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ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment

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BACKGROUND: Most studies examining the use of ICSI for cases of elevated sperm DNA fragmentation report poor pregnancy and implantation rates. ICSI with testicular sperm samples has recently been suggested for these cases. Here we test a less invasive approach based on oral antioxidant treatment prior to ICSI with ejaculated spermatozoa. METHODS: Thirty-eight men with an elevated (≥15%) percentage of DNA-fragmented spermatozoa in the ejaculate were treated with antioxidants (1 g vitamin C and 1 g vitamin E daily) for 2 months after one failed ICSI attempt. In 29 (76%) of these cases this treatment led to a decrease in the percentage of DNA-fragmented spermatozoa, and a second ICSI attempt was performed. Outcomes of the two attempts were compared. RESULTS: No differences in fertilization and cleavage rates or in embryo morphology were found between the ICSI attempts performed before and after the antioxidant treatment. However, a marked improvement of clinical pregnancy (48.2% versus 6.9%) and implantation (19.6% versus 2.2%) rates was observed after the antioxidant treatment as compared with the pretreatment ICSI outcomes. CONCLUSIONS: Oral antioxidant treatment appears to improve ICSI outcomes in those patiens with sperm DNA damage, in whom this treatment reduces the percentage of damaged spermatozoa.

Key words: antioxidant treatment/ejaculated spermatozoa/ICSI/sperm DNA damage/sperm fertilizing ability

Introduction

Several studies have reported impaired clinical outcomes of ICSI with ejaculated spermatozoa in men with elevated nuclear DNA damage (Lopes *et al.*, 1998; Twigg *et al.*, 1998; Host *et al.*, 2000; Larson *et al.*, 2000; Benchaib *et al.*, 2003; Virro *et al.*, 2004; Greco *et al.*, 2005a). With the use of the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay, two recent independent studies have reported the absence of pregnancy when the sperm subpopulation showing DNA fragmentation was >20% (Benchaib *et al.*, 2003), and the absence of birth when the sperm subpopulation with DNA fragmentation was ≥15% (Greco *et al.*, 2005a). However, when testicular biopsy was used to recover spermatozoa from patients showing ≥15% of DNA-fragmented sperm in the ejaculate, significantly lower incidence of DNA fragmentation was found in these testicular sperm samples as compared with the ejaculate, and the use of ICSI with testicular spermatozoa led to high pregnancy, implantation and birth rates (Greco *et al.*, 2005a).

Even though these data have suggested that ICSI with testicular spermatozoa is an efficient treatment approach to infertility caused by elevated sperm nuclear DNA damage, continuing search for more conservative treatment approaches, making it possible to avoid recourse to testicular biopsy, was warranted. Previous studies, using indirect assays, have shown that the incidence of DNA fragmentation in ejaculated spermatozoa can be reduced by oral antioxidant treatment (Suleiman *et al.*, 1996; Kodama *et al.*, 1997; Geva *et al.*, 1998; Comhaire *et al.*, 2000; Keskes-Ammar *et al.*, 2003). A recent prospective randomized study using a direct evaluation of sperm DNA fragmentation by TUNEL assay (Greco *et al.*, 2005b) has confirmed these findings. However, some reports questioned the usefulness of antioxidants in the treatment of male infertility (Martin-Du Pan and Sakkas, 1998; Bolle *et al.*, 2002; Agarwal *et al.*, 2004), and *in-vitro* effects of antioxidants on sperm DNA integrity are inconsistent (Hughes *et al.*, 1998; Donnelly *et al.*, 1999). To our knowledge, no evaluation of the effect of oral antioxidant treatment on ICSI clinical outcomes in cases with elevated sperm DNA damage has yet been reported.

In this study, clinical outcomes of two sequential ICSI attempts with ejaculated spermatozoa, one performed shortly before and the other immediately after oral antioxidant treatment, are compared.

