CHAPTER III

MATERIALS AND METHODS

Experiment 1

1. Animals

Eight mature female beagles with body weight of 8.7 $\pm$ 0.4 kg were used in the experiment. The Cross-over design was used for this experiment. All dogs were housed individually in the metal cages (1.0 x 1.2-m) with a plastic slat floor at temperature between 25.7 and 30.3°C. Each cage was cleaned twice daily. In the pretest period, all dogs were fed a basal diet for 2 weeks. The purpose of this period was to reduce the variability of Zn stores of the dogs. At the end of this pretest period, four dogs were randomly allotted to one of two treatments. However, dogs receiving the basal diet would be removed from the test if sign of Zn deficiency were severe. The dogs were fed the basal diets with Zn supplement in the form of solution for 3 weeks (test period). Fresh water containing 0.15 ppm Zn was available ad libitum throughout the experiment.

2. Feed and Feeding

The commercial extruded dog diet formulated with no Zn supplementation in accordance with the AAFCO (2000) nutrient guide for adult dog was used as the basal diet. The major ingredients of basal diet composed of broken rice, corn gluten meal, chicken meal, soybean meal, salt, limestone, monocalciumphosphate, and potassium chloride. The chemical analysis of nutrient composition in basal diet is presented in Table 2. This basal diet contained Zn at the level 58.5 ppm DM as composition of raw materials. Each treatment was received the basal diet with supplied Zn 61.5 ppm DM (to meet minimum Zn requirement for maintenance (120 ppm DM) according to AAFCO (2000)) either in the form of zinc methionylglycinate (ZnMG; 20% Zn; 2 mol Met-Gly : 1 mol Zn) or zinc sulfate (35% Zn; $ZnSO_4 \cdot H_2O$) for treatment 1 and 2, respectively (Table 3). Each form of Zn was prepared as solution

by dissolving 0.8 g of either ZnMG or $ZnSO_4$ with 20 ml of deionized water. The amount of food was calculated by using standard equations to determine energy requirements of active adult dogs (ME requirement, kcal = $132BW_{kg}^{0.67}$; Case et al., 2000) and adjusted every 2 weeks. Each day, food was weighed and divided into two equal portions and fed to dogs at 0700 and 1530 hours in stainless steel bowls. Zinc solution was served at 15 minutes after offering each meal.

Table 2 **Chemical analysis of nutrient composition in the basal diet (DM basis)**

Item	
Metabolizable energy (kcal/g)[a]	3.7
Crude protein (%)[b]	25.8
Ether extract (%)[b]	7.6
Crude fiber (%)[b]	0.8
Ash (%)[b]	5.5
Calcium (%)[b]	1.0
Phosphorus (%)[b]	0.8
Zinc (ppm)[c]	58.5

[a] Calculated by use of equation from NRC (2006): ME, kcal/g = [(3.5 x CP) + (8.5 x EE) + (3.5 x NFE)]/100

[b] Analyzed according to the AOAC (1990) procedures

[c] Analyzed according to Sullivan and Carpenter (1993)

Experiment 2

1. Animals

Nine mature female beagles with body weight of 8.3 ± 0.1 kg were used in the experiment. The experiment was design as a 3 x 3 Latin square. All dogs were housed and managed as the experiment 1 at temperature between 24.1 and 32.4 °C. They were fed the basal diet for 2 weeks (pretest period). At the end of this pretest period, three dogs were randomly allotted to one of three treatments. Dogs were fed the basal diets with Zn supplement in the form of solution for 3 weeks (test period).

Fresh water containing 0.15 ppm Zn was available ad libitum throughout the experiment.

2. Feed and Feeding

The basal diet was the same as experiment 1. Each treatment was received the basal diet with supplied Zn 21.5, 61.5, and 101.5 ppm DM (to meet 80, 120, and 160 ppm DM, respectively) in the form of ZnMG for treatment 1 to 3, respectively (Table 4). Feeding method was the same as experiment 1.

Table 3 Description of the treatments in the experiment 1

Treatment	Description
1	Basal diet + 61.5 ppm Zn DM from ZnMG
2	Basal diet + 61.5 ppm Zn DM from $ZnSO_4$

Table 4 Description of the treatments in the experiment 2

Treatment	Description
1	Basal diet + 21.5 ppm Zn DM from ZnMG
2	Basal diet + 61.5 ppm Zn DM from ZnMG
3	Basal diet + 101.5 ppm Zn DM from ZnMG

Data Collection

Data collection of both experiment 1 and 2 were similar. Throughout of the experiment, body weight of dogs were recorded twice weekly for adjusting the amount of food. Food intake were measured daily.

