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APPENDICES

APPENDICES

Appendix I: chemical reagents

1.1 Radioimmunoassay kits

- 17β-estradiol standard (Batch number 79/11) : WHO RIA Reagent Programme,Switzerland.
- antiserum to 17β-estradiol (Cloned 2F9) : Professor Kohen Fortune, Department of Biology Regulation, Weizmann Institute of Science, Israel.
- 17β-estradiol tracer (2,4,6,7-^{3}H) (Batch number 2 Nov 2001) : Amersham International, PLC, England.
- testosterone standard (Batch number K079810) : WHO RIA Reagent Programme, Switzerland.
- antiserum to testosterone (Batch number K888510) : WHO RIA Reagent Programme, Switzerland.
- testosterone tracer (1,2,6,7-^{3}H) (Lot TRK 402) : Amersham International, PLC, England.
- FSH standard (Batch number NIDDK-rFSH-RP-2 (AFP-4621B)) : National Institute of Diabetes and Digestive and Kidney Disease (NIDDK), Japan.
- antiserum to FSH (Batch number NIDDK-anti-rFSH-S-11 (AFPO 972881)) : NIDDK, Japan.

- FSH tracer (Batch number NIDDK-rFSH-I-5 (AFP-11454B)) : NIDDK, Japan.
- LH standard (Batch number NIDDK-rLH-RP-3 (AFP-7187B)) : NIDDK, Japan.
- antiserum to LH (Batch number NIDDK-anti-rLH-S-11) : NIDDK, Japan.
- luteinizing hormone tracer (NIDDK-rLH-I-5 (AFP-11536B)) : NIDDK, Japan.

1.2 Hormones

- testosterone propionate : Sigma Chemical Company, Merck, USA.

1.3 Others

- charcoal reagent : WHO RIA Reagent Programme, Switzerland.
- dextran reagent : WHO RIA Reagent Programme, Switzerland.
- gelatin : Difco laboratory, USA.
- diethyl ether $(C_2H_5)_2O$: E. Merck, Damstadt, Germany.
- natrium dihydrogen phosphate-monohydrated ($NaH_2PO_4 . H_2O$) : E. Merck, Damstadt, Germany.
- toluene P.a. (C_7H_8) : E. Merck, Damstadt, Germany.
- ethanol (95%) : E. Merck, Damstadt, Germany.
- ethanol (Absolute) : E. Merck, Damstadt, Germany.

- methanol (CH_3OH) : E. Merck, Damstadt, Germany.
- art.3115-1-4-Dioxane ($C_7H_8O_2$) : E. Merck, Damstadt, Germany.
- art.2946 [(2,5-diphenyloxazol)-phenyl-oxazolyl phenyl anhydrous)] ($C_{15}H_{11}NO$) : E. Merck, Damstadt, Germany.
- formalin (40%) : E. Merck, Damstadt, Germany.
- xylene ($C_6H_4(CH_3)_2$) : E. Merck, Damstadt, Germany.
- n-butyl alcohol (absolute) : E. Merck, Damstadt, Germany.
- hematoxylin : E. Merck, Damstadt, Germany.
- eosin : E. Merck, Damstadt, Germany.
- glacial acetic acid : E. Merck, Damstadt, Germany.
- glycerine : E. Merck, Damstadt, Germany.
- POPOP [1,4-bis(2-(5-phenyloxazol))]-Benzene, phenyl-oxazolyl-phenyl-oxazolyl phenyl anhydrous : Sigma Chemical Company, USA.
- thiomersal (merthiolate) : Sigma Chemical Company, USA.
- sesame oil : Sigma Chemical Company, USA.
- sodium hydroxide (NaOH) : BDH Chemical Ltd. England.
- disodium hydrogen phosphate-anhydrous (Na_2HPO_4) : BDH Chemical Ltd. England.
- sodium chloride (NaCl) : BDH Chemical Ltd. England.
- paraffin
- egg albumin

Appendix II: equipments

- beta counter : Model 1218 Rack Beta LKB Wallac, Findland.
- dunoff incubator shaker : Model 3575-1, Lab-Line Instrument Inc., USA.
- dynac centrifuge : Clay Adams, Becton Dickinson and Company, USA.
- ultrasonic cleanser : Right A Weight, WM, Benver, USA.
- magnetic stirrer bars S-18520 : Thermolyne Corporation Iowa, USA.
- micropipette size 100 ul : Nichiyo Model 5000 Japan.
  200 ul : Nichiyo Model 8100 Japan.
  1,000 ul : Eppendorff 3130 Germany
- vortex mixer: M-16715 : Thermolyne Corporation Iowa, USA.
- pH meter : Corning pH meter 240 Cat No. 476530, Corning-EEI Scientific Instrument, England.
- refrigerated centrifuge : Model PR-J, International Equipment Company, USA.
- machinery weight : Right A Weight, WM, Benver, USA.
- foam decanting rack : DPC, USA.
- machinery weight : Right A Weight, WM.
- microtome : Model 820 serial 66305: American optical, Scientific Instrument Division, Buffalo, New York, USA.
- microtome blade : S 35, USA.
- hot air oven : Griffin Grundy.
- refrigerator : J-elegance Mitsubishi MR-F51GY.

