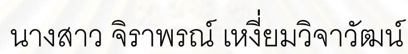
การตรวจหาความผิดปกติอนูพลอยดีของโครโมโซมเพศของอสุจิที่ได้จากท่อนำอสุจิส่วนอีพิดิไดมิส ในผู้ป่วยที่ตรวจไม่พบอสุจิในน้ำอสุจิจากการอุดตันของท่อนำอสุจิ



นางสาว จิราพรณ์ เหงี่ยมวิจาวัฒน์

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
หลักสูตรวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2545

ISBN 974-17-1161-1

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

DETECTION OF SEX CHROMOSOME ANEUPLOIDY OF EPIDIDYMAL SPERMATOZOA FROM OBSTRUCTIVE AZOOSPERMIC MEN

Miss Jiraporn Ngeamvijawat

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Medical Science
Program of Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2002
ISBN 974-17-1161-1

Thesis Title	Detection of Sex Chromosome Aneuploidy of Epididymal Spermatozoa from Obstructive Azoospermic Men
By	Miss Jiraporn Ngeamvijawat
Field of Study	Medical Science
Thesis Advisor	Professor Nares Sukcharoen, M.D.

Accepted by the Faculty of Medicine, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master 's Degree

……………………………………………….. Dean of Faculty of Medicine

(Professor Pirom Kamolratanakul, M.D.)

THESIS COMMITTEE

……………………………………………….. Chairman

(Associate Professor Wilai Chentanez, M.D.)

……………………………………………….. Thesis Advisor

(Professor Nares Sukcharoen, M.D.)

……………………………………………….. Member

(Professor Anek Aribarg, M.D.)

……………………………………………….. Member

(Associate Professor Apiwat Mutirangura, M.D.)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

จิราพรณ์ เหงี่ยมวิจาวัฒน์ :การตรวจหาความผิดปกติอนูพลอยดีของโครโมโซมเพศของอสุจิที่ได้จากท่อนำอสุจิส่วนอีพิดิไดมิสในผู้ป่วยที่ตรวจไม่พบอสุจิในน้ำอสุจิ จากการอุดตันของท่อนำอสุจิ(Detection of Sex Chromosome Aneuploidy of Epididymal Spermatozoa from Obstructive Azoospermic Men) อ.ที่ปรึกษา : ศ.นพ.เนรศร สุขเจริญ ; 67 หน้า ISBN 974-17-1161-1

การฉีดอสุจิเข้าในไข่ (intracytoplasmic sperm injection:ICSI) เป็นวิธีการรักษาภาวะที่ไม่พบอสุจิในน้ำอสุจิซึ่งเกิดจากการอุดตันของท่อนำอสุจิโดยใช้อสุจิจากท่อนำอสุจิ (epididymal spermatozoa) ได้อย่างมีประสิทธิภาพ อย่างไรก็ตามยังคงมีความกังวลถึงความเสี่ยงต่อการเกิดความผิดปกติของโครโมโซมชนิด aneuploidy ในทารกที่เกิดจากวิธีดังกล่าวจากบิดา ในปัจจุบันยังมีข้อมูลน้อยมากเกี่ยวกับอุบัติการณ์ของความผิดปกติของโครโมโซมของ epididymal spermatozoa ในผู้ป่วยที่ไม่พบอสุจิในน้ำอสุจิซึ่งเกิดจากการอุดตันของท่อนำอสุจิ ดังนั้นจึงได้ทำการศึกษาถึงอุบัติการณ์ของความผิดปกติของโครโมโซมชนิด aneuploidy และ diploidy ของepididymal spermatozoa ในผู้ป่วยที่ไม่พบอสุจิในน้ำอสุจิซึ่งเกิดจากการอุดตันของท่อนำอสุจิ ทำการตรวจ epididymal spermatozoa ที่เก็บได้จากผู้ป่วยที่ไม่พบอสุจิในน้ำอสุจิซึ่งเกิดจากการอุดตันของท่อนำอสุจิจำนวน 24 คน และผู้ชายปกติซึ่งเคยมีบุตรมาแล้วจำนวน 24 คน ด้วยเทคนิค three colour fluorescence in-situ hybridization (FISH) เพื่อตรวจหาอุบัติการณ์ของความผิดปกติของโครโมโซม18,X และ Y ของ epididymal spermatozoa พบว่า epididymal spermatozoa จากผู้ป่วยที่ไม่พบอสุจิในน้ำอสุจิซึ่งเกิดจากการอุดตันของท่อนำอสุจิ พบอุบัติการณ์ของ total sex aneuploidy,disomy 18 และ diploidy สูงกว่าอสุจิของผู้ชายปกติอย่างมีนัยสำคัญทางสถิติ (1.44% vs 0.14%; 0.11% vs 0.02 และ 0.18 vs 0.02%ตามลำดับ, $p<0.005$) ไม่พบความแตกต่างกันอย่างมีนัยสำคัญทางสถิติของ total sperm aneuploidy และ diploidyในผู้ป่วยซึ่งทำหมันชายและไม่สำเร็จในการแก้หมัน (n=12) กับผู้ป่วยที่มีการอุดตันของท่อนำอสุจิจากสาเหตุอื่น (n=12) โดยสรุปผู้ป่วยที่มีการตรวจไม่พบอสุจิจากการอุดตันของท่อนำอสุจิพบมี aneuploidy และ diploidy rateใน epididymal spermatozoa สูงกว่าผู้ชายปกติ ผลการศึกษานี้มีประโยชน์ในการนำมาให้คำปรึกษาผู้ป่วยที่มีการตรวจไม่พบอสุจิจากการอุดตันของท่อนำอสุจิก่อนการรักษาด้วยการฉีดอสุจิเข้าในไข่

หลักสูตร วิทยาศาสตร์การแพทย์	ลายมือชื่อนิสิต....................................
สาขา ภาควิชาวิทยาศาสตร์การแพทย์	ลายมือชื่ออาจารย์ที่ปรึกษา.....................
ปีการศึกษา 2545	

437 52102 30: MAJOR MEDICAL SCIENCE

KEY WORDS : SPERMATOZOA / FISH / SEX CHROMOSOME/ANEUPLOIDY/ AZOOSPERMIA

JIRAPORN NGEAMVIJAWAT : DETECTION OF SEX CHROMOSOME ANEUPLOIDY OF EPIDIDYMAL SPERMATOZOA FROM OBSTRUCTIVE AZOOSPERMIC MEN. THESIS ADVISOR : PROF. NARES SUKCHAROEN, M.D. 67 pp. ISBN 974-17-1161-1

Intracytoplasmic sperm injection (ICSI) is in efficient treatment for obstructive azoospermia using epididymal spermatozoa. However, serious concern about the risk of chromosomal aneuploidies from paternal origin in the children still exist. The data on the frequencies of chromosomal abnormalities in epididymal spermatozoa from obstructive azoospermic men are very few, therefore the frequencies of sperm aneuploidy and diploidy of epididymal spermatozoa from obstructive azoospermic men were evaluated. Epididymal spermatozoa retrieved from 24 obstructive azoospermic men by percutaneous epididymal sperm aspiration (PESA) and ejaculated spermatozoa from 24 healthy fertile donors were analyzed using three colour fluorescence in-situ hybridization (FISH) techniques to evaluate the rates of diploidy and aneuplodiy for chromosome 18,X and Y. Epididymal spermatozoa form obstructive azoospermic men had total sex aneuploidy,disomy 18 and diploidy rates significantly higher than ejaculated spermatozoa from normozoospermic fertile controls (1.44 % vs 0.14 %, 0.11 % vs 0.02 % and 0.18 %, respectively; $p<0.005$). The total sperm aneuploidy and diploidy rates were not statistically different between the vasectomized patients with failed vasectomy reversal (n=12) and the patients with other causes of obstructive azoospermia (n=12). In conclusion, obstructive azoospermic patients had an elevated epididymal sperm aneuploidy and diploidy rate. These results may be useful in counseling the obstructive azoospermic patients before ICSI treatment.

Department - Student's signature……………………………

Field of study Medical Science Advisor's signature……………………………

Academic year 2002

Acknowledgement

This thesis was carried out successful by though many valuable advises,helpful guidances, suggestions and intensive review from my advisor, Professor Dr. Nares Sukcharoen, Department of Obstetrics and Gynecology, whom I would like to express my deep gratitude.

I would like to express my sincere thank to the Andrology Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University for supplying the donor semen samples and the FISH laboratory.I also express my thank to IVF laboratory, Department of Obstetrics and Gynecology and Navabutra Center for Assisted Reproduction and Embryology for supplying the patient semen samples.

This study was supported by the Thai National Budget for Research and Development

Table of Contents

Page

Abstract (Thai)……………………………………………………………………………iv

Abstract (English)…………………………………………………………………………v

Acknowledgement…………………………………………………………………………vi

Table of Contents…………………………………………………………………………vii

List of Tables………………………………………………………………………………ix

List of Figures………………………………………………………………………………x

List of Abbreviations………………………………………………………………………xi

Chapter

1. Introduction………………………………………………………………………1

2. Theory and Literature review…………………………………………………9

3. Material and Methods……………………………………………………………28

    Specimens………………………………………………………………………28

    Materials…………………………………………………………………………28

    Equipment………………………………………………………………………29

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Reagents……………………………………………………………………..30

Method……………………………………………………………………..32

4. Results……………………………………………………………………………43

5. Discussion and Conclusion……………………………………………………49

References…………………………………………………………………………………54

Appendix……………………………………………………………………………………65

Biography…………………………………………………………………………………..67

List of Table

Table Page

1. Incidence of chromosomal abnormalities of ICSI-derived births …………..2
2. Common methods of sperm retrieval …………………………………………13
3. The frequencies of X and Y-bearing sperm, sex disomy, disomy 18 and diplody of ejaculated spermatozoa from the 24 healthy fertile controls ……………………………46
4. The frequencies of X and Y-bearing sperm, sex disomy, disomy 18 and diplody of epididymal spermatozoa from the 24 obstructive azoospermic men …………………..47
5. Results of fluorescence in situ hybridization analysis in 24 obstructive azoospermic patients compared with health fertile controls …………………………………………48
6. Comparison of chromosomal abnormalities between vasectomized men and the remaining obstructive azoospermic patients……………………………………………..48

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

List of Figures

Figure Page

1. Intracytoplasmic sperm injection (ICSI) procedures……………………………………2
2. Schematic diagram of the normal physiology of male fertility…………………………9
3. Percutaneous Epididymal Sperm Aspiration (PESA)…………………………………..13
4. Microsurgical Epididymal Sperm Aspiration (MESA)…………………………………14
5. Testicular Sperm Extraction (TESE)……………………………………………………..15
6. Diagrammatic representation of meiotic non-disjunction……………………………..18
7. The principle of fluorescence *in situ* hybrydization (FISH) ………………………… 20
8. Untreated sperm (a) and decondensed sperm head after treatment (b)…………..23
9. Result of locus specificity with mitotic chromosome spreads were used as positive controls in each FISH procedure ………………………………………………………44
10. Three-probe FISH applied to the epididymal sperm from obstructive azoospermic men …………………………………………………………………………..45

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

List of abbreviation

FISH	Fluorescence *in situ* hybridization
DNA	Deoxyribonucleic acid
rpm	Revolution per minute
g	Gram
ml	Millilitre
M	Molar
pH	The negative logarithm of the concentration of hydrogen ions
µl	Microlitre
DAPI	4,6-diamidino-2-phenylindole
FITC	Fluorescein isothiocyanate-conjugated
TRICT	Tetramethylrhodamine isothiocyanate isomer R-conjugated
DABCO	1,4-diazo bicyclo-(2,2,2) octane

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

1. Background and Rationale

In the first 15 years of clinical application of in vitro fertilization to create successful human pregnancies, it became clear that the IVF procedure of simply placing eggs and sperm in a culture dish had its limitations in helping couples with very severe male factor infertility to conceive.[1] If the male partner had a very low sperm concentration or motility, the sperm was unlikely to bind and penetrate the egg. In 1992,the first successful pregnancy by intracytoplasmic sperm injection (ICSI) was announced in Brussels, Belgium.[2] This technique, was so successful in achieving normal fertilization (about 60-70% of all injected eggs fertilized normally) that it rapidly spread to IVF laboratories world wide.[2,3] At the same time, the researchers were careful to study the results of their work. They performed genetic testing on a large number of babies born after ICSI-assisted fertilization. Today, these studies have demonstrated that a small percentage of children conceived with ICSI may have a slightly higher risk of a specific type of chromosome abnormality known as sex chromosome aneuploidy. [4-6]

ICSI is a high efficiency method for the treatment of severe male factor infertility.[7] ICSI is one of a group of assisted reproductive laboratory procedures refer to as micromanipulation which shown in Figure 1. The procedures are performed on sperm and eggs under very controlled environmental conditions using specialized equipment under high power magnification. The equipment is specially designed, one glass tool (holding pipet) is used to stabilize the egg while a second microscopic glass tube containing sperm (injection pipet) is use to penetrate the zona pellucida and egg membrane. Thus, a single sperm is deposited into the egg cytoplasm. [3] It allows the use of spermatozoa from men with severely compromised semen parameters and currently is even in some case of azoospermia where can be retrieved from the epididymis.[8,9] testis [10,11] or spermatid. [12] With ICSI ,resulting in a high fertilization rateswere obtained, even in males with severe sperm parameters. [13,14]

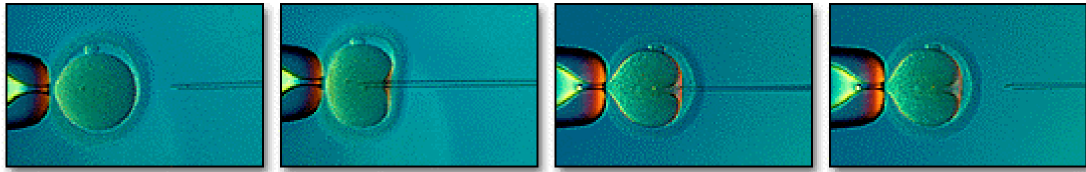


Figure 1: Intracytoplasmic sperm injection (ICSI) procedures [3]

Although ICSI will become a successful technique for the treatment of male infertility but serious concerns have been raised about passing genetic abnormalities to the offsprings of these men. There is a report of the men with very severe sperm defects with a higher frequency of chromosomal aneuploidies, especially conceptions from ICSI procedures which natural sperm selections do not occur .[15] This concern has been confirmed by several reports of a higher incidence of sex chromosomal aneuploidies of paternal origin in children conceived after ICSI, compared to the general population. [16-18] Each reports shown differences in the incidence of chromosomal abnormalities (2.6%-33%)(Table 1)[16,18-21]

Table 1: Incidence of chromosomal abnormalities of ICSI-derived births

References	No. of ICSI-dirived births	Chromosomal abnormalities
In't Veld et al.,1995 [16]	15	33.3
Van Opstal et al., 1997 [18]	71	12.8
Loft et al., 1999 [19]	206	3.4
Bonduelle et al., 1999 [20]	1,082	2.6
Aboulghar et al., 2001 [21]	430	3.5

However, only few studies of chromosomal abnormalities have been reported if compared with the children conceived after ICSI.

