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The Effect of Calcium Deficient Intake for Premenopausal Age Rat Model

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Abstract

The calcium deficient rat is condition of rat with calcium deficient in its body, one reason of this condition is intake with calcium deficient diet. The aim of this study was to analyze the effect of calcium deficient diet in premenopausal age female rat model of strain *Sprague dawley*. Research methods consist of: formulation of purified diet and calcium deficient diet, preparation of calcium deficient rat model strain *Sprague dawley* of premenopausal age; parameters measured were: consumption of dry matter; consumption of calcium, calcium concentration in feces, liver, kidney, and bone; calcium absorption; serum calcium concentration; progesterone hormone concentration; and daily body weight gain. Treatment with calcium deficient diet for 12 weeks could reduce serum calcium concentration into 7.72±1.08 mg dL^{-1} in calcium deficient rat and 11.60±0.85 mg dL^{-1} in the control. Thus, rat model strain *Sprague dawley* of premenopausal age given calcium deficient diet could be calcium deficient rat based on the condition of serum calcium concentration. Serum progesterone hormone concentration in control rat and calcium deficient rat was not affected by treatment of diet, but it was affected by the age of rat, at the end of the study, the serum progesterone concentration of 15 month old rat was decreasing.

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1. Introduction

White rat (*Rattus norvegicus*) is an animal model widely used in research, such as to study the effect of food consumption, drugs, toxicity, bioactive components of food, supplements, metabolism, embryology and behavior study. Calcium deficient rat model is a rat which have calcium deficient concentration compared with normal rat.

Preparation for calcium deficient rat model can be conducted using 6 weeks old of *Sprague dawley* female rat which were ovariectomized [1], and female rat strain *Sprague dawley* aged 50 days which were ovariectomized [2]. Thus, the rat experienced the estrogen deficiency and had a hormonal condition similar to postmenopausal women [2]. Premenopausal conditions of female rat strain *Sprague dawley* occured at 18 month old which were characterized by decreased concentration of the progesterone hormone, bone density, and the ratio of Ca/P tibia bone [3]. The condition of postmenopausal rat occured on 36 months old or 12 months old after the rat were ovariectomized for 3 months, marked by a drastic reduction in the concentration of the progesterone hormone, bone calcium concentration, the percentage ratio of Ca/P tibia concentration, bone collagen concentration, and bone density [3]. Longitudinal bone growth increase after the rat were ovariectomized at young age, but the bone growth of 9-12 month old mice was minimal [4].

Preparation of calcium deficient rat model with ovariectomized method can caused pain and stress. The use of animals in biomedical research has to fulfil internationally standardized scientific principles; one of them is the fulfilment of animal welfare principle [5]. Hence, to minimize mentioned negative impacts, the treatment of calcium deficient diet intake was conducted to prepare calcium deficient rat model.

Calcium deficient diet was given until the rat experience the calcium deficient marked with serum calcium concentration was less than 9.2 mg dL^{-1} [14]. The animal osteoporosis caused by calcium deficiency factor became the main factor, while other factors were malnutrition and phosphorus deficiency [6]. The use of female rat to calcium deficient rat model, because young male rat were not suitable for animal model osteoponia because growth bone had not been closed under 30 months old [7].

The calcium deficient rat is condition of rat with calcium deficient in its body, one reason of this condition is intake with calcium deficient diet. The aim of this study was to analyze the effect of calcium deficient diet in premenopausal age female rat model of strain *Sprague dawley*.

2. Materials and Methods

2.1. Materials

Materials used include: 12 months female *Sprague dawley* rats from the “Satwa Harapan” Ranch of Faculty of Animal Science, Bogor Agricultural University. Fed used were purified diet [8] and calcium deficient diet for rats [9]. Raw materials used for the production of purified diet consisted of rice flour, casein, corn oil, sugar

flour, vitamin mix, DL-methionine, mineral mix, Carboxy Methyl Cellulose (CMC), salt and distilled water obtained from a chemical store in Bogor, Indonesia. Calcium deficient diet was made with the same material as purified diet without calcium addition the mineral mix. Calcium kit reagen O-C FAST® and progesterone kit reagen DRG.

