

Use of Herb Combination (*Jamu Atoke*) Before Mating to Improve Rat Pup Health

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Abstract

Mortality and health disturbances in children often correlate with maternal health and fertility. Avocado, mung bean sprouts, and holy basil have been traditionally used to improve maternal health, before and during pregnancy. This study was aimed to assess the efficacy of herbal combination of avocado, mung bean sprouts, and holy basil (*Jamu ATOKE*) in optimizing reproductive health. Eighteen female Sprague Dawley rats (9–10 weeks old, BW: 180–250 g) were randomly divided into 3 treatment groups (n=6 rats for each group) of control, group I and II. ATOKE were added into rats drinking water and consumed for 30 days before pregnancy. After the rats were pregnant and gave birth, pup per parent ratio, pup mortality, and pup health performance (body weight gain, feed and drink consumption, motor activity, pup speed in finding light and feed, red blood cell (RBC) count, white blood cell (WBC) count and differential, SGPT, SGOT, blood urea nitrogen (BUN), creatinine, and proinflammatory mediators (TNF- α and IL-6) were analyzed. Pups born to in group I had a generally better health performance compared to the control group. There was a 100% mortality in the litter of group II due to the excessive aggressive behavior of the dams as a sign of toxicity. Administration of 2.5% *Jamu ATOKE* showed the best effects on the health and intelligence of the rat pup.

Keywords: Avocado, holy basil, *Jamu ATOKE*, maternal problems, mung bean sprouts

Introduction

Child mortality and morbidity is still a problem that is quite alarming. Research in 2012 reported that 3.1 million perinatal mortality occurred in 193 countries in 2010 was due to health problems during pregnancy. Moreover, cases of death as a result of health and developmental disorders are still very high, especially in developing countries. Data from 2000–2010 obtained by WHO on causes of death in children under 5 years in Indonesia stated that the majority of deaths were neonatal deaths. Neonatal mortality is caused by several factors, mainly premature births, infections, and congenital malformations. In

other developing countries, there are still a large number of cases of low birth weight, stunting, wasting, and cognitive development disorders in children.

Maternal health and endogenous secretion of estrogen and progesterone before mating and during pregnancy were correlated to child's birth weight, child mortality, milk production, and the child's growth from birth until weaning.^{1,2} Women in developing countries with poor socio-economic conditions are common to have a low height and will give birth to children with low weight. Subfertile women and in vitro fertilization (IVF) patients have a high risk of uterine bleeding, placental complications, hospitalization during pregnancy, and cesarean section compared to fertile women. In IVF patients, the risk of birth outcome such as low birth weight and preterm birth are even higher.³ There are report from other studies which stated that reproductive

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problem such as hormonal imbalance, and lack of nutrition of the mother contributed to the increasing incident of congenital malformations and developmental disorders such as autism.^{4,5,6}

Improving nutrition is an approach that can be used to overcome fertility problems.⁷ Indonesian have been utilized natural ingredients from nutritious plants in the form of herbal formulations or *jamu*. For generations, *jamu* has been used for medical treatment and health maintenance. Primary Health Research (*Riskesdas*) 2010 reported that 49.53% of the Indonesian population consumed herbal medicine both for health care and for treatment due to illness. Ingredients such as avocado, mung bean sprouts and holy basil can be formulated in the form of *jamu* to help improve health and reproductivity of female parent. Avocado (*Persea americana*) is beneficial as supplements for pregnancy since it contains omega 9, omega 7, omega 6, and omega 3 fatty acids.⁸ *Toge* or mung bean sprouts (*Vigna radiata*) is high in phytoestrogen content in the form of isoflavones which function to mimic the action of estrogen.⁹ Holy basil (*Ocimum sanctum*) contains stigmasterol which functions in estrogen and progesterone synthesis.¹⁰ This research was conducted to evaluate long term effects of *jamu* consisted of avocado (*alpukat*), mung bean sprouts (*toge*), and holy basil (*kemangi*) combination (*Jamu* ATOKE) on health and intelligence of rat pup.

Methods

This research was conducted at the research facility of the Laboratory Animal Management Unit (UPHL) of the Faculty of Veterinary Medicine IPB University. The study was conducted from September 2019 to January 2020. This research has been approved by the Animal Ethics Commission Bogor Agricultural University with number 145/KEH/SKE/VIII/2019. Eighteen adult female Sprague Dawley rats, aged 8–10 weeks old, weighing around 180–250 g, and had not been pregnant were acclimatized for two weeks. Feed and drink were given ad libitum. Rats were then randomly divided into three treatment groups (n=6 rats), namely control group (only given solvent), 2.5% *Jamu* ATOKE (group I), and 5% *Jamu* ATOKE (group II).

