

EFFECT OF CRYOPRESERVATION AND SPERM CONCENTRATION ON LIPID PEROXIDATION IN HUMAN SEMEN

Y. WANG, R. K. SHARMA, AND A. AGARWAL

ABSTRACT

Objectives. To assess (1) the effect of increased lipid peroxidation on sperm membrane damage that occurs during the freeze-thaw process of cryopreservation; (2) the effect of sperm concentration on lipid peroxidation in normal fertile men; and (3) the relation between sperm motility, morphology, and lipid peroxidation.

Methods. Lipid peroxidation was measured as levels of malonaldehyde by the thiobarbituric acid method. Malonaldehyde levels in fresh and frozen-thawed semen specimens from normal donors ($n = 20$) were compared at a sperm concentration of $20 \times 10^6/\text{mL}$. Sperm concentrations of 5, 10, 20, and $40 \times 10^6/\text{mL}$ were used to assess the effect of sperm concentration on malonaldehyde levels in frozen specimens. A computer-assisted semen analyzer was used to analyze sperm concentrations and motility. Comparison of the effect of TEST-yolk buffer, glycerol, and Ham's F-10 buffer on lipid peroxidation was studied in fresh semen from normal fertile men ($n = 5$).

Results. Malonaldehyde levels did not differ between fresh and cryopreserved semen specimens ($P = 0.99$). There was no difference in malonaldehyde levels from TEST-yolk buffer, glycerol, and Ham's F-10 buffer. As sperm concentrations increased, so did malonaldehyde levels, from 1.21 ± 0.05 nM/hr at $5 \times 10^6/\text{mL}$ to 3.50 ± 0.46 nM/hr at $40 \times 10^6/\text{mL}$ ($P = 0.000007$). Sperm motility, morphology, and malonaldehyde levels in freeze-thaw specimens were not correlated before or after removal of the cryoprotective medium.

Conclusions. Malonaldehyde levels do not differ between fresh and frozen semen in normal men. Cryopreservation-induced membrane damage is not related to lipid peroxidation but may be associated with stress induced during freezing and thawing. Malonaldehyde levels increase as sperm concentrations increase. There is no correlation between sperm motility, morphology, and malonaldehyde levels. UROLOGY 50: 409-413, 1997. © 1997, Elsevier Science Inc. All rights reserved.

Cryopreservation of semen specimens has become necessary for assisted reproductive procedures in patients with a wide variety of diagnoses, but poor fertilization and pregnancy rates are seen with cryopreserved semen.^{1,2} Freeze-thaw procedures induce sublethal damage to the sperm plasma membrane, resulting in poor motility and

functional characteristics.² Human spermatozoa are particularly susceptible to lipid peroxidation because cellular and intracellular sperm membranes are rich in polyunsaturated fatty acids.³ Unlike somatic cells that rely on cytoplasmic enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase, for their defense, spermatozoa discharge most of their cytoplasm immediately before spermiation and, as a consequence, lose such protection.⁴ Lipid peroxidation can cause irreversible loss of sperm motility, and it serves as a biochemical index of semen quality.^{5,6}

The reasons that sperm motion and functional characteristics decrease during the freeze-thaw process are not clear. Cryopreservation-induced sperm membrane damage in normal semen specimens could be caused by lipid peroxidation or membrane stress during the phase transitions that occur during freeze-thaw. The purpose of this

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From the Andrology Research and Clinical Laboratories, Department of Urology, Cleveland Clinic Foundation, Cleveland, Ohio

Reprint requests: Ashok Agarwal, Ph.D., Andrology Research and Clinical Laboratories, Department of Urology, A19, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195

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study was to determine (1) the effect of lipid peroxidation on the sperm membrane damage that occurs during the freeze-thaw process; (2) the effect of sperm concentration on lipid peroxidation in normal fertile men; and (3) the relation between sperm motility, morphology, and lipid peroxidation levels.

