Human sperm head vacuoles are physiological structures formed during the sperm development and maturation process

Atsushi Tanaka, M.D.,[a] Motoi Nagayoshi, M.D.,[a] Izumi Tanaka, Phar.B.,[a] and Hiroshi Kusunoki, Ph.D.[b]

[a] Saint Mother Obstetrics and Gynecology Clinic and Institute for Assisted Reproductive Technology, Fukuoka; and [b] Faunal Diversity Sciences, Graduate School of Agriculture, Kobe University, Kobe, Japan

Objective: To clarify whether human sperm vacuoles affected intracytoplasmic sperm injection (ICSI) success rates.
Design: Retrospective study.
Setting: A private infertility clinic.
Patient(s): Spermatozoa and spermatids were obtained from 11 normozoospermic, 10 oligozoospermic or asthenozoospermic, 4 obstructive azoospermic, and 3 nonobstructive azoospermic men.
Intervention(s): Differential interference contrast observation and intracytoplasmic injection of morphologically selected sperm.
Main Outcome Measure(s): Incidence, size, and position of vacuoles of sperm cells were recorded. Ability of fertilization and blastocyst development were compared between cells with and without vacuoles.
Result(s): More than 97.4% of ejaculated, 87.5% of epididymal, 87.5% of testicular spermatozoa, and more than 90.0% of Sc-Sd2 spermatids had vacuoles of various sizes. The incidence of vacuoles on ejaculated cells was significantly higher than that on the other types of cells, but there was no difference between sperm from normozoospermic men and those from the other donors. Removal of plasma membrane and/or acrosome did not affect the incidence of vacuoles. Although more than 60% of spermatozoa had small vacuoles in the acrosomal regions, 52.6% of Sb1-2 spermatids had large vacuoles. After injection of a motile spermatozoon with large and small vacuoles, 60.9% and 85.7% of metaphase II oocytes could be normally fertilized, respectively, and almost half of the zygotes developed to the blastocyst stage. When using sperm without vacuoles, the fertilization rate was 80.0%, but only 25% of them developed to the blastocyst stage.
Conclusion(s): Human sperm head vacuoles did not affect ICSI outcomes. (Fertil Steril® 2012;98:315–20. ©2012 by American Society for Reproductive Medicine.)
Key Words: Vacuole, spermatozoa, spermatid, spermiogenesis, epididymal maturation

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Along with a dramatic improvement in the quality of microscopes, intracytoplasmic sperm injection (ICSI) has become feasible at high optical resolution, and the significance of vacuoles of human sperm heads (1, 2) has been reevaluated. The vacuole is a concavity extending from the surface of the sperm head to the nucleus through the acrosome. According to Fawcett (3), the universal presence of vacuoles in the human sperm nucleus was documented in a report written by Eimer (4) more than a century ago. In the 1950s, transmission electron microscopy (TEM) of an ultrathin cross-section of the sperm head was carried out, which showed that a human sperm nucleus usually contained one or more vacuoles at different locations (5–7). On the basis of their universal presence in human sperm heads, most researchers concluded that intranuclear vacuoles should not be considered as degenerative structures with no physiological significance but instead should be regarded as a normal feature of the sperm head (3, 8–10). Meanwhile, after IVF techniques, including ICSI, were introduced in human infertility treatments, Mundy et al. (11) reported that sperm nuclei in subfertile men

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Reprint requests: Atsushi Tanaka, M.D., Saint Mother Obstetrics and Gynecology Clinic and Institute for ART, 4-9-12, Orio, Yahata-Nishi, Kitakyushu, Fukuoka 807-0825, Japan (E-mail: incho@stmother.com).

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contained significantly more intranuclear vacuoles than did fertile controls. More recently, it has been reported that ICSI of sperm with vacuoles tended to result in decreased pregnancy rates (PR) and cause early miscarriage, and it has been proposed that vacuoles are not just a polymorphism but pose a risk for an abnormality that is accompanied by DNA injury (12–19).

