Comparison of Two-layer Percoll Gradient and Mini-Percoll Methods for Sperm Preparation

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Abstract

To compare the efficiency of sperm preparation between the two-layer Percoll gradient and mini-Percoll methods, 50 normal and 33 abnormal semen samples from male partners of infertile couples were studied. The number of recovered spermatozoa, percentage of motility, percentage of normal morphology, and their survival at 24 and 48 hours were assessed. Both Percoll gradient techniques resulted in a significantly higher percentage of motility and percentage of normal morphology compared with the original semen samples ($p<0.0001$). The two-layer Percoll gradient showed a higher sperm recovery than the mini-Percoll method ($p<0.001$), but the latter resulted in a higher percentage of motility ($p>0.001$) and a higher sperm survival rate at 24 hours ($p<0.05$) than the former, regarding normal semen samples. These differences did not appear with abnormal semen samples when analyzed as a group. Considering each of the abnormal parameters separately, sperm recovery was significantly higher after the two-layer Percoll gradient in the case of astheno - and teratozoospermia ($p<0.05$), but sperm survival at 48 hours was higher after the mini-Percoll gradient in the case of teratozoospermia ($p<0.05$). It is concluded that both the two-layer Percoll gradient and mini-Percoll method can be used effectively for sperm preparation. The former yields a higher sperm recovery, but the latter should be considered regarding teratozoospermic samples and semen samples of very low volume.

Suitable and effective techniques of sperm preparation have been necessary for the success of assisted reproductive techniques such as intrauterine insemination (IUI), *in vitro* fertilization and embryo transfer (IVF-ET), and gamete intrafallopian transfer (GIFT). An ideal technique should be able to select a high proportion of spermatozoa with normal morphology and fast progressive moti-

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lity, while eliminating non-motile or dead sperm, cellular debris, and seminal plasma.

Several methods of sperm preparation have been developed, including self-migration[1], washing and swim-up[2], albumin gradients[3], Percoll gradients[4,5], and glass wool filtration[6]. Among all these techniques, the washing and swim-up, and discontinuous two-layer Percoll gradient methods have been widely used in Thailand. Percoll, a medium composed of a colloidal solution of silica particles coated with polyvinylpyrrolidone, has been found to improve the isolation of motile spermatozoa, generally free from contamination by other seminal constituents. Recently, most IVF laboratories in Thailand have been using the Percoll gradient as the sperm preparation method for couples with male factor. Although the Percoll gradient method has been claimed to improve fertilization rates *in vitro*[7,8] and high clinical pregnancy rates[9], sperm recovery rate is low in very poor semen samples[8]. Ord et al[10] developed a small volume (mini-Percoll) gradient method and obtained good fertilization and pregnancy rates for severe oligoasthenozoospermic samples.

The purpose of this study was to compare sperm parameters and *in vitro* sperm survival between conventional two-layer Percoll gradient and mini-Percoll techniques for both normal and abnormal semen samples.

MATERIAL AND METHOD

Semen samples

Eighty-three semen samples were obtained by masturbation after 3 to 5 days of sexual abstinence from male partners of couples attending the infertility clinic from January to September 1996. After liquefaction, semen analyses were carried out following the World Health Organization recommendations[11]. Samples were defined as normal if the sperm concentration was > 20 x 10^6/ml, the percentage of motility was > 50 per cent, and normal morphology was > 30 per cent. Samples were considered abnormal if one or more of the semen parameters were outside the normal range.

Two-layer Percoll gradient

The 100 per cent stock Percoll solution was prepared by diluting nine parts Percoll (Pharmacia, Uppsala, Sweden) with one part of 10 times concentrated Ham's F-10 medium (GIBCO, Life Technologies, U.S.A.). The 80 and 40 per cent Percoll solutions were prepared by diluting the 100 per cent stock Percoll solutions with human tubal fluid (HTF) medium supplemented with 10 per cent human serum albumin (HSA).

