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Clinical Assisted Reproduction

Sperm Single-Stranded DNA, Detected by Acridine Orange Staining, Reduces Fertilization and Quality of ICSI-Derived Embryos

Irma Virant-Klun,[1,2] Tomaz Tomazevic,[1] and Helena Meden-Vrtovec[1]

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***Purpose*:** The aim of this study was to evaluate the effect of sperm single-stranded DNA, detected by acridine orange (AO), and classical sperm parameters on embryonic quality after ICSI.

***Methods*:** Before ICSI, the spermatozoa of 183 infertile patients with oligo-, astheno-, teratozoospermia ($n = 147$), or more than one previous unsuccessful conventional IVF attempt ($n = 36$) were stained by AO to assess the presence of single-stranded DNA. Two days after ICSI, the embryos of 135 patients were scored for morphology, fragmentation included. Embryos of 48 couples were cultured for 4 days to develop to the morula or blastocyst stage. At most 2 embryos were transferred on Day 2 or 4.

***Results*:** When the level of spermatozoa with single-stranded DNA was increased, there was a significantly lower fertilization rate after ICSI. Besides, increased sperm single-stranded DNA resulted in a higher proportion of heavily fragmented embryos on Day 2 ($P < 0.05$). In patients with an increased level of spermatozoa with single-stranded DNA, a significantly higher number of embryos were arrested in spite of prolonged culturing ($P < 0.05$). Classical sperm parameters did not affect the quality and developmental potential of ICSI-derived embryos. No correlation was found between the level of spermatozoa with single-stranded DNA, pregnancy rate, and live-birth rate achieved by ICSI, except in patients with 0% of spermatozoa with single-stranded DNA, in whom the pregnancy rate was significantly higher.

***Conclusions*:** Sperm single-stranded DNA provides additional data on sperm functional capacity in terms of fertilization and embryonic quality after ICSI.

KEY WORDS: Embryos; human; ICSI; single-stranded DNA; spermatozoa.

INTRODUCTION

Infertile men possess anomalies in the composition of their sperm nuclei, displaying higher levels of loosely packaged chromatin and damaged DNA (1–11). Gopalkrishnan (12) observed 60% of spermatozoa with single-stranded DNA in men with in vitro fertilization (IVF) and intrauterine insemination failures, this percentage being higher than in normal recently fertile men.

Acridine orange (AO) staining is an established cytochemical method for determining sperm DNA integrity, allowing the differentiation between normal, double-stranded and abnormal, single-stranded sperm DNA, using the metachromatic properties of the dye (2). The fluorochrome AO intercalates into double-stranded sperm DNA as a monomer and binds to a single-stranded sperm DNA as an aggregate. The monomeric AO, bound to normal double-stranded DNA, fluoresces green, whereas the aggregated AO on single-stranded DNA fluoresces yellow to red.

[1] Department of Obstetrics and Gynecology, Medical Centre Ljubljana, Šlajmerjeva 3, SI-1000 Ljubljana, Slovenia.
[2] To whom correspondence should be addressed; e-mail: irma.virant@kclj.si.

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Some studies show that sperm single-stranded DNA, detected by AO staining, affects the fertilization process in a classical IVF program negatively (13–16). The negative effect of sperm single-stranded DNA on embryonic quality after intracytoplasmic sperm injection (ICSI), the method excluding the natural selection of spermatozoa, has not been proven yet.

Many authors suggest that semen quality and embryo development may be related (17–20). Janny and Menezo (21) have found that abnormal spermatozoa give rise to embryos with a significantly lower cleavage rate to develop to the blastocyst stage. Observations of some other authors show inferior quality and developmental potential of embryos obtained by ICSI (22–28). On the other hand, Yang (29) and Oehninger (30) have reported an identical quality between IVF- and ICSI-derived embryos.

The aim of this study was to evaluate the effect of sperm single-stranded DNA, detected by AO, and classical sperm parameters on embryonic quality after ICSI.