To clarify these contradictory evaluations of the significance of human sperm vacuoles and establish whether this feature is physiological and normal, we have used the Normarski differential interference contrast (DIC) system to examine the incidence of vacuoles in male germ cells at various developmental and maturation stages (elongating and elongated spermatid, testicular, epididymal, and ejaculated spermatozoa) with or without initial demembranating treatments. Furthermore, we performed intracytoplasmic injection of morphologically selected sperm (IMSI) using motile normal-shaped sperm with or without vacuoles, and compared the results to our clinical data using conventional ICSI.

MATERIALS AND METHODS

Subjects and Ethical Aspects

This retrospective study was conducted with the informed consent of all participating patients. Round and elongating spermatid, testicular, epididymal, and ejaculated spermatozoa were obtained from 28 men (mean age, 37.7 ± 5.6 years; range, 31–50 years), including normozoospermic (11 men), oligozoospermic or asthenozoospermic (10 men), obstructive azoospermic (4 men), and nonobstructive azoospermic patients (3 men). Metaphase II oocytes were collected from 18 women (mean age, 34.6 ± 1.9 years; range, 28–37 years) undergoing controlled ovarian hyperstimulation (COH) according to the long GnRH agonist protocol (20). The Institutional Review Board of the Saint Mother Obstetrics and Gynecology Clinic approved the experimental use of such gametes.

Preparation of Ejaculated Spermatozoa

Semen samples were collected by masturbation and allowed to liquefy for 30 minutes at 37°C, followed by routine assessment of sperm characteristics.The patients were classified as giving oligozoospermia, asthenozoospermia, and normozoospermia according to the World Health Organization laboratory manual (21). These semen samples were centrifuged at 1,300 rpm for 8–10 minutes to remove the seminal plasma, and the spermatozoa were resuspended in homemade human tubal fluid (HTF) medium supplemented with 0.3% human serum albumin (HAS; Irvine Scientific) and kept at 37°C before observation for vacuoles and IMSI.

To estimate the depth of vacuoles, the following three treatments were performed to remove the plasma membrane and/or the acrosome from ejaculated spermatozoa from normozoospermia men: 1) induction of the acrosomal reaction, 2) freezing-thawing, and 3) demembranation with a detergent. The sperm acrosome reaction was chemically induced by the starvation method, which was originally developed for farm animals (goat, bull, and boar) (22, 23). Briefly, washed spermatozoa were resuspended in a substrate-free salt solution ($NeoK_3$; 148 mM NaCl, 2 mM $CaCl_2$, 20 mM *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid, pH 7.4) at a high sperm concentration and incubated in a sealed vessel for 2–3 hours at 40°C. The acrosome reaction was evaluated by the modified triple-stain technique that combined 0.1% trypan blue vital staining (0.1% dye in HSA-HTF, for 15 minutes at 37°C), with Bismark brown counterstaining (0.5% dye in 30% ethyl alcohol, pH 2.8, for 10 minutes at 40°C) and rose Bengal acrosomal staining (0.8% dye in 0.1 M Tris buffer, pH 5.3, for 20 minutes at 24°C) (24). For freezing-thawing, washed spermatozoa were resuspended in HSA-HTF without any cryoprotectants, frozen in liquid nitrogen (−196°C), and thawed in a water bath (37°C). This freezing and thawing sequence was repeated five times. For demembranation, washed spermatozoa were permeabilized with 0.1% polyethylene glycol mono-*p*-iso-octylphenyl ether (T-8787, Triton X-100; Sigma-Aldrich) supplemented HTF for 15 minutes at room temperature.

