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The Effect of Maternal Separation on Anxiety Behavior in of White Mice (Mus Musculus L.) Male Swiss Webster Strain

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ABSTRACT

Introduction. Maternal separation (MS) is a commonly utilized model of early-life stress to explain the influence on brain development and psychological behavior. MS has been demonstrated to elevate the likelihood of developing anxiety disorders as a consequence of alterations in hypothalamic- pituitary-adrenal (HPA) functionality and neurotransmitter levels. This study aimed to evaluate the impact of maternal separation (MS) duration on anxiety behavior in male Swiss Webster strain white mice (Mus musculus L.). **Methods.** The study used a true experimental method with a posttest- only control group design. A total of 28 male mice aged 2 days were randomly assigned to one of four groups: a control group that did not undergo maternal separation (MS) and three MS treatment groups with a duration of 2 hours, 4 hours, and 6 hours per day for 21 days. Anxiety behavior was evaluated using the Elevated Plus Maze (EPM), with the number and duration of entries into the closed and open arms serving as the primary analysis metrics. The data were analyzed using one-way ANOVA and post-hoc LSD statistical tests. The duration of MS significantly impacted the anxiety behavior of the mice. **Results.** The MS groups of 2, 4 and 6 hours demonstrated a notable increase in the number and duration of entries into the closed arm in comparison to the control group ($p < 0.05$), which is indicative of increased anxiety. In contrast, the number of entries into the open arm decreased in the 6 h MS group compared to the control. **Conclusion.** The results indicated that MS negatively affected the anxiety behavior of mice, with longer duration exacerbating the trend.

1. Introduction

Maternal Separation (MS) is a trauma or stress model used to understand the influence of early life stress on brain development and individual psychopathological adaptation.[1,2] MS is often used to observe early life stress responses and their impact on an individual's vulnerability or resilience to mental health problems.[3] MS can result from a variety of factors, such as maternal death, parental divorce, working mothers, or educational needs.[4]

Research indicates MS can contribute to various psychological and behavioral disorders in adulthood, including schizotypal personality traits, recurrent nightmares, social difficulties, and a heightened risk of alcohol and substance abuse.[5–8] In addition, MS also impacts children's mental health, including the risk of depression and anxiety.[9] The effects of MS are experienced not only by the child but also by the mother. MS can influence brain function, alter parenting behaviors, and trigger prolonged activation of the corticotropin-releasing factor (CRF) system, heightening stress responses. Additionally, changes in neurotransmitter systems caused by MS may contribute to behavioral disorders, including anxiety and depression, in both the mother and child.[2,10,11]

Anxiety is a common emotional disorder characterized by fear for no apparent reason and can affect the ability to think logically and concentrate.[12] Globally, anxiety is experienced by approximately 5% to 25% of the population, with anxiety disorders due to MS common in children.[13] These disorders can disrupt multiple aspects of life and may persist into adulthood if not properly addressed. Around 4.1% of children experience anxiety related to MS at a severity that warrants clinical intervention.[8]

Previous research in mice has demonstrated that MS can impact emotional function and provoke anxiety-related behaviors.[8,15] The studies by Komada et al., Knight et al., and Kambali et al.

consistently found a link between MS and anxiety.[16–18] However, other research, such as the work by He et al., reported contrasting findings, suggesting that MS was not associated with anxiety.[7] Therefore, this study aims to examine the effect of different duration of maternal separation on anxiety-like behaviors in male Swiss Webster mice using the elevated plus maze (EPM) test.

2. Methods

Experimental Design

This study is a true experimental study with a posttest only control group design that aims to examine the effect of maternal separation (MS) on anxiety behavior in white mice (Mus musculus L.) male Swiss Webster strain. The exclusion criteria inferred to animals with apparent illness or physical abnormalities. This research was conducted from July to October 2024 at the Animal House of the Faculty of Medicine, Sriwijaya University Palembang. The samples were randomly divided into four groups, namely the group of mice without MS (K1), the MS group for 21 days with a duration of 2 hours (K2), the MS group for 21 days with a duration of 4 hours (K3), and the MS group for 21 days with a duration of 6 hours (K4), each consisting of 7 mice.

