ORIGINAL ARTICLE
https://doi.org/10.5653/cerm.2019.46.1.8
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2019;46(1):8-13

CERM

Evaluation of normal morphology, DNA fragmentation, and hyaluronic acid binding ability of human spermatozoa after using four different commercial media for density gradient centrifugation

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Objective: Density gradient centrifugation (DGC) is frequently used to isolate high-motility fractions of spermatozoa. We compared the efficacy of four DGC media in terms of the percentage of morphologically normal spermatozoa, DNA fragmentation level, and hyaluronic acid (HA) binding ability.
Methods: Thirty men with a total motile spermatozoa count > 80 million participated. Semen samples were divided into four aliquots, which were processed using PureSperm, PureCeption, Sidney, and SpermGrad media, respectively. The DNA fragmentation level was measured using the Halosperm assay kit and HA binding ability was measured using the HBA assay kit.
Results: The mean percentage of morphologically normal spermatozoa was significantly enhanced after DGC using all four media (10.3%, 9.9%, 9.8%, and 10.7%, respectively; $p < 0.05$ for each when compared with 6.9% in raw semen). The DNA fragmentation level was significantly reduced after DGC using PureSperm, PureCeption, and SpermGrad media (6.0%, 6.5%, and 4.9%, respectively; $p < 0.05$ for each when compared with 11.2% in raw semen), but not after DGC using Sidney media (8.5%, $p > 0.05$). HA binding ability did not change after DGC using any of the four media.
Conclusion: The four media were equally effective for obtaining a sperm fraction with highly motile, morphologically normal sperm. PureSperm, PureCeption, and SpermGrad media were equally effective for acquiring a sperm fraction with less DNA fragmentation.

Keywords: Centrifugation, density gradient; DNA fragmentation; Hyaluronic acid; Spermatozoa

Introduction

Density gradient centrifugation (DGC), a method of separating spermatozoa by density, is frequently used to isolate high-motility sperm fractions. The density of morphologically normal or mature spermatozoa is usually > 1.10 g/mL, whereas the density of morphologically abnormal or immature spermatozoa is between 1.06 and 1.09 g/mL. The centrifugation of semen using DGC media produces a higher-motility fraction at the bottom [1]. DGC has been reported to be a more suitable method for semen processing than simple washing or swim-up in normozoospermic samples [2]. However, concerns have been raised about whether DGC damages the membranes of spermatozoa during high-speed centrifugation or during formation of the hard pellet [3,4].

In the past, the Percoll gradient, composed of polyvinylpyrrolidone (PVP)-coated silica particles, was widely used as a DGC medium. However, due to concerns regarding the toxicity of PVP, its use in hu-

Received: Oct 17, 2018 · Revised: Nov 20, 2018 · Accepted: Nov 22, 2018
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mans was discontinued in 1996 [5]. Thereafter, silane-coated silica colloid media have emerged, and several commercial DGC media are now widely used for infertility treatment.

In an earlier study, no significant difference was observed between PureSperm and ISolate media in terms of vitality, progressive motility, normal morphology, and normal chromatin condensation [6]. It has been reported that PureSperm is superior to Sil-Select Plus or SpermGrad in terms of acquisition of a larger fraction of motile spermatozoa [5,7]. However, in the study of Chiamchanya et al. [7], both PureSperm and Sil-Select Plus were superior to other media in terms of sperm maturity and DNA fragmentation. Recently, PureCeption and ISolate have been reported to be superior to SpermGrad in terms of acquisition of a higher-motility fraction; however, DNA fragmentation was similar across the three media [8].

Collectively, PureSperm and PureCeption media appear to be better for the acquisition of a fraction with intact spermatozoa, but a direct comparison of the efficacy between PureSperm and PureCeption media has not yet been conducted. Moreover, SpermGrad showed contradictory results; an increased DNA fragmentation level was reported [7], but a decreased DNA fragmentation level and no reduction of the DNA fragmentation level have also been reported [8,9]. Sydney medium is used at several infertility centers, but its efficacy has not yet been reported. In our previous study, the DNA fragmentation level decreased when the swim-up method was used, but there was no change after DGC using Sidney medium [10]. Therefore, an experiment should be conducted to objectively compare this medium with other DGC media.

In the present study, we compared the efficacy of four commonly used DGC media (PureSperm, PureCeption, Sydney, and SpermGrad) in terms of the percentage of morphologically normal spermatozoa, DNA fragmentation level, and hyaluronic acid (HA) binding ability.

