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Original Article

The efficiency of conventional microscopic selection is comparable to the hyaluronic acid binding method in selecting spermatozoa for male infertility patients

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ABSTRACT

Objective: To evaluate if hyaluronic acid (HA)-bound spermatozoa surpassed conventional microscopy-selected spermatozoa in the status of sperm DNA integrity by acridine orange (AO) fluorescence staining.
Materials and methods: Spermatozoa obtained from couples with indication for the intracytoplasmic sperm injection (ICSI) procedure due to male infertility ($n = 34$) and control males with normal sperm parameters ($n = 12$) were analyzed using AO fluorescence staining after density-gradient centrifugation (DGC), polyvinylpyrrolidone (PVP)-microscopic selection, and HA-binding selection to determine sperm DNA integrity.
Results: Percentages of DNA intact spermatozoa with green fluorescence were significantly higher in both PVP-microscopic selected spermatozoa (82.1 ± 24.0%) and HA-bound spermatozoa (83.9 ± 21.1%) than in spermatozoa prepared by DGC (66.8 ± 24.0%). However, there was no significant difference between the PVP-sperm and HA-sperm groups. When the percentage of green fluorescent spermatozoa prepared by DGC fell initially below 68%, both PVP-microscopic and HA-binding selection failed to select over 90% spermatozoa with intact DNA for ICSI in the male infertility group. Compared to control males with normal sperm parameters (99.3 ± 1.8%), the proportion of green fluorescence sperm after HA-binding selection from couples with male infertility (83.9 ± 21.1%) did not reach the range of > 99% reported by Yagci et al.
Conclusion: The percentages of DNA intact spermatozoa between the PVP-sperm and HA-sperm groups were not significantly different. In an ICSI procedure, a well-trained embryologist will have the same ability to choose sperm with intact DNA by conventional microscopic selection as with HA-bound spermatozoa selection.

Introduction

The quality of the oocyte and spermatozoon influences subsequent fertilization and embryo development. As spermatogonia transform into mature spermatozoa, a complex process involving meioses and mitoses occurs. Aberrant chromatin packaging and defective apoptosis during spermatogenesis [1,2], high levels of reactive oxygen species (ROS) produced by immature spermatozoa [3], and environmental toxicants [4] all result in sperm DNA damage. As soon as spermatozoa with damaged DNA enter the oocytes, fertilization may lead to defects in embryonic development, implantation failure, or unexplained pregnancy loss [5–8]. In the era of assisted reproductive technologies (ART), intracytoplasmic sperm injection (ICSI) has clearly overshadowed conventional *in vitro* fertilization (IVF) procedures and become the ultimate option for the treatment of severe male factor infertility. This technology bypasses the barriers of natural selection by introducing an

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apparently intact motile spermatozoon into the ooplasm and may transport the DNA-damaged sperm to the oocyte. Furthermore, males with fertility problems show a higher rate of chromosomal abnormalities [9] and often need ICSI treatment. However, the shape of spermatozoa does not predict the absence of chromosomal aberrations [10].

In the natural fertilization process, hyaluronic acid (HA) in the extracellular matrix of the cumulus oophorus complex surrounding the oocyte seems to be a "physiological" selector of mature spermatozoa. *In vitro* it has been demonstrated that only mature spermatozoa that have completed plasma membrane remodeling, cytoplasmic extrusion, and nuclear maturity are able to bind with HA because they have a high density of HA receptors [11,12]. These HA-bound spermatozoa show low frequency of chromosomal aneuploidies and DNA fragmentation, have good nuclear morphology, and result in good embryo quality [9,13]. Hence, sperm selection with the HA binding method prior to ICSI may help optimize the outcome of ART.

Sperm DNA damage can be evaluated in a variety of ways, including the *in situ* nick translation (ISNT) assay, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, single cell gel electrophoresis (COMET) assay, sperm chromatin dispersion (SCD) test, aniline s (AB) test, chromomycin-A3 (CMA3) staining, and acridine orange (AO) fluorescence by microscopy or flow cytometry (sperm chromatin structure assay, SCSA). In most infertility laboratories, methods requiring expensive equipment like flow cytometry and dedicated software are often unavailable. The AO fluorescence that is based on the principle that damaged DNA denatures much faster than undamaged DNA when subjected to stresses such as heat and pH changes distinguishes sperm with intact double-stranded DNA (green fluorescence) or single-stranded DNA (red fluorescence) [14]. The AO fluorescence staining is a simple rapid microscopic procedure introduced as the AO test (AOT) by Tejada et al [15] and has been widely used for evaluating male infertility [16].

