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RESEARCH ARTICLE

Expression of Caspase 9 in The Cerebrum and Cerebellum Newborn Rats from The Model of Maternal Death

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ABSTRACT

Neonatal stress occurred due to the maternal death. It causes the cells apoptotic function to be damaged. Caspase 9 is a cysteine protease enzyme whose role is to regulate physiological cell death. This study aims to determine the effect of parental separation model maternal death on caspase 9 expression in the cerebrum and cerebellum of newborn rats. The study was divided into two groups (n=18), the control group (C) of newborn rats which were not separated from their mothers. While, the treatment group (T) of newborn rats which were separated from their mothers and being given formula milk as nutrition. After 3 days, they were taken decapitated. Then, the brains were taken for histopatological assay. The results of caspase 9 expression of newborn rats in the control group average 1.04±0.79 in the cerebrum and 3.16±1.28 in cerebellum. The average of caspase 9 expression treatment group 1.44±0.25 in cerebrum and 1.99±0.54 in cerebellum. The results showed a significant difference between the control and treatment groups. It analyzed by using the Mann-Whitney test with a value of $P \leq 0.001$ in the cerebrum and the Independent T test with a value of $P \leq 0.001$ in the cerebellum. Expression of caspase 9 in the cerebrum and cerebellum of newborn rats were separated from their parents as a model of maternal mortality was higher than not separated from their parents.

INTRODUCTION

The issue of high maternal mortality remains a significant concern in developing nations, such as Indonesia (Utomo et al., 2021). The World Health Organisation (WHO) (2019) defines the maternal mortality rate as the quantification of maternal fatalities that occur as a consequence of pregnancy, delivery, and postpartum processes. This metric serves as a valuable indication of the overall health condition of women. The reduction of Maternal Mortality Rate (MMR) to 70 per 100,000 live births by 2030 is a key objective within the global Sustainable Development Goals (SDGs) framework, as outlined by the World Health Organisation (WHO, 2016). According to the World Health Organization's statistics from 2019, the global number of individuals affected by measles, mumps, and rubella (MMR) stands at 303,000. Following data provided by the ASEAN Secretariat in 2020, the Maternal Mortality Ratio (MMR) throughout the ASEAN region is at 235 per 100,000 live births. Based on the data obtained from the Indonesian Demographic and Health Survey (SDKI), the maternal mortality ratio (MMR) in Indonesia exhibited an upward trend, rising from 228 per 100,000 live births from 2002 to 2007 to 359 per 100,000 live births in the subsequent period of 2007-2012. However, a subsequent decline was seen in the MMR, with a fall to 305 per 100,000 live births from

2012 to 2015. According to data from the Ministry of Health of the Republic of Indonesia (2019), the total number of maternal fatalities in Indonesia in 2019 was recorded at 4,221 instances. Based on the aforementioned statistics, it was determined that the maternal death rate exhibited a decline; nonetheless, this fall fell short of achieving the national objective.

According to the Ministry of Health of the Republic of Indonesia (2019), the primary factors contributing to maternal mortality in Indonesia during the year 2019 were hemorrhage, hypertensive disorders during pregnancy, infections, metabolic diseases, and several other reasons. According to the WHO (2019), a significant proportion, ranging from 25% to 50%, of maternal mortality cases may be attributed to complications arising from pregnancy, delivery, and the postpartum period. Scott et al. (2017) claim that the occurrence of maternal mortality during the early stages of a child's life might result in the termination of the mother-child attachment and the interruption of nursing, hence impacting the child's chances of survival. Mothers have heightened sensitivity in their responses to communication signals directed towards newborn infants. The establishment of a bonding connection between mother and baby may occur via several means, such as tactile stimulation, skin-to-skin contact, and visual engagement, all of which contribute to a soothing impact. The occurrence of maternal mortality may lead to diminished or absent maternal-infant interaction, which may contribute to elevated cortisol levels in infants compared to those who receive direct maternal care. Hence, it is probable that this factor contributes to the occurrence of stresses throughout the newborn period of a child's development (Angelhoff et al., 2018).

