normal dose (20 mg/kg), and high dose (100 mg/kg) caffeine citrate group.

Conclusions: It was determined that caffeine citrate, used in the treatment of apnea of prematurity, did not cause intracranial bleeding in premature rats at high doses (100 mg/kg) in an experimental setting. It provides clues about whether high doses of caffeine will cause intracranial hemorrhage in humans.

Key words: Caffeine citrate, prematurity, intracranial hemorrhage, rat, experimental, high dose treatment

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1. Introduction

The normal duration of pregnancy is between 38 and 42 weeks. Premature birth is defined as birth occurring before the 37th week of pregnancy or 259 days after the first day of the mother's last menstrual period [1]. Although many children are born prematurely, that is, before the 37th week of pregnancy, every year, this number is increasing day by day. Many children die due to premature birth

complications. Some of the surviving patients also have problems such as learning difficulties, vision and hearing problems [2]. 5-9% of births in Europe and more than 12% of births in the United States are premature births [3, 4]. According to the data of the Turkey Demographic and Health Survey in 2013, the rate of low-birth-weight babies was seen as 10% [5].

Premature babies face serious problems after birth. These problems can be listed as apnea, respiratory distress syndrome (RDS), intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), and sepsis [6]. Apnea of prematurity is a temporary respiratory arrest that lasts more than 20 seconds and is usually accompanied by bradycardia (heart rate below 100 beats per minute) and cyanosis. The number of apneas increases inversely with gestational age. The frequency of pathological apnea is seen to be more than 50% in babies with a gestational age of less than 32 weeks [6]. Measures such as maintaining normal temperature, appropriate positioning of the head and neck, protecting nasal patency, and providing oxygen support can help reduce the frequency of apnea. Medication and mechanical ventilator support can be used for treatment of symptomatic apneas. Methylxanthines (aminophylline, theophylline and caffeine) are preferred in drug treatment. Methylxanthines increase the sensitivity of the central nervous system to CO2. In addition, it contributes to the improvement of ventilation by strengthening the functioning of the respiratory muscles [7]. Caffeine is preferred more frequently compared to theophylline and aminophylline because it has a long half-life, a wide therapeutic index and a rarer side effect profile [8].

One of the important problems of premature babies is respiratory apnea. Methylxanthines are the most commonly used drugs in premature babies. It has been shown that early caffeine initiation is associated with a decrease in the incidence of BPD and PDA requiring treatment in very low birth weight babies and a decrease in neonatal morbidity, including a shorter duration of mechanical ventilation [9]. Caffeine stimulates the central nervous system and cardiovascular system; it increases catecholamine secretion, has a diuretic effect and alters glucose homeostasis. Caffeine achieves this effect by functioning as an antagonist of the A1 and A2a receptor. Thus, it undertakes the task of modulating many neurotransmitters such as noradrenaline, dopamine, serotonin, acetylcholine, glutamine and gamma aminobutyric acid. It also increases cyclic adenosine 3'-5' monophosphate and cyclic guanosine monophosphate, leading to bronchodilation. Additionally, caffeine increases peripheral chemoreceptor activity, thus able to terminate apnea and initiate normal breathing. Caffeine may also have an anti-inflammatory effect on the immature lung [10].

In addition to all these positive effects, there are also studies suggesting that it may have negative effects. Animal studies have also raised concerns about the long-term consequences of exposure to caffeine citrate on the growing brain. A study conducted in rodents showed that maternal caffeine consumption during pregnancy and lactation may have negative effects on the neural development and adult behavior of their offspring, and that postnatal caffeine treatment in neonatal mice was associated with changes in astrocytogenesis [11]. Another study revealed a significant decrease in cerebral oxygenation and cerebral blood flow rates 1 hour after administration of a loading dose of 20 mg/kg caffeine citrate [12]. Another remarkable study was conducted with caffeine citrate given in two different dosages as 20 mg/kg and 80 mg/kg, and a statistically significant increase in the incidence

of cerebellar hemorrhage was found in newborns receiving 80 mg/kg caffeine citrate in cranial MR imaging [13].

Due to alarming results in animal studies, we investigated whether high doses of caffeine citrate cause intracranial hemorrhage. Since it is not possible to apply high dose (100 mg/kg) caffeine citrate to newborn babies, we preferred newborn rat modeling. We also aimed to estimate the risk of intracranial hemorrhage if newborn babies are exposed to high doses of caffeine citrate (100 mg/kg) for any reason.