Preparation of Epididymal and Testicular Spermatozoa and Spermatids

Epididymal and testicular spermatozoa and spermatids were obtained from azoospermic men according to the method described elsewhere (25–27). The results of preliminary biopsies indicated that spermatogenesis had been arrested at the level of the round spermatid in all nonobstructive azoospermic men. In contrast, a small number of spermatozoa were found in epididymis or testis of all obstructive azoospermic patients. After spinal anesthesia, the epididymis was exposed with a median incision of the scrotum, and spermatozoa were collected under microscopic observation by direct aspiration from the epididymal ducts using a fine glass pipette (Keisei SE-4 surgical loupes; Keisei Medical Industrial). For collection of testicular spermatozoa and spermatids, small pieces of testicular tissue were obtained through microtesticular sperm extraction (TESE). After being washed with erythrocyte-lysing buffer (28), the testicular tissues were finely minced with ophthalmic knives on ice, digested with 0.125% collagenase (Type IV-S; Sigma-Aldrich) solution containing 0.001% deoxyribonuclease (DNase) I (Sigma-Aldrich) for 20 minutes at 32.5°C with gentle agitation, washed with Dulbecco's phosphate-buffered saline (PBS) supplemented with 1% glucose and 6 mM sodium pyruvate and antibiotics (29) by centrifugation at 1,300 rpm for 3 minutes at 4°C, and finally filtered using two types (30 and 15 μm) of nylon filter sheet (NRS-030 and NRS-015; Nippon Rikagaku Kikai). Isolated spermatogenic cells were microscopically observed and spermatids were distinguished by the cytologic criteria according to Mansour et al. (30) and classified into six phases (Sa, Sb1, Sb2, Sc, Sd1, and Sd2) (31–33).

Epididymal or testicular spermatozoa and spermatids were suspended in HTF supplemented with 10% serum protein substrate (10% SPS-HTF; Sage In-Vitro Fertilization Inc., A Cooper Surgical Company) and stored at 32.5°C before observation of vacuoles, fixation for electron microscopy, and chromosomal maturation (only epididymal spermatozoa).

Observation of Vacuoles by Light Microscopy

Spermatozoa and spermatids were placed on a glass slide using a Pipetman (P-20; Gilson) or a micromanipulator (Three-axis joystick oil hydraulic micromanipulator MO-202U; Narishige), overlaid with a cover slip, and sealed with rubber cement (Paper Bond; Kokuyo) to prevent the cells from drying (wet mount). Another sample of these cells was placed into a microdrop of 10% SPS-HTF on a sterile, glass-bottomed culture dish under sterile mineral oil (mouse embryo tested, M-8410; Sigma-Aldrich). In these two samples, the number, size, and position of the vacuoles in normally shaped sperm head or spermatid nucleus were observed using two types of inverted microscope equipped with the Normarski DIC system (Olympus IX71-ARCEVA; Olympus and Nikon Eclipse TE300; Nikon) at a magnification of ×1,000 with an oil immersion lens or ×600 with a dry lens. If the spermatozoa moved vigorously, glutaraldehyde (final concentration: 0.2%) was added to stop the movement.

The number of vacuoles was counted, and they were divided into three types based on their size (large; >50% of surface area, medium; 50%–25% of surface area, and small; <25% of surface area of total sperm head) and based on their location, namely acrosomal, equatorial, or postacrosomal regions.

Intracytoplasmic Injection of Selected Spermatozoon With or Without Vacuole

Washed ejaculated spermatozoa from asthenozoospermic men were transferred into a microdrop of HTF supplemented 3% polyvinylpyrrolidone on a glass-bottomed dish, and a motile, morphologically normal spermatozoon with or without vacuole was picked up using micromanipulators. After the magnification was lowered to ×200 in the same visual field, the selected spermatozoon was injected into a metaphase II oocyte. After ICSI, oocytes were cultured in two sequential media (Quinn's Advantage Cleavage and Blastocyst Media; Sage In-Vitro Fertilization Inc., A Cooper Surgical Company), fertilization and subsequent embryonic development were investigated, and compared with our clinical data obtained from conventional ICSI with a nonselected spermatozoon.

Statistical Analysis

The χ^2 test was used to evaluate whether the incidence of vacuoles among various types of sperm cells, and the rate of fertilization and embryo development after IMSI and conventional ICSI using ejaculated sperm with or without vacuoles were significantly different.

