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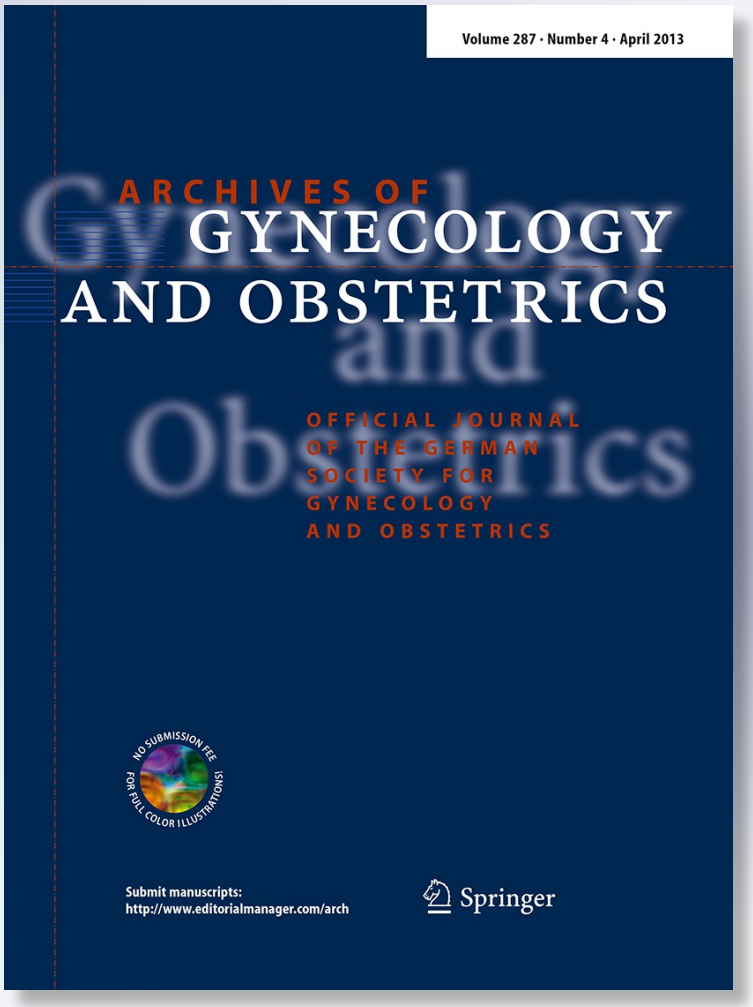

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REPRODUCTIVE MEDICINE

Evaluation of subgroups of the human sperm hypoosmotic swelling test in normozoospermic male cases with recurrent fertilization failure: a prospective case-controlled study

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Abstract
Purpose To compare subgroups of the human sperm hypoosmotic swelling test subgroups in both recurrent fertilization negative infertile cases with normal semen analysis and fertilization positive controls.
Methods This was a prospective case-controlled study performed with normospermic 33 previously fertile male (secondary infertility) and 41 infertile men who had undergone two or three unsuccessful in vitro fertilization attempts. HOS test was investigated in 4 subgroups including HOS 1, HOS 2, HOS 3, and HOS 4 according to the degree of sperm tail swelling and compared between the two groups.
Results Four subgroups were compared and statistical significance was demonstrated in HOS 1, HOS 3 and HOS 4 tests ($p < 0.001$) in fertile and infertile men. Highest HOS 1 and lowest HOS 4 grades were determined in Group A. However, no statistical significance was determined between two groups in HOS 2 test which was minimal swelling in sperm tails.
Conclusions HOS 1, HOS 3 and HOS 4 subgroups of HOS test are reliable and useful methods providing important information regarding the sperm function. A high HOS test 1 grade plus a low HOS test 4 grade should suggest a fertility problem, despite a normal semen analysis. HOS test subgroups provide additional information in normospermic cases with unexplained infertility.

Keywords Hypoosmotic swelling test · Male infertility · Hypoosmotic swelling test grades · Fertilization failure