The two-layer discontinuous gradient was formed by layering 1.5 ml of the 40 per cent Percoll on 1.5 ml of the 80 per cent Percoll in a 15-ml Falcon conical centrifuge tube (No. 2001, Becton Dickinson, U.S.A.) After liquefaction, 1 ml aliquots of semen were gently layered onto the top of the Percoll gradient and contrifuged in a swinging bucket rotor for 20 minutes at 500 x g. After centrifugation, semen and 40 per cent Percoll layers were aspirated off until the 80 per cent Percoll interface was reached. The remaining 80 per cent layer was washed once with 2 ml HTF medium by centrifuging at 200 x g for 10 minutes. The final sperm pellet was resuspended in 0.5 ml of the 10 per cent HSA supplemented HTF medium. The number of spermatozoa was counted, and motility and morphology were estimated. The preparation was incubated at 37°C with 5 per cent CO_2 concentration. The percentage of motility was assessed again after 24 and 48 hours of incubation.

Mini-Percoll gradient

The 95, 70, and 50 per cent Percoll solutions were prepared by dilution the 100 per cent stock Percoll solution with 10 per cent HSA-supplemented HTF. The mini-Percoll gradient was established by carefully pipetting 0.3 ml of 95, 70, and finally 50 per cent Percoll in a 5- ml Falcon conical centrifuge tube (No. 2003). The 0.3 ml aliquots of the same semen sample were layered onto the Percoll gradient and centrifuged for 20 minutes at 500 x g. After centrifugation, semen and the upper two layers of Percoll were aspirated off. The remaining 95 per cent layer was washed once by diluting it in 2 ml of HTF medium, and centrifuged at 200 x g for 10 minutes. The final pellet was resuspended in 0.5 ml of the 10 per cent HSA-supplemented HTF medium. The sperm count, motility, and morphology were determined. Again, the percentage of motility was assessed after 24 and 48 hours of incubation at 37°C with 5 per cent CO_2 concentration.

Statistical analysis

Results were expressed as the mean value ± standard deviation (SD). Parameters that showed normal distribution were compared by the *t*-test or

ANOVA for comparison of the mean values. As for the parameters that did not have a normal distribution, the non-parametric test of Wilcoxon was performed.

RESULTS

Of the 83 semen samples, 50 were found to be normal, 8 were oligozoospermia, 8 were asthenozoospermia, 4 were teratozoospermia, 8 had double parameter defects, and 5 had triple defects. Semen samples with abnormal parameters are analysed as a group in Table 2, and separately in Table 3 and 4.

In the normal and abnormal semen samples, both the two-layer Percoll and mini-Percoll gradient centrifugation resulted in a significantly higher percentage of motility and percentage of normal morphology ($P<0.0001$) compared with the original semen samples (Table 1 and 2).

However, in the normal semen samples, comparison between the two preparation methods showed a significantly higher sperm recovery (37.8 ± 18.9 *versus* 20.0 ± 14.3 ; $p < 0.001$) applying the two-layer Percoll gradients, but higher percentage of motility (77.2 ± 12.0 *versus* 69.0 ± 11.4 ; $p < 0.001$) and *in vitro* survival after 24 hours (66.5 ± 16.2 *versus* 58.0 ± 16.4 ; $p < 0.05$) applying the mini-Percoll method (Table 1). There were no statistically significant differences in semen parameters between these two methods with respect to the abnormal semen samples (Table 2).

Considering each of the abnormal parameters, the results showed that in astheno - and teratozoospermic samples, sperm recovery was significantly higher after the two-layer Percoll gradient compared to that obtained after the mini-Percoll method ($P<0.05$). However, *in vitro* survival of the sperm after 48 hours was higher applying the mini-Percoll gradient with teratozoospermic samples ($p < 0.05$, Table 3).

Regarding semen samples with double and triple parameter defects, there was no statistically significant difference as to sperm recovery, motility, *in vitro* survival, and normal morphology between two-layer Percoll and mini-Percoll gradient techniques (Table 4).

DISCUSSION

A variety of sperm preparation methods and function tests are available for new reproductive technology. Several studies have compared different procedures of sperm preparation, especially

Table 1. Semen parameters before and after separation procedures from normal semen samples (n = 50).

	Original	2-layer Percoll	mini-Percoll
Concentration (x 10^6/ml)	82.9 ± 44.8	37.8 ± 18.9*	20.0 ± 14.3*
Motility (%)	54.9 ± 5.8	69.0 ± 11.4*	77.2 ± 12.0*
24 h		58.0 ± 16.4**	66.5 ± 16.2**
48 h		44.1 ± 16.2	48.0 ± 16.0
Normal morphology (%)	42.8 ± 9.7	70.7 ± 11.1	71.6 ± 10.4

* $P < 0.001$
** $P < 0.05$

Table 2. Semen parameters before and after separation procedures from abnormal semen samples (n = 33).

