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정자의 밀도 차 원심분리 시행 시 사용하는 네 가지
밀도 차 용액이 정자의 모양, DNA 분절, 하이알루론산
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Evaluation of normal morphology, DNA fragmentation, and
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centrifugation

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Evaluation of normal morphology, DNA
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ABSTRACT

Evaluation of normal morphology, DNA fragmentation, and hyaluronic acid binding ability of human spermatozoa after using 4 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 4 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 4 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 4 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 4 media.

Conclusion: The 4 media were equally effective for obtaining a sperm fraction with highly motile and morphologically normal sperm. PureSperm®, PureCeption®, and SpermGrad® media were equally effective for acquiring a sperm fraction with less DNA fragmentation.

Keywords: spermatozoa / centrifugation / density gradient / DNA fragmentation / hyaluronic acid

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CONTENTS

Abstract	i
Contents	iv
List of tables	v
Introduction.....	1
Materials and methods.....	5
Results.....	15
Discussion	19
References	25
Abstract in Korean.....	30

LIST OF TABLES

Table 1. Concentrations of upper and lower layers of the gradient and centrifugation-specific criteria recommended by each manufacturer	10
Table 2. Semen parameters before and after density gradient centrifugation using 4 media in 30 normozoospermic men.....	17
Table 3. Correlation coefficients between various parameters in the raw semen samples	18

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 humans 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 Chiamchany et al., 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 3 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 Sydney® 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 4 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.

MATERIALS AND METHODS

Subjects

A prospective study was performed during 2017. Thirty men (24 healthy volunteers and 6 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 3 of them had biological children.

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 5 aliquots, 1 of which was not processed and served as the control sample (raw semen). The remaining 4 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 4 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 million, 282 million]; and mean percentage of morphologically normal spermatozoa, 6.9% [5.2%, 8.3%].

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 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 $300 \times g$ for 5 minutes. After 2 rounds of centrifugation, the supernatant was discarded and the final pellet was re-suspended in 3 mL of Ham's F-10 medium with 10% SSS.

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

	Pure Sperm®	Pure Ception®	Sidney®	Sperm Grad®
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

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 $\times 200$ 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.

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- μ L 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 $\times 400$ 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 \times 100 (%).

Statistical analysis

Statistical analysis was performed using SPSS version 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 4 DGC fractions. Spearman rank correlation analysis was used to analyze correlations between continuous variables.

RESULTS

The semen parameters in the raw samples and the 4 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 4 media.

In all 4 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 relationship with motility, but a significant positive linear relationship with the DNA fragmentation level.

Table 2. Semen parameters before and after density gradient centrifugation using 4 media in 30 normozoospermic men

	Raw semen	Pure Sperm [®]	Pure Ception [®]	Sidney [®]	Sperm Grad [®]
TMC (million)	144.1 [113.5, 282.4]	3.0* [1.7, 7.4]	5.1* [1.4, 11.7]	5.2* [3.1, 10.4]	0.3* [0.1, 1.3]
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]
Morphologically normal spermatozoa (%)	6.9 [5.2, 8.3]	10.3* [7.1, 14.2]	9.9* [7.6, 12.6]	9.8* [7.9, 15.3]	10.7* [7.7, 15.9]
DNA fragmentation (%)	11.2 [7.9, 21.2]	6.0* [2.7, 12]	6.5* [2.9, 13.8]	8.5 [3.9, 14.4]	4.9* [1.7, 10.6]
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]

Median [interquartile range]

*p <0.05 when compared with raw semen

TMC = total motile spermatozoa count, HA = hyaluronic acid.

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

	Motility (%)	Morphologically normal spermatozoa (%)	DNA fragmentation level (%)	HA binding ability (%)
Age (years)	$r = -0.435$ $p = 0.016$	$r = -0.084$ $p = 0.658$	$r = 0.470$ $p = 0.009$	$r = -0.252$ $p = 0.180$
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.

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 4 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 4 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.

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국 문 초 록

목적: 보조생식술을 시행할 때 운동성이 좋은 정자를 선별하기 위한 방법으로 밀도 차 원심분리법이 사용된다. 본 연구에서는 네가지 밀도 차 원심분리 용액에 따른 정자 선별 능력에 차이가 있는지를 밝히기 위해 정상 모양 정자 선별, DNA 분절 감소, 하이알루론산 결합능 증가의 측면에서 각 용액을 비교하였다.

방법: 총 운동성 정자 수가 8 천만 개 초과인 30 명의 남성으로부터 정액을 기증받아 실험하였다. 각 대상자의 정액 샘플을 대조군 및 4 개의 실험군으로 균등하게 나눈 다음, 각각의 실험군에 대하여 4 종의 상용화된 밀도 차 원심분리 용액(PureSperm®, PureCeption®, Sidney®, SpermGrad®)을 이용한 밀도 차 원심분리를 시행하였다. DNA 분절도는 Halosperm® 측정 키트를 이용하였고, 하이알루론산 결합능은 HBA® 측정 키트를 이용하였다.

결과: 네 가지 용액으로 정액을 처리하였을 때 정상 모양을 가진 정자의 비율은 네 용액 별로 각각 10.3%, 9.9%, 9.8%, 10.7%였으며, 실험 전 정액 샘플의 평균치인 6.9%와 비교하여 유의하게 상승하는 소견을 보였다 ($p < 0.05$). DNA 분절도는 PureSperm®, PureCeption®, SpermGrad® 용액을 이용하여 밀도 차 원심분리를 하였을 때 각각 6.0%, 6.5%, 4.9% 였으며, 실험 전 정액 샘플의 11.2%와 비교하여 유의하게 감소하였다 ($p < 0.05$). 하지만 Sidney® 용액을 이용하여 처리하였을 때는 이러한 효과가 관찰되지 않았다 (8.5%, $p > 0.05$). 하이알루론산 결합능은 네 용액으로 처리를 하였을 때 모두 유의한 차이를 보이지 않았다.

결론: 네 가지 원심분리 밀도 차 용액은 정상 모양을 가지는 정자를 선별하는 효과의 측면에서 대등한 효과를 나타내었다. 이 중 PureSperm®, PureCeption®, SpermGrad® 용액은 DNA 분절도가 낮은 정액을 선별하는데 있어서 효과를 보였으나

Sidney® 용액에서는 이러한 효과가 관찰되지 않았다.

주요어: 정자 / 원심분리 / 밀도 차 / DNA 분절 / 하이알루론산

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