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Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: a randomized, placebo-controlled, double-blind study

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In a randomized, placebo-controlled, double-blind study we investigated whether high-dose oral treatment with vitamins C and E for 56 days was able to improve semen parameters of infertile men. Ejaculate parameters included semen volume, sperm concentration and motility, and sperm count and viability. Thirty-one patients without genital infection but with asthenozoospermia (<50% motile spermatozoa) and normal or only moderately reduced sperm concentration (>7×10^6 spermatozoa/ml) (according to WHO criteria) were examined. To investigate the influence of the epididymal storage period on semen parameters, the patients were asked to deliver two semen samples with abstinence times of 2 and 7 days both before and at the end of vitamin treatment. After randomization, the patients received either 1000 mg vitamin C and 800 mg vitamin E ($n = 15$) or identical placebo capsules ($n = 16$). No changes in semen parameters were observed during treatment, and no pregnancies were initiated during the treatment period. Combined high-dose antioxidative treatment with vitamins C and E did not improve conventional semen parameters or the 24-h sperm survival rate. Prolonged abstinence time increased ejaculate volume ($P < 0.05$), sperm count ($P < 0.05$), sperm concentration ($P < 0.05$) and the total number of motile spermatozoa ($P < 0.05$).

Key words: alpha-tocopherol/ascorbic acid/asthenozoospermia/male infertility/vitamins

Introduction

Until now, no appropriate treatment for idiopathic male infertility has been found; neither have any controlled, double-blind studies demonstrated any significant improvement in pregnancy rates following the use of hormones or hormone analogues (Nieschlag and Leifke, 1997). In addition to endocrine aspects, the influence of reactive oxygen species (ROS) on fertility has become of increasing interest. In patients with asthenozoospermia, an elevated production of ROS in seminal plasma and increased ROS-mediated damage of sperm membranes has been detected, but the origin of these effects is unknown (Iwasaki and Gagnon, 1992; Kovalski *et al*., 1992; Agarwal *et al*., 1994; Aitken, 1995; Aitken *et al*., 1995; Suleiman *et al*., 1996; Yeung *et al*., 1996). Neither is it known at which point the peroxidative damage to spermatozoa occurs, whether within semen (during the time required for liquefaction), in the epididymis (where spermatozoa are stored before ejaculation), or in the testis. By altering membrane integrity, ROS may impair sperm motility as well as sperm viability (Aitken, 1995). Therefore, protective agents against ROS may be useful therapeutic agents in the treatment of male infertility.

Ascorbic acid is a water-soluble ROS scavenger with high potency. In seminal plasma, ascorbic acid concentrations are 10-fold higher than in serum (Dawson *et al*., 1987; Jacob *et al*., 1992). In semen samples exhibiting ROS activity, ascorbate concentrations in the seminal plasma are significantly reduced (Lewis *et al*., 1997). With a pharmacological supplementation of vitamin C (1 g/day), a more than 2-fold increase in plasma ascorbic acid concentrations can be achieved (Wen *et al.*, 1997). Moreover, ascorbic acid concentrations in seminal plasma are also positively related to the percentage of morphologically normal spermatozoa, and it has been suggested that ascorbic acid is a protective vitamin in the epididymis (Thiele *et al*., 1995). Furthermore, it has been shown that ascorbic acid protects human spermatozoa against endogenous oxidative DNA damage (Fraga *et al*., 1991). The amount of DNA damage is significantly greater in infertile male patients than in control patients (Kodama *et al*., 1997).

Alpha-tocopherol (vitamin E) is lipid-soluble and acts mainly within cell membranes (Ford and Whittington, 1998). Following oral administration of vitamin E at doses of 300 to 1200 mg per day for 3 weeks, concentrations in seminal plasma became slightly elevated in infertile men (Moilanen *et al*., 1993; Moilanen and Hovatta, 1995). In contrast, Ford and Whittington (1998) were unable to detect increased vitamin E concentrations in seminal plasma after 3 months treatment with 400 mg vitamin E per day. The concentration of alpha-tocopherol in spermatozoa is not significantly related to the concentration, nor to the total amount of alpha-tocopherol in seminal plasma, though the percentage of motile spermatozoa is significantly related to sperm alpha-tocopherol content (Therond *et al*., 1996).

