CHAPTER III

RESEARCH METHODOLOGY

Animals

Twenty four adult male Sprague-Dawley rats (8 weeks old) (National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand) weighing between 200-250 g were housed one per cage at 24±1 °C and dark/light cycle 12 hours. The protocol for this study was approved by the Animal Research Committee of Naresuan University, Thailand. All animals were divided into 4 groups, each group contains 6 animals, including control group, acute dose-METH binge (AB-METH) group, escalating dose-METH (ED-METH) group and escalating dose-METH binge (ED-METH binge) group.

Chemical

D-methamphetamine hydrochloride (Lipomed AG, Arlesheim, Switzerland) with the consent from the Ministry of Public health was used in this experiment.

Methamphetamine administration

The animals were treated with METH or saline by intraperitoneal (i.p.) injection. In the control group, animals were treated with 0.9% saline every 3 hours interval, for 14 days. On day 15, animals were treated with 0.9% saline every 2 hours for 4 times interval before sacrificed. In the AB-METH group, animals were treated with 0.9% saline every 3 hours interval, for 14 days. On day 15, animals were treated with METH 6 mg/kg every 2 hours for 4 times interval before sacrificed (Table 2). In the ED-METH group, animals were treated with METH, initial intensity of METH was treated at 0.1-3.9 mg/kg every 3 hours interval by gently increase the concentration of METH at 0.1 mg/kg on day 1-13, for 3 times each day and treated with METH at 4.0 mg/kg every 3 hours interval on day 14 for 3 times. On day 15, animals were treated with 0.9% saline every 2 hours for 4 times interval before sacrificed (Table 3). In the ED-METH binge group, animals were treated with METH,

initial intensity of METH was treated at 0.1-3.9 mg/kg every 3 hours interval by gently increase the concentration of METH at 0.1 mg/kg on day 1-13, for 3 times each day and treated with METH at 4.0 mg/kg every 3 hours interval on day 14 for 3 times. On day 15, animals were treated with METH 6 mg/kg every 2 hours for 4 times interval before sacrificed (Table 4) (Figure 16).