The rapid growth and maturation of a child's brain commence during the prenatal period and continue throughout the first 1000 days of life, encompassing the age range from birth to three years. This critical developmental phase encompasses various essential processes, such as the proliferation of neurons, the formation of synapses, the myelination of neural pathways, and the growth of axons and dendrites. The optimal development and evolution of the brain have a profound impact on cognitive, social, and emotional capacities, serving as a fundamental basis for the advancement and progression of future generations. The cerebral hemisphere, also known as the cerebrum, is a significant component of the brain that is involved in many cognitive and affective activities. Additionally, the cerebellum, or the little brain, plays a crucial role in the development of motor skills, cognitive abilities, emotional regulation, and behavioral patterns (Pieterman et al., 2017). The transmission of information throughout the nervous system in the brain encompasses intricate methods of innervation characterized by optimal nerve function and structure since it is crucial for all human actions to rely on this process (Koop and Tadi, 2023). The process of brain development commencing during prenatal development encompasses many key stages, including proliferation, migration, differentiation, and synaptogenesis.

Caspase, a cysteine aspartate-specific protease, plays a crucial role in the beginning and progression of apoptosis. It is composed of two types of caspases: initiator caspases (Caspase 8, Caspase 9, Caspase 10) and executioner caspases (Caspase 3, Caspase 6, and Caspase 7) (McIlwain et al., 2013). Caspase 9 is a cysteine protease enzyme that is recognized for its function as an activator of intrinsic apoptosis, regulating the process of programmed cell death in normal physiological conditions and the degeneration of tissues in pathological contexts. The non-optotic activities of the mentioned entity include the control of cellular differentiation and maturation, innate immunity, maintenance of mitochondrial homeostasis, and the process of autophagy (Avrutsky and Troy, 2021). An elevation in oxidative stress levels leads to an augmentation in mitochondrial permeability, resulting in the release of cytochrome c from the mitochondrial intermembrane space into the cytosol (Zorov et al., 2014). The formation of the apoptosome involves the interaction of cytochrome c, apoptotic protease activating factor-1 (Apaf-1), dATP, and caspase 9. The conversion of procaspase 9 into caspase 9 leads to the activation of caspase 3, resulting in the degradation of proteins and subsequent induction of cell death (Pfeffer and Singh, 2018).

According to Brentnall et al. (2013), the activity of caspase 9 has been associated with the induction of heightened reactive oxygen species (ROS) generation. The impact of oxidative stress conditions extends to the structural integrity of cellular organelles, including mitochondria. Mitochondrial dysfunction, characterized by impaired energy generation in cellular units, leads to a reduction in adenosine triphosphate (ATP) synthesis. Consequently, the functionality of mitochondrial enzymes is hindered, resulting in the destabilization and aggregation of proteins within the cell. This phenomenon marks the initiation of the apoptosis process (Elmore, 2007). According to Avrutsky et al. (2020), Caspase 9 exhibits dual functionality in the context of ischemic neurovascular damage, including both apoptotic and non-apoptotic activities. Caspase 9 can inhibit the development of tumors by initiating intrinsic apoptosis in response to cellular damage, including genomic instability, oxidative stress, and abnormal proliferation. Nevertheless, several tumors use the inhibition of caspase 9 as a tactic to evade apoptosis (Chee et al., 2013). Furthermore, it is worth noting that apoptosis serves not only as a mechanism for cell death but also as a catalyst for cell proliferation within the neighboring tissue, hence potentially facilitating the progression of tumor formation (Fogarty and Bergmann, 2017). This study aims to determine the effect of parental separation model maternal death on caspase 9 expression in the cerebrum and cerebellum of newborn rats.

