SHOTGUN PROTEOMICS ANALYSIS OF PROTEIN RESPONDING TO DRUG ADDITION IN RAT TESTIS.

JITNAPAR THONGLEART

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Anatomy
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By JITNAPAR THONGLEART

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for the Master of Science in Anatomy of Naresuan University

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ABSTRACT

Drug addiction is one of the major problems in the world that affects human health in various systems, including potentially affecting the male reproductive system. The most common of drug abuses are methamphetamine (METH) and dextromethorphan (DXM). Previously, METH can induce apoptotic cells within the seminiferous tubule and reduce testosterone that affect to spermatogenesis. Moreover, both chemicals reduced gonadotropin releasing hormone (GnRH) in the hypothalamus, affecting spermatogenesis, and causing poor sperm quality in the testis. Diazepam is a common drug used to treat the symptoms of drug addiction. However, diazepam causes adverse effects on germ cells in male reproductive system. Interestingly, pre-germinated brown rice (PGBR); a high GABA natural substance, can reduce the effects of drug addiction. As mentioned above, drug addiction and its treatments can affect on several cell signaling cascades that can induce changes in several proteins. These findings provide the hypothesis that drug addiction and treatments might be associate with changes of proteins in the testis. Therefore, the aim of this study is to investigate changes of protein profiles in rat testis in drug addiction and its treatments using proteomics analysis. Male Sprague-Dawley rats were divided in to 2 groups of addiction model. METH model, those rats in a control group were received normal saline for 15 days, whereas those rats in an ED-binge METH group were received an escalating dose of METH for 14 days following a binge dose of METH on day 15. DEX model, the control group, those rats were received normal saline for 15 days and treated with distilled water for

60 days. In the DXM, those rats were received 30 mg/kg DXM. Withdrawal group, those rats were received DXM and distilled water for drug-withdrawal. Diazepam group, those rats were received DXM and treated with 10 mg/kg diazepam. Synthetic GABA group, those rats were received DXM and treated with 0.8 mg/kg synthetic GABA. Pre-germinated brown rice group, those rats were received DXM and treated with 5 mg/kg PGBR. After the end of treatments, animals were sacrificed and the testis was collected. Proteins were extracted from the rat testis, which were pooled from three samples in each group. The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed to identify the protein profiles. Moreover, expression of calbindin in rat sperm after treated with METH-addiction was examined using immunohistochemistry. The protein expression profiles after drug addiction and treatments are enriched in calcium-binding protein pathways and ATP binding, which play critical roles in calcium homeostasis, cell functions in the testis and sperm function. The present study showed that alteration of the protein profile after drug addiction, which is associated with the calcium signaling pathway, causes disrupted intracellular Ca^{2+} homeostasis in the testis and might be related to spermatogenesis deficiency, resulting in the reduction of sperm quality. In addition, alteration of the protein profile in diazepam treatment indicated an adverse effect, while GABA and PGBR treatments showed a recovery effect on the testis via intracellular Ca^{2+} signal. These findings examined the effects of drug addiction and addiction treatments on spermatogenesis and sperm quality through Ca^{2+}homeostasis.

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ABBREVIATIONS

µg	=	microgram
µl	=	microliter
µm	=	micrometer
APB	=	androgen binding protein
ATP	=	adenosine triphosphate
BCA	=	bicinchoninic acid
°C	=	degree Celsius
Cm	=	centimeter
CNS	=	central nervous system
Da	=	Dalton
DAB	=	3,3’-Diaminobenzidine
DNA	=	deoxyribonucleic acid
DXM	=	dextromethorphan
ERβ	=	estrogen receptor beta
ERα	=	estrogen receptor alpha
FA	=	ferulic acid
FSH	=	follicular stimulating hormone
g	=	gram
g/kg	=	gram per kilogram
GABA	=	gamma aminobutyric acid
GnRH	=	gonadotropic releasing hormone
H_2O_2	=	hydrogen peroxide
i.p.	=	intraperitoneal injection
LS-MS/MS	=	Liquid chromatography-tandem mass spectrometry

LH	=	lutinizing hormone
M	=	molar
METH	=	methamphetamine
MeV	=	MultiExperiment Viewer
mg/kg	=	milligram per kilogram
ml	=	milliliter
mM	=	millimolar
NaCl	=	sodium chloride
ng	=	nanogram
NGS	=	normal goat serum
NMDAR	=	N-methyl-D-aspartate receptor
PANTHER	=	Protein Analysis Through Evolutionary Relationships
PBS	=	phosphate buffer saline
PGBR	=	pre-germinated brown rice
PR	=	progesterone receptor
ROD	=	reactive optical density
rpm	=	round per minute
SDS	=	sodium deoxycholate
SEM	=	standard error of mean
STITCH	=	Search Tool for Interacting Chemicals
Tris-HCl	=	tris-hydrochloride
UniProt KB	=	UniProt Knowledgebase
WHO	=	World Health Organizatio

CHAPTER I
INTRODUCTION

Rationale and Significance of the study

Drug addiction is one of the major problems in today's world. That is a chronic relapsing disorder that is characterized by compulsive drug seeking and taking despite severe adverse effects (Hyman, et al., 2006; Kalivas, et al., 2005; Koob and Kreek, 2007). It's considered to be both a mental illness and a complex brain disorder caused by some neurobiological abnormalities affecting specific brain regions known to be involved in processes such as reward seeking, habit formation, memory connections, and stress are at the root of these maladjusted behaviors (Benedetto, 2021). Drug addiction has also been linked to an increased risk of cancer, heart, liver, and lung illness, as well as infections and reproductive system dysfunction. (Ghanbari and Sumner, 2017). Within the past few years, it has been reported in illicit drug use and drugs of abuse especially methamphetamine and dextromethorphan.

Methamphetamine (METH) is an addictive drug that is often abused which potent effects on the central nervous system (CNS). Chronic or heavy METH use may induce symptoms resembling paranoid schizophrenia, including delusions, ideas of reference, and auditory hallucinations (McKetin, et al., 2006; Ziedonis, et al., 2003 and Zweben et al., 2004). Previous studies also suggest that methamphetamine induces apoptosis in seminiferous tubules in male mice testis (Yamamoto, et al., 2002; Alavi, et al., 2008; Nudmamud-Thanoi, et al., 2011), decreased testosterone levels and a reduced sperm count, sperm quality, sperm motility and sperm morphology (Lin, et al., 2014; Nudmamud-Thanoi, et al, 2016).

Dextromethorphan (DXM) is an antitussive dextrorotatory morphinan. The side effects of dextromethorphan are included vomiting, diarrhea, respiratory depression, hallucinations and hypertension. Previous studies have indicated that dextromethorphan also has neuroprotective effects in diverse neurodegenerative diseases (Shin, et al., 2010, 2011; Werling, et al., 2007; Zhang, et al., 2007). In addition, dextromethorphan could induce significant reductions in gonadotropin releasing-hormone immunoreactivity in the hypothalamus and affect to increased sperm abnormalities, decreased sperm viability and sexual behavior (Nam, et al., 2012).

As previously stated, both chemicals cause drug addiction and affect various abnormal processes in the male reproductive system including spermatogenesis, which involves complete sperm quality: sperm count, sperm morphology, and sperm motility (Oghbaei, et al., 2020). Normally, the process of spermatogenesis is regulated by hormones through the hypothalamic-pituitary-testicular axis (Tilbrook, et al., 2001; Cheng, et al., 2010; Schlatt, et al., 2014). Another layer of regulation, spermatogenesis is also regulated by signal transduction through proteins. In many biological systems, protein is known to play a role in the regulation of cell proliferation, cell differentiation, and cell death (Cooper, 2000). Many proteins play critical roles in spermatogenesis, such as testis-specific Y-encoded protein 1 (Tspy1), the members of the cullin (Cul) family, midkine (Mkd) and serine/threonine-protein kinase PLK1 (Plk1), which have a role in cell cycle regulation, male germ cell proliferation and sperm differentiation, which are related to spermatogenesis (Zhang, et al., 1992; Schnieders, et al., 1996; Kueng,

et al.,1997; Arama, et al., 2007). Also, several proteins are associated with various functions of the male reproductive system that reflect male fertility, including a-kinase anchoring proteins (Akap), protein kinase-A like (Pka) and dual specificity mitogen-activated protein kinase (Map2k1), which are associated with energy in mitochondria and activating signal transduction producing cAMP, which plays a role in the initiation, maintenance, and control of sperm motility (Muratori, et al., 2009; Ashrafzadeh, et al., 2013). Likewise, the calcium binding protein family regulates many biological functions, including activating the signal transduction required for sperm motility and capacitation/acrosome reaction, such as calretinin and calmodulin (Kawashima, et al., 2009; Dressen, et al., 2013; Hossam, et al., 2019; Leclerc, et al., 2020).

Currently, there are drugs used to treat drug addiction. One of the drugs used is diazepam, which is a common drug used to treat the symptom of drug addiction. However, diazepam causes adverse effects on various body systems, including male reproductive systems. A previous study has reported that diazepam induced germ cell aneuploidy, which may affect germ cell morphology (Compton a Volkow, 2006). Therefore, the present study has researched natural substances that can reduce drug addiction and does not affect other systems of the body. Currently, the study of our research group has reported that pre-germinated brown rice can reduce drug withdrawal behavior in a model of drug addiction (Weerasakul, et al., 2011). In addition, the previous studies showed that GABA is involved in maturation of germ cell and spermatogenesis (Kanbara, et al., 2005), increasing sperm concentration

and sperm motility (Galatioto, et al., 2008; Calogero, et al., 1996; Thanoi, et al., 2018).

As mentioned above, drug addiction and treatment can affect on several cell signal cascades that can induce changes of several proteins. Currently, proteomics is a useful technique for identifying and quantifying proteomes in cells, tissues, and organisms. Furthermore, proteomics has been used to evaluate the differential protein expression and functional output of protein in biological systems on male reproductive system such as oxidative stress response, reproduction, and cellular process (Mallick, et al., 2010; Aslam, et al., 2017). Previous studies, proteomic was used to employed to evaluate the toxicity from other substances on male reproductive system E.g., the study changes sperm protein patterns in mice exposed to sodium fluoride (Sun, et al., 2018), the study proteomic view of Atrazine-induced effects on male infertility (Chen, et al., 2015) and proteomic analysis of mouse testis reveals Perfluorooctanoic acid- induced reproductive dysfunction (Zhang, et al., 2014).

Therefore, in this research to investigate change of protein profile in rat testis associated with methamphetamine and dextromethorphan addictions including investigating the alterations of protein expression in rat testis associated with effects of GABA and PGBR treatment of drug addiction by shotgun proteomic technique.

Purposes of the Study

A general experiment objective

The core purpose of this study is to investigate change of protein profile in rat testis in drug addiction and addiction treatment.

Specific objectives

1. To examine the differential expression of protein in rat testis after exposure to methamphetamine.
2. To examine the differential expression of protein in rat testis after exposure to dextromethorphan.
3. To examine the differential expression of protein in rat testis after treatment with GABA standard.
4. To examine the differential expression of protein in rat testis after treatment with pre-germinated brown rice (PGBR).
5. To examine the differential expression of protein in rat testis after treatment with diazepam.

Hypotheses

1. Drug addictions could affect protein expressions in male rat testis.
2. GABA and PGBR treatment could affect protein expressions in male rat testis of drug addictions.

Scope of this study

This study investigated the effect of drug addiction and addiction treatment in rat testis on change of protein profiles by using proteomic techniques. Moreover, expression of calbindin in rat sperm following METH-addiction was examined using immunohistochemistry.

Keywords

Methamphetamine, Dextromethorphan, Drug addiction, Testis, Proteomic

Conceptual framework

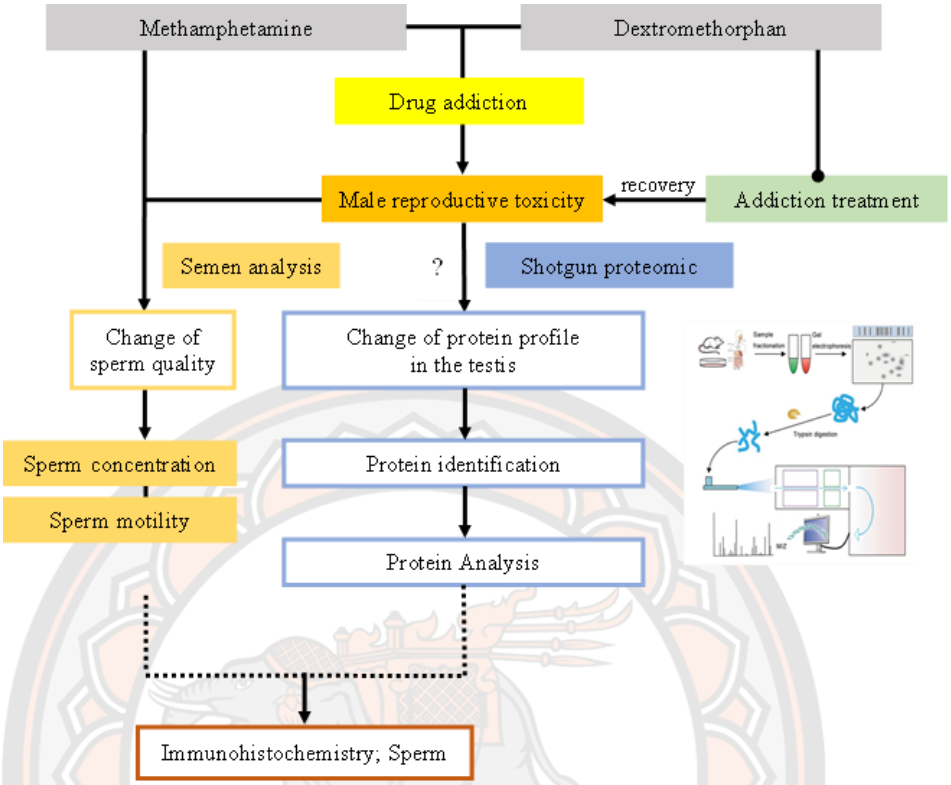


Figure 1 Conceptual framework of this study

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Male reproductive system

Testis

The testes are important organs in male reproductive system which have two main functions are production of sperm and production of the male hormones for reproductive functions. The testes are a pair of oval-shaped organs, which are contained within the scrotal sac. Each testis is enclosed by a fibrous capsule called the tunica albuginea and tunica vaginalis. Inside the testis has the compact of seminiferous tubule and vascularized interstitial tissue (Harold, 2014). Within the seminiferous tubules, contain spermatogonia and sertoli cells that envelop and support germ cells undergoing progressive differentiation and development into mature spermatozoa. In addition, inside the testes are also found leydig cells, which produce testosterone (Meng, et al., 2000). Testosterone is a male sex hormone that is responsible for the growth and maintenance of the germinal epithelium as well as the development of secondary sex characteristics (Feng, et al., 2012).

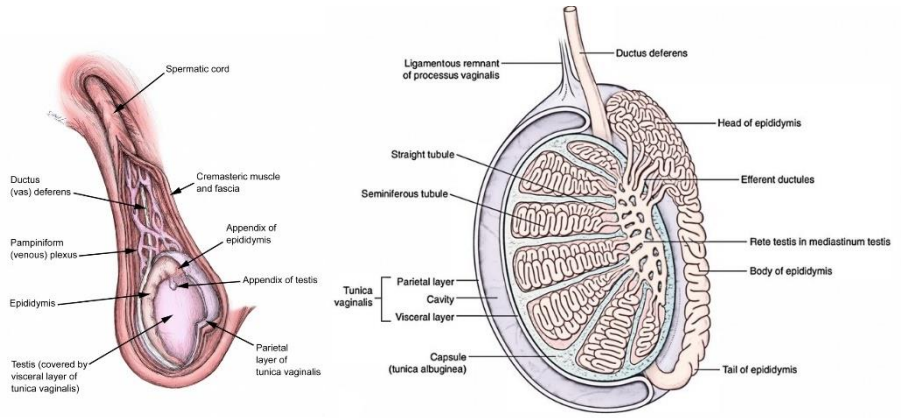


Figure 2 Structure of testis

Source: https://www.earthslab.com/anatomy/testis-and-epididymis

Seminiferous tubule

Seminiferous tubules are tubes that lie on the basal lamina. Seminiferous tubules are covered with connective tissue and flat cells resemble smooth muscle cells called myoid cells that can release spermatozoa. The seminiferous epithelium is a stratified epithelium that contains different stages of spermatogenic cells (Forsman, et al., 2012). Seminiferous epithelium can be divided into three compartments: the basal compartment, the adluminal compartment, and the luminal compartment. The basal compartment consists of spermatogonial cells and sertoli cells. The adluminal compartment consists of spermatocytes and spermatid. The luminal compartment consists of spermatid (Fawcett, et al., 1986). In addition, between each seminiferous tubules is called interstitial spaces, which have interstitial fluid and Leydig cells or interstitial cells (Panpan, et al., 2019).

