THE EFFECT ON MALE RATS' REPRODUCTIVE FUNCTION AFTER LONGTERM ADMINISTRATION OF METHAMPHETAMINE HHYDROCHLORIDE AND ITS WITHDRAWAL

by

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ADD	Attention-deficit disorder
ATS	Amphetamine-type stimulants
cAMP	Adenosine 3':5'-cyclic monophosphate
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
HIV	Human immunodeficiency virus
HPG	Hypothalamus-pituitary-gonadal
H&E	Haematoxylin and eosin
GnRH	Gonadotrophin releasing hormones
LH	Luteinizing hormone
MA	Methamphetamine hydrochloride
P2P	Phenyl-2-propanone
MDMA	Ecstacy
MAT	Methamphetamine treated
mRNA	Messenger ribonucleic acid
MRW	Mean relative weight
M12	Methamphetamine treated of 12 weeks
NIDA	National Institute of Drug Abuse
SEH	Seminiferous epithelial height
STD	Seminiferous tubular diameter
T	Testosterones hormone
TUNEL labelling	Terminal deoxynucleotidyl tranferase (TdT)-mediated dUTP-biotin nick end
W2	2 weeks withdrawal
W4	4 weeks withdrawal
W8	8 weeks withdrawal
W12	12 weeks withdrawal

LIST OF PUBLICATIONS & SEMINARS

Oral presentation

Yusof, M. F. H., Islam, M. N. & Hasnan Jaafar. 2009. Effect Of Mehtamphetamine (MA) And Its Withdrawal On Pituitary Gonadal Hormones And Sperm Count. 2nd USM-Penang International Postgraduate Convention: 2nd Health and Medical Sciences Conference. USM

KESAN RAWATAN JANGKA PANJANG METHAMFETAMIN HIDROKLORIDA DAN PEMBERHENTIANNYA TERHADAP FUNGSI REPRODUKTIF TIKUS JANTAN

ABSTRAK

Eksperimen ini mengkaji kesan rawatan jangkapanjang methamfetamin hidroklorida (MA) dan pemberhentiannya terhadap serum gonadotrofin tikus jantan iaitu hormone perangsang folikel (FSH), hormone peluteinan (LH) dan testosterone (T), berat relatif organ-organ pembiakan, morfologi testis, kiraan dan morfologi sperma.

120 ekor tikus jantan Wistar berumur 12 minggu dibahagikan kepada tiga kumpulan; kawalan, placebo dan MA terawatt. Tikus terawat MA menerima rawatan harian 5 mg/kg berat badan melalui intraperitoneal, kumpulan placebo menerima rawatan salin normal. Setiap kumpulan mempunyai 5 subkumpulan; iaitu M12, W2, W4, W8 dan W12 yang telah ditetapkan dengan lima tempoh pemberhentian berbeza iaitu sifar, dua, empat, lapan dan 12 masing-masing. Apabila tempoh pemberhentian tamat, tikus akan dibius dengan eter dan dimatikan dengan dislokasi servikal dan dituruti laparotomi. Darah diperolehi dari vena kava inferior dan diukur aras FSH, LH dan T serum. Epididmis kanan dipicit dengan halus dan sperma dikumpulkan dan ditempatkan di dalam 2 ml salin normal. Kemudian, epididimis dicincang dan dicampurkan dengan sperma yang terkumpul dan akhirnya ditapis. Bahan yang tertapis diwarnakandengan 1% eosin untuk kajian morfologi sperma dan pengiraan sperma. Testis, epididimis, kelenjar prostat dan vesikel simen ditimbang untuk ditentukan berat

organ relative. Hirisan 4µm tisu benaman paraffin testis diwarnakan dengan haematoxilin dan eosin setelah 24 jam diawet dengan larutan Bouin. Setelah itu, garispusat tubul seminiferus (STD) dan ketinggian epitelium seminiferus diukur menggunakan penganalisis imej. Didapati rawatan MA mengurangkan aras FSH dan LH serum dengan signifikan pada kumpulan W8 dan W12. Aras T serum juga rendah secara signifikan. Berat relatif testis dan epididimis dari kumpulan W2, W4 dan W8 turut rendah dengan signifikan. Bagaimanapun, berat relatif kelenjar prostat dan vesikel semen tidak terkesan dengan rawatan MA. STD untuk W2 dan W4 serta SEH untuk kumpulan W2, W4 dan W8 menunjukkan pengurangan ukuran yang signifikan. Morfologi sperma dan kiraan sperma juga berkurang dengan signifikan untuk kumpulan M12, W2, W4 dan W8 masing-masing. Setelah 12 minggu pemberhentian, aras FSH dan LH kembali kepada aras normal. Aras T serum kembali pulih kepada aras normal setelah 4 minggu pemberhentian. Berat relatif testis dan epididimis juga kembali ke jajaran normal setelah lapan minggu pemberhentian. Morfologi dan kiraan sperma turut pulih setelah 12 minggu pemberhentian.