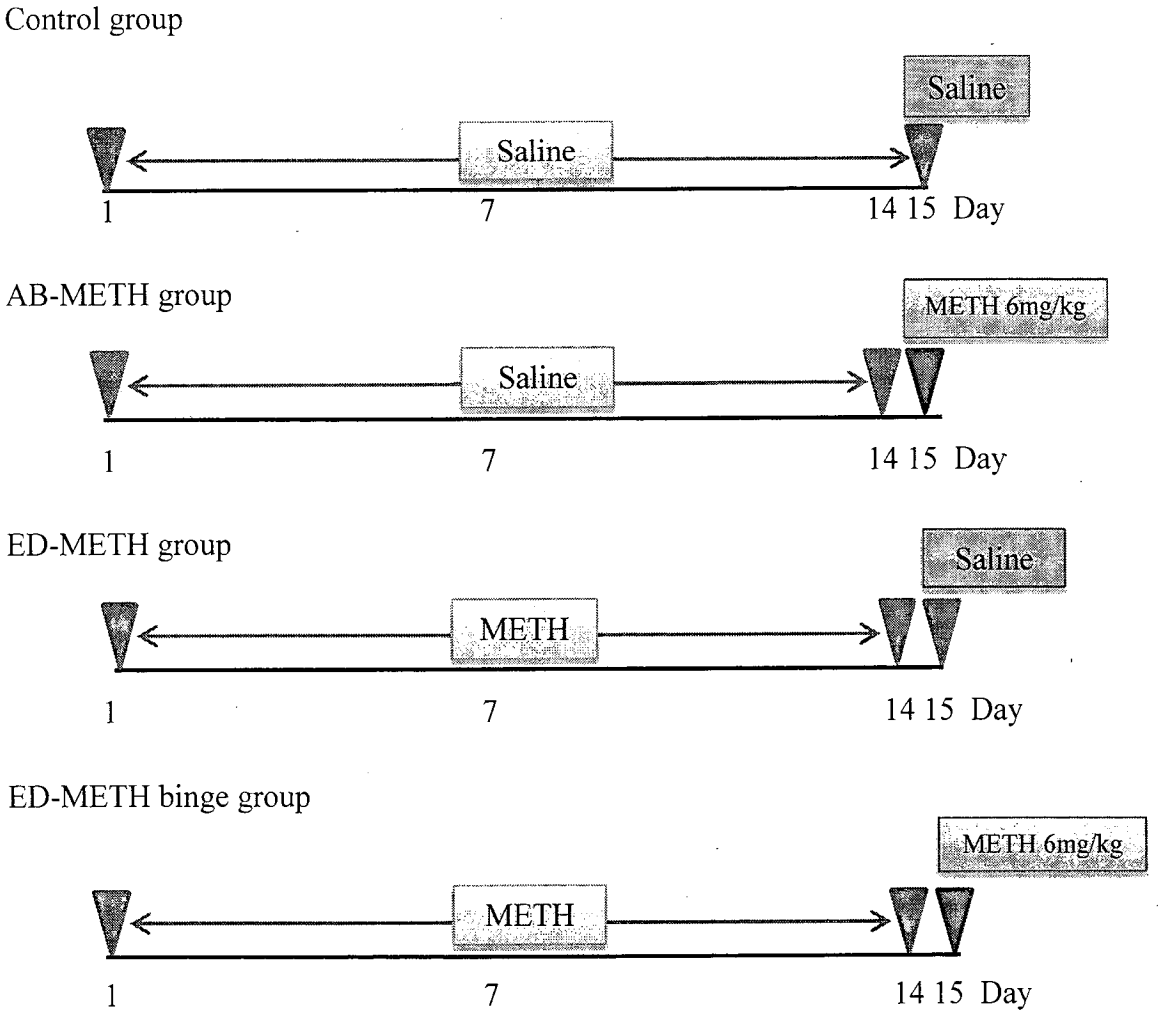

Figure 16 Animals were treated with METH or saline in 4 groups (Control group, AB-METH group, ED-METH group and ED-METH binge group)

Table 2 Schedule of saline administration in AB-METH group on days 1-14 and a high dose binge of METH on day 15

Days	Methamphetamine dose (mg/kg)			
	7.30(a.m.)	**10.30(a.m.)**	**13.30(p.m.)**	
1	saline	saline	saline	
2	saline	saline	saline	
3	saline	saline	saline	
4	saline	saline	saline	
5	saline	saline	saline	
6	saline	saline	saline	
7	saline	saline	saline	
8	saline	saline	saline	
9	saline	saline	saline	
10	saline	saline	saline	
11	saline	saline	saline	
12	saline	saline	saline	
13	saline	saline	saline	
14	saline	saline	saline	
Day	**7.30(a.m.)**	**9.30(a.m.)**	**11.30(a.m.)**	**13.30(p.m.)**
15	6.0	6.0	6.0	6.0

Source: Adapted form Segal, et al., 2003

Table 3 Schedule of METH administration in ED-METH group on days 1-14 and a saline on day 15

Days	Methamphetamine dose (mg/kg)			
	7.30(a.m.)	**10.30(a.m.)**	**13.30(p.m.)**	
1	0.1	0.2	0.3	
2	0.4	0.5	0.6	
3	0.7	0.8	0.9	
4	1.0	1.1	1.2	
5	1.3	1.4	1.5	
6	1.6	1.7	1.8	
7	1.9	2.0	2.1	
8	2.2	2.3	2.4	
9	2.5	2.6	2.7	
10	2.8	2.9	3.0	
11	3.1	3.2	3.3	
12	3.4	3.5	3.6	
13	3.7	3.8	3.9	
14	4.0	4.0	4.0	
Day	**7.30(a.m.)**	**9.30(a.m.)**	**11.30(a.m.)**	**13.30(p.m.)**
15	saline	saline	saline	saline