	Original	2-layer Percoll	mini-Percoll
Concentration (x 10^6/ml)	49.7 ± 48.8	22.4 ± 22.0	11.8 ± 15.0
Motility (%)	41.3 ± 14.3	65.6 ± 11.3	64.0 ± 18.1
24 h		56.2 ± 16.3	56.4 ± 14.5
48 h		36.9 ± 11.5	40.5 ± 14.5
Normal morphology (%)	30.7 ± 18.0	62.8 ± 13.0	62.7 ± 11.9

Table 3. Results of sperm preparation from oligozoospermic, asthenozoospermic, and teratozoospermic samples.

	Oligozoospermia (n=8)		Asthenozoospermia (n=8)		Teratozoospermia (n=4)	
	2-layer Percoll	mini-Percoll	2-layer Percoll	mini-Percoll	2-layer Percoll	mini-Percoll
Concentration (x 10^6/ml	11.3 ± 17.5	7.4 ± 10.4	42.4 ± 24.8*	16.5 ± 15.7*	32.3 ± 14.3*	9.8 ± 2.7*
Motility (%)	66.9 ± 13.2	74.8 ± 12.2	67.0 ± 12.8	66.5 ± 13.7	70.5 ± 6.8	69.5 ± 21.9
24 h	61.8 ± 10.9	61.9 ± 12.0	52.0 ± 15.8	59.1 ± 17.5	70.3 ± 19.4	63.3 ± 6.2
48 h	48.9 ± 10.3	44.1 ± 14.0	33.1 ± 13.0	42.3 ± 11.8	38.0 ± 5.0*	57.8 ± 15.4*
Normal morphology (%)	68.9 ± 12.3	69.4 ± 7.8	65.2 ± 7.6	64.5 ± 9.3	56.5 ± 18.1	52.0 ± 13.5

* $p < 0.05$

Table 4. Results of sperm preparation from double and triple defects semen samples.

	Double defects (n = 8)		Triple defects (n = 5)	
	2-layer Percoll	mini-Percoll	2-layer Percoll	mini-Percoll
Concentration (x 10^6/ml)	21.4 ± 15.8	18.9 ± 22.0	2.0 ± 1.3	1.4 ± 1.2
Motility (%)	58.8 ± 10.1	53.1 ± 21.8	68.6 ± 9.5	56.0 ± 15.7
24 h	48.8 ± 16.6	47.1 ± 13.7	54.4 ± 17.6	52.4 ± 15.2
48 h	32.8 ± 8.4	29.6 ± 11.6	32.2 ± 9.2	36.0 ± 9.3
Normal morphology (%)	60.0 ± 14.0	62.0 ± 14.0	55.3 ± 16.2	58.7 ± 16.0

the two most widely used : the swim-up and the Percoll gradients. However, the two-layer discontinuous Percoll gradient method is gaining popularity as it yields greater recovery of sperm with higher motility and improved sperm function[5,7,12,13,14]. Its reported application for oligoasthenozoospermic samples has been variable, and the mini-Percoll gradient method has been claimed to produce better results with severe oligoasthenozoospermic samples[10]. In this study, sperm preparation by the conventional two-layer Percoll gradient was compared to the mini-Percoll method. Sperm parameters were evaluated for motility, normal morphology and *in vitro* sperm survival because these are known to correlate with the *in vitro* fertilization potential[15-17].

Our results show that for normal semen samples, the two-layer Percoll gradient yielded better sperm recovery, but less sperm motility and survival after 24 hours than the mini-Percoll gradient methods. When 33 abnormal semen samples were pooled and analysed as a single group, there were no statistically significant differences between sperm parameters obtained by these two methods of sperm preparation. However, when abnormal parameters were analysed separately, the recovery of spermatozoa was significantly higher with the two-layer Percoll gradient method for astheno-and teratozoospermic samples, but the sperm survival after 48 hours was higher with the mini-Percoll method for teratozoospermic samples. The results were slightly different according to the study of Ng et al[18] which showed no significant differences between the Percoll and mini-Percoll methods for all groups of abnormal semen parameters. Ruiz-Romero et al[19] compared five different combinations of Percoll concentrations and showed that two-layer gradients with low volume appeared to be superior to other combinations regarding samples with oligo - and/or asthenozoospermia. The mini-Percoll method was also reported to be advantageous in handling sperm surgically retrieved from epididymis[20].