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Introduction

Infertility affects approximately 20 % of the couples, and among these the male factor prevents successful conception even when in vitro fertilization (IVF) is used [1]. Standard semen analysis has long been the primary laboratory test used in infertile couples. Semen analysis, however, cannot demonstrate the functional capacity of sperm and often fails to predict the outcome of male infertility [2]. Sometimes, fertilization might occur despite an abnormal semen analysis, or it might fail to occur in the presence of a normal analysis [2]. Impaired fertilization is one of the causes of subfertility; it might play a role in failure of intrauterine insemination (IUI) treatment, and could result in failure of an IVF procedure in these patients [3]. In addition, in vitro fertilization might be the ultimate test of sperm and/or oocyte function [3]. Specific analysis of sperm functions, including motility, capacitation, acrosome reaction, sperm–zona binding ability, sperm–oocyte fusion and nucleus decondensation might be necessary to evaluate male fertility and to understand fertilization failures in IVF [4]. Sperm function tests are important to determine the better type of treatment in infertile couples. Among the sperm function tests, hypoosmotic swelling (HOS) test has first been reported by Jeyendran et al. [5]. The HOS test was developed to investigate the ability of the sperm

membrane to transport fluids. During the test, sperms are incubated in a hypoosmolar medium and those sperm with a functional membrane undergo swelling of the cytoplasmic space and the sperm tail fibers curl. These changes are visualized easily under light microscopy. Those sperm with damaged or chemically inactive plasma membranes will not have cytoplasmic swelling and their tails will remain uncurled. On the other hand, total fertilization failure (TFF) is observed in 5–20 % of couples undergoing IVF with normal sperm count, with a substantial recurrence rate of 30–67 % [6–9]. In this study, we investigated hypoosmotic swelling test subgroups, including HOS 1, HOS 2, HOS 3, HOS 4 in normospermic male subjects with fertilization failure.

Materials and methods

Participants

The study was performed in the In Vitro Fertilization Practice and Research Center of Istanbul University Cerrahpasa School of Medicine. Patients who admitted to In Vitro Fertilization Practice and Research Center were recruited in the study consecutively. The 41 infertile men, who had undergone two or three unsuccessful IVF attempts with total fertilization failure were enrolled in Group A. Couples had no children in Group A. 33 normozoospermic men, who already had at least one child (proven fathers) were enrolled in Group B as controls (secondary infertility). None of the couples had any obvious causes of infertility according to physical examination and routine diagnostic investigations. All of these couples in groups A and B had been unable to conceive for at least 12 months. Semen samples were collected by masturbation in IVF Practice and Research Center. The semen analysis was performed after at least 3 days of sexual abstinence by the methods described in the laboratory manual of the World Health Organization [10]. All semen analyses, including measurement of the ejaculation volume were performed manually within 1 h of collection of the sample. All samples met the following standards: $>20 \times 10^6$ spermatozoa/ml semen, >50 % spermatozoa with progressive motility and >14 % morphologically normal forms according to Kruger strict criteria. The protocol and potential risks and benefits of the study were explained to participants before they provided written informed consent. The study was performed in accordance with the Declaration of Helsinki. Institutional ethics committee approval was obtained, all subjects were informed of the study and a written informed consent was obtained from each subject prior to the performance of any study procedures.

The hypoosmotic swelling test technique

Semen were placed in a culture dish containing a hypoosmotic solution (fructose 150 mOsm, Na citrate 150 mOsm) and incubated at 37 °C for approximately 30 min. The hypoosmotic swelling test was performed using an Olympus phase-contrast microscope (Olympus Optical CO., Japan). The results of hypoosmotic swelling test were subdivided into four subgroups (HOS 1, HOS 2, HOS 3, HOS 4) according to the different shapes of hypoosmotically affected spermatozoa tails (Fig. 1) [11, 12]. HOS 1 indicated that there is no change, HOS 2 indicated minimal swelling in spermatozoa tail, HOS 3 indicated moderate swelling in spermatozoa tail, and HOS 4 indicated appearent swelling in spermatozoa tail. The percentage of spermatozoa that showed typical tail abnormalities indicative of swelling (HOS 1, 2, 3, and 4) was calculated.

In vitro fertilization procedures

Multiple follicular stimulation, cycle monitoring, and oocyte retrieval were performed using standard protocols. Ovulation was stimulated in all women using a long GnRH agonist protocol with 1 mg SC leuprolide acetate (LA; Lucrin; Abbott Pharmaceuticals, Turkey) starting in the mid-luteal phase until day 3 of the subsequent cycle. Estradiol (E2) measurements were performed. If serum E2 was <45 pg/ml (150 pmol/L) on day 3, the LA dose was cut in half and human menopausal gonadotrophin (HMG) (Menogon, Ferring pharmaceuticals, Saint-Prex, Switzerland) was started. Human chorionic gonadotropin (hCG; 10,000 IU IM, Pregnyl, Organon, The Netherlands) was administered when more than two follicles of ≥18 mm diameter were observed. Oocyte retrieval was performed by ultrasound-guided transvaginal aspiration under general anesthesia 36 h after administration of hCG (Siemens Medical Solutions, Germany, 5 MHz vaginal probe). Each