2.2. Formulation of purified diet and calcium deficient diet

The formulation refers to [9], the raw material used to preparation the purified diet were: rice flour, casein, corn oil, glucose, vitamin mix, DL-methionine, mineral mix with calcium, mineral mix without calcium, *Carboxy methyl cellulose* (CMC), salt, and aquadest. Calcium deficient diet was made from the same material except for the use of mineral mix without calcium. Composition of purified diet and calcium deficient diet are presented in Table 1 and the composition of the mineral mix constituent are presented in Table 2.

Table 1. Composition of purified diet and calcium deficient diet

Composition		Purified diet	Calcium deficient diet
Rice flour	(%)	25.00	25.00
Casein	(%)	18.00	18.00
Corn oil	(%)	3.50	3.50
Glucose	(%)	49.00	49.00
DL-Methionine	(%)	0.30	0.30
Carboxy methyl cellulose (CMC)	(%)	3.00	3.00
Mineral mix with calsium	(%)	0.50	0.00
Mineral mix without calcium	(%)	0.00	0.50
Vitamin mix	(%)	0.50	0.50
Salt	(%)	0.20	0.20
Total	(%)	100.00	100.00

Source: Modified AIN-93 M

Female rat in growth period requires energy such as 3800 to 4100 Kcal ME g^{-1}, 50 g of fat, 150 g of protein, minerals from 5 g of calcium and 3 g of phosphorus. In the reproductive period 3800 to 4100 Kcal ME g^{-1} requires energy, 50 g of fat, 150 g of protein, minerals from 6.3 g of calcium and 3.7 g of phosphorus. The calcium and phosphorus minerals requirement on the reproductive period was higher than growth period [8].

2.3. Calcium deficient rat model making

Preparation of calcium deficient rat model has received ethical approval from the Animal Ethics Committee at Bogor Agricultural University (No. 12-2013). Rats used were 14 female rats strain *Sprague dawley* aged 12 months with average body weight of 235.00±27.94 g. Rats were adapted for 4 days with a given purified diet and ad libitum drinking. The initial weight of the rat was weighed and then they were caged individually. Rats were divided two groups: 7 rats fed by purified diet [8] as a control group and 7 rats fed by calcium deficient

diet [9]. The amount of diet given was 15 g per day in a paste form and the drinking water provision was with ad libitum way. These treatments were given for 12 weeks.

The research utilizing experimental method with completely randomized experimental design (CRD) with 2 treatments and 7 replications. Treatment group of calcium deficient rat model could be seen in Table 3.

Table 2. Composition of mineral mix constituent

Composition of mineral		Mineral mix with calcium	Mineral mix without calcium
Sodium chloride	NaCl	139.300 g	139.300 g
Potassium hidrofosfat	KH_2PO_4	389.000 g	389.000 g
Magnesium sulfate anhydrous	$MgSO_4$	57.300 g	57.300 g
Calcium carbonate	**$CaCO_3$**	**381.400 g**	**0 g**
Corn starch		**0 g**	**381.400 g**
Ferric sulfate.$7H_2O$	$FeSO_4.7H_2O$	27.000 g	27.000 g
Manganese sulfate.H_2O	$MnSO_4.H_2O$	4.010 g	4.010 g
Potassium iodide	KI	0.790 g	0.790 g
Zinc sulfate.$7H_2O$	$ZnSO_4.7H_2O$	0.548 g	0.548 g
Copper sulfate	$CuSO_4.5H_2O$	0.477 g	0.477 g
Cobalt chloride	$CoCl_2.6H_2O$	0.023 g	0.023 g

Source: Modified AIN-93 M

Table 3. Treatment group for calcium deficient rat model making

Group	Treatment	Amount
C	Normal rats fed with purified diet	7 rats
CD	Normal rats fed with calcium deficient diet	7 rats

2.4. Parameters measured

(1) Consumption of dry matter was calculated daily by weighing the residual diet, substracted the diet given (g rat^{-1} day^{-1}) by it and multiplied by dry matter of diet

(2) Consumption of calcium, daily calcium intake was calculated by weighing given diet, substracted it by the residual diet (g rat^{-1} day^{-1}) and multiplied by the calcium concentration of the diet resulted by the proximate analysis.

