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A New Human Spermatozoon Selection Method Based on Penetration of Cervical Mucus for Intracytoplasmic Sperm Injection

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Abstract: *Spermatozoa used for intracytoplasmic sperm injection (ICSI) are selected based on their motility and morphology. To explore other methods for selecting better spermatozoa, we developed a spermatozoa-sorting method using a physiologically natural selection system involving penetration into cervical mucus (CM). In addition, we analyzed the spermatozoa that penetrated the CM (CM-penetrating spermatozoa). The results were as follows. The CM-penetrating spermatozoa traveled a longer distance with better linear motility than spermatozoa in semen. Also, in comparison with spermatozoa obtained by density gradient centrifugation (DGC) and the swim-up method, the proportion of spermatozoa with normal morphology was higher, although, the proportion of spermatozoa with the head vacuole did not change. No DNA fragmentation was detected in the CM-penetrating spermatozoa. This method has several advantages. The technical procedure is simple and easy. Physical damage to spermatozoa is reduced because it does not require any centrifugation or washing procedure. A higher collection ratio of morphologically normal spermatozoon is achievable compared to the DGC and swim-up method, and it is a physiological selection method. We conclude that the CM penetration-based spermatozoa-sorting method is a promising new technique for ICSI because it is possible to collect physiologically better spermatozoa than those selected in the conventional selection method.*

Key words: *ICSI, Human spermatozoa, Cervical mucus*

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Introduction

The achievement of successful pregnancy in humans by intracytoplasmic sperm injection (ICSI) was first reported by Palermo *et al.* in 1992 [1]. Since then, ICSI has been used as an essential technique in assisted reproductive technologies. However, there has been concern since the inception that natural selection in fertilization might be eliminated because the fertilization process in ICSI, which is performed when fertilization fails in IVF, involves many artificial elements. Spermatozoon selection is a very important step in the ICSI procedure. However, in the procedure as it is currently performed, an operator selects a spermatozoon to be injected into eggs from a couple dozen to a few hundred million spermatozoa based only on criteria of motility and morphology. This eliminates the physiological selection of spermatozoa that normally occurs in the natural fertilization process. Therefore, we have attempted to establish a non-invasive method to evaluate the quality (abilities) of individual spermatozoa in order to select good spermatozoa for ICSI that would result in the birth of a healthy baby. In human fertilization *in vivo*, a few thousands to a few hundred million spermatozoa are ejected into the vagina at the same time in order to fertilize a mature egg released during ovulation. The first stage of natural selection of ejaculated spermatozoa occurs as they pass through the uterine cervix during the ovulation period. Spermatozoa first pass through cervical mucus (CM), which is secreted from the uterine cervix, and they swim forward to the uterine cavity. It is believed that, when spermatozoa enter the uterus, they receive the biochemical modification that is needed for separation from the seminal plasma

and fertilization of an egg [2]. Ejaculated spermatozoa are attached or fused to many substances included in the epididymal fluid or the seminal plasma, one of which is the decapacitation factor (DF). The DF is known to inhibit a spermatozoon's fertilizing ability if added to spermatozoa with fertilizing ability [3]. Thus, the components of the seminal plasma need to be removed in order to impart to spermatozoa the ability to fertilize an egg. The amount of CM secretion decreases and the viscosity of CM increases in the follicular and luteal phases of the menstrual cycle, which prevents bacteria from entering the uterus. The amount of CM secretion increases and the viscosity soften along with the amount of estrogen secretion when the ovulation period approaches. The CM produced during the ovulation period contains mucin, which has a high viscosity. This allows only the spermatozoa with good motility and morphological traits to progress, and limits the progression of the other morphologically abnormal spermatozoa [4, 5]. Subsequently, many of the spermatozoa are subjected to other natural selection systems such as phagocytosis by white blood cells as they migrate through the female reproductive organs (vagina, uterine cervix, uterus, and uterine tube) [6]. The percentage of spermatozoa that reach the ampulla of the uterine tube where fertilization occurs is less than 0.00005%, i.e. the number of spermatozoa that reach the egg is about 100–500 [7, 8]. Moreover, only the first spermatozoon that reaches the egg can penetrate the egg. The female reproductive organs and cervical mucus (CM) thereby naturally select the spermatozoa with the best quality, and eliminate spermatozoa with poor motility or abnormalities [9]. Because of these reasons, it is considered that better spermatozoa could be selected by analyzing CM-penetrating spermatozoa, which are selected in the normal fertilization process through physiological selection based on their ability to penetrate the CM.

