의학석사 학위논문

사람 정자에서 DNA 분절 정도의 예측 인자로서 저장액 내에서 보이는 특이 꼬리 팽창 유형

Specific tail swelling pattern in hypo-osmotic solution as a predictor of DNA fragmentation status in human spermatozoa

2020년 8월

서울대학교 대학원
의학과 산부인과학전공
김 성 우

사람 정자에서 DNA 분절 정도의 예측 인자로서 저장액 내에서 보이는 특이 꼬리 팽창 유형

지도교수 지 병 철

이 논문을 의학석사 학위논문으로 제출함

2020년 5월

서울대학교 대학원

의학과 산부인과학전공

김 성 우

김성우의 석사 학위논문을 인준함

2020년 7월

위 원 장 _____ (인)

부 위 원 장 _____ (인)

위 원 _____ (인)

사람 정자에서 DNA 분절 정도의 예측 인자로서
저장액 내에서 보이는 특이 꼬리 팽창 유형

2020년

김성우

Abstract

Specific tail swelling pattern in hypo-osmotic solution as a predictor of DNA fragmentation status in human spermatozoa

Sung Woo Kim
Department of Obstetrics and Gynecology
The Graduate School
Seoul National University

The aim of this study was to investigate DNA fragmentation status in human spermatozoa according to specific tail swelling patterns determined via hypo-osmotic swelling test (HOST).

Frozen semen samples from 21 healthy donors were thawed and prepared by the swim-up technique for use in intracytoplasmic sperm injection. The semen samples were treated for 5 minutes as part of the HOST procedure and then underwent the sperm chromatin dispersion test using a Halosperm kit. DNA fragmentation status (large halo, medium halo, small halo, no halo, or degraded) and the specific tail swelling pattern ("a" -"g") were assessed at the level of a single spermatozoon. A total of 42,000 spermatozoa were analyzed, and the percentage of spermatozoa without DNA fragmentation (as evidenced by a large or medium halo) was assessed according to the specific tail swelling patterns observed.

The HOST examinations showed that 98% of spermatozoa across all types displayed no DNA fragmentation. The percentage of spermatozoa without DNA fragmentation was 100% in type "d", 98.67% in type "g", and 98.17% in type "f" spermatozoa.

We found that the type "d" spermatozoa displayed no DNA fragmentation, but the other types of spermatozoa also displayed very low rates of DNA fragmentation. This result may be associated with the

processing of the spermatozoa by density gradient centrifugation and the swim-up technique.

Keywords: human spermatozoa, DNA fragmentation, tail swelling pattern, hypo-osmotic solution

Student Number: 2013-21667

목차

List of Figures

List of Tables

Introduction

When performing intracytoplasmic sperm injection (ICSI), spermatozoa are typically selected based on their motility and morphology as determined by a visual inspection under a light microscope. However, this conventional procedure still allows the selection of spermatozoa with DNA fragmentation. A high level of sperm DNA fragmentation (SDF) has been reported to be associated with a decreased fertilization rate, poor embryonic development, and poor pregnancy outcomes [1-7]. Selection of spermatozoa that display either no DNA fragmentation or the least amount of fragmentation possible may be an important component of ICSI in the future and may improve embryo quality and reproductive outcomes.

Several methods can be used to increase the prevalence of spermatozoa with low SDF levels in a given sample. These include sperm preparation methods, such as the swim-up method and density gradient centrifugation, as well as several sperm sorting methods, including annexin V binding-based magnetic-activated cell sorting and hyaluronic acid binding-based physiological ICSI [8]. However, methods of selecting a single live spermatozoon without DNA damage are currently unavailable.

Several tests to examine SDF levels exist, such as the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, the comet assay, and sperm chromatin dispersion (SCD). However, spermatozoa tested using these techniques are not viable because they are processed by fixation and staining during the examination and thus are not useful for clinical purposes. Therefore, noninvasive techniques to select a single live spermatozoon without DNA damage are needed.

The hypo-osmotic swelling test (HOST) is a simple vitality test used to assess the tail membrane function of spermatozoa [9,10]. When semen samples are incubated in a hypo-osmotic solution (150mOsm), the tails of

the sperm in solution may or may not become swollen. Among sperm tails that do swell, the specific swelling pattern can be used to classify the spermatozoon as one of six types. The presence of any swelling (as denoted by spermatozoa types “b”–“g”) represents healthy cell membrane function, and these spermatozoa are thus considered to be viable. The lower reference limit for vitality (i.e., membrane-intact spermatozoa) is 58% [10]. The tested spermatozoa can be used directly in ICSI because the test does not harm the head or neck of the spermatozoa.

