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RESEARCH ARTICLE

Maternal Separation as Model Maternal Mortality Induced Caspase-3 Expression of Cerebrum and Cerebellum in Rattus Norvegicus Newborn

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ABSTRACT

Maternal mortality results from the breakdown of the bonding-attachment between mother and child, affecting the development of psychological factors and the expression of initiator-activating caspase-3. In this study, we described maternal separation from neonates using a posttest-only control group experimental design. The research sample was randomly split into two groups: Rattus norvegicus newborns aged 3 days and those that were not separated. The assessment employed immunohistochemistry and was evaluated using the Rammele Scale Index (Immunoreactive Score / IRS) analysis. These findings demonstrate a notably elevated expression of caspase-3 in the cerebrum of Rattus norvegicus neonates subjected to maternal separation therapy in comparison to the control group, attributed to neuropsychiatric disorders such as anxiety and depression in infants, as well as the prolonged impact of parental loss on neurodevelopmental and behavioral well-being, which may enhance susceptibility to psychopathology. Additionally, there are only minor variations in caspase-3 expression in the cerebrum of newborn Rattus norvegicus between those separated and not separated from their mother, influenced by the duration and frequency of separation. To our knowledge, there is no documentation of maternal separation that might trigger caspase-3 expression in the cerebrum and cerebellum. These findings present novel insights that could be significant for clinical applications in newborns.

INTRODUCTION

Maternal mortality is defined as death of women during pregnancy until termination of pregnancy or 42 days after delivery, regardless of place or stage of pregnancy (1, 2). The number of maternal deaths during the pregnancy, childbirth or another caused like accidents was known as the *maternal mortality rate* (MMR). According to statistics data from Indonesian health profile in 2015, there were 305 maternal reported as MMR for every 100,000 live births (3). In 2019, MMR for East Java Province reached 89.81 per 100,000 live births and followed by increasing in 2020 which reached 98.39 per 100,000 live births (4). Postpartum (PP) hemorrhage, eclampsia and pre-eclampsia, obstructed labor, infection, and indirect causes are the main causes of maternal mortality (5). Maternal mortality in early few months life of child will cause a loss of bonding between mother-baby and disrupt of breastfeeding process (2). In addition, maternal mortality caused an absence of bonding and attachment between mother-baby, which affects psychology and physical development of children which has leading to neonatal stressors (6, 7).

Neonatal stress can activate of *hypothalamic pituitary adrenal* (HPA) axis followed secrete of cortisol and increasing cortisol will have an impact on the development of brain in baby (8, 9). Hypothalamus

secreted *corticotropin-releasing hormone* (CRH), thereby stimulating releasing of *adenocorticotrophin hormone* (ACTH) which is secreted by the pituitary gland. Secretion of ACTH hormone through bloodstream reached adrenal glands, secrete a hormone as an adaptive response to stress called *glucocorticoids* (GC), namely hormone cortisol can be used as a benchmark for stress sensitivity (10). GC-induced mitochondria lead to disruption of membrane potential and release of important apoptosis-inducing factors such as *cytochrome-C* (11). While in cytosol, *cytochrome-C* binds to the C-terminal region of Apaf-1, a cytosolic protein with an *N-terminal caspase-recruitment domain* (12). The binding between cytochrome-C with Apaf-1 can recruited and activated Caspase-9, followed by activated caspase-3 to the apoptosome and cleaves key substrates in cells to generate many cell and biochemical apoptotic events (13, 14). Several researchers have been reported that the activation of caspases to be a sign of apoptosis cells. Apoptosis in neonatal neuron cells hindered information processing in the brain and control center, impairing cognitive and language development in neonates (15). However, maternal mortality events should not only focus on reducing mortality but also consider to health of the mother, growth and development of babies such as expression caspase-3 activity in cerebrum and cerebellum who have been abandoned by their mothers. Thus, as one of strategy potentially address these multifaceted challenges is understanding of the maternal separation affect in clinical application newborn through *in vivo* study.

