Outcome of Sperm Preparation Using Double-Gradients Technique Study in Siriraj Hospital

Somsin Petyim MD*,
Roungsin Choavaratana MD*, Singpetch Suksompong MD*,
Pitak Laokirkkiat MD*, Orawan Makemaharn MSc*

** Infertility Unit, Faculty of Medicine, Siriraj Hospital, Bangkok, Thailand*

Objective: *To determine the succession of sperm preparation using the double-gradients technique on sperm quality: sperm recovery rate, sperm concentration, sperm motility and percentage of post wash total motile sperm count.*
Study Design: *Retrospective descriptive study.*
Settings: *Infertility clinic, Faculty of Medicine, Siriraj hospital.*
Material and Method: *During the period of January 1, 2002 through December 31, 2007, data including semen analysis before and after IUI procedure were reviewed in all male patients who were referred to the andrology laboratory for sperm washing and IUl. Comparison of semen parameters such as total sperm concentration, total motile sperm count before and after sperm preparation as well as total sperm recovery rate and total motile sperm recovery rate was evaluated.*
Results: *After sperm preparation, both sperm concentration and progressive sperm motility significantly increased, while total motile sperm count significantly decreased. Moreover, the percentage of motile sperm recovery rate and total sperm recovery rate was higher after sperm preparation at around 59.88 $\pm$ 19.26% and 34.03 $\pm$ 14.58% respectively. When categorizing semen parameters to 4 groups: normozoospermia, oligozoospermia, astenozo-ospermia and oligo-astenozoospermia, sperm motility in each group, comparing with sperm motility prior preparation, significantly improved after sperm preparation. Furthermore, motile sperm recovery rate in each group significantly increased except for astenozoospermia. Total sperm recovery rate in oligozoospermia was significantly higher than normozoospermia, yet the others were significantly lower.*
Conclusion: *Sperm preparation using double gradient percoll provided a high percentage of motile sperm recovery rate and total sperm recovery rate. It also dramatically improved progressive sperm motility in normozoospermia, oligozoospermia, astenozoospermia and oligo-astenozoospermia.*

Keywords: *Sperm count, Sperm motility, Semen preservation*

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Intrauterine insemination has been developed for more than 40 years[1,2]. Comparing it with other advanced reproductive technology procedures, intrauterine insemination offers a simple, less expensive, and more acceptable treatment for infertility couples especially for treatment of male infertility problems. Until now intrauterine insemination has become one of the most widely used and is routinely performed in most assisted reproduction centers. There are many causes of male infertility which were suitable to treat by IUI such as oligozoospermia, asthenozoospermia, teratozoospermia, ejaculatory dysfunction, cervical factors, and endometriosis as well as unexplained infertility. In case of sperm cryopreservtion, which is routinely performed in many ART clinics, it provides a poor sperm quality after thawing, so IUI is an important procedure for improving and selection of the high sperm quality[3]. In addition, many previous publications presented the usefulness of this procedure and effectiveness as well. Overall pregnancy rates with IUI were around 10-20%[4-6]. Before performing intrauterine insemination, sperm preparation was

Correspondence to: Petyim S, Infertility Unit, Faculty of Medicine, Siriraj Hospital, Bangkok 10700, Thailand

required to improve fertilization capacity of the spermatozoa. The ideal sperm preparation method should be able to select normal sperm including normal motility and normal morphology from the ejaculated semen and to minimize contamination and iatrogenic damage to sperm during processing[7]. Moreover, many sperm preparation techniques, which were created such as discontinuous percoll gradient, swim up, sephadex beads filtration[8-10] and glass wool filtration[11,12], provide a high recovery of motile spermatozoa. But some of those methods can't remove the bulk of seminal plasma efficiently as well as, sperm damage which can be caused by sperm preparation and will generate reactive oxygen species[13]. Now there are a few methods which are commonly performed. Firstly, the swim up method is mostly used and regularly performed for sperm preparation. Although this method, which uses self-migration of spermatozoa as a method principle completely removes seminal plasma and debris. They, after sperm preparation, often have a low yield especially in oligozoospermia or asthenozoospermia[14,15], so that they need a sufficient number of motile spermatozoa prior to preparation for such a method. The other method is Percoll gradient centrifugation. This method selects spermatozoa on the basis of their specific gravidity and normal morphology and effectively removes the bulk of seminal plasma, and eliminates round cells and debris. It provides low reactive oxygen species activity[13,16,17] as well. The advantage of this method, more over, can be used in cases with low sperm concentration. Since Percoll preparation is greatly simplified, sperm selection using this technique is easily and routinely performed in many clinics.