The independent variable in this study is maternal separation (MS), while the dependent variable is anxiety behavior in male Swiss Webster white mice, which is measured using Elevated Plus Maze (EPM) by counting the number of closed and open arm entries and calculating the duration of closed and open arm entries.

Animal Preparation

The animal preparation process began with breeding 20 female mice and 5 male Swiss Webster mice aged 2-3 months. Mice cages were lined with chaff and room conditions with a 12:12 light-dark cycle, maintained room temperature, and unrestricted access to food and water. After birth, the offspring were separated from their mothers on day 2 postpartum (PND2) and placed in separate cages lined with husks. The MS procedure was carried out for 21 days and during behavioral tests only male mice were used as seen from the presence of scrotum.

Elevated Plus Maze

The Elevated Plus Maze (EPM) is a behavioral testing tool used to measure anxiety levels in animals. It consists of a "+" sign-shaped platform with two open arms and two closed arms. The EPM measures 36 cm x 6 cm x 15 cm. In the experiment, white mice that experienced maternal separation for 21 days were tested on day 22 to evaluate the impact of early life stress on their behavior. Mice were placed in the center of the maze, and their behavior was observed for 5 minutes, recording the time spent in the open and closed arms and the number of entries into each arm. Mice with higher levels of anxiety tended to be in the closed arms more often, reflecting a preference for safer places. This research helps understand the relationship between early life stress and anxiety disorders.

Data Processing and Analysis

Data were analyzed using Microsoft Excel and SPSS version 27 for Mac analytically. This study was approved by the Ethics Committee for Medical and Health Research (KEPKK) Faculty of Medicine, Sriwijaya University with ethical number 265-2024.

3. Results

Closed Arm Entries

Analysis using one-way ANOVA. Table 1 presents the results of the comparison of the number of closed arm entries between groups, with p values <0.05 indicating a significant difference in the number of closed arm entries between each MS group.

To determine the relationship between groups, the LSD post-hoc test was further analyzed. Figure 1 shows the difference in the mean number of closed arm entries in various MS groups tested using EPM. The results show that each MS group had an increase in the number of closed arm entries, indicating that the longer the duration of MS, the higher the level of anxiety behavior in mice.

Figure 1 shows the difference in the number of closed arm entries in EPM. One-way ANOVA test resulted in a significant value [$F(3,24) = 15.506$; $p = 0.000$]. Significant differences between groups were then analyzed using the LSD Post-Hoc test, with results of $p<0.05$ compared to the control group. Significant differences were found in the 2, 4 and 6 hours MS groups.

Table 1. One-way ANOVA test results between MS groups on EPM

No.	Group	n	Closed Arm Entries ± Standard Deviation	*p*
1.	Control	7	5.000 ± 1.155	0.000
2.	MS 2 hours	7	7.140 ± 1.676	
3.	MS 4 hours	7	8.140 ± 0.900	
4.	MS 6 hours	7	9.860 ± 1.574	

Notes: One-way ANOVA test (p = 0.05)