RESULTS

Table 1 shows the incidence of vacuoles in untreated (Fig. 1A), acrosome-reacted (Fig. 1B), frozen-thawed or demembranated ejaculated spermatozoa (Fig. 1C), untreated epididymal (Fig. 1D), and testicular spermatozoa (Fig. 1E) and Sb1-Sd2 spermatids (Fig. 1F–1I) detected under the DIC microscope. The triple staining revealed that 84.1% of ejaculated spermatozoa underwent the acrosome reaction (live spermatozoa without an intact acrosome) by the Starvation technique, whereas 87.7% had an intact acrosome before treatment. After simple freezing-thawing, all cells were dead with damaged acrosomes, and after treatment with Triton X-100 they had lost the plasma membrane and the acrosome. The DIC observations showed that the incidence of vacuoles did not change after the various pretreatments (97.4–99.4%, 1.58–1.70 vacuoles per spermatozoon). There was no significant difference between oligozoospermic or asthenozoospermic patients and normozoospermic men. The incidence of vacuoles on both epididymal and testicular spermatozoa were lower than on ejaculated cells, and almost the same as on Sc-Sd2 spermatids. However, on spermatids of the earlier stages, such as Sb2 and Sb1, these values were markedly lower.

Table 2 shows the distribution of different sizes of vacuoles in ejaculated and epididymal spermatozoa and

TABLE 1

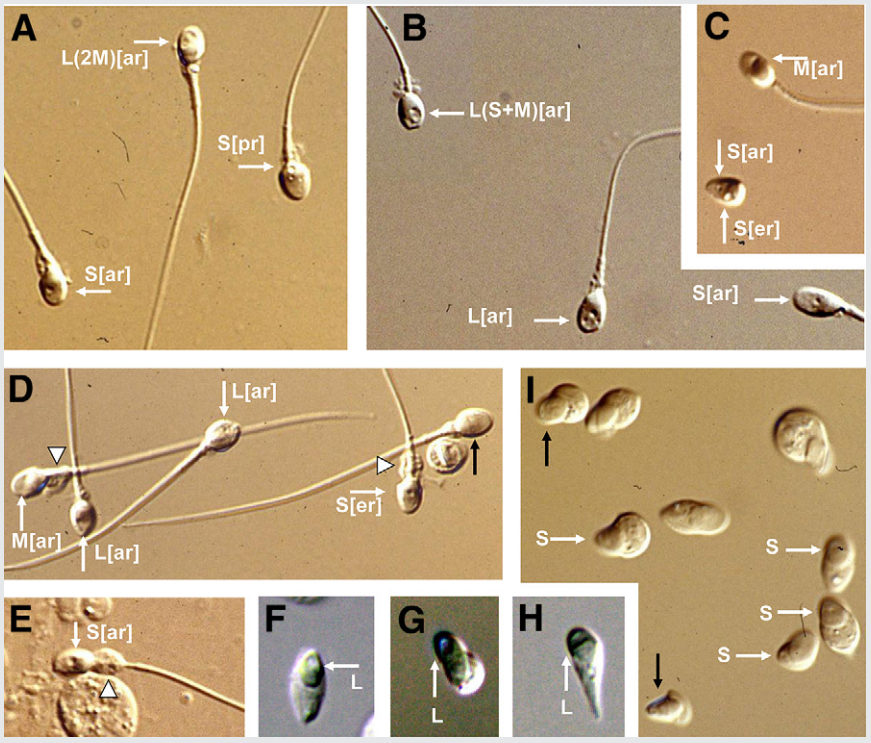
Incidence of vacuoles in ejaculated, epididymal, and testicular spermatozoa and spermatids after various treatments, observed under the Normarski differential interference contrast microscope.

Type of cells	Case (n)	Before treatment	No. cells observed	% cells with vacuoles	No. of vacuoles/cell
Ejaculated sperm	Oligo- or asthenozoospermia (10)	None	596	98.8[a]	1.59
	Normozoospermia (11)	None	570	97.7[a,b,c]	1.49
	Normozoospermia (3)	Acrosome-reacted	154	97.4[a,b,c]	1.69
	Normozoospermia (3)	Simply frozen-thawed	154	98.7[a,b]	1.70
	Normozoospermia (3)	Demembranated	154	99.4[a]	1.58
Epididymal sperm	Obstructive azoospermia (2)	None	104	87.5[d]	1.22
Testicular sperm	Obstructive azoospermia (2)	None	16	87.5[d]	1.35
Spermatid					
Sd2	Nonobstructive azoospermia (2)	None	10	90.0[c,d]	1.40
Sd1	Nonobstructive azoospermia (3)	None	66	90.9[d]	1.21
Sc	Nonobstructive azoospermia (2)	None	32	93.8[b,c,d]	1.21
Sb2	Nonobstructive azoospermia (3)	None	41	51.2[e]	0.67
Sb1	Nonobstructive azoospermia (3)	None	48	18.8[f]	0.19

[a–f] Percentage of cells with different superscripts in the same file were significantly different ($P<.05$, χ^2 test).

