CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

Blood samples were withdrawn from the jugular vein of the female goats and it was done at the Institute Science and Biology (ISB) farm, University Malaya. There were three phase of blood samples collection. For the first phase, blood samples were collected daily during oestrous cycle from 25 mature female goats of different genotypes, aged 1 to 7 years old between 0700 hours – 0800 hours for 50 consecutive days from April 1998 until June 1998. For the second phase of blood collection, blood samples were collected every 8 hours daily, 2 days before oestrus until 2 days after oestrus from 5 mature female goats of Jermasia genotype, aged 1 to 4 years old from early December 1998 until end of December 1998. In the third phase of blood collection, blood samples were collected daily, one day before the insertion of CIDR implant until a few days after the withdrawal of the CIDR implant (that is one day after the detection of oestrus in the female goats) from 15 mature female goats of three different genotypes, aged between 1 to 4 years old from March 2002 until April 2002. The blood samples were taken to the Radioimunoassay Laboratory, Institute of Biological Sciences (ISB), Faculty of Science, University of Malaya, to be centrifuged and the sera were kept in the freezer at -40°C before use. The frozen sera were analyzed for the reproductive hormone concentrations using the radioimunoassay (RIA) procedure. The main objective of the study was to characterize the patterns of

progesterone and oestradiol hormones in the blood of different goat genotypes with normal and abnormal oestrous cycles and after oestrus synchronization.

3.2 EXPERIMENTAL ANIMALS

The experimental animals were divided into three major groups. For the frist group, a total of 25 female goats were divided into three different genotypes, namely the local Katjang goats (5 animals), the Jermasia goats (10 animals) and the Boer crossbred goats (10 animals). Katjang goats are from local breed goat (Plate 1) while the Jermasia were goats from synthetic breed produced at University of Malaya, after crossing between the imported German Fawn males and the local Katjang females (Plate 2). The Boer crossbred goats were resulted from crossing between the Boer males (imported from South Africa) and the local Katjang females (Plate 3). Each genotype was divided into normal and abnormal oestrous cycles. For the abnormal oestrous cycle, it was further divided into long oestrous cycle and short oestrous cycle (Table 2). For the second group, a total of 5 mature female goats from Jermasia genotype aged 1 to 4 years old were grouped together for blood collection. For the third group, a total of 15 female goats were divided into three different genotypes; Jermasia goats (5 animals), the mix bred (5 animals) and the Boer crossbred goats (5 animals). The age of the animals ranged between 1 to 4 years old. All the experimental female goats used in this experiment were isolated from the male goats during the entire experiment to avoid mating. The animals were fed daily with pellets(concentrates) such as; Napier grass and were given clean water *ad libitum.*

Plate 1. A typical Katjang female goat

Plate 2. A typical Jermasia female goat

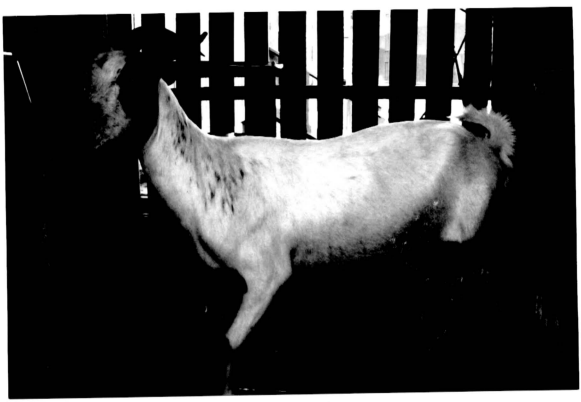
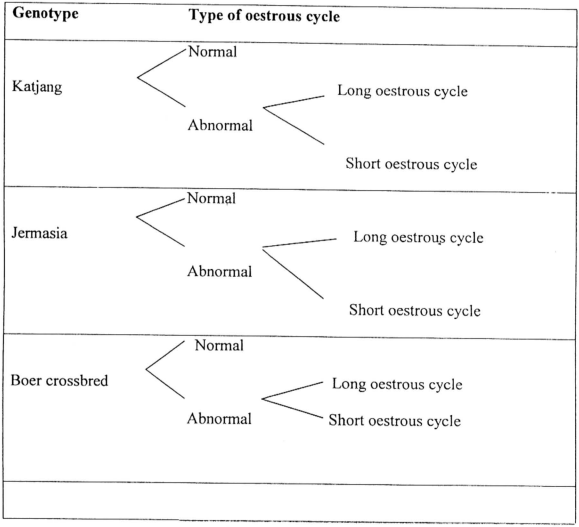