MATERIALS AND METHODS

Study Population

In this prospective study, 183 men were included: 147 men affected by male factor of infertility (oligo-, astheno-, teratozoospermia, OAT) and 36 normozoospermic men with more than one previous unsuccessful classical IVF attempt. Two to six months before ICSI the patients were clinically observed. The mean age was 35 ± 6 years and duration of infertility 8 ± 5 years. In 107 couples only male infertility was diagnosed, whereas in 76 couples male infertility was combined with female infertility (tubal, endometriosis, and/or hormonal disturbances such as polycystic ovaries).

According to the percentage of spermatozoa with single-stranded DNA the men were divided into two ICSI groups: Group I ($n = 64$), with normal percentage (<56%) of spermatozoa with single-stranded DNA, and Group II ($n = 119$), with increased percentage (≥56%) of spermatozoa with single-stranded DNA, according to the data in the literature and our own experience. Liu and Baker (14) proposed the increased level of sperm single-stranded DNA to be at ≥56% of spermatozoa with single-stranded DNA and Hoshi (16) at ≥60%. The cutoff value of 56% of spermatozoa with single-stranded DNA had been confirmed also in our IVF patients with normal semen quality, tubal sterility, and successful transfers in our own program.

All men were informed of the research and all gave their consent for participation.

After ICSI, the embryos of 135 couples were cultured for 2 days to *low-cell embryos* (*2–6 cells*) *stage*, and the embryos of 48 couples were cultured for 4 days to the *morula* or *blastocyst stage*.

Spermiogram

Semen was obtained by masturbation after 3–5 days of sexual abstinence. In liquefied semen, classical parameters of sperm quality—semen volume, sperm concentration, rapid progressive motility, morphology, vitality, and leukocytes—were determined (by the same technicians) using the conventional methods proposed by the World Health Organization guidelines (31).

Semen Preparation and AO Staining

Semen was prepared on Pure Sperm (Nidacon, International AB, Sweden) discontinuous concentration gradient (80%/40%) and then washed in 5 mL of SpermPrep medium (Medi-Cult, Denmark). After centrifugation the sperm pellet was resuspended in 0.5 mL of SpermPrep medium. A small aliquot (20 μl) of sperm suspension was glass smeared. According to Liu and Baker (15) it was air dried and then fixed overnight in Carnoy's solution (methanol/acetic acid, 3 : 1). Once rinsed and air dried, the slides were stained for 5 min with freshly prepared AO stain as follows: 10 mL of 1% AO in distilled water was added to a mixture of 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M $Na_2HPO_47H_2O$. The AO solution was stored in dark at 4°C for 4 weeks. After washing and drying, the slides were examined using a fluorescent microscope (Leitz, Germany; excitation of 450–490 nm). After AO staining, the samples were immediately observed. Each field was observed for some seconds under the fluorescence microscope. On each slide an average of 100 spermatozoa were counted. The percentage of spermatozoa with single-stranded DNA was calculated from the ratio of spermatozoa with red, orange, or yellow fluorescence to all spermatozoa counted per sample.

Interindividual variability for the AO test was 23.8% and intraindividual variability 18%. Variabilities were comparable to those for classical parameters of sperm quality (32).

Ovarian Preparation and Oocyte Retrieval

Before ICSI, the female partners with the mean age 33 ± 5 years were stimulated using first a desensitizing protocol of gonadotropin-releasing hormone agonist (GnRHa) (subcutaneous buserelin acetate 0.6 mg once daily; Suprefact: Hoechst AG, Germany), started on Day 22 of the menstrual cycle. After 14 days, if serum estradiol concentrations were <40 pg/mL and no ovarian cystic structures were observed on ultrasound (US) examination, stimulation was started with 225 IU/day of human menopausal gonadotropin (Pergonal: Serono, Switzerland) or FSH (Metrodin; HP 75 IU: Serono, Switzerland). Human chorionic gonadotropin (HCG, Pregnyl: Organon, The Netherlands) was administered when the leading follicle measuring more than 18 mm in diameter was obtained. Transvaginal US-guided aspiration of the ovarian follicles was performed 36 h after HCG administration using a single lumen needle.

