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Does the presence of nuclear vacuoles in human sperm selected for ICSI affect pregnancy outcome?

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BACKGROUND: To verify whether or not microinjection of sperm with a normal nuclear shape but large vacuoles affects IVF–ICSI pregnancy outcome. METHODS: A comparative study testing IVF outcome parameters of IVF–ICSI, based on morphological selection of spermatozoa with normal nuclei against those based on microinjection of sperm with a normal nuclear shape but large vacuoles. An experimental group, including 28 IVF–ICSI cycles, where only embryos obtained from microinjection of spermatozoa with a normal nuclear shape but large vacuoles were transferred, was matched with a control group, including 28 IVF–ICSI cycles, where only embryos obtained from microinjection of spermatozoa with a strictly defined morphologically normal nuclear shape and content were transferred. The main outcome was IVF–ICSI pregnancy rate. RESULTS: The experimental group exhibited a significantly lower pregnancy rate per cycle and significantly higher abortion rate per pregnancy compared to the control group (18 versus 50%, and 80 versus 7%, respectively, *P* = 0.01). CONCLUSION: Microinjection of vacuolated sperm appears to reduce the pregnancy rate and appears to be associated with early abortion.

Key words: IVF–ICSI/IVF pregnancy outcome/vacuolated spermatozoa

Introduction

Based on morphological selection of motile spermatozoa (MSOME) (Bartoov *et al*., 2002) ICSI was found to be an effective treatment for male infertility (Bartoov *et al*., 2001, 2003). Microinjection into retrieved oocytes of individually selected spermatozoa with a strictly defined morphologically normal nuclear shape and content resulted in significantly higher pregnancy rates compared to the conventional IVF–ICSI (Bartoov *et al*., 2001, 2003; Junca *et al*., 2004a,b). In our recent publication, we demonstrated that sperm cells with certain nuclear abnormalities may still be able to cause pregnancy following ICSI, based on MSOME (Berkovitz *et al*., 2005). The present study concerns a specific sperm nuclear content malformation: the appearance of large vacuoles. Previous ultramorphological investigation revealed that this sperm malformation has a clear negative association with natural male fertility potential (Bartoov *et al*., 1994; Mundy *et al*., 1994). Thus, in cases where spermatozoa with a normal nuclear shape but large vacuoles were the only ones available, and where the patients agreed to use these spermatozoa for microinjection into the oocytes, we examined the effect of this sperm malformation on ICSI, based on MSOME pregnancy outcome.

The aim of this study was to verify whether microinjection of sperm cells with normal nuclear shape but large vacuoles may affect ICSI outcome.

Materials and methods

Experimental ICSI group

In this case-controlled retrospective analysis, the experimental group was selected from 878 IVF–ICSI cycles, based on MSOME, which fit the following criteria: female partner younger than 40 years; three or more retrieved M-II oocytes in the present IVF–ICSI cycle and ICSI outcome, confirmed 3 months after the microinjection. Twenty-eight IVF–ICSI cycles (3.2%) based on MSOME, where spermatozoa were with a normal nuclear shape but large vacuoles (Figure 1c), were the only ones available for microinjection into the ova, were included in the experimental group. All the participants in this group agreed to perform this compromised sperm selection prior to ICSI by means of a signed, informed consent form.

Control group

Twenty-eight ICSI cycles, based on MSOME, were selected from 878 IVF–ICSI trials, as described above. Only spermatozoa with strictly defined morphologically normal nuclei, including shape and content (Figure 1a), were used for microinjection into the ova, and these were

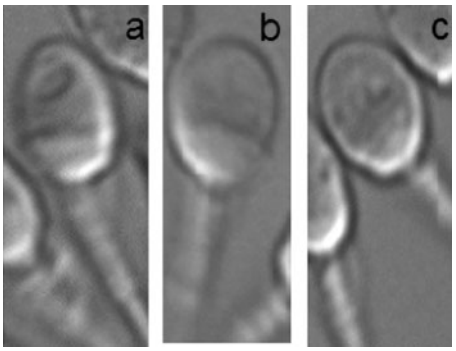

Figure 1. Micrograph of motile spermatozoa with: (**a**) Morphologically normal nuclear shape and content. (**b**) Normal nuclear shape + small nuclear vacuoles. (**c**) Normal nuclear shape + large nuclear vacuoles.

matched with the experimental group, according to the date of ICSI performance (≤3 days) and to female's age. The above 28 cycles were designated as the control group. To avoid any bias, the statistician who made the matching was not allowed to know the pregnancy outcome prior to group selection.

