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The Ratio of Bax And Bcl-2 in the Cerebrum of Newborn Rattus Novergius Given Mackerel Oil During Pregnancy

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Abstract

The quality of a nation is determined by nutritional intake during pregnancy. Pregnancy is the right time to prepare the brain's potential optimally, one of which is the intake of mackerel oil which is rich in omega-3. Omega-3 fatty acids can stimulate gene expression and nerve activity, increase synaptogenesis and neurogenesis, and prevent apoptosis. This study aims to analyze the intake of mackerel oil during pregnancy to the ratio of Bcl-2 / Bax in the cerebrum. This research is a true experiment with a posttest-only control group design. 30 pregnant Rattus norvegicus mothers aged 2-3 months were divided into 3 groups, namely the control group (K), the mackerel fish oil group (P1), and the omega-3 supplement group (P2). This research was conducted at the Experimental Animal Cages and Pathology Laboratory, Faculty of Veterinary Medicine, Airlangga University. The mean Bcl-2 / Bax cerebrum ratio was 1.127 ± 0.342 (K), 3.928 ± 1.984 (P1), and 2.526 ± 1.122 (P2). The ANOVA test on the Bcl-2 / Bax ratio in the cerebrum showed a significant difference with a p-value of 0.000. The Bcl-2 / Bax ratio in the cerebrum of Rattus norvegicus newborns treated with mackerel oil on Rattus norvegicus mothers during pregnancy showed higher results than the other groups.

Keywords: Cerebrum; Mackerel Oil; Bax, Bcl-2

Kualitas suatu bangsa ditentukan oleh asupan nutrisi selama masa kehamilan. Kehamilan merupakan masa yang tepat untuk menyiapkan potensi otak dengan optimal, salah satunya dengan asupan minyak ikan kembung yang kaya akan omega-3. Asam lemak omega-3 dapat merangsang ekspresi gen dan aktivitas saraf, meningkatkan sinaptogenesis dan neurogenesis, serta mencegah terjadinya apoptosis. Penelitian ini bertujuan untuk menganalisis asupan minyak ikan kembung selama kebuntingan terhadap rasio Bcl-2/Bax di *cerebrum*. Penelitian ini merupakan *true experiment* dengan *posttest-only control group design*. 30 induk *Rattus norvegicus* bunting usia 2-3 bulan dibagi menjadi 3 kelompok yaitu kelompok kontrol (K), kelompok minyak ikan kembung (P1), dan kelompok suplemen omega-3 (P2). Penelitian ini dilakukan di Kandang Hewan Coba dan Laboratorium Patologi, Fakultas Kedokteran Hewan, Universitas Airlangga. Rerata rasio Bcl-2/Bax *cerebrum* yaitu 1,127±0,342 (K), 3,928±1,984 (P1), dan 2,526±1,122 (P2). Uji ANOVA pada rasio Bcl-2/Bax di *cerebrum* menunjukkan bahwa terdapat perbedaan signifikan dengan *p-value* 0,000. Rasio Bcl-2/Bax di *cerebrum Rattus norvegicus* baru lahir yang diberi minyak ikan kembung pada induk *Rattus norvegicus* selama kebuntingan menunjukkan hasil lebih tinggi dibanding kelompok lain.

Kata Kunci: Cerebrum; Minyak Ikan Kembung; Bax; Bcl-2

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Introduction

The success of national development of a nation is determined by the availability of good quality Human Resources (HR) from the fields of education and health. Efforts that can be made to improve the quality of human resources include improving nutritional status and brain quality, starting from the conception phase (UNDP, 2018). Adequate nutrition is the main and most important factor that has a role in determining the success of body and brain growth in the fetus. Nutritional intake affects fetal brain development during its early life in the womb (Syari et al., 2015).

