EFFECTS OF METHAMPHETAMINE ON DOPAMINE, DOPAMINERGIC RECEPTORS, NOREPINEPHRINE AND ADRENERGIC RECEPTORS IN RAT TESTIS

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This thesis entitled "Effect of methamphetamine on dopamine, dopamine receptors, norepinephrine and adrenergic receptors in rat testis" submitted by Siriporn Janphet in partial fulfillment of the requirements for the Master of Science degree in Anatomy is hereby approved.

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ABSTRACT

Methamphetamine (METH) is an illicit drug that affects central nervous system by stimulates catecholamine release such as dopamine (DA) and norepinephrine (NE). It also has effects on other peripheral organ systems including reproductive system. Moreover, dopamine and adrenergic receptors expression were found in testes involving with spermatogenesis and sperm quality. Furthermore, METH may have an effect on reproductive system via action of those two receptors. Thus, the aim of this study was to investigate the alteration of DA, NE, dopamine D2 receptor, α1 adrenergic receptor and stage of seminiferous tubules after METH administration. Twenty two male rats were divided into four groups; control, acute binge (AB), escalating dose (ED), escalating binge dose (ED-binge). Animals in control group were received saline through intraperitoneal (i.p.) injection for 15 days. For acute binge group, animals were injected (i.p.) with saline for 14 days and on day 15, animals were injected with 6 mg/kg METH four times a day (every 2 hours). In escalating dose group, animals were received METH 0.1-4 mg/kg for 14 days (three times a day at 3 hours interval) and on day 15, animals were injected with 0.9 % saline. Animals in escalating binge dose group were injected with METH 0.1-4 mg/kg for 14 days (three times a day at 3 hours interval) and on day 15, animals were treated with 6 mg/kg METH four times a day (every 2 hours). After treatment, animals were sacrificed and testes were removed. The alteration of 3, 4-Dihydroxyphenylacetic acid

(DOPAC), main metabolite of DA, NE and its metabolite, 3, 4-dihydroxyphenylethyleneglycol (DHPG) in testis were measured by high performance liquid chromatography (HPLC). The expression of dopamine D2 receptor and α1 adrenergic receptor were determined by immunohistochemistry. The overview of spermatogenesis was observed via alteration of stage of seminiferous tubules by hematoxylin and eosin staining. The results of the present study showed a significant increase of DOPAC in AB group. Moreover, the concentration of NE was decreased in ED-binge group. In addition, decrease of DHPG was observed in ED and ED-binge groups when compared with control. The development of stage of seminiferous tubules in stage II was significantly decreased in ED and ED-binge groups. While, percentage of stage V was found only in ED group. Moreover, stage XI was significantly increased in ED-binge group. In addition, increase of stage XIII was observed in ED group when compared with control. We also categorize stage of seminiferous as early stage (I-V), middle stage (VI-VIII) and late stage (IX-XIV). The results showed reduction in early stage when compared with control group. The reduction of dopamine D2 receptor expression in Sertoli cells was showed in ED-binge group. Furthermore, the expression was significantly decreased in all METH treated groups. Additionally, the expression of dopamine D2 receptor in round spermatid was increased in ED and ED-binge groups. On the other hand, the dopamine D2 expression in elongated spermatid was significantly reduced in ED and ED-binge groups. The Alpha-1 adrenergic receptor expression was found in Sertoli cells and spermatogenic cells. The percentage of Alpha-1 adrenergic receptor expression in Sertoli cells was significantly increased in ED-binge group. But, the expression of this receptor in spermatogonia was decreased ED-binge group. Moreover, the expression of Alpha-1 adrenergic receptor in spermatocyte was increased in all METH treated groups. In addition, increase of Alpha-1 adrenergic receptor in round and elongated spermatid was found in ED and ED-binge groups when compared with control group. Additionally, percentage of total positive cell expression was also increased ED and ED-binge groups.

In conclusion, the results of this study indicate that METH can cause DA and NE concentration changes. Moreover it affects to dopamine D2 receptor and α1 adrenergic receptor expression in testis. Thus, METH may disrupt DA and NE

function that lead to development of seminiferous epithelium change. Accordingly, METH administration may lead to spermatogenesis impairment and poor sperm quality.

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CHARPTER I

INTRODUCTION

Rationale and significance of the study

Methamphetamine (METH) is a central nervous system (CNS) stimulant drug. It has been popular in teenage because of cheapness and long activation. METH can rapidly cross blood brain barrier because of lipophilic attribute. In Thailand, METH is an illicit drug abuse that cause family problem, economics and public health problems. METH can affect on CNS by increased dopamine level. Moreover, chronic administration of METH can cause dopaminergic neuron toxicity (Woolverton, Ricaurte, Forno, & Seiden, 1989) and an α_{1D} adrenergic receptor is involved in METH-induced hyperthermia and neurotoxicity (Kikuchi-Utsumi et al., 2013). Another study has reported that low dose of METH can increase α_{2A} adrenergic receptor in hippocampus (Nishio, Kanda, Mizuno, & Watanabe, 2002). METH can cause neurotransmitters change especially dopamine, norepinephrine (Rothman et al., 2001). For example, acute METH administration can activate dopamine and norepinephrine release but long term METH administration can cause depletion of dopamine (Wagner, Seiden, & Schuster, 1979). Though, METH is central nervous system drug stimulant but it also has an effect on peripheral organ.

METH also has an effect on reproductive system. A previous study found that METH altered serum testosterone concentration and increased apoptotic cells in seminiferous tubule in mice (Y. Yamamoto et al., 2002). METH has also been reported to decrease proliferation of spermatogonia in rat testis (Alavi, Taghavi, & Moallem, 2008), induce abnormal sperm morphology, decrease sperm concentration and increase apoptotic cells in rat seminiferous tubule (Nudmamud-Thanoi & Thanoi, 2011). In our research team we have been studying about effects of METH on the effect to reproductive system. We found that METH not only decreased progesterone receptors, estrogen receptor alpha and estrogen receptor beta in rat testis but also reduced sperm concentration and normal sperm morphology (Nudmamud-Thanoi, & Thanoi, 2011).

In addition, METH has an effect on reproductive system by alteration of sex-hormone such as estrogen and progesterone (Nudmamud-Thanoi, Sueudom, Tangsrisakda, & Thanoi, 2016b) which can induce of reproductive system abnormality subsequently. METH results in the interference function of hypothalamic-pituitary-gonadal axis (Shen et al., 2014). In addition, METH also activates sympathetic nervous system (Haile, De La Garza, Mahoney, & Newton, 2013). Therefore, these two mechanisms can cause sex-hormone change. So, METH may have direct effect on reproductive system.

As mentioned above, METH is dopamine agonist. Thus, we hypothesized that METH may has direct effects on testicular cell. Because, prior studies suggest that dopamine receptor and adrenergic receptors express in testis and spermatozoa (Adeoya-Osiguwa, Gibbons, & Fraser, 2006; Huo, Zhong, Wu, & Li, 2012; Otth et al., 2007). In α_{1B} adrenergic receptor knockout male mice resulted in abnormal morphology of sertoli cell and stopped development of spermatozoa (Mhaouty-Kodja et al., 2007). The other studies have reported that norepinephrine is involved in development of leydig cell (Huo et al., 2012) and activated sperm capacitation (Adeoya-Osiguwa & Fraser, 2005). High dose of dopamine was found decreased sperm motility (Ramirez et al., 2009). Studies have been reported that dopamine and adrenergic receptors expression were found in testis. Moreover, these receptors have an influence in spermatogenesis and sperm quality that can cause abnormal spermatogenesis and low sperm quality. So, these abnormalities may occur from these dopamine and adrenergic receptor dysfunction. Therefore, the expression of dopamine and adrenergic receptor in testis were investigated in this study.

It is interesting that METH may induce alterations of dopamine, dopamine receptor, norepinephrine and adrenergic receptors in rat testis that can cause male reproductive functions abnormality. This study provided information for effects of METH on reproductive system. In addition, it is be advantage for development of quality of life and medical science research.

Purpose of the study

A general experiment objective

This study was investigated the effects of METH on dopamine and norepinephrine functions in male reproductive system.

Specific objectives

1. To determine alteration of dopamine D2 and adrenergic receptors α1 subtype expressed cell in rat seminiferous tubule after exposure to METH.

2. To examine changes of dopamine and norepinephrine levels in rat testis after exposure to METH.

3. To determine alteration of stage of seminiferous tubules in rat testis after exposure to METH.

Hypotheses

1. METH, an agonist of dopamine has directly effects on male reproductive system via dopamine and adrenergic receptors expressed in testicular cells.

2. Male reproductive functions may be consequently affected from changes of dopamine and norepinephrine levels in testis which is induced from METH administration. For example, disruption spermatogenesis, reduction of sperm quality and cause male infertility.

Scope of this study

The animal model of METH addiction was used to investigate the alteration of dopamine and adrenergic receptors, dopamine and norepinephrine levels and stage of seminiferous epithelium by using immunohistochemistry, high performance liquid chromatography (HPLC) techniques and hematoxylin and eosin staining, respectively.

Key words

Methamphetamine, dopamine, dopamine receptors, norepinephrine, adrenergic receptors and seminiferous tubules

Anticipated outcome

1. To provide an information about effects of METH to alteration of dopamine, dopamine receptor, norepinephrine and adrenergic receptor in testis.

2. To provide knowledge for effects of METH dependence on male reproductive system.

3. To provide an information about effects of dopamine and norepinephrine on male reproductive system.

Conceptual framework

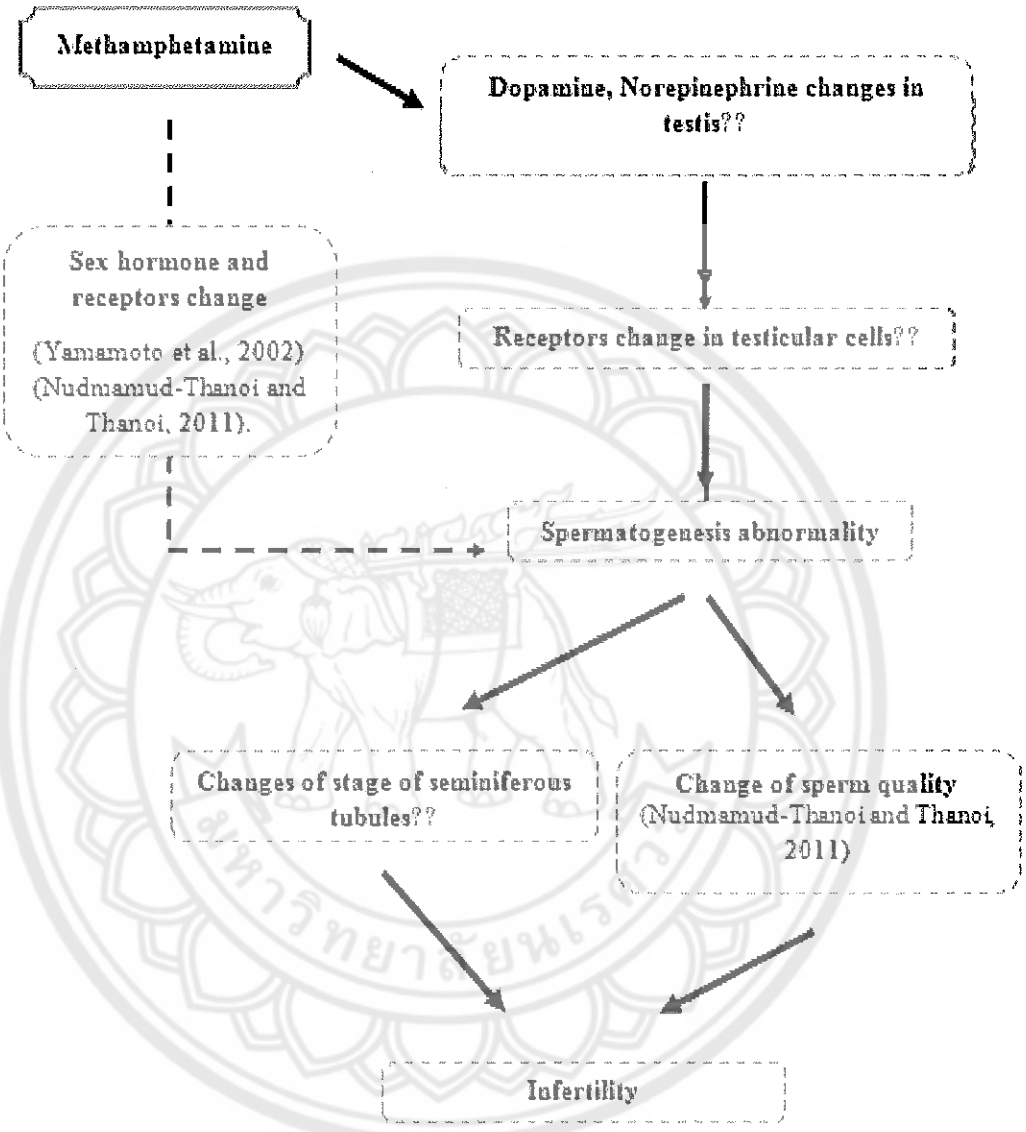

Figure 1 The conceptual framework of this study

CHARPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Methamphetamine

Methamphetamine (METH) is an addictive psychostimulant drug of the phenethylamine and amphetamine class. In 1893, METH was firstly synthesized from ephedrine by Nagai Nagayoshi, Japanese scientist. It has a chemical structure related to amphetamine (see figure 1). METH has been used for treating attention deficit hyperactivity disorder (ADHD), extreme obesity, or to treat narcolepsy. The drug increasingly abused mainly in young adults, as a result of its wide availability, rather low cost, and long duration of psychoactive effects (Panenka et al., 2013). METH has been known in many different names such as ice, meth, crank, jib, speed and crystal, depend on the geographical and characteristic of drug. In Thailand, it is called "Yaba". Forms of METH were classified as powder METH, damp or oily METH, crystalline METH and other forms of the drug for example liquid and pills (Topp, Degenhardt, Kaye, & Darke, 2002). According to a United Nations report, approximately 33.4 million people use METH in Eastern and Southeast Asia. The routes of METH ingesting include snuffle, smoking or injection. After taking the drug, METH rapidly crosses blood brain barrier because of lipophilic nature (Lake & Quirk, 1984).

The acute effects of METH consist of increased heart rate and blood pressure, vasoconstriction, bronchodilation hyperglycemia (Cruickshank & Dyer, 2009), euphoria, hypersexuality, decreased anxiety and increase energy (Homer et al., 2008; Meredith, Jaffe, Ang-Lee, & Saxon, 2005). These effects can last for several hours. METH has a longer duration of action than cocaine due to METH has a longer half-life, 10 to 12 hours (Schepers et al., 2003). The chronic effects of METH administration involve in anxiety, depression, aggressiveness, antisocial, psychosis, mood disturbances, and psychomotor dysfunction (Darke, Kaye, McKetin, & Duflou, 2008; Homer et al., 2008; Scott et al., 2007). The previous study have detected deficit in attention, working memory, and decision in chronic METH abuser (William et al., 2013). A previous study reveals that METH can induce neuronal apoptosis in many

brain regions, consisting of striatum, hippocampus, cortex, indusium griseum and medial habenular nucleus (Deng, Wang, Chou, & Cadet, 2001). Cause of neuronal apoptosis may be arise from METH can increase reactive oxygen species (ROS) levels which involve cell apoptosis and DNA damage (Li, & Trush, 1993). This proposition has been supported by changing in expression of gene associate with DNA repair and DNA mismatch protein in mice after taken toxic dose of METH (Cadet, McCoy, & Ladenheim, 2002).