(3) Concentration of calcium in the feces, liver, kidney, and bone. Sampling of feces was conducted before treatment (t_0), 4^{th} week (t_1), 8^{th} week (t_2), and 12^{th} week (t_3). Sampling of liver, kidney, and bone was conducted before treatment (t_0) and after 12^{th} week treatment (t_3). Sample preparation and analysis used the wet ashing method and then the sample was read by Atomic Absorption Spectrophotometer (AAS) Shimadzu AA-6300 to know the calcium concentration.

(4) Calcium absorption was calculated by subtracting the amount of calcium consumed by the amount of calcium released together in feces divided by the amount of calcium consumption and multiplied by 100%.

(5) Serum calcium concentration. Blood sampling was conducted before treatment (t_0), 4^{th} week (t_1), 8^{th} week (t_2), and 12^{th} week (t_3), by using cardiac puncture or blood sampling in the heart. Blood samples were centrifuged to obtain serum, test tubes and O-C FAST® calcium reagent kits were prepared, added 1000 µl of calcium-1 as a solvent then added 5 µl serum blood samples and vortexed for 10 seconds and incubated for 5 minutes, added 250 µl calcium-2 (an enzyme) then vortexed again for 10 seconds and finally read the wavelengths using UV-Vis spectrophotometer with a wavelength of 570 to 580 nm [10].

(6) Concentration of the progesterone hormone. Sampling of serum using a syringe and tube berheparin was conducted before treatment (t_0), 4^{th} week (t_1), 8^{th} week (t_2) and 12^{th} week (t_3). Serum samples were centrifuged at 2000 rpm for 15 minutes to obtain serum which was then used to determine the concentration of progesterone. Kit used is DRG 17-α-OH Progesterone ELISA (DRG Instruments GmbH, Germany). Plasma progesterone concentrations were conducted through Enzyme Linked Immuno Sorbent Assay (ELISA) [11].

(7) Daily body weight gain was calculated by weighing the initial and final body weight of the study, and then multiplied by the maintenance time (days).

2.5. *Statistical analysis*

All the data obtained were shown as mean value + standard of deviation (Mean ± SD). Statistical analysis was conducted with Microsoft Excel 2007 and SPSS version 15. Data of parameter measurement were analyzed by ANOVA at 95% confidence interval, followed by Duncan's multiple range test

3. Results and Discussion

3.1. Fed formulasi

Purified diet is a fed formulation with selected raw material, unvary concentration and more easily to be controlled [8]. This fed can be made specifically to prepare an animal model which is deficient to one of nutrient (macro or micro), including calcium deficient. The need for calcium in normal rat is 0.5% [8]. Fed formula

made to a calcium deficient animal model should contain calcium which is less than 0.5% of the normal standard needs. Thus this animal experiences calcium deficient. Table 4 present the results of fed formulation analysis result of purified diet for rat model as control and calcium-deficient diet for calcium deficient rat.

Table 4. The results of the analysis of fed formulation

Composition of diet		Purified diet	Calcium deficient diet
Dry matter	(%)	76.89	77.31
Protein	(%)	17.78	17.90
Dietary fiber	(%)	0.44	0.49
Fat	(%)	3.11	3.07
Calcium	(%)	0.60	0.40
Phosphorus	(%)	0.20	0.20

The analysis results of calcium concentration in purified diet of 0.6% and calcium deficient diet of 0.4%. If the animal is given calcium deficient diet during the growth period, it will experience osteomalacia [12]. The absorption of calcium occurs in the small intestine duodenum, in normal conditions approximately 30-35% of calcium consumed is absorbed by the body and circulated throughout the body whereas calcium excretion is mostly through feces [13]. One factor that affects the nutritional requirements for rat is interaction of nutrition [8].