The two matched study groups were statistically similar with regard to the age of the female and male partners (33.4 ± 3.6 versus 33.5 ± 3.9 years, and 37.8 ± 4.3 versus 36.9 ± 6.8 years, respectively).

As mentioned in our previous article (Bartoov *et al*., 2003) the IVF–ICSI treatment, based on MSOME, was approved by the Meir Hospital ethical committee.

Routine preparation of motile sperm fraction

Only freshly ejaculated semen was used in this study. As described in our previous studies, the routine MSOME was performed on the basis of a two-layer Sil-Select density gradient system, which consists of 1-ml upper (low density) and 1-ml lower (high density) layers of saline-coated colloidal silica particles suspended in HEPES-buffered Earle's balanced salt solution (EBSS; Ghent, Belgium). The sperm cells suspension was used for further MSOME preparation (Bartoov *et al*., 2003; Berkovitz *et al*., 2005).

Sperm preparation for individual retrieval based on MSOME

Sperm preparation for individual retrieval based on MSOME was described in detail in our previous studies (Bartoov *et al*., 2003; Berkovitz *et al*., 2005). The sperm cells, aimed for examination, were suspended in a microdroplet and used for individual retrieval by MSOME. For this purpose, the sterile glass bottom dish containing the microdroplet (observation droplet) was placed on a microscopic stage over the top of an Uplan Apo ×100 oil/1.35 objective lens previously covered by a droplet of immersion oil. In this way the motile sperm cells, suspended in the observation droplet, could be examined at high magnification by the inverted microscope (Olympus IX 70, Tokyo, Japan) equipped with Nomarski differential interference contrast optics. The total calculated magnification was ×6600.

MSOME criteria for spermatozoa selected for ICSI

In our previous study (Bartoov *et al*., 2002), we came to the conclusion that the MSOME criteria for normally shaped nuclei are smooth, symmetric and oval configuration, with average length and width limits of 4.75 ± 0.28 and 3.28 ± 0.20 μm, respectively, and homogeneity of the nuclear chromatin mass, with no regional nuclear disorders, and containing no more than one vacuole, with a borderline diameter of 0.78 ± 0.18 μm from the front view (Figure 1a and b). During selection, a fixed, transparent, celluloid form of a sperm nucleus fitting the normal nuclear shape was superimposed on each examined cell. Spermatozoa which varied in length or width by two standard deviations from the normal mean axes values were considered as abnormal shape cells.

Under Nomarski optics, the sperm cells are transparent; nevertheless, using the front and side views of the differential interference contrast (DIC) optics, we were able to distinguish between the topography of the chromatin vacuoles, which are always surrounded by a smooth chromatin mass, and the topography of the regional disorders of the nucleus, which look like craters or extrusions. No other observation methods, including electron microscopy, can do so, since only MSOME is performed in real time on motile spermatozoa, which, during observation, change position. Thus, sperm motility becomes an advantage in the detection of nuclear content.

A transparent, celluloid form of a vacuole with a borderline diameter of 0.78 ± 0.18 μm was superimposed on each examined cell with a vacuole (Figure 1b). A vacuole which varied in length or width by 1 SD from the above normal mean axes values was considered as a large vacuole (Figure 1c).

Sperm retrieval for IVF–ICSI, based on MSOME

As previously described (Bartoov *et al*., 2003; Berkovitz *et al*., 2005), selected sperm cells were retrieved from the observation droplet and placed into a recipient selection droplet, containing 4 μl of SPERM medium in the same WillCo dish (Willco wells BV, Amsterdam, The Netherlands). This procedure was performed using the Eppendorf Micromanipulation System 5 177 000.010 and Celltram oil 5 176 000.02 (Eppendorf-Nethelei-Hinz GmbH, D-2000, Hamburg, Germany), which is equipped with a sterilized, non-angulated, glass microcapillary with a 12-μm diameter tip. The glass microcapillary was inserted from above into the observation droplet from the condenser lens direction.

As mentioned above, all the selected sperm cells in the control group fit the strict morphological sperm selection MSOME criteria (Bartoov *et al*., 2002) while the selected spermatozoa in the experimental group exhibited normal nuclear shape but large nuclear vacuoles (Figure 1c), since no other cells were available in the ejaculates of the males included in this group.