MATERIAL AND METHODS

SEMEN COLLECTION AND FREEZING

Semen samples from 20 healthy donors were evaluated according to World Health Organization criteria.⁷ All subjects abstained from ejaculation for 48 hours. The samples were obtained by masturbation into a sterile specimen cup. The ejaculate was allowed to liquefy at 37°C for 30 minutes, and 5 μ L of the sample was loaded on a 20- μ m counting chamber (Conception Technologies, La Jolla, Calif). Samples were analyzed in a computer-assisted semen analyzer (CASA, Motion Analysis Corporation, Model VP 110, Santa Rosa, Calif). To ensure the accuracy of the CASA results, a manual assessment was done each time, along with the CASA analysis. For cryopreservation of the semen specimens, semen samples were diluted by a dropwise addition of the TEST-yolk buffer freezing medium (Irvine Scientific, Santa Ana, Calif), each time with uniform mixing for 5 minutes. Four additions were carried out to give a final semen with a freezing medium ratio of 1:1 (vol/vol). The cryopreservation vials were placed in a -20°C freezer for 8 minutes and thereafter in liquid nitrogen vapor at -79°C for 2 hours. The vials were then transferred to liquid nitrogen at -196°C. For post-thaw analysis, each semen specimen vial was removed from liquid nitrogen and thawed at room temperature for 5 minutes, followed by incubation for 20 minutes at 37°C.

SPERM MORPHOLOGY DETERMINATION

Seminal smears were made from 10 μ L of each fresh semen specimen and air dried. Smears were stained with the Diff-Quik stain (Baxter Healthcare Corporation, Miami, FL). Slides were examined by light microscopy at 100 \times magnification, and at least 100 spermatozoa were counted and classified as normal or abnormal according to the criteria of Menkveld *et al.*⁸ Spermatozoa were considered normal when the head was smooth and oval, with a well-defined acrosome comprising 40% to 70% of the sperm head area. Heads that were borderline normal were considered abnormal.

LIPID PEROXIDATION MEASUREMENT

Lipid peroxidation levels were measured using the thiobarbituric acid method.^{9,10} The seminal plasma in fresh semen samples and the cryomedium in cryopreserved semen specimens were removed after thawing by centrifugation at 300g for 7 minutes, followed by washing in Ham's F-10 buffer (pH 7.4) (Life Technologies Inc., Grand Island, NY). Sperm concentrations of 5, 10, 20, and 40 $\times 10^6$ /mL were used to study the effect of sperm concentration on malonaldehyde levels in frozen semen specimens (n = 10). Ferrous sulfate and sodium ascorbate (Sigma Chemical Company, St. Louis, Mo) were used to promote lipid peroxidation. Malonaldehyde levels in fresh and frozen specimens were compared at a sperm concentration of 20 $\times 10^6$ /mL.

The washed sperm were incubated with 0.25 mL of ferrous sulfate (2.5 mM) and 0.25 mL of sodium ascorbate (12.5 mM) for 1 hour in a 37°C water bath. Control sperm contained only ferrous sulfate and sodium ascorbate during the incubation period. To precipitate proteins after incubation, 500 μ L of 40% ice-cold trichloroacetic acid (Sigma Chemical

Company) was added. To maintain identical assay volumes, an equal volume of sperm suspension was added to the control samples. The sample was centrifuged at 1600g for 12 minutes, 1 mL of clear supernatant was collected, and 500 μ L of 2% thiobarbituric acid (Sigma Chemical Company) in 0.2 N NaOH (Sigma Chemical Company) was added to the supernatant. The test tubes were boiled at 100°C for exactly 10 minutes. The sample was cooled in crushed ice, and the optical density was measured at 534 nm with an Ultrospec III spectrophotometer (Pharmacia Biotech Inc., Piscataway, NJ). The malonaldehyde level of the sample was calculated by comparing it with the optical density produced by malonaldehyde standard (Sigma Chemical Company). The lipid peroxidation level was expressed as nM MDA/10⁸ sperm/hr.

EFFECT OF TEST-YOLK BUFFER, GLYCEROL, AND HAM'S F-10 BUFFER ON LIPID PEROXIDATION

The effect of TEST-yolk buffer, glycerol, and Ham's F-10 buffer on lipid peroxidation was studied in fresh semen specimens from normal fertile men (n = 5). The specimens were divided into three aliquots. Each aliquot was then diluted 1:1 with the TEST-yolk buffer freezing medium, 5% glycerol, or Ham's F-10 buffer (pH 7.4). The samples were then incubated at 37°C for 2 hours. Seminal plasma and the medium were removed by centrifugation at 300g for 7 minutes, followed by washing in Ham's F-10 buffer. The sperm concentration was adjusted to 20 $\times 10^6$ /mL, and the rest of the lipid peroxidation assay was performed as described above.

