Original Article

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Evaluation of Propolis and Milk Administration on Caffein-Induced *Mus musculus* Fetus Skeletal

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ABSTRACT

Caffeine consumption by pregnant women at doses above 300 mg/day was suggested to cause skeletal damage. Propolis with high flavonoids concentration could increase the number of osteoblasts. This research aims to evaluate the effect of propolis and milk administration on fetal skeletal of caffeine-induced female mice (*Mus musculus*). Mice were divided into six groups: negative group, positive group of caffeine (a dose of 75 mg/kg BW), positive group of propolis (a dose of 1400 mg/kg BW), positive group of milk (200 ml), group D1 (caffeine 75 mg/kg BW and propolis 1400 mg/kg BW) and group D2 (caffeine 75 mg/kg BW and milk 200 ml). Data were processed using one-way ANOVA. The results showed that administration of propolis and milk on caffeine-induced mice during pregnancy does not affect the mice body weight, the number of fetuses and fetal weight significantly (P> 0.05). No skeletal defects detected in group D1 and D2 (observation with Alizarin solution) compared to the negative group. In conclusion, the admnistration of propolis at the dose of 1400 mg/kg BW and 200 ml of milk can repair skeletal damage caused by caffeine induction.

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INTRODUCTION

In general, pregnant women still consume caffeine every day (FDA, 2010). Caffeine can be consumed through coffee, colas, chocolate, tea, soft drinks, food, and medicine (Nawrot *et al*., 2002). Numerous studies in animals have shown that caffeine can cause birth defects, premature birth, reduced fertility, and increase the risk of birth with low weight that leads to spontaneous abortion (Committee on Toxicity, 2001; Weng *et al*., 2008).

Caffeine (1,3,7-trimethylxanthine) is an active compound which has a variety of pharmacological effects (Nawrot *et al*., 2002). Caffeine has beneficial effects as a stimulant for the central nervous system and respiratory system. The effect is characterized by reduced fatigue and weak bronchodilation effect (Welsh *et al*., 2001; Burns *et al*., 2014). On the other hand, the adverse effects of caffeine can vary such as insomnia, anxiety, increased blood pressure and pulse rate as well as a risk factor for osteoporosis (Mesas *et al*., 2011; Drake *et al*., 2013; Marquinaa *et al*., 2013).

Although it has not been ascertained fetal defects with caffeine consumption in humans, but it was the fact that caffeine can come across the placenta to amniotic fluid, umbilical cord blood, plasma and urine of newborns, and across breastmilk barrier, both in mice and humans. Those facts about caffeine can potentially interfere with fetal development (Mose *et al*., 2008). Caffeine consumption limit in pregnant women is less than 300 mg/day (OTIS, 2015). Pregnant women who consume caffeine more than 300 mg/day are increasing the risk of fetal to suffer growth retardation, skeletal abnormalities, and miscarriage (Committee on Toxicity, 2001). This is evidenced by research on the teratogenic effects of caffeine on fetal mice that had been done previously. Caffeine is bound to cause skeletal system abnormalities and cleft palate (Nishimura & Nakai, 1960).

Caffeine is able to intervene mitosis which decreases the activity of DNA polymerase enzyme, induces mitotic cells mammal prior to synthesis of DNA replication phase, inhibit the enzyme activity phosphodiesterase, inhibit osteogenesis process and potentially cause abnormal development of the embryo (Beck & Urbano, 1991). The negative influence of caffeine on fetal

exacerbated by the extension of the half-time of caffeine in pregnant women from 3.5 hours to 16 hours. The extension of half-time period will result in a substantial accumulation of caffeine potentially disrupt the fetus and placenta (European Food and Safety Authority, 2015).

One solution to reduce the negative effects caused by caffeine is the use of herbal preparations in pregnant women. Most people assume that herbal preparations are safer than modern chemical drugs (Broussard *et al*., 2010). At the moment, one of the most popular herbal preparations is propolis. Propolis is a substance produced by honey bees which contains flavonoids chrysin (5,7-dihidroxy-2-phenyl-4H-chromen-4-one) [16], caffeic phenetyl acid ester (50%), wax (30%), essential oils (10%), pollen (5%), and other organic components (5%) (Franz, 2008). Propolis is sticky pollen collected from plants, especially from flower, leaves, and shoots. Then, pollen is mixed with bees' saliva (Marcucci *et al*., 2001).