Microinjection

As we have already demonstrated (Bartoov *et al*., 2003; Berkovitz *et al*., 2005), the transferred, retrieved, cumulus-free ova were placed into drops of SPERM medium prepared in the same glass dish with the recipient droplet. The latter contained the sperm cells morphologically selected for ICSI. The majority of these cells located in the recipient droplet were easily found by the embryologist at the usual magnification of ×200–400. Each microinjected oocyte was immediately transferred to a 4-well dish (Nunc, Hereford, UK), incubated in 0.5 ml of IVF or ISM 1 medium (Medi-Cult, Jyllinge, Denmark), and covered with 0.5 ml of mineral oil (Medi-Cult) in 37°C with an atmosphere of 5% CO_2.

Definitions

Fertilization rate was calculated as the percentage of fertilized ova resulting from the number of injected ova. An embryo was considered to be a top-quality embryo if there were four or five blastomeres on day 2 and six to eight blastomeres on day 3 with <10% of fragments and the total absence of multinucleated blastomeres at any stage of early cleavage. The percentage of top-quality embryos was defined as the number of top-quality embryos obtained from the total number of cleaved embryos. Embryo transfer was usually performed on day 3. However, when day 3 fell on Saturdays or Israeli holidays, embryo transfer was performed on day 2. The implantation rate was calculated as the percentage of gestational sacs, observed by ultrasound, resulting

from the total number of transferred embryos. Clinical pregnancy was confirmed at six weeks by ultrasound examination. Early abortion rate was calculated as the percentage of abortions which appeared within 12 weeks of pregnancy from the total number of pregnancies obtained.

Statistical analysis

All statistical analyses were performed using SPSS for Windows Version 11.0 (SPSS, Chicago, IL, USA). Data were presented as mean ± SD for continuous variables with normal distribution, including the numbers of retrieved and injected oocytes, fertilization rate, percentage of top-quality embryos and number of transferred embryos. Comparisons between the experimental and control matched groups in the above variables were performed using univariate analysis of variance (ANOVA).

Non-parametric statistics, involving Mann–Whitney *U*-tests were performed in parallel to the described statistical analysis in order to avoid possible artefacts due to the small number of cases in each group. The non-parametric methods revealed results similar to those reported here.

The only continuous variable, which exhibited abnormal distribution, was the implantation rate. This phenomenon may be explained by the fact that in non-pregnancy cases (50 and 72% in the control and experimental groups, respectively) the implantation rate value was 0%, while in the pregnancy cases (50 and 18% in the control and experimental groups, respectively) the range of the implantation rate was 20–100%. Comparison between the two study groups in this variable was performed using the non-parametric Two-Sample Kolmogorov-Smirnov test.

The discrete variables included ICSI pregnancy rate per cycle, abortion rates per pregnancies obtained. These variables were presented as percentages. Comparison between the matched groups in the discrete variables was made by Chi-square tests.

Results

The total and mean values per cycle of the IVF–ICSI outcome parameters obtained in the matched experimental and control groups are demonstrated in Table I. Comparison between these two groups in continuous variables with normal distribution revealed no significant differences between them in any of these parameters. The experimental and control groups were also statistically similar in the implantation rate, which exhibited abnormal distribution (median = 0 versus median = 0; range 0–100% versus range 0–100%; 95% confidence interval 0.00–0.10).

The general pregnancy outcome of the 28 IVF–ICSI experimental cycles included one ongoing pregnancy (1 gestational sac) and 4 first trimester missed abortions (5 gestational sacs). In the control group, 10 deliveries (11 healthy children), 3 ongoing pregnancies (6 gestational sacs) and 1 first trimester missed abortion of one gestational sac were obtained. The pregnancy rate per cycle in the experimental group was significantly lower, and the early spontaneous abortion rate per pregnancy significantly higher, than those of the control group (18 versus 50%, Pearson's Chi-square = 6.4, and 80 versus 7%, Pearson's Chi-square = 10.9, respectively, $P = 0.01$, Table I).