Plate 3. A typical Boer crossbred female goat

Table 2. The grouping of female goats according to genotypes and types of oestrous cycle



3.3 COLLECTION OF BLOOD SAMPLES

There were three phase of blood samples collection. For the first phase, blood samples were collected daily during oestrous cycle from 25 mature female goats of different genotypes, aged 1 to 7 years old between 0700 hours–0800 hours for 50 consecutive days. The collection of blood samples were done at the Institute of Biological Science, University of Malaya, from April 1998 until June 1998. For the second phase of blood collection, blood samples were collected every 8 hours daily, 2 days before oestrus until 2 days after oestrus from 5 mature female goats of Jermasia genotype, aged 1 to 4 years old. The collection of blood samples were done at the Institute of Biological Science, University of Malaya, from early December 1998 until end of December 1998. In the third phase of blood collection, blood samples were collected daily, one day before the insertion of CIDR implant until a few days after the withdrawal of the CIDR implant (that is one day after the detection of oestrus in the female goats). Blood samples were collected from 15 mature female goats of three different genotypes, aged between 1 to 4 years old. The collection of blood samples were done at the Institute of Biological Science, University of Malaya, from March 2002 until April 2002.

The blood collections were done in the area of the jugular vein of the goat. This particular area of the goat was rubbed with 70% alcohol and then the jugular vein area was pressed using our finger to block the blood flows until the jugular vein can be seen clearly. Then a needle size 23 G X 31/2" (0.60 X 90.00 mm) was injected into the area of the jugular vein and 7 ml of blood was collected into the plain blood collecting tubes size 15 X 100 mm (Venoject, Japan) (Plate 4). The blood samples were taken

Plate 4. Collection of blood samples

back to the Radioimmunoassay Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, to be centrifuged and analyzed using the radioimmunoassay (RIA) procedure.

3.4 PREPARATION OF SERUM

The blood samples collected were centrifuged at 3000 rpm for 10 minutes at 4°C. The sera were transferred into 2 ml microcap tubes and kept at - 40°C in the freezer until assay.

3.5 SYNCHRONIZATION OF OESTRUS

Oestrus synchronization of the animals was done for all the animals in the third group, using CIDR implant (Plate 5). The treatment procedure for oestrus synchronization for the animals are shown in Figure 7.

3.5.1 Insertion of the CIDR

CIDR implant was inserted through the vagina of the animals on the second day of the blood collection. The CIDR applicator was lubricated with the KY lubricating jelly and was inserted into the anterior vagina (Plate 6). The CIDR applicator was pushed through the vagina until it reached 4-5 cm in depth. Once it is in position, the applicator was withdrawn from the vagina allowing the drawstring of the implant hanging outside the vagina (Plate 7). The function of this string is for easy removal of the implant from the vagina. After the insertion, the applicator was cleaned