Oocyte and Sperm Preparation Before ICSI

The cumulus–corona cell complexes were isolated from the follicular fluid and put into 5-mL Falcon tubes with 1 mL of Universal IVF medium (Medi-Cult, Denmark); the tubes were gassed in 5% O_2, 5% CO_2, and 90% N_2. The removal of the cells of the cumulus and the corona radiata by hyaluronidase has been described extensively in the literature (33–36). Oocytes were put in 80 IU hyaluronidase (Type VIII; Sigma, USA) and the cells of the cumulus and the corona radiata were removed by denudation pipette (Swemed Lab, Sweden) and washed by Flushing medium (Medi-Cult, Denmark).

After sperm preparation on PureSperm discontinuous concentration gradient and washing, 5-μl droplets of sperm suspension were prepared under paraffin oil (Sigma, USA).

ICSI Procedure

Metaphase-II oocytes were placed in 5-μl droplets of IVF medium under paraffin oil. ICSI was performed by the conventional method (34) without using polyvinyl pyrrolidone (PVP). A metaphase-II oocyte was aspirated with a slight negative pressure by the holding pipette (Swemed Lab, Sweden), and a single spermatozoon was injected into the ooplasm by the microinjection pipette (Swemed Lab, Sweden) under inverted microscope (Diaphot—TMD, Nikon, Japan) equipped by hydraulic micromanipulator (Narishige, Japan). Before microinjection, no special selection of spermatozoa was performed. Spermatozoa with normal morphology and motility were microinjected, if possible.

Assessment of Fertilization

About 18 h after the sperm microinjection (Day 1), the oocytes were checked for the presence of two clearly distinct pronuclei and polar bodies under the inverted microscope with magnification of $\times 200$.

Assessment of Embryonic Quality on Day 2

About 48 h after the ICSI procedure (Day 2), in 135 couples (108 with OAT and 27 with an unsuccessful classical IVF attempt) *each low-cell embryo with 2–6 cells* was scored according to the number, size and shape of blastomeres, and the extent of anucleate fragments (37). Embryos were defined as low-fragmented when anucleate fragments were present in less than 10% of the volume of the embryo, and as heavily fragmented when anucleate fragments were present in at least 10% of the embryonic volume. According to Bolton (38), Type IV or *excellent* embryos were defined as embryos with regular, spherical blastomeres of an equal shape and size, with no fragmentation. Type III or *good* embryos had regular, spherical blastomeres of an equal shape and size, with some fragmentation (<30%). Type II or *fair* embryos had blastomeres, slightly irregular in size and shape with considerable fragmentation (30–50%). Type I or *bad* embryos were embryos with barely defined blastomeres with considerable fragmentation (>50%).

Cleaved embryos with less than 50% of their volume filled with anucleate fragments were considered suitable for transfer. Embryos were loaded into 5 μl of Universal IVF medium (Medi-Cult, Denmark) and into a TDT catheter (TDT set; Prodimed, Neuilly-en-Thelle, France) and at most 2 embryos were transferred into the uterus approximately 48 h after microinjection (Day 2). Supernumerary embryos of good quality (Types IV and III) were cryopreserved for later use.

Prolonged Embryo Culturing

In 48 couples—39 with OAT and 9 normozoospermics with more than one previous unsuccessful classical IVF attempt—ICSI-derived embryos were

cultured for 4 days in multiwall petri dish: first 2 days in 0.5 mL of Universal IVF medium (Medi-Cult, Denmark) followed by M3 medium (Medi-Cult, Denmark) to develop to the *morula* or *blastocyst* stage. Morula was defined as embryo with 16–32 cells and blastocyst as embryo with trophectoderm, inner cell mass, and cavity *(*from compact to expanded blastocyst*)*. Each day the embryos were transferred into 0.5 mL of fresh medium. On Day 4 the embryos were observed and divided into 5 groups according to the developmental stage (2–6-cell arrested embryos, 7–10-cell embryos, >10-cell embryos, morulae, and blastocysts) and at most 2 of the most developed embryos were transferred into the uterus. Arrested embryos were not transferred. Supernumerary embryos of good developmental potential were cryopreserved for later use.

