# CHAPTER III

# MATERIALS AND METHODS

## Animals and management

Ten, late pregnancy 87.5% crossbred Holstein cattle were used in the experiment. They were divided into two groups of five animals each. All animals were housed in tie stall type sheds, having a solid floor and open sides. Animals in each group were fed with rice straw treated with 5% urea as the source of roughage throughout the experiment. Animals individually received an average of a 4 kg/day of roughage in combination with the same concentrated mixture (7kg/day) to maintain a moderate body condition score (2.5 scale = 1 to 5). The chemical composition of feeds is presented in Table 1. The maximum temperature in the shed at noon was 34±1°C and the minimum temperature at night was 26±1°C. The relative humidity was 68±12%. Food was given in equal portions at about 06.00 h and 17.00 h when animals were milked. Water were available for *ad libitum* intake.

## Experimental procedures

Animals were random divided into control (n = 5) and experimental (n = 5) groups. Two consecutive period of experiments were carried out in each group, consisting of the pretreatment period (45 days postpartum), and treatment periods of 105 days postpartum (early lactation). In the treatment period, animals in the experimental group were biweekly injected subcutaneously until the end of experiment with 500 mg of recombinant bovine somatotropin (rbST) suspended in 792 mg of a prolonged-release formulation in sesame oil (POSILAC, Monsato, USA) at day 60 of lactation, while animals in the control group were biweekly injected subcutaneously

with 800 mg of sterile sesame oil without rbST as placebo. An injection was administered at postscapular.

**Table 1**: Chemical composition of feed components (% on dry matter basis).

| Particulars | Urea-treated rice Straw | Concentrate |
|---|---|---|
| Dry matter | 57.7 | 89.4 |
| Crude protein | 7.0 | 17.2 |
| Acid detergent fibre | 43.5 | 20.7 |
| Neutral detergent fibre | 70.2 | 28.4 |

Concentrate formulation: fresh weight (kg/100% kg) consisted of soy bean meal 26.3 kg, cotton seed 37 kg, cassava 28.5 kg, rice bran 3.3 kg, limestone 1.3, dicalcium phosphate 1.5 kg, sodium bicarbonate 1.1 kg, potassium chloride 0.8 kg and premix 0.2 kg.

(5 kg urea dissolved in 100 water per 100 kg dry rice straw). Rice straw sprayed with urea solution was mixed thoroughly and stored under airtight conditions in a cement pit for 21 days. A continuous supply of treated rice straw was made available by using a two pit x 21 day system of urea treatment. After 21 days, the rice straw treated with 5% urea was offered to the animals.

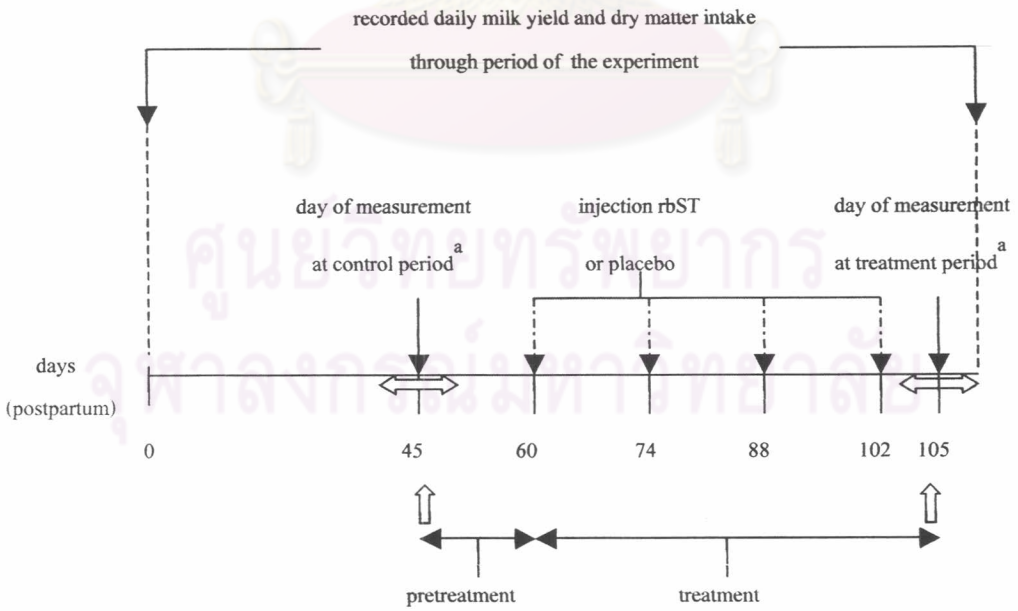
From the beginnig of pretreatment to the end of treatment period, animals of both groups were fed the same ration from before parturition through the completion of experiment. The dry matter intake of each animal was determined by measuring both the concentrate and roughage offered and refused each day. On the day of experiment in each period, measurements of the total body water, water turnover rate, mammary blood flow, plasma volume and extracellular fluid were carried out. The rate of milk secretion was recorded by hand milking in the afternoon and measurement of mammary blood flow was carried out. In each period of study animals were weighed after collecting the milk sample.