Jamu ATOKE formulation consists of a combination of avocado, mung bean sprouts, and holy basil leaves (1:1:1/4) which have been previously sorted and cleaned. The three

ingredients were mashed and then added with water in a ratio of 1:2. The mixture was heated while stirred until the temperature reaches 60 °C and this step was repeated 3 times. Then, the mixture were cooled and filtered. Filtrate were put into a bottle and stored in a freezer/refrigerator at 4°C. *Jamu* ATOKE that had been made then diluted into a concentration of 2.5 and 5% in female rats drinking water. Treatment of ATOKE was given to the rats for 30 days.

After *Jamu* ATOKE treatment had been completed, rats estrous were synchronized using the whiten effect method i.e. brought female rat near to the male rat for 48 hours. Following the synchronized estrous, female rats were mated with male rats by mixing them with a ratio of 1 male and 2 females for about 48 hours.

Pregnant rats then gave birth after 20–25 days of pregnancy. Pup per parent ratio was calculated by comparing the number of children born to number of mothers giving birth. Body weight of pups was measured every five days for 30 days. Number of pup mortality was the number of children surviving until the 30th day post-birth. On the 30th day of post-birth, rat pups blood were taken for RBC count (number of RBC, hematocrit (Hct), hemoglobin (Hb) also WBC count and differential (neutrophils, lymphocytes, monocytes, basophils, eosinophils). RBC and WBC count was carried out according to the following standard procedure: Hct level was measured using a microhematocrit technique, Hb level was measured using the cyanmethemoglobin method, number of RBC and WBC was calculated using a hemasitometer, leukocyte differentiation count was performed by using Leishman method i.e. made thin blood smear over a glass object, each type of white blood cell was then counted. Blood serum was used to measure SGPT, SGOT, BUN, and creatinine values. Values of these four parameters was measured using the UV spectrophotometer method.

Rat pups intelligence and motor activity were measured afterwards. Evaluation of rat pups intelligence was performed by placing pups in a rat simple maze with a length of 1 meter and formed the letter, then pups speed in finding light and feed the measurements were done 3 times. After that, motor activity of the pups was observed by using forced swim test. During the test, pups swimming duration was measured.

Rat pups were then sacrificed and the spleen were taken. Spleen was made into a suspension then stained with extracellular (CD4 and CD68) and intracellular (IL-6 and TNF- α) antibodies staining. Samples were analyzed using the BD

FACSCalibur™ flowcytometer. Data obtained were analyzed with one-way ANOVA followed by Duncan's test at 95% accuracy level. Data were also analyzed using one t-test. Data analysis was performed using Microsoft Excel 2013 and Minitab 16.

Results

Jamu ATOKE made of a combination of avocado, mung bean sprouts, and holy basil can be used to improve maternal health conditions before pregnancy hence the pups born have better health quality and intelligence. Effects of ATOKE on rat pups health performance are presented in Table 1.

Mother rats of control group and mother rats of group I gave birth to a total of 40 pups each group. That number was higher than number of children born to mother rats group II which only gave birth to 17 pups, hence pup-per-parent ratio of group II was the lowest compared to the other groups. Average body weight of 5-day-old pups born to dams of group I was almost the same as control and was higher compared to pups born to dams of group II. Toxic phenomena and behavioral abnormalities occurred in 5 group II. This was indicated by the decrease in body weight of pups observed in the next measuring, as well as changes in the behavior of the dams—which was getting more temperament, and even ate

all of the pups. Thus, the observation of pups of group II could not be continued. Behavior of the dams who ate their own offspring also occurred in control and group I. However, mortality of pups in both groups did not reach 50%, with smallest percentage of mortality was possessed by pups whose mothers had been supplemented with 2.5% *Jamu* ATOKE.

At 30 days post-birth, pups of group I had higher average body weight and weight gain, with longer duration of motor activity compared to control ($p < 0.05$). Pups born to mothers that were given 2.5% *Jamu* ATOKE also had higher intelligence. Pups duration in finding light and feed were 85.23 and 77.36% faster, respectively, compared to control pups. Furthermore, *Jamu* ATOKE effects on RBC profile, WBC count and differential, liver function, and kidney function of rat pups were presented in Table 2.