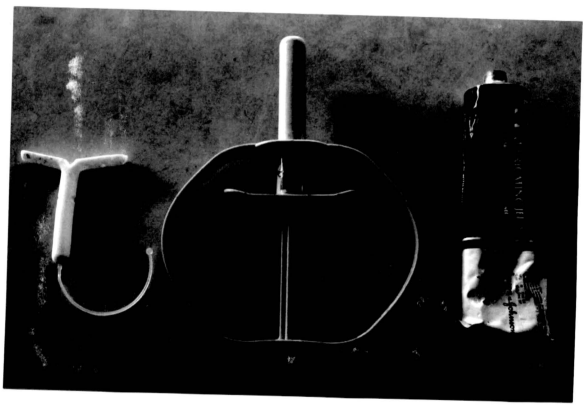


Plate 5. CIDR and its applicator

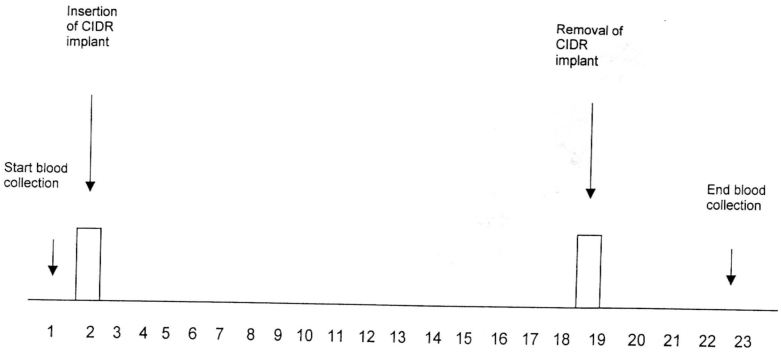


Figure 7. Treatment procedure for oestrus synchronization

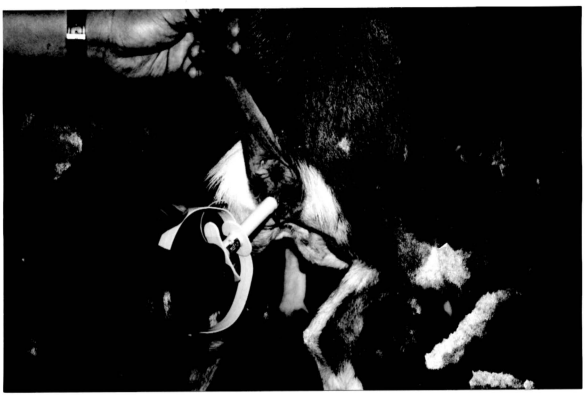


Plate 6. Insertion of CIDR implant through the anterior vagina of the goat

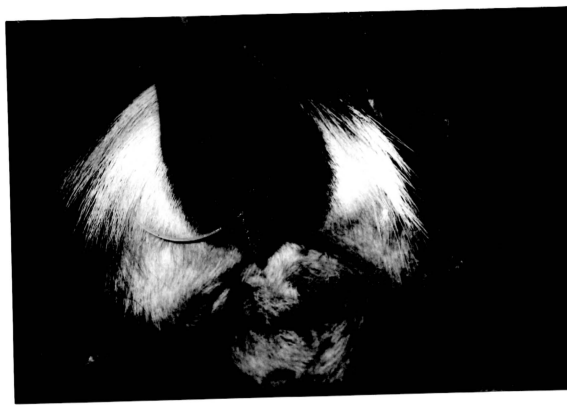


Plate 7.The drawstring of the CIDR implant hanging outside the vagina

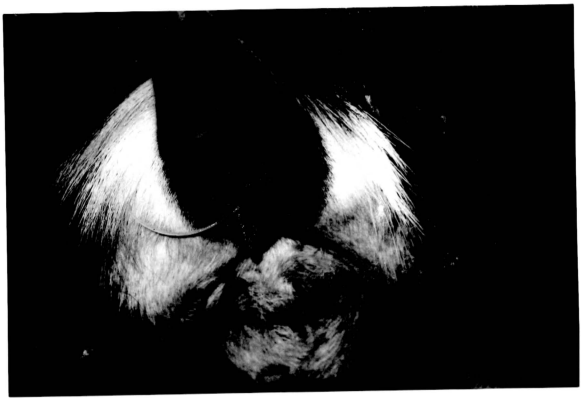


Plate 7.The drawstring of the CIDR implant hanging outside the vagina

using a disinfectant solution before being used for the next animals. The vagina of the animal was checked every morning to make sure that the CIDR implant was still in the place and any discharge from the vagina was also noted.