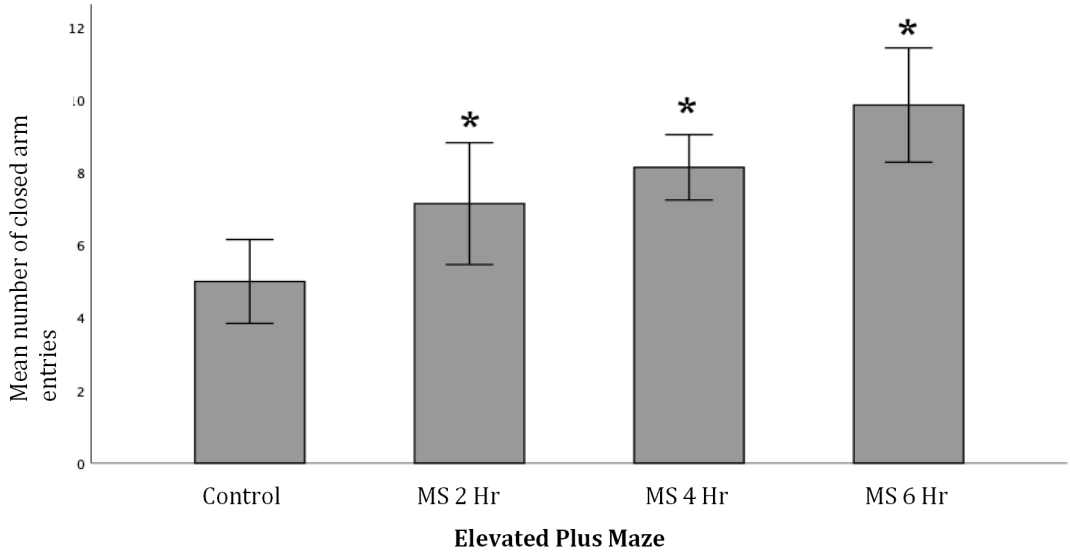

Figure 1. Closed arm entries in EPM

Table 2. One-way ANOVA test results between MS groups on EPM

No.	Group	n	Duration of Closed Arm Entries ± Standard Deviation	*p*
1.	Control	7	191.710 ± 48.685	0.045
2.	MS 2 hours	7	207.860 ± 30.284	
3.	MS 4 hours	7	238.710 ± 31.117	
4.	MS 6 hours	7	245.000 ± 38.432	

Notes: One-way ANOVA test (p = 0.05)

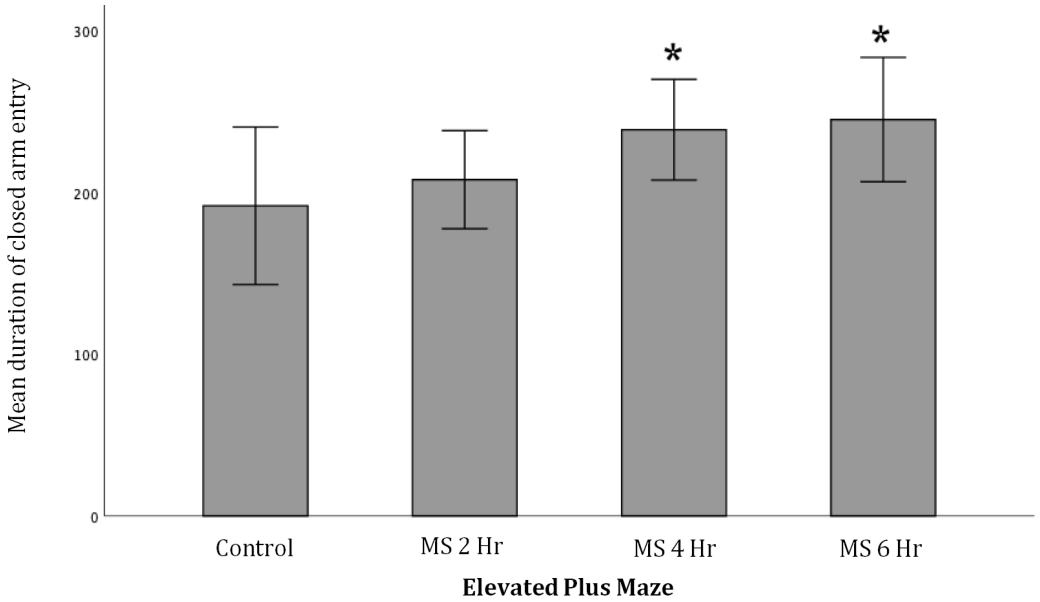

Figure 2. Duration of closed arm entries in EPM

Duration of Closed Arm Entries

Analysis using one-way ANOVA. Table 2 presents the results of the comparison of the duration of closed arm entries between groups, with p values <0.05 indicating a significant difference in the number of closed arm entries between each MS group.

To determine the relationship between groups, further analysis of the LSD post-hoc test was conducted. Figure 2 shows the difference in the mean duration of closed arm entry in various MS groups tested using EPM. The results show that each MS group had an increase in the duration of closed arm entry, indicating that the longer the duration of MS, the higher the level of anxiety behavior in mice.

The one-way ANOVA test resulted in a significant value [F(3,24) = 3.126; p = 0.045]. Significant differences between groups were then analyzed using the LSD Post-Hoc test, with results

of p<0.05 compared to the control group. Significant differences were found in the 4 and 6 hours MS groups.