Since then, many studies have reported the chromosomal aneuploidies in men with abnormal sperm parameters, and especially those with oligoasthenoterato-zoospermia (OAT), those results shown a significantly higher rates of sex chromosomal aneuploidy than autosomal aneuploidy.[21,22] In addition, molecular cytogenetic analysis de-novo chromosome abnormalities in virtually all sperm cell with normal haploid cell (< 2%) and the presence of diploid (40%) and triploid (24%) sperm cell and hyperhaploid spermatozoa aneuploidy for the sex chromosome (22%). [23] However, the incidence of diploidy or disomy was differences in such studies, some studies found an increased incidence of chromosomal anomalies [24] while others found no difference in disomy or diploidy compared to control group. [25] Today, ICSI has been successfully used to treat men with fresh sperm collected directly from the testis and epididymis. With this technique, it was possible to achieve a pregnancy in infertile male with sperm retrieval. Although, babies from ICSI are increasingly, but very few sex chromosomal abnormalities were studies in epididymal and testicular spermatozoa.

In a study of sex chromosomal aneuploidy of spermatozoa obtained from epididymis found an incidence of 2.89% and 0.44% with diploidy, however, this report analyzed only six patient.[26] Another study of the chromosomal aneuploidy for sex chromosome were 6.27% and 1.9% were chromosome 18 from epididymal sperms of ten patients. [27] However, most studies were carried out on small number of spermatozoa in each patient that led to an error in the incidence of chromosomal abnormalities.

The study of chromosomal abnormalities in testicular sperms of three patients have demonstrated the incidences with 32.4% for sex chromosome aneuploidy. However, in this group it was difficult to determine whether the cell analyzed were dysmorphic spermatozoa or spermatids. [23] Nevertheless, the controversy from one author reported no significant difference in testicular sperm aneuploidy compared to control group.[28]

According to these data, very few studies have reported the risk of sex chromosomal abnormalities in epididymal spermatozoa from obstructive azoospermic men, and the incidence seemed to be higher when compared to normal controls. Therefore, we would like to

detect the sex chromosome aneuploidy of epididymal spermatozoa from obstructive azoospermic men.

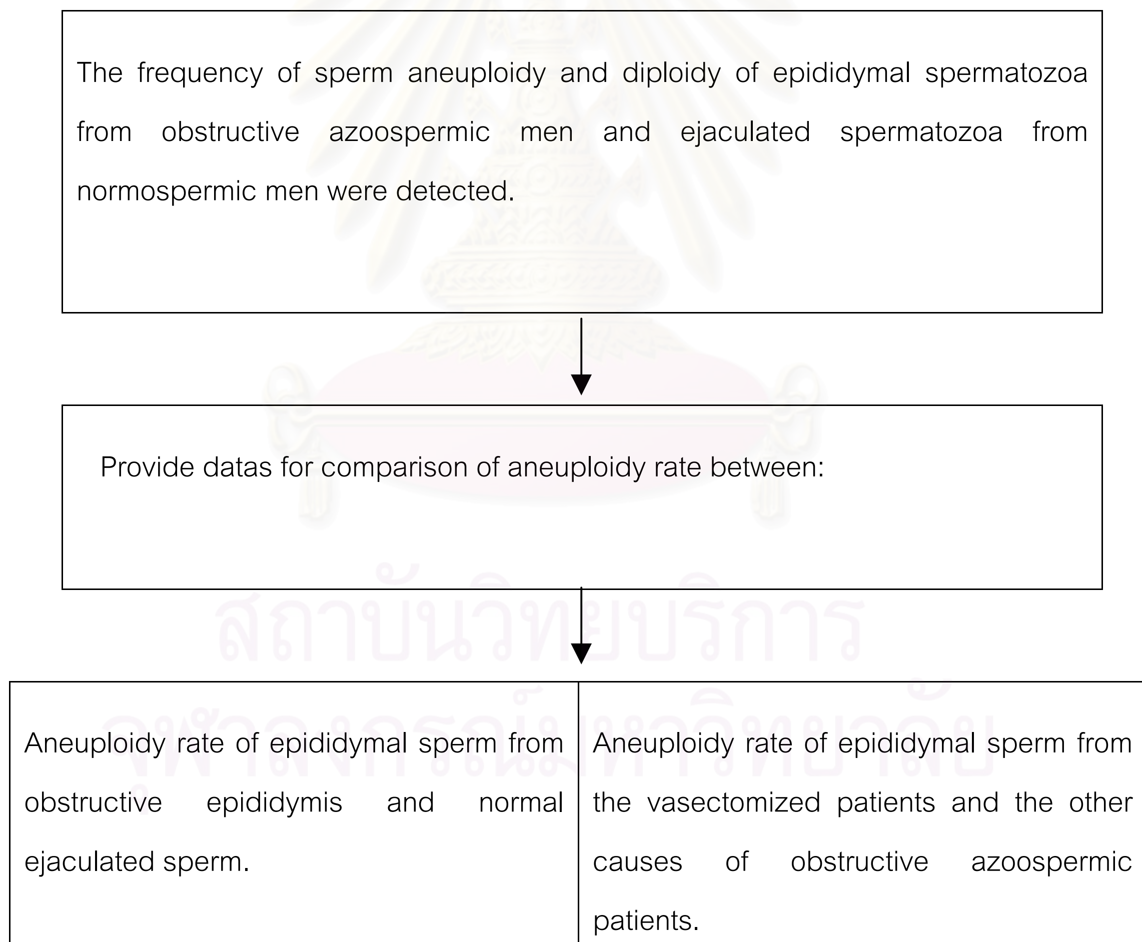
สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

2.Objectives

1.1 To compare the incidence of sex chromosomal aneuploidy of epididymal spermatozoa from obstructive azoospermic men with ejaculated spermatozoa from normozoospermic men.

1.2 To compare the incidence of sex chromosomal aneuploidy of the vasectomized patients with failed vasectomy reversal with the patients with other causes of obstructive azoospermia.

3 .Conceptual Framework



4. **Key Words**

Epididymal Spermatozoa

FISH

Sex Chromosomal Aneuploidy

Azoospermia

5. **Operational Definition**

$$\text{Hybridization efficiency} = \frac{\text{The number of hybridized spermatozoa} \times 100}{\text{Total number of spermatozoa}}$$

Sex chromosomal aneuploidy rate =

$$\frac{\text{The number of sex chromosomal aneuploidy spermatozoa} \times \text{Hybridization efficiency}}{\text{The number of hybridized spermatozoa}}$$

6. **Expected Benefit & Application**

1. The data can be provided for counseling the patients about the potential paternal contribution to the risk of fetal chromosomal anomalies after intracytoplasmic sperm injection (ICSI) in obstructive azoospermic men.

2. Overall findings suggest the need for future researches to study whether long duration of vasectomy may increase the frequency of sperm aneuploidies.

7. **Research methodology**

The study of chromosomal abnormalities in testicular sperms of three patients have demonstrated the incidences with 32.4% for sex chromosome aneuploidy. However, in this group it was difficult to determine whether the cell analyzed were dysmorphic spermatozoa or

spermatids.[28] Nevertheless, the controversy from one author reported no significant difference in testicular sperm aneuploidy compared to control group.[31,32]

- Sample collection

Epididymal sperm (study group)

The epididymal sperm samples from azoospermic men were retrieved by percutaneous epididymal sperm aspiration (PESA).

Ejaculated sperm (control group)

The semen samples from normal healthy men were produced by masturbation

- Process of study

Step 1. Sperm preparation

Study group cryopreservation - The remained epididymal spermatozoa after treatment by ICSI and were dropped onto slides. The slides were then stored at – 20 °C.

Control group - To analyze the liquefy semen samples using standard procedures and computer assisted semen analyzer (CASA).

- The sperm samples were washed three times in phosphate-buffered saline (PBS) and the sediment was then dropped onto pre-clean slides.

Both of study and control group slides were stored at – 20 °C until evaluation.

Step 2. Sperm decondensation

Slides were incubated for 5 minutes in 1 M dithiothreitol (DTT) in 0.1 M Tris, pH 8.0, and then for 10 minutes in 0.1 M DTT and 0.01 M lithium 3,5- diiodosalicylic acid (LIS) in 0.1 M Tris, pH 8.0. After pretreatment, slides were rinsed in 2 X SSC, pH 7.0 and air dried.

Step 3. Fluorescence *in situ* hybridization

- All slides were treated with 100 µg / ml of RNase A for 60 min at 37 $^{\circ}$C, rinsed three times in Milli Q H_2O, dehydrate through an ethanol series (80 %, 95% and 100 %ethanol) and air dried.

- A mixture of the X,Y and 18 probes was prepared in Vysis hybridization buffer then applied 10 µl of probes mixture to target site on each slide and cover slip was sealed with rubber cement.

- Simultaneously denatured slides and probes by heating at 75 $^{\circ}$C for 5 minutes then hybridization processed at 37 $^{\circ}$C for 4 –18 hours.

- The slides were washed three times in 50 % formamide/ 2X SSC at 45 $^{\circ}$C and air dried. They were mounted with DAPI II.

Step 4. Fluorescence microscopy and scoring criteria

FISH signals were analyzed with epifluorescence microscope equipped with an appropriate filters. This enabled simultaneous observation of the orange (X), green (Y) and aqua (18) hybridization signals. Sperm nuclei scoring was done according to the strict criteria.

Step 5. Evaluation and comparison of sex chromosome aneuploidy rate.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

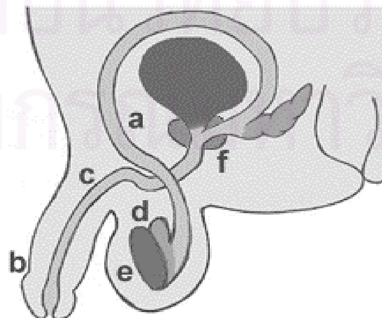
THEORY AND LITERATURE REVIEW

Approximately 15% of all married couples present with infertility. [1]Of these, up to 50% were due male factors. [29]

In men, fertility requires normal function and coordination of complex physiological process resulting in production transport, and ejaculation of adequate numbers of normally functional spermatozoa and deposition of sperm into the female genital tract at the appropriate time in relationship to ovulation. [30] Thus the processes required for the normal male fertility (Figure 2) are (1) the production of adequate numbers of spermatozoa by the testes (spermatogenesis), (2) the transport and maturation of spermatozoa produced in the testis through the male genital tract, (3)adequate penile erection on complete intercourse and ejaculation, (4) forward ejaculation of adequate numbers of spermatozoa, and (5) appropriate frequency and timing of intercourse and delivery of adequate numbers of normally functional motile sperm into the female genital tract to permit fertilization of an ovum. Each of these processes is under separate physiologic regulation, and disorders in any these functions may result in male infertility. [30]

Figure 2. Schematic diagram of the normal physiology of male fertility. [30]

A. Vas deferences, B. Penis, C. Urethra, D. Epididymis, E. Testis, F. Prostate, G. Sperm



สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Azoospermia

The most severe expression of male factor infertility is azoospermia, where no sperm are present in the ejaculate. Azoospermia found in 15-20 % of men evaluated for infertility.[31]

There are two main reasons why sperm are absent from the semen:[30-34]

1. Obstructive azoospermia (Blockage of sperm transport from the testis)
2. Non-obstructive azoospermia (Abnormal sperm production)

Obstructive azoospermia

Sperm from the testis must pass through the epididymis and vas deferens on their journey to the ejaculate. Blockage, or obstruction, of sperm transport from the testis can cause a zero sperm count, even though the testis continues to produce sperm. Obstruction in one (unilateral) or both (bilateral) sides may be due to either missing or blocked tubes (ducts).[31] Obstruction may occur at any point from the epididymis up to the ejaculatory ducts in the prostatic urethra.[31,32]

Obstructions can result from a variety of causes and include:

* Congenital absence of vas deferens
* Infection
* Vasectomy
* Obstructions in region of prostate

Congenital absence of the vas deferens

Absence of the vas deferens prevents sperm from passing the testis into the ejaculate. When men are born with this condition it is known as congenital absence of vas deferens and these men are usually missing both their vas deferens and two third of their epididymis. In most cases, congenital absence of the vas deferens results from a

genetic change or mutation to a gene called the " cystic fibrosis transmembrane regulator" (CFTR).[31,32] Men with cystic fibrosis also have congenital absence of the vas deferens. Men with congenital absence of the vas deferens are now regarded as having an extremely mild form of the cystic fibrosis syndrome.[31]

In men with congenital absence of the vas deferens, it is possible to collect sperm directly from the testis or from the small remaining part of the epididymis.[31-35]

Infection

Infections of the reproductive tract may lead to blockage of sperm transport and preventing sperm from being ejaculated.

Sexually transmitted diseases such as gonorrhoea and chlamydia may result in damage and blockage of the epididymis, preventing sperm passing from the testis to be ejaculated. Because the testis only contributes a small part of the quantity of the normal ejaculate, blockage at this level does not change the ejaculate volume.[31-33]

Vasectomy

Vasectomy involves the cutting and removal of a section of the vas deferens, the tube which carries sperm from the testis to the urethra at the bladder. Vasectomy is safe and effective long-term contraceptive option.

Evidence does not support long term changes in testosterone levels following vasectomy. However, with the passage of time, damage to the sperm producing tubes of testis may occur. In addition, scarring and blockages in the epididymal tubes can also occur. Sperm antibodies develop in 80 % of men who have had a vasectomy. [34]For these reasons, vasectomy reversal surgery is less successful if 10 years have elapsed since the original vasectomy.[31,33,34]

Reversal of vasectomy

The most common form of obstructive problem results from a previous vasectomy. Associated problems of epididymal blow-out and/or sperm antibodies may also require consideration.

A reversal of vasectomy operation is available to men who may wish to become fertile again. Microsurgery is used to region the cut ends of the vas deferens. Sperms are seen again in the ejaculate after this operation. However, The true success of vasectomy reversal surgery is pregnancy and not all men with sperm in the ejaculate succeed in this regard.[31-34]

Epididymal blow-out. In some cases where the vasectomy reversal is unsuccessful, a further blockage of the epididymis may have occurred. This secondary blockage is referred to as an “epididymis blow-out”. Following a vasectomy, the testis continues to produce sperm which

travel down the epididymis and vas deferens and pool where the vas deferens has been cut. The vas deferens is a thick wall of muscular tube that cannot expand, causing back pressure which expands the epididymis. In some men, this expansion may lead to a bursting of the epididymis that can cause a blockage when it heals.[31]

Obstructions in region of prostate

Infections of prostate can cause swelling and blockage of the ejaculatory duct as this duct passes through the prostate gland. Blockage in this region will prevent sperm from being ejaculated. Occasionally, a man have a congenital abnormality in which or "cysts" in the prostate gland have formed. By pressing on the ejaculatory ducts, these cysts can also cause blockage of sperm at this level.[31,33]

Non-obstructive azoospermia

Non-obstructive azoospermia is a condition in which no sperm are present in the ejaculate because of severely abnormal sperm production.

Production problems: The three major causes for lack of sperm production are hormonal problems, testicular failure and varicocele.