The HOST score, or sperm vitality as measured by the HOST in raw semen, has been reported to be correlated with sperm motility, percentage of normal form, and SDF level (as measured by acridine orange staining) [11]. In that study, when individual spermatozoa were sequentially treated by HOST and then TUNEL staining, types “d”, “e”, and “f” spermatozoa showed significantly lower SDF levels than types “a” and “g” spermatozoa. In another study, the SDF level (as assessed by the TUNEL method) was lowest in type “b” spermatozoa (mean, 3.9%), which was significantly lower than in type “a” (mean, 16.8%) or “g” spermatozoa (mean, 13%) [12]. In that report, type “d” spermatozoa also displayed a significantly lower SDF level (mean, 5%) than type “a”. However, in those studies, spermatozoa were tested in raw semen that had not been prepared for ICSI use. Visualization of spermatozoa after TUNEL staining requires expensive immunofluorescence equipment. In contrast, SCD testing using a Halosperm kit (Halotech DNA, Madrid, Spain) does not require immunofluorescence equipment.

In the present study, frozen semen samples were thawed and prepared by swim-up for ICSI use and were sequentially treated using the HOST and the SCD tests. The DNA fragmentation status was assessed at the level of a single spermatozoon, and the SDF level was assessed according to the specific tail swelling pattern observed.

Methods

Semen samples were obtained from 21 healthy donors between 2008 and 2014. The semen samples were processed by density gradient centrifugation (SAGE gradient kit, 40%/80%; CooperSurgical, Copenhagen, Denmark) and were rapidly frozen using the liquid nitrogen (LN2) vapor method. A commercial yolk buffer was used as a basic freezing medium (Difco Egg Yolk Enrichment 50%; BD, Franklin Lakes, NJ, USA). The median age of the male donors at the time of semen collection was 27 years (interquartile range, 26–29 years). Unfortunately, no information was known regarding the marriage or fertility status of the sperm donors, their medical or surgical history, or their prescribed medications, if any. At the time of semen collection, informed consent was obtained from all subjects for the later therapeutic or experimental use of the samples.

A prospective study was performed from 2016 to 2017. The Institutional Review Board of Hamchoon Women's Clinic and Seoul National University Bundang Hospital approved this study (IRB No. B-1804-463-301). The semen samples were thawed by rapidly immersing the straws in a water bath at 37°C for 5 minutes. The thawed samples were mixed with 3 mL of Ham's F-10 solution containing 10% human serum albumin (HSA; Irvine Scientific, Santa Ana, CA, USA). The basic sperm concentration and motility were then assessed using motion analysis equipment (SAIS-PLUS 10.1; Medical Supply Co., Seoul, Korea). Strict criteria for the definition of normal sperm were used during the morphological assessment. According to the 2010 guidelines from the World Health Organization (WHO) [10], a sperm count ≥ 15 million/mL, motility ≥ 40%, and a percentage of normal form ≥ 4% were defined as normal. After thawing, the semen displayed a median sperm count of 20 million/mL (interquartile range, 10–33

million/mL), a median total motility of 30.8% (interquartile range, 19.2%-51.8%) and a median percentage of normal form of 6.7% (interquartile range, 3.9%-8.5%).

The semen samples were centrifuged three times at 250×g for 5 minutes after mixing with 3 mL of Ham's F-10 solution containing 10% HSA. The semen samples were then processed using the swim up method in the same procedure used for the preparation for ICSI in our center; 0.5 -1 mL of Ham's F-10 containing 10% HSA was gently layered on the pellet and then incubated at 37°C in a 5% CO2 atmosphere for 30 minutes. The supernatant (0.5 mL) was transferred to a conical tube and was then used for HOST.

HOST was performed using the method described previously [10,11]. A hypo-osmotic solution was prepared by dissolving 0.735 g of sodium citrate dihydrate and 1.351 g of D-fructose in 100 mL of purified water. The final solution was set at 150 mOsm and was then frozen at -20°C until later use. For each semen sample, 0.1 mL of semen was added to 1 mL of warmed hypo-osmotic solution and incubated at 37°C for 5 minutes. In HOST, 30 minutes of incubation is suggested for routine diagnostic procedures, but 5 minutes of incubation is recommended when spermatozoa are to be processed for therapeutic use [10].