Open Arm Entries

Analysis using one-way ANOVA. Table 3 presents the results of the comparison of the number of open arm entries between groups, with p values <0.05 indicating a significant difference in the number of closed arm entries between each MS group.

To determine the relationship between groups, the LSD post-hoc test was further analyzed. Figure 3 shows the difference in the mean number of open arm entries in the various MS groups tested using EPM. The results show that each MS group experienced a decrease in the number of open arm entries.

One-way ANOVA test resulted in a significant value [F(3,24) = 4.436; p = 0.013]. Significant differences between groups were then analyzed using the LSD Post-Hoc test, with results of p<0.05 compared to the control group. Significant differences were found in the 6-hour MS group.

Duration of Open Arm Entries

Analysis using one-way ANOVA. Table 4 presents the results of the comparison of the duration of open arm entry between groups, with p values <0.05 indicating a significant difference in the duration of open arm entry between each MS group.

To determine the relationship between groups, an LSD post-hoc test was conducted. Figure 4 shows the difference in the mean duration of open arm entry in the various MS groups tested using EPM. The results show that each MS group had an increase in the duration of open arm entry.

The one-way ANOVA test resulted in a significant value [F(3,24) = 3.126; p = 0.045]. Significant differences between groups were then analyzed using the LSD Post-Hoc test, with results of p < 0.05 compared to the control group. Significant differences were found in the 4 and 6 hours MS groups.

Table 3. One-way ANOVA test results between MS groups on EPM

No.	Group	n	Open Arm Entries ± Standard Deviation	*p*
1.	Control	7	6,710 ± 1,604	0.013
2.	MS 2 hours	7	5,710 ± 1,704	
3.	MS 4 hours	7	5,000 ± 1,915	
4.	MS 6 hours	7	3,710 ± 0,951	

Notes: One-way ANOVA test (p = 0.05)

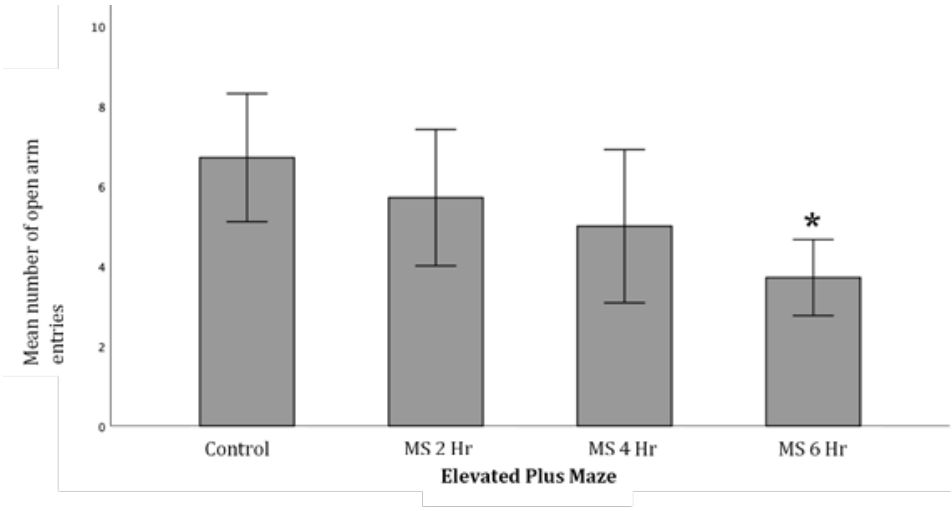

Figure 3. Open arm entries

Table 4. One-way ANOVA test results between MS groups on EPM

No.	Group	n	Duration of Open Arm Entries ± Standard Deviation	*p*
1.	Control	7	108.290 ± 48.685	0.045
2.	MS 2 hours	7	92.140 ± 30.284	
3.	MS 4 hours	7	61.290 ± 31.117	
4.	MS 6 hours	7	55.000 ± 38.432	

Notes: One-way ANOVA test (p = 0.05)

