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RESEARCH ARTICLE

Folic Acid Supplementation in Pregnant Mice: An Approach to reduce the Expression of TNF-A and Placental Apoptosis Index in Maternal Stress

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ABSTRACT:

Introduction: Maternal stress is harmful to the placenta. It induces apoptosis. Maternal folic acid (FA) supplementation is mandatory in Indonesia and other countries. FA is known as an antioxidant and probably reduces the apoptosis index in placenta with maternal stress. **Aim:** To investigate the folic acid supplementation in pregnant stress mice (Mus Musculus) on the apoptosis index and placental TNFα expression. **Methods:** Twenty-one *Mus musculus* strainded balb/c female mice were randomly divided into three groups. The first group was normal mice, the second group was given stress, and the third group was given stress and folic acid supplementation (3 mg/kg body weight/day in 0,5ml suspension). The ovulation was induced by pregnant mare's serum gonadotropin and HCG. The stress was given by immobilizing the mice in a transparent pipe and giving bright light twice a day on gestational day 10-15. On gestational day 16, the mice were sacrificed, and the placenta tissue was taken. The expression of TNF-α and apoptotic index was calculated by multiplying the score for % stained cells and staining intensity. **Result:** The expression of TNF-α (mean±SD) of the normal, second, and third groups were 2.1 ± 0.37, 5.9 ± 1.01, and 3.2 ± 1.78. The apoptotic index (mean±SD) of the normal, second, and third groups were 2.7 ± 1.09, 6.3 ± 0.87, and 3.2 ± 0.65. **Conclusion:** Folic acid supplementation reduces TNF-α and apoptotic index expression in the placenta with maternal stress, and there is a positive correlation between TNF-α and apoptotic index.

KEYWORDS: Folic acid supplementation, TNF-α, placental apoptosis index, maternal stress, maternal mortality.

INTRODUCTION:

The most critical factors for reducing maternal neonatal mortality and morbidity can be started during pregnancy. In the gestation period, there are many physiological and psychological changes[1,2]. Maternal stress has been conceptualized as a teratogen, a mediator that can damage the perinatal period and development of the product of conception[3–5]. The estimated prevalence of stress and depressive symptoms was 7.4% - 20% in the antenatal period and 19.2% in the first three months after birth[6,7].

Pregnancy should be the gladdest time for a mother-to-be. However, sometimes, as a mother-to-be, there is an elevated sense of anxiety regarding the birth process[8,9].

During pregnancy, the development of the placenta is vital for fetal survival in maternal-fetal transport[10]. The placenta has a variety of functional activities, including complex synthetic capabilities for normal infant development. Placental dysfunction is closely related to complications in the fetus[11–13]. It can activate the HPA (Hypothalamus-Pituitary-Adrenal) axis in prenatal stress conditions, resulting in increased glucocorticoids (cortisol). This can enhance Reactive Oxygen Species (ROS). It triggers the secretion of proinflammatory cytokines by regulating NF-kB, notably the Tumor Necrosis Factor (TNFα). TNF cytokines have specific

receptors in the cell membrane, whose results can be apoptosis[10,11].

Recent research has explained that apoptosis in placental cells is correlated with a critical event responsible for the placenta's abnormal function. In humans, placental apoptosis increases intrauterine growth retardation (IUGR) and other fetal disorders[10,12]. Proinflammatory cytokine levels have been linked to stress and depressive symptoms. The natural relationship between stress, cytokine levels, and placental apoptosis during pregnancy has not been delineated, especially in efforts to improve the quality of pregnancy outcomes[14,15].

In Indonesia, the government has programmed iron tablets supplemented by 90 tablets of folic acid during pregnancy, operational implementation of the minimum standard of antenatal care[16,17]. This program that has been implemented has a coverage rate of 83.3% of the national target of 86%. However, when viewed from pregnant women's compliance to consume supplemented iron tablets and folic acid, only 19.3%[18]. The government program that has been running is expected to be more optimal if giving folic acid that is already in the iron supplemented tablet is not only to help reduce anaemia in pregnant women but also to prevent adverse impacts on prenatal stress conditions[19,20].

Providing folic acid as one of the antioxidants in plasma increases 5-methyltetrahydrofolate (5-MTHF), reducing ROS and homocysteine levels to reduce TNF inflammatory cytokines and apoptosis[20,21]. To investigate folic acid administration in stress-exposed pregnancies in this study, mice (Mus Musculus) were used because of ethical constraints if the study sample was of pregnant women. Based on the description above, we aim to investigate the folic acid supplementation's effect in pregnant stress mice (Mus Musculus) on the apoptosis index and placental TNFα expression.

