

# Role of male factor in early recurrent embryo loss: do antioxidants have any effect?

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**Objective:** To evaluate whether increasing antioxidant intake in men with high levels of DNA damage or lipid peroxidation improves gestational results in couples with history of recurrent embryo loss.

**Design:** Descriptive study (case series).

**Setting:** Early recurrent embryo loss program at the University of Antioquia, Medellín, Colombia.

**Patient(s):** Seventeen men whose spouses had a history of two or more embryo losses before 12 weeks of gestation.

**Intervention(s):** Male partners with increased DNA fragmentation index (%DFI) or high thiobarbituric acid reactive substances (TBARS) were instructed to consume a diet rich in antioxidants or commercial multivitamins containing  $\beta$ -carotene, vitamin C, vitamin E, and zinc for at least 3 months.

**Main Outcome Measure(s):** Pregnancy outcome was recorded in the spouses of men with increased %DFI or TBARS who received antioxidant supplementation.

**Results:** Of the 17 men, 9 (53%) presented with an increased %DFI or TBARS. They were started on an antioxidant supplementation regimen. Of these nine men, six of their spouses became pregnant. All couples whose male partners accepted antioxidant supplementation achieved a successful pregnancy.

**Conclusions:** Our study demonstrates the benefits of an increased intake of antioxidant-rich food or antioxidant supplements by men who show high levels of sperm DNA fragmentation or lipid peroxidation, which could result in an improvement in gestational outcomes in couples with history of recurrent embryo losses. (Fertil Steril® 2008; ■: ■–■. ©2008 by American Society for Reproductive Medicine.)

**Key Words:** Spermatozoa, embryo loss, DNA damage, oxidative stress, antioxidants

The male gamete contributes 50% of the genomic material to the embryo and contributes as well as to placental and embryonic development (1–3). Genetic and epigenetic alterations of the sperm may therefore have important consequences on early pregnancy. Epigenetic alterations in the sperm, such as altered chromatin packing, imprinting errors, absence or alteration of the centrosome, telomeric shortening, and absence of sperm RNA, can affect some of the functional characteristics leading to early embryo loss (4).

Recurrent pregnancy loss is defined as the miscarriage of two or more consecutive pregnancies in the first or early second trimester of gestation. Although recurrent pregnancy loss may be associated with endocrine, anatomical, psychological, infectious, thrombotic, genetic, or immunological causes, more than 50% of cases remain unexplained, even after an extensive diagnosis (5). The frequency with which sperm defects contribute to recurrent pregnancy loss has

not been established (5), and the relation between standard semen parameters and recurrent miscarriage has been a controversial subject (6–9). Partners of recurrent pregnancy loss patients show a significant increase in sperm chromosome aneuploidy, abnormal chromatin condensation, DNA fragmentation, increased apoptosis, and abnormal sperm morphology compared with fertile men (10–17).

Spermatozoa are particularly susceptible to damage induced by reactive oxygen species (ROS) because sperm plasma membrane is rich in polyunsaturated fatty acids, the cytoplasm contains low concentrations of the scavenging enzymes, and the sperm has limited capacity for DNA repairs (18). Seminal plasma contains high amounts of antioxidants that protect the spermatozoa from DNA damage and lipid peroxidation (19). Excessive amounts of ROS cause DNA damage, leading to formation of 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG), the major oxidative product of sperm DNA, which causes DNA fragmentation (20, 21). Reactive oxygen species are produced by morphologically abnormal spermatozoa and leukocytes and result in peroxidative damage. This alters sperm quality and function, sperm-oocyte interaction, implantation, and early embryo development (22–25), all of which are good indicators of successful pregnancy.

A rich daily dietary intake of antioxidants, such as vitamin C, vitamin E, and  $\beta$ -carotene, has been proposed as an alternative to improve male reproductive capacity by reducing the

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extent of oxidative damage (26, 27). Previously published data have shown contradictory results when evaluating the effects of antioxidants on sperm DNA integrity, both in vitro (28–32) and in vivo (33–35).

