Blackwell Publishing

Asian J Androl 2005; 7 (1): 86–92
DOI: 10.1111/j.1745-7262.2005.00008.x

AJA

·*Original Article*·

Comparative study on density gradients and swim-up preparation techniques utilizing neat and cryopreserved spermatozoa

Shyam S.R. Allamaneni, Ashok Agarwal, Sreedhar Rama, Pavithra Ranganathan, Rakesh K. Sharma

Center for Advanced Research in Human Reproduction, Infertility and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, Cleveland Clinic Foundation, Cleveland, Ohio, USA

Abstract

Aim: To 1) compare post-wash and post-thaw parameters of sperm processed with PureSperm density gradient technique and swim-up method; and 2) test the efficacy of two commonly available density gradient media PureSperm and ISolate. **Methods:** This prospective study used semen specimens from 22 patients. Specimens from nine patients were processed by both PureSperm density gradient and swim-up method. These specimens were then cryopreserved. Thirteen specimens were processed by both PureSperm (40 % and 80 %) and Isolate (50 % and 90 %) double density gradient techniques. The two fractions processed by both PureSperm and swim-up were analyzed for post-wash sperm characteristics. Post-thaw analysis was done after 24 hours. Sperm fractions obtained after processing with PureSperm and ISolate were compared for post-wash sperm characteristics and ROS levels. **Results:** Specimens prepared with PureSperm had significantly higher median total motile sperm counts (TMSC) (32.2×10^6 *vs*. 17.6×10^6), recovery rates (69.2 % *vs*. 50.0 %), and longevity at 4 hours (83.0 % *vs*. 55.0 %) compared to specimen prepared by swim-up. Post-thaw specimens also had a higher recovery and longevity at 4 hours with PureSperm as compared to the swim-up. Semen specimens processed by PureSperm had significantly higher total sperm count, TMSC, and percentage recovery rates (30.0 % *vs*. 19.7 %) than ISolate. **Conclusion:** Semen quality is better preserved in fresh and cryopreserved semen prepared with PureSperm density gradient compared to swim-up. A significant enrichment of sperm is observed with PureSperm compared to ISolate. Higher recovery rates of mature motile sperm obtained after PureSperm sperm preparation may be beneficial for successful ART. *(Asian J Androl 2005 Mar; 7: 86–92)*

Keywords: spermatozoa; cryopreservation; density gradient centrifugation; sperm motility

1 Introduction

The rapid increase of assisted reproductive techniques (ART) as treatment modalities for infertility during the last two decades has led to the development of a wide range of different sperm preparation methods. Following the development of the classical swim-up method, more complicated techniques have been developed to improve the number of motile spermatozoa recovered

Correspondence to: Ashok Agarwal, Professor of Surgery; Director, Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Desk A19.1, Cleveland, OH 44195, USA.
Tel: +1-216-444-9485/444-4402
Fax: +1-216-636-3118/445-6049
E-mail: agarwaa@ccf.org
Received 2004-05-19 Accepted 2004-12-06

even in cases of severe andrological pathologies.

An ideal sperm preparation technique should involve the removal of seminal plasma efficiently and quickly. Although seminal plasma protects the spermatozoa from stressful conditions such as oxidative stress [1], it is endowed with senescent sperm, leukocytes, epithelial cells, particulate debris, and microbial contamination. Seminal plasma contains factors that inhibit the fertilizing ability of the spermatozoa and reduce the induction of capacitation [2, 3]. In addition, an ideal sperm preparation method should be cost-effective and allow for processing of a large volume of the ejaculate, which in turn maximizes the number of spermatozoa available [4]. The sperm preparation technique should also minimize the risk of reactive oxygen species (ROS) generation, as excessive ROS could adversely affect DNA integrity and sperm function *in vitro* [1, 5].

Sperm preparation methods are essentially integrated in semen cryopreservation protocols. Freezing of spermatozoa is a routine technique prior to ART, cancer therapy, or vasectomy [6]. Cryopreservation of spermatozoa results in a considerable decrease in motility and pregnancy rate compared to fresh semen [7, 8]. Therefore, pre-freeze specimens should be processed in order to enrich the specimen with higher-quality sperm cells [6, 7].