2. Materials and metods

2.1. Ethical statement

Our study was approved by the decision of the DETAB Ethics Committee within Tokat Gaziosmanpaşa University Faculty of Medicine, numbered HADYEK-04, dated May 7, 2021. The BAP number was accepted as 2021-50 by Tokat Gaziosmanpaşa University Scientific Research Projects Unit on 30.05.2021 and the necessary support was provided.

2.2. Selection and features of groups

This study was conducted with Wistar Albino rats obtained from the DETAB center within Tokat Gaziosmanpaşa University. A one-day-old Wistar Albino rat baby is 22-24 days old in humans. 28-32 weeks of pregnancy, when it is three days old. Since it coincided with the gestational week, the study was conducted with one-day-old rat pups [14]. Three pregnant rats were obtained from DETAB center for the study and they were allowed to breed under the same environmental conditions (12 hours of light-12 hours of darkness, 23^{o}C room temperature). Mother rats were fed with standard pellet mouse chow containing 21% protein. The rats were determined as 3 groups and consisted of 8 baby rats in each group. Pregnant rats underwent pregnancy under the same conditions (temperature, humidity, nutrition, light, sound and living space).

In the group 1, there were 4 males and 4 females, and the lowest weight was 5,122 grams and the highest weight was 5,365 grams. This group was considered the control group and no medication was used. Feeding of the baby rats and preventing heat loss were provided by their mothers.

In the group 2, there were 5 males and 3 females, and the lowest weight was 5,224 grams and the highest weight was 5,852 grams. This group was recorded as the group administered normal dose (20 mg/kg) caffeine citrate. Feeding of the baby rats and preventing heat loss were provided by their mothers.

In the group 3, there were 3 males and 5 females, and the lowest weight was 5,205 grams and the highest weight was 5,988 grams. This group was recorded as the group administered high dose (100 mg/kg) caffeine citrate. Feeding of the baby rats and preventing heat loss were provided by their mothers.

In our study, the groups were determined randomly; all of the offspring of the first mother gave birth to the control group, all of the offspring of the second mother constituted the normal dose (20 mg/kg) group, and all of the offspring of the third mother formed the high dose (100 mg/kg) group.

2.3. Drugs used and administration method

- ***Caffeine Citrate:*** In order to conduct an experimental study, PEYONA 20 mg/ml infusion and oral solution (Chiesi, Istanbul) with caffeine citrate active ingredient was administered orally to rats with the help of an orogastric tube.
- ***Xylazine Hydrochloride*****:** Xylazin Bio 2% vial (Bioveta, Czech Republic) was administered intraperitoneally at a dose of 1 mg/kg to sacrifice the baby rats whose first and second brain CT imaging was completed.

- ***Ketamine Hydrochloride:*** Ketalar 500 mg vial (Zentiva, Lüleburgaz, Kırklareli, licensed by Phizer) was administered intraperitoneally at a dose of 20 mg/kg to sacrifice the baby rats whose first and second brain CT imaging was completed.
- ***Formaldehyde 10%:*** All sacrificed rats were delivered to the Medical Pathology unit in a transport container containing 10% formaldehyde solution (Net chemical, Ankara) to block the brain tissues.

2.4. Radiological Imaging

All rats in our study were imaged with the same brain tomography device. In the study, axial, coronal and sagittal reformatted images with a section thickness of 0.625 mm were created from a helical scan of a section thickness of 1.25 mm in the axial plane using a CT device with 128 slices (Optima CT660, 2016, GE, Tokyo, Japan). Parameters for CT scan performed in the brain parenchyma window; tube current: 150 mA, tube voltage 120 kV, field of view (FOV) 16 cm, window width (WW) 30 and window level (WL) 120. Images were interpreted by a radiologist with 10 years of experience. Mechanical fixation was made using elastic bandages and thumbtacks to reduce the movements of the baby rats during brain tomography.

2.5. Histopathological evaluation

Since ICHs may be too small to be detected on brain CT imaging, brain tissue pathology was subsequently performed on the sacrificed rats. Blocking of the brain tissue of all rats in our study was done with a microtome device called LEICA RM 2245 (USA). Tissue samples from all rats were cut into blocks with a thickness of 3 microns. All of the preparations made into blocks were stained with Hematoxylin-Eosin (H&E) dye. Brain tissue samples of all rats in our study were examined using the NIKON ECLIPSE 80i (2003, Japan) device. The evaluation was made by a specialist pathologist using X1-X100 magnifications under the microscope.