Sample Collection and Determination

Sample collection and determination of both experiment 1 and 2 were similar as follow:

1. Food

Throughout the experiment, the food sample was collected daily and pooled into plastic bags and stored at -20°C until nutrient content analysis. Food sample was ground through a 1-mm screen mill (cyclotec 1093 sample mill). It was analyzed in duplicate for DM, CP, EE, CF, ash, Ca and P using AOAC (1990) procedures. Zinc concentrations were determined by atomic absorption spectrophotometer (AAS; Model spectr AA - 300) using Sullivan and Carpenter (1993) method (nitric-perchloric acid digestion). Food sample was digested by boiling in 20 ml HNO_3 plus 10 ml 70 – 72% $HClO_4$. Until solution is colorless or nearly so and dense white fumes appear. The resulting cool solution was diluted to 100 ml volumetric flask with deionized water.

2. Blood

At the initiation of the experiment, the end of the pretest period (d 14 of the experiment), and the end of test period (d 35 of the experiment), 6 ml of blood was collected from cephalic vein. These collections were performed between 3 and 4 hours after offering the meal at 0700 hours (Swinkels et al., 1996). Blood samples 5 ml were collected into heparinized polypropylene (PP) tubes and placed on ice then centrifuged at 2000 x g for 15 minutes at 6°C (modified from Brinkhaus et al., 1998). Another 1 ml of blood samples were collected into nonheparinized PP tubes then placed on ice until it was analyzed for serum ALP activity. The separated plasma was stored at -20°C in 5 ml PP tubes until plasma Zn analysis was performed. Plasma was prepared for analysis of Zn concentration by diluting plasma with deionized water (modified from Wedekind et al., 1994) using AAS (Model spectr AA – 300;). Serum ALP activity was determined by automated analyzer (BT2000Plus, Biotecnica Instrument S.p.A.) and test kit Cat. No. AD711AP and AD701AP (Audit diagnostics).

3. Hair

A patch of similar colored hair was shaved from the dog's neck using a 20 cm^2 template on the day before the beginning of the test period (d 14 of the experiment). Thereafter the same patch was shaved on the last day of the experiment (d 35). The hair was handled, collected in plastic bag, weighed and stored at room temperature until Zn analysis was performed. At the end of the experiment (d 35), chest hair was shaved using a 10 cm^2 template and collected in plastic bag for analysis of hair condition. Neck hair samples were weighed for determination of hair growth rate (Lowe and Wiseman, 1998) and analyzed for Zn concentrations by AAS (Model spectr AA – 300) using Van den Broek (1988) method. Neck hair samples were digested by boiling in 3 ml HNO_3 plus 1 ml 70 – 72% $HClO_4$. The resulting cool solution was diluted to 50 ml volumetric flask with deionized water. The Zn deposition in hair values were calculated by equation: Zn deposition in hair (μg/21d·20cm^2) = [Zn concentration in neck hair sample (μg/g)] x [weight of neck hair sample (g/21d·20cm^2)]. Chest hair samples were screened and photographed under scanning electron microscopy photographic (JSM-5800LV, JEOL). Briefly, approximately 10 strands of hair from each individual dog were adhered on the stub using double-stick tape, and then the surface of these stubs were coated with gold for electrical conductivity (Toya et al., 1986). Hair coat condition were evaluated base on physiological appearance under scanning electron microscopy at x 1,500 magnification (Kuhlman and Rompala, 1998).

4. Feces

From d 25 to 32 of the experiment, the dogs were dosed orally twice a day, before each feeding, with a gelatin capsule containing 250 mg of chromic oxide, in order to use as an indigestible marker. On the first day of fecal collection (d 29), all feces before 0700 hours were removed from the cages and discarded. Fecal output after that was collected until 2000 hours on d 32 from individual dog and placed into labeled plastic bags. Fecal samples of each dog were stored at -20°C and dried at 60°C in a forced-air oven. After drying, the sample was ground through a 1-mm screen mill (cyclotec 1093 sample mill) and collected in labeled

plastic bottles at room temperature until further analysis. Fecal samples were examined for Zn by the similar method as food sample. They were examined for Cr using Williams et al. (1962) procedures. Both Zn and Cr concentrations were determined by AAS (Model spectr AA – 300). Apparent absorption of Zn was calculated as described by Maison (2001). The percentages of apparent Zn absorption and fecal Zn, daily fecal Zn excretion, and daily Zn absorption formulas are shown below.