For the isolation of functionally normal spermatozoa, sperm migration techniques and gradient centrifugation remain the most popular methods [2]. Swim-up or sperm migration, which separates the sample into motile and non-motile fractions, and double density gradient media such as PureSperm (Nidacon, International AB, Gothenburg, Sweden), which separates spermatozoa according to their density, favors the isolation of motile and morphologically normal sperm [9]. Sperm preparation with the use of density gradient centrifugation has become a standard technique for sperm preparation for use in ART [10]. For density gradient centrifugation, sperm preparation media such as PureSperm, ISolate (Irvine Scientific, Santa Ana, CA), and IxaPrep (Medicult, Copenhagen, Denmark) were introduced to replace Percoll (Pharmacia Biotech AB, Uppsala, Sweden) [11] and concern has arisen over the utility and efficacy of these new products.

In this study, we compared the efficacy of the density gradient technique using PureSperm and standard swim up method and after cryopreservation of prepared sperm. We also compared the post-wash sperm characteristics including ROS levels of the sperm fractions obtained after processing of PureSperm and ISolate density gradient media.

2 Materials and methods

2.1 Subjects

The Cleveland Clinic Foundation Institutional Review Board approved this study. Semen specimens were obtained from 22 men who were evaluated for infertility in our laboratory. All specimens were collected by masturbation at the clinical andrology laboratory after a period of 48–72 hours of abstinence.

After liquefaction, each semen sample was analyzed manually for semen analysis. We performed two experiments. In the first experiment nine specimens were divided into two equal parts and processed by either density gradient technique using PureSperm medium or swim-up sperm preparation method (Figure 1). In the second

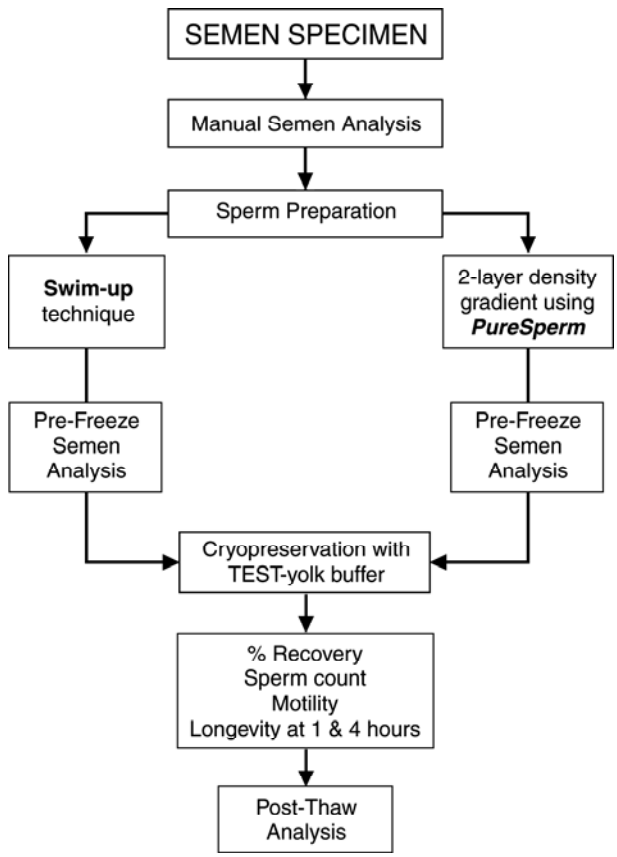

Figure 1. Flow diagram describing comparison between PureSperm density gradient technique and swim-up method.

experiment, after initial semen analysis (n = 13) two equal aliquots were prepared by density gradient technique using either PureSperm or ISolate (Figure 2). Sperm recovered by these preparation techniques were assessed for concentration and motility (type a + b) according to the World Health Organization guidelines [12]. Percentage recovery was calculated by dividing post-wash total motile sperm count with pre-wash total motile sperm count and multiplying by 100. Sperm recovered by the two density gradients in the second experiment were also assessed for levels of ROS. In addition, we compared the recovery rates of motile sperm in normospermic and oligospermic patients.