In this study, in order to explore the possibility of finding a better method for selecting spermatozoa for ICSI based on CM penetration, we developed the CM penetration-based spermatozoa-sorting method. We evaluated the effectiveness of this method by analyzing the motility, morphology and DNA fragmentation.

Study Subjects and Methods

Study subjects

After obtaining informed consent, semen and CM samples were obtained from outpatients who had been receiving infertility treatment at the Fertility Clinic Tokyo. For the analysis of CM-penetrating spermatozoa, the collected semen samples were tested first, and only samples containing at least 20×10^6 spermatozoa with good linear motility were analyzed.

The CM samples were collected during natural cycles as well as induced ovulatory cycles. However, induced ovulatory cycles differ depending on the drugs used for stimulation; therefore, samples were collected during ovulatory cycles induced by gonadotropin using FSH products or HMG products. Clomiphene-induced ovulatory cycles were excluded due to their anti-estrogen effect on the cervical duct.

Experiment 1: Collection of CM-penetrating spermatozoa and motility analysis

We first analyzed the motility of spermatozoa in semen and the spermatozoa that were able to migrate to the culture medium. On the basis of our hypothesis that these traits could be used to define good spermatozoa, we conducted detailed objective motility analysis using the SMAS (Sperm Motility Analysis System, DITECT Co., Ltd., Tokyo, Japan) [10].

Measurement of spermatozoa motility

The motility of spermatozoa was measured using the SMAS sperm imaging device. The parameters used for the measurement were as follows. Straight line velocity (VSL, μm/s): the linear distance traveled by spermatozoa from the starting point to the final point was calculated and then divided by time. Curvilinear velocity (VCL, μm/s): the sum of the actual lengths of the curvilinear paths traveled by spermatozoa was divided by time. Linearity was expressed as VSL/VCL. Linearity increases as the VSL/VCL ratio approaches 1.0.

Semen samples collected from 32 patients were analyzed for motility. CM samples were collected during the ovulation period in natural cycles or at the time of egg collection during assisted reproductive technology (ART). Using a 1ml tuberculin syringe (without needle), CM was aspirated from the cervical canal. The outcome of the spermatozoa penetration experiment is largely influenced by the properties of the CM. Thus, based on the WHO laboratory manual, the minimally viscous and watery CM with a spinnbarkeit of at least 9 cm, which corresponds to the mid-cycle (ovulation period) CM, was used [11].

Ejaculated semen samples were left at room temperature for 30 to 60 min. After liquefaction, the semen was tested, and a portion of it was used to observe the penetration of spermatozoa through the CM by the CM penetration-based spermatozoa -sorting method.