- light microscope : Olympus B071, Japan.
- hot plate : Model PS-D, Sakura Finetechnical Co.Ltd., Tokyo, Japan.
- paraffin dispenser : Ashcroft, USA.
- laminar flow
- syringe terumo with needle size 1, 2.5 and 5 ml : Terumo, Inc., Japan.
- gamma counter

Appendix III: reagent preparations

3.1 Preparation of reagents for determination of sex steroid hormone by RIA technique

The reagent preparations were followed WHO (1986) procedure.

3.1.1 Steroid assay buffer (buffer S)

Natrium dihydrogen phosphate-monohydrated: $NaH_2PO_4 . H_2O$	3.05	g
Disodium hydrogen phosphate anhydrous: Na_2HPO_4	11.6	g
Sodium chloride: Nacl	8.8	g
Thiomersal	0.1	g
Gelatin	1.0	g

The gelatin must be dissolved in 300 ml of warm di-distilled water. After the solution was cool, the rest of the certain reagents was added. The volume was made up to 1 liter and the pH of this buffer is adjusted to 7.2 to 7.4 by dropwise addition of sodium hydroxide (NaOH) or hydrochloric acid (HCl).

The buffer was stored at 4°C. It should be stable for at least 1 month. This buffer was used as the diluent for all reagents in sex steroid assays of hormone.

3.1.2 Charcoal suspension

Charcoal	0.625	g
Dextran	0.0625	g
Assay buffer	100	ml

Dextran was dissolved in 100 ml of assay buffer in a stoppered container, then charcoal was added and shaked vigorously for 30 seconds. The charcoal reagent should be stable at 4°C for at least 1 month. The settle down suspension should be stirred vigorously during use at 4°C.

3.1.3 Scintillation fluid

2,5-diphenyloxazole (PPO)	5.0	g
1,4-bis(2-(5-phenyloxazol))-Benzene (POPOP)	0.3	g
Toluene	1.0	g
Dioxane	200	ml

These constituents were homogenously mixed and stored in the dark bottle. The solution could be stable at the room temperature. Scintillation fluid should be prepared before use at least 7 days.

3. 2 Preparation of estradiol tracer, antiserum and standards

3.2.1 Estradiol tracer

The stock solution (concentration 10 μCi/ml) was prepared from estradiol tracer [(2,4,6,7-^{3}H) estradiol] in amounts of 250 μCi by mixing with toluene:ethanol (9:1). 100 μl of stock solution was pipetted and evaporated, then redissolved in 10 ml of assay buffer. The final concentration contained 100 nCi/ml or 10,000 cpm. The estradiol tracer was stored at 4°C.

3.2.2 Estradiol antiserum

Lyophilized form of estradiol antiserum obtained from Prof. Kohen Fortune, Israel was added with 0.5 ml of di-distilled water two times, then transferred 100 μl of the solution in each microfuge tube and stored at 0°C. Each microfuge tube was added with 900 μl of di-distilled water, this concentration was 1:10. One-hundred microlitters of this solution was transferred in each microfuge tube and stored at 0°C. The solution was dissolved again as mentioned above, but this time the assay buffer was used instead of di-distilled water. The concentration was 1:1,000. This concentration served as stock solution of estradiol antiserum and stored at at 4°C.

When working solution was required, stock solution was added with assay buffer and mixed until the final concentration was 1:20,000.

3.2.3 Estradiol standard

Estradiol standard batch number 79/11 at the concentration of 10 ng/ml obtained from WHO RIA Reagent Programme, Switzerland was served as stock solution of estradiol standard. The concentration of estradiol standard serial dilution was 9.8, 19.6, 39, 78, 156.5, 312.5, 625, 1250, 2500 and 5,000 pg/500µl/tube

3.3 Preparation of testosterone tracer, antiserum and standards

3.3.1 Testosterone tracer

The stock solution (concentration 10 µCi/ml) was prepared from testosterone tracer [(1,2,6,7-^{3}H) testosterone] in amounts of 250 µCi by mixing with toluene:ethanol (9:1). 100 µl of stock solution was pipetted and evaporated, then redissolved in 10 ml of assay buffer. The final concentration contained 100 nCi/ml or 10,000 cpm. The testosterone tracer was stored at 4°C.

