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Fertility of ejaculated and testicular megalohead spermatozoa with intracytoplasmic sperm injection

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In this study the fertility and outcome of intracytoplasmic sperm injection (ICSI) using megalohead spermatozoa from the ejaculates and testicles was evaluated. Seventeen males with megalohead and pinhead sperm forms in their ejaculate were studied in 22 cycles. A high number of sperm heads without tails and abundant round spermatid forms were commonly observed. Round-headed spermatozoa were seldom accompanied by these severely abnormal spermatozoa. The majority of megalohead spermatozoa were observed to have multiple tails, were predominant in the sample, and were used for ICSI. Ejaculated megalohead spermatozoa were used for ICSI in 15 cycles, while testicular spermatozoa were used in seven cycles where there were no vital spermatozoa or spermatozoa of low vitality in the ejaculate. The same abnormal morphology was observed in the testicles as in the ejaculated spermatozoa in the same males. Mean (± SD) low motility 4.7 ± 5.6% and sperm count ($3.8 \pm 4.19 \times 10^6$) were common findings in these severely teratozoospermic patients. A low fertilization rate (43.2%) was achieved by using megalohead sperm forms (group I, $n = 17$) in comparison with the control group (60.2%) which had zero normal sperm morphology according to strict criteria (group II, $n = 30$) ($P < 0.01$). Furthermore, a low pregnancy rate (9.1%) was obtained in the megalohead sperm group in comparison with the control group (40%) ($P < 0.05$). Low fertilization and pregnancy rates may be due to a high incidence of chromosomal abnormalities from severely defective spermatozoa in the ejaculate. Couples should be counselled and warned about possible low fertilization and pregnancy rates with ICSI when only pinhead and megalohead forms with a high number of sperm heads without tails are present in the ejaculate.

Key words: intracytoplasmic sperm injection/megalohead/pinhead spermatozoa

Introduction

Sperm motility, concentration and percentage of normal morphology are the criteria for semen quality. Although abnormalities of these parameters strongly affect the fertilization rate, intracytoplasmic sperm injection (ICSI) has been offered as a successful treatment mode in cases of severe male infertility. It has been suggested that spermatozoa with severe morphological defects do not affect the fertilization rate once ICSI has been performed. Moreover, high fertilization and pregnancy rates after ICSI treatment with ejaculated spermatozoa are not found to be related to sperm concentration and morphology in the semen sample on the day of oocyte retrieval. It has been stated that the fertilization rate after ICSI is only dependent on the injection of a motile or immotile spermatozoon (Nagy *et al*., 1995). However, in rare cases with severe abnormal spermatozoa forms such as tail stump and round-headed acrosomeless spermatozoa, the fertilization and pregnancy rates may be low. In this study, the fertility and outcome of ICSI using megalohead spermatozoa from ejaculates and testicles was evaluated.

Materials and methods

Patients

This study was undertaken between April 1996 and March 1997 in Sevgi Hospital Assisted Reproductive Technology Unit.

Seventeen patients with 22 cycles (group I, $n = 17$) having megalohead and pinhead spermatozoa, and a high number of sperm heads without tails in their ejaculates, were included in this study after two semen analyses were performed 2 months apart. A zero normal morphology group (group II, $n = 30$) was selected as a control group since a normal sperm morphology group would provide a more drastic comparison.

Semen analysis

Semen samples were obtained after 3–4 days of sexual abstinence. Samples were allowed to liquefy for at least 20 min at 37°C before analysis. Sperm concentration and motility were evaluated according to the recommendations of the World Health Organization (1992) and sperm morphology was evaluated by strict criteria (Kruger *et al*., 1996).

Different forms of megalohead and pinhead spermatozoa and spermatids are shown in Figures 1–3. An electron microscopic evaluation was performed in one case with only pinhead and megalohead sperm in the ejaculate. Figures 4 and 5 show the electron microscopic appearance of spermatozoa with amorphous head and pinheads. The viability of immotile spermatozoa was also evaluated using an eosin-Y test (WHO, 1992). Semen was washed in SpermPrep™ (Scandinavian IVF Science AB, Gothenburg, Sweden) by centrifuging for 10 min at 1800 *g*. The pellet was placed on a single-layer PureSperm™ (Scandinavian IVF Science AB) gradient (90%). The 90% fraction was washed twice with SpermPrep™ for

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Figure 1. A double-tailed spermatozoon (small arrows) with well-defined attached heads (large arrow).