In conclusion, both the conventional two-layer Percoll gradient and the mini-Percoll methods can be used effectively in assisted reproductive technologies, because they result in a significantly higher percentage of motility and normal morpho-

logy, with good sperm survival up to 48 hours. The former seems to be superior to the latter with respect to sperm recovery. But the latter should be used in teratozoospermic samples, with a semen volume of less than 0.5 ml, including sperm specimens obtained by epididymal aspiration or testicular extraction.

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เปรียบเทียบการเตรียมอสุจิระหว่างวิธีปั่นแยกด้วยน้ำยาเปอร์คอลล์สองชั้น และน้ำยาเปอร์คอลล์จำนวนน้อย

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ได้ศึกษาเปรียบเทียบถึงประสิทธิผลของการเตรียมอสุจิระหว่างวิธีปั่นแยกด้วยน้ำยาเปอร์คอลล์สองชั้น และน้ำยาเปอร์คอลล์จำนวนน้อย โดยศึกษาจากน้ำอสุจิของคู่สมรสฝ่ายชายที่มารักษาที่คลินิกผู้มีบุตรยากตั้งแต่เดือนมกราคมถึงเดือนกันยายน 2539 จำนวน 83 ราย เป็นน้ำอสุจิที่ปกติ 50 ราย และผิดปกติ 33 ราย ได้ประเมินเกี่ยวกับจำนวนอสุจิที่แยกได้ อัตราการเคลื่อนที่ อัตราของอสุจิที่มีลักษณะปกติ และอัตราการอยู่รอดที่ 24 และ 48 ชั่วโมง พบว่าการเตรียมอสุจิทั้ง 2 วิธี ทำให้ได้อสุจิที่มีอัตราการเคลื่อนที่ และลักษณะปกติสูงขึ้นอย่างมีนัยสำคัญ เมื่อเปรียบเทียบกับน้ำอสุจิเดิมก่อนเตรียม ($p < 0.0001$) ในน้ำอสุจิที่ปกติพบว่าแม้วิธีเปอร์คอลล์สองชั้นจะสามารถแยกได้จำนวนอสุจิที่สูงกว่า ($p < 0.001$) แต่อัตราการเคลื่อนที่และอัตราการอยู่รอดที่ 24 ชั่วโมง กลับต่ำกว่าวิธีเปอร์คอลล์จำนวนน้อย ($p < 0.001$ และ $p < 0.05$ ตามลำดับ) ในน้ำอสุจิที่ผิดปกติ ไม่พบความแตกต่างอย่างมีนัยสำคัญระหว่างวิธีการเตรียมอสุจิทั้ง 2 วิธี อย่างไรก็ตาม เมื่อแยกพิจารณาความผิดปกติแต่ละชนิดของอสุจิพบว่า วิธีเปอร์คอลล์สองชั้นจะแยกได้จำนวนอสุจิสูงกว่าวิธีเปอร์คอลล์จำนวนน้อย ในกลุ่มที่มีความผิดปกติในการเคลื่อนที่และกลุ่มที่อสุจิลักษณะผิดปกติ ($p < 0.05$) แต่อัตราการอยู่รอดที่ 48 ชั่วโมง ของอสุจิกลุ่มที่มีลักษณะผิดปกติ กลับได้ผลดีกว่า เมื่อแยกด้วยวิธีเปอร์คอลล์จำนวนน้อย ($p < 0.05$) สรุปได้ว่าทั้งวิธีเปอร์คอลล์สองชั้น และวิธีเปอร์คอลล์จำนวนน้อย ต่างสามารถใช้เตรียมอสุจิอย่างได้ผลดี วิธีแรกแยกได้จำนวนอสุจิที่มากกว่า แต่วิธีหลังใช้ได้ผลดีในน้ำอสุจิที่มีลักษณะผิดปกติ และน้ำอสุจิที่มีปริมาตรต่ำมาก

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