2.6. Experimental study design

It was made with 24 rats of the Wistar Albino breed, each consisting of 8 rats, born from 3 mother rats breeding under the same conditions. The groups were determined so that the rats born from the same mother were in the same group; randomly divided into 3 groups. Brain CT was performed on the rats immediately after birth to rule out congenital ICH. Elastic bandages and pins that provide mechanical stabilization were used to reduce the movement of the rats during brain CT imaging. Brain CT was performed separately for each group; Brain CT scans were performed among the groups simultaneously. No medication was administered to the first group, which was designated as the control group. To the second group, designated as the normal dose group, caffeine citrate was administered via orogastric tube at a dose of 20 mg/kg immediately after the first brain CT scan. Caffeine citrate was administered via orogastric tube at a dose of 100 mg/kg to the third group, which was designated as the high dose group, immediately after the first brain CT scan.

All rats treated with caffeine citrate remained next to their mother for 2 hours. The task of feeding the baby rats and maintaining their body temperature is provided by the mother rat during this period.

In rats, the half-life ($T_{1/2}$) of caffeine citrate in blood is 1.9 hours and the maximum concentration time (T_{max}) is 1 hour. Considering these values, control brain CT was taken 2 hours after caffeine citrate application [15, 16].

At the end of the 2nd postnatal hour, the baby rats were separated from their mothers in 3 groups for control brain CT scan. Following control brain CT, all baby rats were administered 1 mg/kg xylazine and 20 mg/kg ketamine intra-abdominally and sacrificed. Sacrificing rats with

high doses of anesthetic was chosen as a method instead of cervical dislocation, considering the possibility of causing ICH. All brain CT images were evaluated by a specialist radiologist for the presence of ICH.

The sacrificed rats were delivered in groups, in containers containing 10% formaldehyde, to the pathology unit for brain tissue pathology. In the pathology department, brain tissues were made into blocks and H&E staining was performed. Brain tissue pathology was performed in case there was an undetectable level of ICH on brain CT images. Brain tissues were made into blocks and evaluated by a specialist pathologist for the presence of ICH at the microscopic level.

2.7. Statistical analysis

Statistical analysis was performed using commercial software (IBM SPSS Statistics 19, SPSS inc., an IBM Co., NY, USA). Categorical measurements were summarized as numbers and percentages, and numerical measurements were summarized as mean, median, minimum, maximum and standard deviation. Pearson Chi Square test statistics were used to compare categorical measurements between groups. Mann Whitney U and Kruskal Wallis tests were used to compare numerical measurements between groups. $P<0.05$ was considered statistically significant for the difference between the detected results.

3. Results

3.1. Gender and birth weight

Our study was conducted with a total of 24 Wistar Albino rats born from 3 pregnant rats. Each mother rat gave birth to 8 baby rats, and each group was determined as rats born from the same mother and divided into groups randomly.

- Control group: 4 males, 4 females
- Normal dose (20 mg/kg) group: 5 males, 3 females
- High dose (100 mg/kg) group: Consists of 3 males and 5 females.

There was no statistically significant difference between groups in terms of gender (p=0.607) (Table 1).

Table 1. Gender distribution by groups.

Gender	Groups												χ^2	*p*
	1			2			3			Total				
	n	K %	S %	n	K %	S %	n	K %	S %	n	K %	S %		
Female	4	50,0	33,3	3	37,5	25,0	5	62,5	41,7	12	50,0	100,0	1,000	*0,607*
Male	4	50,0	33,3	5	62,5	41,7	3	37,5	25,0	12	50,0	100,0		
Total	8	100,0	33,3	8	100,0	33,3	8	100,0	33,3	24	100,0	100,0		

Pearson chi-square test was used. K%: Column percentage, S%: Row percentage. p<0.05 was considered statistically significant, 1: control group, 2: normal dose group, 3: high dose group.

Table 2. Distribution of body weight according to qualitative variables.

Parameters		Weight		z,χ^2	*p*
		Mean ± SD	Median (min-max)		
Groups	1	5,22±0,08	5,23 (5,16-5,27) (a)	9,695	*0,008*
	2	5,5±0,21	5,47 (5,34-5,64) (ab)		
	3	5,61±0,28	5,67 (5,37-5,8) (b)		
Gender	Female	5,49±0,28	5,45 (5,26-5,71)	0,866	*0,386*
	Male	5,4±0,23	5,29 (5,22-5,64)		

Mann Whitney U test or Kruskal Wallis test was used. p<0.05 was considered statistically significant. Mean: average, SD: Standard deviation Group 1: Control group, Group 2: Normal dose group, Group 3: High dose group.

