

Ascorbic acid protects against endogenous oxidative DNA damage in human sperm

[vitamin C/oxidants/8-hydroxy-2'-deoxyguanosine (8-oxo-7,8-dihydro-2'-deoxyguanosine)/DNA adducts/mutation]

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Contributed by Bruce N. Ames, September 9, 1991

ABSTRACT Damage to the DNA of germ cells can lead to mutation, which may result in birth defects, genetic diseases, and cancer. The very high endogenous rate of oxidative DNA damage and the importance of dietary ascorbic acid (AA) in preventing this damage has prompted an examination of these factors in human sperm DNA. The oxidized nucleoside 8-hydroxy-2'-deoxyguanosine (8-oxo-7,8-dihydro-2'-deoxyguanosine; oxo⁸dG), 1 of ≈20 major products of oxidative damage to DNA, was measured in DNA isolated from human sperm provided by healthy subjects and compared to the seminal fluid AA levels. This relationship was studied in two groups. In a group of 24 free-living individuals 20–50 years old high levels of oxo⁸dG were correlated with low seminal plasma AA. The endogenous level of oxo⁸dG in this group was 13 fmol per μg of DNA or ≈25,000 adducts per sperm cell. The second group of individuals was maintained on a controlled diet that varied only in AA content. When dietary AA was decreased from 250 to 5 mg/day, the seminal fluid AA decreased by half and the level of oxo⁸dG in sperm DNA increased 91%. Repletion of dietary AA for 28 days (from 5 mg/day to 250 or 60 mg/day) caused a doubling in seminal fluid AA and reduced oxo⁸dG by 36%. These results indicate that dietary AA protects human sperm from endogenous oxidative DNA damage that could affect sperm quality and increase risk of genetic defects, particularly in populations with low AA such as smokers.

The damage produced by endogenously generated oxygen radicals has been proposed to be a major contributing factor in aging and the many degenerative processes associated with it including cancer, heart disease, and cognitive dysfunction (1–6). Under physiological conditions, endogenous oxidants are produced at a high rate, resulting in extensive oxidative damage to proteins, lipids, and DNA (5–8). Oxidative damage to DNA, based on the urinary excretion of DNA adducts, occurs at an estimated rate of 10⁵ hits per cell per day in the rat (8) and ≈10⁴ hits per cell per day in the human (9).

Endogenous oxidative damage to germline DNA is likely to lead to heritable mutations and increased incidence of birth defects, genetic diseases, and cancer in offspring. Ionizing radiation, an oxidative mutagen, damages gamete DNA, resulting in mutations that are transmitted to the progeny in experimental animals (10). Most, but not all, DNA damage is expected to be repaired, and damaged residues that remain may be converted to mutations during the DNA replication that accompanies cell division in spermatogenesis, oogenesis, and embryogenesis. Studies of genetic abnormalities and cancers believed to arise from germline mutations show a higher frequency of paternal than maternal origin (11), such as the increased frequency of paternal origin of germline

mutations in the retinoblastoma gene (12). This is consistent with the markedly higher number of cell divisions that occur during spermatogenesis (relative to oogenesis), resulting in an increase in the risk for mutation in sperm. Once spermatogenic cell division is completed, maturation of the spermatid into the spermatozoon involves condensation of the DNA accompanied by the apparent loss of DNA repair activity (13). Damaged residues remaining in sperm DNA may be delivered to the egg upon fertilization, increasing the possibility of mutations when the egg divides.

The dietary antioxidant ascorbic acid (AA) may play a critical role in protecting male germ cells against oxidative damage. AA is present at a high concentration in seminal fluid compared to blood plasma (400 vs. 60 μM), presumably reflecting an important physiological role. In addition, AA is an effective defense against peroxyl radical-induced lipid peroxidation in blood plasma (14, 15) and should prevent oxidative damage to DNA. Thus, the relationship between oxidative damage to sperm DNA that could result in improper sperm function, infertility, and birth defects and seminal fluid AA levels is of great interest. We report in this communication the finding that the level of oxidative damage to human sperm DNA, determined by the measurement of 8-hydroxy-2'-deoxyguanosine (8-oxo-7,8-dihydro-2'-deoxyguanosine; oxo⁸dG), is inversely related to seminal plasma AA levels.