Methods

1. Subjects

A prospective study was performed during 2017. Thirty men (24 healthy volunteers and six male partners from couples who underwent an infertility evaluation) participated and showed a total motile spermatozoa count (TMC) of at least 80 million. Informed consent was obtained from all participants. This study was approved by the Institutional Review Board of Bundang Seoul National University Hospital (IRB No. B-1705/395-307). None of the subjects had a history of genital inflammation or surgery, no subjective symptoms or self-reported medical risk factors were identified, and none of the subjects were taking any prescription medications. The participants ranged in age from 23 to 45 years, and three of them had biological children.

2. Semen collection and analysis

Semen samples were obtained by masturbation into a sterile container. Semen samples were liquefied for 30 minutes at room temperature and then divided equally into five aliquots, one of which was not processed and served as the control sample (raw semen). The remaining four aliquots underwent DGC procedures using PureSperm (Nidacon Laboratories, Gothenburg, Sweden), PureCeption (Sage Biopharma, Bedminster, NJ, USA), Sydney (William A. COOK, Queensland, Australia), and SpermGrad (Vitrolife, Englewood, CO, USA), respectively.

The raw semen sample and the four processed semen fractions were examined for TMC, motility, the mean percentage of normal morphology, DNA fragmentation level, and HA binding ability. Semen analysis was performed using motion analysis equipment (SAIS-PLUS 10.1; Medical Supply, Seoul, Korea). Strict criteria, as indicated in the World Health Organization (WHO) guidelines (fifth edition, 2010) were used to define normal spermatozoa in the morphological assessment.

The basic semen characteristics were as follows (median with interquartile range): semen volume, 3 mL (2–4 mL); sperm count, 111 million/mL (59–138 million/mL); total motility, 70.8% (60%–80%); TMC, 144 million (114–282 million); and mean percentage of morphologically normal spermatozoa, 6.9% (5.2%–8.3%).

3. Procedure for DGC

DGC was performed as described in the WHO guidelines, but was modified appropriately according to the situation of our laboratory as described in our previous study [10]. The concentrations of the upper- and lower-phase gradient and centrifugation-specific criteria recommended by each manufacturer are summarized in Table 1. The

Table 1. Concentrations of upper and lower layers of the gradient and centrifugation-specific criteria recommended by each manufacturer

Variable	PureSperm	PureCeption	Sidney	SpermGrad
Upper-layer density (%)	40	40	40	45
Lower-layer density (%)	80	80	80	90
Centrifugation time (min)	20	20	20	20
Centrifugal force (×*g*)				
Recommended	300	350–400	300	300–600
In this experiment	300	350	300	300

recommended centrifugal force for PureCeption medium was 350–400×*g*, and we used 350×*g* to minimize the procedural differences across the medium types.

Each semen sample was mixed with 3 mL of Ham's F-10 media (Gibco, Paisley, UK) with 10% synthetic serum substitute (SSS; Irvine Scientific, Santa Ana, CA, USA) and then centrifuged at 300×*g* for 5 minutes to remove seminal plasma. The precipitated pellet was suspended in 1.5 mL of Ham's F-10 medium with 10% SSS. Two milliliters of lower-phase gradient medium was placed in a 15-mL conical tube and the same amount of upper-phase gradient medium was gently loaded. The suspended semen samples were placed onto the upper-phase gradient medium. Next, the conical tube was centrifuged at 300×*g* for 20 minutes. The pellet collected from the bottom layer was washed twice by resuspension in 4 mL of Ham's F-10 medium with 10% SSS, followed by centrifugation at 300×*g* for 5 minutes. After two rounds of centrifugation, the supernatant was discarded and the final pellet was resuspended in 3 mL of Ham's F-10 medium with 10% SSS.

4. Assessment of DNA fragmentation

The DNA fragmentation level was measured using the Halosperm assay kit according to the manufacturer's instructions with minor modifications. In brief, each aliquot of semen sample was diluted to 10 million/mL in F-10 medium. Agarose was melted by heating in a water bath at 90°–100°C for 5 minutes and incubated in a water bath at 37°C for 5 minutes. After the temperature of agarose was equilibrated to 37°C, 25 µL of the semen sample was mixed with fused agarose. Twenty microliters of the mixture was pipetted onto a slide and covered with a 22×22 mm coverslip. The slide was cooled in the refrigerator at 4°C for 5 minutes to solidify the agarose. The coverslip was gently removed and the slide was treated with a denaturation solution (a mixture of 80 µL HCl and 10 mL of distilled water) for 7 minutes. The slide was treated with lysis buffer for 25 minutes and washed for 5 minutes in a tray containing distilled water. Stepwise dehydration was performed with increasing concentrations of ethanol (70%, 90%, and 100%; 2 minutes for each concentration), followed by air-drying. After Diff-Quick staining (Eosin G for 7 minutes, Azure B for 7 minutes), the slide was washed in tap water and air-dried.

