# THE ROLE OF GABAERGIC SYSTEM ON TESTICULAR AND SPERM FUNCTIONS

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**in Partial Fulfillment of the Requirements**
**for the Doctor of Philosophy Degree in Anatomy**
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## ABSTRACT

Testicular and sperm functions are estimated by spermatogenesis and sperm quality such as sperm concentration, motility, morphology, and DNA integrity. It is very well known that the main regulation of these functions is hypothalamic–pituitary–testicular (HPT) axis. Gonadotropins including follicle–stimulating hormone (FSH) and luteinizing hormone (LH) act directly to maintain spermatogenesis through their receptors, FSH and LH receptors, in testis. Nevertheless, those functions are regulated not only by HPT axis but also by neurotransmitter systems such as GABAergic system. The GABAergic system has been reported the role in spermatogenesis, spermatogonial stem cell and Leydig cell proliferation, testosterone production, as well as sperm functions (i.e., sperm motility, capacitation, hyperactivation, and acrosome reaction). The abnormalities in testicular and sperm functions are the main cause of male infertility. Intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) are the most used methods for infertility treatment. Usage of healthy sperm in these methods has an impact on the treatment outcomes, i.e., fertilization rate, embryo quality, and pregnancy rate; however, sperm sorting using density gradient centrifugation (DGC) and swim–up (SU) methods are ineffective to select high–quality sperm. Nowadays,

microfluidic device technologies have been developed and applied in sperm sorting to use instead of those conventional methods. This study aimed to demonstrate the alteration of the GABAergic system in testis and sperm under condition of poor sperm quality. Concerning the testicular function, its correlation with gonadotropin receptors in the testis was examined, whereas concerning the sperm function, correlation between the expression of GABA receptors in human sperm and ICSI outcomes was also investigated. Additionally, sperm sorting using a novel microfluidic device compared with DGC and SU methods was studied.

In an animal study, the changes in GABAergic components as well as FSH and LH receptors were studied in methamphetamine (METH)–administrated rats. The impairments of spermatogenesis, sex steroid hormone function, and sperm quality in these rats have been reported in the previous studies. In this study, a significant increase in GABA concentration in testis was found in the METH–administrated rats. These rats had a high mRNA expression of GABA synthesizing enzyme (GAD1) and GABA A–α1 receptor as well as a low mRNA expression of GABA transporter (GAT1) compared with controls. Interestingly, a significant increase in the expression of GAD1 and GABA A–α1 receptor was especially found in the ED–binge METH group which mimics the human METH use. The findings of the change in GAD1 but not in GAD2 indicate that GAD1 is more effective than GAD2 to control the production of GABA in testis. This study also found a significant increase of GABA A–α1 receptor expression, localized in an anterior acrosomal segment of sperm head, in METH–administrated rats. Besides, this study found a decrease in FSH receptor protein expression in testis after METH exposure, although there were no significant changes in the mRNA expression of FSH and LH receptors. A negative correlation between FSH receptor expression and GABAergic components in testis and sperm was also found. All results demonstrate that the increase in GABA synthesis and function might occur to compensate for the testicular and sperm impairments because of METH. The decrease in FSH function, which can lead to the downregulation of sex steroid hormones function, might relate to the compensatory effect of GABA.

In a human study, a significant increase in mRNA expression of GABA A–α1 and GABA B–R2 receptors in sperm were found in oligoasthenoteratozoospermic (OAT) men compared to normozoospermic (NOR) men; however, there was no

significant difference in those changes between teratozoospermic (TER) and NOR men. A negative correlation between mRNA expression of these receptors and sperm parameters including sperm concentration, motility, and normal morphology was found. After ICSI, patients having the female partner with > 50% Good quality embryo (GQE) on cleavage stage had significantly lower levels of GABA A–α1 receptor than those with ≤ 50% GQE; however, it was not found in the GABA B–R2 receptor expression. There was a negative correlation between the percentage of good–quality embryo on cleavage stage and the expression of GABA A–α1 receptor in sperm but not GABA B–R2 receptor. There was no significant correlation between the expression of GABA receptors and other ICSI outcomes including fertilization rate as well as embryo quality on morula and blastocyst stages. All results demonstrate that the increased expression of GABA receptors in sperm, especially in OAT men, might be the compensatory response to GABA function. The levels of GABA A–α1 receptor expression in sperm have a stronger effect on embryo quality on cleavage stage after ICSI.

A novel microfluidic device (MFD), which selects sperm according to their behaviour and motility characteristics, was used in this study. The results show that the increase of sperm progressive motility, total motility, normal morphology, and DNA integrity was found in the sperm selection by a novel MFD compared to DGC and SU methods. The devices can isolate high–quality sperm from both normal and abnormal (low progressive motility) unprocessed ejaculates using a small amount of sample (60 and 100 μl). Interestingly, the high recovery rate of sperm selected by the devices was also found. The highlight of this study is the selection of sperm with low DNA fragmentation (approximately 1%) using a novel MFD. These results demonstrate the potential of a novel MFD for sperm selection.

Overall conclusion of this study, the findings of this study provide the role of the GABAergic system on testicular and sperm functions. GABAergic components in testis and sperm were changed in the condition of poor sperm quality. The results also provide their relationship to FSH receptor expression in testis (animal study) and embryo quality on cleavage stage after ICSI (human study). A novel MFD is more efficient in selecting high–quality sperm than DGC and SU methods and might be useful for ICSI and IVF treatments.

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# ABBREVIATIONS

| | | |
|---|---|---|
| % | = | percentage |
| °C | = | degree Celsius |
| ART | = | assisted reproductive technology |
| ATS | = | amphetamine–type stimulants |
| BSA | = | bovine serum albumin |
| cDNA | = | complementary DNA |
| DAPI | = | 4',6–diamidino–2–phenylindole |
| DFI | = | DNA fragmentation index |
| DGC | = | density gradient centrifugation method |
| DNA | = | deoxyribonucleic acid |
| EBSS | = | Earle's Balanced Salt Solution |
| ECD | = | electrochemical detector |
| FSH | = | follicle–stimulating hormone |
| GABA | = | gamma–aminobutyric acid |
| GABA A–α1 | = | GABA–A receptor α1 subunit |
| GABA B–R2 | = | GABA–B receptor R2 subunit |
| GAD | = | glutamate decarboxylase |
| GAT | = | GABA transporter |
| GQ | = | good quality |
| GQE | = | good–quality embryo |
| hCG | = | human chorionic gonadotropin |
| HPLC | = | high–performance liquid chromatography |
| HPT | = | hypothalamic–pituitary–testicular |
| ICM | = | inner cell mass |
| ICSI | = | intracytoplasmic sperm injection |
| IVF | = | *In vitro* fertilization |
| LH | = | luteinizing hormone |
| M | = | molar |
| max | = | maximum |

# ABBREVIATIONS (CONT.)

| | | |
|---|---|---|
| METH | = | methamphetamine |
| MFD | = | microfluidic device |
| mg | = | milligram |
| min | = | minimum |
| ml | = | milliliter |
| mm | = | millimeter |
| mM | = | millimolar |
| MQ | = | moderate quality |
| mRNA | = | messenger ribonucleic acid |
| mV | = | millivolt |
| n | = | number |
| ng | = | nanogram |
| NOR | = | normozoospermia |
| ns | = | not significant |
| OAT | = | oligoasthenoteratozoospermia |
| OPA | = | ortho–phthalaldehyde |
| PBS | = | phosphate–buffered saline |
| PDMS | = | polydimethylsiloxane |
| PQ | = | poor quality |
| PVDF | = | polyvinylidene fluoride |
| RNA | = | ribonucleic acid |
| ROD | = | relative optical density |
| ROS | = | reactive oxygen species |
| rpm | = | revolutions per minute |
| RSD | = | relative standard deviation |
| RT–PCR | = | reverse transcription–polymerase chain reaction technique |
| SCSA | = | sperm chromatin structure assay |
| SCD | = | sperm chromatin dispersion |

# ABBREVIATIONS (CONT.)

| | | |
|---|---|---|
| SEM | = | standard error of the mean |
| SSC | = | spermatogonial stem cell |
| SU | = | swim–up method |
| TBS | = | Tris–buffered saline |
| TdT | = | terminal deoxynucleotidyl transferase |
| TE | = | trophectoderm |
| TER | = | teratozoospermia |
| TUNEL | = | terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling |
| µg | = | microgram |
| µl | = | microliter |
| µm | = | micrometer, micron |
| µM | = | micromolar |
| WHO | = | World Health Organization |

# CHAPTER I

# GENERAL INTRODUCTION

**Rational for the study**

The regulation of testicular and sperm functions has been reported not only by hormonal regulation but also by neurotransmitters such as catecholamines (stimulant neurotransmitters) and gamma–aminobutyric acid (GABA) (an inhibitory neurotransmitter). Hypothalamic–pituitary–testicular (HPT) axis is a main regulation on testicular functions (i.e., spermatogenesis and sex steroid hormone production) and sperm quality. Gonadotropins including follicle–stimulating hormone (FSH) and luteinizing hormone (LH) act through their receptors (FSH and LH receptors) in testis. These hormones control the function of testicular cells (i.e., Leydig and Sertoli cells) and sex steroid hormones (i.e., testosterone, estrogen, and progesterone) on spermatogenesis in testis (George, Dille, & Heckert, 2011; Sofikitis et al., 2008).

The expression of GABAergic components (including GABA receptors, GABA synthesizing enzymes, and GABA transporter) was found both in testis and sperm. GABA receptors (GABA–A, –B, and –C receptors), GABA synthesizing enzymes (glutamate decarboxylase (GAD) 67 and 65), and GABA transporter type 1 (GAT1) have been reported their expression in the testis (Geigerseder et al., 2003; He, Hu, Wu, Yan, & Koide, 2001; Hu, & Yan, 2002; Li, Zhang, Liu, Yan, & Li, 2008; Li et al., 2005). Interestingly, in sperm, only the expression of GABA–A and –B receptors as well as GAT1 have been reported (He, Zhang, Yan, Li, & Koide, 2003; He et al., 2001; Hu, He, Wu, Yan, & Koide, 2002; Hu, He, & Yan, 2000; Li et al., 2005; Ritta, Calamera, & Bas, 1998; Zhang, Gui, Yuan, Bian, & Guo, 2009). Several studies have revealed that GABAergic components are involved in spermatogenesis, spermatogonial stem cell and Leydig cell proliferation, as well as testosterone production (Du et al., 2013; Geigerseder, Doepner, Thalhammer, Krieger, & Mayerhofer, 2004; Hu et al., 2004; Ritta et al., 1998; Zhang et al., 2009). Moreover, GABA receptors play a role in sperm functions (i.e., sperm motility, capacitation, hyperactivation, and acrosome reaction) through controlling the opening of calcium and potassium channels, calcium influx, and

cAMP accumulation (Burrello et al., 2004; Calogero et al., 1999; Calogero et al., 1996; Jin et al., 2009; Kon, Takei, Fujinoki, & Shinoda, 2014; Kurata, Hiradate, Umezu, Hara, & Tanemura, 2019; Puente, Tartaglione, & Ritta, 2011; Ritta, Bas, & Tartaglione, 2004). The impairments of testicular and sperm functions mainly occur by endocrine disruption and oxidative stress. Many endocrine disruptors (including environmental agents such as pesticides and heavy metals; pharmacologic agents such as anabolic steroids, estrogens, chemotherapeutic agents, and radiation therapy) have been reported the adverse effects on testis and sperm (Sikka, Kendirci, & Naz, 2004). Oxidative stress in sperm contributes to approximately 30-80% of male infertility cases. It occurs by lifestyle factors such as smoking, drinking, and using illicit drugs (i.e., methamphetamine).

Methamphetamine (METH), a central nervous system stimulant, is an addictive drug that is often abused. There is strong evidence supporting the adverse effects of METH on testis and sperm. A previous study has shown that METH administration can induce apoptotic germ cells in testis (Nudmamud-Thanoi, & Thanoi, 2011). METH–administrated rats reveal the decrease in sperm concentration and normal morphology (Nudmamud-Thanoi, Sueudom, Tangsrisakda, & Thanoi, 2016; Nudmamud-Thanoi, & Thanoi, 2011). Changes in sex steroid hormone receptors including progesterone and estrogen receptors in testis were found after METH exposure (Nudmamud-Thanoi et al., 2016). Interestingly, the changes in levels of catecholamines (monoamine neurotransmitters) in the testis of METH–administrated rats has also been reported (Janphet, Nudmamud-Thanoi, & Thanoi, 2017). This finding suggests that GABA, an amino acid neurotransmitter, might be involved in testicular and sperm functions as same as other neurotransmitters. Interestingly, there is evidence of the improvement in testicular functions and sperm quality by GABA. GABA is a nutrient finding in pre–germinated brown rice. Receiving of GABA standard and pre–germinated brown rice can improve testicular structure, androgen receptor expression, and sperm quality in dextromethorphan–administrated rats (Thanoi, Roboon, & Nudmamud-Thanoi, 2018).

The impairments of sperm quality (i.e., low sperm concentration, motility, normal morphology, and DNA integrity) are the main cause of infertility in males, an inability to conceive after 12 months of regular sexual intercourse without the contraception (Borges et al., 2019). Poor sperm quality contributes to 50–80 million

infertile couples worldwide. (reviewed by Babakhanzadeh, Nazari, Ghasemifar, & Khodadadian, 2020; Fainberg, & Kashanian, 2019). Several terms are referring to men with poor sperm quality. Oligoasthenoteratozoospermic (OAT) men are defined as men revealing low three sperm parameters including sperm concentration, motility, and morphology, whereas teratozoospermic (TER) men are defined as men revealing only low sperm morphology. Infertile men with OAT and TER are most often found in patients attending infertility clinics (da Silva, Wessler, Madeira, & da Silva, 2017). There are many treatments for male infertility such as surgical, medical, and assisted reproductive technologies (ARTs), i.e., intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) treatments. For ICSI treatment, ICSI procedure bypasses the processes of natural sperm selection for the fertilization, i.e., sperm capacitation, hyperactivation, and acrosome reaction, as well as the process of sperm–oocyte plasma membrane fusion (Neri, Lee, Rosenwaks, Machaca, & Palermo, 2014). The failures of fertilization and embryo development after ICSI because of poor sperm quality have been reported (Bhilawadikar et al., 2013). One of the major causes of those failures is the delayed oocyte activation which occurs after triggering intracellular calcium release and oscillations (reviewed by Anifandis, Messini, Dafopoulos, Daponte, & Messinis, 2016). The successful ICSI outcomes are influenced by the timing of disintegration of the sperm plasma membrane and acrosome which occurs within injected oocyte (Katayama et al., 2005; Morozumi, Shikano, Miyazaki, & Yanagimachi, 2006; Seita, Ito, & Kashiwazaki, 2009; Takeuchi, Colombero, Neri, Rosenwaks, & Palermo, 2004). The removal of sperm plasma membrane and acrosome before ICSI has been reported to improve the timing of oocyte activation and the first cleavage division (Morozumi et al., 2006). It is possible that the remaining components (i.e., GABA receptors) of the intact sperm plasma membrane and acrosome in ooplasm after ICSI might have an impact on fertilization and embryo development.

Because using poor–quality sperm for ICSI and IVF treatments causes a decrease in fertilization rate and embryo development, sperm selection is an important process for those treatments (Chapuis et al., 2017; Loutradi et al., 2006). Density gradient centrifugation (DGC) and swim–up (SU) methods are generally used for sperm selection; however, they are not effective to select sperm with high DNA integrity and requires careful training. In the case of DGC method, it requires the use of high

centrifugal forces which have the potential to damage sperm (reviewed by Beydola, Sharma, Lee, & Agarwal, 2013). Nowadays, microfluidic devices have been developed for selecting sperm with high quality than DGC and SU methods, easy to use, low-cost, and available for small quantities of sperm and the samples with low sperm motility.

**Scope of the present study**

The present study is interested in the role of the GABAergic system in testicular and sperm functions. Changes in the GABAergic system in testis and sperm were investigated in the condition of poor sperm quality both in animal and human studies. METH–administrated rats were used in the animal study. Because FSH and LH receptors are involved in testicular function, it is interesting to draw attention to changes in these receptors and their relationship to the GABAergic components in the testis of METH–administrated rats. High–performance liquid chromatography (HPLC) technique was used to estimate GABA concentration in rat testis. The alteration of mRNA expression in rat testis was evaluated by using reverse transcription–polymerase chain reaction (RT–PCR) technique whilst the changes of protein expression in rat testis and sperm were examined by using immunohistochemistry technique. In the human study, changes in GABA receptors were studied in OAT and TER men compared with normozoospermic (NOR) men. Semen analysis was assessed according to World Health Organization (WHO) 2010 guidelines, Laboratory Manual for the Examination and Processing of Human Semen (World Health Organization, 2010). The mRNA expression of GABA receptors in human sperm was examined using RT–PCR technique. Fertilization rate and embryo quality after ICSI have been investigated in each group of patients. The association of GABA receptors with semen quality, fertilization rate, and embryo quality was investigated by using correlation statistics. In microfluidic device study, sperm concentration, motilities, morphology, and DNA fragmentation (using the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay) of sperm selected by a novel microfluidic device were compared to those selected by DGC and SU methods.

**Overall aims**

The core objective of this study is to examine the changes in GABAergic components in testis and sperm in the condition of poor sperm quality. Concerning the testicular and sperm functions, the changes in gonadotropin receptors in testis and ICSI outcomes were also examined. Moreover, the present study also aimed to develop a novel microfluidic device for selecting high–quality sperm. Therefore, the specific aims in this study are as follows:

**Animal study**

1. To assess the change of GABA concentration in testis of METH–administrated rats compared with controls using HPLC technique

2. To examine the changes in mRNA expression of GABAergic components and gonadotropin receptors, FSH and LH receptors, in testis of METH–administrated rat compared with controls using RT–PCR technique

3. To examine the changes in protein expression of FSH receptor in testis of METH–administrated rat compared with controls using immunohistochemistry technique

4. To examine the changes in protein expression of GABA receptor in sperm of METH–administrated rat compared with controls using immunohistochemistry technique

5. To study the relationship between GABAergic components and gonadotropin receptors

**Human studies**

1. The mRNA expression of GABA receptors in human sperm

1.1 To examine the changes in mRNA expression of GABA receptors in sperm of OAT and TER men compared with NOR men using RT–PCR

1.2 To study their relationship with sperm parameters as well as fertilization rate and embryo quality after ICSI

2. A novel microfluidic device for sperm selection

To examine sperm concentration, progressive motility, total motility, morphology, and DNA fragmentation (using TUNEL assay) of sperm selected by a novel microfluidic device compared to DGC and SU methods from normal and abnormal (low progressive motility) semen samples

## Hypothesis

Changes in GABAergic components in testis and sperm of METH–administrated rats compared with controls and their relationship to gonadotropin receptors in testis might be found. Expression of GABA receptors in sperm of OAT and TER men might be changed compared with NOR men. These changes might correlate with: 1) sperm quality including sperm concentration, motility, and morphology; and 2) fertilization rate and embryo quality after ICSI. A novel microfluidic device can isolate sperm with higher progressive motility, total motility, normal morphology, and DNA integrity compared to DGC and SU methods. These devices could be applied in both normal and abnormal (low progressive motility) semen samples.

## Conceptual framework

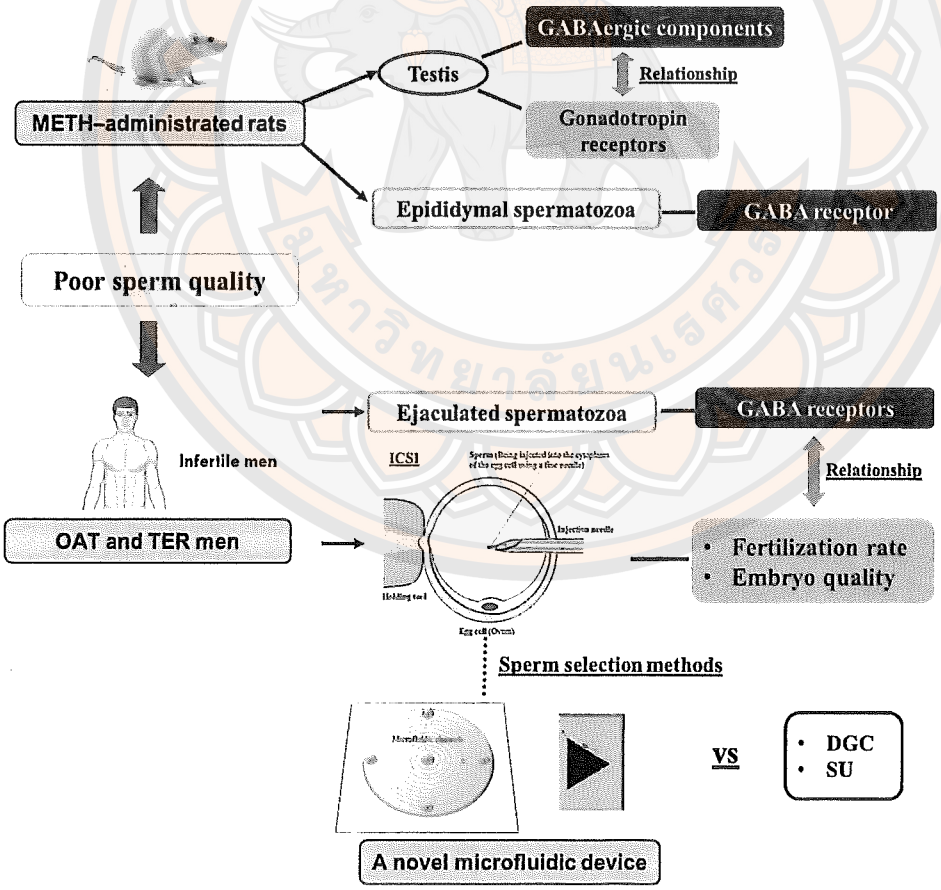



**Figure 1 Conceptual framework of this study**

# CHAPTER II

# REVIEW OF RELATED LITERATURE AND RESEARCH

## Testis and its functions

Testis is a principle male reproductive organ containing many seminiferous tubules inside (Figure 2). Sperm are produced within the seminiferous tubules and then transported into the epididymis. These sperm are matured and stored in the epididymis before ejaculation into the female genital tract for fertilization. The main function of testis is sperm production (spermatogenesis and spermiogenesis) and the production of sex steroid hormones (steroidogenesis). There are two compartments in testis including seminiferous tubule and interstitial compartments. The seminiferous tubule compartment (including basal and adluminal compartments) containing spermatogenic and Sertoli cells is the main compartment of spermatogenesis while the interstitial compartment containing principally Leydig cells plays a role in the testosterone production, see Figure 3 (Weinbauer, Luetjens, Simoni, & Nieschlag, 2010). Sertoli cell is located close to the basal lamina of seminiferous tubule. Its functions are necessary for sperm production, i.e., supporting structure of the germinal epithelium, maintaining testicular volume, creating blood–testis–barrier, and providing proper condition for meiotic process and sperm development. Besides, Leydig cell acts mainly in the production and function of testosterone hormone.

## Spermatogenesis and spermiogenesis

There are two processes in sperm production including spermatogenesis and spermiogenesis occurring within seminiferous tubules in testis. Spermatogenesis is a complex process consisting of mitosis and meiosis of spermatogenic cells. The process of spermatogenesis begins with the division of spermatogonial stem cells (SSCs). Firstly, SSCs proliferate by mitosis and differentiate to form type B spermatogonium. Then, it transforms to be a preleptotene spermatocyte. These processes arise near the basal lamina in the basal compartment of seminiferous tubule. After moving from the basal lamina into the adluminal compartment, the preleptotene spermatocyte (primary spermatocyte) still divides by meiosis I and II to form a secondary spermatocyte and

round spermatid, respectively. Lastly, the round spermatid transforms to be a mature spermatozoon through the spermiogenesis process. A process that sperm are released from seminiferous epithelium into the seminiferous tubule lumen is called "spermiation" (Figure 4) (Xiao, Mruk, Wong, & Yan Cheng, 2014).

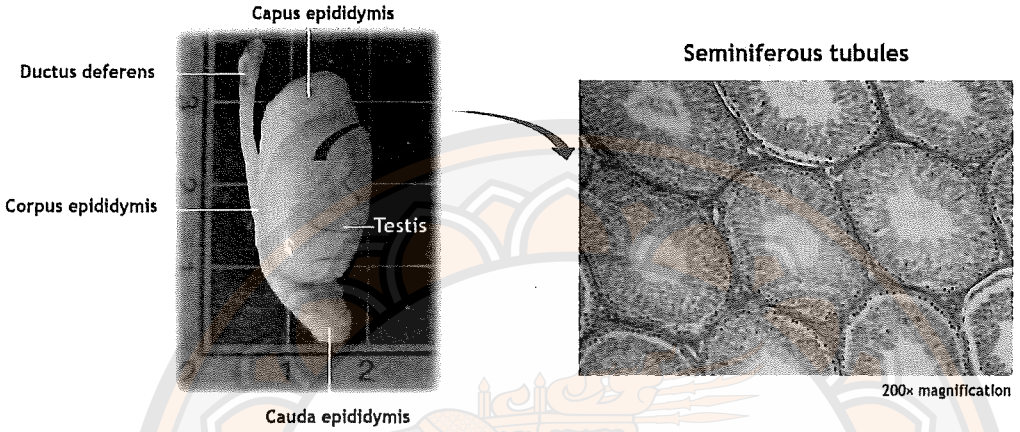



**Figure 2 Illustration of testicular structure**

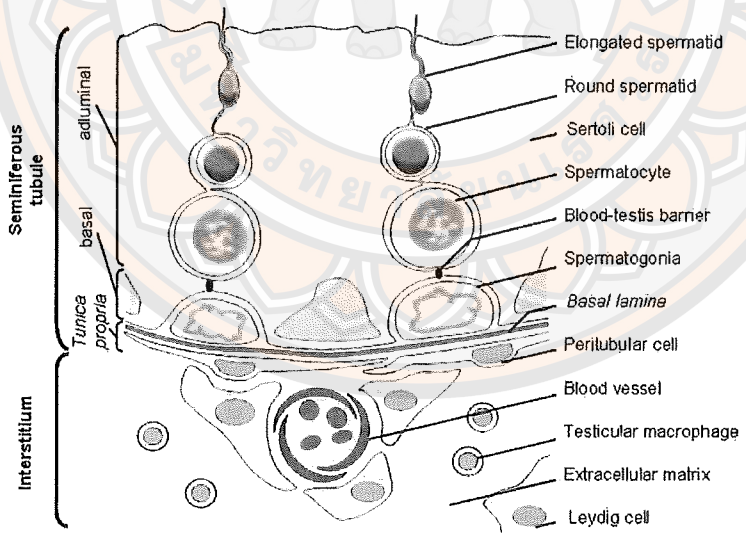



**Figure 3 Illustration of seminiferous tubule and interstitial compartments**

**Source:** Lagarrigue et al., 2011

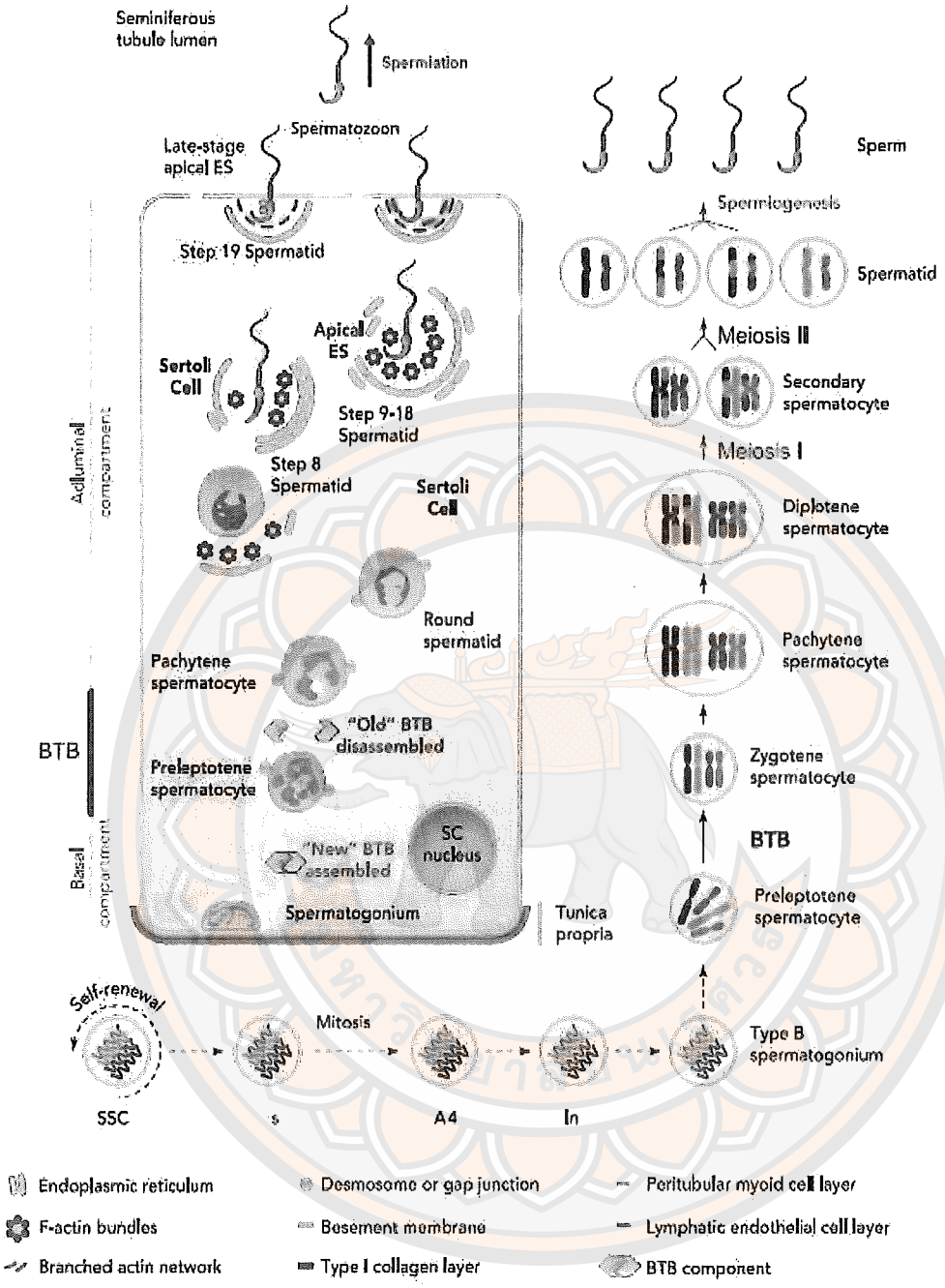



**Figure 4 Illustration of spermatogenesis and spermiogenesis processes in seminiferous tubule**

**Source:** Xiao et al., 2014

**Sperm and its functions**

Mature sperm are produced via spermatogenesis and spermiogenesis processes in the testis. Sperm structure consists of three main regions including head, midpiece, and tail (Figure 5). Sperm head contains a large sperm nucleus and acrosome. Acrosome, Golgi apparatus–derived organelle, is located in an anterior end of sperm head and important for fertilization because it contains hydrolytic enzymes necessary for penetration of egg cell (Berruti, & Paiardi, 2011). A process that includes the disruption of acrosome and the releasing of hydrolytic enzymes is called acrosome reaction. Sperm midpiece is the compartment that mitochondria are formed in a spiral shape. It provides energy to support sperm movement. Moreover, sperm tail is an end region of sperm which consists of long flagellum covering with the thin plasma membrane. Sperm movement occurs by beating of the flagellum in the sperm tail (Alberts et al., 2002).

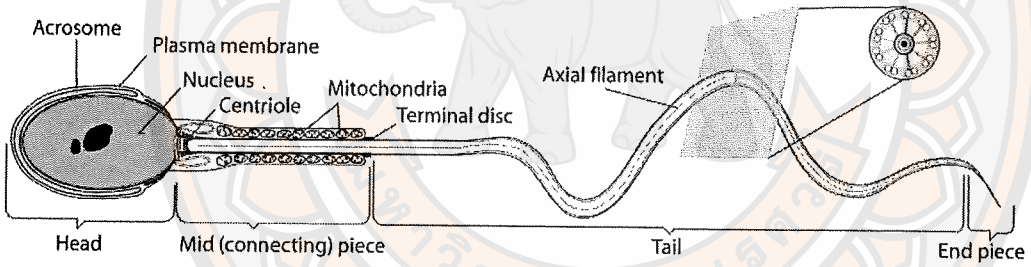



**Figure 5 Illustration of sperm structure**

**Source:** https://upload.wikimedia.org/wikipedia/commons/thumb/1/13/Simplified_spermatozoon_diagram.svg/1920px–Simplified_spermatozoon_diagram.svg.png, 7 March 2020

Sperm in different species alter in size and shape, especially the shape of sperm head and acrosome. Human sperm have a round–shaped head, whereas rodent sperm such as mouse and rat sperm have a hook–shaped head, see Figure 6. Besides those differences, the fraction in each compartment of sperm differs between rodents and humans. In human sperm, a midpiece is much shorter than a tail; however, it is converted to a finding in the rodent sperm. Moreover, a cytoplasmic droplet is normally observed in testicular, epididymal and ejaculated human sperm, whereas it is only observed in testicular and epididymal mouse sperm (reviewed by Darszon, Nishigaki, Beltran, & Treviño, 2011). Sperm head in all species is divided into 3 parts including anterior acrosomal, equatorial, and postacrosomal segments. Figure 7 shows the microstructure in each part of the rat sperm head.

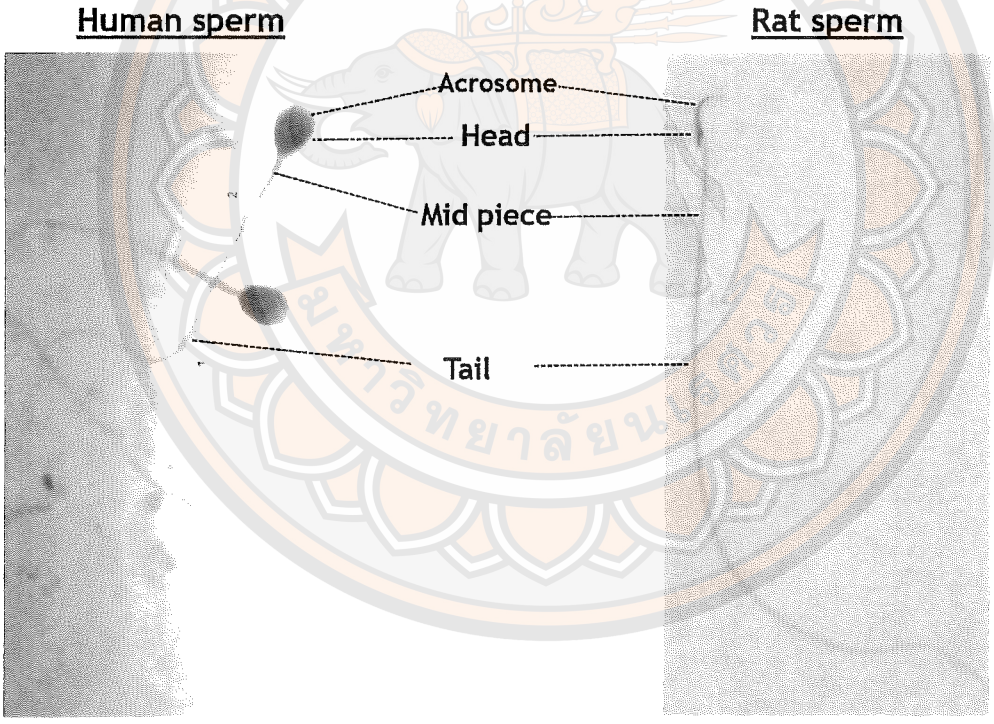
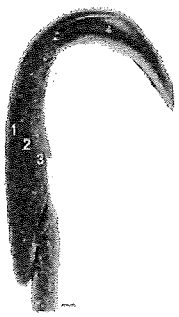



**Figure 6 Illustration of the sperm structure in human and rodent (rat)**




1 AAS (anterior acrosomal segment)
2 ES (equatorial segment)
3 PAS (postacrosomal segment)

**Figure 7 Illustration of structure of rat sperm in head region**

**Source:** http://www.onlineveterinaryanatomy.net/sites/default/files/original_media /image/asset_9414_spearm_head.jpg, 7 March 2020

The main sperm function is the fertilization which occurs in the oviduct of females. Only motile sperm can move into the female genital tract to reach the oviduct for the fertilization. The capability of sperm including sperm motility, capacitation, hyperactivation, and acrosome activation is important for the natural fertilization processes, see Figure 8. Sperm that are unable to properly go through these processes will not be able to fertilize an oocyte. In the acrosome reaction process, sperm must first fuse with the oocyte plasma membranes before penetrating into an oocyte. Then, sperm nucleus and factors but not sperm acrosome and plasma membranes can enter the oocyte for fertilization. On the other hand, in case of intracytoplasmic sperm injection (ICSI) which bypasses the natural fertilization processes, a single sperm with intact acrosome and plasma membranes is injected into an oocyte. Therefore, the disintegration of the sperm acrosome and plasma membranes must be occurred in an oocyte after ICSI. It is interesting that ICSI without sperm acrosome and plasma membranes results in much better outcomes (Roldan, 2006). The remaining of sperm factors in sperm acrosome and plasma membranes can affect the fertilization and embryo development. Besides, the sperm DNA integrity and chromatin condensation are referred to as the biological functions of sperm. These factors have a clinical impact on the outcome of fertilization (Henkel et al., 2005).

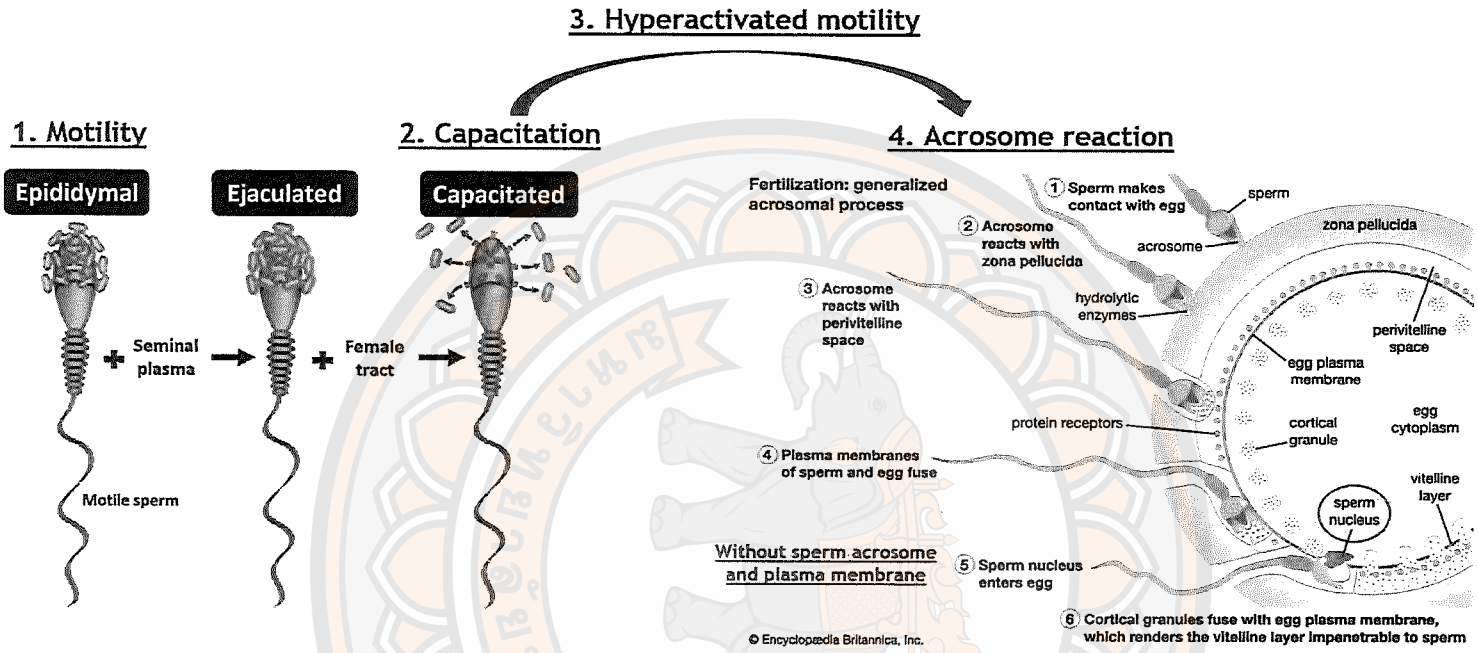



**Figure 8 Illustration of the natural fertilization processes**

**Source:** Adapted from https://cdn.britannica.com/22/194822-050-336EB737/sperm-cell-changes-layers-contact-prominence-spermatozoa.jpg and https://o.quizlet.com/Doqy9biOb.7sP8FYsVo3-A.jpg, 7 March 2020

**The regulation of testicular and sperm functions**

Testicular functions including spermatogenesis and testosterone production are controlled by the hypothalamic–pituitary–testicular (HPT) axis. Gonadotropin–releasing hormone (GnRH) from the hypothalamus is involved in the synthesis and releasing of gonadotropins from the anterior pituitary. Gonadotropins including follicle–stimulating hormone (FSH) and luteinizing hormone (LH) are glycoprotein hormones that act mainly to regulate spermatogenesis and testosterone production in the testis. The negative feedback of these hormones is regulated by inhibin (inhibition of FSH secretion), and estradiol (inhibition of LH secretion). (Figure 9) (Alves et al., 2013). Interestingly, several neurotransmitters such as catecholamines (stimulant neurotransmitters) and gamma–aminobutyric acid (an inhibitory neurotransmitter) have been reported their involvement in testicular and sperm functions.

**GABAergic system**

Gamma–aminobutyric acid (GABA) is a main inhibitory neurotransmitter that plays a role in the central nervous system. GABAergic components consist of GABA receptors, GABA synthesizing enzymes (glutamate decarboxylase; GAD), and GABA transporters (GAT). Several studies have reported that an amphetamine–type stimulant (ATS) leads to the reduction of GABAergic function in the central nervous system. There is evidence of a relationship between levels of GABAergic components and ATS use disorders (Jiao, Liu, Li, Liu, & Zhao, 2015). Long–term use of high–dose ATS results in the impairment of GABA synthesis, the decrease in GAD and GABA concentrations (Jayanthi, Deng, Noailles, Ladenheim, & Cadet, 2004). Nevertheless, the compensatory enhancement of GABA is occurred by short–term use of ATS (Pereira et al., 2008). Changes in GABA differ in various brain regions. Interestingly, the GABAergic system is found in several peripheral tissues including testis, ovary, uterus, pancreas, and adrenal glands (Gladkevich, Korf, Hakobyan, & Melkonyan, 2006; Watanabe, Maemura, Kanbara, Tamayama, & Hayasaki, 2002).

**GABAergic system in testis and sperm**

On the male reproductive system, several studies have reported the expression of the GABAergic system in testis and sperm. GABA–A, –B, and –C receptors are expressed in testis (Geigerseder et al., 2003; He et al., 2001; Li et al., 2008). GABA–A receptor subunits are localized in several testicular cells including Leydig cells, elongated spermatids, and spermatozoa while GABA synthesizing enzymes (GAD65 and 67) is exclusively expressed in Leydig cells (Geigerseder et al., 2003; Geigerseder et al., 2004; Hu, & Yan, 2002; Li et al., 2005). The findings of those enzymes reflect that GABA might be synthesized in the testis by Leydig cells. Moreover, the expression of GABA–A and –B receptors has been found in a sperm head, whereas GABA–C receptor expression has been found in a sperm tail (Li et al., 2008). The expression of GABA–A receptor has been reported both in rat epididymal sperm and ejaculated human sperm (Hu et al., 2002; Li et al., 2005; Ritta et al., 1998). In human sperm, the GABA–A receptor alpha subunits are found the localization in the equatorial segment of acrosome (Wistrom, & Meizel, 1993), while those receptors in rodent sperm are found in the acrosomal region (Hu et al., 2002; Kurata et al., 2019). Additionally, the localization of GABA–B receptor subunits are also found in the acrosomal region of rat sperm head (He et al., 2003; He et al., 2001). Expression of GAT1 has been reported in the testis and sperm (Hu et al., 2000; Hu et al., 2004; Zhang et al., 2009). Interestingly, GABA functions on the male reproductive system have been reported not only inhibitory effect but also excitatory effect. *In vitro* studies indicate that GABA controls the proliferation and differentiation of Leydig cells as well as testosterone production via GABA–A receptor (Ritta et al., 1998). Furthermore, GAT1 overexpressing transgenic mice express a decrease in testis mass and testosterone level, the impairment of spermatogenesis, and an increase in abnormal sperm morphology (Geigerseder et al., 2004; Hu et al., 2004; Zhang et al., 2009). These findings showed that spermatogenesis is regulated by the GABAergic system. Nevertheless, only an *in vitro* study has demonstrated the inhibitory effect of GABA in maintaining the homeostasis of spermatogenesis in the testis. It has been reported that GABA acts as a negative regulator of spermatogonial stem cell proliferation (Du et al., 2013).

The role of GABA receptors on sperm function has been reported in several studies. GABA–A and –B receptors control calcium signaling in the modulation of

sperm kinetic parameters (including sperm motility) (Calogero et al., 1996). *In vitro* studies using agonist and antagonist of GABA receptors show that GABA regulates sperm acrosome reaction, hyperactivated motility, and capacitation through GABA receptors. Both GABA and progesterone interact with GABA–A receptor to control sperm acrosome reaction and hyperactivation (Calogero et al., 1999; Calogero et al., 1996; Kon et al., 2014; Puente et al., 2011). Additionally, not only the GABA–A receptor but also the GABA–B receptor are involved in the human follicular fluid–stimulated acrosome reaction (Burrello et al., 2004). A previous study has revealed that GABA can induce sperm capacitation through a specific GABA–A receptor by an intracellular mechanism depending on calcium influx and cAMP accumulation (Ritta et al., 2004). This mechanism is controlled by tyrosine phosphorylation of sperm protein (Kurata et al., 2019). Supporting evidence for the mechanism of GABA on sperm capacitation and hyperactivation indicates that GABA plays a role in the changes in calcium, chloride, and bicarbonate ions via GABA–A receptor (Jin et al., 2009). In the female genital tract, the highest values of GABA have been reported in the oviduct than other female genital organs, i.e., ovary, uterus, and vagina. Different levels of GABA in each stage of the estrous cycle are also found (Louzan, Gallardo, & Tramezzani, 1986). GABA receptors are involved in progesterone signaling controlling cilial activity in the oviduct (Jung et al., 2018). Together, these data support the relationship between GABA and the fertilization process.