EFFECT OF HYDROGEN PEROXIDE ON LIPID PEROXIDATION

Final concentrations of 0.01% and 0.05% H₂O₂ were used to study the effect of H₂O₂ on lipid peroxidation in fresh semen from normal fertile men (n = 5). Seminal plasma was removed by centrifugation at 300g for 7 minutes, followed by washing in Ham's F-10 buffer. The sperm concentration was adjusted to 20 $\times 10^6$ /mL. The specimens were divided into three aliquots of equal volume. One aliquot did not contain H₂O₂ and was used as a control. The second and third aliquots contained the final H₂O₂ concentrations of 0.01% and 0.05%, respectively. All aliquots were incubated at 37°C for 15 minutes. The washed sperm were incubated with 0.25 mL of ferrous sulfate (2.5 mM) and 0.25 mL of sodium ascorbate (12.5 mM) for 15 minutes in a 37°C water bath. The remaining procedure was carried out according to the standard protocol described above.

STATISTICAL ANALYSIS

A paired Student's *t* test was used to compare the malonaldehyde levels between fresh and cryopreserved semen. Repeated measures analysis of variance was used to analyze the effect of different freezing media on malonaldehyde levels. Repeated measures analysis of variance on ranks was used to analyze the effect of sperm and H₂O₂ concentrations on malonaldehyde levels; an alpha value of 0.009 with the Bonferroni correction was used in the pairwise sperm concentration comparisons. Pearson's correlation test was used to assess the correlation between sperm motility, morphology, and malonaldehyde levels. A *P* value of less than 0.05 was considered statistically significant. Results are expressed as mean value \pm standard error or median (interquartile range). The SAS statistical software package (SAS Institute Inc., Cary, NC, 1992) was used for data analysis.

RESULTS

Semen analysis [median (interquartile range)] of fresh ejaculates showed a sperm count of 50.6 \times

$10^6/\text{mL}$ (37.2, 84.8), motility of 55.8% (50.7%, 62.5%), and morphology of 13% (7%, 17.8%). Malonaldehyde levels did not differ significantly between fresh ($24.81 \pm 1.12 \text{ nM}/10^8 \text{ sperm/hr}$) and cryopreserved specimens ($24.86 \pm 1.43 \text{ nM}/10^8 \text{ sperm/hr}$) at a sperm concentration of $20 \times 10^6 \text{ sperm/mL}$. Malonaldehyde levels also did not differ among buffers ($P = 0.80$): $30.81 \pm 2.11 \text{ nM}/10^8 \text{ sperm/hr}$ for TEST-yolk buffer freezing medium, $29.53 \pm 1.47 \text{ nM}/10^8 \text{ sperm/hr}$ for glycerol, and $29.58 \pm 1.20 \text{ nM}/10^8 \text{ sperm/hr}$ for Ham's F-10 buffer.

Malonaldehyde levels increased linearly with increases in H_2O_2 concentrations. A significant difference was seen in malonaldehyde levels in relation to H_2O_2 concentrations ($P = 0.024$). Malonaldehyde levels increased from $16.10 \pm 1.29 \text{ nM/hr}$ (absence of H_2O_2) to $19.90 \pm 2.23 \text{ nM/hr}$ at 0.01% H_2O_2 and $46.00 \pm 3.55 \text{ nM/hr}$ at 0.05% H_2O_2 .

Levels of malonaldehyde increased with increases in sperm concentrations, rising from $1.21 \pm 0.05 \text{ nM/hr}$ at $5 \times 10^6 \text{ sperm/mL}$ to $1.62 \pm 0.12 \text{ nM/hr}$ at $10 \times 10^6 \text{ sperm/mL}$, $2.31 \pm 0.23 \text{ nM/hr}$ at $20 \times 10^6 \text{ sperm/mL}$, and $3.50 \pm 0.46 \text{ nM/hr}$ at $40 \times 10^6 \text{ sperm/mL}$ ($P = 0.000007$) (Fig. 1).

No significant correlation was seen between sperm motility and malonaldehyde levels in fresh and cryopreserved semen specimens before or after the removal of cryomedium (Fig. 2). Similarly, normal sperm morphology did not correlate with malonaldehyde levels in fresh semen ($r = -0.23$; $P = 0.36$). Similarly, in cryopreserved specimens, sperm morphology did not correlate with malonaldehyde levels ($r = 0.16$; $P = 0.50$).