Because of the apparently low probability of success with IUI, many clinicians will not recommend the IUI procedure as an initial treatment for a male patient who has poor semen quality especially with severe oligozoospermia for which IVF or ICSI was recommended as a treatment instead. However, some couples who have a severe male factor and are suitable for IVF or ICSI treatment cannot afford its high cost. In addition, the invasiveness of IVF procedure is a factor that affects the patient's decision-making. For the reasons above, they, therefore, often decide to undergo IUI instead of IVF despite the poor outcome. Improving sperm recovery rate by enhancing sperm motility, after sperm preparation, is one of important factors and is necessary for IUI outcome especially in cases with abnormal semen-ejaculated oligozoospermia, oligo-asthenozoospermia and asthenospermia.

The objective of the present study was to determine the effect of sperm preparation using two-layer discontinuous Percoll gradient method on sperm quality: sperm recovery rate, post wash percentage motile sperm count, and percentage of post wash total motile sperm count.

Material and Method

During the period of January 1, 2002 through December 31, 2007, data including semen analysis before and after IUI procedure were reviewed in all male patients who were referred to the andrology laboratory for sperm washing and IUl. Only complete data for semen analysis and post wash sperm analysis was included in the present retrospective study.

Semen specimens were collected from 1400 male partners, with abstinence for 3-5 days and attended the infertility clinic for IUI, by masturbation in sterile containers. After being liquefied at room temperature, semen analysis was performed within 60 minutes after delivery to the laboratory. Semen volume, sperm concentration, percentage of sperm motility and viability were measured according to World Health Organization (WHO) guidelines[18,19]. Consequently, each semen sample was processed by two-layer discontinuous percoll gradient method after that sperm evaluation was done.

Two-layer discontinuous Percoll gradient

Semen preparation was performed by double-gradients technique, with two-layer (80%, 40%) discontinuous Percoll. After semen was completely liquefied, 1 mL of this semen was layered on the top of the tube which contained 1 mL of 80% percoll on the top and 1 mL of 40% percoll on the below. After centrifugation for 20 min at 600 g, the upper layer seminal plasma mixing with percoll was aspirated and the pellet, which contained the selected spermatozoa, was collected from the bottom of the tube. Then, washing with Hams F10 medium and centrifuged at 300 g for 10 min were done twice for removable of residual percoll. After last centrifugation, the media on the top were removed, and the pellet on the bottom was re-suspended in an IVF medium. Since the incubation at 37°C, 5% CO_2 for 30 minutes, sperm parameters were re-evaluated before IUI.

Assessment of sperm concentration

Approximately 10 µl drop of the semen was placed on the lower section of the 10 □m deep Makler counting chamber, of which the lower surface was

engraved with a grid covering a total area of 1 mm^2, subdivided into 100 small squares each of 0.01 mm^2, and immediately covered with a glass cover slip upper part. Sperm was counted in 10 small squares using a bright-field light microscope magnification of x 400. The number of sperm count is a sperm concentration (Million per milliliter).

Assessment of sperm motility

Approximately 10-20 µl drop of semen was placed on a slide then covered with a glass cover slip. A total of 100 sperm was counted at 400x with bright field microscopy. A motile sperm was estimated and divided into 4 categories; category A - Rapid forward progression spermatozoa, category B - Movement with forward motion spermatozoa, category C - Motion with no forward progression, categorie D - No motion.

Assessment of sperm Viability

The test was performed by mixing a drop of semen with equal drop of eosin Y and nigrosin. The mixture was then smeared on a slide and wait for air-dried. A total 100 spermatozoas were scored per smear with a bright field microscope. Abnormal spermatozoa which exhibited abnormal membrane structure permitted dye to enter the cell, the sperm was dye stained, while spermatozoa with normal membrane structure remained unstained (75% or more live spermatozoa is considered normal).

Data analysis

The data were analyzed using Computer program SPSS for Microsoft Windows version 10.0 (Chicago, IL). Baseline characteristic was presented using descriptive statistics. Comparison of quantitative variables such as total sperm concentration, total motile sperm count before and after sperm preparation was done by pair t-test. To test the difference in total sperm recovery rate, total sperm motility rate between four different semen groups, one way ANOVA was applied with the Dunnette test for multiple comparisons of each group against control. A simple logistic regression was used to assess the relationship between log 10 (total motile sperm count before sperm preparation) and total motile sperm count post sperm preparation $\geq$ 10 million. The level of significance was set at $p < 0.05$.