Figure 2. A non-vital, multi-tailed megalohead spermatozoon defined with an eosin-Y test.

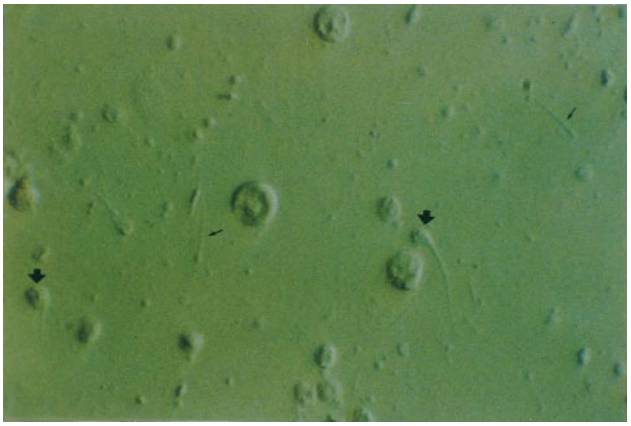


Figure 3. Megalohead and pinhead forms (large and small arrows) of spermatozoa in the same ejaculated sample.

5 min at 1800 *g* and the pellet placed in an incubator until used for microinjection.

Testicular tissue sampling

The testicular biopsy group included patients with spermatozoa of zero or low vitality found in the ejaculate in the megalohead sperm group (group I), and whose testicular sperm were found to have the same abnormal morphology.

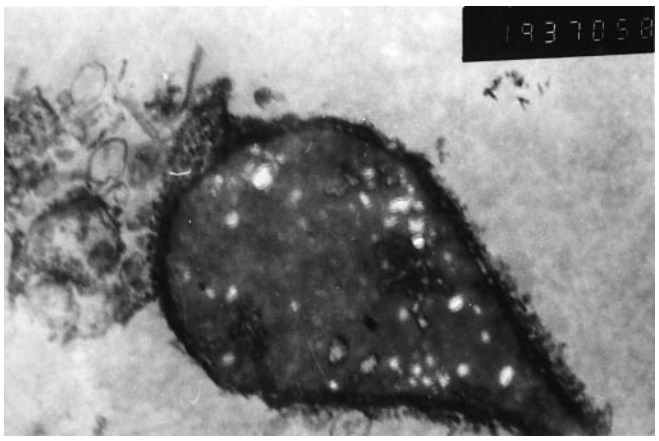


Figure 4. A megalohead spermatozoon with intranuclear vacuoles (large arrows) and abnormal pattern of chromatin condensation. Note the hypoplasia (small arrows) and fragility of acrosome, indicating the existence of abnormal remodelling patterns during spermiogenesis. (Original magnification, ×10 000).

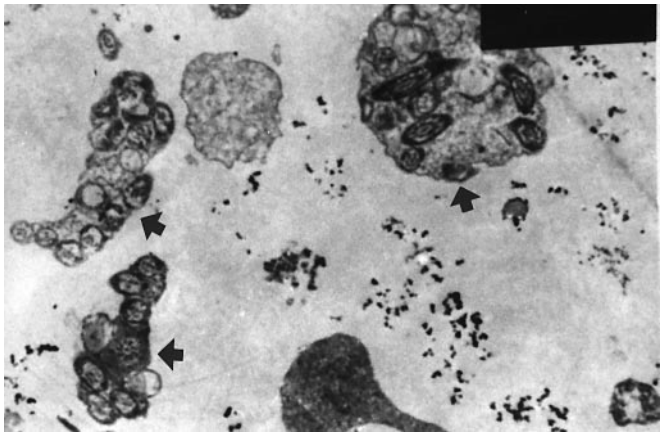


Figure 5. Decapitated sperm flagella (small arrows) with cytoplasmic droplet (large arrow) surrounding their cranial segment. (Original magnification, ×29 000)

Testicular tissue samples were obtained either by needle aspiration or open biopsy. The testicular tissue was transferred to the laboratory in a 2 ml culture medium (IVF-50; Scandinavian IVF Science AB) and divided progressively using two sterile forceps under a stereomicroscope in order to extract the cellular elements of the tissue. The suspension was gently layered over a 0.5 ml single gradient (90%) PureSperm™ fraction. More than one Falcon tube was prepared in order to supply sperm preparations from the suspension. The pellet was gently aspirated after 20 min centrifugation (300 *g*), and then washed and re-centrifuged twice (5 min, 1800 *g*) in 8 ml of SpermPrep™. The final pellet was resuspended with 50 µl of culture medium until used for microinjection. If no spermatozoon was observed during the first evaluation of the sample, the suspension was centrifuged for 10 min at 2500 *g*, the supernatant discarded, and the pellet resuspended in 0.2 ml culture medium. The presence of spermatozoa was evaluated under an inverted microscope.