3.5.2 Removal of CIDR

The CIDR was removed from the animals after 19 days of blood collection. It was done by pulling out the string from the vagina (Plate 8).

3.6 DETECTION OF OESTRUS IN THE FEMALE GOATS

The detection of oestrus in the female goats was done continuously every morning at about 0730 hours, immediately after the blood collection. It was done by observing the behavioural patterns of the animals and also with the aid of a mature buck. Signs of oestrus that were observed included:

I. Secretion from the vagina.

II. Frequent wriggling of the tail.

III. Behavioural patterns, such as sexual receptivity and interest in the male goat.

The first day detection of oestrus in the female goats was considered as Day 0 of the oestrous cycle.

Plate 8. The removal of CIDR implant from the vagina

3.7 RADIOIMMUNOASSAY (RIA) PRINCIPLES

Two types of reproductive hormones were analyzed using the radioimmunoassay procedure. These two types of hormones were:

1. Progesterone (P_4)

2. Oestradiol (E_2)

The basic principle of RIA technique was based on the competition between the labelled and unlabelled antigens for the limited binding sites on antibodies. The antigens will combine with the antibodies to form the antigen - antibody complex. In this experiment, tracer isotope was used to label the antigens and was referred to as the 'hot' antigens. The unlabelled antigens were known as the 'cold' antigens. Tritium-3 (H-3) was a common isotope used for labelling the steroid hormones such as progesterone and oestrogen whereas radioactive iodine-125 (^{125}I) was for labelling the protein hormones such as LH, FSH, TSH, hCG, PRL and GH. However, in this experiment, ^{125}I was used to label all the reproductive hormones (P_4 E_2 and LH). When the labelled and unlabelled antigens were incubated together with the antibodies, the labelled antigens would compete with the unlabelled antigens in the animal's blood samples for a limited binding sites on antibodies thus produced the antibody-antigen complex. The reaction occur is shown as:

Ag +	*Ag +	Ab --------→	AgAb*Ag +	
(unlabelled antigen or 'cold' antigen)	(labelled antigen or 'hot' antigen)	(antibody)	(Antigen-antibody complex)	
			Ag +	*Ag
			(unlabelled antigen)	(labelled antigen)

For the reactions to reach the equilibrium, the assays were incubated for 3 hours at room temperature (27°C). It could also be incubated for only one hour but at higher temperature (37°C) to reach the equilibrium. After 3 hours of incubation, there were still unlabelled and labelled antigens remained in the assay. All the free antigens were tdiscarded, whereby the bound complexes (antigen-antibody complex) remained in the antibody-coated tubes. Then the tubes were counted using the gamma counter to determine the radioactivity in the tubes. The count was inversely related to the amount of unlabelled antigens present in the blood samples. The quantities of unlabelled antigens in the samples were determined by comparing the counts to the calibration/standard curve.

The calibration/standard curve was obtained by using known amounts of the same hormone but at several different concentrations (supplied by the manufacturer). Using the standard curve, the unknown amounts of hormones (in the blood samples collected) were compared with the amount of radioactive present in the known amounts of hormones.

3.7.1 Principle Procedure for Progesterone and Oestradiol Assay Estimation

The principle of assay estimation for progesterone and oestradiol was based on the Coat-A-Count's of progesterone and oestradiol procedure. Radioactive Iodine, ^{125}I labelled progesterone and oestradiol competes with the respectively unlabelled antigens for progesterone and oestradiol in the serum (blood samples collected from the animals) for the limited antibody binding sites. After three hours of incubation, separation of antigen-antibody complex from the free antigens (the labelled and

unlabelled antigens) was achieved by simply decanting the remaining into the sink. All the free antigens were discarded whereby the bound complexes remained in the antibody-coated tubes. The tubes were counted in a gamma counter.

