Comparison of Hyaluronan Binding Assay Scores of Spermatozoa Using Swim-Up Techniques and Density Gradient Centrifugation

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Objective: *To compare the hyaluronan binding assay (HBA) scores of sperm preparation using two different methods, the swim-up technique and density gradient centrifugation (DGC).*
Materials and Method: *This experimental study used semen specimens from 54 volunteer subjects with normal semen analysis according to the 2010 World Health Organization criteria. Each semen specimen was split into two portions: one was prepared using the swim-up method and the other the DGC method. The prepared sperm were counted in the sperm-HBA slide to determine bound and unbound motile sperm. The HBA scores between the two methods were compared using matched analysis.*
Results: *The HBA scores by either preparation method were >80%. There was no statistically significant difference in HBA scores between the swim-up preparation [median 97%, interquartile range (IQR) 94, 98] and density gradient centrifugation [median 96%, IQR 95, 98] (p = 0.96). Ten of the 54 specimens received the same HBA scores following the two methods and none differed by more than ±7%.*
Conclusion: *Both preparation methods gave high HBA scores with no apparent difference in the proportions between methods.*

Keywords: *Hyaluronan binding, Spermatozoa, Swim-up, Density gradient centrifugation*

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Infertility occurs in about 15% of couples[1,2], with male factors being responsible in about 50% of cases[3]. In assisted reproductive technologies (ART), the aim of sperm preparation is to maximize the chance of fertilization and provide as many normally-fertilized oocytes as possible for transfer to the uterus or cryopreservation[4]. Sperm preparation has been used to obtain spermatozoa with a high potential for normal fertilization and separate normal spermatozoa from the abnormal ones or retrieve normal spermatozoa from samples obtained directly from the male genital tract[5]. Several methodologies are available for sperm preparation including migration techniques (swim-up, underlay and migration-sedimentation methods), density gradient centrifugation (DGC) and filtration techniques. In all of them, the aim is to select viable, mature and healthy sperm without or with only a low level of DNA damage.

The popular methods in our center are the swim-up technique and DGC using SilSelect®. Different sperm preparation methods have their own advantages and disadvantages. In general, DGC will result in better sperm motility with less mitochondria and DNA damage than other methods[6-9], but a lower percentage of progressive motile sperm and normal-morphology sperm than the swim-up method[4,10-14]. Many studies have attempted to identify the most effective sperm preparation method; however, it has not been possible to recommend a superior method[15].

Currently, a novel test, which is convenient and not time-consuming, the hyaluronan binding assay (HBA), has been developed as a diagnostic kit

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for assessing sperm maturity and functional competency[16-18]. Hyaluronic acid (HA) or hyaluronan, a high molecular weight glycosaminoglycan, is a major component in the cumulus oophorus complex (COC) and zona pellucida (ZP). During fertilization process, the mature sperm needs to bind and react with ZP to start the process. Commercial HBA has HA coated onto a slide. Mature sperm that have successfully completed spermiogenesis, are devoid of DNA fragmentation[18-20], have low aneuploidy frequency[19] and possess a receptor for hyaluronan on the plasma membrane, which can bind to HA on the slide. Conversely, immature sperm have cytoplasmic retention, higher frequency of aberrant morphology, lower DNA integrity[17], and fewer HA receptors; thus, they cannot bind to HA and remain free-swimming on the HBA slide.

Although one would assume that different methods of sperm preparation might give different percentages of hyaluronan-binding sperm, no study on this topic has been conducted to date. The present study aimed to compare the percentages of sperm binding to HA or HBA scores between the swim-up and DGC (SilSelect®) sperm preparation methods.

Material and Method

Subjects

This experimental study was approved by the Institutional Ethics Committee of Faculty of Medicine, Prince of Songkla University, and conducted between 1 July 2012 and 31 October 2012 at the Infertility Clinic of our university's hospital. Semen specimens were obtained from volunteer men aged between 19 and 30 years of age, who had normal sperm analysis according to the criteria of the World Health Organization[21]. The volunteers were asked to provide semen samples after 2-4 days of ejaculatory abstinence. Semen specimens were produced by masturbation directly into a sterile plastic container, in a room specially provided for this purpose and located adjacent to the laboratory of infertility unit.