The number of RBC, Hct, monocytes, neutrophils, eosinophils, basophils, neutrophil/lymphocyte ratio, and the values of SGPT, SGOT, BUN, and creatinine in pups born to mothers in group I were similar to control ($p > 0.05$). Whereas Hb levels, WBC count, and number of lymphocytes of rat pups of 2.5% ATOKE group were significantly higher compared to control ($p < 0.05$).

Overall, *Jamu* ATOKE did not trigger inflammation. This can be seen by the concentration of TNF- α and IL-6 in male and female pups of group I, that were not significantly

Table 1 Health Performance, Motor Activity, and Intelligence of Rat Pups Born to Mothers Given *Jamu* ATOKE Prior to Mating

Parameters	Control	ATOKE Concentration (%)		P-value
		2.5 (Group I)	5 (Group II)	
Number of pups born (head)	40	40	17	-
Pup-to-parent ratio	10	10	5,67	-
Pup mortality (%)	43 (17 out of 40)	30 (12 out of 40)	100 (17 out of 17)	-
Average weight of pups 5 days postnatal (g)	11.00±0.00 ^a	11.50±2.20 ^a	7.38±1.77 ^b	0.00
Average weight of pups 30 days postnatal (g)	65.00±5.40 ^b	83.25±8.03 ^a	*	0.00
Pup weight gain for 30 days (g)	54.00±5.40 ^b	71.75±6.69 ^a	*	0.00
Pup motor activity (seconds)	163.30±15.70 ^b	211±17.00 ^a	*	0.00
Pup speed of finding light (s)	82.60±51.40 ^b	12.20±1.92 ^a	*	0.04
Pup speed in finding feed (s)	63.30±28.90 ^a	14.33±3.06 ^b	*	0.01

Note: Different superscript (a)(b) letters within same row showed significant difference in one Way ANOVA method followed by Duncan test ($p < 0.05$)

Table 2 RBC Profile, WBC count and differential, Liver Function, and Kidney Function of Rat Pups Born to Mother Given *Jamu ATOKE*

Parameters	Normal Value*	Control	Group I Pups	P-value
RBC count (× 10 ⁶ /μL)	2.9–6.8	6.76±1.05 ^a	5.68±0.61 ^a	0.05
Hct (%)	10–47	27.17±3.54 ^a	27.67±5.32 ^a	0.85
Hb (g%)	8.6–15.38	11.53±0.70 ^b	13.60±1.27 ^a	0.01
WBC cell count (× 10 ³ /μL)	3.6–14.5	6.43±1.05 ^b	7.77±0.91 ^a	0.04
Neutrophils (× 10 ³ /μL)	1.9–28.7	0.42±0.10 ^a	0.41±0.13 ^a	0.79
Lymphocytes (× 10 ³ /μL)	6.7–15.7	4.91±0.92 ^b	6.27±0.93 ^a	0.03
Monocytes (× 10 ³ /μL)	0.3–1.4	1.06±0.37 ^a	1.05±0.47 ^a	0.09
Eosinophils (× 10 ³ /μL)	0.05–0.51	0.04±0.03 ^a	0.05±0.06 ^a	0.75
Basophils (× 10 ³ /μL)	0–0.1	0.00±0.00 ^a	0.00±0.00 ^a	–
Neutrophil/lymphocyte ratio	-	0.22±0.09 ^a	0.18±0.10 ^a	0.42
SGPT (Unit/L)	20.8–470.0	52.03±1.61 ^a	51.43±1.61 ^a	0.729
SGOT (Unit/L)	2.1–426.5	85.18±4.33 ^a	83.38±3.07 ^a	0.523
BUN (mg/dL)	12.33–77.6	48.3±2.16 ^a	50.75±2.25 ^a	0.167
Creatinine (mg/dL)	0.2–1.2	0.61±0.04 ^a	0.57±0.02 ^a	0.243

Note: Different superscript (a)(b) letters within same row showed significant difference one way ANOVA method followed by Duncan test (p < 0.05); *Delwatta *et al.* 2018; Nemzek *et al.* 2001

different (p>0.05) compared to control. Concentration of rat pups proinflammatory mediators TNF-α and IL-6 are presented in Table 3.