Luteal Support and Follow-Up of Pregnancy

On the day of embryo transfer (ET), the luteal phase support was started using dydrogesterone per os, 300 mg/day (Dabroston: BELUPA, Croatia). Biochemical pregnancy was confirmed by the serum beta-HCG determination 15 days after ET. Clinical pregnancy was confirmed by US demonstration of at least one gestational sac at 7 weeks of pregnancy. An abortion was considered preclinical when HCG levels did not reach 1000 mIU/mL and no gestational sac was detected on US.

Statistical Analysis

For statistical analysis the SPSS program (SPSS Inc., Chicago, IL, USA) was used.

In couples with embryos cultured for 2 days, the two ICSI groups were compared: percentages of 2-, 3-, 4-, 5-, and 6-cell embryos, percentage of heavily fragmented embryos with at least 10% of fragmentation, and percentage of bad quality embryos (Type I) by means of Wilcoxon nonparametric test. Sperm single-stranded DNA and classical sperm parameters were correlated with the number of heavily fragmented embryos by Spearman correlation coefficient.

In couples with embryos cultured for 4 days, Spearman's rank correlation coefficient was used to evaluate the relationship between the percentage of spermatozoa with single-stranded DNA, classical sperm parameters, and number of embryos at each developmental stage, including arrested embryos (2–6-cell arrested embryos, 7–10-cell embryos, >10-cell embryos, morulae, and blastocysts). At each developmental stage, the differences in number of embryos between the two ICSI groups were evaluated by Mann–Whitney U test.

In all couples the *fertilization rate* (no. of fertilized oocytes per all oocytes), *embryonic implantation rate* (no. of implanted embryos per transferred embryos), *pregnancy rate* (no. of pregnancies per ET), and the *outcomes of pregnancy*—live-birth rate and spontaneous abortion rate—of both ICSI groups were compared by means of Wilcoxon nonparametric test. Statistical significance was set at $P < 0.05$.

RESULTS

Level of Sperm Single-Stranded DNA

The level of sperm single-stranded DNA was increased in 119 of the 183 (65%) infertile patients. After sperm preparation the mean percentage of spermatozoa with single-stranded DNA was $65 \pm 25\%$ (range 0–100%). In 14 patients (8%) we did not observe any spermatozoa with sperm single-stranded DNA, whereas in 21 patients (11%) all spermatozoa had single-stranded DNA.

Between the couples with normal percentage of spermatozoa with single-stranded DNA ($n = 64$) and the couples with increased percentage of spermatozoa with single-stranded DNA ($n = 119$), there were no statistical differences in clinical parameters (male age, female age, tubal factor of infertility, endometriosis, and hormonal disturbances in the female partner), as shown in Table I.

Table I. Male and Female Clinical Parameters in Two ICSI Groups According to the Percentage of Spermatozoa with Single-Stranded DNA

ICSI group	Spermatozoa with single-stranded DNA	Mean no. of obtained oocytes	Mean male age (years)	Mean female age (years)	No. of females with tubal infertility (%)	No. of females with endometriosis (%)	No. of females with hormonal disturbances (%)
I ($n = 64$)	0–55%	6 ± 4	$37 \pm 6^{*a}$	$34 \pm 5^{*c}$	14 (22)^{*e}	6 (9)^{*g}	12 (19)^{*i}
II ($n = 119$)	56–100%	6 ± 4	$34 \pm 5^{*b}$	$32 \pm 5^{*d}$	22 (18)^{*f}	5 (4)^{*h}	17 (14)^{*j}
Total ($n = 183$)	$65 \pm 25\%$	6 ± 4	35 ± 6	33 ± 5	36 (20)	11 (6)	29 (16)

$^{*a,b/c,d/e,f/g,h/i,j}$ Statistically nonsignificant differences by means of Wilcoxon test.

