The impact of spermatozoa preincubation time and spontaneous acrosome reaction in intracytoplasmic sperm injection: a controlled randomized study

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Objective: To determine the optimum time interval between semen processing and incubation before intracytoplasmic sperm injection (ICSI) and correlate it with the acrosomal reaction rate.
Design: Controlled randomized study.
Setting: The Egyptian IVF-ET Center.
Patient(s): Couples with male factor infertility undergoing ICSI using ejaculated semen.
Intervention(s): The patients were prospectively randomized according to differences in sperm preincubation time before ICSI into 1-hour, 3-hour, and 5-hour groups. The status of the acrosome was studied using electron microscopy.
Main Outcome Measure(s): The primary outcome measures were fertilization rate and acrosome reaction rate. Secondary outcome measures were the implantation and pregnancy rates.
Result(s): The rate of acrosomally reacted spermatozoa was the highest (68.2%) after 5 hours of incubation and lowest (25.6%) after 1 hour of incubation. The difference was statistically significant. The fertilization rate was the highest (74%) using spermatozoa incubated for 3 hours as compared with 1 hour (70%) and 5 hours (67%), but the difference was not statistically significant.
Conclusion(s): Acrosome reaction is time dependent; the optimum incubation time of spermatozoa before ICSI was 3 hours, which resulted in the highest fertilization rate. (Fertil Steril® 2008;90:584–91. ©2008 by American Society for Reproductive Medicine.)

Key Words: Acrosome reaction, ICSI, fertilization, electron microscopy, sperm preincubation time

Many hours elapse between ejaculation and in vivo fertilization. During this time, sperm capacitation and the acrosome reaction occur. These crucial steps in gamete interaction allow the penetration of the zona pellucida and fusion with the oocyte membrane (1, 2). The acrosome reaction and the release of (pro) acrosin have been studied extensively both in vivo (2–5) and in vitro (6–9). The ability of spermatozoa to undergo a normal acrosome reaction and the rate of this reaction are important indicators of fertilizing ability (9, 10). The acrosome reaction score following ionophore challenge was found to be a predictor of pregnancy (10).

With intracytoplasmic sperm injection (ICSI), zona pellucida penetration and oolemmal fusion are bypassed, and the acrosome reaction may be seen as unnecessary. The introduction of ICSI in humans (11, 12) and the achievement of a high fertilization rate has led to the assumption that male nuclear decondensation and pronuclear formation do not need prior interaction between gamete membranes. The introduction of an artificial acrosome reaction for ICSI has been suggested following animal studies that showed increased fertilization rates and accelerated pronucleus formation (13, 14). The question remains, however, whether the acrosome reaction is necessary for sperm to undergo nuclear decondensation. The introduction of an acrosome into the ooplasm by ICSI seems to physically disturb sperm chromatin decondensation (15). It was found that the delay of sperm chromatin decondensation is associated with that of acrosomal disassembly, and that acrosomes appear to disintegrate in the ooplasm whether or not the acrosome reaction has taken place (15).

To optimize ICSI outcomes, several recent studies have examined the effect of different oocyte preincubation times on fertilization, cleavage, and implantation rates (16–18). The effect of different preincubation times of spermatozoa before ICSI on the fertilization and pregnancy rates was previously studied by our group (19).

Based on the observation that the acrosome reaction occurs spontaneously during incubation in a defined medium (20, 21) and is time dependent (22), we hypothesized that extending the preincubation time of spermatozoa might improve the fertilization rate in ICSI. The aim of this study was to correlate the acrosomal status of the spermatozoa at the time of ICSI and the fertilization rate to determine the optimum time interval between semen processing and incubation before ICSI.

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MATERIALS AND METHODS

Patients

The study enrolled 256 couples undergoing ICSI treatment for male factor infertility at the Egyptian IVF-ET center. The female partners were younger than 40 years, had normal hormonal profiles, and no pelvic pathologic conditions. Cases requiring surgical retrieval of spermatozoa were excluded from the study. The study included male factor infertility from oligoasthenoteratospermia with sperm count from 5 to 10 million per mL. The couples signed informed consent forms, and approval of the institutional review board was obtained. Randomization was done using sealed, dark envelopes, and distributed patients equally between three groups according to the time interval between sperm processing and ICSI: 1 hour, 3 hours, and 5 hours.