3.8 MATERIALS AND REAGENTS FOR PROGESTERONE AND OESTRADIOL ASSAY

All reagents used in this experiment were from Coat-A-Count's, Diagnostic Product Corporation, USA. After each experiment, the reagents were stored in a refrigerator at 2-8°C. The radioactive iodine-125, ^{125}I labelled for progesterone and oestradiol stabled for only 30 days after reconstitution whereby the other reagents could be used until expired.

3.8.1 Materials and Methods for Progesterone

3.8.1.1 Materials for Progesterone

1. Progesterone antibody-coated tubes

100, 200, 500 or 1000 polypropylene tubes coated with rabbit antibodies to progesterone were packed in zip-lock bags and stored in the refrigerator at 2-8°C until assay or until expired date marked on the bag. The colour of the tubes for progesterone were orange (Plate 9). It was kept in the zip-lock bags to protect the tubes from moisture.

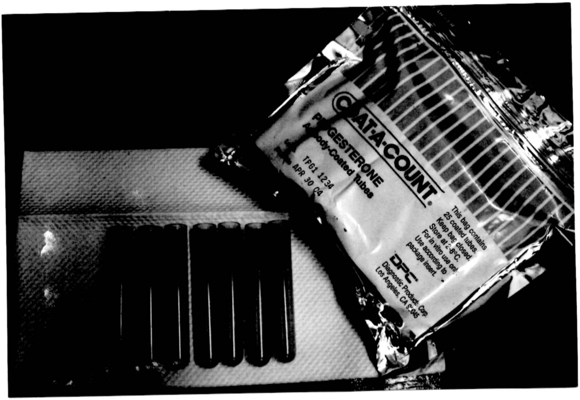


Plate 9. Progesterone tubes

2. Radioactive Iodine-125,(^{125}I) labelled for progesterone

One vial contained 105 ml of iodinated progesterone, with preservative, was supplied in liquid form and ready to be used (Plate 10). If not been used, it was kept in the refrigerator at 2-8°C and stabled for 30 days after opening. The colour of the liquid is clear .

3. Progesterone calibrators

One set containing seven vials of progesterone calibrator, labelled A to G represent, respectively, 0, 0.1, 0.5, 2, 10, 20 and 40 nanograms of progesterone per milliliter (ng/ml) in processed human serum, with preservative, were supplied in liquid form and were ready to be used (Plate 11). The zero calibrator, vial A, contained 4 ml and the remaining vials, vial B through G contained 2 ml each. After each experiment, all vials were stored in the refrigerator at 2-8°C and stabled until expiration date marked on the label. The calibrator represents, respectively, 0, 0.1, 0.5, 2, 10, 20 and 40 ng/ml. This is equivalent to 0, 0.3, 1.6, 6.4, 31.8, 63.6 and 127.2 nanomoles per liter (nmol/L).

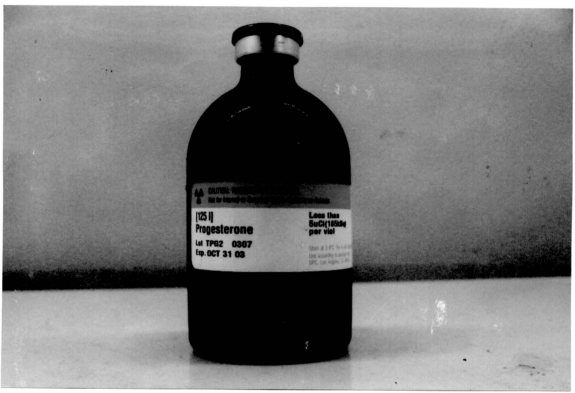


Plate 10. Radioactive Iodine-125

Plate 11. Progesterone calibrators

4. Control (pool) serum

One set containing 3 vials of lyophilized material (from human serum) of each level (Low, Medium and High) were supplied by the manufacturer (Cat NO. CON6, Diagnostic Product Corporation, USA) (Plate 12). Each vial was reconstituted by adding exactly 6.0 ml distilled or deionized water, using the volumetric pipette. Then the serum was let stand for 30 minutes. After 30 minutes, the sera were gently mixed by gentle swirling or inversion until it was completely dissolved. These control sera were stabled for seven days at 2-8°C or two month if frozen at -20°C. Before assay, the control serum were allowed to come to the room temperature (27°C) and were mixed by gentle swirling or inversion.