Kesimpulannya, rawatan 5 mg/kg berat badan selama 12 minggu menunjukkan mempunyai kesan yang negatif terhadap fungsi reproduktif tikus jantan. Bagaimanapun, setelah menjalani tempoh pemberhentian yang tertentu, fungsi reproduktif kembali normal dan kerosakan yang dilihat berjaya dipulihkan.

THE EFFECT ON MALE RATS' REPRODUCTIVE FUNCTIONS AFTER LOMG TERM ADMINISTRATION OF METHAMPHETAMINE HYROCHLORIDE AND ITS WITHDRAWAL

ABSTRACT

This experiment investigate the effect of methamphetamine hydrochloride (MA HCl) administration and its withdrawal on the male rat reproductive functions. We studied the serum gonadotrophins which are follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone, relative reproductive organs weight, testis morphology and sperm count and sperm morphology.

One hundred and twenty 12 week old male Wistar rats were divided into three groups; control, placebo and MA treated. MA treated rats received 5 mg/kg body weight (bw) dose of MA daily intraperitoneally whereas the placebo rats were administered normal saline. Each group consists of five subgroups; M12, W2, W4, W8 and W2, which were assigned with five different withdrawal weeks of 0, 2, 4, 8 and 12 weeks respectively. At the end of withdrawal, the rats were anesthetized by ether and sacrificed by cervical dislocation followed by laparotomy. The blood was obtained from inferior vena cava and later analysed the serum concentration of FSH, LH and testosterone. The isolated right epididymis was gently pressed and sperms were collected. Later the epididymis was minced and mixed with the collected sperms and filtered. The filtered material was stained with 1 % eosin for sperm count and sperm morphology assessment. The testes, left epididymis, prostate gland and seminal vesicle were weighed and determined for their relative organ weight. The testes were stained with haemotixylin and eosin for histological analysis. Consequently, seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH) were measured using image

analyzer. Our results showed that the rats of MA administration group has significantly reduced serum FSH and LH concentration of W8 and W12 .The serum testosterone concentration in W2 and W4 were also significantly reduced. The testis and epididymis relative weight of W2, W4 and W8 were significantly reduced. However the prostate gland and seminal vesicle relative weight were not affected by the MA. The STD of W2 and W4 and SEH of W2, W4 and W8 demonstrated significantly reduced measurement. The sperm morphology and sperm count were also significantly lowered in W2, W4, W8 and W12 groups respectively. After 12 weeks of withdrawal, the serum FSH and LH return to normal. Serum testosterone concentration was restored following 4 weeks of withdrawal. The relative weight of testis and epididymis returned to normal range after 8 weeks of withdrawal. The STD and SEH measurement also returned to normal range after eight and four weeks of withdrawal respectively. Sperm count and sperm morphology also improved after 12 weeks of withdrawal.

In conclusion, the administration of 5 mg/kg bw MA 12 weeks appears to be harmful to the male rat reproductive function. However, after certain withdrawal weeks, the functions restored to normal and the damages are reversible.

CHAPTER ONE
INTRODUCTION

1.1 History of methamphetamine

There is a recent increasing trend of substance issues; a preference of abusers of synthetically manufactured drug and also huge increase in reported use of illicit designer drugs globally as well regionally. Amphetamine-type stimulants (ATS) accounts for most of the statistic and methamphetamine (MA) falls in ATS class. This critical situation reflects the MA's "hi" neuropsychiatric effect, easy availability, relative affordability, presumed safety and in some instances, perceived legality (Babu *et al.,* 2005). According to Anglin *et al.,* (2000) historically, methamphetamine was initially produced in Japan during the late 1800s. The drug became extensively used during the World War II by the Japanese, United States and German army to overcome fatigue and increase alertness (Scott *et al.,* 2007). This illicit psychostimulant is amongst the most abused drug recognized internationally (Krasnova & Cadet, 2009).

In Malaysia, this substance is better known as syabu while in Thailand, this drug name is called yaa baa. Recently, Abdul Muhid (2009) reported MA worth of Ringgit Malaysia 254 million was seized in Pahang, Malaysia. The Narcotic Criminal Investigation Department (Royal Malaysia Police 2008) reported that during 2007, 65.49 kg, 50.20 litre and 121,629 pieces of solid, liquid and tablet of MA respectively

were seized. In 2007, there were 1235 MA abusers, 8.52 % from the total recorded drug addicts registered at the rehabilitation centers (Buletin Dadah 2007).

With the increase abuse of MA, it has resulted for more demands on treatments services. However, the effective treatments of MA dependence are limited. Several reports noted the treatments for MA (Baker *et al*., 2004; Shearer and Gowing 2004) are relatively less effective than the other drug dependence. Present approaches to the management of MA withdrawal and dependence include the prescription of benzodiazepines, antipsychotics and/or antidepressants along with symptomatic medications.

In this study we evaluated the alterations which occurred due to MA administration and withdrawal effects. The documentation of changes and amelioration of the withdrawal would be of significant achievements to observe the effect of MA to the reproductive functions particularly. And to date, there is no literature that reports the withdrawal effect of MA administration to the male rat reproductive functions.