Herein, our study aims to activity of caspase-3 expression in cerebrum and cerebellum towards *Rattus norvegicus* newborns within 3 days compared with who were not separated. The analysis showed significant differences expression of caspase-3 in the cerebrum of *Rattus norvegicus* newborn after separated from maternal separation by immunohistochemistry method, compared to control which is not separated due to maternal separation has long-term effects on neuro developmental and behavioral health that may increase vulnerability to psychopathology. Moreover, only slightly differences between caspase-3 expression in the cerebellum of *Rattus norvegicus* newborn with control group due to duration and frequency of separation. To the best of our knowledge, there is no report of maternal separation could induce caspase-3 expression in cerebrum and cerebellum. Therefore, this reported clearly provide important knowledge of maternal separation in clinical application newborn.

MATERIALS AND METHODS

Material

Rattus norvegicus female and *Rattus norvegicus* male, *Human chorionic gonadotropin, Pregnant mare serum gonadotropin,* animal milk for *Rattus norvegicus* newborn, 10% paraformaldehyde buffer, xylol, ethanol, and anti-caspase-3 recombinant.

Methods

Insemination *Rattus norvegicus*

Rattus norvegicus female and male were purchased and kept in standard environmental condition, given standard rodent food (formulated) and water ad libitum in the animal house of the Department of Medical Science in Airlangga University. *Rattus norvegicus* female 2.5-month-old was injected with 10 IU *Pregnant mare serum gonadotropin* (PMSG). After 48h, *Rattus norvegicus* female has injected 10 IU *Human chorionic gonadotropin* (hCG) via intraperitoneally and collected in the same cage as male rats (mating). Then, 17h after mating, a vaginal plug examination was conducted to confirm pregnancy. If a vaginal plug is found, it is calculated as 0 day of pregnancy. Pregnant rats were cared for and observed until term (20-21 days) and gave birth. Finally, randomly grouping including *Rattus norvegicus* newborn that remains with mother as control and *Rattus norvegicus* newborn separated from mother as treatment within 3 days.

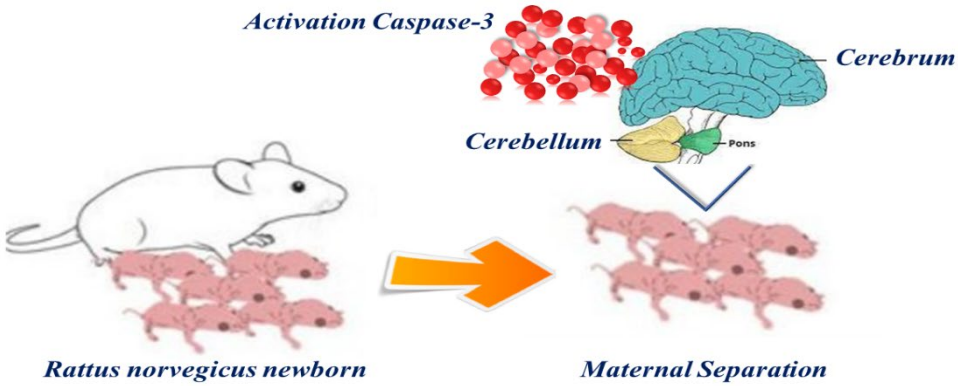

Figure 1: Illustration of maternal mortality induced caspase-3 expression of cerebrum and cerebellum in *rattus norvegicus* newborn

Caspase-3 Expression analysis by immunohistochemistry

Rattus norvegicus was sacrificed and taken brain tissue with placed on a 10% paraformaldehyde tube. Then, cut with a rotary microtome with a thickness of 4 µm and placed in a poly-L-lysine object glass and left at room temperature.