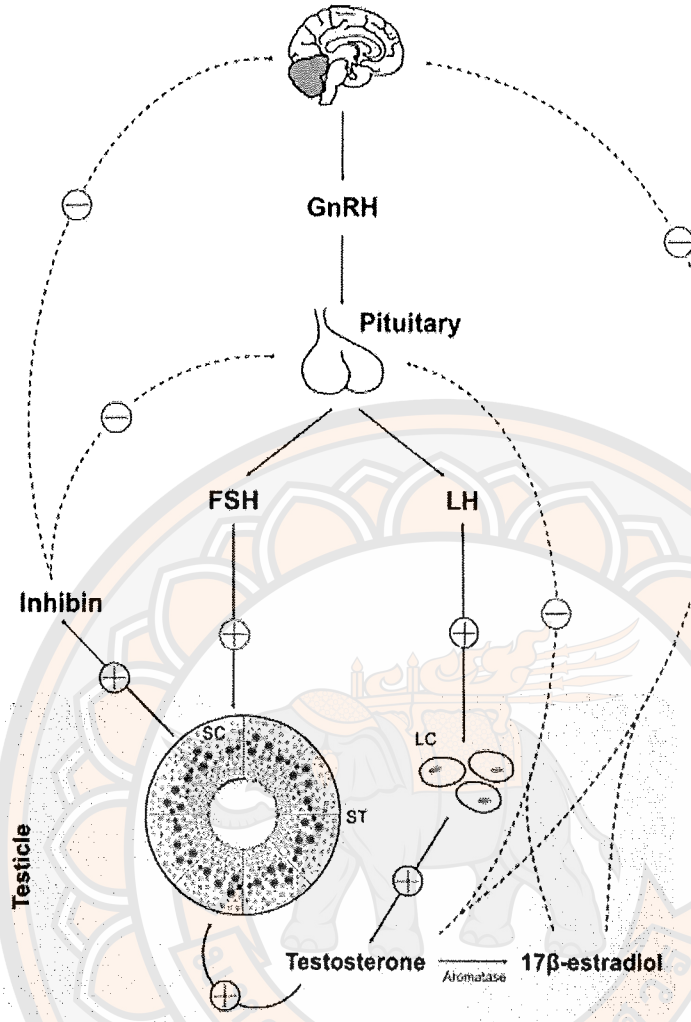



**Figure 9 Diagram of hypothalamic–pituitary–testicular axis. Gonadotropin–releasing hormone (GnRH), follicle–stimulating hormone (FSH), luteinizing hormone (LH), Sertoli cells (ST), and Leydig cells (LC). Circled plus: positive feedback, Circled minus: negative feedback**

**Source:** Alves et al., 2013

**Follicle–stimulating hormone (FSH) receptor in testis**

FSH plays a role to regulate the proliferation and function of Sertoli cells, and hormonal production in testis which is necessary for spermatogenesis. FSH acts through the FSH receptor (FSHR), a member with the family of G protein–coupled receptors, which is localized on the plasma membrane of Sertoli cells (George et al., 2011). Nevertheless, the study of FSHR mRNA expression using in situ hybridization has reported that FSHR expresses not only on the plasma membrane but also in the nucleus or scattered in the cytoplasm of Sertoli and round germinal cells (Baccetti et al., 1998). Additionally, expression analysis of FSHR protein using monoclonal FSHR antibody has shown the FSHR localization at the basal part of the Sertoli cell and around the spermatogonia (Vannier, Loosfelt, Meduri, Pichon, & Milgrom, 1996). Interestingly, the levels of FSHR mRNA expression differ according to the stages of spermatogenesis within seminiferous tubules, 14 stages in the rat (stage I–XIV). The highest levels of FSHR mRNA expression have been found in stage XIII, XIV, I, and II, while the lowest levels of those have been found in stage VII–VIII, see Figure 10 (Heckert, & Griswold, 1991). These findings indicate that FSHR expression is decreased during spermiation whereas it is increased in the stages containing undifferentiated spermatogonia and early spermatids (Simoni, Gromoll, & Nieschlag, 1997). It has been suggested that the highest FSHR expression is found in those stages because FSH acts chiefly to control the proliferation and survival of undifferentiated spermatogonia (especially A3–A4 spermatogonia) in those stages (Meachem, McLachlan, Stanton, Robertson, & Wreford, 1999; Ruwanpura, McLachlan, & Meachem, 2010). Moreover, FSH is also necessary for the progression of meiosis and spermiogenesis occurring in those stages (Vihko, LaPolt, Nishimori, & Hsueh, 1991). Interestingly, the study in FSHR knockout mice also supports the role of FSH in testicular and sperm functions. It has been reported that the aberration of FSHR expression causes the reduction of sperm production and quality (O'Shaughnessy, Monteiro, & Abel, 2012).

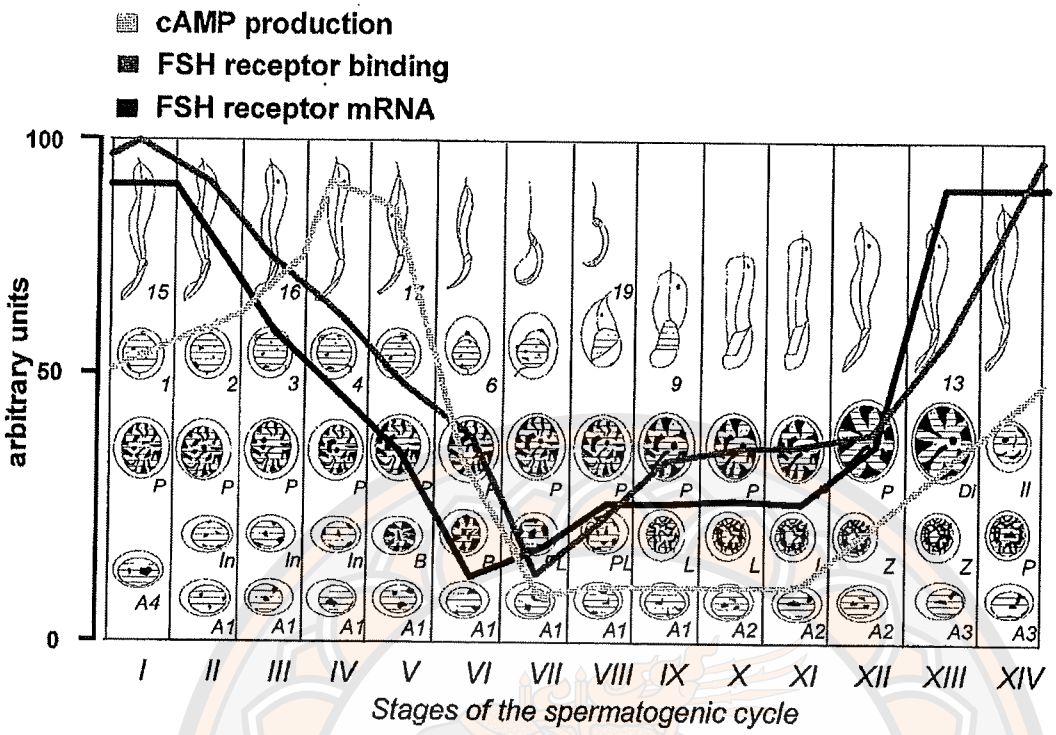



**Figure 10 Illustration of levels of FSH receptor expression in each stage of seminiferous epithelial cycles of rat spermatogenesis**

**Source:** Simoni et al., 1997

**Luteinizing hormone (LH) receptor in testis**

LH plays a role exclusively to stimulate Leydig cell proliferation and testosterone production. LH acts by interaction with the LH receptor (LHR) located on the plasma membrane of Leydig cell (De Kretser, Catt, & Paulsen, 1971; Wahlstrom, Huhtaniemi, Hovatta, & Seppala, 1983). LH also supports the spermatogonial maturation and spermatocyte development in testis as the same function as FSH; however, LH appears potentially less effective than FSH in these functions (Matthiesson et al., 2006). Loss of LHR function in mice causes a decrease in testosterone levels and the impairment of spermatogenesis and spermiogenesis (the arrest of spermatid cells development at the round spermatid stage) (Lei et al., 2001)

**Semen analysis**

Semen analysis, the first step in monitoring male infertility, consists of two main examinations including macroscopic and microscopic examination. The macroscopic examination consists of the examination of the liquefaction, viscosity, volume, pH, and appearance of the semen, whereas microscopic examination consists of the examination of sperm quality (Vasan, 2011; World Health Organization, 2010). Sperm quality is mainly estimated using three sperm parameters: 1) sperm concentration; 2) sperm motility, the assessment of sperm direction and movement, including sperm progressive motility and total motility; and 3) sperm morphology, the assessment of the sperm structure, i.e., sperm head, neck and midpiece, tail, and residual cytoplasm. Nowadays, the low reference value of sperm concentration ($15 \times 10^6$ sperm per ml), progressive motility (32%), total motility (40%) and morphology (4% in normal forms) have been provided by WHO 2010 guidelines (World Health Organization, 2010). Interestingly, the cutoff value for normal sperm morphology has been suggested according to the analytical and staining methods. 30% normal form of sperm morphology has been suggested to use as the cut off value for normal sperm morphology in traditional (manual) method (Eliasson, 2010; Loutradi et al., 2006; Menkveld, 2010). The ejaculates revealing the value of sperm parameters less than the low reference value in each parameter are defined as having abnormal sperm quality. According to the WHO guidelines, poor–quality sperm are classified into several terms. Normozoospermia (NOR) refers to semen with normal sperm concentration, motility, and morphology. Conversely, oligoasthenoteratozoospermia (OAT) refers to those with abnormal values. Moreover, there are several terms of ejaculate with abnormal in a single parameter such as oligozoospermia (having abnormal sperm concentration), asthenozoospermia (having abnormal sperm motility), and teratozoospermia (TER, having abnormal sperm morphology). Most infertile men express poor sperm quality; however, approximately 15% of those men have been reported the normal in sperm parameters (reviewed by Agarwal, & Allamaneni, 2005). Interestingly, infertile men have been reported higher levels of sperm DNA fragmentation than fertile men (Zini, Bielecki, Phang, & Zenzes, 2001). Currently, sperm DNA fragmentation has been suggested to be one of the sperm parameters for the evaluation of male fertility potential.

**Sperm DNA fragmentation**

Sperm DNA fragmentation occurs by several factors, i.e., environmental factors such as radiation, high temperature, and chemical substances; pathological factors such as male genital tract infection, testicular torsion, and hypospermatogenesis; genetic factors such as chromosomal aberrations, genetic variations, and chromosome structural abnormalities; lifestyle factors such as smoking, alcohol and illicit drug use; and oxidative stress. There are several methods for the assessment of sperm DNA fragmentation (Evgeni, Charalabopoulos, & Asimakopoulos, 2014). Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) and the alkaline comet assays are methods that detect DNA fragmentation directly in the DNA fragmented area. On the other hand, the sperm chromatin structure assay (SCSA) and the sperm chromatin dispersion (SCD) assay are the indirect types of the sperm DNA fragmentation test (Kim, 2018). Nowadays, the TUNEL assay is the most widely used method in the research field. Several studies by Henkel et al. and Benchaib et al. have investigated the relationship between sperm DNA fragmentation detecting by TUNEL assay and the outcomes of infertility treatments such as ICSI and *in vitro* fertilization (IVF). Their results indicate that sperm DNA fragmentation affects the pregnancy rate in these treatments (Benchaib et al., 2003; Henkel et al., 2003). The assessment of sperm DNA fragmentation using the TUNEL and alkaline comet assays showed a stronger correlation with the pregnancy rate than using SCSA and SCD assays (reviewed by Chi et al., 2017).

Interestingly, patients with high sperm DNA fragmentation undergoing ICSI have higher pregnancy rate than those undergoing IVF. The sperm DNA fragmentation has been reported the inverse correlations with sperm motility (especially rapid motility) and morphology (reviewed by Chi et al., 2017). Moreover, Benchaib et al. have reported the correlation between a high sperm DNA fragmentation (>10%) and a low fertilization rate in patients undergoing ICSI and IVF. A similar result has been found in the correlation between sperm DNA fragmentation and embryo formation rate on day 3 after IVF/ICSI (Kim, Kim, Jee, & Kim, 2019).

**Male infertility**

Three aspects including 1) patient history, especially genital tract surgery, testicular trauma, and medications; 2) testing of endocrine and sperm antibody; and 3) semen analysis are used for the clinical evaluation of male infertility (Iammarrone, Balet, Lower, Gillott, & Grudzinskas, 2003). Semen analysis is the main aspect of male infertility diagnosis that useful not only in the clinical evaluation but also in the research study. Infertility is defined as an inability of sexually active, non–contracepting couples to achieve spontaneous pregnancy in one year (World Health Organization, 2010). Recently, approximately 50–80 million people worldwide have been reported as an infertile population. Interestingly, up to 70% of these cases are contributed by male factor, mostly in poor sperm quality. (reviewed by Babakhanzadeh et al., 2020; Fainberg, & Kashanian, 2019). Male infertility can cause by several risk factors. Hormonal impairment and oxidative stress are commonly found in male infertility. Several studies have published that environmental toxicants, many pharmacological agents, and biological agents can induce an endocrine disruption in the male reproductive system. Alteration of the synthesis and release of male sex hormones such as testosterone and gonadotropins is the main cause of abnormal sperm quality (Sikka et al., 2004). Moreover, environmental toxicants, lifestyle factors, systemic pathologies, and pathologies in male reproductive organs can enhance oxidative stress and excessive levels of reactive oxygen species (ROS). The induction of oxidative stress results in sperm damage and male infertility (Figure 11) (reviewed by Esteves, & Agarwal, 2011).

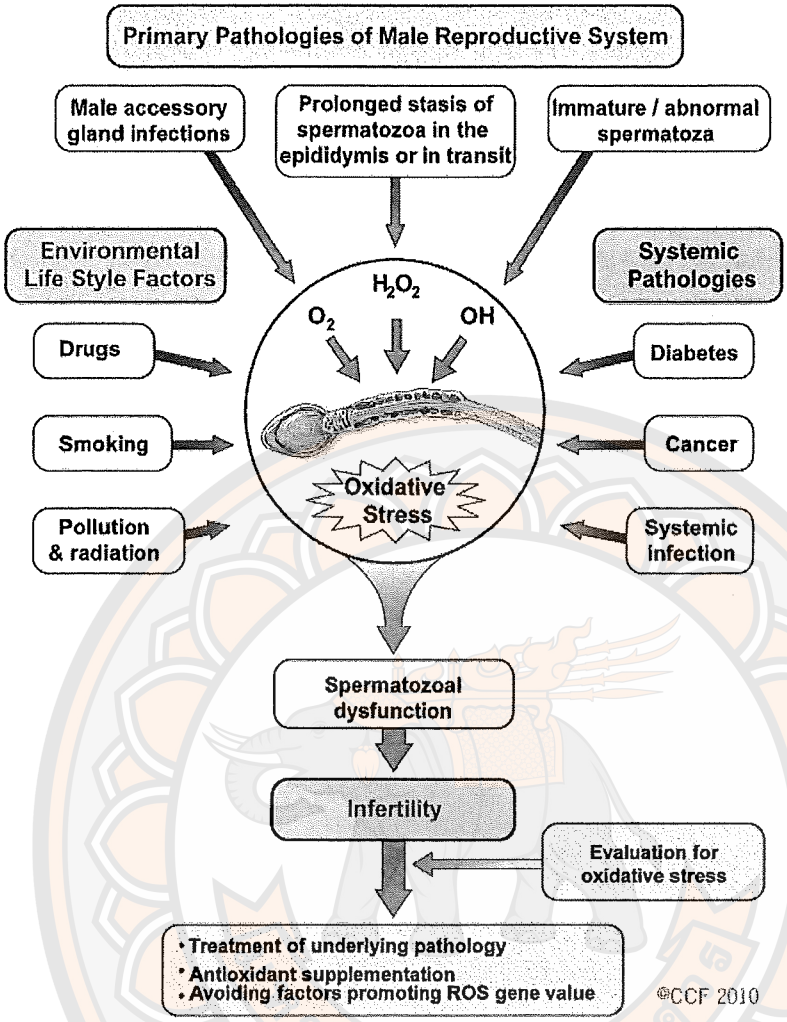



**Figure 11 Illustration of the relation of oxidative stress, its causes and male infertility**

**Source:** Esteves, & Agarwal, 2011

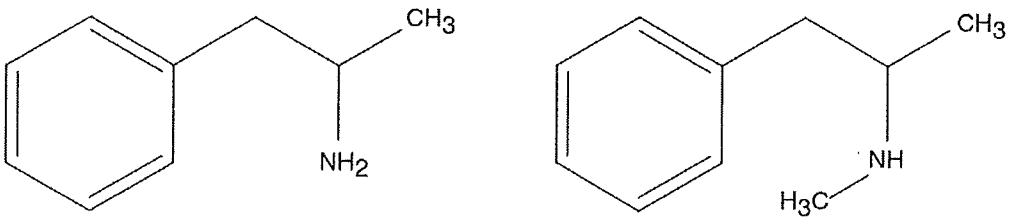
**Risk factors in male infertility**

The risk factors causing male infertility have been reported such as environmental toxicants, lifestyle factors, pharmacological agents, systemic pathologies, and pathologies of the male reproductive system. These factors relate to unexplained or idiopathic infertility, infertility caused by several factors, and the lack of an obvious cause (Sadeghi, 2015). Idiopathic infertility has been reported as the most frequent cause of male infertility, approximately 30–60% of infertile men (Jungwirth et

al., 2012; Punab et al., 2017; Sadeghi, 2015). The lifestyle factors, especially psychoactive substances use, are powerful factors influencing male infertility. Psychoactive substances are divided into depressants (e.g. alcohol), stimulants (e.g. nicotine, amphetamine, and several illicit substances), opioids (e.g. morphine and heroin), and hallucinogens (e.g. dextromethorphan). Interestingly, the changes in neurotransmitters including dopamine, serotonin, norepinephrine, GABA, glutamate, and endogenous opioids have been found after psychoactive substance exposure (World Health Organization, 2004). Psychoactive substances, especially METH and dextromethorphan cause adverse effects on the male reproductive system. Moreover, the infections on male reproductive system such as *Chlamydia trachomatis* infection can cause harmful effects on sperm quality. *In vitro* studies indicate that the adherent of *Chlamydia trachomatis* on sperm can cause premature sperm death and induce apoptosis in ejaculated sperm (Eley, Hosseinzadeh, Hakimi, Geary, & Pacey, 2005; Hosseinzadeh, Brewis, Eley, & Pacey, 2001).

**Methamphetamine (METH)**

METH is a psychoactive substance that can cause male infertility. It is a central nervous system stimulant and a member of the amphetamine–type stimulants. The chemical structure of METH occurs from the addition of a methyl group into the structure of amphetamine (Figure 12) (Dasgupta, 2010). METH usually comes in the form of base, powder, and crystal. Moreover, it is mixed with other chemicals and compressed in the form of tablet. Accordingly, METH has various names in different countries depending on its form and purity. It also known as crystal, ice, chalk, speed, and meth. It is commonly mixed with caffeine and ephedrine and comes in form of tablet. In Thailand, the tablet form of METH, called "yaba", is the most commonly abused (reviewed by Chomchai, & Chomchai, 2015).




Amphetamine Methamphetamine

**Figure 12 Illustration of the structures of amphetamine and METH**

**Source:** Dasgupta, 2010

**METH abuse**

METH is an illicit drug abused in many countries around the world. METH abuse is a serious problem in many contries. It contributes to approximately 35 million METH abusers worldwide (Talloczy et al., 2008). In Southeast Asia, it is the most common addictive drug in many countries. Approximately 3.5–20.9 million of METH abusers are estimated in this region. Thailand is a country with the highest METH abusers in the Southeast Asia region (reviewed by Chomchai, & Chomchai, 2015). METH is an addictive drug that usually taken orally, smoked, snorted, and injected. The most popular pattern for METH abuse is "binge" pattern, continuous use of multiple high doses to maintain the high METH concentration in the blood (reviewed by Cho, & Melega, 2002). METH abuse relates to neurological and physical effects and it is a public health problem worldwide (Volkow, 2013). Several studies have been reported the acute and chronic effects of METH abuse. Acute effects of METH abuse consist of increased alertness, heart rate, blood pressure, body temperature, and decrease appetite. Nevertheless, chronic effects of METH abuse include paranoid thinking, deficits in thinking and motor skills hallucinations, violent behavior, psychosis, severe anxiety, weight loss, kidney and damages, and severe dental problems (Kirkpatrick et al., 2009; Mendelson, Newton, de Wit PhD, & Urschel III, 2008; Shaner, Kimmes, Saini, & Edwards, 2006).

**METH effects on the nervous system**

METH abuse can lead to the adverse effects on the nervous system. It can cause an increase in blood–brain barrier permeability (Park, Kim, Lim, Wylegala, & Toborek, 2013). The changes in several proteins in the hippocampus after METH exposure have been reported and associated with neuronal cell death, inflammation, oxidative stress, and apoptotic pathways (Zhu et al., 2016). METH administration leads to the alteration of several neurotransmitter systems in the brain including dopamine, norepinephrine, serotonin (reviewed by Cho, & Melega, 2002), glutamate, (Kerdsan, Thanoi, & Nudmamud-Thanoi, 2009) and GABAergic systems (Jiao et al., 2015). The neurotoxicity effects of METH on the striatal dopamine system can cause an increase in dopamine release in the brain. Because dopamine plays a critical role in the reward pathway and motor function, the change in dopamine levels causes behavioral changes. Accordingly, chronic METH abuse can cause motor skill impairment, cognitive defect, and an increase of anxiety, psychotic disorders, hallucination, delusions, as well as depression (Rusyniak, 2011). A study in METH–administrated rats has demonstrated that cognitive defects are associated with the change in expression of inflammatory marker, peripheral benzodiazepine receptor (Veerasakul, Thanoi, Watiktinkorn, Reynolds, & Nudmamud-Thanoi, 2016). In addition, a previous study has reported the changes of calcium binding proteins especially parvalbumin and GABAergic neuronal markers after METH exposure (Veerasakul, Thanoi, Reynolds, & Nudmamud-Thanoi, 2016). Interestingly, the association between the polymorphisms in GAD1 and GAD2 genes and METH dependence has been reported (Veerasakul, Watiktinkorn, Thanoi, Reynolds, & Nudmamud-Thanoi, 2017).

**METH effects on testicular and sperm functions**

The adverse effects of METH have been found not only on the nervous system but also on the reproductive system. METH has been popularly used by teenagers and young adults which are a group of reproductive age. The studies in METH–administrated rats have reported a decrease in testicular germ cell proliferation and an increase of apoptotic germ cells in testis (Alavi, Taghavi, & Moallem, 2008; Nudmamud-Thanoi, & Thanoi, 2011). Receiving a single dose of METH results in an increase in the percentage of apoptotic seminiferous tubules, and a decrease in serum testosterone levels (Yamamoto et al., 2002). Moreover, the significant decreases in the

epididymal sperm numbers and the percentage of normal sperm morphology have also been reported after METH administration in a single dose (Nudmamud-Thanoi, & Thanoi, 2011). Additionally, the study of METH administration in escalating dose (gradually increasing doses of METH) and binge dose (multiple high doses) patterns has represented the impairment of sperm quality after METH exposure. This previous study has shown a significant decrease in the percentage of normal sperm motility in all METH–administrated groups including acute binge, escalating dose, and escalating dose–binge groups. Nevertheless, the percentage of normal sperm morphology is significantly decreased in the escalating dose and escalating dose–binge groups (Nudmamud-Thanoi et al., 2016). The study in hormonal receptor changes after METH exposure has demonstrated a decrease in the expression of progesterone and estrogen receptors in Sertoli cells and several types of testicular germ cells of METH–administrated rats (Nudmamud-Thanoi et al., 2016). Interestingly, the concentration of catecholamine in testis has been changed because of METH exposure (Janphet et al., 2017).

**Treatment of male infertility**

Male infertility has been reported to cause up to 70% of infertility in couples worldwide (reviewed by Babakhanzadeh et al., 2020). The main cause of male infertility is the impairment of sperm quality. Poor sperm quality is caused by several factors. Accordingly, methods for treating male infertility are different depending on individual causes of infertility. Surgery and medicine have been used in male infertility treatment. Surgical treatment is normally used in case of the obstruction of male genital ducts, i.e., epididymal, ejaculatory duct, and vas deferens as well as the cystic fibrosis (Jungwirth et al., 2012). The main target of medical treatment is to achieve the improvement of hormone production, germ cell maturation, and sperm production and quality.

Medicines, hormones, and other supplements are used in the medical treatments of male infertility. The anti–estrogens, aromatase inhibitors, androgens, gonadotropins (recombinant FSH, and human chorionic gonadotropin (hCG)), pentoxyphylline, arginine, carnitine, glutathione, vitamins (A, C and E), oligominerals (folic acid, zinc, selenium), etc. have been used for the treatment in case of idiopathic (unexplained) infertility (Isidori, Latini, & Romanelli, 2005). There are several reports

of the harmful side effects of hormonal treatments such as gonadotropins. The treatment with gonadotropins results in gynecomastia, acne, influenza–like symptoms, and weight gain (reviewed by Dabaja, & Schlegel, 2014). Additionally, anti–estogens such as clomiphene citrate have also been reported the side effects including nausea, vomiting, breast discomfort, blurred vision, and headache (reviewed by Dabaja, & Schlegel, 2014). Treatment with antioxidants such as β–carotine, tocopherol (vitamin E), ascorbic acid (vitamin C), retinoids (vitamin A), oligominerals (folic acid), acetylcysteine, and glutathione have been reported to improve male infertility (reviewed by Esteves, & Agarwal, 2011; Isidori et al., 2005). Not only these substances but GABA has also been reported the improvement effects on the impairment of testicular and sperm functions. Interestingly, GABA standard and pre–germinated brown rice containing GABA can improve the poor sperm quality, the impairment of seminiferous tubules, and androgen receptor expression because of the antidepressant drug treatment (Thanoi et al., 2018). Nevertheless, surgical and medical treatments are not effective in some cases of male infertility, especially unexplained infertility. Therefore, the assisted reproductive technologies (ARTs) such as ICSI and IVF are used to improve the success of those treatments.

**Intracytoplasmic sperm injection (ICSI)**

ICSI is one of the most commonly used ARTs in male infertility treatment. Sperm are collected and prepared in the first step for ICSI treatment. Only one selected spermatozoon is injected directly into an oocyte, see Figure 13. After ICSI, the injected oocytes are allowed to develop to the blastocyst embryos in culture for 5 days. Finally, these embryos are transferred into the female uterus for implantation. Nowadays, ICSI treatment is a powerful treatment for male infertility. The success of ICSI outcomes, especially the fertilization rate and embryo quality, depends on the quality of injected sperm (Loutradi et al., 2006). The selection of highly–motile sperm is essential for the success of the ARTs. Thus, the process of sperm selection is important for the treatment; however, density gradient centrifugation (DGC) and swim–up (SU) methods are the most common method of sperm selection in the clinic today.

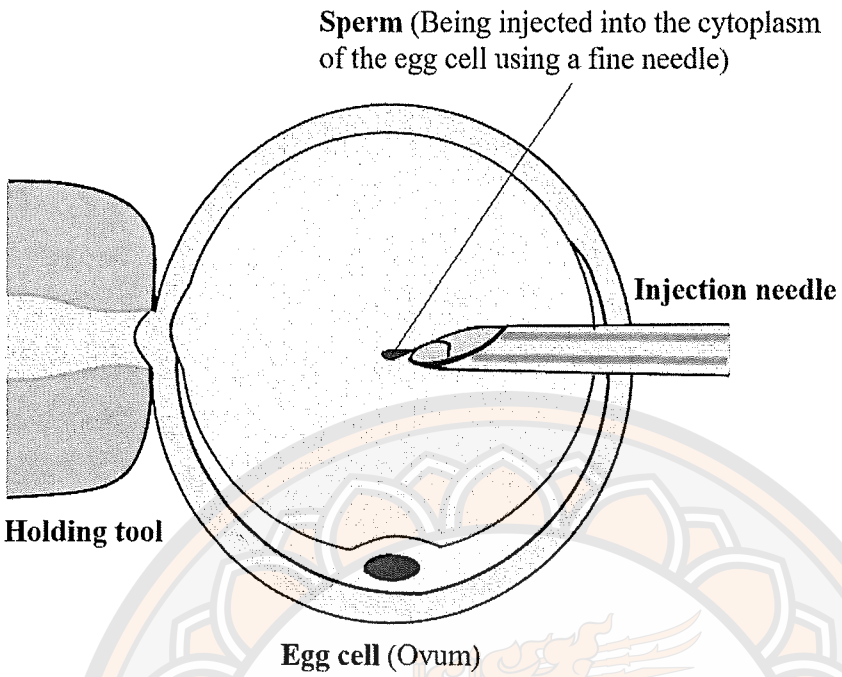



**Figure 13 Intracytoplasmic sperm injection (ICSI) method**

**Fertilization rate and embryo development in culture after ICSI**

The success of ICSI treatment can evaluate by the fertilization rate and embryo development. The morphological assessment of embryo in each stage of the development is used to monitor embryo quality after ICSI. The appearance of two pronuclei formation is defined as normal fertilization, see Figure 14, which is observed on day 1. Generally, the fertilization process will occur between 12 and 20 hours after ICSI. Observation of the fertilization in culture has been suggested in two periods including the first observation at 16 to 18 hours for the appearance of the pronuclei cytoplasm and the second observation at 24 and 26 hours for the breakdown of the nuclear membrane and cell division (reviewed by Abeyta, & Behr, 2014). The embryo development in culture after the fertilization shows in Figure 15. Firstly, a fertilized oocyte (a two–pronuclear zygote; 2PN) is cleaved into a two–cell embryo in the early cleavage stage. The study of Meseguer et al. has suggested that the cleavage of an embryo before 25 to 28 hours fails embryo implantation (Meseguer et al., 2011). The assessment of embryo on the cleavage stage is considered in cell number and symmetry, as well as embryo fragmentation. In the cleavage stage (on days 2 and 3 after ICSI),

two–cell embryos are developed into the four–cell embryos on day 2 and the six or eight–cell embryos on day 3. Moreover, the percentage of embryo fragmentation is also important to monitor the embryo quality. There is evidence of the positive correlation between embryo fragmentation and chromosomal abnormalities (Munne, 2006). Additionally, Pelinck et al. have suggested that the embryos on the cleavage stage with $< 10\%$ fragmentation have higher implantation rate than those with $\geq 10\%$ fragmentation (Pelinck et al., 2010). Thus, several studies showed that the cutoff value of the embryo fragmentation on the cleavage stage should be 10% fragmentation (reviewed by Abeyta, & Behr, 2014). The compaction formation of the cleavage embryos occurs in the morula stage on day 4 post–insemination. Last stage of the embryo development in culture, the compacting embryos develop to form the blastocyst embryos on day 5 which called the blastocyst stage. The component of blastocyst embryo consists of the inner cell mass (ICM), the trophectoderm (TE), blastocyst cavity (blastocoel), and zona pellucida, see Figure 16.

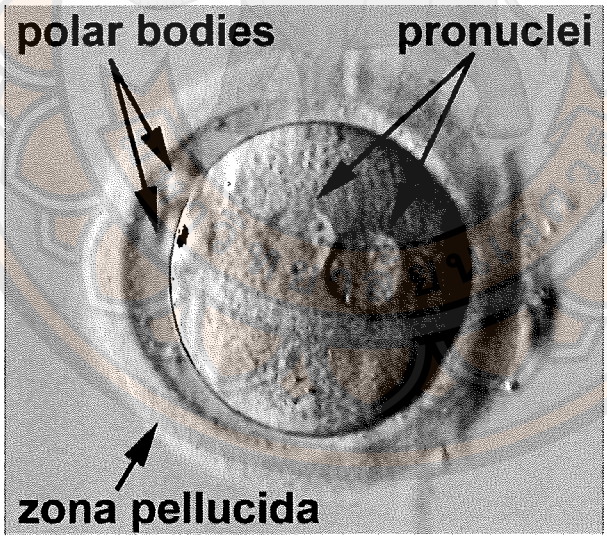



**Figure 14 Illustration of fertilized oocyte at day 1 after ICSI**

**Source:** https://embryology.med.unsw.edu.au/embryology/index.php/File:Human_zygote_two_pronuclei_22.jpg, 7 March 2020

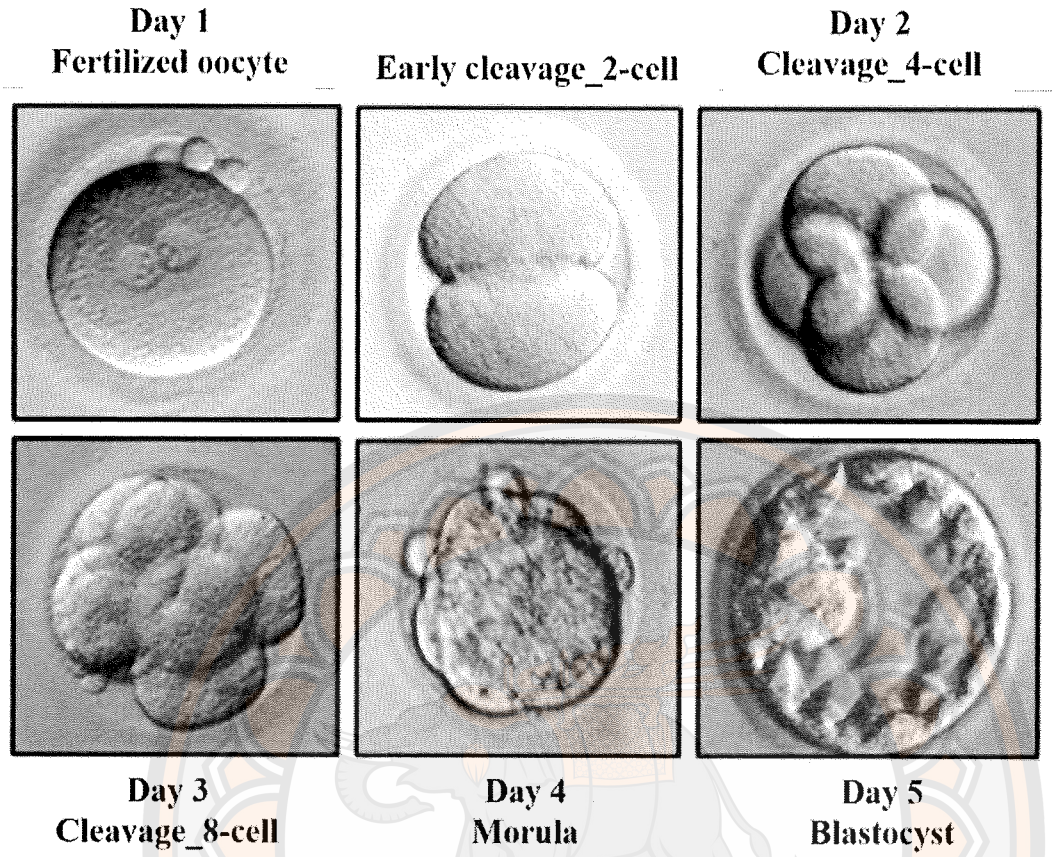



**Figure 15 The development of embryo in culture after ICSI (day 1–5)**

**Source:** Adapted from https://www.utahfertility.com/wp–content/uploads/2017/09/grading–blog–table–1.jpg, 7 March 2020

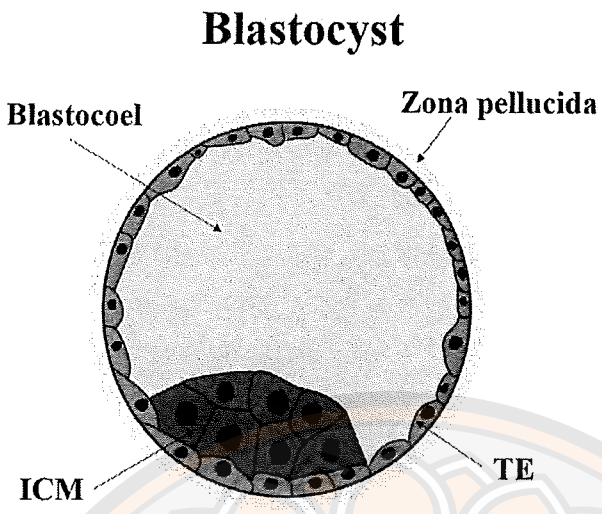



**Figure 16 Components of blastocyst embryo (expanded blastocyst) including the inner cell mass (ICM), the trophectoderm (TE) and blastocyst cavity (blastocoel)**

**Sperm selection**

The sperm selection is the main process in male infertility for ARTs, i.e., ICSI and IVF. The clinical outcomes of ARTs depend on sperm quality. Several studies have been reported the relationship between sperm quality and the success of ART treatment. The high quality of sperm has an impact on the success of ICSI and IVF. The treatment using poor sperm quality, especially a low motility, affected the decrease in the fertilization rate and embryo development after ICSI and IVF treatments (Chapuis et al., 2017; Loutradi et al., 2006). For this reason, the motile sperm is required for the ARTs. These sperm are selected by the sperm selection methods. Currently, DGC and SU methods are generally used in infertility clinics. Those methods can separate motile sperm from immotile sperm, round cells, and debris. Not only sperm motility but also sperm DNA fragmentation has an impact on the success of ART treatment. The percentage of sperm DNA fragmentation in infertile men was higher than in normozoospermic men. A previous study by Cohen–Bacrie et al, which studied sperm DNA fragmentation using TUNEL assay, reported the inverse correlation between sperm DNA fragmentation and sperm progressive motility, total motility, and vitality.

Conversely, they reported the positive correlation between the sperm DNA fragmentation and an abnormal form of sperm morphology including atypical forms, abnormal necks, and coiled tails (Cohen-Bacrie et al., 2009). A high sperm DNA fragmentation resulted in the impairment of fertilization rate, embryo development, and pregnancy rate (Asghar et al., 2014). Benchaib et al. reported that the fertilization rate was increased in ICSI and IVF treatments using sperm with less than 10% DNA fragmentation (Benchaib et al., 2003). These results indicate that the selection of sperm with high quality, especially high motility and low DNA fragmentation is effective for the ART treatment.

**Conventional methods for sperm selection**

In clinics today, the conventional methods (DGC and SU) are commonly used for sperm preparation in the ART treatment. These methods select sperm from the ability of sperm motility. The selection of sperm with high motility, morphology, and DNA integrity is necessary for the ARTs. There was reported that high motile sperm is more effective than low motile sperm for fertilization in these treatments. Thus, the sperm selection for IVF and ICSI is focused on the selection of sperm with high motility. Moreover, sperm with high quality in terms of sperm motility, morphology, and DNA integrity are also required for these treatments. However, DGC and SU methods have been reported their limitation of use (reviewed by Beydola et al., 2013). DGC separates sperm based on sperm density. At the end of DGC, normal sperm with high motility are centrifuged to be pellet at the bottom of a centrifuge tube whilst abnormal sperm with poor motility and other cells are located in the gradient solutions. There was reported that the centrifugation process resulted in the DNA damage of sperm which affected the fertilization and embryo quality (Zini, Nam, Mak, Phang, & Jarvi, 2000). For the SU method, after the incubation, motile sperm naturally move out of the semen samples into the medium solution without the centrifugal force. The disadvantage of SU is unable to separate sperm from the low sperm motility samples. Moreover, there were reported that both conventional methods are not effective in the selection for samples with very low sperm concentration (less than $4 \times 10^6$ sperm per ml) and low sperm motility (Henkel, & Schill, 2003).

**Microfluidic devices for sperm selection**

The principle of microfluidic technology is the separation of the particles from a small volume of fluids (milliliter to nanoliter). Many researchers take advantage of this technology for the fabrication of microfluidic devices (reviewed by Samuel et al., 2018). Nowadays, the microfluidic device has been widely developed and used in the research field of sperm analysis and sorting); however, several studies mostly focused on the evaluation of microfluidic devices for sperm sorting using in ICSI and IVF treatments. Because the currently conventional methods for sperm selection including DGC and SU have been reported several disadvantages: the centrifugation steps in DGC cause the sperm damage; ineffective selection in sperm with high DNA integrity, the microfluidic device is designed to be simple, rapid, low cost and efficient in high–quality sperm selection. There are several principles which use to create microfluidic devices for sperm sorting, i.e., passively driven, chemoattractant driven, flow–driven, and thermotaxis–driven. Moreover, the combination of these strategies is utilized which called multi–principle–driven microfluidics. The chemoattractant and thermotaxis–driven microfluidic devices have been created by mimicking the microenvironments in the female genital tract. The passively and flow–driven microfluidic devices have been designed based on the behaviour of sperm movement. The main part of the microfluidic device for sperm isolation is the microchannels. There are two types of the microfluidic device which have been mostly fabricated such as polydimethylsiloxane (PDMS)–based with a glass (PDMS–glass) and paper–based microfluidic devices. PDMS has been used in many biological studies and accepted as non–toxic, physiologically inert, and biocompatible (Halldorsson, Lucumi, Gómez-Sjöberg, & Fleming, 2015). The behaviour of sperm movement has been studied in several research teams. As evidence from Kantsler et al., the strategy of boundary–following behaviour of sperm, and cell scattering in microfluidic ratchets are reported, see Figure 17a and Figure 17b (Kantsler, Dunkel, Polin, & Goldstein, 2013). Moreover, the direction of sperm movement is navigated by the surface scattering of sperm flagellar, see Figure 17c (Denissenko, Kantsler, Smith, & Kirkman-Brown, 2012). These strategies are used to design a ratchet microfluidic device for sperm isolation in this study.

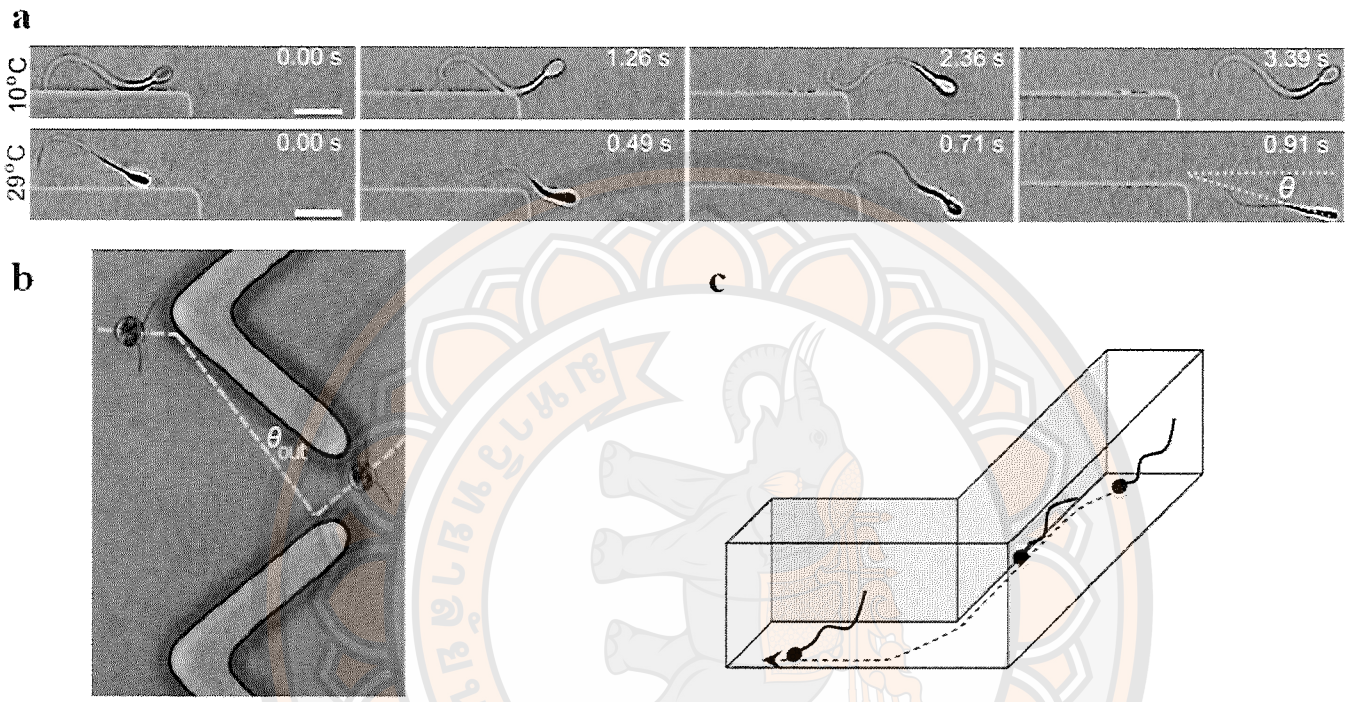



**Figure 17 The strategy of (a) boundary–following behaviour of sperm, (b) cell scattering in microfluidic ratchets, and (c) the surface scattering of sperm flagellar**

**Source:** Denissenko et al., 2012; Kantsler et al., 2013

The study of the microfluidic device has been mostly focused on the isolation of sperm with high motility which is useful for the ART treatment. Some studies developed microfluidic devices to isolate or capture sperm based on size, shape, and charge. Additionally, the individual cell observation and selection by a microfluidic device have also been studied (reviewed by Samuel et al., 2018). To date, many studies of the microfluidic device for sperm selection aim to isolate sperm not only with high motility but also with high DNA integrity. The main advantage of the microfluidic device is the isolation of sperm without centrifugation. The centrifugal force in the DGC method causes the damage of sperm. The previous study in the comparison of the DNA fragmentation of sperm selected by DGC and direct SU, which is SU method without centrifugation, demonstrated the adverse effect of the centrifugation. The sperm selected by DGC had higher DNA fragmentation compared with unprocessed semen and sperm selected by SU (Zini, Finelli, Phang, & Jarvi, 2000). Nowadays, several studies have reported the efficiency of their microfluidic devices to select high motile sperm. Two studies are using the passively driven microfluidic devices which revealed the isolation of high–quality sperm from raw human semen with normal sperm quality. Nosrati et al. develop a microfluidic device based on the boundary–following behaviour of sperm (Nosrati et al., 2014). They show that approximately 600,000 sperm with high vitality and DNA integrity are recovered from 113 million sperm of 1 ml of unprocessed semen using the device with 7.5mm microchannel length for 20 minutes incubation time. The recovery rate of their microfluidic devices is approximately 0.5%. However, they did not report other sperm parameters including sperm motility and morphology. There is no comparison of sperm selection by their devices and the conventional methods, i.e. DGC and SU. Asghar et al. have reported the efficiency of a microfluidic device which designed based on the ability of sperm migration through micropores within a polycarbonate membrane filters using paper–based microfluidic technologies (Asghar et al., 2014). Their devices can isolate sperm with high motility and DNA integrity, which is higher than the selection by SU. Currently, this paper-based microfluidic device was developed to be a commercial product for sperm selection: the Fertile Plus® microfluidic sperm sorting chips, see Figure 18. However, because the commercial product still has a high cost, it is not widely used in andrology clinics. Interestingly,

there is no evidence in the study of microfluidic devices for sperm selection using human semen samples with poor sperm quality, i.e low sperm motility.

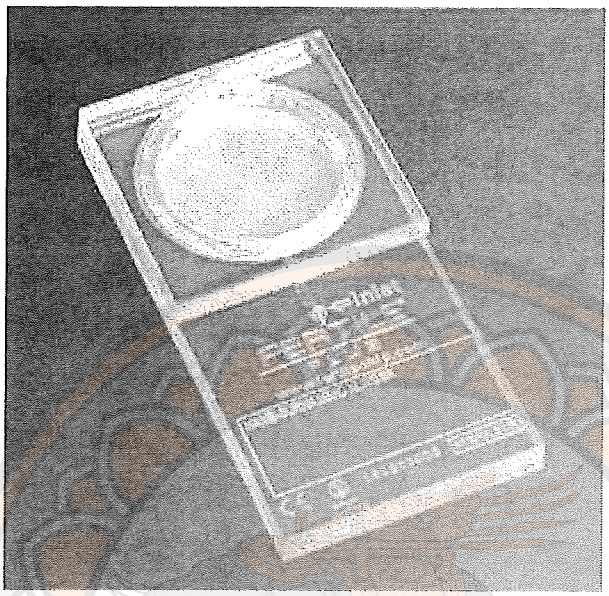


**Figure 18 Image of a commercial product for the sperm selection: the Fertile Plus® microfluidic sperm sorting chips.**

**Source:** https://www.koekbiotech.com/images/products/fertile–plus.jpg, 18 March 2020

# CHAPTER III

# RESEARCH METHODOLOGY

## Animal study

Methamphetamine (METH)–administrated rats were used in this study. These rats exposed the impairment of testicular and sperm functions after METH exposure (Nudmamud-Thanoi et al., 2016). Rat testis was used to study the GABA concentration using high–performance liquid chromatography (HPLC) technique, the mRNA expression of GABAergic components and gonadotropin receptors (FSH and LH receptors) using reverse transcription–polymerase chain reaction (RT–PCR) technique, and the protein expression of FSH receptor using immunohistochemistry technique. Moreover, rat sperm were used to study the protein expression of the GABA receptor using the immunohistochemistry technique.