3.2. Calcium deficient rat model

Preparation of calcium deficient rat model was with provision of calcium deficient diet for 12 weeks [9]. Calcium deficient period was made up until the rat experience calcium deficient characterized by a serum calcium concentration which is less than 9.2 mg dL^{-1} [14]. Provision of calcium-deficient diet containing 0.4% calcium was lower than rat calcium requirement of 0.5% in accordance with NRC [8]. Osteoporosis could be worse not only caused by low calcium consumption and absorption but also due to the very high ratio of phosphate and calcium in diet [2]. The high phosphate consumption leads the secondary hyperparathyroidism that disrupts calcium homeostasis, especially in the elderly [15].

The results of the calculation for consumption of dry matter, consumption of calcium, calcium absorption, calcium in feces, bone, liver, kidney were presented in Table 5. The result showed that there was no significant difference between treatments. Serum calcium concentration of rat fed by calcium deficient diet was lower than rat fed by purified diet as the control treatment ($P < 0.05$).

At the end of 12 weeks treatment, serum calcium concentration was 7.72±1.08 mg dL^{-1} in calcium deficient rats group and 11.60±0.85 mg dL^{-1} in control group. Normal serum calcium concentration ranged from 9.2 to 10.4 mg dL^{-1} [14]. Serum calcium concentration of rat fed with calcium deficient diet was significantly lower than rat fed with purified diet as a control ($P < 0.05$). Serum calcium concentration in *Sprague dawley* female rat were

approximately 13.60 mg dL^{-1} [16], whereas in human, normal serum calcium concentration were among 8.5-10.5 mg dL^{-1} [17].

Table 5. Measurement parameter of normal and calcium deficient rats group

Parameters		Normal rats	Calcium deficient rats
Consumption of dry matter	(g rat^{-1} day^{-1})	7.997 ± 0.877	7.886 ± 0.647
Consumption of calcium	(g rat^{-1} day^{-1})	0.048 ± 0.005	0.032 ± 0.003
Calcium in feces	(g rat^{-1} day^{-1})	0.004 ± 0.001	0.003 ± 0.001
Calcium absorption	(g rat^{-1} day^{-1})	0.044 ± 0.006	0.028 ± 0.003
Calcium absorption	(%)	90.732 ± 3.478	89.948 ± 4.028
Serum calcium	(mg dL^{-1})	11.600 ± 0.850[a]	7.720 ± 1.080[b]
Calsium in bone	(%)	32.900 ± 2.652	31.588 ± 0.887
Calsium in liver	(%)	0.001 ± 0.001	0.001 ± 0.001
Calsium in kidney	(%)	0.003 ± 0.002	0.002 ± 0.001
Daily body weight gain	(g rat^{-1} day^{-1})	0.100 ± 0.090	0.060 ± 0.040

Different superscripts in the same row indicate significant differences in test ($P < 0.05$) by ANOVA

Serum concentration of calcium deficient rat were 34.38% lower than the control treatment, without the clinical disorders. This looked from the activity of normal eating. The higher concentration of calcium in serum, the higher calcitonin secretion by the thyroid, it would enhance the bone mineralization [18]. When serum calcium or phosphorus concentration were too high, the hormone of calcitonin would reduce the absorption in the small intestine, increased the absorption of calcium and phosphorus in bone and increase the excretion in renal. Conversely when the serum calcium deficient, parathyroid hormone followed by calcitriol would increase the concentrations of calcium serum or without phosphorus through absorption in the small intestine or reabsorption in bone and reduces the calcium excretion of kidney [19].

The presence of calcium in the body was a homeostatic function that causes calcium concentration in serum unchange too much. To maintain serum calcium concentrations within the normal range, the system homeostasis parathyroid hormone stimulated the kidneys to increase the reabsorption of calcium and lower phosphate absorption. It was characterized by lower calcium excretion [20].

3.3. Progesterone hormone condition

Serum progesterone hormone concentration in normal rat and calcium deficient rat at the beginning and end of treatment were presented in Table 6, showed that rat aged 15 months began to decline in progesterone concentration. Statistical analysis showed that the significant effect of rat age ($P < 0.05$) in the serum

concentration of the progesterone hormone. The average serum progesterone concentration could decline with age.