**Animals preparation**

On the day before the experiment in each period, two catheters (i.d. 1.0 mm, o.d. 1.3 mm, length 45 mm) were inserted into either the left or right milk vein by using intravenous polymer catheter (Jelco, Critikon; Johnson & Johnson, U.K.) under local anesthesia. This was carried out on standing animal for measurement of mammary blood flow and for the collection venous blood. The tip of the catheter was positioned near the sigmoid flexure anterior to the point at which the vein leaves the udder. The other catheter was positioned downstream about 20 cm from the first one. The catheter for both isotope and dye injection was inserted into and ear vein under local anesthesia. All catheters were flushed with sterile heparinized normal saline and were left in place during the experiment.

**Protocol of the experiment**

The protocol of the experiment is shown as following.




[a] On the day of the experiment : injected tritiated water, 0.5%Evan's blue dye and 10%NaSCN

⟺ : recorded water intake 7 days consecutive on each experiment period

⇧ : sampling period

**Determinations of water intake, total body water, water turnover and empty body water (EBW)**

An estimation the rate of water intake values of each animal in each period of experiments was recorded by an averaged over seven days from weighing daily water consumption in water bowl.

The water turnover rate and total body water were determined in each animal by tritiated water dilution techniques. The animal was injected intravenously via the ear vein with carried free tritiated water (TOH) in normal saline at a single dose of 2,500 μCi per animal. The equilibration time was determined by taking blood samples for 3 days after the injection. Blood samples for water turnover measurements were collected 4, 8, 20, 26, 32, 44, 50, 56, 68 and 74 h subsequent to the injection (Chaiyabutr et al., 1997). The preparation for sample counting was achieved by the internal standardization technique as described by Vaughan and Boling (1961). The corrected activity of the samples, in d.p.m., were plotted on semi-logarithmic paper against time, in hours after dosing, and the extrapolated activity at theoretical zero time of complete mixing of radio-isotope was used to determine the TOH space by the following equation.



TOH space (l) = [standard count (dis/min) x dose (ml)]/[sample counts at zero time (dis/min)].



The biological half-life of tritium (T1/2) was determined from the slope of the linear regression line obtained from plot on semi-logarithmic paper of the activity of the samples taken over the period of 3 days against time.

The water turnover rate was calculated from the equation.

$$\text{Water turnover rate (l/d)} = \frac{0.693 \times \text{TOH space}}{\text{biological half-life}}$$

The total body water was calculated by using the corrected factor (1 – fraction of plasma solids) x TOH space (Chaiyabutr et al., 1997).

Empty body water (EBW) did not include water associated with gastrointestinal contents or the water in the fetus. The EBW was estimated from the disappearance curve of tritium in blood plasma for each animal. Two compartment open system model was used to estimate the EBW (Shipley and Clark, 1972). The exponential equation describing the two compartment model was calculated from the equation.

$$Y = Ae^{-k1t} + Be^{-k2t}$$

Where Y = concentration of tritium in plasma at time t

A = plasma concentration intercept of the fast phase of the plasma curve

B = plasma concentration intercept of the slow phase of the plasma curve

k1= first order rate constant of the fast phase

k2= first order rate constant of the slow phase

t = time in minutes

**Determinations of plasma volume, extracellular fluid and plasma solids concentration**

In each period of study, plasma volume was measured by dye dilution technique using of Evans blue dye (T-1824) (E. Merck, Darmstadt, Germany) and extracellular fluid volume was measured using sodium thiocyanate (NaSCN). The injection of 20 ml of the 0.5% T-1824 (0.5 g/100 ml normal saline), and 20 ml of the

10% NaSCN solution (10 g/100 ml normal saline) were given into the ear vein catheter. Venous blood samples from the jugular vein were taken at 20, 30, 40 and 50 min after dye injection. The dilution of dye at zero time was determined by using semi logarithmic concentration on time extrapolation. Blood volume was calculated from the plasma volume and packed cell volume (PCV) from the equation.

$$\text{Blood volume (l)} = \frac{\text{plasma volume}}{1 - \text{PCV}}$$

Intracellular fluid could be calculated by subtracting ECF from TBW.

Plasma osmolality was measured using the freezing point depression method (Advance Osmometer model 3, Massachusetts, USA). The plasma solids concentration was determined by a refractometer.

**Determination of mammary blood flow**

Blood flow through half of the udder was determined by measuring the dilution of dye T-1824 (Evans blue) by a short term continuous infusion (Chaiyabutr et al., 1997). The dye was infusion by peristaltic pump, at a constant rate of 80 ml/min into the milk vein for 10 sec. Before infusion, blood was drawn from downstream in the milk vein as a pre – infusion sample. About 10 sec after starting the infusion, 10 ml of blood was drawn from downstream in the milk vein at a constant rate into heparinized tube. Three consecutive blood samples were taken during dye infusion. The mammary blood flow was therefore calculated by doubling the flow measured in one milk vein. Pack cell volume was measured after centrifugation of the blood in a microcapillary tube. Mammary blood flow was calculated from the equation.