Discussion

Jamu ATOKE consumed by mother rats before pregnancy affected pups health. Pups whose mother were given 2.5% *Jamu ATOKE* had lower mortality compared to control group, although pup-per-parent ratio of two groups were the same. Mortality in rats generally occurs

in the first week post-birth, either because of pup's inadequate body condition or eaten by its mother. Usually, mother rats will eat pups who have low weight so they can breastfeed children who have higher body weight efficiently. However, the mortality in group II pups reached 100% because the mothers ate all of their pups. This behavior could be triggered by high concentrations of progesterone in mother's body. Research conducted by Saltzman *et al.* found that female gerbils that attacked their offspring had higher plasma progesterone concentrations compared to mothers who did

Table 3 Concentration of Proinflammatory Mediators (TNF-α dan IL-6) in Rat Pups Born to Mother That were Given *Jamu ATOKE*

Parameters	Groups	Control	Group I pups	P-value
CD ₄ ⁺ /TNFα ⁺ (pg/mL)	Male	13.20±5.33 ^a	15.64±6.88 ^a	0.653
	Female	12.79±0.82 ^a	14.22±1.94 ^a	0.305
CD ₆₈ ⁺ /TNFα ⁺ (pg/mL)	Male	43.52±3.74 ^a	35.49±9.38 ^a	0.240
	Female	32.02±12.94 ^a	32.29±11.69 ^a	0.980
CD ₄ ⁺ /IL6 ⁺ (pg/mL)	Male	12.85±5.54 ^a	9.05±2.37 ^a	0.347
	Female	12.96±2.43 ^a	13.14±2.11 ^a	0.928
CD ₆₈ ⁺ /IL6 ⁺ (pg/mL)	Male	13.60±4.34 ^a	8.14±2.77 ^a	0.140
	Female	14.80±8.96 ^a	19.61±3.13 ^a	0.430

Note: Different superscript (a)(b) letters within same row showed significant difference in one way ANOVA method followed by duncan test (p<0.05).

not. Administration of high concentrations of *Jamu ATOKE* resulted in an excess increase of plasma progesterone concentrations in mother rats, thus rats maternal aggression became more severe.

There was no difference in average weight of 5 day post-birth between control pups and group I pups. However, in the 30 day body weight measurement, it was found that pups whose mother had been given *ATOKE* had a significantly higher average body weight compared to control pups ($p < 0.05$). This is probably correlated to the effect of *Jamu ATOKE* in stimulating appetite of the mother during gestation which can affect child birth weight and child weight in growth period. Study reported that dietary behavior and maternal nutrition are correlated to body weight, height, and body mass index of children under 3 years old.¹¹ The combination of avocado, mung bean sprouts, and holy basil could probably increase mothers feed intake, thus resulted in increased dams and pups body weight. Good appetite is correlated to low stress level. Avocado has antihypertensive activity, which can reduce stress and depression. Holy basil contains eugenol, alkaloids, saponins, tannins, and glycosides that can work as anti stresses and affect the hypothalamic-pituitary-adrenal axis (HPAA). In addition, Yeap et al.¹² reported that mung bean sprouts contain antistress and antioxidant compounds. Antidepressant contained in the ingredients could increase the appetite by regulating the hunger center mechanism in the hypothalamus. A good diet of the mother also affects the nutrients contained in breast milk.¹³ The milk produced by the mother rats supplemented with *Jamu ATOKE* was more nutritious thus pups growth were better.

Female parent stress level may be correlated to the stress level of the children born. According to Bartha et al.¹⁴, maternal cortisol level in early pregnancy affect HPAA activity as a regulator of body's biological systems including stress mechanisms of children. Forced swim test conducted on rat pups is a method to test the effectiveness of antidepressants in reducing stress and depression. When rat experiences stress, there is an increase secretion of corticosterone which can affect the motor performance of rat. One of them is to cause abnormal arms and legs movements. Administration of 2.5% *Jamu ATOKE* was able to prevent high level of stress in mother rats before and during pregnancy. The pups born had lower stress level, thus had better motor skill and swam for longer period of time. This result corresponds with the previous paragraph which

mentioned that the combination of benefits contained in avocado, bean sprouts and holy basil can reduce stress and depression levels, although the mechanism of maintaining motor activity is not yet known.

A good pregnancy can be achieved by improving nutritional conditions and optimizing concentrations of estrogen and progesterone in the serum. Avocado contains cholesterol and monounsaturated fatty acids, especially oleic acid.¹⁵ Cholesterol plays an important role in the biosynthesis of steroid hormones especially estrogen and progesterone. Isoflavones, one of the phytoestrogen contained in mung bean sprouts interact and bind to ER α and ER β receptors of estrogen, hence will optimize estrogen function.¹⁶ Fertility and adequate intake of nutrition before and during pregnancy can affect children's intelligence. According to a study by Carson et al. children born to fertile mothers whose planning a pregnancy had higher verbal and non verbal intelligence compared to children born to infertile mothers (IVF patients or ovulation-induced mothers) and mothers who did not plan for pregnancy. Provision of 2.5% *Jamu ATOKE* was able to improve reproductive and health performance of mother rats thus gave birth to healthy and intelligent pups.