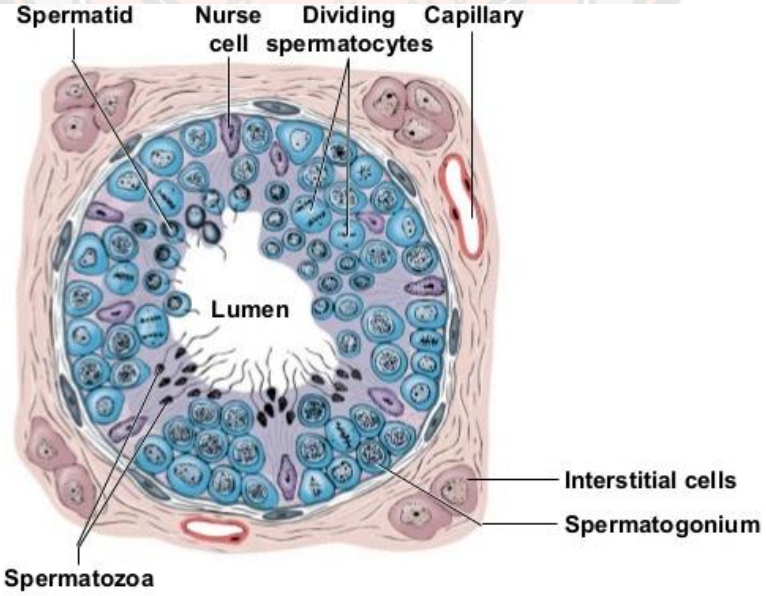


Figure 3 Seminiferous tubule

Source: https://www.slideshare.net/gwrandall/ch-28lecturepresentation-25361578

Spermatogenesis

Spermatogenesis is the proliferation and maturation of germ cells leading to the formation of spermatozoa occurring in the seminiferous tubules (Yoshinaga, et al., 1991). Spermatogenesis starts with the mitotic division of the spermatogonial cells into primary spermatocytes. Then, the primary spermatocyte divides meiosis I into secondary spermatocytes. After that, the secondary spermatocytes have moved to meiosis II, resulting in haploid round spermatids (Lehti, et al., 2016; Yongli, et al., 2020). The spermatids are transform structure into spermatozoa by the spermiogenesis.

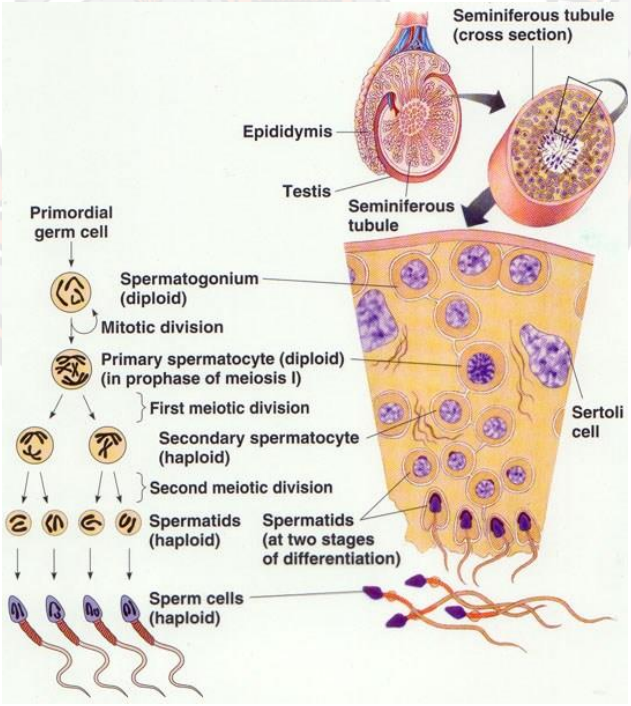


Figure 4 Spermatogenesis: spermatogonia divide by mitotic to primary spermatocytes, divide by meiosis I to secondary spermatocytes, divide by meiosis II to spermatids and transform into spermatozoa

Source: https://pornsawanthaoblog.wordpress.com

Spermiogenesis

Spermiogenesis is a stage that changes the structure of the round spermatid into mature spermatozoa (O'Donnell, 2014). The spermiogenesis has been divided into 4 phases including golgi phase, cap phase, acrosomal phase and maturation phase, respectively. In the golgi phase, the golgi apparatus created a proacrosomal granule, which developed into an acrosomal granule. The cap phase is characterized by the distribution of acrosomal granules to the front of the spermatozoa's head, which is referred to as the head cap. The acrosomal phase is characterized by changes in the structure of the spermatozoa's head and tail. Furthermore, the nuclear condensation and mitochondria move into the midpiece of the spermatozoa's tail. The maturation phase of spermiogenesis is the final stage, during which the structure of spermatozoa is changed to its most complete state and excess cytoplasm is removed via sertoli cell phagocytosis (Leblond, et al., 1952).

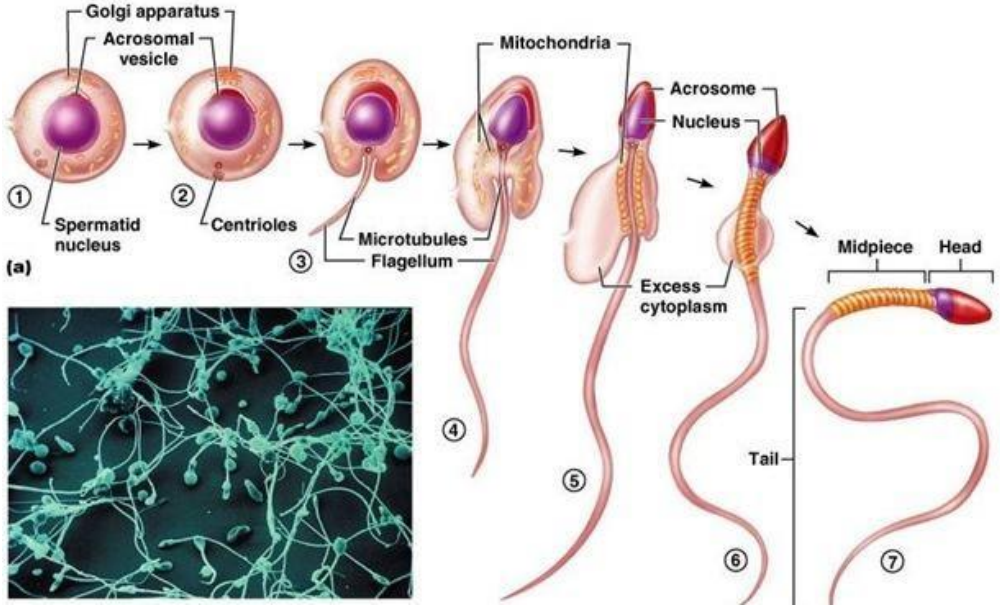


Figure 5 The phases of spermiogenesis
Source: https://www.majordifferences.com

Spermatogenic cells

Spermatogonia

Spermatogonia is largest germ cell type located in the basal portion of the seminiferous tubules in the testis. The spermatogonia cells have been divided into 2 types: type A spermatogonia and type B spermatogonia. Type A spermatogonia are divided into type B spermatogonia during mitosis. Type B spermatogonia were small and had a large amount of scattered chromatin (Dadras, et al., 2019).

Spermatocyte

Spermatocytes are developed from type B spermatogonia. The spermatocyte has been divided into 2 types: the primary spermatocyte is a diploid cell, and the secondary spermatocyte is a haploid cell. The secondary spermatocytes are divided into the spermatids (Van der Horst, 2019).

Spermatid

Spermatids are developed from the secondary spermatocytes that are haploid male gametes. The spermatid has been divided into 2 types: round spermatid and elongated spermatid. During the haploid round spermatids differentiate into elongated spermatozoa stage, several critical cell remodeling events occur including DNA condensation in the spermatid nucleus, production of the acrosome, mitochondrial remodeling, formation of the flagellum, and removal of cytoplasm (Fawcett, et al., 1971; Struijk, et al., 2018).

Spermatozoa

Spermatozoa is a mature sperm cell which consists of a head, mid-piece and tail (Mortimer, 2018). The head contains a nucleus and is

covered with acrosome. The mid-piece contains mitochondria that produces adenosine triphosphate (ATP) for movement of the spermatozoa. The tail contains a flagellum that is waved for movement of the spermatozoa (Gerton, et al., 2018).

Sertoli cells

The Sertoli cell is a supporting cell in the seminiferous tubule which provides key features for spermatogonia to progress successfully into spermatozoa (Sofikitis, et al., 2008). The function and development of the sertoli cell have been stimulated by the follicular stimulating hormone (FSH) produced from the anterior pituitary gland (Fritz, et al., 1976). In addition, the sertoli cell acts as the blood-testis barrier to maintain the balance of the growth factors or nutrients within the testis for sperm production (Griswold, 1998).

Leydig cell

Leydig cells or interstitial cells are located in the interstitial space surrounding the seminiferous tubules. Under the regulation of pituitary luteinizing hormone (LH), leydig cells produce testosterone in the testes (Vasta, et al., 2006).

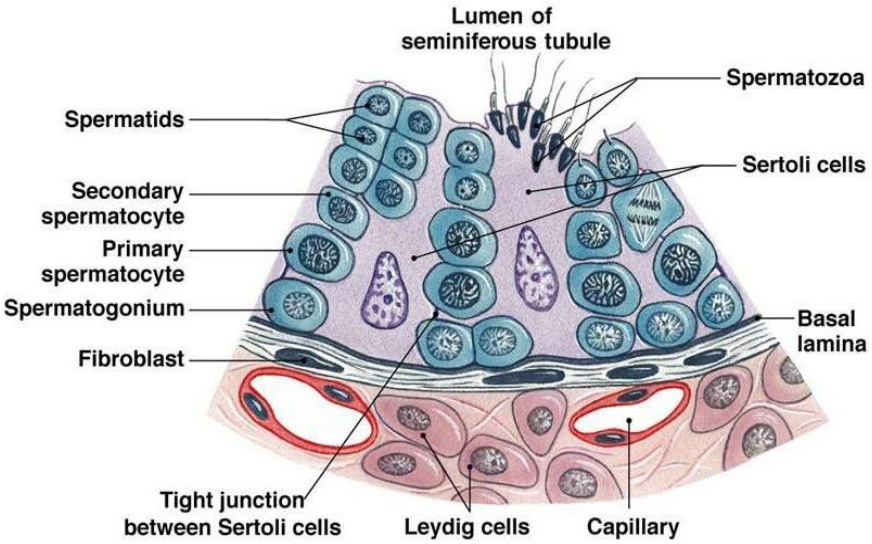


Figure 6 Spermatogenic cells, Sertoli cell and Leydig cell
Source: https://i.pinimg.com/originals/

Hormonal regulation of spermatogenesis

Spermatogenesis is a process of producing spermatozoa caused by the increase of gonadotropin releasing hormone (GnRH) from the hypothalamus and stimulating the anterior pituitary gland to secrete lutinizing hormone (LH) to stimulate leydig cells to produce testosterone (Mendis-Handagama, 1997; Sofikitis, et al., 2008). In addition, gonadotropic releasing hormone (GnRH) also stimulates the production and function of follicle stimulating hormone (FSH), which is important for spermatogenesis and the function of the sertoli cell (Sharpe, 1994).

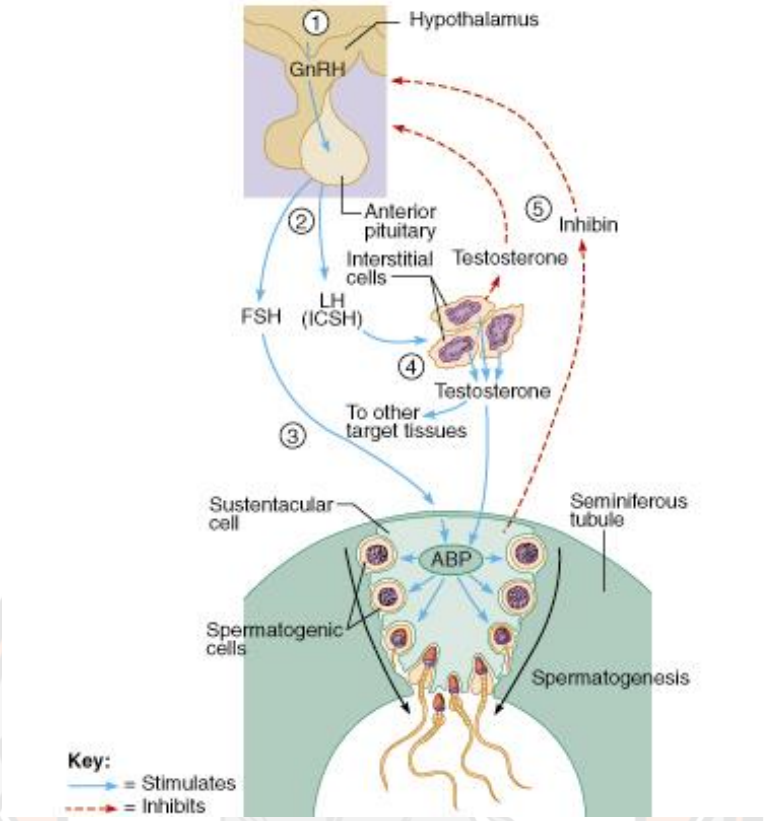


Figure 7 Hormonal regulation of spermatogenesis
Source:
https://www.apsubiology.org/anatomy/2020/2020_Exam_Reviews/Exam_5/CH27_Reproductive_Hormones.htm

Semen Analysis

Semen analysis is also known as a sperm count test that is a process to evaluate the viability of a men's sperm and male fertility. The semen analysis is estimated the volume, pH, viscosity, liquefaction, sperm quality and other cells contaminated with semen such as white blood cells and red blood cells (De Jonge, 2012). The evaluation criteria of human semen parametesr have been assigned by World Health Organization (WHO) (Table 1).

The 3 majors of parameters measured in a semen analysis are sperm concentration, sperm motility and sperm morphology collectively referred to as sperm quality. Ascertainment of sperm quality has been used for evaluating the basic properties of sperm cells, which applied in both human and animal.

Table 1 Normal criteria of semen parameters for human (WHO, 2010)

Parameter	Lower reference limit
Sperm volume (ml)	1.5
Total sperm number (10^6/ejaculate)	39
Sperm concentration (10^6/ml)	15
Total motility (Progressive & Non progressive motility, %)	40
Progressive motility (%)	32
Vitality (live spermatozoa, %)	58
Sperm morphology (normal form, %)	4
pH	≥7.2
Peroxidase-positive leukocytes (10^6/ml)	<1.0

Sperm qualities

Sperm motility

Sperm motility or movement of spermatozoa is the ability of sperm to move efficiently and is an associated with the pregnancy rate. Sperm motility is determined by counting the total spermatozoa in the Makler counting chamber and under the microscope, which counting both motile and immotile spermatozoa (Manual on Basic Semen Analysis, 2002). Progressive motility refers to the forward movement of sperm, that classified the sperm motility grades into 4 grades comprising A grades; rapid progressive motility, B grade; slow progressive motility, C grade;

non-progressive motility and D grade; immotility. Normal sperm motility is 40% motile spermatozoa (WHO, 2010).

Sperm concentration

Sperm concentration is a method for evaluate the number of spermatozoa. The sperm concentration can indicate the rate of pregnancy (Eliasson, 2010). The sperm concentration is calculated by dividing the number of spermatozoa counted in the counting chambers under the microscope, with depending on the number of squares counted and the dilution factor (Manual on Basic Semen Analysis, 2002). Normal sperm concentration is least $15x10^6$ spermatozoa/ml (WHO, 2010).

Sperm morphology

Sperm morphology is a method of assessing the shape of spermatozoa by smearing and staining with eosin. Normally, the sperm morphology is divided into 3 main regions: head, mid-piece and principal piece or tail. The head of spermatozoa contains the nucleus with DNA and covered by the acrosome, a cap-shaped that contains enzymes and receptors for oocyte binding and fertilization. The mid-piece region is surrounded by spiral mitochondria that generates ATP for the movement of flagella. The principal piece or tail region is a long flagellum used for movement through the female reproductive tract and penetration of the oocyte (Rothmann and Bort, 2018). However, the sperm morphology in different animal is also different in size and shape such as the head of a human spermatozoa have oval-shaped, and the head of a rat have hook-shaped (Mustika et al, 2018).