Hormonal problems: The testicular need pituitary hormones to be stimulated to make sperm. If these are absent or severely decreased, the testis will not maximally produce sperm.[33,34]

Testicular failure: This generally to the inability of the sperm producing part of the testicle (the seminiferous epithelium) to make adequate numbers of mature sperm.[33,34] This failure may occur at any stage in sperm production for a number of reasons. Either the testicle may completely lack the cells that divide to become sperm (this called "Sertoli cell-only syndrome.") or there may be an inability of the sperm to complete their development (this is termed a "maturation arrest.") This situation may be caused by genetic abnormalities.[31,33,34]

Varicocele: A varicocele is dilated veins in the scrotum, (just as an individual may have varicose veins in their legs.)These veins are dilated because the blood does not drain properly from them. These dilated veins allow extra blood to pool in the scrotum, which has a negative effect on sperm production.[31,33,34,36]

Sperm retrieval techniques

Men with obstructive azoospermia include with congenital bilateral absence of the vasa deferentia (CBAVD), failed vasectomy reversals and other causes of obstructive azoospermia. In these situation, sperm can retrieved by the techniques following

Table 2.[35]

Table 2: Common methods of sperm retrieval [35]

Acronym	Method
PESA	Percutaneous epididymal sperm aspiration
MESA	Microsurgical epididymal sperm aspiration
TESE	Testicular sperm extraction

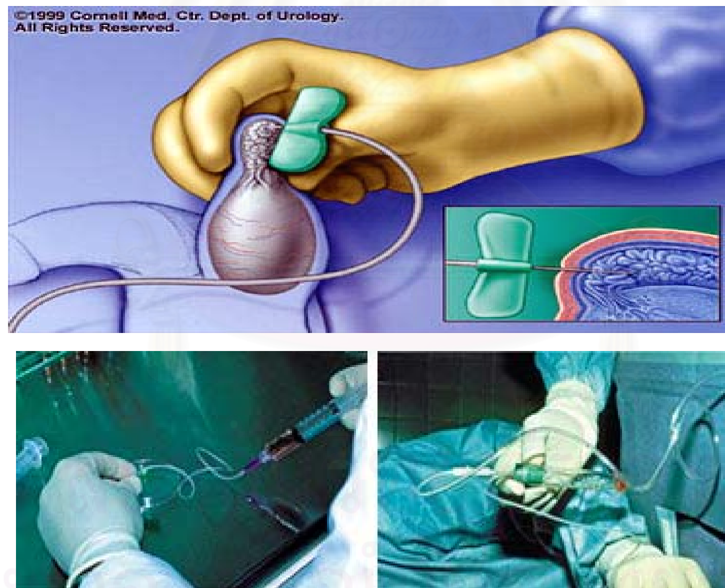


Figure 3: Percutaneous Epididymal Sperm Aspiration (PESA)[35]

PESA has been advocated because it can be performed without surgical scrotal exploration, it is repeated easily at low cost, and it does not require an operating microscope or expertise in microsurgery.[35-36]

The procedure can be performed under local or general anesthesia. The testis is stabilized and the epididymis is held between the surgeon' s thumb forefinger. A 26-gauge was connected to a 1 ml disposable syringe is inserted into the caput eppididymis and withdrawn gently until fluid can be seen entiring the syringe. The procedure is repeated until adequate numbers of sperm retrieved. PESA yields very small amount of epididymal fluid and contamination with blood cells is frequent.[35-37]

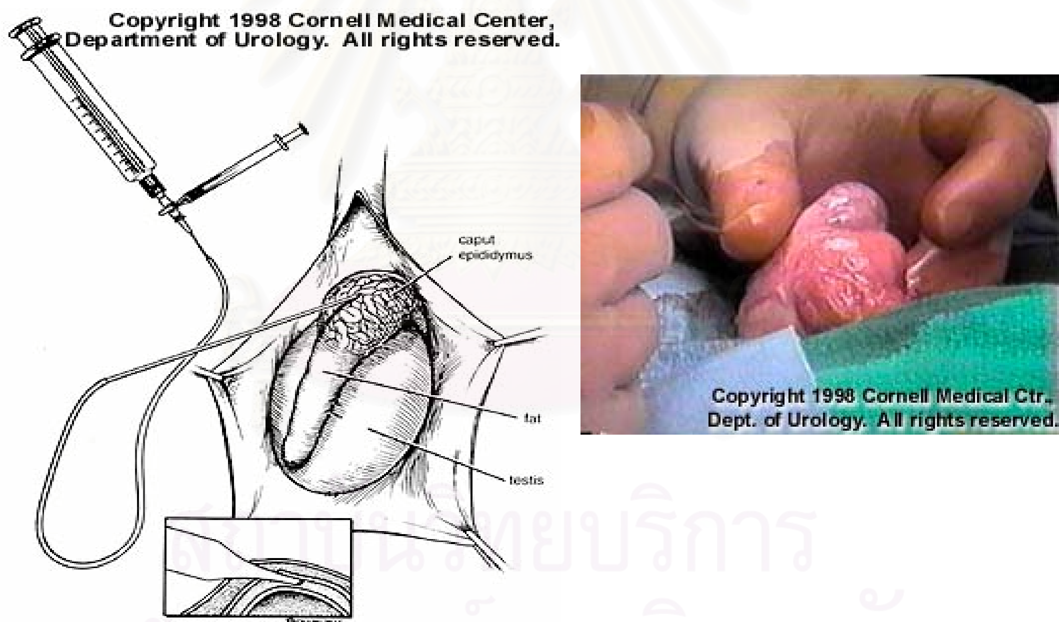


Figure 4: Microsurgical Epididymal Sperm Aspiration (MESA)[35]

MESA is performed as open operation under the operating microscope.[37-39] Individual tubules of the epididymis are isolated and micropuncture aspiration is taken. This approach has the advantages of reliable retrieval of large numbers of epididymal spermatozoa that can be readily frozen and for subsequent attempts at fertility. Since MESA involves direct retrieval of

spermatozoa from epididymal tubule, it minimizes contamination of the epididymal fluid by blood cells, which may affect spermatozoa fertilizing capacity during the IVF. [38]

MESA is performed with a micropuncture siliconized glass pipette connected to sterile medical grade silicone tubing that is attached to an aspiration device consisting of a 1-ml tuberculin syringe and a 10-ml syringe. [36] Epididymal sperm can also be retrieved by an opening individual epididymal tubule with a micro-knife and collecting the sperm through a simple micropipette / capillary action technique during some epididymal reconstructive microsurgeries. [36-39]

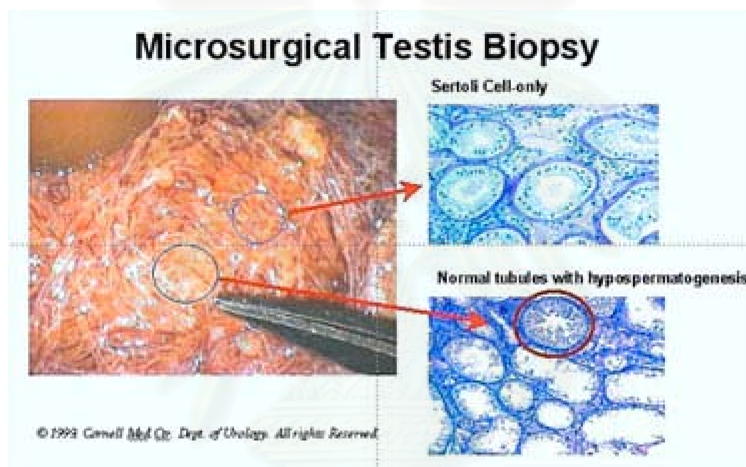


Figure 5: Testicular Sperm Extraction (TESE) [35]

TESE is an effective method for sperm retrieval from men with non-obstructive azoospermia for ICSI. [37] However, the conventional TESE technique requires multiple blind testis biopsies, with excision of large volumes of testicular tissue and risks permanent to damage. Using optical magnification, a relatively avascular region of the testis is located for an incision. After the testicular capsule has been opened, individual tubules are examined. [37-39]

Chromosomal abnormalities in sperm retrieval

For a long time, it has been recognized that constitutional chromosomal abnormalities are much more frequent in infertile men in the general male population. Furthermore, men with a normal peripheral karyotype may have chromosomal abnormalities limited exclusively to the germ cell probably arising from non-disjunction during spermatogenesis. Most numerical chromosome

anomalies originate at meiosis; [40,41] de-novo structural rearangments are also of meiotic origin, [38] while inherited duplications and/or deficiencies (also know as partial trisomies and monosomies) result from the abnormal segregation of structural rearrangements at meiosis. [42] Meiotic pairing disorders result from mutations affecting the meiotic process [43-45] or from the influence of an abnormal testicular environment.[46]

Meiosis is a complex process that includes two successive cell divisions, without DNA replication between them. In the first meiotic division, which takes place in the diploid (2n = 46) primary spermatocytes, homologous chromosomes pair, and an exchange of genetic information may take place (recombination) to produce new genetic combinations in the offspring; at anaphase, whole chromosomes (with two chromatids each) migrate to the cell poles to produce haploid (n = 23) secondary spermatocytes, in which the each chromosome still has two chromatids.

During the second meiotic division, the chromatids of each chromosome migrate to the cell poles, to produce haploid (n = 23) spermatid, which each chromosome has only one chromatid. The spermatids will change their shape and reorganize the location of their organelles to produce spermatozoa, but replication of the genetic material will only take place after fertilization.

Any of these steps may go wrong, giving rise to different types of chromosome abnormalities such as aneuploidy (Figure 6A, 6B) [47] or diploidy. If the non-disjunction occurs during the first meiotic division (meiosis I), all the gametes derived will be abnormal. Half of them will contain neither members of the chromosome pair, while the other half will contain both homologous chromosomes (Figure 6A).If non-disjunction occurs in meiosis II, only two of the four gametes will be abnormal. Two gametes will have an extra chromosome the other, two will have an absence of the chromosome (Figure 6B).

Sex chromosome aneuploidies

The most common numerical chromosome anomaly in infertile men is 47, XXY; 90% of affected males display the condition of 47,XXY, and mosaic forms 46, XY, 47, XXY also exist. [46] The classic Klinefelter syndrome are of paternal and maternal origin in similar proportion, and in most cases result from non-disjunction in meiosis I of the X chromosome. Mosaic forms are caused by non-disjunction meiosis of X chromosome after fertilization. [46] While XYY males are obviously of paternal origin, and the error is produced at meiosis II or in the post –zygotic stage. [46]

The result of human chromosomal abnormalities whose trisomic conditions are tolerated involving the sex chromosomes. X chromosome inactivation means that only one X chromosome is active in any cell, no matter how many X chromosomes exist. The Y chromosome appears to be inactive in most all cells except for those involving the formation of the male gonads and gametes. Therefore, sex chromosome monosomies and trisomies can produce infertility, but they are not lethal. XXY individuals have the Klinefelter Syndrome. They are males, but the presence of the extra X appears to interfere with gametogenesis. The phenotype varies, but many XXY individuals are unaware of having anything wrong with them except that they are sterile and have larger than average breasts for men. Others have a mild mental retardation or learning disability. XO individuals (monosomy X) are said to have Turner' s Syndrome. They are females who are usually short, broad-necked, and sterile. Most other functions, including mental abilities, are normal. XYY individuals (due to a nondisjunction of the Y chromosome) are often taller than their counterparts, and they may have learning disabilities as well. Although there once had been thought to be an association of these XYY individuals with aggressive behavior, these associations have not held up. Most XYY males probably neither large nor aggressive, and those imprisoned XYY persons are usually not there for violent crimes. [6,46,47]

(Figure 6A) (Figure 6B)

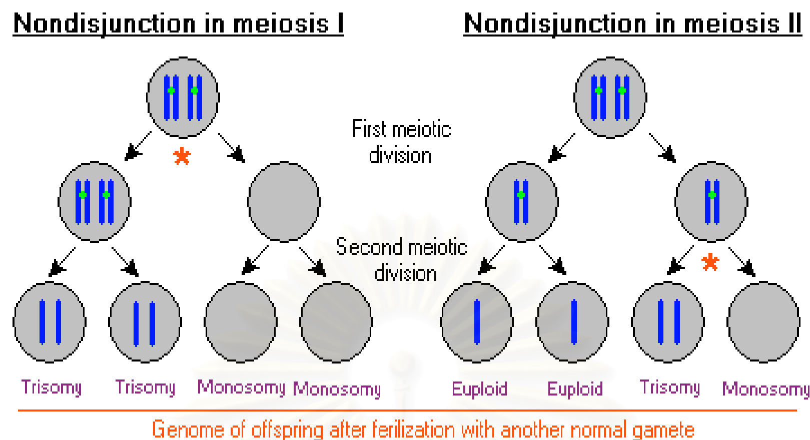


Figure 6 : Diagrammatic representation of meiotic nondisjunction [47]

Another serious concerns, including the risk of transmission of chromosomal or genetic diseases. It is established that some cases of male infertility, particularly those with severe oligozoospermia or azoospermia of testicular origin, are associated with an increased incidence of peripheral karyotype abnormalities, microdeletions of the Y chromosome, and sperm aneuploidy.[41-44]

There is evidence of possible " functionally immature " or " structurally abnormal" spermatozoa for ICSI that could theoretically be associated with gamete genomic imprinting anomalies and even transmission of damaged DNA.[48]

Analysis of spermatozoal chromosomes

In the initial study of chromosomal constitution of human spermatozoa was quinacrine mustard staining of the long arm of the chromosome.[48] The analysis of fluorescent (F)-body distribution in spermatozoa is based on the principle of differential staining of metaphase chromosomes with quinacrine dihydrochloride [49] and its high affinity for the human Y chromosome.[50] The distal portion of the long arms of the Y chromosome is rich in adenine and thymine, thereby enhancing quinacrine fluorescence. The distal portion of a bright fluorescing

spot in interphase nuclei.[51] However, the use of this staining method has been criticized because of lack of specificity.

Since then, the zona-free hamster oocyte-sperm fusion technique was introduced. [52] Because after meiotic metaphase II sperm chromosomes do not reappear until the formation of male and female pronuclei of the fertilized egg prepared for the first cleavage division. The use of hamster eggs to activate human sperm to the point where their chromosomes can be studies directly. This technique has been developed for the chromosomal component of spermatozoa.[53] Despite this technique provides valuable information of the sperm karyotypes, there were significant disadvantages : it was extremely time consuming, provided low yields of karyotypes and was only successfully applied by few laboratory. Thus, in-situ hybridization with radiolabeled probes has been applied.[54]

However, the results obtained with radiolabeled probes were also limited importantly involved the handling of hazardous material. Therefore, this technique was improved with the use of fluorescent dyes known as fluorescence in-situ hybridization (FISH).[55-56] FISH is the detection of highly specific DNA probes which have been hybridized to either interphase or metaphase chromosome using fluorescence microscopy. The technique, uses two starting DNA materials (Figure 7): [57] target spermatozoal chromosomes and probe DNA. Sperm carrying the target chromosome; are placed on glass slides. The sperm chromatin is chemically treated, or decondensed ,so that DNA probe can penetrate the chromatin to reach the target chromosomes. The probe consists of DNA fragments prepared by attaching a fluorescent dye, and denatured to yield single-strand DNA (Figure7). When the labeled probes hybridize with the complementary single strand of target sperm chromosomal DNA (Figure7), and the cells are reviewed using a fluorescence microscopy. The advantages of fluorescent dyes has made it possible to visualize multiple probes at the same time. This has opened up how to detect many chromosomes of human spermatozoa.[58] In addition, FISH can be used to analyse the chromosome aberrations in the sperm-derived pronuclei [59] and to determine the sex selection protocols.[60]

However, studying the spermatozoal chromosome is more difficult than that of other cell types (lymphocytes, uncultured amniotic cells) as the chromatin packed tightly within the nucleus of spermatozoa.