Propolis works to improve the pathological condition of the sick body, works as an antioxidant and boost the immune system both humoral and cellular (El Sohaimy & Masry, 2014). In an experiment conducted before (Bereket *et al*., 2014), propolis can improve skeletal abnormalities in rats by accelerating osteogenesis. That occurrence may also apply to the fetus. The fetus may encounter osteogenesis process due to exposure to caffeine. Based on previous research (Sartika *et al*., 2013), it is also proven that provision of propolis extracts have an influence on bone repairment time of orthodontic tooth movement. The mechanism was by increasing significantly the number of osteoblasts. The results are consistent with research conducted before (Elwakkad *et al.*, 2008) which showed propolis effect on bone formation. Then, the latest research (Jeong *et al*., 2016) showed that the provision of propolis can significantly lower the maximum concentration of caffeine in plasma.

Another solution to prevent the adverse effects of caffeine for pregnant women is consuming milk (Winarno, 1993). Milk is a nutritious liquid produced by the mammary glands of female mammals. Milk contains fat and fat-soluble vitamins such as vitamin A, vitamin D and vitamin E (Burger *et al*., 2007). Milk is a source of calcium and phosphorus which is very good for growing bones and teeth (Weinsier & Krumdieck, 2000). Additionally, milk is safe for consumption by pregnant women (Mehta, 2007) and is an antidote to the toxic substances that can harm pregnant women (Guharaj & Chandran, 2003). According to the American Academy of Neurology's Annual Meeting in 2010, the risk of multiple sclerosis in the infant is lower whose mothers consumed milk (Paul, 2010). Then, according to the Canadian Medical Association Journal in 2007, pregnant women who consume milk gave birth to babies whose weight increases as much as 41 g (Mehta, 2007).

Reproductive toxicity is one of the toxicity tests for the preparations of herbs and chemicals that will be consumed by humans. Reproductive toxicity test which is frequently used is the teratogenicity test. Teratology is the study of the causes, mechanisms and manifestations of embryonic defect (abnormal) (Wilson & Fraser, 1973). Teratogen is a chemical that can significantly affect fetal development and the effects of changes start from lethality until deformity (malformations) and growth retardation (Wilson & Fraser, 1973). Teratology principle is the provision of test compounds in animals during pregnancy and sees its effect on fetal development so that the ability or potential toxicity of compounds against a developing fetus cells is known (Almahdy, 2012).

Therefore, researchers wanted to prove that propolis can reduce the negative effects of caffeine on the fetal skeletal system during the period of organogenesis. Researchers also want to compare the provision of milk that has proven good effect on bone and is safe for consumption by pregnant women.

METHOD

The tools used are micropipette (Eppendorf®), vortex (Etech®), analytical balance, refrigerator, rubber gloves, masks, treatment cage, cage maintenance, surgical scissors, a petri dish, toilet roll, watch glass, tweezers, pipette, measuring cups, beaker glass.

Materials used are pure caffeine powder (Brataco), propolis (Melia Propolis®) from PT. Melia Sehat Sejahtera, sterilized milk (Nestle® Bear Brand), distilled water, and the solution containing Alizarin Red (1% KOH and alizarin red 6 mg / L).

Experimental animals were white mice (Webster) females aged less than two months with a weight of 20-30 grams, healthy and had never experienced treatment of the drug. Also, the male mice also required for mating. Male mice that used to be healthier and live approximately three months.

Preparation of the Test Solution

The test substance used for this study was caffeine, propolis and milk. Caffeine stock solution was made by dissolving 300 mg of caffeine in 60 ml of distilled water, propolis stock solution was made by dissolving 4 ml of propolis (1 ml propolis = 900 mg) in 40 ml of distilled water and milk was pack in 10 mL solution).

Acclimatization of Experimental Animal and Determination Estrous Cycle

Acclimatization conducted for ten days to familiarize the animals with the experimental environment. They were given adequate food and drink, bodies were weighed daily, and behavior was being observed. During the acclimatization, estrous cycle is determined by mice vagina visual observation. Mice in estrous period showed vagina with red-colored and gummy texture.