Tanaka. Human sperm head vacuoles. Fertil Steril 2012.

FIGURE 1



Normarski differential interference contrast photomicrographs of untreated (A), acrosome-reacted (B), and demembranated (C) ejaculated spermatozoa, untreated epididymal (D) and testicular spermatozoa (E), and Sb1–Sd1 spermatids (F–I). Original magnification, ×1,000. *White arrows* indicate vacuoles; *black arrows* indicate spermatozoa or spermatids without vacuoles. Large vacuoles could be observed on three spermatids without arrow and out of focus (I). *White arrowheads* indicate cytoplasmic droplets (D and E). S, M, L = small, medium, and large sizes of vacuoles; [ar], [er], [pr] = vacuole was observed in the acrosomal, equatorial, and postacrosomal regions of the sperm head, respectively.

Tanaka. Human sperm head vacuoles. Fertil Steril 2012.

Sb1–Sb2 spermatids as observed under the DIC microscope. About 70% of the vacuoles found in ejaculated and epididymal spermatozoa were small, whereas large vacuoles accounted for less than 15% of the total (Fig. 1A–1D). Almost all vacuoles were observed in the acrosomal region of these spermatozoa (Fig. 1A–1D). On the other hand, large vacuoles were most frequently found on spermatids (Fig. 1F–1H), and small and medium-sized vacuoles were observed less frequently (Fig. 1G) among the three size groups.

Table 3 indicates the outcomes of IMSI using ejaculated spermatozoa with or without vacuoles. In this study, the time required for IMSI was similar to that for the conventional

TABLE 2

Distribution of different sizes of vacuoles in ejaculated and epididymal spermatozoa and spermatids, observed under the Normarski differential interference contrast microscope.

Type of cell	No. cells observed	% cells with vacuoles	Size of vacuoles	% vacuoles located in Acrosomal region	Equatorial region	Postacrosomal region	Total
Ejaculated sperm[a]	1,166	98.3[c]	Small	60.3	5.1	2.6	68.0
			Medium	16.8	1.8	1.2	19.8
			Large	11.4	0.3	0.5	12.2
Epididymal sperm	104	87.5[d]	Small	68.4	4.4	1.1	73.9
			Medium	13.0	0.0	0.0	13.0
			Large	12.0	0.0	1.1	13.1
Spermatids[b]	89	33.7[e]	Small	–	–	–	22.6
			Medium	–	–	–	24.8
			Large	–	–	–	52.6

[a] Including oligo- or asthenozoospermic patients and normozoospermic men.
[b] Including Sb1 and Sb2 spermatids.
[c–e] Percentage of cells with different superscripts in the same file were significantly different ($P < .05$, χ^2 test).

Tanaka. Human sperm head vacuoles. Fertil Steril 2012.

TABLE 3

Comparison of outcomes of conventional ICSI and IMSI using ejaculated spermatozoa with or without various sizes of vacuoles.

	IMSI using sperm with			
No. [%] of oocytes or embryos	**Large vacuoles**	**Small vacuoles**	**No vacuoles**	**Conventional ICSI[c]**
Injected	23	63	20	236
Fertilized[a]	14 (60.9[e,f])	54 (85.7[d])	16 (80.0[d,e])	167 (70.8[d,e])
Developed to the blastocyst stage[b]	7 (50.0[d,e])	28 (51.9[d,e])	4 (25.0[e])	86 (51.5[d])

Note: ICSI = intracytoplasmic sperm injection; IMSI = intracytoplasmic injection of morphologically selected sperm.
[a] Percentages were per oocyte injected.
[b] Percentages were per oocyte fertilized.
[c] Routine ICSI outcomes using nonselected sperm in our clinic.
[d–f] Values with different superscripts in the same row were significantly different ($P<.05$, χ^2 test).