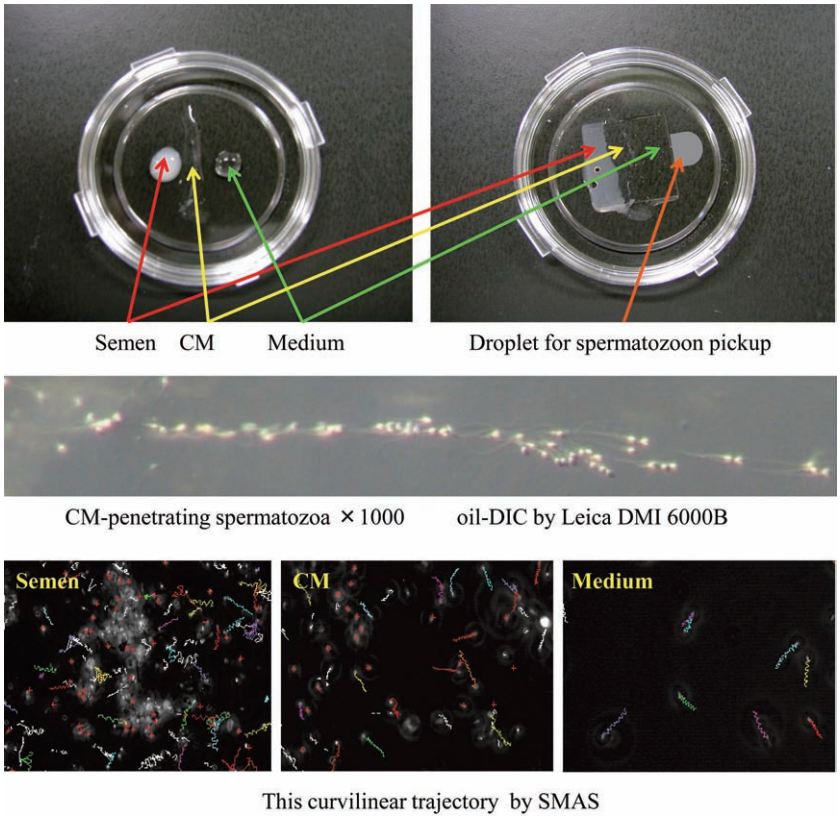

Fig. 1. The CM penetration-based spermatozoa-sorting method
The CM penetration-based spermatozoa-sorting method was created as follows. A CM line was drawn at the center of a glass-bottomed dish using an 18G syringe needle. A drop of semen and a drop of the culture medium were placed on the right and left sides, respectively, of the CM line. A coverslip was placed over the system. In addition, a droplet for spermatozoon pickup was placed on the same side as the drop of culture medium. The spermatozoa moves from the semen to the culture medium.

CM penetration-based spermatozoa-sorting method

We developed a method of collecting CM-penetrating spermatozoa by modifying the Miller-Kurzrok test [12], which is used to test CM penetration by spermatozoa.

The observation system of the CM penetration-based spermatozoa-sorting method was created as follows. A line was drawn with CM on a glass-bottomed dish (Matsunami Glass, Tokyo Japan or WPI Fluoro Dish) using an 18G syringe needle. A drop of semen and a drop of the culture medium were placed on the right and left sides respectively, of the CM line. A coverslip (18 mm × 18mm, Matsunami Glass, Tokyo Japan) was placed over the system (semen→CM→culture medium). In addition, another droplet of medium for spermatozoon pick-up was placed on the same side as the drop of culture medium (Fig. 1). When too much semen or culture medium was placed on the dish, it caused a small flood disturbing the separation (CM line), creating a shortcut between the semen and culture medium. Thus, after the preparation, it was necessary to check if any shortcut had been created around the coverslip between the semen and the medium using an inverted microscope. Mineral oil can be used to maintain the osmotic pressure of the culture medium; however, it sometimes created a shortcut by pushing the semen or culture medium when the mineral oil was applied. Thus, we stopped using mineral oil.

The glass bottom dish was incubated in 5% CO_2, 5% O_2 at 37°C in a humidified incubator for 30 to 90 min. The motility of spermatozoa was analyzed on the same glass-bottomed dish using the SMAS. Both the semen portion and the culture medium portion of the system were measured.

The statistical difference was determined using the two-sided Student's *t*-test. Differences with $P < 0.05$ were considered to be significant.

Experiment 2: Morphological analysis of CM-penetrating spermatozoa

We analyzed the morphology of spermatozoa using the criteria for normal spermatozoon morphology is described in the WHO laboratory manual for examination and processing of human semen, Fifth edition [13]. Semen samples were collected from 13 patients. Among the spermatozoa selection methods, a combination of density gradient centrifugation and swim-up techniques, which are used to remove bacteria blood cells and seminal plasma and to collect good spermatozoa, was selected as the method for comparison. The effectiveness of the CM penetration-based spermatozoa-sorting method was compared with this method. Additionally, since the publication of reports on IMSI (High magnification ICSI) [14–16], the presence of the head vacuole in spermatozoa has attracted considerable attention. Thus, based on the study published by Berkovitz *et al.* [15], we investigated the effect of removing spermatozoa with the head vacuole based on the spermatozoon classification criteria used at the Fertility Clinic Tokyo. The results of the CM penetration-based spermatozoa-sorting method and the density gradient centrifugation and swim-up method were compared.

Spermatozoa sample preparation

1. Collection of CM-penetrating spermatozoa and motility analysis

Using the CM penetration-based spermatozoa-sorting method described above, spermatozoa samples were incubated in 5% CO_2, 5% O_2 at 37°C in a humidified incubator for 30 to 90 min. After the incubation, motile spermatozoa (100%) were collected and fixed in 10% neutral formalin.