COMMENT

The thiobarbituric acid reaction for malonaldehyde is the most convenient and widely used assay of lipid peroxidation; the outcome of this assay correlates well with other lipid peroxidation assay methods, including chemiluminescence, pentane or ethane formation, and colorimetric reactions based on the reduction of phospholipid hydroperoxides with potassium iodide.^{5,11} Malonaldehyde is a stable product of lipid peroxidation and therefore can be used as a measure of cumulative lipid peroxidation.^{9,10,12} The combination of ferrous sulfate and sodium ascorbate has been used to induce lipid peroxidation as well as to promote the breakdown of lipid peroxides into smaller alkenals like malonaldehyde, the product most often measured in studies involving lipid peroxidation.^{10,13,14}

Comparison of lipid peroxidation results among various reports is difficult, mainly because different investigators used various assay methods.^{5,9,12,15-18} Some variables studied include the

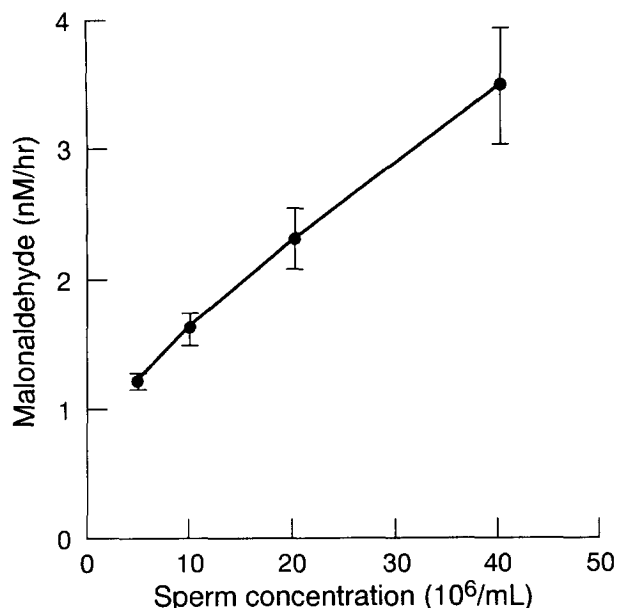


FIGURE 1. Effect of frozen sperm concentration on malonaldehyde level. Values represent mean \pm SE ($n = 10$). Malonaldehyde levels differed significantly among different sperm concentrations ($P = 0.000007$).

type and concentration of lipid peroxidation promoters, duration of sperm incubation, amount of trichloroacetic acid used, centrifugation speed, concentration and amount of thiobarbituric acid used, boiling period, and spectrophotometer wavelength. Our study helps to standardize lipid peroxidation measurement and suggests a normal range of lipid peroxidation in both fresh and frozen semen in normal men. This standardization will make the comparison of malonaldehyde results among different investigators possible.

Hydrogen peroxide is the primary toxic reactive oxygen species for human spermatozoa and can induce lipid peroxidation.¹⁹ In our study, H_2O_2 significantly affected malonaldehyde generation in fresh semen, confirming that H_2O_2 can also produce malonaldehyde.

Investigators have used sperm concentrations ranging from 10 to $200 \times 10^6/\text{mL}$ in determining the level of lipid peroxidation.^{4,6,20,21} The optimal sperm concentration for lipid peroxidation assay is not known. Although all four different sperm concentrations examined by us can be used to measure malonaldehyde, a concentration of $20 \times 10^6/\text{mL}$ is optimal for studies using a computer-assisted semen analyzer because this is the minimum concentration that can be measured with accuracy. Malonaldehyde levels increased linearly with increases in sperm concentration, indicating that sperm generate malonaldehyde.²²

The lack of a significant difference in malonaldehyde levels between fresh and frozen semen specimens in normal men suggests that lipid per-