2.2 Sperm preparation by density gradients

PureSperm gradients 40 % and 80 % and ISolate gradients 50 % and 90 % were used for the experiment. All procedures were conducted under sterile conditions. Media were brought to 37 °C temperature. Using a sterile pipette 2.0 mL of the "lower layer" (80 % PureSperm gradient or 90 % ISolate gradient) was transferred into a conical centrifuge tube. Using a new sterile pipette 2.0 mL of the "upper layer" (40 % PureSperm gradient or 50 % ISolate gradient) was gently dispensed on top of the lower layer. A liquefied semen sample was then placed on top of the upper layer and the tube was centrifuged for 20 minutes at 330 ×g. The upper and lower layers were carefully aspirated without disturbing the pellet. Using a transfer pipette, 2–3 mL of modified human tubal fluid (mHTF, Sage BioPharma, Bedminster, NJ) was added and the re-suspended pellet was centrifuged for 7 minutes at 330 ×g. The supernatant was then removed and the pellet suspended in a volume of 0.5 mL of mHTF. Sperm count, motility, and ROS levels were estimated in the recovered fractions.

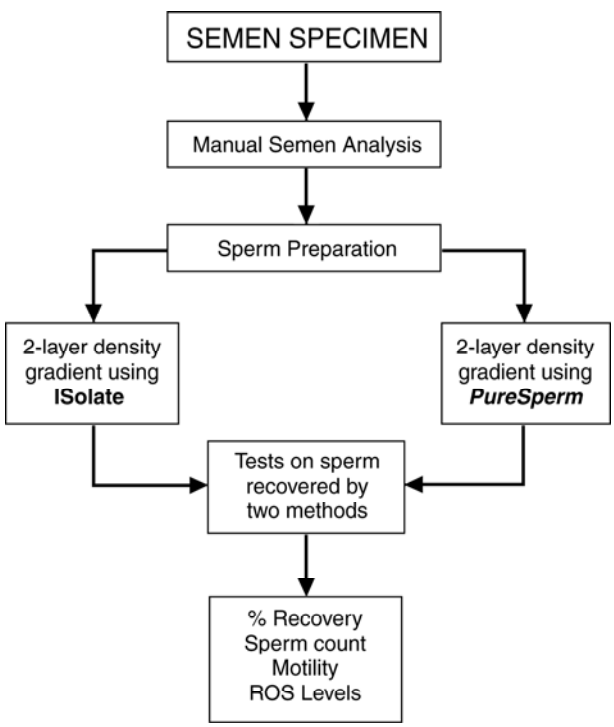

Figure 2. Flow diagram describing comparison between PureSperm and ISolate density gradient media.

2.3 Sperm preparation by the swim-up method

After liquefaction, an aliquot of the specimen was mixed with mHTF (1:4 v/v) using sterile Pasteur pipette and centrifuged at 330 ×g for 10 minutes. The supernatant was carefully aspirated and the pellet re-suspended in 3 mL of fresh mHTF. The re-suspended sample was transferred into two 15 mL sterile round bottom tubes using plastic pipettes and centrifuged at 330 ×g for 5 minutes. The tubes were then incubated at an angle of 45° for 1 hour in the incubator at 37 °C. After the incubation period, the entire supernatant was aspirated and centrifuged at 330 ×g for 7 minutes. The supernatant was aspirated and the pellet re-suspended in 0.5 mL of mHTF. The final volume was measured and the semen analysis performed on an aliquot of the sample.

2.4 Cryopreservation of spermatozoa

The semen specimen processed either by density gradient (n = 9) or swim-up (n = 9) was mixed with an aliquot of TES [N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid] and Tris (TEST) yolk buffer equal to 25 % of the original specimen volume to the centrifuge tube with a sterile pipette [13]. The mixture was then gently mixed in an aliquot mixer for 5 minutes and the process was repeated three times or until the TEST yolk buffer volume equaled the original specimen volume. The mixture was transferred into cryovials and frozen after loading them into cryocanes in the liquid nitrogen tanks. The specimen was thawed after 24 hours and post-thaw semen parameters were analyzed.

2.5 Reactive oxygen species measurement

Levels of ROS were measured by a chemiluminescence assay using luminol (5-amino-2, 3, -dihydro-1, 4-phthalazinedione; Sigma, St. Louis, MO, USA) as a probe.

The measurement used 400 μL aliquot of specimen, 10 μL of luminol, prepared as 5 mmol·L^{-1} stock in dimethyl sulfoxide (DMSO) were added. A negative control was prepared by adding 10 μL of 5 mmol·L^{-1} luminol to 400 μL phosphate buffered saline (PBS).