Animals used in this experiment are animal with the healthy body. Animal considered as healthy if having decrease body weight not more than 10%, visually show normal behavior and having 4-5 days estrous cycle (Dillasamola *et al*., 2016).

Mating Animal Experiments

At the time of estrous, animals mated. The ratio of male and female is 1:4. Male mice were put in female mice cage at four o'clock in the afternoon and separated again tomorrow morning. On the morning, examination of vaginal plugs is conducted.

Test Preparation

The preparation in the form of caffeine and propolis with the aid of a needle in oral sonde. Mice were divided into six treatments; one group consisted of 5 mice. The negative group was given distilled water, the positive group of propolis was given propolis at the dose of 1400 mg/kg BW, positive group of caffeine was given caffeine at the dose of 75 mg/kg BW, positive group of milk was given milk 200 ml, group D1 were given propolis at the dose of 1400 mg / kg and caffeine at the dose of 75 mg/kg, group D2 were given 200 ml milk and caffeine at the dose of 75 mg/kg. The test preparation was given 10 days in a row starting on the 6th until the 15th day of pregnancy (Almahdy, 2012).

Observations During the Administration of Test preparation

Daily weighing was done to retrieve weight gain data. In the event of a drastic weight loss and accompanied by bleeding around the vagina, it is likely the animal have a miscarriage or abortion then the animal must be killed and examined. At the time of test preparation administration, the mice that were sick due to treatment or disease then it is not included anymore (Dillasamola *et al*., 2016).

Preparation of Alizarin Red solution

Alizarin red solution is made by adding 6 mg of alizarin red in one liter of 1% aqueous KOH. This solution is used for coloring mice skeletal system (Manson *et al*., 1982).

Laparotomy

Laparotomy performed on day 17 of pregnancy. Mice were killed by cervical dislocation, then do the laparotomy to remove the fetuses of mice. How mice were dissected at the abdomen upward until it looks uterus containing a fetus. Fetus removed by cutting the uterus and placenta. Furthermore, the presence or absence of resorption site marked by a red blob as the implantation point of the fetus was observed. After the fetus is dried with paper towels, the weight of each fetus weighed to determine the average birth weight. Then observe the presence or absence of visual abnormalities eg tail, earlobes, eyelids, the number of fronts and rear feet (Almahdy, 2012).

Fixation and Observations of Morphology Defect

Having visually observed, the fetus was soaked with a solution of alizarin red, allow two to three days, while occasionally shaken until the fetus becomes transparent and the bone look red. Then observe bone abnormalities such as the size and the distance between the ribs, bone abnormalities of the skull, and coccyx. Then compare all the observations with controls .

Data examinations are to be taken as follows:

1. Parent body weight of mice during pregnancy after being treated to laparotomy
2. Number of fetuses
   a. The amount of fetal alive
   b. Number of dead fetuses in the uterus (intrauterine fetus)
3. Fetal body weight
4. Observation types of disabilities
5. The number of fetal defects
6. Observation of the result of the fixation with a solution of Alizarin Red

Data analysis

Data from this study were statistically analyzed using one-way analysis of variance (ANOVA) for the parameters of the parent body weight of mice, count of the fetus and fetal body weight. If the result is significant, the analysis continued by using Duncan's multiple range test (Duncan Multiple Range T-Test).

RESULTS

After comparing the effect of propolis and milk to the fetal mice skeletal (*Mus musculus*) induced by caffeine, positive group propolis, the positive group milk, dose groups 1 and 2 do not have skeletal abnormalities when compared with the negative group. Bone abnormalities found in the positive group of caffeine.