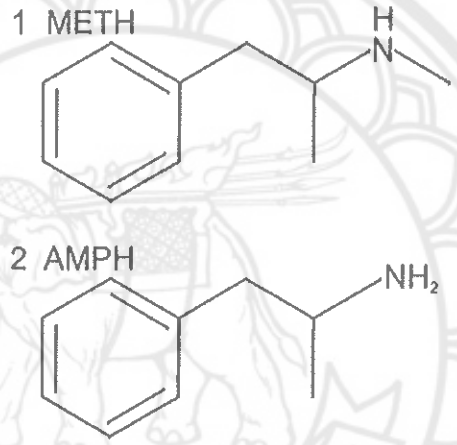

Figure 2 Chemical structure of METH (1), as well as the closely related structure amphetamine (2).

Note: Applied form Panenka et al., 2013

Methamphetamine and neurotransmitter system

METH is a psychostimulant drug that causes the release of dopamine, norepinephrine and serotonin (Rothman et al., 2001). METH can affect on the brain because it can cross the blood brain barrier. Then, the drug enters monoaminergic neuron via monoamine transporters for example dopamine transporter (DAT) which is located on neuronal membrane. METH can transport into monoamine vesicles by vesicular monoamine transporter (VMAT) and induce dopamine release (Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007; Schep, Slaughter, & Beasley, 2010). In addition, METH can cause an increase of monoamine in cytoplasm by inhibiting activity of monoamine oxidase (MAO), enzyme degradation and blocking monoamine reuptake

(See figure 2) (Suzuki, Hattori, Asano, Oya, & Katsumata, 1980). Long term effects of METH treatment to rhesus monkeys have been reported to decrease norepinephrine and dopamine levels in several brain regions such as frontal cortex, caudate, pons-medulla and midbrain (Seiden, Fischman, & Schuster, 1976). In addition, the effects of chronic METH administration in animal models, can contribute dopamine and serotonin nerve terminals damage because this drug increases dopamine accumulation in cytosol which initial oxidative stress and neuroinflammation (B. K. Yamamoto, Moszczynska, & Gudelsky, 2010).

METH-induced neurotoxicity on behavioral outcomes,(Jablonski, Williams, & Vorhees, 2016) such as cognitive deficits, such as decreased attention and working, and spatial memory impairments (Kiblawi et al., 2013; Piper et al., 2011; Roussotte et al., 2011). In addition, Chronic METH exposure involves in cognitive impairment and various psychiatric symptoms (Zhong et al., 2016). Moreover, acute METH exposure increases locomotor activity and anxiety-like behavior in the open field (Rud, Do, & Siegel, 2016).

In addition, high dose of METH have been decreased ability of mating and number of births(Y. Yamamoto, Yamamoto, & Hayase, 1999). Furthermore, there is a study has been reported that METH can cause increasing of prevalence of erectile dysfunction (Chou, Huang, & Jiann, 2015). Moreover, it also has an effect on sexual function such as erectile function, orgasmic function, and overall satisfaction (Chou et al., 2015).

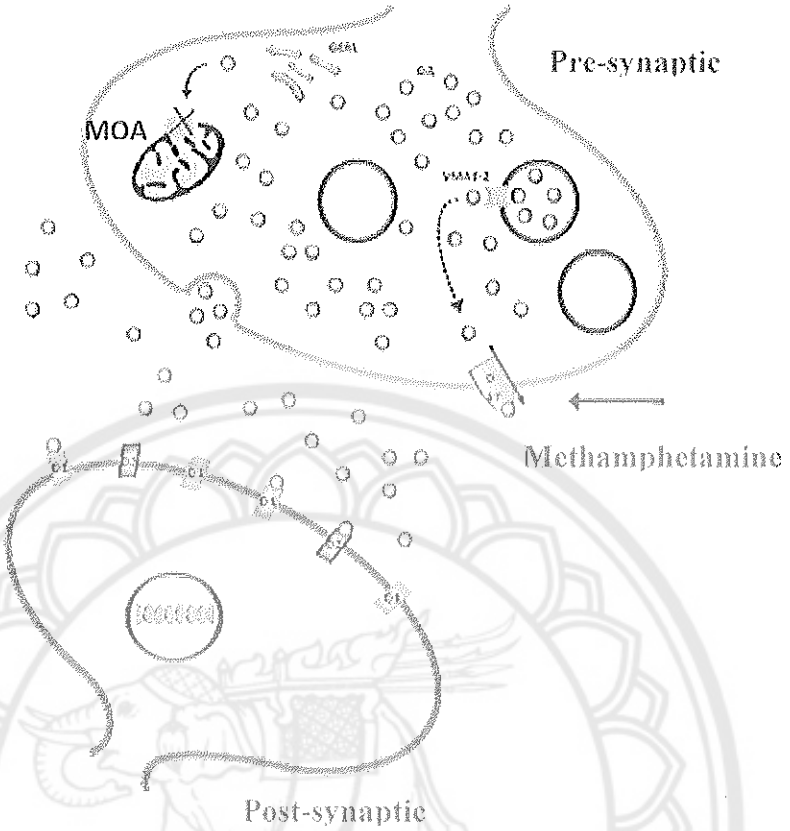

Figure 3 METH alters dopamine neurotransmission in two ways. METH enters the dopamine- or norepinephrine-containing vesicles and causes the release of neurotransmitter. METH also blocks the dopamine transporter from pumping dopamine back into the neuron.

Note: Applied from Ferrucci et al., 2013

Dopamine

Dopamine is a catecholamine neurotransmitter that is found in CNS. Dopamine neurons are mainly located in substantia nigra and project to the other areas to form four different pathways (Lindvall, & Bjorklund, 1978) that are nigrostriatal, mesolimbic, mescocortical and tuberoinfundibular pathways. The dopamine functions are associated with cognition, emotion, appetite regultation, locomotion and operation of endocrine system (Cristina et al., 1998). Dopamine is synthesized from non-essential amino acid tyrosine. Then, tyrosine is converted in to

dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH) enzyme. DOPA can be transformed to dopamine by aromatic L-amino acid decarboxylase (AAAD) (see figure 3). After synthesis, dopamine is transported to store in vesicle by vesicular monoamine transporter 2 (VMAT2) until it is released to synaptic cleft. Degradation of dopamine to inactive form can be occurred by using enzymes, monoamine oxidase (MAO), and catechol-O-methyl transferase (COMT). The inactive main metabolite of this process is 3,4-Dihydroxyphenylacetic acid (DOPAC) and Homovanillic acid (HVA)(Sharp, Zetterstrom, & Ungerstedt, 1986). The other metabolite is 3-Methoxytyramine (3-MT). The alteration of dopamine has been reported to be involved in neurological diseases such as Parkinson's disease and schizophrenia (Missale, Nash, Robinson, Jaber, & Caron, 1998) and drug addiction (Miller, Dackis, & Gold, 1987). In addition, DA can be released from sympathetic nervous system in a small amount; Goldstein (2011) indicates this to be 2-4 % of catecholamine released by sympathetic stimulation (Goldstein et al., 2011).

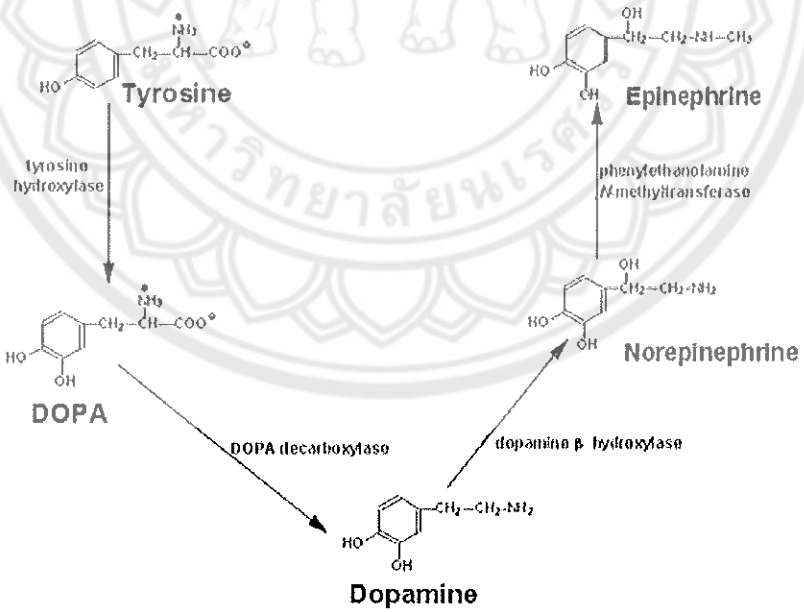

Figure 4 Dopamine synthesis

Source: Adapted from Ganong, 2001

Norepinephrine

Norepinephrine is one of catecholamine neurotransmitters. It is synthesized from dopamine by dopamine β-hydroxylase in adrenal medulla and is then released into blood circulation to act as hormone. In addition, norepinephrine is also released from noradrenergic neurons in the locus coeruleus to act as a neurotransmitter in the central nervous system and sympathetic nervous system. Signal of norepinephrine can be terminated either by transportation to neuron or degradation. Norepinephrine is degraded to various metabolites such as 3, 4-dihydroxymandelic acid, vanillylmandelic acid, 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) and 3,4-dihydroxyphenylethyleneglycol (DHPG). 3,4-dihydroxyphenylethyleneglycol is normally used for studying norepinephrine activity (Karege, & Gaillard, 1986). These activities are occurred by using monoamine oxidase, (MAO). An important function of norepinephrine is released from the sympathetic neurons to affect to the heart (Baker, Boyd, & Potter). Moreover, alteration of norepinephrine level involved in depression (Remy, Doder, Lees, Turjanski, & Brooks, 2005), schizophrenia (Lake et al., 1980) and hypotension(Maxwell, Heber, Waks, & Tuck, 1994).

Dopamine receptors

Dopamine can mediate its physiological effects through dopamine receptors. Dopamine receptors are a member of seven transmembrane G-protein coupled receptors family (Civelli, Bunzow, & Grandy, 1993). There are five different types of dopamine receptors which can be subdivided into two groups of receptors: D1-like including D1 and D5 receptors, and D2-like comprising D2, D3 and D4 receptors. The principle of classification is based on the homology in their protein structure, pharmacology and functional properties (Sokoloff, & Schwartz, 1995). Functions of dopamine receptors have been analyzed: D1-like receptors stimulate signal transduction by activation of adenylyl cyclase to increase cyclic AMP levels whereas, D2-like receptors inhibit the cyclic AMP accumulation (Missale et al., 1998).

Dopamine receptors are predominant spread in the central nervous system. Each subtypes of dopamine receptors the specific areas. For example, the highest distribution of dopamine D2 receptor is found in the caudate nucleus, putamen and nucleus accumbens (Hurley, & Jenner, 2006). Distributions of dopamine receptor in

peripheral organs are also investigated. The dopamine receptors are found in heart, blood vessels, kidney and adrenal gland (Missale et al., 1998). Recent studies indicate that dopamine D2 receptors are also expressed in testis and spermatozoa (Adeoya-Osiguwa et al., 2006; Huo et al., 2012; Otth et al., 2007).

Adrenergic receptors

The actions of norepinephrine are presented via the binding to adrenergic receptors that are G-protein-coupled receptors consisting of α and β subtypes. α- and β-adrenergic receptors can be subdivided into α1A, α1B, α1D, and β1, β2, β3, respectively. β2 adrenergic receptor is more widely expressed (Dixon et al., 1986; McCune, Voigt, & Hill, 1993; Nagatomo & Koike, 2000). Adrenergic receptors are expressed throughout the brain and peripheral organs such as heart, blood vessel and smooth muscle. Moreover, the expression of adrenergic receptors in male reproductive organs has been investigated. Several studies have reported that both α and β adrenergic receptors are expressed in mammalian spermatozoa (Adeoya-Osiguwa et al., 2006) and mice Leydig cells (Huo et al., 2012). These results may imply that the functions of adrenergic receptors are involved in spermatogenesis and/or sperm functions.

Male reproductive system

Testis and seminiferous tubule

Testes are in scrotum suspended outside abdominal cavity to keep the temperature lower than body temperature that is appropriate for spermatogenesis. The main functions of the testes are to produce sperm and androgens especially, testosterone. Seminiferous tubules are tortuous tubule inside testis. A cross section of seminiferous epithelium presents spermatogonia which are located at the basement membrane of the seminiferous tubule, one or several layers of spermatocytes in the middle, and groups of spermatids located next to lumen of seminiferous tubule. It can indicate that developmental progression of less mature to more mature germ cells as they move toward the lumen (Amann, 1970).

Spermatogenesis

Spermatogenesis is a complex process consisting of maturation and differentiation of spermatogonial cells until give rise spermatozoa through mitosis and meiosis division. Spermatogenesis occurs within seminiferous tubule of testes (de Kretser, & Kerr, 1983). The spermatogenesis involves four major processes including spermatogonial development, meiosis, spermiogenesis and spermiation.

The whole processes of spermatogenesis are briefly described below.

Firstly, the initial cells of these processes are called spermatogonia, which yield primary spermatocytes by mitosis. After that, the primary spermatocytes are divided into two secondary spermatocytes by first meiosis division. Then, each secondary spermatocyte undergoes second meiotic division which creates haploid round spermatids (Handel, & Schimenti, 2010). Subsequently, these change into mature spermatozoa via spermiogenesis process. Finally, each secondary spermatocyte can be subdivided to give rise four spermatozoa. (See figure 4.)

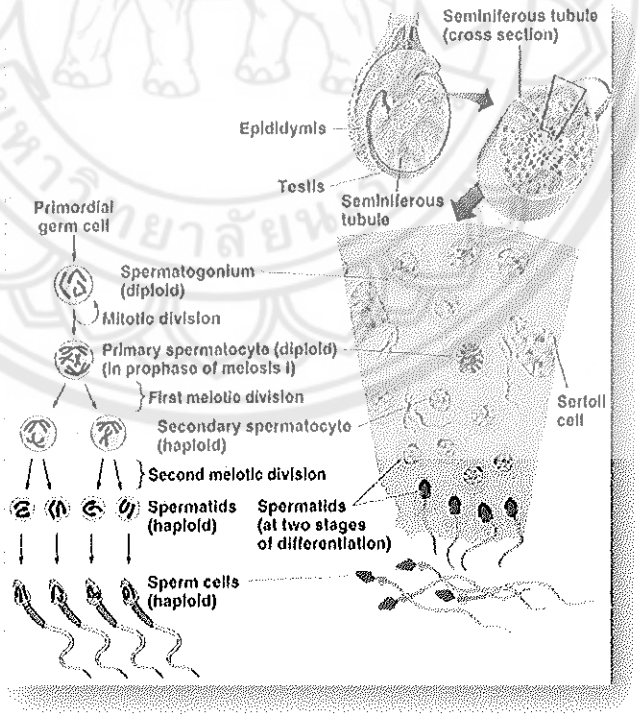

Figure 5 Spermatogenesis

Source: http://instruction.cvhs.okstate.edu/histology/mr/himrp3.htm

Spermatogonia is a stem cell that are recognized to be a single cells located at basement membrane of seminiferous tubule. Spermatogonia comprising of A pale and A dark spermatogonia. The A pale spermatogonia can undergo self-renewal division to keep balance of stem cell population, or they can go through differentiation pathway to produce type B spermatogonia (Jan et al., 2012) (see figure 5)

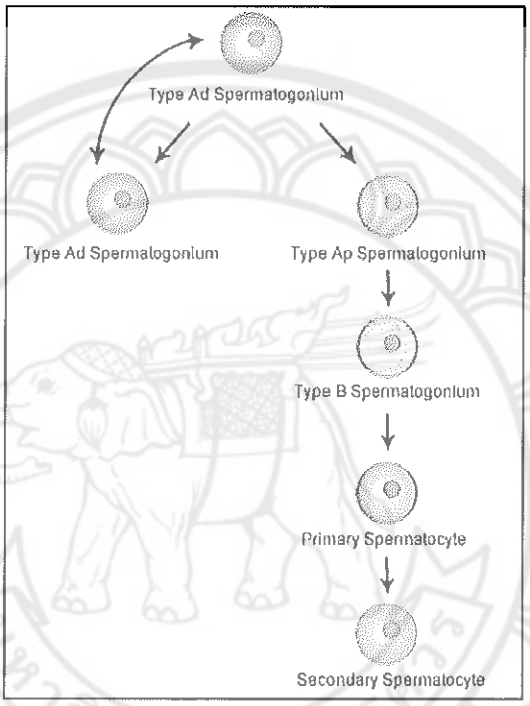

Figure 6 Proliferation and differentiation of spermatogonia called spermatocytogenesis, developing of spermatogonia to form spermatocyte.