Autolumat LB 953 luminometer (Berthold Technologies, Bad-Wildbad, Germany) was used to measure chemiluminescence in the integrated mode for 15 minutes. The results were expressed as $\times 10^4$ counted photons per minute (cpm) per 20×10^6 sperm.

2.6 Statistical analysis

Comparisons between groups were performed with Wilcoxon matched-pairs signed-ranks test. $P < 0.05$ was considered significant using the two-tailed test. Data was analyzed using GraphPad Software Version 3.20 (GraphPad Software, Inc., San Diego, CA, USA).

3 Results

3.1 Sperm preparation by PureSperm density gradient and swim-up

The initial (pre-wash) semen characteristics for comparison between PureSperm density gradient and swim-up method were sperm concentration (million·mL^{-1}) 78 (61.8–140.5) and percentage motility 54.4 (42.0–64.4). Results comparing the sperm prepared by PureSperm density gradient and swim-up method are illustrated in Table 1.

3.1.1 Post-wash semen analysis

Total motile sperm (TMS) recovered by PureSperm density gradient method was significantly higher than by the swim-up method ($P = 0.003$). The percent recovery of sperm processed by PureSperm was significantly higher than swim-up ($P = 0.003$). Initial motility after processing by the two methods was comparable ($P = 0.84$). The median (25th, 75th percentile) motility at one hour was significantly higher with PureSperm than with swim-up [82 % (64.3 %–90.0 %) *vs.* 63 % (56 %–71 %) ($P = 0.027$)]. Similarly, sperm motility at 4 hours was higher after PureSperm than for swim-up [83 % (67 %–90 %) *vs.* 55 % (37 %–67 %) ($P = 0.011$)] (Figure 3A). The recovery rate for asthenospermic specimens processed with PureSperm was significantly higher than with swim-up method [66.2 % (51.1 %–79.1 %) *vs.* 47.0 % (27.8 %–52.5 %) ($P = 0.014$)].

3.1.2 Post-thaw semen analysis

The total motile sperm count after cryopreservation was significantly higher for specimens processed following preparation by PureSperm compared to swim-up ($P = 0.003$) (Table 1). In addition, the percent recovery for PureSperm processed specimens was higher than swim-up ($P = 0.027$). Initial motility (at 0 hour) in post-thaw specimens was higher with PureSperm than swim-up ($P = 0.007$). Similarly, the post-thaw motility at 4 hours was significantly higher with PureSperm than with swim-up method [31.0 % (12.4 %–36.5 %) *vs.* 7.6 % (3.2 %–14.0 %) ($P = 0.003$)] (Figure 3B).

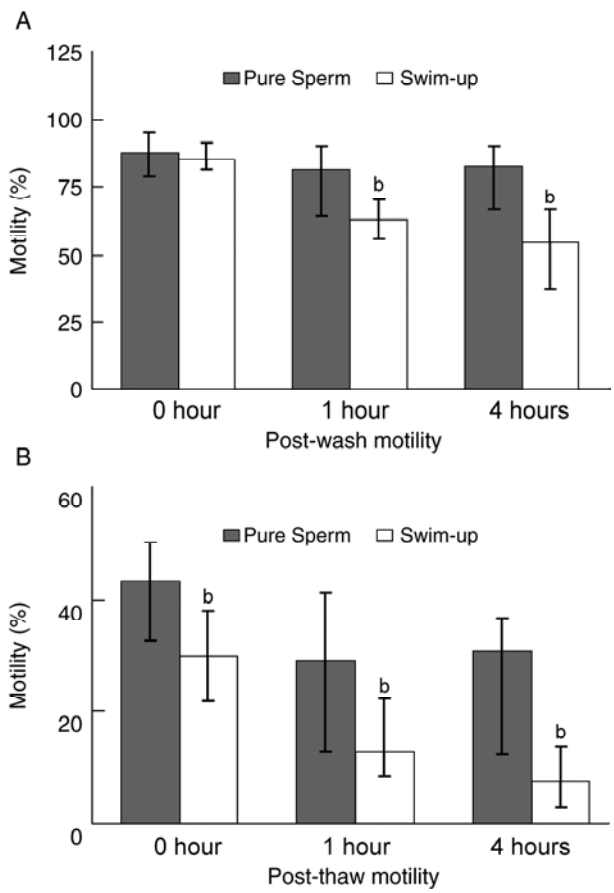

Figure 3. (A) Comparison of median post-wash motility in sperm prepared by PureSperm and by swim-up method at 0, 1, and 4 hours. (B) Comparison of median post-thaw motility in sperm prepared by PureSperm and by swim-up method at 0, 1 and 4 hours. $^bP < 0.05$ was considered significant by Wilcoxon matched-pairs signed-ranks test. Error bars represent 25th, 75th percentiles.

