ORIGINAL ARTICLE:

Apoptosis index of cerebrum and cerebellum neuronal cells in *Rattus norvegicus* neonates born from mothers treated with 50% food restriction during gestation

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ABSTRACT

Objective: To analyze the difference of neuronal apoptotic index on *Rattus norvegicus* offspring in cerebrum and cerebellum between food restriction 50% group and control.
Materials and Methods: An analytical experimental study with single blind randomized post test only control group using animals subjects *Rattus norvegicus*. This study was conducted at animal laboratory, Veterinary Faculty, Universitas Airlangga. Animal subjects were divided into food restriction 50% group and control. The apoptotic index was analyzed using comparison test, with significancy p<0.05.
Results: There was significant difference of neuronal apoptotic index on *Rattus norvegicus* offspring between FR 50% group and control in cerebrum with p= 0.002 (mean 6,12±3,51 in FR 50% group, 2,81±2,16 in control group), and cerebellum with p= 0.026 (mean 5,56±3,03 in FR group, 3,43±2,58 in control group).
Conclusions: Food restriction 50% during gestation has significant influence on neuronal apoptotic index in cerebrum and cerebellum of *Rattus norvegicus* offspring.

Keywords: Food restriction 50%; prenatal; apoptotic index; *Rattus norvegicus.*

ABSTRAK

Tujuan: Menganalisis perbedaan indeks apoptosis sel neuron pada cerebrum dan cerebellum *Rattus norvegicus* baru lahir pada kelompok dengan perlakuan food restriction 50% selama kebuntingan dan tidak mendapat perlakuan.
Bahan dan Metode: Penelitian analitik eksperimental dengan desain single blind randomized post test only control group menggunakan hewan coba *Rattus norvegicus* di kandang hewan coba Fakultas Kedokteran Hewan Universitas Airlangga. Kelompok hewan coba dibagi dua yaitu kelompok kontrol tanpa diberi perlakuan, dan kelompok perlakuan yang mendapat perlakuan food restriction 50% sejak dinyatakan bunting. Penelitian ini menggunakan uji komparasi dalam menganalisis indeks apoptosis
Hasil penelitian: Didapatkan perbedaan bermakna indeks apoptosis sel neuron di cerebrum dengan nilai p=0.002 (mean 6,12±3,51 pada kelompok perlakuan, 2,81±2,16 pada kelompok kontrol), begitu pula di cerebellum dengan nilai p=0.026 (mean 5,56±3,03 pada kelompok perlakuan dan 3,43±2,58 pada kelompok kontrol)
Simpulan: Food restriction 50% selama kebuntingan berpengaruh terhadap indeks apoptosis sel neuron di cerebrum dan cerebellum *Rattus norvegicus* baru lahir secara bermakna.

Kata kunci: Food restriction 50%; cerebrum; cerebellum; indeks apoptosis; *Rattus norvegicus*

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INTRODUCTION

The quality of human resources (HR) is strongly influenced by various factors early in life of the fetus in the mother's womb, even since the preconception phase. One factor is the health status and nutrition of pregnant women. One way to obtain an intelligent generation is to prepare the fetus from the inside of the womb. Intelligence is influenced by nature (genetic) and nurture (environment) factors. Of the two factors, the factor that can be modified is the nurture (nutrition, stimulation, and disease). Poor prenatal environmental conditions will result in long-term effects on the individual during post-natal development. The number of neurons, glia, dendrites and the ratio of glia per neuron is the benchmark of intelligence from the prenatal psychoneurological side. Thus, one attempt to educate the fetus is to increase the number of neurons and reduce the apoptosis of neuron cells. The process of human brain development starts from the 17th day of gestation.

The human brain comes from a layer of ectoderm cells called neural plates. In the womb, brain cells undergo proliferation, migration, synaptogenesis and apoptosis. Since the age of 6 months of pregnancy, there is a process of the formation of connection between cells (synapses) that play a role in various functions. The process of migration to areas of the brain, such as cerebrum, cerebellum, hippocampus and other structures, is almost entirely completed at the time of term pregnancy. In the growth proliferation phase, neuron cell form as much as 250.000 per minute starting at week 7 and 8, whereas in adult humans the number of brain cells reaches 10 billion neurons that are interconnected to form the brain organ. This number of neurons is less than the number of neurons formed during the proliferation phase of human brain growth.[1,2]

At the prenatal period there is a massive cutting of the number of neuron cells. This is thought to be due to the presence of programmed cell death known as apoptosis. Only half of the neuron cells will survive. In the organizational phase of brain development, neuronal cells form connections between cells known as synaptogenesis processes. The process of apoptosis occurs a lot in this phase. The more rich the neuron cells with dendritic site, the more synapses are formed, so the number of cells undergoing apoptosis will also decrease.[2]