Total sperm recovery rate and Total motile sperm recovery rate were accordingly calculated as follows[20]:

$$\text{Total sperm recovery rate (\%)} = \frac{\text{Volume X Sperm concentration after sperm preparation X 100}}{\text{Volume X Sperm concentration before sperm preparation}}$$

$$\text{Total motile sperm recovery rate (\%)} = \frac{\text{Volume X Sperm concentration X Sperm motility after sperm preparation X 100}}{\text{Volume X Sperm concentration X Sperm motility before sperm preparation}}$$

Results

Mean ages of the semen donor were 33.6 $\pm$ 4.51 years old. The semen characteristics before sperm preparation are presented in Table 1. The sperm quality, comparing between pre- and post sperm preparation using bi-layers percoll technique, is shown in Table 2. After sperm preparation, both sperm concentration and progressive sperm motility significantly increased, while total motile sperm count significantly decreased. Moreover, the percentage of motile sperm recovery rate and total sperm recovery rate was higher after sperm preparation, around 59.88 $\pm$ 19.26% and 34.03 $\pm$ 14.58% respectively.

Categorizing semen parameters into 4 groups: normozoospermia, oligozoospermia, astenozoospermia and oligo-astenozoospermia, sperm motility in each group, compared with sperm motility prior to preparation, significantly improved after sperm preparation (Table 3). Furthermore, the motile sperm recovery rate in each group, which is presented in Table 4, significantly increased except for astenozoospermia. Total sperm recovery rate in oligozoospermia was significantly higher than normozoospermia, yet the others were significantly lower.

Table 5 shows comparison between 2 groups of sperm post preparation using cut off level as 10

Table 1. Baseline characteristics of semen analysis (mean $\pm$ SD)

	Mean $\pm$ SD
Age (yr)	33.60 $\pm$ 4.52
Semen parameter	
Volume (mL)	1.85 $\pm$ 0.78
Sperm concentration ($x10^6$/mL)	30.23 $\pm$ 14.43
Total motile sperm count ($x10^6$)	24.57 $\pm$ 17.88
Sperm motility (%)	
Progressive motility	43.66 $\pm$ 8.74
A	19.12 $\pm$ 5.83
B	24.54 $\pm$ 4.89
C	23.66 $\pm$ 5.26
D	32.68 $\pm$ 8.81
WBC ($x10^6$)	0.28 $\pm$ 0.38

Table 2. Semen parameters before and after sperm preparation

	Before		After		p-value*
	Mean	SD	Mean	SD	
Total sperm concentration ($x10^6$/mL)	30.29	14.42	35.97	24.78	<0.001
Total motile sperm count ($x10^6$)	24.57	17.88	13.42	8.85	<0.001
Progressive sperm motility (%)	43.66	8.74	81.12	18.74	<0.001
Calculated value (%)					
Total sperm recovery rate	34.03 (SD 14.58)				
Motile sperm recovery rate	59.88 (SD 19.26)				

* Paired t-test

Table 3. Semen parameters presenting as 4 different categories before and after sperm preparation

	Motility before (%)		Motility after (%)		p-value*
	Mean	SD	Mean	SD	
Normozoospermia	55.39	5.50	84.71	16.22	<0.001
Oligozoospermia	56.21	5.31	86.24	6.86	<0.001
Astenozoospermia	41.24	5.59	79.72	41.24	<0.001
Oligoastenozoospermia	37.44	7.66	81.27	15.29	<0.001

* Paired t-test

Table 4. Recovery rate before and after sperm preparation for 4 different categories of abnormality

	Total sperm recovery rate (%)			Motile sperm recovery rate (%)		
	Mean	SD	p-value#	Mean	SD	p-value#
Normozoospermia	39.05	15.21	-	57.61	19.72	-
Oligozoospermia	44.10	14.15	<0.001	66.84	18.54	<0.001
Astenozoospermia	33.26	14.45	<0.001	59.45	18.89	0.05
Oligoastenozoospermia	29.52	11.88	<0.001	62.40	19.69	<0.001

Pairwise comparison of each group vs. normozoospermic group as a control using Dunnett

Table 5. Comparison of total motile count before sperm preparation (TMC1) between total motile sperm count after sperm preparation (TMC2) of less than and more than 10 million per milliliter

	Total motile count before sperm preparation (TMC1)				
	Mean	SD	Median	Range	p-value
TMC2 < 10 mil/mL	13.57	8.91	11.75	0.34-94.75	-
TMC2 ≥ 10 mil/mL	31.71	18.60	26.88	10.56-223.65	<0.0001

million, total motile count before sperm preparation in total motile sperm count equal or exceed 10 million after preparation was higher than those below 10 million. The difference between them reached statistical significance. Total motile sperm count before sperm preparation, which was the product of sperm concentration, progressive motility and semen volume, was a good predictor of total motile sperm count after preparation as shown in the result of logistic regression analysis (Table 6). The total motile sperm count before preparation can be used to predict the probability of having total motile sperm count after preparation of ≥ 10 million as shown in Fig. 1.