Source: Adapted form Segal, et al., 2003

Table 4 Schedule of METH administration in ED-METH binge group on days 1-14 and a high dose binge on day 15

Days	Methamphetamine dose (mg/kg)			
	7.30(a.m.)	10.30(a.m.)	13.30(p.m.)	
1	0.1	0.2	0.3	
2	0.4	0.5	0.6	
3	0.7	0.8	0.9	
4	1.0	1.1	1.2	
5	1.3	1.4	1.5	
6	1.6	1.7	1.8	
7	1.9	2.0	2.1	
8	2.2	2.3	2.4	
9	2.5	2.6	2.7	
10	2.8	2.9	3.0	
11	3.1	3.2	3.3	
12	3.4	3.5	3.6	
13	3.7	3.8	3.9	
14	4.0	4.0	4.0	
Day	**7.30(a.m.)**	**9.30(a.m.)**	**11.30(a.m.)**	**13.30(p.m.)**
15	6.0	6.0	6.0	6.0

Source: Adapted form Segal, et al., 2003

Sperm collection

After treatment with METH or saline, animals were sacrificed by cervical dislocation. Testis and epididymis were immediately dissected. The cauda epididymis was separated and minced in phosphate buffer saline (PBS) 2,000 µl to release spermatozoa.

Sperm motility

The spermatozoa in PBS were immediately loaded in Makler counting chamber at 10 µl and counted under bright field microscope. Motile and non-motile sperm in ten squares of Makler counting chamber were examined and evaluated in percentage of normal sperm motility.

Sperm morphology

Spermatozoa in PBS were fixed in 200 µl 10% formaldehyde solution after evaluated sperm motility. Fixed sperm were used to evaluate sperm morphology. Sperm morphology was examined with eosin staining in dilution 5:1 and incubated for 60 minutes. After that, stained sperm were loaded at 50 µl and smeared on glass slides. Stained sperm on glass slides were allowed to dry at room temperature for 60 minutes. Three slides were prepared from each animal. Then, slides were mounted with mounting media (Fisher scientific, New Jersey, USA). Each slide of stained sperm was examined with a Nikon eclipse 80i (Hollywood International Ltd., BKK, Thailand) and taken a picture with Nikon DXM1200c. Size and shape of head, midpiece and tail of sperm were examined under bright field microscope. Two hundred spermatozoa of each animal were counted. The result of sperm morphology was reported in percentage of normal sperm morphology.

Sperm concentration

To examine sperm concentration, hemacytometer was used and placed on flat surface with coverslip. Fixed sperm in 10% formaldehyde solution were gently agitated and loaded at 10 µl on hemacytometer. Sperm were evaluated under bright field microscope. Head sperm were counted in five small squares pattern (Figure 17) of large center square (1mmx1mmx0.1mm). The result of sperm concentration was reported in epididymal sperm number ($x10^6$ cells/ml). Epididymal sperm number/ml = counted sperm in 0.1 mm^3 x dilution factor x 10 x 1000