Materials and methods

Study design and participants

Thirty-eight couples in which the male partner had ≥15% of ejaculated spermatozoa with fragmented DNA, as detected by TUNEL assay, were enrolled in this study. Patients with varicocele, genitourinary inflammation or infection and smokers were excluded. According to WHO criteria for basic semen parameters (World Health Organization, 1999), 26 males had oligoasthenoteratozoospermia, six had oligoteratozoospermia and six were normal. The female partners underwent standard investigation including endocrine profile and evaluation of ovarian reserve, pelvic ultrasound examination, hysterosalpingography, hysteroscopy, and search for autoantibodies and coagulation factor deficiencies. Seven patients had tubal factor. No female pathology was detected in the remaining 31 couples. Female age ranged between 25 and 35 years.

An ICSI attempt with ejaculated spermatozoa was performed in all of these cases. In those cases in which no ongoing clinical pregnancy was established, oral antioxidant treatment was administered to the male partner. These men were given a 2-month oral treatment with two antioxidants, vitamin C and vitamin E, both at a daily dose of 1 g (500 mg twice a day). In those cases in which a reduction of the incidence of sperm DNA damage was detected at the end of the treatment, a new ICSI attempt with ejaculated spermatozoa was performed. These cases were defined and those in which the difference in the percentage of DNA-fragmented spermatozoa before and after treatment was >10% and in which the percentage of DNA-fragmented spermatozoa after the treatment was <10%. The interval between the two sequential attempts did not exceed 5 months. In those cases in which no improvement of sperm DNA status was achieved, the second ICSI attempt was performed with testicular spermatozoa. The outcomes of this attempt are not included in this study. This design allowed us to begin ovarian stimulation during the period of the husbands' oral antioxidant treatment so as to perform the second ICSI attempt immediately after the antioxidant treatment, thus avoiding the risk of post-treatment impairment of sperm DNA integrity status.

Evaluation of basic sperm parameters

Basic sperm parameters, including sperm count, concentration, motility and morphology, were evaluated according to World Health Organization recommendations (World Health Organization, 1999).

Evaluation of sperm DNA fragmentation

Visualization of fragmented sperm nuclear DNA was performed with the use of Cell Death Detection Kit with tetramethylrhodamine-labelled dUTP (Roche, Monza, Italy), according to the manufacturer's instructions. Ejaculated sperm samples were washed from seminal plasma by low-speed centrifugation (200 *g*; 10 min), smeared on microscope slides, air-dried, fixed with 4% paraformaldehyde in phosphate-buffered saline at 4°C for 25 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Fixed sperm smears were further processed for TUNEL as described previously (Tesarik *et al.*, 2004a). Spermatozoa with fragmented DNA were detected in an epifluorescence microscope with a ×100 oil immersion objective. For quantitative evaluation, 500 spermatozoa in 50 randomly selected areas on three different miscroscope slides were evaluated for each sample, and the percentage of TUNEL-positive spermatozoa was determined.

Assisted reproduction techniques and embryo evaluation

Controlled ovarian hyperstimulation, oocyte recovery and ICSI were performed as described previously (Tesarik *et al.*, 2001). Zygotes and embryos were evaluated with the use of previously described scoring systems (Tesarik and Greco, 1999; Tesarik *et al.*, 2000; Mendoza *et al.*, 2002) on days 1, 2 and 3 after ICSI. Two to four best-scoring embryos were transferred to the patient's uterus on day 3 after ICSI.

Statistical analysis

Differences between groups were assessed by two-tailed χ^2-test with Yates' correction and Fisher's exact test All analyses were performed using the Statistica 5.0 package, version 5.1 (Statsoft, Hamburg, Germany).