The cerebrum is the largest part of the brain and occupies about 85% of human brain, which is divided into right and left hemispheres. The cerebrum is heavily involved in somato-sensory processes and motor information as well as intellectual awareness and function. Cerebellum is known as the center of motor and balance, while the role of cerebellum in cognitive function is still a debate. Timman and Daum (2007), who used PET (Positron Emission Tomography) scans and MRI (Magnetic Resonance Imaging), proved cerebellum involvement in aspects of cognitive functioning, particularly verbal memory, which ultimately affects cognitive function, although it is debatable and further studies are needed.[8.9]

Adequate nutrition is a good factor for fetal growth. Nutrition will affect brain function, namely cognitive, motor, and socio-emotional (an adult skill that has been pre-programmed during the prenatal period). Provision of complete and balanced nutrition from the womb until the age of 3 years will optimize the number of cells in the baby's brain as well as improve the quality of the development of brain circuits and the complexity of the synapse circuit that is formed.[3,5,6,28]

Lack of maternal nutrition is a condition in which there are one or more essential nutrients the body needs is present in less or no amount at all. Maternal perinatal undernutrition (MPU) may affect central nervous system (CNS) maturation, both morphologically and physiologically.[13]. The impact of these poor nutrients provides both long-term and short-term effects that depend on the window of incidence time during the period of rapid brain development among species. Cellular and molecular cellular disruption during this critical period can cause permanent damage and long-term disruption to adulthood, including behavioral and cognitive impairment with impaired fine motor skills, low IQ scores and attention deficit disorders.[10] Poor nutrition during pregnancy affects increased apoptosis of neuron cells. Antonow (2010) in his study who gave 30% food restriction (FR 30%) in baboons during pregnancy will lower the brain-derived neurotrophic factor (BDNF) by about 13.4% -26.3% and increase the neuronal cell apoptosis by 118% in baboon offspring.[11]

This study was a continuation of a series of research on enrichment in the womb in order to optimize fetal brain development, which aims to determine the effect of nutritional deficiencies on neuron cell apoptosis index on cerebellum and cerebellum of *Rattus norvegicus* offspring.

MATERIALS AND METHODS

This study was a laboratory experimental study with single blind randomized post test only control group design. The experimental animals used were *Rattus norvegicus* treated with 50% food restriction. Subjects were divided into two groups at random. The treatment group received a 50% food restriction treatment since it

was declared pregnant, and control group was not treated. The study was conducted at Experimental Animal Cage and Pathology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, from November 2016 to January 2017. The inclusion criteria were 2-month-old healthy *Rattus norvegicus*, weighing 134-160 grams, never giving birth, and newborn *Rattus norvegicus*. The exclusion criteria of the study were *Rattus norvegicus* females with anatomic disorders and illness before treatment.

Sample size was calculated based on replication formula of Steel and Torrie. The number of samples per group was found as many as 16. From each *Rattus norvegicus* mothers, two offsprings with the highest bodyweight were taken. Once born, the offsprings of the *Rattus norvegicus* were sacrificed by using chloroform, weighed, their brains were taken and histochemical preparations were made by TUNEL assay method. The apoptotic cells were stained dark brown to black. The apoptotic indexes was calculated on each sample, then compared between groups using statistical tests. Ethical eligibility was obtained from the Research Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga.

RESULTS AND DISCUSSION

Characteristics of the subjects

Rattus norvegicus mothers in this study gave birth at gestation day 19-21.

Table 1. *Rattus norvegicus* mothers' characteristics based on gestational age

Pregnancy Age	Control		Treatment	
	N	%	N	%
19 days	4	25	5	31.25
20 days	12	75	10	62.5
21 days	-	-	1	6.25

The mean gestational age of the *Rattus norvegicus* mothers in control and treatment group was 19.75 days. The weights of *Rattus norvegicus* mothers measured prior to the study were in accordance with those in Table 2. Distribution of the data was found by Saphiro Wilk test to be normal in both groups ($p>0.05$).

In order to identify data distribution of *Rattus norvegicus* offsprings' weights, normality test was performed using Shapiro-Wilk with significance $p=0.331$ ($p>0.05$) for control group and $p=0409$ ($p>0.05$) for treatment group, showing normal distribution ($p>0.05$). Then, homogeneity test was performed on body weight of treatment group and control group with Independent T test, which showed no significant result with $p=0.001$ ($p<0.05$). These results indicate that the offsprings' weight in treatment and control groups showed significant difference.