Ovarian Stimulation

In all cycles, ovarian stimulation was performed using a gonadotropin-releasing hormone analogue (GnRH-a, Decapeptyl; Ferring Pharmaceuticals, Copenhagen, Denmark) long protocol and menotropins. The GnRH-a was administered as 0.1 mg SC on day 20 and was given daily until the day of human chorionic gonadotropin (hCG) injection. After 2 to 3 weeks, when down-regulation was confirmed, human menopausal gonadotropin (hMG, Menogon; Ferring Pharmaceuticals) was given IM at 150–300 IU/day, according to the patient's age, weight, and previous response. Daily monitoring of ovarian response was performed using ultrasound and serum estradiol measurement. The hCG was given as 10,000 IU (Pregnyl; NV Organon, Oss, the Netherlands) when the mean diameter of the leading three follicles reached 18 mm. Oocyte retrieval was performed 36 hours after hCG injection by ultrasound-guided transvaginal aspiration.

Oocyte Preparation for ICSI

The oocytes were denuded of their surrounding cumulus cells (2 hours after ovum pick-up) using 80 IU/mL of hyaluronidase in N-2-hydroxyethylpiperazine-N′ -2-ethanesulfonic acid (HEPES)-buffered Earle's Balanced Salt Solution (EBSS; cat. no. 1200; Medicult, Copenhagen, Denmark) for 10 to 15 seconds, and then the oocytes were transferred to tissue culture media (Vitrolife AB, Kungsbacka, Sweden) for complete removal of the corona cells by repeated aspiration in a finely pulled pipette. The oocytes were then rinsed and incubated in tissue culture media under mineral oil (Squibb, Princeton, NJ) until the time of injection (2 to 3 hours after pick-up), which was done for oocytes at the metaphase II stage.

Semen Processing and ICSI

Semen samples were collected by masturbation after 2 to 3 days of abstinence. The samples were allowed to liquefy at room temperature for 20 to 30 minutes. Tissue culture media was added in a 2:1 ratio and mixed with the semen. Centrifugation was done at 400 $\times g$ for 8 minutes; the supernatant was discarded, and the pellet was resuspended in tissue culture media and centrifuged again. Then the supernatant was discarded, and the pellet was resuspended in 0.2–0.5 mL of tissue culture medium. The tissue culture medium used for semen processing was Ham's F10 equilibrated at 37°C and 5% CO_2 in the air. It is a carbonated medium to allow for capacitation. The resuspended pellet was distributed carefully in the surrounding ring of a Tea tube to separate the motile spermatozoa (23). The Tea tube was placed in an incubator at 37°C and 5% CO_2 in air until the time of ICSI, as determined by the randomization process. The motile spermatozoa swim up from the pellet in the surrounding ring of the Tea tube, then form a sediment in the bottom of the central cone of the tube. All the oocytes from each patient were allocated to one of three study groups according to differences in sperm preincubation time: 1 hour, 3 hours, and 5 hours. After the allocated preincubation period had elapsed, a microdroplet of spermatozoa was taken from the bottom of the central cone of the Tea tube and added to a 10% polyvinylpyrrolidone droplet in the microinjection dish. Then sperm selection, immobilization, and oocyte injection were performed.

Spermatozoa Preparation for Electron Microscopy Study

The status of the acrosome was studied using electron microscopy. After semen processing, aliquots were taken at intervals of 1, 3, and 5 hours for fixation to be sent for electron microscopic evaluation. The semen sample was processed as previously described. The motile sperms were collected after exactly 1, 3, and 5 hours from the bottom of the central cone of the Tea tubes. The sperm suspension was put in labeled Eppendorf tubes (Eppendorf AG, Hambury, Germany) and centrifuged at 400 $\times$ g for 8 minutes. One mL of phosphate buffer was put on the pellet in the Eppendorf tubes. The phosphate buffer was removed after 6 hours and replaced by 1 mL of glutaraldehyde fixative.