Results

Incidence of sperm DNA fragmentation before and after oral antioxidant treatment

Of the 38 couples enrolled in the study, two achieved a clinical pregnancy after the first ICSI attempt performed before the beginning of the oral antioxidant treatment. However, the two pregnancies were spontaneously lost shortly after the detection of embryonic cardiac activity. All of the 38 male partners thus uderwent the antioxidant treatment (1 g vitamin C and 1 g vitamin E daily for 2 months). At the end of this treatment only 29 out of the 38 patients (76.3%) met the criteria required to consider the treatment result to be satisfactory (difference before and after treatment of >10% and <10% DNA-fragmented spermatozoa). The overall patient group was thus retrospectively divided into an antioxidant-responsive group and an antioxidant-non-responsive group.

Basic sperm parameters and the incidence of TUNEL-positive spermatozoa before the beginning of the oral antioxidant treatment were similar in the antioxidant-responsive and the antioxidant-non-responsive group (Table I). In the antioxidant-responsive group no difference was found in basic sperm characteristics after the treatment as compared with the before-treatment figures, in spite of a marked reduction of the percentage of TUNEL-positive spermatozoa (Table II). The antioxidant-non-responsive group did not show any differences in basic sperm parameters. (Table III).

ICSI outcomes with ejaculated spermatozoa before and after oral antioxidant treatment

Comparison of the two sequential ICSI attempts performed before and after the oral antioxidant treatment did not show any diference in the number of oocytes injected, fertilization

Table I. Basic sperm parameters and the incidence of TUNEL-positive spermatozoa in the antioxidant-responsive and antioxidant-non-responsive patient groups[a]

Group	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Normal sperm forms (%)	TUNEL-positive spermatozoa (%)
Responsive	17.9 ± 16.3	40.6 ± 24.8	10.5 ± 8.3	24.0 ± 7.9
Non-responsive	19.1 ± 17.4[b]	39.9 ± 24.8[b]	11.4 ± 7.9[b]	25.1 ± 8.5[b]

[a]Results of examinations performed before antioxidant treatment. Data are mean ± SD.
[b]$P > 0.05$.

Table II. Comparison of basic sperm parameters and the incidence of DNA fragmentation in the antioxidant-responsive group before and after the treatment period[a]

Time of analysis	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Normal sperm forms (%)	TUNEL-positive spermatozoa (%)
Before treatment	17.9 ± 16.3	40.6 ± 24.8	10.5 ± 8.3	24.0 ± 7.9
After treatment	18.3 ± 17.9[b]	39.9 ± 19.0[b]	9.6 ± .4[b]	8.2 ± 4.3[c]

[a]Data are mean ± SD.
[b]$P > 0.05$.
[c]$P < 0.001$.

Table III. Comparison of basic sperm parameters and the incidence of DNA fragmentation in the antioxidant-non-responsive group before and after the treatment period[a]

Time of analysis	Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)	Normal sperm forms (%)	TUNEL-positive spermatozoa (%)
Before treatment	19.1 ± 17.4	39.9 ± 24.8	11.4 ± 7.9	25.1 ± 8.5
After treatment	27.5 ± 24.6[b]	41.6 ± 22.0[b]	8.0 ± 7.1[b]	23.8 ± 9.2[b]

[a]Data are mean ± SD.
[b]$P > 0.05$.

and cleavage rate and the proportion of good-mophology embryos (Table IV). Similar numbers of embryos were transferred in the two ICSI attempts (Table V). However, only two clinical pregnancies, both of which were singleton and were later spontaneously lost, resulted from the 29 ICSI attempts performed before the antioxidant treatment, whereas the 29 ICSI atempts performed after the oral antioxidant treatment resulted in 14 clinical pregnancies (Table V). Ten of these pregnancies were singleton and four were twin. Consequently, significant differences in the clinical pregnancy ($P < 0.05$) and implantation ($P < 0.01$) rates were observed between the two sequential ICSI attempts (Table V).