MATERIALS AND METHODS:
This research design is a laboratory experiment using a randomized post-test control group design. Twenty-one Mus musculus (Balb/c female mice) were used. These animals were approved for experimental use by the Animal Care and Use Committee (Ref no:458-KE), Airlangga University. Mice were randomly divided into three groups, with 7 mice in each group. The first group was normal mice, the second group was given stress, and the third group was given stress and folic acid supplementation (3mg/kg body weight/day in 0.5ml suspension).

The study's inclusion criteria were healthy female mice, 10-12 weeks old, 25-30mg mice body weight, and 10 days of gestation. While the exclusion criteria were used as experimental animals in other studies and the drop-out sample criteria were mice giving birth before 16 days' gestation, mice were sick, and mice died. The research was conducted at the Laboratory of Experimental Animal and Veterinary Pathology, Faculty of Veterinary Medicine, Airlangga University, Surabaya Indonesia.
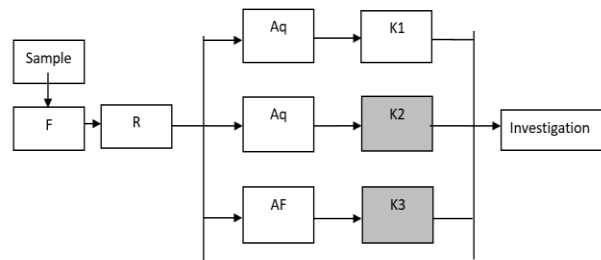

Figure 1: Research Design Framework

Information:
S: Samples
R: Randomization
F: Fertilization
Aq: Aquades
AF: Folic Acid
K1: Pregnant mice that do not receive stress treatment and do not get folic acid
K2: Pregnant mice who received stress treatment and did not get folic acid
K3: Pregnant mice that receive stress treatment and get folic acid

The ovulation was induced by pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotrophin (HCG) two days after PMSG. All mice were paired with males of their strain overnight. To check whether they mated or not, the vaginal plug was checked in the morning, if the plug was confirmed, the gestational day was termed as gestational day 1. The stress was given by immobilizing the rats in transparent pipes (the diameter was 3,5cm and the length was 10 cm) and gave bright light (300-400 lux) for 30 minutes, twice a day on gestational day 10-15. On gestational day 16, the mice were sacrificed, and the placenta tissue was taken.

The expression of TNF-α and apoptotic index was evaluated by immunohistochemistry assay. The pattern of TNF-α and apoptotic index was categorized as positive or negative. Degree of TNF-α and apoptotic index was expressed as % positive cells. Depending on % stained cells, a score was given (1: <10%, 2: 11-15%, 3: 51-80%, and 4: >80%). The intensity of staining was graded as 1+, 2+, and 3+. Immunoreactivity score was calculated by multiplying the score given for % stained cells and the intensity of staining. The immunoreactive scores of TNF, TNF-α, and apoptotic index were analyzed using one-way ANOVA. Significant

differences obtained in the one-way ANOVA were followed by Games Howel post hoc analysis. All significance test was considered significant if the P-value <0.05.

RESULTS:
Body Weight of Pregnant Mice:
The following is the data on body weight of pregnant mice in the three experimental groups on day 10. The table above shows that the samples are normally distributed, and there is no difference in the mice's body weight in the three groups.

Table 1: Body weight of mice

Data	K1	K2	K3
Mean	27,93	27,56	27,75
SD	1,65	1,47	1,78
p (kolmogorof-smirnov)	0,991	0,998	0,933
p (anova)	0,914		

Placental TNFα Expression:
The immunohistochemical assessment of TNFα expression using the Immuno Reactive Score (IRS) method or the Remmele scale Index is shown in the following table.