The electron microscopy specialist was specifically asked to examine and comment on the acrosomal status of the perinuclear sheath. The examiner was blinded to the groups.

Statistical Analysis

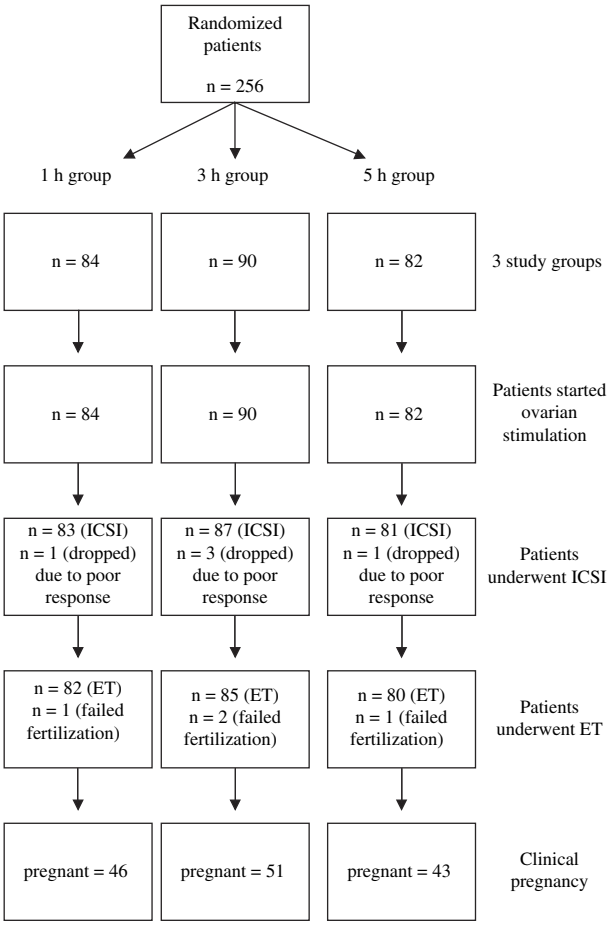
All statistical tests were performed using the Arcus Quickstat, version 1.0 (StatsDirect Ltd, Altrincham, Cheshire, United Kingdom). $P<.05$ was considered statistically significant. For comparison of the means of variables, an unpaired Student's t-test was used. For comparison of fertilization rates between the groups, a chi-square analysis was performed.

RESULTS

The study included 256 patients undergoing ICSI for male factor infertility, and the flow sheet illustrates the follow-up period of the randomized patients until the pregnancy results (Fig. 1). Semen count varied from 3.5 to 11 $\times$ 10^6/mL, with a mean of 6.8 $\pm$ 5.2, and motility varied from 5% to

FIGURE 1

Flowchart of patients from time of randomization to clinical pregnancy.



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40% with a mean of 10.4 ± 8.5; abnormal forms varied from 40% to 100% with a mean of 80.3 ± 10.3. There were no statistically significant differences in the semen parameters among the three groups. The female partners' ages and infertility periods were comparable in the three groups. The overall ICSI outcome is shown in Table 1. There was no statistically significant difference in the number of oocytes retrieved or metaphase II oocytes between the three groups. The fertilization rates were 70%, 74%, and 67% in the 1, 3, 5 hour groups, respectively. The difference was not statistically significant.

The number of embryos per transfer, the implantation rate, and the clinical pregnancy rates were not significantly different among the groups. The rate of acrosomally reacted spermatozoa was only 25.6% after 1 hour of incubation, which was statistically significantly lower than in the 3–hour group (40.9%, P=.005, OR = 0.62; 95% CI, 0.45–0.86) and the 5-hour group (68.2%, $P \leq .001$, OR = 0.22; 95% CI, 0.15–0.31). The 3-hour group's acrosome reaction was statistically significantly higher than that of the 5-hour group (P=.0005, OR 0.6; 95% CI, 0.45–0.80) (Table 2). Figures 2, 3, and 4 show electron microscopy studies of sperm heads after various incubation times.