2. Collection and fixation of spermatozoa using the density gradient centrifugation + swim-up technique

After an ART capillary (NIPRO, Osaka, Japan) had been added to a centrifuge tube containing 80% Percoll solution, semen was layered slowly on the top of the 80% Percoll to make a continuous density gradient. This gradient sample was centrifuged at 300×*g* for 20 min. After centrifugation, a 1-ml disposable syringe was attached to the ART capillary, and 0.3 ml of the pellet was collected and suspended in a Falcon tube (#2003). The 80% Percoll spermatozoa suspension was then transferred to the bottom of another Falcon tube (#2003) that was wet with the culture medium beforehand. To this tube, 1 ml of sperm culture medium was slowly added to create another layer on top of the suspension layer. The tube was then incubated for the swim-up process in a humidified incubator in 5% CO_2 + 5% O_2 at 37°C for 30 to 60 min. After the density gradient centrifugation + swim-up procedure, only 100% motile spermatozoa were collected and fixed in 10% neutral buffered formalin liquid.

Morphological analysis

1. The proportion of spermatozoa with normal morphology

A small amount of the fixed spermatozoa was transferred to a glass-bottomed dish, and a cover slip was placed over it. Using a Leica DMI 6000B microscope, the ratio of spermatozoa with normal morphology was calculated using Nomarski differential interference contrast (DIC) microscopy with an oil immersion lens (1000×). The spermatozoa were not stained, and we used the criteria for normal spermatozoon morphology described in the WHO laboratory manual for the examination and processing of human semen [13]. Statistical analysis was conducted using the χ^2-test, and differences with $P < 0.05$ were considered to be significant.

2. The proportion of spermatozoa with a head vacuole

Semen samples were collected from 10 patients. Based on the appearance of the head vacuole, the spermatozoa were classified as follows.

A) Morphologically normal spermatozoa without a head vacuole.
B) Morphologically normal spermatozoa with a single small head vacuole (smaller than 4% of the area of the spermatozoon head).
C) Morphologically normal spermatozoa with a large head vacuole.
D) Morphologically normal spermatozoa with small and large head vacuoles (Fig. 2).

Statistical analysis was conducted using the χ^2-test, differences with $P < 0.05$ were considered to be significant.

Experiment 3: DNA fragmentation analysis of CM-penetrating spermatozoa

For quality assessment of CM-penetrating spermatozoa, DNA fragmentation was analyzed. Semen samples were collected from 15 patients. The collected samples were subjected to the CM penetration-based spermatozoa-sorting method, and the density gradient centrifugation + swim-up method (for comparison). For both methods, the presence/absence of DNA fragmentation was analyzed by the Sperm Chromatin Dispersion test (SCD test) using prepared samples containing 100% motile spermatozoa.

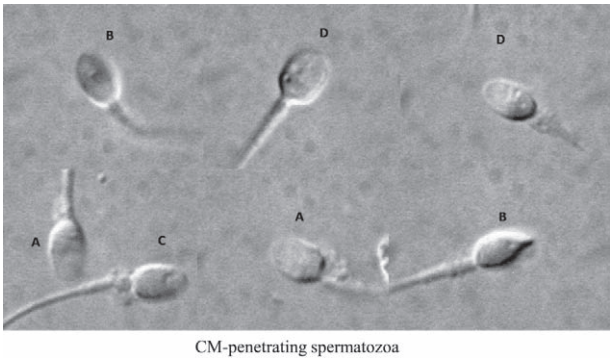

Fig. 2. Categorization of the sperm head vacuole
The human spermatozoon head vacuole is observed in most spermatozoa. The photograph shows the head vacuoles seen in a morphologically normal human spermatozoa. Based on the appearance of the head vacuole, the spermatozoa were classified as follows: A) morphologically normal spermatozoa without a head vacuole; B) morphologically normal spermatozoa with a single small head vacuole (smaller than 4% of the size of the nucleus); C) morphologically normal spermatozoa with a large head vacuole; D) morphologically normal spermatozoa with small and large head vacuoles.

SCD test

Although SCD test kits are available, we modified and used the method described by Fernández *et al.* [17]. The method is summarized below. Preparation of pre-coated slides: a slide was coated with 0.65% standard agarose and dried at 80°C. Reagents: 0.08 N HCl, 1% low melting point agarose, lysis solution 1 (0.4 M Tris, 1% SDS and 50 mM EDTA, pH 7.5, 0.8 M DTT; DTT was added just before use), lysis solution 2 (0.4 M Tris, 2 M NaCl, pH 7.5), Tris borate EDTA buffer (0.09 M Tris borate and 0.002 M EDTA, pH 7.5).