Research data in this study were tested statistically using one-way analysis of variance test as follows:

1. Test preparation given in the positive group of propolis, positive group of caffeine, milk positive group, dose 1 group and dose 2 group does not significantly affect the mice body weight during pregnancy (F count <F Table 0.05). The average weight gain during pregnancy in the negative group, positive propolis, caffeine positive group, positive milk group, dose 1 and dose 2 was 32.3 grams; 31.93 grams; 31.59 grams; 31.25 grams; 30.44 grams; 29.97 grams respectively.
2. The provision of propolis and milk to the caffeine-induced fetus mice does not significantly affect the average number of fetuses (F count <F Table 0.05). An average number of fetuses to the negative group, positive propolis group, caffeine positive group, positive milk group, dose 1 and dose 2 was 12.2 tail; 11.6 tail; 10.8 tail; 9.6 tail; 9 tail and tail 8.5, respectively.
3. The provision of propolis and milk to the caffeine-induced fetus mice does not significantly affect the average weight of fetuses (F count <F Table 0.05). The average weight of the fetus to the negative group, positive propolis group, caffeine positive group, positive milk group, dose 1 and dose 2 was 0.98 grams; 0.89 grams; 0.79 grams; 0.69 grams; 0.67 grams; and 0.66 grams, respectively.

DISCUSSION

In this research, the test was conducted to see the effect of propolis and milk on the mice fetal skeletal induced by caffeine. Besides, observation was done to see other effects such as abnormal fetal shape, their growth inhibition, haemorrhage or other abnormalities that may occur.

Caffeine is a methylxanthine class of compounds that can cross the placenta to substantially amniotic fluid, umbilical cord blood, plasma and urine. In pregnant women, caffeine half-life increased from 3.5 hours to 16 hours (European Food and Safety Authority, 2015). The pharmacokinetic changes occur due to an increased number of steroid hormones (estrogen and progesterone) during pregnancy. Steroid hormones (estrogen and progesterone) was acting as a competitive inhibitor of microsomal enzyme oxidase. When this enzyme is inhibited can cause a decrease in the amount of drug eliminated. This causes the longer the half-life of caffeine and caffeine also increases bioavailability, so that caffeine is in the plasma for a long time and the accumulation of substantial caffeine are potentially disrupt the fetus and placenta (Lee, 2009; European Food and Safety Authority, 2015).

The use of caffeine as inducer compounds based on studies in animals during pregnancy, caffeine can cause skeletal abnormalities in the mice fetus (Nishimura & Nakai, 1960; Santoso, 2004). The use of propolis in this study was based on the role of propolis can accelerate osteogenesis (Bereket *et al*., 2014), significantly increase the number of osteoblasts (Sartika *et al*., 2013), and affect bone metabolism (Elwakkad *et al*., 2008). The use of milk in this study was based on the knowledge that milk is a source of calcium and phosphorus which is very good for the growth of bones and teeth. In addition, milk is safe for consumption by pregnant women and is an antidote to the toxic substances that can harm pregnant women by chelating these harmful substances. Experiment D1 between propolis with caffeine was done because the content based on propolis chrysin can reduce the maximum concentration of caffeine in plasma (Jeong *et al*., 2016), thus being a protective agent for the negative effects caused by caffeine. Later experiments D2 between milk and caffeine was done because milk is an antidote to neutralize the negative effects caused by caffeine.

Caffeine dose used in this study was 75 mg/kg BW, it is based on previous research that says that mice are more sensitive and malformations occurred in mice induced by caffeine at a dose of 75 mg/kg BW (Anderson *et al*., 2005). Propolis dose used in this study were taken from 1/5 of the LD_{50} in mice. Propolis LD_{50} in mice is 7340 mg/kgBW (Burdock, 1998), so we get 1/5 of the LD_{50} dose is 1400 mg/kgBW. The use of propolis dose of 1400 mg/kgBW as the positive group based on the research conducted by Burdock in 1998 stated that the NOAEL (No Adverse Effect Level) of the mice were given a dose of 1400 mg propolis/kgBW for 90 days. And the milk dose used in this study was 200 ml by human daily consumption patterns in general.

Experimental animals used in this study were female white mice at the age of approximately two months, had a body weight of 20-30 grams (Kementerian Kesehatan RI, 1979) and had never given birth. White mice were used as experimental animals because they have a short gestation period (Almahdy, 2012), the high number of fetuses (Almahdy, 2012), simple handling, the price is relatively cheap and easy to obtain. In addition, in some research, mice are more susceptible to teratogens than other experimental animals (Dillasamola *et al*., 2016).