DISCUSSION

Low sperm–zona pellucida binding or low zona pellucida–induced acrosome reaction was found to be associated with a low fertilization rate (24). In this study, we investigated the affect of the time interval between semen processing and ICSI on the rate of acrosome reaction and fertilization. We hypothesized that increasing the incubation time of the spermatozoa would increase the rate of acrosome reaction and improve the fertilization rate.

It seems physiologically correct to that we should use acrosome-reacted spermatozoa for ICSI to avoid irregular sperm decondensation. During ICSI, it is very difficult to obtain a sperm that is acrosomically reacted and theca-removed like the in vivo condition when sperm reacts with the oolemmal membrane.

Capacitation is a postejaculatory modification of the sperm surface, which is a species-specific and time-dependent process that varies markedly among men (25, 26). It involves the mobilization and/or removal of certain surface components from the sperm plasma membrane, such as glycoproteins, decapacitation factor, acrosome-stabilizing factor, and acrosin inhibitor. It has been demonstrated that proteasome is present in human sperm (27), and it may participate in activating Ca^{2+} channels (28, 29). Subsequently, an increase in membrane fluidity and permeability occurs (3). These events are followed by or simultaneous with [1] a decrease in net surface charge, [2] devoided area of intramembrane protein and sterols, and [3] increased concentrations of anionic phospholipids. These areas are thought to be the sites of fusion and vesiculation during acrosome reaction (3).

In vivo, the acrosome reaction seems to be induced by appropriate stimuli, which are believed to be follicular fluid, progestin, progesterone, and hydroxyprogesterone. Follicular fluid and cumulus cells in particular have been proven to induce the acrosome reaction due to the fact that they have sufficient protein-bound progesterone concentration, which has been identified as the acrosome reaction-inducing agent in follicular fluid (5, 30). It was demonstrated recently that follicular fluid stimulated the acrosome reaction in a dose-dependent manner (31). The maximally effective concentration of follicular fluid was 30% vol/vol.

Recently, a link between locally produced estradiol from ejaculated spermatozoa, and acrosome reaction and sperm capacitation was described (32). Evidence has been provided that environmental estrogen can significantly stimulate mammalian sperm capacitation, acrosome reactions, and fertilizing ability (33). Antisperm antibodies have a variable effect on the acrosome reaction and capacitation. Some antisperm antibodies may adversely affect the ability of sperm to undergo capacitation or acrosome reaction, but other antisperm

TABLE 1

Outcome of intracytoplasmic sperm injection (ICSI) according to different incubation times of spermatozoa.

Sperm incubation time before ICSI	1-hour group	3-hour group	5-hour group
Randomized patients	84	90	82
ICSI cycles	83	87	81
ET cycles	82	85	80
Age in years (mean ± SD)	32.34 ± 2.52	32.05 ± 3.35	33.01 ± 1.34
Infertility period (mean ± SD)	7.92 ± 2.85	8.12 ± 1.99	7.64 ± 2.33
Oocytes retrieved (mean ± SD)	810 (9.9 ± 2.5)	840 (10.2 ± 3.2)	795 (10.04 ± 1.55)
M2 oocytes (mean ± SD)	648 (8.5 ± 4.1)	672 (7.9 ± 2.5)	640 (8.2 ± 2.3)
2PN oocytes (mean ± SD)	453 (4.6 ± 0.5)	498 (6.5 ± 1.9)	428 (5.9 ± 1.5)
Fertilization rate	70%	74%	67%
Embryos per ET (mean ± SD)	3.00 ± 0.43	2.99 ± 0.63	3.02 ± 0.24
Implantation rate	22.52%	21.42%	20.55%
Clinical pregnancies pregnancy rate	46 (54.8%)	51 (56.7%)	43 (52.4%)

Note: ET, embryo transfer; M2, metaphase II; 2PN, two pronuclear (oocyte).