Spermatozoa preparation: 15 µl of spermatozoa suspension was added to 35 µl of 1% low-melting point agarose kept at 38°C (for a final agarose concentration of 0.7%), and the solution was mixed using a vortex mixer. Using a micropipette, 15 µl of the sample was placed on a pre-coated slide glass, and promptly covered with an 18 × 18 mm coverslip. The sample was solidified by incubation at 4 °C for 4 min. The coverslip was carefully removed, and the slide was immediately immersed in a tray containing 0.08 N HCl for the reaction. The slide was kept in solution at 22 °C for 7 min. The slide was then reacted with lysis solution 1 for 10 min at room temperature. Subsequently, the slide was reacted with lysis solution 2 for 5 min at room temperature, washed with a 2-min immersion in Tris borate EDTA buffer, and dehydrated by 2-min treatments with 70%, 90%, and 100% ethanol. After this, the slide was air-dried, and stained with Diff-Quik (Sysmex, Cat No. 16920, Kobe, Japan). After the slide was air-dried, clear embedding was applied. Spermatozoa in the prepared specimen were counted using a microscope (400×), and evaluated based on the halos formed by DNA fibers dispersed around the spermatozoon nuclei as follows: (A) a big halo or medium halo: without DNA fragmentation, (B) a small, no halo and degraded halo: with DNA fragmentation.

Results

Experiment 1: Motility analysis of CM-penetrating spermatozoa

With regard to spermatozoa motility in semen, the total migration distance measured using the SMAS were between 0 and 60 µm for 54.2% of the spermatozoa, and between 60 µm and 150 µm in 45.8% of the spermatozoa. In CM-penetrating spermatozoa, the total migration distance measured using the SMAS were between 0 and 60 µm for 16.1% of the spermatozoa, and between 60 and 150 µm for 83.9% of the spermatozoa (Fig. 3A). The mean total migration of CM-penetrating spermatozoa was 91.3 µm, which was significantly higher ($P < 0.01$) than that of non-treated semen (63.4 µm). Moreover, the largest proportion of all spermatozoa in semen was observed to have linearity (VSL/VCL) of between 0.3 and 0.4, whereas in the case of CM-penetrating spermatozoa, the largest proportion was observed to have a linearity of between 0.4 and 0.5 (Fig. 3B). This shows that CM-penetrating spermatozoa had a better linearity. The mean linearity of CM-penetrating spermatozoa was 0.37, which was significantly higher ($P < 0.01$) than that of non-treated semen (0.39).

Experiment 2: Morphological analysis of CM-penetrating spermatozoa

The mean percentage of spermatozoa exhibiting normal morphology was 41.9% in the CM penetration-based spermatozoa-sorting method, and 58.0% in the density gradient centrifugation + swim-up method, showing a statistically significant difference ($P < 0.01$; Fig. 4). The proportion of spermatozoa containing a head vacuole was comparable between the spermatozoa in semen and CM-penetrating spermatozoa (Fig. 5).

Experiment 3: DNA fragmentation analysis of CM-penetrating spermatozoa

DNA fragmentation of spermatozoa was analyzed by the SCD test. When motile spermatozoa were collected by the CM penetration-based spermatozoa-sorting