Source: http://en.wikipedia.org/wiki/File:Spermatocytogenesis.png

Type B spermatogonia are divided into primary spermatocytes. Then, these germ cells are ready to differentiate by meiosis division, in order to transform diploid germ cells into haploid spermatids (Jan et al., 2012).

After achievement of meiosis, the round spermatids are presented. These cells are ready to enter spermiogenesis process for morphological and cytological changes. Morphological changes are composed of development of head, midpiece and tail regions. The result of these changes is that round spermatids become elongated spermatids and release to the lumen of the seminiferous tubule during spermiation. Cytological changes comprise of chromatin remodelling, develop an acrosome, a hydrolytic enzyme vesicle necessitate for oocyte penetration and remove almost all of their cytoplasm.

Spermatozoa are a yield from spermatogenesis. Mature spermatozoa consist of three main regions that are head; middle piece and tail (see figure 6). The head has pack of DNA material that contains half of genetic information. Moreover, the head is covered with acrosome of hydrolytic enzymes that are essential to penetrate the zona pellucida of the oocyte. The middle piece, a source of energy for sperm motility is included numerous mitochondria which are wrapped around the dense fibers and surrounded the axoneme. The tail has a distinctive axoneme of a flagellum with its nine outer doublets and a central pair of microtubules that are used for movement of sperm.

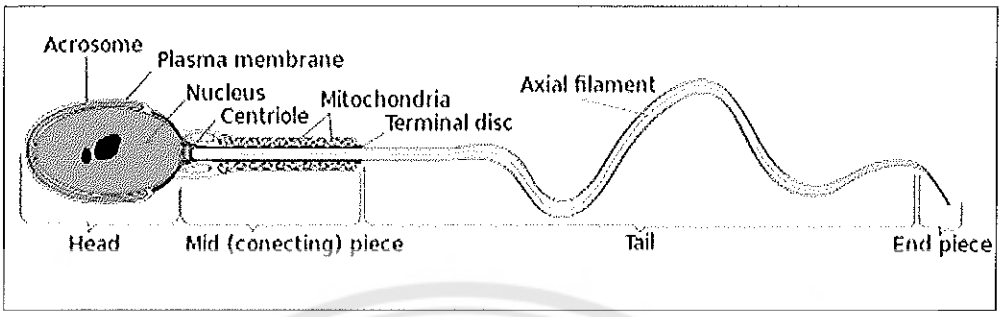

Figure 7 Morphology of human spermatozoa

Source: http://en.wikipedia.org/wiki/File:Complete_diagram_of_a_human_spermatozoa_en.svg

There are two important non-spermatogenic cells consist of Sertoli cells and Leydig cell. Sertoli is a columnar cell which has long and thin mitochondria. Moreover, Sertoli cells usually have lipofuscin and lipid droplets at the base of their cytoplasm (Johnson, 1991, Russell, 1993). Sertoli cell nuclei exhibit a variety of shapes, but they are usually oval or pear shaped with significant indentations in the nuclear membranes. Location of sertoli cells is closed to basement membrane of seminiferous tubules. The Sertoli cells play an important role in regulation of spermatogenesis and changing spermatozoa production (Cooke, Zhao, & Bunick, 1994). The functions of Sertoli cells consist of providing structural support and nutrition to developing germ cells, phagocytosis of degenerating germ cells and residual bodies, release of spermatids at spermiation process and production of essential proteins that control and/or react to pituitary hormone for mitotic division of spermatogoni (Johnson, Thompson, & Varner, 2008).

Leydig's cell is another non-spermatogenic cell that is important for spermatogenesis. It has also known as interstitial cells. A Leydig cell is a group of polyhedral shape cell that located in interstitial space. Functions of Leydig' cell are associated with production and secretion of testosterone by stimulation of luteinizing hormone (LH) from pituitary gland (Saez, 1994). Prolactin can induce the response of Leydig cells to LH by increasing amount of LH receptors expressed on Leydig cells (Lejeune et al., 1998). (figure 7)

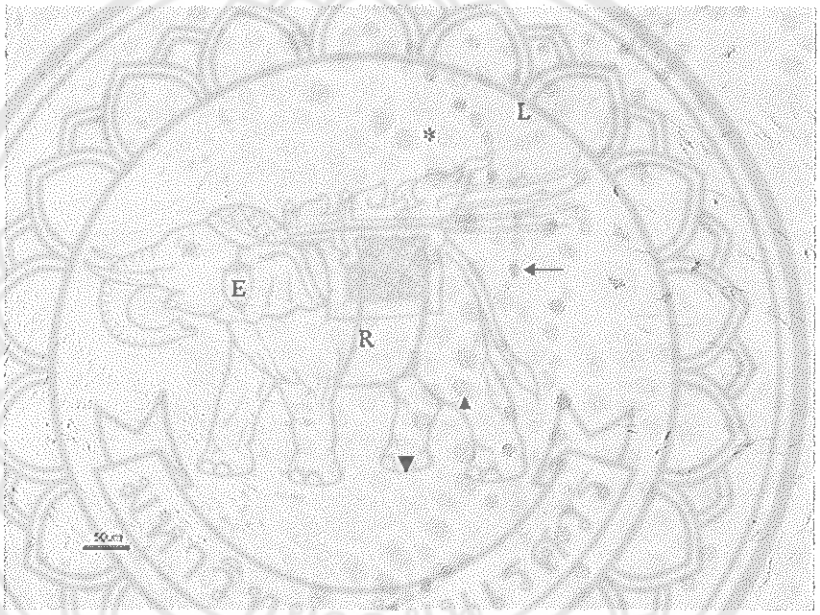

Figure 8 Cross section of rat seminiferous tubules (40x) consists of spermatogenic cells and non-spermatogenic cells, sertoli and Leydig cell. spermatogonia (arrow), spermatocyte (arrow head), round spermatid (R), elongated spermatid (E), Sertoli cells (*), Leydig cell (L)

Stages of seminiferous epithelium

Development of spermatogenic cells that occurs in seminiferous tubules is called spermatogenic cycle or cycle of seminiferous epithelium. It shows the whole process of spermatogenesis. In rat, it takes about thirteen ours for a cycle of spermatogenesis. The systemic arrangement of spermatogenic cells in seminiferous tubules may be defined as stages. So, in rat seminiferous epithelium consists of fourteen stages (Leblond, & Clermont, 1952) (figure 8) that can divide into three categories such as early stage (I-V), middle stage (VI-VIII) and late stage (IX-XIV). Moreover, Spermiogenesis can be divided into 19 steps (figure 8).

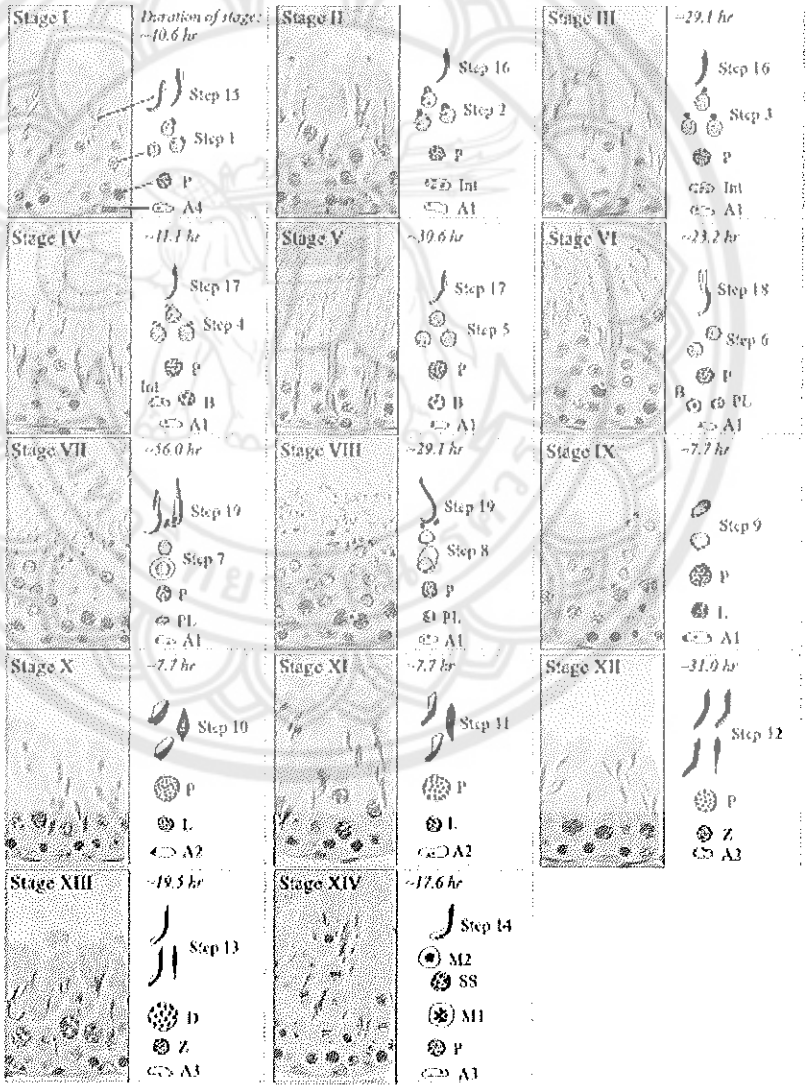

Figure 9 Stages of seminiferous epithelium in rat testis

Source: Murk et al.,2012

Methamphetamine and reproductive system

METH not only affects on the central nervous system but also disturbs other systems especially reproductive system. A single treatment of METH has been reported to increase apoptotic cells in mice seminiferous tubules (Nudmamud-Thanoi, & Thanoi, 2011). In addition, the DNA fragmentation was detected in 15 mg/kg METH treated-mice (Y. Yamamoto et al., 2002). In accord with Alavi et al., 2008, METH can induce apoptosis in spermatogonia and primary spermatocyte (Alavi et al., 2008). Moreover, the adverse effects of METH lead to reduction of cell proliferation and alteration of proliferation/apoptosis ratio(Alavi, et al., 2008). It has also been reported that METH can cause abnormal sperm morphology, low sperm concentration (Nudmamud-Thanoi & Thanoi, 2011) and decreased sperm motility (Y. Yamamoto et al., 1999). 15 mg/kg METH administration results in inhibition ability of male mice to mate with female and decreasing in total number of offspring (Yamamoto et al., 1999). In our research team, we also examine adverse effects of METH on reproductive functions. The results indicate that METH can inhibit expression of progesterone receptors, estrogen receptor alpha and estrogen receptor beta in rat testis(Nudmamud-Thanoi, Sueudom, Tangsrisakda, & Thanoi, 2016a). METH has also shown to induce reductions of sperm concentration and normal sperm morphology (Nudmamud-Thanoi, & Thanoi, 2011). High doses of METH exposure can induce a change of plasma testosterone concentration (Yamamoto et al., 1999). Testosterone is accepted to be essential for copulation (Hull, Du, Lorrain, & Matuszewich, 1997) that can induce releasing of dopamine and enhance dopamine receptors in medial preoptic area, which is a crucial area for male sexual behaviors (Hull et al., 1997). Cathine, an amphetamine related compound can enhance capacitation but inhibit acrosomal reaction in mice and human spermatozoa (Adeoya-Osiguwa, & Fraser, 2005). A study has also reported that cathine affects on mammalian spermatozoa via adrenergic receptors (Adeoya-Osiguwa, & Fraser, 2007).

CHARPTER III

RESEARCH METHODOLOGY

Animals

Twenty-two Spargue-Dawley rats were obtained from Nation Animal Center, Salaya, Nakorn Pathom and were used in this study. The animals were placed in cage (28 x 18 x 19 cm) and were handled within control temperature at 24 ± 1 °C with 12 hours light and dark cycle. Before treatment, the animals were allowed to acclimatize for 5 days. The protocol of this study was approved by the Animals Research Committee of Naresuan University.

Methamphetamine administration

D-methamphetamine hydrochloride (Lipome AG, Arlesheim, Switzerland) was used in this study. The animals were divided into four groups consisted of control group (n=6), acute binge group (n=6), escalating dose group (n=4) and escalating binge dose group (n=6). The animals in control group were received 0.9 % normal saline through intraperitoneal (i.p.) injection for 15 days. For acute binge group, animals were injected (i.p.) with 0.9 % normal saline for 14 days and day 15 animals were injected with 6 mg/kg METH four times a day (every 2 hours). In escalating dose group, animals were received METH 0.1-4 mg/kg for 14 days (three times a day at 3 hours interval) and day 15, animals were injected with 0.9 % saline. Animals in escalating binge dose group were injected with METH 0.1-4 mg/kg for 14 days (three times a day at 3 hours interval) and day 15 animals were treated with 6 mg/kg METH four times a day (every 2 hours).

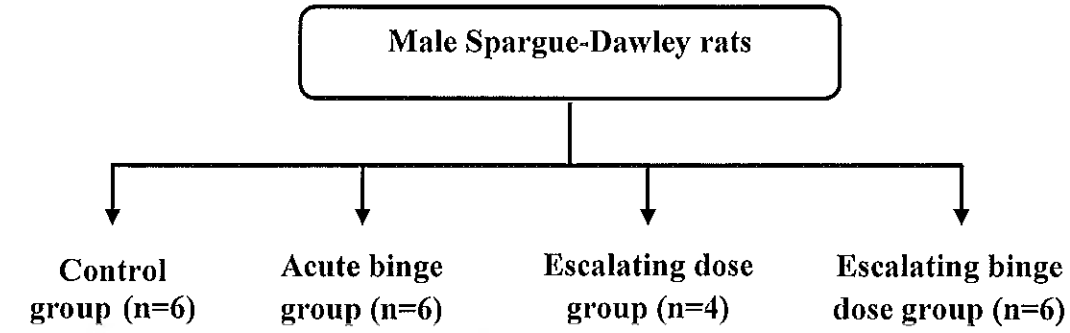

Figure 10 Diagram showing experimental design; four groups of animals

Table 1 Showing methamphetamine administration in each experimental group

Day/time	METH (mg/kg)			
	07:30	10:30		13:30
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
	07:30	09:30	11:30	13:30
15 (binge dose)	6.0	6.0	6.0	6.0

Source: Adapted from Segal, 2003

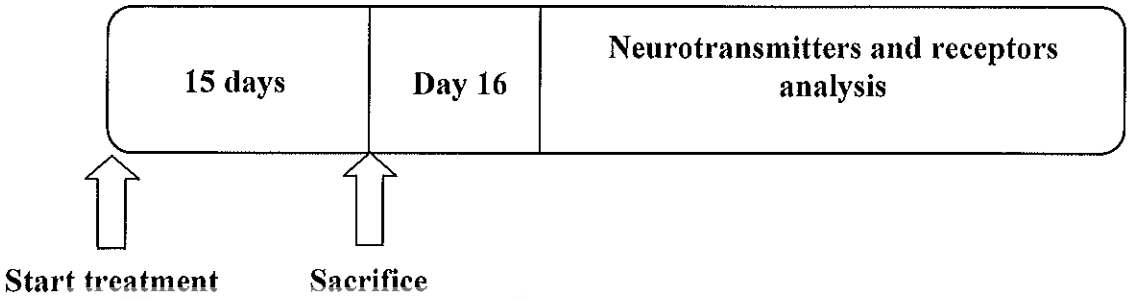

Figure 11 Diagram showing METH effects investigation

Dopamine and norepinephrine levels analysis

Animals were sacrificed by cervical dislocation and testes were removed immediately. Removed testis were fixed in 10% neutral formaldehyde solution while another testis were kept in -80 °C. DOPAC, norepinephrine and 3,4-dihydroxyphenylglycol (DHPG) were analyzed using high performance liquid chromatography with electrochemical detector. Testes were moved from -80 °C and allowed to thaw at 4 °C. Then, the testes were homogenized in 0.1 M perchloric acid and sonicated for 1 minute on ice. After that, samples were centrifuged at 18,000 x g for 15 minutes at 4 °C. Then, supernatants were selected and filtered through 0.25 µm nylon filters before injecting to pump by auto-sampling injection. C18 reverse phase column (sigma) was used. Mobile phase is consisted of LiH2PO4 100 mM, octane sulfinic acid 1.5 mM and 10 % methanol. Electrochemical conditions for this study, guard cell potential is +10 mV, E1 is +10 mV and E2 is +440 mV. Flow rate 0.6 ml/min was used for peak separation. Column temperature and auto-sample injection chamber was controlled at 30 °C and 4 °C respectively.