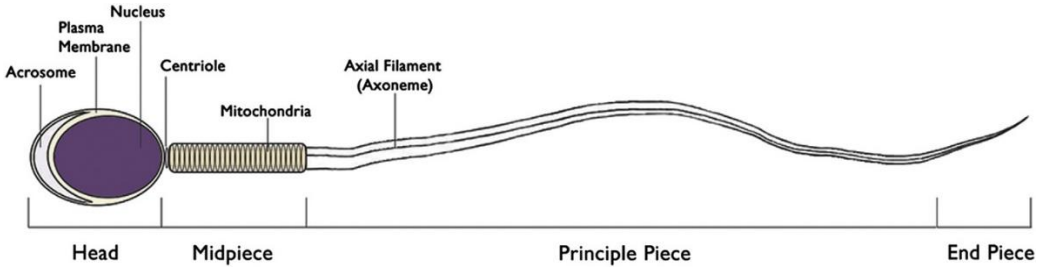


Figure 8 Basic anatomy of a human spermatozoa
Source: Rothmann and Bort, 20

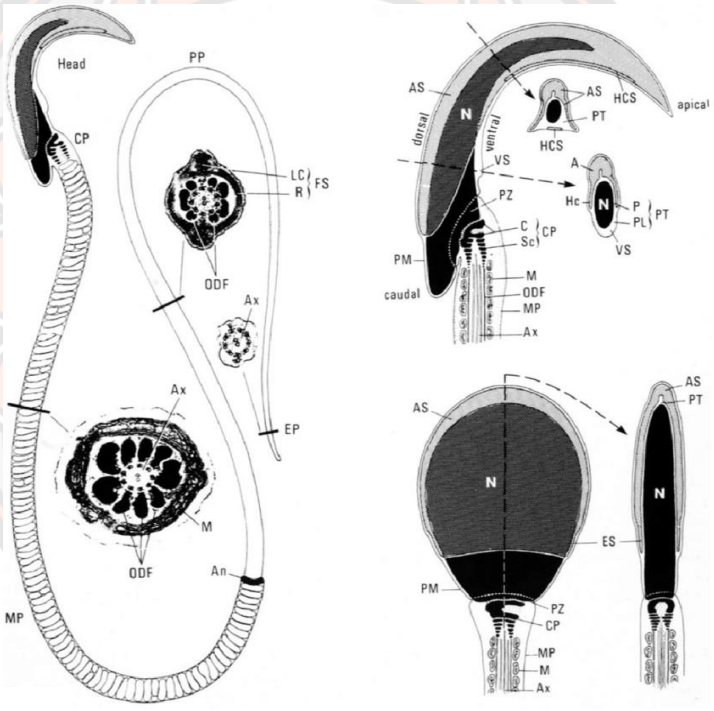


Figure 9 The structure of rat spermatozoa
Source: Hermo, et al., 2009

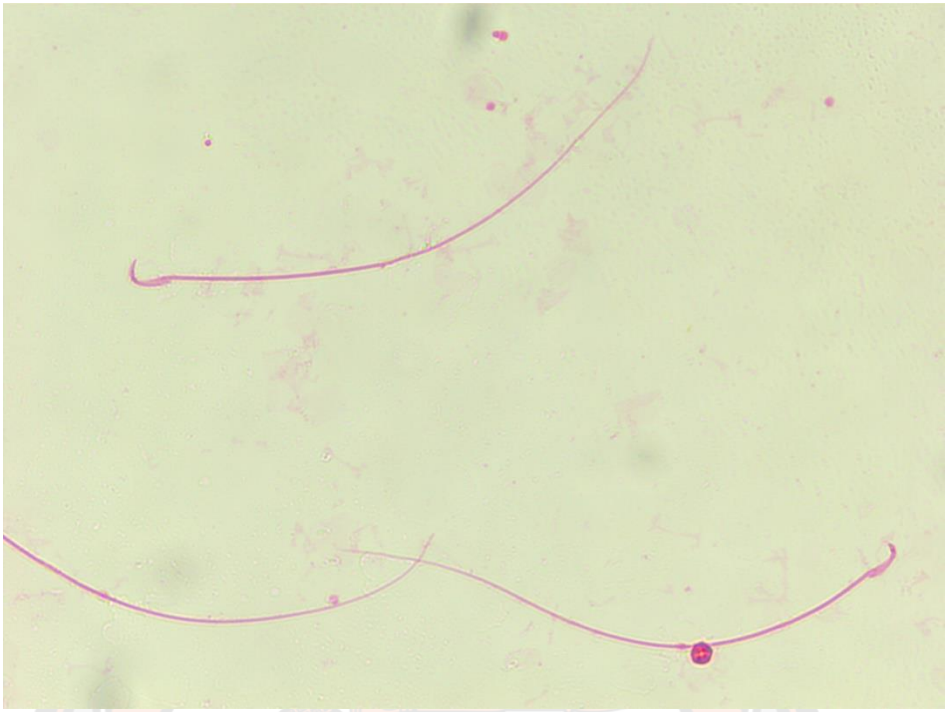


Figure 10 The normal morphology of rat spermatozoa

Abnormalities of sperm morphology has observed in many variations such as head defection, neck defection, tail defection and cytoplasmic droplet. Head defections are including large head, small head, double head, pin head, amorphous, small acrosomal and absent head. Defective neck and mid-piece are including bent neck and thin or thick of mid-piece. Tail defections are including short, double, bent, coil and absent tail (Manual on Basic Semen Analysis, 2002). Moreover, abnormalities of rat spermatozoa have also observed as in human such as small head, flat head, amorphous head, absent head, bent neck, bent tail, coil tail and absent tail.

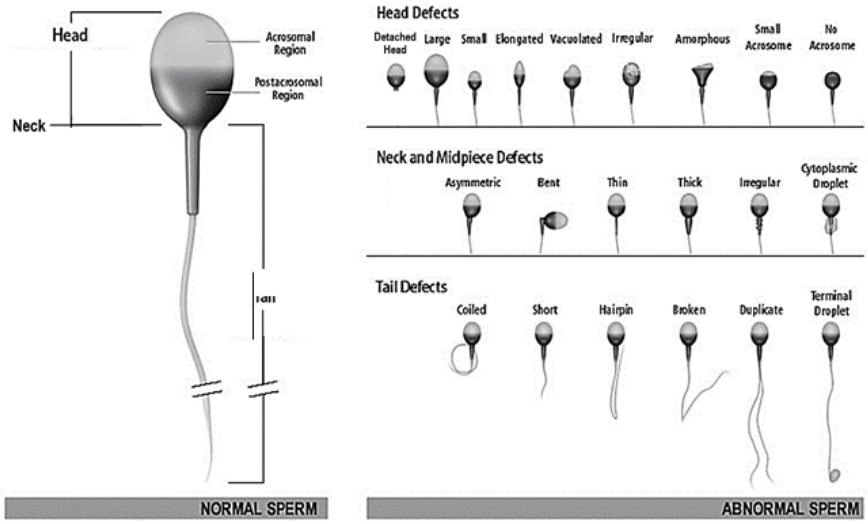


Figure 11 Abnormal sperm morphology of human spermatozoa
Source: https://carolinaconceptions.com/understanding-sperm-morphology/

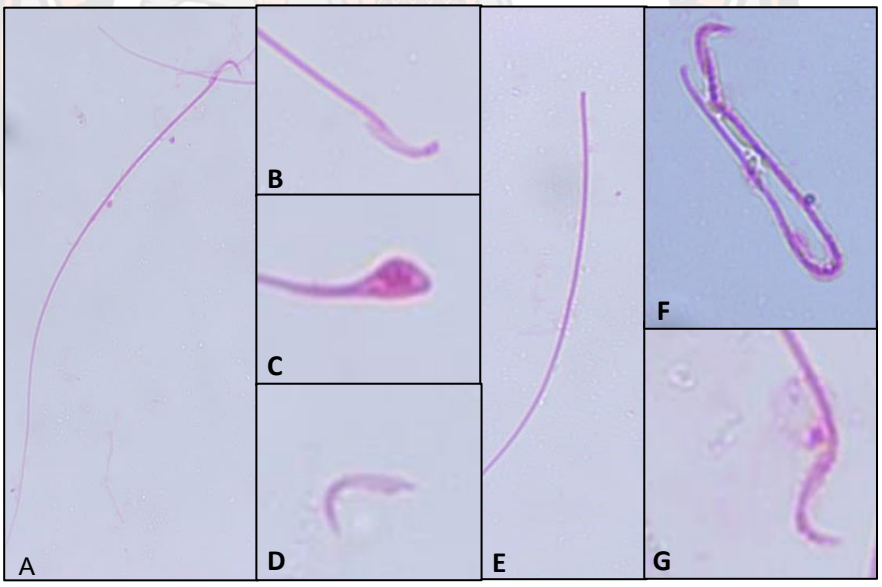


Figure 12 Abnormal sperm morphology of rat spermatozoa: normal morphology (A), Flat head (B), amorphous head (C), absent tail (D), absent head (E), coil tail (F) and bent neck (G)

Drug addiction

Drug addiction is defined as a brain disease characterized by persistent drug-seeking, drug-taking behaviours, loss of control in limiting intake despite the effects are dangerous, and a negative emotional state when lack of the drug (Feltenstein and See, 2008; Ruisoto and Contador, 2019). After drug addiction, the body to change from the effects of the drug. First, tolerance stage means the brain and body have changes in response to the drug. It can no longer produce the same mental or physical effects and, as a result, the individual further increases the dosage and the frequency of use to bring about the feelings that they are seeking out. Second, dependence stage is an adaptive state that develops from repeated drug administration. Third, drug withdrawal stage is the group of symptoms that occur on the abrupt discontinuation or decrease in intake of drugs such as fatigue, anxiety, irritability, nausea, tremors and seizures. The last stage of addiction is relapse, often occurring as the withdrawal symptoms become too overwhelming. So in this stage, the person struggling with drug addiction and drug dependence once again to reclaim what they feel is a normal emotional and physical state. During over the years have been reported of illicit use and drug abuse have risen especially methamphetamine and dextromethorphan.

Methamphetamine

Methamphetamine (METH) is a stimulant drug usually used as a white, powder or a pill. It is a highly addictive psychostimulant drug, which are many addicts around the world. The addiction to METH is a major public concern because it is associated with serious health complications including deficits in attention, memory, and executive functions in humans (Krasnova and Cadet, 2009).

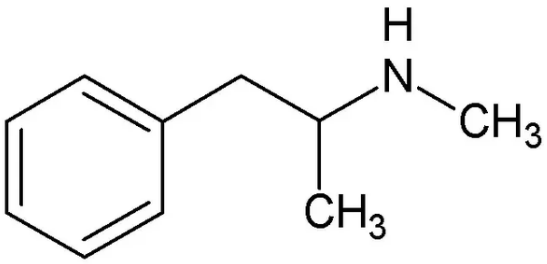


Figure 13 Chemical structure of Methamphetamine
Source:https://www.quora.com/Does-methylphenidate-have-methamphetamine-in-it

Methamphetamine and male reproductive system

The previous studies were considered that Methamphetamine could induce apoptosis of spermatogenic cells and compromises antioxidant defense in the seminiferous tubule in rat testis, which results in decreased testosterone concentration in the serum and reduced sperm count and sperm quality in rats (Yamamoto, et al., 2002; Alavi, et al., 2008; Nudmamud-Thanoi and Thanoi, 2011; Lin, et al., 2014). Moreover, Methamphetamine Drug abuse is a chronic disease induce a reduction of PR, ERβ and ERα proteins and gene expression of spermatogenic cells and Sertoli cells in Methamphetamine-treated rats, which result to dysfunctions of progesterone and estrogen in spermatogenesis (Nudmamud-Thanoi, et al., 2016).

Dextromethorphan

Dextromethorphan (DXM) is an antitussive drug. While it is one of the active ingredients in many over the counter cold and cough medicines, in exceeding maximum dosages, dextromethorphan acts as a dissociative hallucinogen. Its mechanism of action is as an NMDA receptor antagonist, producing effects similar to those of ketamine and phencyclidine (WHO, 2012). The side effects of dextromethorphan are

vomiting, diarrhea, respiratory depression and hypertension (Lachover, 2007).

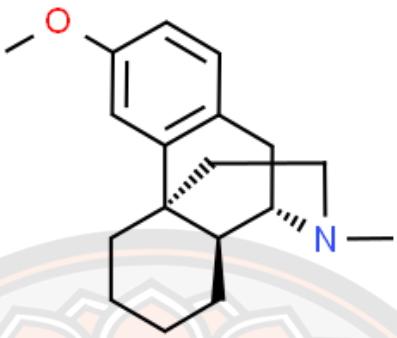


Figure 14 Chemical structure of Dextromethorphan
Source: http://www.chemspider.com/Chemical-Structure.13109865.html

Dextromethorphan and male reproductive system

The previous studies suggested that the use of addictive substances may damage to male reproductive system which can cause reducing the testosterone levels (Ragni, et al,. 1988; Yamamoto, et al., 2002). Moreover, Dextromethorphan psychotoxicity leads to male mice reproductive system injury by activating α-1 receptors can cause increased sperm abnormalities and decreased sperm viability and sexual behavior (Nam, et al., 2012).

Drug treatment for drug addictions

Diazepam and male reproductive system

Diazepam is a common drug treatment for addiction patient. Diazepam belongs to a class of drugs known as benzodiazepines which act on the brain and central nervous system. Diazepam increases the activity of gamma aminobutyric acid (GABA) in the brain that increases

calming effect and results in sleepiness, a decrease in anxiety and relaxation of muscles. Thus, Diazepam is mainly used to treat anxiety, skeletal muscle spasms and symptoms caused by alcohol withdrawal. However, if using the drug for a long time, causing side effects such as drowsiness, fatigue, muscle weakness, headache, tremor, dizziness, nausea, constipation.

Moreover, the previous studies have been reported diazepam decrease sexual behavior (Hernandez-Alvarez, et al., 1998), increase germ cell aneuploidy which affect to spermatogenesis (Baumgartner, et al., 2001).

Gamma-aminobutyric acid (GABA) and male reproductive system

GABA is a major inhibitory neurotransmitter in mammalian central nervous system (CNS) (Tillakaratne, et al., 1995). GABA has not only affected on central nervous system (CNS), but also has affected on male reproductive system. The previous studies have been reported GABA effect on agglutination and motility of spermatozoa (Boldizsar, et al., 1992), involved maturation of spermatogenic cells in spermatogenesis and spermiogenesis (Kanbara, et al., 2005; Kanbara, et al., 2010), acrosome reaction (Blackmore, et al., 1994; Cao, et al., 1997). In addition, the previous study has been reported GABA regulate the gonadotropin releasing hormone (GnRH), luteinizing hormone (LH) and testosterone (Ritta, et al., 1987; Jackson and Kuehl, 2002). These results suggested that GABA may regulate the testicular function.

Pre-germinated brown rice (PGBR) and male reproductive system

Pre-germinated brown rice (PGBR) is produced by dipping brown rice (BR) in water to induce germination (Zhang, et al., 2007; Sakamoto,

et al., 2007). PGBR contains various dietary fiber, vitamins and minerals. PGBR has high alpha-tocopherol (Vitamin E), gamma-oryzanol, inositol, ferulic acid (FA) and gamma-aminobutyric acid (GABA) (Ohtsubo,et al., 2005). The previous studies have been reported alpha-tocopherol (Vitamin E) can regulate sexual maturation, spermatogenesis (Santymire, et al., 2015), induce sperm motility and sperm concentration (Keskes-Ammar, et al., 2003; Echeverria-Alonzo, et al., 2009). Moreover, Gamma-oryzanol has been reported increases sperm concentration and sperm motility (Arlas, et al. 2008). In addition, ferulic acid (FA) has been reported increases sperm viability, sperm count and sperm motility (Zheng and Zhang, 1997; Roy, et al., 2014). These results suggested that PGBR may regulate the sperm morphology and testicular function.

Proteomics

Proteomics is a widely used technology for the identification and quantification of proteomes in cell, tissue and organism. The application of proteomic techniques are widely used in diagnostic and scientific research to understand of the structural system and function to be studied. Recently, proteomics has been used to evaluate the differential protein expression and functional output of protein in biological system on male reproductive system such as oxidative stress response, reproduction, and cellular process (Mallick, et al., 2010; Aslam, et al., 2017). Although the proteomics has been employed to evaluate the toxicity from other substances on male reproductive system. For example, proteomic analysis of mouse testis reveals Perfluorooctanoic acid-induced reproductive dysfunction (Zhang, et al., 2014). Proteomic analysis of gonad tissue after chronic atrazine exposure (Chen, et al., 2015). Proteomics and metabolomics analysis of arsenic-induced rat testis (Huang, et al., 2016).

Proteome profile of spermatozoa in mice after Bisphenol-A exposure (Rahman, et al., 2017). However, the proteomics study of the differential protein expression in testis because of drug addiction remained unclear.

CHAPTER III

RESEARCH METHODOLOGY

This chapter presents the methodology of this study including materials, equipment and methods. This study is divided into 2 sub-projects.