a) Furthermore, deparaffinization was carried out, with slide was preheated at 600 ^{0}C for 60 mins. Then, the following solutions were added sequentially: xylol (2x10 mins), ethanol (2x10 mins), 90% ethanol (1x5 mins), 80% ethanol (1x5 mins), 70% ethanol (1x5 mins), sterile D_2O (3x5 minutes). Antigen retrieval process with citrate buffer. The slides were immersed in a chamber containing citrate buffer pH 6.0 and heated in a water bath until 95 ^{0}C for 20 mins. The slides were removed from water bath and waited until room temperature (±20 mins) then washed with PBS (3x2 mins).
b) Immunohistochemical staining was carried out by slides dripping with 3% H_2O_2 in methanol and incubated for 15 mins, then washed with PBS for 3 times.
c) *Blocking unspecific protein* with drops of *sniper background*, incubated for 15 mins at room temperature, and washed with PBS for 2 mins 3 times.
d) Dropped primary antibody (anticaspase-3 dissolved in PBS buffer with a ratio of 1:50 and 5% FBS) for 1 night at 40 ^{0}C. The slides were then incubated with the secondary antibody for 30 mins at room temperature and washed with PBS for 2 mins 3 times.
e) Then, SA-HRP enzyme was incubated for 20 mins at room temperature, washed with PBS for 2 mins 3 times and rinsed with distilled water.
f) Then, DAB and DAB buffer were dripped with a ratio of 1:50 and incubated for 3-10 mins at room temperature, washed with PBS for 2 mins 3 times and washed with distilled water for 2 mins 3 times.
g) Mayer and tap water are dripped with a ratio of 1:10 and incubated for 5-10 minutes at room temperature, then rinsed with tap water, dried and observed under a microscope with 400x magnification.

RESULTS AND DISCUSSION

Rattus norvegicus which became pregnant consisted of 2 parents from each group and randomization was carried out into 2 control and treatment groups by experimental posttest-only control group design. The body weight of the *Rattus norvegicus* before insemination was about 160-180 gr, compared to the end pregnancy showed between 230 - 260 gr. Next, the average body weight of *Rattus norvegicus* newborn in control group showed 3.530 ± 0.557 gr obtained from a ratio of 0 and 3 days. In reverse, *Rattus norvegicus* newborn has body weight significantly decrease to 1.112 ± 0.338 gr as treatment group. These results indicate that *Rattus norvegicus* newborn with maternal separation has stress-related with eating disorders (16, 17). To further evaluate maternal separation as a model maternal mortality-induced caspase-3 expression cerebrum and cerebellum in *Rattus norvegicus* newborns were examined by statistical analysis. The body weight of *Rattus norvegicus* newborn was tested by the normality test. The results of *Rattus norvegicus* newborn obtained by

Shapiro-Wilk test showed p=0.198 (control) and p=0.824 (treatment) > α=0.05 respectively, indicating that data in this study are normally distributed. To find out the differences between groups, we employed an *independent sample t-test*. There was a significant difference of *Rattus norvegicus* newborn between the control and the treatment group with a value of $p = 0.000$ ($\alpha < 0.05$). Overall, these findings indicate that maternal separation from neonatal caused eating-disordered individuals, to subsequently decreasing of body weight in *Rattus norvegicus* newborn.