Each slide was examined under a light microscope at ×200 magnification and at least 300 spermatozoa were scored. Each spermatozoon was assessed as having a large halo, a medium halo, a small halo, or no halo; degraded spermatozoa were also counted. A halo with a thickness equal to or greater than the minor diameter of the core was considered large, a halo with a thickness smaller than the minor diameter of the core and greater than one-third of the minor diameter of the core was considered medium, and a halo with a thickness equal to or smaller than one-third of the minor diameter of the core was considered small. Spermatozoa with a small halo or no halo, as well as degraded spermatozoa, were considered to have fragmented DNA. The DNA fragmentation level was calculated as the percentage of spermatozoa with DNA fragmentation out of all observed spermatozoa.

5. HA binding assay

HA binding ability was assessed using the HBA Sperm Hyaluronan-Binding Assay kit (Origio, Måløv, Denmark) according to the manufacturer's instructions. The semen samples were diluted with F-10 medium to a concentration of approximately 10 million/mL. A 10-µL drop of each semen sample was placed in the assay chamber, and a coverslip was carefully placed over the drop to avoid bubble formation. After 20 minutes, each slide was examined under a light microscope at ×400 magnification, and at least 300 spermatozoa were scored. The bound spermatozoa showed typical tail-beating without any progressive movement, which could be easily distinguished from the unbound spermatozoa. HA binding ability was calculated using the following equation: bound motile spermatozoa/total motile spermatozoa ×100 (%).

6. Statistical analysis

Statistical analysis was performed using IBM SPSS ver. 22.0 (IBM Corp., Armonk, NY, USA). All data were presented as median with interquartile range. The paired Wilcoxon signed-rank test was used to compare the semen parameters of the raw samples and each DGC fraction. The Kruskal-Wallis test was used to compare the semen parameters among the four DGC fractions. Spearman rank correlation analysis was used to analyze correlations between continuous variables.

Results

The semen parameters in the raw samples and the four DGC fractions are presented in Table 2. The mean percentage of morphologically normal spermatozoa was significantly enhanced after DGC using PureSperm, PureCeption, Sidney, and SpermGrad media when compared with the raw semen. The DNA fragmentation level was significantly reduced after DGC using PureSperm, PureCeption, and SpermGrad media, but not after DGC using Sidney medium. HA binding ability did not change after DGC using any of the four media. In all four DGC fractions, the mean percentage of morphologically normal spermatozoa, DNA fragmentation level, and HA binding ability were all similar.

In the raw semen samples, HA binding ability had no correlation with the age of the participants, motility, the percentage of morphologically normal spermatozoa, or the DNA fragmentation level (Table 3). The participants' age showed a significant negative linear relation-

https://doi.org/10.5653/cerm.2019.46.1.8

Table 2. Semen parameters before and after density gradient centrifugation using four media in 30 normozoospermic men

Variable	Raw semen	PureSperm	PureCeption	Sidney	SpermGrad
TMC (million)	144.1 (113.5–282.4)	3.0 (1.7–7.4)[a]	5.1 (1.4–11.7)[a]	5.2 (3.1–10.4)[a]	0.3 (0.1–1.3)[a]
Motility (%)	70.8 (60–80)	67.0 (45.6–78.7)	62.2 (49.6–77.2)	61.3 (41–78.2)	50.0 (40.8–64.7)[a]
Morphologically normal spermatozoa (%)	6.9 (5.2–8.3)	10.3 (7.1–14.2)[a]	9.9 (7.6–12.6)[a]	9.8 (7.9–15.3)[a]	10.7 (7.7–15.9)[a]
DNA fragmentation (%)	11.2 (7.9–21.2)	6.0 (2.7–12)[a]	6.5 (2.9–13.8)[a]	8.5 (3.9–14.4)	4.9 (1.7–10.6)[a]
HA binding ability (%)	48.9 (28.1–72.8)	55.3 (26–76.9)	58.3 (38.7–76.4)	51.5 (33.6–85.7)	49.7 (20.9–72.3)

Values are presented as median (interquartile range).
TMC, total motile spermatozoa count; HA, hyaluronic acid.
[a] $p < 0.05$ when compared with raw semen.