The development of brain cells undergoes a series of processes such as proliferation, migration, synaptogenesis, and apoptosis during pregnancy (Stiles & Jernigan, 2010). Pregnancy is the right time to prepare brain potential optimally and is a window of opportunity for parents (Hidayati & Joewono, 2018). Providing optimal nutrition is one of the factors that plays a strong role in brain development during pregnancy (Cusick & Georgieff, 2016).

Nutrition plays an important role in the human life cycle, especially the brain because it determines the quality of human resources in the future as the next generation of the nation. Adequate nutrition is needed to achieve good growth and development. Food that is lacking both in quality and quantity will cause nutritional problems. The state of malnutrition can result in structural and functional changes in the brain (Georgieff, 2007). In addition, malnutrition can affect a low level of intelligence because brain growth and development have started from the time in the womb (Cao et al., 2009).

Brain development is influenced by the mother's nutritional intake during pregnancy. One of the most important nutrients for brain growth is omega-3 fatty acids. Omega-3 is a polyunsaturated fatty acid (PUFA) that contains Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA). This fatty acid is not produced by the body so it requires intake of omega-3 fatty acids from outside. Fatty acids consumed during pregnancy will be transferred by the mother to the fetus through the placenta. Studies on the consumption of fish oil containing EPA and DHA have been shown to increase fetal brain growth and development (Arterburn et al., 2006).

Omega-3 fatty acids can induce the expression and activation of Uncoupling Proteins (UCPs) to reduce Reactive Oxygen Species (ROS), reduce neuronal dysfunction, and induce neuroprotective effects. Omega-3 fatty acids can also activate Peroxisome Proliferator-Actived Receptor α (PPARα) and induce up-regulation of energy transcripts so as to increase energy reserves, stabilize synapse function, and limit hyperexcitability, and can reduce brain edema, release of cytochrome c, and decrease pro-apoptotic protein Bax and increased anti-apoptotic Bcl-2, strengthen AMPK which can regulate cell synthesis thereby inhibiting aerobic glycolysis (Tan et al., 2019).

Programmed cell death in the process of apoptosis provides signals and is mediated by several genes that code for proteins. Apoptotic events occur after the disruption of the mitochondrial membrane barrier function by releasing cytochrome c through the caspase-3 pathway in normal cells (Xiao & Zhang, 2008). The main process of apoptosis is controlled by Bcl-2 group proteins. Changes in mitochondrial membrane conformation depend on the ratio between the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 (D'Archivio et al., 2008).

Omega-3 fatty acids can be found in significant quantities in marine fish such as mackerel. The highest amount of omega-3 mackerel is 414.7 and 956.0 mg/100 g for EPA and DHA, respectively (Rincón-Cervera et al., 2020). Based on data released by the Nutrition Institute of the Ministry of Health of the Republic of Indonesia, several types of Indonesian sea fish have a high content of omega-3 fatty acids (up to 10.9 g/100 g) including mackerel (Hafiludin, 2011). In this study mackerel was chosen because it has a high omega-3 content, is easy to obtain, and has a relatively low price. Ethically, this research could not be carried out on humans, so this research was carried out on experimental animals, Rattus norvegicus.

Methods

This research is an experimental laboratory with a posttest-only control group research design. This study used experimental animals which were divided into 3 groups. The first group was not given any treatment acting as the control group (K), while the other 2 groups were given a different treatment, namely the second group was given mackerel oil (P1) and the third group was given omega-3 supplements (P3).

The experimental animals in this study were adult female Rattus norvegicus aged 2-3 months of the Sprague Dawley strain with a pre-pregnancy weight of 120-130 grams. Giving treatment to the mackerel oil group (K2) and the omega-3 supplement group (K3) at the age of 1-17 days of gestation, and the group without treatment were only given standard feed. Before the treatment was given, the rats were adapted for one week and randomly divided (simple random sampling) to be included in the study group. Each group consisted of 10 Rattus norvegicus broodstock. The inclusion criteria in this study were normal and healthy female rats, had never given birth, and had never been used as experimental animals for other studies.