MATERIAL AND METHODS

Handling, mating, and breeding of female rats

Three adult female Wistar rats (130-150 g), three per cage, were housed in a temperature of 26 ± 2°C, a humidity level of 50-60% and lighting was regulated by a light-dark cycle with 12-hour light and 12-hour dark cycles (08:00–20:00) (Rejeki et al., 2021; Antoni et al., 2022). The rats were healthy, unmated, and older than three months. Every handling technique followed the animal care guidelines.

Female Wistar rats' estrous cycles have previously been observed using a vaginal swab. Small cotton swabs were employed, which were dampened with physiological NaCl. It then performed a test on a glass slide. Methanol was used to fix the cells, and a 10% Giemsa solution was used to stain them (Merck-1.09204.0500). The estrous cycle was seen using a light microscope at a 100x magnification. The estrous cycle was then synchronized in female rats by administering injections of Pregnant Mare Serum Gonadotropin (PMSG-Intervet). To stimulate superovulation, it also included human chorionic gonadotropin (hCG-Chorulon). After 48 hours, 10 IU of hCG was given intraperitoneally, then 10 IU of PMSG. Following that, the rats were bred using the monomating technique with male rats. Using a vaginal plug, mating was confirmed for the following 17 hours. The vaginal plug, which stopped spermatozoa from spilling, was made of coagulated gelatinous fluids. A vaginal plug indicated successful copulation, which was noted as the first day of pregnancy.

Animal maternal mortality model

This research was a true laboratory experimental study design. In addition, this study used a randomized posttest only control group design. The Animal Care and Use Committee (ACUC), Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia, gave its approval to this study. Moreover, this research has obtained a certificate of ethical eligibility (No. 2.KEH.061.05.2022; May 27th, 2022) from Animal Care and Use Committee in Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia. The research group on the maternal mortality model was divided into two groups (n = 18). The C group of newborn rats which were not separated from their mothers could breastfeed directly from their mothers. However, the T group of newborn rats were separated from their mothers and being given animal formula milk as nutrition. There were 3 parents in each group. The control group was six newborn rats 3 days old were still being with their parents. While, another six newborn rats 3 days old in the treatment group were separated from their parents and given formula milk. Rats were anesthetized with a combination of ketamine HCL and 2% xylazine

HCL in the ratio of 3:1, in a dose of 0.1 mL per 100 g of body weight. Then, t brains were taken and put in 10% formalin for 24 hours.

The examination of caspase 9 expression

Histopatological preparations were made and then immonohistochemical examination of Caspase 9 expression was carried out. The brain pups tissue that had been sliced was deparaffinated with xylene 3 times for 3 minutes each. After that, the preparation of rehydration used 100% ethanol, 95% ethanol, and 70% ethanol for two minutes, two minutes, one minute respectively. Then, it used water for one minute and then soaked in a peroxidase blocking solution at room temperature for 10 minutes. The preparations were incubated in a predilution inhibitor serum 25°C for 10 minutes and soaked the preparations in 25°C polyclonal anti-Caspase-9 antibody (ab2013), Abcam Inc. (1:300) for 10 minutes. It has to wash with Phosphate Buffer Saline (PBS) for 5 minutes. All samples were first treated with biotin-labeled secondary antibodies (Trekkie Universal Link), followed by an overnight incubation with DAB as the chromogen and streptavidin conjugated peroxidase (Trekavidin-HRP Label). Caspase 9 sample samples should be stained with Mayer hematoxylin and eosin. Cleaning was done before mounting medium was dropped over the preparations. A coverslip was used as its final covering.

Measurement of caspase 9 expression

Tissue examination under a light microscope at 5x field of view with 400x magnification. The evaluation of Caspase 9 expression was conducted based on the percentage of the caspase 9. It expressed cerebrum and cerebellum of the brain using the Semi-quantitative Immuno Reactive Score (IRS) method. Under a microscope at 400× magnification in 5 microscopy fields, the mean percentage of the antibody-positive cerebrum and cerebellum was analyzed. By dividing the positive cell percentage score by the color reaction intensity score, the Remmele scale index was calculated. A score of 0 meant there were no positive cells, a score of 1 meant there were less than 10% positive cells, a score of 2 meant there were 11-50% positive cells, a score of 3 meant there were 51- 80% positive cells, and a score of 4 meant there were more than 80% positive cells. There was no color reaction for score of 0, low color intensity for score of 1, medium color intensity for score of 2, and strong color intensity for score of 3.