1.2 Manufacturing of methamphetamine

Methamphetamine production is relatively cost-efficient and easy. It can be synthesised in clandestine laboratory with over-the-counter ingredients. The most commonly utilised, simple and cheap method to manufacture methamphetamine is the reduction of ephedrine or pesudoephedrin (Figure 1.1).

According to Martyny *et al.*, (2008), prior to restriction placed on phenyl-2-propanone (P2P) with the Federal Chemical Diversion and Trafficking Act of 1998, the predominate early production method in the clandestine laboratory used P2P as the precursor. This kind of method can be very aromatic and very tricky and the person who handles it must have some background and knowledge in chemistry. Though this method was quite popular, it yields a low quality MA with less addictive properties compared to the present production process.

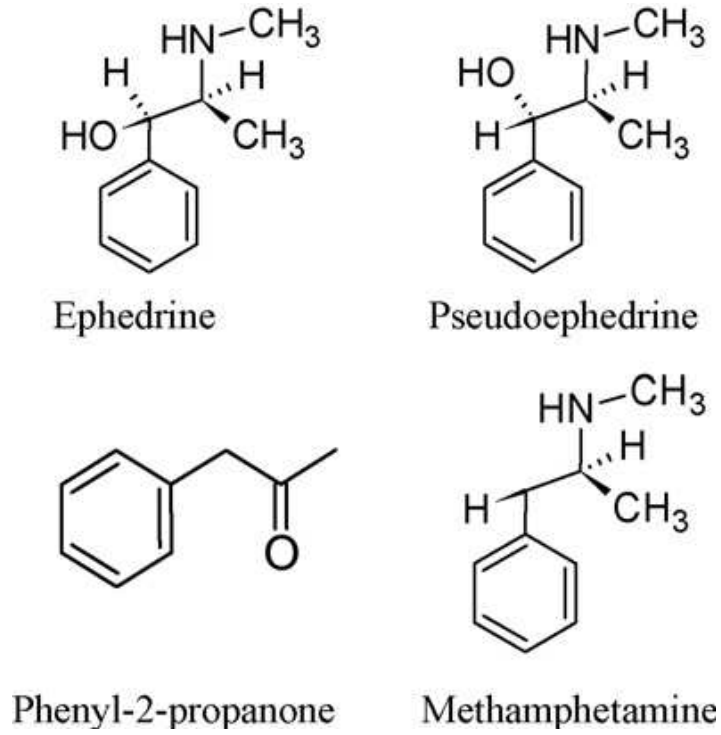


Figure 1.1: The chemical structures of ephedrine, pseudoephedrine, phenyl-2-propanone and methamphetamine

As the act is enacted, obtaining of the precursor became difficult. The illegal manufacturers switched off their preference and started to use ephedrine or pseudoephedrine as precursors in the production formula. Structurally, these compounds are analogous to methamphetamine with ephedrine differing only by a single hydroxyl group. Because this method can produce higher purity d-methamphetamine; which is more physiologically active, most of the clandestine laboratories today use ephedrine or pseudoephedrine method and are the most commonly found by the enforcement (Gibson 2002).

Burgess *et al.*, (2003) reported that as ephedrine and pseudoephedrine are getting more difficult to be acquired, the MA manufacturers nowadays extract these precursors from tablets prescribed for common cold. The method is performed by blending the crushed pills with light solvent (water or alcohol) to extract the essential parts. Compared to water, alcohol is more “user-friendly” because it evaporates faster, however there are few disadvantages; this compound is flammable and can result in fire.

1.3 Prevalence of methamphetamine abuse

Methamphetamine abuses are not only face by Malaysia (Daniel 2008), (Jebanesan 2008), but has become a regional and global attention (United Nation 2008).

In the past decade, Southeast and East Asia have experienced a methamphetamine abuse which began around 1997 and peaked in 2000 – 2001 (McKetin et al. 2008). He also noted that South East and East Asia are one of the global hubs for MA production and trafficking over the past decade. And for this reason, there is a major concern to minimise the potential consequences of spreading methamphetamine production, trafficking and use in the Mekong region and in the peninsular and archipelago of Southeast Asia (McKetin et al. 2008). According to Milesi-Hallé at al. (2005), another explanation of the increasing abuse in of MA around the world is due to its ease of manufacture.

United Nation (2008) explained that MA abuse is unique. The market of the substance is mostly the youth age group, the price is relatively cheap compared to other drugs plus the low cost of production. Statistically, in 2007, the global consumers of the ATS are approximately 25 million people which is larger than the markets for cocaine and heroin. From this figure, United Nation estimated that around 15-16 million are MA abusers.

According to Buxton and Dove (2008), there are users sex-dependant which influence the public to use MA. The men consumed MA to improve sexual performance while for women, the bodyweight issue encouraged the abuse. Several studies associated the use of MA with unprotected sexual activities among the HIV “at-risk” gay and bisexual men (Frosch 1996). Semple et al (2002) reported that MA motivated users to experience more pleasurable sex, assist them to be confident to approach for

sexual partner and to have sex without being emotionally connected. Methamphetamine is also being used for self-medication of negative effects associated with HIV+ serostatus and to make them feel better physically and push away negative perception about being HIV+.