3.3.2 Testosterone antiserum

Lyophilized form of testosterone antibody batch number K888510 obtained from WHO RIA Reagent Programme, Switzerland was stable for several years if

stored at 4°C. One bottle of testosterone antibody was added with 10 ml of assay buffer when required with the final dilution in 1:210,000.

3.3.3 Testosterone standard

Testosterone standard batch number K079810 obtained from WHO RIA Reagent Programme, Switzerland at the concentration of 220 nmol/l or 2,200 fmol/l was aliquoted to the vials provided, each vial contained 100 µl of testosterone standard. These aliquotes were stored at 4°C until need. When required, 10 ml of assay buffer was added to 100 µl of testosterone standard and heated at 40°C in water bath for 30 minutes. After the solution was mixed vigorously, it allowed to cool at the room temperature before use. The concentration of testosterone standard serial dilution was 17.2, 34.4, 68.8, 137.5, 275, 550 and 1,100 fmol/500µl/tube.

3.4 Preparation for determination of gonadotropins by RIA technique

The reagent preparations were followed Watanabe *et al.* (1990) procedure.

3.4.1 Assay buffer for 0.5 M PBS-0.1% NaN_3 pH 7.6

1. Preparation of solution A and B was as followed;

   - 900 ml of 0.05 M of solution A was prepared from 35.814 grams of $Na_2HPO_4.12H_2O$ (MW: 358.14) which it was dissolved in 2 liters of di-distilled water

   - 250 ml of 0.05 M of solution B was prepared from 7.801 grams of

$NaH_2PO_4.2H_2O$ (MW: 156.10) which it was dissolved in 1 liters of di-distilled water.

2. Solution B was poured into solution A , then the pH of this mixed solution should be checked to be 7.6
3. NaN_3 2 grams / 2 liters was added.
4. 0.14 M of NaCl 16.364 grams / 2 liters was added.
2. The solution was stored at 4°C. It should be stable for 1 month.

3.4.2 Assay buffer for 0.5 M PBS-0.1% NaN_3-0.05 M EDTA-1% NRS pH 7.6

1. The assay buffer above was added with EDTA, then the pH of this mixed solution should be checked to be 7.6 by dropwise addition of 5 M NaOH.
2. NRS was added and mixed.

3.5 Preparation of FSH tracer, antiserum and standards

3.5.1 FSH tracer

Lyophilized form of FSH tracer (Batch number NIDDK-anti-rFSH-S-11 (AFPO 972881)), which it obtained from NIDDK, Japan and contained its substance 100 μg/ampule, was dissolved in 1 ml of assay buffer, then the solution was aliquoted into vials. 20-25 μg of FSH tracer per vial. The solution was stored at 4°C, it should be stable for 4-6 months. FSH tracer was dissolved in 0.05 M PBS-0.1% NaN_3-0.1% BSA. The value of cpm used in this assay was 4,000-5,000 cpm/50 μl.

3.5.2 FSH antiserum

FSH antisera (NIDDK-anti-rFSH-S-11 (AFPO 972881)) obtained from NIDDK, Japan was dissolved in 1 ml of 1:12.5 (2% normal rabbit serum (NRS):assay buffer), then the solution was lyophilized. The lyophilized form of FSH antisera was mixed with 1 ml of di-distilled water served as stock solution. This solution was stored at 0°C. When working solution was required, stock solution of FSH antisera was added with 0.05 M PBS-0.1% NaN_3-0.05 M EDTA-1% NRS (pH 7.6) and mixed until the final concentration was 1:125,000.

3.5.3 FSH standard

Lyophilized form of FSH standard (NIDDK-rFSH-RP-2 (AFP-4621B)) obtained from NIDDK, Japan was dissolved in 1 ml of 1% BSA phosphosaline buffer. Its concentration was 10 μg/ml, then 25 μl of the solution was aliquoted into each vial and stored at 0°C. It should be stable for 3-5 months. The concentration of FSH standard serial dilution was 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1,000 pg/100μl/tube.

3.6 Preparation of LH tracer, antiserum and standards

3.6.1 LH tracer

Lyophilized form of LH tracer (NIDDK-rLH-I-5 (AFP-11536B)), which it obtained from NIDDK, Japan and contained its substance 100 μg/ampule, was

dissolved in 1 ml of assay buffer, then the solution was aliquoted into vials. 20-25 μg of LH tracer per vial. The solution was stored at 0°C, it should be stable for 2-3 months. LH tracer was dissolved in 0.05 M PBS-0.1% NaN_3-0.1% BSA. The value of cpm used in this assay was 4,000-5,000 cpm/50 μl.