Statistical analysis

Assessment of samples from the control and treatment groups used the IRS based on the average value. Then, it analyzed the data using the SPSS 22 software (IBM Corp., Armonk, NY, USA). The Shapiro Wilk test was used to conduct a normality test to determine if the data was normally distributed or not. Normality test results aimed to determine the further analysis. It could be using the parametric analysis if the data was normally distributed. Yet, non-parametric if the data was abnormally distributed. The normally distributed data was continued with the Independent T test. It can be performed if the data was not normally distributed so that it will be continued with the Mann Whitney test. This study variables used a significance level of 0.05 with a 95% confidence level.

RESULTS

Expression of Caspase 9 in cerebrum tissue using immunohistochemical staining with yellow arrows indicated the maximum expression area of chromogen brown color shown in the Figure 1 and Figure 2.

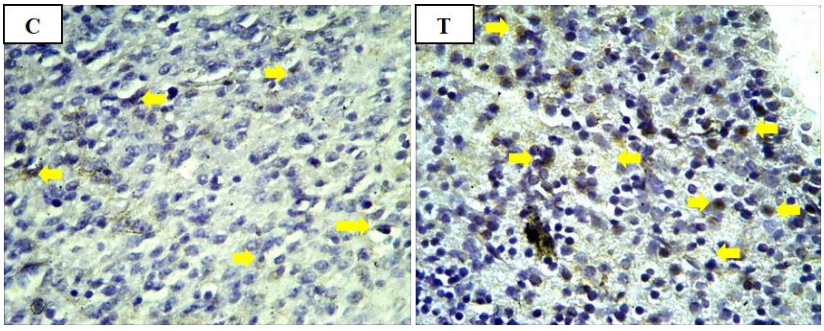

Figure 1. Comparison of Caspase 9 expression in rat cerebrum cells. Arrows indicate Caspase 9 expression which is indicated by the presence of brown chromogen (arrows). C: Control group; T: Treatment group (IHC; Miconos MCX50LED 400x magnification and Optilab Plus camera).

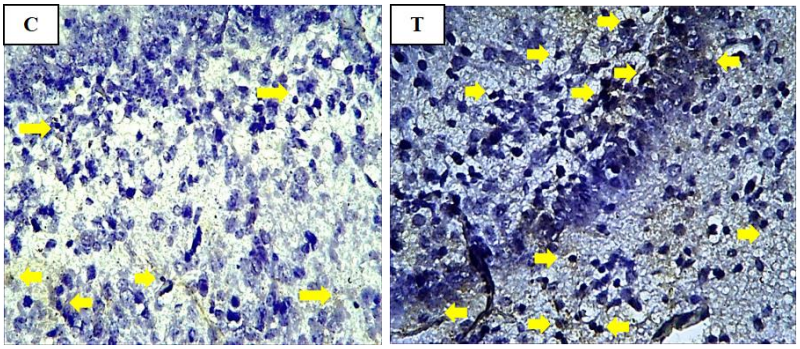

Figure 2. Comparison of Caspase 9 expression in rat cerebellum cells. Arrows indicate Caspase 9 expression which is indicated by the presence of brown chromogen (arrows). C: Control group; T: Treatment group (IHC; Miconos MCX50LED 400x magnification and Optilab Plus camera).