The addition of antioxidants in vitro significantly decreased the amount of DNA damage induced by ROS generation (20). The antioxidant properties of flavonoids can protect the integrity of human sperm DNA from ROS induced by estrogenic compounds (36). Supplementation with the antioxidants, such as ascorbate, urate,  $\alpha$ -tocopherol, and Ham's F-10 medium, when used individually has shown beneficial effects in improving sperm DNA integrity. However, acetyl cysteine or ascorbate and  $\alpha$ -tocopherol when used in combination were reported to increase DNA damage (28, 31, 37). Therefore the design of clinically effective antioxidant treatments will depend largely on the source of oxidative stress. For cases involving excessive intracellular ROS generation, albumin appears to be an important means of neutralizing lipid peroxide-mediated damage to the sperm plasma membrane and DNA (30). Addition of glutathione and hypotaurine, either individually or in combination, to sperm preparation medium did not show significant effect on progressive motility or baseline DNA integrity of the spermatozoa. Despite this, it conferred significant protection to the sperm against hydrogen peroxide-induced damage and ROS generation (38).

Oral antioxidant treatment has been reported to reduce the percentage of damaged spermatozoa and improved intracytoplasmic sperm injection (ICSI) outcomes in patients with sperm DNA damage (34). However, a dose–response association was not seen between any of the DNA fragmentation index (%DFI) outcomes and the antioxidant intake measures. Nondose-related associations were seen among  $\beta$ -carotene intake and SD of DFI and percentage of immature sperm. Participants with moderate, but not high,  $\beta$ -carotene intake showed an increase in SD DFI compared with participants with low  $\beta$ -carotene intake (adjusted means 206.7 and 180.5, respectively;  $P=.03$ ), as well as an increase in the percentage of immature sperm (adjusted means 6.9% and 5.0%, respectively;  $P=.04$ ). If antioxidant intake is indeed beneficial for fertility in healthy men, it does not appear to be mediated through sperm chromatin integrity. The results of this study do not preclude possible beneficial effects of high antioxidant intake on sperm chromatin integrity for men with fertility problems (33).

On the other hand, %DFI and the degree of sperm decondensation were measured using the sperm chromatin structure assay before and after 90 days of treatment with zinc and selenium. Antioxidant treatment led to a decrease in sperm DNA fragmentation (19.1%,  $P<.0004$ ), suggesting that this decrease was partially linked to ROS. However, it also led to a similar increase in sperm decondensation (22.8%,  $P<.0009$ ). This may be explained by the opening of interchain disulfide bridges in protamines as antioxidants. In particular, vitamin C is able to open the cystine net, thereby interfering with paternal gene activity during preimplantation

development. This observation might help explain the discrepancy observed regarding the role of these antioxidant treatments in improving male fertility (35).

The objective of this study was to measure %DFI and lipid peroxidation as measured by the presence of thiobarbituric acid reactive substances (TBARS) in the male partners of spouses with a history of repeated miscarriages. An antioxidant (food rich in antioxidants and commercial multivitamins) regimen was initiated in men with elevated DFI or TBARS, and pregnancy outcome was recorded.

## MATERIALS AND METHODS

### Patient Selection

After approval by the Research Ethics Committee of the University of Antioquia, 17 couples were screened at the Habitual Abortion Program in the Reproduction Lab, University of Antioquia, from October–December 2005. This was part of an ongoing research project studying sperm DNA damage and plasma membrane changes in men with spouses with a history of more than two miscarriages before week 12 of gestation (Gil-Villa A and Cadavid A, unpublished manuscript) as diagnosed by ultrasound. To rule out antiphospholipid syndrome as a cause of recurrent embryo loss, titers of IgG and IgM antibodies against cardiolipin, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidic acid were measured using a qualitative method, described by Kwak et al. (39). Diagnosis of alloimmunity was determined in one woman by the absence of blocking factors in a mixed lymphocyte reaction using the technique adapted in our laboratory (40). This woman received lymphocyte immunotherapy with her husband's lymphocytes (40).