There was a statistically significant difference between the groups in terms of birth weight between the control group and the high dose caffeine citrate group (p= 0.008) (Table 2).

After the groups were divided into separate groups as male and female, there was no significant difference in the statistical evaluation made in terms of birth weight (Table 3).

3.2. Radiological Imaging Findings

There was no appearance compatible with ICT in the brain CT imaging at the end of the first and 2nd postnatal hours of all rats in the control group, normal dose (20 mg/kg) caffeine citrate and high dose (100 mg/kg) caffeine citrate groups (Figure 1).

3.3. Histopathological Findings

All of the rats in the first group, the control group, were not given any medication, and there was no bleeding focus in the brain tissue examination of all of them (Figure 2.).

There was no bleeding focus in the brain tissue examination of all rats in the second group, which was administered normal dose caffeine citrate (20 mg/kg) (Figure 3.).

There was no bleeding focus in the brain tissue examination of all rats in the 3rd group, the high dose caffeine citrate (100 mg/kg) administered group (Figure 4.).

In our study, there was no focus of bleeding in the first brain CT scan taken to rule out

Table 3. Body weight variable distribution by group and gender.

Groups	Gender	Weight		Z	*p*
		Mean±SD	Median (min-max)		
1	Female	5,19±0,08	5,18 (5,12-5,26)	0,866	*0,386*
	Male	5,26±0,08	5,24 (5,2-5,31)		
2	Female	5,43±0,08	5,42 (5,36-5,52)	0,447	*0,655*
	Male	5,54±0,26	5,62 (5,32-5,66)		
3	Female	5,75±0,19	5,74 (5,69-5,86)	1,938	*0,053*
	Male	5,37±0,25	5,26 (5,21-5,65)		

Mann Whitney U test was used. Mean: mean, SD: Standard deviation. p<0.05 was considered statistically significant. Group 1: Control group, Group 2: Normal dose group, Group 3: High dose group.