The more optimal nutrients intake of the mother, the more important essential nutrients that can be distributed to the fetus across the placenta for its brain development. The intelligence of pups whose mothers were supplemented with 2.5% *Jamu ATOKE* was significantly higher compared to the control pups ($p < 0.05$), as seen from the speed of the pups in finding light and feed that were much faster. The result of this study is also corresponds with research conducted by Melo et al.¹⁷ which reported that the administration of avocado oil and porridge in 7th day of pregnancy until the 21st day of lactation could improve the reflexes, physical development, and memory of rat pups.

Jamu ATOKE components have the potential to increase RBC count of the mother rats. Avocado contains folic acid, riboflavin and vitamin B6 which are important for iron absorption, RBC and hemoglobin formation. Mung bean sprouts contain anti anemia compounds, such as iron or Fe.¹⁸ Study by Das et al.¹⁹ also reported that holy basil could increase the number of RBC. However, the results showed that the RBC count of pups born to mothers given 2.5% *Jamu ATOKE* was not significantly different compared to control pups. Hemoglobin was the only parameter that had significant different value. Even the

number of RBC of ATOKE pups was lower. That being said, 2.5% *Jamu* ATOKE did not have long term effects on RBC count of rat pups, although theoretically nutritional content contained in *Jamu* ATOKE could increase number of RBC, Hct, and Hb of mother rats. The result could be linked to a study which stated that only Hb, mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) levels of mother and children had a significant correlation between each other.²⁰

Results of WBC evaluation on rat pups showed that pups of 2.5% ATOKE group had a significantly higher WBC count compared to pups of control group. Aside from increasing nutrient intake, *Jamu* ATOKE also had the ability to increase antibodies formation of the mother rats that would affect the ability of antibodies in the pups, which in this case was the WBC. Holy basil contains flavonoids and terpenoids which can act as immunomodulators by increasing the number of WBC. Moreover, folic acid and vitamin B6 contained in avocado could also help to boost immune system by increasing antibody production.²¹ Fetal development including the development of the immune system is strongly influenced by maternal health, nutritional adequacy, and environmental factors.

Results showed that administration of 2.5% *Jamu* ATOKE did not cause liver damage on rat pups, as indicated by the SGPT and SGOT values of 2.5% ATOKE pups which were not much different compared to that of control pups. SGPT and SGOT levels usually increase in children who experience impaired liver function and metabolic disorders such as obesity.^{22,23} Although the body weight of 2.5% ATOKE rat pups were higher than control pups, it did not reach the level of obesity. Pups liver was in good condition thus the levels of SGPT and SGOT were still normal. Higher than normal levels of BUN and creatinine indicate that there is a damage to kidney function.²⁴ Results showed that pups born to mothers that were provisioned with 2.5% *Jamu* ATOKE did not experience kidney damage. It can be seen that BUN and creatinine levels in ATOKE pups were not different compared to control. Maternal diet greatly influences fetal development, one of which is kidney development. *Jamu* ATOKE supplied more nutrition for the mother and help preparing the body to be ready for pregnancy, thus fetal organ development including the kidneys could be optimal.

Inflammation has been linked as a component that plays a role in the pathogenesis of diseases related to malnutrition. A healthy child with

good nutritional status will have low levels of proinflammatory mediators. Research conducted by Abd El-Maksoud et al.²⁵ reported that stunted children had high levels of proinflammatory mediators IL-6 and TNF- α compared to children who grew normally. This is due to stunted children undergoing micronutrient deficiencies which will result in an increase of cell-mediated immune responses as well as cytokine production. Pups whose mothers were treated with 2.5% *Jamu* ATOKE had good nutritional status and therefore had generally lower levels of IL-6 and TNF- α compared to control, although there were no significant differences between the two. *Jamu* ATOKE consisted of a combination of avocado, mung bean sprouts, and holy basil can be effective for improving health and reproductivity of mother rats prior to pregnancy thus gave birth to smarter, healthier, and stronger pups. Concentration of 2.5% *Jamu* ATOKE is recommended for mother because it could increase body weight, motor activity, intelligence, and WBC, as well as maintained normal level of liver enzymes, kidney metabolites and proinflammatory mediators in pups born.

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