3.2 Sperm preparation by PureSperm and ISolate density gradients

The initial (pre-wash) semen characteristics for comparison between PureSperm and ISolate density gradients were sperm concentration (million/mL) 41.5 (32 – 49) and percentage motility 51(35.7– 65). Semen specimens processed by PureSperm gave higher recovery of total sperm count (P = 0.0002) and total motile sperm count (P = 0.0002) compared to ISolate (Table 2). The recovery rate for PureSperm was 37 % higher than ISolate (P = 0.0002). Recovery rates in semen specimens processed with PureSperm was significantly higher than those processed with ISolate in both normozoospermic [43 % (27.8 %–67 %) *vs.* 22.3 % (18.3 %–43.4 %) (P = 0.04)] and asthenozoospermic patients [28.9 % (18.9 %–31.6 %) *vs.*18.2 % (11.4 % – 20.8 %) (P = 0.003)]. Percentage motility and ROS levels were similar in the spermatozoa processed by both gradients (P = 0.9 and 0.492, respectively).

4 Discussion

The need for effective sperm preparation methods has increased with the increased use of assisted reproductive techniques. Sperm preparation techniques vary greatly in terms of recovery rates, motility, morphology, and degree of DNA damage [10, 14, 15]. Due to their simplicity, reproducibility, and excellent yields in motile spermatozoa, Percoll gradients became very popular for processing semen specimens. Percoll was withdrawn due to safety concerns [16]. New sperm preparation media such as PureSperm and Isolate, have been claimed to have low endotoxin levels and provide similar yields of motile sperm as Percoll [10, 11, 17, 18].

Studies have shown no significant differences between PureSperm and Percoll as sperm preparation media in terms of recovery of progressively motile spermatozoa [10, 17]. PureSperm 3-layer gradients for sperm preparation have been reported to result in specimen with

Table 1. Comparison of post-wash and post-thaw sperm parameters after semen preparation by PureSperm density gradient and swim-up methods.

Variable	PureSperm	Swim-up	*P* value
	Post-wash		
Concentration ($\times 10^6 \cdot mL^{-1}$)	39.0 (27.2, 48.0)	30.3 (13.0, 33.9)	0.007
Motility (%)	88.0 (78.7, 95.0)	85.5 (82.1, 92.0)	0.840
TSC ($\times 10^6$)	38.0 (24.1, 48.0)	25.4 (13.0, 33.9)	0.007
TMSC ($\times 10^6$)	32.2 (16.0, 45.1)	17.6 (10.0, 26.6)	0.003
Recovery (%)	69.2 (60.0, 75.0)	50.0 (44.0, 55.0)	0.003
	Post-thaw		
Motility %	43.4 (32.5, 50.0)	30.0 (21.8, 38.2)	0.007
TSC ($\times 10^6$)	38.4 (17.7, 42.3)	21.2 (8.4, 29.0)	0.007
TMSC ($\times 10^6$)	15.2 (5.2, 23.8)	4.1 (2.3, 10.6)	0.003
Recovery (%)	44.8 (32.8, 51.5)	29.0 (23.4, 38.4)	0.027

Values are expressed as median and 25th, 75th percentile. $P < 0.05$ was considered significant by Wilcoxon matched-pairs signed-ranks test. TSC = Total sperm count; TMSC = Total motile sperm count.