The advantages of techniques for spermatozoal chromosome analysis

Analysis of sperm karyotypes using human sperm-hamster egg fusion system must be done several times to get sufficient number of spermatozoas (normally one hundred spermatozoas). This problem is managed easily using multi-color fluorescence in situ hybridization because the technique allows rapid detection analysis of thousands of sperms. The FISH technique has great advantages over sperm karyotyping because it is rapid and simple so that very large samples of spermatozoa can be tested. However, the limitation of FISH is that analysis of interphase chromosome cannot detect structural abnormalities.[61]

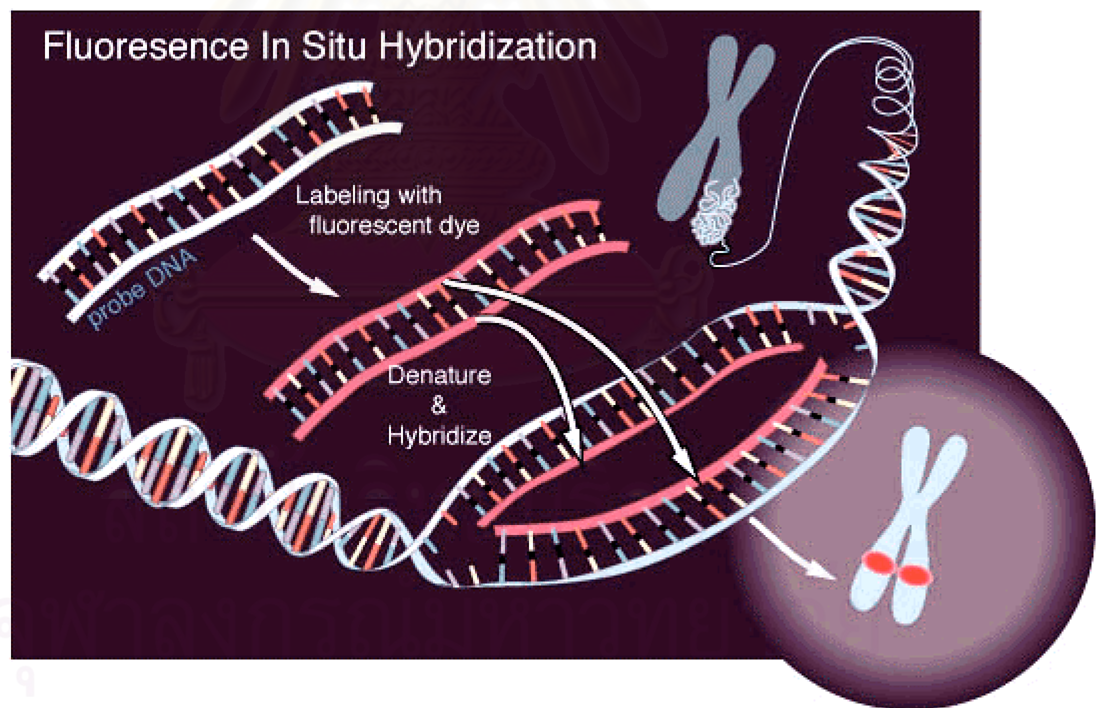


Figure 7: The principle of fluorescence in situ hybridization (FISH)[57]

The limitation of the techniques for spermatozoal chromosome analysis

Because of the tightly packed of sperm nucleus, decondensed sperm heads has to be carried out prior to hybridization in order for specific DNA probe to hybridize with DNA target.[62,63] The efficiency of decondensation of sperm head correlates directly to a sharper signal which provides a reliability of detection.

Following, the use of double- probe FISH protocols for spermatozoal chromosome detection, disabled us to distinguish between disomy and diploidy, and between nullisomy and hybridization failure.[64-66] Chromosomal abnormalities might be found during cell division, such as chromosome lag.[67,68]

According to the previous data, they have frequently found nullisomy from technical error,[69] and the findings related to effective type of probes such as locus-specific probe has higher nullisomy than centromic probe [70] as this probe may hybridize to specific chromosome only one position more difficult than a repetitive chromosome position.

The limitation of FISH technique is that it is possible to detect chromosomal structural abnormalities (accept for abnormal positions are complementary probe).[71] The hamster- egg method provides valuable information on the whole chromosomal complement of spermatozoa. Whereas the partial chromosome detected by FISH using DNA probe.

Until now, using the multi-color FISH for simultaneous detection of sperm carrying chromosomal abnormalities, but there are technical limitations such as the decondensed sperm head areas. This data suggest that the incidence of disomic detected in sperm are higher, compared with trisomic offspring. However, the trisomic abberations detected in sperm by the hamster-egg method, have similar incidence compared with FISH technique, and they are also higher than trisomic offspring.

Technical aspects

Some of the factors that may influence the final result analyze some conflicting situations that can arise from the improper use of the technique metaphases untreated, or

subject to the same decondensing treatment used to decondense sperm heads. It is indicated that the probes were chromosome specific after decondensation but the quality of the signals was affected, producing a dispersion of the fluorescent label that, in interphase nuclei, could result in a difficult estimation of the frequency of disomies.

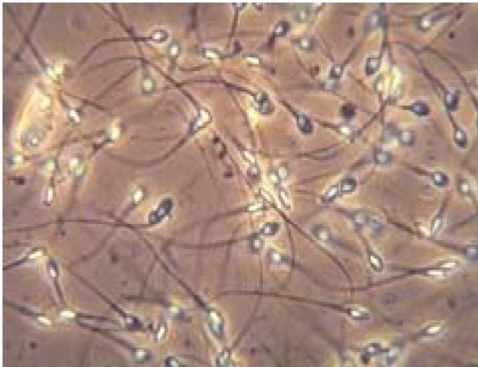
The analysis of spermatozoal chromosome using FISH technique

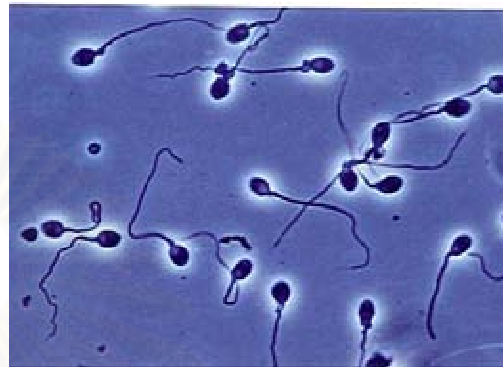
Sperm decondensation

The decondensation treatment is crucial to obtain a proper hybridization efficiency: this make it possible for the probes to access the extremely highly histamine-packed DNA of the spermatozoa which allowed the typical sperm morphology to be preserved as far as possible. The use of reducing agents such as dithiothreithol (DTT) to decondense sperm nuclei is one of several techniques, among several used for DNA decondensation, which allows the maintenance of the cell structure without disrupting the tail and with low cell swelling. DTT breaks the disulphide bonds of protamines and makes the DNA accessible for DNA probe.[62,63] Using 2 M NaOH, after hybridization the sperm morphology appeared a less increased in size compared with DTT.[72] The insufficient swelling of the sperm heads can result in overestimation of nullisomy[73] whereas excessive swelling results in an overestimation of disomy due to excessive swelling signal splitting. The FISH technique for studying human sperm aneuploidies should be included other autosomes in order to distinguish between disomic and diploid spermatozoa. An analysis using only sex chromosome (X and Y) can not distinguish disomic from diploid spermatozoa because both categories would display two signals.

Although some groups tried to distinguish diploid spermatozoa by there large,[55,74] with the differential chromatin package of each spermatozoa,[75] so the nucleus size after swelling head depend on an amount and package DNA.

Figure 8: (a) Untreated sperm (b) Decondensed sperm head after treatment





Scoring criteria

The sperm aneuploidy scoring depended on number and characteristic of the detectable fluorescent signals within sperm head. The different results are probably due to the different scoring criteria adopted in each laboratory. Therefore, scoring criteria were defined as the following:[76]

1. Disrupted or overlapping spermatozoa were excluded from analysis

2. Considered to be the cells with disomy or diploidy if two distinct signals for the same chromosome each equal in intensity and a distance of at least one diameter of the signal size within the cell.

3. Consider to be diploid if it exhibited two signals for each tested chromosome (two-color FISH) and if the tail was evident as well as the normal oval shape

4. The incidence of nullisomy for each chromosome should be estimated conservatively to be similar to the incidence of disomy for the same chromosome.

The specificity of probes

The other factors may effect the studies by FISH technique involved the type of probe used. Probes are complementary sequences of nucleotide bases to the specific RNA or DNA sequence of interested. These probes can be as small as 20-40 base pairs, up to a 1,000 bp. The strengh decreases in the order RNA~RNA,

DNA~ RNA, DNA~ DNA. The stability is in turn influenced by various hybridization conditions such as concentration of formamide, salt concentration, hybridization temperature and pH.[77]

The types of probes[77]

1. Locus specific probes

Locus specific probes hybridize to a particular region of chromosome. These probes are useful in microdeletion studies, where the presence or absence of a region of a chromosome can be diagnostic of certain syndromes or abnormalities.

2. Alpha-satellite probes (chromosome enumeration probes)

Alpha satellite DNA is composed of highly repetitive sequence element found near human chromosome centromeres, and most cases are chromosome-specific. FISH probes made of this type of DNA are mainly used to determine aneuploidy and to identify the origin of small " mark " chromosome.

3. Whole chromosome probes

Whole chromosome probes are actually collections of small probes, each of which hybridizes a different sequence along the length of the same chromosome. Using these libraries of probes, they are able to paint an entire chromosome and generate a spectral karyotype. These full color image of the chromosomes allows to distinguish between the chromosomes based on their colors, rather than based on their dark and light banding patterns, views in black and white

through traditional karyotyping. Whole chromosome probes are particularly useful for examining chromosomal abnormalities, when a piece of one chromosome attached to the end of another.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Literature review

Although ICSI provides a way of treating azoospermic men, concern has been raised about the potential risk for transmission of genetic abnormalities to the offspring.[16-18] So far, follow-up studies of children born after ICSI have not revealed more congenital malformations as compared with the general population, but there seems to be a slightly increased risk of transmission of chromosomal aberrations, mainly sex chromosomal abnormalities.[16,18-21]
Nowadays, the FISH method can be performed even when spermatogenesis is impaired and allows the analysis of several thousands of sperm per patient. The simultaneous hybridization of three chromosome specific DNA probes enables an accurate estimation of disomy and diploidy rates. Utilizing this method, several studies have demonstrated that men with suboptimal semen quality have a higher incidence of chromosomal abnormalities in their semen.[16-28] Nevertheless, very few studies have reported the incidence of sex chromosomal abnormalities in epididymal spermatozoa from obstructive azoospermic men and controversial.

In't Veld et al,[16] present a case history of oligoasthenoteratozoospermia (OAT) patient with multiple phenotypic abnormalities in sperm cells. They investigated the sperm aneuploidy for chromosome 18, X and Y. The result showed the virtual absence of normal haploid cells (<2 %) and the presence of diploid (40 %) and triploid (24 %) sperm cells and hyperhaploid spermatozoa aneuploid for the sex chromosomes (22 %).

Bernardini et al,[26] analyzed in term of sperm aneuploidy and diploidy in an additional series of infertile patients presenting unexplained infertility (n= 3), congenital absence of vas deferens (CAVD) (N= 6) and non-obstructive azoospermia (= 3) undergoing IVF, microsurgical epididymal sperm aspiration (MESA/ICSI) and testicular sperm extraction (TESE/ICSI) cycles respectively. FISH for chromosome 1,17,X and Y were performed on these samples.The results found an incidence of 2.89 % and o.44 % sex chromosomal eneuploidy and diploidy in epididymal sperm respectively.

Levron et al, [27] investigated the potential paternal contribution the risk of fetal chromosomal anomalies after ICSI. Testicular sperm were collected by testicular biopsies from non-obstructive azoospermic patients (n= 9), epididymal sperm were retrieved by percutaneous needle biopsy from obstructive azoospermic patients (congenital agenesis of the vas deferens, n= 6), ejaculated sperm from severe oligoasthenoteratospermia patients and normal fertile donors were analyzed for chromosomes X, Y and 18 by FISH. The results of sex chromosomal aneuploidy obtained from epididymis found an incidence of 6.27 % and 1.9 % were chromosome 18. The disomy rate in obstructive azoospermic patient was also significantly higher (P<. 001) than in the controls.

Recently, Palermo et.al, [78] quantified the incidence of chromosomal abnormalities in epididymal and testicular sperm retrieved from azoospermic patients undergoing ICSI.
Individual testicular sperm were collected from testicular biopsies with an ICSI pipette in five patients, and epididymal sperm were retrieved by microsurgical epididymal sperm aspiration (MESA) in eight patients. Samples were processed by FISH for chromosomes 18,21,X and Y. The results shown overall aneuploidy rate of 11.4 % in men with non-obstructive azoospermia was significantly higher (P= 0.0001) than the 1.8 % detected in epididymal sperm from men with obstructive azoospermia and also the 1.5 % found in ejaculated sperm. They reported that no significant difference was found between the epididymal and ejaculated samples. The obstructive azoospermic group displayed a modest increase of nullisomy, however significant (0.43 versus 0.27 % in the control group, P< 0.05), with the remaining sex chromosome disomy as the most common abnormality.

CHAPTER III

MATERIALS AND METHODS

1.Specimens

The epididymal spermatozoa retrieved by percutaneous epididymal sperm aspiration (PESA) from twenty-four obstructive azoospermic men. The remained epididymal spermatozoa after treatment by ICSI and cryopreservation were used. The ejaculated sperms of the controlled group were collected by masturbation after 2-5 days of abstinence.