Table 6. Logistic regression analysis of total motile sperm count (TMC) post preparation ≥ 10 million based on T total motile count before sperm preparation (TMC1)

	Regression coefficient	SE	p-value
Constant	-10.449	0.342	<0.0001
Log 10 (TMC1)	8.582	0.269	<0.0001

Note: Logistic regression equation:

$$\Pr(\text{TMC post preparation} > 10 \text{ million}) = \frac{e^{Z}}{1 + e^{Z}}$$

where Z = -10.449 + 8.582 log 10 (TMC1)

TMC1 = Total motile sperm count before sperm preparation

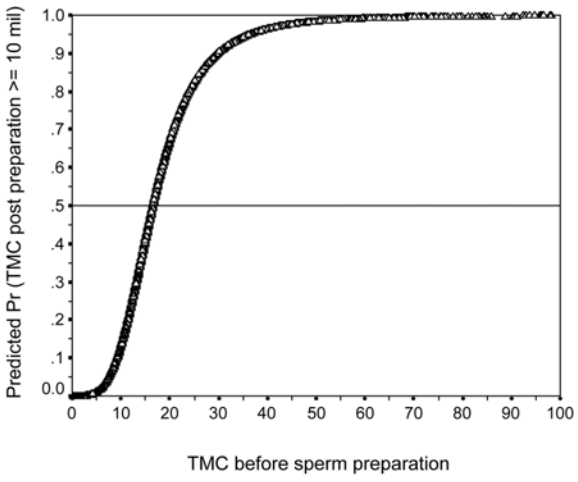

Fig. 1 Predicted probability of TMC post preparation ≥ 10 million based on TMC before preparation

Discussion

The present study was designed to determine not only outcome after sperm preparation, but also the chance to rescue poor semen quality. More than half of the husbands who seek infertility treatment have problems related to abnormal semen qualities. Although advance technology such as *in vitro* fertilization or intracytoplasmic sperm injection can be used for infertility treatment in a couple who have poor semen quality, cost of treatment is one of the greatest obstacles. Instead, some of them may choose to do IUI despite the lower chance of achieving conception. The factors which could certainly be a predictor of the succession of IUI are semen parameters especially sperm motility after preparation. Previous studies reported that poor sperm motility was related to poor pregnancy rate[6,21]. Sperm preparation before IUI, therefore, which possibly improves the poor semen quality by selecting only motile sperm and increasing concentration of sperm, could increase the chance of pregnancy[4].

In the present study, all sperm parameters such as total sperm concentration and progressive sperm motility were significantly increased, but total motile sperm count was significantly decreased. These results may relate to the effectiveness of sperm preparation in separating immotile or poor motility; the total progressive sperm count, therefore, was decrease. However, total sperm and motile sperm recovery rate were high. When categorizing following WHO guide, the present study also confirmed that sperm motility dramatically recovered in all 4 groups of semen samples- normozoospermia, oligozoospermia, astenozoospermia and oligo-astenozoospermia. This corresponded to a previous study[20].

Compared with motile sperm recovery rate of normozoospermia semen sample, sperm preparation also provided a high effectiveness for recovering sperm motility on the others as showing higher percentage of motile sperm recovery rate, but only oligozoospermia and oligoastenozoospermia groups reach statistical significance. Moreover, in the aspect of total sperm recovery, only semen samples in the oligozoospermic group significantly improved after sperm preparation. In contrast, sperms, which is abnormal motility-factor related, such as astenozoospermia, oligo-astenozoospermia significantly decreased, compared with normozoospermic group.

Following sperm preparation process, total motility sperm was expected to be normal[18] or exceed 10 million which was accepted as the threshold value

for performing IUI[21]. The present study demonstrated that sperm parameters before sperm preparation including semen volume, sperm concentration, progression sperm motility and total motile count strongly associated with total motile sperm count after sperm preparation using logistic regression analysis. In addition, when comparing with the group of total motile sperm count below 10 million after sperm preparation, mean and median in the group of total motile count equal or more than 10 million were higher.

Although sperm preparation of poor semen quality was routinely performed, it cannot guarantee the success of sperm recovery. The predictable value for succession of sperm recovery, therefore, was important information especially for counseling to the patients. The present study demonstrated a predicting curve of the probability of the succession of sperm recovery. It showed that a 50 percent chance of succession of sperm recovery has to have total motile sperm count before sperm preparation of around 18 million. Although the curve cannot directly predict the pregnancy rate, it can be used as an indirect predictor-tool for indirectly predictor of achieving conception especially in a couple who has only a male factor. Further studies should be performed including the effectiveness of sperm preparation relating to the other characteristics of sperm such as sperm apoptosis and DNA fragmentation.