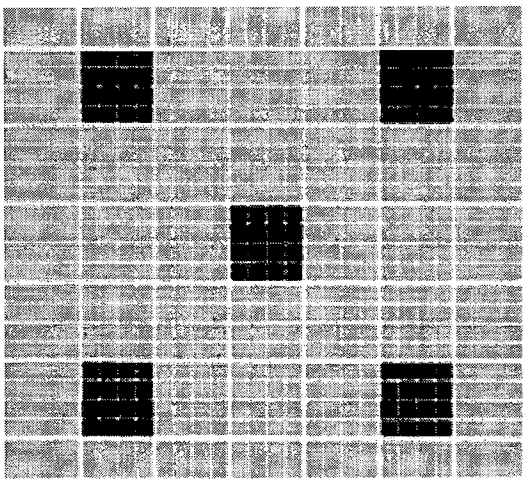

Figure 17 Five small squares of large center square were used for sperm count

Source: http://fg.cns.utexas.edu/fg/protocol%3A_hemacytometer.html

Tissue preparation

Testis was immediately immersed in 10% formaldehyde solution at least 3 days to preserve tissue structure. After that, mid testis were cut and placed in cassettes and immersed in PBS 3 times for 5 minutes. Tissues were processed by dehydration with gradual series of alcohol concentration (70%, 80%, 85%, 90%, 95%, 100%, respectively) to eliminate water (30 minutes each). Alcohol was removed from testis by xylene 2 times (1 hour and 2 hours, respectively). Then, tissues were infiltrated with paraffin 2 times (2 hours and 2 hours, respectively). Subsequently, tissue were placed in mold and embedded in paraffin. The tissue blocks were kept at 4 °C until section. Tissue blocks were coronally sectioned using microtome at 5 μm thickness. The sections were floated on warm water at 45 °C before mounting onto silane coated slide. Tissue sections were allowed to dry at room temperature overnight.

Immunohistochemistry study

Immunohistochemistry study is used for localization of antigen expression on tissue. Principle of this technique depends on interaction between antigen-antibody complex. Androgen receptor (AR) expression was detected in male rat testis by using indirect immunohistochemistry method (Figure 18).

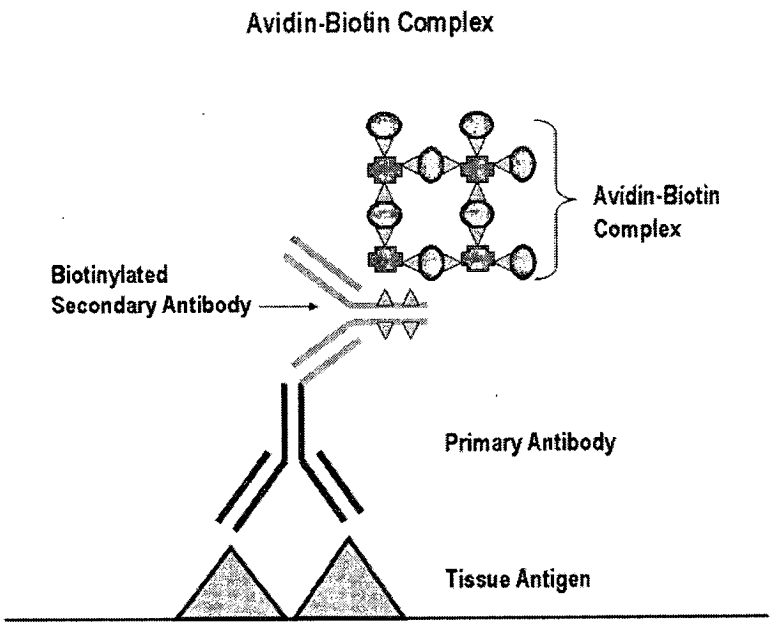

Figure 18 Indirect immunohistochemistry method

Source: http://www.labce.com/immunohistochemistry-ihc-basics-in-histology.aspx

Paraffin sections were deparaffinized by xylene 2 times for 5 minutes and rehydrated by gradual series of alcohol dilution (100%, 95%, 80%, 70%, respectively) to distilled water for 5 minutes. After that, antigen was retrieved with high temperature heating in microwave 3 times for 5 minutes at 560 Watt and allowed to cool down at room temperature for 30 minutes. Then, tissue sections were washed in PBS 3 times for 5 minutes and incubated for 30 minutes with endogenous peroxidase blocking solution including 10% methanol, 0.3% hydrogen peroxide (H_2O_2) and 1% triton X diluted in PBS and washing with PBS 3 times for 5 minutes. Tissue sections were blocked non-specific protein with 5% normal goat serum (Vector Laboratories, Burlingame, California, USA) diluted in PBS for 2 hours. Then, tissue sections were

incubated with anti-androgen receptor primary antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) 1:25 diluted with PBS containing 5% normal goat serum overnight at 4 °C following washing with PBS 3 times for 5 minutes. The dilution of AR staining in the testis was determined from the standard curve (Figure 19).