Table 2: TNFα Expressions

S. No.	K1	K2	K3
1.	1,7 ± 1,66	7,5 ± 1,58	3,7 ± 2,04
2.	1,9 ± 0,31	5,4 ± 0,96	2,2 ± 2,09
3.	2,1 ± 0,31	5,2 ± 1,03	1,6 ± 1,89
4.	2,4 ± 0,66	5,2 ± 1,03	5,7 ± 2,62
5.	2,5 ± 0,66	6,3 ± 0,94	5,7 ± 1,70
6.	2,5 ± 0,66	6,9 ± 1,44	2 ± 0,66
7.	1,8 ± 0,42	4,8 ± 1,03	2 ± 4,08
	Average 2,1 ± 0,668	**Average 5,9 ± 1,144**	**Average 3,2 ± 1,978**

The results of the examination showed that the mean value of the control group (K1) was 2.1±0.668; the treatment group with stressors (K2) of 5.9±1.144; and the treatment group with the administration of stressors and folic acid 3 mg/kg BW (K3) of 4.5±2.154. The Kolmogorov-Smirnov normality test results on K1 showed p = 0.542, while K2 showed p = 0.689 and K3 showed p = 0.637 (p> 0.05), which means that the data from the three groups were normally distributed but not homogeneous. The results of the statistical analysis using the One Way Anova (Brown Forsthy) test showed a significant difference with p = 0.000 (p<0.05). To determine the differences between groups, it was continued with the Post Hoc (Games Howel) test, which resulted in a significant difference between K1 and K2 (p = 0.000) and K2 and K3 (p = 0.019) but there was no difference between K1 and K3 (p = 0.283).
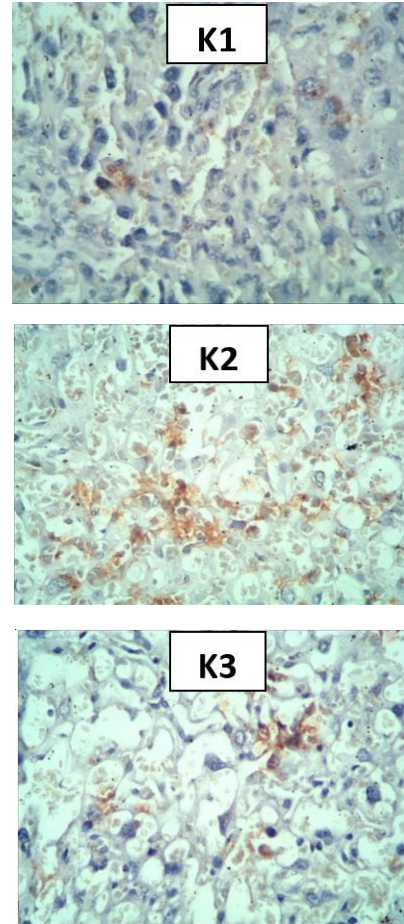

Figure 2: Examination Image of TNFα Expression

The picture above shows placental trophoblast cells in the three research groups. Positive TNF-α expression is characterized by expression in the form of chromogen chocolate on celltrophoblasts. (IHC: 400x).

Placental Apoptosis Index:
Assessment of the placenta's apoptotic index by counting the number of cells experiencing apoptosis in 10 different fields of view seen with a microscope magnification 1000 times divided by the total number of cells, multiplied by 1000. With the TUNEL assay method, apoptotic cells will be stained from dark brown to blackish.

Table 3: Placental Apoptosis Index 5.2, Placental Apoptosis Index

No.	K1	K2	K3
1.	1,8 ± 1,31	5,4 ± 0,96	2,5 ± 0,84
2.	4,5 ± 2,12	6,9 ± 1,44	2,8 ± 1,03
3.	2,1 ± 0,32	7,2 ± 1,54	2,6 ± 0,84
4.	2,9 ± 1,19	5,0 ± 1,05	3,9 ± 0,31
5.	2 ± 0	5,8 ± 0,63	4,0 ± 0
6.	1,7 ± 1,06	6,9 ± 1,44	3,6 ± 0,84
7.	3,8 ± 1,75	6,9 ± 1,44	2,7 ± 0,94
	Average 2,68 ± 1,107	**Average 6,3 ± 1,214**	**Average 3,2 ± 0,685**