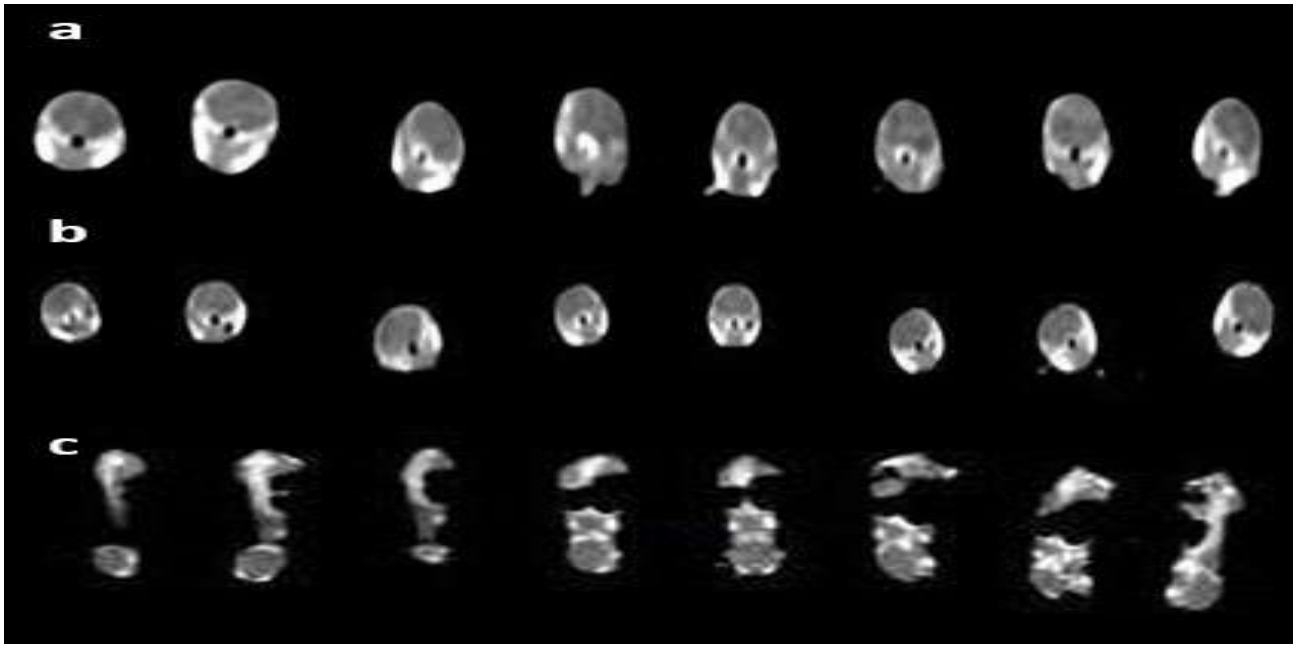

Figure 1. a: Brain CT images of control group rats after caffeine. b: Brain CT images of normal dose group rats after caffeine. c: Brain CT images of high dose group rats after caffeine.

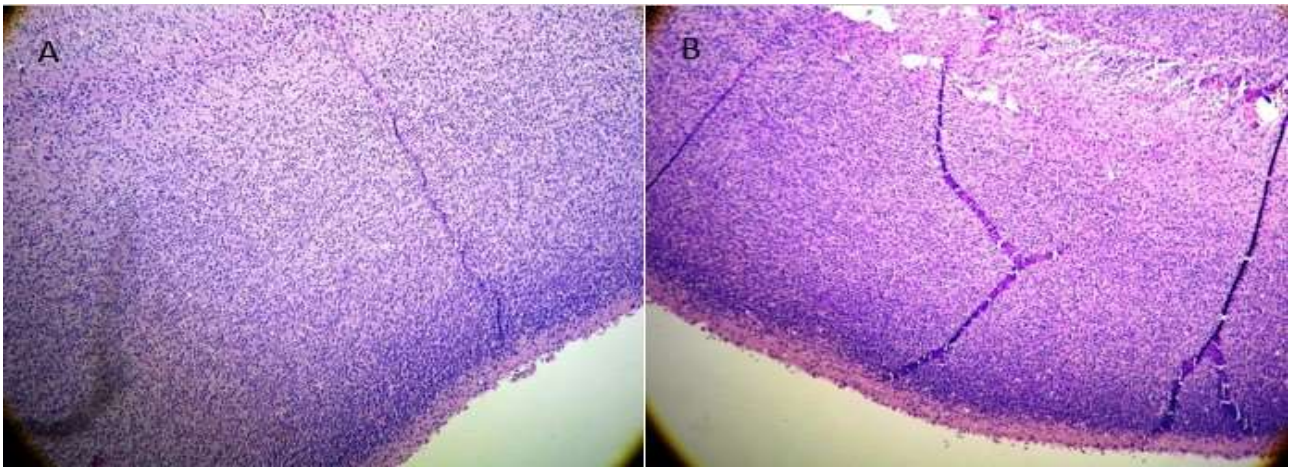

Figure 2. A: Female rat, Control group, H&E staining, X100 brain tissue pathology. B: Male rat, Control group, H&E staining, X100 brain tissue pathology.