Ovarian stimulation

Controlled ovarian hyperstimulation was executed in 17 females with 22 cycles. A gonadotrophin releasing hormone analogue (Supracur; Hoechst, Frankfurt, Germany) was given as a nasal spray, starting in

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the luteal phase, and continued for 14 days until sufficient pituitary downregulation was achieved. Follicular development was then stimulated with an injection of follicle stimulating hormone (FSH; Metrodin, Serono, Rome, Italy) in a step-down manner. Ovulation was induced by 10 000 IU human chorionic gonadotrophin (HCG; Pregnyl, Organon, Istanbul, Turkey).

Injection and oocyte aspiration were performed 36 h later under guidance using vaginal ultrasound.

Oocyte preparation

After oocyte retrieval, the cumulus cells were dissected by using 25 G needles. The remaining cumulus cells and corona radiata were removed by a brief exposure to culture medium containing 80 IU/ml hyaluronidase (Type 8, specific activity 320 IU/ml; Sigma Chemical Co., St Louis, MO, USA). The oocytes were completely denuded by aspiration of the cell complex into and out of a hand-drawn glass pipette, rinsed several times in culture medium, and assessed under an inverted microscope to determine their stage of maturity. Oocytes were incubated in the culture medium at 37°C in an atmosphere of 5% CO_2 in air and covered by paraffin oil. Only metaphase II oocytes were microinjected.

ICSI procedure

A single living and motile spermatozoon, if present, was injected into an oocyte as described previously (Kahraman *et al*., 1996). The injected oocytes were incubated for fertilization and further cleavage.

Further evaluation of injected oocytes and establishment of pregnancy

Oocytes were observed for the presence of pronuclei 16–18 h after ICSI. Fertilization was assessed as normal when two clearly distinct pronuclei containing nucleoli were present. Cleavage of the fertilized oocytes was assessed at 24 h after fertilization. The embryos were evaluated according to blastomere size equality and the relative proportion of anucleate fragments. A maximum of four embryos was transferred in exceptional cases when all the available embryos had >50% fragmentation. Embryo transfer was performed routinely on the third day after a selective assisted hatching procedure with acidified Tyrode's solution. An assisted hatching procedure was applied when the age of the female was >35 years or when the basal FSH concentration was found to be >10 mIU/ml.

All patients underwent an initial test for serum oestradiol and progesterone at 8–12 days after embryo transfer. Pregnancy was confirmed when serum HCG concentrations were rising on at least two separate occasions between the 12th and 14th days. Clinical pregnancy was diagnosed by ultrasonography at 7 weeks of pregnancy (a prenatal diagnosis was performed by amniocentesis at 16 weeks of pregnancy). All couples were counselled about a possible risk of chromosomal abnormality and also informed about a prospective follow-up study of the children born after ICSI.

Statistical analysis

A statistical analysis was performed by chi-square and Fisher Exact tests. Significance was defined as $P < 0.05$.

Results

The mean (± SD) period of infertility was 11.5 ± 4.0 and 9.3 ± 4.8 years respectively in the two groups ($P > 0.05$). The mean (± SD) age of the females was 31.5 ± 2.99 and 31.5 ± 3.96 years respectively ($P > 0.05$). Interestingly, more than half of the male patients had a history of male family members with infertility. The patients did not belong to any particular ethnic group or geographical area. Cytogenetic analysis was performed in 10 out of 17 males in group I. Cytogenetic studies were performed on GTG-banded chromosomes using standard techniques after culture of lymphocytes obtained from peripheral blood. The results were all found to be normal. Ejaculated spermatozoa were used in 12 cases with 15 cycles. In the remaining five cases, a testicular biopsy or aspiration was performed due to a low number of vital spermatozoa in the ejaculate in seven cycles. The testicular samples showed the same abnormal sperm morphology. The mean (± SD) vitality of abnormal spermatozoa in group I was found to be 21.9 ± 12.4%, with rates of megalohead, pinhead and globozoospermic sperm being 68.5%, 20.5% and 10.8%, respectively. The mean concentration of spermatozoa in groups I and II was 3.8 ± 3.68×10^6 and 25.8 ± 30.3×10^6, and mean motility was 4.7 ± 5.59% and 18.3 ± 12.2%, respectively ($P < 0.01$). In five couples with megalohead spermatozoa, the ICSI procedure was repeated twice and the same severe morphological defect was observed both times, highlighting a permanent sperm abnormality. The same abnormal morphology was found in repeated testicular sperm extraction and ICSI cycles.