2. Materials

2.1 Polystyrene conical centrifuge - tube: 15 ml, 50 ml (Falcon ®,USA)

2.2 Liquipette (Elkey, USA)

2.3 Cell culture flask : 50 ml, 250 ml (Falcon ®, USA)

2.4 Precleaned microscopic slides (SuperFrost color; MENZEL-GLASER®, Germanny)

2.5 Glass coverslips : 22X22 mm, 22X50 mm (MENZEL-GLASER ®, Germany)

2.6 Forceps

2.7 Graduated cylinder: 250, 500, 1000 ml

2.8 Diamond-tipped scribe

2.9 Coplin jar (Vertical staining jar; Wheaton Product No. 900620)

2.10 0.45, 0.22 μm pore filtration unit (Sartorious, Germany)

2.11 Reagent bottles : 250 ml, 500 ml (Duran ®, Germany)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

2.12 Test tube racks

2.13 Dispoable gloves

2.14 Timer

2.15 Laboratory sealing film (Whatman ®, USA)

2.16 Aluminum foil (Diamond ®, USA)

2.17 Microscope slide box

2.18 Humidified chamber

2.19 Slide film (Elitechrome 400, Kodak)

3. Equipments

3.1 Biohazard larminar flow (Labservice Ltd., Part, Thailand)

3.2 Low-speed centrifuge (Kubota 5400, Japan)

3.3 Refrigerator : 4 °C, -20 °C (Whirlpool)

3.4 CO_2 Cell culture incubator (Forma Scientific, USA)

3.5 Vortex mixer

3.6 pH meter (Orion, USA)

3.7 Air incubator : 37 °C (Fisher ®, England)

3.8 Microlitre pipette : 1-10 µl, 50-1000 µl and sterile tips (Eppendorf)

3.9 Computer assisted semen analysis (CASA IVOSE Version 10; Hamilton Thorne, USA)

3.10 Counting chamber (Makler ®, Sefi-Medical Instruments, Israel)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

3.11 Thermometer

3.12 Laboratory counter (Clay Adam ®, USA)

3.13 Fume hood (Labservice Ltd., Part, Thailand)

3.14 Water baths: 45 ± 1°C and 73 ± 1°C (Memmert, Germany)

3.15 Phase contrast light microscope (Olympus, BX50, Japan)

3.16 Fluorescence microscope equipped with an appropriate filter set and camera (Olympus, BX50, Japan)

3.17 Triple-bandpass (DAPI/FITC/Texas red) and single-bandpas filters (Vysis Inc., Framingham, MA, USA)

4. Reagents

4.1 Reagents for culturing white blood cells

1.1.1 Fetal calf serum (Gibco BRL, Germany)

1.1.2 Colcemid (Boehringer mannheim)

1.1.3 Phythemaglutinin, PHA-L (Seromed, M5030)

1.1.4 Penicillin /Streptomycin (Sigma, USA)

1.1.5 L-glutamine (Gibco BRL)

1.1.6 Heparin (LEO)

1.1.7 Absolute methanol (Merk, Germany)

1.1.8 Acetic acid (Merk, Germany)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

1.1.9 Potassium chloride (Merk, Germany)

1.1.10 RPMI 1640 medium (Gibco BRL)

4.2 Reagents for sperm preparation for FISH

4.2.1 Calcium chloride (Sigma, USA)

4.2.2 Magnesium chloride (Sigma, USA)

4.2.3 Potassium chloride (Sigma, USA)

4.2.4 Sodium chloride (Merk, Germany)

4.2.5 Potassium phosphate monobasic (anhydrous)-KH_2PO_4

4.2.6 Sodium phosphate dibasic (anhydrous)-Na_2HPO_4

4.2.7 Trisodium citrate (Merk, Germany)

4.2.8 Ethylenediaminetetra acetic acid (EDTA; Amresco ®, USA)

4.2.9 Dithiothreitol (DTT; Sigma, USA)

4.2.10 3,5-Diiodosalicylic acid (Lithium salt; Sigma, USA)

1.1.11 Absolute ethanol (Merk, Germany)

4.3 Reagents for hybridization

4.3.1 Formamide (Unilab, Australia)

4.3.2 20X SSC (Sodium chloride, trisodium citrate; Merk, Germany)

4.3.3 RNase, DNase free (Boehringer Mannheim)

4.3.4 1,4-diazobicyclo[2,2,2] octane (DABCO; Sigma, USA)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

4.3.5 4,6-diamidino-2-phenylindole (DAPI; Sigma, USA)

4.3.6 Glycerol (Mallinckrodt, USA)

4.3.7 CEP® X Spectrum Orange™ / Y Spectrum Green™ DNA Probe Kit (Vysis Inc., Framingham, MA, USA)

4.3.8 CEP ® 18 Alpha Spectrum Aqua™ DNA Probe Kit (Vysis Inc., Framingham, MA, USA)

5. Methods

● Subjects

Obstructive azoospermic patients

Twenty-four obstructive azoospermic men recruited in this study. All of the patients were treated by ICSI using the epididymal spermatozoa retrieved by PESA. The mean age of the azoospermic men was 43.2 ±8.7 years. All males had normal testicular volume and normal serum FSH concentration. Azoospermia was found on at least two occasions. The study was approved by the Ethics Committee of Medical Faculty and in formed consent was obtained all patients.

Normal control donors

Twenty-four normal healthy men with a history of having children were volunteers. All donors gave their informed consents prior to this study.

● Sperm collection and preparation

Percutaneous sperm aspiration (PESA)

PESA took place in the operating room using intravenous sedation anesthesia with Propofol and local anesthesia with Bupivacaine injected at the skin and underneath the epididymal caput. The epididymal caput was identified and held firmly between thumb and index finger. A small needle (26 gauge) was connected to a 1 ml disposable syringe. The proximal part of the

epididymal caput was punctured. Suction was applied to the syringe and the needle was withdrawn gradually to the point where segments of fluid from the epididymis were seen entering the syringe. The aspirated fluid was then transferred and flushed out of needle and syringe into a sterile petri dish using HEPES-buffered Ham's F10 supplemented with 10 percent patient 's serum. This procedure could be performed as many times as necessary until sufficient sperms were recovered and prepared for FISH.

Ejaculated sperm

Semen samples were produced by masturbation after 2-5 days of abstinence, allowed to liquefy at room temperature for 30 minutes and then analysed using WHO criteria, [79] and a CASA. The results (mean $\pm$SD) from the 24 samples were : volume 2.7$\pm$0.9 ml; sperm concentration 195.2$\pm$45 $\times 10^{6}$ ml; progressive motility 51.0 $\pm$ 7 %; normal sperm morphology 30.5 $\pm$ 9 % , which is in the normal ranged for WHO standard.

- Sperm preparation and fixative

The sperm samples were washed three times in PBS, centrifuge at 280 g for 10 minutes and the sediment was then dropped onto pre-cleaned glass slides and air dried. At least two slides were prepared for each subject. The slides were stored at –20 °C until FISH examination.

- Sperm decondensation

A modification of technical of Williams et al [80] was used.

1. Slides were incubated for 5 minutes in 1 M dithiotreitol (DTT) in 0.1 M Tris, pH 8.0.
2. Incubated for 10 minutes in 0.1 M DTT and 0.01M lithium 3,5-diiodosalicylic acid (LIS) in 0.1 M Tris, pH 8.0.
3. The slides were rinsed in 2$\times$SSC, pH 7.0 and allowed to air dry.

● Metaphase chromosome preparation

Metaphase chromosome spreads from human male lymphocytes were used as positive control for each hybridization procedure.

1. Collected 2-5 ml human blood in a heparinized vacutainer tube.
2. Added 5 ml of RPMI 1640 medium to a cell culture flask.
3. Invert the container with the blood several times. Added 0.3 ml of whole blood to the cell culture flask with the medium.
4. Mixed the medium and blood then incubated culture for 72 hours in CO_2 cell culture incubator, mixed flask 1-2 times per day.
5. Stopping the cell division at metaphase

   - pre-warmed the Colcemid in the incubator at 37°C .

   - added 50 µl of pre-warmed 37°C Colcemid to the culture, mixed gently and put the culture back into the incubator.

   - incubated for 45 minutes.
6. Put the entire contents of the flask into a conical centrifuge tube.
7. Centrifuged for 10 minutes at 1000rpm.
8. Removed supernatant with Liquipette until 0.2-0.5 ml remain. Be careful not to disturb the cells at the bottom.
9. Gently added 10 ml 0.075 M KCl (prewarmed 37°C), first 1 ml drop by drop (hypotonic treatment).
10. Incubated for 25 minutes in 37°C incubator.

11. Centrifuged 10 minutes at 1000 rpm, then removed supernatant, left about 0.2-0.5 ml, resuspended pellet.

12. Fixing the cells by added 2 ml of cold fixative, mixed well and added fixative until 5 ml.

13. Repeated steps 11-12 until the pellet is white (at least 4 times).

14. Removed the supernatant leaving about 0.5 ml. Vortexed the tube for a few seconds.

15. Making the chromosome slides

    - cool slides to -20°C

    - took a cool slide and hold at an angle. Coated the slide with fix-solution. Set the slide down and immediately placed 1-2 drops of the cell suspension per slide.

    - when the slide is dry, checked chromosomes under a phase contrast microscope. Made adjustments to the concentration of the cell suspension as necessary.

    - let the slides sit at room temperature overnight. The next day, take the slides in a 60°C oven (dry incubator) for 3-4 hours.

    - marked the target site on the slide with a diamond-tipped scribe.

- Chromosome specific DNA probes

Simultaneous three-probe three-color-FISH was performed using probe-sets for either chromosomes X, Y and 18. Three-probe FISH was used to differentiate disomy from diploidy. Simultaneous scoring of three chromosomes also provided an internal control to differentiate nullisomy from hybridization failure (no signals).

The DNA probes used in this study recognize the satellite III DNA of the chromosomes Y (Yq12, locus DYZ 1), alpha satellite DNA of the centromeric region of the human chromosomes X (Xp11.1-q11.1,locus DXZ 1), and chromosomes 18 (18p11.1-q11.1, locus D18Z1).The probes

detecting chromosome X,Y and 18 were labeled with fluorescent haptens CEP (chromosome enumeration probe) SpectrumOrange, CEP SpectrumGreen and CEP SpectrumAqua, respectively.

● Test the specific performance characteristics of DNA probes

Locus specificity studies were performed with metaphase spreads according to standard Vysis protocols. Select a minimum of 20 good quality, complete metaphase spreads with well defined, non-overlapping chromosomes for chromosome enumeration and analysis.

1. Slide preparation

- mark the target site on the slide with a diamond-tipped scribe

- dehydrated through an ethanol series (80%, 95% and 100% ethanol) and air dried

2. Probe preparation

- allowed the probe to warm to room temperature

- mixed the following in an eppendorf tube

+ 7 µl hybridization buffer

+ 1 µl X/Y enumeration probes (1:1 premix of X centromeric probe and Y satellite III probe)

+ 1 µl CEP 18

+ 2 µl Milli Q H_2O

- vortexed and microfuged for 1-3 seconds, and left at room temperature for short time.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

3. Setting the HYBrite™ Parameter

- moistened two strips of a towel water and place the towels in the troughs along the heating surface of the HYBrite unit.

- turned on the HYBrite. Program the Melt Temp to 75°C and Melt Time to 5 minutes. Set the Hyb Temp to 37°C and the Hyb Time to 16 hours (overnigh)

4. Simultaneous hybridization

- applied 10 μl of probes mixture to target site on a slide

- added glass coverslip (22×22 mm) and sealed the edge with rubber cement

- placed the specimen slide(s) on the HYBrite surface.

- closed the lid of the HYBrite and start the Hyb/melt program. The program will begin the melt phase of the program and will then carry the process through the 16 hours hybridization.

5. Washing the slides

- removed the rubber cement and coverslip from the slides

- immersed slides in three changes of 50 % formamide / 2×SSC for 10 minutes each, and then washed for 5 minutes in 2×SSC. The jars were setting in a 45°C water bath. Each jar contained 50 ml.

- after air dried, applied 10 μl of DAPI II counterstain (Vysis Inc.,Framingham, MA, USA) or antifade medium to the target area of the slide and placed a glass coverslip over the DAPI II, avoiding air bubbles.

- stored the slide in the dark prior to signal examination.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

6. Fluorescence microscopy and analysis

FISH signals were analysed with epifluorescence microscope (Olympus, BX50, Japan) equipped with an appropriate filter sets: single band pass filter for DAPI II SpectrumAqua,™ SpectrumGreen,™ and SpectrumOrange™ (DAPI,Aqua,FITC and Texas red respectively) and a triple-band pass filter (DAPI/FITC/TRICT).

- Select a minimum of 20 good quality, complete metaphase spreads with well defined, non-overlapping chromosomes for chromosome enumeration and analysis.

- The CEP X/Y,18 Probe signal will be visible as a distinct fluorescent signal locate near the centromere region of the X,18 chromosomes and the Yq 12 region of chromosome Y.

- Visualization of SpectrumOrange, SpectrumGreen,SpectrumAqua and DAPI counterstain may be performed separately or simultaneously.

- An X or Y chromosome was recognized by a green or an orange fluorescent spot respectively.

- Chromosome 18 was recognized by the presence of an aqua fluorescent spot in the sperm nucleus.

● In situ hybridization to decondensed sperm

1.One set of slides for each FISH procedure were consisted of:

- nine slides of decondensed sperm

- one slide of metaphase slide

2. Slide preparation for FISH

- all slides were treated with 100 µg / ml of Rnase A for 60 minutes at 37°C in humidified chamber, rinsed three times in Milli Q H_2O.

- dehydrated through an ethanol series (80 %, 95 % and 100 % ethanol) for a minute each and air dried.

3. Probe preparation for 10 slides

- prepared the following while the slides were in the ethanol dehydration series.

- mixed the following in an eppendorf tube

+70 µl hybridization buffer

+2.5 µl X/Y enumeration probes (1:1 premix of X centromeric probe and Y satellite III probe)

+2.5 µl CEP 18

+25 µl Milli Q H_2O

- briefly vortexed and microfuged for 1-3 seconds, and left at room temperature for short time.

4. Simultaneous hybridization

- applied 10 µl of probes mixture (made in following step 2 above) to target site on each slide.

- added glass coverslip (22×22 mm) and sealed the edges with rubber cement

- placed slides on HyBrite™ or humidified chamber

- simultaneously denatured slides and probes by heating in a 75°C incubator for 5 minutes

- incubated overnight (4-18 hours) in 37°C incubator or HYBrite.™

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

5. Post-hybridization

- removed the rubber cement and coverslip from the slides

- immersed slides in three changes of 50 % formamide / 2×SSC for 10 minutes each, and then washed for 5 minutes in 2×SSC. The jars were setting in a 45°C water bath. Each jar contained 50 ml.

- after air dried, added 10 µl of DAPI II (Vysis Inc.,Framingham, MA, USA) or a glycerol-based solution containing 0.1 µl / ml 4,6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain and 20 mg /ml 1,4-diazobicyclo[2,2,2] octane (DABCO) as an anti-fade.

- added a glass coverslip and sealed with nail vanish

- stored in the dark at -20°C until examination

6. Fluorescence microscopy

FISH signals were analysed with epifluorescence microscope following the metaphase analysis. A spectrum nucleus was scored only if it was intact and not overlapped.

- An X or Y chromosome in a sperm nucleus was recognized by a green or an orange fluorescent spot respectively.

- Chromosome 18 was recognized by the presence of an aqua fluorescent spot in the sperm nucleus.

- Sperm nuclei were scored disomic for sex chromosomes when an extra X or Y and a signal aqua fluorescent spot were clearly visible within the nucleus, were comparable in brightness and size, and were at least one domain apart.

- Sperm nuclei were scored as nullisomic for sex chromosome when only a single chromosome 18 signal was visible.

- Sperm nuclei were considered diploid when an extra X or Y chromosome signal and two chromosome 18 signals were present.

- At least 2000 spermatozoa were evaluated in each sample from health fertile donor and all epididymal sperms retrieved left from ICSI and cryopreservation were evaluated.