An immunohistochemical examination was carried out to see the expression of Caspase 9 in the cerebrum and cerebellum of newborn rats. Assessment of the control and treatment group samples using the IRS score was presented based on the average value and standard deviation with the following results in Figure 3. The results of the description of Caspase 9 expression in the cerebrum in the control group obtained an average of 1.04±0.79 and in the treatment group obtained an average of 3.16±1.28. Then, the expression of Caspase 9 in the cerebellum in the control group obtained an average of 1.44±0.25 and in the treatment group obtained an average of 1.99±0.54 (Figure 3).

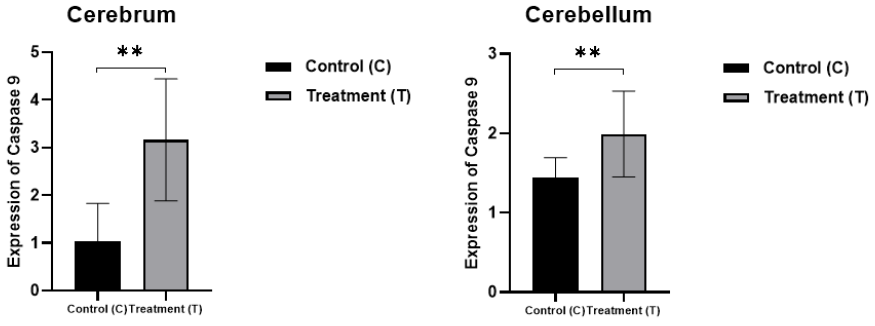

Figure 3. Results of Cerebrum and Cerebellum Caspase 9 Expression Analysis. (**) Significant with the control group (C) ($P \leq 0.001$)

Since the Mann Whitney test yielded a significant value of less than $P \leq 0.05$, the results of the normality test utilizing the Shapiro-Wilk test on Caspase 9 expression in the cerebrum revealed that the data were not normally distributed. In the cerebellum in each group the data showed a normal distribution because a significant value of more than $P \leq 0.05$ was obtained and independent T-test was conducted. The results of the Mann-Whitney test on Caspase 9 expression in the cerebrum showed that there was a significant difference between the C and T groups with a value of $P \leq 0.0001$. While the independent T-Test on Caspase 9 expression in the cerebellum showed that there was a significant difference between the C and T groups with a value of $P \leq 0.001$ (Figure 3).

DISCUSSION

Early maternal deaths can lead a baby to lose contact with its mother and stop nursing, both of which have an impact on the child's survival (Ahmed and Fullerton, 2019). Mothers are quite perceptive when it comes to responding to signals sent by newborns. It is because the mother and newborn bond through physical contact, skin-to-skin contact, and eye contact. The result is a relaxing influence from those. Babies with little or no interaction with their mothers as a result of maternal death may have greater cortisol levels than those who receive direct care from their mothers. As a result, it is probably to blame for neonatal stresses, which appear early in a child's life (Avrutsky and Troy, 2021; Tesfay et al., 2022).

The growth and development of a child's brain takes place rapidly from inside the womb, namely in the first 1000 days of the child's life window up to the age of 3 years. Neuron proliferation, synapse formation, myelination, axon and dendrite growth are included as several important developmental processes. The development and growth of good brain will affect cognitive, social and emotional abilities for the foundation of the future generations' development. The cerebrum and cerebellum are the part of brain. Cerebrum plays a role in cognitive and affective functions. However, the cerebellum plays a role in the development of motor, cognitive, emotional and behavioral (Jumsheleishvilli and Dididze, 2019). The transmission of nervous system information to the brain includes complex neural mechanisms with good nerve function and shape since all human activities depend on this nerve transmission. Brain development starting from still in the womb includes proliferation, migration, differentiation and synaptogenesis (Thau et al., 2022).