For the SCD test, a Halosperm kit was used as described previously [13,14]. After assessment of tail swelling, the semen samples (25 μL) were mixed with pre-warmed agarose gel and were then dropped onto slides. The slides were covered with a glass coverslip and were kept for 5 minutes at 4°C in a refrigerator to create a microgel with implanted sperm. The coverslip was then removed, and the slides were immersed into a prepared acid solution (80 μL of HCl in 10 mL of distilled water) for 7 minutes at room temperature (RT). The slides were then transferred to the tray with a lysis solution and incubated for 25 minutes at RT. The slides were rinsed with distilled water for 5 minutes, followed by dehydration in increasing

concentrations of ethanol (70%, 90%, and 100%, for 2 minutes each). After drying, the slides were stained with Diff-Quik (Baxter Diagnostics Inc., McGaw Park, IL, USA), rinsed under tap water, and were allowed to dry at RT.

Each slide was examined under the light microscope at × 400 or × 1,000 magnification, and the tail swelling patterns and head halo patterns were simultaneously assessed for 200 spermatozoa per slide (Figure 1). Ten slides were prepared for each participant; thus, the final count of spermatozoa was 2,000 per person. Each sperm head was counted as either a large halo, medium halo, small halo, no halo, or degraded; a spermatozoon was classified as having a large halo if it produced a halo with a thickness equal to or greater than the length of the minor diameter of the core, as having a medium halo if the spermatozoon produced a halo with a thickness smaller than the length of the minor diameter of the core and greater than one-third of the minor diameter of the core, and as having a small halo if the spermatozoon produced a halo with a thickness equal to or smaller than one-third of the minor diameter of the core. Spermatozoa with a small halo or no halo, and those classified as degraded, were considered spermatozoa with fragmented DNA.

All statistical analyses were performed using IBM SPSS ver. 22.0 (IBM Corp., Armonk, NY, USA). The Spearman correlation test was used to evaluate the correlations between parameters. The results were considered significant when the p-value was < 0.05.

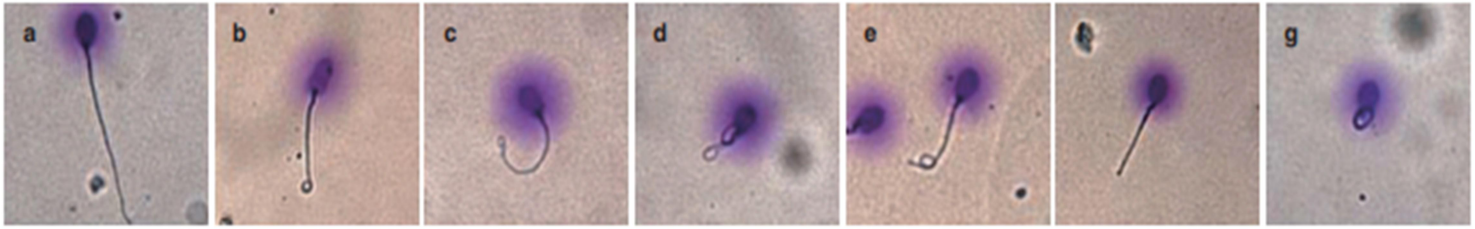

Figure 1. Representative photographs showing sperm heads with large halos after the sperm chromatin dispersion test. Each letter (a–g) corresponds to the specific tail swelling type after the hypo-osmotic swelling test.

Results

In this study, the median HOST score was 16.7% (interquartile range, 10.4%–20.7%), and the median SDF level was 1.40% (interquartile range, 0.65%–2.83%). The HOST score was not correlated with the age of the man, sperm concentration, total motility, or the percentage of normal form. The SDF level was not correlated with the age of the man, sperm concentration, or motility, but it was negatively correlated with the percentage of normal form (Spearman coefficient of rank correlation, $r = -0.473$; $p = 0.034$). The HOST score was not correlated with the SDF level.

The results of a pooled analysis after sequential treatment using the HOST and the SCD test are presented in Table 1. Across all types of spermatozoa as classified by the HOST procedure, > 93% of spermatozoa lacked DNA fragmentation (i.e., had a large or medium halo), and no statistical significance was noted among the seven types from HOST. However, the proportion of spermatozoa without DNA fragmentation was highest in type “d” (100%), followed by type “g” (98.67%) and type “f” (98.17%).