Evaluation of neurotransmitter levels and statistical analysis

The levels of DOPAC, norepinephrine and 3,4-dihydroxyphenylglycol were recorded and expressed in term of ng/g wet tissue weight. The data was analyzed by using one way ANOVA and followed by post hoc Dunnett test. Statistically significant was considered at $p<0.05$.

Tissue preparation

After formaldehyde infiltrate through testicular tissue, testes were cut and placed in cassettes. Tissues were then washed in distilled water for 10 minutes (three times) to clean formalin off. The testicular tissues were prepared by automatic tissue processor (LEICA TP 1020). Tissue dehydration was done a used series alcohol concentration (70%, 80%, 85%, 90%, 95% and 100% respectively) in order to remove water. Then, tissue was infiltrated in xylene, a clearing agent, for removing alcohol and followed by melted paraffin wax, an infiltration agent. Next, tissue was moved from the cassettes and placed in mold to embed in paraffin. The embedded tissue was left on cool plate and finally, the tissue block was removed from molds.

Table 2 Showing procedure of tissue preparation by using auto-tissue processor

Processing		solution	Time
Dehydration	1	70 % ethanol	30 min
	2	80 % ethanol	30 min
	3	85% ethanol	30 min
	4	90 % ethanol	30 min
	5	95 % ethanol	30 min
	6	100% ethanol	30 min
	7	100 % ethanol	30 min
	8	100 % ethanol	30 min
Clearing	9	xylene	1 h
	10	xylene	2 h
Infiltration	12	palaplast	2 h
	13	palaplast	2 h

Tissue sectioning

The tissue block was sectioned by using microtome with thickness 5 μm. Then, sectioned tissue was floated in warm water (40 °C) and mounting on silane coated slides. Finally, section was left to dry at room temperature for 2 days.

Hematoxylin and eosin staining for stage of seminiferous tubule analysis

Paraffin sections were deparaffinized by placed in xylene 2 times (5 minutes/time) and rehydrated by using series ethanol (100%, 95%, and 80%). Then, place slides in to deionize H_2O for 5 minutes. Slides were placed in Hematoxylin for 5 minutes and rinsed with tap water about 10 minutes. After that, slides were dipped in lithium carbonate 30 dip and rinsed with deionized H_2O. Then, slides were placed in Eosin for 30 seconds and dipped in series ethanol (80%, 95%, and 100%) for tissue dehydration. Then, slides were placed in xylene for 5 minutes 2 times to remove ethanol. Slides were mounted with mounting medium (Fisher Scientific, New Jersey, U.S.A) and covered by cover glasses. Finally, slides were dried overnight in room temperature.

Evaluation of the stage of seminiferous tubule and statistical analysis

The slide was captured by image capture system joining with computer. Stages of seminiferous epithelium in cross section of testis were identified. The data was analyzed by one way ANOVA and followed by LSD post hoc test. The data was investigated as P-values less than 0.05

Table 3 Showing procedure of hematoxylin and Eosin staining

Processing		solution	Time
Deparaffin and rehydration	1	Xylene	5 min
	2	Xylene	5 min
	3	100% ethanol	5 min
	4	95 % ethanol	5 min
	5	80 % ethanol	5 min
	6	deionized water	5 min
Hematoxylin staining	9	Hematoxylin	5 min
	10	Tap water	10 min
Destain	11	lithium carbonate	30 dip
	12	deionized water	30 dip
Eosin staining	13	Eosin	30 dip
Rehydration	11	80 % ethanol	5 min
	12	95 % ethanol	5 min
	13	100% ethanol	5 min
	14	Xylene	5 min
	15	Xylene	5 min
Cover slides	16	permount	

Immunohistochemistry analysis

The expression of dopamine D_2 receptor and α_1-adrenergic receptors in testis was investigated by using immunohistochemistry technique. The sectioned testis was deparafinized with xylene for 5 minutes (two times). Then, the tissue sections were rehydrated by using serial alcohol concentrations (100%, 95%, 80%, and 70% respectively) and distilled water, to preserve water to tissue. After that, the tissue sections were permeated by employing citrate buffer (pH 6.0) and using high temperature 70P (560 watt) in microwave for 5 minutes (three times), for antigen retrieval. Then, the sections were left to cool down at room temperature about 30 minutes. Next, the testicular tissues were doused with endogenous peroxidase blocking solution, consisting of 10% methanol, 0.3% H_2O_2 and 0.1% triton X for 30 minutes. Afterwards, the testis sections were washed in PBS for 5 minutes (three times) and incubated with 5% bovine serum albumin (BSA) for an hour, for blocking non-specific proteins. Testis sections were then incubated in specific primary antibodies

that are anti- D2 dopamine receptor (Merck Millipore, California, U.S.A) and anti α1-adrenergic receptor (abcam, UK) for 1 hour at room temperature and then put in refrigerator for 12 ours . After incubating with each primary antibody, the testicular sections were washed with PBS and were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, California) for 2 hours and avidinbiotinylated horseradish peroxidase complexes (ABC kit) (Vector Laboratories, Burlingame, California, U.S.A) for an hour to enhancing signal. Next, the testis sections were washed with PBS and the specific proteins were visualized by chomogen 3, 3 – diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, California) for 5 minutes for D2 receptor and for 3 minutes for α1-adrenergic receptor. Then, reaction was stopped by immerse in distilled water for 5 minutes. After that the testicular sections were dehydrated by using serial alcohol concentrations, 70%, 80%, 95% and 100%, then immersed in xylene, respectively. Finally, the sections were mounted with mounting media (Fisher Scientific, New Jersey, U.S.A) and cover with cover glasses (See figure 11). Moreover rat brain section was also investigated the expression of D2 receptor and α1-adrenergic receptor that was used as positive control.

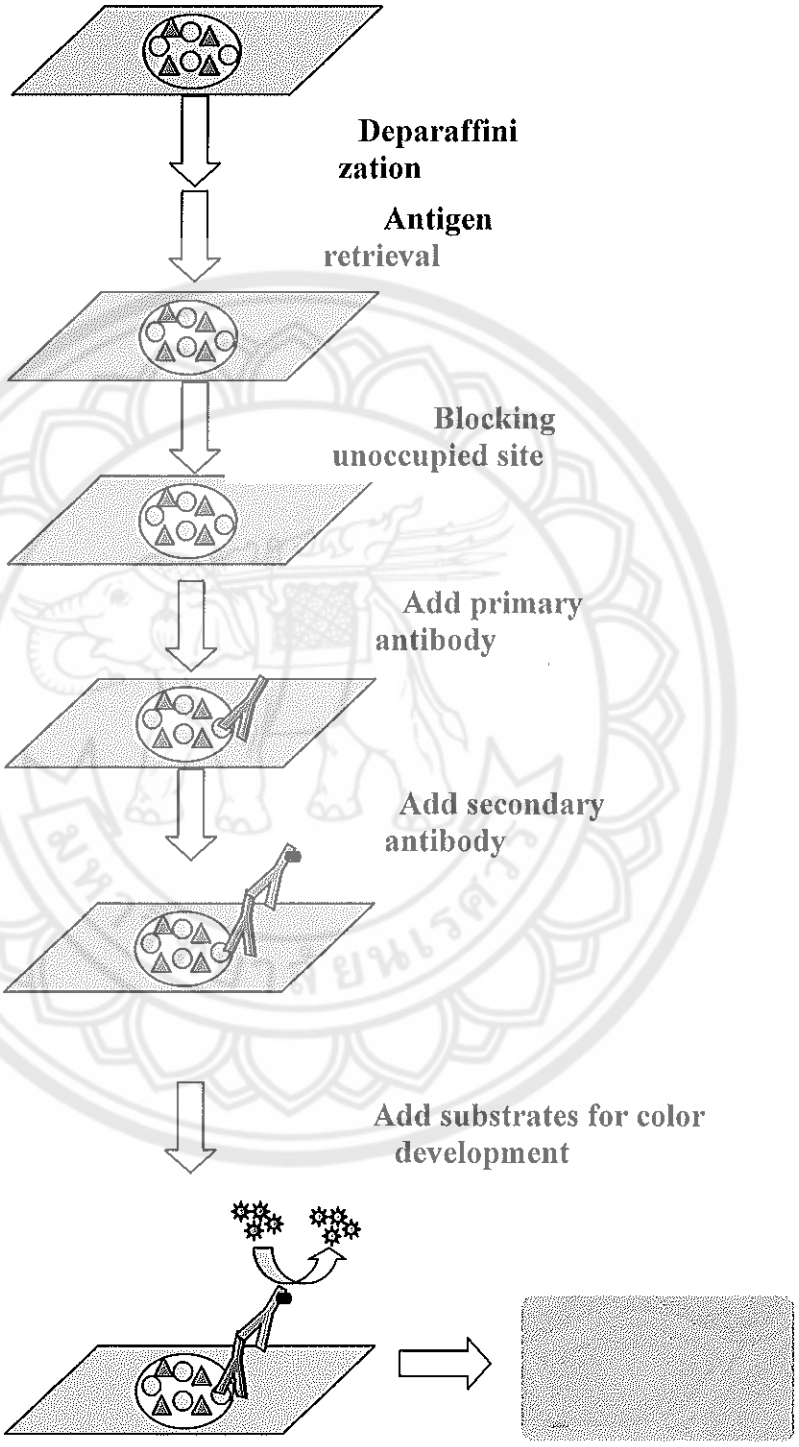

Figure 12 Immunohistochemical technique

Evaluation of the immunopositive cells and statistical analysis

The immunoreactive detected slides were captured by image capture system joining with computer. The immonopositive cells were measured by image J program (version 1.45). Ten seminiferous tubules per section were selected randomly under light microscope by using 200X magnification. The quantity of specific protein expression in testes was counted. The data was analyzed by one way ANOVA and followed by LSD post hoc test. The data was investigated as P-values less than 0.05.

Research place

This study was proceeded at Molecular Neurobiology Unit, Department of Anatomy Faculty of Medical Science, Naresuan University.

CHARPTER IV

RESULTS AND DISCUSSION

Effects of METH on monoamine concentration

The concentration of monoamine such as DOPAC, NE and DHPG was investigated. DOPAC, NE and DHPG were eluted out within 6 minutes, the retention time of these neurotransmitters are: 4.6 min (DOPAC), 5.9 (NE) and 3.6 min (DHPG), respectively (figure 13). Recovery rate of these three monoamines were DOPAC 104%, NE 89% and DHPG 98%. Increased DOPAC concentration was observed in all METH treated rats with a significant increase in the AB group ($p<0.05$) (figure 14). The concentration of NE was decreased in AB and ED groups but did not reach significance. In addition, NE concentration was significantly decreased in ED-binge group ($p<0.05$) when compared with control (figure 15). The level of DHPG was significantly decreased in ED and ED-binge groups when compared with the control group ($p<0.05$) (figure 16) but was not significantly reduced in the AB group. The ratio between DHPG and NE was also calculated. The result showed no significant difference (figure 17).

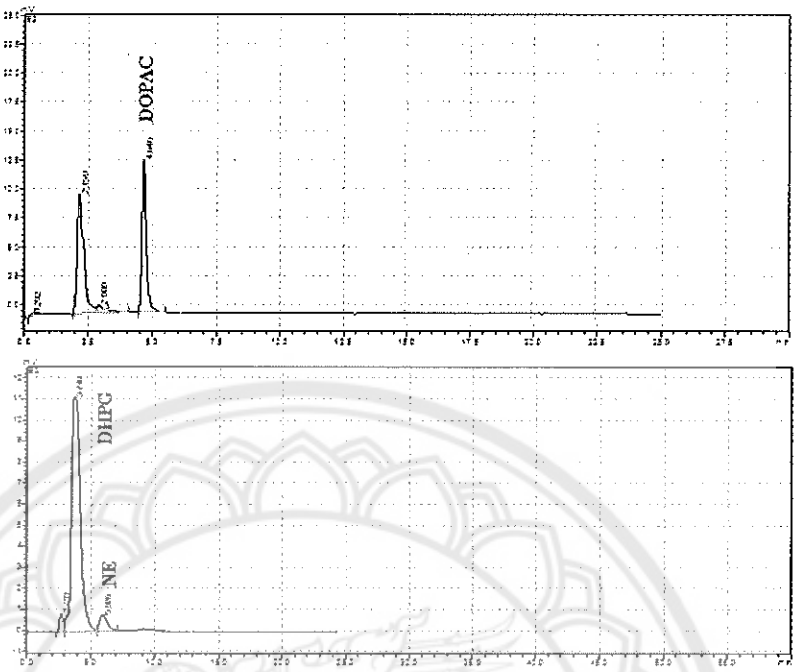

Figure 13 The chromatogram of DOPAC, NE and DHPG

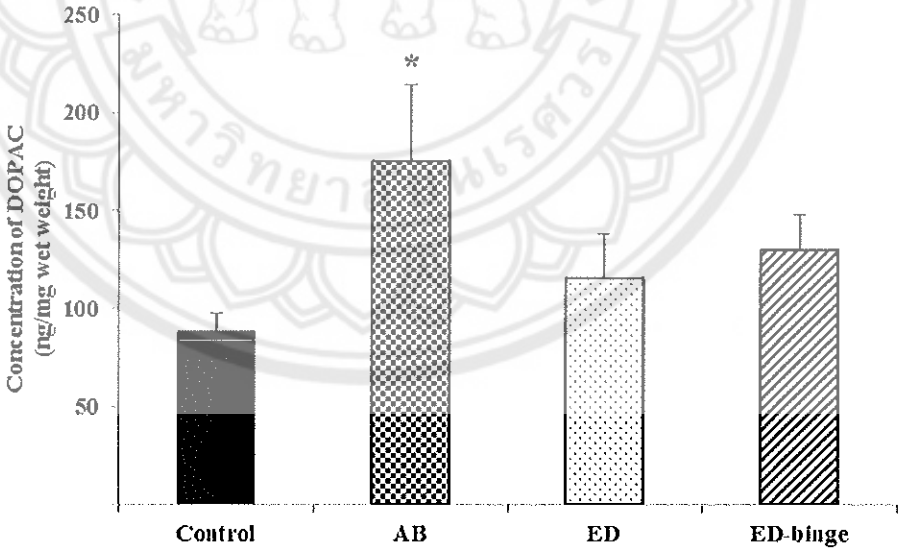

Figure 14 Concentrations of DOPAC in testis of METH-administered and control rats. Data are presented as mean ± SEM. in ug/mg tissue. One way ANOVA and followed by post hoc Dunnett test * $p<0.05$