### Animals

Male Sprague–Dawley (SD) rats were obtained from the National Animal Center, Mahidol University, Thailand. The rats were housed 1–3 per cage and maintained at 24 ± 1°C under a 12 h light/dark cycle with free access to water and food. All animal procedures were carried out in compliance with Mahidol University Code of Practice and the National Institutes of Health (USA) Guidelines for the treatment of laboratory animals by the Center for Animal Research Naresuan University. The experimental protocol for 1) the studies of GABAergic components in rat testis and sperm were approved by the ethical committee of Naresuan University, Thailand (Naresuan University Animal Care and Use Committee (NUACUC), Ref: 60 02 004), and 2) the study of gonadotropin receptors in the testis was approved by the ethical committee of Naresuan University, Thailand (Naresuan University Animal Care and Use Committee (NUACUC), Ref: 55 04 003).

### METH administration

METH administration method were adapted from Segal et al. (Segal, Kuczenski, O'Neil, Melega, & Cho, 2003) was used as previously described (Nudmamud-Thanoi et al., 2016), see Figure 19. Briefly, the rats were divided into 4

groups including control, acute binge (AB) METH, escalating dose (ED) METH, and escalating dose–binge (ED–binge) METH groups. The rats in the control group were injected intraperitoneally (i.p.) with 0.9% normal saline for 15 days. In AB METH group, rats were injected (i.p.) 0.9% with normal saline for 14 days and with METH binge dose, 6 mg/kg METH in 4 times a day at 2 h intervals (4 × 6 mg/kg at 2 h intervals), on day 15. The rats in the ED METH group were treated in parallel with the ED–binge METH group. They were injected (i.p.) with escalating dose, gradually increasing doses of METH (0.1–4 mg/kg), for 14 days and either 0.9% normal saline or METH binge dose (4 × 6 mg/kg at 2 h intervals) on day 15 for the ED METH and ED–binge METH groups, respectively. The schedule for METH administration in the ED–binge METH group including the administration of escalating dose followed by binge dose of METH is shown in Table 1.

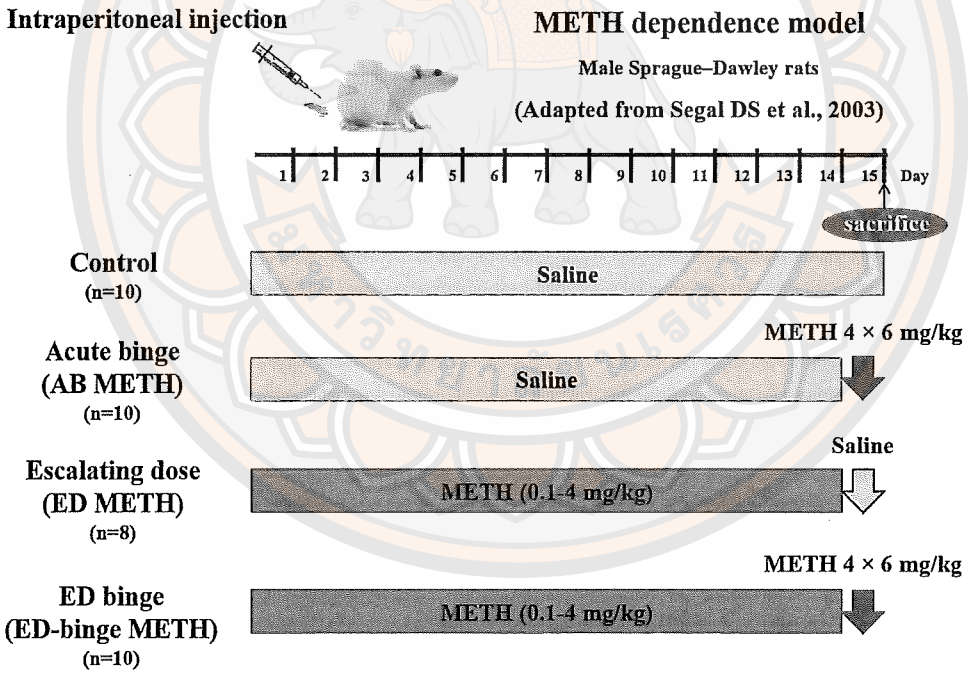



**Figure 19 Schematic diagram of the METH administration in METH dependence model**

**Table 1 Schedule for METH administration in ED–binge METH group including the administration of escalating dose followed by binge dose**

| Day/time | METH (mg/kg) | | | |
|---|---|---|---|---|
| | **07:30** | **10:30** | **13:30** | |
| 1 | 0.1 | 0.2 | 0.3 | |
| 2 | 0.4 | 0.5 | 0.6 | |
| 3 | 0.7 | 0.8 | 0.9 | |
| 4 | 1.0 | 1.1 | 1.2 | |
| 5 | 1.3 | 1.4 | 1.5 | |
| 6 | 1.6 | 1.7 | 1.8 | |
| 7 | 1.9 | 2.0 | 2.1 | |
| 8 | 2.2 | 2.3 | 2.4 | |
| 9 | 2.5 | 2.6 | 2.7 | |
| 10 | 2.8 | 2.9 | 3.0 | |
| 11 | 3.1 | 3.2 | 3.3 | |
| 12 | 3.4 | 3.5 | 3.6 | |
| 13 | 3.7 | 3.8 | 3.9 | |
| 14 | 4.0 | 4.0 | 4.0 | |
| | **07:30** | **09:30** | **11:30** | **13:30** |
| 15 (binge dose) | 6.0 | 6.0 | 6.0 | 6.0 |

**Source:** Adapted from Segal et al., 2003

**Testis and sperm collections**

At the end of the treatment, both sides of testis were removed immediately after sacrifice. The right testis was frozen in dry ice and kept at –80°C until used. The left testis was fixed with 10% neutral buffered formalin. For sperm collection, cauda epididymis (tail of epididymis) was removed and minced in ×1 phosphate–buffered saline (PBS, pH 7.4). Then, the PBS containing only sperm were extracted by pipetting. These sperm were immediately assessed sperm concentration and motility using a Makler counting chamber. The remaining sperm were fixed with 10% neutral buffered formalin and kept at 4°C for the study of sperm morphology and protein expression.

**GABA concentration study in rat testis**

The frozen testis was thawed and homogenized in deionized water on ice. After that, the homogenate was centrifuged at 10,000 rpm for 20 minutes at 4 °C. The supernatant was collected and then precipitated with 50% methanol (HPLC grade). Following precipitation, the sample was centrifuged at 10,000 rpm for 20 minutes at 4 °C again. The supernatant was collected and filtrated with 0.22 μm polyvinylidene fluoride (PVDF) membrane to use for HPLC analysis. GABA concentration was analysed by using HPLC with an electrochemical detector (ECD). The ortho–phthalaldehyde (OPA)/sodium sulfite, derivatizing reagent, was added into filtrated supernatant. Then, samples were injected into the HPLC system by an auto sample injector. C18 reverse–phase (pore size 5 μm) column was used. The mobile phase consists of 0.1 M monosodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4.H_2O$) in 8% methanol (HPLC grade), pH 5.84. Electrochemical conditions for this study were set as + 700 mV for guard cell potential, +400 mV for E1 and +650 mV for E2. Flow rate 1 ml/minute was used for peak separation. Column temperature and auto–sample injection chamber was controlled at 25 °C and 4 °C, respectively. The peak area was recorded and analysed. Besides, this method was validated by precision, linearity, and accuracy test. The precision test was investigated by the value of relative standard deviation (RSD) of samples in intra–day and inter–day measurements. The acceptable value of RSD should be less than 10%. Moreover, the linearity test was investigated and calculated from four different GABA standard concentrations. The acceptable value for the linearity test is $R^2 = 1$. Additionally, the accuracy test was

determined from the percentage of the recovery. The acceptable value for the accuracy test is 100 ± 5% (Monge-Acuna, & Fornaguera-Trias, 2009).

**The mRNA expression study of GABAergic components and gonadotropin receptors in rat testis**

Total RNA from the right testis was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) and chloroform for RNA isolation following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized by an iScript™ cDNA Synthesis kit (Bio–Rad, Hercules, CA) or a qScript™ XLT cDNA Supermix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's protocol. Finally, cDNA was stored at –80°C until used. For the mRNA expression study of GABAergic, oligonucleotide primers were prepared by Macrogen Inc. (Seoul, South Korea), see Table 2. The primer for GABA A–α1 receptor according to published sequences was used (Wearne, Parker, Franklin, Goodchild, & Cornish, 2016), whereas the primers for GAD1 (GAD67), GAD2 (GAD65), GAT1 and glyceraldehyde–3–phosphate dehydrogenase (GAPDH; reference gene) were designed by using Primer3 software (freely available at http://bioinfo.ut.ee/primer3–0.4.0/) and Oligo Analyzer 1.0.3 (Teemu Kuulasma, 2001–2002). The complementary with other sequences in the rat genome was investigate by using PRIMER BLAST. The PCR sequences were amplified using PerfeCTa® SYBR® Green FastMix®, ROX™ (Quanta Biosciences, Gaithersburg, MD), and performed on a PCRmax Eco 48 real–time PCR system (PCRmax, Staffordshire, UK). For the mRNA expression study of gonadotropin receptors, the primers of FSHR and LHR genes were as follows: FSHR–Forward, 5'–CAT–CAC–TGT–GTC–CAA–GGC–CA–3' and FSHR–Reverse, 5'–TGC–GGA–AGT–TCT–TGG–TGA–AAA–3' (Romero, Paredes, Dissen, & Ojeda, 2002); LHR–Forward, 5'–GTT–CAC–CCA–AGA–CAC–TCC–AAT–G–3' and LHR–Reverse, 5'–TCA–GCC–AAA–TCA–GGA–CCC–TA–3' (designed by Oligo Analyzer 1.0.3 (Teemu Kuulasma, 2001–2002) and investigated complementary with other sequences in the rat genome by using PRIMER BLAST). The primers for glyceraldehyde–3–phosphate dehydrogenase (GAPDH; reference gene): GAPDH–Forward, 5'–AAT–GTA–TCC–GTT–GTG–GAT–CTG–A–3' and GAPDH–Reverse, 5'–GCC–TGC–TTC–ACC–ACC–TTC–T–3' (Barlow et al., 2003) was used as an internal control for FSHR and LHR genes expression study. The PCR sequence was amplified using

Ssofast™ EvaGreen supermix (Bio–Rad, Hercules, CA) and analysed on a CFX96 Touch™ Real–Time PCR system (Bio–Rad, Hercules, CA). Then, the gene expression levels were represented as relative mRNA expression values of interesting genes that were normalized with the reference gene (Livak, & Schmittgen, 2001).

**Protein expression study of FSHR in rat testis**

The left testis was dehydrated with serial ethanol and embedded in paraffin. 5 μm thickness sections were used for immunohistochemistry study. Goat anti–FSHR polyclonal antibody (Santa Cruz, CA, USA) was used as primary antibodies. Then, the sections were incubated with biotinylated secondary antibody, and signals were enhanced by using avidin–biotinylated horseradish peroxidase complexes (ABC kit) (Vector, Burlingame, CA). The immunoreaction of FSHR was visualized using DAB (3,3'–Diaminobenzidine) (Vector, Burlingame, CA). After that, the sections were dehydrated with serial ethanol and mounted with mounting media. The expression of FSHR proteins was obtained from a picture that was taken with a Nikon Eclipse 80i microscope. The specific protein intensity was measured by the Scion Image program based on NIH image (v. Alpha 4.0.3.2; www.scioncorp.com; 2000–2001).

**Protein expression study of GABA A–α1 receptor in rat sperm**

Rat sperm were smear on silane coated slides and then air–dried at temperature. Rabbit polyclonal anti GABA A–α1 antibody (Abcam, MA, USA) was used as primary antibodies. Then, the sections were incubated with biotinylated secondary antibody, and signals were enhanced by using avidin–biotinylated horseradish peroxidase complexes (ABC kit) (Vector, Burlingame, CA). The GABA A–α1 immunoreactivities were visualized using DAB (3,3'–Diaminobenzidine) (Vector, Burlingame, CA). After that, the sections were dehydrated with serial ethanol and mounted with mounting media. The expression of proteins was obtained from digital images that were taken by the microscope connected to a microscope camera. 400 sperm from two slides (200 sperm per slide) were used to investigate the expression of GABA A–α1. The percentage of positive sperm, sperm with strong positive immunoreaction, was calculated. Additionally, the intensity values of immunoreaction were measured by ImageJ software (NIH, Bethesda, MD, freely available at https://imagej.nih.gov/ij/). They were represented as normalized relative optical density (ROD).

**Table 2 Primer sequences for RT–PCR in rat testis**

| Gene | Primer sequence (5'–3') | Annealing temp. (°C) | PCR product size (bp) |
|---|---|---|---|
| GABA A–α1 | F: TGTCTTTGGAGTGACGACCGTTCT<br>R: ACACGAAGGCATAGCACACTGCAA | 63°C | 125 |
| GAD1 (GAD67) | F: CCTGGAGCTGGCTGAATACC<br>R: TGTGCTCAGGCTCACCATTG | 60°C | 83 |
| GAD2 (GAD65) | F: GATCGGAACAGACAGCGTGAT<br>R: GCACTCACCAGGAAAGGAACA | 66°C | 123 |
| GAT1 | F: GCAGATGACACCACTCACCA<br>R: ATGACCTGGAGACGCTGCTT | 66°C | 159 |
| GAPDH (reference) | F: AGTGCCAGCCTCGTCTCATA<br>R: GACTGTGCCGTTGAACTTGC | 63°C | 200 |

**Note:** F = Forward, R = Reverse

**Human studies**

There are two experiments in the human study. In the first experiment, the mRNA expression of GABA receptors in human sperm was studied using RT–PCR. The relationship between these expressions and the fertilization rate and embryo quality after intracytoplasmic sperm injection (ICSI) treatment were analysed. Another experiment, a novel microfluidic device for sperm selection which is an important process in the assisted reproductive technology (ART) treatments, i.e., ICSI and *in vitro* fertilization (IVF), was studied.

**The mRNA expression study of GABA receptors in human sperm**

**1. Volunteer patients**

In this study, 113 semen samples were obtained from infertile men who are assessed semen analysis at the Naresuan Infertility Centre, Faculty of Medicine, Naresuan University, Thailand. The experimental protocols were approved by the ethical committee of Naresuan University (Naresuan University Institutional Review Board, IRB No. 0549/60). The consent form was signed and obtained from all patients. The schematic overview of the volunteer patient's composition is shown in Figure 20. Fourteen azoospermic and oligozoospermic men who had sperm concentration less than $5 \times 10^6$ sperm per ml were excluded from the study. Ninety–nine patients that met the inclusion criteria were divided into 3 groups including normozoospermic (NOR, n = 32), oligoasthenoteratozoospermic (OAT, n = 22) and teratozoospermic (TER, n = 45) groups. The first group, normozoospermic men were defined as those had normal sperm concentration ($\geq 15 \times 10^6$ sperm per ml), progressive motility ($\geq 32\%$), and morphology (normal form $\geq 4\%$). Second group, oligoasthenoteratozoospermic men were defined as those had abnormal sperm concentration ($< 15 \times 10^6$ sperm per ml), progressive motility ($< 32\%$) and morphology (normal form $< 4\%$). In the last group, teratozoospermic men were defined as those had normal sperm concentration, normal progressive sperm motility, and abnormal sperm morphology. The age of these patients was 26–69 years.

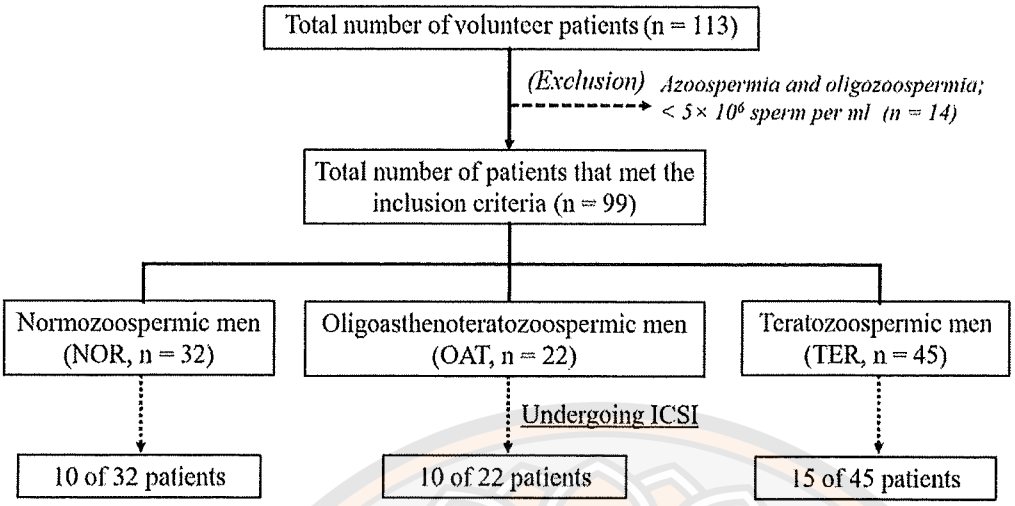



**Figure 20 Schematic overview of the volunteer patient's composition**

**2. Semen samples and semen analysis**

Semen samples were collected by masturbation and allowed to liquefy for 30–60 minutes at room temp. Macroscopic and microscopic examinations were observed according to WHO 2010 recommendations, Laboratory Manual for the Examination and Processing of Human Semen (World Health Organization, 2010). Semen liquefaction, viscosity, appearance, volume, and pH were assessed. After liquefaction, all samples were split into 2 parts for semen analysis and the study of gene expression. For semen analysis, a drop of semen, approximately 10 µl, was loaded into a Makler counting chamber. Sperm concentration and motilities were recorded using an Optikam microscope camera and software (OPTIKA Microscopes, Italy) under a bright–field microscope at 200× magnification. At least 200 sperm were measured. The average sperm per ten squares was defined as the sperm concentration (× $10^6$ sperm per ml). Sperm motility was graded into progressive motility (PR), non–progressive motility (NP), and immotility. The percentage of total motility (PR + NP, %) and progressive motility (PR, %) was used to assess sperm quality. For sperm morphology analysis, a drop of semen was smeared on a glass slide, allowed to air dry, fixed, and stained using the Diff–Quick stain (a Wright Rapid Stain set; RVL Supply, Bangkok). Approximately 200 sperm were observed sperm morphology by a bright–field microscope at 1000× magnification. The blinding of the label in each morphological slide was done for the

analysis. The percentage of normal and abnormal forms of sperm morphology was calculated. Categories of abnormal sperm morphology were estimated according to the WHO 2010 guidelines including the defects of the sperm head, neck and midpiece, principal piece, and excess residual cytoplasm (ERC). A low reference value for normal sperm morphology is 4% normal form (World Health Organization, 2010). Semen having $\geq$ 4% normal form of sperm morphology was regarded as normal sperm morphology. For the study of gene expression, the semen samples were frozen in liquid nitrogen and stored at –80°C until used.

**3. The mRNA expression study of GABA–A and –B receptors in human sperm**

Approximately $10 \times 10^6$ sperm in each semen sample were used for mRNA isolation. Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) and chloroform. Then, complementary DNA (cDNA) was synthesized by a qScript™ XLT cDNA Supermix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's protocol. Finally, the cDNA was stored at –80°C until used for the RT–PCR technique. The sequences of oligonucleotide primers including GABA A–α1 receptor (Chen et al., 2012), GABA B–R2 receptor (Plummer et al., 2011), and glyceraldehyde–3–phosphate dehydrogenase (GAPDH; reference gene) (Yang, Yu, Hong, Yang, & Shao, 2015) were used according to the previous studies for the study of gene expression, see Table 3. They were synthesized by Macrogen Inc. (Seoul, South Korea). The complementary with other sequences in the human genome was tested by PRIMER BLAST. The cDNA were amplified using 2× SensiFAST™ SYBR® No–Rox (BIO–98005, Bioline, London, UK) and performed on the LineGene 9600 Plus QPCR system (Bioer, Hangzhou, China). Then, the gene expression levels were represented as relative mRNA expression values of interesting genes that were normalized with the reference gene (Livak, & Schmittgen, 2001).

**Table 3 Primer sequences for RT–PCR in human sperm**

| Gene | Primer sequence (5'–3') | Annealing temp. (°C) | PCR product size (bp) | Reference |
|---|---|---|---|---|
| GABA A–α1 | F: AGAAAAACAACACTTACGCTCCA<br>R: GGGCTTGACCTCTTTAGGTTC | 57 | 119 | Chen et al., 2012 |
| GABA B–R2 | F: GGAAGAGGTCACCATGCAG<br>R: AGTTTCCCAGGTTGAGGATG | 66 | 101 | Plummer et al., 2011 |
| GAPDH (reference) | F: GCCTCAAGATCATCAGCAATGCCT<br>R: TGTGGTCATGAGTCCTTCCACGAT | 63 | 104 | Yang et al., 2015 |

**Note:** F = Forward, R = Reverse

**4. The relationship between the mRNA expression of GABA receptors and sperm quality, fertilization rate and embryo quality after ICSI**

Parameters of semen quality were observed in the process of semen collection and preparation. The female partner of all patients undergoing ICSI was operated controlled ovarian stimulation, using a standard protocol (the GnRH antagonist protocol), and oocyte retrieval as previously described (ART et al., 2019). After incubation for three hours, oocyte denudation was taken by a hyaluronidase solution (80 IU/ml Hyaluronidase in FertiCult™ flushing medium, FertiPro NV, Belgium). Sperm were prepared using two–layers density gradient centrifugation (DGC), the 45% and 90% of Sil–Select solutions (FertiPro NV, Belgium), following standard swim–up (SU) method. After ICSI, the fertilization rate and embryo quality were assessed. The development of embryo was observed after ICSI from day 1 to day 5. On day 1 (16–18 hours after ICSI), the fertilization of oocyte was observed. The criteria of successful fertilization are the development of embryos into the zygote stage with two polar bodies (first and second polar bodies) and two pronuclei. Moreover, the embryo quality was observed in each stage of embryo development including cleavage stage on day 2 and 3, morula stage on day 4 and blastocyst stage on day 5 after ICSI. The cell number, symmetry and fragmentation were used for the grading of embryo quality on the cleavage stage. Good–quality embryos on cleavage stage were defined as having 6–8 cells on day 3 combined with grade 1–2 of cell symmetry and fragmentation, see Table 4 (Bhilawadikar et al., 2013; Medicine, & Embryology, 2011). For the grading of embryo quality on the morula stage, the embryo with evidence of compaction on day 4 was defined as a good–quality embryo. For the quality of embryo on the blastocyst stage, the blastocyst formation, the inner cell mass (ICM), and the trophectoderm (TE) were used to assess the embryo quality according to Gardner's guideline. The development of blastocyst was graded into 6 grades (grade 1–6) based on their degree of expansion and hatching status, see Table 5 and Figure 21. The ICM and TE were assessed into 3 grades (grade A, B, and C), see Table 6 (Gardner, Lane, Stevens, Schlenker, & Schoolcraft, 2000). Briefly, the ICM is assessed into grade A, tightly packed with many cells; grade B, loosely grouped with several cells; and grade C, very few cells. The TE scores include grade A, many cells forming a cohesive epithelium; grade B, few cells forming a loose epithelium; and grade C, very few cells. The embryos on the blastocyst stage were

graded into good, moderate, and poor quality according to Irani et al. guideline (Irani et al., 2018). In this study, the embryo quality on blastocyst stage were divided into good (3–6 with AA, AB, BA or BB), moderate (3–6 with BC, CB, or CC), and poor (1–2 regardless of ICM and TE grades and arrested embryos; defecting to reach the blastocyst stage). To analyse the relationship between the mRNA expression of GABA receptors and the fertilization rate after ICSI, patients were divided into 2 groups based on the percentage of fertilized oocytes: including 1) High group, patients who had a couple with high fertilization rate (≥ 50% fertilized oocytes); and 2) Low group, patients who had a couple with low fertilization rate (< 50% fertilized oocytes). To analysed the relationship between the mRNA expression of GABA receptors and embryo quality after ICSI in each stage of the embryo development (cleavage, morula and blastocyst stages), patients were divided into 2 groups based on the percentage of the good–quality embryo: including 1) Good group, patients who had a couple with > 50% good–quality embryo; and 2) Poor group, patients who had a couple with ≤ 50% good–quality embryo.

**Table 4 Four grades of cell symmetry and fragmentation of embryos on cleavage stage after ICSI**

| | |
|---|---|
| Grade 1 | Blastomeres of equal size without cytoplasmic fragmentation |
| Grade 2 | Blastomeres of equal or unequal size with low cytoplasmic fragmentation (less than 10%) |
| Grade 3 | Blastomeres of equal or unequal size with moderate cytoplasmic fragmentation (10–50%) |
| Grade 4 | Few blastomeres of any size with severe or complete fragmentation (more than 50%) |

**Source:** Bhilawadikar et al., 2013; Medicine, & Embryology, 2011

**Table 5 Six grades of blastocyst formation on blastocyst stage after ICSI including early blastocyst (Grade 1), blastocyst (Grade 2), full blastocyst (Grade 3), expanded blastocyst (Grade 4), hatching blastocyst (Grade 5) and hatched blastocyst (Grade 6)**

| | |
|---|---|
| Grade 1 | Early blastocyst: A blastocyst with a small size of blastocoel (less than half of the embryo volume) |
| Grade 2 | Blastocyst: A blastocyst with large size of blastocoel (half or greater than half of the embryo volume) |
| Grade 3 | Full blastocyst: A blastocyst with a complete blastocoel (blastocoel completely filling the embryo) |
| Grade 4 | Expanded blastocyst: A blastocyst with a huge of blastocoel (larger than the early embryo) and a thinning zona pellucida |
| Grade 5 | Hatching blastocyst: A blastocyst with a moving of the TE through the zona pellucida |
| Grade 6 | Hatched blastocyst: A blastocyst which completely moves out of zona pellucida |

**Source:** Gardner et al., 2000

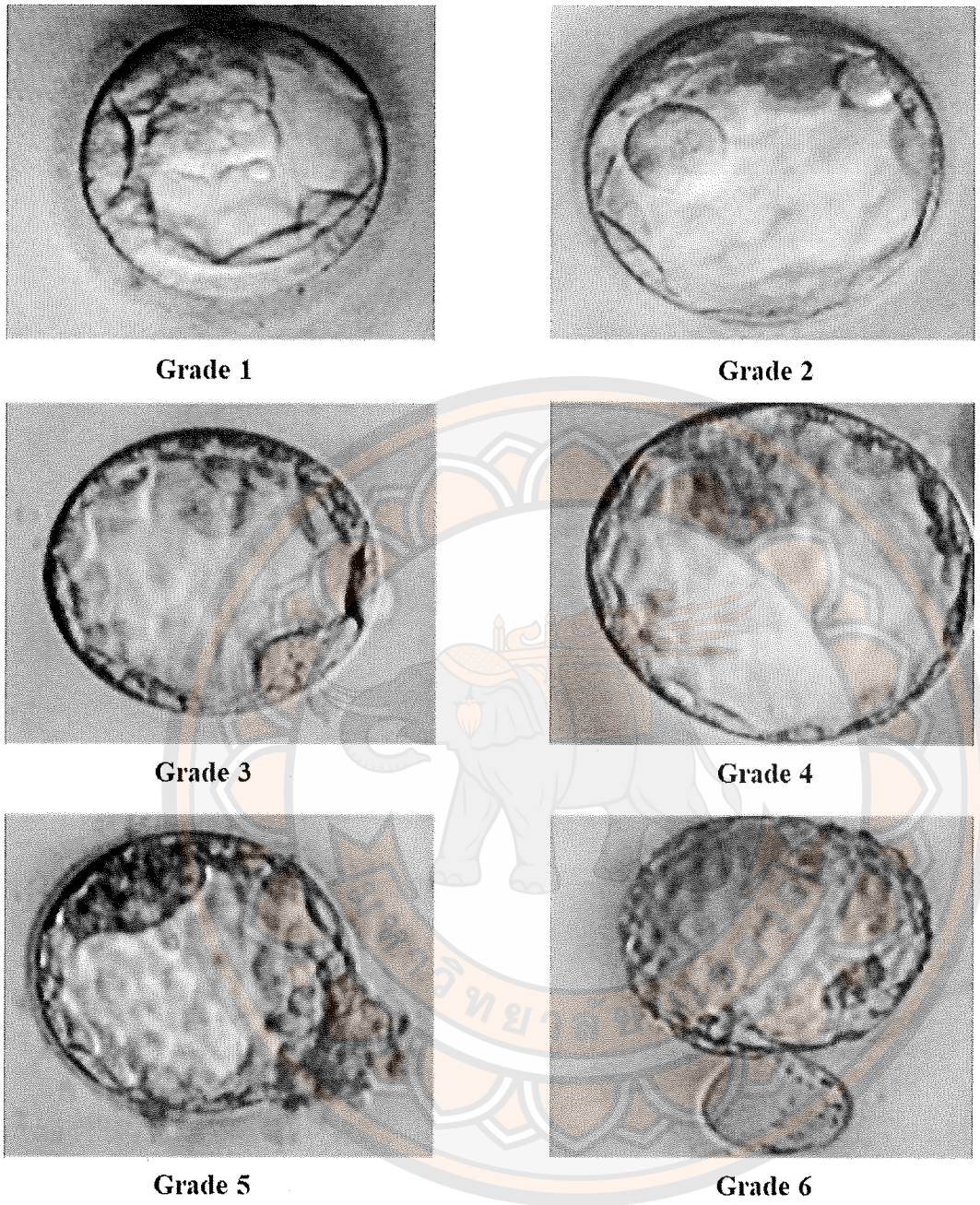



**Figure 21** **Six grades of the blastocyst formation on blastocyst stage including early blastocyst (Grade 1), blastocyst (Grade 2), full blastocyst (Grade 3), expanded blastocyst (Grade 4), hatching blastocyst (Grade 5) and hatched blastocyst (Grade 6)**

**Source:** Adapted from Talwar, 2012

**Table 6 Grading of the inner cell mass (ICM), the trophectoderm (TE) on blastocyst stage after ICSI**

| Grade | ICM | TE |
|---|---|---|
| A | Tightly packed with many cells | Many cells forming a cohesive epithelium |
| B | Loosely packed with several cells | Few cells forming a loose epithelium |
| C | Very few cells | Very few cells |

**Source:** Gardner et al., 2000

**A novel microfluidic device for sperm selection**

**1. Semen samples**

An overview of the semen sample composition in this study is shown in Figure 22. Forty–four semen samples were donated from 21 volunteer donors at the University of Sheffield, United Kingdom (University of Sheffield Research Ethics Committee. Ref: SMBRER293). Those were produced by masturbation, following 3–4 days of sexual abstinence. Then, donors brought ejaculates to the laboratory within 1 hour of production. All ejaculates, n = 44, were left for 30–60 minutes at room temperature for liquefaction. The examination of appearance, viscosity, and volume were done according to the WHO 2010 recommendations, Laboratory Manual for the Examination and Processing of Human Semen (World Health Organization, 2010). Samples with normal appearance (i.e. no blood) and liquefaction were included in this study (n = 41) while unliquefied samples were excluded from the study (n = 3). Forty–one samples which met the inclusion criteria were assessed for sperm concentration, motilities, morphology, and aggregation, see below for details. Samples were divided into Normal and Abnormal groups based on the percentage of progressive motility (PR). The ejaculates in both groups had normal sperm concentration ($\geq 15 \times 10^6$ per ml) and semen volumes between 0.35–5.5 ml. The Normal group, n = 22, was defined as having normal sperm motility (PR $\geq$ 32%) while the Abnormal group, n = 19, was defined as

having abnormal sperm motility (PR < 32%). Each ejaculate in the Normal group was split for selecting by two–layer Percoll DGC, direct SU, and microfluidic device tests. Those in the Abnormal group were split for DGC and microfluidic device tests only because of the low quantities of sperm present in these ejaculates. Interestingly, after the selection using microfluidic device, a very low sperm concentration was found in two ejaculates in the Abnormal group having < 10% PR and ≤ 40% total motility, with high viscosity or large aggregation. Thus, in the Abnormal group, only 17 samples were analysed.

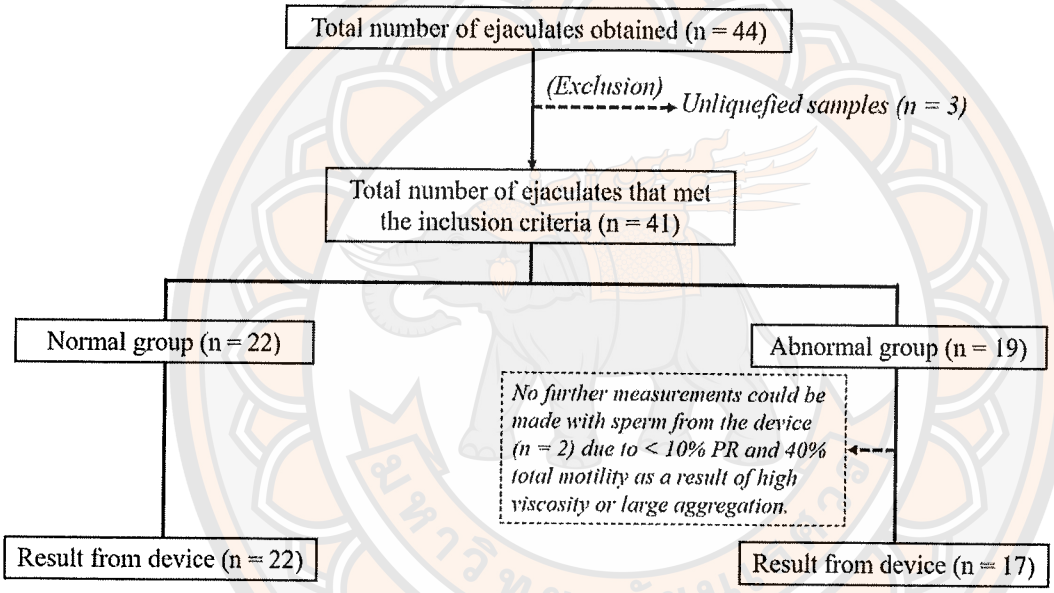



**Figure 22 Schematic overview of the sample composition**

**2. Semen analysis**

Semen samples and sperm selected by all methods were assessed and analysed according to the WHO 2010 recommendations, Laboratory Manual for the Examination and Processing of Human Semen (World Health Organization, 2010). For sperm concentration and motility tests, 3 µl of sperm samples were added into disposable 10 µm depth, 4–cell Leja chamber (Leja, Nieuw–Vennep, The Netherlands) using a positive displacement pipette. Sperm concentration, PR, and total motility were examined using computer–assisted sperm analysis (CASA. SCA, Microptic, Barcelona, Spain) system as previously described (Calvert et al., 2019). For sperm morphology test,

one drop, 10–20 µl, of sperm was smeared onto an adhesive–coated microscope slide, allowed to air dry, and fixed in absolute methanol. Sperm were stained using Shandon Kwik–Diff™ stain kit (Thermo Fisher Scientific, Shandon, UK). Stained slides were examined by bright–field microscope (Microtec microscopes; TEC Microscopes Ltd, Axbridge, UK) at 1000× magnification. At least 200 sperm were counted in normal and abnormal forms of sperm morphology. The researcher was blinded to the sperm selecting methods for each of the morphology slides.

**3. Sperm DNA fragmentation analysis**

The terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay was used for the analysis of sperm DNA fragmentation. Semen samples and sperm selected by all methods were assessed and analysed. 10–20 µl of the sperm samples were smeared in a small square (22mm × 22mm) onto a polysine microscope adhesion slide (VWR, Lutterworth, UK) and allowed to air dry. After air–drying, a Hydrophobic Barrier Pen (ImmEdge, Vector Laboratories, Peterborough, UK) was used to bound around the specimen. Then, the sperm samples were fixed in absolute methanol for one minute and allowed to air dry. Finally, the slides were stored at –20°C until used. For the sperm DNA fragmentation detection, the smeared slides were stained using the fluorescent FragEL™ DNA fragmentation detection kit (Merck Chemicals Ltd., Nottingham, UK) following the manufacturer's instruction. Briefly, the specimen was rehydrated with ×1 Tris–buffered saline (TBS, pH 7.6. Sigma Chemical Co. Ltd, Poole, UK) for 15 minutes and then permeabilized using 100 µl of 20 µg/ml proteinase K for 5 minutes. After washing the slides three times with ×1 TBS, these slides were incubated with 100 µl of ×1 terminal deoxynucleotidyl transferase (TdT) equilibration buffer for 30 minutes at room temperature followed by 60 µl of TdT labeling reaction mixture for 90 minutes at 37°C in a humidified chamber. After incubation, the slides were washed three times with ×1 phosphate–buffered saline (PBS, pH 7.4. Fisher Scientific, Loughborough, UK) for one minute. Finally, the slides were mounted using Fluorescein–FragEL™ mounting media, containing 4',6–diamidino–2–phenylindole (DAPI), protected with coverslips, with the edges sealed with clear varnish. A manufacturer supplied control slide containing a mixture of DNA–intact (normal cells) and DNA–fragmented cells (apoptotic cells) was stained to confirm the accuracy of the staining procedure. The visualization of DNA–

intact and DNA–fragmented sperm was performed with a fluorescence microscope (Olympus IX73, London, UK) at 600× magnification using a DAPI filter (excitation wavelength of 330–380 nm) and a fluorescein filter (excitation wavelength of 465–495 nm). DNA–fragmented sperm (the TUNEL–positive sperm) exhibited bright green fluorescence, while DNA–intact sperm (the TUNEL–negative sperm) exhibited blue fluorescence. The DNA fragmentation index (DFI) was defined as the number of DNA–fragmented sperm divided by the number of total sperm. At least 500 sperm in each slide were counted.

**4. Density gradient centrifugation method**

Two–layer Percoll gradients were used for the DGC method, see Figure 23. Briefly, 40% and 80% Percoll solutions (GE Healthcare, Buckinghamshire, UK) were prepared in Earle's Balanced Salt Solution (EBSS; Sigma Chemical Co. Ltd, Poole, UK). Then, the two–layer Percoll gradients were prepared. 1.5 ml of 40% Percoll solution was layered onto an equal volume of 80% Percoll solution in a 13 ml polypropylene tube with a ventilation cap (Sarstedt, Leicester, UK). For the sperm selection, 1 ml of unprocessed semen was layered on top of the gradients. After centrifugation at 300g for 20 minutes, the sperm pellet was separately resuspended in ×1 EBSS, pH 7.4 in a new tube. The tube was recentrifuged at 500g for 10 minutes. Finally, approximately 0.5 ml of the pellet containing the motile sperm was resuspended and used for sperm analysis, see above.

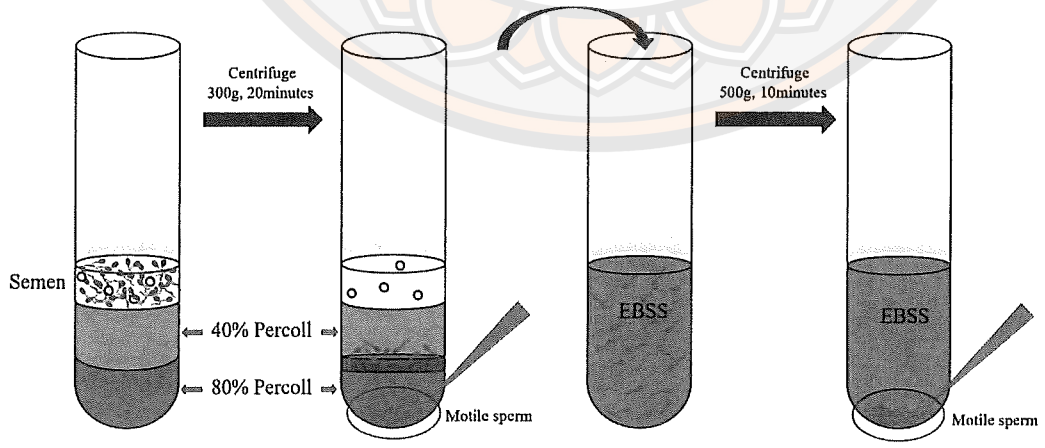



**Figure 23 Two–layer Percoll DGC method for perm selection (an upper phase (40% Percoll) and a lower phase (80% Percoll)**

**5. Swim–up method**

A direct SU method was used for sperm separation, see Figure 24. Briefly, approximately 0.3 ml of unprocessed semen sample was gently added at the bottom of a 13 ml polypropylene tube with a ventilation cap containing 1 ml of ×1 EBSS, pH 7.4. After incubation in a 37°C, 5% CO2 incubator for 1 hour by placing the tube at 45° angle, approximately 0.5 ml of the EBSS solution containing the motile sperm was collected from the top of the sample, taking care not to disturb the semen layer and used for sperm analysis, see above.

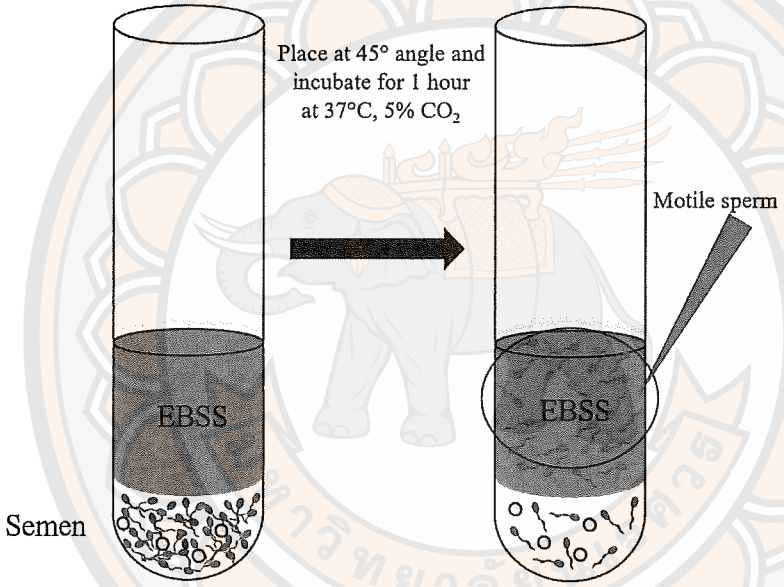



**Figure 24 Direct SU method for sperm selection**

**6. Microfluidic device method**

In this study, a novel microfluidic device for sperm selection was produced by the research team at the University of Warwick, UK. Its microfluidic channel was designed to select sperm base on the behaviour of sperm movement (Denissenko et al., 2012; Kantsler et al., 2013). Theoretically, motile sperm move from the inlets along the boundaries of the microfluidic channels (containing an asymmetric triangle arrayed microstructure) that directs them toward the outlet, as shown in Figure 25. For the fabrication, polydimethylsiloxane (PDMS), non–toxic polymers, was used as the

material of the devices. Each device was mounted onto glass plates using the covalent bonding of silicon and oxygen, the formation of silicon dioxide. The PDMS–glass microfluidic device has a circle shape, 3.5 cm in diameter, with four inlets (I) and one outlet (O) ports. In this study, there are two types of microfluidic devices, including 5*5 and 5*3 devices. These devices have the same outlet size, 5 mm, but different inlet size. The 5*5 device has 5 mm in diameter while the 5*3 device has 3 mm in diameter, see Figure 26. All inlet ports are connected to the outlet port by microfluidic channels that are designed to separate motile sperm from non–motile/immotile sperm.

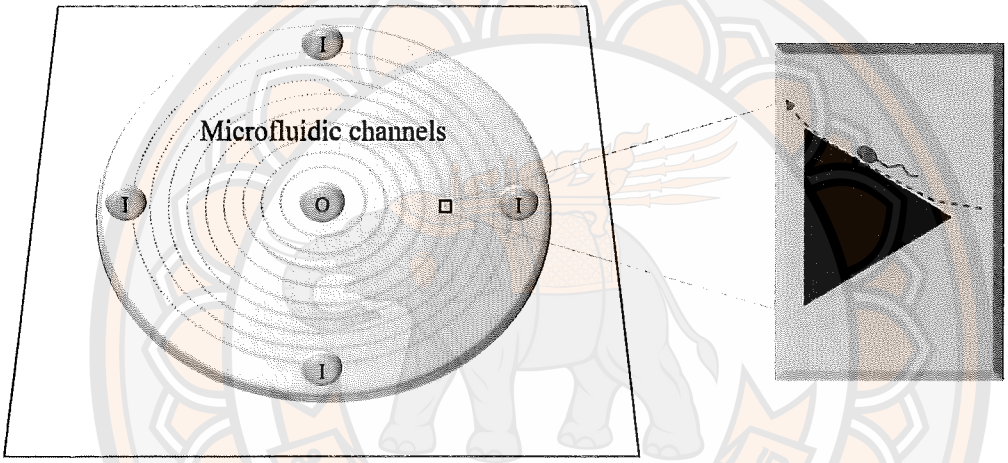



**Figure 25 Theoretical movement of sperm within microfluidic channels**

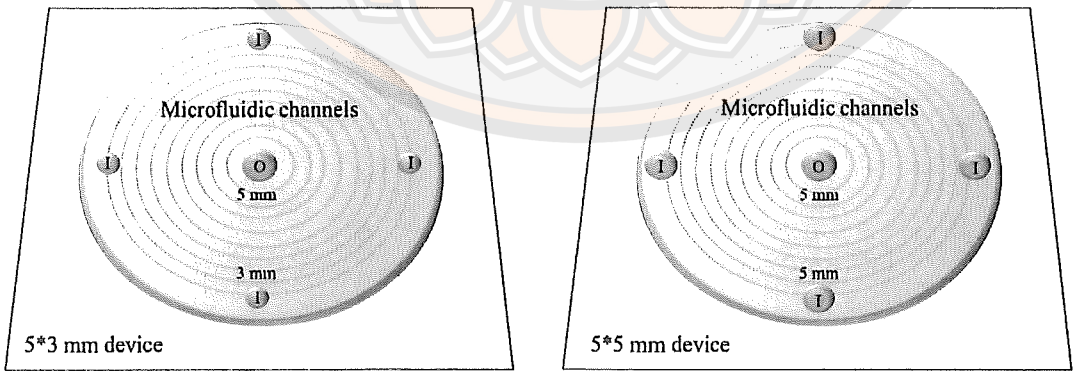



**Figure 26 Two microfluidic devices with different inlet size; 5*3 device (Left) and 5*5 device (Right)**

All devices were prepared before use. Firstly, the devices were flushed with ×1 EBSS containing 0.2%w/v Bovine serum albumin (BSA; Sigma Chemical Co. Ltd, Poole, UK). After flushing, the prepared devices without air bubbles were prewarmed for 2 minutes on a 37°C hot plate. Figure 27 shows the microfluidic device method. For the sperm selection in Normal group, 100 µl (25 µl per inlet) and 60 µl (15 µl per inlet) of unprocessed semen were loaded into 5*5 and 5*3 devices, respectively. In the Abnormal group, 100 µl (25 µl per inlet) of unprocessed semen was loaded into a 5*5 device. Then, the device was placed into a humidified chamber inside a 5% CO2 incubator at 37°C for 1 hour (Normal group) or 2 hours (Abnormal group). After the incubation, motile sperm were extracted from the outlet by pipetting for sperm analysis, see above. The extraction volume was between 30–75 µl.