MATERIALS AND METHODS

Subjects and Experimental Design. The study consisted of three parts. Participants in experiments A and C were nonsmokers; there were both smokers and nonsmokers in experiment B. In experiment A, five semen samples were obtained from a 36-year-old individual at 1-week intervals and one sample was obtained 15 months later. Experiment B consisted of 50 free-living individuals (20–50 years old) with normal sperm who were being studied at the Laboratory of Biochemical Cytology (School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina). In experiment C, as part of a study on the effects of AA deficiency, 10 volunteer participants 25–43 years old were housed in the metabolic unit of the U.S. Department of Agriculture–Western Human Nutrition Research Center and maintained on controlled diets supplemented with various amounts of AA according to the study design shown in Table 1 (16). One semen sample was collected on the last day of each AA regimen. The Research Protocol and Informed Consent were

Abbreviations: oxo⁸dG, 8-hydroxy-2'-deoxyguanosine (8-oxo-7,8-dihydro-2'-deoxyguanosine); AA, ascorbic acid.

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Table 1. Dietary AA intake, semen AA, and oxo⁸dG in DNA from sperm of individuals in experiment C

	Baseline	Depletion	Marginal	Repletion
AA intake, mg/day	250	5	10 or 20	60 or 250
n	10	8	6	4
Length, days	7-14	32	28	28
Semen AA, μ M	399 \pm 55 ^a	203 \pm 72 ^b	115 \pm 25 ^{b,c}	422 \pm 100 ^a
oxo ⁸ dG, fmol per μ g of DNA				
Analysis a	13.2 \pm 1.0			
Analysis b	34.0 \pm 2.4 ^d	66.9 \pm 8.5 ^e	84.4 \pm 22.3 ^a	53.8 \pm 16.8 ^{d,e}

Individuals were maintained on controlled diets supplemented with various amounts of AA. Samples were collected and analyzed for oxo⁸dG and AA as described. Values are expressed as the mean \pm SEM. Mean values in the same row with different superscripts are significantly different at $P < 0.01$ (d vs. e, a vs. c) or $P < 0.05$. Trends of oxo⁸dG levels in individuals are consistent with the group means: of the 18 individual transitions (8 + 6 + 4), 15 matched the trend of the group mean.

approved by the Institutional Review Committee of Letterman Army Medical Center (Presidio of San Francisco, CA) and by the Human Studies Review Committee of the Agricultural Research Service (U.S. Department of Agriculture).

DNA Isolation and Enzymatic Hydrolysis. Sperm samples used for the determination of oxo⁸dG in experiments A and B were obtained by masturbation and frozen promptly at -20°C . In experiment C, aliquots of whole semen were taken for AA and oxo⁸dG analysis after routine fertility tests on the semen and sperm were performed. For isolation of DNA, samples were thawed, allowed to liquefy at room temperature for 30 min, and diluted with 1 vol of buffer (150 mM NaCl/10 mM Tris-HCl, pH 7.4/1 mM EDTA). Sperm cells were pelleted from the suspensions by centrifugation at $600 \times g$ for 10 min. Where indicated the supernatant (seminal plasma) was used for AA determinations. The pellet was washed by resuspension in 1 ml of buffer, repelleted as described above, and resuspended in 200 μ l of buffer. The DNA was isolated by a model 340A Applied Biosystems DNA extractor using helium-pressurized vessels and Applied Biosystems reagents. The extraction was carried out by diluting the cell suspension with Applied Biosystems lysis buffer containing 1% 2-mercaptoethanol (Eastman Kodak). The isolated DNA (200–400 μ g; about 70% recovery from sperm) was resuspended in 200 μ l of 20 mM sodium acetate (pH 4.8) and digested to nucleotides with 20 μ g of nuclease P1 (Sigma) at 37°C for 30 min. This was followed by the addition of 20 μ l of 1 M Tris-HCl (pH 7.4) and treatment with 1.3 units of *Escherichia coli* alkaline phosphatase (Sigma) at 37°C for 60 min. After digestion, the samples were passed through a 30,000 molecular weight cut-off filter (Ultrafree MC; Millipore) and analyzed by HPLC with electrochemical detection as described below.

Determination of oxo⁸dG in DNA. The amount of oxo⁸dG in the DNA was measured by HPLC with electrochemical detection (17) using an ESA model 5100 Coulochem detector equipped with a 5011 analytical cell. The potentials for electrodes 1 and 2 were adjusted to +0.10 and +0.30 V, respectively. The amount of oxo⁸dG in the samples was expressed relative to the amount of DNA. The amount of DNA in the samples was calculated from the peak area of dG detected by UV. This was accomplished by comparing dG peak areas to those obtained from known amounts of calf thymus DNA or by applying the conversion factor 0.62 nmol of dG = 1 μ g of DNA.