The experimental animals performed estrus synchronization using PMSG and hCG and then mated with male rats with the same age range. After mating, vaginal obstruction was observed as a sign of pregnancy. There were no sick mother rats, dead rats in utero (IUFD), and born before 18 days of gestation (premature) in this study. Furthermore, soon the child Rattus norvegicus was born by sectio caesarea at the 18th day of gestation. All pregnant Rattus norvegicus parents were weighed before being sacrificed. Three children were selected from each parent with the largest weight, one medium weight, and the smallest weight, then decapitation was carried out. Each of the three brains of Rattus norvegicus children from one parent was made into one preparation and stained. The brain tissue was taken for examination of the Bcl-2/Bax ratio by immunohistochemistry.

The research was located at the Experimental Animal Cages and Pathology Laboratory, Faculty of Veterinary Medicine, Airlangga University, Surabaya, which was conducted from April to September 2020. This research received ethical approval from the Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University, Surabaya with number 2. KE. 034.04.2020.

The dosage calculation is based on the Food and Agriculture Organization of the United Nations (FAO) for pregnant women, namely EPA+DHA of 300 mg/day. For administration to experimental animals, it has been converted by multiplying by 0.018 per 200 grams of rat body weight, so that the dose of mackerel oil in experimental animals is 3.24 mg/120 grams BW/day. The fatty acid content of mackerel extract was determined by GC-MS. The omega-3 supplement group was given a dose based on supplements containing 360 mg EPA and 240 mg DHA, namely 6.48 mg/120 BW/day after being converted to experimental animals.

The data for each research subject is the average Immuno Reactive Score (IRS) observed in five visual fields with 400x magnification. Statistical calculation using SPSS software tools. A normality test was performed with Shapiro-Wilk. If the data is normally distributed, the analysis of differences in the Bcl-2/Bax ratio between groups uses the one-way Analysis of Variance (ANOVA) test and is continued with a different test for each group. The significance limit is 0.05 with a 95% confidence level.

Results and Discussion

This study used the parent Rattus norvegicus Sprague Dawley strain with an initial weight range of 120-130 grams. During pregnancy, the weight of the Rattus norvegicus brood ranges from 231-240 grams. After being treated for 17 days with mackerel oil and omega-3 supplements, all pregnant Rattus norvegicus parents were weighed before being sacrificed. Based on Table 1, the results of the descriptive analysis of characteristic data showed that the highest mean initial and final body weight of Rattus norvegicus came from the group that was given mackerel oil treatment, namely 127.00 grams and 253.70 grams, respectively.

After weighing the mother rat's body weight, the baby rats were born by sectio caesarea. Based on Table 2, shows that the highest average body weight, body length, and head weight of Rattus norvegicus children came from the treatment group given mackerel oil with respective averages of 4.27 grams, 4.59 cm, and 1.22 grams

Table 1. Characteristics of the parent Rattus norvegicus

Animal Group	Mean ± SD Initial Body Weight	Mean ± SD Final Body Weight
K	124.50±3.60	231.60±45.87
P1	127.00±2.75	253.70±94.49
P2	125.60±3.17	240.00±52.01

Table 2. Characteristics of newborn Rattus norvegicus

Animal Group	Mean ± SD Body Weight	Mean ± SD Body Length	Mean ± SD Head Weight
K	2.78±1.26	3.55±0.71	0.85±0.26
P1	4.27±1.29	4.59±0.41	1.22±0.40
P2	3.66±1.27	3.88±0.72	1.01±0.41