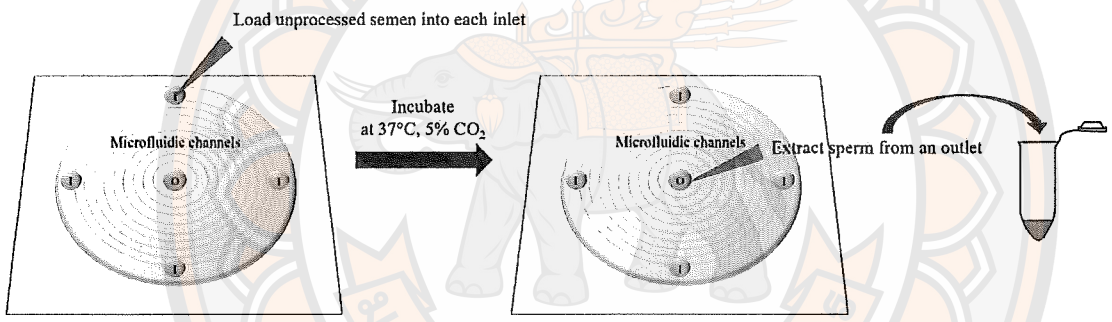



**Figure 27 The microfluidic device method for sperm selection.**

### 6. The dilution test for the validation of microfluidic device method

The dilution test was done to evaluate the operating range of sperm concentration using three semen samples. The serial dilution of each sample was made in a dilution factor of 2 up to 5 times, i.e., 100%, 50%, 25% 12.5, 6.25%, 3.125%. The seminal plasma prepared by DGC was used for the semen dilution. Briefly, 1 ml of unprocessed semen were layered onto 40% Percoll–EBSS solution in a 13 ml polypropylene tube with a ventilation cap and centrifuged for 20 minutes at 300g. After centrifugation, approximately 800 µl of seminal plasma was removed into a new microcentrifuge tube and then recentrifuged for 10 minutes at 500g. Finally, the supernatant which was the seminal plasma without sperm were removed into a new microcentrifuge tube. One drop of prepared seminal plasma was loaded on a glass slide

(VWE Collection, VWR, Leicestershire) and observed the remaining of sperm under a bright–field microscope (Microtec microscopes; TEC Microscopes Ltd, Axbridge, UK) at 200× magnification. The undiluted and diluted semen in each sample were tested in separate 5*5 devices using the microfluidic device method for the Normal group as above. Sperm concentration and motilities were determined for each dilution.

# CHAPTER IV

# THE ALTERATIONS OF GABAERGIC SYSTEM AND GONADOTROPIN RECEPTORS IN TESTIS AND SPERM OF MALE RATS WITH POOR SPERM QUALITY: A MODEL OF METHAMPHETAMINE DEPENDENCE

## Introduction

GABAergic system consisting of GABA, GABA receptors, GABA synthesizing enzyme (glutamate decarboxylase; GAD), and GABA transporters (GAT) has been found in testis and sperm. There are many findings of GABAergic components in testis including GABA, GABA receptors (especially GABA–A (Geigerseder et al., 2004; Hu, & Yan, 2002; Li et al., 2005), GABA–B (He et al., 2001; Kanbara et al., 2005), and GABA–C receptors (Li et al., 2008)), GAD65/67 (Geigerseder et al., 2003), and GAT1 (Hu et al., 2004; Zhang et al., 2009). Interestingly, the finding of GAD65 and GAD67 localized on Leydig cells has demonstrated that GABA might be synthesized by Leydig cells in testis. Expression of GABA–A and –B receptors but not GABA–C receptor has been found in sperm (He et al., 2003; He et al., 2001; Hu et al., 2002; Hu, & Yan, 2002; Li et al., 2005; Ritta et al., 1998); however, GAT1 expression in sperm has also been reported (Hu et al., 2000; Zhang et al., 2009). GABAergic components have been reported to be involved in Leydig cell proliferation, spermatogenesis, and testosterone production in testis (Geigerseder et al., 2004; Hu et al., 2004; Zhang et al., 2009). GABA–A and –B receptors play a role in fertilization processes including sperm capacitation, hyperactivated motility, and acrosome reaction (Burrello et al., 2004; Calogero et al., 1999; Calogero et al., 1996; Jin et al., 2009; Kon et al., 2014; Puente et al., 2011). Besides, the level of GAT1 expression is associated with sperm quality (Hu et al., 2004; Zhang et al., 2009).

It is well known that gonadotropins including follicle–stimulating hormone (FSH) and luteinizing hormone (LH) are released by the anterior pituitary gland of the brain and act through their receptors (FSHR and LHR). Those receptors are located in testicular cells (especially, Sertoli and Leydig cells) to maintain spermatogenesis in

testis (Holdcraft, & Braun, 2004). The expression levels of FSHR and LHR in the testis differ in each stage of spermatogenesis. FSHR levels are highest in stage XIII–II and lowest in stage VII–VIII of spermatogenesis (Heckert, & Griswold, 1993).

Methamphetamine (METH) is one of the psychoactive substances causing the addiction and adverse effects on the nervous system as well as the male reproductive system. METH abuse induces oxidative stress and the increase in apoptotic seminiferous tubules and germ cells in testis (Alavi et al., 2008; Barenys et al., 2009; Nudmamud-Thanoi, & Thanoi, 2011; Yamamoto et al., 2002). Additionally, it causes testicular changes and the impairment of sperm quality. The significant decrease of epididymal sperm numbers and the percentage of sperm with normal morphology have been found after METH administration in a single dose (Nudmamud-Thanoi, & Thanoi, 2011). Furthermore, rats receiving METH in escalating dose (gradually increasing doses of METH) and binge dose (multiple high doses of METH) patterns have also shown a reduction in sperm motility and a number of normal sperm morphology as well as the expression of progesterone and estrogen receptors in spermatogenic and Sertoli cells (Nudmamud-Thanoi et al., 2016). Several studies have reported that METH causes endocrine disruption. Gonadotropins are involved in modulating neurotoxicity which causes by METH (reviewed by Dluzen, Anderson, & Pilati, 2002; Dluzen, & McDermott, 2002). Moreover, the administration of amphetamine derivatives can disrupt the mRNA expression of gonadotropin–releasing hormone (GnRH) which is a hormone for directly controlling the release of FSH and LH (Dickerson, Walker, Reveron, Duvauchelle, & Gore, 2008). The changes in gonadotropin and testosterone levels were occurred because of METH administration (reviewed by Fronczak, Kim, & Barqawi, 2012). Interestingly, METH also affects the changes in catecholamine levels in testis (Janphet et al., 2017). Taken together, these findings indicate the powerful adverse effects of METH on testicular and sperm functions. Therefore, the present study used METH–administrated rats as the model of poor sperm quality to study the changes in the GABAergic system in testis and sperm and its association with gonadotropin receptors. The study hypothesized that if the GABAergic system is directly involved in the testicular and sperm functions, the changes in expression of GABAergic components and its association with gonadotropin receptors should be found in METH–administrated rats.

**Methods**

1) Animals
(see, Chapter III; Animal study)

2) Testis and sperm collections
(see, Chapter III; Animal study)

3) Experiments
- GABA concentration
(see, Chapter III; Animal study)
- The mRNA expression
(see, Chapter III; Animal study)
- The protein expression
(see, Chapter III; Animal study)

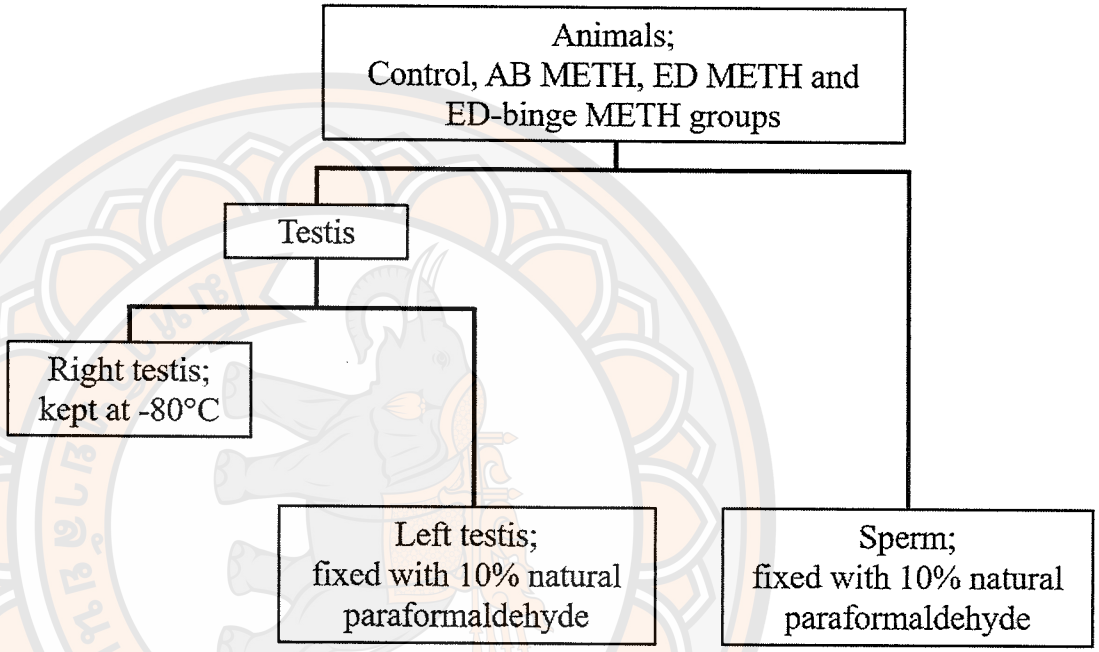



**Figure 28 Schematic representation of methods in animal study; the study of the alteration of GABAergic system and gonadotropin receptors in testis and sperm of male rats with poor sperm quality: a model of METH dependence**

### Statistical analysis

Normal distribution of the data was determined using Shapiro–Wilk test. The statistical difference between three or more groups was analysed using One–way ANOVA followed by Dunnett's post hoc test (parametric data) and The Kruskal–Wallis test followed by Dunn's multiple comparison test (nonparametric data). Statistically significant was considered at $P \leq 0.05$. Data are shown as mean ± SEM. Moreover, Pearson's correlation coefficient was used to investigate the relationship in all parameters.

## Results

### Sperm quality

The results of sperm quality in each group were shown in the previous study, (Nudmamud-Thanoi et al., 2016). METH–administrated rats revealed a significant reduction in normal sperm motility and morphology.

### GABA concentration in rat testis

The results of HPLC method validation including precision, accuracy, and linearity tests indicated that this method is suitable for GABA concentration analysis in testis. The values of RSD of samples in intra–day and inter–day measurements were 0.240% and 1.925%, respectively. The linearity and accuracy test results were acceptable ($R^2$ = 0.999 and recovery rate = 100%). The chromatograms of GABA standard solution and GABA in rat testis samples are shown in Figure 29a and Figure 29b, respectively. The concentration of GABA was significantly increased in AB METH, ED METH and ED–binge METH groups compared with control group (26.97 ± 2.05, 26.92 ± 1.28, 26.47 ± 2.01 vs 20.49 ± 1.46 for AB METH, ED METH and ED–binge METH vs Control), see Figure 30.

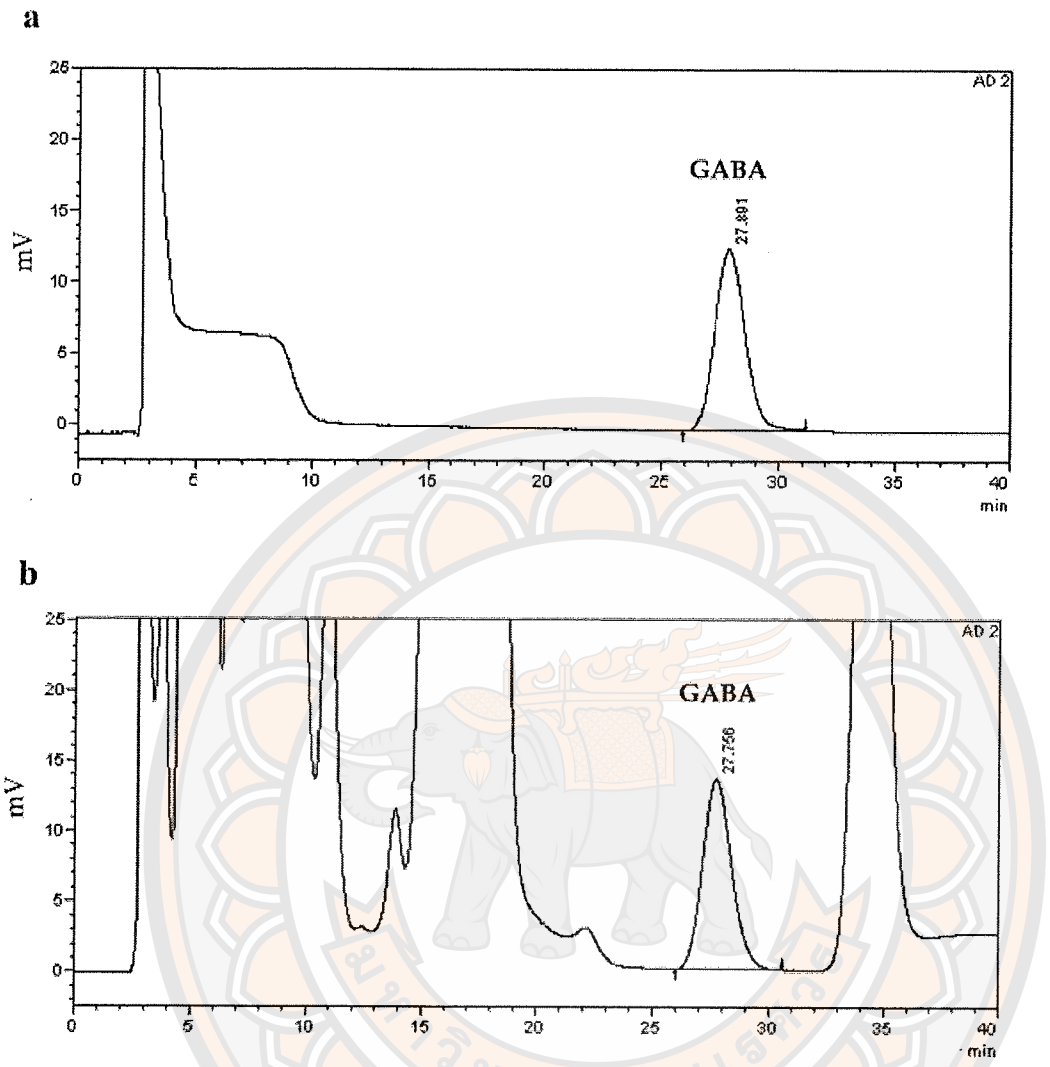



**Figure 29 Representative the chromatograms of (a) GABA standard solution, 2.5 ng/µl and (b) GABA in rat testis sample**

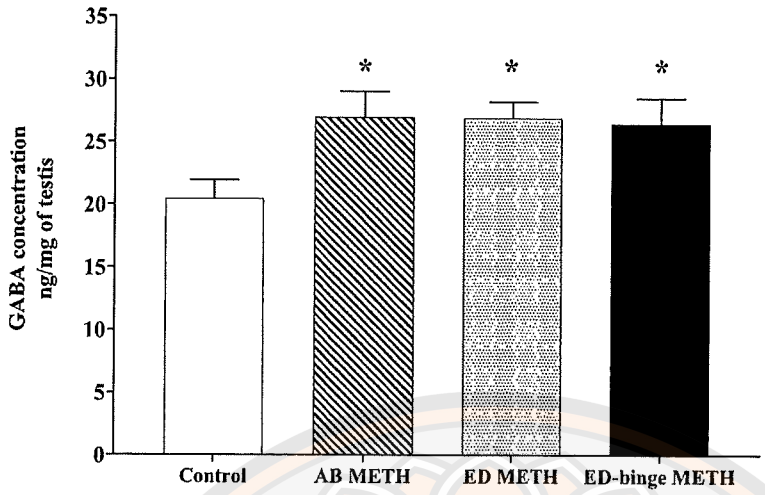



**Figure 30 GABA concentration in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 8–10 per group (*P ≤ 0.05; Dunnett's post hoc test).**

**GABA A–α1 receptor, GAD1 (GAD67), GAD2 (GAD65) and GAT1 mRNA expression in rat testis**

METH–administrated rats in ED–binge METH group had a significant increase in mRNA expression levels of GABA A–α1 receptor and GAD1 (GAD67) in testis compared with control group (GABA A–α1 receptor, 1.29 ± 0.08 vs 1.02 ± 0.06; GAD1 (GAD67), 1.23 ± 0.03 vs 1.00 ± 0.03 for ED–binge METH vs Control), see Figure 31 and Figure 32, but had no significant difference in GAD2 (GAD65) in the testis (Figure 33). A significant decrease in the GAT1 mRNA expression in the testis was found in the ED METH group compared with control group (0.77 ± 0.07 vs 1.01 ± 0.04), see Figure 34. Moreover, a negative correlation between the mRNA expression of GAT1 and GABA concentration in testis ($r = -0.5821$, $p = 0.0002$) is shown in Figure 35.

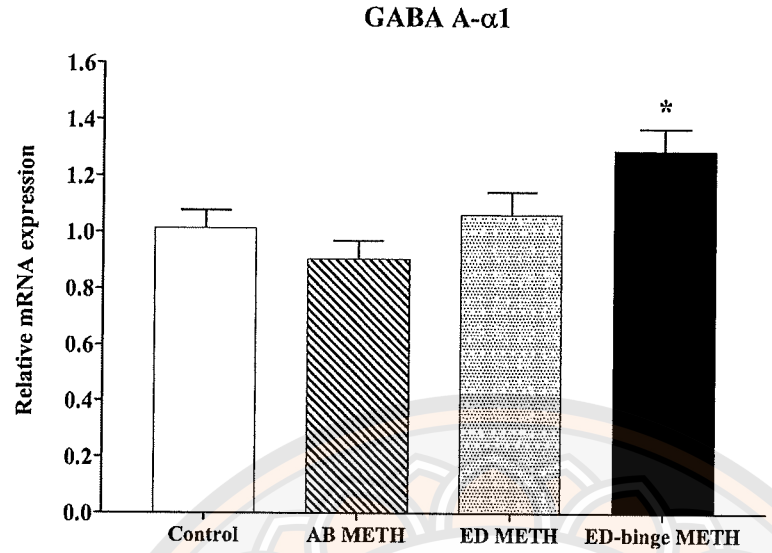



**Figure 31** **GABA A–α1 receptor mRNA expression in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 9–10 per group (*P < 0.05; Dunnett's post hoc test).**

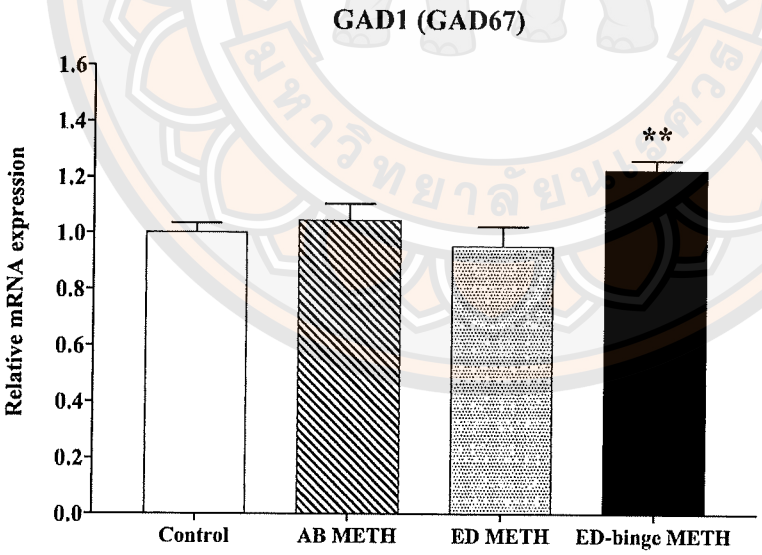



**Figure 32** **GAD1 (GAD67) mRNA expression in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 9–10 per group (**P < 0.01; Dunnett's post hoc test).**

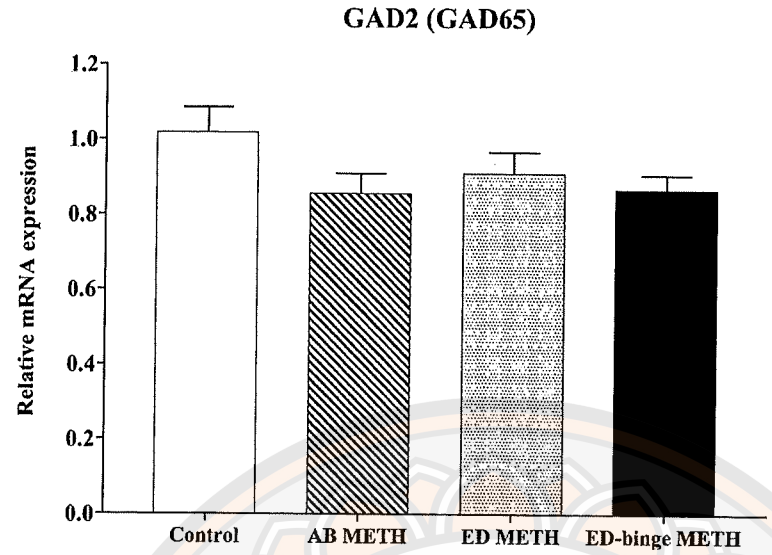



**Figure 33** **GAD2 (GAD65) mRNA expression in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 9–10 per group.**

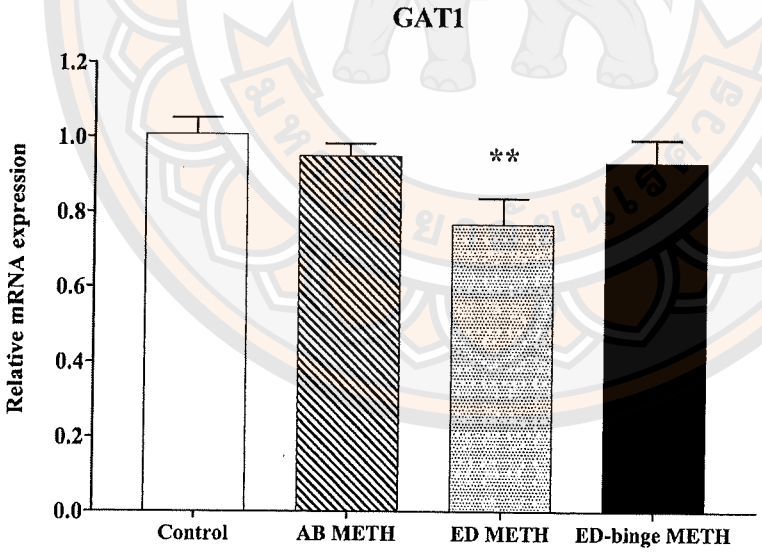



**Figure 34** **GAT1 mRNA expression in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 9–10 per group (**P < 0.01; Dunnett's post hoc test).**

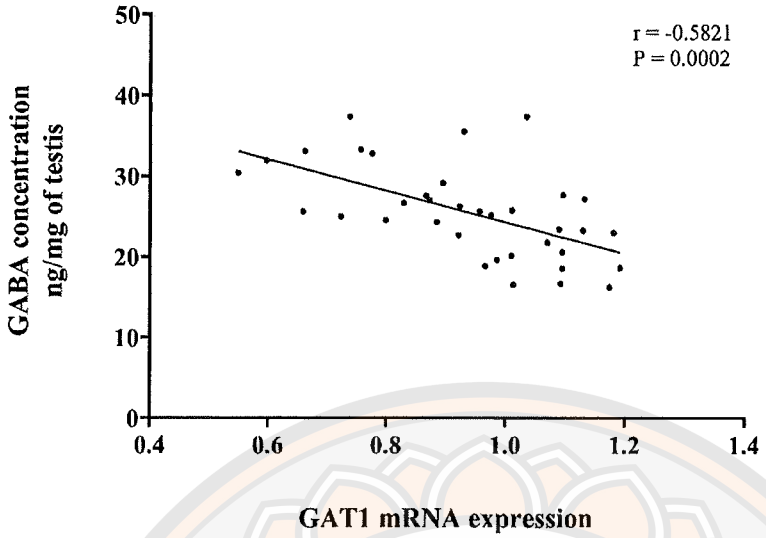



**Figure 35 Correlation between GAT1 mRNA expression and GABA concentration in testis. Linear regression line (black line) fitted to all data points.**

**The relationship between GABAergic system in testis and sperm parameters**

For total sperm motility, this study found a negative correlation between the percentage of normal sperm motility and GABA concentration in testis ($r = -0.4687$, $p = 0.0039$; Figure 36), whereas a positive correlation between the percentage of total sperm motility and the mRNA expression of GAT1 in testis was found ($r = 0.4930$, $p = 0.0022$; Figure 37). Similarly, the mRNA expression of GAT1 in testis was positively related to the normal form of sperm morphology ($r = 0.4613$, $p = 0.0046$; Figure 38).

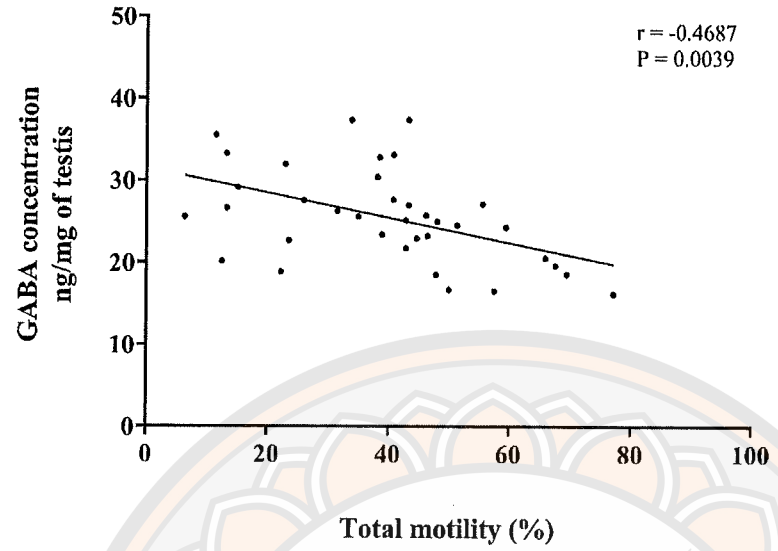



**Figure 36** Correlation between the percentage of total sperm motility and GABA concentration in testis. Linear regression line (black line) fitted to all data points.

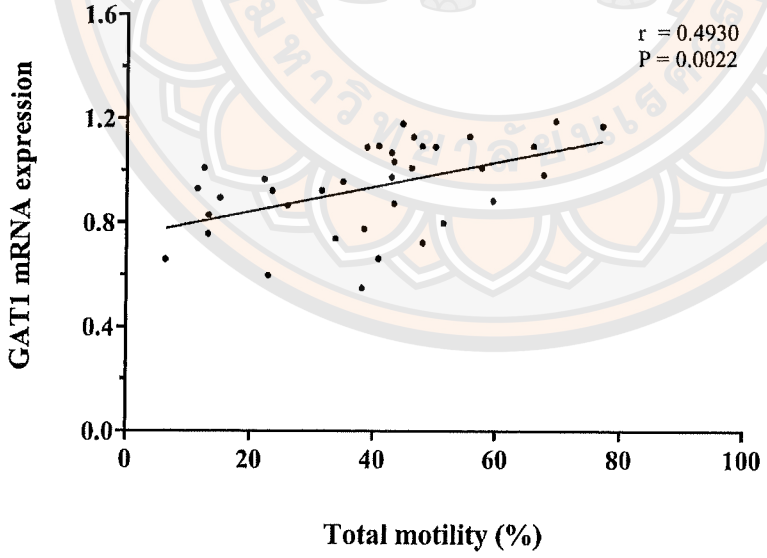



**Figure 37** Correlation between the percentage of total sperm motility and GAT1 mRNA expression in testis. Linear regression line (black line) fitted to all data points.

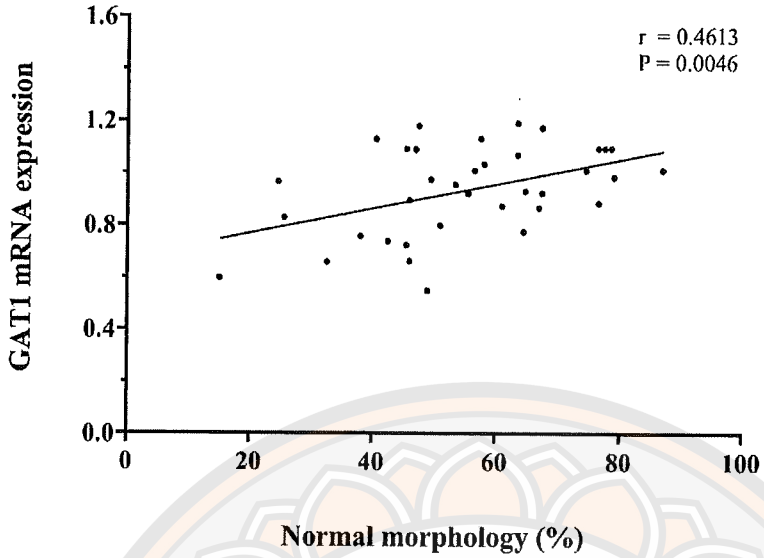



**Figure 38 Correlation between normal sperm morphology and GAT1 mRNA expression in testis. Linear regression line (black line) fitted to all data points.**

**GABA A–α1 receptor protein expression in rat sperm**

As shown in Figure 39, in immunohistochemistry analysis, GABA A–α1 receptor was located in the anterior acrosomal segment of the head region of rat sperm. The staining intensity of GABA A–α1 receptor on the sperm head was used to divide those sperm into strong–positively stained, weak–positively stained and negative, see Figure 40. The percentage of the strong–positively stained sperm for GABA A–α1 receptor immunostaining was significantly increased in METH–administrated groups, AB METH (72.06 ± 5.29) and ED–binge METH (71.34 ± 4.15) groups, compared with control group (32.19 ± 4.80), see Figure 41. Moreover, the relative optical density (ROD) of GABA A–α1 receptor on sperm was significantly increased in AB METH (2.12 ± 0.21) and ED–binge METH (2.13 ± 0.16) groups compared with control group (1.00 ± 0.10), see Figure 42.

**The relationship between GABA A–α1 receptor expression in sperm and sperm parameters**

This study found a negative correlation between the percentage of normal sperm motility and the protein expression of GABA A–α1 receptor in sperm (ROD: r = –0.4745, p = 0.0143; the percentage of strong–positively stained sperm: r = –0.5868, p = 0.0016, Figure 43). Moreover, those expression of GABA A–α1 receptor were negatively related to the normal form of sperm morphology (ROD: r = 0.4619, p = 0.0175; the percentage of strong–positively stained sperm: r = –0.5418, p = 0.0043, Figure 44)

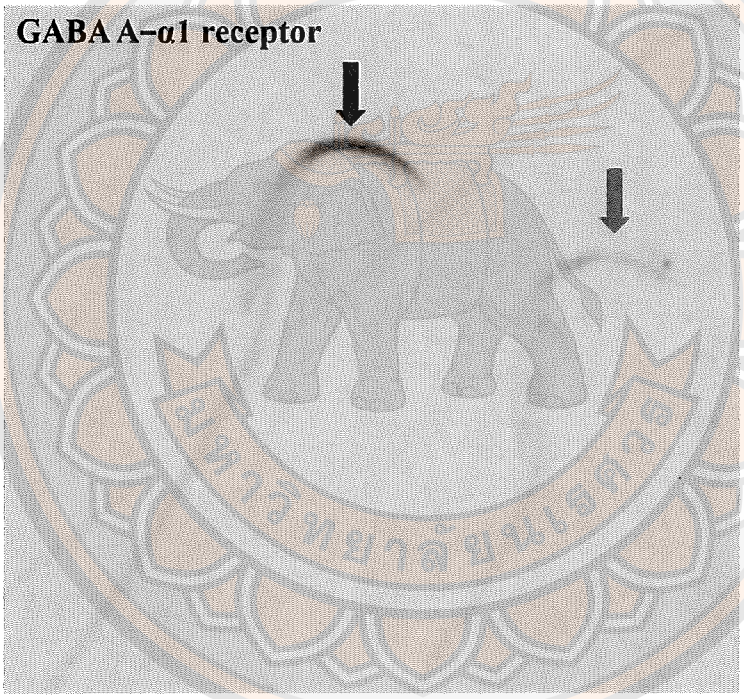



**Figure 39 Immunostaining for GABA A–α1 receptor in rat epididymal sperm at 1000× magnification. A black arrow indicates an immunopositive spermatozoon, whereas a red arrow indicates an immunonegative spermatozoon.**

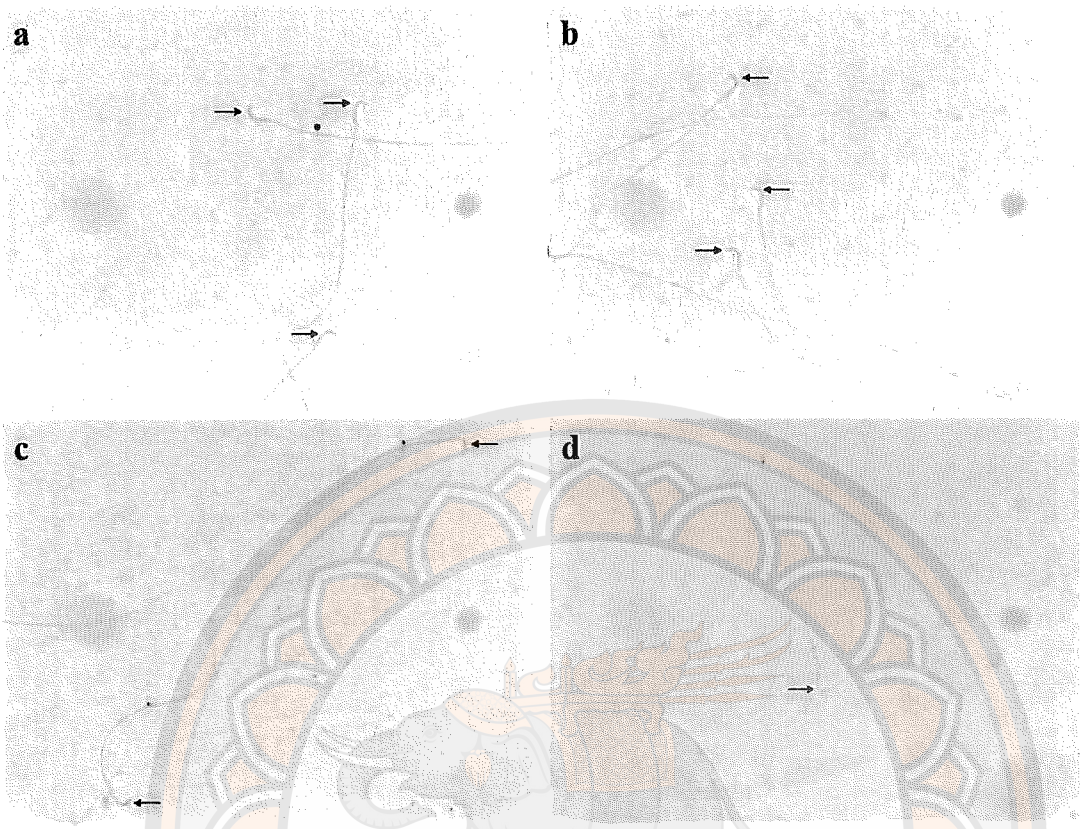



**Figure 40** **Immunostaining for GABA A–α1 receptor in rat epididymal sperm at 200× magnification (a–d). Black arrows in a–c indicate the strong–positively stained sperm, whereas blue arrows in a–b indicates the weak–positively stained sperm. A red arrow indicates a negatively stained spermatozoon (d).**

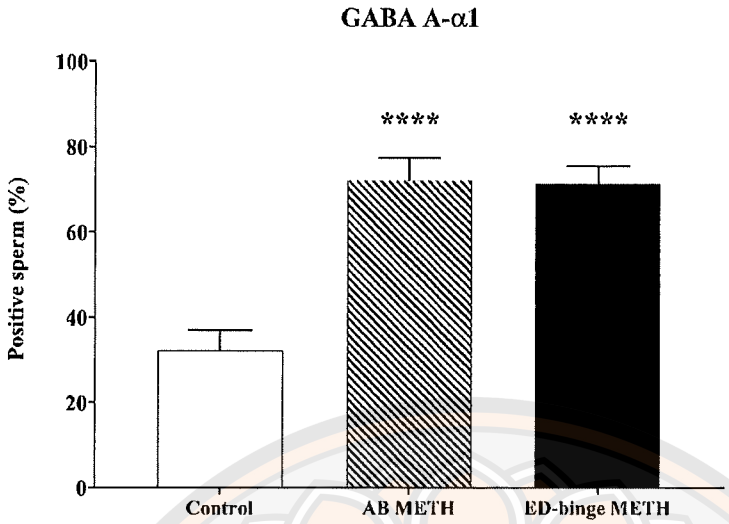



**Figure 41** **The percentage of strong–positively stained sperm for GABA A–α1 receptor immunostaining in METH–administrated group compared with control group. Values are shown as mean ± SEM, n = 8–9 per group (****P < 0.0001; Dunnett's post hoc test).**

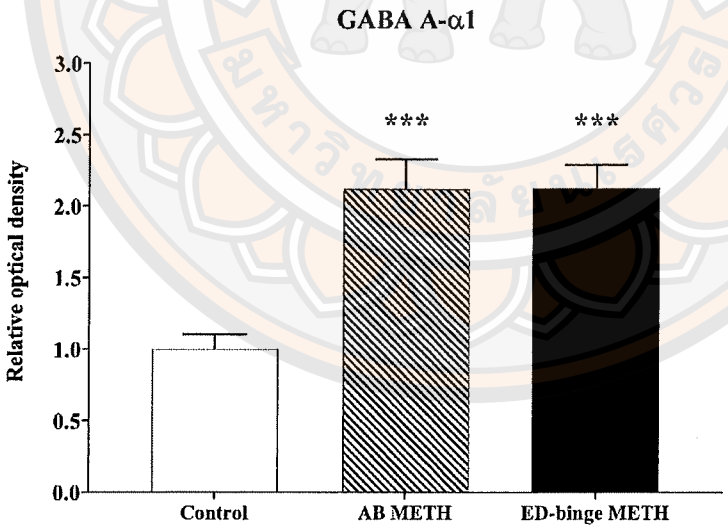



**Figure 42** **Relative optical density of GABA A–α1 receptor in rat sperm of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 8–9 per group (***P < 0.001; Dunnett's post hoc test).**

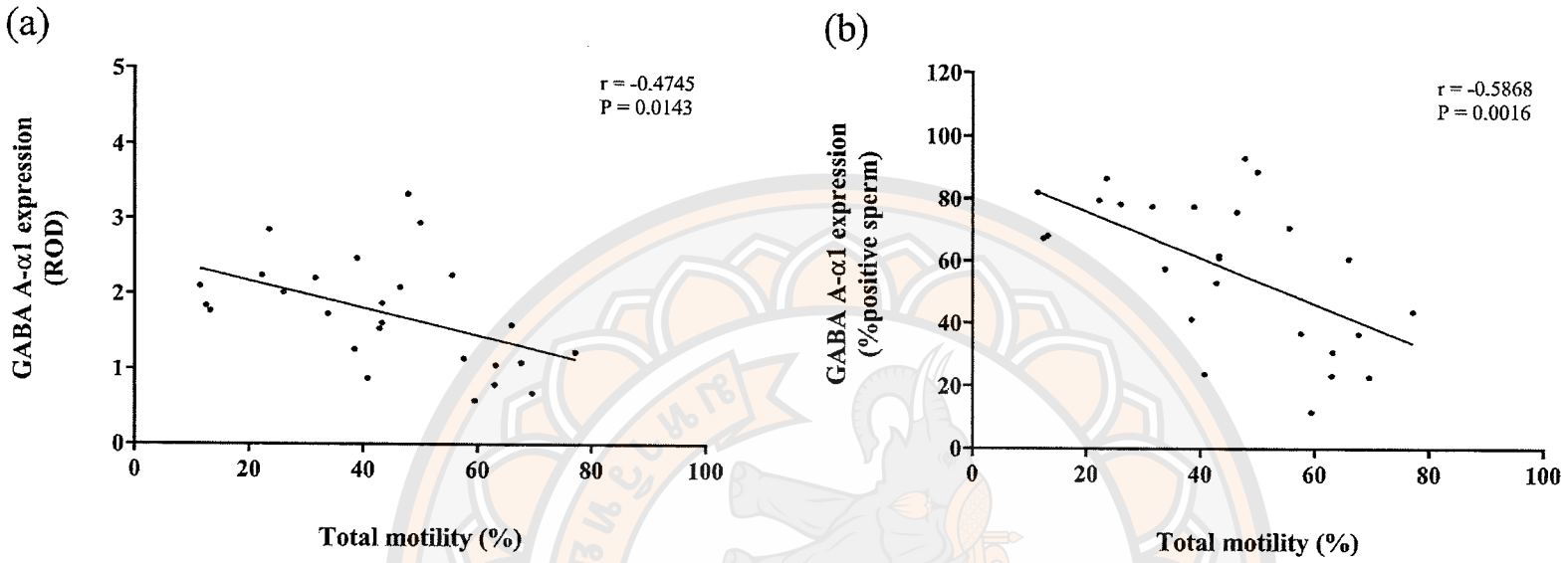



**Figure 43** **Correlations between the percentage of total sperm motility and GABA A–α1 receptor expression in sperm including (a) relative optical density (ROD) and (b) the percentage of strong– positively stained sperm for GABA A–α1 receptor immunostaining. Linear regression line (black line) fitted to all data points.**

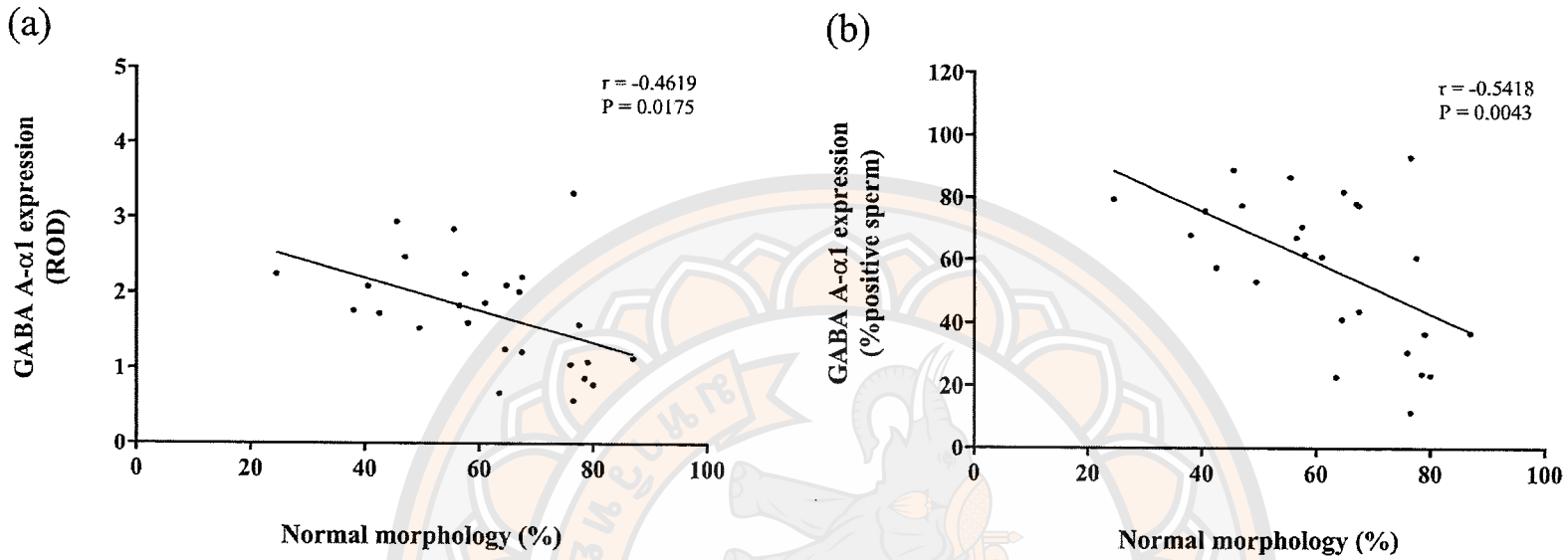



**Figure 44** **Correlations between the percentage of normal sperm morphology and GABA A–α1 receptor expression in sperm including (a) relative optical density (ROD) and (b) the percentage of strong– positively stained sperm for GABA A–α1 receptor immunostaining. Linear regression line (black line) fitted to all data points.**

**FSHR and LHR expressions in rat testis**

There was a decrease in the mRNA expression of FSHR and LHR in METH–administrated groups compared with control group, these were not significantly different. (Figure 45 and Figure 46). The results of immunohistochemistry indicated the strong expression of FSHR protein on Sertoli cell and testicular germ cells in stage XII, XIII, XIV, and I of the spermatogenic cycle in the rat testis. In stage XII and XIII, the FSHR expression was found in various cell types within a seminiferous tubule that consist of Sertoli cell, spermatogonia, zygotene spermatocyte, and elongated spermatid but not pachytene and diplotene spermatocytes, see Figure 48a and Figure 48b. There was the finding of FSHR expression on Sertoli cell, spermatogonia, and elongated spermatid in stage XIV and I of the spermatogenic cycle which is performed in Figure 48c and Figure 48d. Besides, the FSHR protein also expressed on secondary spermatocyte (SS) in stage XIV and round spermatid (RS) in stage I. Interestingly, the FSHR expression on pachytene spermatocyte was detected in stage XIV and I but was not found in stage XII. The levels of the FSHR protein expression in the rat testis were analysed in stage XII, XIII, XIV, and I of the spermatogenic cycle. The protein expression of FSHR was significantly decreased in binge METH–administrated groups, AB METH ($0.85 \pm 0.04$), and ED–binge METH groups ($0.87 \pm 0.02$), compared with control group ($1.00 \pm 0.02$); however, those in ED METH group did not reach statistical significance (Figure 47).