Table 3. Correlation coefficients between various parameters in the raw semen samples

Variable	Motility (%)	Morphologically normal spermatozoa (%)	DNA fragmentation level (%)	HA binding ability (%)
Age (yr)	$r=-0.435$ $p=0.016$	$r=-0.084$ $p=0.658$	$r=0.470$ $p=0.009$	$r=-0.252$ $p=0.180$
Motility (%)	-	$r=0.187$ $p=0.323$	$r=-0.613$ $p=0.391$	$r=0.277$ $p=0.138$
Morphologically normal spermatozoa (%)	-	-	$r=-0.126$ $p=0.509$	$r=0.179$ $p=0.344$
DNA fragmentation level (%)	-	-	-	$r=-0.032$ $p=0.865$

HA, hyaluronic acid.

ship with motility, but a significant positive linear relationship with the DNA fragmentation level.

Discussion

In the present study, the mean percentage of morphologically normal spermatozoa was significantly enhanced after DGC using PureSperm, PureCeption, Sidney, and SpermGrad media. This morphological improvement was also observed in a previous report, in which PureSperm and SpermGrad media were used [7].

The DNA fragmentation level was significantly reduced after DGC using PureSperm, PureCeption, and SpermGrad media, but not after DGC using Sidney medium. An absence of improvement in the DNA fragmentation level in the fraction treated using Sidney medium was also observed in our previous study [10].

In fact, contradictory results have been found regarding the effects of various media on the DNA fragmentation level after DGC. No reduction of the DNA fragmentation level was reported in fractions obtained using PureCeption and SpermGrad media [8]. In contrast, the DNA fragmentation level was reduced in fractions obtained using PureSperm media, but increased in fractions obtained using SpermGrad [7]. In another report, the DNA fragmentation level was reduced in fractions obtained using SpermGrad [9]. In a previous study, the DNA fragmentation level was reduced in fractions obtained using PureCeption, but that study included only teratozoospermic samples [11]. In the present study, PureSperm, PureCeption, and SpermGrad media appeared to be equally effective for the acquisition of a sperm fraction with less DNA fragmentation in normozoospermic men.

Because the sperm DNA fragmentation level may affect the fertilization rate, embryo cleavage, implantation rate, abortion rate, and offspring health, DNA fragmentation measurements have been recognized as an important test for assessing the quality of spermatozoa, and further studies are underway to obtain sperm fractions with less DNA fragmentation [12,13]. The terminal deoxynucleotidyl transferase dUTP nick end labeling assay, the comet assay (single-cell gel electrophoresis), the sperm chromatin dispersion (SCD) test, and the sperm chromatin structure assay have been proposed as methods for evaluating DNA damage. SCD testing, as exemplified by the Halosperm assay, are advantageous because the DNA fragmentation level can be easily examined under light microscopy without the need for fluorescent light [14].

In the present study, HA binding ability did not change after DGC using any of these four media. To the best of our knowledge, this is the first study to measure HA binding ability before and after DGC processing. It has been suggested that a HA binding ability of $<65\%$ poses a high risk for spontaneous abortion after *in vitro* fertilization [15]. However, no consensus yet exists regarding the normal range of HA binding ability, and the WHO guidelines (fifth edition, 2010) do not present reference values. In the present study, the median value

of HA binding ability was 48.9% in raw semen from 30 normozoospermic men, and the values for the lower fifth and 10th percentiles were 9.6%, and 10.6%, respectively. In another study, the mean value of HA binding ability was 39.2% (range, 0%–87%) [16]. Since there are no reference values for HA binding ability, further research on this topic is warranted.

We observed that HA binding ability in raw semen samples had no correlation with the age of the participants, motility, the percentage of morphologically normal spermatozoa, or the DNA fragmentation level. We also observed that HA binding ability did not change after DGC using any of the four media. Thus, the clinical significance of HA binding ability appears to require further investigation.

In this study, the participants' age showed a significant negative linear relationship with motility, but a significant positive linear relationship with the DNA fragmentation level. A recent meta-analysis similarly reported a positive association between age and the DNA fragmentation level of spermatozoa [17]. While the exact mechanisms are not fully understood, increased reactive oxygen species (ROS) levels have been proven to be correlated with nuclear DNA fragmentation [18,19]. Decreased sperm function has been correlated with the excessive production of ROS by sperm mitochondria. Spermatozoa have limited antioxidant potential and are easily overwhelmed by excessive ROS generation, resulting in damage to the DNA of the nucleus and mitochondria [20].

A limitation of this study is that it was conducted on men with a TMC of at least 80 million. The experiment involved dividing semen samples into several aliquots, and samples with a small number of spermatozoa could not be processed. The effects of DGC might be more pronounced in men with a low TMC or abnormal semen analysis results. Accordingly, further experiments should be conducted to elucidate these issues.