Table I. Fertilization characteristics of injected oocytes in the megalohead sperm group (I) and the control group (II)

	Group I	Group II	*t*	*P*
Injected oocyte (%)	192/247 (77.7)	420/493 (85.1)	5.89	<0.01
Fertilization with 2PN (%)	83/192 (43.2)	253/420 (60.2)	4.7	<0.01

PN = pronuclei.

Table II. Distribution of embryo grades according to the megalohead sperm group (I) and the control group (II)

Embryo grade	Group I (n = 64)	Group II (n = 122)	*P*
Grade 1 (%)	25 (39.1)	68 (55.7)	>0.05
Grade 2 (%)	18 (28.1)	30 (24.5)	>0.05
Grade 3 (%)	10 (15.6)	17 (13.9)	>0.05
Grade 4 (%)	2 (3.1)	7 (5.7)	>0.05

A total of 247 oocytes were retrieved of which 192 were injected in the study group (Table I); numbers of injected oocytes and fertilized oocytes with two pronuclei (2PN) are also shown in Table I. The fertilization rate was 43.2% and a high rate of abnormal fertilization (12%) was observed in group I; 4% of oocytes were degenerate. The distribution of embryo grades in the megalohead spermatozoa and control groups is shown in Table II. In three cycles, embryo transfer could not be performed due to a total fertilization failure and, from a total of 64 embryos, 55 were transferred. An average of 3.4 embryos was transferred in 19 cycles in group I. The grade of embryos was found to be similar in the two groups (NS). A total of two pregnancies was achieved in 22 cycles (9.1%) and resulted in two deliveries, including one singleton offspring and one set of twins. The fertilization rate (43.2%) and pregnancy rate (9.1%) were found to be low in group I,

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Table III. Outcome of intracytoplasmic sperm injection in the megalohead sperm group (I) and the control group (II)

	Group I	Group II	*t*	*P*
Pregnancy per cycle (%)	2/22 (9.1)	14/35 (40.0)	4.71	<0.05
Pregnancy per embryo transfer (%)	2/19 (10.5)	9/35 (25.7)	25.7	<0.05

and the difference was significant in comparison with group II (pregnancy per cycle, 40%, pregnancy per embryo transfer, 25.7%; $P < 0.05$) (Table III).

Discussion

This study was designed to evaluate the fertility and outcome of ICSI using only megalohead ejaculated and testicular spermatozoa. Pinhead spermatozoa were very often found to be accompanied by megalohead forms, while globozoospermic cells were rarely seen among these abnormal spermatozoa. The most significant morphological signs were the high number of sperm heads without tails, and abundant immature spermatids in the ejaculate or in the testicular sperm extraction solution. Interestingly, many abnormal forms were present in the same samples, such as spermatids, megalohead and pinhead spermatozoa, a high number of sperm heads without tails and, more rarely, globozoospermic spermatozoa.

According to Fawcett (1975), an anomaly during the early and late phase of the spermiogenesis is highly likely in these cases. Perotti and Giorgia (1981) and Chemes *et al*. (1987a) have found that the connecting piece of spermatozoa evolves independently from the nucleus in immature spermatids. These findings indicate that there is a defect in the mechanism of migration or positioning of the flagellum in relation to the nucleus. Anucleate forms may not originate by a late separation of normally developed heads and tails as a consequence of contact with the nucleus. Therefore, Chemes *et al*. (1987b) proposed that anucleate spermatozoa result either from a defect in the migration of the tail to the caudal pole of the nucleus or as a consequence of some nuclear defect that interferes with the formation of the implantation fossa and basal plate. In either case, the nucleus and the flagellum would develop separately within the spermatid cytoplasm and would become separated at the end of spermiogenesis. An abnormal fragility of the head connecting piece attachment might be the reason, as suggested previously by Fawcett *et al*. (1975). The formation of the neck structures begins very early, before the centrioles are established. Possibly, the heads are phagocytosed by Sertoli cells, epididymal cells or luminal macrophages, but the tails are not. This is a chronic condition and responsible for the patients' primary sterility, as has been shown in five cases in our study. In the patients' sample many tails without heads (pinhead) or many heads without tails were observed in the same ejaculated or testicular samples.