Table II. ICSI Procedure in 183 Couples According to the Percentage of Spermatozoa with Single-Stranded DNA in Male Partner

ICSI group	Spermatozoa with single-stranded DNA	All obtained oocytes	Mean no. of oocytes/couple	Degenerated oocytes	Immature (GV, MI) oocytes	Microinjected oocytes	Fertilized oocytes (%)
I ($n = 64$)	0–55%	394	6 ± 4	44	59	291	177 (61)*[a]
II ($n = 119$)	56–100%	772	6 ± 4	69	74	629	293 (46)*[b]
Total ($n = 183$)	$65 \pm 25\%$	1166	6 ± 4	113	133	920	470 (51)

*[a,b] Statistical by significant difference as means by Wilcoxon test ($P < 0.05$).

Outcome of ICSI

One hundred and eighty-three couples underwent ICSI. We obtained 1166 oocytes (mean 6 ± 4 per couple); 920 oocytes were microinjected and the remaining were either immature (133 oocytes) or degenerated (113 oocytes). Of the 920 microinjected oocytes 470 (51%) were fertilized and 411 fertilized oocytes developed to the embryo stage. The outcome of ICSI (Table II) shows lower fertilization rate in the group of men with increased level of sperm single-stranded DNA than in the normal group (46 vs. 61%; $P < 0.05$).

All 411 ICSI-derived embryos were included in this study. Of the 183 ICSI cycles 160 (87%) resulted in ET and 40 pregnancies were achieved, i.e. a 25% pregnancy rate per ET or a 22% pregnancy rate per cycle.

Embryo Quality 2 Days After ICSI

In 135 couples 256 embryos were scored 2 days after ICSI. Between the ICSI group I (<56% sperm single-stranded DNA; $n = 40$) and the ICSI group II (≥56% sperm single-stranded DNA; $n = 95$), there were no statistically significant differences in the percentages of 2-, 3-, 4-, 5-, and 6-cell embryos.

In the ICSI group II the percentage of heavily fragmented embryos was significantly higher than in the ICSI group I (41 vs. 23%, $P < 0.05$). In all couples there was a significant positive correlation between the number of heavily fragmented embryos and the percentage of spermatozoa with single-stranded DNA in male partner ($r = 0.211$, $P < 0.05$). Besides, in patients with increased percentage of spermatozoa with single-stranded DNA there was a significantly higher percentage of bad quality (Type I) embryos than in the patients with a normal percentage of spermatozoa with single-stranded DNA (9 vs. 3%, $P < 0.05$; Table III).

There was no correlation between the percentage of heavily fragmented embryos and classical parameters of sperm quality in the male partner: sperm total number ($r = 0.044$, $P = 0.664$), rapid progressive motility ($r = 0.030$, $P = 0.766$), morphology ($r = 0.098$, $P = 0.335$), vitality ($r = 0.037$, $P = 0.839$), and seminal leukocytes ($r = 0.045$, $P = 0.657$).

Developmental Stage of Embryos 4 Days After ICSI

In 48 couples 155 embryos were cultured for 4 days after ICSI. Of the 155 embryos 31 (20%) arrested at the 2–6-cell stage and did not develop further in spite of prolonged culturing.

In all 48 couples there was a statistically significant positive correlation between the percentage of spermatozoa with single-stranded DNA in the male partner and number of arrested embryos after ICSI, but there were no correlations between the number of arrested embryos and classical sperm parameters described above (Table IV). In the ICSI group II there was a significantly higher percentage of embryos arrested at the stage of 2–6 cells, whereas the percentage of morulae and blastocysts was approximately the same as in the ICSI group I (Table V).

Table III. Quality of 256 Low-Cell Embryos in 135 Couples Two Days After ICSI According to the Percentage of Spermatozoa with Single-Stranded DNA

ICSI group	Excellent embryos, Type IV (%)	Good embryos, Type III (%)	Fair embryos, Type II (%)	Bad embryos, Type I (%)
I ($n = 40$)	71 (78)	15 (16)	2 (2)	3 (3)*[a]
II ($n = 95$)	95 (58)	43 (26)	12 (7)	15 (9)*[b]
Total ($n = 135$)	166 (65)	58 (23)	14 (5)	18 (7)

*[a,b] Statistical by significant difference as means by Wilcoxon test ($P < 0.05$).