In conclusion, PureSperm, PureCeption, Sidney, and SpermGrad media appear to be equally effective for obtaining a sperm fraction enriched with morphologically normal sperm. The PureSperm, PureCeption, and SpermGrad media proved to be comparably effective for acquiring a sperm fraction with less DNA fragmentation.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

The authors thank Ms. Jang Mi Lee and Ms. Ye Eun Lee, Researchers, Fertility Center, Seoul National University Bundang Hospital (Seongnam, Korea), for their technical assistance.

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Conceptualization: BCJ. Data curation: DL. Formal analysis: DL. Methodology: BCJ. Writing - original draft: DL. Writing - review & editing: BCJ.

References

1. Beydola T, Sharma RK, Lee W, Agarwal A. Sperm preparation and selection techniques. In: Rizk B, Aziz N, Agarwal A, Sabanegh E, editors. Male infertility practice. New Delhi: Jaypee Brothers Medical Publishers; 2013. p. 244-51.
2. Moohan JM, Lindsay KS. Spermatozoa selected by a discontinuous Percoll density gradient exhibit better motion characteristics, more hyperactivation, and longer survival than direct swim-up. Fertil Steril 1995;64:160-5.
3. Arcidiacono A, Walt H, Campana A, Balerna M. The use of Percoll gradients for the preparation of subpopulations of human spermatozoa. Int J Androl 1983;6:433-45.
4. Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. Fertil Steril 1992;57:409-16.
5. Mousset-Simeon N, Rives N, Masse L, Chevallier F, Mace B. Comparison of six density gradient media for selection of cryopreserved donor spermatozoa. J Androl 2004;25:881-4.
6. 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.
7. Chiamchanya C, Kaewnoonual N, Visutakul P, Manochantr S, Chaiya J. Comparative study of the effects of three semen preparation media on semen analysis, DNA damage and protamine deficiency, and the correlation between DNA integrity and sperm parameters. Asian J Androl 2010;12:271-7.
8. Malvezzi H, Sharma R, Agarwal A, Abuzenadah AM, Abu-Elmagd M. Sperm quality after density gradient centrifugation with three commercially available media: a controlled trial. Reprod Biol Endocrinol 2014;12:121.
9. Enciso M, Iglesias M, Galan I, Sarasa J, Gosalvez A, Gosalvez J. The ability of sperm selection techniques to remove single- or double-strand DNA damage. Asian J Androl 2011;13:764-8.
10. Kim SW, Jee BC, Kim SK, Kim SH. Sperm DNA fragmentation and sex chromosome aneuploidy after swim-up versus density gradient centrifugation. Clin Exp Reprod Med 2017;44:201-6.

11. Xue X, Wang WS, Shi JZ, Zhang SL, Zhao WQ, Shi WH, et al. Efficacy of swim-up versus density gradient centrifugation in improving sperm deformity rate and DNA fragmentation index in semen samples from teratozoospermic patients. J Assist Reprod Genet 2014;31:1161-6.
12. Agarwal A, Majzoub A, Esteves SC, Ko E, Ramasamy R, Zini A. Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios. Transl Androl Urol 2016;5:935-50.
13. Simon L, Zini A, Dyachenko A, Ciampi A, Carrell DT. A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. Asian J Androl 2017;19:80-90.
14. Zhang LH, Qiu Y, Wang KH, Wang Q, Tao G, Wang LG. Measurement of sperm DNA fragmentation using bright-field microscopy: comparison between sperm chromatin dispersion test and terminal uridine nick-end labeling assay. Fertil Steril 2010;94:1027-32.
15. Worrilow KC, Eid S, Woodhouse D, Perloe M, Smith S, Witmyer J, et al. Use of hyaluronan in the selection of sperm for intracytoplasmic sperm injection (ICSI): significant improvement in clinical outcomes: multicenter, double-blinded and randomized controlled trial. Hum Reprod 2013;28:306-14.
16. Rashki Ghaleno L, Rezazadeh Valojerdi M, Chehrazi M, Sahraneshin Samani F, Salman Yazdi R. Hyaluronic acid binding assay is highly sensitive to select human spermatozoa with good progressive motility, morphology, and nuclear maturity. Gynecol Obstet Invest 2016;81:244-50.
17. Johnson SL, Dunleavy J, Gemmell NJ, Nakagawa S. Consistent age-dependent declines in human semen quality: a systematic review and meta-analysis. Ageing Res Rev 2015;19:22-33.
18. Aitken RJ, De Iuliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. Int J Androl 2009;32:46-56.
19. Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Hum Reprod 2010;25:2415-26.
20. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol Reprod 1989;41:183-97.