Brain tissue collected after death from depressed patients revealed DNA fragmentation and neuronal apoptosis. This suggests that depression increases neuronal susceptibility. The death of neurons most likely happens via two different pathways. They can be the swiftly occurring acute form (necrosis) or the delayed form (apoptosis). Several pro-apoptotic genes, specifically cysteine proteases or caspases, can regulate apoptosis in mammalian cells (Eskandari et al., 2022). The production of ROS may rise as a result of caspase 9. Oxidative stress situations also negatively impact the health of cellular organelles like the mitochondria. A decrease in ATP synthesis will result from the occurrence of mitochondrial malfunction, which serves as a source of energy for body cells. Then, it prevents mitochondrial enzymes from doing their jobs. As a result, the process of apoptosis begins with the instability and aggregation of intracellular proteins (Cui et al., 2021; Huiting et al., 2020). Both apoptotic and non-apoptotic functions of caspase 9 are involved in ischemic neurovascular damage. Early life stress in children is also linked to oxidative stress, which results from an imbalance between ROS and antioxidants in the brain. The function of GC receptors is disrupted as a result of ROS-induced translocation of these receptors from the cytoplasm to the nucleus. NADPH oxidase is produced as a result of an imbalance in the mitochondria (NOX). Cellular stresses suggest that NOX enzymes are the primary source of ROS in the brain. In addition, how much NOX stimulation causes the activation of stress signaling pathways like ERK1 and p38 MAPK, which affects cell damage (Pua et al., 2022; Wen et al., 2019; Avrutsky et al., 2020).

In a group of newborn rats used as a model for maternal mortality, Caspase 9 expression in the cerebrum and cerebellum was higher than in another group. The newborn rat's cerebellum and

cerebellum both had greater apoptotic indices of neuron cells that had split from the mother than those that had not. The newborn lost bonding ties after the mother died, which was the first effect of maternal death. The stimulation of the baby's sensory system, which could have a direct impact on brain development, is thus lost. It can influence a baby's growth in terms of emotions, cognition, and general mental health. The development of the baby's brain may be directly impacted by sensory system stimulation (Damayanti et al., 2020; Sullivan et al., 2011).

Infant rats exposed to stress brought on by maternal mortality experience attachment loss that affects the maturation and expansion of the developing brain. The HPA axis was stimulated by stressors. The paraventricular hypothalamus started to secrete CRH, which then prompted the pituitary gland to secrete ACTH. An sign of stress, cortisol is released by the adrenal gland cortex as a result of ACTH activation. A glucocorticoid was shown to be bound to the glucocorticoid receptor in the cerebellum and brain of newborn rats (GR) (Pua et al., 2020; Wen et al., 2019).

Brain-derived neurotrophic factor (BDNF) expression is inhibited by prolonged stress and interferes with the basic process of apoptotic cell death. A BDNF is made up of pro- and mature BDNF. TrkB would be bound by mature BDNF. Through protein kinase B, it triggers pathways for cell growth and survival (Akt). It can also activate ERK, MEK, and MAPK (Holopainen et al., 2020). But Sortilin was involved in the activation of the apoptotic pathway by pro-BDNF binding to the p75NTR receptor. Sortilin activated pro-apoptotic proteins including as p53, Bad, BIM, and BAX through jun-N terminal kinase (JNK), which then released them. It induced the release of cytochrome-C from the mitochondria and activated caspases 3, 6, and 9, which started the intrinsic cascade of apoptosis (Damayanti et al., 2020). Therefore, if a mother passes away after giving birth, the stimulation and bonding relationship between mother and kid will be lost, there will be no touch with the mother, which causes stress in the child's early years, and apoptosis will be activated, leading to greater Caspase 9 expression (Damayanti et al., 2020; Avrutsky et al., 2020).

CONCLUSION

Expression of Caspase 9 in the cerebrum and cerebellum of newborn rats which were separated from their parents as a model of maternal mortality was higher than not separated.

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AUTHOR CONTRIBUTIONS

Septi Andriana and Widjiati assisted in conducting the experiments, performed the statistical analysis and data visualization and wrote the manuscript**.** Hermanto Tri Joewono designed and conducted all of the experiments and wrote the manuscript**.** All authors have read and approved of the final manuscript.

CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

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