Conclusion

Sperm preparation using double-gradient percoll provided a high percentage of motile sperm recovery rate and total sperm recovery rate. It also dramatically improved progressive sperm motility in normozoospermia, oligozoospermia, astenozoospermia and oligo-astenozoospermia.

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ความสำเร็จของการปั่นแยกน้ำเชื้ออสุจิเพื่อคัดเลือกตัวอสุจิที่ดีโดยวิธีการปั่นแยกแบบดับเบิลเกรเดียนในโรงพยาบาลศิริราช

สมสิญจน์ เพ็ชรยิ้ม, เรืองศิลป์ เชาวรัตน์, สิงห์เพชร สุขสมปอง, พิทักษ์ เลาหเกริกเกียรติ, อรวรรณ เมฆมหรรณพ์

วัตถุประสงค์: *เพื่อศึกษาถึงผลสำเร็จของการปั่นแยกน้ำเชื้ออสุจิเพื่อคัดเลือกตัวอสุจิที่ดีก่อนที่จะนำไปใช้โดยวิธีการปั่นล้างแบบดับเบิลเกรเดียน (Double gradients technique) ด้วยน้ำยา Percoll*

วัสดุและวิธีการ: *ทำการรวบรวมข้อมูลผลการตรวจน้ำเชื้ออสุจิก่อนและหลังการเตรียมก่อนฉีดเชื้อเข้าสู่โพรงมดลูก จำนวน 1,400 ราย ทำการเปรียบเทียบและคำนวณข้อมูลของการตรวจน้ำเชื้ออสุจิก่อนเตรียมกับผลของการตรวจน้ำเชื้ออสุจิ หลังการเตรียม*

ผลการศึกษา: *ผลการศึกษาพบว่าหลังจากเตรียมน้ำเชื้ออสุจิโดยวิธีปั่นแยกแบบดับเบิลเกรเดียน จะมีความเข้มข้นของตัวอสุจิทั้งหมดรวมถึงร้อยละของตัวอสุจิที่เคลื่อนไหวปกติเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ในขณะที่จำนวนตัวอสุจิที่เคลื่อนไหวทั้งหมดลดลงอย่างมีนัยสำคัญทางสถิติ ในส่วนของอัตราการแยกตัวอสุจิที่ดี พบสูงถึงร้อยละ 59.88 ± 19.26 และอัตราการแยกตัวอสุจิที่เคลื่อนไหวปกติ พบร้อยละ 34.03 ± 14.58 เมื่อแบ่งกลุ่มน้ำเชื้อเป็น 4 กลุ่ม ได้แก่ กลุ่มที่น้ำอสุจิปกติ กลุ่มน้ำอสุจิที่มีความเข้มข้นน้อย กลุ่มน้ำอสุจิมีการเคลื่อนไหวช้า และกลุ่มน้ำอสุจิมีความเข้มข้นน้อยและเคลื่อนไหวช้า พบว่าร้อยละของตัวอสุจิที่เคลื่อนไหวปกติเพิ่มขึ้นอย่าง มีนัยสำคัญทางสถิติในทุกกลุ่มเมื่อเปรียบเทียบกับผลของตัวอสุจิก่อนเตรียม ในขณะที่อัตราการแยกตัวอสุจิที่ดี ในกลุ่มที่น้ำอสุจิมีความเข้มข้นน้อยพบสูงกว่าน้ำอสุจิในกลุ่มที่ปกติ อย่างมีนัยสำคัญทางสถิติ*

สรุป: *การปั่นแยกน้ำเชื้ออสุจิโดยวิธีแบบดับเบิลเกรเดียน สามารถคัดเลือกตัวอสุจิที่มีการเคลื่อนไหวดี ทำให้จำนวนความเข้มข้นของตัวอสุจิที่ดีอยู่ในเกณฑ์ปกติ นอกจากนี้การปั่นแยกน้ำอสุจิในกลุ่มที่ปกติ น้ำอสุจิที่มีความเข้มข้นน้อยหรือ มีการเคลื่อนไหวช้ารวมถึงในรายที่น้ำอสุจิมีความเข้มข้นน้อยและเคลื่อนไหวช้า สามารถปั่นแยกตัวอสุจิโดยสามารถคัดตัวที่เคลื่อนไหวปกติโดยมีอัตราความสำเร็จที่สูง*