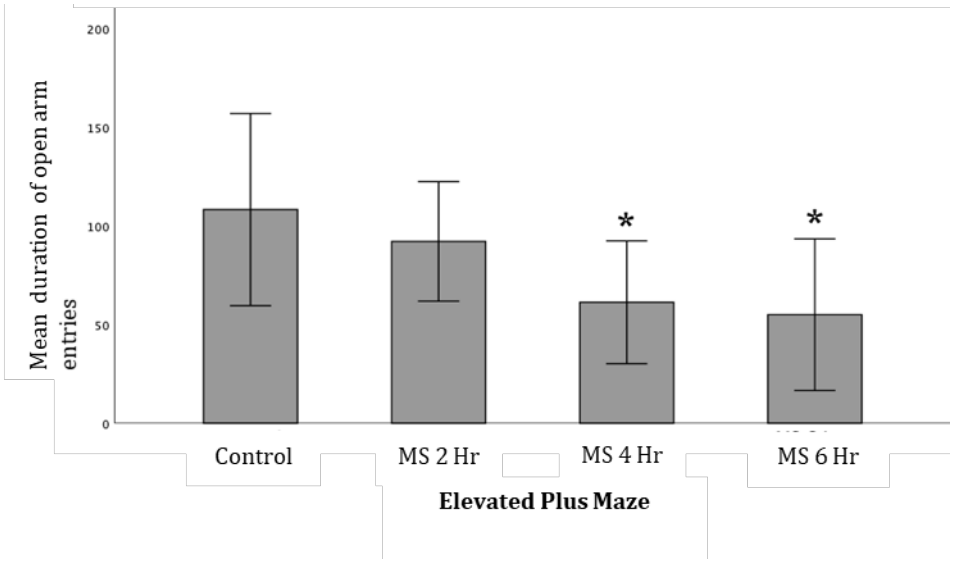

Figure 4. Duration of open arm entries

4. Discussion

This study aims to assess the impact of Maternal Separation (MS) on anxiety-related behavior in male Swiss Webster white mice during the neonatal period. The findings indicate that mice in the 2-hour, 4-hour, and 6-hour MS groups exhibited an increased number and duration of entries into the closed arms compared to the control group. The mice's behavior was analyzed by measuring their time spent in both the closed and open arms. Naturally, mice tend to avoid open spaces and prefer enclosed areas, a response that reflects anxiety when exposed to unfamiliar or threatening environments. The longer a mouse remains in the closed arm, the higher its anxiety level. The study revealed significant differences in anxiety levels between the control group and those subjected to MS, with longer separation periods correlating with heightened anxiety behaviors.[16,19–21]

The findings of this study suggest that a 2-hour duration of Maternal Separation (MS) can elevate anxiety-related behavior in mice, though its impact remains less pronounced compared to longer MS durations. A meta-analysis by Wang et al. found that prolonged MS correlated with heightened anxiety, while a 2-hour separation had a relatively minimal effect. In the 4-hour MS group, a more noticeable increase in anxiety behavior was observed, as indicated by a higher frequency of entries into the closed arm. This aligns with previous studies showing that daily 4- hour separations heighten anxiety in mice, leading to a preference for enclosed spaces in the Elevated Plus Maze (EPM). However, research by He et al. suggests that a 4-hour MS duration does not always result in a significant anxiety increase. The most substantial rise in anxiety behavior was seen in the 6-hour MS group, where mice exhibited the highest frequency and duration of entries into the closed arm. This supports findings by Troakes et al. and Kambali et al., who linked extended separation with activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased anxiety in rodents.[15,16,20,23]

Maternal separation (MS) during development has a significant impact on the neuroendocrine system, brain structure and neurotransmitter pathways that play a role in anxiety regulation. Research shows that the duration of MS affects anxiety levels through changes in hypothalamic-pituitary-adrenal axis (HPA) activity.[15,24,25] In the control group without separation, stress responses and HPA axis regulation remained normal. However, separation for 2, 4 and 6 hours resulted in different levels of HPA axis activation. A 2-hour separation generally caused only transient stress, with corticosterone levels that increased moderately and could still be compensated by the body.26 In contrast, a 4-hour separation triggers a stronger hyperactivation of the HPA axis, disrupts the normal development of the neuroendocrine system, and results in a higher increase in corticosterone levels, as reported by Levine et al.[27] A more serious impact was found at the 6-hour separation, which caused corticosterone levels to remain high for a long time and the hyperactivation of the HPA axis to be more severe. McEwen et al. noted that these effects could significantly affect the brain's ability to regulate stress, both during development and in adulthood.[28]