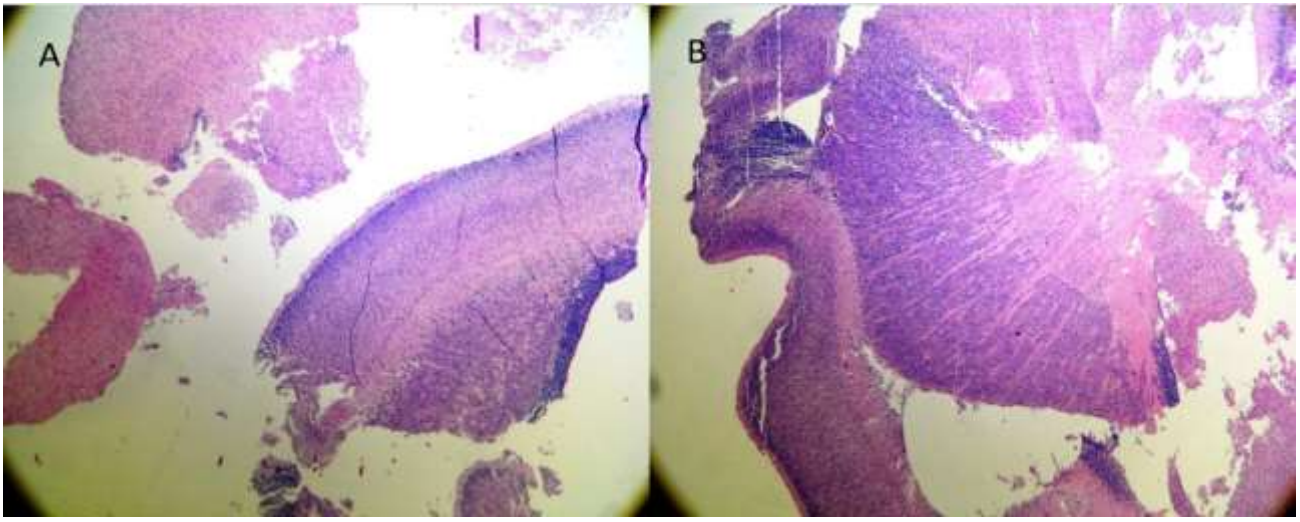

Figure 3. A: Female rat, Normal dose group, H&E staining, X40 brain tissue pathology. B: Male rat, Normal dose group, H&E staining, X40 brain tissue pathology.

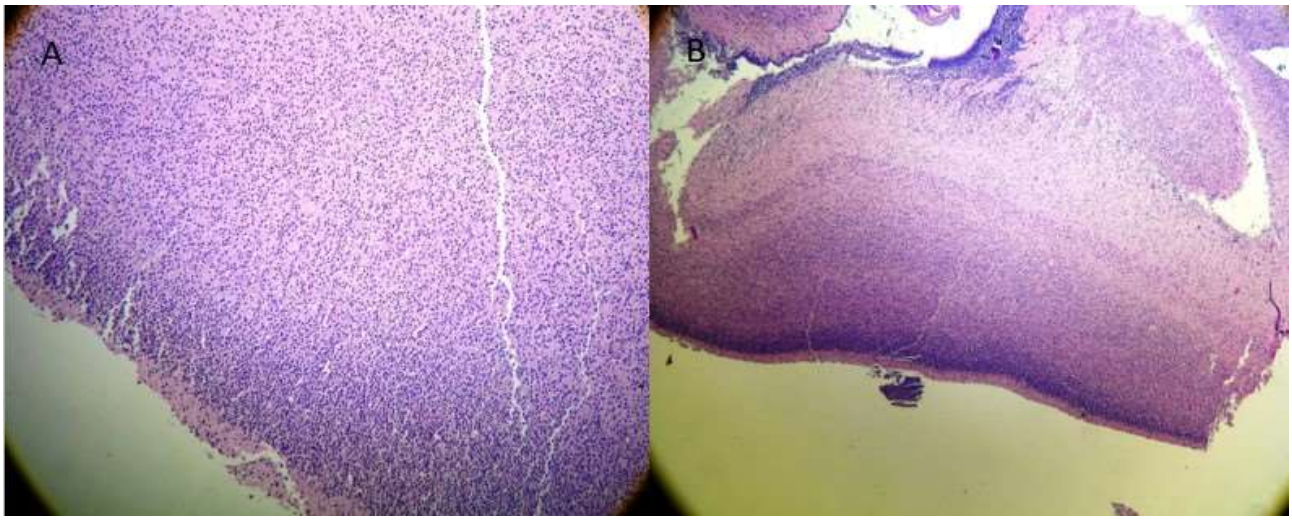

Figure 4. A: Female rat, High dose group, H&E staining, X100 brain tissue pathology. B: Male rat, High dose group, H&E staining, X40 brain tissue pathology.

congenital ICH in Wistar albino newborn rats, which we considered premature.

1st Group: In the group we formed as the control group, no bleeding focus was detected in the brain CT image taken at the end of the 2nd postnatal hour, and there was no bleeding focus in the examination of brain pathology.

2nd Group: In the group given normal dose caffeine citrate (20 mg/kg), no bleeding focus was detected in the brain CT image taken at the end of the 2nd postnatal hour, and there was no bleeding focus in the examination of brain pathology.

3rd Group: In the group given high dose caffeine citrate (100 mg/kg), no bleeding focus was detected in the brain CT image taken at the end of the 2nd postnatal hour, and there was no bleeding focus in the examination of brain pathology.

4. Discussion

In our study, we aimed to estimate the risk of intracranial bleeding in human newborns if high doses of caffeine citrate are administered for any reason. Today, the potential benefits and harms of caffeine citrate when given in high doses in newborn babies are still unclear.