$$\text{Mammary plasma flow (MPF; ml/min)} = \frac{I\,(C_I - C_V)}{(C_V - C_A)}$$

$$\text{Mammary blood flow (MBF; ml/min)} = \frac{MPF}{1 - PCV}$$

Where I = dye infusion rate

$C_I$ = concentration of dye infusion

$C_V$ = concentration of dye in plasma milk vein

$C_A$ = concentration of dye in plasma artery

**Determination of mammary uptake of metabolites**

Blood samples were collected from milk vein by venipuncture with a No. 21 needle and coccygeal artery by venipuncture with a No. 18 needle into heparinized tube. Blood samples were kept in crushed ice and then centrifuged at 3,000 rpm for 10 min for plasma collection. Plasma samples from both milk vein and coccygeal artery were kept at –20°C until measurements of the concentration of plasma metabolites.

Plasma samples from both milk vein and coccygeal artery were used to determine the concentrations of glucose, triglyceride, β-hydroxybutyrate, and acetate.

**Determinations of the plasma glucose concentration, mammary arterio–venous difference, mammary extraction ratio and uptake of glucose**

The plasma glucose concentration was assayed by colorimetric method using enzymatic oxidation in the presence of glucose oxidase. The formed hydrogen peroxide reacts under the catalysis of peroxidase with phenol and 4 aminophenazone to a red–violet quinoneimine dye as an indicator. The mammary extraction ratio of glucose was calculated by divided the arterio–venous concentration difference by the

arterial plasma glucose. Mammary uptake of glucose was calculated using mammary plasma flow multiplied by glucose arterio–venous difference (equation 1).

**Determination of the plasma triglyceride concentration, mammary arterio–venous difference, mammary extraction ratio and uptake of triglyceride**

The plasma triglyceride concentration was assayed by an enzymatic method using enzymatic hydrolysis with lipase. The indicator is the quinoneomine formed hydrogen peroxide, 4–aminoantipyrine and 4–chlorophenol under the catalytic influence of peroxidase. The arterio–venous concentration difference and mammary extraction ratio of triglyceride were calculated as described in equation 1.

**Determination of the plasma β-hydroxybutyrate concentration, mammary arterio-venous difference, mammary extraction ratio and uptake of β-hydroxybutyrate**

The plasma β-hydroxybutyrate concentration was assayed using an enzymatic reaction in the presence of β-hydroxybutyrate dehydrogenase. The arterio–venous concentration difference and mammary extraction ratio of β-hydroxybutyrate were calculate as described in equation 1.

**Determination of the plasma acetate concentration, mammary arterio–venous difference, mammary extraction ratio and uptake of acetate**

The plasma acetate concentration was assayed by an enzymatic method (Boehringer Mannhelm). The arterio–venous concentration difference and mammary extraction ratio of acetate were calculated as described in equation 1.

**Calculations**

Equation 1

Arterio – venous concentration difference (mmol/l) = A – V

Mammary extraction ratio (%) = [(A – V)/A] x 100

Mammary uptake (μmole/min) = MPF x (A – V)

A = arterial plasma metabolites concentration

V = venous plasma metabolites concentration

**Milk collection**

Milk was collected by hand milking and kept in formaldehyde. The formalinized milk sample (300 μl of 40% formalin in 30 ml of fresh milk) was kept at 4°C for determinations of lactose, fat and protein concentrations.

**Determination of milk compositions**

Milk samples were analyzed for concentration of protein, lactose and milk fat as followed.



The milk protein concentration was analyzed using milkoscan.



The milk lactose concentration was analyzed by the colorimetric method, as described by Tele et al.(1978). The color development was based on the combined action of phenol, sodium hydroxide, picric acid and sodium bisulfite with lactose. The concentrations of samples were read the optical density at wavelength 520 by spectrophotometer.

The milk fat concentration was measured using Gerber method. This method was used sulphuric acid of 91 mass% and amylalcohol. The sulphuric acid oxidizes and hydrolyzes the organic components of the fat globule protective coating the lactalbumin fractions and the lactose. The fat was released and separated by centrifuging whereby the addition of amyl alcohol makes the phase separated easier and a sharp delineation is produced between the fat and the acid solution. The fat content of the milk can be read off on the scale on the butyrometer as a mass content in percent (gm%).

**Determination of the plasma IGF-1 concentration**

Arterial plasma samples were used to determine the plasma IGF-1 concentration by the IMMULITE® analyzer. The IMMULITE® analyzer is automated chemiluminescent immunoassays for the quantitative determination of the plasma IGF-1 concentration. The assay utilized a monoclonal murine anti-IGF-1 antibody on the solid phase-a polystyrene bead. The bead was coincubated with pretreated sample for 30 minutes and, after removal of unbound material by a centrifugal wash procedure. It was incubated in a subsequent 30 minute incubation with alkaline phosphatase-conjugated polyclonal rabbit anti-IGF-1 antibody.



**Statistical analysis**

All data were presented as the means ± SD. Statistical significant difference between period in the same group was determined by the student' s paired t–test. The student's unpaired t–test was used to estimate the statistical significant difference between groups.