AA Determinations. The content of AA in semen and seminal plasma was determined by HPLC with electrochemical detection from aliquots of those used for oxo⁸dG determinations (18). Seminal fluid samples were prepared for HPLC analysis from subjects in experiment B after separating sperm cells by centrifugation as described above and were diluted with 9 vol of 5% (wt/vol) metaphosphoric acid containing 1 mM EDTA and 2 mM cysteine to reduce

dehydroascorbic acid to AA. Semen from the subjects in experiment C were diluted with 1 vol of 10% (wt/vol) metaphosphoric acid containing 0.45 mM EDTA.

Statistical Analysis. Values in the text and table represent the mean \pm SEM. Statistical significance (regression analysis and one-way analysis of variance) was calculated according to the routines available in Statview 512+ (Brainpower, Calabasas, CA).

RESULTS

Experiment A. To establish the content of oxo⁸dG in human sperm DNA, five samples from a healthy individual, obtained at 1-week intervals, were analyzed. The average content of oxo⁸dG in these sperm samples was 13.8 ± 1.6 fmol per μ g of DNA (values ranging from 9.5 to 18.4 fmol per μ g of DNA) corresponding to 2.2 oxo⁸dG residues per 10^5 dG or 2.7×10^4 oxo⁸dG residues per spermatozoon. The content of oxo⁸dG in the sperm obtained 15 months later from the same individual was 10.6 fmol per μ g of DNA, within the range of values described above.

Experiment B. The average content of oxo⁸dG in sperm DNA from 50 free-living individuals was determined. The values ranged from 3 to 90 fmol of oxo⁸dG per μ g of DNA and averaged 12.6 ± 2.7 , corresponding to 2.1 oxo⁸dG residues per 10^5 dG. The seminal plasma AA concentrations were examined for 24 of the individuals in this group and were 215 ± 43 μ M (range, 4–880 μ M). The sperm cell populations obtained from these individuals (L_{AA} , <200 μ M AA; H_{AA} , >200 μ M AA) exhibited, according to the guidelines of the World Health Organization, normal physiological characteristics: percent progressive motility (L_{AA} , 31; H_{AA} , 46), percentage of living cells (L_{AA} , 87; H_{AA} , 91), total number of sperm cells per ejaculate (L_{AA} , 167×10^6 ; H_{AA} , 294×10^6), number of contaminating leukocytes (L_{AA} , 0.41×10^6 ; H_{AA} , 0.36×10^6), and percentage of cells with normal morphology (L_{AA} , 32; H_{AA} , 31). A plot of oxo⁸dG levels in sperm DNA and seminal plasma AA, as shown in Fig. 1, reveals possible threshold for AA protection. The level of oxo⁸dG was not correlated with sperm motility ($r^2 = 0.023$) or age of the donor ($r^2 = 0.013$).

The possible influence of semen AA concentration on the content of oxo⁸dG and the effect of storage on seminal fluid AA levels were examined. Samples from experiment B containing low AA were spiked with AA (60–1400 μ M), incubated for 1 h at 37°C , and analyzed for oxo⁸dG. The oxo⁸dG content of DNA from sperm samples treated in this manner was not significantly different from untreated paired control samples (data not shown). Thus, AA does not appear to participate in *in vitro* prooxidant reactions to produce artifacts of oxidative DNA damage. The stability of AA during storage of seminal plasma was examined, since the samples in experiment B were stored for ≈ 10 weeks (range,

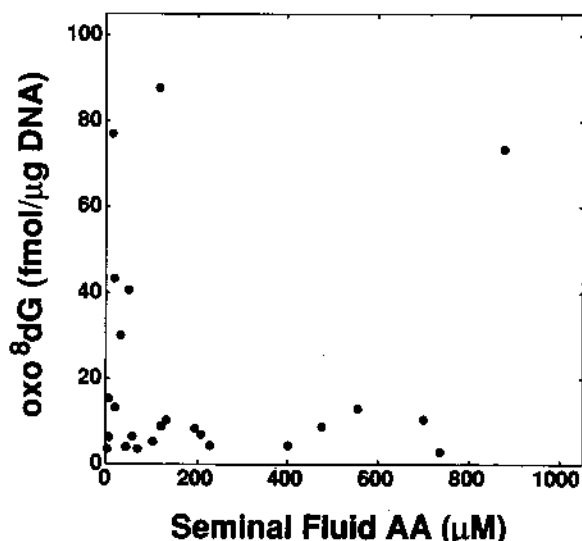


FIG. 1. AA concentrations in seminal fluid and oxo⁸dG in sperm DNA of 24 free-living individuals. Semen was collected and analyzed for AA and oxo⁸dG as described. The AA concentrations are uncorrected for losses during storage (see Results).