By combining their hydrophilicity and lipophilicity, vitamins C and E—although being ineffective *in vitro*—may act synergistically *in vivo* to reduce peroxidative attack on spermatozoa (Baker *et al*., 1996). Furthermore, if these agents act directly on spermatozoa to prevent damage by ROS, such improvement may be rapid, provided that the vitamins gain access to the spermatozoa either at ejaculation (benefit within days) or in the epididymis (benefit within weeks). The present study was performed to clarify whether supplementation with vitamins C and E can protect human semen from possible damage by oxygen free radical species in the epididymis, and thus improve semen parameters. Different, albeit fixed, periods of abstinence

Table I. Clinical parameters of patients before treatment. Results are expressed as mean ± SD

	Placebo treatment	Vitamins C + E treatment
Patient age (years)	35.2 ± 4.8	36.1 ± 5.0
Age of female partner age (years)	31.5 ± 4.4	33.0 ± 3.5
Sperm motility (% WHO grade a + b)	29.5 ± 12.6	33.5 ± 8.1
Sperm concentration ($\times 10^6$/ml)	25.5 ± 21.6	29.5 ± 21.7
Sperm morphology (% normal)	12.7 ± 8.6	12.8 ± 5.3
Luteinizing hormone (U/l)	4.6 ± 2.2	4.1 ± 1.9
Follicle stimulating hormone (U/l)	5.3 ± 2.6	5.6 ± 2.1
Testosterone (nmol/l)	18.3 ± 5.2	20.3 ± 4.3
Oestradiol (pmol/l)	61.2 ± 20.2	60.0 ± 19.3

were selected to investigate the potential adverse effects of prolonged exposure of spermatozoa to ROS in the epididymis and the potential beneficial influence of ascorbic acid and alpha-tocopherol on the maturation of spermatozoa and sperm motility, and thereby on their potential for fertilization.

Materials and methods

Study protocol

The investigation was a single-centre, double-blind, placebo-controlled, truly randomized study. The protocol was approved by the Ethics Committee of the University of Munster and the State Medical Board. Before enrolment in the study, each subject's written informed consent was obtained in response to a fully written and verbal explanation of the nature of the study.

The potential participants, each with infertility persisting longer than one year, were examined twice before recruitment to the study. The main inclusion criterion was asthenozoospermia (<50% motile spermatozoa, grade a and b) diagnosed at both examinations with normal or only moderately reduced spermatozoa concentration (>7×10^6 spermatozoa/ml or >20×10^6 spermatozoa per ejaculate) and without infection of accessory glands. Infections were diagnosed by aerobic and anaerobic microbiological culture, and by leukospermia according to WHO criteria (>1×10^6/ml) (WHO, 1992). Complete physical, hormonal and semen examinations were performed in the screening examination. In addition, the medical histories of patients and their female partners were recorded.

To investigate the influence of epididymal storage on semen parameters, patients were asked to deliver two semen samples with fixed abstinence times of 2 and 7 days [upper and lower recommendations of abstinence time according to WHO (1992)].

After delivering the second semen sample, patients were given either 1000 mg vitamin C and 800 mg vitamin E daily, or identical placebo capsules. Both medication and the placebos were prepared by the university pharmacy. Randomization was performed with random numbers without further stratification by the pharmacist, and the code was withheld from researchers and patients. Two additional semen samples were delivered after 7 and 8 weeks of treatment, with abstinence times of 2 and 7 days respectively.

Patients

Thirty-three men attending the authors' institute were recruited for the study. Clinical data and hormone parameters of the patients are presented in Table I. During the pre-treatment phase, no differences in the age of patients or of their spouses, duration of infertility, serum sex hormone values or semen parameters were found between the placebo and vitamins C + E groups (Table I). Tubal patency was investigated in 16 female partners (eight each in the placebo and vitamins C + E groups); tubal blockage was diagnosed in one woman of the vitamin group. None of the female partners presented with known ovulation disturbances, nor did any patient report as being vegetarian or to consume additional vitamins or other nutritional supplements.

Eleven of the patients (five in the placebo group, six in the vitamin group) reported smoking 10–40 cigarettes per day. The ejaculate parameters of smokers and non-smokers were in the same range.