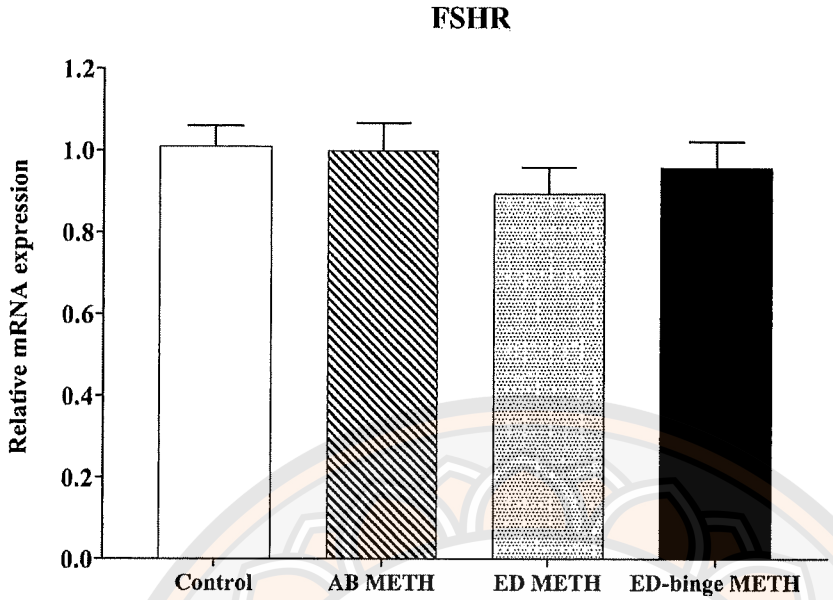



**Figure 45** **FSHR mRNA expression in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 9–10 per group.**

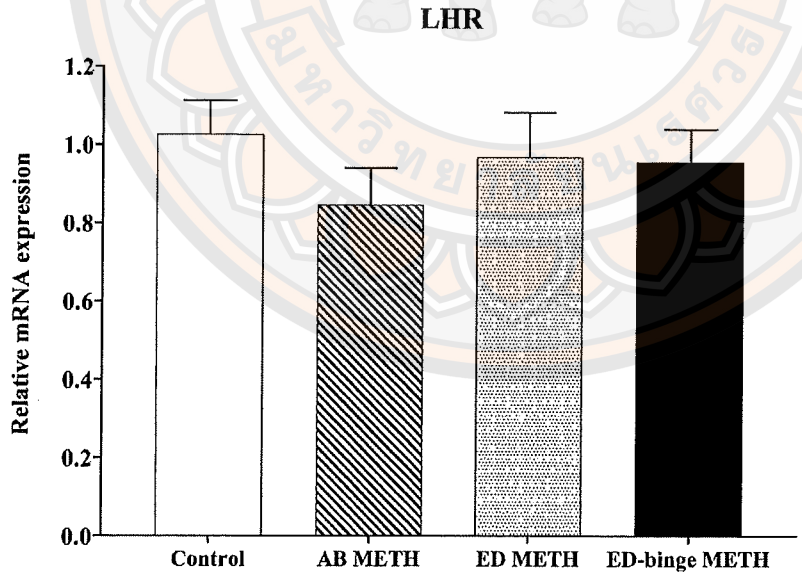



**Figure 46** **LHR mRNA expression in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 9–10 per group.**

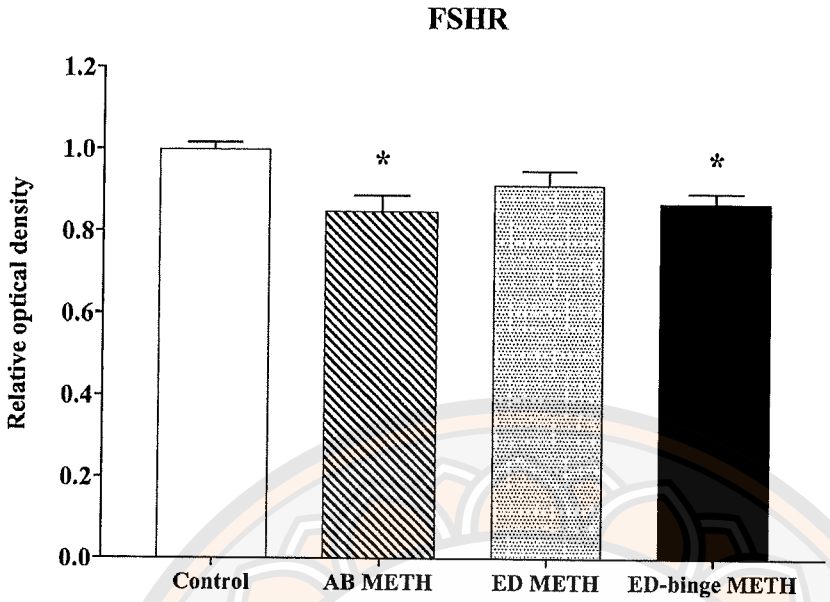



**Figure 47** **Relative optical density of FSHR expression in rat testis of METH–administrated groups compared with control group. Values are shown as mean ± SEM, n = 4–5 per group (*P < 0.05; Dunnett's post hoc test).**

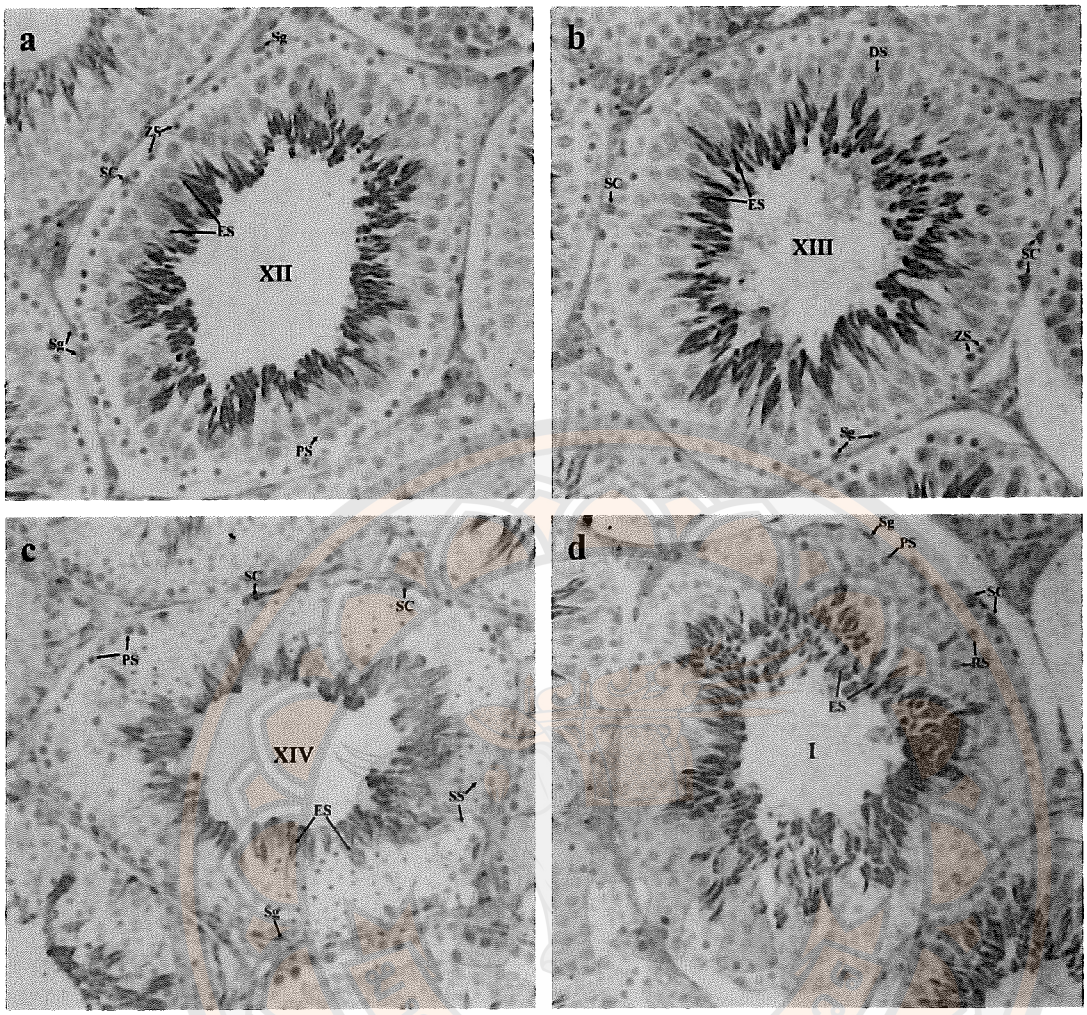



**Figure 48** Illustrating FSHR protein expression in stage XII (a), XIII (b), XIV(c) and I (d) of the spermatogenic cycle in rat testis. Sertoli cells (SC), Leydig cells (asterisks), as well as testicular germ cells including spermatogonia (Sg), zygotene spermatocytes (ZS), pachytene spermatocytes (PS), secondary spermatocytes (SS), diplotene spermatocytes (DS), round spermatids (RS), and elongated spermatids (ES)

**The relationship between FSHR in testis and sperm motility as well as GABAergic system in testis and sperm**

The high levels of FSHR protein expression were significantly correlated with the high percentage of normal sperm motility ($r = 0.5627$, $p = 0.0150$), see Figure 49. The finding of a negative correlation between FSHR protein expression and the relative mRNA expression of GAD1 (GAD67) in testis ($r = -0.8422$, $p = 0.0427$) is shown in Figure 50. Similarly, a negative correlation between the FSHR expression in testis and the protein expression of GABA A–α1 receptor in sperm were found (ROD: $r = -0.5748$, $p = 0.0315$; the percentage of strong–positively stained sperm: $r = -0.5698$, $p = 0.0334$), see Figure 50.

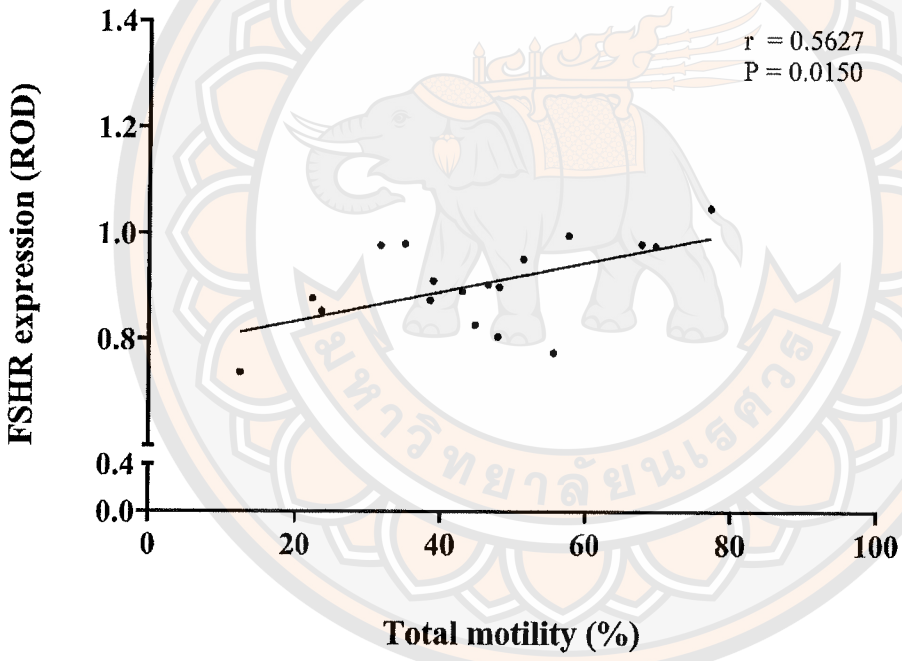



**Figure 49 Correlation between the percentage of total sperm motility and relative optical density (ROD) of FSHR expression in testis. Linear regression line (black line) fitted to all data points.**

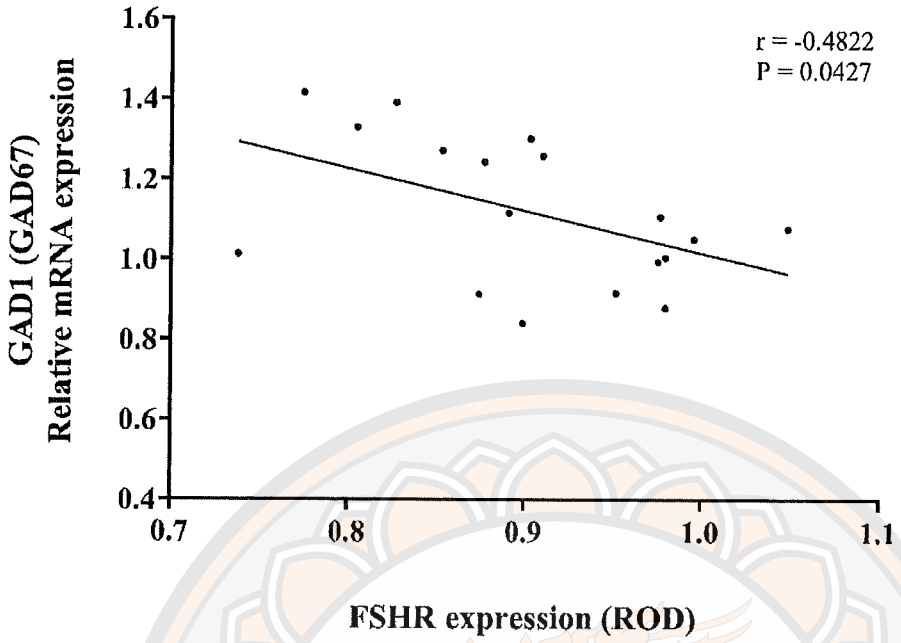



**Figure 50** **Correlation between relative optical density (ROD) of FSHR expression and of GAD1 (GAD67) mRNA expression in testis. Linear regression line (black line) fitted to all data points.**

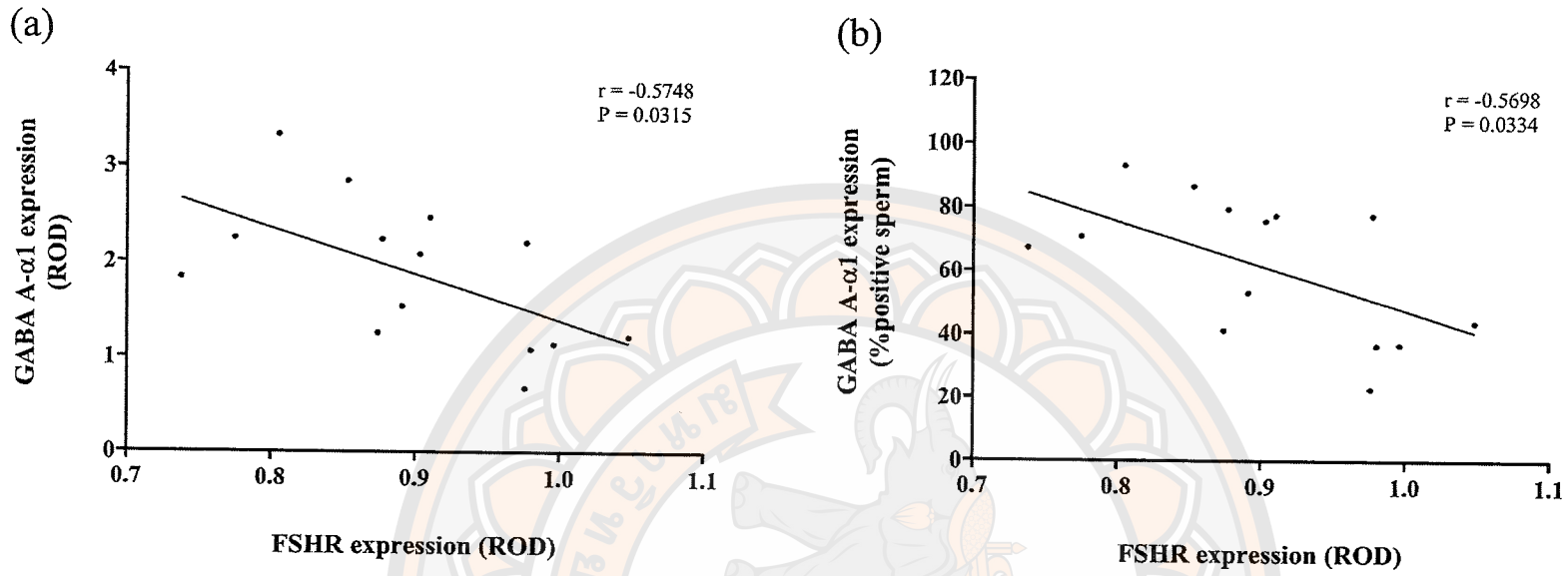



**Figure 51** **Correlations between relative optical density (ROD) of FSHR expression in testis and GABA A–α1 receptor expression in sperm including (a) relative optical density (ROD) and (b) the percentage of strong– positively stained sperm for GABA A–α1 receptor immunostaining. Linear regression line (black line) fitted to all data points.**

**Discussion and conclusion**

The findings of the present study showed that METH exposure slightly disturbed the transcription process of FSH and LH receptors in the testis because there was no significant difference in the mRNA expression of those receptors. However, a significant decrease in the FSHR protein expression in the testis was found after METH administration. These results indicate that METH exposure strongly affected only the translation process of FSHR but not its transcription process. The previous studies showed that the exposure of amphetamine derivatives, including METH and ecstasy, causes the decrease in serum testosterone but not LH (Dickerson et al., 2008; Lin et al., 2014). Because LH plays a role in testosterone production by Leydig cells, the impairment of LHR in the testis can cause the reduction of testosterone although there is no change in LH levels. Unfortunately, this study does not measure the protein expression of LHR in the testis. Nevertheless, Ruwanpura et al. reviewed that both FSH and LH, act together to regulate spermatogenesis; however, FSH mainly controls spermatogonial development and apoptosis via FSHR in testis (reviewed by Ruwanpura et al., 2010). The study in FSHR knockout mice has also supported the importance of FSHR expression in testis, they reported that the aberration of FSHR resulted in the reduction of sperm production and quality (Dierich et al., 1998; O'Shaughnessy et al., 2012). The present study also reported that the low FSHR expression in testis was significantly related to the low normal sperm motility. These results support previous reports of FSHR in testis on sperm function. Altogether, the results revealed the reduction of FSHR might be involved in the impairment of testicular and sperm function.

This study found that GABA concentration and GABA synthesizing enzyme in testis were increased after METH administration. These results reflect the increase in GABA production. Interestingly, a significant increase in GAD1 (GAD67) was found after METH exposure but not in GAD2 (GAD65). This result demonstrates that the GAD1 gene has stronger responsiveness to the adverse effect of METH than the GAD2 gene in testis. In parallel with these changes, the reduction of the GAT1 expression in METH–administrated rats indicated the inhibition of GABA reuptake which increases the GABA concentration (Soudijn, & van Wijngaarden, 2000), as supported by the finding of negative correlation between the relative mRNA expression of GAT1 and

GABA concentration in testis. The present study found the changes not only in GABA production but also in GABA function. The increase in GABA A–α1 receptor was found in METH–administrated rats. As mentioned above, the previous studies have been reported poor sperm quality and testicular damage (the apoptosis induction in spermatogenic cells) in METH–administrated rats (Lin et al., 2014; Nudmamud-Thanoi et al., 2016; Nudmamud-Thanoi, & Thanoi, 2011). These rats also revealed the functional impairment of sex steroid hormones, including testosterone, estrogen, and progesterone (Nudmamud-Thanoi et al., 2016). Additionally, the present study also indicates the decrease of FSH function in the testis.

GABA plays a role in Leydig cell proliferation and testosterone production (Geigerseder et al., 2004; Taherianfard, Bahaddini, Keshtkar, Fazeli, & Shomali, 2013). Additionally, it has been reported that GABA inhibits the proliferation of spermatogonial stem cells (SSCs), the initial spermatogenic cells in spermatogenesis, to maintain SSC homeostasis during spermatogenesis (Du et al., 2013). These results indicated a compensatory upregulation of GABA production and its functions in testis after METH exposure. Therefore, these changes might represent a homeostatic response of GABAergic to the adverse effects of METH. Moreover, the results in the relationship between the GABAergic system and sperm parameters (sperm motility and morphology) demonstrated that sperm quality influences the responsiveness of the GABAergic system in the testis.

The immunohistochemistry study of the GABA A–α1 receptor strongly supports the previous finding (Kurata et al., 2019). This study found the immunoreaction of GABA A–α1 receptor on the acrosomal segment in the head region of rat sperm. In parallel with the results in the testis, an increase in GABA A–α1 receptor was found in the ejaculated sperm of rats in the ED–binge METH group. This finding indicates that the levels of GABA A–α1 receptor remains expressed in the epididymal sperm after spermiation. Interestingly, a significant change of the GABAergic system was found in rats receiving METH in an escalating dose–binge pattern, ED–binge METH groups. These rats were administered METH by mimicking the METH use in humans; moreover, the previous study revealed that these rats had more severe poor sperm quality (Nudmamud-Thanoi et al., 2016). As shown in the correlation analysis results, the high levels of GABA A–α1 receptor expression in sperm were significantly related

to the low normal sperm motility. These results suggest that the responsiveness of the GABAergic relies on the severity of sperm impairment. Additionally, these results provide evidence for the correlation between the expression of FSHR and the expression of the GABAergic system in testis and sperm. The finding of negative correlation between the expression of GAD1 (GAD67) and FSHR in testis suggests that the increase in GABA synthesis in testis occurs because of the reduction of FSH function.

The improvement of sex steroid hormone function is the main target of the medical treatment. Using medicine, hormones, and other supplements can improve the function of sex steroid hormones and lead to the improvement of sperm production and quality. The infertility treatments using medicine and hormones, such as anti–estrogens, and gonadotropins, have been reported the side effects (reviewed by Dabaja, & Schlegel, 2014). Accordingly, the nutrient supplements that act as an antioxidant such as β–carotene, tocopherol (vitamin E), ascorbic acid (vitamin C), retinoids (vitamin A), oligominerals (folic acid), acetylcysteine and glutathione are used to improve male infertility (reviewed by Esteves, & Agarwal, 2011; Isidori et al., 2005); however, the current nutrient supplements are not sufficient for the treatment of male infertility. Nowadays, other nutrient supplements have been studied the effect on male infertility. According to the finding of the GABAergic system function in testis and sperm, the supplements that relate to the GABAergic system are interested to study. The GABA supplements such as GABA standard and pre–germinated brown rice has been reported that these supplements can improve morphological changes in seminiferous tubules, androgen receptor expression, and sperm quality that cause by antidepressant, psychoactive drugs, and drug addiction: dextromethorphan (Roboon, Nudmamud-Thanoi, & Thanoi, 2017; Thanoi et al., 2018). Nevertheless, the understanding of the physiological relevance of GABA in testicular and sperm functions remains unclear and is needed to elucidate. In conclusion, the present study provides novel data to support the role of the GABAergic system in testicular and sperm functions.

# CHAPTER V

# THE CHANGE OF GABAERGIC RECEPTORS IN SPERM OF INFERTILE MEN WITH POOR SPERM QUALITY

## Introduction

On the male reproductive system, sperm quality is performed by sperm parameters: including sperm concentration, motility, and morphology. The reference values of these parameters according to the WHO guidelines 2010, Laboratory Manual for the Examination and Processing of Human Semen (World Health Organization, 2010) are used to identify sperm quality. Men with normal sperm concentration, motility, and morphology are defined as normozoospermic (NOR) men. Furthermore, various clinical terms were used to define the types of abnormal sperm quality. Interestingly, oligoasthenoteratozoospermia (OAT) and teratozoospermia (TER) are most often found in the infertile patients attending an infertility clinic (da Silva et al., 2017). OAT is a condition in which abnormal in three sperm parameters: including sperm concentration, motility, and morphology, whereas the presence of only abnormal sperm morphology is defined as TER (World Health Organization, 2010).

GABA plays a role in the regulation of the testicular and sperm functions through its receptors (especially GABA–A and –B receptors). Several subunits of GABA–A and –B receptors have been detected in sperm. As reported in Chapter IV, this study found the localization of GABA–A receptor alpha 1 subunit (GABA A–α1) on the head of sperm whereas the GABA B receptor R2 subunit (GABA B–R2) has been found in the previous studies (He et al., 2003; Kurata et al., 2019). Moreover, the changes in the mRNA expression of GABA A–α1 receptor in testis and epididymal sperm have been found in methamphetamine (METH)–administrated rats, which revealed poor sperm quality. Although these results provided evidence supporting the role of the GABAergic system in testis and sperm; however, there is no prior information about the levels of GABA function in ejaculated human sperm in the condition of poor sperm quality. Therefore, the present study aimed to determine the levels of expression of GABA receptors, including GABA A–α1 and GABA B–R2

receptors, in sperm of men representing poor sperm quality (OAT and TER men) compared with normozoospermic (NOR) men. The results of this study might support the evidence of the function of GABA on sperm functions.

Generally, medical, surgical, and ARTs (especially ICSI and IVF) are used for infertility treatment. To date, the ICSI treatment is widely used in cases of male factor infertility because the ICSI procedure bypasses the processes of natural sperm selection for the fertilization (naturally occur within the female genital tract), such as capacitation, hyperactivation, and acrosome reaction. Nevertheless, the failure of fertilization and embryo development was also found. A major cause of those failures is the delayed oocyte activation which occurs after triggering intracellular calcium release and oscillations by the sperm–borne oocyte activating factors (SOAFs) (reviewed by Neri et al., 2014; Palermo et al., 2017). These findings demonstrated the importance of calcium signaling in the success of ICSI treatment. Interestingly, GABA–A and –B receptors are involved in the calcium signaling in the modulation of sperm kinetic parameters (including sperm motility) (Calogero et al., 1996), as well as the stimulation of sperm capacitation (Jin et al., 2009; Kurata et al., 2019; Ritta et al., 2004), hyperactivation (Binh, Van Thuan, & Miyake, 2009; Calogero et al., 1996; Kon et al., 2014; Ritta et al., 1998), and acrosome reaction (Burrello et al., 2004; Gramajo-Buhler, Zelarayan, Lopez Luis, & Sanchez-Toranzo, 2012; Hu et al., 2002; Kurata et al., 2019; Puente et al., 2011); however, their physiological relevance remains elusive. Indeed, both GABA and progesterone act through GABA receptors to promote those sperm functions by triggering the increase in intracellular calcium and chloride ions, cyclic 3',5' adenosine monophosphate (cAMP), and protein tyrosine phosphorylation in the sperm head (Puente et al., 2011; Ritta et al., 2004). The activation of GABA–A receptor, a chloride ion channel, induces hyperpolarization of the cell membrane, whereas the GABA–B receptor, a G–protein–coupled receptor, implicates in the opening of calcium and potassium channels (Kurata et al., 2019).

As mentioned earlier, the delayed oocyte activation is a major cause of ICSI failure. Morozumi et al. showed that the removal of the sperm plasma membrane and acrosome before ICSI can improve the timing of oocyte activation and the first cleavage division. During ICSI, the timing of disintegration of the sperm plasma membrane and acrosome which occur within the oocyte has been reported to influence the successful

ICSI outcomes because it controls the releasing of SOAFs (Katayama et al., 2005; Morozumi et al., 2006; Seita et al., 2009; Takeuchi et al., 2004). Taken together, these findings bring into the hypothesis that the remaining components of the intact sperm plasma membrane and acrosome in ooplasm following ICSI, including GABA receptors, might have an impact on fertilization and embryo development. Therefore, in support of the hypothesis, this study also evaluated the correlation of the expression of these receptors with sperm parameters as well as ICSI outcomes, including the fertilization rate and embryo quality.

**Methods**

1) Volunteer patients (see, Chapter III)

2) Semen samples and semen analysis (see, Chapter III)

3) The mRNA expression (see, Chapter III)

4) The relationship between the expression with sperm quality, fertilization rate and embryo quality after ICSI (see, Chapter III)

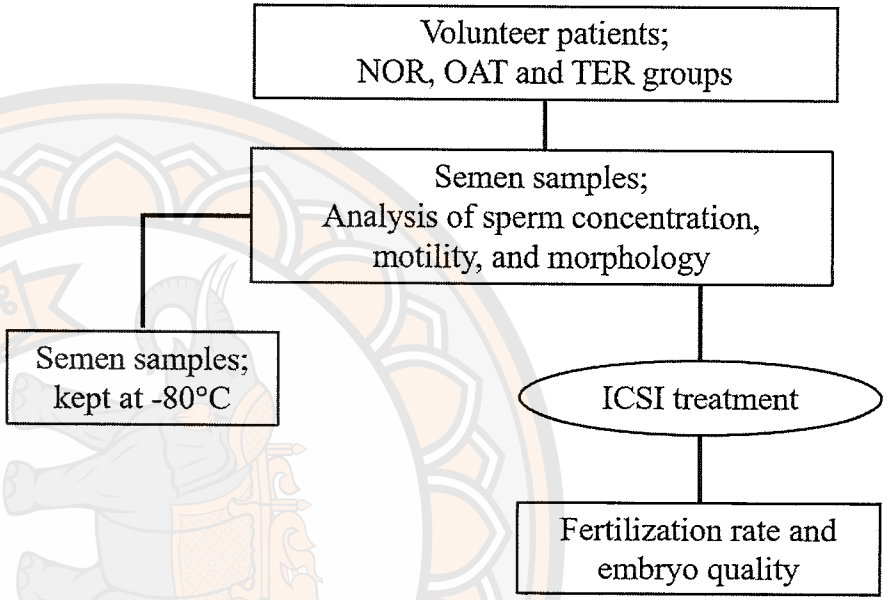



**Figure 52 Schematic representation of methods for the study of the changes in GABAergic system in sperm of infertile men with poor sperm quality**

**Statistical analysis**

Normal distribution of the data was determined using Shapiro–Wilk test. The statistical difference between two groups was analysed using Student's t–test (parametric data) and Mann–Whitney test (nonparametric data). The statistical difference between three or more groups was analysed using One–way ANOVA followed by Dunnett's post hoc test (parametric data) and The Kruskal–Wallis test followed by Dunn's multiple comparison test (nonparametric data). Moreover, the Pearson's correlation coefficient was used to investigate the relationship of the relative mRNA expression of GABA–A receptor α1 subunit and GABA–B receptor R2 subunit to sperm parameters, fertilization rate, and embryo quality. The chi–square test was used to compare the categorical variables of groups of volunteer patients on the rate of fertilization and embryo quality. Statistically significant was considered at $P < 0.05$.

## Results

**Volunteer patients**

In this study, the total number of volunteer patients receiving ICSI treatment was 35 patients including 10 from 32 patients in NOR group, 10 from 22 patients in OAT group and 15 from 45 patients in TER group, see Figure 53 and Figure 54.

**Semen analysis**

The results of semen analysis in all parameters are shown in Table 7. There were no significant differences in male patient age and semen volume in OAT and TER groups compared with NOR group. OAT group had significantly lower sperm concentration, progressive motility, total motility, and normal morphology compared with NOR group. A significant decrease in the normal form of sperm morphology was found in TER group compared with NOR group.

**The mRNA expression of GABA receptors in each group of volunteer patients**

The increase in the mRNA expression of GABA A–α1 and GABA B–R2 receptors was found in OAT and TER groups compared with NOR group, see Figure 55 (GABA A–α1; 1.7 ± 0.2 and 1.4 ± 0.2 vs 1.0 ± 0.1 for OAT and TER vs NOR) and Figure 56 (GABA B–R2; 2.2 ± 0.3 and 1.3 ± 0.2 vs 1.0 ± 0.2 for OAT and TER vs

NOR). Nevertheless, a significant increase in the mRNA expression of both receptors was found only in OAT group compared with NOR group.

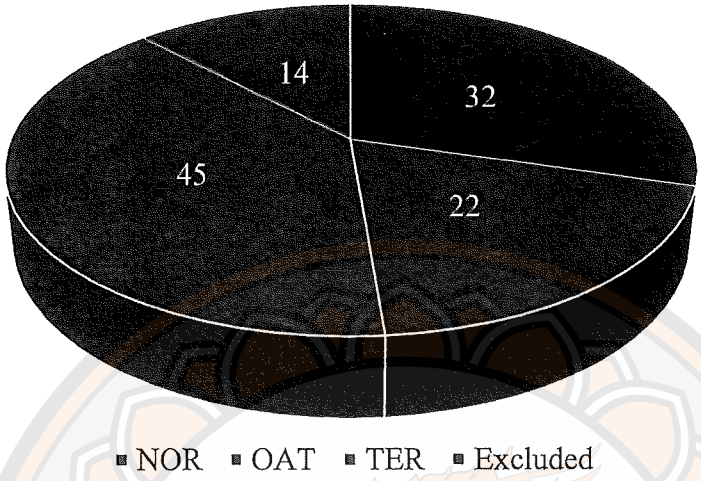



**Figure 53 Proportions of volunteer patients: NOR group (n = 32); OAT group (n = 22); TER group (n = 45) and excluded patients (n = 14)**

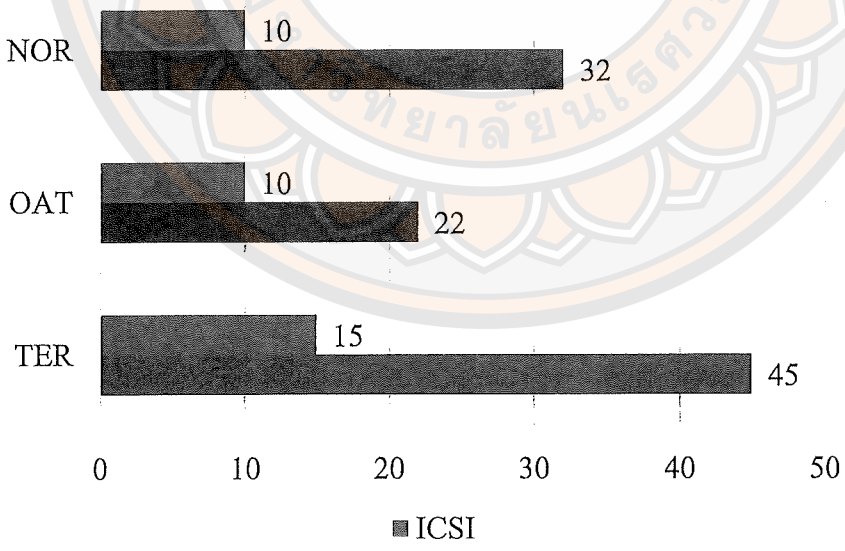



**Figure 54 Proportions of volunteer patients receiving ICSI treatment: NOR group (n = 10); OAT group (n = 10); TER group (n = 15)**

**Table 7 Comparison of semen parameters in each group of volunteer patients. All data are shown as mean ± SEM.**

| Parameter | NOR (n = 32) | OAT (n = 22) | TER (n = 45) |
|---|---|---|---|
| Male age (years) | 36.0 ± 0.8 | 37.5 ± 1.3 | 37.7 ± 1.1 |
| **Sperm concentration ($10^6$ per ml)** | 104.5 ± 14.0 | **10.2 ± 0.5**$^{****}$ | 91.7 ± 11.2 |
| **Progressive motility (%)** | 57.8 ± 2.9 | **20.6 ± 1.9**$^{****}$ | 52.8 ± 1.8 |
| **Total motility (%)** | 72.3 ± 1.9 | **41.6 ± 3.4**$^{****}$ | 67.2 ± 1.9 |
| **Normal morphology (%)** | 8.6 ± 0.4 | **1.0 ± 0.2**$^{****}$ | **1.2 ± 0.2**$^{****}$ |
| Semen volume (ml) | 3.0 ± 0.4 | 2.9 ± 0.3 | 2.6 ± 0.2 |

**Note:** Significant differences as compared with NOR group (****P < 0.0001); Kruskal–Wallis Test followed by Dunn's multiple comparison test. NOR = Normozoospermia, OAT = Oligoasthenoteratozoospermia, TER = Teratozoospermia

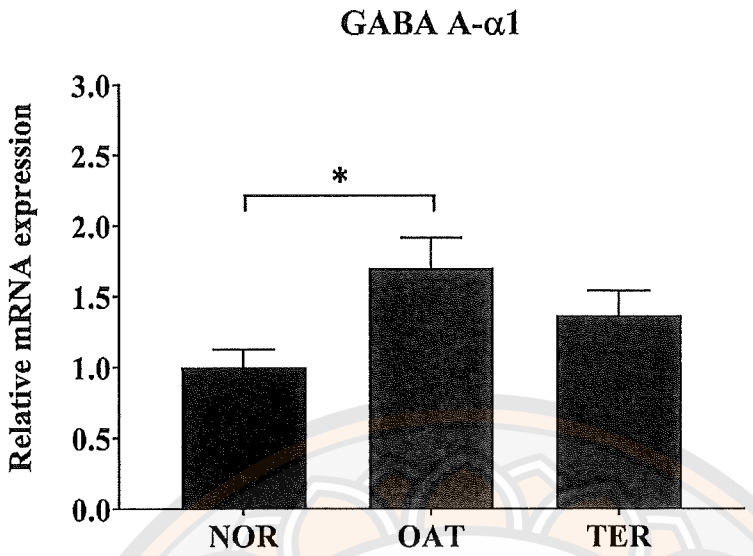



Figure 55 GABA A–α1 mRNA expression in human sperm. Values are shown as mean ± SEM. The nonparametric Kruskal–Wallis test was used to compare between groups, *P < 0.05.

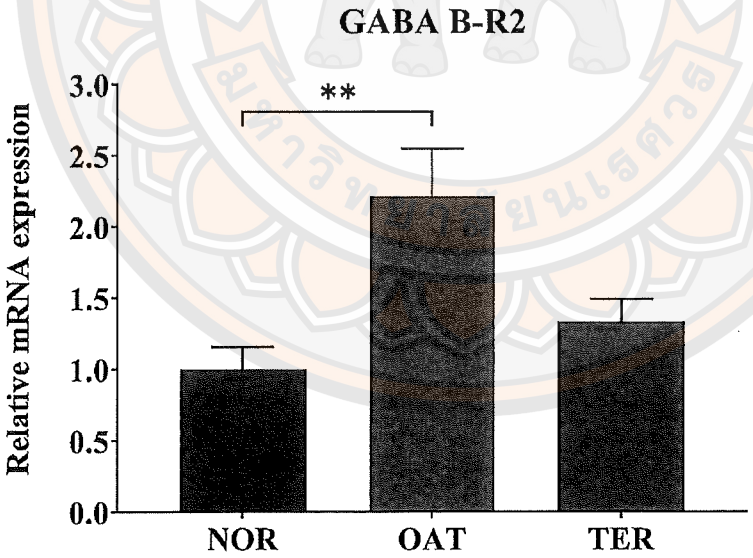



Figure 56 GABA B–R2 mRNA expression in human sperm. Values are shown as mean ± SEM. The nonparametric Kruskal–Wallis test was used to compare between groups, **P < 0.01.

**The relationship between the mRNA expression of GABA receptors and sperm parameters**

The finding of negative correlation between the GABA A–α1 expression and sperm concentration, progressive motility, total motility, and normal morphology are shown in Figure 57, Figure 59, Figure 61, and Figure 63, respectively. The negative correlation between the mRNA expression and those sperm parameters were found not only in the GABA A–α1 receptor but also in the GABA B–R2 receptor, see Figure 58 for sperm concentration, Figure 60 for sperm progressive motility, Figure 62 for total sperm motility and Figure 64 for normal sperm morphology.

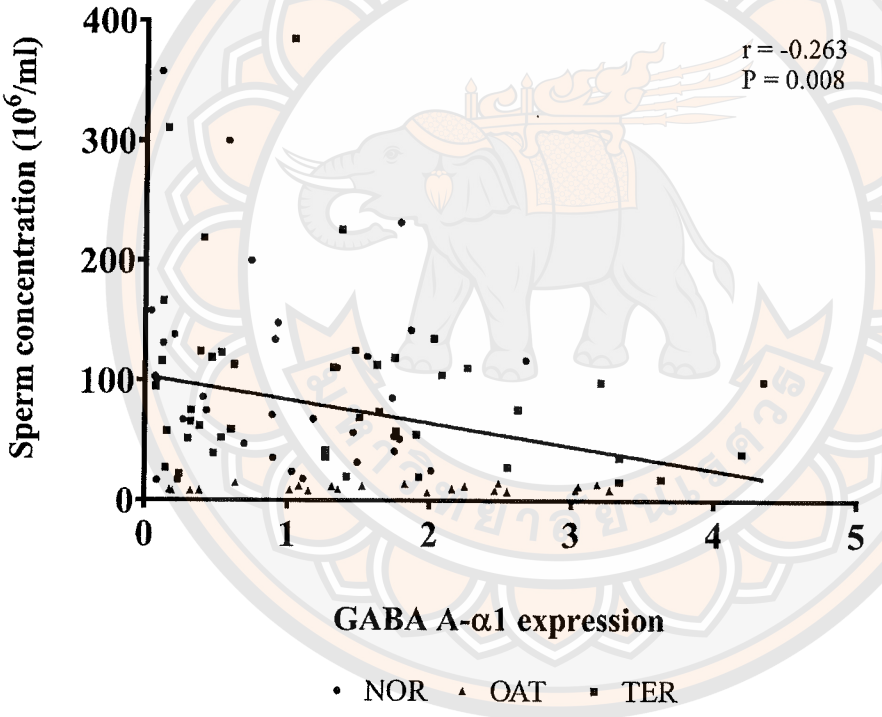



**Figure 57 Correlation between GABA A–α1 mRNA expression and sperm concentration. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

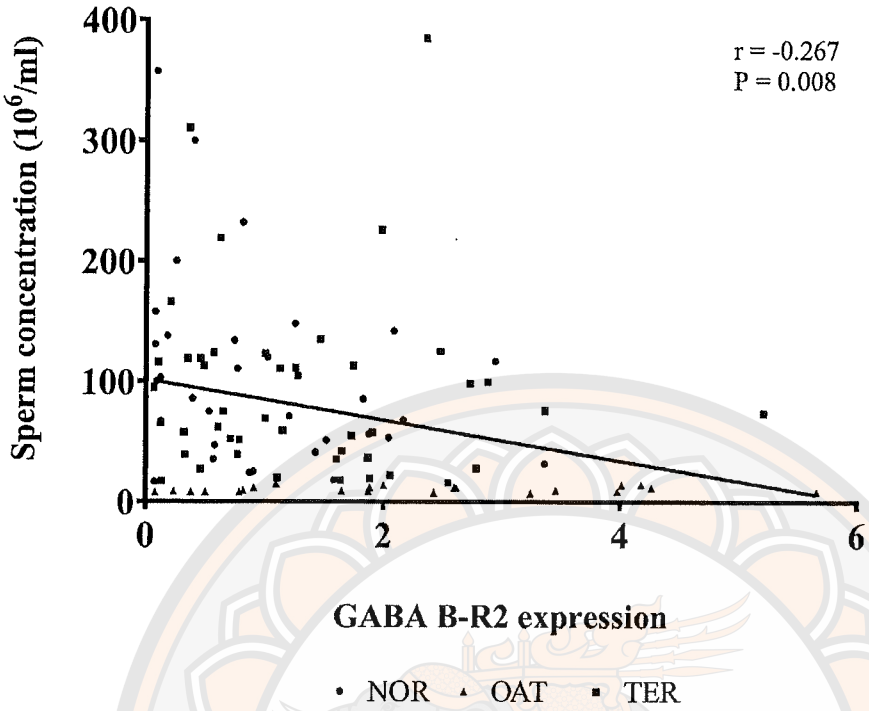



**Figure 58** **Correlation between GABA B–R2 mRNA expression of and sperm concentration. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

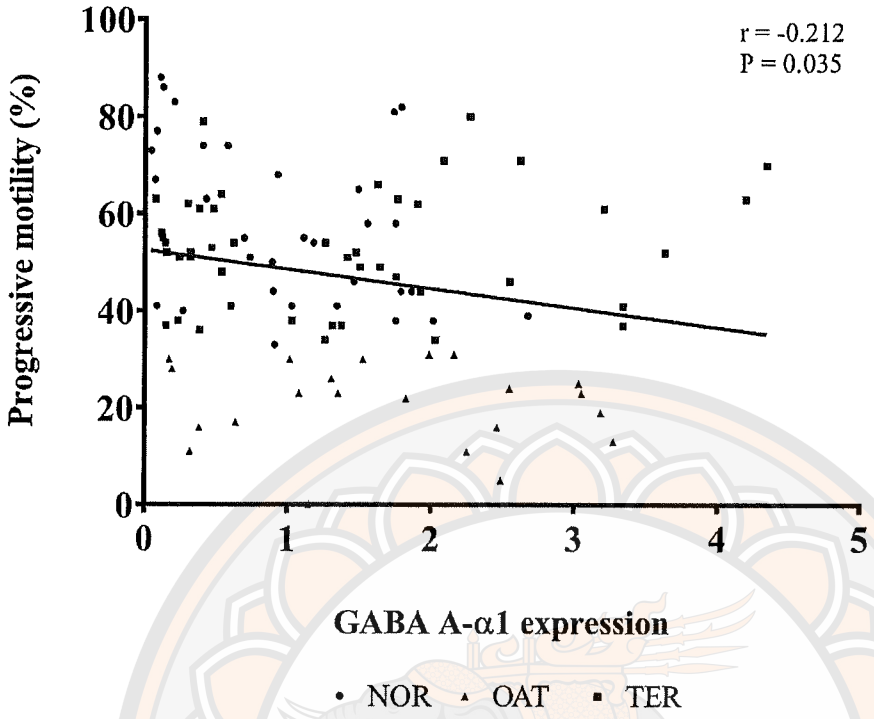



**Figure 59** **Correlation between GABA A–α1 mRNA expression and sperm progressive motility. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

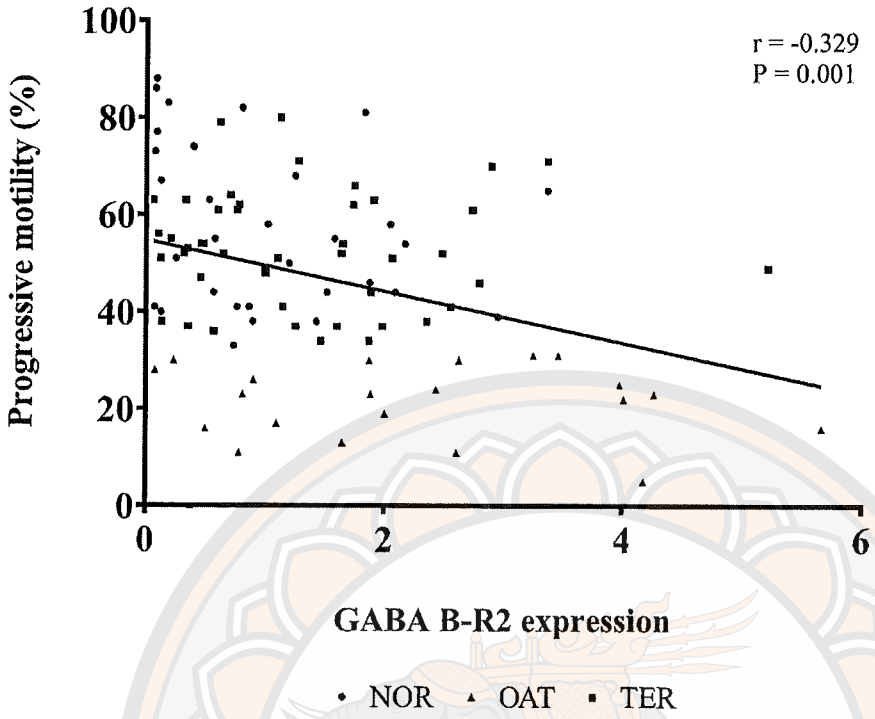



**Figure 60** **Correlation between GABA B–R2 mRNA expression and sperm progressive motility. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