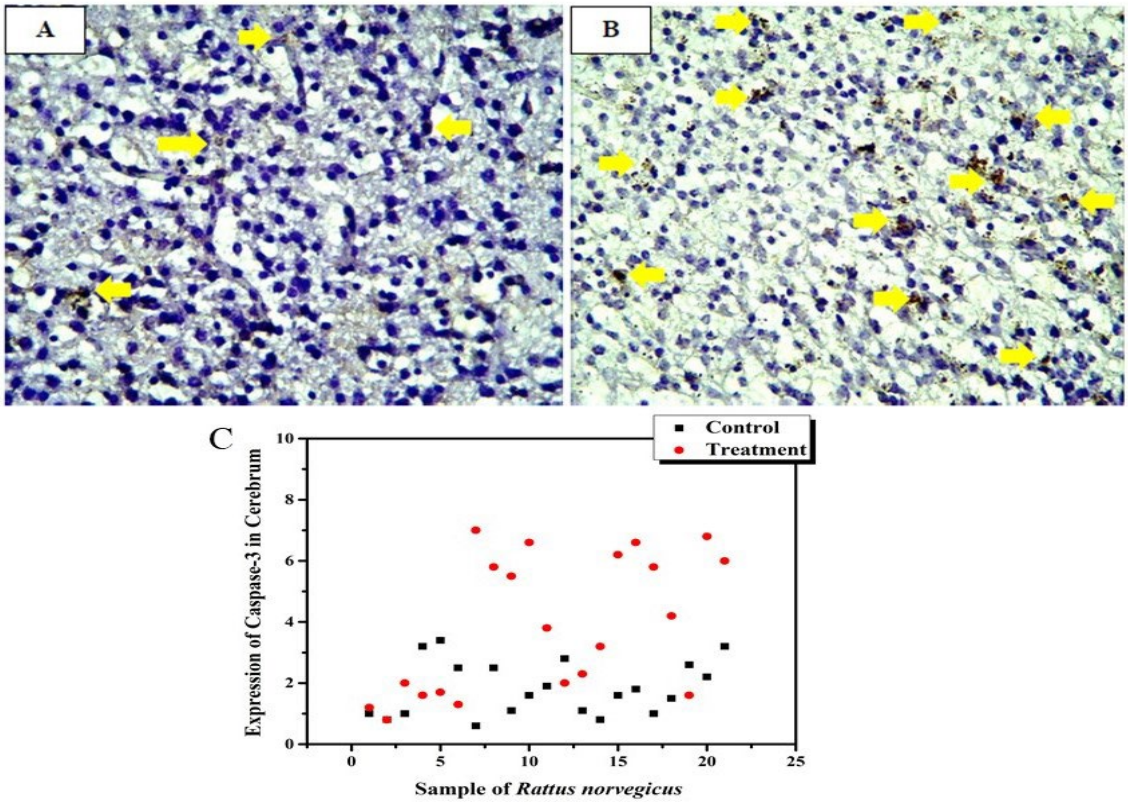

Figure 2: Comparison of the expression of caspase-3 in cerebrum *Rattus norvegicus* newborns. The arrows indicate caspase-3 expression which is marked in brown chromogen (arrows). A control group; B treatment group (400x). C Scatter plot of caspase-3 expression in the cerebrum of *Rattus norvegicus* newborn.

Furthermore, we analyzed expression of caspase-3 in the cerebrum and cerebellum of *Rattus norvegicus* newborn with maternal separated to calculate the index of apoptosis in neuronal cells and samples were assessed by *Rammele Scale Index* (Immunoreactive Score / IRS). As shown in **Figure 1A,** caspase-3 expression in the cerebrum of *Rattus norvegicus* newborns significantly increases after maternal separation within 3 days as indicated by brown chromogen compared to control **(Figure 1B).** To calculate the quantitative evaluation of caspase-3 expression by plotting IRS, we found an immensity value with highest score of 7.0 while lowest value is 1.2. In reverse, control groups exhibited only the highest value of caspase-3 expression about 3.2 and lowest value is 0.8 **(Figure 1C)**. These results imply that maternal separation could induce activation of caspase-3 expression in cerebrum of *Rattus norvegicus* newborns. In addition, these results further confirm that maternal separation can lead to neuropsychiatric disorders, including anxiety and depression in newborns, and long-term isolation during the early postpartum period increases susceptibility to neuroendocrine and other stress-related disorders (18, 19) which caused significantly increased the percentage expression of caspase-3. Next, we analyzed expression of caspase-3 in the cerebellum of *Rattus norvegicus* newborn. *Rattus norvegicus* newborn has expression of caspase-3 in cerebellum showed only slight differences after maternal separation within 3 days compared to control groups. To confirm these findings, quantitative analysis also displayed that the average caspase-3 expression was about 1.8 after maternal separation and 1.6 for control groups. These results imply that might be due to cerebellum of *Rattus norvegicus* newborn has several conditions such as duration and frequency of separation, ages of the child, and factor of neurotropic (20-22).