Table 2. Characteristics of *Rattus norvegicus*' body weight

Groups	Mother's BW(gram)	
	Mean	S/D
Control	146.25	± 6.94
Treatment	144.94	± 7.01

Table 3. Characteristics of *Rattus norvegicus* offsprings' body weight

Groups	Offspring BW (gram)	
	Mean	S/D
Control	4.73	± 0.49
Treatment	4.30	± 0,50

Analysis of the results on cerebrum

Normality test was performed using Shapiro-Wilk to observe the distribution of apoptotic index data in the cerebrum of both groups. The results showed $p=0.001$ in control group and $p=0.034$ in treatment group, which showed no normal distribution ($p<0.05$). To determine the difference of apoptotic index on the cerebrum of control and treatment group with abnormal data distribution, an analysis using Mann Whitney test was performed.

Table 4. Mean apoptotic index on cerebrum in both groups

Groups	Mean ± S/D	p value
Control	2.81±2.16	0.002
Treatment	6.12±3.51	

Table 4 shows significant differences in the apoptotic index in cerebrum between control and treatment groups (2.81±2.16 vs 6.12±3.51) with $p=0.002$ ($p<0.05$).

Analysis of results on cerebellum

Normality test was performed with Shapiro-Wilk to see the distribution of apoptotic index data on the cerebellum in both groups. The test yielded $p=0.003$ for control group and $p=0.132$ for treatment group. This shows in the apoptotic index that data on *Rattus norvegicus*' cerebellum had no normal distribution ($p<0.05$). To determine the difference of apoptotic index

of cerebellum in control and treatment groups with no normal data distribution, the Mann Whitney's test was performed.

Table 5. Mean apoptotic index of the cerebellum in both groups

Groups	Mean ± S/D	p value
Control	3.43±2.58	0.026
Treatment	5.56±3.03	

Table 5 shows that the apoptotic index in cerebellum demonstrated significant differences between control and treatment groups (3.43±2.58 vs 5.56±3.03) with p = 0.026 (p<0.05).

All of the *Rattus norvegicus* mothers used in this study gave birth at term, ie between 19-21 days of gestation. In control and treatment groups, mean age of gestation was 19.75 days. No mothers of *Rattus norvegicus* has given preterm, abortive or stillbirth. All treatment groups received less nutritional treatment with a 50% food restriction model. The weight of the mothers *Rattus norvegicus* was measured prior to the study. In this study, we did not measure the weight of the mothers' body after treatment or before delivery so the influence of food restriction 50% during pregnancy on the mothers' weight was not identified.

The weighing of *Rattus norvegicus* offsprings was done as soon as possible after *Rattus norvegicus*'s offsprings were born, with the aim of reducing weight bias. Subsequently, two *Rattus norvegicus* newborns were chosen with the heaviest weights and then immediately sacrificed by means of decapitation. 50% food restriction would theoretically reduce the weight of *Rattus norvegicus*'s offspring. The weight data of *Rattus norvegicus* offsprings were then analyzed using an comparative Independent T-test to determine differences between control and treatment groups. The results showed that the weight of *Rattus norvegicus* offsprings in treatment group was significantly lower than those in control group, ie p=0.001 (p<0.05). The differences were obtained because in the treatment group 50% food restriction had effect on the offsprings' weight. This was in accordance with the results of a study from Jahnke (2007), who found that mice birth weights were found lower and the weights decreased significantly from birth to day 21 in group receiving less nutritional treatment than in control.[24]

The composition of nutrients in food has 6 main components, the carbohydrates, proteins, fats, minerals, vitamins, and water. The main sources of calories are carbohydrates and fats, while protein is needed for growth and development. Different food combinations are important to meet all nutritional needs. In this study, a 50% food restriction led to a decrease in all nutritional components received by mice during gestation.

This study was conducted from the beginning of the *Rattus norvegicus* gestation until the birth of the *Rattus norvegicus*' offspring to determine the effect of nutritional deficiencies with 50% food restriction model during gestation on the apoptotic index of *Rattus norvegicus* offspring. The study of the effect of nutritional deficiencies during gestation in mice on apoptosis index of the offsprings was conducted by Sani (2007), who conclude that nutritional deficiencies during gestation and breastfeeding may lead to an increase in apoptotic cells on TUNEL examination in mice dentate gyrus on day 21.[24] In the study the treatment group received daily dietary restriction intake, which was about half of the amount of food eaten by the control group. A previous quantitative research conducted by Bedi (1991) suggests that nutritional deficiencies during early life cause a significant deficit in the total number of granular cells of the dentate gyrus. Taking into account these findings, Sani and Bedi conclude that the increased number of observed apop-totic cells in their study strongly suggests that most of these micro neurons have experienced cell death due to the lack of nutrients.[24]