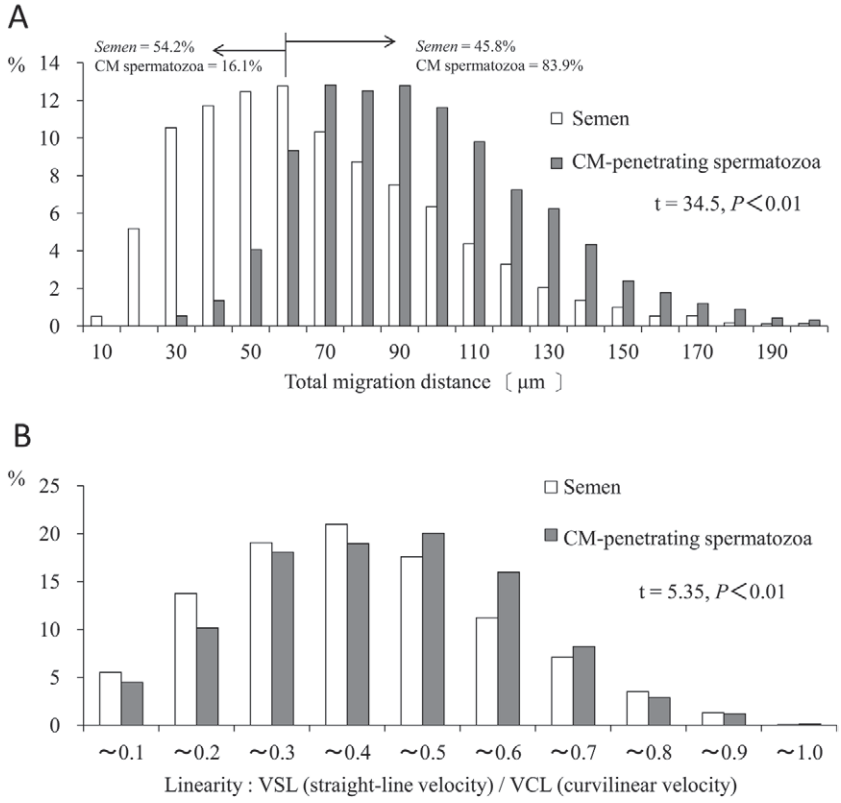

Fig. 3. CM penetration-based spermatozoa-sorting method
Using the SMAS, spermatozoa motility was analyzed with regard to 2 parameters: total migration distance (A) and linearity (B). A total of 5094 spermatozoa from semen samples, and 2581 from CM-penetrating spermatozoa samples (n=23 patients) were analyzed.

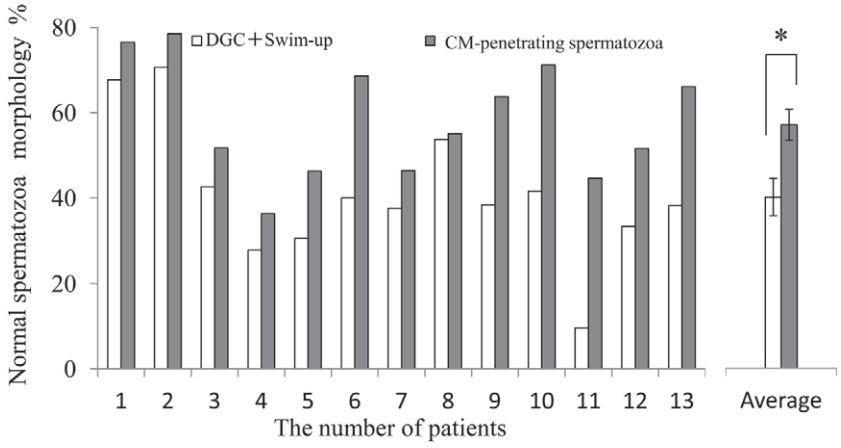

Fig. 4. Normal spermatozoa morphology
Semen samples were collected from 13 patients, and the proportion of spermatozoa with normal morphology was compared between the CM penetration-based spermatozoa-sorting method and the density gradient centrifugation (DGC)+swim-up method. *: Significantly different ($P < 0.01$).

method or the density gradient centrifugation + swim-up method, DNA fragmentation in the spermatozoa was not detected in most samples (Table 1).

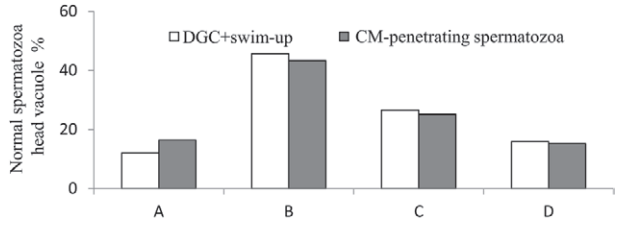

Fig. 5. Categorization of the normal morphological spermatozoa head vacuole
The comparison of the rate of removal of spermatozoa with head vacuole between the CM penetration-based spermatozoa-sorting method and the density gradient centrifugation (DGC) + swim-up method.
Semen samples were collected from 10 patients. Based on the appearance of the head vacuole, the spermatozoa were classified as belonging to the A, B, C or D group.

Discussion

In this study, to explore alternative methods for selecting better spermatozoa for ICSI, we developed a method of collecting CM-penetrating spermatozoa, and multidimensionally evaluated the properties of the spermatozoa.