3.8.1.2 Radioimmunoassay Procedure for Progesterone

1. Preparation of reagent

Before each experiment, all reagents (^{125}I progesterone, progesterone calibrator and control serum) were place at room temperature (27 °C).

2. Labelling of the progesterone antibody-coated tubes (Table 3).

Tubes were labelled as follow:

a) Plain tubes

Two plain tubes (uncoated) sized 12 x 75 mm were labelled as follows:

Tube 1 = TC (total count)

Tube 2 = NSB (nonspecific binding)

Plate 12. Control (pool) serum

Table 3. Concentration of calibrators and volume of solutions for progesterone and oestradiol calibration curve

Tube Number	Tube Code	Progesterone concentration ng/mL	Oestradiol Concentration pg/mL	Standard/ Sample (µL)	P_4 or E_2 I^{125} tracer (mL)	Total Volume (mL)
1	TC	-	-	0	1	1
2	NSB	0	0	100	1	1.1
3	A(MB)	0	0	100	1	1.1
4	B	0.1	20	100	1	1.1
5	C	0.5	50	100	1	1.1
6	D	2	150	100	1	1.1
7	E	10	500	100	1	1.1
8	F	20	1800	100	1	1.1
9	G	40	3600	100	1	1.1
10	LOW	-	-	100	1	1.1
11	MEDIUM	-	-	100	1	1.1
12	HIGH	-	-	100	1	1.1
13	Sample			100	1	1.1
etc.						

b) Seven progesterone antibody-coated tubes

Tubes 3 to 9 were for progesterone calibrator (standard). Tube 3 is equal tp maximum binding (MB) follow by tube 4 to 9 respectively, increasing in the amount of progesterone. The increasing amount of progesterone is shown as below:

Tube 3 = 0 ng/ml

Tube 4 = 0.1 ng/ml

Tube 5 = 0.5 ng/ml

Tube 6 = 2.0 ng/ml

Tube 7 = 10 ng/ml

Tube 8 = 20 ng/ml

Tube 9 = 40 ng/ml

c) Three tubes labelled as tube 10, 11 and 12 were for control (pool) serum

Tube 10 = Low level control serum

Tube 11 = Medium level control serum

Tube 12 = High level control serum

d) Tubes 13 onwards were for unknown samples (i.e. blood samples collected from the goats).

3. Preparation for TC, NSB, standard curve, controls and unknown tubes (Table 3).

a) 100 µl of the zero calibrator (from vial A) was pipetted into NSB tube (tube 2) and tube 3.

b) 100 µl of each of the calibrator (from vial B through G) was pipetted into correspondingly labelled tubes (tube 4 to tube 9), respectively.

c) 100 µl of each control (pool) serum was pipetted into tube 10, 11 and 12. Tube 10 was for Low level control serum whereby tube 11 was for medium level control serum and tube 12 was for high levels control serum.

d) 100 µl of unknown sample (i.e. blood samples collected from the goats) was pipetted into designated tubes (tubes 13 onwards).

All of the reagents and sera were pipetted using the micropipette directly to the bottom of the tubes and after each step, micropipette tip was changed in order to avoid contamination.

4. 1.0 ml of ^{125}I progesterone was added into every tube. All tubes were then mixed using the vortex mixer for 2 minutes and were incubated for 3 hours at room temperature (27°C). During incubation, all tubes were covered with parafilm to avoid dust or other contamination from surrounding (a one hour incubation at 37°C may also been done using a waterbath).

5. All tubes, except for TC tube (tube 1), were carefully decant thoroughly into the sink. It was done using foam decanting rack. The tubes were allowed to drain for two to three minutes. Then the tubes were striked sharply on the absorbent paper to remove the last drop of the liquid.