Biotinylated secondary antibody and avidin-biotinylated horseradish peroxidase complexes (ABC kit) (Vector Laboratories, Burlingame, California, USA) were used for enhancing signal. Tissue sections were incubated with biotinylated secondary antibody 1:200 diluted in PBS containing 5% normal goat serum for 2 hours and washing with PBS 3 times for 5 minutes. The ABC kit was prepared before use for 30 minutes and tissue sections were incubated with ABC kit for 60 minutes and washing with PBS 3 times for 5 minutes. Eventually, tissue sections were incubated with 3, 3'-Diaminobenzidine (DAB) (Vector Laboratories, Burlingame, Carifornia, USA) for 15 minutes to visualization androgen receptor protein on tissue sections. The reaction between tissue sections and DAB were stopped by distilled water for 5 minutes. After that, tissue sections were counterstained with hematoxylin 2 dips and stop reaction with tap water for 5 minutes. Then, tissue sections were dehydrated by gradual series of alcohol concentration (70%, 80%, 95%, 100%, respectively) and removed alcohol by xylene 2 times for 5 minutes. Mounting media (Fisher scientific, New Jersey, USA) were used to mount the tissue sections and coverslip. After that, tissue sections were allowed to dry at room temperature overnight.

Each tissue section was obtained with a Nikon eclipse 80i microscope (Hollywood International Ltd., BKK, Thailand) and taken a picture with Nikon DXM1200c.

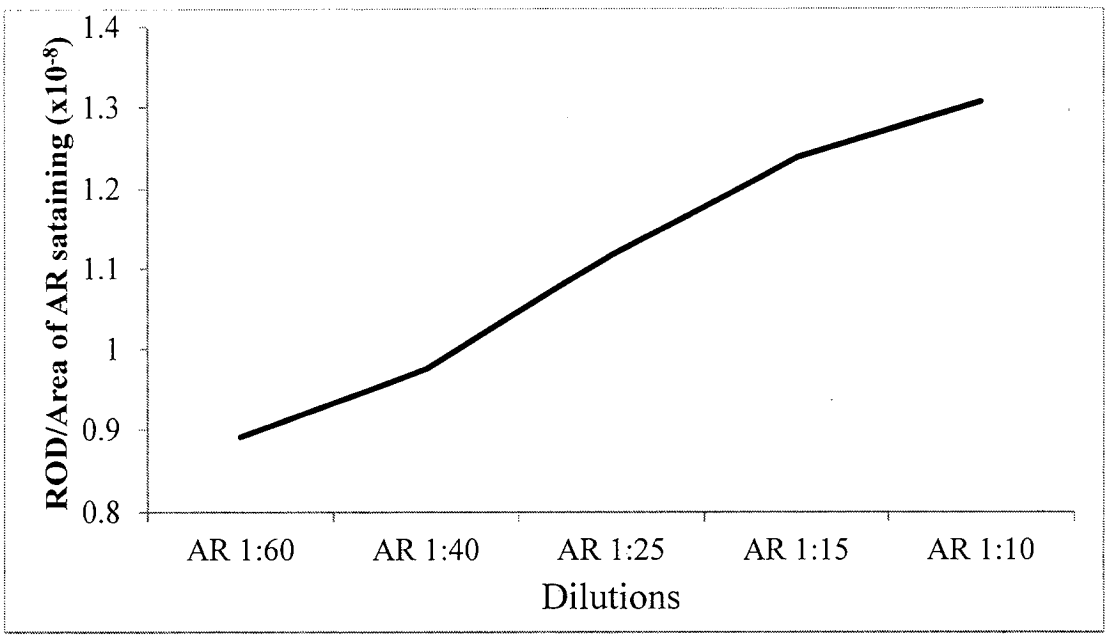

Figure 19 Relative optical density (ROD) per area of AR expressions in seminiferous tubule stage VII-VIII in each dilution was examined. The standard curve was generated by plotting the ROD/area of AR staining against the dilution of AR staining.