For the analysis of spermatozoa motility using the CM penetration-based spermatozoa-sorting method in Experiment 1, the following points were considered. It was difficult to retrieve spermatozoa suspensions with sufficient concentration in a Makler counting chamber (Sefi-Medical, Israel), which is typically used for measurement by SMAS. Thus, although the thickness of the material cannot be determined precisely, in order to confirm that the focus of the microscope was adjusted to the motile spermatozoa, we carried out the measurements using a glass-bottomed dish, which can create a relatively uniform thickness in the test sample. As a result, we were able to detect the migration path of spermatozoa from start to finish. Since we were able to measure different test samples under the same conditions, we concluded that a thickness did not affect the motility analysis.

Spermatozoa with various degrees of motility were

Table 1. DNA fragmentation analysis of CM-penetrating spermatozoa

Patient	DGC Swim-up			CM-penetrating spermatozoa		
	Total number of spermatozoa	DNA non-fragmentation		Total number of spermatozoa	DNA non-fragmentation	
		The number of spermatozoa	%		The number of spermatozoa	%
1	125	125	100	44	44	100
2	349	349	100	485	484	99.8
3	450	450	100	98	98	100
4	506	506	100	481	481	100
5	371	371	100	309	309	100
6	520	520	100	169	167	98.8
7	390	388	99.5	320	320	100
8	354	354	100	301	299	99.3
9	440	440	100	414	410	99
10	253	253	100	261	261	100
11	353	353	100	304	304	100
12	530	529	99.8	418	418	100
13	217	217	100	204	204	100
14	281	281	100	407	407	100
15	303	303	100	200	200	100
Total	3,505	3,503	99.9	2,621	2,612	99.7

DNA fragmentation analysis of CM penetrating spermatozoa (by CM penetration-based spermatozoa-sorting method), and selected motile spermatozoa (by density gradient centrifugation (DGC)+swim-up method). The spermatozoa mobility ratio was 100%. The DNA fragmentation of the motile spermatozoa was analyzed by the SCD test.

observed in semen. The ratio of spermatozoa with normal morphology increased in both the density gradient centrifugation+swim-up method and the CM penetration-based spermatozoa-sorting method. In particular, the ratio of spermatozoa with normal morphology was high among spermatozoa selected by the CM penetration-based spermatozoa-sorting method. Thus, the CM penetration-based spermatozoa-sorting method was considered a promising method. With regard to the mechanism of spermatozoa selection, mucin, which is present in the CM, is a highly viscous polymer, and in order to move forward in such polymer, it is important for spermatozoa to have left-right symmetry; moreover, in order to break the mucus and swim continuously, spermatozoa probably need good linear motility and strong propulsion. Density gradient centrifugation is often used independently, and is a good selection method for washing and separating spermatozoa. The principle of density gradient centrifugation is to separate spermatozoa based on differences in the densities of seminal components. If the immotile spermatozoa, weakly motile spermatozoa, or morphologically abnormal spermatozoa have greater density, they will precipitate. However, density gradient centrifugation can be combined with the swim-up technique to separate spermatozoa with good motility using the self-propelling mobility of the spermatozoa. However, because the spermatozoa are collected in a less viscous, watery culture medium, it appears that morphologically abnormal motile spermatozoa are also collected as mixture.

The chemical composition and physical properties of cervical mucus have been reviewed by Schumacher [18], and by Katz [19]. More recently, the biochemical analysis of cervical mucus was carried out by Rohr *et al.* [20] using nuclear magnetic resonance spectroscopy. The mucus is a visco-elastic gel consisting of solid and liquid phases. The solid phase is a glycoprotein polymer known as mucin whose macromolecules interact to form a network and is the agent responsible for the rheological properties of mucus. The liquid phase of cervical mucus contains low molecular weight substances such as electrolytes, carbohydrates, amino acids, lipids and other compounds dissolved in it. The physical properties of CM may assist the physiological selection of good spermatozoa.