1. Methamphetamine project

Animal

The animals that were used in this research has been received from "The role of glutamate receptor modulation in drug addiction" of Miss Sri-arun Iamjan.

Male Sprague-Dawley rats from National Animal Center, Salaya, Nakorn Pathom, Thailand were used as experimental animals. The animals ageing 5 weeks and weighing 200-250 g. The animals were housed in cages, temperature at 24 ± 1 ◦C under a 12 hours dark-light cycle at Center for Animal Research of Naresuan University. Food and water were ad libitum. The body weights of animal were recorded daily until sacrificed.

All animals were carried out in according to the guideline for animal care and use of laboratory animal. All procedures were approved by the Animal Research Committee of Naresuan University, Thailand.

Drug and reagents administration

D-Methamphetamine hydrocholoride (Alletch, palatine & DC, IL, USA) was used for the experiment with permission from the Thai Ministry of Public Health. Methamphetamine will be dissolved in normal saline.

Experimental design

The animals were divided into 2 groups as follow;

1. Control group (C): The rats were injected intraperitoneal (i.p.) with normal saline for 15 days.
2. Escalating dose methamphetamine binge group (ED-METH Binge): The rats were received three injections per day at 3 hours intervals of increasing doses of METH from 0.1 to 4.0 mg/kg for 2 weeks. The last day, the rats in this group were received four injections per day at 2 hours intervals 6.0 mg/kg METH by i.p.

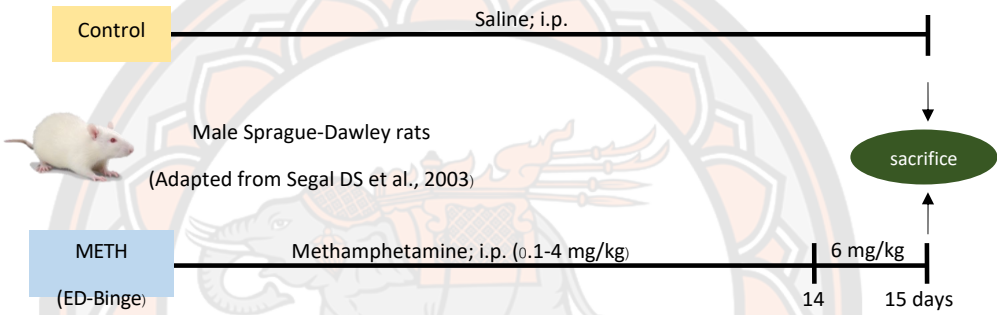


Figure 15 Schematic diagram of METH administration

Table 2 Schedule for METH administration in ED-Binge METH 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	
15	**07:30**	**09.30**	**11.30**	**13.30**
	6.0	6.0	6.0	6.0

Source: Adapted from Segal et al., 2003

2. Dextromethorphan project

Animal

The animals that were used in this research has been received from "Effect of pre-germinated brown rice on spermatogenesis and sperm quality in animal models of depression and addiction" of Miss Siriluk Veerasakul.

Male Sprague-Dawley rats from National Animal Center, Salaya, Nakorn Pathom, Thailand were used as experimental animals. The animals ageing 5 weeks and weighing 200-250 g were used in all experiments. The animals were housed in cages, temperature at 24 ± 1 ◦C under a 12 hours dark-light cycle at Center for Animal Research of Naresuan University. Food and water were ad libitum. The body weights of animal were recorded daily until sacrificed.

All animals were carried out in according to the guideline for animal care and use of laboratory animal. All procedures were approved by the Animal Research Committee of Naresuan University, Thailand.

Drug and reagents administration

1. Dextromethorphan, Dextromethorphan hydrobromide was purchased from Sigma-Alorich®
2. Diazepham was got form Naresuan University Hospital
3. Gamma aminobutyric acid, GABA was purchased from Sigma Chemical Company, ST. Louis, USA. Amount of synthetic GABA were equaled with the GABA found in PGBR, GABA was dissolved in distilled water.
4. Pre-germinated brown rice, PGBR was obtained from Faculty of Agriculture Natural Resources and Environment, Naresuan University.

Experimental design

The animals were divided into 17 groups as follows;

1. Control group (C): The rats were treated with normal saline by oral administration via gavage for 75 days.
2. Dextromethorphan group (DXM): The rats were treated with DXM 30 mg/kg by intraperitoneal injection (i.p.) for 15 days.
3. Pre-germinated brown rice control 15 days group (CR15): The rats were treated with normal saline for 15 days (i.p.) and treated with PGRB 5 mg/kg for 15 days by oral administration via gavage.
4. Pre-germinated brown rice control 30 days group (CR30): The rats were treated with normal saline for 15 days (i.p.) and

treated with PGRB 5 mg/kg for 30 days by oral administration via gavage.

5. Pre-germinated brown rice control 60 days group (CR60): The rats were treated with normal saline for 15 days (i.p.) and treated with PGRB 5 mg/kg for 60 days by oral administration via gavage.
6. DXM and withdraw 15 days group (DW15): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and withdraw for 15 days
7. DXM and withdraw 30 days group (DW30): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and withdraw for 30 days
8. DXM and withdraw 60 days group (DW60): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and withdraw for 60 days
9. DXM and diazepam 15 days group (DD15): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with diazepam 10 mg/kg for 15 days by oral administration via gavage.
10. DXM and diazepam 30 days group (DD30): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with diazepam 10 mg/kg for 30 days by oral administration via gavage.
11. DXM and diazepam 60 days group (DD60): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with diazepam 10 mg/kg for 60 days by oral administration via gavage.

12. DXM and GABA standard 15 days group (DS15): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with GABA standard 0.8 mg/kg for 15 days by oral administration via gavage.
13. DXM and GABA standard 30 days group (DS30): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with GABA standard 0.8 mg/kg for 30 days by oral administration via gavage.
14. DXM and GABA standard 60 days group (DS60): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with GABA standard 0.8 mg/kg for 60 days by oral administration via gavage.
15. DXM and PGBR 15 days group (DR15): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with PGBR 5 g/kg for 15 days by oral administration via gavage.
16. DXM and PGBR 30 days group (DR30): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with PGBR 5 g/kg for 30 days by oral administration via gavage.
17. DXM and PGBR 60 days group (DR60): The rats were treated with DXM 30 mg/kg for 15 days (i.p.) and treated with PGBR 5 g/kg for 60 days by oral administration via gavage.

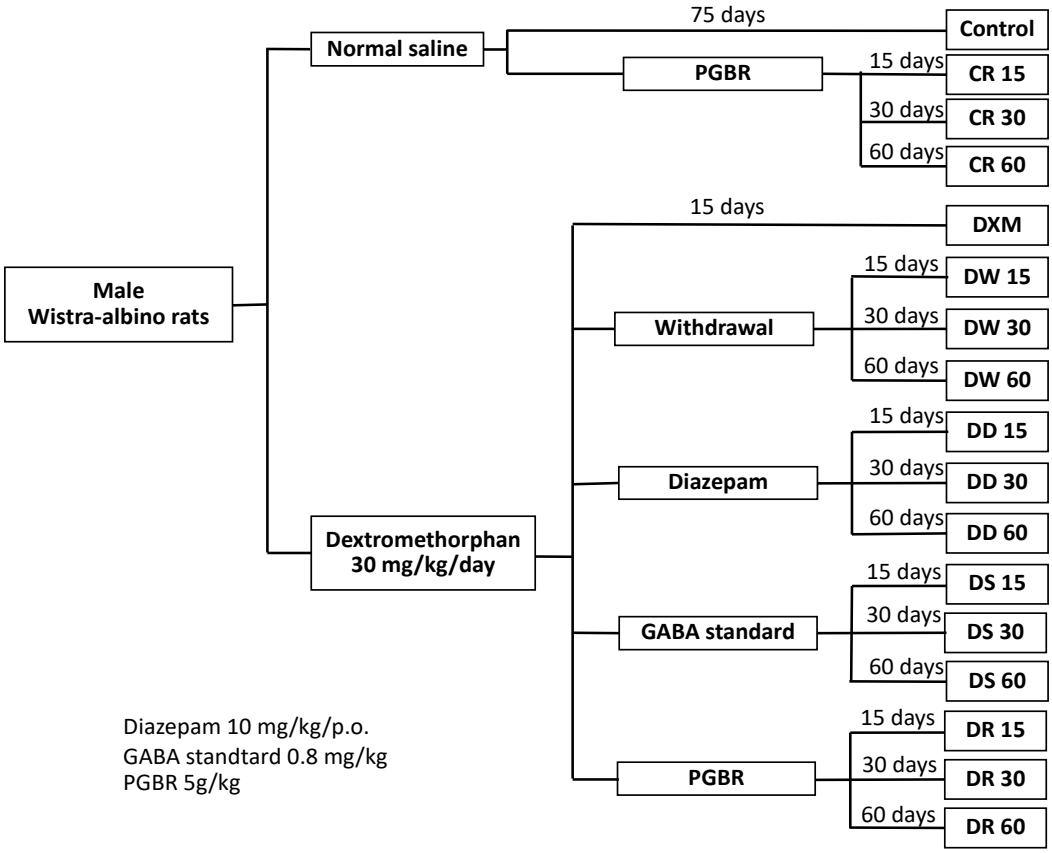


Figure 16 Schematic diagram of DEX administration

Testis and sperm collections

After the end of treatments, testes and epididymis were removed immediately after sacrifice. The left testis and epididymis were fixed in 10% neutral buffered formalin. The right testis was separated from epididymis and kept at -80°C for proteomics. For sperm collection, cauda epididymis (tail of epididymis) was separated and minced in phosphate buffer saline (PBS) to release spermatozoa. These spermatozoa were fixed in 10% neutral buffered formalin and kept at 4°C for immunohistochemistry.

Sperm morphology

Spermatozoa in PBS that fixed in 10% formaldehyde solution were examined with eosin staining in dilution 4:1 and incubated for 1 hour at room temperature. Then, stained spermatozoa were smeared on glass slides. The total 200 spermatozoa were captured under the microscope and were counted the number of normal sperm morphology. The result of sperm morphology was reported in percentage of normal sperm morphology that was calculated according to following ratio:

% Normal sperm morphology = (Number of normal sperm morphology)/(Total spermatozoa) x100

Tissue preparations for proteomic analysis

Protein extraction

Testis tissues 30 mg was homogenized in 5 mM Tris-HCl containing 25 mM NaCl, pH 8.0 and homogenate was centrifuged for 10 minutes at 14000 rpm. After that, the pellet was collected and homogenized again in lysis buffer containing 50 mM Tris-HCl, 0.15 M NaCl, 0.1% SDS, 0.25% Sodium deoxycholate and 1% Protease inhibitor cocktail.

Protein concentration

The tissue lysates were measured by Lowry protein assay for determining the total level of protein in a solution.

Protein digestion

Protein samples of each group was pooled for LS-MS/MS analysis. A total protein sample was reduced with 5 mM Dithiothreitol in 10 mM Ammonium bicarbonate and incubated at 56 °C for 1 hour. Next, the alkalation with 15 mM Idoacetamine in 10 mM Ammonium bicarbonate

and incubated at room temperature for 1 hour in dark box. To perform in-solution digestion, the proteins were digested with 50 ng trypsin in 10 mM ammonium bicarbonate and incubated at 39 °C on overnight. The protein digestion was kept at -80 °C for further mass spectrometric analysis.

Proteomic analysis of protein expression profile

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

After the proteins were digested, the elution peptides were dried and resuspended in 0.1% formic acid in LC/MS water. The solution was centrifuged at 10,000 rpm for 10 min and transferred to vial tubes. The resuspended peptides were analyzed with Impact II UHR-TOF MS system (Bruker Daltonics Ltd., Germany) coupled with nanoLC system (Thermo Fisher Scientific, USA). Peptides were separated into nanocolumn (Acclaim PepMap column 75μm x15cm, nanoViper C18). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The peptides were separated with a linear gradient from 10% to 45% mobile phase B over 8.5 minutes at a flow rate of 1 μl/minute.

Protein quantification and identification

The protein quantification was analyzed by using the DeCyder MS Differential analaysis software (DeCyderMS, GE Healthcare). The NCBI database was searched with the LC–MS/MS data from the DeCyder MS analysis and the proteins were identified with the MASCOT search engine (Matrix Science, London, UK), with the following parameters: taxonomy (*rattus*), enzyme (trypsin), variable modifications (carbamidomethylating of cysteine residues, oxidation of methionine

residues, mass values (monoisotopic), peptide mass tolerance (1.2 Da), MS/MS tolerance (0.6 Da) and peptide charge states (1 +, 2 +, 3 +). The maximum value of each group was used to define the presence or absence of the identified proteins. Protein Analysis Through Evolutionary Relationships database (PANTHER) and Database for Annotation, Visualization, and Integrated Discovery (DAVID version 6.8) were used to classify the function of protein such as the biological process, cellular component, molecular function, and protein class. The UniProt Knowledgebase (UniProt KB) was used to create the information of the identified proteins (Parrilla, et al., 2019). Comparison of protein quantification was analysed by using the MultiExperiment Viewer (MeV, Version 4.9) software and shown as heatmap. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (https://string-db.org) 11.0 database was used to analyze protein-chemical interactions and generate the network.

Immunohistochemistry analysis

The expression of calbindin in rat spermatozoa was investigated by using immunohistochemistry technique. Spermatozoa in PBS were smeared on glass slides. After dried, the smeared sperm were retrieved antigen with high temperature heating at 70P (560 Watt) in microwave for 5 minutes, 3 times. After that, the smeared sperm were left to cool down at room temperature about 30 minutes. Then, the smeared sperm were incubated with endogenous peroxidase blocking solution (10% methanol, 3% H_2O_2, 1% triton-X and PBS) for 30 minutes at room temperature. After washing with PBS 2 time for 3 minutes, the smeared sperm were incubated with 5% normal goat serum (NGS) for 2 hours at room temperature, for blocking non-specific proteins. Next, the smeared

sperm were incubated with primary antibody of calbindin (rabbit polyclonal IgG, Sigma-Aldrich, USA) by diluted 1:250 in PBS containing 1% NGS for 3 hours at room temperature and 4 °C overnight. Then, the smeared sperm were washed with PBS for 3 minutes, 2 times. After that, the smeared sperm were incubated with biotinylated secondary antibody (Goat anti-rabbit, Vector Laboratories, Burlingame, California) for 2 hours at room temperature. For the enhancing signal, the smeared sperm were incubated with avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, California) for an hour at room temperature and washing with PBS 2 times for 3 minutes. The specific proteins were visualized by chromogen 3,3'-Diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, California) for 10 minutes and stop reaction with distilled water for 5 minutes. Finally, the smeared sperm were dehydrated by using a series ethanol concentration: 70%, 95%, 100% and then immersed in xylene, respectively. Lastly, the smeared sperm were mounted with mounted media (Fisher scientific, New Jersey, USA).

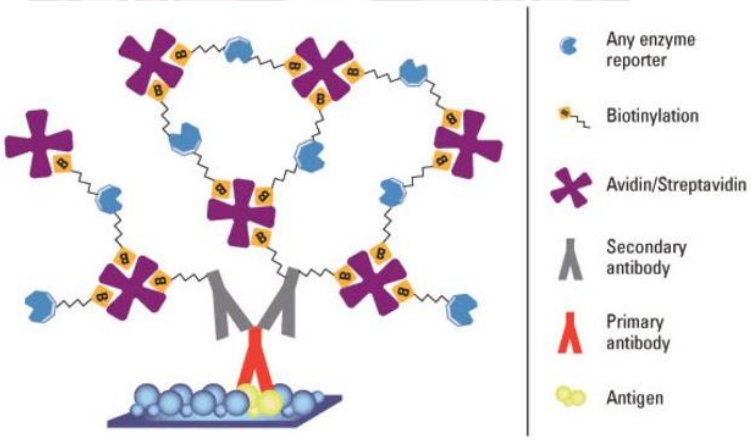


Figure 17 Immunohistochemistry technique
Source:
https://www.thermofisher.com/order/catalog/product/29129#/29129

Evaluation of the immunopositive cell and statistical analysis

The immunoreactive detected slides were captured by the microscope connected to a computer. 200 spermatozoa from each slide were used to investigate the expression of calbindin. The intensity values of immunoreaction were measured by imageJ fiji software. The result was represented as normalized reactive optical density (ROD).

Statistical analysis

The data were shown as mean ± standard error of mean (SEM). SPSS program version 17.0 (SPSS Inc., USA) was used to analyse the data. The statistical difference between groups were determined using independent sample t-test. The significant of all data was considered at $P \leq 0.05$.