No concomitant medications were permitted during the study. The patients were instructed not to take any additional vitamins, or to change their dietary habits during the study.

Semen analysis

Semen samples were obtained by masturbation. Ejaculate analysis was performed according to WHO guidelines (WHO, 1992) and included physical parameters (ejaculate volume, pH, colour) as well as spermatozoa concentration, motility and morphology (Papanicolaou staining). Internal quality control of semen parameters was carried out according to Cooper *et al*. (1992); in addition, the authors' laboratory is enrolled in an external quality assurance scheme (UKNEQAS, Manchester, UK).

24-h survival test

Sperm survival was examined by monitoring the percentage of viable cells before and after storage. After liquefaction, one volume of semen was processed immediately, and another was incubated at 37°C for 24 h. Both samples were treated as follows: a 50 µl aliquot of semen was diluted with 400 µl phosphate-buffered saline (PBS; Dulbecco, Gibco; Eggenstein, Germany) containing 4 mg/ml bovine serum albumin (fraction V) and propidium iodide to a final concentration of 5 µg/ml. Samples were kept in the dark for 5 min at room temperature and then approximately 5000 spermatozoa, gated by forward and side scatter, were analysed in an Epics XL flow cytometer (Coulter, Krefeld, Germany) using argon laser excitation at 488 nm and fluorescence emission detection at 605–635 nm (620 BP band pass filter). The percentage of cells not stained by propidium iodide was recorded as viable.

Statistical evaluation

Before beginning the study, a statistical power analysis was performed based on random data from unselected patients of the Institute on a level of significance of a = 5% and b = 80%. The minimum number of patients needed in each study group to detect an improvement in sperm motility of 20% as significant was determined to be 30. As 33 patients had been included in the study, a post-priori statistical power analysis revealed that, due to the unexpected small standard deviation with the actual number of patients treated, the study was able to detect a 20% improvement in sperm motility

All variables were checked for normal distribution by applying the Kolmogorov–Smirnov one-sample test for goodness-of-fit. Where appropriate, the variables were logarithmically or arcsine transformed before analysis to achieve normal distributions. Factorial analysis of variance (ANOVA) for repeated measurements was applied for testing significant differences between the study groups. Two-sided *P* values < 0.05 were considered significant. Confidence intervals on differences in sperm motility before and during treatment were calculated from the binomial distribution. Computations were performed using the statistical software package SPSS for Windows version 6.1 (SPSS, Chicago, IL, USA).

Results

Of 31 patients who completed the study, 16 received placebo and 15 vitamins. Results from two patients were rejected from

Table II. Ejaculate parameters before and after treatment. Results are expressed as mean ± SD

Group		Days of abstinence	Semen volume (ml)	Progressive motility[a] (%)	Sperm concn ($\times 10^6$/ml)	Sperm count ($\times 10^6$/ejaculate)	Sperm morphology (% normal)	Eosin test[b] (% unstained)
Placebo	Before treatment	2.1 ± 0.3	3.9 ± 2.3	33.6 ± 15.2	23.6 ± 20.4	101.6 ± 116.4	14.7 ± 9.9	48.5 ± 16.7
Placebo	Before treatment	6.6 ± 0.7	4.9 ± 2.4	30.1 ± 12.3	28.2 ± 23.5	150.4 ± 160.1	12.7 ± 7.4	49.3 ± 10.9
Vitamin C +E	Before treatment	2.1 ± 0.4	3.5 ± 1.7	36.3 ± 9.6	18.1 ± 7.0	66.7 ± 48.2	11.0 ± 4.3	53.6 ± 12.7
Vitamin C +E	Before treatment	6.8 ± 1.4	4.1 ± 1.9	36.7 ± 9.8	26.2 ± 16.9	116.4 ± 108.5	12.9 ± 5.6	50.5 ± 10.9
Placebo	After treatment	2.2 ± 0.4	3.9 ± 1.7	33.9 ± 16.3	25.0 ± 17.8	114.1 ± 131.0	13.3 ± 11.4	50.2 ± 16.2
Placebo	After treatment	6.3 ± 0.9	4.6 ± 2.0	35.3 ± 15.7	31.5 ± 21.8	155.2 ± 170.1	14.4 ± 8.6	49.4 ± 15.1
Vitamin C +E	After treatment	2.1 ± 0.3	3.3 ± 2.0	34.1 ± 11.8	20.6 ± 13.5	75.9 ± 84.4	12.2 ± 5.4	49.3 ± 11.7
Vitamin C +E	After treatment	6.5 ± 1.1	4.3 ± 1.8	37.6 ± 11.1	28.3 ± 16.0	108.3 ± 90.4	13.4 ± 7.8	55.8 ± 17.7

[a]WHO grade a + b.
[b]Sperm viability.