The objective of the present investigation was to evaluate if HA-bound spermatozoa surpassed conventional microscopy-selected spermatozoa in terms of sperm DNA integrity by AO fluorescence staining in the patient with male infertility.

Materials and methods

Patient selection

Thirty-four couples with indication for ICSI procedure due to male infertility were enrolled. Semen samples for investigation were obtained only after the ICSI procedure was completed. Twelve semen samples with normal parameters that were collected from husbands of infertile couples referred for semen analysis were considered the control group. The experiments were started after the semen analysis was completed. The use of discarded spermatozoa was approved by the Institutional Review Board and all participants provided informed consent.

Sperm preparation

Forty-six semen samples were collected by masturbation after 2–3 days abstinence. After liquefaction for at least 30 minutes, routine semen analysis was conducted. The motility and concentration of spermatozoa were evaluated according to the 2010 World Health Organization (WHO) criteria, while sperm morphology was assessed according to Kruger's strict criteria. The semen samples were processed using density-gradient centrifugation (PureSperm 40/80, Nidacon, Sweden). After the ICSI procedure, the remaining semen was collected for the experiments.

Experimental design

The 46 collected spermatozoa that were processed with PureSperm 40/80 were labeled as Pure-sperm. Twenty spermatozoa were chosen from Pure-sperm by a well-trained embryologist using conventional polyvinylpyrrolidone (PVP, SAGE Media, USA)-ICSI procedures and labeled as PVP-sperm. For HA selection of mature spermatozoa, the PICSI dish (PICSI Sperm Selection Device; Mid-Atlantic Diagnostics Inc.) with three micro-dots of HA attached to the interior bottom were used. Approximately 10–20 μL of pure sperm suspension was applied to one micro-dot and incubated for at least 30 minutes. The same embryologist selected 20 HA-bound spermatozoa (HA-sperm) that exhibited no progressive migration despite a vigorous tail cross-beat frequency in addition to intact morphology under a microscope (Olympus SZX7, Tokyo, Japan).

Sperm fixation for AO fluorescence

The Pure-sperm, PVP-sperm, and HA-sperm of each sperm sample were smeared respectively on three precleaned glass slides and air-dried. Carnoy's solution (3:1 ratio of methanol and glacial acetic acid) was used to fix the sperms for 6 hours, which were then stained with freshly prepared 0.19 mg/mL AO (Acridine Orange, Sigma, Aldrich, USA) stain (10 mL of 1% AO in distilled water added to a mixture of 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M Na_2HPO_4, $2H_2O$; Polysciences, Warrington, PA) for 5 minutes in a dark room. Subsequently, the glass slides were rinsed with distilled water, covered with glass cover slips, and immediately evaluated under an epifluorescence microscope (Olympus BX40) equipped with an Olympus DP-70 digital camera at an excitation wavelength of 450–490 nm.

Each slide with 20 sperms was assessed by the same examiner within 40 seconds. Spermatozoa showing green fluorescence were scored as containing intact DNA, whereas those displaying yellow/orange to red fluorescence were considered as having denatured or relaxed DNA induced by citric acid (Fig. 1). The percentage of spermatozoa with green fluorescence was then recorded.

Statistical analysis

The nonparametric Friedman test was used to examine the differences in the percentages of green fluorescence spermatozoa in Pure-sperm, PVP-sperm, and HA-sperm groups. Statistical significance was set at $p < 0.05$. All data analyses were performed with MedCalc Version 10.2.0.0.

Results

Characteristics and outcomes of ART

Thirty-four couples with indication for the ICSI procedure due to male infertility were included, but 10 failed to participate in the ICSI procedures due to economic considerations or hesitation regarding the procedure itself, and received conventional IVF treatments instead. Data on ART and clinical outcomes were described in Table 1. The mean age of the females was 35.0 ± 4.1 years (mean ± standard deviation, SD) at oocyte retrieval and the mean number of eggs retrieved was 11.0 ± 7.4. After the ICSI procedure, 71.1% of oocytes were fertilized and 25.2% of embryos were graded as good-quality embryos. The mean number of embryos transferred was 2.8 ± 1.0 and the implantation rate was 24.2%. Clinical pregnancy was defined by ultrasonographic visualization of the gestational sac and the clinical pregnancy rate per embryo transfer cycle reached 41.7% in this series. However, the spontaneous abortion rate was 20%.