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antibodies do not have this effect (34). It is also a very common feature to have an absence of the acrosome in globozoospermia, which is known to be associated with very low or complete failure of fertilization in ICSI (35).

In vitro ionophore and increased albumin concentrations have also been used to induce acrosome reaction but cannot be used for human IVF or ICSI because of their toxicity. It was demonstrated that, in the absence of serum albumin, hamster egg zonae pellucidae cannot efficiently induce acrosome reactions (36). In a recent study, lead levels in seminal plasma positively correlated with spontaneous acrosome reaction and negatively correlated with mannose receptors and mannose-induced acrosome reaction (37). Reduced acrosome reaction was also observed in spermatozoa incubated in hydrosalpinx epithical cell culture conditioned medium (38). Sperm defects associated with low or impaired zona pellucida–induced acrosome reaction are some of the major causes of failure of fertilization in standard IVF (39).

A nontoxic method for induction of the acrosome reaction that can be used in actual IVF is to expose the spermatozoa

TABLE 2

Rate of acrosome reaction in relation to the sperm incubation time.

Sperm incubation time	Semen parameters (mean ± SD)	Total number of sperm heads studied	Number of sperm heads with acrosomal reaction	Rate of acrosomal reaction
1 hour	Count: 7.5 ± 2.4 × 10^6/mL Motility: 11.2 ± 5.5% Abnormal forms: 79.5 ± 11.2%	308	79	25.6%[a]
3 hours	Count: 8.2 ± 1.2 × 10^6/mL Motility: 10.5 ± 6.4% Abnormal forms: 80.7 ± 9.5%	298	122	40.9%[b]
5 hours	Count: 9.2 ± 6.5 × 10^6/mL Motility: 12.3 ± 5.8% Abnormal forms: 78.6 ± 9.8%	251	171	68.2%

[a] Statistically significant difference as compared with 3-hour group: OR = 0.63; 95% CI, 0.45–0.87; P=.005. Statistically significant difference as compared with 5-hour group: OR = 0.22; 95% CI, 0.15–0.31; P=.0001.
[b] Statistically significant difference as compared with 5-hour group: OR = 0.6; 95% CI, 0.45–0.8; P=.0005.

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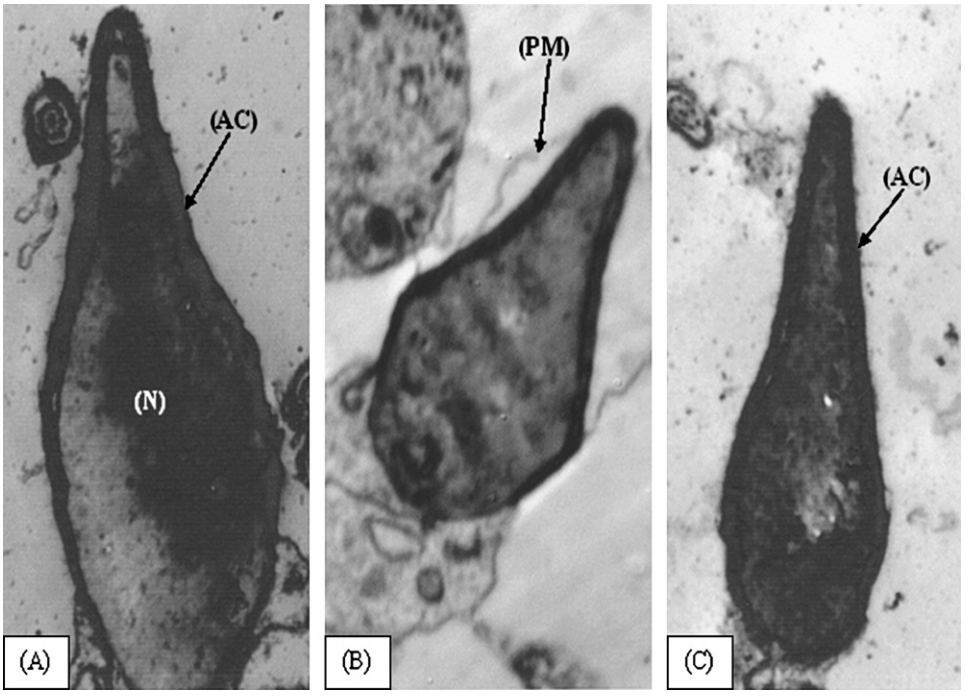
FIGURE 2