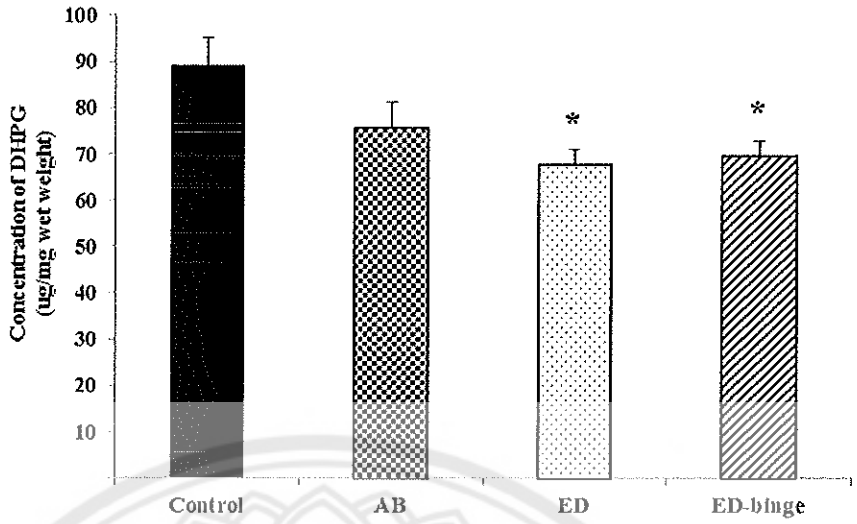

Figure 15 Concentrations of DHPG in testis of METH-administered and control rats. Data are presented as mean ± SEM. in ug /mg tissue. One way ANOVA and followed by post hoc Dunnett test * p<0.05

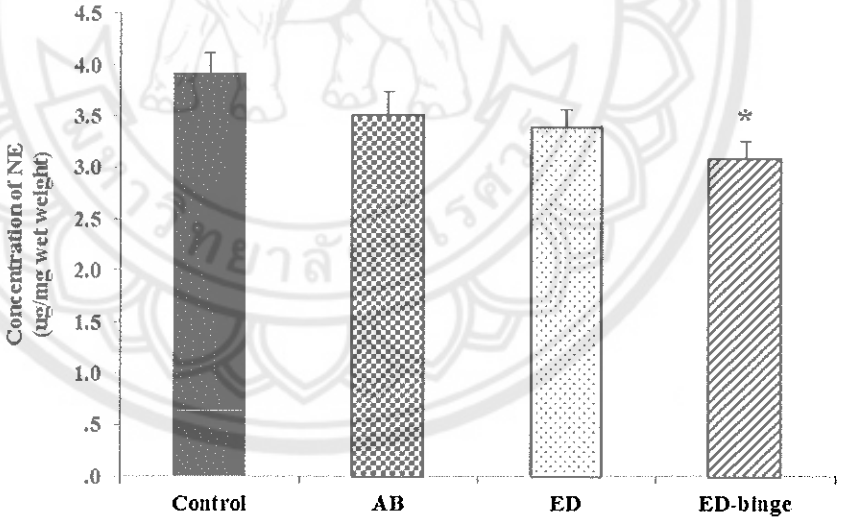

Figure 16 Concentrations of NE in testis of METH-administered and control rats. Data are presented as mean± SEM. in ug /mg tissue. One way ANOVA and followed by post hoc Dunnett test *p<0.05

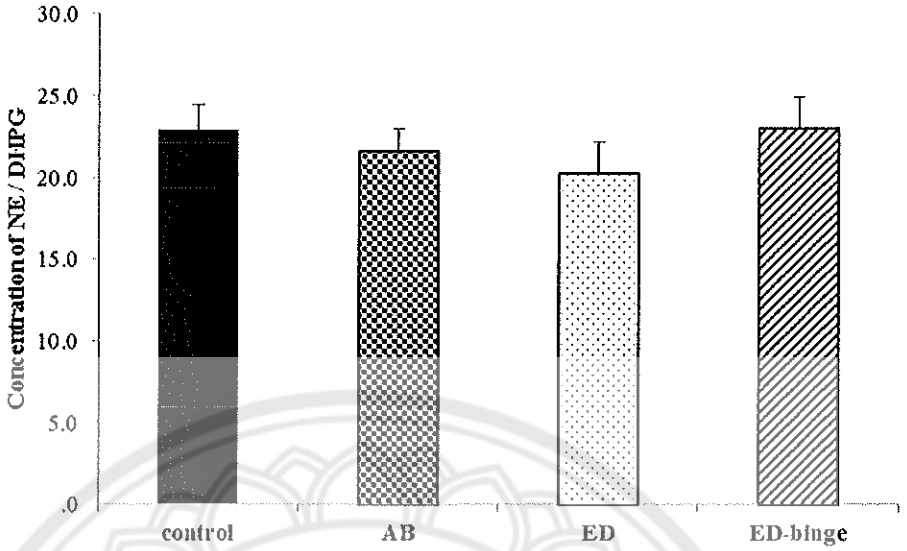

Figure 17 Ratio between NE and DHPG, data are presented as mean± SEM. in ug /mg tissue one way ANOVA and followed by post hoc Dunnett test

Changes of stage of development in seminiferous tubule

The hematoxylin and eosin staining section of testis contains numerous of seminiferous tubules (figure 17). The development of germ cells is occurred in seminiferous tubules that show different arrangement of epithelium. In addition, in rat seminiferous tubules can be divided into fourteen stages (figure 18-31).

Percentage of stages of seminiferous tubules all fourteen stages were shown in figure 32. After METH exposure, stages of seminiferous tubules were changed (figure 33). Stage II of seminiferous tubules was significantly decreased in ED and ED-binge groups ($p<0.05$) when compared with control. A significantly decrease of stage V was observed in ED group ($p<0.05$). Moreover, stage XI was significantly increased in ED-binge group ($p<0.05$). Stage XIII was significantly increased in ED group ($p<0.05$) when compared with control.

Stages of seminiferous epithelium in rat also can divide into three categories such as early stage (I-V), middle stage (VI-VIII) and late stage (IX-XIV). The result showed that the early stage of seminiferous epithelium was reduced in all METH-treated groups and reached a statistical significant in ED group when compared with control ($P<0.05$) (figure 34).

Figure 18 A cross section of rat testis consisting of numerous of seminiferous tubules

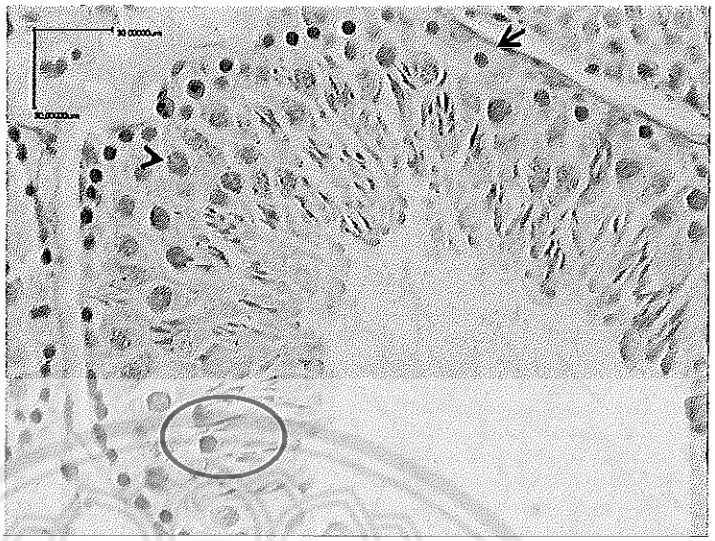

Figure 19 Stage I of rat seminiferous tubule, cells in tubule was listed form basement membrane to lumen, consisting of spermatogonia (arrow), spermatocyte (arrow head), round spermatid and group of bundle of immature spermatozoa (circle) which lo cated on lumen side.

Figure 20 Stage II of seminiferous rat tubule, consisting of all types of spermatogenic cells and the clusters of elongated spermatid (circle) was inserted into epithelium, betaween round spermatid near lumen side

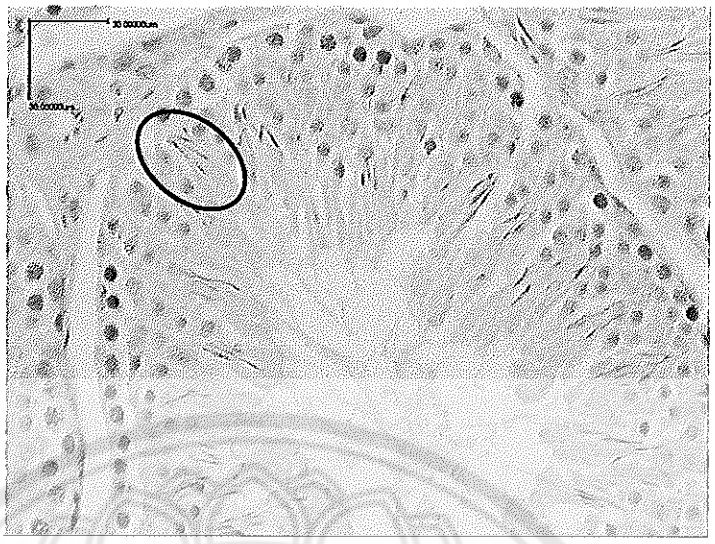

Figure 21 Stage III of rat seminiferous tubule, consisting of all types of spermatogenic cells and the bundle of elongated spermatid (circle) was more clarely and deeply inserted into epithelium.

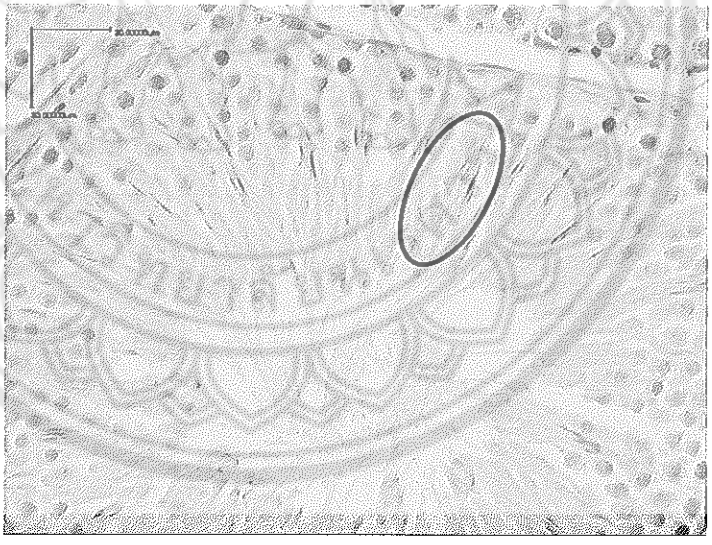

Figure 22 Stage IV of rat seminiferous tubule, the immature spermatozoa (circle) were inserted closer to Sertoli cells.

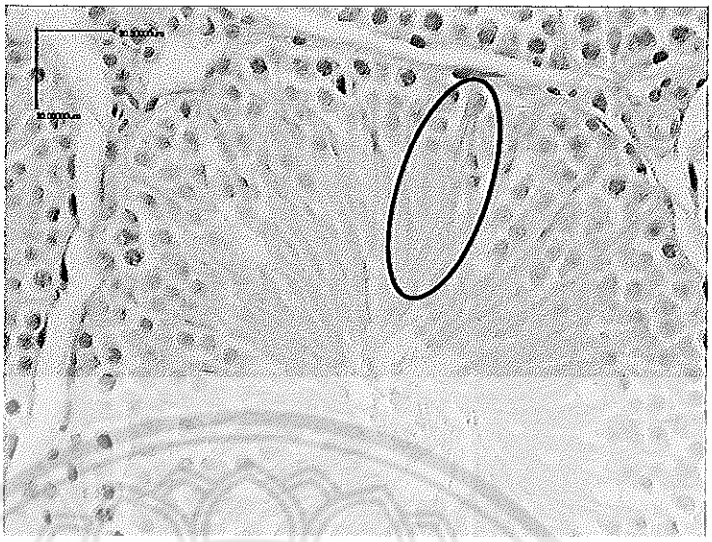

Figure 23 Stage V of rat seminiferous tubule, round spermatid is separated by bundle of immature spermatozoa (circle) and it was attached to Sertoli cells.

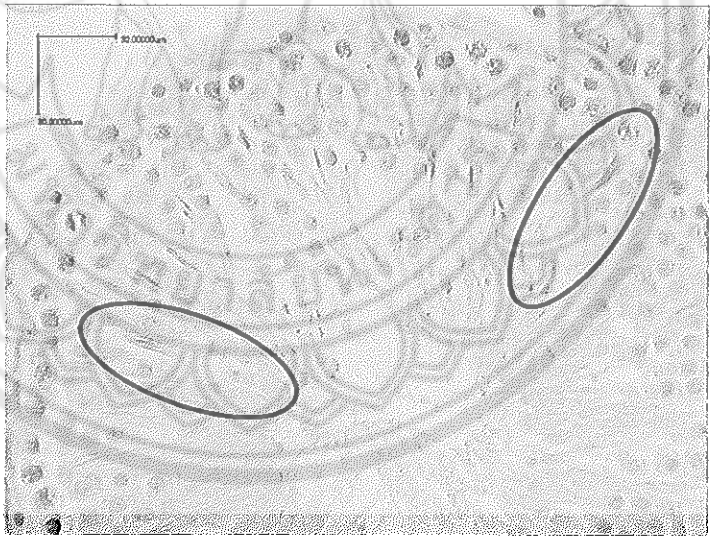

Figure 24 Stage VI of rat seminiferous tubule, immature spermatozoa (circle) was released from Sertoli component and they was found in different levels of seminiferous epithelium.

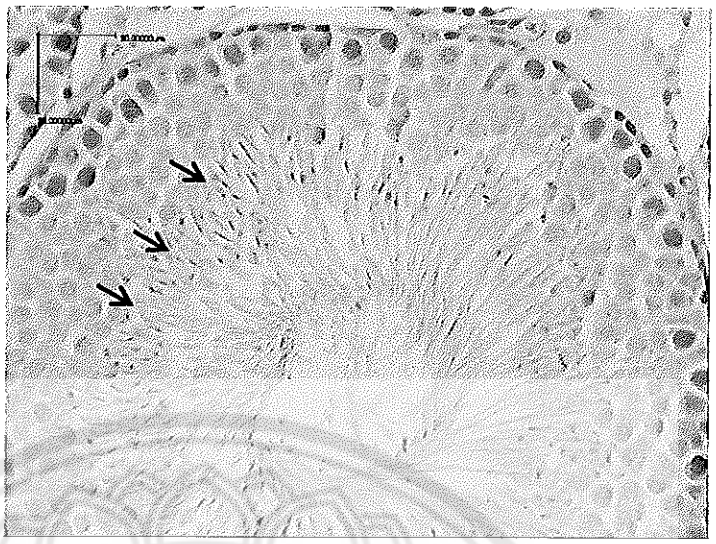

Figure 25 Stage VII of rat seminiferous tubule, there was no bundle arragement of immature spermatozoa. Several layers of immature spermatozoa were located on lumen side (arrow).

Figure 26 Stage VIII of rat seminiferous tubule, there was only one layer of immature spermatozoa (arrow) lined up the lumen side of seminiferous tubules.

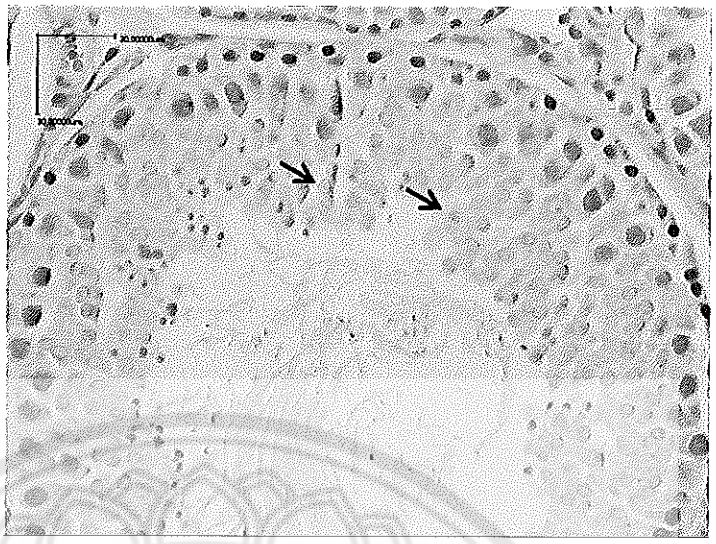

Figure 27 Stage IX of rat seminiferous tubule, immature spermatozoa were release from lumen. Round spermatid (arrow) was found in lumen.