Semen analysis

Within 1 hour, the liquefied semen sample was processed and analyzed according to the recommendation of the World Health Organization[21]. Sperm concentration was assessed by a conventional method using a Makler counting chamber. Sperm motility was assessed in at least 100 spermatozoa per replicate for the percentage of different motility categories (progressive, non-progressive, immotile). Sperm morphology was assessed via Papanicolaou stain according to strict Kruger's criteria[22], and sperm vitality was assessed using the Eosin-nigrosin staining technique. All of the analysis procedures were performed in 2 replicates and counting followed the World Health Organization[21] recommendations. Bias was reduced by calculating acceptable differences between two percentages for a given average reported.

Sperm preparation technique

After examination, the rest of the semen sample was split into two parts and processed by swim-up technique and the DGC as described below:

Swim-up

An 1 ml aliquot of whole semen was placed in a test tube, and gently layered on 1.2 ml of pre-warmed sperm preparation medium, (Earle's media, Biochrome Ltd, Cambridge, UK) supplemented with human serum albumin (Sigma-Aldrich, St. Louis, USA) over it. The tube was inclined at an angle of about 45° and incubated for 1 hour at 37°C in a 5% CO_2 incubating chamber. The upper most 1 ml of medium was removed and diluted with 2.0 ml of supplemented medium. Centrifugation was then performed at 500 g for 5 minutes and the supernatant discarded. The sperm pellet was resuspended in supplemented medium to assess sperm concentration, motility, morphology and HBA score.

Density gradient centrifugation

Gradients of 40% and 80% of SilSelect® (FertiPro N.V.8730, Beeman, Belgium) were prepared from an isotonic gradient medium and Earle's media (Biochrome Ltd., Cambridge, UK) supplemented with human serum albumin (Sigma-Aldrich, St. Louis, USA). One milliliter of 40% density-gradient medium was layered over 1 ml of 80% density-gradient medium followed by 1 ml of semen placed above the density-gradient medium and centrifuged at 300 g for 20 minutes. The pellet was washed twice (200 g, 5 minutes) in 5 ml of pre-warmed sperm preparation medium. The sperm pellet was resuspended in supplemented medium to assess sperm concentration, motility, morphology and HBA score.

Sperm-hyaluronan-binding assay

The procedure was performed by researcher following the manufacturer's instructions (Origio, Inc., 2011) using an HBA kit slide (Biocoat, Inc., PA, USA); the HBA was performed immediately after sperm preparation. The prepared semen was gently mixed and

10 µL of it pipetted onto the center of the HBA slide, covered with a cell-Vu gridded cover slip and incubated at 37°C in a 5% CO_2 incubating chamber for 10 minutes. The spermatozoa were evaluated under a phase contrast microscope 400x magnification, counted twice by 2 embryologists blinded from each other and the average percent bound recorded. If less than 30 motile spermatozoa were present within the counting area, a second HBA slide and the same sperm were used. Bound sperm demonstrated vigorous tail beating with no progressive movement while unbound sperm swam freely. The predominant class (bound or unbound) of motile sperm was counted first (at least 100 spermatozoa). Immediately, the count was repeated in exactly the same number of grid squares, counting the other class of motile sperm. If the total spermatozoa were less than 100, counting in 100 grid squares was performed. The percentage of HA-bound spermatozoa was calculated by dividing the bound motile spermatozoa by the total of bound and unbound motile spermatozoa and then multiplied by 100.

Statistical analysis

The statistical analysis was performed using the computer program 'STATA version 10.0' (stataCorp, College Station, TX, USA). Comparisons between groups were performed via the Wilcoxon matched-pairs signed-ranks test. A *p*-value of <0.05 was considered significant using the two-tailed test. The percentages of HA-bound sperm following the two methods were plotted in a two-way scatterplot. The continuous variables were summarized as median and interquartile range.

Results

A total of 54 volunteer men with normal sperm analysis according to the criteria of the World Health Organization[21] participated in the present study. The patients' characteristics are demonstrated in Table 1 and the initial semen characteristics are demonstrated in Table 2.

In comparison with sperm parameters after preparation, only concentration resulted in a significant difference between the methods, with DGC being higher ($p<0.001$). The sperm-HBA outcome was not significantly different within specimen between the two methods of sperm preparation (Wilcoxon matched-pairs signed-ranks test, $p = 0.96$), as shown in Table 3 and Fig. 1 and its value in all specimens was more than 80%.