Many studies have compared the benefits and harms of methylxanthines in premature newborns. Examples of well-known side effects of methylxanthines include tachycardia, cardiac arrhythmia, seizure, food intolerance, increased metabolic rate, and increased O2 consumption. It has been shown that caffeine has fewer side effects than aminophylline and theophylline (40). Caffeine is preferred more frequently than aminophylline and theophylline due to its long half-life, wide therapeutic index and less side effect profile [15]. Atik et al. state that caffeine shortens the duration of respiratory support in premature newborns, increases the chance of survival, and reduces the incidence of cerebral palsy and cognitive delay, but states that there is little evidence about the short- and long-term effects of caffeine on the developing brain, especially at the cellular and molecular levels. Based on experimental data, studies showing harmful or beneficial effects of caffeine on the developing brain are conflicting. Most experimental studies suggest that caffeine causes harmful changes in the developing brain, regardless of dose or duration of administration. For this reason, they stated that there is an urgent need to evaluate the effects of caffeine on the developing brain in preclinical studies, especially using animal models [17]. In our study, we aimed to investigate whether caffeine citrate has an effect on intracranial bleeding due to high dose (100 mg/kg) use by using premature rat modeling.

Yazdani et al., in their study to investigate the effect of caffeine on the developing central nervous system, examined whether caffeine intake would affect the saturated and monounsaturated fatty acids in the growing cerebellum in newborn rats during the breastfeeding period. They reported that chronic caffeine exposure in offspring through breast milk from birth to day 10 caused a decrease in cerebellum weight, a significant increase in saturated fatty acids, and an increase in monounsaturated fatty acids. Additionally, there was a slight increase in some polyunsaturated fatty acids [18]. Staphane et al. investigated the effect of adding caffeine to the culture medium of brain astrocytes in newborn rats on glial cell development and hyaluronic acid secretion. In primary glial cell cultures, 20 mg/L caffeine had no apparent effect on cell number or hyaluronic acid secretion, while 50 mg/L caffeine resulted in a significant reduction in the number of hyaluronic acid-producing cells. As a result, they showed that caffeine, found in high

concentrations in the brain, has an effect on the composition of the extracellular matrix, which may affect the number of proliferating glial cells (astrocytes and oligodendrocytes) and the onset of myelination [19]. As a result of these studies, it has come to mind whether caffeine citrate, especially when used in high doses, has unknown effects on the central nervous system that is not yet fully developed. In our study, in the group given high dose caffeine citrate (100 mg/kg), no bleeding focus was detected in the brain CT image taken at the end of the 2nd postnatal hour, and there was no bleeding focus in the examination of brain pathology.

In the study conducted by Desfrere et al., it was accepted that the brain structures of mice on the 3rd and 10th postnatal days mimic the brain development of human newborns at the 24th and 38th weeks. With this acceptance, caffeine citrate 10 mg/kg was administered from postnatal day 3 to postnatal day 7 and they showed that there was a decrease in cell proliferation in the subventricular region and dentate gyrus in mice. The same study suggests that postnatal caffeine exposure causes a transient and dose-dependent decrease in the expression of GFAP and S100, a marker of astrocytogenesis [11]. Another study indicating another negative effect is that Kang et al., in their study on rats, suggest that following the administration of 50 mg/kg caffeine citrate, it may cause caspase-3-dependent neuronal cell apoptosis in newborn rats. In the study conducted with the Tunnel staining method, an increase in the number of Tunnel stained cells was observed, especially in the parietal cortex, temporal cortex, caudate nucleus, putamen, dentate gyrus, thalamus and hypothalamus. This proves that the stained cells experience increased apoptosis [20]. Similarly, in a 2008 study by Black et al., it was observed that the administration of high doses of caffeine (100 mg/kg) in rats caused an increase in apoptosis in various brain regions, including the cerebral cortex and caudate nucleus, on the 3rd postnatal day [21]. In a study conducted by Pan and Chen, it was determined that caffeine caused behavioral disorders (hyperalgesia, anxiety disorder, learning disorder) by affecting the brain regions associated with adenosine in newborn rats exposed to caffeine citrate (20 mg/kg) [22]. This study argues that even at accepted doses, it may have negative effects on the developing brain. Compared to studies in the literature, in our study, no bleeding focus was observed in the brain CT image taken at the end of the 2nd postnatal hour and in the brain tissue examined histopathologically for both normal dose and high dose caffeine citrate.