Methamphetamine is also associated with the criminal incidence. Tyner & Fremouw (2008) implied that the use of methamphetamine is perceivably associated with violence. One of the signs and long term effects of MA use is the action of violence by the MA user (Buxton & Dove 2008). Dluzen & Liu (2008) reviewed that violent behaviour problems of the female abusers under the influence of the MA was more characteristic compared to the male abusers. However, Zweben et al (2004) found that the weapon charges and quantity of assaults appeared to be more in males and Cohen et al. (2003) reported that females are subjected to more violence such as sexual abuse and the females too significantly have experienced sexual abuse during their childhood.

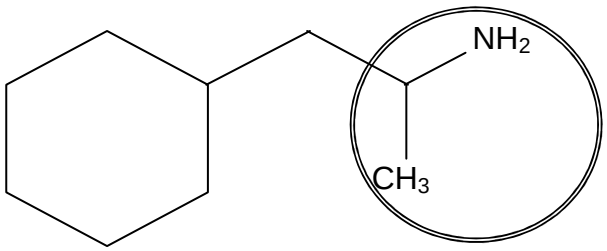
The innocent children who lived with the caretaker operating the clandestine laboratories are also exposed the threat of MA. This threat is not only the hazard from the chemical ingredients to manufacture the MA, it was may also observed the violent behaviour, involuntarily eyewitnessed when the police force apprehend their caregiver (Horton et al 2003). This extreme condition could affect the children emotionally as well as affect the cognitive functions and behaviour (Swetlow 2003).

1.4 Pharmacology and pharmacokinetic of methamphetamine

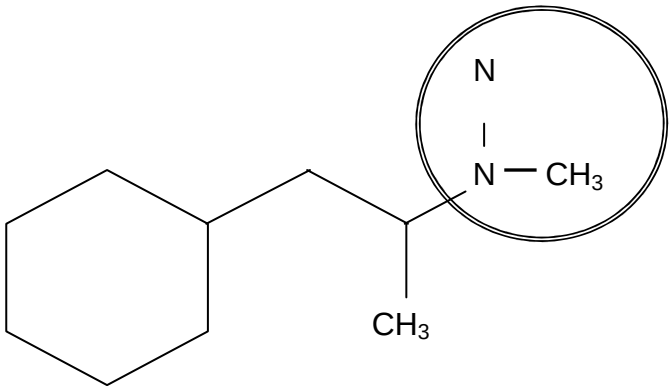
Methamphetamine Free Base is N, α-Dimethylbenzeneethanamine $C_6H_6CH_2CH(NHCH_3)CH_3$. Its molecular weight is 149.24. It is highly hydrophilic because of the hydrochloride salt. Physically, MA is crystalline powder, white in color, odourless and bitter and can be dissolved in water and alcohol (Yu et al 2003). Methamphetamine is structurally related to amphetamine, with a variant of the methyl group addition (Figure 2).

Methamphetamine is popular among the recreational users and it is sold illicitly. While there are a lot of hazardous threats reported, this drug is legally available with its trade name Desoxyn® since the 1940 in various dosages to treat patients with Attention-Deficit Disorder (ADD), narcolepsy as well as obesity

For oral prescription, Abbot Laboratories of The United States provide the tablet at the dosage of 5 mg. The recommended treatment regime for ADD is 20-25 mg daily. To treat the obesity, the effective dose is one tablet of 5 mg which has to be taken prior to each meal. However, this medication is not suitable to treat obesity in children below 12 years old and also not applicable to for treating ADD in children below six years old. Under this few accepted medical reasons, this drug is only available as a prescription (Bray 1993; Mitler et al.1993; Jirovsky *et al.*, 1998; National Institute on Drug Abuse 2006).



Amphetamine



Methamphetamine

Figure 1.2 : The different between the structure of methamphetamine and amphetamine

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According to Jirovsky *et al*., (1998), the utilisations of methamphetamine were wide in human medicine during 1930s. Because of the high toxicity and unwanted side-effects (dependence), it was then replaced by other less hazardous drugs. Ironically, methamphetamine is still prescribed for certain illnesses. Unlike methamphetamine related substances such as 3, 4- (methylenedioxy) methamphetamine (ecstasy) and methcathione which are by no means marketed and used for medication; methamphetamine is a remedy to treat narcolepsy as well as children associated with attention-deficit disorder.

The use of amphetamine as anorectics has been substituted by a more suitable drugs due to unfavourable properties. Since 1991, in the Unite States, pure ( )-MAP is an ingredient in nasal inhalers which serves as decongestant. The current development, however, is to establish amphetamines only as virtual drug; it performs as an intermediate in the other drugs manufacturing. ( )-MAP acts as a precursor for producing of (+)-selegiline (deprenyl), an effective antidepressant and antiparkinson.

A study by Cook et al (1992) and Cook et al (1993) investigated the pharmacokinetic of MA in humans and the drug is shown to have extensive extravascular distribution (volume of distribution = 3.7 l/kg) and a long terminal elimination half-life ($t_{1/2\lambda} \approx 3.7$ l/kg). In humans, the average plasma half-life is ~ 12 hours, with nine to thirteen hours range. Lynch et al. (2002) reported that women during

the luteal phase have greater gastrointestinal transit time which then suggested greater absorption in women.