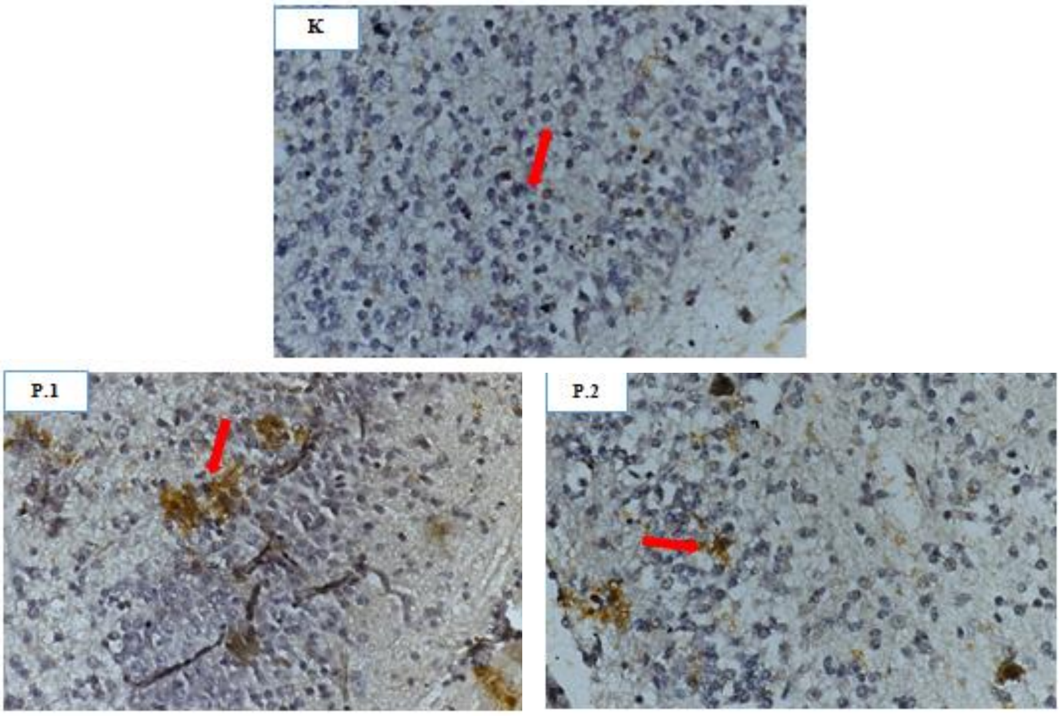

Figure 1. Histological picture of Bcl-2 expression in the cerebrum of newborn Rattus norvegicus (K, P.1, P.2) by immunohistochemical examination. 400x magnification. The red arrow indicates the presence expression of Bcl-2 in the cerebrum which is marked by the presence of brown chromogen.