CHAPTER IV
RESULTS

Sperm quality

The studies from our team have been reported that exposure to methamphetamine and dextromethorphan reduce sperm quality (Nudmamud-Thanoi and Thanoi, 2016; Thanoi, et al, 2018). The sperm concentration was decreased after the METH administration. Moreover, the percentages of normal sperm motility and normal sperm morphology were significantly decreased in METH-treated groups (Nudmamud-Thanoi, et al, 2016). Either way, the present study has been also observed that the percentages of normal sperm morphology was significantly decreased ($p<0.01$) in METH group when compared with the control group (Figure 18). Furthermore, our previous study found that sperm motility, the percentage of normal sperm morphology, and sperm concentration was highly significant decreased in DXM-treated when compared with the control group (Thanoi, et al, 2018). In addition, the previous study also found that animals treated with GABA and PGBR showed recovery in sperm quality, including sperm concentration, sperm motility, and the percentage of normal sperm morphology after exposure to DXM (Thanoi, et al, 2018).

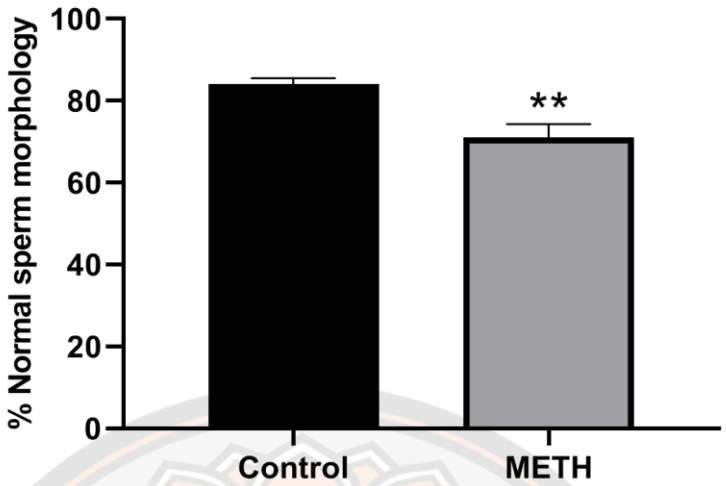


Figure 18 Effect of methamphetamine on rat sperm morphology when compared with control group. Data are presented as mean ± SEM. **Significantly different at p<0.01; Independent t-test.

The proteomic analysis

The protein profile of the rat testis was identified by an LC-MS/MS machine and analyzed by a bioinformatic tool. The proteomic analysis in this study was divided into 2 parts, including the protein profile of drug addiction and the protein profile of drug addiction treatment.

The protein profile of drug addiction induced by methamphetamine and dextromethorphan.

The protein profiles were analyzed with Venn diagrams for protein expression in each group and overlap. First, a total of 2,501 proteins in the Rattus database in the METH model were identified, including 1,389 proteins in the control group, 729 proteins in the ED-binge METH group, and 383 proteins in both groups (Figure 19A). A total of 2,699 proteins were identified as DXM models. 1,035 and 1,019 proteins were uniquely expressed in the control and DXM groups respectively. Moreover, 645 proteins were expressed in both groups (Figure 20A). Only the expressed

proteins with a fold change ≥ 1.2 in both groups were focused on as significantly expressed proteins. The present study found that there were 212 proteins in the METH model; 111 proteins were upregulated, and 101 proteins were downregulated (Figure 19B). In the DXM model, 341 proteins were significantly expressed; 184 proteins were upregulated, and 157 proteins were downregulated (Figure20B). The significantly differentially expressed proteins in both models were selected and analyzed by Venn diagram. The results showed that 184 proteins were expressed in the METH model and 313 proteins were expressed in the DXM model. In addition, 28 proteins were expressed in both models, which were considered as responded proteins in drug addiction (Figure 21). The 28 significantly differentially expressed proteins in drug addiction were further analyzed by Multiple Experiment Viewer and showed in heatmap (Figure 22).

The PANTHER system classification was used to gain a better understanding of these protein functions. Protein binding (50%), catalytic activity (32%) and transport activity (11%) were mainly enriched in the molecular functional category, which is shown in Figure 23A. The biological process category of these proteins is mainly in the cellular process (32%), biological regulation (17%) and localization (17%) (Figure 23B). As shown in Figure 23C, the top of protein class category includes transporter protein (18%), cytoskeleton protein (14%) and metabolite interconversion enzyme (14%).

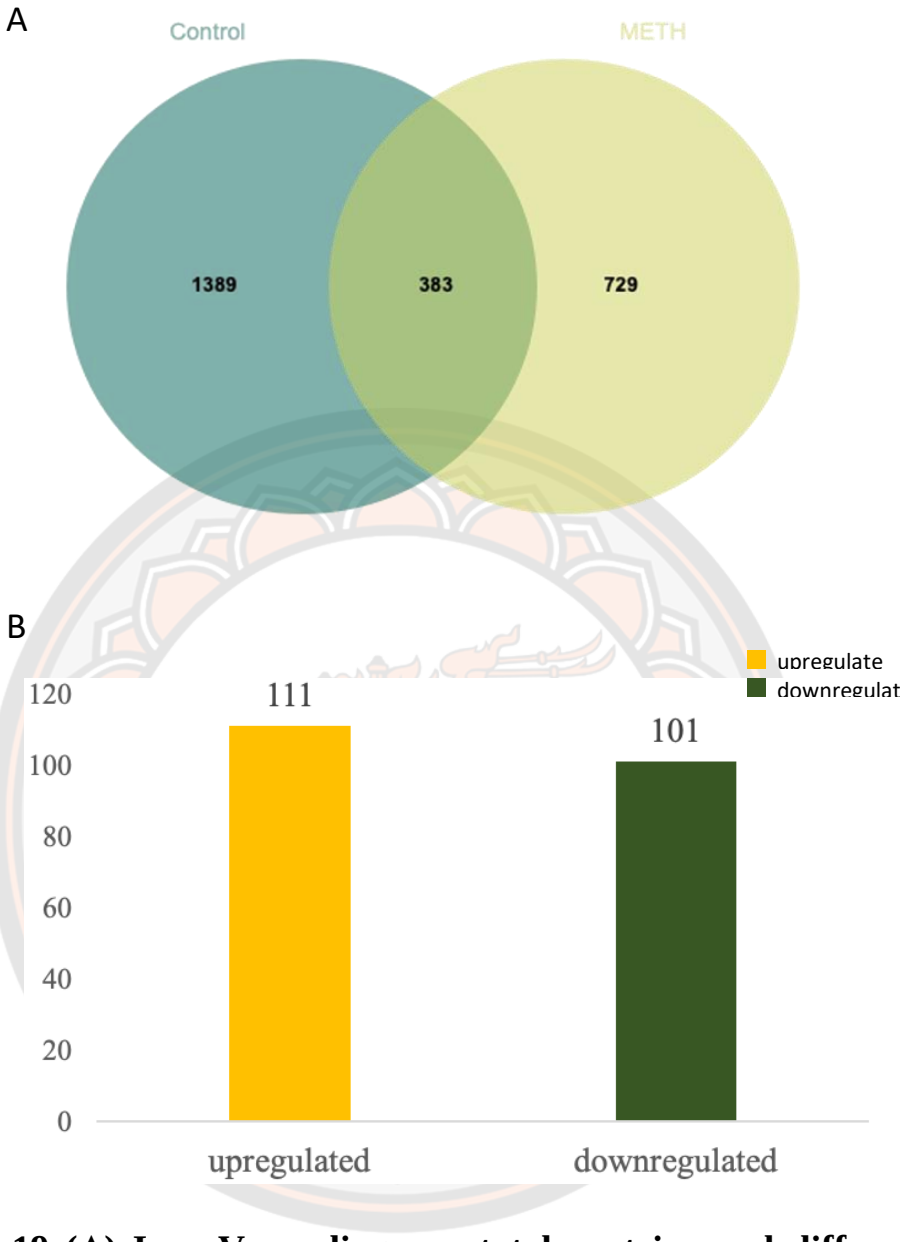


Figure 19 (A) In a Venn diagram, total proteins and differentially expressed proteins overlap in METH model. (B) The expressed proteins with fold change ≥ 1.2 with upregulated (Yellow) and downregulated (Green) expression in both groups.

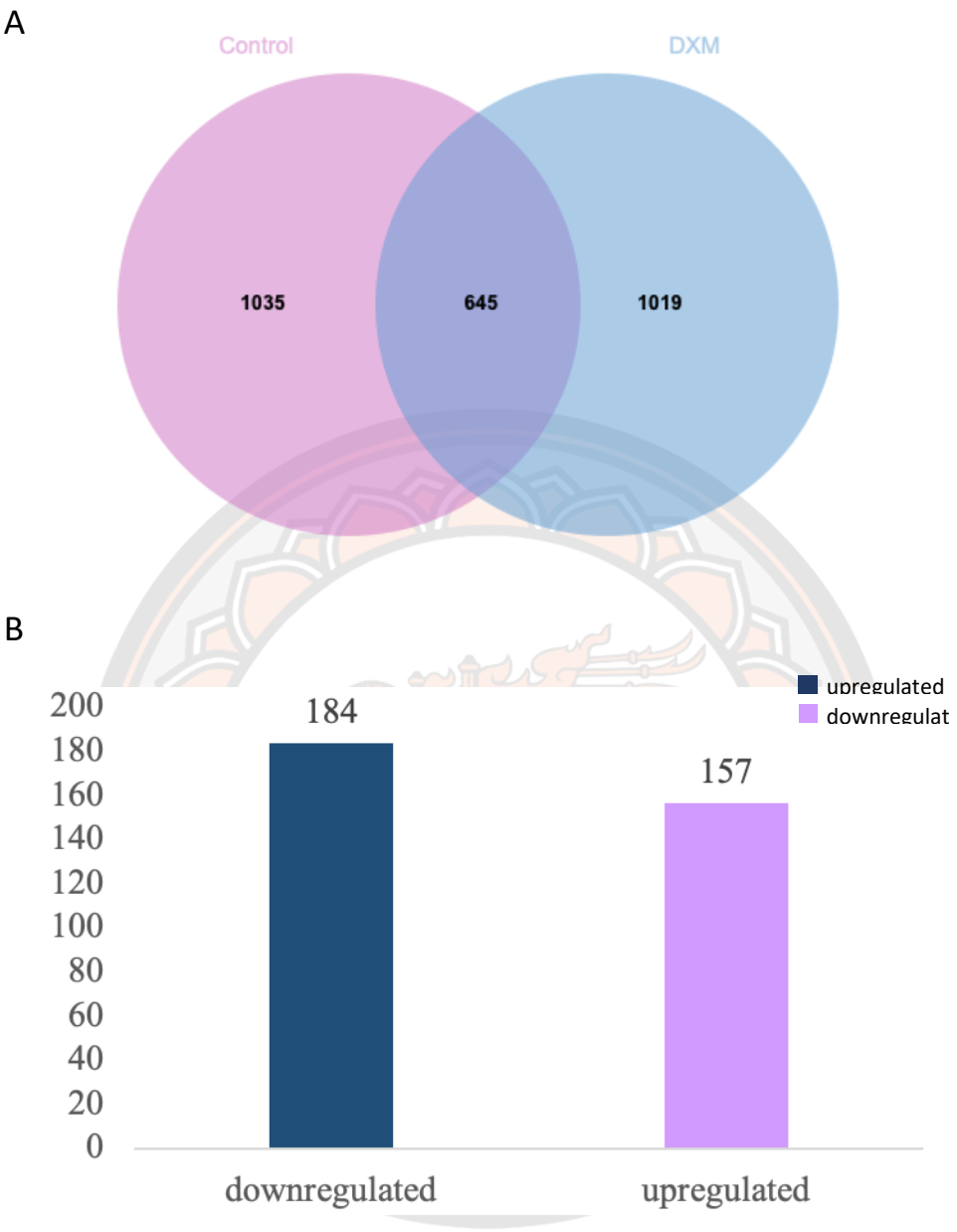


Figure 20 (A) In a Venn diagram, total proteins and differentially expressed proteins overlap in DXM model. (B) The expressed proteins with fold change ≥ 1.2 with upregulated (Blue) and downregulated (Purple) expression in both groups.

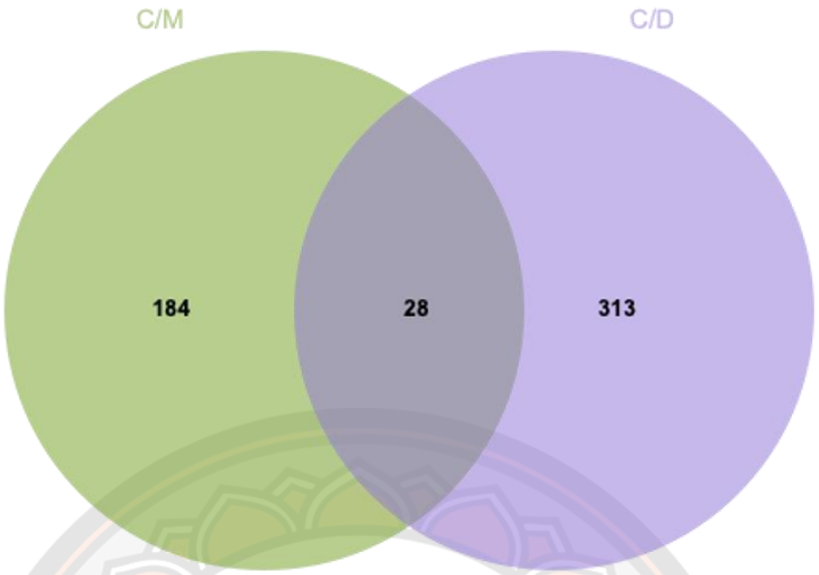


Figure 21 The significantly differentially expressed proteins were analyzed by the Venn diagram. In the green circle shows the proteins were only expressed in METH model. The purple circle shows the proteins were only expressed in the DXM model. The presence of overlap indicates that the proteins were expressed in both.

To investigate the important pathways by differentially expressed proteins in the drug addiction, 28 differential proteins were mapped to the KEGG database in DAVID. 5 pathways were significantly enriched in the differential proteins of drug addiction. The dominant pathway was the calcium signaling pathway, and was followed by retrograde endocannabinoid signaling, glutamatergic synapse, cGMP-PKG signaling pathway and cAMP signaling pathway, respectively (Figure 24). Interestingly, most of the differential proteins in drug addiction were classified by binding function and were mainly involved in the calcium signaling pathway, which play critical roles for testicular and sperm functions (Table 3).

Using Pathway Studio analysis, 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1 (Plec1), Inositol 1,4,5-trisphosphate receptor type 2 (Itpr2), Voltage-dependent L-type calcium channel subunit alpha-1D (Cacna1d) and Plasma membrane calcium-transporting ATPase 1 (Atp2b1) were shown directly related to the calcium molecule that plays a critical role in spermatogenesis, spermatid development and sperm function (Figure 25).

Proteins in the calcium signaling pathway were focused on and searched for using STRING software for protein interaction. The interactions between the identified proteins in the PPI network offer information on biological processes. 2 sub-network clusters were retrieved for the PPI network. The distribution of the proteins of the sub-network in the red circle involves the transporter. Signal transduction and biological regulation are involved in the distribution of proteins in the sub-network in the red square. (Figure 26). Surprisingly, Calbindin (calb1) was founded on the PPI network and is the link between the 2 sub-network clusters. From this result, 2 proteins, including Atp2b1 and Cacna1d, showed direct interaction with Calb1.

Figure 22 The heat map of significantly differential expressed proteins in drug addiction was analyzed by Multiple Experiment Viewer (MeV). Proteins with low, moderate, and high levels of expression are represented by green, black, and red colors, respectively.