The results of the examination showed that the mean value of the control group (K1) was 2.68±1.107; the treatment group with stressors (K2) was 6.3±1.214, and the treatment group with the administration of stressors and folic acid 3 mg/kg BW (K3) of 3.2±0.685. The Kolmogorov-Smirnov normality test results on K1 showed p = 0.729, while K2 showed p = 0.857 and K3 showed p = 0.741 (p> 0.05), which means that the data from the three groups were normally distributed. The statistical analysis results using the ANOVA test showed a significant difference with p = 0.000 (p<0.05). To determine the differences between groups, it was continued with the Post Hoc test, which resulted in a significant difference between K1 and K2 and K2 with K3 (p = 0.000) but there was no difference between K1 and K3 (p = 0.336).
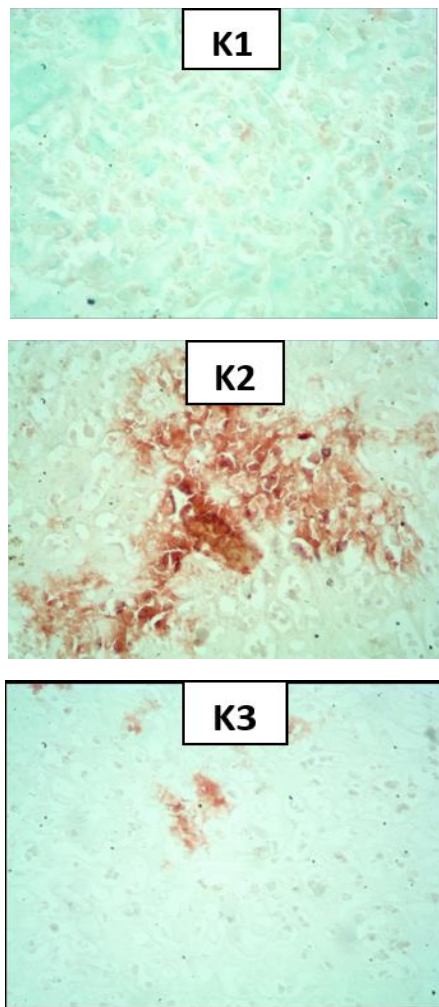

Figure 3: Image of Apoptosis Index Examination

The picture above shows the placental trophoblast cells in the three research groups; the change in colour to brown shows the cells undergoing apoptosis. (IHC: 400x)

Correlations between TNFα expression and placental apoptotic index in pregnant mice were given stressors and folic acid:
Statistical analysis to determine the relationship between variables using the Pearson correlation test. The statistical test results showed no significant relationship with p = 0.078 (p<0.05). There is no relationship between TNFα expression and placental apoptotic index in pregnant mice given stressors and folic acid.

DISCUSSION:
This study discusses folic acid's effect on TNFα expression and placental apoptotic index in pregnant mice that receive stressors. The study was conducted on 21 pregnant mice, which were divided into three groups: the control group (K1), the stressor group (K2), and the stressor and folic acid group (K3). The research design used a randomized post-test control group design. The experimental animals used were female balb/c white mice (Mus musculus) that had never been pregnant. The mice were acclimatized first for one week. Mice were injected with Pregnant Mare's Serum Gonadotropin (PMSG), 48 hours later, Human Gonadotropin (HCG) was injected, which functioned to synchronize ovulation. Female mice are mated with male mice. Each pair is placed in a different cage. In the next 17 hours a vaginal plug was seen, female mice with a vaginal plug were sampled and considered the 0th day of pregnancy. The number of each group was seven samples of pregnant mice.

In our research proposal, the stress exposure used is immobilization and the bright light of 300-400 lux on a transparent cylinder with a diameter of 3.5cm and a length of 10cm. for 45 minutes, 3 times a day, at the age of 10-15 days of gestation which is expected to have an impact on stress both physically and psychologically[22,23]. The results of stressor exposure to 14 pregnant mice showed that 3(21.43%) mice died, 3(21.43%) mice gave birth prematurely. On the 16th day of surgery, there were 6(42.86%) abortions and 2(14.28%) there were fetuses defects. In these preliminary studies, we reduced stress exposure in the form of immobilization and bright light from 300-400 lux on a transparent cylinder with a diameter of 3.5cm and a length of 10cm. for 30 minutes, 2 times a day, morning and evening. Performed 10-15 days of gestation.

Giving folic acid as one of the antioxidants in plasma can reduce the inflammatory cytokines TNF and apoptosis[14,24,25]. The dose of folic acid given to the parent animal during pregnancy is 3mg/kg/day, 1 time a day before morning stressors and given per round from the 10-15th day of gestation. Folic acid is used in tablets that have been crushed and emulsified with 0.5% CMC to make the solution homogeneous[26]. The maximum

volume given is 0.5ml for each mouse. Surgery for mice was performed at 16 days of gestation. At 17 to 21 days of gestation, the placenta's function is useful in distributing nutrients, producing hormones to maintain pregnancy, and transfer water used to expand the fluid space[10,27]. In the preliminary study, there were many preterm births, so that surgery was performed at 16 days of gestation to anticipate a reduced sample.