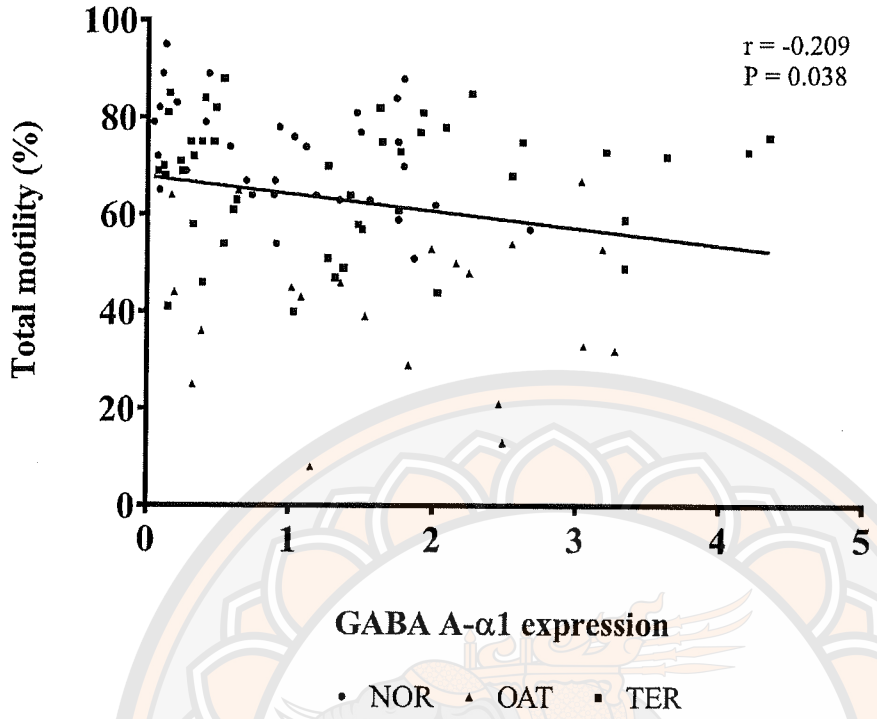



**Figure 61** **Correlation between GABA A–α1 mRNA expression and total sperm motility. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

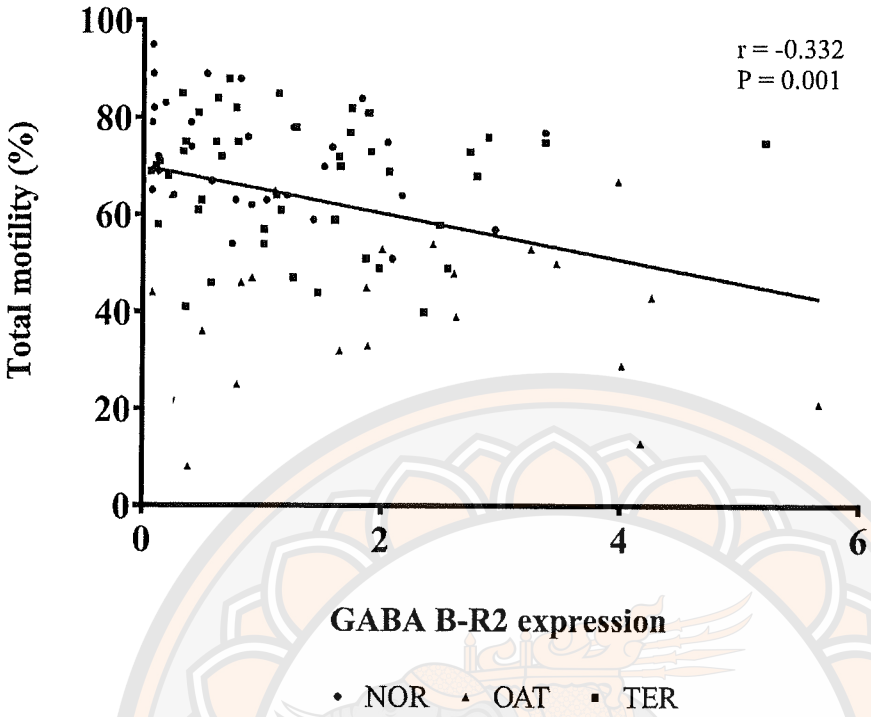



**Figure 62** **Correlation between GABA B–R2 mRNA expression and total sperm motility. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

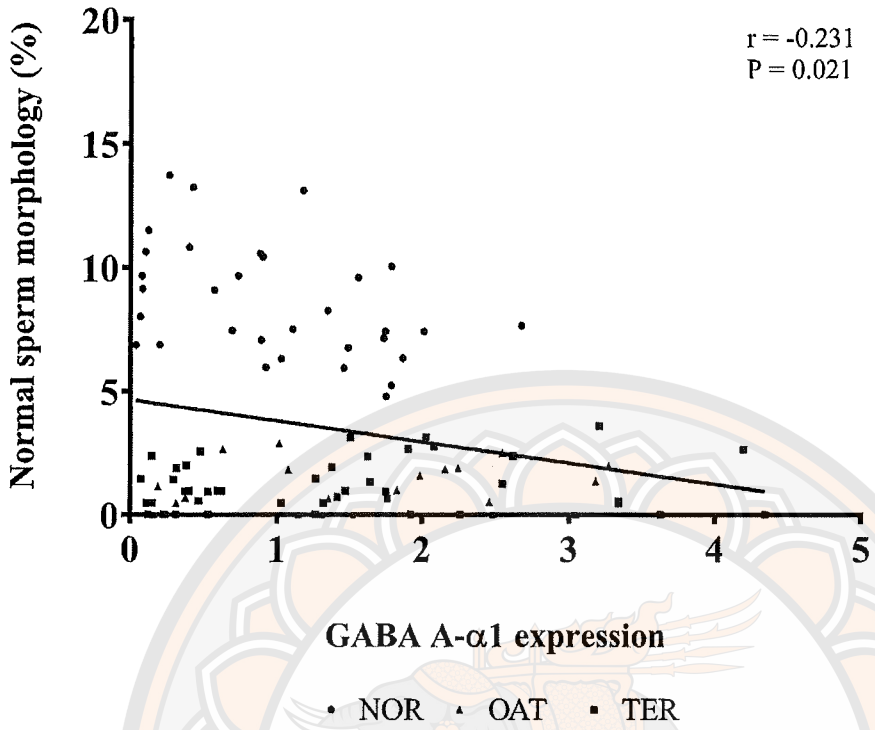



**Figure 63 Correlation between GABA A–α1 mRNA expression and normal form of sperm morphology. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

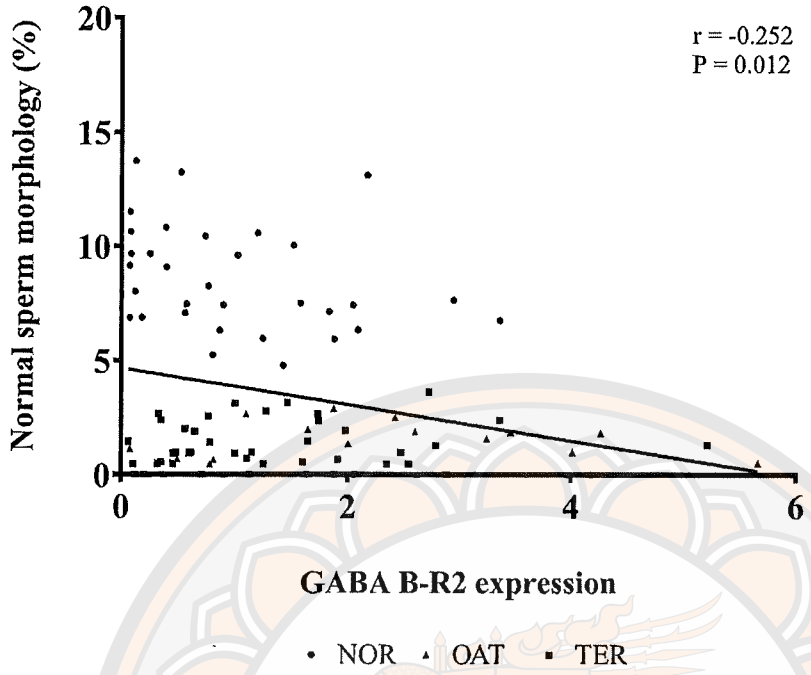



**Figure 64 Correlation between GABA B–R2 mRNA expression and normal form of sperm morphology. All data points in NOR, OAT, and TER are shown. Linear regression line (black line) fitted to all data points.**

**Fertilization rate and embryo quality after ICSI in each group of volunteer patients**

There were no significant differences in the age of the female couple, fertilization rate, and embryo quality on morula and blastocyst stages among all groups, see Table 8. Conversely, after ICSI, patients in the NOR group yielded a significantly increased good–quality embryo on cleavage stage than patients in OAT and TER group (76.8% vs 55.8% for NOR vs OAT and 76.8% vs 42.9% for NOR vs TER). The proportion of the fertilization rate and embryo quality on all stages after ICSI in each group of volunteer patients is shown in Figure 65, Figure 66, Figure 67, and Figure 68: for fertilization rate, embryo quality on cleavage, morula and blastocyst stages, respectively.

**Table 8 Comparison of baseline characteristics of volunteer patient couple and clinical outcomes of fertilization rate and embryo qualities after ICSI.**

| | | NOR | OAT | P–value | TER | P–value |
|---|---|---|---|---|---|---|
| Number of patients with ICSI | | 10 | 10 | | 15 | |
| Female age (year) mean ± SEM (minimum – maximum) | | 34.3 ± 1.5 (29 – 40) | 36.5 ± 1.3 (32 – 44) | ns† | 36.7 ± 1.1 (30 – 46) | ns† |
| Number of cycles | | 10 | 10 | | 15 | |
| Number of retrieved oocytes | | 98 | 66 | | 155 | |
| Number of MII oocytes injected | | 94 | 63 | | 147 | |
| Fertilization rate (%) | | 87.2 (82/94) | 82.5 (52/63) | ns‡ | 84.4 (124/147) | ns‡ |
| **Embryos on cleavage stage (%)** | **GQ** | **76.8 (63/82)** | **55.8 (29/52)** | **0.01‡** | **42.9 (63/124)** | **<0.001‡** |
| | PQ | 23.2 (19/82) | 44.2 (23/52) | | 57.1 (61/124) | |
| Embryos on morula stage (%) | GQ | 48.8 (40/82) | 46.2 (24/52) | ns‡ | 40.3 (50/124) | ns‡ |
| | PQ | 51.2 (42/82) | 53.8 (28/52) | | 59.7 (74/124) | |
| Embryos on blastocyst stage (%) | GQ | 22.0 (18/82) | 19.2 (10/52) | ns‡ | 22.6 (28/124) | ns‡ |
| | MQ | 9.8 (8/82) | 5.8 (3/52) | | 11.3 (14/124) | |
| | PQ | 68.3 (56/82) | 75.0 (39/52) | | 66.1 (82/124) | |

**Note:** † One–way ANOVA followed by Dunnett's post hoc test, ‡ Chi–square test. ns = not significant

GQ = Good quality, MQ = Moderate quality, and PQ = Poor quality

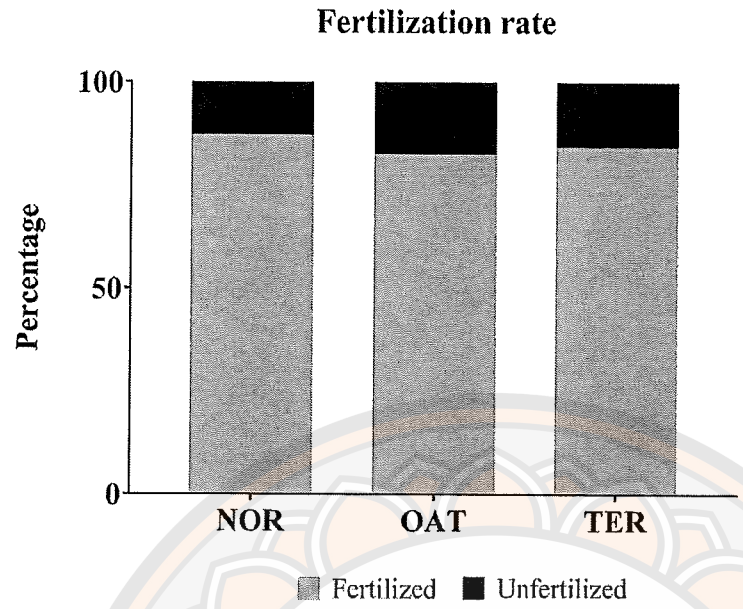



**Figure 65** The percentage of fertilized and unfertilized oocytes after ICSI in each group of volunteer patients

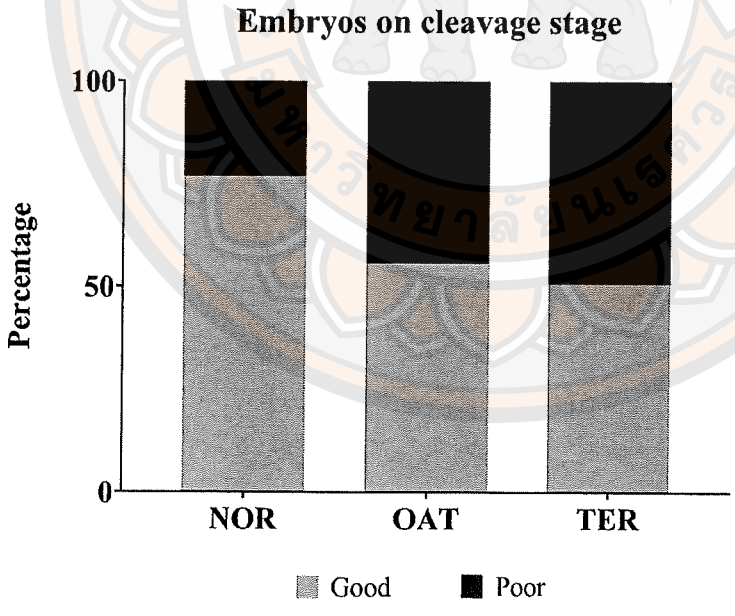



**Figure 66** The percentage of embryos with good and poor qualities on the cleavage stage after ICSI in each group of volunteer patients

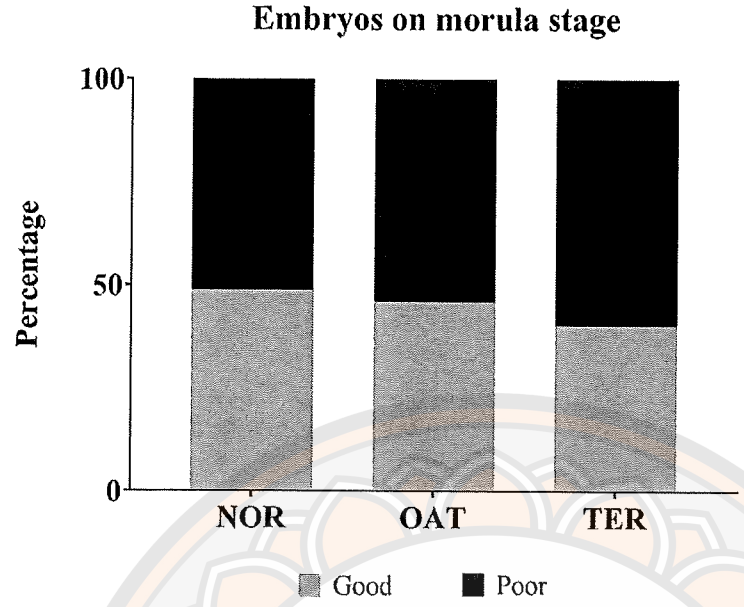



**Figure 67** The percentage of embryos with good and poor qualities on the morula stage after ICSI in each group of volunteer patients

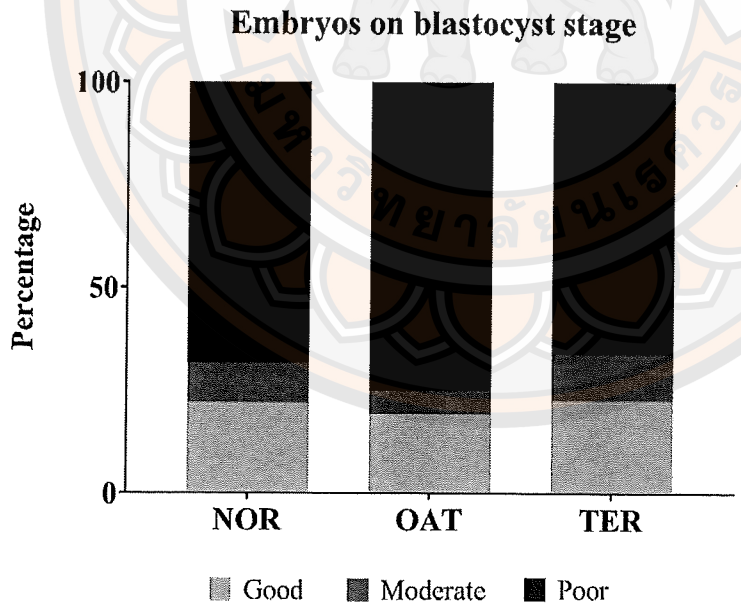



**Figure 68** The percentage of embryos with good, moderate and poor qualities on the blastocyst stage after ICSI in each group of volunteer patients

**The relationship between the mRNA expression of GABA receptors and fertilization rate and embryo quality after ICSI**

There was a significant increase in the mRNA expression of GABA A–α1 receptor in Poor group (patients who had a couple with ≤ 50% good–quality embryo) compared with Good group (patients who had a couple with > 50% good–quality embryo) in the analysis of embryo quality on cleavage stage (1.00 ± 0.16 vs 2.24 ± 0.40), see Figure 70a. The increase in the mRNA expression of GABA B–R2 receptor was also found, but it was not significantly different (1.32 ± 0.27 vs 1.72 ± 0.40), see Figure 70b. Comparisons between Good and Poor groups in the analysis of embryo quality on morula and blastocyst stages found that there were no significant differences in the mRNA expression of GABA receptors between groups, see Figure 71 and Figure 72 for morula and blastocyst stages. Similarly, there were no significant differences between High (patients who had a couple with ≥ 50% fertilized oocytes) and Low (patients who had a couple with < 50% fertilized oocytes) groups in the mRNA expression of GABA receptors, see Figure 69.

In the correlation analysis, a negative correlation between the mRNA expression of GABA receptors and the percentage of good–quality embryos on cleavage stage was found in the GABA A–α1 receptor ($r = -0.464$, $p = 0.005$; Figure 74a) but not in the GABA B–R2 receptor (Figure 74b). Conversely, there was no correlation between the mRNA expression of GABA receptors and the fertilization rate (Figure 73), the embryo quality on the morula (Figure 75), and blastocyst (Figure 76) stages.

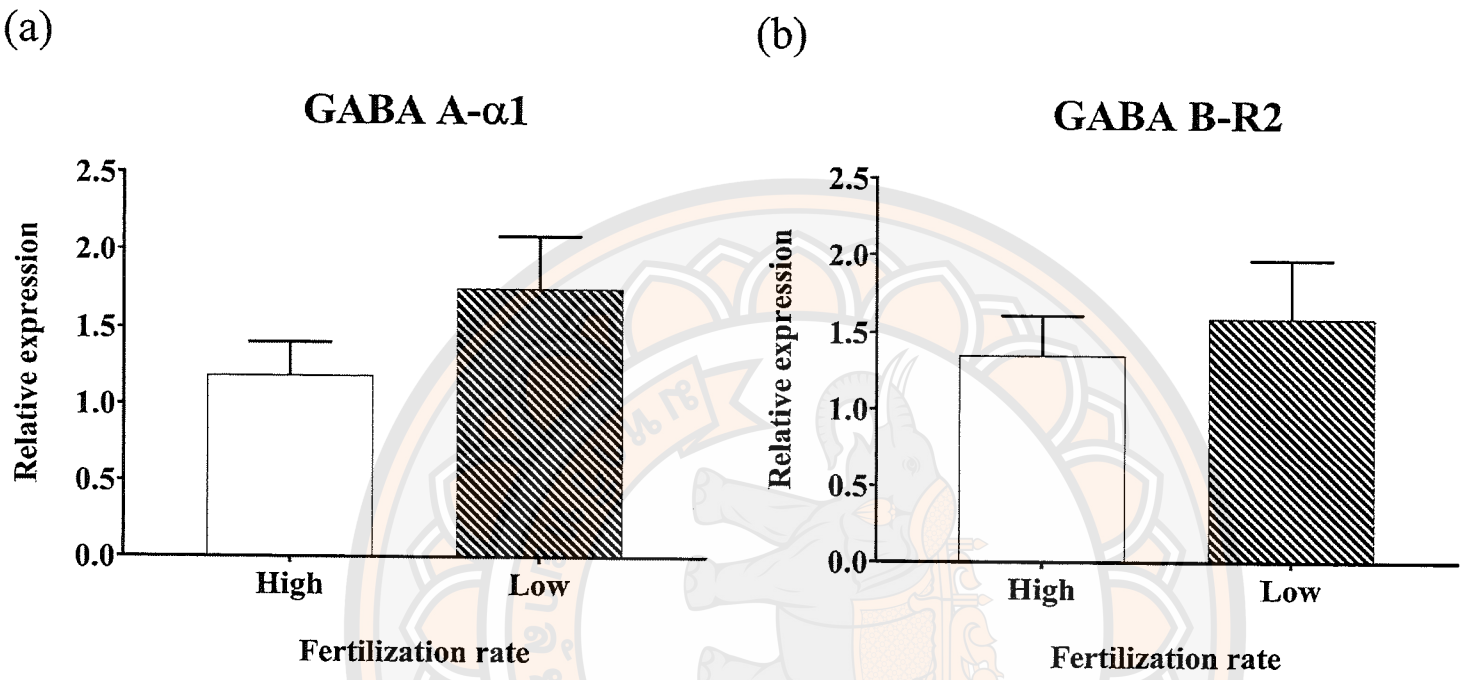



**Figure 69** **The mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 in patients who have a couple with high (≥ 50% fertilized oocytes) and low (< 50% fertilized oocytes) fertilization rate. Values are shown as mean ± SEM. The t–test was used to compare between groups.**

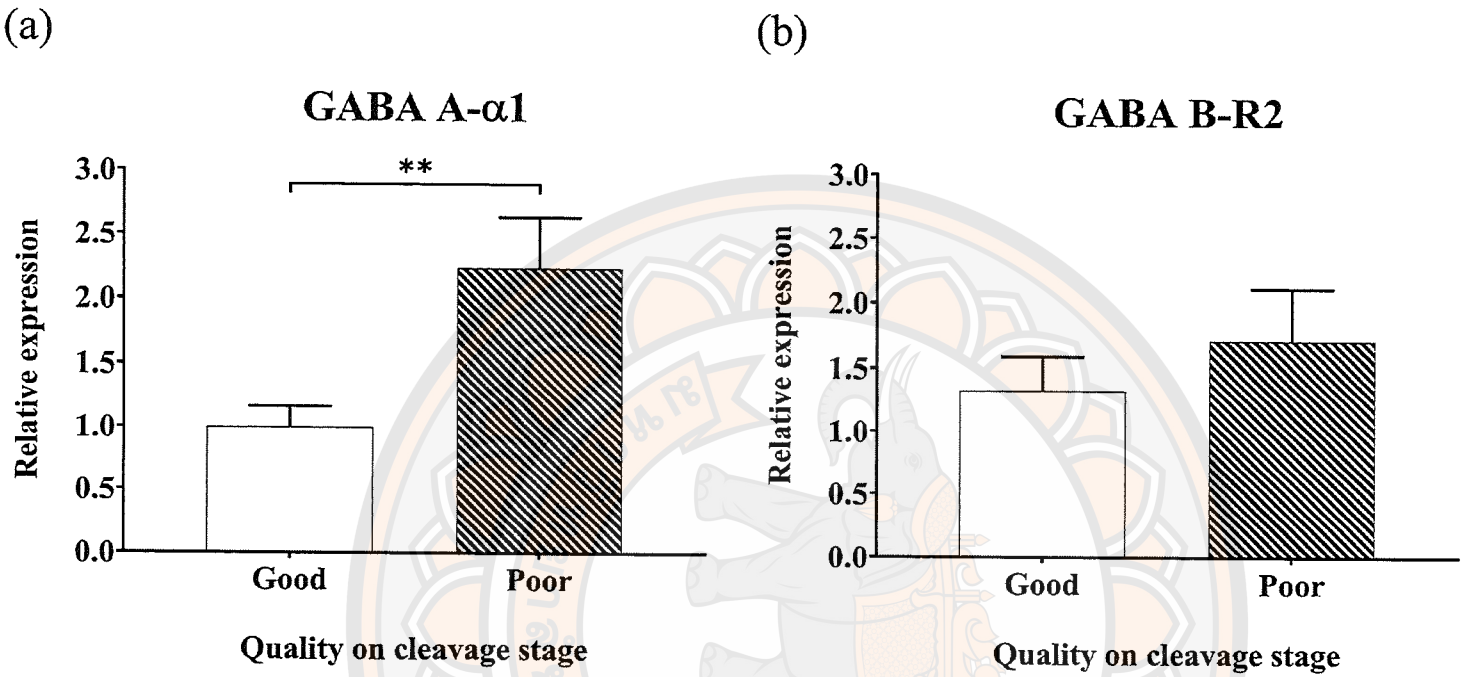



**Figure 70** **The mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 in patients who have a couple with good (> 50% GQE) and poor (≤ 50% GQE) on cleavage stage. Values are shown as mean ± SEM. The t–test was used to compare between groups; **P < 0.01. GQE = good–quality embryo**

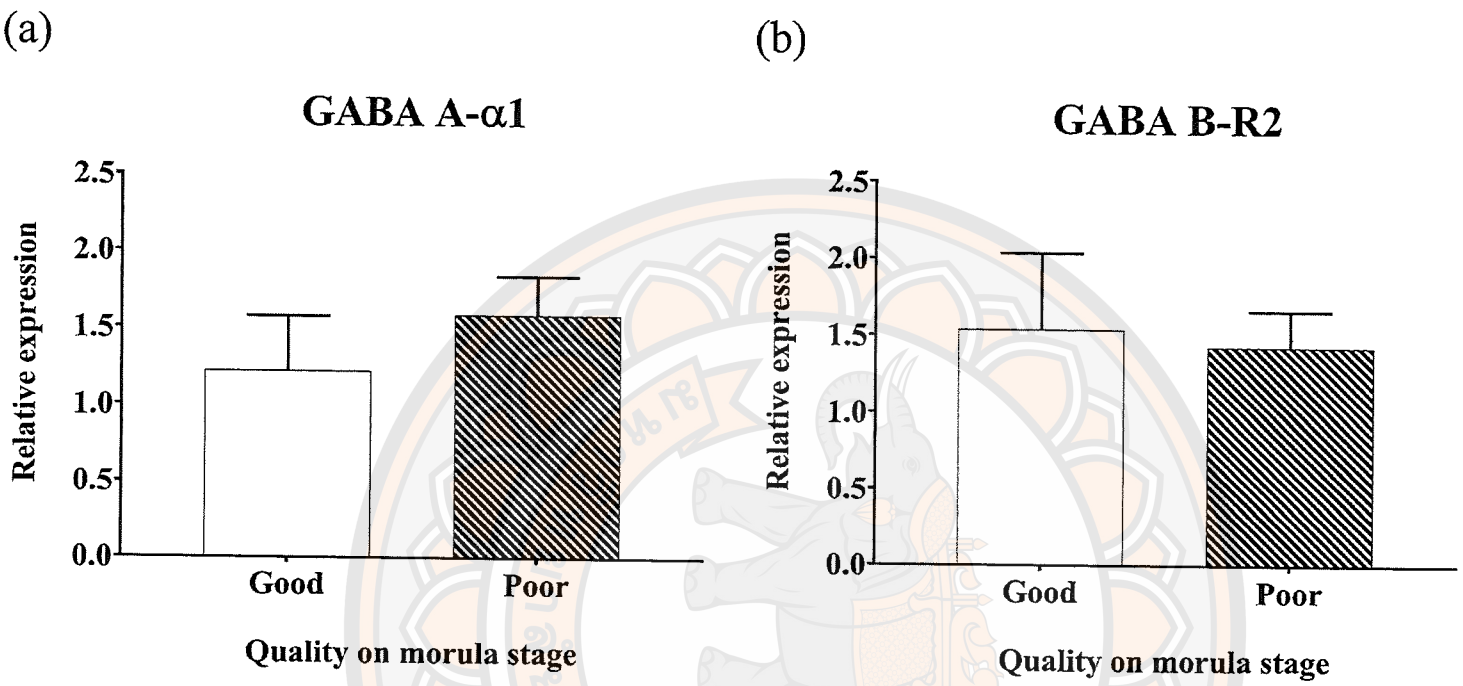



**Figure 71** **The mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 in patients who have a couple with good (> 50% GQE) and poor (≤ 50% GQE) on morula stage. Values are shown as mean ± SEM. The t–test was used to compare between groups. GQE = good–quality embryo**

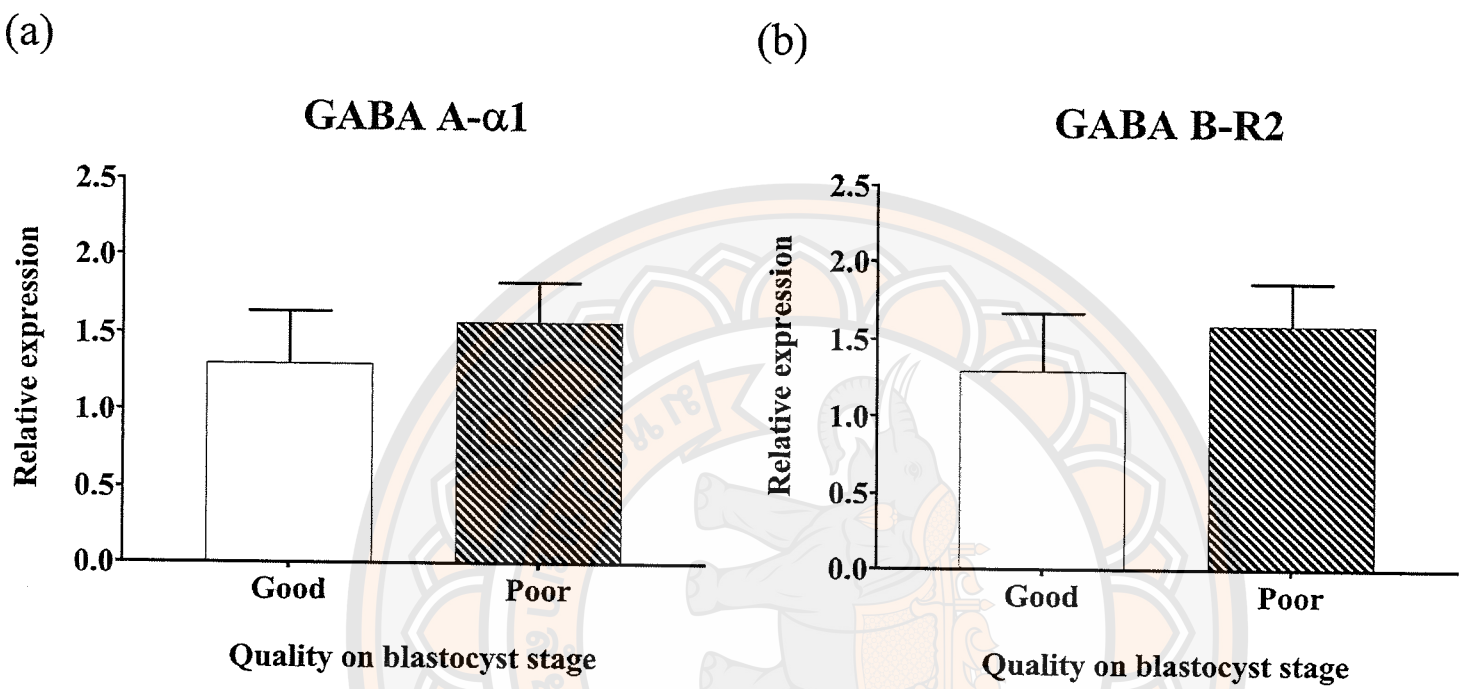



**Figure 72** **The mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 in patients who have a couple with good (≥ 10% GQE) and poor (< 10% GQE) on blastocyst stage. Values are shown as mean ± SEM. The t–test was used to compare between groups. GQE = good–quality embryo**

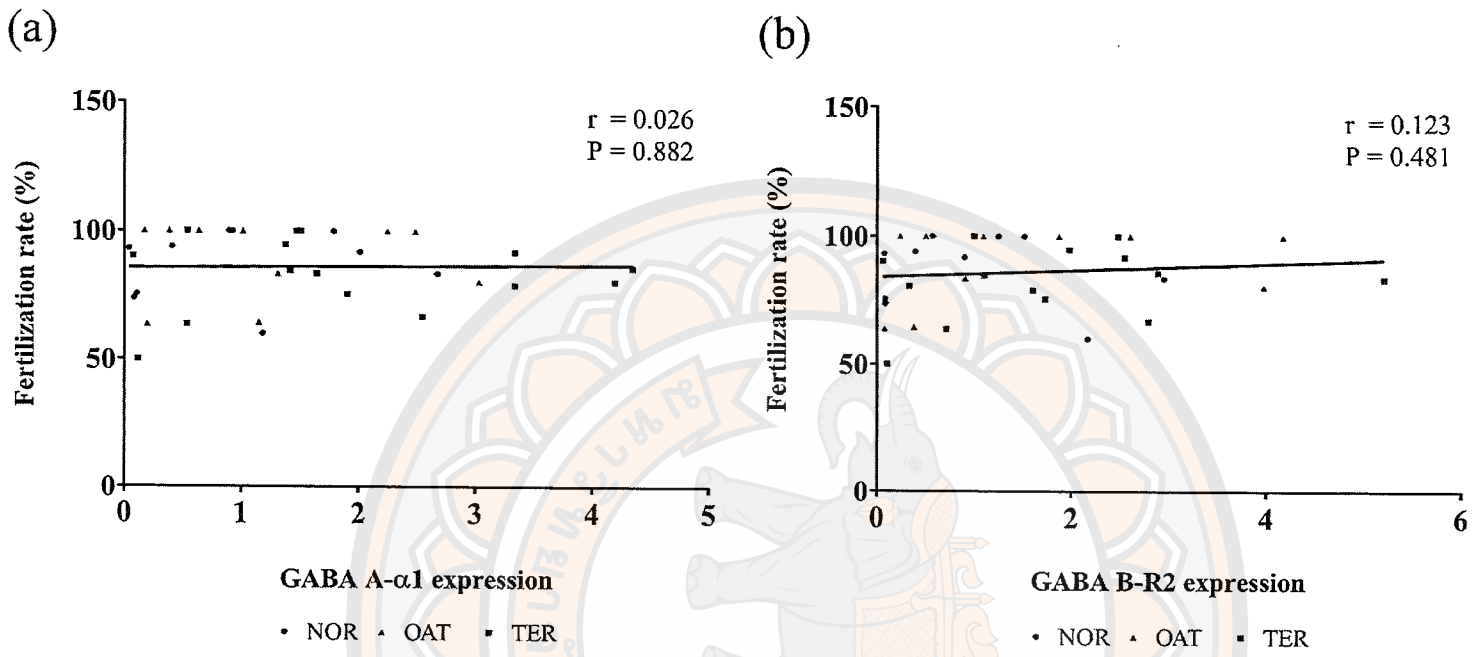



**Figure 73 Correlation between the mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 and fertilization rate.**

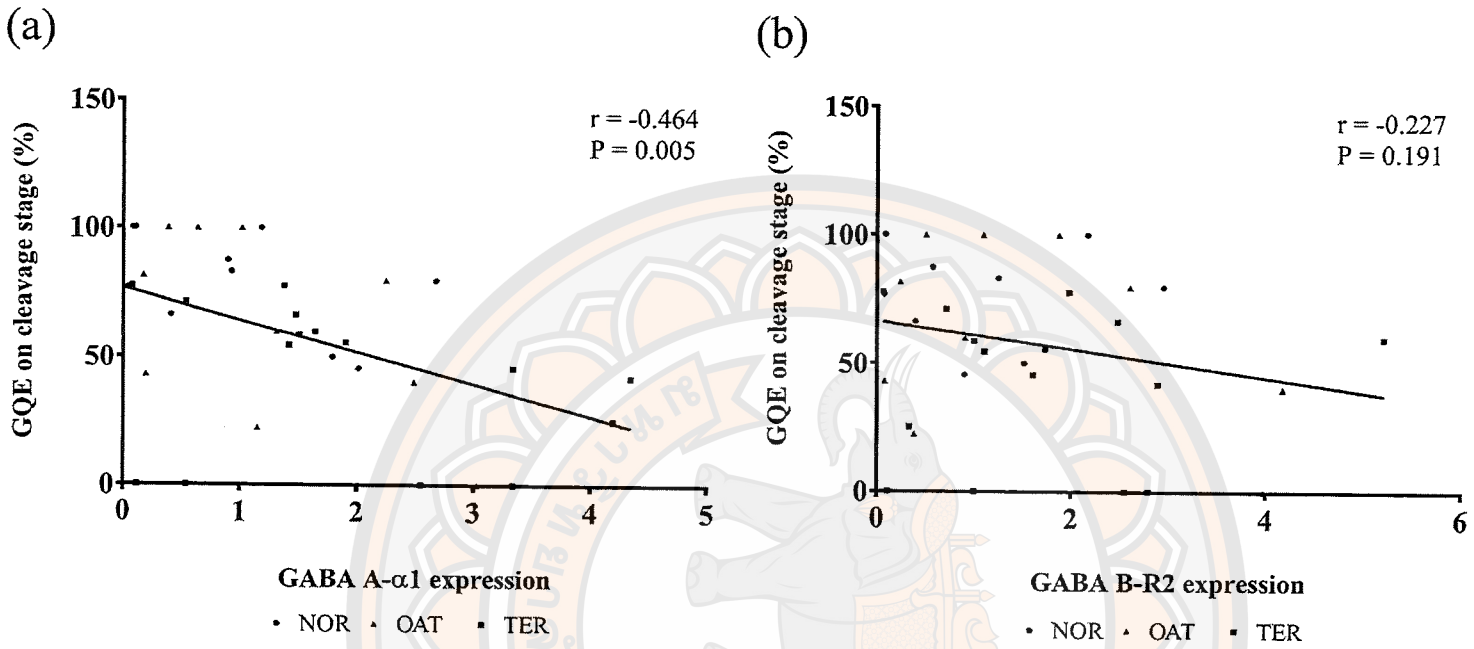



**Figure 74** **Correlation between the mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 and the percentage of good–quality embryos on cleavage stage.**

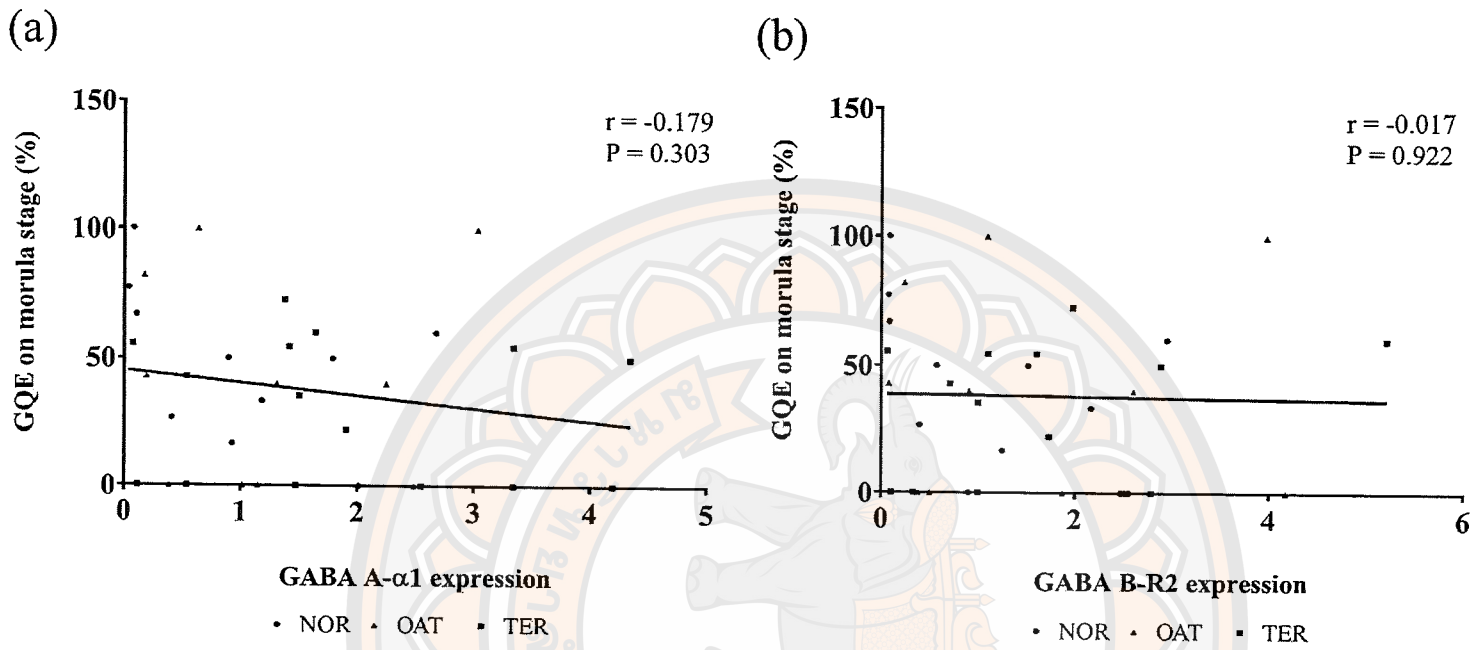



**Figure 75** **Correlation between the mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 and the percentage of good–quality embryos on morula stage.**

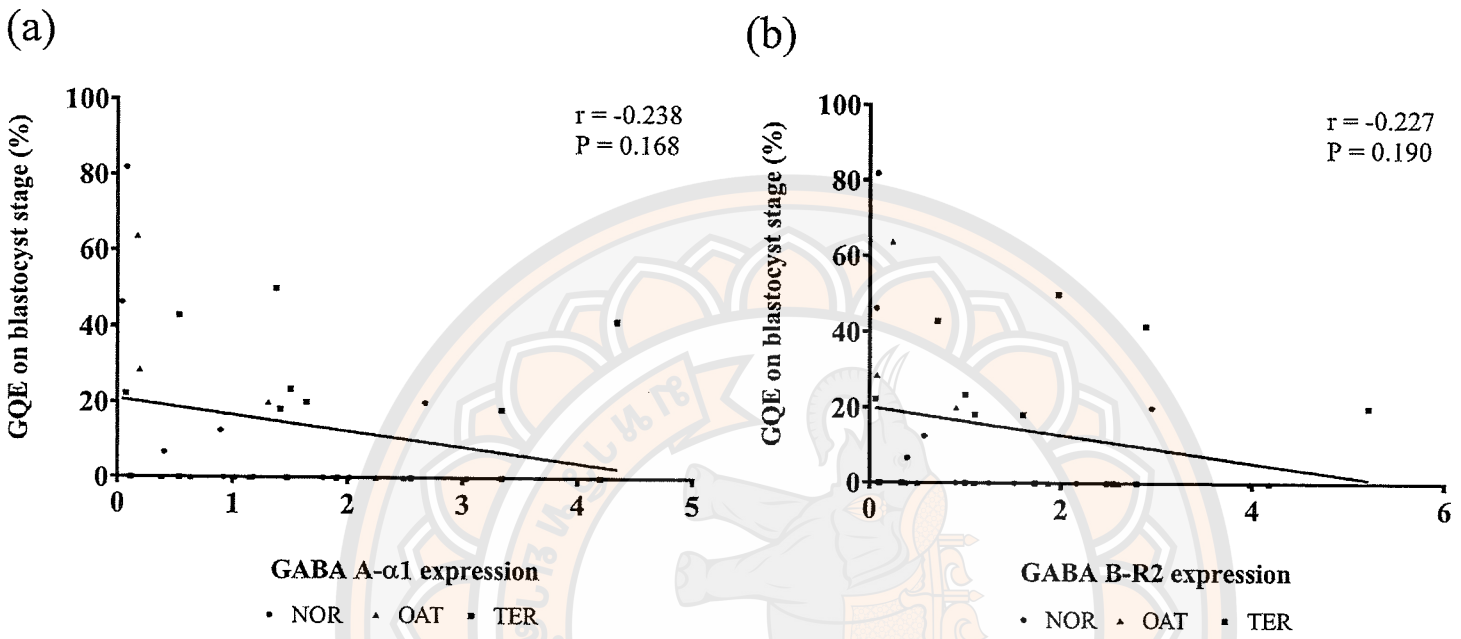



**Figure 76 Correlation between the mRNA expression of (a) GABA A–α1 and (b) GABA B–R2 and the percentage of good–quality embryos on blastocyst stage.**

**Discussion and conclusion**

In the present study, the expression of GABA A–α1 and GABA B–R2 receptors was increased in OAT and TER patients. However, a significant increase in those receptors was found only in OAT patients which revealed the severe impairment of sperm quality. This result is consistent with the results in an animal model of poor sperm quality, METH–administrated rats. These rats had the significantly increased GABA concentrations as well as the mRNA expression of GABA A–α1 receptor and GABA synthesizing enzyme in the testis. Additionally, an increase in GABA A–α1 receptor expression in epididymal sperm is also found, see Chapter IV. Taken together, these results demonstrated the alteration of GABA receptors in testis and sperm, both epididymal and ejaculated sperm, in the condition of poor sperm quality. As mentioned above, the function of GABAergic in testis has been reported including the stimulation of spermatogenesis, Leydig cell proliferation, and testosterone production (Geigerseder et al., 2004; Hu et al., 2004). Therefore, it should be noted that the increase in GABA receptors is supposed to occur earlier in the testis to compensate for the sperm impairment and maintain the homeostasis of testicular function. Moreover, those receptors remain expressed in the epididymal sperm after spermiation as well as in the ejaculated sperm. Interestingly, this study found not only the high expression levels of GABA A–α1 and GABA B–R2 receptors in the ejaculated sperm of OAT and TER men but also significant correlation between the high levels of these receptors and the low sperm parameters. These results demonstrate that the levels of those receptors depend on the severity of sperm impairment.