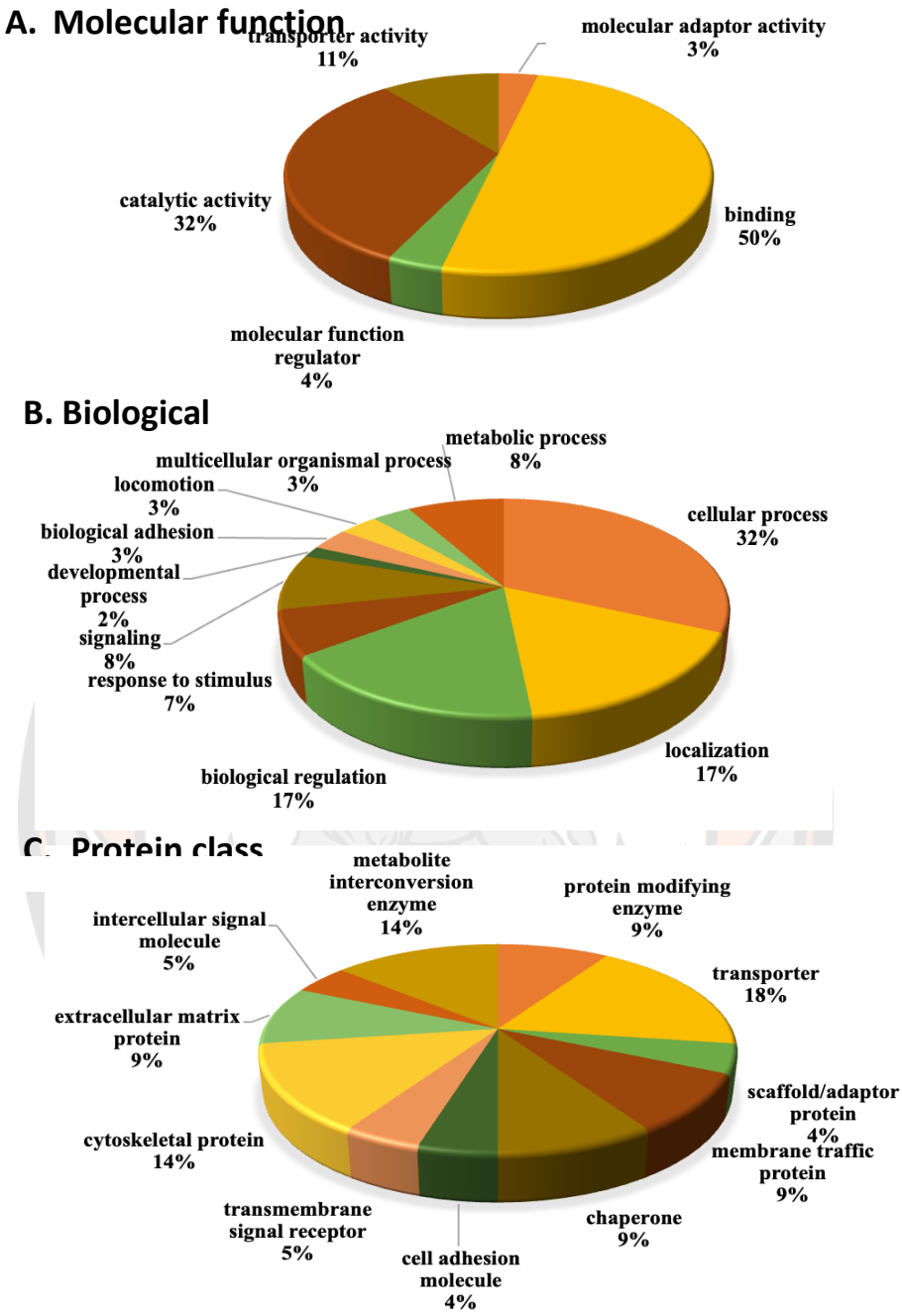


Figure 23 PANTHER showed that the classification of the differentially expressed protein in drug addiction involves in molecular function (A), biological process (B) and protein class (C).

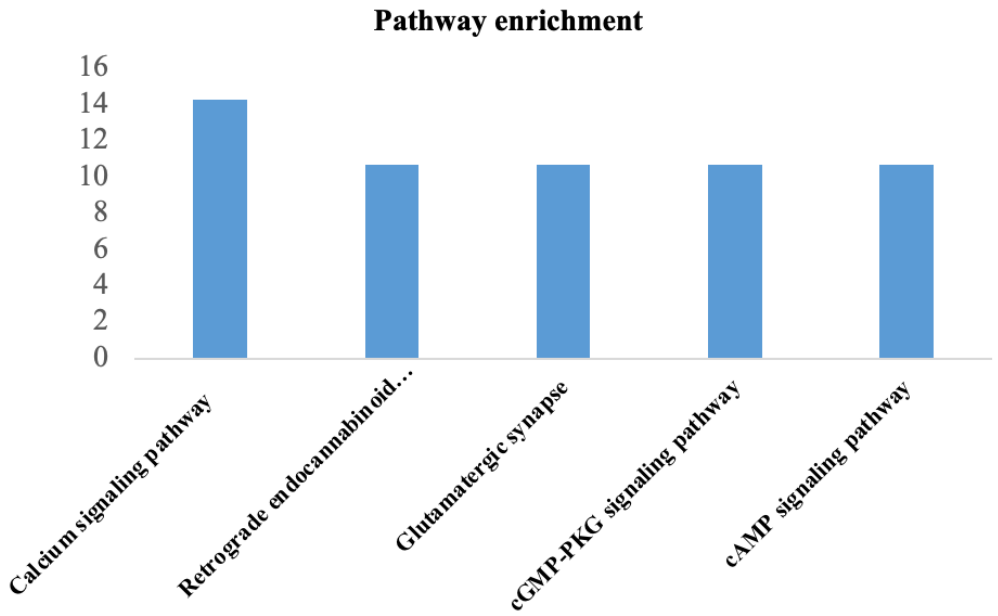


Figure 24 KEGG pathway enrichment of the differentially expressed proteins in drug addiction. 5 pathways were statistically significant with $p < 0.05$.

Table 3 The description of 4 proteins that are differentially expressed proteins in drug addiction involving in the calcium signaling pathway.

Accession No.	Abbreviation	Full name	Function
gi|81872439	Plce1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1	Activation of MAPK activity
gi|266389	Itpr2	Inositol 1,4,5-trisphosphate receptor type 2	Calcium ion transport
gi|6165987	Cacna1d	Voltage-dependent L-type calcium channel subunit alpha-1D	Regulation of calcium ion transport
gi|1476413370	Atp2b1	Plasma membrane calcium-transporting ATPase 1	Regulation of calcium ion transport, calcium ion export

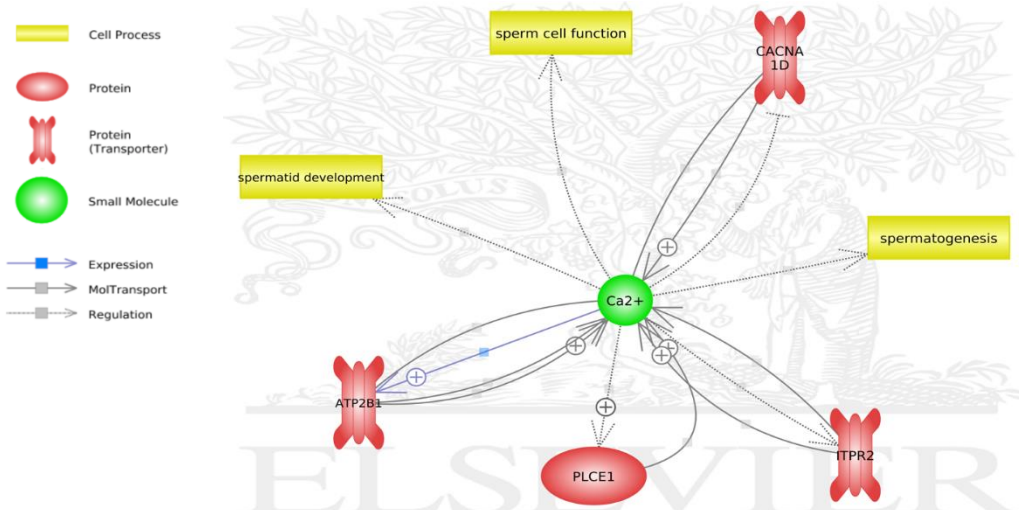


Figure 25 Analysis of the regulatory network of 4 differentially expressed proteins identified in drug addiction and related to the calcium signaling pathway. Pathway analysis was performed using Pathway Studio (https://mammalcedfx.pathwaystudio.com/login/form) analysis.

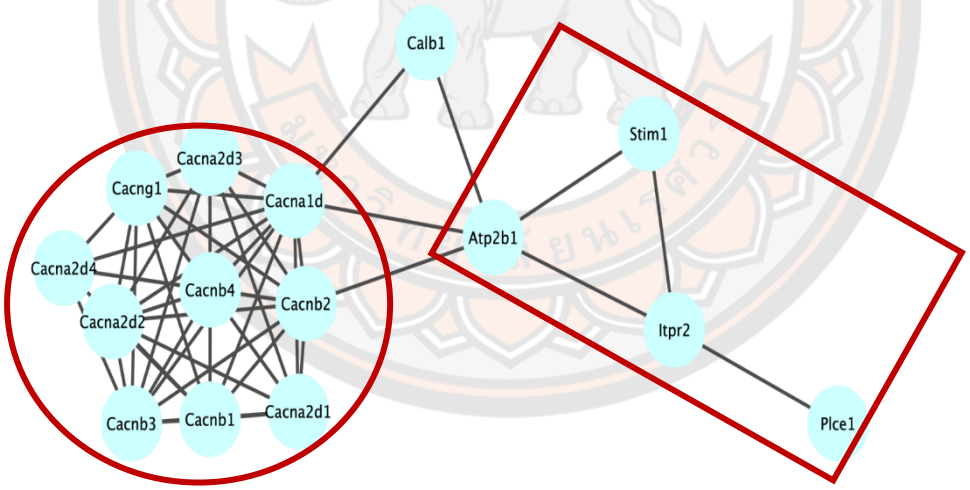


Figure 26 The network of protein-protein interactions (PPI) of the differentially expressed proteins in drug addiction to be specifically in the calcium signaling pathway. The STRING database was used to create the network. 2 sub-network clusters were shown in the red circle and square.

The protein profile of addiction treatments is withdrawal, diazepam, GABA and PGBR.

The protein profiles of withdrawal, diazepam, GABA and PGBR were analyzed with Venn diagrams for protein expression in each group and overlap. A total of 10,560 proteins in the Rattus database in all groups were identified. 630, 330, 513 and 640 proteins were uniquely expressed in the withdrawal group, diazepam group, GABA group and PGBR group, respectively. Moreover, 485 proteins were expressed in all groups (Figure 27). Then, 485 proteins were selected and analyzed with Venn diagrams for protein response to addiction treatments. A total of 499 proteins in the Rattus database in drug addiction and treatments were identified. The results showed 14 and 471 proteins were uniquely expressed in the drug addiction and treatment groups. In addition, 14 proteins were expressed in both, which were regarded as the responded proteins in addiction treatment (Figure 28).

14 proteins which response to addiction treatments were focused and classified their functions using the PANTHER system. The molecular function category of these proteins is mainly in protein binding (56%), catalytic activity (31%), molecular adaptor activity (7%) and transport activity (6%) (Figure 29A). Cellular process (28%), biological regulation (17%), localization (17%), signaling (9%), response to stimulus (7%), biological adhesion (5%), locomotion (5%), multicellular organismal process (5%) and metabolic process (5%) were mainly enriched in the biological process category, which is shown in Figure 29B. As shown in Figure 29C, the protein class category includes cytoskeletal protein (28%), membrane traffic protein (18%), transporter (9%), extracellular

matrix protein (9%), protein modifying enzyme (9%), cell adhesion molecule (9%), chaperone (9%) and scaffold/adaptor protein (9%).

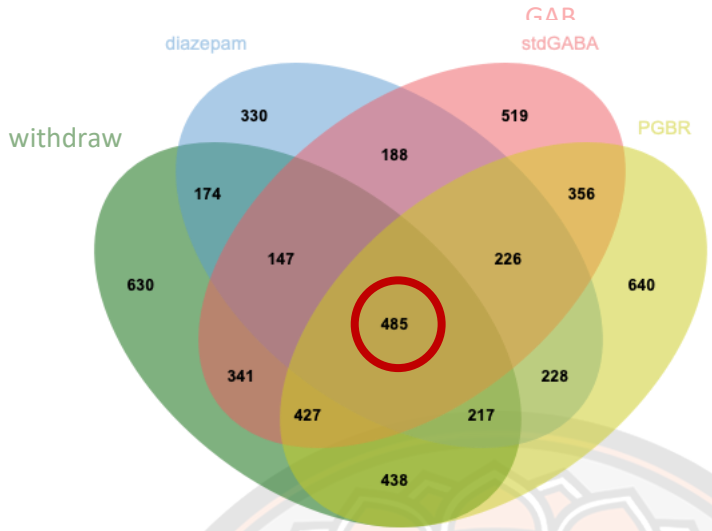


Figure 27 Total proteins and overlap differentially expressed proteins presented in a Venn diagram. The red circle shows the proteins were expressed in all groups.

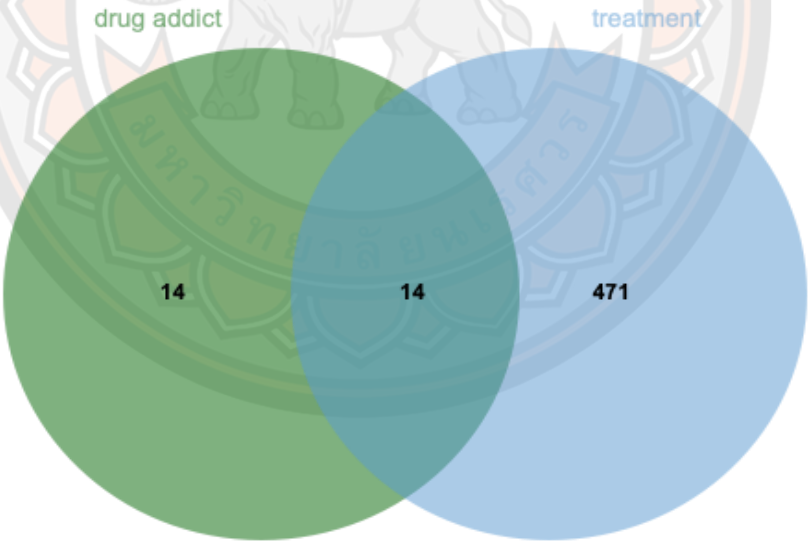


Figure 28 The differentially expressed proteins were analyzed by the Venn diagram. In the green circle shows the proteins were only expressed in drug addiction. The bule circle shows the proteins were only expressed in the addiction treatments. The presence of overlap indicates that the proteins were expressed in both.

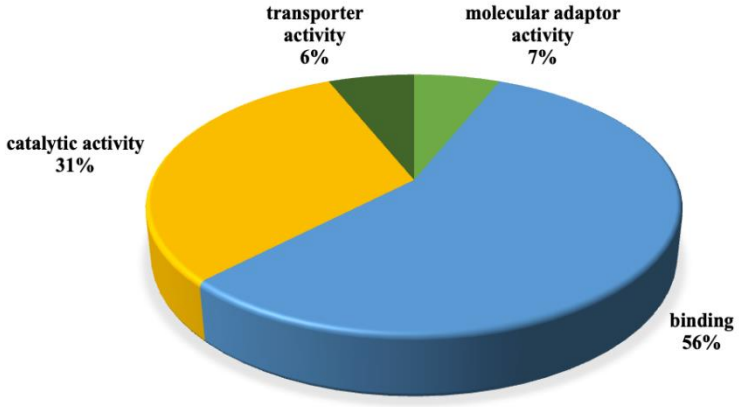
The DAVID program was used to analyze the specific functions of these proteins. The differentially expressed proteins in addiction treatments were categorized to the following Gene Ontology (GO) classes: biological process, cellular process, and molecular function. In all, 14, 6 and 4 categories were significantly enriched in biological process, cellular process, and molecular function, respectively ($p < 0.05$). In the biological process, the dominant functions were determination of left/right symmetry, coronary vasculature development, heart development and cilium morphogenesis (Figure 30). As shown in Figure 31, extracellular exosomes, cell junctions, and synapse were the dominant function of these proteins in the cellular process. The differentially expressed proteins in addiction treatments appear to play a critical role related to energy resource through molecular functions involving ATP binding, ATPase activity, ion channel binding and microtubule motor activity (Figure 32). As shown in Table 4, 6 proteins associated with microtubule motor activity, ATP binding, ATPase activity and ion channel binding were ATPase plasma membrane Ca2+ transporting 1 (Atp2b1), DEAD-box helicase 46 (Ddx46), dynein cytoplasmic 2 heavy chain 1 (Dync2h1), dynein, axonemal, heavy chain 5 (Dnah5), heat shock protein 90, alpha (cytosolic), class A member 1 (Hsp90aa1), myosin IE (Myo1e), regulating synaptic membrane exocytosis 1 (Rims1) and regulating synaptic membrane exocytosis 2 (Rims2). The 6 differentially expressed proteins in addiction treatments were analyzed by Multiple Experiment Viewer and showed in heatmap.