Before being treated, mice were acclimatized for ten days to get the animals in the experimental conditions and avoid stress during treatment. Acclimatization for 10 days was also aimed to observe the estrous cycle. Female animals should have regular estrous cycles. This needs to be done in order to avoid false pregnancy even if the vaginal plug were found during mating. Observations of

Table 1. The Average Body Weight of Mice During Pregnancy

Day to-	Average Body Weight of Mice					
	K-	**K + Caffeine**	**K + Propolis**	**K + Milk**	**D1**	**D2**
6	25.9	27.02	27.1	26.28	26.6	26
7	27.2	28.16	28	27.48	26.76	26.46
8	27.7	28.9	28.12	28.04	27.08	26.9
9	28.4	29.02	28.78	28.78	28.02	27.58
10	29.8	29.54	29.8	29.88	28.68	28.18
11	31.3	30.2	30.4	30.86	29.38	28.94
12	32.3	31.02	30.8	32.66	29.98	30.42
13	33.8	32.86	32.42	34.12	30.48	31.88
14	35.6	33.04	33.18	35.38	31.54	33.04
15	37	36.02	35	36.58	33.46	34.34
16	38.7	37.6	36.44	37.62	36.08	35.48
17	39.9	38.8	39.1	38.34	37.88	37.4
X ± SD	32.3 ± 4.71	31.85 ± 3.86	31.51 ± 3.59	32.17 ± 4.2	30.49 ± 3.66	30.55 ± 3.83

Information:
K-: Negative Group (Aquadest)
K + Caffeine: Propolis Positive Group dose of 75 mg / kgBW
K + Propolis: Positive Group dose of 1400 mg caffeine / kgBW
K + Milk: Milk Positive Group dose of 200 ml
D1 (Dose 1): The caffeine dose of 75 mg / kgBW + Propolis dose of 1400 mg / kg BW
D2 (Dose 2): The caffeine dose of 75 mg / kgBW dose + Milk 200 ml

estrous cycle can be done visually by looking at the mice vagina as reddish, open and slightly damp (Dillasamola *et al*., 2016).

Giving the test preparation carried out for ten days beginning on the 6th day and ending on the 15th day because at this time the fetus is during the period of organogenesis (Harbinson, 2001). In this organogenesis period, the fetus is very vulnerable to teratogenic compounds and at this time the embryo organ began to take shape such as the eye, brain, heart, skeleton, urogenital (Harbinson, 2001). This period is referred to as the critical period of pregnancy (Harbinson, 2001). On day 1 until the 5th of gestation, mice were not given the test preparation because at the moment there are totipotency properties in the fetus that can repair damaged tissue. On day 16 and onwards, teratogen compound did not cause morphological defects but resulted in dysfunctional disorders that can not be detected immediately after birth (Lu, 1995).

Observation of the increase in parent body weight during gestation to see the state of the parent of nutrition and health in general. In this case, the weighing is done

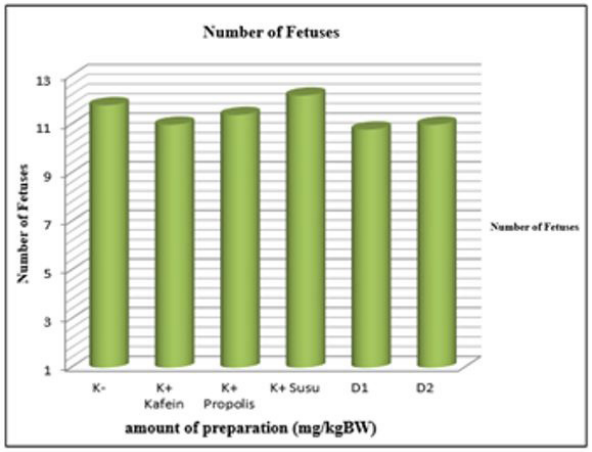

Figure 1. Number of Fetuses

from the time of administration of the compound to laparotomy, as it aims to see how the influence of the compound on mice (Table 1).