Prenatal stress can cause placental dysfunction and fetal endocrine disorders[28,29]. The results showed that the samples for TNFα expression were normally distributed in each group, but not homogeneous. The statistical test used was One Way Anova (Brown Forsthy), which showed significant differences between the three groups. The expression of placental TNFα in stress pregnant mice was higher than in normal pregnancy. In several other studies, prenatal stress conditions can activate the HPA (Hypothalamus-Pituitary-Adrenal) axis resulting in increased glucocorticoid (cortisol). This can increase Reactive Oxygen Species (ROS). It triggers the secretion of proinflammatory cytokines by regulating NF-kB, especially Tumor Necrosis Factor (TNFα)[10,30].

In pregnant mice exposed to stressors and given folic acid, placental TNFα expression was lower than those without folic acid. Placental TNFα expression in normal pregnancy was lower than stress pregnancy with folic acid, but there was no statistically significant difference. This is according to the administration of folic acid as an antioxidant in plasma to increase 5-methyltetrahydrofolate (5-MTHF), which reduces ROS and homocysteine levels and it can reduce TNF inflammatory cytokines[21,31]. Folic acid can significantly reduce lipopolysaccharide (LPS), which can increase TNFα, interleukin (IL) -1β, and IL-6 in the placenta, maternal serum, and amniotic fluid. Supplements with high doses of folic acid during pregnancy can protect the fetus from anti-inflammatory and antioxidative stress. Pregnant women with a high risk of developing NTD benefit more from giving high doses of folic acid (4-5 mg/day). Giving folic acid to high-risk pregnant women can reduce the risk of NTD and the level of protection increases with increasing doses. Folic acid can decrease placental TNFα expression in stressor-exposed pregnancy[20,32].

The placental apoptotic index of mice in the three treatment groups also showed a significant difference. All samples in each group were previously stated to be normally distributed and homogeneous. Due to stressors, high placental TNFα expression through the extrinsic pathway can increase the apoptotic index[10,33]. The placental apoptosis index results in stressed pregnant mice were higher than those in normal pregnancy. In pregnant mice exposed to stressors and given folic acid, the placenta apoptosis index was lower than those without folic acid. Although there were no significant differences in statistical tests, the apoptotic index of the placenta in normal pregnancy was lower than in those exposed to stressors and received folic acid. However, other studies on chronic stress exposure through immobilization in mice did not significantly affect the apoptotic process[7,14].

Long-term folic acid administration during pregnancy protects against various complications associated with decreased placental development and is also associated with decreased apoptosis in the placenta[22,34]. Previous research data showed that the administration of a combination of DHA and 5-MTHF (primary biologically active folic acid) in pregnant women impacts placental proliferation[3,35]. In stressful conditions, it can increase cortisol levels in plasma and be followed by an increase in homocysteine levels. Folic acid administration (20nmol/l) inhibited the effects of homocysteine on human placental trophoblasts[1,10]. These results suggest that folic acid administration reduces trophoblast apoptosis associated with homocysteine. Other studies have also explained that increased homocysteine levels in the serum of pregnant women are a risk factor for placental malfunction and abnormalities in the fetus. Apoptotic activation inhibits proliferation, damaging the placental trophoblast. Even in experimental studies on human trophoblast cultures, homocysteine thiolactone increases apoptosis, and this effect can be reduced by administering antioxidants[36,37].

In this study, the TNFα expression value was directly proportional to the placental apoptotic index, but there was no significant relationship between these variables. TNFα via extrinsic pathway induces apoptosis. TNF cytokines have specific receptors in the cell membrane, whose results can be apoptosis. Our findings are expected to become a reference in similar research that is more comprehensive and at a higher level of experimental animals and can also be used as a basis for epidemiological research. There are still several limitations, including preliminary studies specifically for stressors and the type of examination used.

CONCLUSION:
Folic acid supplementation reduces the expression of TNF-α and apoptotic index in placenta with maternal stress. There is a positive correlation between TNF-α and apoptotic index in placenta with maternal stress. Further research is warranted to confirm these results before its recommendation to clinical using.

CONFLICT OF INTEREST:
The authors declared there were no competing interests in the study.

ACKNOWLEDGEMENTS:

ETHICAL CLEARANCE:
This research was declared an ethical pass test by the Committee on Animal Care and Use (ACUC) of Faculty of Veterinary Medicine Universitas Airlangga, Surabaya East Java Indonesia with Reference number: 458-KE.

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