Table 1. Patients characteristics

Characteristics	N = 54
Age (year), median (IQR)	21.0 (21, 22)
Body mass index (kg/m^2), median (IQR)	22.8 (20.4, 25)
Drinking, No. (%)	29.0 (53.7)
Smoking, No. (%)	24.0 (44.4)
Abstinence day, median (IQR)	3.0 (2, 3)

Table 2. Initial semen characteristics (N = 54)

	Median (IQR)
Volume, ml	4.5 (4, 5)
pH	8.0 (8, 8.5)
Liquefaction, min	30.0 (30, 30)
Concentration, x 10^6/ml	66.0 (39, 100)
Vitality, %	62.0 (60, 70)
Total motility, %	79.0 (72, 91)
Progressive motility, %	67.0 (62, 77)
Normal morphology, %	11.5 (8, 18)

Table 3. Comparison of semen characteristics after semen preparation by swim-up and density gradient centrifugation techniques

Variables	Swim-up	DGC	*p*-value*
Concentration, x10^6/ml	6 (2, 12)	10 (5, 21)	<0.001
Total motility, %	99 (97, 100)	98 (94, 100)	0.089
Progressive motility, %	89 (62, 95)	67 (62, 76)	0.334
Morphology, %	18 (11, 26)	17 (12, 23)	0.456
HBA score, %	97 (94, 98)	96 (95, 98)	0.955

DGC = density gradient centrifugation techniques; HBA = hyaluronan binding assay
Values were expressed as median and 25th, 75th percentile.
* Wilcoxon matched-pairs signed-ranks test.

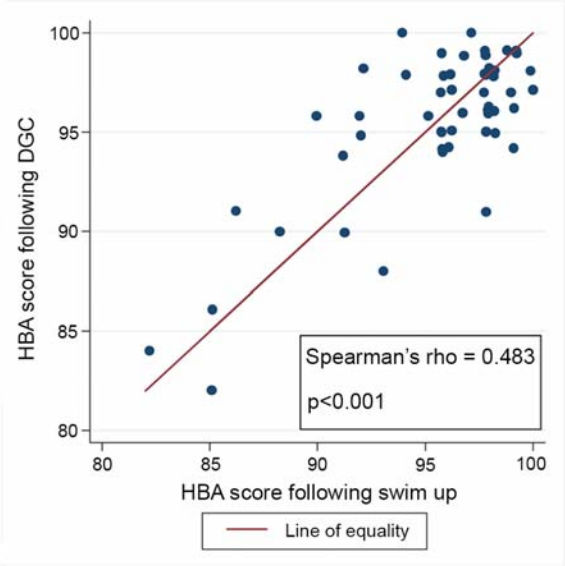

Fig. 1 Two way scatterplot of HBA scores from swim-up and density gradient centrifugation techniques (N = 54).

Table 4. The frequency of difference in HBA scores between the swim-up and density gradient centrifugation techniques

Difference in HBA scores (swim-up-DGC)	N = 54 n (%)
-6	3 (5.6)
-5	1 (1.8)
-4	2 (3.7)
-3	4 (7.4)
-2	5 (9.3)
-1	6 (11.1)
0	10 (18.5)
1	6 (11.1)
2	9 (16.7)
3	5 (9.3)
5	2 (3.7)
7	1 (1.8)

HBA = hyaluronan binding assay; DGC = density gradient centrifugation techniques

Ten of the 54 specimens received the same HBA scores following the two methods and none differed by more than ±7% (Table 4).

Regarding interobserver variation in HBA scores, the mean difference was 0.120 (95% confidence interval -0.444 to 0.685) and the limits of agreement (mean difference ±1.96 x standard deviation of difference) were -5.8 to 6.0 over the range of mean values from 82-100%. Differences between the methods were lower when the average value approached 100%.

Discussion

Sperm preparation is used to obtain spermatozoa with a high potential for normal fertilization and separate normal spermatozoa from abnormal ones[5]. The aim of sperm preparation in ART is to maximize the chance of fertilization and provide as many normally fertilized oocytes as possible for transfer to the uterus or cryopreservation[4]. The recovery rates, motility, morphology, and degree of DNA damage vary greatly among sperm preparation techniques[14,23,24]. A recent meta-analysis resulted in no consensus to recommend any specific sperm preparation technique for intrauterine insemination [IUI][15]. Therefore, the present study aimed to compare two common sperm preparation techniques, the swim-up and DGC using HBA.