The increase in mice body weight, seen in the 6th to 8th gestation, yet an increase in mice body weight is large enough. The increase in mice body weight is likely to increase at day 11 to 17 of pregnancy. The increase is due

to the development of mice fetal and increased volume of amniotic fluid, placenta, and amniotic membranes (Guyton, 1990). The number of fetus also affect the increase in body weight of mice parent, generally, the greater the increase of total fetus, the greater increase in body weight of the mother (Figure 1).

To see the effect of the test preparation to the fetal mice, then on day 17 of pregnancy performed laparotomy is spending parent fetus from the uterus. This is done because the mice gave birth spontaneously, tend to eat the defective, dead and nearly die offspring so that it can affect the results of the calculation data. Besides, laparotomy was conducted to observe whether or not the resorption site that is the red or brownish-yellow blob on the former site of fetus implantation of the uterus (Wilson & Fraser, 1973).

Hemorrhage happen spontaneously due to platelet dysfunction. Hemorrhage is a discharge of blood from the cardiovascular system is accompanied by accumulation of tissue (Wilson & Fraser, 1973). A foreign substance in the network can change the osmotic pressure. Osmotic imbalance can be caused due to an interruption of pressure and viscosity of the liquid in different parts of the fetus such as the blood plasma and capillary extra space (Wilson & Fraser, 1973). This osmotic imbalance was caused by the concentration of methylxanthine in high fetal blood vessels. Methylxanthine high concentration can inhibit enzymes that hydrolyze cAMP phosphodiesterase, thereby increasing the amount of cAMP (Beck & Urbano, 1991). The increase in cAMP causes blood vessels to dilate (Katzung, 2004). It could also be the cause of hemorrhage. In pregnancy frequent hemorrhage shock. Hemorrhage shock can also be diagnosed with changes in body temperature. Hyperthermia is a failure condition regulation of body temperature (thermoregulation) due to the inability of the body to release heat or excessive heat production by the body to release heat at a normal rate. Therefore, the possibility of hyperthermia can lead to hemorrhage.

Growth inhibition occurs when a teratogen agents affecting cell proliferation, cell interaction, and a reduction in the rate of biosynthesis-related barriers to the synthesis of nucleic acids, proteins or mucopolysaccharides (Wilson & Fraser, 1973). Teratogen compounds with low dose capable of causing the death of some cells and can also cause cell turnover because of fetal cells have high regeneration ability. If one or a group of cells damaged by the interruption of toxic agents, the surrounding normal cells will divide and replace cells that are damaged. Substitution of damaged fetal cells will be maintained during the period of organogenesis in order to form a normal morphology. If that fails or does not reach the target in organogenesis phase, it will cause fetal malformations.

Fetal growth restriction may be caused by disruption of cell division, so that the synthesis of nucleic acids and proteins are disturbed. Fetal growth proliferation by mitosis and cell proliferation rate is a function of the speed of growth (Herbold, 1985). Giving caffeine during organogenesis is potentially causing delayed embryonic development because caffeine can decrease the activity of DNA polymerase enzyme, induces mitotic cells before DNA replication perfect ending, as well as inhibiting the enzyme phosphodiesterase (Beck & Urbano, 1991). Therefore it can be restricted growth fetuses.

From the results of fetuses with alizarin red soaking dosing 1 and 2, bone disorders are not found. Such as those disorders found in the positive group of caffeine. In normal fetuses the skull, ribs, and coccyx normal. All observed after the fetus is immersed in a solution of alizarin red - KOH 1% that causes the fetus becomes transparent and the bone becomes dark red so that shape of bones can be observed. In the administration of caffeine at the dose of 75 mg/kg, it was found that the ribs were not fused, abnormalities of the fetal skull, and no tail. These is caused by caffeine, due to reduced amount of calcium, thus deficiency of calcium (Figure 2, 3, 4).