6. The tubes were placed in the gamma counter (Plate 13) to determine the radioactivity in each tube for one minutes.

Plate 13. Gamma counter

3.8.2 Materials and Methods for Oestradiol

3.8.2.1 Materials for Oestradiol Assay

1. Oestradiol antibody-coated tubes

100, 200, 500 or 1000 polypropylene tubes coated with rabbit antibodies to oestradiol were packed in the zip-lock bag and were stored in refrigerator at 2-8°C until assay or until expired date. After opening the bag, it must carefully resealed to protect the tubes from moisture. The colour of the tubes for oestradiol were purple (Plate 14).

2. Radioactive Iodine-125,(^{125}I) labelled for oestradiol

One vial contained 105 ml of iodinated oestradiol, with preservative, was supplied in liquid form and ready to be used. The vial must be stored in refrigerator at 2-8°C and stabled for only 30 days after opening the cap. If the tracer was transferred from its original container, it is important to use container made of glass rather than plastic. This is because oestradiol has a strong tendency to absorb untreated plastic surfaces. The colour of the liquid is clear.

3. Oestradiol Calibrators

One set contained seven vials of oestradiol calibrator, labelled A through G represent, respectively, 0, 20, 50, 150, 500, 1800 and 3600 pictograms of oestradiol per milliliter (pg/ml) in processed human serum, with preservative, were supplied in liquid form and were ready to be used. This is equivalent to 0, 0.07, 0.18, 0.55, 1.84, 6.61 and 13.20 nanomoles per liter (nmol/L). The zero calibrator, vial A contained 4 ml and the remaining vials, vial B through G contained 2 ml each. After each experiment, all vials were stored in refrigerator at 2-8°C and stabled until expired date marked on the label.

Plate 14. Oestradiol antibody-coated tubes

4. Control (pool) serum

Control (pool) serum used in this experiment were the same control (pool) serum used for progesterone.

3.8.2.2 Radioimmunoassay Procedure for Oestradiol

1. Preparation of reagent

Before each experiment, all reagents (^{125}I Oestradiol, oestradiol calibrator and control serum) were placed at room temperature (27°C).

2. Labelling of the oestradiol antibody-coated tubes (Table 3).

Tubes were labelled as follow:

a) Plain tubes

Two plain tubes (uncoated) sized 12 x 75 mm were labelled as follows:

Tube 1 = TC (total count)

Tube 2 = NSB (nonspecific binding)

b) Seven oestradiol antibody- coated tubes

Tubes 3 to 9 were for oestradiol calibrator (standard). Tube 3 was equal to maximum binding (MB), followed by tube 4 to 9, respectively, increasing in amount of oestradiol. The increasing amount of oestradiol is shown as follows:

Tube 3 = 0 pg/ml

Tube 4 = 20 pg/ml

Tube 5 = 50 pg/ml

Tube 6 = 150 pg/ml

Tube 7 = 500 pg/ml

Tube 8 = 1800 pg/ml

Tube 9 = 3600 pg/ml

c) Three oestradiol antibody-coated tubes were labelled as tube 10, 11 and 12.

These tubes were for control (pool) serum. It is shown as below:

Tube 10 = Low level control serum

Tube 11 = Medium level control serum

Tube 12 = High level control serum

d) Tubes 13 onwards were for unknown samples (i.e. blood samples collected from goats).

3. Preparation for TC, NSB, calibrators, controls and unknown tubes (Table 3)

a) 100 µl of the zero calibrator (from vial A) was pipetted into NSB (tube 2) and tube 3

b) 100µl of each of the calibrator (from vials B through G) was pipetted into correspondingly labelled tubes (tube 4 to tubes 9) respectively.

c) 100 µl of each control (pool) serum (low, medium and high level) was pipetted into tubes 10, 11 and 12. Tube 10 was for low level control serum whereby tube 11 was for medium level control serum and tube 12 was for high level control serum.

d) 100 µl of unknown sample (i.e. blood samples collected from the goats) was pipetted into designated tube (tube 13 onwards).