● Scoring criteria

Sperm nuclei scoring was done according to the strict criteria.[68] Nuclei were scored only if they were not over-decondensed, did not overlap and were intact with clearly defined borders. Normal haploid sperm nuclei carried one signal for a sex chromosome and one signal for an autosome. If the distance between the two fluorescent signals was equal to or greater than the diameter of one fluorescent domain, the sperm was scored as disomic for that particular chromosome. Two spots seperated by less than the diameter of one domain were scored as a single chromosome. Consistent with the scoring criteria of Martin and Rademarker.[65] two spots seperated by less than the diameter of one domain were scored as a single signal. The absence of signal for a single chromosome was scored as nullisomy for that chromosome. Spermatozoa showing signal for none of the chromosomes of a probe set were scored as such, but were not included in the calculation of nullisomy frequencies as this outcome may be an artefact resulting from unsuccessful hybridization due to inadequate nuclear decondensation. A cell was scored as diploid if there were two signals for both probe chromosome pairs. Diploid cells with a clearly defined round shape and without a tail were considered spermatogenetic or other cells and were not scored. Scoring was done blindly on coded samples whose origins were unknown to the individuals involved in the scoring. For each healthy fertile donor control 2000 sperms were evaluated.

- Statistical analysis

Descriptive statistics have been performed for each variable, quantitative results were presented by using mean and deviations, quanlitative results were summarized by using distribution of frequencies.

Before comparing the two groups, each variable was tested in order to check the normality distribution using Kolmogorov-Smirnov test, the comparison between sperm aneuploidy and diploidy of epididymal spermatozoa from obstructive azoospermic men and ejaculated spermatozoa from normospermic men was determined by unpaired t-test.

The χ^2 - test was used to analyze the differences in frequency of aneuploidy for sex chromosomes and for chromosome 18 among epididymal sperm derived from obstructive azoospermic patients with failed reversal vasectomy and other causes. All statistical analysis were performed using the SPSS for Windows version 10.05 (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was considered to be statistically significant.

CHAPTER IV

RESULTS

Results of the specificity of probes

No cross- hybridization to other chromosome loci was observed in any of the 50 cells examined; hybridization was limited to the centromere of chromosome X, 18 and the Yq12 region of chromosome Y were shown in Figure 9.

Results of the sperm aneuploidy

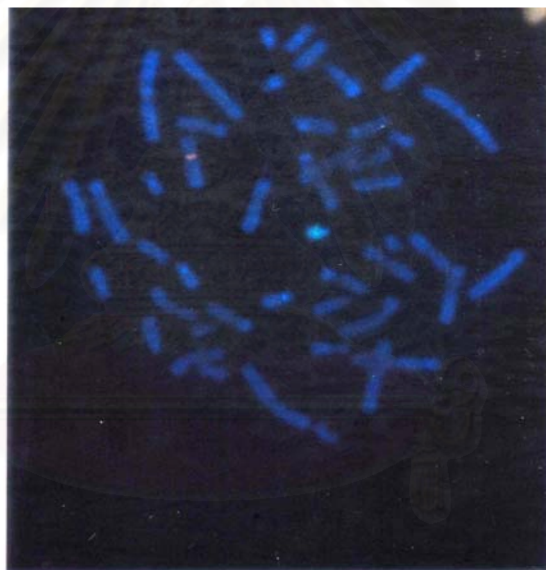
A total of 23,267 epididymal spermatozoa from the 24 obstructive azoospermic patients and 48,000 ejaculated spermatozoa from the 24 healthy fertile controls were analyzed by three-color- FISH with DNA probes for chromosomes X, Y and 18. A mean of 969 spermatozoa (rang, 100-3,057 spermatozoa) and 2,000 spermatozoa were analyzed per sample in study group and control group, respectively. Hybridization was efficient, with an overall frequency of hybridization of 99.51 % (rang, 98.77 %-100 %) and 99.68 % (rang, 99.25 %-99.85 %) in study group and control group, respectively. No difference in hybridization efficiency between the patients and controls was observed. The details of the frequencies of X and Y-bearing sperm, sex disomy, disomy 18, and diploidy of epididymal spermatozoa from the obstructive azoospermic men were shown in Table 4.

The results of FISH analysis of epididymal spermatozoa from the obstructive azoospermic patients compared with the results obtained from healthy fertile controls are shown in Table 6. Statistically significant differences were observed in the incidence of haploidy X, haploidy Y, sex aneuploidy, disomy 18 and diploidy between the study group and the control group ($p<0.005$).

The patients were divided into two groups according to the causes of obstructive azoospermia: the patients with history of vasectomy and failed vasectomy (n= 12) and the remaining patients with other causes of obstruction (n= 12). A total of 23,267 spermatozoa were scored: 13,524 in the vasectomized men and 9,743 in the remaining obstructive

azoospermic patients. There were no significant differences in sex disomy rate (1.37 %VS 1.54 %), disomy 18 rate (0.10 % VS 0.13 %), diploidy rate (0.16 % VS 0.21 %) between vasectomized men and the remaining obstructive azoospermic patients, respectively. However, the total aneuploidy rate did not correlate to the duration of vasectomy. (r=0.048, *p*= 0.89).

Figure 9: Result of locus specificity with mitotic chromosome spreads from human male peripheral blood lymphocytes were used as positive controls in each FISH procedure. The CEP X DNA probe (DXZ1) is a SpectrumOrange specific for alpha satellite at the centromeric region of chromosome X (X p11.1-Xq11.1). The CEP Y DNA probe (DYZ1) is SpectrumGreen specific for the satellite III at the Yq12 region of chromosome Y. The CEP 18 probe (D18Z1) is SpectrumAqua specific for alpha satellite at the centromeric region of chromosome 18.

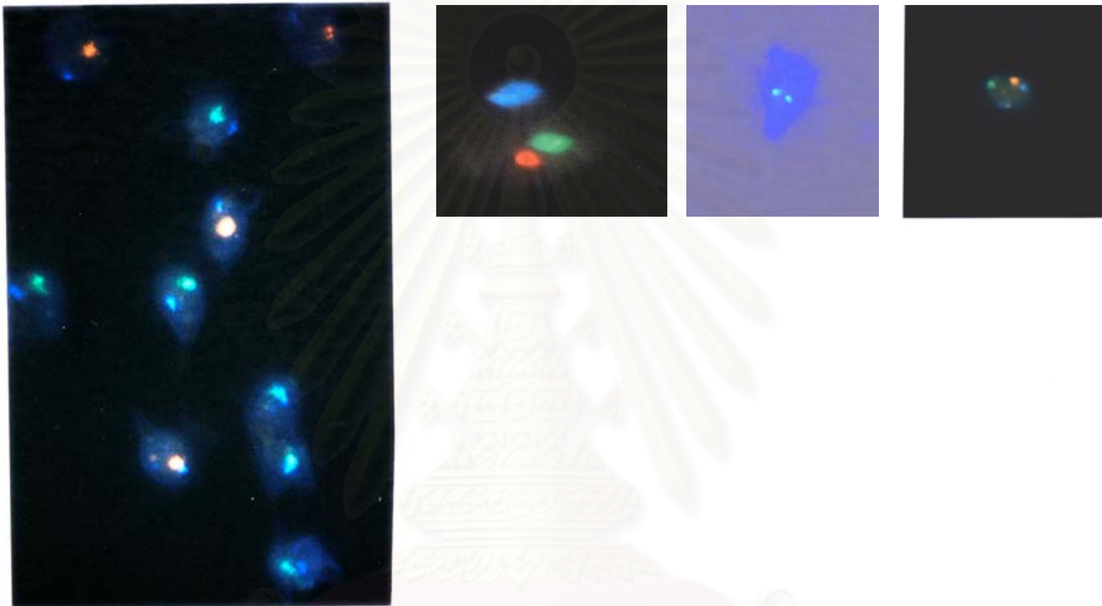


สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Figure 10: Three-probe FISH applied to the epididymal sperm from obstructive azoospermic men. Chromosome 18 (aqua), X (orange) and Y (green). (a) Normal sperm with single copies of chromosome X or Y and one copy of chromosome 18. (b,c) Abnormal sperm (aneuploid) with XY,18 and X,18,18 respectively. (d) Abnormal sperm (diploid) with XY,18,18.

Normal human sperm | Aneuploid sperm | Diploid sperm

(a) X,18 and Y,18 | (b) XY,18 | (c) X,18,18 | (d) XY,18,18



สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Control group	Normal		Disomy			Toltal sex aneuploidy	Normal Ch 18 (%)	Disomy Ch (18)	Diploidy (%)	Labellin g (%)	Total Cell
	X (%)	Y (%)	XX(%)	XY (%)	YY (%)						
D1	50.700	49.050	0.000	0.050	0.050	0.100	99.800	0.000	0.000	99.250	2000
D2	49.800	50.000	0.050	0.000	0.000	0.050	99.850	0.000	0.000	99.850	2000
D3	50.450	49.100	0.000	0.000	0.000	0.050	99.602	0.000	0.050	99.650	2000
D4	50.550	49.151	0.000	0.050	0.000	0.050	99.751	0.000	0.000	99.750	2000
D5	49.800	50.000	0.000	0.000	0.000	0.000	99.804	0.000	0.050	99.851	2000
D6	49.990	49.500	0.000	0.050	0.050	0.100	99.452	0.000	0.000	99.452	2000
D7	49.450	50.251	0.050	0.050	0.050	0.151	99.700	0.100	0.000	99.804	2000
D8	50.880	48.800	0.050	0.100	0.000	0.151	99.550	0.000	0.000	99.552	2000
D9	49.450	49.700	0.100	0.050	0.000	0.151	99.251	0.050	0.100	99.403	2000
D10	49.650	49.400	0.050	0.000	0.050	0.100	99.150	0.000	0.000	99.151	2000
D11	49.500	50.000	0.000	0.100	0.100	0.201	99.701	0.050	0.100	99.754	2000
D12	49.950	49.700	0.050	0.000	0.000	0.050	99.700	0.000	0.000	99.701	2000
D13	50.230	49.570	0.050	0.050	0.050	0.151	99.90	0.050	0.050	99.650	2000
D14	50.050	49.850	0.050	0.000	0.050	0.100	100.00	0.000	0.000	99.700	2000
D15	50.600	49.250	0.050	0.100	0.000	0.150	100.00	0.000	0.000	99.800	2000
D16	49.850	49.950	0.050	0.050	0.050	0.150	99.95	0.000	0.050	99.800	2000
D17	50.880	48.920	0.051	0.101	0.050	0.201	100.00	0.000	0.000	99.450	2000
D18	50.080	49.670	0.100	0.100	0.000	0.201	100.00	0.000	0.050	99.650	2000
D19	49.170	49.970	0.100	0.150	0.050	0.301	99.90	0.050	0.050	99.650	2000
D20	50.250	49.400	0.050	0.201	0.050	0.302	99.90	0.050	0.050	99.400	2000
D21	49.200	50.600	0.050	0.050	0.050	0.151	99.95	0.000	0.050	99.500	2000
D22	49.970	49.450	.0100	0.250	0.000	0.351	99.95	0.050	0.000	99.850	2000
D23	50.350	49.170	0.050	0.150	0.000	0.200	100.00	0.000	0.000	99.800	2000
D24	50.680	49.170	0.050	0.050	0.050	0.150	100.00	0.000	0.000	99.750	2000

Table 4: The frequencies of X and Y - bearing sperm, sex disomy, disomy 18 and diploidy of ejaculated spermatozoa from the 24 healthy fertile controls.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Patient group	Normal		Disomy			Toltal sex aneuploidy	Normal Ch 18 (%)	Disomy Ch 18 (%)	Diploidy (%)	Labellin g (%)	Tolal Cell
	X (%)	Y (%)	XX (%)	XY (%)	YY (%)						
P1	48.670	46.010	2.010	2.690	1.001	5.712	99.670	0.333	1.010	99.330	300
P2	47.760	50.170	0.690	1.550	0.520	2.760	99.483	0.170	0.350	99.820	580
P3	48.580	49.777	0.746	1.046	0.298	2.087	99.404	0.149	0.447	99.850	671
P4	50.000	42.000	2.020	4.040	1.010	7.070	99.000	0.000	1.010	99.000	100
P5	49.591	48.363	0.710	0.994	0.293	1.997	98.830	0.117	0.117	99.060	1710
P6	50.213	48.904	0.131	0.525	0.164	0.818	99.509	0.100	0.033	99.640	3057
P7	47.598	50.503	0.112	1.006	0.112	1.230	98.883	0.224	0.335	99.440	895
P8	49.888	49.101	0.337	0.787	0.000	1.124	99.775	0.000	0.000	99.770	890
P9	49.400	49.533	0.133	0.600	0.130	0.863	99.467	0.067	0.200	99.730	1500
P10	49.500	49.333	0.250	0.750	0.333	1.333	99.583	0.083	0.168	99.750	1200
P11	48.878	49.898	0.306	0.816	0.204	1.326	99.490	0.102	0.204	99.750	980
P12	49.546	48.910	0.365	1.091	0.273	1.728	99.546	0.000	0.000	99.540	1100
P13	44.680	45.850	0.168	0.754	0.084	1.006	99.749	0.168	0.084	99.417	1200
P14	49.600	48.640	0.159	0.797	0.159	1.116	99.522	0.000	0.478	99.366	631
P15	48.460	49.750	0.297	1.288	0.099	1.685	99.604	0.297	0.099	99.704	1012
P16	47.840	45.580	0.282	0.658	0.470	1.410	99.643	0.188	0.094	99.346	1071
P17	49.160	49.940	0.112	0.561	0.224	0.898	100.00	0.000	0.000	99.553	895
P18	50.000	48.990	0.202	0.606	0.101	0.909	99.899	0.000	0.101	99.497	995
P19	49.140	49.340	0.305	0.814	0.203	1.322	99.797	0.000	0.203	99.293	990
P20	48.170	50.150	0.305	1.067	0.305	1.677	99.238	0.152	0.610	99.394	660
P21	49.060	48.280	0.781	1.719	0.156	2.656	100.00	0.000	0.000	100.00	640
P22	48.480	49.720	0.450	0.900	0.112	1.462	99.438	0.225	0.337	98.778	900
P23	50.060	49.150	0.226	0.452	0.113	0.791	99.774	0.226	0.000	99.438	890
P24	47.360	48.870	0.756	1.511	0.504	2.771	98.741	0.252	1.008	99.250	400

Table 5: The frequencies of X and Y - bearing sperm, sex disomy, disomy 18 and diploidy of epididymal spermatozoa from the 24 obstructive azoospermic men.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 6: Results of fluorescence *in situ* hybridization analysis in 24 obstructive azoospermic patients compared with healthy fertile controls.

Findings	No.(%) in control group	No.(%) in study group	*P* value
Haploid X	23,975 (49.95)	11,314 (48.63)	<0.001
Haploid Y	23,763 (49.51)	11,311 (48.61)	<0.005
Sex aneuploidy	69 (0.14)	335 (1.44)	<0.001
Disomy 18	8 (0.02)	26 (0.11)	<0.001
Diploidy	11 (0.02)	42 (0.18)	<0.001
Total	48,000	23,267	

Table 7: Comparison of chromosomal abnormalities between vasectomized men and the remaining obstructive azoospermic patients.