HBA is a simple method and not time-consuming to detect functionally mature sperm. This diagnostic kit is based on the principle that functionally mature sperm will bind to HA that coat the slide because, after spermiogenesis, only functionally mature sperm which have completed plasma membrane remodeling develop ZP and HA binding sites[25]. To start the fertilization process, sperm needs to bind and react with ZP[26], of which a major component is hyaluronan or HA. Conversely, immature sperm and intermediate-maturity ones still have cytoplasmic retention due to incomplete plasma membrane remodeling. Therefore, immature sperm will not bind to HA and the intermediate-maturity sperm will bind briefly and release, and rebind briefly again because it does not develop HA receptors and has a low density of HA receptors, respectively[17]. Furthermore, HBA is likely to be a non-direct measurement test of DNA fragmentation. Mature sperm, which bind to HBA due to the presence of HA receptors, are devoid of DNA fragmentation. The percentage of HA-bound sperm correlates with low levels of DNA fragmentation[25,27-30], high DNA integrity[28] and low sperm aneuploidies[19,31]. Many studies have demonstrated a correlation between TUNEL and HBA-bound sperm[20,27]. The immature sperm have cytoplasmic retention, higher frequency of aberrant morphology and lower DNA integrity[17]. Even though a myriad of tests to detect DNA fragmentation exist, e.g., TUNEL, comet, CMA3, in-situ nick translation, DBD-FISH (DNA breakage detection fluorescence in situ hybridization), sperm chromatin

dispersion test (SCD) and sperm chromatin structure assay (SCSA)[32], they are time-consuming. HBA may help in identifying sperm with low-level DNA fragmentation faster.

The present study found that both methods of sperm preparation had no statistically significant difference in HBA scores. Other studies on only the DGC method for sperm preparation have reported high HBA score as well (91.3%[33] and 71.4%[34]). Moreover, the difference in HBA scores in our investigation was not big; the maximum was 7% and most scores were equal. Both methods yielded HBA scores higher than the threshold value that discriminates between higher and lower fertility expectation (80%) according to the manufacturer. This may be because the initial semen samples were normozoospermic; therefore, the proportion of normal spermatozoa was larger than that of abnormal ones. Based on this finding, it may be assumed that both of these sperm preparation methods yield a high level of functionally mature sperm with little DNA damage.

To date, even a recent meta-analysis[15] did not find enough evidence to recommend a specific preparation technique for intrauterine insemination (IUI). Henkel and Schill[35] reviewed the advantages and disadvantages of both of these sperm preparation methods. They concluded that the swim-up method had an advantage in that it was easy to perform, cost-effective and made possibly the recovery of a very clean fraction of highly motile spermatozoa. Nevertheless, it was suitable for a high initial sperm count and motility level. Furthermore, spermatozoa were massively damaged by reactive oxygen species (ROS) and had a significantly lower percentage of normally chromatin-condensed spermatozoa. Regarding DGC, its advantages consisted of its ability to separate even a very low sperm density of spermatozoa from ejaculates, usual yield a clean fraction of highly motile spermatozoa and a significantly reduced ROS level. The disadvantages of DGC were time-consuming, high cost and carrying a potential risk for endotoxins. The present study was also found that only the concentration parameter was significantly higher in DGC than in the swim-up. In contrast, Amiri et al[36], found that DGC was better than the swim-up techniques in terms of concentration, motility, normal morphology and even DNA fragmentation detected by comet assay. Other studies have found that spermatozoa recovered after the swim-up preparation had significantly less denatured DNA[37] and a reduced proportion of sperm with chromosomal aberrations[38]; yet, some studies have reported no significant differences in the rates of the DNA-damaged sperm[39]. Additionally, sperm recovery using DGC resulted in a significant decrease in the percentage of sperm with DNA damage, whereas the swim-up treated sperm showed no significant improvement[40]. The different outcome in DNA damage of sperm after recovery between studies may be due to the use of different techniques for detection. Nevertheless, the techniques of sperm preparation used for IUI or ART depend on the facilities of the medical institutes.

The effects of HA-bound sperm on ART outcome are still controversial. Previous studies have reported no correlation between HBA score and fertilization, cleavage, good-quality embryos, miscarriage and pregnancy rate in couples undergoing IVF[27]. Moreover, HBA scores in patients with clinical pregnancy and those without pregnancy were comparable[27]. In contrast, Parmegiani et al[41], who compared sperm selection using PVP-ICSI and HA-ICSI, found that the rate of best-quality embryos in the HA-ICSI group was significantly higher than that in the PVP-ICSI group, and fertilization, pregnancy, and implantation rates had a tendency to be better in the HA-ICSI group, but this was not a statistically significant difference. In a different study, Nasr-Esfahani et al[30], found that the percentage of fertilization was significantly higher in oocytes inseminated by HA-selected sperm while pregnancy and implantation rates were insignificantly increased. Pregl Breznik et al[33], and Ye et al[42], found that the fertilization rate after conventional IVF correlated with the hyaluronan-binding ability. Currently, there are no cutoff values for HBA scores to discriminate between higher and lower fertility expectation[43].