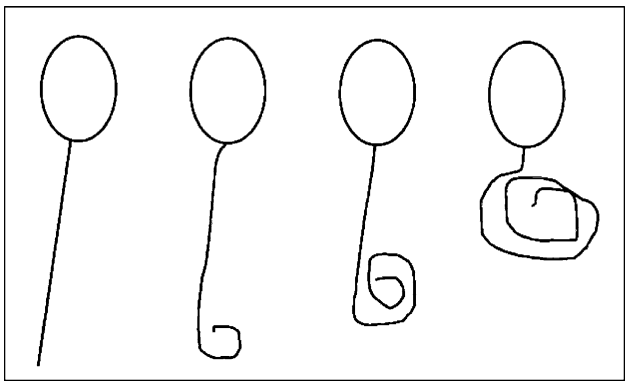

Fig. 1 Hypoosmotic swelling test was divided into four subgroups (HOS 1, 2, 3, 4) according to the different shapes of hypoosmotically affected spermatozoa tails

oocyte was inseminated in 1 ml of human tubal fluid (HTF) Irvine medium (Santa Ana, CA, USA) with 2×10^6 spermatozoa prepared by instructions 24 h after insemination; oocytes with two pronuclei were considered fertilized and were then cultured further with fresh HTF medium with 10 % human serum for cleavage and 30 % human serum for transfer.

Statistical analysis

Statistical Package for Social Sciences version 13.0 for windows (SPSS, Chicago, IL, USA) was used to perform statistical analyses. Non-parametric analyses were performed with Kruskal–Wallis and χ^2 tests, variance analyses were performed with analysis of variance. For all statistical analyses, a $p < 0.05$ was considered statistically significant.

Results

Mean age of female participants was 34.2 ± 2.1 in Group A, and 33.4 ± 3.6 in Group B, and there were no statistically significant differences in terms of mean female age between the two groups ($p > 0.05$). Infertility duration was 4.1 ± 2.3 years in Group A, 3.1 ± 1.5 years in Group B, respectively. Level of follicle stimulating hormone (FSH) was 7.9 ± 4.6 in Group A and 6.4 ± 3.1 in Group B at day 3 of the cycle. There were no statistically significant differences in terms of the FSH level determined before the stimulation, and also between the numbers of antral follicles observed in transvaginal sonographic examination before stimulation (Table 1). The results of HOS 1, 3, and 4 tests were statistically significantly different between groups A and B (Table 2). There were no statistically significant differences between groups A and B in HOS 2. Correlation of HOS test grades between groups A and B are shown in Table 2. HOS 1 grade sperm pattern suggestive of bad-functioning sperm was 80.13 ± 18.11 in Group A who had undergone two or three unsuccessful IVF attempts because of total fertilization failure, and consequently it was the highest HOS 1 pattern among the groups. However, lowest HOS 4 pattern suggestive of well-functioning sperm was also determined in the same group with a grade of 0.65 ± 2.66. A marked reduction was observed in the amount of well-functioning sperms in Group A in HOS test subgroups decreasing from 1 to 4. A sharp decrease from 80.13 ± 18.11 to 0.65 ± 2.66 was noted in Group A, whereas a mild decrease from 34.85 ± 18.25 to 17.95 ± 11.3 was noted in Group B. HOS 4 pattern was statistically significantly different between groups A and B with grades of 0.65 ± 2.66 and 17.95 ± 11.3, respectively (Table 2).

Table 1 Age of female participants, body mass index and other features

	Group A ($n = 41$)	Group B ($n = 33$)	p
Age of female participants	34.2 ± 2.1	33.4 ± 3.6	$p > 0.05$
Infertility duration (year)	4.1 ± 2.3	3.1 ± 1.5	$p > 0.05$
Body mass index (kg/m^2)	24.4 ± 2.9	25.1 ± 3.3	$p > 0.05$
Serum FSH level (IU/ml)	7.9 ± 4.6	6.4 ± 3.1	$p > 0.05$
Number of antral follicles before stimulation	9.6 ± 3.1	9.8 ± 2.8	$p > 0.05$

Values are given as mean ± SD

Table 2 Correlation of HOS grades in fertilization (+) and (−) groups

HOS test subgroups	Group A fertilization (−) ($n = 41$)	Group B fertilization (+) ($n = 33$)	p
1	80.13 ± 18.11	34.85 ± 18.25	<0.001
2	25.12 ± 19.65	27.17 ± 11.73	>0.05
3	5.20 ± 5.15	18.17 ± 12.56	<0.001
4	0.65 ± 2.66	17.95 ± 11.3	<0.001

Values are given as mean ± SD

Discussion

The outcome of IVF might be considered as the ultimate test of sperm fertilizing ability. A patient is considered fertile if at least one oocyte is fertilized with spermatozoa in vitro. The HOS test provides information on the capacity of spermatozoa to pump water into cell indicating that the membrane of mid-piece and tail is functionally intact and active [6]. The hypoosmotic swelling test is a functional test of sperm membrane integrity. An intact membrane allows the hypoosmotic solution to be taken up into the sperm followed by the typical tail swelling. Although the HOS test is inexpensive, easy to perform, and contrary to other semen parameters is stable over time once subnormality has been determined; it is rarely performed by most clinicians [2].