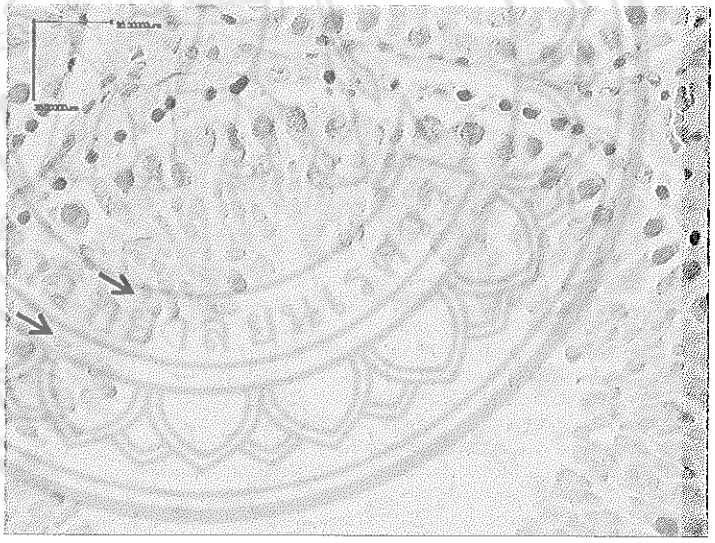

Figure 28 Stage X of rat seminiferous tubule, shape of nuclei of spermatid starting to change into ellipse shape. They were starting separated by spermatocyte.

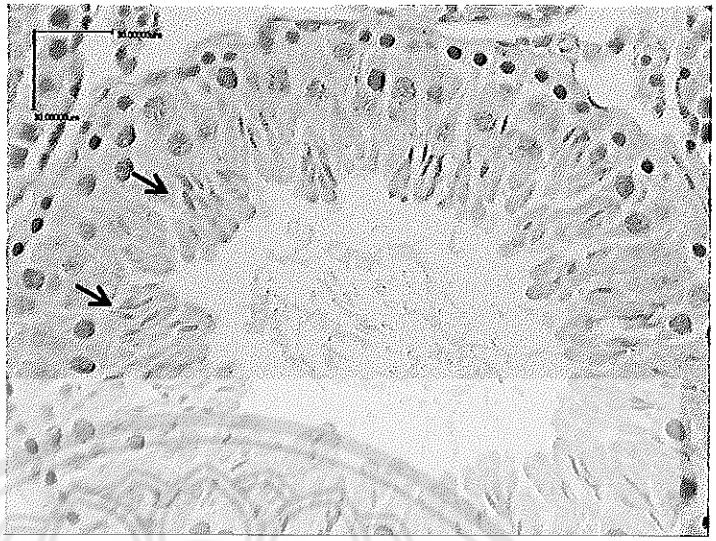

Figure 29 Stage XI of rat seminiferous tubule, the nuclei of spermatid became elongated (arrow) and formed bundle between spermatocyte.

Figure 30 Stage XII of rat seminiferous tubule, nuclei of spermatid lost their cuveture (arrow) and bundle are clearly appearance.

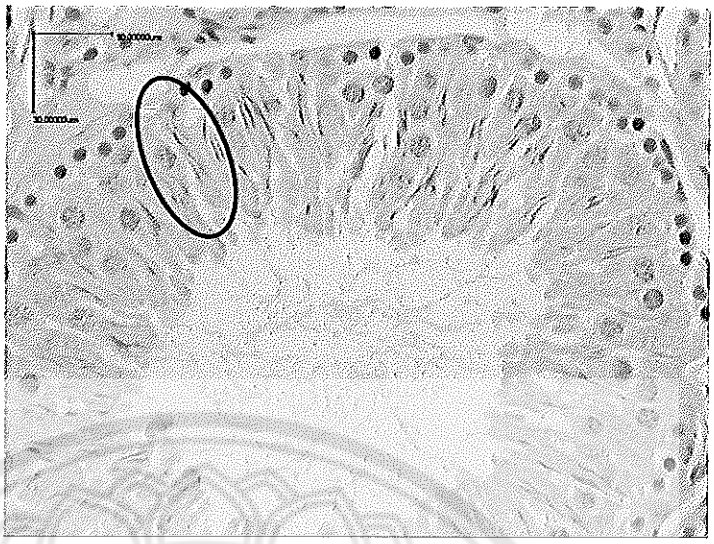

Figure 31 Stage XIII of rat seminiferous tubule, curved and thin nuclei of spermatid (circle) was appeared and tip of bundle directed toward to Sertoli cell.

Figure 32 Stage XIV of rat seminiferous tubule, secondary spermatocytes (arrow) were presented and spermatid was not group in bundle.

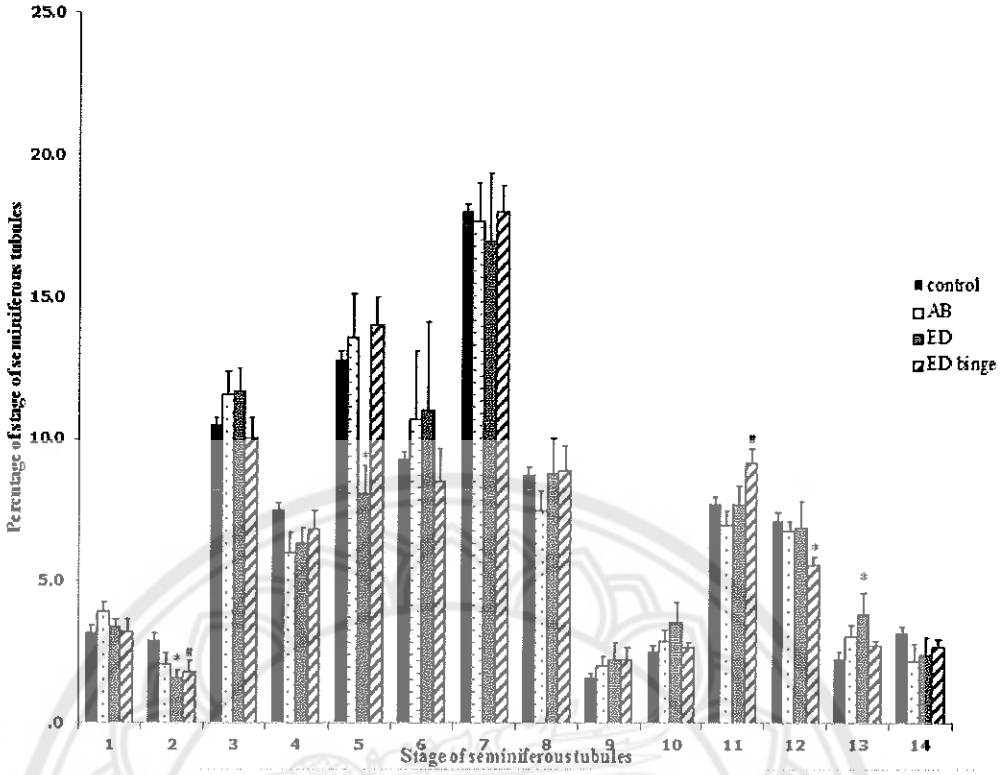

Figure 33 Percentage of stage of seminiferous tubules in control, AB, ED and ED binge group. Data are presented as mean± SEM. * P<0.05 vs control, one-way analysis (ANOVA LSD post-hoc test)

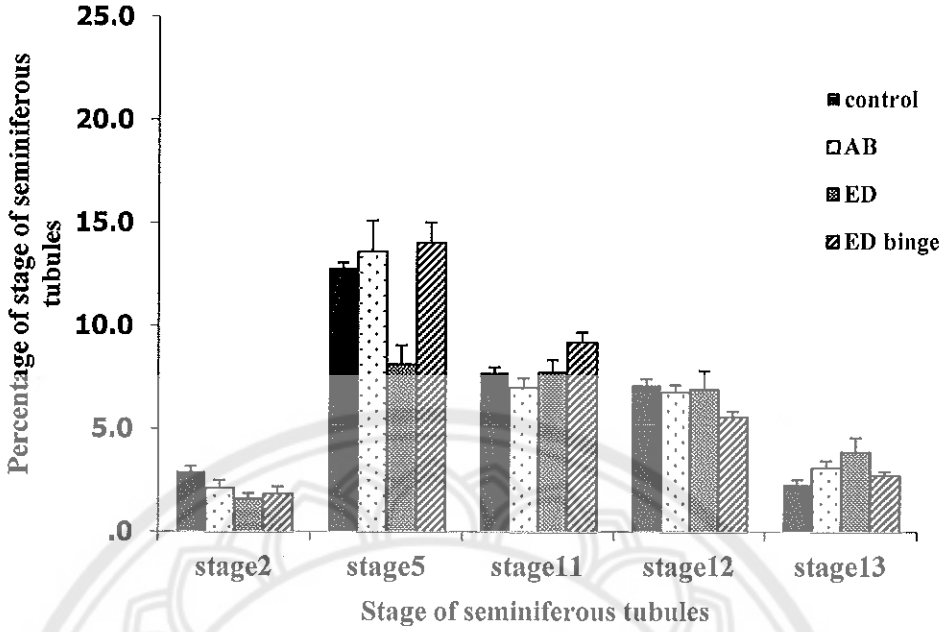

Figure 34 Changes of stages of percentage of stage of seminiferous tubules after METH exposure in control, AB, ED and ED binge group. Data are presented as mean± SEM. * P<0.05 vs control, one-way analysis (ANOVA LSD post-hoc test)

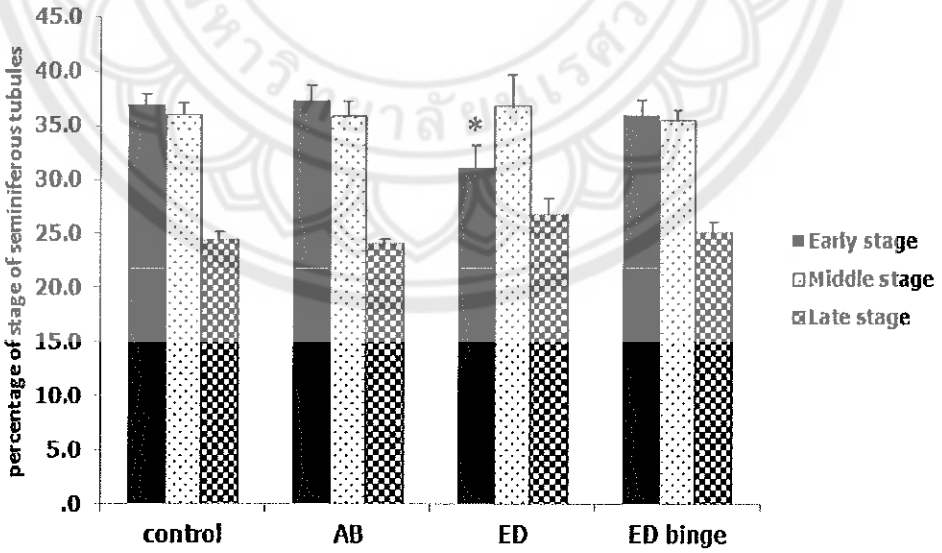

Figure 35 The number of seminiferous epithelium in rat testes treated with METH. Data are presented as the mean ± SEM. * P<0.05 vs control, one-way analysis (ANOVA LSD post-hoc test)

METH on the expression of D2 receptor

The immunohistochemical staining was used to determine the localization of D2 receptor in rat seminiferous tubules. The result showed that the D2 receptor was localized in all spermatogenic cells, especially spermatocyte as well as in Sertoli cells (figure 35). Moreover the expression of D2 receptor in each group was shown in Figure 36 C-F. The expression of D2 receptor was also found in rat brain (Figure 36 B).

The quantitative study of expression of D2 receptor (figure 37) by immunohistochemistry demonstrated that the percentage of expression of D2 receptor in Sertoli cells was decreased in all METH treated groups and reached significance in ED-binge group ($P<0.05$) when compared with control group. In addition, significantly decreased of D2 receptor expression in spermatocyte was observed in all METH treated groups ($P<0.05$). Moreover, the expression of D2 receptor in round spermatid was significantly increased in ED and ED-binge groups ($P<0.05$). But, decreased of D2 receptor expression in elongated spermatid was found in ED and ED-binge groups when compared with control group ($P<0.05$). Additionally, there were no significance differences in percentage of D2 receptor expression in spermatogonia. Percentage of total positive cells in seminiferous tubules was changed but did not reach significance.

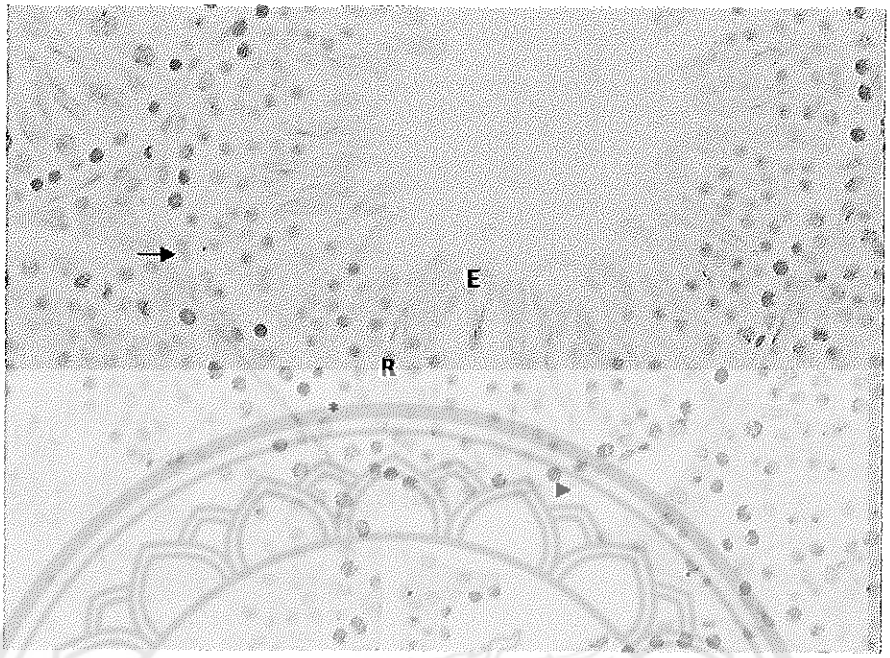

Figure 36 Immunolocalization of dopamine D2 receptor was found in seminiferous tubule of rat testis. That is expressed in Sertoli cells (*), spermatogonia (arrow), spermatocyte (arrow head) round spermatid (R) and elongated spermatid (E)