In cases with absolute teratozoospermia (100% morphologically abnormal spermatozoa) with abnormal head morphology, although high fertilization and cleavage rates were achieved, the implantation and ongoing pregnancy rates were low with a high incidence of early pregnancy loss (Tasdemir *et al*., 1997).

A lower fertilization with 2PN was observed in the megalohead group than in the control group, and the difference was found to be statistically significant ($P < 0.01$).

In't Veld *et al*. (1997) reported a high rate of diploidy and triploidy of the autosomes in addition to aneuploidy for the sex chromosomes in a case with only macrocephalic sperm heads in the ejaculate. ICSI using abnormal spermatozoa and multiple phenotypic abnormalities can result in a low fertilization rate because of a high percentage of chromosomally abnormal spermatozoa.

Idiopathic infertile men have an increased risk of constitutional chromosome aberrations (Chandley *et al*., 1975), interstitial deletions of the Y chromosome and an increased frequency of sperm cells disomic for the sex chromosomes (Moosani *et al*., 1995). In addition, oligozoospermic patients often display increased frequencies of phenotypically abnormal spermatozoa. Very few studies have indicated a possible correlation between the sperm structure and chromosomal constitution (Lee *et al*., 1996; In't Veld *et al*., 1997). The aneuploidy rates for these chromosomes in sperm heads, as estimated by differential staining procedures, appeared to be very high (range 1–5%). The incidence of structural chromosome aberrations was approximately 4-fold higher in spermatozoa with amorphous heads than in those with morphologically normal heads. It is possible that abnormal sperm head morphology reflects abnormality in spermatogenesis. This is manifested by embryos with a low potential for establishing a normal pregnancy. The use of in-situ hybridization to evaluate sperm nuclei has allowed more reliable results to be obtained (Joseph *et al*., 1984). In this study, by using chromosome-specific probes on human mitotic and meiotic cells, the frequency of aneuploidy in mature sperm nuclei was determined in three patients, all of whom showed a high diploidy incidence with the fluorescein isothiocyanate hybridization (FISH) procedure.

Controversy exists as to whether sperm phenotype abnormalities are indicative of genotype abnormalities (Engel *et al*., 1996). Our study showed that in oligoasthenozoospermic patients with multiple phenotypic abnormalities in their sperm cells, low fertilization and ongoing pregnancies were achieved. At present, the couples are counselled about the use of macrocephal, multi-tailed spermatozoa, as the possibility of fertilization and ongoing pregnancy with these cells is low.

As suggested by In't Veld *et al*. (1997), a FISH procedure should be performed on phenotypically abnormal sperm cells to estimate the genetic risk involved, as aneuploidy could be a possibility. However, to evaluate spermatozoa by using multicolour FISH probes does not allow us to select spermatozoa with a normal chromosomal structure for ICSI. The FISH procedure, however, can be carried out to define the rate of abnormal spermatozoa with diploidy, triploidy or aneuploidy. In terms of implantation and pregnancy, a blastomere biopsy could be beneficial to select normal embryos and to discriminate abnormal embryos.

Although Lee *et al*. (1996) have shown that no increase in chromosome aberrations was found when mouse oocytes were

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injected with human spermatozoa with large heads, the number of cases reported on this subject is very limited. Furthermore, Engel *et al*. (1996) concluded that ICSI would not result in either a significant increase in genetic-based diseases or in an increase in the number of infertile males. If however, mutations of X and Y chromosomal genes should play a major role in male fertility disorders, one could expect a very slight increase in the number of infertile males in future generations.

In three cases in group I, embryo transfer was not possible as no embryo developed. In two cycles, only one embryo was transferred due to a very low fertilization rate, and no pregnancy was achieved. A pregnancy rate per cycle of only 9.1% was obtained in the megalohead sperm group, compared with a rate of 40.0% in the control group ($P < 0.05$). However, with megalohead spermatozoa, both the fertilization and pregnancy rates were found to be lower.

A further explanation for low fertilization may be the immaturity of the spermatozoa used in ICSI in the megalohead group. It seems remarkable that so many round spermatids were present in the sample; however, the presence of multi-tailed megalohead spermatozoa in the sample could be due to missegregation of chromosomes during meiosis.

Couples should be counselled about low rates of fertilization and pregnancy with ICSI when only pinhead and megalohead forms are present in the ejaculate.

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