Table 1. The number of spermatozoa exhibiting each of the five head halo patterns and categorized as each of the seven types after the HOST

HOST type	Head halo pattern							Spermatozoa without DF (%)[a]
	Large halo	Medium halo	Small halo	No halo	Degraded	Total	Large halo+medium halo	
a	30,316	3,380	505	154	49	34,404	33,696	97.94
b	884	242	25	2	0	1,153	1,126	97.66
c	288	119	11	0	0	418	407	97.37
d	60	24	0	0	0	84	84	100.00
e	130	78	7	1	0	216	208	96.3
f	1,439	489	30	5	1	1,964	1,928	98.17
g	2,685	1,026	49	1	0	3,761	3,711	98.67
Total	35,802	5,358	627	163	50	42,000	41,160	98.00

The data above were pooled from 21 men.

HOST, hypo-osmotic swelling test; DF, DNA fragmentation.

[a]The pooled percentages of spermatozoa without DF by type after the HOST are listed in the last column.

Discussion

In the present study, type “d” spermatozoa displayed no DNA fragmentation (i.e., all type “d” spermatozoa exhibited a large or medium halo). Unfortunately, type “d” spermatozoa were rare (0.2% of the total pool of spermatozoa), which is consistent with the findings of Bassiri et al. [12]. The percentage of spermatozoa without DNA fragmentation was 98.67% in type “g” spermatozoa and 98.17% in type “f” spermatozoa. Since type “g” and type “f” spermatozoa were not rare (8.9% and 4.7% of the total pool of spermatozoa, respectively), it would be reasonable to choose type “g” or type “f” spermatozoa for ICSI use, assuming no type “d” spermatozoa are available.

In one study, types “d”, “e”, and “f” spermatozoa showed a significantly lower SDF level (as assessed by the TUNEL method) than types “a” or “g” spermatozoa [11]. In another study, the SDF level (as assessed by the TUNEL method) was lowest in type “b” (mean 3.9%), which was significantly lower than that found in type “a” (mean, 16.8%) or type “g”(mean, 13%) spermatozoa [12]. In that report, type “d” spermatozoa also displayed a significantly lower SDF level (mean 5%) than type “a”.

In those reports, type “g” spermatozoa displayed a relatively high SDF level; however, we found that type “g” spermatozoa displayed a relatively low SDF level [11,12]. The explanation for this difference in findings is not known for certain, but possible reasons could be that we used a different sperm processing and different assay method for SDF. Nonetheless, type “d” spermatozoa showed a good SDF level in our study and in both other reports.

The lower reference limit for the HOST score is 58% [10]. In the present study, however, the median value of the HOST score was 16.7%

(interquartile range, 10.4%–20.7%). This lower median HOST score observed in our study might have been related to our relatively short incubation time of 5 minutes. The WHO manual recommends 30 minutes of incubation for routine diagnostic procedures, but it recommends only 5 minutes of incubation for therapeutic purposes [10]. A shorter incubation time for the HOST procedure would result in less swelling of the spermatozoa tail; thus, the HOST score is expected to be lower after 5 minutes of incubation than after 30 minutes of incubation.

Additionally, the median SDF level was very low in the present study (1.40%; interquartile range, 0.65%–2.83%). We believe that the semen samples used were relatively good, as they were obtained from young, healthy donors. Furthermore, the semen samples were processed by density gradient centrifugation before freezing and were processed by the swim-up method after thawing.

In our experiment, we could not simultaneously evaluate the sperm head morphology and the tail swelling pattern of an individual spermatozoon because the sperm head was already swollen due to the SCD test. However, in a previous study, types “d” and “c” spermatozoa showed a significantly higher percentage of normal form than type “a” spermatozoa [15]. In another study conducted by the same researchers, the percentage of normal form was significantly higher in type “d” spermatozoa than in the other types [12]. In that report, type “d” spermatozoa, followed by type “c”, exhibited the highest quality in terms of early and late apoptosis, nuclear maturity, and membrane integrity [12].

A limitation of the present study is that the spermatozoa were obtained only from donors for the purpose of artificial insemination. Additionally, all of the samples were frozen. Further studies will be required to ascertain the relationship between the HOST pattern and SDF level in an infertile population and/or in fresh, unfrozen spermatozoa.

A sibling oocyte study in couples with severe male infertility

revealed that embryos exhibited better quality and a higher implantation rate when type “c”, “d”, or “e” spermatozoa were preferentially selected, compared to the results of conventional selection [16]. We recommend routine HOST during ICSI and subsequent selection of the specific types of spermatozoa that have been found to have the lowest SDF level. However, our recommendation should be further confirmed by a clinical trial. HOST is a simple and convenient technique that does not destroy the structure of the spermatozoon. The application of HOST may be a valuable tool in the routine identification and selection of viable, DNA-intact individual spermatozoa for ICSI.

References

1. Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, et al. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. Arch Androl 2003;49:49-55.