Discussion

Previous studies have shown that oral antioxidant treatment improves sperm nuclear DNA integrity in men with elevated sperm DNA damage (Suleiman *et al.*, 1996; Kodama *et al.*, 1997; Geva *et al.*, 1998; Comhaire *et al.*, 2000; Keskes-Ammar *et al.*, 2003; Greco *et al.*, 2005b). However, reports concerning the clinical usefulness of antioxidants in the treatment of male infertility are controversial (reviewed in Agarwal *et al.*, 2004). This is the first study in which the possible effect of this treatment on ICSI outcomes is tested in a clearly defined group of patients meeting two selection criteria: first, an elevated percentage of DNA-damaged spermatozoa in the ejaculate; and second, a reduction of this percentage after 2 months of antioxidant treatment. Even though this is an uncontrolled clinical study, the data obtained show clearly that ICSI outcomes are markedly improved after the antioxidant treatment in these cases. It is not clear whether at least some improvement would be obtained if a second ICSI attempt with ejaculated spermatozoa were performed also in those cases in which no difference in the extent of sperm DNA fragmentation was detected before and after the oral antioxidant treatment. This question was not addressed in this study because we previously found that high pregnancy

Table IV. Fertilization and embryo development in two sequential ICSI attempts performed in the antioxidant-responsive group before and after antioxidant treatment

Time of attempt	Attempts	Oocytes injected	Normal zygotes[a]	Fertilization rate (%)[b]	Cleaved embryos [*n* (%)][c]	Good-morphology embryos [*n* (%)][d]
Before treatment	29	288	199	69.1[e]	188 (94.5)[e]	86 (45.7)[e]
After treatment	29	276	195	70.7[e]	181 (92.8)[e]	92 (50.8)[e]

[a]With two equal-sized pronuclei.
[b]Percentage of injected oocytes that developed to normal zygotes.
[c]Percentages are calculated from the number of normal zygotes.
[d]Embryos with normal pronuclear morphology on day 1, six or more cells on day 3, equal-sized blastomeres, and $<10\%$ of intrazonal space occupied by fragments. The percentages are calculated from the number of cleaved embryos.
[e]The differences between data for the two sperm sources are not significant ($P > 0.05$)

Table V. Implantation and pregnancy in two sequential ICSI attempts performed in the antioxidant-responsive group before and after antioxidant treatment

Time of attempt	Attempts	Embryos transferred	Clinical pregnancies[a]	Pregnancy rate (%)[b]	Gestational sacs[c]	Implantation rate (%)[d]
Before treatment	29	89	2	6.9[e]	2	2.2[f]
After treatment	29	92	14	48.3[e]	18	19.6[f]

[a]With at least one gestational sac with cardiac activity.
[b]Percentage of attempts resulting in a clinical pregnancy.
[c]With cardiac activity.
[d]Percentage of embryos transferred that gave rise to a gestational sac with cardiac activity.
[e]$P < 0.05$.
[f]$P < 0.01$.

and implantation rates could be achieved in cases of elevated sperm DNA damage in the ejaculate by ICSI with less damaged testicular spermatozoa (Greco *et al.*, 2005a), thus supporting the previous hypothesis that ejaculated sperm DNA fragmentation is mainly produced after the release of spermatozoa from the Sertoli cells (Tesarik *et al.*, 2004b). The patients with no apparent improvement of ejaculated sperm DNA fragmentation figures after the oral antioxidant treatment were thus switched to this more invasive treatment alternative.

The cut-off values used in this study were the same as those used in our previous study comparing outcomes of ICSI with ejaculated and testicular spermatozoa from men with elevated sperm DNA fragmentation (Greco *et al.*, 2005a), which in turn were derived from a previous study using the same technique for the determination of sperm DNA damage (Benchaib *et al.*, 2003). It has to be stressed, however, that different laboratories currently use different methods for sperm DNA evaluation and different cut-off values. Even with the same TUNEL technique the suggested cut-off values differ; for instance a cut off of 24.3% TUNEL-positive spernatozoa has been proposed recently in the context of the ICSI treatment (Henkel *et al.*, 2003). On the other hand, differences between laboratories appear to be much lower with the use of sperm chromatin structure assay (SCSA), another technique currently employed for the evaluation of sperm DNA integrity (reviewed in Perreault *et al.*, 2003). It remains to be determined what the reasons for the inconsistencies of TUNEL results are, and whether they can be reduced by unifying methodology and data interpretation.