Maternal separation (MS) impacts key brain structures, particularly the hippocampus and amygdala, which are essential for regulating anxiety. Studies indicate that MS heightens amygdala activity by increasing the expression of glutamate receptors, leading to heightened anxiety responses. The hippocampus, which is crucial for managing stress, also undergoes significant changes. A separation period of six hours can reduce hippocampal volume and lower glucocorticoid receptor expression, impairing the brain's ability to regulate stress effectively. These effects are further intensified by alterations in neuroplasticity caused by prolonged stress exposure.[29–32]

The medial prefrontal cortex (mPFC) and amygdala, which are highly interconnected, play a crucial role in regulating emotional responses, including anxiety. Under normal conditions, the mPFC modulates amygdala activity to prevent the expression of inappropriate emotions. However, prolonged maternal separation (MS) can weaken this regulatory function, leading to excessive amygdala activation and impairing emotional and behavioral control. Similar patterns of dysfunction have been observed in psychiatric patients and animal studies, highlighting this disruption as a key characteristic of stress-related neuropsychiatric disorders.[33]

Chronic stress activates the HPA axis, leading to the release of cortisol—a hormone that helps the body cope with stress by boosting glucose production and regulating immune responses. However, prolonged exposure to cortisol can harm brain function, particularly in the hippocampus, which plays a crucial role in memory, emotional regulation, and stress management. High cortisol levels can impair hippocampal function by increasing oxidative stress, where excessive Reactive Oxygen Species (ROS) overwhelm the brain's antioxidant defenses. This results in cellular damage, inflammation, and disrupted glial cell activity—cells essential for maintaining neuronal health, ATP production, and glutamate clearance. Consequently, neuronal energy metabolism is compromised, leading to reduced ATP production, which is vital for proper cellular function. Additionally, oxidative stress heightens the activity of NMDA (N-Methyl-D-Aspartate) receptors, and excessive glutamate stimulation can cause excitotoxicity, leading to neuronal damage. Cortisol also interferes with the brain's ability to absorb and utilize glucose, a key energy source for neurons. This cascade of oxidative stress, inflammation, and neuronal degeneration exacerbates anxiety symptoms, diminishes the brain's capacity to regulate stress effectively, heightens emotional reactivity, and contributes to the progression of anxiety disorders.[34–38]

Overall, the physiological impact of MS on anxiety suggests a close relationship between the duration of separation and the severity of neuroendocrine, neuroplasticity and neurotransmitter disturbances. Separation for 2 hours may still be within adaptive tolerance limits, but separation for 4 hours and especially 6 hours tends to produce more maladaptive effects. Long-term effects include increased basal anxiety, impaired stress regulation, and increased risk of psychological disorders such as depression and generalized anxiety. Previous research provides strong evidence regarding how early experiences such as MS can shape physiological responses to stress and increase the risk of anxiety disorders in adulthood. Furthermore, longitudinal designs are needed to evaluate the lasting impact of MS on anxiety, cognition, and depression into adulthood. Interventional approaches post-separation, such as pharmacological or environmental enrichment, should also be explored to model clinical treatments.

5. Conclusion

There is an effect of maternal separation (MS) for 2, 4, and 6 hours on anxiety behavior in white mice (Mus musculus L.) male Swiss Webster strain, using the Elevated Plus Maze (EPM) test.

6. Author Contribution

B.S., E.F.Z., S.S.F.P., A.H., A.P.L., N.T., conceived and designed research; A.P.L., N.T., performed experiments; A.P.L., N.T., analyzed data; E.F.Z., S.S.F.P., A.P.L., N.T., interpreted results of experiments; A.P.L., N.T., prepared figures; E.F.Z., S.S.F.P., A.P.L., N.T., drafted manuscript; E.F.Z., S.S.F.P., A.P.L., N.T., edited and revised manu-script; B.S., E.F.Z., S.S.F.P., A.H., A.P.L., N.T., approved final version of manuscript.

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