The results in the ICSI outcomes are consistent with the previous studies. As reported in Loutradi et al. and Li et al, they showed that OAT and TER patients had significantly lower embryo quality on cleavage stage after ICSI compared with NOR patients but had no significant difference in the fertilization rate (Li et al., 2014; Loutradi et al., 2006). Moreover, Loutradi et al. revealed that there was no significant difference in the embryo quality on blastocyst stage of OAT patients undergoing ICSI compared with NOR patients. These findings confirmed the impairment of embryo development in OAT and TER men undergoing ICSI occurs in the cleavage stage. Several studies have suggested that sperm from those men isolated by density gradient centrifugation (DGC) are still represented higher sperm DNA fragmentation and aneuploidies value

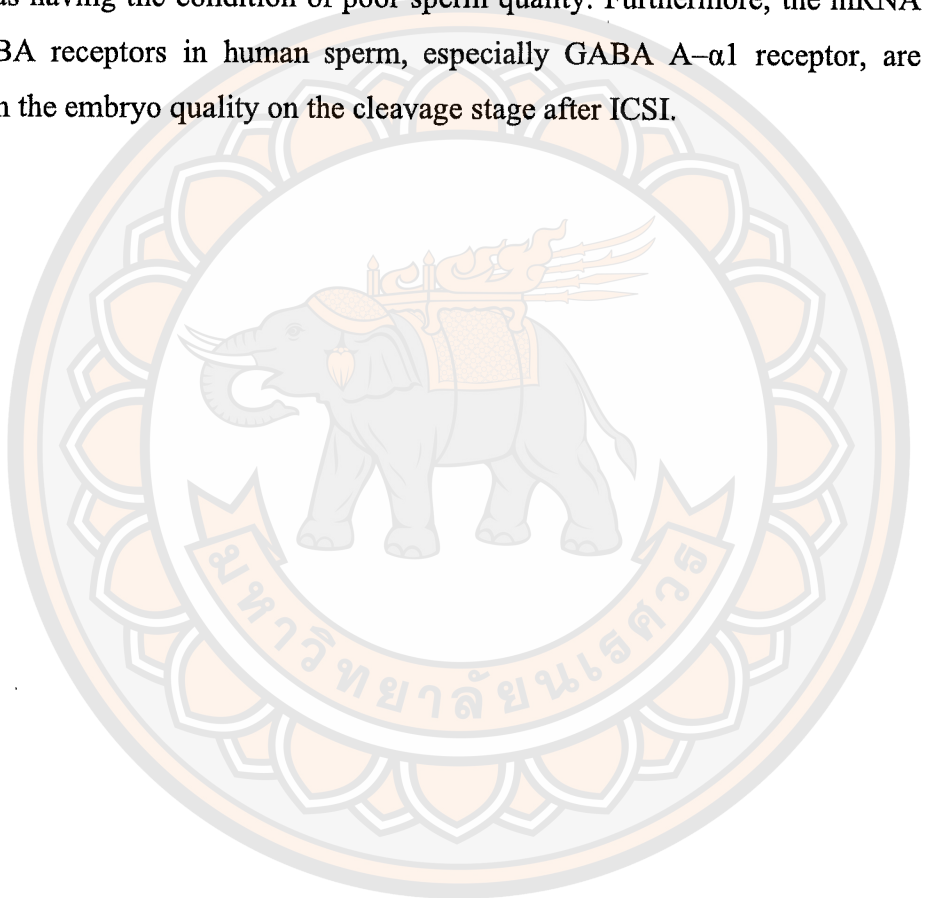
than NOR men which affect the embryonic cleavage after ICSI (Borges et al., 2019; Calogero et al., 2001; Huang et al., 2005; Komiya, Kato, Kawauchi, Watanabe, & Fuse, 2014; Sivanarayana et al., 2014).

To my knowledge, this is the first report in the correlation between GABA receptors in sperm and the ICSI outcomes. This study showed that the significantly increased levels of GABA A–α1 receptor expression in sperm were found in patients who have a female partner with a low percentage of good quality embryos on the cleavage stage after ICSI. Moreover, the high levels of GABA A–α1 receptor were significantly correlated with the low good–quality embryo (GQE) on the cleavage stage after ICSI. These findings support the hypothesis that the GABA receptors, especially GABA A–α1 receptor, in human sperm are involved in the embryonic cleavage after ICSI. However, there was no correlation between GABA receptors and other ICSI outcomes, including the fertilization rate and the embryo development on morula and blastocyst stages.

Because the ICSI procedure bypasses not only the processes of natural sperm selection for the fertilization but also the sperm–oocyte plasma membrane fusion, the disintegration of the sperm plasma membrane and acrosome is important for the ICSI outcomes (reviewed by Neri et al., 2014). However, the sperm plasma membrane and acrosome remain intact for several hours in the oocyte although sperm immobilization is performed immediately before ICSI to induce the sperm plasma membrane disruption (reviewed by Yanagimachi, 2005). Interestingly, a major cause of ICSI failure is the delayed oocyte activation that normally occurs after triggering intracellular calcium release and oscillations by sperm factors in acrosome, the sperm–borne oocyte activating factors (SOAFs). Moreover, the calcium signaling during the cleavage stage has an impact on the timing of the cleavage division and the development (Roldan, 2006). Therefore, it is possible that the levels of remaining GABA receptors on the intact sperm head may affect the calcium signaling in the oocyte after ICSI by leading to the calcium influx from ooplasm into sperm. Indeed, the activation of GABA receptors is associated with the increase in intracellular calcium ions in the sperm head (Calogero et al., 1999; Puente et al., 2011; Ritta et al., 2004). Moreover, the finding of GABA synthesizing enzyme in the midpiece of ejaculated human sperm indicates that GABA receptors might be activated in the oocyte after ICSI by GABA that is synthesized in

sperm (Persson et al., 1990). Using sperm with high levels of GABA receptors in ICSI might disturb the pattern of intracellular calcium oscillations within ooplasm which causes the delay of oocyte activation and cleavage division of embryo; however, the functional role of GABA receptors on those processes remains unknown.

In summary, these findings suggest that the levels of GABA receptors, GABA A–α1 and GABA B–R2 receptors in sperm, can be used as the biomarkers for the prediction of male infertility. The finding of high expression of these receptors in sperm is referred to as having the condition of poor sperm quality. Furthermore, the mRNA levels of GABA receptors in human sperm, especially GABA A–α1 receptor, are associated with the embryo quality on the cleavage stage after ICSI.



# CHAPTER VI

# THE STUDY OF A NOVEL MICROFLUIDIC DEVICE FOR SPERM SELECTION

## Introduction

Currently, up to 50% (20–70%) among an infertile population, ~ 50–80 million people worldwide, are contributed by the male factors which expressed mostly the low sperm quality (reviewed by Babakhanzadeh et al., 2020; Fainberg, & Kashanian, 2019). One of the most major problems in the assisted reproductive technologies (ARTs), i.e., intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF), is the low success rate caused by male factors. Several previous studies have reported that using low–quality sperm in these treatments affects the treatment outcomes, including the fertilization rate and embryo development (Loutradi et al., 2006). There is evidence that the fertilization rate and good–quality embryo were declined because of the employing of low–motile sperm in the ARTs (Zheng et al., 2016). Piccolomini et al. reported that the blastocyst formation rate was decreased in a male patient with low sperm parameters, including sperm concentration, motility, and morphology undergoing IVF (Piccolomini, Bonetti, La Motta, Serafini, & Alegretti, 2018). Additionally, sperm progressive motility (PR) is essential for successful fertilization and embryo development in IVF and ICSI treatments (Chapuis et al., 2017). In parallel with these findings, the results in Chapter V indicate that OAT and TER patients had a low percentage of good–quality embryos on the cleavage stage after ICSI. These results indicate that the sperm factors including GABA receptors located on the sperm head might be involved in the cleavage divisions during embryo development after ICSI. Moreover, their levels are also related to sperm parameters. Interestingly, not only those sperm parameters but also the sperm DNA integrity has been suggested to be one of the most common problems causing the ART treatment failure (Benchaib et al., 2003; Henkel et al., 2003; Jin et al., 2015; Larson-Cook et al., 2003). Taken together, these results demonstrated that sperm quality has a major impact on successful treatment outcomes. Thus, the selection of sperm with high quality is essential for improved the outcomes in ICSI and IVF treatments.

For many years, density gradient centrifugation (DGC) and swim–up (SU) methods which have been known as the conventional methods for sperm preparation are the most widely used methods in the ART treatment. Nevertheless, there are several limitations to the use of these methods (reviewed by Henkel, & Schill, 2003). For example, DGC requires careful training and the use of high centrifugal forces which have the potential to damage sperm. Moreover, the SU method is less effective in high–quality sperm selection for the ejaculates with low sperm count and motility, as well as a high–yield preparation. Importantly, the high levels of sperm DNA fragmentation are still present in the sperm selected by those methods (Xue et al., 2014; Zini et al., 2000). Several studies reviewed that although using the novel methods, such as a magnetic–activated cell sorting, glass wool filtration, and a hyaluronic acid–binding method, conjunction with the conventional methods can improve the sperm DNA fragmentation, these methods are relatively expensive and insufficient for the selection of sperm without DNA damage and aneuploidy (reviewed by Beydola et al., 2013; Kim, 2018). Recently, a microfluidic device is widely developed to use as the sperm sorting method. The chemoattractant–driven and thermotaxis–driven devices are designed their microchannels mimicking the microenvironments in the female genital tract and the fertilization process. Conversely, the microchannels of passively driven and flow–driven devices are designed based on the behaviour of sperm movement (reviewed by Knowlton, Sadasivam, & Tasoglu, 2015). Kantsler and his research team have reported the movement pattern of sperm in the microfluidic ratchets. In the present study, this strategy is used to design and fabricate a novel microfluidic device by using polydimethylsiloxane (PDMS) as material for microfluidic device fabrication (Denissenko et al., 2012; Kantsler et al., 2013). Theoretically, only motile sperm can move along the boundaries of the microfluidic channels and then keep moving towards the outlet. Moreover, several studies have tested the devices for their ability to select sperm from normozoospermic samples, but few have examined their efficiency to isolate sperm from poor quality samples.

Therefore, this study aimed to evaluate the efficiency of a novel PDMS–glass microfluidic device for sperm selection from normal and abnormal (low progressive motility) semen samples compared to DGC and SU methods. Sperm motility (PR and total motility), morphology, and DNA integrity were observed.

**Methods**

1) Semen samples (see, Chapter III)

2) Sperm selection (see, Chapter III)

3) Sperm analysis (see, Chapter III)

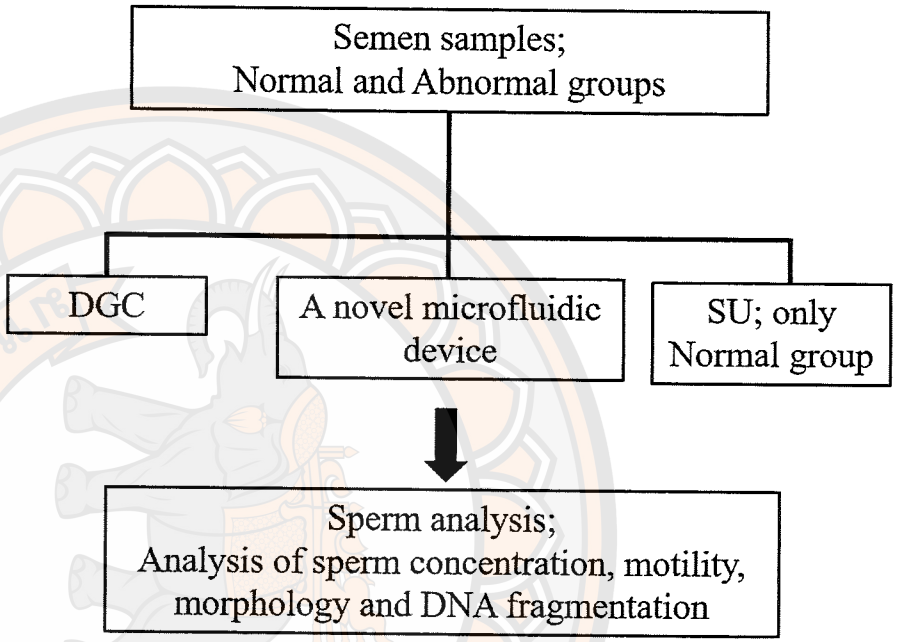



**Figure 77 Schematic representation of methods for the study of a novel microfluidic device for sperm selection**

**Statistical analysis**

Normal distribution of the data was determined using Shapiro–Wilk test. One-way analysis of variance (ANOVA) with post hoc Tukey's test (normally distributed data) and the nonparametric Kruskal–Wallis test (non–normally distributed data) were used to compare sperm concentration, motilities, morphology and DFI for each of the sperm separation methods and unprocessed semen. For a comparison of semen parameters between Normal and Abnormal groups, an independent Student's t–test (normally distributed data) and Mann–Whitney U–Test (non–normally distributed data) were used. Statistical analysis was performed using GraphPad Prism software (version 8, GraphPad Inc., USA). Data is shown as mean ± SEM.

## Results

### Semen analysis

Patients in Abnormal group had significantly lower sperm progressive motility and total motility, but higher sperm DNA fragmentation compared with those in Normal group, see Table 9. There were no significant differences in semen volume, total sperm number, sperm concentration and morphology between Normal and Abnormal groups, see Table 9.

**Table 9 Comparison of semen parameters in Normal and Abnormal groups**

| Parameter | Normal (N = 22) | Abnormal (N = 17) | Significance |
|---|---|---|---|
| Semen volume (ml) | 3.1 ± 0.2 | 2.8 ± 0.3 | 0.384† |
| Total sperm number ($10^6$ per ejaculate) | 263.9 ± 27.9 | 220.3 ± 46.1 | 0.117‡ |
| Sperm concentration ($10^6$ per ml) | 89.6 ± 9.7 | 72.4 ± 8.4 | 0.243‡ |
| **Progressive motility (%)** | **52.1 ± 2.1** | **21.8 ± 1.5** | **< 0.0001†** |
| **Total motility (%)** | **76.4 ± 1.8** | **48.2 ± 1.8** | **< 0.0001†** |
| Normal morphology (%) | 5.3 ± 0.5 | 5.1 ± 0.8 | 0.857† |
| **DFI (%)** | **8.3 ± 0.7** | **12.5 ± 1.4** | **0.003‡** |

**Note:** †, independent Student's t–test; ‡, Mann–Whitney U–Test.

### Sperm concentration after the selection

The result of sperm concentration in unprocessed semen and all sperm selection methods in Normal group was shown in Figure 78, whereas those in Abnormal group was shown in Figure 79. The significant difference of sperm concentration was found in a comparison between DGC and 5*3 device in Normal group ($63.1 \pm 6.7 \times 10^6$ sperm per ml vs $24.8 \pm 3.6 \times 10^6$ sperm per ml), see Figure 78; nevertheless, there was no significant difference sperm concentration after the selection by DGC compared to 5*5 device both in Normal ($63.1 \pm 6.7 \times 10^6$ sperm per ml vs $44.7 \pm 5.6 \times 10^6$ sperm per ml)

and Abnormal (29.0 ± 3.8 × $10^6$ sperm per ml vs 22.7 ± 4.9 × $10^6$ sperm per ml) groups. Interestingly, in Normal group, a significant decrease in sperm concentration was found in sperm selected by SU (19.4 ± 2.9 × $10^6$ sperm per ml) than DGC (63.1 ± 6.7 × $10^6$ sperm per ml) and 5*5 device (44.7 ± 5.6 × $10^6$ sperm per ml) but was not significantly lower than 5*3 device (24.8 ± 3.6 × $10^6$ sperm per ml), see Figure 78.

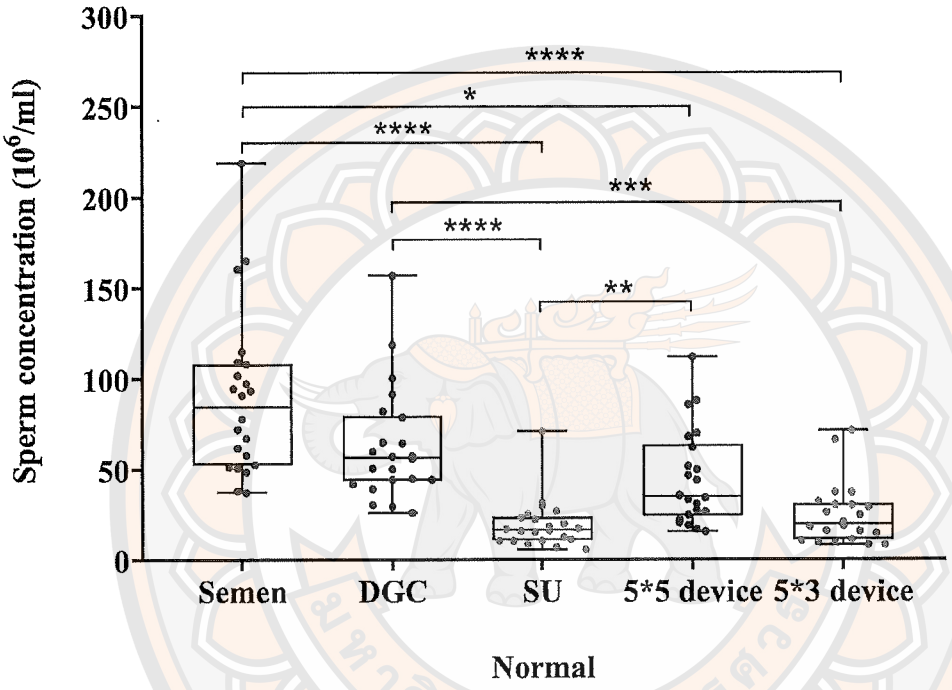



**Figure 78** **Boxplots of sperm concentration (semen and selected sperm) in Normal group. The nonparametric Kruskal–Wallis test was used to compare between sperm selection methods; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).**

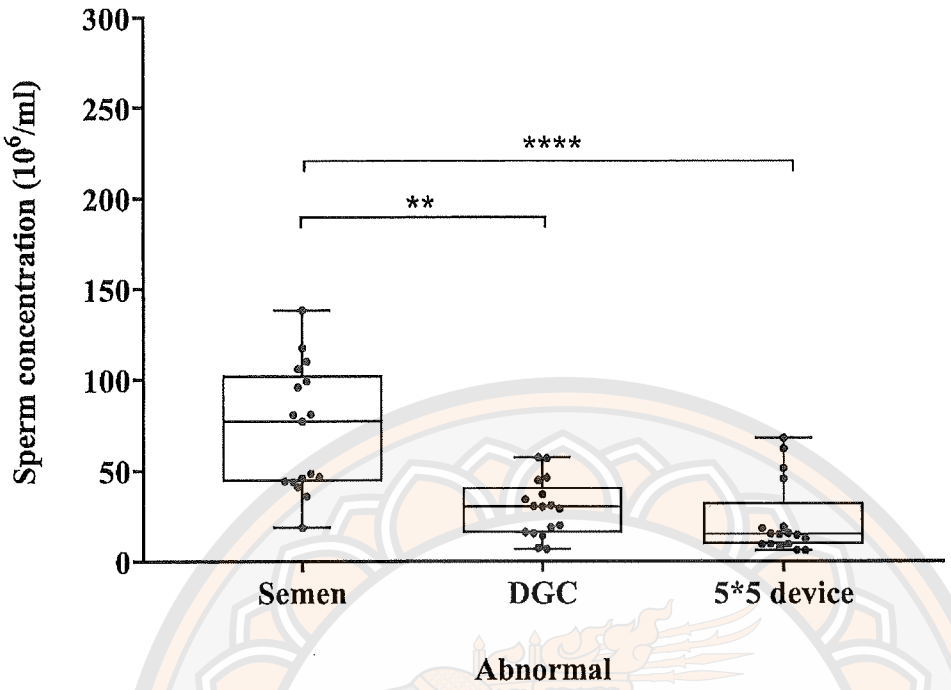



**Figure 79** **Boxplots of sperm concentration (semen and selected sperm) in Abnormal group. The nonparametric Kruskal–Wallis test was used to compare between sperm selection methods; **P < 0.01 and ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).**

**Sperm motilities after the selection**

The results of sperm progressive motility in unprocessed semen and all sperm selection methods in Normal and Abnormal groups were shown in Figure 80 and Figure 81, respectively. The increases in sperm progressive motility and total motility were found after the selection by all methods compared to semen in both groups. Sperm selected by DGC had significantly lower progressive motility compared to other sperm selection methods in Normal (70.4 ± 2.0% vs 84.8 ± 2.2%, 85.6 ± 1.8% and 85.4 ± 1.8% for DGC vs SU, 5*5 and 5*3 devices) and Abnormal (51.9 ± 2.2% vs 76.8 ± 2.0% for DGC vs 5*5 device) groups. On the contrary, there was no significant difference progressive motility between SU (84.8 ± 2.2%) and both devices (85.6 ± 1.8% for 5*5 device and 85.4 ± 1.8% for 5*3 device) in Normal group, see Figure 80.

Total sperm motility in unprocessed semen and all sperm selection methods in Normal and Abnormal groups were shown in Figure 82 and Figure 83, respectively. Sperm selected by DGC had significantly lower total motility compared to the selection using the devices in Normal (89.2 ± 0.9% vs 95.3 ± 0.8% and 95.7 ± 0.7% for DGC vs 5*5 and 5*3 devices) and Abnormal (73.1 ± 2.0% vs 90.4 ± 1.4% for DGC vs 5*5 device) groups. Interestingly, in the Normal group, there was a significant increase in sperm progressive motility after the selection by SU compared to DGC (84.8 ± 2.2% vs 70.4 ± 2.0%), but not in total sperm motility (92.5 ± 1.7% vs 89.2 ± 0.9%), see Figure 80.

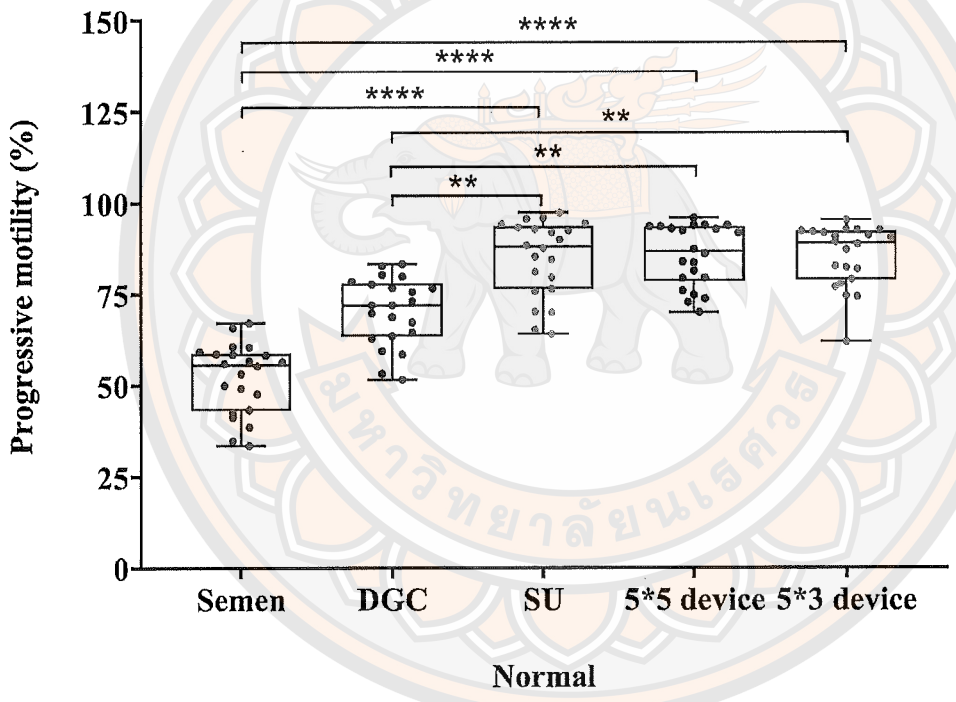



**Figure 80 Boxplots of sperm progressive motility (semen and selected sperm) in Normal group. The nonparametric Kruskal–Wallis test was used to compare between sperm selection methods; **P < 0.01 and ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).**

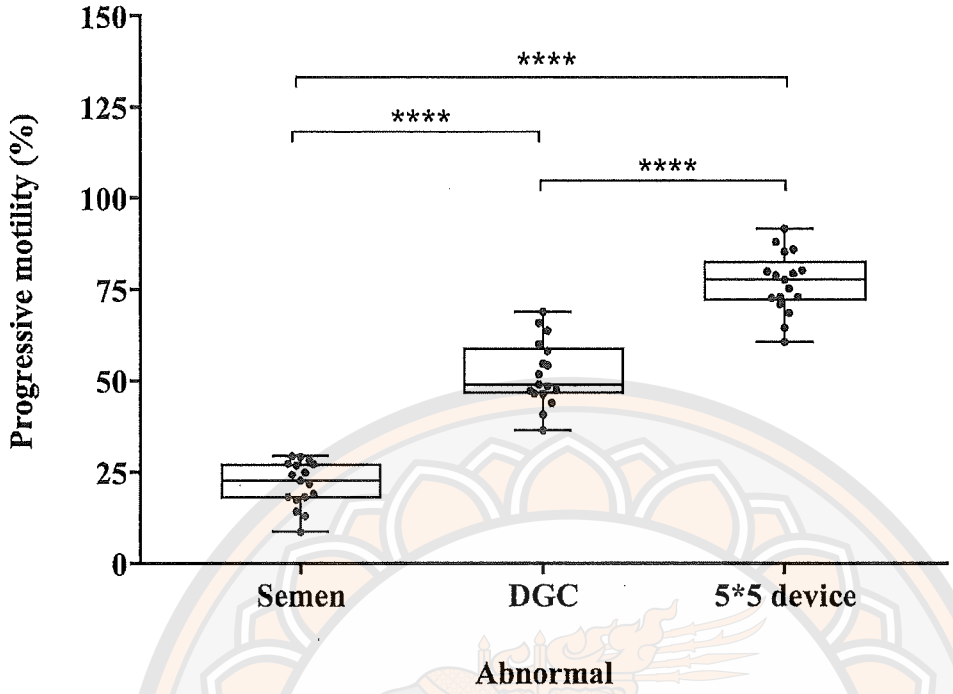



**Figure 81** Boxplots of sperm progressive motility (semen and selected sperm) in Abnormal group. One–way ANOVA with post hoc Tukey's test was used to compare between sperm selection methods; ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).

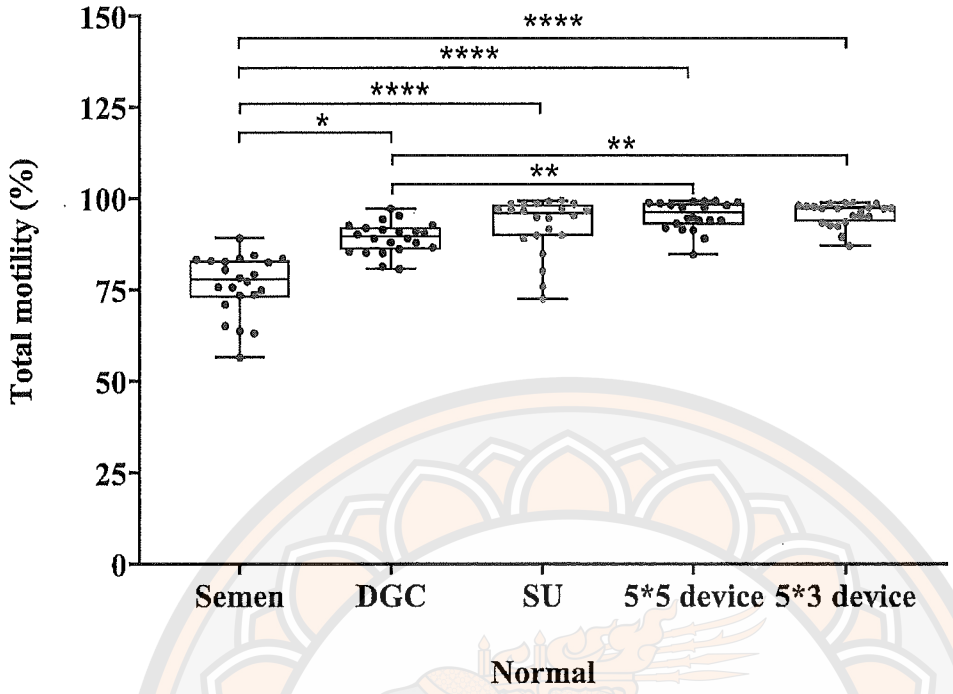



Figure 82 Boxplots of total sperm motility (semen and selected sperm) in Normal group. The nonparametric Kruskal–Wallis test was used to compare between sperm selection methods; *P < 0.05, **P < 0.01 and ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).

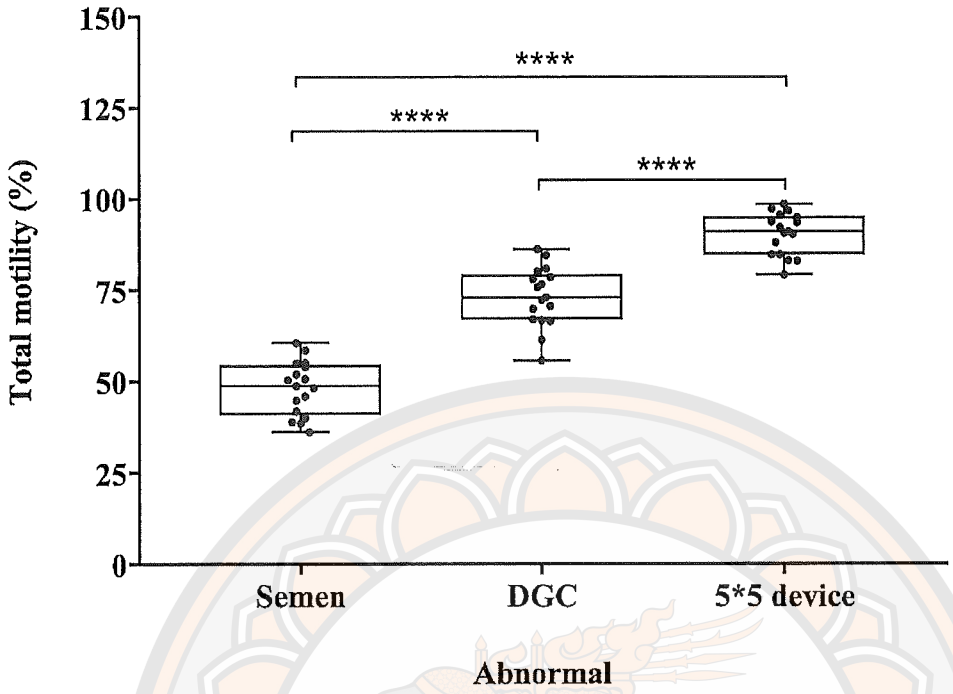



Figure 83 Boxplots of total sperm motility (semen and selected sperm) in Abnormal group. One–way ANOVA with post hoc Tukey's test was used to compare between sperm selection methods; ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).

**Sperm morphology after the selection**

Sperm morphology in unprocessed semen and all sperm selection methods in Normal group was shown in Figure 84, whereas those in Abnormal group was shown in Figure 85. Sperm recovered from the devices had significantly higher normal sperm morphology compared with unprocessed semen (Normal group, 10.2 ± 1.0% and 11.5 ± 1.0% vs 5.3 ± 0.5% for 5*5 and 5*3 devices vs semen; Abnormal group, 9.6 ± 1.3% vs 5.1 ± 0.8% for 5*5 device vs semen). Interestingly, there was a significant increase in normal sperm morphology after the selection by 5*3 device compared to DGC (11.5 ± 1.0% vs 7.4 ± 0.7%) and SU (11.5 ± 1.0% vs 7.7 ± 0.7%) in Normal group, see Figure 84.

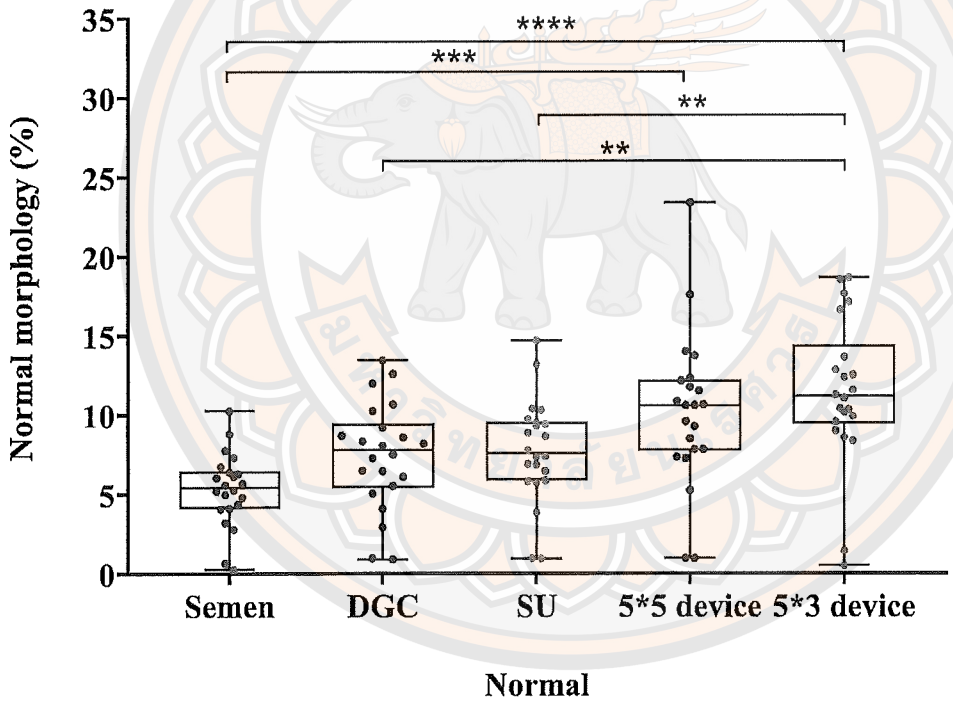



**Figure 84 Boxplots of normal sperm morphology (semen and selected sperm) in Normal group. One–way ANOVA with post hoc Tukey's test was used to compare between sperm selection methods; **P < 0.01, ***P < 0.001 and ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).**

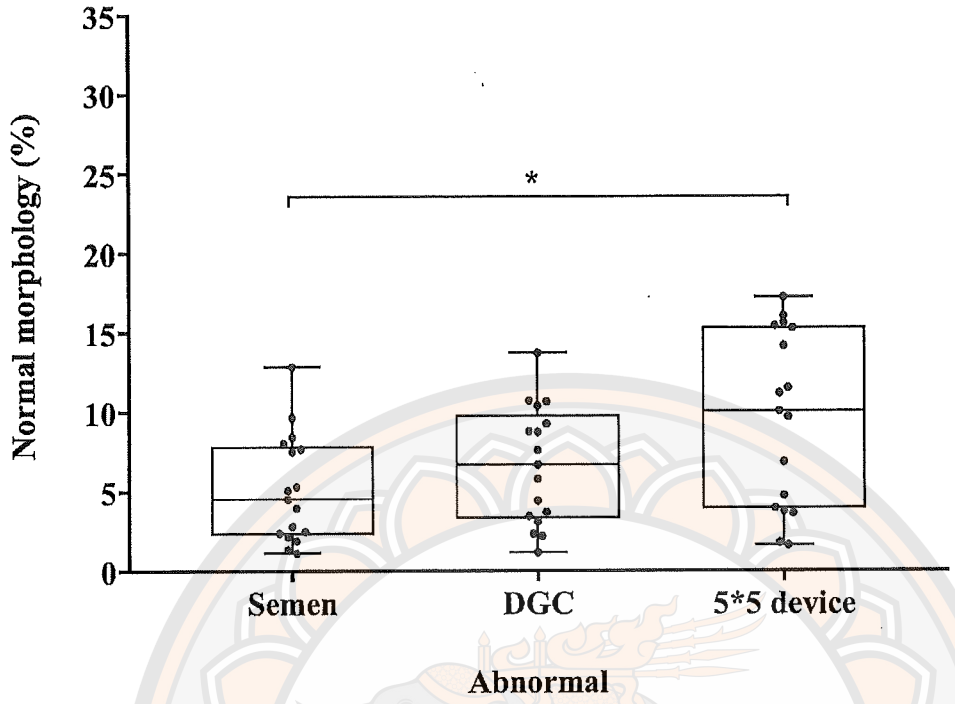



**Figure 85** Boxplots of normal sperm morphology (semen and selected sperm) in Abnormal group. One–way ANOVA with post hoc Tukey's test was used to compare between sperm selection methods; *P < 0.05. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).

**Sperm DNA fragmentation after the selection**

The results of sperm DNA fragmentation in unprocessed semen and all sperm selection methods in Normal group was shown in Figure 86 whereas those in Abnormal group was shown in Figure 87. In Normal group, the DFI was improved by 88% (8.3 to 1.0) in 5*5 device and 95% (8.3 to 0.4) in 5*3 device, whereas that was improved by 94% (12.5 to 0.8) in 5*5 device in Abnormal group. The sperm DNA fragmentation was significantly decreased in sperm selected by 5*5 (1.0 ± 0.2%) and 5*3 devices (0.4 ± 0.1%) compared to DGC (7.0 ± 0.7%), SU (3.7 ± 0.7%) in Normal group; however, there was no significant difference DFI between DGC and SU, see Figure 86. Sperm selected by 5*5 device in Abnormal group had significantly lower the sperm DNA fragmentation compared to DGC (0.8 ± 0.1% vs 10.2 ± 0.7%), see Figure 87. The DNA fragmentation of sperm selected by DGC in both groups was not significantly different from semen samples (Normal group, 7.0 ± 0.7% vs 8.3 ± 0.7%: Abnormal group, 10.2 ± 0.7% vs 12.5 ± 1.4%). The fluorescent visualization of sperm DNA fragmentation in unprocessed semen and all sperm selection methods in Normal and Abnormal groups were shown in Figure 88 and Figure 89, respectively.

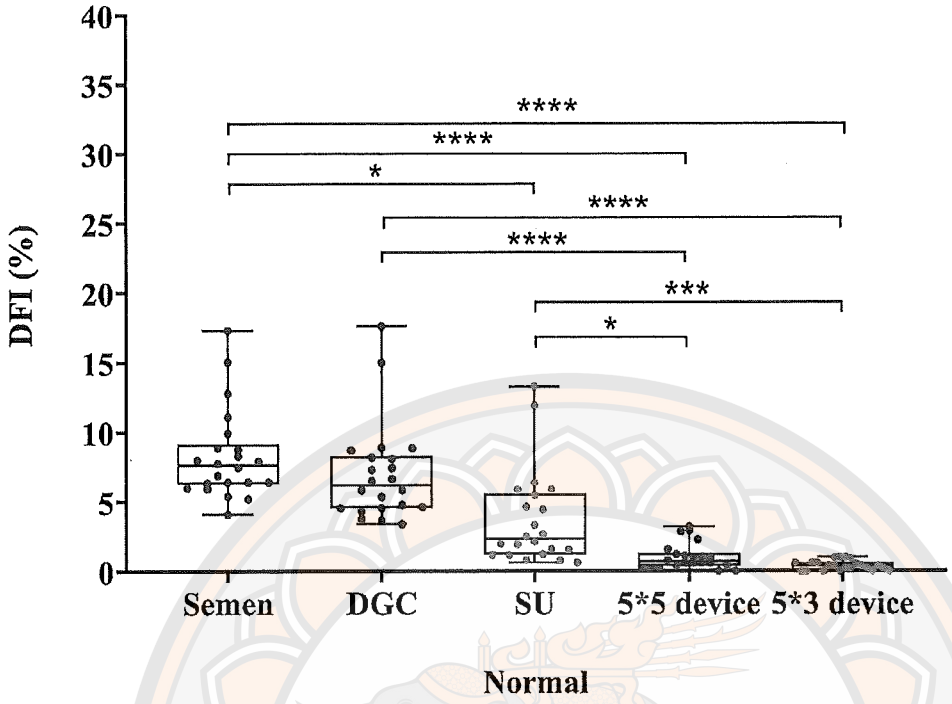



**Figure 86** Boxplots of sperm DFI (semen and selected sperm) in Normal group. The nonparametric Kruskal–Wallis test was used to compare between sperm selection methods; *P < 0.05, ***P < 0.001 and ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).

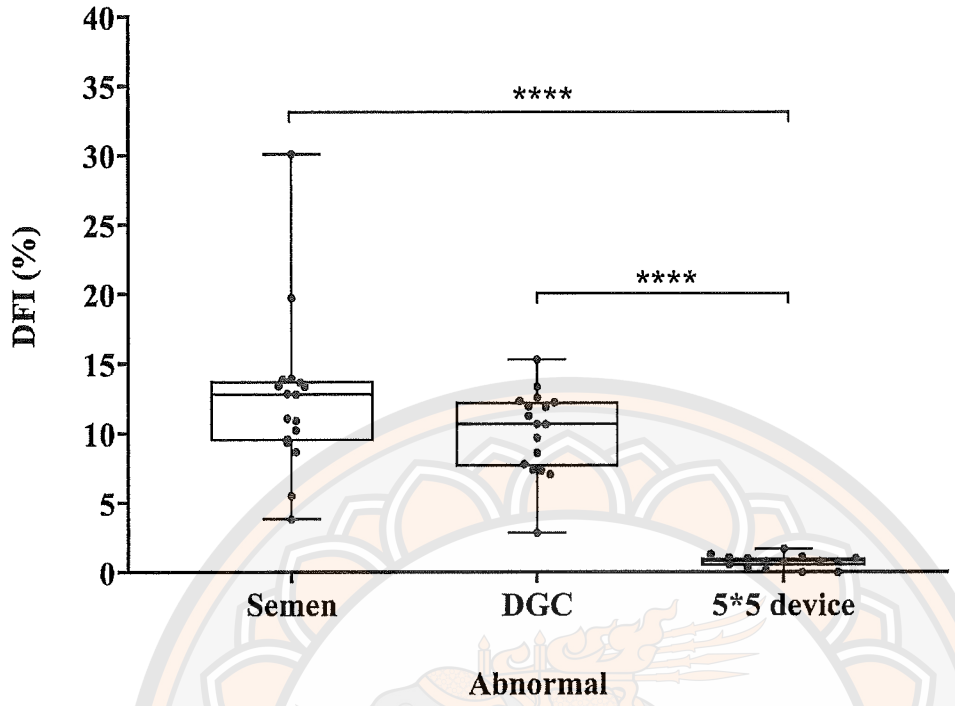



**Figure 87** **Boxplots of sperm DFI (semen and selected sperm) in Abnormal group. The nonparametric Kruskal–Wallis test was used to compare between sperm selection methods; ****P < 0.0001. Whisker boxes show the median, 25%, 75% quartiles, whiskers (min–max).**

**The recovery rate of sperm after application of the microfluidic devices**

The number of loaded and extracted sperm, as well as the recovery rate in the selection using the microfluidic devices both in Normal and Abnormal groups were shown in Table 10. In Normal group, the number of sperm selected by 5*5 device was higher than 5*3 device ($2.1 \pm 0.2 \times 10^6$ sperm vs $1.2 \pm 0.2 \times 10^6$ sperm). Sperm selection using 5*5 device in Normal group had higher sperm number than those in Abnormal group ($2.1 \pm 0.2 \times 10^6$ sperm vs $1.0 \pm 0.2 \times 10^6$ sperm). Moreover, the recovery rate after the sperm selection using 5*5 (25.1 ± 2.5%) and 5*3 devices (22.8 ± 2.1%) in Normal group were higher than those using 5*5 device (15.2 ± 2.5%) in Abnormal group.

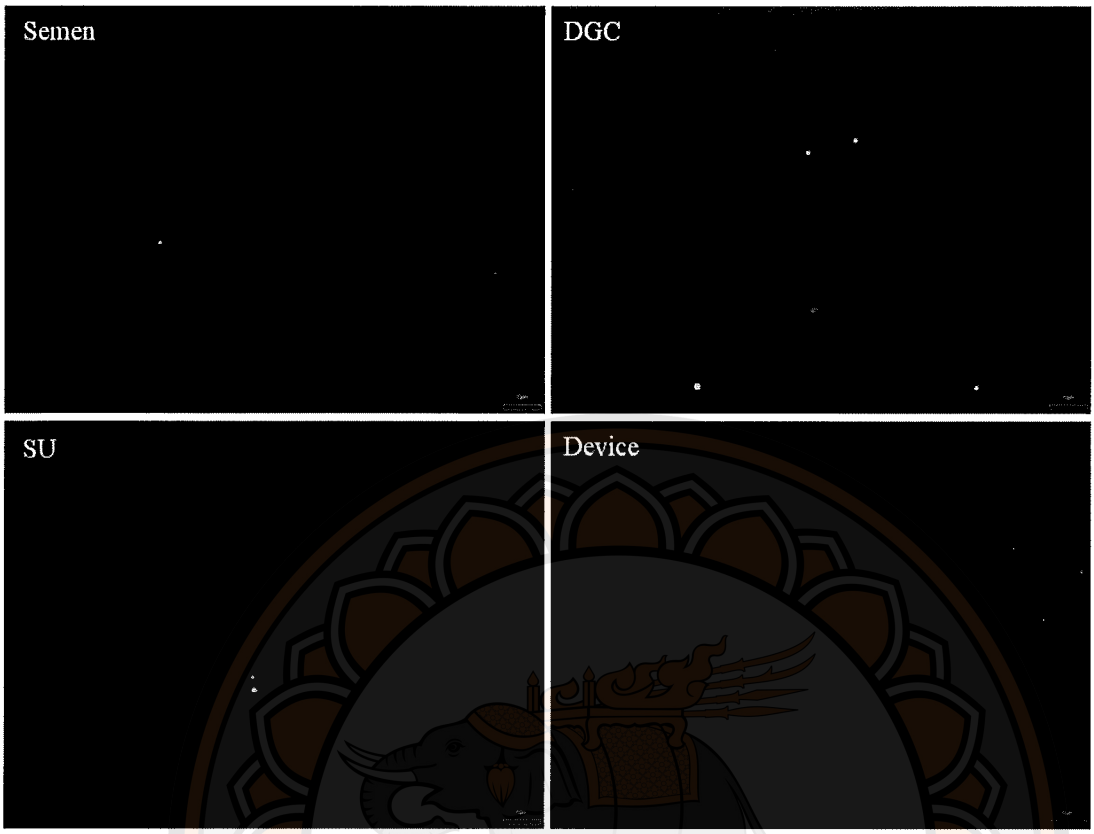



**Figure 88 Fluorescent visualization at 200× magnification of DNA–fragmented sperm (green) and DNA–intact sperm (blue) in semen, after the selection by DGC and the microfluidic device in Normal group.**

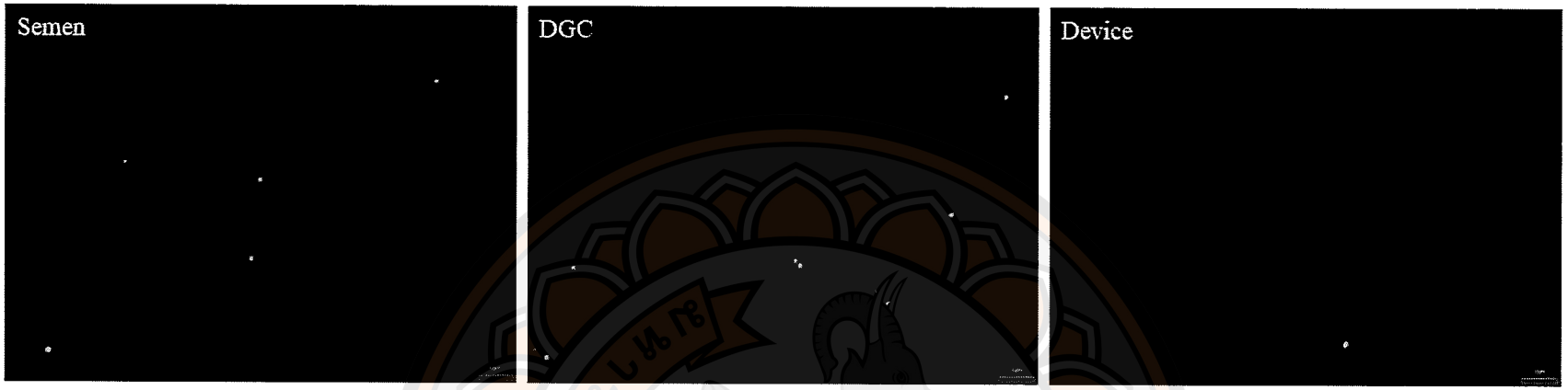



**Figure 89** **Fluorescent visualization at 200× magnification of DNA–fragmented sperm (green) and DNA–intact sperm (blue) in semen, after the selection by DGC and the microfluidic device in Abnormal group.**

**Table 10 Sperm concentration (semen and all sperm selection methods) and the microfluidic device test (the number of loaded, extracted sperm and recovery rate) in Normal (N = 22) and Abnormal (N = 17) groups. Data shown as mean ± SEM.**

| | Group | | Semen | DGC | SU | MFD | |
|---|---|---|---|---|---|---|---|
| | | | | | | 5*5 device | 5*3 device |
| **Sperm concentration ($10^6$ per ml)** | **Normal** | | 89.6 ± 9.7 | 63.1 ± 6.7 | 19.4 ± 2.9 | 44.7 ± 5.6 | 24.8 ± 3.6 |
| | **Abnormal** | | 72.4 ± 8.4 | 29.0 ± 3.8 | – | 22.7 ± 4.9 | – |
| **The MFD test** | **Normal** | Loaded sperm ($10^6$) | | | | 9.0 ± 1.0 (3.7 – 21.9) | 5.4 ± 0.6 (2.2 – 13.2) |
| | | Extracted sperm ($10^6$) | | | | 2.1 ± 0.2 (0.6 – 3.9) | 1.2 ± 0.2 (0.3 – 3.6) |
| | | Recovery rate (%) | | | | 25.1 ± 2.5 (6.9 – 57.8) | 22.8 ± 2.1 (5.0 – 45.3) |
| | **Abnormal** | Loaded sperm ($10^6$) | | | | 7.2 ± 0.8 (1.9 – 13.8) | – |
| | | Extracted sperm ($10^6$) | | | | 1.0 ± 0.2 (0.2 – 3.7) | – |
| | | Recovery rate (%) | | | | 15.2 ± 2.5 (1.8 – 35.2) | – |

**Note:** MFD = microfluidic device

**The dilution test**

The minimum and maximum of loaded sperm in the dilution test were 1.2 × $10^6$ and 37 × $10^6$ sperm, respectively, see Figure 90. A linear relationship between the number of loaded and extracted sperm was found in the dilution test (Pearson's correlation force through zero, r = 0.964, p < 0.0001). The result of linear regression indicates that 17% of sperm could be extracted from the device. Figure 91 demonstrates that all data points of the microfluidic device test in both groups were found inside the range of the dilution test. Moreover, Figure 92 and Figure 92 show the positive correlation between the percentage of progressive sperm motility and the number of extracted sperm and recovery rate from the devices, respectively.