Methamphetamine is an addictive psychostimulant and has powerful effects on the central nervous system. As an effect of the presence of methyl group in MA chemical structure, the substance has a relatively high lipid solubility therefore resulting quicker transport of the drug across the blood-brain barrier (Barr et al. 2006). The blood barrier is very important for metabolic transport, brain homeostasis as well as protection against bacterial infections as well harmful molecules substances.

Methamphetamine affects the nerve endings by elevating the discharge of extracellular monoamine neurotransmitter; dopamine, serotonin and norepinephrine hence increasing the neurotransmitter level (Rothman & Baumann 2003). Zaczek et al. (1990) described that MA administration stimulates the release of dopamine via a number of different molecular mechanisms including displacement of storage vesicles and inhibition of monoamine oxidase, although the dopamine transporter maybe the primary site of action (Giros et al 1996). Sulzer et al (2005) suggested the process involve redistribution of neurotransmitters from synaptic vesicles to the neuronal cytoplasm and the reverse transport of neurotransmitters through the plasma membrane transporter into the extracellular space.

Caldwell et al. (1972) studied the metabolism of MA in the rat, guinea pig and also human. The research examined the metabolites of MA metabolism in the urine of the three models.It was evident that in the rat, the main metabolic process involved is

aromatic hydroxylation. The key process that occurs in the guinea pig is demethylation followed by side-chain degradation. Whereas in human, both metabolic process; aromatic hydroxylation and demethylation are the major processes.

A study conducted by Kanamori et al. (2005) investigated the in vitro metabolism of MA by using rat hepatocytes (Figure 3). They used freshly isolated male Wistar rats' hepatocytes which were to be treated with 2 different initial concentration of MA; 100µM and 10µM. The extracted metabolites from the culture fluids were analyzed by using the GC/MS after derivatization. Amphetamine, p-hydroxymethamphetamine and p-hydroxyamphetamine were detected in the culture fluids of the rat hepatocytes inoculated with MA.

Kanamori *et al*., 2005 found the same processes; p-hydroxylation, N-demethylation and β-hydroxylation took place in the hepatocytes which were identical as reported by Caldwell et al. (1972). And the detected metabolites from the extraction are p-hydroxymethamphetamine, amphetamine and p-hydroxyamphetamine. These metabolites are MA initial concentration dependant (Kanamori et al. 2005). However, in Kanamori at al. (2005) in vitro study, they did not discover certain metabolites which were found by Caldwell et al. (1972) in vivo study.