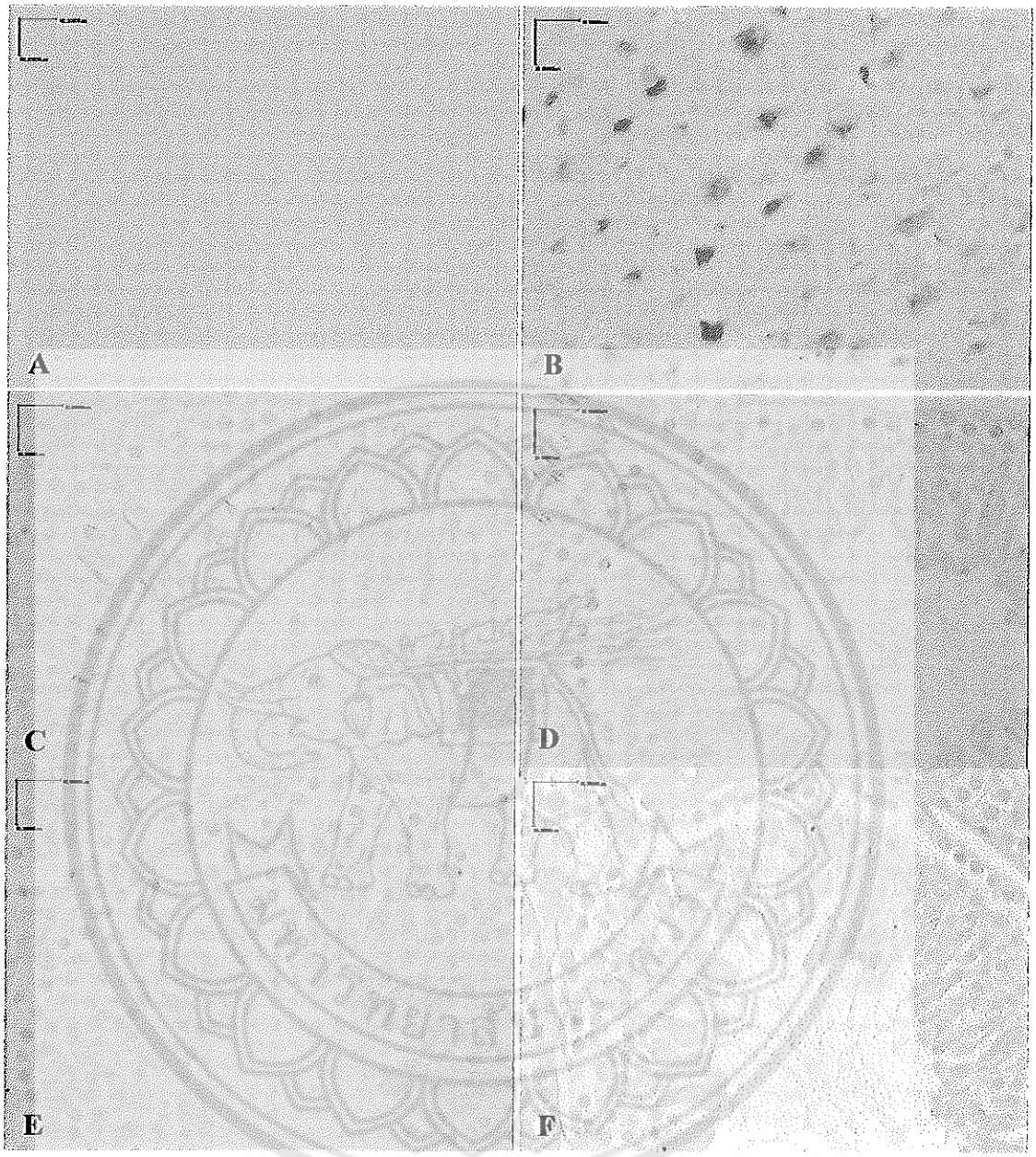

Figure 37 Immunolocalization of dopamine D2 receptor was found in seminiferous tubule of rat testis after METH administration in control (C), acute binge (D), escalating (E), and escalating binge groups (F). D2 receptor was found in rat brain (B). The negative control was shown in (A). Magnification 40X

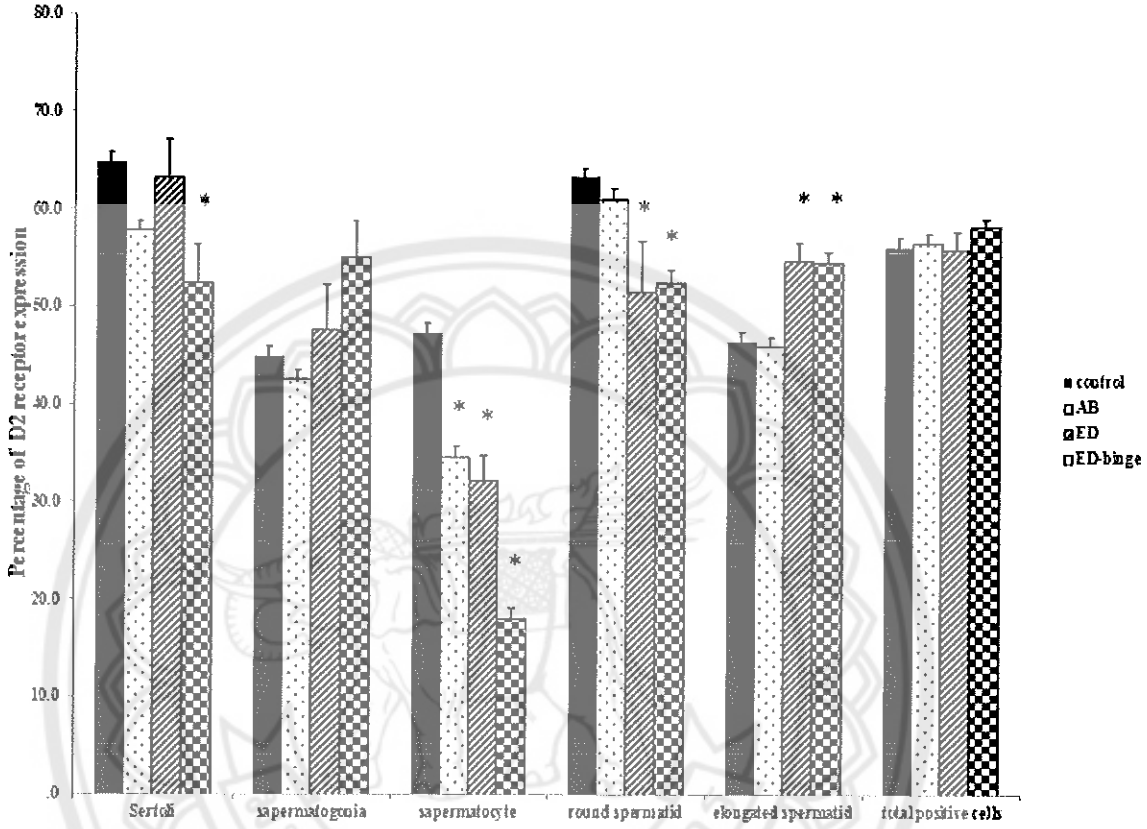

Figure 38 Percentage of D2 expression in Sertoli, each germ cell type and total positive cells in rat seminiferous tubules treated with METH. Each bar represents the mean ± SEM. * P<0.05 vs control, ANOVA followed by LSD post hoc test

Effects of METH on α1-adrenergic receptor expression

The qualitative of α1-adrenergic receptor expression was studied by immunohistochemistry technique. The result showed that the receptor was localized predominantly in spermatocyte and elongated spermatid. It also expressed in the other germ cells and Sertoli cells (figure 38). The expression of α1-adrenergic receptor in each group was shown in figure 39 C-F. In addition, the expression of α1-adrenergic receptor was also found in rat brain section (figure 39 B).

The quantitative of α1-adrenergic receptor expression was also observed and illustrated as percentage of positive cells (Figure 40). Percentage of α1-adrenergic receptor expression in Sertoli cells was increased in all METH treated groups and reached significance in ED-binge group ($P<0.05$) when compared with control group. But, significantly decreased of α1- adrenergic receptor expression in spermatogonia was observed in ED-binge group ($P<0.05$). Furthermore, the percentage of α1-adrenergic receptor expression in spermatocyte was significantly increased in all METH treated groups ($P<0.05$) when compared with control group. Moreover, the α1-adrenergic receptor expression in round spermatid was significantly increased in ED and ED-binge groups ($P<0.05$). A decrease of α1- adrenergic receptor expression in elongated spermatid was also found in ED and ED-binge groups when compared with control group ($P<0.05$). Additionally, there was a significant difference in percentage of α1- adrenergic receptor expression in total positive cells ($P<0.05$) in seminiferous tubules.

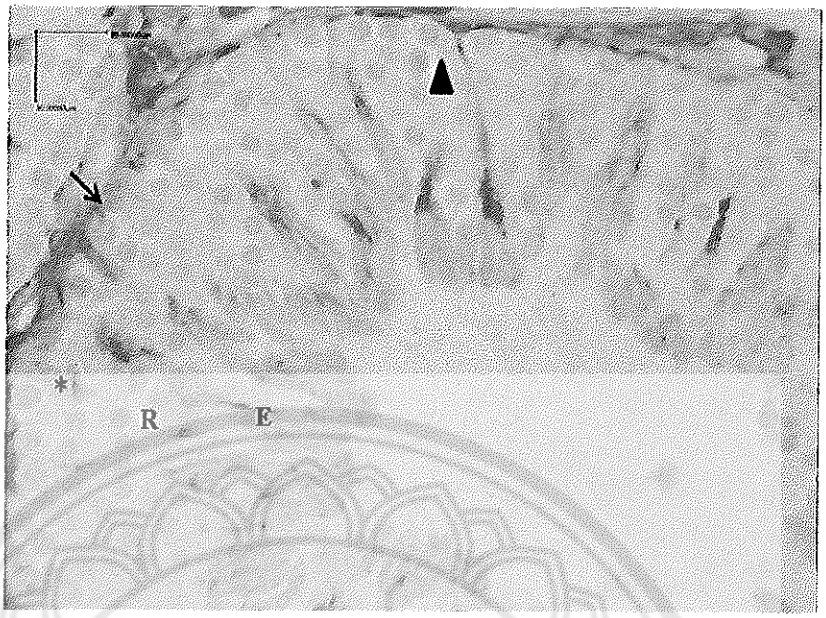

Figure 39 The expression of α1-adrenergic receptor in seminiferous tubules showed α1-adrenergic receptor expressed in sertoli cells (star), spermatogonia (arrow), spermatocyte (arrowhead), round spermatid (R) and elongated spermatid (E). Magnification (20X)

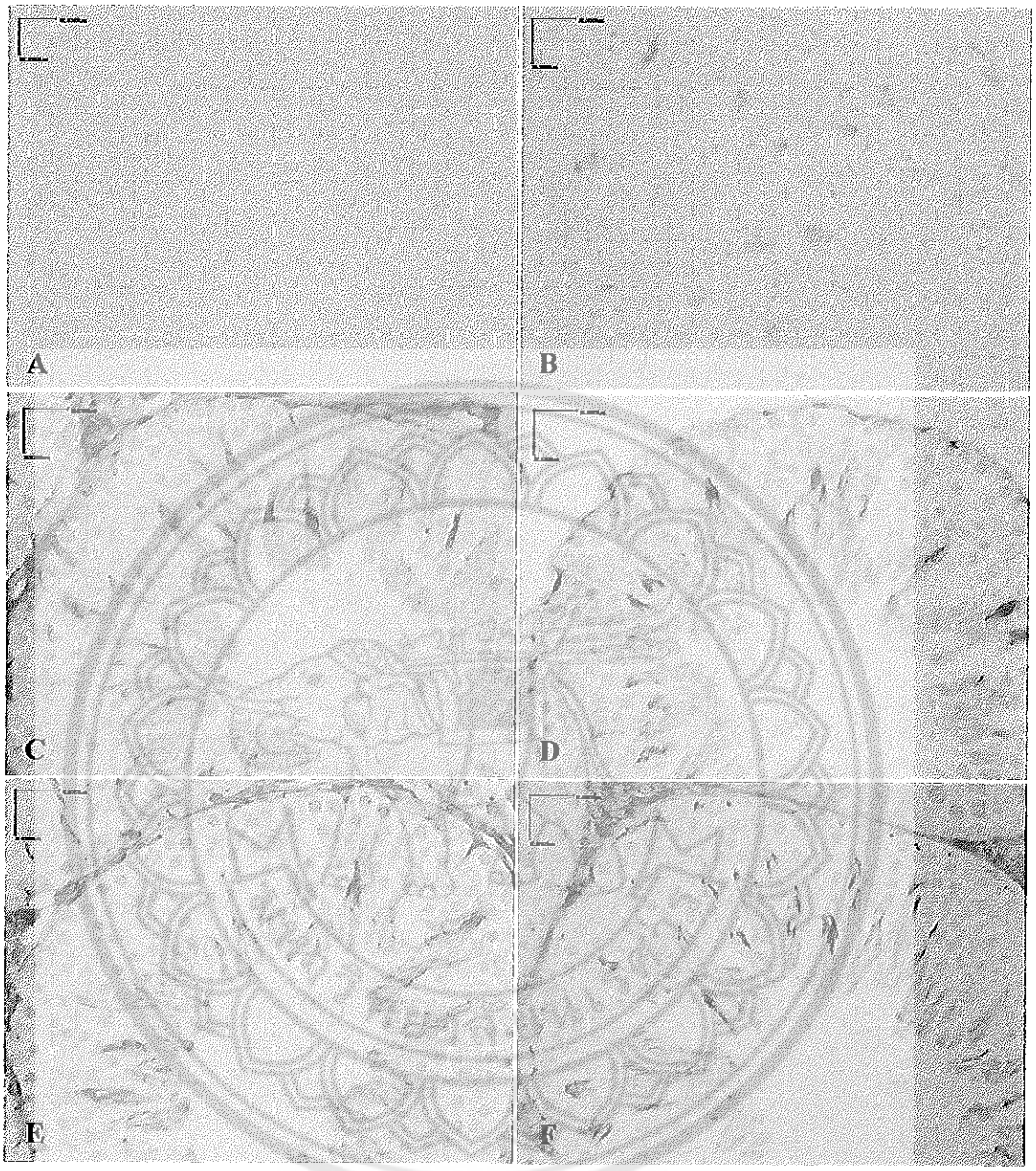

Figure 40 (A) negative control (B) The expression of α1 adrenergic receptor in brain (C) The expression of α1 adrenergic receptor in seminiferous in control group, (D) acute binge, (E) escalating and (F) escalating binge groups. Magnification (40X)

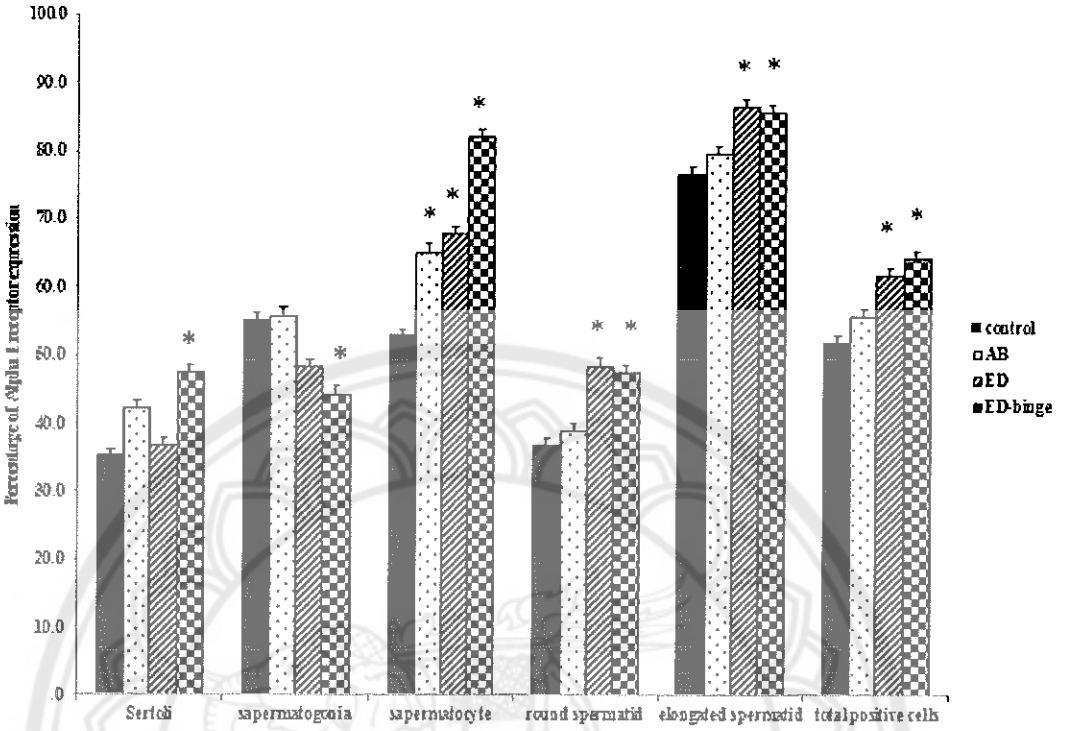

Figure 41 Percentage of α1 receptor expression in Sertoli, each germ cell type and total positive cells in rat seminiferous tubules treated with METH. Each bar represents the mean ± SEM. * **P<0.05** vs control, ANOVA followed by LSD post hoc test