It is not clear why some men responded to antioxidants by reducing the extent of sperm DNA fragmentation while others did not. In a previous study we suggested that the increased percentage of DNA-damaged spermatozoa may be a sequela of different pathophysiological mechanisms in different patients (Greco *et al.*, 2005b), and only some of these conditions may be responsive to antioxidant treatment. This would also explain the discrepancies in the literature concerning the clinical usefulness of antioxidants in the treatment of male infertility (reviewed in Agarwal *et al.*, 2004).

Our previous observations showed that the improvement of the pregnancy and implantation rates after ICSI with testicular spermatozoa, as compared with ICSI using ejaculated spermatozoa in cases of elevated sperm DNA damage, is not accompanied by an improvement of fertilization and cleavage rates or embryo morphology (Greco *et al.*, 2005a). Similarly, no difference in the fertilization and cleavage rates and in the proportion of good-morphology embryos between the ICSI attempts performed before and after the oral antioxidant treatment were found in this study, in spite of a marked improvement of the pregnancy and implantation rates in the post-treatment ICSI attempts.

These observations suggest that the impairment of sperm developmental competence caused by nuclear DNA damage becomes manifest relatively late in the preimplantation embryo development. This late manifestation of the sperm-derived developmental handicap is in agreement with previous observations showing that fertilization, cleavage and embryo morphology during the 3 days following ICSI is not impaired in men with elevated sperm DNA fragmentation, leading to the introduction of the term 'late paternal effect' for this kind of paternally inherited developmental disadvantage (Tesarik *et al.*, 2004a). In fact, we have described previously that a paternal effect can be detected as early as the pronuclear-stage zygote (Tesarik *et al.*, 2002). However, in a subsequent study we observed that this early paternal effect is not associated with an increased sperm DNA fragmentation (Tesarik *et al.*, 2004a). On the other hand, elevated sperm DNA fragmentation caused a late paternal effect by reducing embryo implantation potential, but it was not associated with poor morphological quality of pronuclear zygotes and cleaving embryos (Tesarik *et al.*, 2004a). This is consistent with the observations reported in the present study. The possible mechanisms leading to early and late paternal effects have recently been reviewed (Tesarik, 2005).

The mechanism of embryo developmental impairment in cases of elevated sperm DNA damage is not clear. The threshold of 15% of TUNEL-positive sperm, above which the impairment was observed (Benchaib *et al.*, 2003; Greco *et al.*, 2005a; this study) is too low to explain the embryo demise merely by accidental injection of a TUNEL-positive spermatozoon. It has been speculated that a slight but developmentally relevant degree of DNA-damage, undetectable by TUNEL, may be more frequent in cases in which $>15\%$ TUNEL-positive spermatozoa are found, or that the TUNEL-visualized DNA damage may be a quantitative marker of damage of other sperm components essential for the early embryo development (Tesarik, 2005). This phenomenon has previously been described for SCSA data and termed the 'tip of the iceberg' effect (Evenson *et al.*, 2002).

From the clinical point of view, the present observations make it possible to consider the oral antioxidant treatment followed by ICSI with ejaculated spermatozoa as a less invasive treatment alternative for cases of elevated sperm DNA damage as compared with ICSI with testicular spermatozoa, which has previously been suggested in this indication (Greco *et al.*, 2005a). However, the lack of improvement of sperm DNA integrity after the oral antioxidant treatment in a subgroup of patients suggests that this treatment may not be equally effective in all men suffering this pathology, and the recourse to testicular spermatozoa may thus still be necessary in some cases. Moreover, some cases of sperm DNA damage may be resistent to both of these treatment approaches. A larger prospective controlled study is needed to confirm these data and to design a diagnostic and therapeutic scheme according to which cases of infertility due to sperm DNA damage should be managed.

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