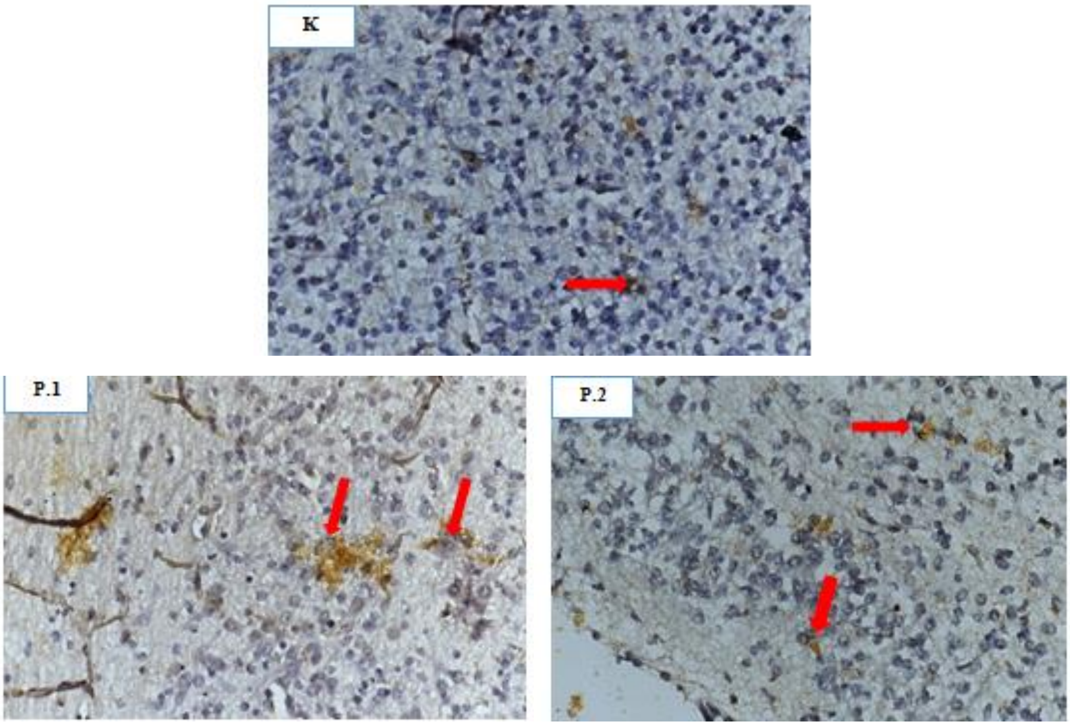

Figure 2. Histological picture of Bax expression in the cerebrum of newborn Rattus norvegicus (K, P.1, P.2) by immunohistochemical examination. 400x magnification. The red arrow indicates Bax's expression on *cerebrum*indicated by the presence of a brown color chromogen.

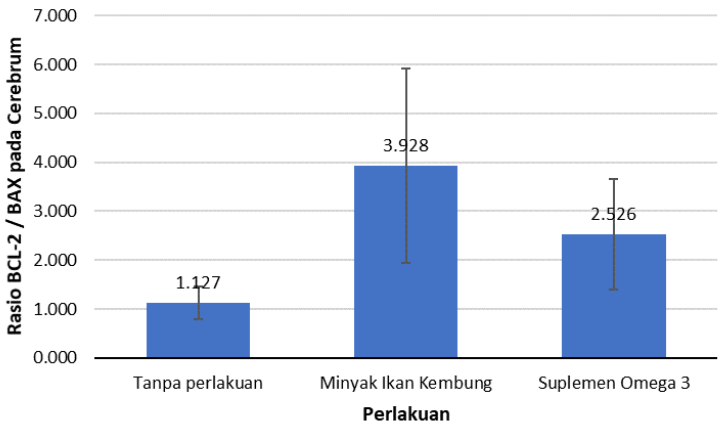

Figure 3. Graph of the mean Bcl-2/Bax ratio in newborn Rattus norvegicus cerebrum

Table 3. Statistical test results of the ratio of Bcl-2/Bax in newborn Rattus norvegicus cerebrum

Animal Group	*Shapiro-Wilk*	ANOVA test	Test Dunnet T3
K	0.167		1.127±0.342
P1	0.074	0.000	3,928±1,984
P2	0.496		2.526±1.122

Histological expression of Bcl-2/Bax in newborn Rattus norvegicus cerebrum (K, P.1, P.2) was performed by immunohistochemical examination (IHC). The red arrows indicate the presence of Bcl-2/Bax expression in the cerebrum which is indicated by the presence of brown chromogen. Figure 3 shows the highest average Bcl-2/Bax ratio in the cerebrum of rats given mackerel oil of 3.928.

Normality test using Shapiro-Wilk on Bcl-2/Bax ratio data in each group. In the normality test, it was obtained that the p-value was > 0.05 in each group, indicating that the data distribution was normal. Furthermore, the statistical analysis used was ANOVA parametric test which aims to assess differences between groups. The results of the ANOVA test obtained a significance value of 0.000 which indicated that there was a significant difference between groups in the Bcl-2/Bax ratio in the cerebrum of Rattus norvegicus. Therefore, it was continued with the Dunnett T3 test (Table 3).

Administration of EPA and DHA during pregnancy is necessary to support the processes of neurogenesis and synaptogenesis which have effects on monoaminergic, cholinergic, and GABAergic neurotransmitters (Healy-Stoffel & Levant, 2018). EPA and DHA are concentrated in the brain and have anti-oxidative, anti-inflammatory, and anti-apoptotic effects (Crupi et al., 2013).