Craciunescu et al (2010) study compared apoptotic index in pregnant mice by dividing 2 groups of controls and treatment with folic acid deficiency. The mother mice were sacrificed at 17 days of gestation, then the brain of the fetus was examined and its apoptotic index was compared to the septum and hippocampus. The study found that the apoptotic cell index increased by 96% in the septal area of the folic acid deficiency group (p<0.01) and 114% in the hippocampus area in the folic acid deficiency group (p<0.01) compared with the control group.[10] In a study conducted by Chi-Liang et al (1999) it was suggested that in PC12 cells cultured on choline-free medium chemically showed the cells stopped growing and/or started to die after 48 hours (data not shown). The percentage of apoptotic cells (calculated on the basis of morphological characteristics) on choline-free media was significantly higher than that in control media (70 µM choline) after 54 hours of choline deprivation (5.2 ± 0.3% vs 1.6 ± 0, 6%, P<0.05, after 72 hours, 27.4±0.6% vs 4.4±1.1%, p<0.01).[26]

Bures et al. (1988) stated that the brain that grows in a stimulus-rich environment has a thicker cortex, larger cell nuclei and more glial cells. It was also found that brain neuron cells grown in a stimulus-rich environment have more dendritic sites that allow for more synapses to form. Rees also stated that the number of cells

undergoing apoptosis depends on the synapse. The more synapses, the less apoptosis that occurs. The richer the dendritic site of a neuron cell, the higher the number of synapses formed so that the number of cells undergoing apoptosis will also decrease. The brain that grows in a rich environment of stimuli and nutrients will experience less apoptosis. Thus, the brain capacity will be further improved.[2]

In the results of our study on cerebrum, we obtained an average of apoptotic index in the treatment group higher than the control group. The total score of the treatment group was 98 and the mean was 6.12 ± 3.51, while the total control group score was 45 and the mean of 2.81 ± 2.16. From the results of the analysis using normality test with the Saphiro-Wilk test, we found that the data on the apoptosis index of *Rattus norvegicus* cerebrum were not normally distributed ($p<0.05$), so that to determine differences in apoptosis index in the cerebrum between the two groups, we carried out analysis using non parametric Mann Whitney test. We found apoptotic index in cerebrum had $p=0.002$ ($p< 0.05$) so that there was significant difference in both groups.

Likewise, the results of our research on the cerebellum showed that the apoptosis index in the treatment group was higher than that in control group with the total score in the treatment group was 89 and mean 5.56±3.03 and the total score in control group was 55 and mean 3.43±2.58. We performed normality tests on both groups' apoptotic index data with the Saphiro-Wilk test. The apoptosis index data distribution in both groups was found to have no normal data distribution ($p<0.05$), so to determine the differences in apoptosis index in the cerebellum in control and treatment groups, we analyzed using non-parametric Mann Whitney test. The results showed significant differences in the apoptotic index in the cerebellum between the treatment group and the control group , with $p=0.026$ ($p<0.05$). The results of these statistical analyses were in accordance with the initial hypothesis that there were differences in the apoptotic index in the cerebrum and cerebellum of between the newly born *Rattus norvegicus* from mothers with FR50 gestation model and those from the control group.

Mechanisms thought to be involved in apoptosis that are induced by nutritional deficiencies may involve an association between nutritional deficiencies and the availability of adequate amounts of neurotrophic factors, the Nerve Growth Factor (NGF), Brain Derived Neurotropic Factor (BDNF) and Neurotropin 3/4/5 (NT).[24] Nutrition and growth factors regulate brain growth during early fetal and post-natal periods. Embryonic period, when the organogenetic process is occuring, including brain development at 24 to 42 weeks of pregnancy, is very susceptible to nutritional deficiencies because the brain is in rapid growth, during which the process of neuron formation, gliogenesis, cell migration, and early differentiation including synapse formation and mielinization, are taking place.[7,14]

Some studies suggest that BDNF has an effect on nerve cell survival, both in vitro and in vivo. A study by Kubo et al. (1995) states that the administration of BNDF to cerebellum nerve cells in vitro can prevent apoptosis and improve nerve cell survival and this ability to prevent apoptosis associates with BDNF doses given. The ability of BDNF to prevent apoptosis in vitro is better than other neurotrophins, the NGF and NT 4/5. BDNF has 2 receptors TrkB and p75NTR. The TrkB receptor is a pro-survival receptor, whereas the p75NTR receptor is a pro-apoptotic cell receptor. The BDNF bond with its receptor, TrkB, activates a series of events that will eventually activate PI3K, which then activates protein kinase B (Akt), which has an important role in cell survival, growth of dendrites and axons in nerve cells. Sheikh (2010) examined the brains of autism patients, and found that in the brains of autistic patients BDNF and protein kinase B (Akt) levels were lower than those in controls, and protein kinase B (Akt) levels were related to BDNF expression.[20] The program of neuronal cell death in the fetus in the womb consists of two processes, a pathological necrosis process and a physiological apoptosis process. The program is affected by the expression of several genes and can be prevented by the absorption of several neurotropic factors, one of which is BDNF.[23]