Evaluation of immunoreactive cell

Twenty images of stage VII-VIII of seminiferous tubules and Leydig cells around stage VII-VIII of seminiferous tubules were chosen from two different tissue sections of each rat and examined under bright field microscope with 20x objective lens. AR expression in Sertoli cells, round spermatids, elongated spermatids and Leydig cells were analyzed by ImageJ program (http://rsb.info.nih.gov/ij/). AR expression was presented as percentage of AR expression in Sertoli cells, round spermatids and elongate spermatids per total cells of each cell type. AR expression in Leydig cells was counted around stage VII-VIII of seminiferous tubule and presented as percentage of AR expression in Leydig cells per total Leydig cells.

Blood collection and preparation

After sacrificed, blood samples were collected from all animals and kept in heparinized tube and immediately centrifuged at 4,000 rpm for 10 minutes at room temperature. Plasma was collected at least 1,000 µl and stored at -80 °C until used for evaluate the testosterone levels by electrochemiluminescence immunoassay.

Electrochemiluminescence immunoassay

Plasma testosterone levels were measured by electrochemiluminescence immunoassay (ECLIA) with Testosterone II kit (Cobas, USA) according to manufacture's protocol. The plasma was incubated with biotinylated monoclonal testosterone-specific antibody. Then, the plasma was added with streptavidin-coated microparticles and a testosterone derivative labeled with a ruthenium complex (Figure 20). After that, the sample was aspirated into the measuring cell. In the measuring cell, the microparticles were magnetically captured onto the surface of the electrode. ProCell were used to remove unbound substances. The samples were induced for electrochemiluminescence emission by application of a voltage to the electrode. The electrochemiluminescence emission was measured by a photomultiplier. Testosterone levels were presented as mean plasma testosterone level (ng/ml).

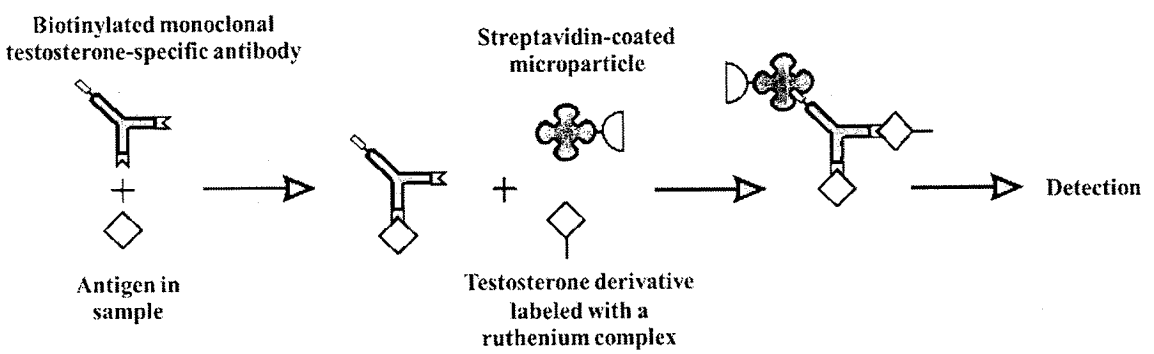

Figure 20 Electrochemiluminescence immunoassay

Source: Adapted from Cobas, 2010

Statistical analysis

Percentage of normal sperm motility, percentage of normal sperm morphology and sperm concentration, AR expression in Sertoli cells, round spermatids, elongated spermatids and Leydig cells were analyzed by One way ANOVA following by Dunnett's post hoc test or LSD test. SPSS program version 11.5 was used to analyze the data. The data were presented as mean±SEM. The significant were determined as P-values less than 0.05.

Research place and acknowledgement

This study was carried out at Neuroscience and Reproductive Biology Research Group, Department of Anatomy, Faculty of Medical Science, Naresuan University. This research was supported by Naresuan University Research Fund.