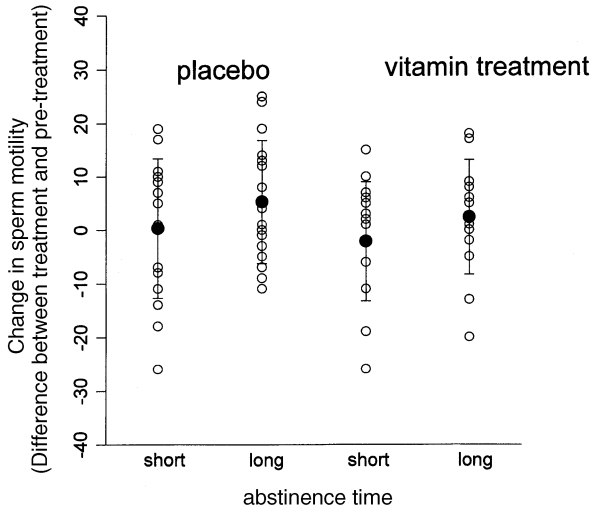

Figure 1. Change in motility of spermatozoa in semen after vitamin or placebo treatment following short and long abstinence times. Data above 0 indicate an improvement; data below 0 indicate an impairment after treatment. A change in sperm motility of 20% was determined as clinically relevant. The mean (●) and the upper and lower limits of the standard deviation (bars) of the differences in the study groups are indicated.

analysis, one in the vitamin group, owing to poor compliance, and one in the placebo group owing to a newly acquired genital infection with obstruction of the ductus deferens that required antibiotic treatment.

The semen parameters in the pre-treatment phase and after 7 and 8 weeks of treatment are presented in Table II. Compared with baseline parameters (before treatment) as well as with the parameters of the placebo group, no changes were observed in any of the conventional ejaculate parameters during vitamin treatment after either short or prolonged abstinence. The main expected improvement was an increase in sperm motility. As sperm motility generally has high intra-individual variation, an improvement of 20% in motility was defined as significant. An improvement in motility exceeding 20% was observed once after treatment in only two patients, both in the placebo group. Results are outlined in Figure 1.

Antioxidant treatment had no influence on ejaculate parameters in those patients who were either smokers or non-smokers, After vitamins C and E treatment in smokers, the percentage of progressively motile spermatozoa decreased slightly (from 37.3 ± 10.7 to 31.2 ± 12.8%) after a short abstinence time, but increased slightly (from 35.2 ± 8.8 to 40.3 ± 13.1%) after prolonged abstinence; neither difference was significant.

Antioxidant treatment did not improve the 24-h survival rate of spermatozoa as measured by flow cytometry (Table II).

No adverse effects during treatment were reported. No pregnancies were initiated in the treatment period in either the placebo or vitamin groups.

In the pre-treatment phase, combining the values obtained from the placebo and vitamin groups indicated that a prolonged abstinence time led to significant increases in ejaculate volume (3.7 ± 1.9 ml after short abstinence versus 4.6 ± 2.2 ml after long abstinence; $P < 0.05$), sperm concentration (20.9 ± 15.4$\times 10^6$/ml versus 27.26 ± 32.6$\times 10^6$/ml; $P < 0.05$), total sperm count (84.7 ± 90.4$\times 10^6$ versus 130.4 ± 138.4$\times 10^6$/ejaculate; $P < 0.05$) and total number of motile spermatozoa (30.2 ± 32.6$\times 10^6$ versus 43.9 ± 45.9$\times 10^6$/ejaculate; $P < 0.05$).