Findings	Vasectomized men	Other causes of obstructive azoospermic patients	*P* value
Sex aneulpoidy	1.37 %	1.54 %	NS
Disomy 18	0.10 %	0.13 %	NS
Diploidy	0.16 %	0.21 %	NS
Total	13,524	9,743	

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER V

DISCUSSION AND CONCLUSION

Aneuploidy is one of the most serious and common chromosomal abnormalities affecting human embryos and offspring. For the general population, numerical chromosomal abnormalities are mostly of maternal origin, usually resulting from non-disjunction,[82] which is strongly related to maternal age. [83] It can also be transmitted via sperm. [84] Recently, the incidence of chromosomal abnormalities from paternal origin in children born after ICSI has been a matter of concern. [23-25]

Fluorescence in situ hybridization (FISH) with the use of DNA probes for specific chromosomes has become an increasingly popular approach for estimating aneuploidy frequencies in spermatozoa because it provides a rapid and reliable results. Using multicolor FISH techniques, a higher frequency of chromosomal aneuploidies in spermatozoa of infertile men compared to normal fertile donors have been reported. [85-89] In this study, we used three-color FISH with centromeric DNA probes for chromosomes X, Y and 18. DNA probes for chromosome 18 was used as an internal autosomal control. It allowed the differentiation between disomic and diploid spermatozoa and also between failure of hybridization and nullisomic spermatozoa.

In this study, we analyzed the rates of aneuploidy for chromosomes 18, X and Y in 24 obstructive azoospermic men and 24 fertile donors using triple color FISH techniques. The overall hybridization efficiency was about 99.6 % which was similar to the previous study (99.8 %). [90] The hybridization efficiency were high in both patients and controls (99.51 % vs 99.68 %, respectively) which were not statistically different.

The reported frequencies of chromosomal abnormalities of ejaculated spermatozoa from healthy fertile men rang from 0.05-0.45 % for disomy, [31,32,86,88-91] 0.23-0.78 % for overall sex chromosome aneuploidy, [31,32,86,88,89] 0-0.17 % for XX disomy, 0-0.62 % for YY disomy and 0-0.3 % for XY disomy; [32,88,91] the diploidy rates rang from 0-0.25 %. [31,86,88-91] The frequencies of sex

chromosome aneuploidy (0.14 %), disomy 18 (0.02 %) and diploidy (0.02 %) observed in our study group was lower than the previous studies [31,86,88-91]because the healthy fertile men were recruited as control and all of them had normal semen analysis according WHO criteria. The variability of these frequencies may be from the various geographical areas, criteria for fertile men selection, the different FISH protocol: decondensation procedures, types of probes and the different scoring criteria. Moreover, several factors have been found to increased a man's risk of producing aneuploid sperm, including advancing paternal age, in particular for sex chromosomes [92] and treatment with cancer chemotherapy. [93]Some lifestyle factors such as smoking, alcohol drinking and caffeine consumption have not been associated with disomy frequency. [94]

The reported frequencies of chromosomal abnormalities of epididymal spermtozoa from obstructive azoospermic men rang from 2.89-4.36 % for overall sex chromosome aneuploidy, o.54 % for disomy 18 and o.44 % for diploidy. [31,32] However, very small numbers of spermatozoa per case were analyzed in these studies. The frequencies of sex chromosome aneuploidy (1.44 %), disomy 18 (0.11 %) and diploidy (0.18 %) observed in our study group were lower than the previous studies. [31,32] The difference in the frequencies of aneuploidy may be caused by the limited numbers of spermatozoa analyzed, the limited numbers of the patients and the differences in methodology. The three chromosomes detected in this study were not equally affected with regard to their relative contribution to the incidence of aneuploidy. In this study, the proportions of aneuploidy involving the sex chromosomes were significantly higher than the rate for chromosome 18 which was similar to the previous studies. [31,32]These results continue supporting the possibility of a paternal origin of sex chromosome abnormalities in the karyotype of ICSI offspring.

We also compared the aneuploidy rates of the sex chromosomes and chromosome 18 between the patients and controls. A higher frequency of the chromosome aneuploidy rate of the epididymal spermatozoa from obstructive azoospermic patients compared to the ejaculated sperm from normal fertile controls ($p<0.001$) (Table 6).

The frequencies of chromosomal abnormalities of epppididymal spermatozoa were significantly increased in the obstructive azoospermic men which is similar to the previous studies [31,95] In previous study, epididymal spermatozoa from obstructive azoospermic men had sex chromosome aneuploidy, total aneuploidy and diploidy rates significantly higher than ejaculated spermatozoa from normozoospermic controls (2.89 % vs 0.81 %,4.84 % vs 1.47 % and 0.44 % vs 0.13 %, respectively). Sex chromosome aneuploidy rate was 2.89 %. However, only 6 obstructive azoospermic men were studied and were congenital agenesis of bilateral vas deferens (CABVD). [31] Recently, epididymal spermatozoa from ten obstructive azoospermic men also had total aneuploidy rate significantly higher than ejaculated spermatozoa from normozoospermic controls (8.2 % vs 1.6 %). [32]

The patients were divided into two groups according to their causes of obstructive azoospermia, 12 were obstructive azoospermic men from vasectomy with failed vasectomy reversal and 12 were obstructive azoospermic men from other causes. There were no significant difference in sex chromosome aneuploidy, disomy 18, diploidy rates between these two groups. Surprisingly, the frequencies of chromosomal abnormalities of epididymal spermatozoa were also significantly increased in obstructive azoospermic men, even in the previous fertile men after vasectomy and faild vasectomy reversal. Although vasectomized men were previously fertile men, the rate of total sex chromosome aneuploidy, disomy 18 and diploidy were significantly higher than fertile healthy controls. However, relationship between the duration of vasectomy and the frequency of chromosome abnormalities can not be demonstrated. Overall findings suggest the need for future research to study whether long duration of vasectomy may increase the frequency of sperm aneuploidies.

Recently, increased aneuploidy rates in epididymal sperm have also been found in the patients with the CABVD. [31,95] The differences in chromosomal abnormalities between ejaculated sperm and epididymal spermatozoa in obstructive azoospermic men may be explained by the sequestration of abnormal epididymal spermatozoa during maturation and passage of sperm in the

epididymis [32] and abnormal spermatogenesis from high back pressure in the seminiferous tubules in the obstructive azoospermic men.

Since aneuploidy might have a negative impact on oocyte fertilization and/or on embryonic development, the rate of epididymal sperm aneuploidy in obstructive azoospermic patient undergoing ICSI and its impact on ICSI results were evaluated by comparing the ICSI outcome between the obstructive azoospermic patients who had the total aneuploidy rate higher and lower than the medium value. There were no statistically significant differences of ICSI outcomes between both groups

In agreement with the previous study about the relation of incidence of ejaculated sperm aneuploidy and assisted reproductive outcome,[96] the increased frequency of chromosomal abnormalities of epididymal spermatozoa did not have a direct effect on the ICSI outcome, including fertilization rate, pregnancy rate, and pregnancy outcome. However, some previous studies found the association between the adverse ICSI outcomes and an increased aneuploidy rate of ejaculated spermatozoa. [87,89] This is probably due to the small sample size and the frequencies of epididymal spermatozoa aneuploidy is not high enough to effect the overall ICSI outcomes. In conclusion, the increased frequency of chromosomal abnormalities did not have a direct effect on the fertilization rate, pregnancy characteristics or outcomes in our study. Although the clinical outcomes of ICSI using epididymal spermatozoa from the groups that had different sperm aneuploidy rates were similar, the long term follow-up of the ICSI outcome using epididymal spermatozoa in larger population is needed.

In summary, our results show increased rates of diploidy and disomy in epididymal spermatozoa from obstructive azoospermic men compared with ejaculated spermatozoa from healthy fertile controls. Moreover, there were no differences in the incidence of epididymal spermatozoa chromosomal aneuploidy between the vasectomized men and men with other causes of obstructive azoospermia. Where epididymal spermatozoa were used for ICSI in obstructive azoospermia, the differences of percentage of chromosome abnormalities did not influence ICSI

outcome. Pregnancy occurred even in the offspring with chromosomal abnormalities in this group. This can provide the data for counseling the patients about the potential paternal contribution to the risk of fetal chromosomal anomalies after intracytoplasmic sperm injection (ICSI) in obstructive azoospermic men.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

REFERENCES

1. Michelmann HW. Minimal criteria of sperm quality for insemination and IVF therapy. Int J Androl 1995 ;18 (Suppl 2) : 81-87.
2. Palerno G, Joris H, Devoey P, Van Steirteghem AC. Pregnancies after intracytoplasmic sperm injection of single spermatozoon into an oocyte. Lancet 1992; 340: 17-18.
3. Diamond MP. Intracytoplasmic sperm injection [Online]. Available from: http://obg.med.wayne.edu/fertility.htm [2000, June 12].
4. Tarlatzis BC, Bili H. Survey on intracytoplasmic sperm injection : report from the ESHRE Task Force. Hum Repord 1998;13(Suppl 1):165-177.
5. Sutcliffe AG, Taylor B, Sauaders K, Thornton S, Lieberman BA. Outcome in the second year of life after in-vitro fertilization by intracytoplasmic sperm injection: a UK case-control study. Lancet 2001; 357:2080-2084.
6. Van Blerkom J. Developmental failure in human reproduction associated with chromosomal abnormalities and cytoplasmic pathologies in meiotically mature hhuman oocytes. In: Van Blerkom J. (ed.) The Biological Basis of Early Reproductive Failure in The Human: Applications to Medically to Assisted Conception. Oxford University Press, Oxford, 1994: 283-325.
7. Van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smitz J, et al. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum Reprod 1993;8:1061-1066.
8. Liu J, Lissens W, Silber SJ, Devroey P, Liebaers I, Van Steirteghem A. Birth after preimplantation diagnosis of the cystic fibrosis DF508 mutation by polymerase chain reaction in human embryos resulting from intracytoplasmic sperm injection with epididymal sperm. JAMA 1994;272:1858-1860.
9. Tournaye H, Devroey P, Liu J, Nagy Z, Lissens W, Van Steirteghem A. Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection : a new effective approach to infertility as a result of congenital

bilateral absence of vas deferens. Fertil Steril 1994;61:1045-1051.

10. Devroey P, Liu J, Nagy Z, Tournaye H, Silber SJ, Van Steirteghem AC, Normal fertilitzation of human oocytes after testicular sperm extraction and intracytoplasmic sprem injection. Fertil Steril 1994;62:639-641.
11. Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H, Devroey P, High fertilitzation and pregnancy rate after sperm injection with spermatozoa obtained from testicle biopsy. Hum Reprod 1995;10:148-152.
12. Tesarik J, Mendoza C, Testart J. Viable embryos from injection of round spermatids into oocytes, N Engl J Med 1995;333:525.
13. Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M, et al. The result related to any three basic sperm parameters. Hum Reprod 1995;10:1123-1129.
14. Sukcharoen N, Sithipravej T, Promviengchai S, Chinpilas V, Boonkasemsanti W. Sperm morphology evaluated by computer (IVOS) cannot predict the fertilization rate in vitro after intracytoplasmic injection. Fertil Steril 1998;69:564-568.
15. Chandley AC, Hargreave TB. Genetic anomaly and ICSI. Hum Reprod 1996;11:930-932.
16. In't Veld R, Brandenburg H, Verhoeff, A dhont M, Los F. Sex chromosomal abnormalities and intracytoplasmic sperm injection. Lancet 1995;346:773.
17. Liebaers I, Bonduelle M, Van Assche E, Devroey P, Van Steirteghem A. Sex chromosome abnormalities after intracytoplasmic sperm injection. Lancet 1995;346-1095.
18. Van Opstal D, Los FJ, Ramlakhan S, Van Hemel JO, van den Ouweland AMW, Brandenburg H, et al. Determination of the parent of origin in nine cases of prenatally detected chromosome aberrations found after intracytoplasmic sperm injection : Hum Reprod 1997;12:682-686.
19. Loft A, Peterson K, Erb K, Mikkelsen AL, Grinsted J, Hald F, et al, A Danish national cohort of 730 infants after intracytoplasmic sperm injection (ICSI) 1994-1997. Hum Reprod 1999;14:2143-2148.
20. Bonduelle M, Camus M, De Vos A, Staessen C, Tournaye H, van Assche E, et al.

Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. Hum Reprod 1999;14:243-264.

21. Aboulghar H, Aboulghar M, Mansour R, Serour G, Amin Y,Al-Inany H. A pros[ective controlled study of karyotypeing for 430 consecutive babies conceived through intracytoplasmic sperm injection. Fertil Steril 2001; 76:249-253.
22. Moosani N, Pattinson HA,Carter MD, Cox DM, Rademaker AW, Martin RH. Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence in situ hybridization. Fertil Steril 1996;64:811-817.
23. In't Veld P, Broekmans FJM, de France HF, Pearson PL, Pieters MH, van Kooij RJ. Intracytoplasmic sperm injection (ICSI) and chromosomally abnormal spermatozoa. Hum Reprod 1997;12:752-754.
24. Lahetie J, Saari N, Ajosenpaa-Saari M, Mykkanen J. Incidence of aneuploid sprmatozoa among infertile men studied by multicolor fluorescence in situ hybridization. Am J Med Genet 1997;71:155-161.
25. Guttenbach M, Martinez-Exposito MJ, Michelmann HW, Engel W, Schmid M. Incidence of diploid an disomic sperm nuclei in 45 infertile men. Hum Reprod 1997;12:468-473.
26. Bernardini L, Gianaroli L, Fortini D, Conte N, Magli C, Cavani S, et al. Frequency of hyper-hypohaploidy and diploidy in ejaculate, epididymal and testicular germ cells of infertile patients. Hum Reprod 2000;15:2165-172.
27. Levron J, Aviram-Goldring A, Madgar I,Raviv G, Barkai G, Dor J. Sperm chromosome abnormalities in men with severe male factor infertility who are undergoing in vitro fertilization with intracytoplasmic sperm injection. Fertil Steril 2001; 76:479-484.
28. Martin RH, Greene C, Rademaker A, Barclay L, Ko E, Chernos J. Chromosome analysis of spermatozoa extracted from testes of men with non-obstructive azoospermia. Hum Reprod 2000;15:1121-1124.
29. Baker HWG, Burger HG, De Krester DM, Hudson B. Relative incidence of etiologic disorders in male infertility. In: Santen RJ, Swerdloff RS, editors.

Male Reproductive Dysfunction : Diagnosis and Management of Hypogonadism, Infertility, and Impotence. New York : Marcel Dekker, 1986:342-72.

30. Matsumoto AM. Pathophysiology of male infertility : In : Keye WR, Chang RJ, Rebar RW, Soules MR. Infertility: Evaluation and Treatment. Philadelphia: WB Saunders, 1995: 555-573.

31. De Kretser DM. Andrology Australia [Online]. Available from: http://www.andrologyaustralia.org/infertility.htm [2002,February 28].

32. Foresta C, Ferlin A, Betella A, Rossalo M, Varotto A. Diagnostic and clinical features in Azoospermia. Clin Endocr 1955; 43: 537-543.

33. Jarow JP, Espeland MA, Lipschnltz LI. Evaluation of the azoospermic patient. J Urol 1989; 142: 62-65.

34. Kamischke A and Nieschlag E. Analysis of medical treatment of male infertility. Hum Reprod; 14 (Suppl 1): 1-23.

35. Werner MA. Male infertility specialists.com [Online]. Available from : http://www.maleinfertilitymds.com [Update 2002, April].

36. Matthews GJ, Goldstein M. A simplified method of epididymal sperm aspiration. Urology 1996; 47: 123-125.

37. Weill IS. Male infertility / Sperm retrieval techniques [Online]. vailable from:http://www.cornellurolog.com. [1999]

38. Schlegel PN. Microsurgical techniques of epididymal and testicular sperm retrieval. In: Golstein M. Surgery for male Infertility-Atlas of the Urologic Clinic of North America. Philadelphia: WB Saunders, 1999; 109-129.