We found 9 of 17 individuals (53%) with elevated %DFI as measured by sperm chromatin structure assay or elevated lipid peroxidation as measured by TBARS production. Increased antioxidant intake was recommended for these men. This included a diet rich in antioxidants such as  $\beta$ -carotene (carrots, spinach, tomatoes, papaya, guava, cherries, melons, peaches), vitamin C (guava, kiwi, mango, pineapple, melons, strawberries, berries, tomatoes, broccoli, cabbage, oranges, lemons and other citrus fruits), vitamin E (lettuce, peanuts, almonds, coconut, corn, soy or olive oil; wheat and corn germ; cereals), zinc (asparagus, potatoes, vegetables, eggs, fish), or commercial multivitamins containing  $\beta$ -carotene (5000 IU), vitamin C (60 mg), vitamin E (30 UI), and zinc (15 mg) for at least 3 months. After 1 year, the new reproductive outcome of the couple (i.e., successful pregnancy) was confirmed by phone interview, and the results are presented in this report.

### Semen Samples

Semen samples were obtained by masturbation after 2–5 days of sexual abstinence. After liquefaction, semen analysis was performed using World Health Organization guidelines (41) and morphology was analyzed following Kruger's strict

criteria (42). The patients were numbered according to their chronological order of entry into the Habitual Abortion Program.

### Sperm Chromatin Structure Assay

The sperm chromatin structure assay was done to measure the %DFI, as previously described (43). Briefly, semen samples were chilled on ice and diluted with TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4) to  $2 \times 10^6$  cells/mL. Two hundred-microliter aliquots of diluted sample were mixed with 400  $\mu$ L of a low pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15 M NaCl, and 0.08 N HCl for 30 seconds; this was followed by staining with 1.2 mL of 6 mg/mL chromatographically purified acridine orange (AO) in a phosphate citrate buffer (pH 6.0). Immediately at the moment of staining, the cells were analyzed using a FACS XL flow cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with an air-cooled argon laser. Measurements were collected on 5,000–10,000 cells per sample. Under these conditions, AO intercalated with double-stranded DNA emits green fluorescence, and AO associated with single-stranded DNA emits red fluorescence. To avoid instrument drift, reference samples were used to set the red and green photomultiplier tube voltages. In addition, a reference sample of semen was run in every assay. WinMDI 2.9 software (Scripps Research Institute, La Jolla, CA) was used for off-line analysis of the flow cytometry data.

The percentage of sperm with high levels of DFI was determined as the increase in the ratio of red-to-[red + green] fluorescence. Fertility potential was categorized as: excellent,  $\leq 15$  %DFI; good, 15%–24 %DFI; fair, 25%–30 %DFI; and poor,  $>30\%$  DFI, as described earlier (44).

### Measurement of Lipid Peroxidation

Lipid peroxidation was measured by determining TBARS production, according to the method described by Laudat et al. (45). Briefly,  $10 \times 10^6$  cells were incubated for 1 hour at 37°C in 5% CO<sub>2</sub>. After placing the cells in ice cold water for 15 minutes, they were mixed with 1 mL of thiobarbituric acid reagent 0.6% and trichloroacetic acid 40%. The solution was incubated at 95°C in a dry bath for 15 minutes and immediately cooled by placing on ice. The reaction mixture was extracted by adding 3 mL of butanol and centrifuged at 1,600  $\times$  g for 7 minutes. The absorbance was read on a spectrophotometer at a wavelength of 535 nm absorbance against a blank containing butanol. The results of sperm TBARS were expressed in nanomoles per  $10^6$  spermatozoa. Normozoospermic semen samples have a mean value of TBARS less than 0.13 nmoles/ $10 \times 10^6$  spermatozoa (19).

### Selection of Patients for Antioxidant Supplementation

Male partners presenting %DFI  $>24\%$  or TBARS values  $\geq 0.13$  nmoles/ $10 \times 10^6$  spermatozoa were selected to receive a diet enriched with food sources known to have a high content

of antioxidants or commercially available multivitamins for at least for 3 months. This time period was chosen to more accurately reflect antioxidant exposure during the period of spermatogenesis (46–49). In vivo antioxidant treatment may act during the testicular period of germ cell development by exerting a beneficial effect on germ cells, Sertoli cells, or both, leading to better functioning of the defense mechanisms that protect germ cells and spermatozoa against DNA damage (34).