Discussion

On the basis of the results of our study, combined high-dose antioxidative treatment with vitamins C and E for an 8-week period did not improve either conventional semen parameters or 24-h sperm survival rate in patients with asthenozoospermia or moderate oligoasthenozoospermia. These disappointing results were in agreement with those reported by other (Giovenco *et al.*, 1987; Moilanen *et al.*, 1993; Kessopoulou *et al.*, 1995), but were at variance with those reported elsewhere in the literature (Dawson *et al.*, 1992; Suleiman *et al.*, 1996).

Treatment with antioxidants is a widely used therapy for several medical indications, though for most indications its efficacy is not yet proven (Meyers *et al.*, 1996). Vitamin E doses of <3200 mg/day and vitamin C doses of <4000 mg/day are regarded as being safe. In the present study, we chose a dose which was 25% of the recommended upper limit of safe daily oral vitamin intake (Meyers *et al.*, 1996).

Whether antioxidant therapy in men can be improved is an unsolved question, as high doses of certain antioxidants, including vitamin A, may have embryotoxic and teratogenic effects (Tarin *et al.*, 1998). After exposure to ROS, the sperm

Table III. Twenty-four-hour survival rate of spermatozoa[a] before and after antioxidant treatment. Results are expressed mean ± SD

Abstinence time (days)	Treatment	Pre-treatment				End of treatment			
		Viability (% unstained)				Viability (% unstained)			
		0 h	24 h	Difference (0 h–24 h)	24-h survival 24 h/0 h (%)	0 h	24 h	Difference (0 h–24 h)	24-h survival 24 h/0 h (%)
2	Placebo	69.9 ± 15.1	51.5 ± 20.4	18.4 ± 13.3	72.4 ± 21.5	70.4 ± 15.4	45.1 ± 22.3	25.4 ± 19.2	63.4 ± 25.7
	Vitamins C +E	67.3 ± 13.2	37.4 ± 9.0	29.8 ± 17.9	58.8 ± 21.9	70.7 ± 14.9	37.5 ± 10.5	33.2 ± 16.7	55.8 ± 17.7
7	Placebo	61.1 ± 16.7	38.8 ± 20.7	21.7 ± 13.6	62.9 ± 26.9	65.9 ± 10.7	41.8 ± 16.7	24.1 ± 14.9	63.0 ± 22.2
	Vitamins C +E	65.2 ± 12.2	37.4 ± 11.3	27.8 ± 13.7	58.3 ± 15.3	70.1 ± 6.2	35.3 ± 18.3	34.8 ± 17.5	50.2 ± 24.6

[a]Expressed as % of spermatozoa unstained by propidium iodide.

membrane becomes more fragile and antioxidant treatment may prevent lipoperoxidation of sperm membranes (Lenzi *et al*., 1998). Despite intracellular and extracellular antioxidant defence mechanisms, ROS may invoke DNA damage which then causes mutations (Geva *et al*., 1998). It is suspected that DNA damage may lead to an increased risk of miscarriage and chromosomal abnormalities, and this should be further investigated (Geva *et al*., 1998).

In some previous studies, attempts have been made to improve semen parameters of infertile men using comparable doses of vitamin C (1 g/day) (Dawson *et al*., 1987, 1992) or vitamin E (600 mg/day) (Kessopoulou *et al*., 1995), whereas other studies have used lower doses of vitamin E, such as 300 mg/day (Giovenco *et al*., 1987; Moilanen *et al*., 1993) or 200 mg/day (Suleiman *et al*., 1996).

In a placebo-controlled study on the sperm quality of smokers, only the groups receiving ascorbic acid at a dose of 200 or 1000 mg/day sustained an improvement in sperm quality, and greatest improvement was seen in the group receiving the higher dose for 4 weeks (Dawson *et al*., 1992). In our study, however, we could not detect any changes in semen parameters in the subgroup of smokers who underwent antioxidant treatment.

Suleiman *et al*. (1996) reported that vitamin E treatment improved sperm motility in asthenozoospermic men in a placebo-controlled, double-blind study. Eleven of 52 spouses in the treatment group became pregnant during the 6-month treatment period, but none in the placebo group.

It is possible that the relatively short treatment time used in our study explains why, in contrast to the report of Suleiman *et al*. (1996), we did not find any improvement during patient treatment. We cannot exclude the possibility that an 8-week treatment period is too short to achieve an improvement in semen parameters, particularly if the effect is on the testis. Lenzi *et al*. (1993), however, showed that 4 weeks of antioxidative treatment with glutathione was sufficient to improve sperm motility, while Dawson *et al*. (1987, 1992) observed an improvement in sperm quality within days of the initiation of ascorbic acid administration.