39. Sheynkin YR, Ye Z, Menendez S, Liotta D, Veeck LL and Schegel PN. Controlled comparison of percutaneous and microsurgical sperm retrieval in men with obstructive azoospermia. Hum Reprod 1998;13 (11): 3086-3089.

40. Egozcue S, Blanco J, Vendrell JM, Garcia F veiga A, et al.Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. Hum Repro Update 2000; 6 (No.1): 93-105.

41. Martin RH. Genetics of human sperm. J Assist Reprod Genetics 1998; 15: 240-245.

42. Chandeley AC. Genetic contribution to male infertility. Hum Reprod 1998; 13 (supple 3): 76-83.

43. Kent-First MG, Kol S, Muallem A. The incidence and possible revalence of Y-linked microdeletions in babies born after intracytoplasmic sperm injection and their infertility fathers. Mol Hum Reprod 1996; 2: 943-950.

44. Pfeffer J, Pang MG, Hoegerman SF. Aneuploidy frequencies in pellete and swim-up semen fractions from ten oligoasthenoteratospermia patients donating sperm for intracytoplasmic sperm injection (ICSI). Fertil Steril 1999; 72: 472-478.

45. Cummis J, Jequier A. Treating male infertility needs more clinical andrology not less. Hum Reprod 1994; 6: 1214-1219.

46. Kupker W, Schwinger E, Hiort O, Ludwis M, Nikolettos N, et al. Genetics of male subfertility: consequences for the clinical work-up. Hum Reprod 1999; 14 (Suppl 1): 24-37.

47. Gillbert SF. Human meiosis. Development Biology 6 rd edn [Online].Available from: http://www.devbio.com/chap19/ink 1904.shtml [2001,June 10].

48. Barlow P, Vosa CG. The Y chromosome in human spermatozoa. Nature 1970;226: 961-962.

49. Caspersson L, Farber G, Foley SE, Kudynowski J, Modest EJ,et al. Chemical differentiation along metaphase chromosomes. Exp Cell Res 1967; 49: 219-223.

50. Zech L. Investigation of metaphase chromosomes with DNA binding fluorochromes. Exp Cell Res 1969; 58: 463-466.

51. Ribbins EP, Lipshultz IL, Ward BJ, Legator SM. Fluorescent body distribution in spermatozoa in the male with exclusively female offspring. Fertil Steril 1988; 49: 670-675.

52. Rudak R, Jacobs PA, Yanagimachi R. Direct analysis of the chromosome constitution of human spermatozoa. Nature 1978;274:911-913.

53. Martin RH, Balkan W, Burns K, Linn CC. Direct chromosomal analysis of human spermatozoa. Am J Hum Genet 1982;34:459-468.

54. Pieters MHEC, Geraedts JPM, Meyer H, Dumoulin JC, Evers JL, Jongbloed RJ,

et al. Human gametes and zygotes studies by nonradioactive in situ hybridization. Cytogenet Cell Genet 1990;53:15-19.

55. Joseph AM, Gosden JR, Chandley AC. Estimation of aneuploidy levels in human spermatozoa using chromosome specific probes and in situ hybridization. Hum Genet 1984;66:234-238.

56. Guttenbach M, Schmid M. Determination of Y chromosome aneuploidy in human sperm nuclei by nonradioactive in situ hybridization. Am J Hum Genet 1990;46:553-558.

57. Wyrobek AJ. Sperm's chromosomal contribution to embryo and infant heath. Science & Technology Review [Online].Available from: http://www.llnl.gov/str/pdf [2001, November 11].

58. Wyrobek AJ, Robbins WA, Mehraein Y, Pinkel D, Weier HU. Detection of sex chromosomal aneuploidies X-X, Y-Y, and X-Y in human sperm using two-chromosome fluorescence in situ hybridization. Am J Med Genet 1994;53:1-7.

59. Martínez-Pasarell O, Márquez C, Coll MD, Egozcue J, Templado C. Analysis of human sperm-derived pronuclei by three-colour fluorescent in situ hybridization. Hum Reprod 1997;12:641-645.

60. Aribarg A, Ngeamvijawat J, Chanprasit Y, Sukcharoen N. Determination of the ratio of X and Y bearing spermatozoa after albumin gradient method using double-labeled fluorescence in situ hybridization (FISH). J Med Assoc Thai 1997;79 (Suppl 1):88-95.

61. Van Hummelen P, Lowe XR, Wyrobek AJ. Simultaneous detection of structural and numerical chromosome abnormalities in sperm of healthy men by multicolor fluorescence in situ hybridization. Hum Genet 1996;98:608-615.

62. Wyrobek AJ, Alhborn T, Balhorn R, Stanker L, Pinkel D. Fluorescence in situ hybridization to Y chromosome in decondensed human sperm nuclei. Mol Reprod Dev 1990;27:200-208.

63. Vidal F, Moragas M, Catala V, Torello MJ, Santalo J, Calderon G, et al. Sephadex filtration and human serum albumin gradients do not select spermatozoa by sex chromosome: a fluorescence in situ hybridization study. Hum

Reprod 1993;8:1740-1743.

64. Holmes JM, Martin RH. Aneuploidy detection in human sperm nuclei using fluorescence in situ hybridization. Hum Genet 1993;91:20-24.
65. Lu PY, Hammitt DG, Zinsmeister AR, Dewald GW. Dual color fluorescence in situ hybridization to investigate aneuploidy in sperm from 33 normal males and a man with at (2;4;8) (q23;q27;p21). Fertil Steril 1994;62:394-399.
66. Bischoff FZ, Nyuyen DD, Burt KJ, Shaffer LG. Estimation of a aneuploidy using multicolor fluorescence in situ hybridization on human sperm. Cytogenet Cell Genet 1994; 66:237-243.
67. Nath J, Tucker JD, Hando JC. Y chromosome aneuploidy, micronuclei,kinetochores and aging in men. Chromosome 1995;103:725-731.
68. Zijno A, Leopardi P, Marcon F, Crebelli R, Sex chromosome loss and non-disjunction in women :analysis of chromosomal segregation in binucleated lymphocytes. Chromosome 1996;104:461-467.
69. Márquez C, Egozuce J, Martorell MR, Moreno V, Templado C. Colcemid increases the frequency of chromosome abnormalities in human sperm. Cytogenet Cell Genet 1996;72:164-170.
70. Egozcue J, Blanco J, Vidal F. Chromosome studies in human sperm nuclei using fluorescence in situ hybridization (FISH). Hum Reprod Update 1997;3:441-452.
71. Rousseaux S, Chevret E. In-vitro decondensation of human spermatozoa for fluorescence in situ hybridization. Hum Repord 1995;10:2209-2213.
72. Sbracia M, Baldi M, Cao D, Sandrelli A, Chiandetti A, Poverini R,et al. Preferential location of sex chromosomes, their aneuploidy in human sperm, and their role in determining sex chromosome aneuploidy in embryos after ICSI. Hum Reprod 2002;17:320-324.
73. Martin RH, Rademaker AW. Reliability of aneuploidy estimates in human sperm: results of fluorescence in situ hybridization studies using two different scoring criteria. Mol Reprod Dev 1995;42:89-93.
74. Coonen E, Pieters M, Dumoulin JC, Meyer H, Evers JL, Ramaekers FC, et al.

Nonisotopic in situ hybridization as a method for nondisjunction studies in human spermatozoa. Mol Reprod 1991;28:18-22.

75. Seligman J, Kosower NS, Weissenberg R, Shalgi R. Thiol-disulfide status of human sperm proteins. J Reprod Fertil 1994;101:435-443.

76. Blanco J, Egozue J, Vidal F. Incidence of chromosome 21 disomy in human spermatozoa as determined by fluorescence in-situ hybridization. Hum Reprod 1996;11:722-726.

77. Phimster B. FISH: (Fluorescence In Situ Hybridization). Science & Technology Review [Online].Available from: http://www.llnl.gov/str/pdf [2001,November 11].

78. Palermo GD, Colombero LT, Hariprashad JJ, Schlegel PN and Rosenwaks Z. Chromosome analysis of epididymal and testicular sperm in azoospermic patients undergoing ICSI. Hum Reprod 2002; 17: 570-575.

79. World Health Organization. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 4rd ed. Cambridge University Press, 1999.

80. Williams BJ, Ballenger CA, Malter HE, Bishop F, Tucker M, Zwingman TA. Non-disjunction in human sperm : results of fluorescence in situ hybridization studies using two and three probes. Hum Mol Genet 1993;2:1929-1936.

81. Hassold T, Hunt PA and Sherman S. Trisomy in human: incidence, origin and etiology. Curr Opin Genet Dev 1993; 3:398-403.

82. Nielson J and Wohelert M. Chromosome abnormalities found among 34,910 newborn children: results from a 13-year study in Arhus, Denmark. Hum Genet 1991; 87: 81-83.

83. Wyrobek AJ. Methods and concepts in detecting abnormal reproductive outcomes of paternal origin. J Reprod Toxicol 1993; 7: 3-16.

84. Pang MG, Hoegerman SF, Cuticchia AJ.et al. Detection of aneuploidy for chromosomes 4,6,7,8,9,10,11,12,13,17,18,21,X and Y by fluorescence in-situ hybridization in spermatozoa from nine patients with

oligoasthenoteratozoospermia undergoing intracytoplasmic sperm injection. Hum Reprod 1999; 14: 1266-1273.

85. Ushijimal C, Kumasako Y, Kihaile PE. et al. Analysis of chromosomal abnormalities in human spermatozoa using multi-colour fluorescence in-situ hybridization. Hum Reprod 2000; 15: 1107-1111.
86. Van Dyk Q, Lanzendorf S, Kolm P, et al. Incidence of aneuploid spermatozoa from subfertile men: selected with motility versus hemizona-bound. Hum Reprod 2000;15: 1529-1536.
87. Vegetti W, Van AE, Frias A, et al. Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence in-situ hybridization in infertile men. Hum Reprod 2000; 15: 351-365.
88. Rubio C, Gil-Salom M, Simon C, et al. Incidence of sperm chromosomal abnormalities in a risk population: relationship with sperm quality and ICSI outcome. Hum Reprod 2001; 16: 2084-2092.
89. Aran B, Blanco J, Vidal F. Screening for abnormalities of chromosomes X, Y, and 18 and for diploidy in spermatozoa from infertile men participating in an vitro fertilization-intracytoplasmic sperm injection program. Fertil Steril 1999; 72: 699-701.
90. Calogero AE, De Palma A, Grazioso C, et al. Aneuploidy rate in spermatozoa of selected men with abnormal semen parameters. Hum Reprod 2001; 16: 1172-1179.
91. Robbins WA, Baulch JE, Moore D. Three-probe fluorescence in-situ hybridization to assess chromosome X, Y, and 8 aneuploidy in sperm of 14 men from two healthy groups: evidence for a paternal age effect on sperm aneuploidy. Reprod Fertil Dev 1995; 7: 799-809.
92. Robbins WA, Meistrich MI, Moore D. et al. Chemotherapy induces transient sex chromosomal and autosomal aneuploidy in human sperm. Nat Genet 1997; 16: 74-78.
93. Shi Q and Martin RH. Aneuploidy in human sperm: a review of the frequency and distribution of aneuploidy, effects of donor age and life style factors. Cytogenet Cell Genet 2000; 90: 219-226.

94. Viville S, Warter S, Meyer JM. Histological and genetic analysis and risk assessment for chromosomal aberration after ICSI for patients with CABVD. Hum Reprod 2000; 15: 1613-1618.

95. Colombero LT, Hariprashad JJ, Tsai MC. Incidence of sperm aneuploidy in relation to semen characteristics and assisted reproductive outcome. Fertil Steril 1999; 72: 90-96.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX

Buffers and Reagents

1. Phosphate Buffer Saline (PBS) Dulbecco's formula — 1 liter

Reagent	Amount	Unit
Calcium chloride : $2H_2O$	0.133	g
Magnesium chloride : $6H_2O$	0.1	g
Potassium chloride	0.2	g
Sodium chloride	8.0	g
Potassium phosphate monobasic (anhydrous)-K_2HPO_4	0.2	g
Sodium phosphate dibasic (anhydrous)-Na_2HPO_4	1.15	g

Adjust to pH 7.2 to 7.4 with 1 M HCl

Add ddH_2O to 1000 ml and sterilize by autoclaving.

2. HEPES-buffered Ham's F10 — 1 liter

Reagent	Amount	Unit
Ham's F10 powder	9.89	g
$NaHCO_3$	2.106	g
Ca-lactate	0.303	g

Make up to 1 liter with tissue culture grade water and sterilized by filtering through a 0.22 μm membrane filter.

Adjust to pH 7.3 to 7.5 by bubbling 5 % CO_2 in air (pinkish orange in color).

Check the osmolarlity is 280 to 286 mOsmol.

Reagent	Amount	Unit
Add HEPES free acid	5.67	g
Add human serum albumin	10 %	

3. 1 M Dithiothreitol (DTT)

Reagent	Amount	Unit
DTT	15.4	g
0.1 M Tris, pH 8.0	100	ml

4. 0.1 M Dithiothreitol (DTT) / 0.01 M lithium 3,5-diiodosalicylic acid (LIS)

Reagent	Amount	Unit
DTT	1.54	g
LIS	0.395	g
0.1 M Tris, pH 8.0	100	ml

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

5. 20×SSC, pH 7.0	1	liter
NaCl	175.3	g
Sodium citrate	88.2	g
dd H_2O	900	g
Adjust pH to 7.0		
Add dd H_2O to 1 liter and sterilized by autoclaving		
6. RPMI 1640 stock solution	1	liter
RPMI powder	10.4	g
$NaHCO_3$	2	g
Tissue culture grad water	900	ml

Add dd H_2O to 1 liter and sterilized by filtering through 0.22 μm membrane filter.

7. Hypotonic solution

0.075 M KCl prepared in dd H_2O

8. Fix solution

3:1 methanol : glacial acetic acid

9. 50 % formamide / 2×SSC		
formaminde (straight from bottle)	75	ml
20×SSC, pH 7.0	15	ml
dd H_2O	60	ml

150 ml total pH to 7.0 -7.5 with 1 N HCl

10. Anti-fade medium

12.1 g DABCO in 42.1 ml dd H2O	(12.1 %)
10.5 ml 1 M Tris- H2O, pH 8.0	(0.1 M)
47.35 ml Glyceral	(47.35 %)
50 μg DAPI	(0.0005 %)

Total volume 100 ml

11. RNase A 100 μg/ml		
Rnase A 500 μg/ml	200	μl
2X SSC, pH 7.0	800	μl

Total volume 1000 μl

Biography

Miss Jiraporn Ngeamvijawat was born on December 1, 1965 in Prachuabkirikhun, Thailand. She received the degree of Bachelor of Biology of Science in 1988 from faculty of science, Srinakharinwirot Bangkhen University, Bangkok, Thailand.

Experience and Positions

1989-1990	Scientist	: The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University
1991-Present	Medical Scientist	: Andrology Unit, Department of Obstetric and Gynecology, Chulalongkorn Hospital

She has enrolled at Chulalongkorn University in graduate program for the degree of master of science in Medical Science in 2002.