In Dextromethorphan-induced drug addiction, several proteins acting on microtubule motor activity, ATP binding, ATPase activity and ion channel binding have been shown to be decreased, such as Atp2b1,

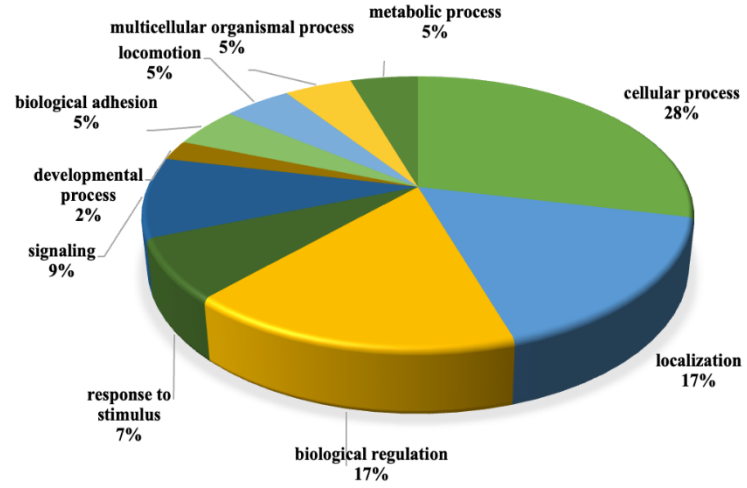
Dnah5, Myo1e and Rims2. In diazepam-treated, the expression of these proteins was highly decreased when compared with the Dextromethorphan-induced drug addiction, especially in the expression of Atp2b1 and Rim2. However, GABA-treated increased the expression of these proteins and a highly increased in PGBR-treated when compared with Dextromethorphan-induced drug addiction such as Atp2b1, Dnah5, Myole and Rim2 (Figure 33).

8 proteins associated with microtubule motor activity, ATP binding, ATPase activity and ion channel binding were focused on and searched for using STRING software for protein interaction. This result was also found Atp2b1 direct interaction with Calb1 (Figure 34).

A. Molecular function



B. Biological process



C. Protein class

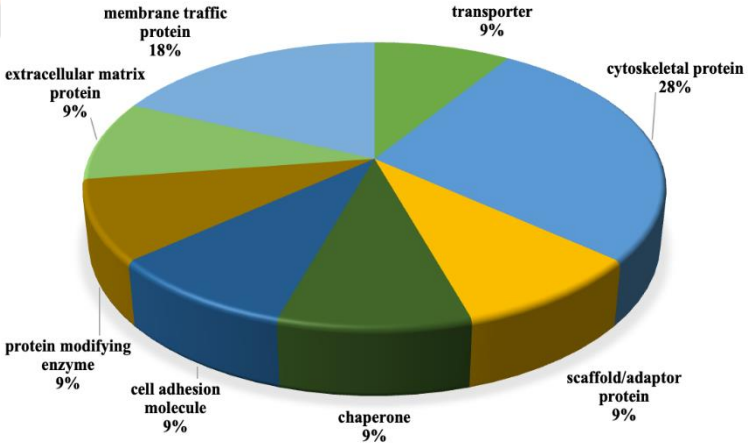


Figure 29 PANTHER showed that the classification of the differentially expressed protein in addiction treatments involves in molecular function (A), biological process (B) and protein class (C).

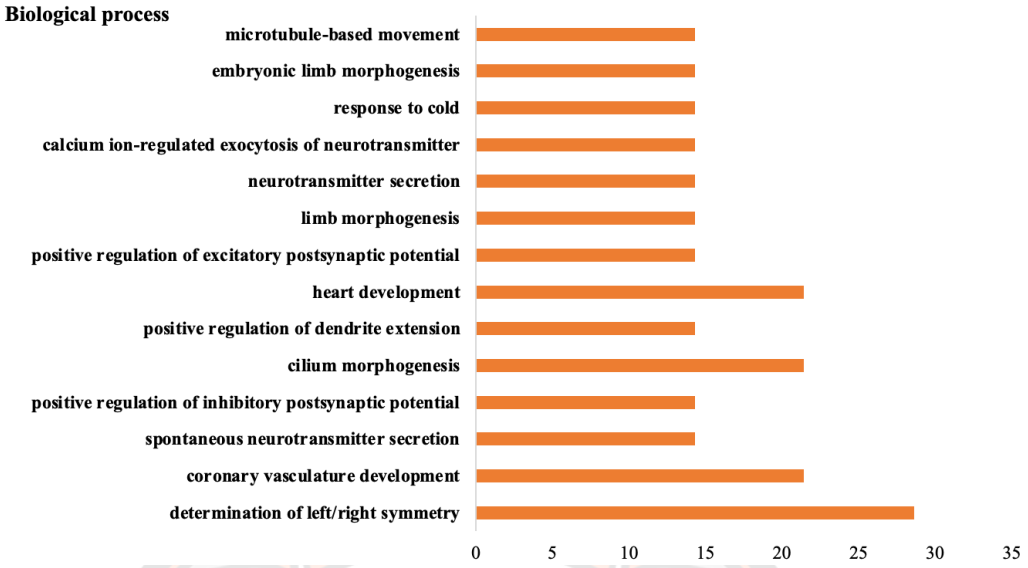


Figure 30 GO biological process analysis of the differentially expressed proteins in addiction treatments. 14 categories GO term enrichment analysis was statistically significant with p< 0.05.

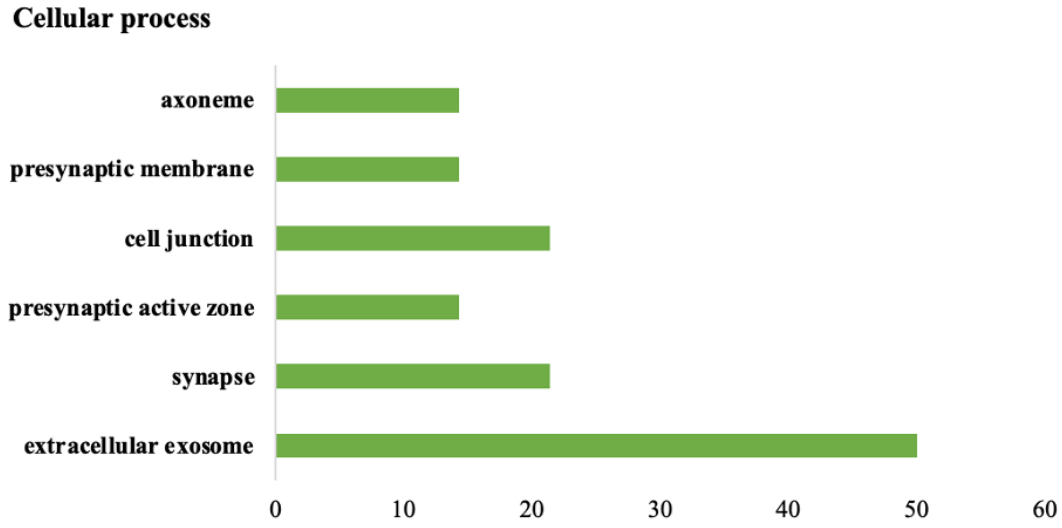


Figure 31 GO cellular process analysis of the differentially expressed proteins in addiction treatments. 6 categories GO term enrichment analysis was statistically significant with p< 0.05.

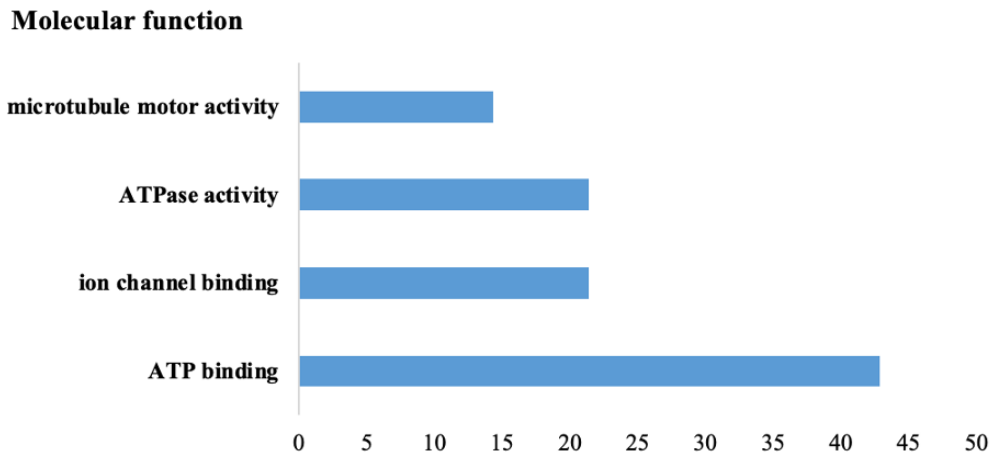


Figure 32 GO molecular function analysis of the differentially expressed proteins in addiction treatments. 4 categories GO term enrichment analysis was statistically significant with p< 0.05.

Table 4 The description of 6 differentially expressed proteins in addiction treatments involving molecular function includes microtubule motor activity, ATP binding, ATPase activity and ion channel binding.

Accession No.	Abbreviation	Full name	Function
gi|1476413370	Atp2b1	ATPase plasma membrane Ca2+ transporting 1	Calcium-transporting, ATPase activity, ATP binding
gi|81890303	Ddx46	DEAD-box helicase 46	ATP binding
gi|81917503	Dync2h1	dynein cytoplasmic 2 heavy chain 1	Microtubule motor activity, ATP binding, ATPase activity
gi|1270743798	Dnah5	dynein, axonemal, heavy chain 5	Microtubule motor activity, ATP binding, ATPase activity,
gi|122065208	Hsp90aa1	heat shock protein 90, alpha (cytosolic), class A member 1	ATP binding, ATPase activity
gi|23821863	Myo1e	myosin IE	ATP binding, ATPase activity

Table 4 (continued) The description of 6 differentially expressed proteins in addiction treatments involving molecular function includes microtubule motor activity, ATP binding, ATPase activity and ion channel binding.

gi|34395745	Rims1	regulating synaptic membrane exocytosis 1	Protein binding, ion channel binding
gi|34395746	Rims2	regulating synaptic membrane exocytosis 2	Protein binding, ion channel binding

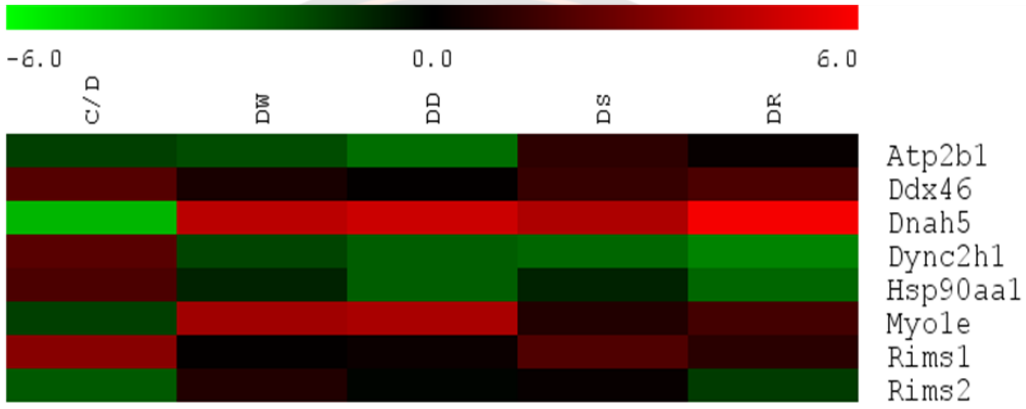


Figure 33 The heat map of differentially expressed proteins in addiction treatments was analyzed by Multiple Experiment Viewer (MeV). Proteins with low, moderate, and high levels of expression are represented by green, black, and red colors, respectively.

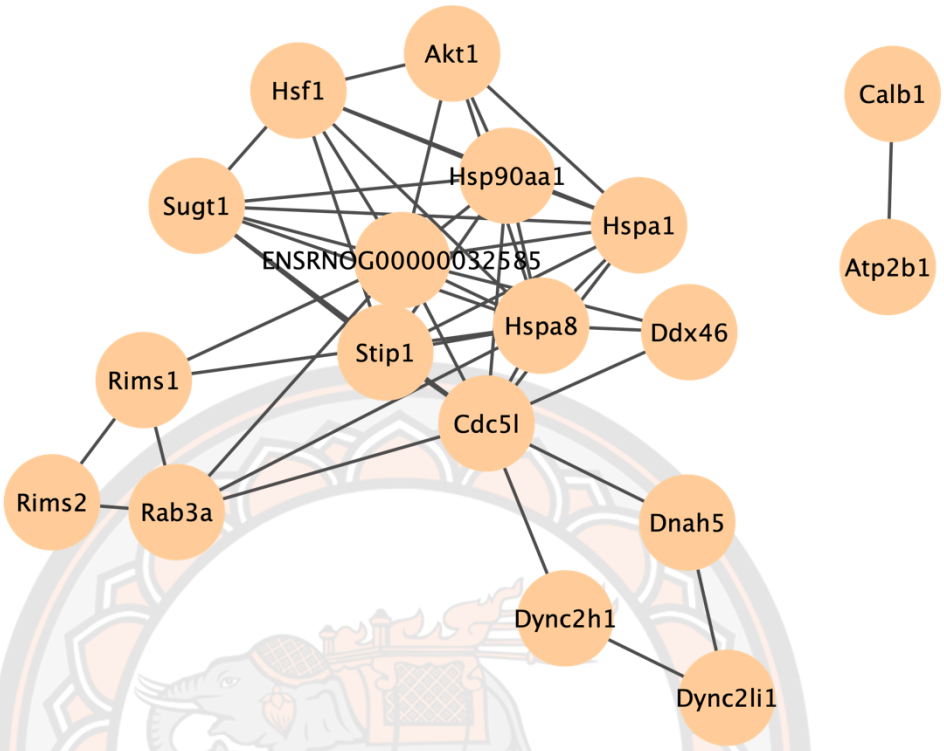


Figure 34 The network of protein-protein interactions (PPI) of the differentially expressed proteins in addiction treatments involves microtubule motor activity, ATP binding, ATPase activity and ion channel binding.

The expression of Calb1 immunoreactivity in spermatozoa

This study found that proteins in drug addiction are most associated with calcium signaling pathway that have a critical role in testicular and sperm function, which can indicate fertility. Additionally, the results of this study found that those proteins were directly interacting with Calb1, a type of protein in calcium-binding proteins.

This study found that Calb1 was expressed in the acrosomal part of the head and the middle piece of rat spermatozoa (Figure 35). Moreover, those expressions of Calb1 were classified into 3 levels: strong-positively stained spermatozoa, moderate-positively stained spermatozoa, and weak-

positively stained spermatozoa (Figure 36). The percentage of strong-positively stained spermatozoa for Calb1 immunostaining was highly significant decreased in METH treated group ($p<0.0001$) when compared with control group (Figure 37).

As shown in Figure 37, the relative optical density of Calb1 immunoreactivity in rat spermatozoa was significantly decreased in METH treated group ($p<0.0001$) when compared with the control group.

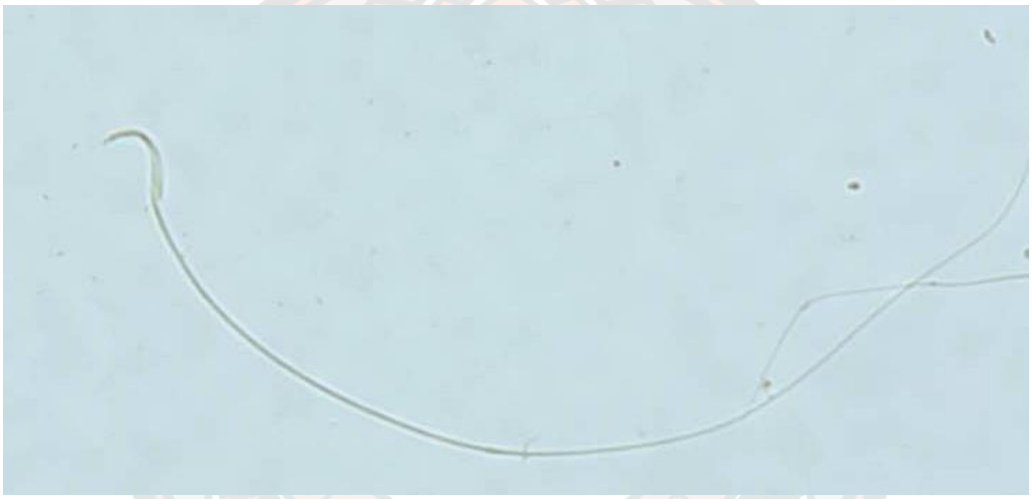


Figure 35 Immunostaining for Calb1 in rat spermatozoa at 200X magnification. A blue arrow indicates Calb1 localized in the acrosomal part and middle piece of rat spermatozoa.