Table IV. Correlations of Percentage of Spermatozoa with Single-Stranded DNA and Classical Sperm Parameters with the Number of Embryos ($n = 117$) at Different Developmental Stages Cultured for 4 Days After ICSI

	r (P)				
Sperm parameters	2–6-cells arrested embryos ($n = 31$)	7–10-cells ($n = 23$)	>10 cells ($n = 8$)	Morulae ($n = 85$)	Blastocysts ($n = 5$)
% spz with single-stranded DNA	0.235 (<0.05) *S	−0.308 (<0.05) *S	0.057 (0.702)	0.007 (0.963)	0.083 (0.576)
Sperm total number ($\times 10^6$ spz)	0.186 (0.211)	0.109 (0.466)	−0.292 (0.460)	−0.185 (0.214)	0.070 (0.642)
Motility (%)	0.279 (0.067)	0.045 (0.764)	−0.131 (0.379)	−0.084 (0.573)	0.074 (0.622)
Morphology (%)	0.175 (0.246)	−0.036 (0.813)	−0.266 (0.074)	−0.076 (0.614)	0.110 (0.466)
Vitality (%)	0.259 (0.283)	−0.071 (0.774)	−0.388 (0.101)	0.135 (0.583)	0.302 (0.209)
Seminal leukocytes	−0.091 (0.549)	−0.266 (0.074)	−0.238 (0.112)	0.145 (0.337)	0.145 (0.336)

Note. spz: Spermatozoa.
*S: statistically significant correlation detected by Spearman's rank correlation coefficient ($P < 0.05$).

Embryo Transfer

In 183 patients 411 embryos (256 low-cell embryos on Day 2, and 155 more-cell embryos on Day 4) were obtained by ICSI. Three hundred and nine (75%) embryos were transferred into the uterus, 74 (18%) embryos were cryopreserved for later use, and 28 (7%) embryos were not transferred because of fragmentation (>50%) or arrest at low-cell stages in spite of prolonged culturing.

In patients with an increased percentage of spermatozoa with single-stranded DNA, a significantly higher percentage of embryos was not transferred or cryopreserved because of bad biological quality than in the patients with normal percentage of spermatozoa with single-stranded DNA (10 vs. 1%, $P < 0.05$).

Pregnancies After ICSI

In the patients with increased percentage of spermatozoa with single-stranded DNA the ET rate was significantly lower than in the patients with normal percentage of spermatozoa with single-stranded DNA (86 vs. 95%, $P < 0.05$). We did not find any correlation between the sperm single-stranded DNA in male partner, embryonic implantation rate ($r = 0.112$, $P = 0.653$), and pregnancy rate ($r = 0.012$, $P = 0.871$) after ICSI. In patients with normal and increased percentages of spermatozoa with single-stranded DNA the embryonic implantation rate and the pregnancy rate were similar (12 vs. 14% and 23 vs. 25%, respectively).

There was no relation between the percentage of spermatozoa with single-stranded DNA and spontaneous abortion. In patients with normal and increased sperm single-stranded DNA the abortion rate was approximately the same (21 vs. 15%).

In patients with normal and in those with increased percentages of spermatozoa with single-stranded DNA, the live-birth rates were approximately the same (79 vs. 81%).

In the studied population there were no correlations between the live-birth rate and classical parameters of sperm quality in male partner: sperm total number ($r = 0.012$, $P = 0.873$), rapid progressive motility ($r = -0.032$, $P = 0.681$), morphology ($r = 0.004$, $P = 0.964$) and vitality ($r = 0.122$, $P = 0.359$).