The control and regulation of apoptosis in the intrinsic pathway occurs via the Bcl-2 family of proteins. This pathway occurs due to increased mitochondrial permeability and the release of pro-apoptotic molecules into the cytoplasm, without the role of death receptors. Growth factors and several other signals stimulate the production of anti-apoptotic proteins, namely the Bcl-2 group. Bcl-2 is an anti-apoptotic protein and Bax is a pro-apoptotic protein. This protein has a special meaning because it can determine whether cells carry out apoptosis or not (Elmore, 2007).

Anti-apoptotic proteins are normally located in the mitochondrial and cytoplasmic membranes. When cells experience a decrease in survival signal or experience stress, Bcl-2 and or Bcl-xl disappears from the mitochondrial membrane and is replaced by pro-apoptotic members such as Bax which were previously present in the cytosol. When an apoptotic signal is present, pro-apoptotic protein translocation occurs from the cytosol to the mitochondrial membrane (Bouchier-Hayes et al., 2005).

The ratio of the anti-apoptotic Bcl-2 family to the pro-apoptotic Bcl-2 determines whether or not an apoptotic process takes place. Cells with more pro-apoptotic proteins will be sensitive to apoptosis. In the mechanism of action Bcl-2 family members demerise to determine the apoptotic pathway. Bcl-XL homodimer will suppress apoptosis or active death cell (ACD) and Bcl-XL/Bax heterodimerization will inhibit apoptosis. Homoditerous Bax will activate apoptosis, and Bcl-XL/Bax heterodimerization will inhibit apoptosis. The combination of the two will induce apoptosis (Ehrlich, 2013).

Bcl-2 and its relatives comprise the Bcl-2 family of proteins, having a role in controlling outer mitochondrial membrane integrity and apoptosis (Chipuk et al., 2010). Over-expression of Bcl-2 prevents permeability transitions in the membrane and prevents apoptosis by preventing the formation of pores in the membrane by Bax and preventing the release of cytochrome c and Apoptosis Inducing Factor (AIF) from mitochondria during apoptosis (Reed, 2000).

Apoptosis is an active form of cell death in which regulation of specific proteins generates anti- or pro-apoptotic signals. Two of the protein families involved in this regulation are the Bcl and caspase proteins. Research by Mooney & Miller, (2000) showed that bax expression increased and the ratio of Bcl-2 expression to Bax expression decreased. Studies of human brain tissue and experimental animal models have proven that the Bcl-2 family regulates cell death by apoptosis in the nervous system. Bcl-2 acts as an anti-apoptotic agent while Bax acts as a pro-apoptotic agent. The Bcl-2 gene is important in the regulation of apoptosis which encodes various proteins that play a key role in the regulation of cell apoptosis. The Bax gene is expressed in the brain and identified as a pro-apoptotic homologue to Bcl-2. Interactions between Bcl2 family members both in the cytosol and in mitochondria determine survival or death (Akhtar et al., 2004). The Bcl-2/Bax ratio determines cell death or survival after an apoptotic stimulus (Mahdavi et al., 2018).

Increased caspase-3/7 activity and changes in the Bcl-2/Bax ratio could be key determinants in cytochrome c release, caspase-3/7 activation, and the initiation of apoptosis. Decreasing this ratio can exacerbate apoptosis, and increasing this ratio can reverse the deleterious effects of cytotoxic stimuli (Eleawa et al., 2014). Whereas Bax has been shown to trigger cell death, anti-apoptotic Bcl-2 can block cytochrome c release and caspase activation (Yiran et al., 2013)

Conclusion

The Bcl-2/Bax ratio in the cerebrum of Rattus norvegicus newborns who were given mackerel oil during pregnancy was higher than those who were not given mackerel oil and omega-3 supplements.

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