Serum progesterone concentration in rat fed of calcium-deficient diet for 3 months had the same pattern seen with the normal rat. Statistical analysis result showed that rat age had a significant effect on the concentration of progesterone. With increasing age, there was a decline in the function of reproductive organs, thus progesterone concentration could decrease. The decreased function of reproductive organs causing decreased concentration of estrogen and progesterone [21]. Rat with age of 12 months still had high progesterone concentration because age of 12 month were in the middle-aged rat [22]. Rat were still able to reproduce the progresterone although fertility conditions began to decrease and still had normal estrous cycles.

Table 6. Measurement parameter for progesterone hormone

Treatment	Serum progesterone (ng mL^{-1})	
	12 months	15 months
Normal rats fed with purified diet	23.97±2.31	19.47±0.34
Normal rats fed with calcium deficient diet	23.97±2.31	19.38±1.20

Different superscripts in the same row indicate significant differences in test ($P < 0.05$) by ANOVA

The older rat, the lower hormone progesterone concentration [3]. An experiment to the female rat strain Wistar on aged 2 months, increased progesterone concentration in pregnant period (48.9±2.6 ng mL^{-1}) than normal period (4.5±0.2 ng mL^{-1}) [23]. In non pregnant women, progesterone was generally secreted by the corpus luteum which period of pregnant the placenta become of main source. Progesterone circulation in the blood was usually binding on *Corticosteroid Binding Globulin* (CBG), *Sex Hormone Binding Globulin* (SHBG) and albumin.

In Figure 1, there are three rats which given calcium deficient diet, suspected on the estrus condition based on the concentration of progesterone.

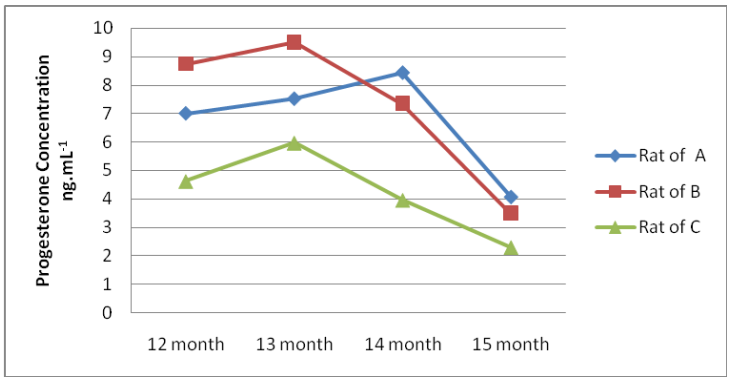

Figure 1. Progesterone concentration on estrus condition

At the age of 15 months, the concentration of progesterone had occurred decreasing compared with rat of 12 months. Premenopausal women, approximately at the age of 40 years, experienced the decline in progesterone hormone secretion [24]. The rat of 18 months experienced the decrease in progesterone hormone that is the basis for determining the condition of premenopausal [3]. The old female rat had long periods of diestrus and increased of gonadotropin secretion [21].

Decreased concentration of estrogen, progesterone, calcium and vitamin D could lead to decrease in bone mass and bone metabolic disorder known as osteoporosis [25]. Premenopausal women generally had normal bone density, but Caucasian women with thin bodies was found osteopenia, abnormal bone density or begins to decline [26]. Stress increases the synthesis of ovarian independently and progesterone release, which could regulate the sensitivity of Ca^{2+} from production in left ventricular cardiomyocytes [27].

4. Conclusion

Treatment with calcium-deficient diet for 12 weeks could reduce serum calcium concentration into 7.72±1.08 mg dL^{-1} in calcium-deficient rats and 11.60±0.85 mg dL^{-1} in the control. Thus, rat model strain *Sprague dawley* of premenopausal age given calcium-deficient diet could be calcium-deficient rat based on the condition of serum calcium concentration and caused the less dense bone in next phase. Serum progesterone concentration in control rats and calcium deficient rats was not affected by treatment of diet, but it was affected by the age of rat, at the end of the study, the progesterone concentration of 15 month old rat was decreasing. The rat require environmental and composition of fed is optimal for growth and development, a lack of calcium in the diet is consumed can affected the condition of health.

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