However, in patients with 0% of spermatozoa with single-stranded DNA a higher percentage of fertilized oocytes, and higher ET and pregnancy rates, were registered than in patients with 100% of spermatozoa with single-stranded DNA and in the entire study population (Table VI). In patients with 0% of sperm single-stranded DNA there were no spontaneous abortions. On the other hand, in patients with

Table V. Number of Embryos and Percentages of Embryos That Reached Different Developmental Stages on Day 4 After ICSI in 2 ICSI Groups According to the Percentage of Spermatozoa with Single-Stranded DNA

ICSI group	All embryos	2–6-cells arrested embryos (%)	7–10-cells (%)	>10 cells (%)	Morulae (%)	Blastocysts (%)	Degenerated embryos
I ($n = 19$)	62	6 (10)*[a]	12 (19)	6 (10)	35 (56)	2 (3)	1 (2)
II ($n = 29$)	93	25 (27)*[b]	11 (12)	2 (2)	50 (54)	3 (3)	2 (2)
Total ($n = 48$)	155	31 (20)	23 (15)	8 (5)	85 (55)	5 (3)	3 (2)

*[a,b] Statistically significant difference by means of Mann–Whitney U test ($P < 0.05$).

Table VI. Clinical Results in Men with 0% ($n = 11$) and 100% of Spermatozoa with Single-Stranded DNA ($n = 22$) in Comparison with Entire Study Population ($n = 183$)

	0%	100%	Entire study population
Cycles	11	22	183
Microinjected oocytes	75	109	920
Fertilized oocytes (%)	55 (73)*[a]	45 (41)*[b]	470 (51)*[c]
Embryo transfers (%)	11 (100)**[a]	15 (68)**[b]	160 (87)**[c]
Pregnancies	5	4	40
Pregnancies per ET	45%***[a]	27%***[b]	25%***[c]
Spontaneous abortions (%)	0 (0)****[a]	2 (50)****[b]	7 (17)****[c]

*[a,b/a,c] and **[a,b/a,c/b,c] and ***[a,b/a,c/b,c] and ****[a,b/a,c/b,c] Statistically significant differences by means of Wilcoxon nonparametric test ($P < 0.05$).

100% of spermatozoa with single-stranded DNA the abortion rate was higher than in the entire study population (Table VI).

DISCUSSION

Increased sperm single-stranded DNA, detected by AO staining, was observed in majority (65%) of infertile patients with OAT and in men with unsuccessful conventional IVF attempts. In patients with increased level of sperm single-stranded DNA we found a lower fertilization rate after ICSI. Besides, in men with increased percentage of spermatozoa with single-stranded DNA, we observed more fragmented embryos and embryos that arrested in spite of prolonged culturing. In the group of men with increased percentage of sperm single-stranded DNA there were significantly more embryos that were not transferred or cryopreserved because of their worse biological quality.

In this study, we tried to evaluate the diagnostic and prognostic value of AO test in terms of fertilization and embryonic quality after ICSI. Sperm single-stranded DNA was evaluated before ICSI on the basis of our former experience that in infertile men an increased percentage of sperm single-stranded DNA detected by acridine orange staining was a permanent situation, since this elevated percentage persisted on subsequent evaluations. If in clinical practice increased sperm single-stranded DNA is evaluated before ICSI, this will enable introduction of treatment to decrease or to prevent sperm single-stranded DNA and to improve the results of ICSI.

Embryos were cultured for 2 days in Universal IVF medium or for 4 days in M3 medium (Medi-Cult, Denmark). On Day 4 about 3% of embryos reached the blastocyst stage and more than 50% of embryos reached the morula stage. After prolonged culturing we transferred embryos on Day 4 to be certain not to miss the uterine implantation window. This was the first step to prolonged culturing in our in vitro fertilization program, which was then followed by embryo transfer on Day 5.

In patients with increased level of sperm single-stranded DNA we observed a higher percentage of embryos arrested at the 2–6-cell stage that did not cleave further in spite of prolonged culturing, but there was the same percentage of embryos that reached the blastocyst stage as in the patients with normal percentage of sperm with single-stranded DNA. We hypothesize that sperm single-stranded DNA, detected by AO test, affects negatively the developmental potential of ICSI-derived embryos, causing developmental retardation at early stages of 2–6 cells. An increased number of embryos arrested at the 2–6 cell stage in the increased sperm single-stranded DNA group is likely to be related to the switch from maternal to embryonic genome at the 4–8 cell stage (38). Embryos arising from fertilization with sperm with single-stranded DNA may not establish normal embryonic genome, but prolonged culturing enables the selection of normal embryos. In this study the percentage of arrested embryos on Day 4 (20%) was approximately the same as in other studies (39).