Since we investigated whether caffeine citrate causes intracranial hemorrhage at high doses in our study, we examined a recent study that found a striking increase in the incidence of cerebellar hemorrhage. In the study conducted by McPherson et al. in 2015, a total of 74 premature newborns were randomly given standard (20 mg/kg) and high (80 mg/kg) doses of caffeine citrate. Although there was no statistically significant difference between the two groups in terms of gestational age, gender, and birth weight, the maternal age of babies exposed to high dose (80 mg/kg) caffeine citrate was found to be higher than the group exposed to standard (20 mg/kg) dose. There was no statistically significant difference in terms of intraventricular hemorrhage or periventricular leukomalacia when evaluated by cranial ultrasound, and brain MRI showed an increased incidence of cerebellar hemorrhage in babies exposed to high doses (80 mg/kg) of caffeine compared to babies exposed to standard doses (20 mg/kg) of caffeine. A total of 58 babies out of 74 were included in the study for various reasons. Cerebellar hemorrhage was detected in 10 of 28 babies receiving high dose (80 mg/kg) caffeine citrate and in 3 of 30 babies

receiving standard dose (20 mg/kg) caffeine citrate (36% in the high dose group, 10% in the normal dose group, and p: 0.02). According to the brain MRI results in the current study, no statistically significant difference was found in white and gray matter damage between the two groups [23]. Continuing the same study, McPherson et al., in their 2022 study, tested the cognitive and neurodevelopmental status of 74 premature babies who participated in the previous study by calling them for a check-up at the end of 5 years. 21 patients who received high dose (80 mg/kg) and 24 patients who received standard dose (20 mg/kg) caffeine citrate were able to participate in the study, which could be done in the surviving part of 74 babies. When both groups were compared, similar rates of general, verbal or non-verbal cognitive scores were obtained. It was reported that there was no statistically significant difference between the groups [24]. With these results, cases in which cerebellar hemorrhage was detected were compared with cases in which cerebellar hemorrhage was not detected, and no significant difference was shown. In our study, there was no evidence of intracanal bleeding in the brain CT and brain pathology results as a result of exposure to high doses of caffeine citrate (100 mg/kg).

A study arguing that high doses of caffeine citrate have a positive effect was conducted by Back et al. In the study, caffeine citrate (300 mg/L) was given to the experimental group of baby mice exposed to a hypoxic environment (10% oxygen) between the 3rd and 12th postnatal days, but not to the control group. It was determined that ventriculomegaly decreased and cerebral myelination increased in mice in the caffeine citrate group, thus reducing periventricular white matter injury (PWMI) [25]. A study that argues that although high doses are beneficial, it should be approached with caution is the study conducted by Yang et al. in 2021, and they showed that high-dose maintenance treatment of caffeine citrate is more effective and safer than low-dose maintenance treatment. However, considering the minimal side effects of the high-dose regimen, they stated that the use of caffeine citrate maintenance dose as 20 mg/kg/day is acceptable [26]. In our study, brain CT was performed immediately after birth to rule out congenital intracranial hemorrhage. No findings suggestive of intracranial hemorrhage were detected in all initial brain CT scans. Control brain CT was taken 2 hours after the drug was given to the groups that were not given caffeine citrate and those that were given caffeine citrate. No findings suggestive of intracranial hemorrhage were detected in all of the second brain CT scans. In addition, brain tissue pathology was performed on all rats, and no intracranial bleeding was observed in all brain pathologies of the rats. Since the number of experiments studied was conducted with the minimum number of subjects that would provide statistically significant information, there is a possibility of different results when using more subjects. However, since it was assumed that the rats in our study did not have additional diseases (respiratory distress, NEC, BPD, PDA, RDS, hydrocephalus), it can provide limited data for premature human newborns with additional diseases.

4.1. Conclusions

In conclusion, later findings of brain CT and pathological evaluations of brain parenchymal tissues may differ as to whether caffeine citrate causes ICH in the long term. We think that our study may contribute to whether there is a risk of ICH, especially in premature newborn babies, if high doses of caffeine citrate (100 mg/kg) are accidentally administered to human newborn babies, other than the generally accepted dose (20 mg/kg). More randomized, controlled studies

are needed to understand the effects of high doses of caffeine citrate on developing brain tissue.

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Conflict of interest: *The authors declare that they have no conflict of interest.*

Ethical statement: *The study was approved by the decision of the DETAB Ethics Committee within Tokat Gaziosmanpaşa University Faculty of Medicine, numbered HADYEK-04, dated May 7, 2021.*

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