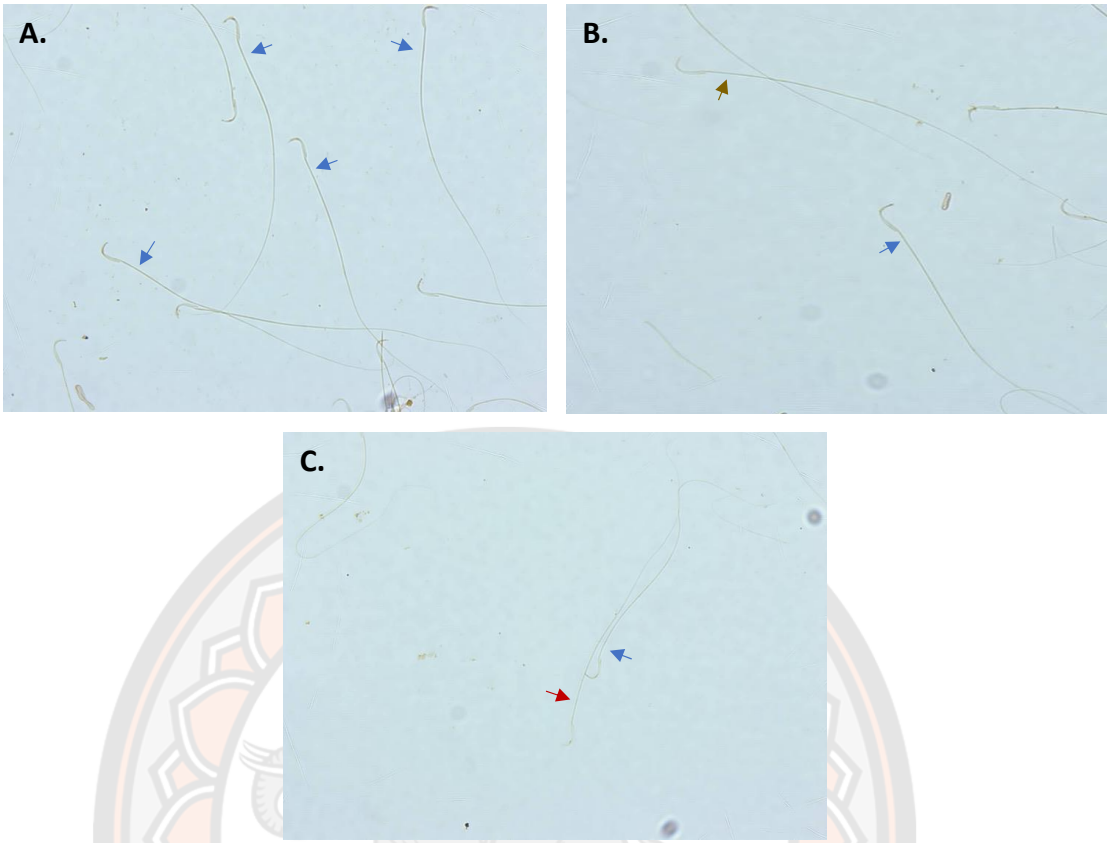


Figure 36 Immunostaining for Calb1 in rat spermatozoa at 200X magnification. Blue arrows in a-c indicate the strong-positively stained spermatozoa. A Brown arrow in b indicates moderate-positively stained spermatozoa. A red arrow in c indicates the weak-positively stained spermatozoa.

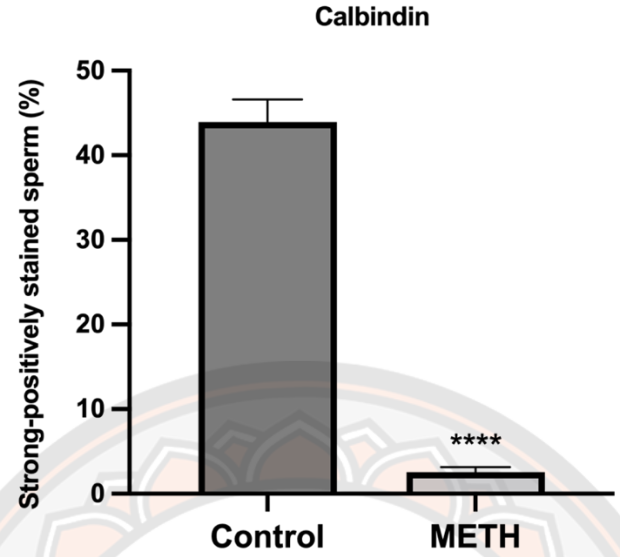


Figure 37 The percentage of strong-positively stained spermatozoa of Calb1 immunostaining in METH treated when compared with control group. Data are presented as mean ± SEM. ****Significantly different at p<0.0001; Independent t-test.

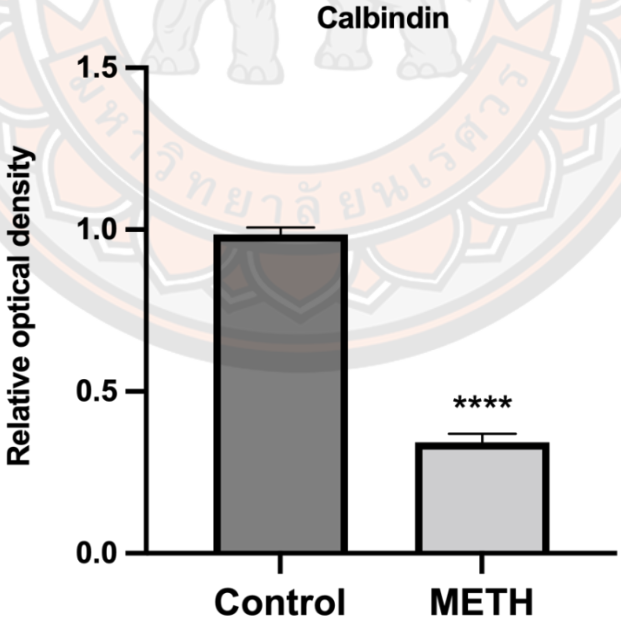


Figure 38 The relative optical density of Calb1 in rat spermatozoa of METH treated when compared with control group. Data are presented as mean ± SEM. ****Significantly different at p<0.0001; Independent t-test.

CHAPTER V
DISCUSSION & CONCLUSION

The result confirmed that the METH administration rat shows abnormal sperm morphology. The result agrees with our previous studies, which showed that METH affects sperm parameters of sperm quality, especially sperm concentration, sperm motility, and sperm morphology (Nudmamud-Thanoi, et al, 2016; Nudmamud-Thanoi & Thanoi, 2011). An increase of abnormal spermatozoa of male mice has also been reported in animal receiving high dose METH-administration (Sabour, et al., 2017). Moreover, our previous study reported that DXM-treated rats showed a reduction in sperm motility, the percentage of normal sperm morphology, and sperm concentration (Thanoi, et al, 2018). The previous study has also found that the sperm quality was reduced in DXM-treated rat (Biose, et al., 2013). As described earlier, METH and DXM administration cause drug addiction and can also reduce sperm quality, which affects male reproductive function. However, the functional and biochemical changes in the male reproductive system remain unclear.

The present study explained the protein expression profiles after drug addiction to evaluate the alterations in the proteome that could be responsible for these functional modifications. 28 differentially expressed proteins in drug addiction were identified and analyzed using bioinformatic tools. This result found that 4 proteins were related to the calcium signaling pathway, which play critical roles in calcium homeostasis, cell functions in the testis and sperm function (Alasmari, et al., 2013; Kong, et al., 2016; Vogl, et al., 2018). In this study, Plce1, Itpr2, Cacna1d and Atp2b1 were found to be related to the calcium

signaling pathway. Plce1 belongs to the phosphoinositol-specific phospholipase C (PLC) family, which plays an important role in the signal transduction processes including activation of intracellular molecules to produce second messenger such as inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), involving regulation of Ca^{2+} intracellular levels and protein kinase C activity (Essen, et al., 1997; Kadamur, et al., 2013). Previous studies have reported that Plcz1 was detected in spermatids in the testis and acrosomal segment of the head and principal piece of the flagellum spermatozoa (Bedford-Guaus, et al., 2011; Aarab, et al., 2012). In addition, abnormal PLC expression caused to abnormal of intracellular Ca^{2+} homeostasis and unable to induce Ca^{2+} oscillations associated with infertility (Yoon, et al., 2008; Heytens, et al., 2009). Itpr2 is an inositol 1,4,5-triphosphate receptor (IP3R) family and a calcium channel associated with intracellular Ca^{2+} stores. (Rosado and Sage, 2000; Kania, et al., 2017). Itpr2 was expressed in the spermatogenic cells and mature spermatozoa that play an important role in the regulation of spermatogenesis and mature sperm function through Ca^{2+} signal (Correia, et al., 2015; Lyon, et al., 2017; Zhang, et al., 2020). Previous studies found that inhibition of IP3R expression blocked spermatocyte proliferation and abnormal morphology of actin protein in the Sertoli-germ cell junction (Alzayady, et al., 2016; Adams and Vogl, 2020). Furthermore, Cacna1d and Atp2b1 were also associated with calcium signaling pathway. Cacna1d is a voltage-dependent calcium channel (VDCC) that regulates calcium ion influx into cells as well as calcium-dependent activities such as cell proliferation and cell death (Bell, et al., 2001). Atp2b1 or PMCA1 is a plasma membrane calcium-transporting ATPase family (PMCAs). There are 4 isoforms such as PMCA1,

PMCA2, PMCA3 and PMCA4 that play an essential role in calcium efflux for intracellular Ca^{2+} homeostasis (Gong, et al., 2018). Previous studies have reported that PMCA1 and PMCA4 are widely distributed in general tissues (Okunade, et al., 2004; Bruce, 2018). PMCA4 expression was discovered in the testis and is controlled by the androgen receptor (Schuh, et al., 2004; Patel, et al., 2013). PMCA4 expression levels are reduced in the testes of androgen receptor knockout mice. which may play a significant role in spermatogenesis (Sun, et., 2021). However, the study has been reported that PMCA1 and PMCA4 are found in a frequently co-express in the same cell (Stauffer, et al., 1995).

Studies from our team have reported that drug addiction reduces androgen receptor expression and induces apoptotic cells in the testis, leading to spermatogenesis impairment (Nudmamud-Thanoi and Thanoi, 2011; Nudmamud-Thanoi, et al., 2016; Thanoi, et al., 2018). That might be related to the result of this study, which showed the downregulated expression of Atp2b1 (PMCA1) in drug addiction indicated lower PMCA activity (Fernandes, et al. 2007). In addition, the present data shows increased Cacna1d expression after METH exposure that might be related to an increase in VDCC activity (Sberna, et al, 2002; Murakami, et al., 2008). The alteration of both results in disruption of intracellular Ca^{2+} homeostasis leading to intracellular Ca^{2+} overload induced ROS production, cell cycle arrest, and apoptosis resulting to spermatogenesis impairment (Schwab, et al., 2002; Cross, et al. 2010; Guo, et al., 2021). Moreover, the result shown that Cacna1d and Atp2b1 directly interaction with calbindin (Calb1) by STRING. Calb1 is one type of calcium-binding proteins (CaBPs) localized to the sperm

acrosome and principal piece of sperm flagellum (Schlingmann, et al., 2007; Dopeso-Reyes, et al., 2014; Shawki, et al., 2014). CaBPs plays an essential role in sperm function including motility and acrosome reaction that indicates sperm quality and male fertility (Chazin, 2007; Valsa, et al., 2015). In the present study, Calb1 expression was also found in the acrosomal region and principal piece of the sperm. There was a highly decrease of Calb1 expression in drug addiction. The reduction of CaBPs was related to a declining level of intracellular Ca^{2+} in sperm, which resulted in sperm dysfunction (Schmid, et al., 2012). However, a decrease of Calb1 expression in the sperm might be the result of spermatogenesis impairment.

Furthermore, this study found decreased Atp2b1 expression in diazepam treatment. The result indicates an adverse effect of diazepam lead to disrupted Ca^{2+} homeostasis that results in cell cycle arrest and apoptosis. Previous studies have been reported that diazepam results in the structural of seminiferous tubule change and reduces sperm quality (Taher, et al., 2015; Thanoi, et al, 2018). However, GABA and PGBR treatments showed an increase in Atp2b1 expression, especially PGBR treatment. These findings could be related to the effect of GABA and PGBR on intracellular Ca^{2+} homeostasis in the testis, resulting in improved spermatogenesis. PGBR has high among of GABA and antioxidants including α-tocopherol (vitamin E), pyridoxine (vitamin B6) and thiamine (vitamin B1), especially γ-Oryzanol (Roboon, et al., 2016; Zhang, et al, 2010). In addition, γ-Oryzanol can block ROS activation and inhibit cell apoptosis, which may relate to spermatogenesis and lead to improved sperm quality (Zhu, et al., 2019; Huang, et al., 2020).

The present study showed that alteration of the protein profile after drug addiction, which is associated with the calcium signaling pathway, causes disrupted intracellular Ca^{2+} homeostasis in the testis and might be related to spermatogenesis deficiency, resulting in reduced sperm quality. In addition, alteration of the protein profile in diazepam treatment indicated an adverse effect, while GABA and PGBR treatments showed a recovery effect on the testis via intracellular Ca^{2+} signal. These findings examined the effects of drug addiction and addiction treatments on spermatogenesis and sperm quality through Ca^{2+} homeostasis.

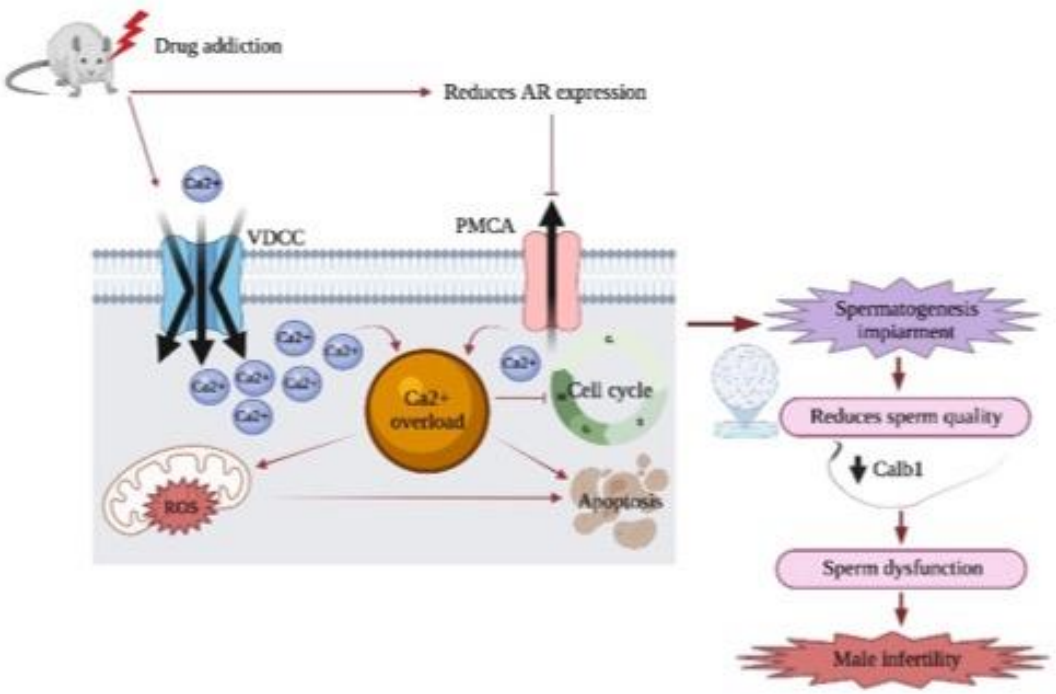


Figure 39 The schematic of drug addiction affects the alteration of intracellular Ca^{2+} homeostasis via the Ca^{2+} channel, leading to spermatogenesis impairment in the testis and causing reduced sperm quality resulting in male infertility.

APPENDIX

APPENDIX I List of solution and buffer

10% formaldehyde solution, 1L

100 ml	37-40% formalin
4 g	NaH_2PO_4
6.5 g	Na_2HPO_4
900 ml	H_2O

Testis homogenate buffer

5 mM	Tris-HCl, pH 8.0
25 mM	NaCl

Lysis buffer

50 mM	Tris-HCl, pH 8.0
0.15 M	NaCl
0.1%	SDS
0.25%	Sodium deoxycholate
1%	Protease inhibitor cocktail, add before use

20X PBS buffer, pH 7.4, 1L

37.1 g	$Na_2HPO_22H_2O$
8.6 g	KH_2PO_4
144 g	NaCl

APPENDIX II certificates of approval from research ethics committee for animal study

เอกสารรับรองโครงการ

คณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์

มหาวิทยาลัยนเรศวร (คกส.)

ชื่อโครงการ	การวิเคราะห์การตอบสนองของโปรตีนต่อการติดสารเสพติดในอัณฑะของหนูทดลองด้วยช็อทกันโปรติโอมิกส์ Shotgun proteomic analysis of proteins responding to drug addiction in rat testis
เลขที่โครงการ	NU-AEE620910
เลขที่เอกสารรับรอง	62 02 012
ประเภทการรับรอง	แบบยกเว้น
ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ	รศ.ดร.สุทิสา รานอย
สังกัดหน่วยงาน /คณะ	วิทยาศาสตร์การแพทย์
วันที่รับรอง	20 พฤศจิกายน 2562
วันสิ้นสุดการรับรอง	20 พฤศจิกายน 2565

ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์
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