### RESULTS

Of the 17 eligible men in the study, antioxidant supplementation was recommended to 9 patients who presented with increased %DFI or TBARS. Six of the nine men followed through with antioxidant supplementation, and the partners of all six of these men became pregnant. Patients with normal %DFI or TBARS did not require antioxidant supplementation (Fig. 1).

The reproductive histories (before and after sperm test) of the six men whose wives became pregnant are summarized in Table 1. The type of sperm alterations indicated by %DFI and TBARS and type of antioxidant supplementation used by each patient are described in Table 1.

Initially, partners of four of the six patients with sperm alterations who were on antioxidant supplementation had a successful pregnancy outcome. However, when patient 15 did not follow the antioxidant diet, his wife had a miscarriage, but when the patient followed this recommendation, they had a baby (Fig. 1 and Table 1). Another patient (#2) did not opt for an antioxidant supplementation, and his wife had a miscarriage.

All of the spouses of the patients were negative for antiphospholipid syndrome. The spouse of patient 3 had a diagnosis of alloimmunity. She had received lymphocyte immunotherapy with her husband's lymphocytes in a previous gestation that ended in a miscarriage. Two of the six women who had a pregnancy (#2 and #15) also had a history of bearing one son with another partner.

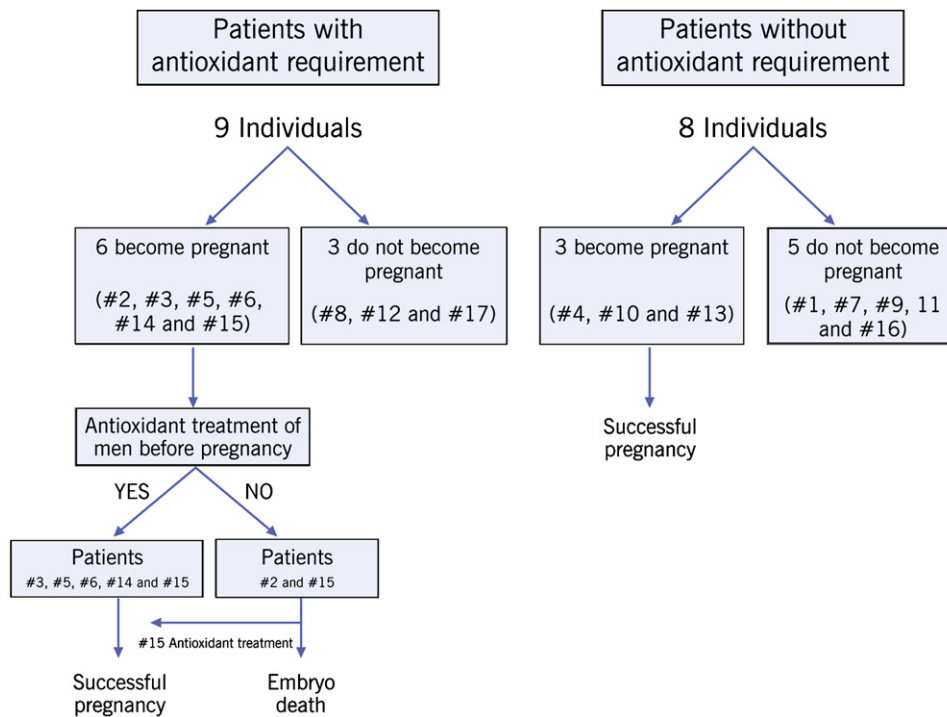
### DISCUSSION

The results of this study appear to show that the increased intake of antioxidant-rich foods or antioxidant supplements by men who presented with high levels of DNA damage or oxidative stress improved the gestational results in a group of couples with history of recurrent embryo loss. All couples whose male partners adopted an antioxidant supplementation achieved a successful pregnancy.

The controlled generation of very low amounts of ROS appears to regulate normal sperm functions, whereas high levels of ROS endanger sperm viability and function (38). Oxidative stress develops as a consequence of excessive ROS production or impairment of the antioxidant defense system. The oxidative process possibly precipitates a range of pathologies currently thought to afflict male reproductive function. In male factor infertility, oxidative stress attacks the fluidity of the

## FIGURE 1

Pregnancy outcome in couples with history of two or more embryo losses, in which the male partner required an antioxidant supplementation.