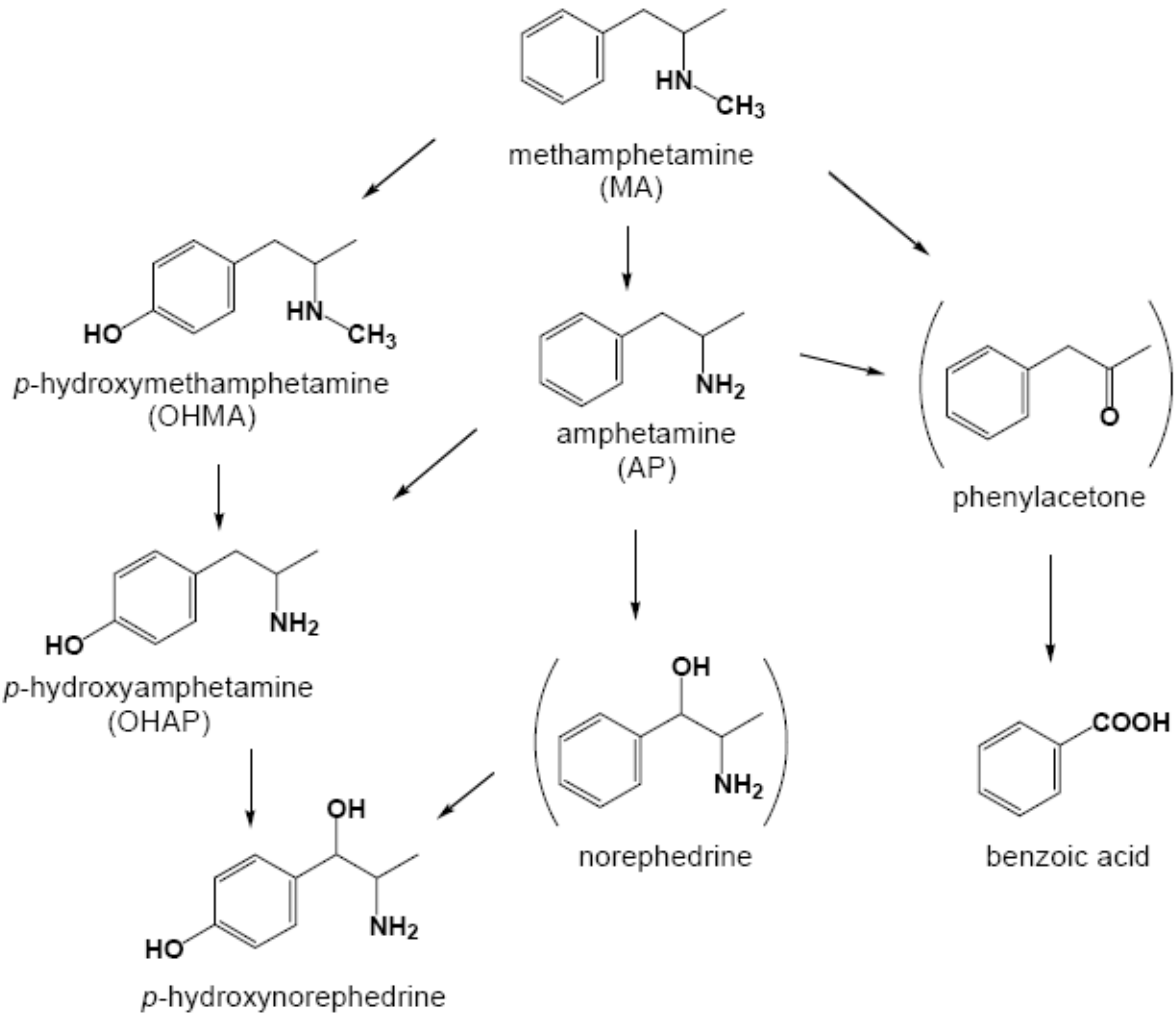

Figure 1.3 : The metabolic pathway of methamphetamine in the rat

There are several typical symptoms of MA intoxication such as increased self-confidence (Beckett and Rowland, 1965) and also impression of internal power and self-belief as reported by Caldwell *et al.,* (1972). The drug is abused for many reasons. If taken, it causes a quick and long lasting high accordance of administration; resulting in intense excitement (Center for Disease Control and Prevention, 1995).

Drug addicts admitted for rehabilitation should be managed with specific treatment. According to Babu et al (2005), the treatment regimes for amphetamine intoxication are provision of supportive care, correction of vital signs, tranquillizing with benzodiazepines and the electrolyte imbalance treatment. Gary and Saidi (1977) reported that droperidol was used to treat MA poisoning combined with acid diuresis. Droperidol acts by antagonizing the amphetamines hence turning the patient calm and cooperative.

1.5 Effect on the methamphetamine on reproductive function

There are studies conducted using animal models to investigate the toxicity of MA administration and NIDA (2002) has reported that neurological, developmental and reproductive effects have been identified as the most sensitive endpoints from methamphetamine exposure. Yamamoto et al. (1991) administered JCI: ICR mouse colony with eight different doses; 0, 11, 13, 14, 15, 17, 19 and 21 mg/kg/day of MA with a single intraperitoneal injection during the eighth of gestational day. The mice foetuses were assessed for external malformation and examined for skeletal irregularities. The external malformations were detected at 19 mg/kg/day dose whereas the skeletal irregularities were observed at 14 mg/kg/day dose.

A study by Cho et al. (1991) examined the effect of MA treatment subcutaneously to Wistar rats. The administration was done at 0, 1, 2, 3 and 4.5 mg/ kg for 14 days from day 7 to 20 of gestation. The dams were inspected for weight gain, health and mortality whereas the pups were examined for weight gain, external anomalies, development of physical characteristics and functional reflexes. The pups which received 3 and 4.5 mg/ kg of MA treatment were found to have significantly reduction in their body weight, delayed testicular descent and incisor eruption. In the other study done by Acuff-Smith et al. (1996), the MA administration could alter behaviour such as delayed development of early locomotion and memory impairment, declining body weight, postnatal mortality and decreased litter size.

Saito et al. (1991) reported that MA affected the copulatory behaviour of male Wistar-Imamichi with sexually receptive female. Mounts, intromissions and ejaculation of semen were significantly reduced when they were given 4 mg/ kg-day. A study using male ICR mice treated with MA paired with female showed that vaginal plugs and birth significantly decreased. Sperm motility was also reduced at the dose of 15 mg/kg/day as well the serum testosterone level (Yamamoto et al. 1999).

Yamamoto et al (2002) conducted a study to examine the possibility of the MA in term of inducing apoptosis in the male mice testis. Four different MA doses were tested; 1, 5, 10 and 15 mg/kg and terminal deoxynucleotidyl tranferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) assay was employed to detect the fragmented DNA from the apoptotic testicular tissue. It appears that spermatogenic cells are influenced by the administration of MA and the cells were apoptotic. The apoptotic tubules are dose dependant from 5 to 15 mg/kg and there is a significant increase of apoptotic tubules even at the dose of 5 mg/kg.

1.5 Methamphetamine Withdrawal

After the effect of MA administration disappear, the substance abusers experience some features of the methamphetamine withdrawal syndromes such as depression, anxiety, sleep disturbance and also improved appetite (McGregor *et al.*, 2005). On the contrary to the effects of MA intoxication, anhedonia, reduced concentration and aggression are among MA abstinence syndromes which are observed (Newton *et al*. 2004). McGregor *et al.*, (2005) also reported that withdrawal severity was greater in those who were older, more dependent and who had been taking MA longer. Murray (1998) suggested that the reason of higher abuse possibility as well the relapse rates among the users is caused by the MA intense and persistent withdrawal effects.