A supportive study was performed by Coupe et al (2008) who suggested that BDNF level in rat fetus was very sensitive to maternal nutritional status. In pregnant mice with FR50%, there was a decrease in BDNF concentration in the offsprings' hippocampus. Observations also showed that there was a disruption of BDNF receptor gene expression, the TrkB, which caused neurotrophic BDNF/TrBB pathways during postnatal period. Several studies have shown that maternal nutritional disorders have a short-term effect on fetal brain BDNF levels. These data show that 70% protein restriction from the day 8 of pregnancy to week 4 post-natal results in the loss of body weight, brain weight, and BDNF concentrations in the hippocampus at P28.[25] The study by Coupe et al. suggested that BDNF played an important role in brain development, where 50% maternal nutrition reduction (50% Food Restriction) during embryonic (E) 14, P7, P14 and P21 affected BDNF levels in fetal rats in the hippocampus and hypothalamus. Maternal perinatal undernutrition affects BDNF expression in the hippocampus and hypothalamus, but differs according to the period of

brain development, whether it was embryonic or postnatal. Antonow (2010) also found that 30% food restriction (FR 30%) in baboons during pregnancy reduced BDNF levels by around 13.4% -26.3% and increased neuronal cell apoptosis by 118% in baboon's offsprings.[4,17]

Other studies, such as those conducted by Roy Sable et al in 2013, showed that micronutrient imbalances (folic acid and vitamin B12) during pregnancy caused an increase in oxidative stress that produced reactive oxygen species (ROS). As a result, if the condition continues, the defense mechanism by superoxide dismutase (SOD) in brain neuron cells cannot compensate for ongoing oxidative stress and a decrease in SOD may occur, which results in decreasing cellular energy with the end result in the form of a decrease in the number of neuron cells. Oxidative stress arises when the production of reactive oxygen species exceeds antioxidants and plays an important role in embryonic, fetal and placental pathophysiology. Oxydative stress is characterized by elevated levels of MDA (malondialdehyde) and decreased antioxidant enzyme superoxide dismuthase (SOD) as well as glutathioneeperoxidase (GPx) in rats' brains. Increased ROS causes epigenetic changes that can interfere with the translation of BDNF, NGF and TrkB genes and will stimulate cell apoptosis, which is a key to some neurological disorders.[19, 22]

In a previous study by Roy et al. about micronutrient deficiencies, such as folic acid and vitamin B12, which can increase brain MDA levels in both mice and offspring at birth, it was also hypothesized that disturbances in intake/metabolism of micronutrients during pregnancy can increase oxidative stress, which may pose a risk of neuronal developmental disorders later on.[19] The findings show that the fetus is very sensitive and responds to the mother's environment, where the inferior prenatal environment is a stress for the fetus.

The results of a study by Craciunescu et al (2010) support our conceptual framework that folate and vitamin B12 deficiency inhibits the regeneration of methionine. SAM levels are also reduced as a consequence of folate or vitamin B12 deficiency. In addition, it also has adverse effects on cells by allowing the accumulation of homocysteine, a potentially toxic substance produced by methionine demethylation. Homocysteine induces DNA damage to neuron cultures by mechanisms that may involve a decrease in DNA transmethylation. Homocysteine induces partial apoptosis through DNA damage.[16] DNA damage activates polymerase (PARP) poly (ribose ADP), resulting in the depletion of ATP and the activation of p53 tumor suppressor protein (intrinsic pathway), which can trigger apoptosis.[16] Yen et al. in 2002 found that deficiency of methionine, tryptophan, isoleucine, or choline induced apoptosis in cultured cells, and stated that the division of caspase-3 and caspase-7 was the most active.[26] Our study showed that the neuronal cell apoptosis index in cerebellum and cerebellum of *Rattus norvegicus* born from 50% food restriction gestational model was higher significantly than that of the control group.

CONCLUSION

Fifty percent food restriction during pregnancy has a significant effect on neuron cell apoptosis index in cerebrum and cerebellum of *Rattus norvegicus* offsprings.

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