3.6.2 Preparation of LH antiserum

LH antisera (NIDDK-anti-rLH-S-11 (AFPO 972881)) obtained from NIDDK, Japan was dissolved in 1 ml of 1:18.75 (2% NRS:assay buffer), then the solution was lyophilized. The lyophilized form of LH antisera was mixed with 1 ml of di-distilled water served as stock solution. This solution was stored at 0°C. When working solution was required, stock solution of LH antisera was added with 0.05 M PBS-0.1% NaN_3-0.05 M EDTA-1% NRS (pH 7.6) and mixed until the final concentration was 1:180,000.

3.6.3 Preparation of LH standard

Lyophilized form of LH standard (NIDDK-rLH-RP-3 (AFP-7187B)) obtained from NIDDK, Japan was dissolved in 1 ml of 1% BSA phosphosaline buffer. Its concentration was 5 μg/ml, the solution was stored at 0°C. It should be stable for 3-5 months. The concentration of LH standard serial dilution was 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1,000 pg/100μl/tube.

3.7 Preparation of histopathological reagents

3.7.1 10% buffer formalin

Formalin (40%)	100	ml
Di-distilled water	900	ml
Natrium dihydrogen phosphate-monohydrated: NaH_2PO_4. H_2O	4	g
Disodium hydrogen phosphate anhydrous: Na_2HPO_4	6.5	g

These chemical substances were mixed together in the dark bottle, the solution was shaked until it was completely dissolved. This solution was stored at the room temperature.

3.7.2 Ehrlich's acid haematoxylin and eosin

Haematoxylin	8	g
Ethanol (absolute)	400	ml
Ammonium alum	8	g
Di-distilled water	400	ml
Glycerine	400	ml
Glacial acetic acid	40	ml

Haematoxylin was dissolved in absolute ethanol in water bath at 40-50°C. When the solution was cool, it was filtered with filtered paper, then ammonium alum was dissolved in warm di-distilled water. These two solutions were mixed together,

then glycerine and glacial acetic acid were added and stirred until these substances were completely dissolved. The solution need to expose to daylight to ripen for at least 6 weeks.

3.7.3 Eosin

Eosin Y	0.5	g
Ethanol (95%)	100	ml

Eosin was dissolved in absolute ethanol until the solution was completely dissolved and stored at the room temperature.

3.8 Preparation for administration of di-distilled water, testosterone propionate and the powder suspension of *M. collettii* to rats

The preparation of di-distilled water and the powder suspension of *M. collettii* treated to rats was shown in Table 16.

The preparation of testosterone propionate treated to rats was shown in Table 17.

Table 16 The preparation of di-distilled water and *M. collettii* administrations at the various body weights in rats.

Body weight (grams)	Mc suspension (ml)	Di-distilled water (ml)
493-500	0.70	-
486-492	0.69	0.01
479-485	0.68	0.02
472-478	0.67	0.03
465-471	0.66	0.04
458-464	0.65	0.05
451-457	0.64	0.06
443-450	0.63	0.07
436-442	0.62	0.08
429-435	0.61	0.09
422-428	0.60	0.10
415-421	0.59	0.11
408-414	0.58	0.12
401-407	0.57	0.13
393-400	0.56	0.14
386-392	0.55	0.15
379-385	0.54	0.16
372-348	0.53	0.17
365-371	0.52	0.18
358-364	0.51	0.19
351-357	0.50	0.20
343-350	0.49	0.21
336-342	0.48	0.22
329-335	0.47	0.23
322-328	0.46	0.24
315-321	0.45	0.25
308-314	0.44	0.26
301-307	0.43	0.27
293-300	0.42	0.28
286-292	0.41	0.29
279-285	0.40	0.30
272-278	0.39	0.31
265-271	0.38	0.32
258-264	0.37	0.33
251-257	0.36	0.34
243-250	0.35	0.35
236-242	0.34	0.36
229-235	0.33	0.37
222-228	0.32	0.38
215-221	0.31	0.39
208-214	0.30	0.40

Table 17 The preparation of testosterone propionate administration at the various body weights in rats.

Body weight (grams)	TP (ml)	Sesame oil (ml)
430-500	0.20	-
408-429	0.19	0.01
385-407	0.18	0.02
363-384	0.17	0.03
340-362	0.16	0.04
318-339	0.15	0.05
295-317	0.14	0.06
273-294	0.13	0.07
250-272	0.12	0.08
228-249	0.11	0.09
205-227	0.10	0.10
183-204	0.09	0.11
160-182	0.08	0.12

BIOGRAPHY

Miss Kwanta Thansa was born on August 24th, 1977 in Bangkok province, Thailand. She received the Bachelor degree of Nursing Science from Mahidol University, Thailand with the first class hornors in 1999. She has enrolled in graduate programme of the Master degree of Science in Physiology at Chulalongkorn University and graduated in 2003.