Gil-Villa. Male factor infertility and early recurrent embryo loss. *Fertil Steril* 2008.

sperm plasma membrane and sperm DNA integrity at both nuclear and mitochondrial levels (50). The ROS-induced DNA damage may accelerate the process of germ cell apoptosis, leading to the decline in sperm counts associated with male infertility. High levels of ROS are detrimental to fertility potential in both natural and assisted conception states (38, 51).

DNA bases are susceptible to oxidative stress, and peroxidation of these structures can cause base modification, DNA strand breaks, and chromatin cross-linking (52). The potential adverse effect of sperm DNA damage on the quality of the postimplantation embryo and spontaneous abortion should be a concern (16, 53). A significant negative association was seen between the percentage of sperm with DNA fragmentation and fertilization rate. Increased red fluorescence indicated impaired fertilization outcome, and good quality embryo rates were significantly lower in the group with high sperm DNA fragmentation. Chromatin damage precedes the loss of fertilization potential and poor embryo quality, resulting in pregnancy loss (54–59).

Cell division in the human preimplantation embryo can be compromised by deficiencies in the sperm nuclear genome or in the sperm-derived developmentally relevant cytoplasmic factors, oocyte-activating substance, and centriole (1, 4). Sperm nuclear deficiencies usually are not detected before the eight-cell stage of embryo development, when a major expression of sperm-derived genes has begun. Sperm cytoplas-

mic deficiencies can be detected as early as the one-cell zygote and then throughout the preimplantation development. The terms late paternal effect and early paternal effect have been suggested to denote these two pathological conditions (reviewed in Ref. 60).

The late paternal effect is associated with an increased incidence of sperm DNA fragmentation. No association with sperm DNA damage has been found for the early paternal effect. The diagnosis of the late paternal effect is thus based on the examination of sperm DNA integrity, which should be performed in cases of recurrent embryo loss or of repeated assisted reproduction failure, even if morphologically normal embryos result from fertilization with the patient's spermatozoa (reviewed in Ref. 60).

The only elements leading to the diagnosis of the early paternal effect are poor zygote and embryo morphology and low cleavage speed. The absence of increased sperm DNA damage does not exclude the presence of this pathology. Intracytoplasmic sperm injection with testicular spermatozoa has recently been shown to be an efficient treatment for the late paternal effect. The use of oral antioxidant treatment in this indication has also yielded promising results (reviewed in Ref. 60).

All male patients in this study with increased %DFI or TBARS production were advised to include foods rich in

**TABLE 1**

**Reproductive outcome before and after sperm tests for altered lipid peroxidation and DNA damage in male partners of couples with history of repeated embryo loss.**

Patient No.	Reproductive history before sperm test		Sperm tests		Reproductive history after sperm test		
	Pregnancy outcome	Treatment during pregnancy	TBARS	% DFI	Antioxidant supplementation	Treatment during pregnancy	Pregnancy outcome
2	1. Embryo loss, 5 wks 2. Embryo loss, 6 wks	Progesterone Progesterone	0.14 <sup>b</sup>	11.5	None <sup>a</sup>	Progesterone and ASA	Miscarriage, 3 wks
3	1. Embryo loss, 8 wks 2. Embryo loss 12 wks 3. Blighted ovum	None None Progesterone, ASA and LIT	0.11	29.8 <sup>b</sup>	Diet + CM	Progesterone and ASA	Term
5	1. Embryo loss, 6 wks 2. Embryo loss, 6 wks	None None	0.27 <sup>b</sup>	24.3 <sup>b</sup>	CM	Progesterone and ASA	Term
6	1. Term 2. Blighted ovum 3. Blighted ovum, 8 wks	None Progesterone None	0.07	26.9 <sup>b</sup>	Diet + CM	Progesterone and ASA	Term
14	1. Embryo loss, 5 wks 2. Embryo loss, 6 wks	Progesterone Progesterone	0.17 <sup>b</sup>	12.6	Diet <sup>a</sup>	Progesterone and ASA	Term
15	1. Embryo loss, 6 wks  2. Embryo loss, 8 wks	Progesterone  Progesterone	0.39 <sup>b</sup>	10.2	None <sup>a</sup>  Diet	Progesterone and ASA  ASA	Embryo loss, 6 wks  Term