All the reagents and sera were directly pipetted to the bottom of the tubes. After each step or process, the micropipette tips were changed in order to avoid carry-over contamination.

4. 1.0 ml of ^{125}I oestradiol was added into every tubes. If a pipette with a plastic syringe was used, it must rinsed twice with the tracer before dispensing. This was because oestradiol has a strong tendency to absorb to untreated plastic surface. Then all tubes were mixed using the vortex mixer for 2 minutes and were incubated for 3 hours at room temperature 27°C. During incubation, all tubes were cover with parafilm to avoid evaporation, dust or other contamination from the air. A one hour incubation at 37°C using the waterbath can also be used.

5. All tubes except TC tube (tube 1), were decant carefully into the sink. It was done using the foam decanting rack. The tubes were allowed to drain for about 2 to 3 minutes. Then the tubes were striked sharply on absorbent paper to remove all the residual droplets.

6. The tubes were placed in the gamma counter (Plate 13) to determine the radioactivity in each tubes for 1 minutes.

3.9 EXPERIMENTAL DESIGN

3.9.1 Progesterone and Oestradiol Levels in Different Goat Genotypes with Different Types of Oestrous Cycle (Experiment 1)

In this experiment, blood samples were collected daily during oestrous cycle between 0700 to 0800 hours every morning for 50 consecutive days from 25 mature female goats of different genotypes namely the Katjang goats (5 animals), Jermasia goats (10 animals) and Boer crossbred goats (10 animals). The blood samples were collected into the plain blood collecting tubes and were taken back to the Radioimmunoassay Laboratory, Institute of Biological Science, University Malaya, to be centrifuged at 3000 rpm for 10 minutes at 4°C. The sera were used to analyzed the progestone and oestradiol hormones using the radioimmunoassay (RIA) procedure. The objective of the study was to characterize the patterns of progesterone and oestradiol hormones in different goats genotypes with normal and abnormal oestrous cycles and also to compare the normal and abnormal goats with reference to hormone secretion during oestrous cycles.

3.9.2 Progesterone and Oestradiol Levels 2 Days before Oestrus until 2 Days after Oestrus for Jermasia Goats with Normal Oestrous Cycle (Experiment 2)

In this experiment, blood samples were collected from 5 mature female goats from Jermasia genotype daily, every 8 hours that is 2 days before oestrus until 2 days after oestrus. The blood samples were collected into the plain blood collecting tubes and were taken back to the Radioimmunoassay Laboratory, Institute of Biological Science, University Malaya, to be centrifuged at 3000 rpm for 10 minutes at 4°C. The sera were used to analyze for progesterone and oestradiol hormones using the radioimmunoassay (RIA) procedure. The objective of the study was to study the patterns of progesterone and oestradiol hormones, 2 days before oestrus until 2 days after oestrus for Jermasia goats with normal oestrous cycle.

3.9.3 Progesterone and Oestradiol Levels in Different Goat Genotypes after Oestrus Synchronization (Experiment 3)

In this experiment, blood samples were collected daily between 0700 hours to 0800 hours from 15 mature female goats of three different genotypes namely, Jermasia goats, Boer crossbred and mixed bred goats. The blood collection was done for 23 consecutive days, that is one days before the insertion of the CIDR implant until a few days after the withdrawal of the CIDR implant from the vagina of the animals(that is, until oestrus was detected in the female goats). The blood samples were collected into the plain blood collecting tubes and were taken back to the Radioimmunoassay Laboratory, Institute of Biological Science, University Malaya, to be centrifuged at 3000 rpm for 10 minutes at 4°C. The sera were used to analyze for progesterone and oestradiol hormones using the radioimmunoassay (RIA) procedure. The objective of the study was to determine the patterns of progesterone and oestradiol hormones in different goats genotypes after oestrus synchronization.

3.9.4 Statistical Analysis

SPSS version 11.0 was used to analyze the data obtained in the studies to determine the significance of treatments on parameters measured.