In our study, however, there was no correlation between classical sperm parameters and developmental potential of ICSI-derived embryos. Arrested embryos and fragmented embryos (over 30% of the volume) have a small chance of implantation and have high rates of chromosome abnormalities (24,40). Embryo fragmentation might be associated with programmed cell death, apoptosis (41,42).

Some other studies show inferior quality of ICSI-derived embryos. Sakkas and coworkers (23) showed that ICSI-derived embryos had significantly lower rates of developing to the blastocyst stage when compared with those developed after conventional IVF. Similarly, Plachot (24) found that the rate of development of supernumerary embryos to the blastocyst stage was lower after ICSI (18%) than after IVF (33%). In some IVF centers higher proportions of lower-cell embryos after ICSI were observed (22,23,25–28). Worse embryo quality and developmental potential after ICSI might be related to bad semen quality (17–21,31), technical problems associated with the ICSI procedure itself (24), or chromatin anomalies. Sakkas reported that sperm

chromatin anomalies and DNA damages could influence decondensation after ICSI (43). In our study worse embryo development after ICSI was due to sperm single-stranded DNA and not to classical sperm parameters.

As spermatozoa are thought to lack the endogenous DNA repair mechanisms of somatic cells, increased levels of impaired DNA integrity have a high likelihood of transmission to the zygote (44). Even if some lesions can be repaired after fertilization has occurred, at least in zona-free hamster oocytes, it seems likely that this is a major source of the paternal contribution to embryonic mortality. If DNA integrity abnormalities are above normal there is a potential risk of embryonic mortality (44).

We did not observe any significant differences in pregnancy rates between the groups of patients with increased and those with normal percentages of spermatozoa with single-stranded DNA. The two groups did not differ in age, incidence of tubal factor, endometriosis, or hormonal disturbances (polycystic ovaries) in the female partner; and in the male, age. In this study low-cell embryos of bad quality with more than 50% of fragmentation were not transferred into the uterus. After prolonged culturing, arrested embryos were not transferred. If fragmented embryos of bad quality and arrested embryos had been transferred, the pregnancy rate might have been lower (39). Also, the embryonic quality is not the only factor affecting the implantation rate. Embryonic implantation is a complex process involving several maternal factors. Additionally, the increased level of sperm single-stranded DNA in infertile men resulted in a fewer embryos suitable for transfer or cryopreservation. However, pregnancies achieved by ICSI, regardless of the level of sperm single-stranded DNA, resulted in approximately the same live-birth rates. On the other hand, in men with 0% of spermatozoa with single-stranded DNA the pregnancy rate was significantly higher than in men with 100% of spermatozoa with single-stranded DNA and in the entire study population.

In men with 100% sperm single-stranded DNA, the spontaneous abortion rate was higher than in men with 0% of spermatozoa with single-stranded DNA and in the entire study population. Similarly, Ibrahim (45) observed increased impairment of sperm DNA integrity in couples with repeated spontaneous abortions. Evenson (46) and Larson (47) have found that sperm single-stranded DNA is predictive for pregnancy loss. Therefore, this might be related to reduced embryo quality found in our study. Furthermore, Larson (47) has found no significant relationship between sperm WHO parameters, fertilization, or embryo development.

In clinical practice we are to select sperm with fully condensed nuclei of normal chromatin structure to minimize the negative effects of sperm single-stranded DNA. Normal DNA integrity of human spermatozoa may be selected by semen preparation before ICSI (48,49). Thus, it might become possible to improve the results of ICSI and avoid the risk of embryonic mortality in patients with increased percentage of sperm single-stranded DNA.

We may conclude that the level of sperm single-stranded DNA provides additional data on sperm functional capacity in terms of fertilization and quality of ICSI-derived embryos. Increased level of sperm single-stranded DNA in infertile men results in fewer embryos suitable for transfer and cryopreservation. AO test performed before ICSI is of prognostic value for the outcome of ICSI.

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