Transmission electron microscopic photomicrographs of 1-hour group. (**A**) Sperm head with disruption of the plasma membrane, intact acrosomal cap (AC), and a nonclear subacrosomal space. The chromatin is compact and dense within the nucleus (N) with no visible vacuoles (magnification × 31,760). (**B**) An acrosome-intact sperm head with the plasma membrane (PM) swollen away from the acrosome (magnification × 26,467). (**C**) A sperm head with ruptured plasma membrane and intact nonreacted acrosome (AC) (magnification × 31,760).



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to a low temperature (4°C) for 24 hours, followed by incubation at 37°C for 3 hours (40). In our study, to avoid any possible cytotoxic effect, we did not use any chemical substance to induce the acrosome reaction. Our idea was to allow the acrosome reaction to occur spontaneously by time and temperature in tissue culture media. The tissue culture media used was carbonating medium in equilibrium with 5% CO_2 in a humidified atmosphere at 37°C. It has been demonstrated that bicarbonate induces capacitative changes (41, 42). Bicarbonate plays a major role in the activation of sperm cells (43). High levels of bicarbonate induce activation of adenylate cyclase, one of the key events in sperm capacitation that occurs in the lumen of the oviduct and is virtually absent in epididymal and seminal plasma (44). Under normal conditions, most of the freshly ejaculated spermatozoa are acrosome unreacted. During sperm incubation in a defined medium, acrosome reaction occurs spontaneously (20, 21). It has been shown that the spermatozoa of fertile men have a significant increase in the percentage of acrosome-reacted spermatozoa, with a maximum peak after 3 hours on average and a variable range among men between 1 to 6 hours (22). A close correlation was found between the number of sperm and the acrosome reaction in response to pentoxifylline treatment, suggesting that the treatment created a greater proportion of the "reactable" population of sperm (45).

The electron microscopic results in our study showed that the rate of acrosome reaction was time dependent, with a maximum of 5 hours. However, the fertilization rate was the highest when the spermatozoa were incubated for 3 hours. Although the acrosome reaction increases with time, there may be an increase in chromatin decondensation in the sperm head that adversely affects fertilization. Recently, a new test was described to allow the identification of the acrosome-reacted sperm (46). It allows acrosome reaction identification by phase-contrast microscopy after a brief sperm incubation in a decondensing solution; the results have been comparable with electron microscopy.

Our study has demonstrated that the rate of spontaneous acrosome reaction of spermatozoa incubated in tissue culture media at 37°C is time dependent and is highest at 5 hours, but the fertilization rate following ICSI is highest with spermatozoa incubated for 3 hours. Therefore, based on the results of this study, it is recommended to allow a 3-hour incubation period for spermatozoa before ICSI to obtain the best fertilization rate.

FIGURE 3

Transmission electron microscopic photomicrographs of 3-hour group. (**A**) Two sperm heads. The upper head has a ruptured plasma membrane, intact nonreacted acrosome (AC), and a compact, dense nucleus (N). The lower head shows complete disruption of the plasma membrane and signs of early acrosomal reaction manifesting by the slight detachment of the acrosome (AC) from the nucleus and a clear subacrosomal space (SS). The nucleus (N) is compact with clear vacuoles (V) (magnification × 31,760). (**B**) Sperm heads with early reacted acrosomes. Plasma membranes (PM) are swollen away from the nucleus and completely ruptured in some areas. The acrosome (AC) is slightly detached from the nucleus, with an irregular outer acrosomal membrane (OM) (magnification × 15,880).