Table I. Comparison between the matched experimental and control IVF–ICSI groups in their outcome parameters

IVF–ICSI outcome parameters	Matched study groups		*P*-value
	Experimental (*n* = 28)	Control (*n* = 28)	
Continuous variables (with normal distribution) [mean ± SD per cycle (total)]			
Retrieved ova	13.0 ± 5.0 (364)	12.1 ± 4.4 (341)	NS
Injected ova	8.1 ± 3.6 (228)	8.4 ± 3.2 (236)	NS
Fertilization rate (%)	68.7 ± 20.3 (148)	72.8 ± 18.5 (168)	NS
Percent of top-quality embryos	23.0 ± 31.1 (36)	27.1 ± 29.4 (51)	NS
Embryos transferred	3.0 ± 1.3 (84)	3.2 ± 0.7 (89)	NS
Continuous variables (with abnormal distribution) [median (range)]			
Implantation rate	0 (0–100%) (*n* = 6)	0 (0–100%) (*n* = 18)	NS
Discrete variables [*n* (%)]			
Pregnancy rate per cycle	5 (18)	14 (50)	≤0.01
Abortion rate per pregnancies obtained	4 (80)	1 (7)	≤0.01

NS, not significantly different.

Discussion

This study dealt with a specific nuclear malformation of the sperm cell, namely 'large nuclear vacuoles'. According to Barth and Oko (1988), only DIC microscopy is an excellent means of detecting this defect. Indeed, our technique of morphological sperm selection, based on MSOME, involves an inverted light microscope equipped with high-power DIC (Nomarski/DIC) optics (Bartoov *et al*., 2002, 2003; Berkovitz *et al*., 2005).

The present study is a phenotypic one, where the only difference between the control and the experimental groups was with regard to the size of the vacuoles. In order to exclude a possible effect of any other nuclear malformation, except nuclear vacuoles, we had to use only cases, where all the sperm cells within the ejaculate exhibited large nuclear vacuoles. Such cases are very rare (only 3% of the ICSI population). On the other hand, MSOME revealed that the ejaculates of males, routinely referred for ICSI, exhibit, on average, 30–40% of spermatozoa with a vacuolated nucleus. This sperm malformation, being a pregnancy risk factor, can easily be missed by the standard selection prior to ICSI, and have, therefore, a chance of at least 30% to be chosen for microinjection. This can be avoided by ICSI, based on MSOME.

The data reported here is, first of all, further evidence that sperm morphology may have a significant impact on pregnancy outcome in IVF using ICSI (Kahraman *et al*., 1999; Osawa *et al*., 1999; Miller and Smith, 2001; De Vos *et al*., 2003). We were able to conclude that in cases of spermatozoa with normal nuclear shapes but large vacuoles, the time axis of embryonic development is probably normal at the beginning (normal fertilization, development of top-quality embryos, and implantation); however, the embryo survival in the later stages is impaired (low pregnancy and high abortion rates). This conclusion was strongly supported by studies performed much earlier with bovine sperm cells. Barth and Oko (1988), Barth (1984) and Thundathil *et al*. (1998) found that nuclear vacuoles in bovine sperm do not prevent fertilization from taking place but increase the rate of early embryonic death. Our conclusion is further supported by studies performed by Bartoov

et al. (1994) and Mundy *et al*. (1994) which revealed a clear negative association between the existence of sperm nuclear vacuoles and natural male fertility potential.

The biochemical mechanism underlying the effect of the large vacuole phenomenon on late embryonic development is not yet clear and may be genetically determined. Indeed, an inverse correlation between sperm aneuploidy and normal sperm cell morphology, in general (Calogero *et al*., 2003; Carrell *et al*., 2003), and normal nuclear shape, in particular (Calogero, 2003; Carrell *et al*., 2004), was reported. Sperm head morphology was also found to be associated with sperm DNA fragmentation. (Sailer *et al*., 1996; Virro *et al*., 2004; Vicari *et al*., 2005).

In order to be completely sure that the large nuclear vacuoles in the sperm cell reflect some underlying chromosomal or DNA defect, sperm cells with and without large vacuoles should be selected from the same ejaculate and examined with different biochemical methods. However, this procedure cannot be easily performed: The sperm selection by MSOME is done in a glass bottom dish under paraffin oil. To perform biochemical analysis of the selected sperm cells each of these cells must be transferred outside the observation system to the external analytic system. This step is still problematic for us; however, we hope to resolve this issue in the future. One should remember that biochemical analysis of a whole ejaculate, even one exhibiting a high percent of spermatozoa with large vacuoles, would not necessarily represent the selected sperm population, since it also may include pin-head, amorphous, tapered, round, or multinucleated sperm cells, which cannot be selected for ICSI.

In conclusion, microinjection of sperm with nuclear vacuoles appears to reduce pregnancy outcome. This drawback can be prevented by morphological sperm selection based on MSOME.

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