2. Saleh RA, Agarwal A, Sharma RK, Said TM, Sikka SC, Thomas AJ Jr. Evaluation of nuclear DNA damage in spermatozoa from infertile men with varicocele. Fertil Steril 2003;80:1431-6.

3. Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, et al. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertil Steril 2004;81:965-72.

4. Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod 2006;21: 2876-81.

5. Evenson D, Wixon R. Meta-analysis of sperm DNA fragmentation using the sperm chromatin structure assay. Reprod Biomed Online 2006;12:466-72.

6. Li Z, Wang L, Cai J, Huang H. Correlation of sperm DNA damage with IVF and ICSI outcomes: a systematic review and meta-analysis. J Assist Reprod Genet 2006;23:367-76.

7. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, et al. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod 2012;27:2908-17.

8. Nadalini M, Tarozzi N, Di Santo M, Borini A. Annexin V magneticactivated cell sorting versus swim-up for the selection of human sperm in ART: is the new approach better then the traditional one? J Assist Reprod Genet 2014;31:1045-51.

9. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ.

Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219-28.
10. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization; 2010.
11. Stanger JD, Vo L, Yovich JL, Almahbobi G. Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation. Reprod Biomed Online 2010;21:474-84.
12. Bassiri F, Tavalaee M, Shiravi AH, Mansouri S, Nasr-Esfahani MH. Is there an association between HOST grades and sperm quality? Hum Reprod 2012;27:2277-84.
13. Tandara M, Bajic A, Tandara L, Bilic-Zulle L, Sunj M, Kozina V, et al. Sperm DNA integrity testing: big halo is a good predictor of embryo quality and pregnancy after conventional IVF. Andrology 2014;2:678-86.
14. Anifandis G, Bounartzi T, Messini CI, Dafopoulos K, Markandona R, Sotiriou S, et al. Sperm DNA fragmentation measured by Halosperm does not impact on embryo quality and ongoing pregnancy rates in IVF/ICSI treatments. Andrologia 2015;47:295-302.
15. Bassiri F, Tavalaee M, Nasr Esfahani MH. Correlation between different patterns of hypo-osmotic swelling and sperm functional tests. Int J Fertil Steril 2013;7:193-8.
16. Charehjooy N, Najafi MH, Tavalaee M, Deemeh MR, Azadi L, Shiravi AH, et al. Selection of sperm based on hypo-osmotic swelling may improve ICSI outcome: a preliminary prospective clinical trial. Int J Fertil Steril 2014;8:21-8.

국문초록

본 연구는 사람 정자에서 저장액팽창검사 (hypo-osmotic swelling test)를 시행하였을 때 보이는 특이 정자 꼬리 팽창 유형에 따라 DNA 분절 정도를 알아보고자 하였다.

21명의 건강한 기증자로부터 채취하여 밀도구배 원심분리 (density gradient centrifugation) 후 동결 보관해두었던 정액 검체를 해동한 후 부유법 (swim-up technique)을 통하여 처리하였다. 처리한 정액 검체에 대하여 5분 동안 저장액팽창검사를 시행한 후, Halosperm kit를 이용하여 정자 염색질 분산 검사 (sperm chromatin dispersion test)를 시행하였다. 단일 정자 수준에서 특이 꼬리 팽창 유형 (“a”-“g”)과 DNA 분절 정도 (large halo, medium halo, small halo, no halo, or degraded)를 평가하였다. 총 42,000개의 정자를 분석하였으며, 특이 꼬리 팽창 유형을 관찰한 뒤 각 유형별로 DNA 분절이 없는 정자 (large halo 혹은 medium halo를 보이는 경우)의 백분율을 분석하였다.

저장액팽창검사 시행 후 DNA 분절이 없는 정자의 백분율은 “d” 유형에서 100%, “g” 유형에서 98.67%, 그리고 “f” 유형의 정자에서 98.17%였다. 본 연구에 이용한 모든 정자를 대상으로 DNA 분절이 없는 정자의 백분율을 분석하였을 때 그 수치는 98%이었다.

본 연구를 통해 “d” 유형의 정자에서 DNA 분절이 없다는 것을 보여주었고, 다른 유형의 정자 또한 매우 낮은 DNA 분절을 보인다는 것을 알 수 있었다. 이러한 결과는 정자를 밀도구배 원심분리와 부유법으로 처리한 것과 관련이 있을 것으로 사료된다.

중심단어: 사람 정자, DNA 분절, 꼬리 팽창 유형, 저장액
학번: 2013-21667