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ICSI (nonselected sperm injection). The fertilization rates after injection of spermatozoa with large or small vacuoles were significantly different. However, these values were not significantly different from IMSI using a spermatozoon without vacuole. The fertilization rates using selected cells with no or large vacuoles were not significantly different from that using nonselected conventional ICSI routinely done in our clinic, whereas the fertilization rate using cells with small vacuoles was significantly higher. In contrast, the percentage of blastocysts obtained from selected cells without vacuoles was significantly lower than those using cells with vacuoles or unselected cells.

DISCUSSION

After doing a study with primates it was reported by Bedford (9) and Zamboni et al. (34) that nuclear vacuoles are structures specific to the human sperm head. In their studies they found by electron microscopic observation that, in contrast to humans, no nuclear vacuoles in cauda epididymal or electroejaculated sperm of several kinds of primates. Therefore these investigators concluded that vacuoles were structures that were specific for the human sperm head. In the present study, DIC observations showed that most spermatozoa in the studied human ejaculates held more than one vacuole of various sizes on the surface of the head, especially in the acrosomal regions, regardless of the quality of apparent structure and motility. The very high incidence of vacuoles we found in normozoospermic men as well as oligozoospermic and asthenozoospermic patients should not be a surprise and matched the results reported by Baccetti et al. (1). Bartoov et al. (13) described that it was not unusual to observe more than one large vacuole on the head of human sperm that actively moves forward.

The incidence of vacuoles did not change after removal of sperm plasma membrane and/or acrosome, and this indicated that most vacuoles were not a cave of the plasma membrane or a hollow on the acrosome, but a cavity in the nucleus.

In the present study, vacuoles were found on the surface of condensing nuclear material, even in early stage spermatids, and the frequency increased, but the size tended to become smaller, during the following spermiogenesis and epididymal transit. Holstein and Roosen-Runger (35) and Johannisson et al. (36) reported that in humans, large chromatin vacuoles were measurable from Step 6 (acrosome phase) and from this step on their relative area did not grow (37). In addition, Golan et al. (38) suggested that human sperm chromatin continued condensation during the passage through the epididymis. These reports support our suggestion that vacuolar formation occurs naturally during the process of condensation of sperm nuclei, and should not be regarded as degeneration but as physiological changes.

Recently, some reports using a modified ICSI technique (12–19) pointed out that human spermatozoa with vacuoles were inferior in quality as male gametes to sperm without vacuoles. And accordingly, PRs should increase with IMSI versus those obtained with conventional ICSI. However, in the present study we were not able to confirm that cells with vacuoles were inferior or that IMSI was superior to unselected conventional ICSI. It must be noticed that our study was not powered to detect a difference because of the limited number of examinations. Furthermore, cells with small vacuoles even showed higher fertility rates than cells without any vacuoles. In addition, Thundathil et al. (39) have shown in bull studies that although bull spermatozoa with multiple nuclear vacuoles were defective in zona binding, they gained access to the ooplasm and apparently participated in fertilization and early embryonic development. Therefore when the ICSI or IMSI systems, in which sperm could bypass the zona pellucid (ZP), were used, the negative effects of vacuoles might not be expressed.

The significance of the universal occurrence of vacuoles in the human sperm head is still not completely known and requires further studies. However, our results strongly indicate that vacuoles are usually present on the surface of the human sperm head, and that they should not be considered as degenerative structures but rather as normal intranuclear elements. In our study, we have found that human sperm vacuoles did not affect ICSI outcomes. The number of spermatozoa without vacuoles in humans is extremely small and trying to find and then inject such cells is a very difficult and time-consuming process. It is almost impossible in practice. Therefore, when performing ICSI/IMSI the sole existence of head vacuoles should not be an issue to discard spermatozoa during the selection process, what is more important is the size of the vacuoles and the use of spermatozoa with large vacuoles should be avoided.

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