Table 2. Comparison of Post-Wash Sperm Characteristics after PureSperm and ISolate density gradient preparation

Variable	PureSperm	ISolate	*P* Value
Concentration ($\times 10^6$/mL)	9.5 (5.0, 11.5)	3.8 (2.7, 8.6)	0.0010
Motility (%)	75.0 (70.0, 83.0)	73.5 (55.0, 84.0)	0.9000
TSC ($\times 10^6$)	10.1 (5.0, 11.4)	3.8 (2.7, 8.6)	0.0002
TMSC ($\times 10^6$)	5.0 (3.1, 9.8)	2.1 (1.9, 7.2)	0.0002
Recovery (%)	30.0 (26.7, 43.0)	19.7 (17.0, 33.8)	0.0002
Log (ROS+1)	0.08 (0.00, 0.43)	0.19 (0.00, 0.99)	0.4920

Values are expressed as median and 25th, 75th percentile. $P < 0.05$ was considered significant by Wilcoxon matched-pairs signed-ranks test. TSC = Total sperm count; TMSC = Total motile sperm count; ROS = Reactive oxygen species.

highly motile and morphologically normal spermatozoa [18]. Few studies have examined the differences in sperm quality in semen specimens prepared with PureSperm or swim-up and between PureSperm and ISolate gradient technique.

We report a significantly higher recovery of total motile sperm and longevity in fresh semen specimens prepared with PureSperm density gradient compared to swim-up method. This is contrary to the finding reported by Soderlund *et al.*, who found a higher percentage of progressive motile spermatozoa recovered after swim-up technique. These differences may be because of the significantly high initial motility of semen specimens prepared by the swim-up technique, compared to preparation by the PureSperm technique [17]. In addition, our results show that PureSperm provides a significantly higher recovery of total motile sperm in asthenozoospermic specimens compared to the swim-up method. This finding assumes importance considering the fact that most of the specimens used in ART are from infertile men with poor semen quality.

We found significantly higher recovery of total motile sperm in specimens cryopreserved after PureSperm density gradient, compared to the swim-up method. This finding is especially relevant when spermatozoa are cryopreserved in donor insemination programs and for routine sperm banking before vasectomy and systemic cancer therapy are utilized for future use. However, Chan *et al.* reported higher post-thaw recovery rate and motion parameters with the swim-up method compared to PureSperm [19]. These higher semen parameters with swim-up in their study may be due to the use of different gradient densities of PureSperm (45 % and 90 %) compared to our study (40 % and 80 %) [19].

We report significantly higher longevity in fresh and cryopreserved semen specimens prepared with PureSperm density gradient compared to the swim-up method. The improved longevity of spermatozoa prepared by PureSperm may be due to the short processing time (35 to 40 min) of the density gradient method as compared to the swim-up technique (90 min).

Our findings are similar to a study by Hammadeh and Kuhnen, who compared the efficacy of swim-up, PureSperm, and glass-wool filtration. These investigators reported a higher percentage of normal spermatozoa obtained with the PureSperm gradient compared to other techniques. However, there was no significant difference in the fertilization, implantation, and pregnancy rates between the three techniques [20]. Sakkas *et al.* studied the ability of different sperm preparation techniques to separate spermatozoa with chromatin and nuclear DNA anomalies. The PureSperm and Percoll techniques resulted in high percentages of spermatozoa with nuclear integrity; sperm preparation using the swim-up technique did not have the same results [9].

In our study, semen specimens processed by PureSperm resulted in a higher recovery of total sperm count and total motile sperm count compared to those separated on ISolate. The improved semen quality with PureSperm was maintained when patients were analyzed based on pre-wash motility as normozoospermic or asthenozoospermic. However, others found no significant difference in percentage recovery between PureSperm, Isolate, and Percoll for both normospermic and oligozoospermic patients [21].

In our study, motility and ROS levels were similar in the spermatozoa processed by both gradients. This is in agreement with the findings of Classens *et al.* who found no differences in motility after sperm preparation with PureSperm, ISolate, Optiprep, and Percoll [21]. The limitations of our study are small sample size. Different gradient densities of PureSperm (40 % and 80 %) compared to ISolate (50 % and 90 %) could be one of the reasons for the difference observed in our study. Multicenter, blinded studies with a large sample size are required to conclusively prove if any sperm preparation technique provides superior results.

In conclusion, PureSperm density gradient method (using 40 % and 80 % PureSperm gradients) is not only superior to the swim-up method for sperm preparation, but is also a rapid and simple technique compared to the swim-up method. Spermatozoa separated in PureSperm gradient yield higher numbers of motile sperm that can withstand cryopreservation changes better than the specimens prepared by swim-up method. A higher enrichment of spermatozoa was observed when PureSperm was used as density gradient media compared to ISolate. The use of PureSperm gradient may provide higher rates of recovery of mature, motile sperm in the specimens processed for ART, which may result in higher fertilization and pregnancy rates. Prospective studies with fertilization and pregnancy rates as end points are needed to conclusively show the effectiveness of PureSperm in enriching functionally competent spermatozoa.