$$\text{Apparent Zn absorption (\%)} = 100 \times \left[1 - \left(\frac{\%\,\text{Cr in food} \times \%\,\text{Zn in feces}}{\%\,\text{Cr in feces} \times \%\,\text{Zn in food}}\right)\right]$$

Fecal Zn (%) = 100 – %Apparent Zn absorption

Daily Zn absorption (mg/d) = %Apparent Zn absorption x Daily Zn intake

Daily fecal Zn excretion (mg/d) = %Fecal Zn x Daily Zn intake

The diagram of sample collection is shown in Figure 9. All glassware used for Zn analysis was soaked in 10% nitric acid and rinsed (3 times) with deionized water.

Statistical Analysis

All data was expressed as mean ± SE. In experiment 1, data were analyzed as a Cross-over using the general linear model. Each dog represented an experimental unit. The model included period, dog, and treatment, and the error was residual error mean square. In experiment 2, data were analyzed as a triplicated 3 x 3 Latin square using the general linear model. Each dog represented an experimental unit. The model included square, dog(square), period, and treatment, and the error was residual error mean square. The mean differences between treatments were tested by least significant difference using the commercially computer program (SAS, 1988). Differences were considered significant when $P < 0.05$ and were regarded as trends if $0.05 \leq P < 0.10$.

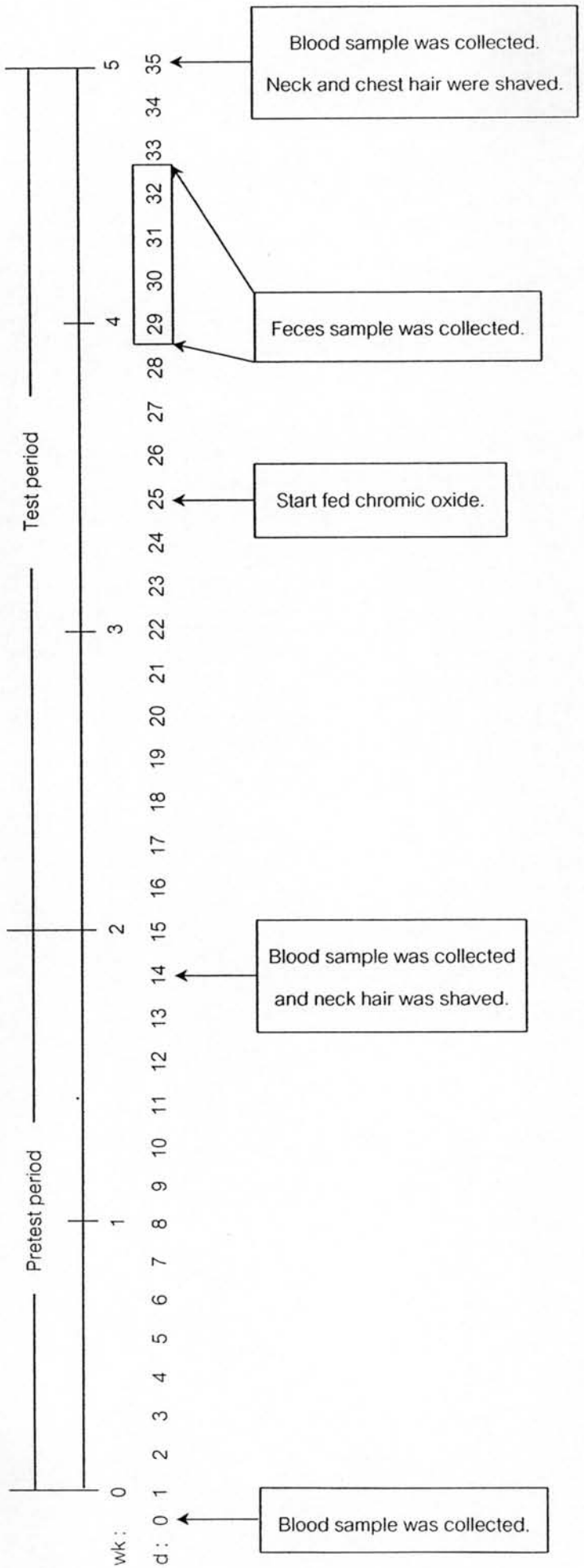

Figure 9. Diagram of sample collection