DISCUSSION

Effects of METH on DOPAC, NE and DHPG concentration

In this study, we found that DOPAC, the main metabolite of dopamine, was elevated in the rat testis after acute METH exposure. This is in consistent with the effect of METH in enhancing the release of catecholamines such as dopamine (Fleckenstein et al., 2007) and in reducing dopamine reuptake and enzyme degradation (Suzuki et al., 1980). However, the concentration of DOPAC that we found in this study is in a very small proportion (approximately 1000-fold lower) of the DHPG concentration. This suggests that there is little, if any, specific dopaminergic innervation and any DOPAC are derived from dopamine as a precursor to NE in sympathetic terminals. Goldstein (2011) indicated that only 2-4 % of catecholamine was released by sympathetic stimulation (Goldstein et al., 2011). In contrast, a reduction of NE and DHPG was found in the group of animals undergoing repeated METH administration followed by a binge dose, while DHPG alone showed a significant reduction in the ED group. A previous study showed that long term administration of METH to rhesus monkeys resulted in a decrease of NE in several brain regions (Seiden et al., 1976). This may reflect an overstimulation lead to adrenergic neuronal apoptosis and nerve terminal degeneration (Schep et al., 2010). METH has been reported to stimulate a release of NE from sympathetic nerve terminals which results in increased activation of peripheral α and β-adrenergic postsynaptic receptors (Schep et al., 2010), although there is no evidence for enhancing the release in our study. Certainly, adrenergic receptors have been found in the testis. α- and β-Adrenergic receptors have been found in Leydig cells (Huo et al., 2012). These receptors are involved in reproductive function; α1B adrenergic receptor-knockout male mice resulted in abnormal Sertoli cell morphology and arrested development of spermatozoa (Mhaouty-Kodja et al., 2007). Furthermore, there are studies has been reported that monoamines influence male reproductive function; NE can influence the development of Leydig cells by promoting the expression of 3β-hydroxysteroid-dehydrogenase, a differentiation marker of precursor cells, and of proliferating cell nuclear antigen, a marker of proliferation (Huo et al., 2012) and can activate sperm capacitation (Way, & Killian, 2002). As mentioned above, METH has toxicity on sympathetic nervous system which can affect NE and

DHPG concentrations in the testis. NE mediates its effects via its receptors in testis. This can cause harmful effects on the reproductive system. METH induced changes of catecholamine in testis may cause adrenergic receptors change. Thus, alteration of adrenergic receptors in testis is important for determining pharmacological effects of catecholamine which may directly bind to its receptors after induced with METH.

Effects of METH on changes of seminiferous tubules stages

Rat seminiferous epithelium has been identified into fourteen stages as previously described by Leblond and Clermont, 1952. In this study, the changes of stages of seminiferous epithelium were defined and it was indicated that percentage of stage II in escalating and escalating binge dose groups were increased. But, a decrease in stage V was observed only in the group of METH mimicking dose in human abuser (escalating binge dose group). Early stage of seminiferous epithelium consisting of stage I-V that has been found the proliferation of spermatogonia by mitotic division and spermatogonia differentiation from type A to type B (Leblond, & Clermont, 1952). Therefore, the finding of changes of distribution of seminiferous epithelium in stage II and V after METH exposure can indicate the effect of METH on spermatogenesis arrest.

Moreover, an increase of percentage of stages of seminiferous epithelium in late stages consisting of stage XI and XIII was found in escalating and escalating binge dose groups, respectively. On the other hand, a reduction of percentage of seminiferous epithelium in stage XII was shown in escalating dose group. Immature spermatids were found within seminiferous epithelium at spermatogenic stages IX-XIV (Lee, Frame, Sykes, & Valentine, 1993). In this study, METH has the effect on late stage changes; consisting of stage XI, XII and XIII of seminiferous epithelium, which can induce abnormal sperm quality such as sperm motility, sperm morphology and sperm concentrations(Nudmamud-Thanoi et al., 2016a). In addition, the results from this study suggested effects of METH on catecholamine (NE and DA) concentration in testis which may involve in testosterone release (Mayerhofer et al., 1992; Stojkov-Mimic et al., 2015). In addition, the spermatogenesis is regulated by endocrine and testicular autocrine/paracrine factors through Leydig cells and Sertoli cells (Huleihel, & Lunenfeld, 2004). Besides, Leydig cells function can modulate the

progression of spermatogenesis which reflects in the stages of seminiferous tubules (Paniagua et al., 1988). Additionally, there is a report on the effect of METH that caused the alteration of progesterone and estrogen receptors expression on Sertoli and spermatogenic cells (Nudmamud-Thanoi et al., 2016b). As mentioned above, toxic effects of METH on testis should be participated in spermatogenesis abnormality which can reflect on the changes of stages of seminiferous epithelium.

Effects of METH on dopamine D2 receptor expression

In this study, the expression of dopamine D2 receptor was investigated and the result showed the expression of D2 receptor in rat seminiferous tubules that is expressed in spermatogenic cells and non-spermatogenic cell, Sertoi cells. It is in agreement with prior study that found the expression of D2 receptor in rat testis and rat spermatogenic cells (Otth et al., 2007) by using immunohistochemistry analysis. In the present study, the expression of dopamine D2 receptor was also found in rat brain section. It is consistent with previous study that using RT-PCR analysis of rat testis also revealed bands that have same isoform in the brain (Otth et al., 2007). In addition, it was confirmed the presence of these protein by using western blot analysis (Otth et al., 2007). The expression of D2 receptor outside the CNS illuminates the interaction between nervous system and reproductive system. It may play an important role on proliferation and/or differentiation of the male germ cells because this present study we have shown the expression of dopamine D2 receptor in all germ cells in pre-meiotic phase and post-meiotic phase of the seminiferous tubule.

After METH exposure, the expression of dopamine D2 receptor in rat seminiferous tubules was changed. The reductions of D2 receptor expression were found in Sertoli cells. Sertoli cells support the development of germ cells to spermatozoa that is controlled by FSH and testosterone (Griswold, 1998). Dopamine agonist affected Leydig cell that caused decrease in cAMP and testosterone production (Dirami, & Cooke, 1998). Consequently, D2 receptor was changed after METH administration may participate in spermatogenesis defect.

In the present study, decrease of dopamine D2 receptor was observed in spermatocyte and round spermatid in chronic METH treated groups. On the other hand, the D2 receptor expression was significantly increased in elongated spermatid.

This appears to be the first study documented the effect of METH on dopamine D2 receptor in rat testis. Previously, there was a study has been reported that dopaminergic neurotoxicity induced by METH is prevented in dopamine D2 receptor knockout mice (Granado et al., 2011). Moreover, METH presence was significantly reduced in dopamine D2 receptor knockout mice. It indicated that dopamine D2 receptor plays an important role in METH uptake in to the cell. Additionally, in this study demonstrated that METH has the effect on dopamine concentration in testis which may be derived from sympathetic innervation (Gnessi et al., 1997) leading to the changes of receptor expression. A study has been reported decreasing levels of dopamine D2 receptor in METH abusers which has been revealed receptor down regulation due to pharmacological effects of METH induced increasing of extracellular dopamine concentration (Wilson, & Kish, 1996). Previously, positron emission tomography (PET) study has been documented that METH can cause decreased level of dopamine D2 receptor availability in brain of METH abuser (Yamamoto et al., 1999). But in this study, we have found either a decrease or an increase of dopamine D2 receptor expression in rat spermatogenic cells due to the different manner of METH administrations. Since, spermatogenesis is a complex process of spermatozoa production and it is not well understood. It may need different levels of dopamine in different steps of development of germ cells. Therefore, up and down regulations may occur to retain homeostasis.

Accordingly, dopamine and D2 receptor may play an important role in spermatogenesis. METH induced changes of dopamine D2 receptor in germ cell and Sertoli cell may consequently lead to abnormal spermatogenesis.

Effects of METH on α1 adrenergic receptor expression

In the present study, the effect of METH on α1 adrenergic receptor expression was examined. The findings showed that the expression of α1 adrenergic receptor in Sertoli cells was increased about 15% in escalating binge dose group. Sertoli cells are important for spermatogenesis and testis formation. Spermatogenesis is regulated by two hormones which are testosterone and FSH. FSH act via Sertoli cells to encourage spermatogenesis by increasing number of Sertoli cells (Griswold, 1998). Increase of α1 adrenergic receptor in Sertoli cells after METH exposure may have an effect on action of Sertoli cells and spermatogenesis. However, nearly 10% reduction of the receptor expression was found in spermatogonia in long term METH treated with binge dose. Moreover, the high percentage of α1 adrenergic receptor expression was observed in spermatocyte in all METH treated groups. Spermatogenesis is a process of sperm production that is started from spermatogonia stem cells. Spermatogonia stem cell can undergo self-renewal division to increase number of itself, and differentiation (Pasha, Rezk, Selim, & Abd El Motteleb, 2016). Thus, the change of α1 adrenergic receptor expression in spermatogonia and spermatocyte may influence to proliferation and differentiation of spermatogonia and spermatocyte. In addition, the expression of α1 adrenergic receptor in spermatid was increased in both escalating groups; high expression was found in elongated spermatid with almost 90%. The percentage of total cells expression was significantly increased in escalating and escalating binge dose (more than 60%). Round spermatid develops to spermatozoa in maturation process is called spermiogenesis which acrosome, head and tail of spermatozoa are formed during this stage. Moreover, it has been reported that NE and adrenergic receptor has been involved in sperm capacitation and acrosome reaction (Cornett, & Meizel, 1978; Way, & Killian, 2002, 2006) and sperm motility (Sliwa, 1994). Therefore, alteration of α1 adrenergic receptor expression after METH administration may result in spermatogenesis abnormality which may affect on sperm morphology and sperm concentration (Nudmamud-Thanoi & Thanoi, 2011)

Previously, there was a study has been reported that NE and alpha-adrenoreceptor agonist, phenylephrine stimulated testosterone secretion in testis. On the other hand, the effect of NE can be blocked by prazosin, alpha-receptor antagonist (Mayerhofer et al., 1992). Moreover, in vitro study demonstrated the role of α1

adrenergic receptor to decreasing Nur77, steroidogenic stimulator transcription and also can cause increase of steroidogenic repressor in Leydig cells. Therefore, α1 adrenergic receptor plays role in the steroidogenic mechanism of Leydig cells (Stojkov-Mimic et al., 2015). Additionally, many studies revealed that the stress involved reproductive dysfunction via α1 adrenergic receptor function (Andric et al., 2013; Stojkov et al., 2014; Stojkov et al., 2013). In this present study, the expression of α1 adrenergic receptor in rat brain was also investigated. This is indicated the reaction between brain and the reproductive system which may act via α1 adrenergic receptor. Besides, Mhaouty-Kodja et al., 2007 indicated that knockout of α1b-adrenergic receptor in male mice caused infertility and spermatogenesis impairment (Mhaouty-Kodja et al., 2007). They has been reported that testosterone concentration was predominantly decreased but LH level was primarily increased in α1b-adrenergic receptor knockout mice.

In the present study, the results indicated that METH can induce NE and α1 adrenergic receptor expression in rat testis due to toxic effect of METH. These results are in agreement with the study of Mhaouty-Kodja S et al., 2007 that found the reduction of testicular weight, spermatogenesis defect and lower level of testosterone in α1b-adrenergic receptor knockout mice. These results were similar with the effect of METH that caused reduction of testosterone levels (Yamamoto et al., 1999). Taken together, these results demonstrated that METH affects male reproductive system via the activation of neurotransmitter changes including NE. It may affect either directly on germ cells such as maturation of spermatocyte or indirectly on Sertoli and Leydig cells communication (Mhaouty-Kodja et al., 2007).

CHAPTER V

CONCLUSION

In summary, the present study revealed the effect of METH that can cause DOPAC, NE and DHPG concentration change in testis. The alteration of these three neurotransmitters may occur because METH has toxic effect on sympathetic nervous system. Moreover, it results in dopamine D2 receptor and α1 adrenergic receptor expression change in Sertoli and spermatogenic cells after METH administration. In addition, changes of frequency of stage of seminiferous tubules were found after METH exposure. The result suggested that catecholamine, DA and NE may play an important role in spermatogenesis process. In addition, METH can disturb action of catecholamine in testis by changing secretion levels of neurotransmitter and expression of their receptors. So, it may lead to spermatogenesis impairment and sperm dysfunction. This study showed toxicity of METH on male reproductive system. Therefore, further study is needed to study because spermatogenesis is a complex process that is not well understands. The effect of METH on spermatogenic cell division and spermatogenic cell proliferation should be studied further.

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APPENDIX

เอกสารรับรองโครงการ

คณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

ชื่อโครงการ	ผลของเมทแอมเฟตามีนต่อโดปามีน, ตัวรับโดปามีน, นอร์อีพิเนฟรินและตัวรับอดรีเนอร์จิกในอัณฑะของหนูแรท Effects of methamphetamine on dopamine, dopamine receptors, norepinephrine and adrenergic receptors in rat testis
เลขที่โครงการ	NU-AE560619
เลขที่เอกสารรับรอง	56 04 0058
ประเภทการรับรอง	ยกเว้น(1)
ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ	รศ.ดร.สุพิสา ถาน้อย
สังกัดหน่วยงาน /คณะ	วิทยาศาสตร์การแพทย์
วันที่รับรอง	20 กันยายน 2556
วันสิ้นสุดการรับรอง	20 กันยายน 2559

ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

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(รองศาสตราจารย์ ดร.รัตติมา จีนาพงษา)
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รองอธิการบดีฝ่ายวิจัย
มหาวิทยาลัยนเรศวร

ประกาศบัณฑิตวิทยาลัย มหาวิทยาลัยนเรศวร
เรื่อง อนุมัติให้นิสิตระดับปริญญาโทดำเนินการทำวิจัย
ครั้งที่ ๐๐๗/๒๕๕๗

บัณฑิตวิทยาลัยอนุมัติให้ นางสาวศิริพร จันหร์เพชร รหัสประจำตัว ๕๕๐๖๑๕๐๒ นิสิตระดับปริญญาโท หลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชากายวิภาคศาสตร์ ดำเนินการทำวิจัยตามโครงร่างวิทยานิพนธ์ที่เสนอ

เรื่อง ภาษาไทย "ผลของเมทแอมเฟตามีนต่อโดปามีน, ตัวรับโดปามีน, นอร์อีพิเนฟริน และตัวรับอดรีเนอร์จิกในอัณฑะของหนูแรท"

ภาษาอังกฤษ "EFFECTS OF METHAMPHETAMINE ON DOPAMINE, DOPAMINE RECEPTORS, NOREPINEPHRINE AND ADRENERGIC RECEPTORS IN RAT TESTIS"

โดยมี รองศาสตราจารย์ ดร.เสมอ ถาน้อย เป็นประธานที่ปรึกษาวิทยานิพนธ์

จึงประกาศมาให้ทราบโดยทั่วกัน

ประกาศ ณ วันที่ ๖ มกราคม พ.ศ.๒๕๕๗

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รองคณบดีฝ่ายบริหารและวางแผน รักษาราชการแทน
คณบดีบัณฑิตวิทยาลัย มหาวิทยาลัยนเรศวร