8–12 weeks). Seminal plasma from a volunteer, who was not in experiment B, was analyzed for AA the day of semen collection and after storage at -20°C for 7 weeks. During this time, the AA had decreased 25%. The AA values in Fig. 1 are therefore likely to be somewhat low compared to fresh material.

These studies cannot be considered definitive because in experiment B no dietary and smoking histories were available. Future work should examine these factors.

Experiment C. The effect of dietary AA on the level of semen AA and oxo⁸dG in sperm DNA was examined. The oxo⁸dG in sperm DNA from 10 individuals maintained on a controlled diet containing 250 mg of AA per day had a mean value of 34.0 fmol per μg of DNA (Table 1, analysis b). When the dietary AA was lowered from 250 to 5 mg/day, the level of oxo⁸dG doubled to 66.9. The corresponding AA concentrations in semen decreased 49%, from 399 to 203 μM . A modest increase in dietary AA (from 5 to 10 or 20 mg/day) did not prevent a further decrease in semen AA concentration, which dropped to 115 μM . This continued depletion of AA in sperm was associated with a further increase in oxo⁸dG in sperm DNA of 248% over initial values. Repletion of AA (to 250 or 60 mg/day) restored seminal AA to 422 μM and diminished the level of oxo⁸dG by 36%. Thus, the data obtained from the dietary study shows an inverse relationship between the level of oxo⁸dG in DNA and the content of AA in semen.

The baseline values for oxo⁸dG reported in Table 1 (analysis b) are higher than expected. This group of samples was analyzed twice: the first analysis (a) was performed immediately after collection, resulting in a mean value of 13.2 fmol of oxo⁸dG per μg of DNA; the second analysis (b) was performed together with the remainder of the samples that make up Table 1, resulting in a mean of 34.0 fmol of oxo⁸dG per μg of DNA. We attribute this difference to a defective standard used for the second set of measurements, which was used up in the analysis along with the samples. We conclude that it is appropriate to compare the values in Table 1 with each other, but that a best estimate of the absolute baseline value is 13.2 fmol of oxo⁸dG per μg of DNA, which is also comparable to the results in experiments A and B.

DISCUSSION

We report in the present study that endogenous levels of oxidative damage to human sperm DNA is extensive. Since

oxo⁸dG is 1 of ≈ 20 major oxidative DNA damage products (19), we multiply the level of this adduct by 20 to approximate the total oxidative adducts. The level of oxo⁸dG in sperm DNA (13 fmol per μg of DNA) corresponds to 2.1 oxo⁸dG per 10^5 dG or $\approx 2.5 \times 10^4$ oxo⁸dG per sperm cell and an estimated total of $\approx 5 \times 10^5$ oxidative adducts per spermatozoon.

The relationship between high oxo⁸dG in DNA and low AA in seminal plasma from the sperm of both free-living (experiment B) and metabolic unit individuals (experiment C) suggests strongly a protective role of AA against oxidative DNA damage. Although the DNA damage observed in sperm occurs before it has been mixed with seminal fluid, the level of AA presumably reflects its level in seminiferous tubules. In addition, the seminal fluid AA would protect the sperm until fertilization. Thus, the very high level of AA that is dependent on diet plays a critical role in protecting the genome. It seems likely that heritable mutations, genetic birth defects, and cancer will be found to be associated with the high levels of oxidative DNA damage in sperm cells under conditions of low dietary AA. The risk for mutations arising from oxidative DNA adducts in sperm is likely to occur during periods of DNA replication: (i) adducts in the mature sperm can give rise to mutation on division of the zygote, and (ii) observed adducts in sperm are indirect evidence that adducts are also formed during spermatogenesis, increasing the probability of mutations in the sperm. Mutations that occur during DNA replication or during unscheduled DNA synthesis could increase the incidence of genetic defects or cancer in the progeny. Oxidative damage that leads to mutation during the early stages of spermatogenesis could result in clonal expansion of a genetically defective sperm population. DNA repair activities are important in all stages of spermatogenesis prior to nuclear condensation (20). (The DNA is not decondensed until pronuclei formation after fertilization.) When the chromatin is condensed, DNA damage induced by endogenous or exogenous sources will accumulate in the absence of DNA repair activity. Oxidative damage to sperm DNA is not always associated with decreased cell viability or motility, so that some sperm containing mutations that arise during spermatogenesis can effectively reach the ova. Ova-derived DNA repair enzymes can efficiently, but not perfectly, repair this damage (13). Damaged bases that are not repaired during fertilization could cause mutations in subsequent cell divisions during embryogenesis.