According to our data, we cannot recommend administration of antioxidant treatment with vitamins C and E for the treatment of unselected men with asthenozoospermia or moderate oligo-asthenozoospermia. Because no pregnancies occurred, we were unable to calculate the necessary number of patients to be treated in order to verify a possible beneficial effect on pregnancy rates. According to Kessopoulou *et al*. (1995), 1670 patients are needed to demonstrate a statistically significant improvement in pregnancy rates. The use of assisted fertilization, especially since the introduction of intracytoplasmic sperm injection, means that effective treatment for male infertility now exists. However, conservative treatment of male infertility is still warranted and further research should be performed to find a more effective treatment for this condition.

We cannot exclude the possibility that antioxidant treatment of selected patients with elevated ROS generation or with reduced protective scavenging capacity in the seminal plasma may be of benefit, as suggested by Kessopoulou *et al*. (1995) and Lenzi *et al*. (1993). However, it is unknown whether ROS production can be used as a criterion to select men for antioxidative therapy, since intracellular sperm antioxidant status, sperm count, abstinence time and other confounding factors must also be considered (Ford and Whittington, 1998). In addition, no reliable, predictive and cheap tests are available to determine the ROS exposure or antioxidant capacity of patients. Therefore, we decided to examine unselected infertile men, because in normal clinical practice patients are selected according to their semen parameters. Moreover, it is almost impossible technically and also very expensive to investigate the individual risk of each patient before treatment.

Ascorbic acid protects human spermatozoa against endogenous oxidative DNA damage (Frage *et al*., 1991) and DNA damage has been reported to be significantly greater in infertile female patients than in control patients (Kodama *et al*., 1997). Therefore, it may be speculated that patients with DNA damage may benefit from antioxidant treatment. In this study, our intention was to compare DNA damage in spermatozoa (as described by Fraga *et al*., 1991) from patients who responded to the treatment (i.e. achieved a pregnancy or demonstrated improved ejaculate parameters) with that in non-responders, and preserved ejaculate samples accordingly. However, since no pregnancy was observed, we did not perform the DNA damage analysis. It may nonetheless be warranted to perform prospective controlled clinical studies in selected patients with identified and known DNA damage for whom antioxidant treatment may be effective.

The influence of abstinence time on sperm quality was also

investigated in this study. We demonstrated that varying the abstinence time from 2 to 7 days [within the range recommended by WHO (1992)] produces significant changes in ejaculate volume, sperm count and total number of motile spermatozoa (grade a + b). A 'significant improvement' could be considered incorrectly to be drug-related if abstinence time is not fixed. Therefore, in clinical studies of declining sperm counts—as well as in retrospective studies—the abstinence time and any changes in sexual habits must be considered as covariables with a potentially large influence on the results.

Longer abstinence times increase the number of spermatozoa ejaculated. Some authors have recommended limiting the frequency of sexual intercourse in order to achieve a pregnancy (Check *et al*., 1991; Magnus *et al*., 1991; Cooper *et al*., 1993), though it cannot be excluded that a prolonged abstinence time might lead to deleterious effects in terms of ageing of spermatozoa, as reflected in decreased motility, reduced normal morphology and decreased acrosin staining (Blackwell and Zanefeld, 1992; Pellestor *et al*., 1994). No data are currently available indicating whether prolonged abstinence time in infertile men increases the pregnancy rate, and an 'improvement in ejaculate parameters' may not reflect an increased fertilizing capacity of spermatozoa. In healthy couples, there is no evidence that frequent ejaculation reduces the chance of impregnating the female partner (Wilcox *et al*., 1995), since conceptions were observed only when intercourse took place during a 6-day period before the day of ovulation. Often, couples are advised to time intercourse to maximize their chance of pregnancy; however, the probability of conception declines soon after ovulation and therefore couples who abstain from intercourse may miss earlier opportunities for conception. From our observations, we conclude that timing intercourse with a prolonged abstinence time does not improve the probability of conception. Rather, we recommend frequent sexual intercourse during the fertile period.

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