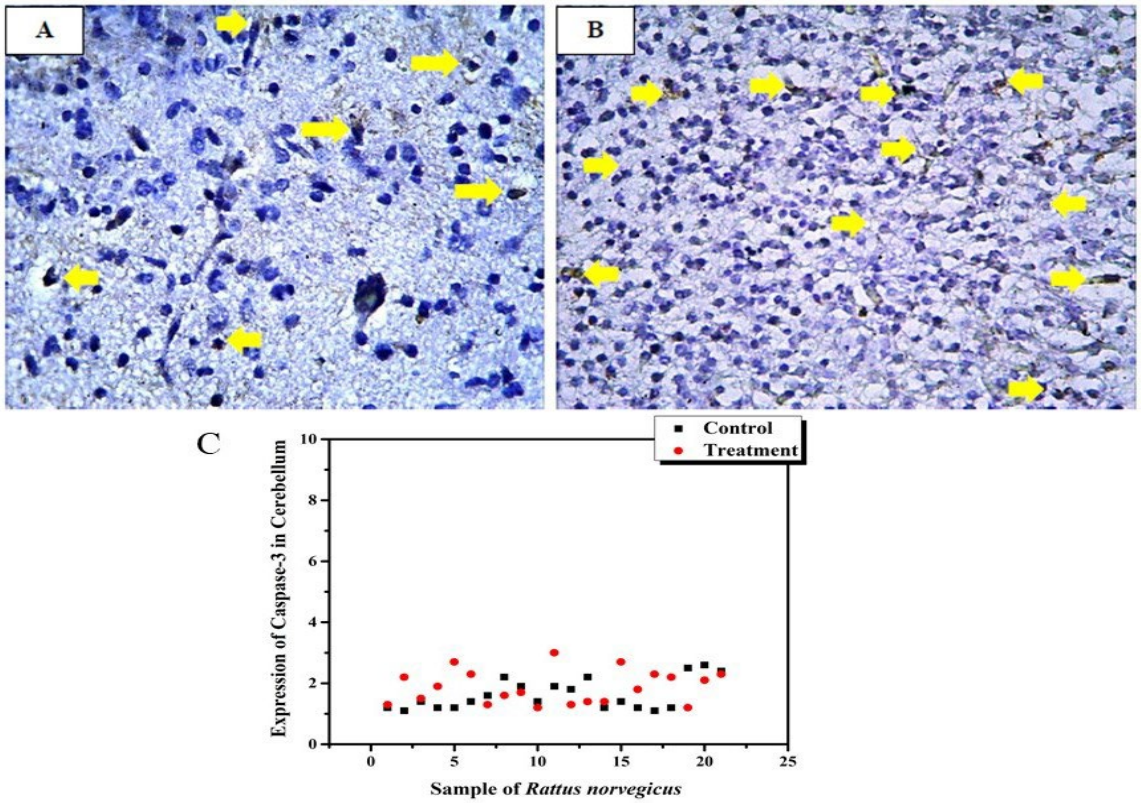

Figure 2: Comparison of the expression of caspase-3 in rat cerebellum *Rattus norvegicus* newborns. The arrows indicate caspase-3 expression which is marked in brown chromogen (arrows). A control group; B treatment group (400x). C Scatter plot of expression caspase-3 in the cerebrum of *Rattus norvegicus* newborn.

In order to verify whether maternal separation can induce caspase-3 expression, we determined a comparative test between treatment group and control group. As present in **Table 1**, comparison test of caspase-3 expression in the cerebrum obtained a significance value of 0.001 ($\alpha < 0.05$), implying that activation of caspase-3 expression after maternal separation is higher than control group. In contrast, activation of caspase-3 expression after maternal separation in the cerebellum obtained a significance value of 0.092 ($\alpha > 0.05$), indicating there was no significant difference with control groups. This work highlights how maternal separation induced caspase-3 expression in the cerebrum and cerebellum of *Rattus norvegicus* newborn.

Table 1: Comparison test caspase-3 expression of *Rattus norvegicus* newborn by *Mann-Whitney* analysis study.

Expression of Caspase-3	Sig	Information
Cerebrum	0,001	Significant
Cerebellum	0,092	No significant

CONCLUSION

We successfully reported activation of caspase-3 expression in cerebrum and cerebellum *Rattus norvegicus* newborns. The analysis by immunohistochemistry showed significant differences of caspase-3 expression in the cerebrum of *Rattus norvegicus* newborns between separated and not separated from maternal separation. Moreover, only slightly differences of caspase-3 expression in the cerebrum of *Rattus norvegicus* newborns between separated and not separated from maternal separation. Therefore, this study clearly provides important knowledge for clinical application newborns.

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Authors contributions: Arinda Dian Anggraini: Writing – original draft, Data curation, Investigation, Methodology. Hermanto Tri Juwono: Investigation, Resources, Validation. Widjiati: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, Visualization.

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