Germline mutations in genes relevant to certain human cancers occur at a higher rate in sperm than in oocytes. It has been estimated (11) that 380 mitotic divisions occur during sperm development, compared to only 23 mitotic divisions for the oocyte. The greater number of mitotic cycles needed for sperm development may explain the much higher mutation rate of the paternal vs. the maternal retinoblastoma allele (12). The prevalence of the defective allele being derived from a paternal germline mutation in hereditary retinoblastoma reinforces the notion that extensive mitogenesis, which accompanies spermatogenesis, is a risk factor for paternally inherited cancers and genetic defects.

The protective role of AA may be critical to those populations at high risk for oxidative damage. For example, the marked decrease in blood plasma AA observed in male smokers (21), due presumably to the very high levels of oxidants in cigarette smoke (22, 23), appears to be associated with an increased incidence of leukemia and lymphoma in their offspring (24). Although the cigarette smoke mutagens responsible for these cancers are not known, increased oxidative damage to sperm DNA, that may result from low AA and increased cigarette-derived oxidants, could play an important role in the development of these cancers.

The importance of AA in protecting against lipid oxidation in human blood plasma has been well documented (14, 15)

and the high concentrations of AA present in semen (25) may play a key role in protecting the easily oxidized sperm lipids (26) from oxidation reactions that would abolish sperm motility and viability. A recent study indicated that dietary AA supplementation improves fertility and sperm quality in male smokers suffering from excessive sperm agglutination and from poor sperm motility and morphology (27). Thus, AA complements the sperm antioxidant defense enzymes superoxide dismutase (28), glutathione peroxidase (29), and catalase (30) that are required for optimal sperm motility. In experiments B (see *Results*) and C (31), low AA was associated with increased DNA damage even though no effect on sperm quality was observed.

In experiment C, it was found that β -carotene levels were generally decreased (32). A low level of β -carotene could in part contribute to the oxidative damage observed. The presence and distribution of other water- and lipid-soluble dietary antioxidants in seminal fluid and spermatozoa is not well characterized, though deficiency of vitamin E has been associated with infertility and decreased sperm motility in rodents (33).

The protective effect of dietary ascorbate on oxidative DNA damage is not inconsistent with the prooxidant role of AA in ferrous ion-catalyzed oxidation reactions that are used to initiate lipid peroxidation-dependent sperm-oocyte fusion *in situ* (34). Seminal plasma iron and copper appear to be bound and unavailable to participate in such prooxidant reactions since oxidative damage is lowered, not enhanced, in the presence of diet-induced increases in seminal plasma AA. Furthermore, incubation of semen with 60–1400 μ M AA, as described in experiment B, did not result in an increase in oxo⁸dG as would be expected if transition metals were available to catalyze this oxidation reaction.

The effect of AA in reducing the level of oxo⁸dG in DNA supports the hypothesis that adequate antioxidant protection is essential to maintain the genetic integrity of sperm cells and to minimize the risks of mutations in germ cells that may lead to birth defects, genetic disease, and/or cancer. Sperm adduct analysis is a noninvasive means to evaluate the genetic toxicity of endogenous and exogenous compounds, and in this regard the measurement of the oxo⁸dG level in sperm DNA may help in understanding optimum antioxidant intakes.

We dedicate this paper to Linus Pauling on his 90th birthday. We thank A. Wyrobek and B. Storey for helpful comments and A. M. Blanco for kindly providing some of the sperm samples used in this study. This work was supported by National Cancer Institute Outstanding Investigator Grant CA 39910 to B.N.A. and by National Institute of Environmental Sciences Center Grant ES01896. C.G.F. was partially supported by Grant RF89033 from the Rockefeller Foundation. P.A.M. was supported by American Heart Association Postdoctoral Fellowship 90-28. M.K.S. was supported by National Institute of Aging Postdoctoral Fellowship AG 05489.

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