Note: TBARS = thiobarbituric acid reactive substances (nmoles/10 x 10<sup>6</sup> spermatozoa); %DFI = DNA fragmentation index; P = progesterone; ASA = aspirin 100 mg/day;

LIT = lymphocyte immunotherapy; Diet = increased intake of food rich in antioxidants; CM = commercial multivitamin ( $\beta$ -carotene, vitamin C, vitamin E, and zinc).

<sup>a</sup> Antioxidant supplementation recommended.

<sup>b</sup> Altered sperm parameters.

Gil-Villa. Male factor infertility and early recurrent embryo loss. *Fertil Steril* 2008.

vitamins C and E,  $\beta$ -carotenes, and zinc in their diet. A list of these foods was given to them; however, the specific amount of these foods to be consumed was not included. Some patients received antioxidant supplements (commercially available multivitamins) at similar concentration to those contained in foods rich in antioxidants. However, patients were not monitored for their diet/multivitamins intake in a regular way.

Only the data on gestational outcome and antioxidant regimen received were obtained. Sperm parameters, such as motility, concentration, morphology, DNA fragmentation, and lipid peroxidation after antioxidant supplementation, were not followed. Although the number of patients in this study is small, the results are encouraging because seven gestations were recorded. Interestingly two couples (#2 and #15) had a gestational loss when the male partner did not switch to antioxidant supplementation.

One of the limitations of our study was the use of progesterone or aspirin by the women during pregnancy. These are commonly used therapies to prevent pregnancy alterations, such as fetal growth retardation, preeclampsia, and recurrent spontaneous abortion (61–63), although some of the women had gestational losses despite having used either of these therapies in their previous pregnancies. This was particularly of interest in couple 15. An antioxidant supplementation was suggested to the husband, but he did not adopt this recommendation. However, the woman received aspirin and P in her subsequent pregnancy. Despite the use of these treatments, only the treatment of the man with antioxidants supplementation resulted in a successful pregnancy (Fig. 1 and Table 1).

Another interesting case was couple 3. The woman had an alloimmunity diagnosis and received multiple treatments (aspirin, P, and lymphocyte immunotherapy). Nevertheless, a successful pregnancy was achieved only when the partner adopted an antioxidant supplementation (Fig. 1 and Table 1). Although the effect of aspirin on pregnancy outcome is not clear, it is possible that aspirin-triggered epilipoxins exhibit a potent anti-inflammatory effect as well as antioxidant activities (64, 65). In addition, none of the spouses of the patients had antiphospholipid antibodies, which is known to be a cause of embryo death (5).

To the best of our knowledge this is the first report that evaluates the management of couples with recurrent embryo loss from the male factor perspective using antioxidant treatment in patients with increased DNA damage (%DFI) or oxidative stress (lipid peroxidation as measured by TBARS). This is especially important because several mechanisms have documented how the paternal component can affect embryo development, implantation, placenta health, birth defects, and recurrent miscarriage (6–9). The preliminary results of this report suggest that semen evaluation in couples with recurrent embryo loss is important and attributed to “abortigenic men,” that is, individuals with seminal-specific characteristics, such as DNA fragmentation and lipid peroxidation, that could be associated with embryo death.

Despite the controversy over whether or not sperm quality is related to recurrent miscarriages, recent data suggest that future studies into more specific sperm quality markers are promising (3). Thus, perhaps antioxidants will not only help reduce sperm DNA fragmentation rates but also will lower pregnancy loss rates (3). Although advances are being made in the field of antioxidant therapy, additional randomized clinical trials with more subjects are needed to define sperm interventions before embryo development. This will provide a strong basis for a better understanding as well as identifying possible diagnoses and treatments for reproductive alterations in DNA damage and oxidative stress that could hinder embryo development.

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