In ICSI, usually, spermatozoa are selected using density gradient centrifugation or the swim-up technique, and then a single spermatozoon is picked up after allowing the spermatozoa to swim in 10% PVP or hyaluronate-added culture media and evaluating them based on the velocity, linearity, and abnormalities in spermatozoon head morphology. However, IMSI, which allows more precise selection of spermatozoon with normal morphology, is recommended [14–16]. The vacuoles found in the spermatozoon head have attracted considerable attention as a factor in spermatozoon selection, and many studies have focused on the head vacuole. Head vacuoles that are visible under a light microscope include intranuclear inclusion bodies [21] and crater defects in the spermatozoon head [22]. Such intranuclear inclusion bodies are called head vacuoles or nuclear vacuoles, which can be spontaneous or physiological vacuoles formed by nuclear condensation, or abnormal vacuoles that are formed by abnormal spermatozoon development, which results in structural abnormalities [23]. With regard to crater defects, Tanaka *et al.* [24] reported that crater defects do not indicate decreased or regressed gamete functionality of spermatozoa, but represent one of the phases in their physiological growth. Both types of vacuoles have been studied in detail using electron microscopy. However, under a light microscope, head vacuoles can be distinguished only by their size. Usually, spermatozoa having small vacuoles or fewer numbers of vacuoles are selected in IMSI. However, there are differing opinions about the effect of selecting such spermatozoa, which need to be resolved. We examined if the presence of these head vacuoles (which are considered to be a fertilization risk) can be minimized by the CM penetration-based spermatozoa-sorting method. However, various head vacuoles were observed in spermatozoa with normal morphology, and effective removal of spermatozoa with head vacuoles was not feasible by any of the methods. This is because head vacuoles do not affect the spermatozoa appearance or motility. Thus, at present, there is no effective way of minimizing the presence of head vacuoles other than by selecting spermatozoa under high magnification.

In Experiment 3, we were able to collect suspensions containing 100% motile spermatozoa using the CM penetration-based spermatozoa-sorting method and the density gradient centrifugation + swim-up method. When the SCD test was performed, virtually no DNA fragments were found in the spermatozoa collected by using the CM penetration-based spermatozoa-sorting method and the density gradient centrifugation + swim-up method. Although the analysis showed DNA fragments in less than 1% of spermatozoa in some subjects, these DNA fragments were considered to be artifacts (for example, samples which had dried during an experiment) that occurred during the preparation of the specimen (Table 1). We previously performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), a method for histologically detecting DNA fragments, on sperma-

tozoa collected using the density gradient centrifugation + swim-up method (the results have not been provided because the sample size was small), but little or no DNA fragments were found in the motile spermatozoa.

The spermatozoa selection method has not fundamentally changed since the first successful performance of ICSI in humans; however, the problem remains that the natural selection in fertilization might be eliminated in this method [25]. Therefore, a spermatozoa selection system using the CM penetration-based spermatozoa-sorting method for ICSI could contribute to the selection of better spermatozoa. CM-penetrating spermatozoa have been studied and discussed in the past; however, there are no easy methods for spermatozoa selection in a clinical setting. Thus, we would like to use the CM penetration-based spermatozoa-sorting method for spermatozoa selection in a clinical setting.

The advantages of the CM penetration-based spermatozoa-sorting method are as follows. (1) The method is simple and easy. (2) It does not require centrifugation or a washing procedure; therefore, physical damage to spermatozoa can be reduced. (3) Because it is a physiological selection method, spermatozoa may gain fertilizing ability in this process [26]. (4) Compared to the density gradient centrifugation + swim-up method, the CM penetration-based spermatozoa-sorting method is associated with a better ratio of spermatozoa with normal morphology. The disadvantages of the CM penetration-based spermatozoa-sorting method are as follows. (1) Only a small number of spermatozoa can be collected with this method. However, ICSI typically requires only a small number of spermatozoa; thus, in most cases, the small number is not problematic. Moreover, more spermatozoa could be collected by adjusting the incubation time or increasing the contact surface between the seminal fluid and the CM. In the future, we may need to evaluate the CM penetration-based spermatozoa-sorting method in patients with oligozoospermia. (2) Because CM is a biological material, it may pose a risk of bacterial infection. However, such infection can be prevented by adding antibiotics to the culture medium. (3) Good-quality CM (a score greater than 10 [11]) may not be obtained at the time of egg collection. In order to obtain good-quality CM, prior to the ovulation induction treatment, ethinyl estradiol can be administered to patients [27]. Also cryopreservation of CM may help to maintain its quality. We previously performed a rapidly CM freezing using liquid nitrogen (−196°C) followed by rapid thawing, and the quality of the CM did not change. Thus, good-quality CM can be cryopreserved in advance and used whenever needed.

On the basis of the results of this study, we conclude that the CM penetration-based spermatozoa-sorting method may be better than conventional methods, because it is a physiological method that enables selection of better spermatozoa. The use of the CM penetration-based spermatozoa-sorting method should be considered for ICSI.

Further studies are needed to analyze how the CM-penetrating spermatozoa gain fertilizing ability and how the acrosome reaction is induced [2, 26], in order to determine the appropriate time to obtain spermatozoa.

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