Hoefer *et al.*, (2006) tested the repeated high doses MA on the Wistar rats and observed its' withdrawal effect. The withdrawal in resulted a significant reduction in responding to a sweet solution reward on a progressive-ratio schedule of reinforcement. The outcome of the experiment may reflect a lack of motivation to seek a naturally rewarding stimulus. Even at as low as 40 mg/kg/day dose, the same withdrawal effect could be observed in 55 mg/kg/day dose group.

Islam *et al.*, (1995) investigated the effect of the 1 mg/kg of MA on the Wistar rats' myocardium and the withdrawal outcome. After 12 weeks, the study found changes in the myocardium changes such as atrophy, hypertrophy, edema and myolysis.

The rats which have undergone withdrawal stage however showed reversible changes in their myocardial tissues and this recovery appeared during the third week of withdrawal.

Although there are few studies investigating the changes of MA administration to the reproductive system, there is paucity of established literatures regarding MA withdrawal effects on the reproductive system. As there are some of the MA addicts involve with the abuse during their sexually matured stage, it is very crucial to study the consequence of the MA consumption and assess its withdrawal effect.

1.5 Objectives of the study

This study aimed to evaluate the effect of long term administration of methamphetamine HCl and its withdrawal effects on male reproductive functions. The parameters that were assesed were

1. Serum levels of FSH, LH and testosterones hormone.
2. The mean reproductive organs weight which included the testes, epididymis, seminal vesicles as well as prostate glands.
3. Histomorphometric analysis of seminiferous tubules which comprises of the measurement of seminiferous tubular diameter and seminiferous epithelial height.
4. Sperm assay (sperm count and morphology).

The hypothesis of this study is the long term administration of MA has negative effects on the reproductive functions and the withdrawals reverse the harmful effects.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Methamphetamine Hydrochloride (MA HCl)

The drug was collected from Malaysian Department of Chemistry Headquarter, Petaling Jaya (Jabatan Kimia Malaysia). It was obtained through official letters which are KKM-55/202/001/01 Bhg. (77) dated 4 May 2005 and KKM-55/202/001/01-(96) dated 20 October 2006. Its purity is 99.99% and in powder form.

2.2 Animal groups

A total of 120 male Wistar rats of 8 weeks old were used for the experiment. The rats were obtained from Animal Research Unit Laboratory, Universiti Sains Malaysia Health Campus and the ethical clearance was awarded for this experiment through letter PPSG/07 (A)/ 044 dated 6 December 2003. The rats were maintained at room temperature and fed with rat pellet and water *ad libitum*.

The animals were divided into three groups; MA-treated group, control group and placebo group (Figure 2.1). The MA-administered group were injected daily (5mg/kg body weight) intraperitoneally for 12 weeks. 0.9 % normal saline was used as the MA vehicle and the concentration of the suspension was 5mg/ml. The placebo groups were treated with only 0.9 % normal saline whereas the control did not get any intervention during the experiment. Each rat was evaluated for its body weight daily. After 12 weeks of administration the MA injection was discontinued. The rats underwent 5 different withdrawal
periods of 0 and 2 and 4 and 8 and 12 weeks.