Acknowledgments

The authors thank Karen Seifarth, MT, Cheryl Ackerman, MT, and Lora Cordek, MT, of the Clinical Andrology Laboratory for technical assistance and Robin Verdi for secretarial support.

References

1 Saleh R, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. J Androl 2002; 23: 737–52.
2 Mortimer D. Sperm preparation methods. J Androl 2000; 21: 357–66.
3 Rogers BJ, Perreault SD, Bentwood BJ, McCarville C, Hale RW, Soderdahl DW. Variability in the human-hamster *in vitro* assay for fertility evaluation. Fertil Steril 1983; 39: 204–11.
4 Yamamoto Y, Maenosono S, Okada H, Miyagawa I, Sofikitis N. Comparisons of sperm quality, morphometry and function among human sperm populations recovered via SpermPrep II filtration, swim-up and Percoll density gradient methods. Andrologia 1997; 29: 303–10.
5 Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J Androl 1988; 9: 367–76.
6 Yogev L, Gamzu R, Paz G, Kleiman S, Botchan A, Hauser R, *et al*. Pre-freezing sperm preparation does not impair thawed spermatozoa binding to the zona pellucida. Hum Reprod 1999; 14: 114–7.
7 Sharma RK, Agarwal A. Sperm quality improvement in cryopreserved human semen. J Urol 1996; 156: 1008–12.
8 Subak LL, Adamson GD, Boltz NL. Therapeutic donor insemination: a prospective randomized trial of fresh versus frozen sperm. Am J Obstet Gynecol 1992; 166: 1597–604; discussion 604–6.
9 Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, Bianchi PG, *et al*. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod 2000; 15: 1112–6.
10 Centola GM, Herko R, Andolina E, Weisensel S. Comparison of sperm separation methods: effect on recovery, motility, motion parameters, and hyperactivation. Fertil Steril 1998; 70: 1173–5.
11 Makkar G, Ng HY, Yeung SB, Ho PC. Comparison of two colloidal silica-based sperm separation media with a non-silica-based medium. Fertil Steril 1999; 72: 796–802.
12 WHO Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press, 1992.
13 Hallak J, Sharma RK, Wellstead C, Agarwal A. Cryopreservation of human spermatozoa: comparison of TEST-yolk buffer and glycerol. Int J Fertil Womens Med 2000; 45: 38–42.
14 Byrd W, Drobnis EZ, Kutteh WH, Marshburn P, Carr BR. Intrauterine insemination with frozen donor sperm: a prospective randomized trial comparing three different sperm preparation techniques. Fertil Steril 1994; 62: 850–6.
15 Claassens O, Kaskar K, Coetzee K, Lombard C, Franken D, Kruger T. Comparison of motility characteristics and normal sperm morphology of human semen samples separated by percoll density gradient centrifugation. Arch Androl 1996; 36: 127–32.
16 Arora M, Carver-Ward JA, Jaroudi KA, Sieck UV. Is Percoll safe for *in vivo* use? Fertil Steril 1994; 61: 979–81.
17 Soderlund B, Lundin K. The use of silane-coated silica particles for density gradient centrifugation in *in-vitro* fertilization. Hum Reprod 2000; 15: 857–60.
18 Chen MJ, Bongso A. Comparative evaluation of two density gradient preparations for sperm separation for medically assisted conception. Hum Reprod 1999; 14: 759–64.
19 Chan CC, Chen IC, Liu JY, Huang YC, Wu GJ. Comparison of nitric oxide production motion characteristics of sperm after cryopreserved in three different preparations. Arch Androl 2004; 50: 1–3.
20 Hammadeh ME, Kuhnen A, Amer AS, Rosenbaum P, Schmidt W. Comparison of sperm preparation methods: effect on chromatin and morphology recovery rates and their consequences on the clinical outcome after *in vitro* fertilization embryo transfer. Int J Androl 2001; 24: 360–8.
21 Claassens OE, Menkveld R, Harrison KL. Evaluation of three substitutes for Percoll in sperm isolation by density gradient centrifugation. Hum Reprod 1998; 13: 3139–43.