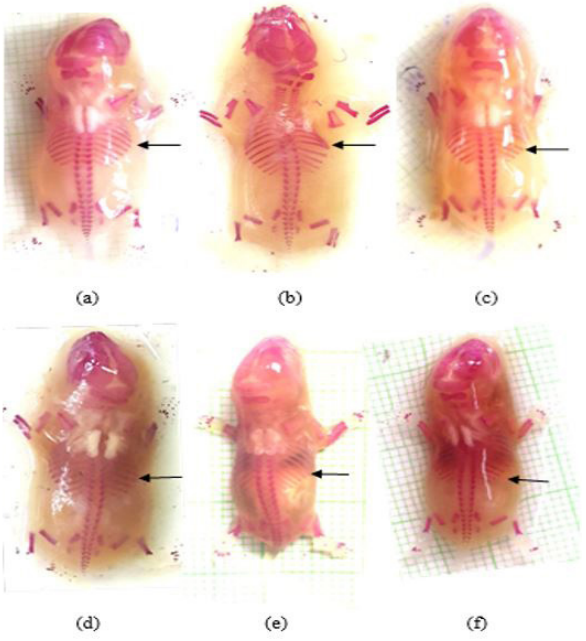

Figure 2. The Observation After The Fetuses Was Fixed With A Solution of Alizarin Red in Each Treatment Group (Differences in Ribs, Pointed By Arrow)

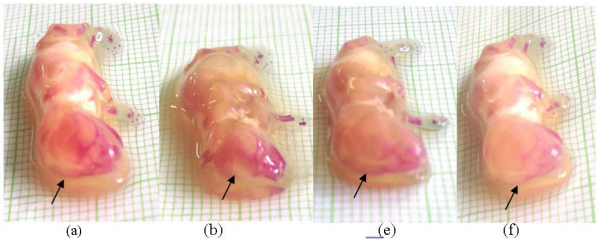

Figure 3. The Observation After The Fetuses was Fixed With A Solution of Alizarin Red in Each Treatment Group (differences in tail, pointed by arrow)

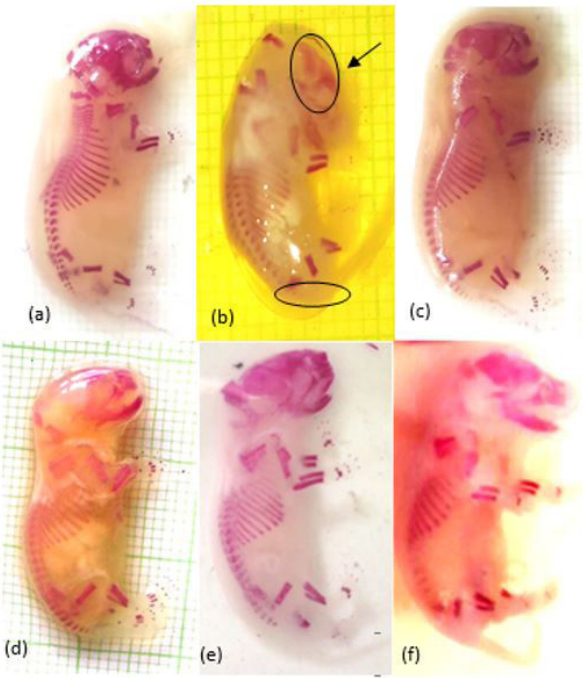

Figure 4. The observation after the fetuses was fixed with a solution of Alizarin red in each treatment group (differences in skull, pointed by arrow)

The absence of bone abnormalities in the first dose group fetus given caffeine is because propolis. Propolis contains flavonoids chrysin to lower the concentration of caffeine in plasma in vivo. Later in the dose group 2 fetus given milk and caffeine are also no bone disorders found, because milk may act as antidotes simultaneously nourish the fetus with calcium contained in milk, so that calcium deficiency caused by caffeine is not the case. On the caffeine positive group, fetus has a skeleton with a perfect ossification. This is due to internal factors, the hormone that may maintain bone mass. A study stated that the hormone is one of the factors that influence whether or not the bones strong. Hormones are natural substances made by specialized cells in the body. Hormones circulate in the bloodstream and can affect the activity of cells in various places in the body. Also, the hormone can also help limit the amount of bone resorption. These is made clear by another study that showed the estrogen hormone deficiency can cause osteoporosis in mice (Masyita, 2006).

CONCLUSION

It can be concluded that the admnistration of propolis at the dose of 1400 mg/kg BW and 200 ml of milk can repair skeletal damage caused by caffeine induction.

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