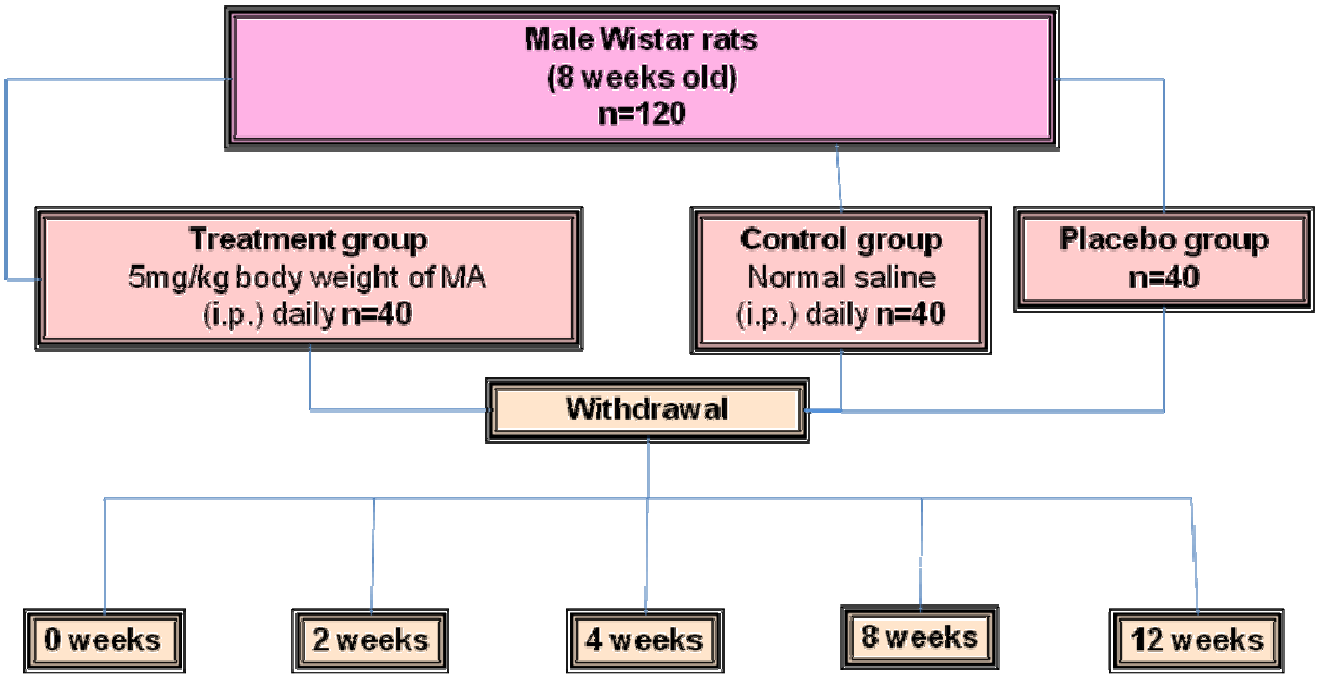

Figure 2.1: Animal division and their respective withdrawal periods.

After completing their respective withdrawal time, the rats were anesthetized with ether (BDH Laboratory Supplies, England) and subsequently sacrificed by cervical dislocation. Laparotomy was carried out and the blood samples were withdrawn from inferior vena cava for the hormones assays. The testis, epididymis, prostate and seminal vesicles were then collected. The right seminal vesicles were pressed smoothly to remove the semen. The collected organs were verified for their weight. Prior the weighing they were made sure to be free of connective tissue as well the fat. After that, the determination of organ weight per 100g body weight was established for each collected organs. Later, these organs were preserved in Bouin's fixative for histological assessment.

The withdrawn blood samples were left to clot for before they were centrifuged at the speed of 3000 rpm to separate its serum. Finally, the serum was stored in the Eppendorf® tubes and keep refrigerated at -78°C and later used for hormones assays.

2.3 Hormone assay

2.3.1 Quantitative evaluation of follicle stimulating hormone and luteinizing hormone

The evaluation of serum FSH and LH was done by using the DRG enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Germany). The reagents and specimens were allowed to reach room temperature prior to the assay procedure. All the procedures were strictly carried out according the protocol from the manufacturer.

The desired number of coated microtiter wells was secured in the holder. The lyophilized contents of the standard vial were reconstituted with 1.0 ml deionized water. 25 µl of standard (FSH standard : 0; 5; 10; 20; 50; 100mIU/ml and LH standard : 0; 10; 20; 40; 100; 200 mIU/ ml), controls and serum specimen were dispensed with new disposable tips into respective well.. Subsequently, 100 µl anti-FSH enzyme-conjugate (for FSH evaluation) and 100 µl anti-LH enzyme-conjugate were dispensed into each well. The combinations were mixed thoroughly for 10 seconds and it is important to have a complete mixing for this step before the plates were incubated for 30 minutes at room temperature. Later, the contents were briskly shaken out from the well and the wells were rinsed with 300 µl deionized water five times. The wells were stricken sharply on the absorbent paper to remove residual water droplets. Next, 100 µl of substrate solution were added to each well and incubated for 10 minutes at room temperature. Finally, 50 µl of stop solution were added to stop the enzymatic reaction at each well followed by measuring the optical density of the specimens by using Ultra Microplate Reader (Bio-Tek Instrument, USA) at the 450 ± nm within 10 minutes following the dispensing of stop solution.

2.3.2 Quantitative evaluation of testosterone hormone in serum

The evaluation of serum testosterone was done by using the DRG enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Germany). The reagents and specimens were allowed to reach room temperature prior to the assay procedure. All the procedures were strictly carried out according the protocol from the manufacturer guide.

Initially the desired number of coated microtiter wells was secured in the holder. Then, 25µl of each standard, controls, placebo and specimens were dispensed with new disposable tips into respective wells and followed by 200µl enzyme conjugate into each well. The combinations were mixed thoroughly for 10 seconds and it is very important to have a complete mixing in this step. Later, the mixtures were incubated for 60 minutes at room temperature. After that, the contents of the wells were shaken out briskly and each well was rinsed 3 times with 400µl diluted wash solution. Wash solution which was earlier prepared by diluting 30 ml of concentrated wash solution with 1170 ml deionized water to a final volume of 1200 ml. Subsequently, the wells were stricken sharply on absorbent paper to remove residual droplets. This washing step is very crucial and needs to be done correctly as it affects the sensitivity and precision of this assay. Then, 200µl of substrate solution were added to each well and incubated for 15 minutes at room temperature. Finally, 100µl stop solution was added to each well to stop the enzymatic reaction and the optical density of the specimens was read at 450±10nm with Ultra Microplate Reader (Bio-Tek Instrument, USA) within 10 minutes after the stop solution was added.