A limitation of the present study is that it was conducted with normal semen samples, which were characterized by the presence of many normal by functional and mature spermatozoa in the original semen; thus, leading to high HBA scores in both methods.

The strength of the study consists in that it is the first study employing the HBA assay to assess sperm and compare between the swim-up and DGC (SilSelect®) methods of sperm preparation. Further studies should be conducted in patients with abnormal semen and compared HBA scores in different preparation methods with clinical correlations such as implantation rate, clinical pregnancy rate and pregnancy loss rate.

In conclusion, the sperm preparation

employing the swim-up and DGC techniques did not have different HBA scores.

What is already known on this topic ?

Hyaluronic acid (HA) or hyaluronanis a major component in the cumulus oophorus complex (COC) and zonapellucida (ZP). During fertilization process, the mature sperm needs to bind and react with ZP to start the process.

Mature sperm that have successfully completed spermiogenesis are devoid of DNA fragmentation, have low aneuploidy frequency and possess a receptor for hyaluronan on the plasma membrane, which can bind to HA on the slide of hyaluronan binding assay (HBA) kit.

DGC will result in better sperm motility with less mitochondria and DNA damage than other methods, but a lower percentage of progressive motile sperm and normal-morphology sperm than the swim-up method. No studies could recommend which one was a superior sperm preparation method.

What this study adds?

The sperm preparation employing the swim-up and DGC techniques did not have different HBA scores and both gave high HBA scores.

Potential conflicts of interest

None.

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การเปรียบเทียบการจับกับไฮยาลูโรแนนของอสุจิจากการเตรียมอสุจิระหว่างวิธีว่ายขึ้นและวิธีการแยกชั้นตามความหนาแน่นหลังการปั่น

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วัตถุประสงค์: *เพื่อศึกษาเปรียบเทียบการจับกับไฮยาลูโรแนนของอสุจิจากการเตรียมอสุจิระหว่างวิธีว่ายขึ้นและวิธีการแยกชั้นตามความหนาแน่นหลังการปั่น*

วัสดุและวิธีการ: *ศึกษาโดยวิธีการวิจัยเชิงทดลองในห้องปฏิบัติการกระทำที่ห้องปฏิบัติการรักษาคู่มีบุตรยาก โรงพยาบาลสงขลานครินทร์ โดยใช้อสุจิที่ได้จากอาสาสมัครชายที่มีอายุระหว่าง 19-30 ปี และมีผลการตรวจวิเคราะห์อสุจิเป็นปกติตามเกณฑ์ขององค์การอนามัยโลกปี พ.ศ. 2553 จำนวน 54 ตัวอย่าง นำอสุจิแต่ละตัวอย่างที่ได้มาเตรียมด้วยวิธีการสองวิธีคือ วิธีว่ายขึ้นและวิธีการแยกชั้นตามความหนาแน่นหลังการปั่น หลังจากนั้นนำอสุจิที่ได้จากการเตรียมทั้งสองวิธีมาดูจำนวนการเคลื่อนไหวและรูปร่างอีกครั้งแล้วนำไปวิเคราะห์ เปรียบเทียบดูเปอร์เซ็นต์การจับของอสุจิกับไฮยาลูโรแนน*

ผลการศึกษา: *การจับกับไฮยาลูโรแนนของอสุจิที่ได้จากการเตรียมแต่ละวิธี มีค่ามากกว่าร้อยละ 80 และพบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างวิธีว่ายขึ้น (ค่ามัธยฐานร้อยละ 97, พิสัยควอไทล์, 94, 98) และวิธีการแยกชั้นตามความหนาแน่นหลังการปั่น (ค่ามัธยฐานร้อยละ 96, พิสัยควอไทล์, 95, 98) (p = 0.96) โดย 10 ตัวอย่างจาก 54 ตัวอย่าง มีการจับของอสุจิกับไฮยาลูโรแนนเท่ากันจากทั้งสองวิธี และไม่มีตัวอย่างใดที่มีค่าการจับของอสุจิกับไฮยาลูโรแนนระหว่างทั้งสองวิธีต่างกันมากกว่าร้อยละ 7*

สรุป: *การเตรียมอสุจิทั้งสองวิธีมีค่าการจับของอสุจิกับไฮยาลูโรแนนสูง และไม่มีความแตกต่างกันระหว่างทั้งสองวิธี*