The test has originally been modified for use in the investigation of infertility by Jeyendran et al. [5], and a score of <60 % sperm with hypoosmotic swelling is usually regarded as suboptimal. Authors have suggested that this test evaluates the functional integrity of the sperm membrane and the in vitro fertilizing ability of spermatozoa. Authors have also suggested that this test might be used in addition to the standardized semen analysis [5]. Mordel et al. [12] have been the first to divide hypoosmotically affected sperms into four subgroups according to the different shapes of spermatozoa tails. They have found the best correlation between HOS test 3 and the different

sperm parameters including motility, total motile sperm fraction, concentration and SPA [12]. In this presenting study we found a significant correlation between HOS grades 1, 3, 4 and fertilization rates; however, there was no superiority of any one of these grades. HOS 1 grade sperm pattern which indicates bad-functioning sperm was highest and also HOS 4 pattern which indicates well-functioning sperm was lowest in subjects in Group A who had undergone two or three unsuccessful IVF attempts because of total fertilization failure. A marked reduction was observed from HOS 1 to 4 in Group A from 80.13 ± 18.11 to 0.65 ± 2.66. However, no significant changes were observed in HOS 2 in Group A, possibly due to the small sample size of this study. Further studies with larger sample sizes should be performed to elucidate the role of HOS 2 test. On the other hand, no significant reductions were seen in HOS tests in Group B.

Chan et al. [7, 8] have attempted to improve the prediction of human sperm fertility by a modification of the HOS test using seven different patterns of sperm tail swelling. They have used a multivariate stepwise discriminate analysis and obtained significant results regarding three of seven sperm tail swelling patterns [8, 9]. Authors have thus concluded that differential evaluation of hypoosmotic tail swelling might be useful for the prediction of human sperm fertility. However, authors have not found any statistically significant correlations between HOS test grades 1 and 4. These results might be related with sperm morphological parameters. Previous studies have demonstrated that the HOS test is able to predict pregnancy rate and outcome in couples undergoing in vitro fertilization and IUI procedures [2, 13, 14].

In this study we tested only normospermic cases with unexplained infertility. Group B was included in the study as the control group since they previously had children, but failed to conceive for 12 months of unprotected intercourse despite a normal spermiogram (secondary infertile). In addition, we encountered highest HOS test 1 pattern suggestive of bad-functioning sperm and lowest HOS 4 pattern suggestive of well-functioning sperm in Group A which previously had total fertilization failure (TFF). A marked reduction was observed in the amount of well-functioning sperms in Group A in HOS test subgroups decreasing from 1 to 4. A sharp decrease from 80.13 ± 18.11 to 0.65 ± 2.66 was observed in this group. Fertilization failure in Group A might be associated with poor sperm HOS test grades and especially HOS test 1 grade. No marked reduction was observed in Group B.

The HOS test might be considered an easy, inexpensive, and reliable test for predicting male fertility potential and for identifying among subfertile men those who have a greater possibility of conceiving with timed intercourse following ovulation induction.

The small sample size was one of the limitations encountered in this study. In addition, investigators evaluating the HOS tests were unblinded to patient groups. Further studies with double-blinded design might provide more objective results. Various techniques have been proposed and used to differentiate between non-viable and viable, but immotile spermatozoa for use in intracytoplasmic sperm injection (ICSI) to maximize the chances of pregnancy [15]. Staining tests lead to cell death and stained spermatozoa cannot therefore be used in this differentiation; however, better methods have been described and used such as the HOS test. The HOS test is the only choice for performing ICSI with nearly or completely non-motile sperm samples, and occasionally in cryopreserved sperm [16].

In conclusion, HOS test subgroups might provide additional beneficial information in normospermic sperm samples. A high HOS test 1 grade plus a low HOS test 4 grade or a sharp decrease from HOS 1 to HOS 4 should suggest a fertility problem even in the presence of a normal semen analysis.

Conflict of interest We declare that we have no conflict of interest.

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