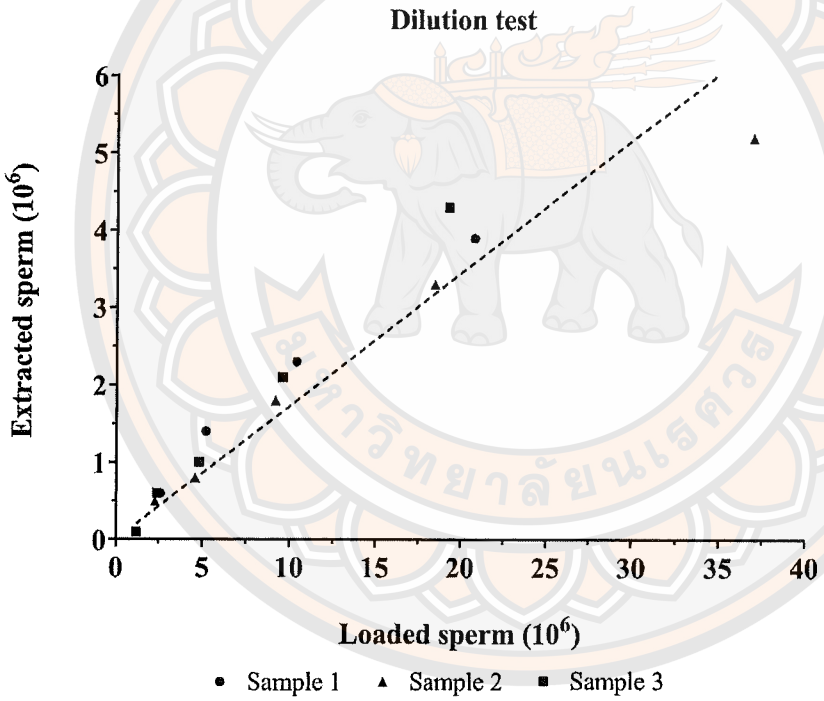



**Figure 90 Correlation between loaded sperm ($10^6$) and extracted sperm ($10^6$) for 3 samples in serial dilution (black points). Linear regression line (dashed black line, r = 0.964, p < 0.0001) fitted to all data points in the dilution test. The result of the regression line is: Extracted sperm = 0.17 (Loaded sperm).**

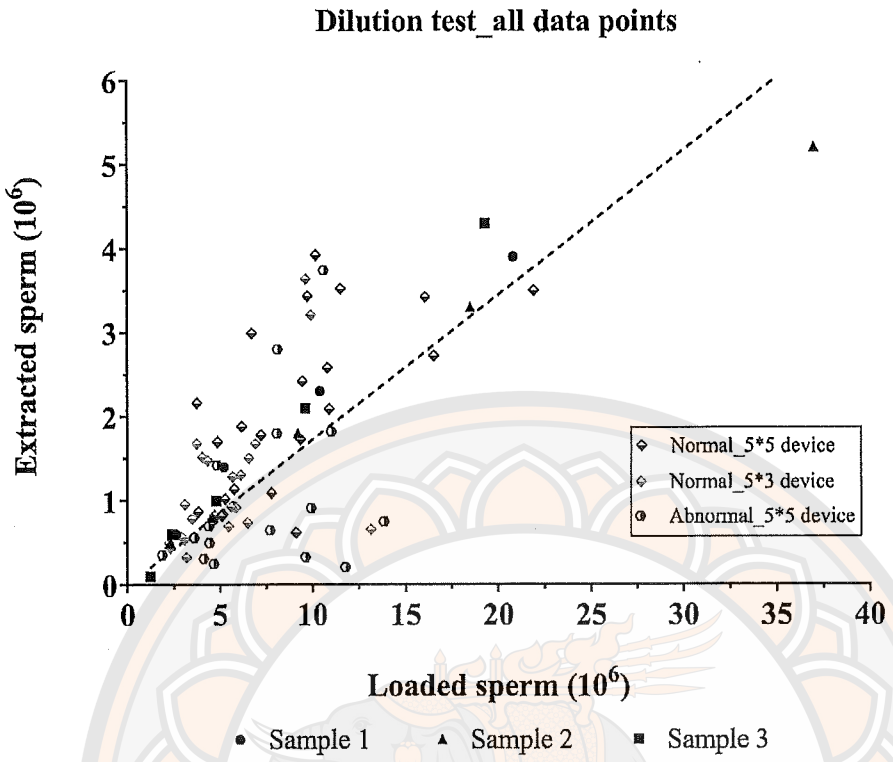



**Figure 91** The quantities of loaded and extracted sperm for all data points in the microfluidic device test including 5*5 device (dark green points) in the Normal and Abnormal groups and 5*3 device (light green points) in the Normal group were plotted in the regression line of the dilution test.

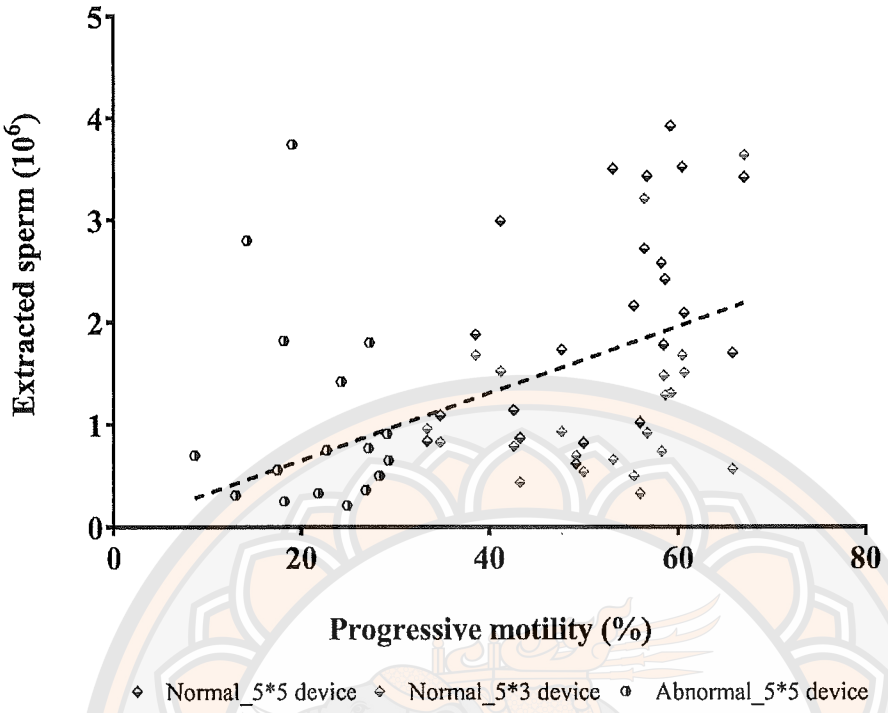



**Figure 92** **Correlation between sperm progressive motility and extracted sperm ($10^6$) for all data points in the microfluidic device test including 5*5 device (dark green points) in the Normal and Abnormal groups and 5*3 device (light green points) in the Normal group. Linear regression line (dashed black line, r = 0.358, p = 0.0046) fitted to those data points. The regression line is: Extracted sperm = 0.03 (% Progressive motility).**

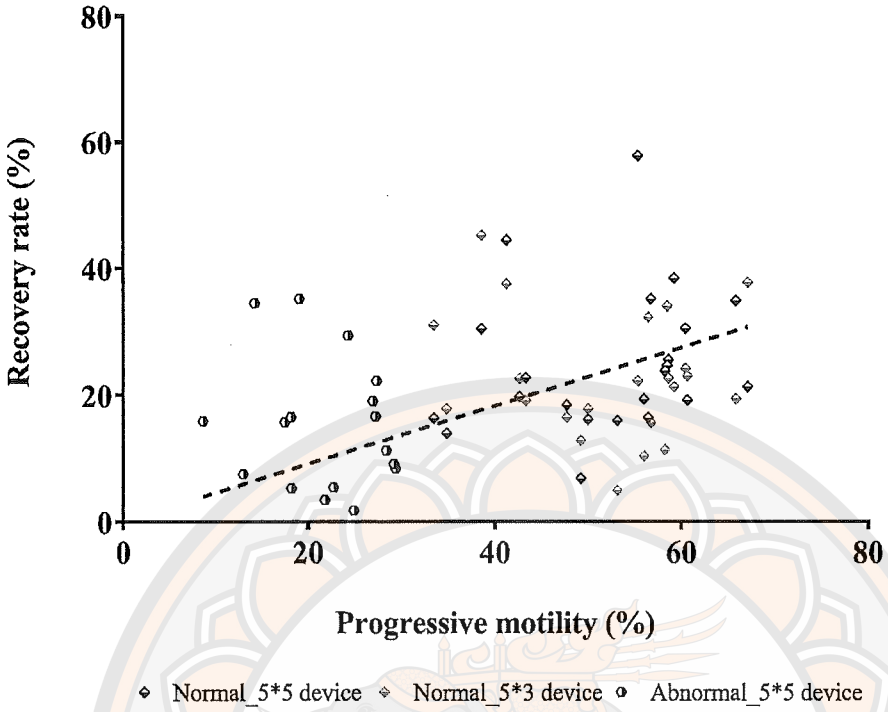



**Figure 93** **Correlation between sperm progressive motility and recovery rate for all data points in the microfluidic device test including 5*5 device (dark green points) in the Normal and Abnormal groups and 5*3 device (light green points) in the Normal group. Linear regression line (dashed black line, r = 0.297, p = 0.02) fitted to those data points. The regression line is: Recovery rate = 0.46 (% Progressive motility).**

**Discussion and conclusion**

In this study, a novel microfluidic device was fabricated using PDMS which is widely used as a low–cost material for the fabrication of the microfluidic device. In contrast to the DGC method, the procedure of the microfluidic device for sperm selection is an uncomplicated procedure with no need for centrifugation. The results of this study showed that sperm selected by a novel microfluidic device from unprocessed semen samples had high sperm progressive motility, total motility, normal morphology, and DNA integrity. These results indicate that the microfluidic channels (containing an asymmetric triangle arrayed microstructure) and the device design are suitable for the separation of intact sperm with high progressive motility. To my knowledge, this is the first report in the sperm selection from the unprocessed semen samples with low sperm progressive motility using the microfluidic device. This study reported that the 5*5 device can isolate sperm with higher sperm quality than using DGC in the Abnormal group, although it is more time–consuming.

The DFI of sperm selected by the devices was significantly lower than those selected by DGC and SU methods. In the present study, there was no significant improvement in the sperm DNA fragmentation after SU. This result is consistent with the previous study which showed no significant difference in the sperm DNA fragmentation after the isolation by direct SU method compared with unprocessed semen, although a significant increase in sperm motility was found (Younglai, Holt, Brown, Jurisicova, & Casper, 2001). Additionally, several studies reported that although DGC and SU can improve the sperm DNA fragmentation, the low level of the improvement was found (Xue et al., 2014; Zini, Finelli, et al., 2000).

As mention earlier, there is evidence that the sperm DNA damage normally occurs during the centrifugation step in DGC because of the centrifugal force; on the other hand, these devices can isolate sperm based on their motility behaviour without the centrifugation. Although SU also selects sperm without the centrifugation, the cell–to–cell contact between sperm and other cells (including cell debris and leukocytes) during the incubation results in the production of ROS which causes potentially the oxidative stress and DNA damages (reviewed by Beydola et al., 2013; Kim, 2018). On the other hand, in the microfluidic device test, the cell debris and leukocytes were stuck immediately at the inlet ports after the semen loading whilst selected sperm immediately

started moving forward into the outlet. This study found that many motile sperm reached the outlet within 5 minutes after loading (data not shown). Altogether, the results of this study suggest that sperm selected by the device have less contact with cell debris and leukocytes than those selected by the SU method. Another possible reason is that sperm recovered from the direct SU method had not only higher DNA fragmentation but also higher abnormal morphology compared to those recovered from the devices. The positive correlation between the levels of sperm DNA fragmentation and abnormal sperm morphology has been reported in several studies (Cohen-Bacrie et al., 2009; Sá, Cunha, Rocha, Barros, & Sousa, 2015). Therefore, it seems that sperm with abnormal morphology which recovered from the direct SU method mostly had DNA damage.

Interestingly, the very low DNA fragmentation, approximately 1% (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay) was found in sperm selected by the microfluidic devices in both groups of samples. The DNA fragmentation of sperm selected by the devices was improved up to 95%, including 88 and 95% (Normal groups), and 94% (Abnormal group) which are higher than the improvement in the normal ejaculates in a previous study. They reported that the highest improvement of sperm DNA fragmentation by their passively driven devices was 81% using 7.5 mm device (Nosrati et al., 2014). In the Normal group, the DFI of sperm selected by 5*5 device was slightly higher than the 5*3 device which is similar to the sperm morphology result. The results of this study suggest that the bigger inlet size of 5*5 device than 5*3 device results in high extraction force during the extraction step. The contamination of sperm with abnormal morphology and DNA damage might occur during the extraction step. Interestingly, it seems that using sperm selected by these devices bypassed the sperm extraction step for the IVF treatment can protect the contamination during the extraction.

In the Normal group, the recovery rate of the sperm selected by 5*5 and 5*3 devices was approximately 25% and 23%, respectively. Moreover, the high recovery rate also found in the Abnormal group using a 5*5 device (15%). The results in this study are higher than the recovery rate in other microfluidic devices used in another study (0.5%) (Nosrati et al., 2014). Interestingly, the novel microfluidic devices can isolate 1–2 million sperm from a small number of unprocessed semen samples (60–100 μl) which is sufficient for ICSI and IVF treatments.

In terms of sperm concentration, sperm selection using the devices in the Normal group had higher sperm concentration than using the SU method. Although a decrease in sperm concentration was found in the use of a novel microfluidic device compared to DGC in the Normal group, it was not found in the Abnormal group. The novel microfluidic devices can isolate sperm with a higher concentration ($44.7 \times 10^6$ sperm per ml and $24.8\times 10^6$ sperm per ml using 100 µl and 60 µl unprocessed healthy semen sample, respectively) compared to other microfluidic devices used in the study of Eamer et al. (less than $2 \times 10^6$ sperm per ml using 200 µl semen) and Nosrati et al. ($0.6 \times 10^6$ sperm per ml using 1 ml semen) (Eamer et al., 2016; Nosrati et al., 2014), although a longer incubation time was taken in the present study. Nabi et al. have reported that the incubation time in culture after 2 hours lead to an increase in the sperm DNA fragmentation; however, less than 2 hours of incubation time were used in the present study (Nabi, Khalili, Halvaei, & Roodbari, 2014).

In summary, the results of this study indicate that a novel microfluidic device is more effective than DGC and SU methods in isolating progressive motile sperm with high DNA integrity from both normal and abnormal (low progressive motility) ejaculates. These microfluidic devices were developed to be easy to use, low-cost, and available for small quantities of sperm and the samples with low sperm motility. However, a comparison between a novel microfluidic device and other microfluidic devices as well as the routine clinical methods for sperm separation in the clinical assessment should be studied further.

# CHAPTER VII

# DISCUSSION AND CONCLUSION

## Findings

The study in a model of methamphetamine (METH) dependence (METH–administrated rats) indicates that the GABA concentration and mRNA expression of GABA A–α1 receptor as well as GAD1 (GAD67) in testis were significantly increased in METH–administrated rats (especially in ED–binge METH group) compared with control group. Moreover, the mRNA expression of GAT1 in testis was significantly decreased in these rats. This study found a negative correlation between GABA concentration and the GAT1 mRNA expression in the testis as well as the percentage of normal sperm motility. Conversely, a positive correlation between the GAT1 mRNA expression in testis and the percentage of normal sperm motility as well as the normal form of sperm morphology were reported. There was a significant decrease in the FSHR protein expression in the testis of METH–administrated rats (AB METH and ED–binge METH groups) compared with control group; however, there was no significant difference in the mRNA expression of FSH and LH receptors. The high levels of FSHR protein expression were significantly correlated with the high percentage of normal sperm motility and the low mRNA expression of GAD1 (GAD67) in testis as well as protein expression of GABA A–α1 receptor in sperm.

In rat sperm, the localization of protein expression of GABA A–α1 receptor was found in the anterior acrosomal segment of the sperm head region. The percentage of the strong–positively stained sperm and ROD of GABA A–α1 receptor immunostaining was significantly increased in METH–administrated groups compared with control group. A negative correlation between those results and the percentage of normal sperm motility was found.

In human sperm, an increase in mRNA expression of GABA A–α1 and GABA B–R2 receptors was found in OAT and TER groups compared with NOR group; nevertheless, the significant increase in mRNA expression of those receptors was found only in the OAT group. A negative correlation between the mRNA expression of GABA

A–α1 and B–R2 receptors and sperm parameters including sperm concentration, progressive motility, total motility, and normal morphology was reported.

The results of ICSI treatment showed that there were no significant differences in the fertilization rate and the embryo quality on morula and blastocyst stages among all groups. Conversely, patients in the NOR group had a significantly increased good–embryo quality on cleavage stage than patients in the OAT and TER groups. Moreover, there was the increase in the mRNA expression of GABA A–α1 and B–R2 receptors in Poor group (patients who had a couple with ≤ 50% good–quality embryo) compared with Good group (patients who had a couple with > 50% good–quality embryo) in the analysis of embryo quality on cleavage stage but not on the morula and blastocyst stages; nevertheless, a significant increase was found only in the GABA A–α1 receptor. Similarly, there were no significant differences in those receptors between High (patients who had a couple with ≥ 50% fertilized oocytes) and Low (patients who had a couple with < 50% fertilized oocytes) groups. In the correlation analysis, a negative correlation between the mRNA expression and the percentage of good–quality embryos on cleavage stage was found in the GABA A–α1 receptor but not in the GABA B–R2 receptor. However, there was no correlation between the GABA receptors and the fertilization rate as well as embryo quality on other stages.

In the study of a novel microfluidic device for sperm selection, there were no significant differences in semen volume, total sperm number, sperm concentration and, morphology between Normal and Abnormal groups. The Abnormal group had significantly lower sperm progressive motility and total motility, but higher sperm DNA fragmentation compared with the Normal group. The sperm concentration in the selection using the novel microfluidic is higher than using SU but lower than using DGC in the Normal group; however, there was no significant difference in the sperm concentration between the device and DGC in the Abnormal group.

Moreover, the increases in sperm progressive motility and total motility were found after the selection by all methods compared with unprocessed semen in both groups. Sperm selected by DGC had significantly lower progressive motility compared to other sperm selection methods in Normal and Abnormal groups. Conversely, there was no significant difference in sperm progressive motility between both devices and SU in the Normal group. Additionally, sperm selected by DGC had significantly lower

total motility compared to the selection using the devices in Normal and Abnormal groups, but it was not different in the comparison with SU method in the Normal group. Sperm recovered from the devices had significantly higher normal sperm morphology compared with unprocessed semen both Normal and Abnormal groups. Interestingly, there was a significant increase in normal sperm morphology after the recovery from the 5*3 device compared to DGC and SU methods in Normal group.

In the Normal group, the DFI was improved by 88% (8.3 to 1.0) in 5*5 device and 95% (8.3 to 0.4) in 5*3 device, whereas that was improved by 94% (12.5 to 0.8) in 5*5 device in Abnormal group. The sperm DNA fragmentation was significantly lower in sperm selected by the 5*5 (1.0 ± 0.2%) and 5*3 devices (0.4 ± 0.1%) compared to DGC (7.0 ± 0.7%), SU (3.7 ± 0.7%) in Normal group; however, there was no significant difference DFI between DGC and SU. Sperm selected by the 5*5 device in the Abnormal group had significantly lower sperm DNA fragmentation compared to DGC (0.8 ± 0.1% vs 10.2 ± 0.7%). The DNA fragmentation of sperm selected by DGC in both groups was not significantly different from semen samples.

The number of sperm selected by 5*5 device was higher than 5*3 device (2.1 ± 0.2 × $10^6$ sperm vs 1.2 ± 0.2 × $10^6$ sperm) in the Normal group; moreover, those recoveries were found higher in the Normal group compared with the Abnormal group. Moreover, the recovery rate after the sperm selection using 5*5 (25.1 ± 2.5%) and 5*3 devices (22.8 ± 2.1%) in the Normal group were higher than those using 5*5 devices (15.2 ± 2.5%) in Abnormal group.

In the dilution test of the microfluidic device study, the minimum and maximum of loaded sperm in the dilution test were 1.2 × $10^6$ and 37 × $10^6$ sperm. 17% of sperm could be extracted from the device. Moreover, the positive correlation between the percentage of progressive sperm motility and the number of extracted sperm and recovery rate were found.

**Discussion and conclusion**

This study found increased GABA concentration and levels of GABA synthesizing enzyme (mainly GAD1 (GAD67)) as well as GABA A–α1 receptor in testis after METH exposure. In parallel with these changes, the reduction of the GAT1 expression in METH–administrated rats reflects the inhibition of GABA reuptake which

increases in the GABA concentration (Soudijn, & van Wijngaarden, 2000), as supported by the finding of negative correlation between the relative mRNA expression of GAT1 and GABA concentration in testis. These results reflect the increase of GABA production and function. As mentioned above, the previous studies have been reported poor sperm quality, testicular damage (the apoptosis induction in spermatogenic cells), and the functional impairment of sex steroid hormones, including testosterone, estrogen, and progesterone in METH–administrated rats (Lin et al., 2014; Nudmamud-Thanoi et al., 2016; Nudmamud-Thanoi, & Thanoi, 2011). Additionally, METH exposure strongly affected the reduction of FSHR expression in the testis which is significantly related to the low normal sperm motility. For many years, FSH has been reported its function in the regulation of spermatogenesis, whereas GABA plays a role in the Leydig cell proliferation, testosterone production, and the maintenance of the proliferation of spermatogonial stem cells (SSCs) during spermatogenesis (Du et al., 2013; Geigerseder et al., 2004; Taherianfard et al., 2013). Altogether, the results of this study suggest that the reduction of FSHR is involved in the impairment of testicular and sperm function and related to the increase of the GABAergic system in the testis.

A negative correlation between the expression of GAD1 (GAD67) and the expression of FSHR in testis suggests that the increase in GABA synthesis in testis might be occurred because of the reduction of FSH function. The results in the relationship between the GABAergic system and sperm parameters (sperm motility and morphology) supported that sperm quality influences the responsiveness of the GABAergic system and results in compensatory upregulation of GABA production and its functions in the testis. In parallel with those findings, the increase in GABA A–α1 receptor was found in the ejaculated sperm of rats in the ED–binge METH group. This finding indicates that the levels of GABA A–α1 receptor remains expressed in the epididymal sperm after spermiation. The significant changes of the GABAergic system were found in ED–binge METH rats which mimicking the METH use in humans. As shown in the correlation analysis results, the high levels of GABA A–α1 receptor expression in sperm were significantly related to the low normal sperm motility; moreover, they had more severe poor sperm quality (Nudmamud-Thanoi et al., 2016). These results suggest that the responsiveness of the GABAergic relies on the severity of sperm impairment. GABA supplements such as GABA standard and pre–germinated

brown rice have been reported that these supplements can improve morphological changes of seminiferous tubules, androgen receptor expression and sperm quality that cause by antidepressant, psychoactive drugs, and drug addiction: dextromethorphan (Roboon et al., 2017; Thanoi et al., 2018). The understanding of the physiological relevance of GABA in testicular and sperm functions remains unclear and is needed to elucidate; however, the present study provides the novel evidence to support the role of the GABAergic system in testicular and sperm functions.

Interestingly, the levels of GABA receptors expression in ejaculated human sperm in the condition of poor sperm quality and their association with the ICSI outcomes were studied. The study found the increase in the expression of GABA A–α1 and GABA B–R2 receptors in OAT and TER patients. This result is consistent with the results in the animal study, METH–administrated rats. Taken together, these results demonstrate the alteration of GABA receptors in testis and sperm, both epididymal and ejaculated sperm, in the condition of poor sperm quality. Therefore, It is possible that the increase in GABA receptors is supposed to occur earlier in the testis to compensate for the sperm impairment and maintain the homeostasis of testicular function; moreover, those receptors remain expressed in the epididymal sperm after spermiation as well as in the ejaculated sperm. Interestingly, a significant correlation between the high levels of these receptors and the low sperm parameters were found. These results demonstrate that the levels of those receptors depend on the severity of sperm impairment which is consistent within the animal study.

The results in ICSI outcomes showed that OAT and TER patients had significantly lower the embryo quality on cleavage stage after ICSI compared with NOR patients but had no significant difference in the fertilization rate and the embryo quality in other stages. The previous studies by Loutradi et al. and Li et al, also supported out results (Li et al., 2014; Loutradi et al., 2006). These findings confirmed that the impairment of embryo development in OAT and TER men undergoing ICSI occurs in the cleavage stage. To my knowledge, this is the first report in the correlation between GABA receptors in sperm and the ICSI outcomes. A significant increase in GABA A–α1 receptor expression in sperm was found in patients who have a female partner with a low percentage of good quality embryos on the cleavage stage after ICSI. The high levels of GABA A–α1 receptor were significantly correlated with the low good–quality

embryo (GQE) on the cleavage stage after ICSI. These findings support the hypothesis that the mRNA levels of GABA A–α1 receptor expression in human sperm are involved in the embryonic cleavage after ICSI.

Because the ICSI procedure bypasses not only the processes of natural sperm selection for the fertilization but also the sperm–oocyte plasma membrane fusion, the disintegration of the sperm plasma membrane and acrosome is important for the ICSI outcomes (reviewed by Neri et al., 2014). A major cause of ICSI failure is the delayed oocyte activation that normally occurs after triggering intracellular calcium release and oscillations by sperm factors in acrosome, the sperm–borne oocyte activating factors (SOAFs). There is evidence that the sperm plasma membrane and acrosome remain intact for several hours in the oocyte although sperm immobilization is performed immediately before ICSI to induce the sperm plasma membrane disruption (reviewed by Yanagimachi, 2005). And, the calcium signaling during the cleavage stage has an impact on the timing of the cleavage division and the development (Roldan, 2006). Indeed, the activation of GABA receptors is associated with the increase in intracellular calcium ions in the sperm head (Calogero et al., 1999; Puente et al., 2011; Ritta et al., 2004). Altogether, this study can suggest that the levels of remaining GABA receptors on the intact sperm head may affect the calcium signaling in the oocyte after ICSI by leading to the calcium influx from ooplasm into sperm. Eventually, using sperm with high levels of GABA receptors in ICSI might disturb the pattern of intracellular calcium oscillations within ooplasm which causes the delay of oocyte activation and cleavage division of embryo; however, the functional role of GABA receptors on those processes remains unknown.

Mainly, these findings suggest that the male factors have a critical impact on the success of ICSI treatment; moreover, the use of high–sperm quality in this treatment has an impact on ICSI outcomes. Currently, DGC and SU methods are generally used to select those sperm in fertility clinics; however, there are several disadvantages in the use of these methods. The efficiency of a novel microfluidic device for sperm selection in normal and abnormal (low progressive motility) semen samples was studied. This study found that sperm selected by these devices had significantly higher sperm progressive motility, total motility, and DNA integrity compared to DGC in both groups; however, an increase in normal sperm morphology also found but it was not

significantly different. Unfortunately, sperm concentration selected by the devices was lower than the DGC method; however, a small number of sperm and low semen volume were used in the device test. The comparison between the devices and the SU method, sperm selected by these devices had significantly increased sperm concentration, normal morphology, and DNA integrity. A novel microfluidic device is more effective than DGC and SU methods in isolating progressive motile sperm with low DNA fragmentation (approximately 1%) from both normal and abnormal (low progressive motility) ejaculates. Moreover, these devices had a higher recovery rate and the number of extracted sperm numbers compared to other microfluidic devices used in the previous studies (Eamer et al., 2016; Nosrati et al., 2014).

In summary, this study found that the high levels of the GABAergic system in testis are referred to as having the condition of testicular damage and poor sperm quality. The levels of GABA A–α1 and GABA B–R2 receptors in human sperm can be used as the biomarkers for the prediction of male infertility and sperm function. Furthermore, these results demonstrate that the levels of GABA receptors in human sperm, especially GABA A–α1 receptor, are associated with male infertility and the embryo quality on the cleavage stage after ICSI. However, further studies of the correlation between the GABAergic system and the outcomes of IVF or IUI treatment are necessary to explain the role of GABA on sperm function for fertilization. The results of this study might be a piece of useful knowledge for the medical development in male infertility. Especially, antioxidant supplements such as GABA standard and antioxidant–rich food such as pre–germinated brown rice might be used as an alternative medical treatment for male infertility in the future. Moreover, a novel microfluidic device used for sperm selection is composed of easy to use, inexpensive, and available for small quantities of sperm and the samples with low sperm motility. A novel microfluidic device usage for sperm selection can select sperm with higher sperm motilities and DNA integrity than using DGC and SU methods.

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Zini, A., Finelli, A., Phang, D., & Jarvi, K. (2000). Influence of semen processing technique on human sperm DNA integrity. *Urology, 56*(6), 1081-1084.

Zini, A., Nam, R. K., Mak, V., Phang, D., & Jarvi, K. (2000). Influence of initial semen quality on the integrity of human sperm DNA following semen processing. *Fertility and Sterility, 74*(4), 824-827.

# APPENDIX

## APPENDIX I Solution preparations

**10% neutral buffered formalin, 1 L**

1. 37% formaldehyde solution — 100 ml
2. Disodium hydrogen phosphate ($Na_2HPO_4$, MW 141.96) — 6.5 g
3. Monosodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4.H_2O$, MW 137.99) — 4.0 g
4. Add distilled water to a final volume of 1 L

**10x Tris-Borate-EDTA (TBE buffer), 1 L**

1. 890 mM Tris (MW 121.14) — 107.82 g
2. 20 mM disodium ethylenediaminetetraacetate dihydrate (EDTA disodium salt, MW 372.24) — 7.45 g
3. 890 mM Boric acid (MW 61.83) — 55.03 g
4. Add distilled water to a final volume of 1 L

**Mobile phase; pH 5.84, 1 L**

1. 0.1 M monosodium dihydrogen orthophosphate monohydrate ($NaH_2PO_4.H_2O$, MW 137.99) — 13.80 g
2. 8% (v/v) Methanol — 80 ml
3. Adjusted to pH 5.84 with sodium hydroxide (NaOH)
4. Add distilled water to a final volume of 1 L

**1 M sodium sulphite ($Na_2SO_3$), 25 ml**

1. 1 M sodium sulphite ($Na_2SO_3$) (MW 126.04) — 3.15 g
2. Add distilled water to a final volume of 25 ml

**0.1 M sodium tetraborate ($Na_2B_4O_7$); pH 10.4, 25 ml**

1. 0.1 M sodium tetraborate ($Na_2B_4O_7$, MW 201.22) — 0.50 g
2. Adjusted to pH 10.4 with sodium hydroxide (NaOH)
3. Add distilled water to a final volume of 25 ml

**O-phthalaldehyde/sodium sulfite, 950ul**

| | |
|---|---|
| 1. o-phthaldialdehyde (MW 134.13) | 11 mg |
| 2. Ethanol | 250 µL |
| 3. 1 M sodium sulphite ($Na_2SO_3$) | 250 µL |
| 4. 0.1 M sodium tetraborate ($Na_2B_4O_7$, MW 201.22); pH 10.4 | 450 µL |

**1x Earle's Balanced Salt Solution (EBSS); pH 7.4, 500 ml**

| | |
|---|---|
| 1. 10x Earle's Balanced Salt Solution (EBSS) | 50 ml |
| 2. Distilled water | 450 ml |
| 3. Adjust pH to 7.4 with sodium hydroxide (NaOH) solution | |

**1x Earle's Balanced Salt Solution (EBSS) with 0.2% BSA, 25 ml**

| | |
|---|---|
| 1. 1x Earle's Balanced Salt Solution (EBSS), pH 7.4 | 25 ml |
| 2. 0.2% (w/v) Bovine serum albumin (BSA) | 0.05 g |

**Isotonic Percoll, 25 ml**

| | |
|---|---|
| 1. Percoll density gradient media | 22.5 ml |
| 2. 10x Earle's Balanced Salt Solution (EBSS) | 2.5 ml |

**80% Percoll solution, 25 ml**

| | |
|---|---|
| 1. Isotonic Percoll | 20 ml |
| 2. 1x Earle's Balanced Salt Solution (EBSS); pH 7.4 | 5 ml |

**40% Percoll solution, 25 ml**

| | |
|---|---|
| 1. Isotonic Percoll | 10 ml |
| 2. 1x Earle's Balanced Salt Solution (EBSS); pH 7.4 | 15 ml |

**10 mM Tris; pH 8, 50 ml**

| | |
|---|---|
| 1. Tris base (MW 121.14) | 0.06 g |
| 2. Adjust pH to 8 with sodium hydroxide (NaOH) solution | |
| 3. Add distilled water to a final volume of 50 ml | |

**1x Tris–buffered saline (TBS); pH 7.6, 1 L**

1. 20 mM Tris base (MW 121.14) 2.42 g
2. 140 mM sodium chloride (NaCl, MW 58.44) 8.18 g
3. Adjust pH to 7.6 with hydrogen chloride (HCL) solution
4. Adjust distilled water to a final volume of 1 L

# APENDIX II certificates of approval from research ethics committee for human and animal studies

เอกสารรับรองโครงการ

คณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

| | |
|---|---|
| ชื่อโครงการ | ผลกระทบของการได้รับสารเสพติดเมทแอมเฟตามีนต่อการเปลี่ยนแปลงของตัวรับฮอร์โมน Follicle Stimulating Hormone (FSHR) และ Lutinizing Hormone (LHR) ในอัณฑะของหนู<br><br>Effects of methamphetamine dependence on the expressions of Follicle Stimulating Hormone Receptor (FSHR) and Lutinizing Hormone Receptor (LHR) in male rat testis |
| เลขที่โครงการ | NU-AE550730 |
| เลขที่เอกสารรับรอง | 55 04 0033 |
| ประเภทการรับรอง | ยกเว้น(1) |
| ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ | รศ ดร.เสมอ ถาน้อย |
| สังกัดหน่วยงาน /คณะ | วิทยาศาสตร์การแพทย์ |
| วันที่รับรอง | วันที่ 14 กันยายน 2555 |

ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

(รองศาสตราจารย์ ดร รัตติมา จีนาพงษา)

ประธานคณะกรรมการกำกับการเลี้ยงและการใช้สัตว์ มหาวิทยาลัยนเรศวร

# เอกสารรับรองโครงการ

## คณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์

## มหาวิทยาลัยนเรศวร (คกส.)

| | |
|---|---|
| ชื่อโครงการ | ผลของสารเสพติดเมทแอมเฟตามีนต่อการเปลี่ยนแปลงระบบสารสื่อประสาทกาบาในอัณฑะและอสุจิของหนูแรท<br>Effects of methamphetamine on the alteration of GABAergic system in rat testis and spermatozoa |
| เลขที่โครงการ | NU-AEE591016 |
| เลขที่เอกสารรับรอง | 60 02 004 |
| ประเภทการรับรอง | ยกเว้น(1) |
| ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ | รองศาสตราจารย์ ดร.เสมอ ถาน้อย |
| สังกัดหน่วยงาน /คณะ | วิทยาศาสตร์การแพทย์ |
| วันที่รับรอง | 25 มกราคม 2560 |
| วันสิ้นสุดการรับรอง | 25 มกราคม 2563 |

ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์
มหาวิทยาลัยนเรศวร (คกส.)

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| ..... | ..... |
| (รองศาสตราจารย์ ดร.รัตติมา จีนาพงษา) | (รองศาสตราจารย์ ดร.รสริน ว่องวิไลรัตน์) |
| ประธานคณะกรรมการกำกับดูแลฯ | รองอธิการบดี |
| มหาวิทยาลัยนเรศวร | มหาวิทยาลัยนเรศวร |

COA No. 496/2017
IRB No. 0549/60

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร
NARESUAN UNIVERSITY INSTITUTIONAL REVIEW BOARD
99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000 เบอร์โทรศัพท์ 05596 8642

## เอกสารรับรองโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากล ได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

| | |
|---|---|
| ชื่อโครงการ | : การเปลี่ยนแปลงการแสดงออกของยีนและโปรตีนตัวรับสารกาบา และตัวขนส่งสารกาบา ชนิดที่ 1 ในอสุจิของเพศชายที่มีภาวะมีบุตรยาก |
| Study Title | : Differential gene and protein expressions of GABA receptors and GABA transporter subtype 1 in spermatozoa from infertile men. |
| ผู้วิจัยหลัก | : รองศาสตราจารย์ ดร.เสมอ ถาน้อย |
| Principal investigator | : Associate Professor Dr. Samur Thanoi |
| สังกัดหน่วยงาน | : คณะวิทยาศาสตร์การแพทย์ |
| ผู้ร่วมวิจัย | : รองศาสตราจารย์ ดร.สุทิสา ถาน้อย นางสาวปรียา แก้วมั่น<br>ผู้ช่วยศาสตราจารย์ แพทย์หญิงพัชรดา อมาตยกุล |
| วิธีทบทวน | : แบบเร่งรัด (Expedited Review) |
| รายงานความก้าวหน้า | : ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการเสร็จสิ้นก่อน 1 ปี |

เอกสารรับรอง

1. AF 01-10 เวอร์ชั่น 1.0 วันที่ 17 กรกฎาคม 2560
2. AF 02-10 เวอร์ชั่น 1.0 วันที่ 17 กรกฎาคม 2560
3. AF 03-10 เวอร์ชั่น 1.0 วันที่ 17 กรกฎาคม 2560
4. AF 04-10 เวอร์ชั่น 2.0 วันที่ 28 สิงหาคม 2560
5. AF 05-10 เวอร์ชั่น 1.0 วันที่ 14 กรกฎาคม 2560
6. สรุปโครงการเพื่อการพิจารณาทางจริยธรรมการวิจัยในมนุษย์ เวอร์ชั่น 2.0 วันที่ 28 สิงหาคม 2560
7. โครงการวิจัยฉบับเต็ม เวอร์ชั่น 2.0 วันที่ 28 สิงหาคม 2560
8. ประวัติผู้วิจัย เวอร์ชั่น 1.0 วันที่ 14 กรกฎาคม 2560
9. สรุปงบประมาณที่ได้รับ เวอร์ชั่น 1.0 วันที่ 14 กรกฎาคม 2560
10. แบบบันทึกข้อมูลอาสาสมัคร เวอร์ชั่น 1.0 วันที่ 14 กรกฎาคม 2560

ลงนาม
(รองศาสตราจารย์ ดร.พญ.สุธาทิพย์ พงษ์เจริญ)
รองประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยนเรศวร

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|---|---|
| วันที่รับรอง | : 07 กันยายน 2560 |
| Date of Approval | : September 07, 2017 |
| วันหมดอายุ | : 07 กันยายน 2561 |
| Approval Expire Date | : September 07, 2018 |

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

COA No. 496/2017
IRB No. 0549/60

**คณะกรรมการจริยธรรมการวิจัยในมนุษย์**
**มหาวิทยาลัยนเรศวร**
99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000 เบอร์โทรศัพท์ 05596 8637

**หนังสือรับรองเอกสารที่เกี่ยวข้องกับโครงการวิจัย**

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร ดำเนินการให้การรับรองเอกสารที่เกี่ยวข้องกับโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากลได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

| | |
|---|---|
| ชื่อโครงการ | : การเปลี่ยนแปลงการแสดงออกของยีนและโปรตีนตัวรับสารกาบา และตัวขนส่งสารกาบา |
| | : ชนิดที่ 1 ในอสุจิของเพศชายที่มีภาวะมีบุตรยาก |
| Study Title | : Differential gene and protein expressions of GABA receptors and GABA |
| | transporter subtype 1 in spermatozoa from infertile men. |
| ผู้วิจัยหลัก | : รองศาสตราจารย์ ดร.เสมอ ถาน้อย |
| Principal investigator | : Associate Professor Dr. Samur Thanoi |
| สังกัดหน่วยงาน | : คณะวิทยาศาสตร์การแพทย์ |
| ผู้ร่วมวิจัย | : รองศาสตราจารย์ ดร.สุทิสา ถาน้อย นางสาวปวีณา แก้วมั่น |
| | : ผู้ช่วยศาสตราจารย์ แพทย์หญิงพัชรดา อมาตยกุล |

**เอกสารที่ได้รับการรับรอง**

1. แบบรายงานการแก้ไขเพิ่มเติมโครงการวิจัย (AF 01-13) เวอร์ชั่น 1.0 วันที่ 2 พฤศจิกายน 2560
2. ตารางสรุปการเปลี่ยนแปลง (AF 02-13) เวอร์ชั่น 1.0 วันที่ 2 พฤศจิกายน 2560
3. AF 04-10 เวอร์ชั่น 3.0 วันที่ 2 พฤศจิกายน 2560
4. สรุปโครงการเพื่อการพิจารณาทางจริยธรรมการวิจัยในมนุษย์ เวอร์ชั่น 3.0 วันที่ 2 พฤศจิกายน 2560
5. โครงการวิจัยฉบับเต็ม เวอร์ชั่น 3.0 วันที่ 2 พฤศจิกายน 2560

ลงนาม

(นายแพทย์สมบูรณ์ ตันสุภสวัสดิกุล)
ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยนเรศวร

**วันที่รับรอง** : 17 พฤศจิกายน 2560
Date of Approval : November 17, 2017

**หมายเหตุ** ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

COA No. 496/2017
IRB No. 549/60

คณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยนเรศวร
99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000 เบอร์โทรศัพท์ 05596 8642

## เอกสารรับรองโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากล ได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

| | | |
|---|---|---|
| ชื่อโครงการ | : | การเปลี่ยนแปลงการแสดงออกของยีนและโปรตีนตัวรับสารกาบา และตัวขนส่งสารกาบาชนิดที่ 1 ในอสุจิของเพศชายที่มีภาวะมีบุตรยาก |
| Study Title | : | Differential gene and protein expressions of GABA receptors and GABA transporter subtype 1 in spermatozoa from infertile men. |
| ผู้วิจัยหลัก | : | รองศาสตราจารย์ ดร.เสมอ ถาน้อย |
| สังกัดหน่วยงาน | : | คณะวิทยาศาสตร์การแพทย์ |
| วิธีทบทวน | : | แบบเร่งรัด (Expedited Review) |
| รายงานความก้าวหน้า | : | ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือ ส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการ |
| | : | เสร็จสิ้นก่อน 1 ปี |

**เอกสารรับรอง**

1. แบบรายงานความก้าวหน้าของโครงการวิจัย (AF01-14) เวอร์ชั่น 1.0 วันที่ 30 สิงหาคม 2561

ลงนาม

(นายแพทย์สมบูรณ์ ตันสุภสวัสดิกุล)
ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยนเรศวร

| | | |
|---|---|---|
| **วันที่รับรอง** | : | 07 กันยายน 2560 |
| **วันหมดอายุ** | : | 07 กันยายน 2561 |
| **วันที่รับรองต่อเนื่องครั้งที่ 1** | : | 07 กันยายน 2561 |
| **วันที่หมดอายุการรับรองครั้งที่ 1** | : | 07 กันยายน 2562 |

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

COA No. 496/2017

IRB No. 549/60

**คณะกรรมการจริยธรรมการวิจัยในมนุษย์**

**มหาวิทยาลัยนเรศวร**

99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000 เบอร์โทรศัพท์ 05596 8642

**เอกสารรับรองโครงการวิจัย**

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากล ได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

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| **ชื่อโครงการ** | : การเปลี่ยนแปลงการแสดงออกของยีนและโปรตีนตัวรับสารกาบา และตัวขนส่งสารกาบาชนิดที่ 1 ในอสุจิของเพศชายที่มีภาวะมีบุตรยาก |
| Study Title | : Differential gene and protein expressions of GABA receptors and GABA transporter subtype 1 in spermatozoa from infertile men. |
| **ผู้วิจัยหลัก** | : รองศาสตราจารย์ ดร.เสมอ ถาน้อย |
| **สังกัดหน่วยงาน** | : คณะวิทยาศาสตร์การแพทย์ |
| **วิธีทบทวน** | : แบบเร่งรัด (Expedited Review) |
| **รายงานความก้าวหน้า** | : ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือ ส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการ : เสร็จสิ้นก่อน 1 ปี |

**เอกสารรับรอง**

1. แบบรายงานความก้าวหน้าของโครงการวิจัย (AF01-14) เวอร์ชั่น 1.0 วันที่ 10 กันยายน 2562

**ลงนาม**

**(รศ.ดร.พญ.สุธาทิพย์ พงษ์เจริญ)**

**รองประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์**

**มหาวิทยาลัยนเรศวร**

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| **วันที่รับรอง** | : 07 กันยายน 2560 |
| **วันหมดอายุ** | : 07 กันยายน 2561 |
| **วันที่รับรองต่อเนื่องครั้งที่ 1** | : 07 กันยายน 2561 |
| **วันที่หมดอายุการรับรองครั้งที่ 1** | : 07 กันยายน 2562 |
| **วันที่รับรองต่อเนื่องครั้งที่ 2** | : 07 กันยายน 2562 |
| **วันที่หมดอายุการรับรองครั้งที่ 2** | : 07 กันยายน 2563 |

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)