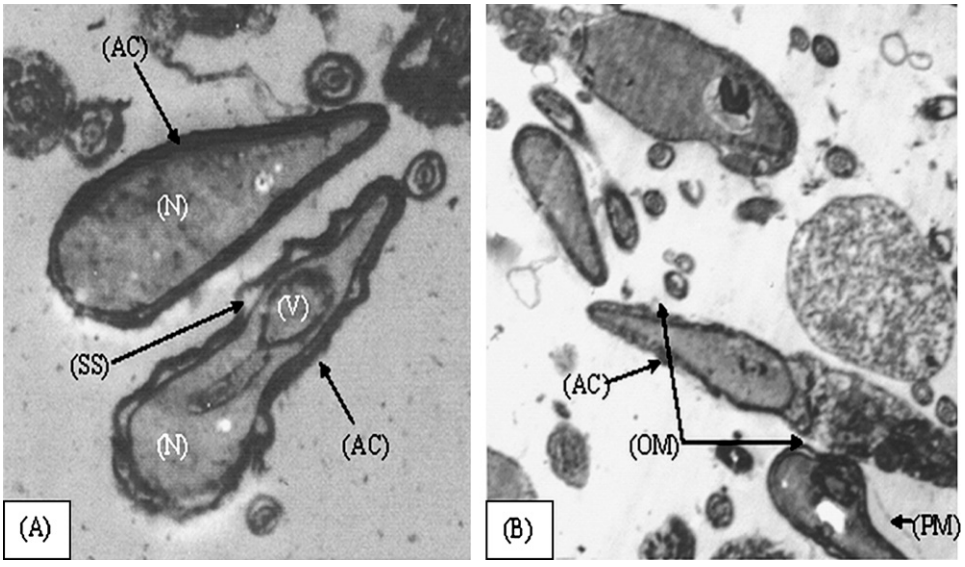

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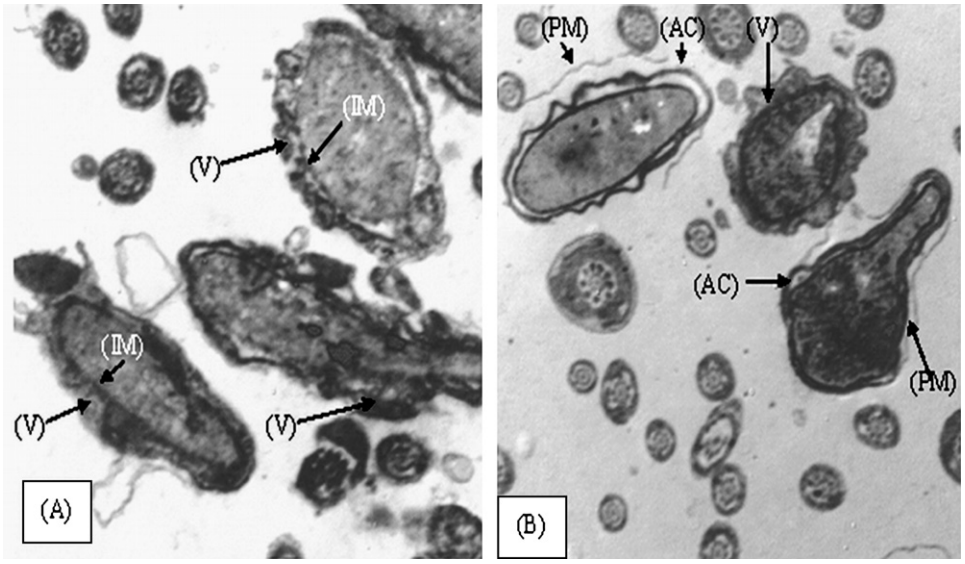
FIGURE 4

Transmission electron microscopic photomicrographs of 5-hour group. (**A**) Magnification × 26,467. (**B**) Magnification × 21,173. Most of the sperm heads have reacted acrosomes, as manifested by the complete disappearance or swelling of the plasma membranes (PM), detachment of the acrosome (AC) from the nucleus, and internal vesiculation (V) adhering to the internal acrosomal membranes (IM).



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