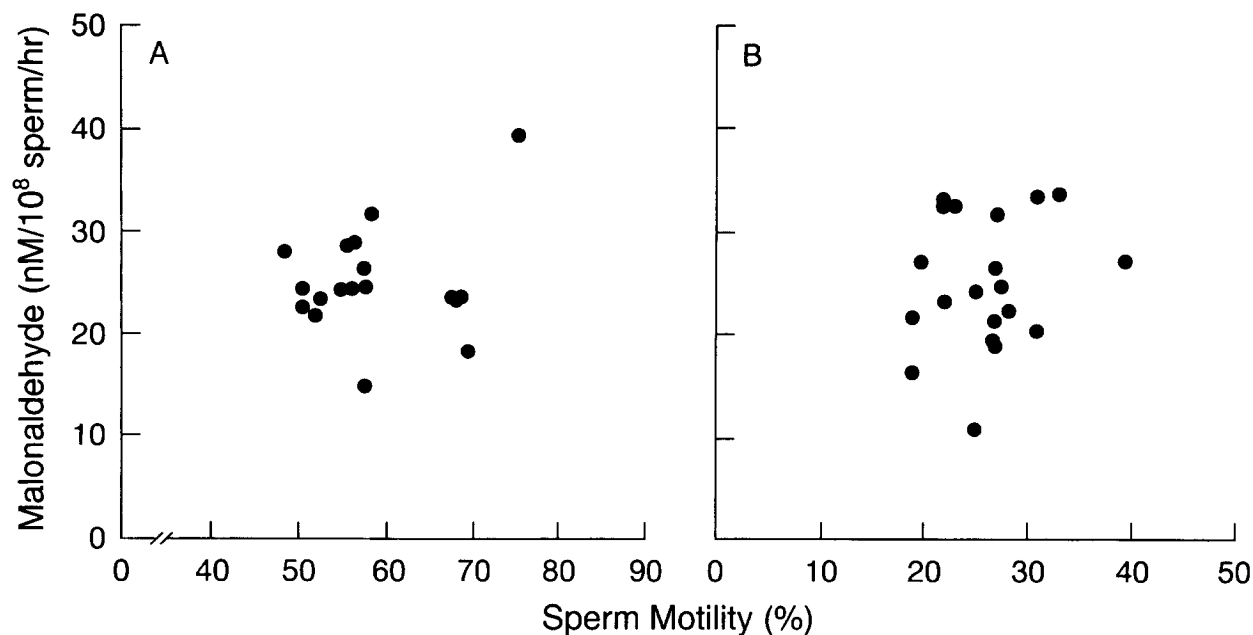


FIGURE 2. Correlation of sperm motility with malonaldehyde levels in fresh (A) and cryopreserved semen (B). Malonaldehyde levels and sperm motility showed no correlation with fresh ($r = 0.26$; $P = 0.28$) or cryopreserved semen ($r = 0.16$; $P = 0.50$).

oxidation is not involved in membrane damage during the freeze-thaw process. The results from our study apply only to semen specimens from normal subjects; however, in subfertile men, higher malonaldehyde levels were observed.²³ Sperm motility is highly correlated with fertilization outcome²¹; however, after cryopreservation, sperm motility decreases by 25% to 75%.²⁴ Cryopreservation-induced membrane damage and the decrease in sperm motility are not related to lipid peroxidation but may be linked to membrane stress induced by freezing and thawing. Our findings support earlier reports that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in frozen-thawed human sperm and that the effect of lipid peroxidation on sublethal cryodamage is negligible.²⁵

Our finding of no difference in malonaldehyde levels between cryopreserved and fresh human semen is contrary to previous reports that malonaldehyde levels are higher in cryopreserved semen.²⁶ Malonaldehyde levels may vary as much as 10-fold among individual donors, and considerable variation does occur within different ejaculates from the same subject in fresh semen samples.¹⁵ In our study, the variation in malonaldehyde levels was small (only threefold) among individual donors both in fresh and cryopreserved semen samples; this variation may be due to the different assay methods used by other investigators.

In addition to the freeze-thaw process itself, cryopreserved sperm differ from fresh sperm in that they must be placed in a freezing medium

(TEST-yolk buffer). The buffer used in the assay may induce malonaldehyde generation in human sperm.²⁷ In normal donors ($n = 5$), lipid peroxidation did not differ in fresh semen incubated with TEST-yolk buffer compared with Ham's F-10 buffer or glycerol, indicating that the freezing medium did not contribute to malonaldehyde generation.

Malonaldehyde levels show poor correlation with ultramorphologic changes,²⁸ indicating that lipid peroxidation is not due to changes in the subcellular organelles. Others have reported negative and positive correlations between malonaldehyde levels and sperm motility.^{15,25,29} We did not find a significant correlation between sperm motility, normal sperm morphology, and malonaldehyde levels in fresh or cryopreserved semen. This lack of correlation indicates that sperm handling, freezing, and freezing medium used do not cause an increase in the level of lipid peroxidation. Increased levels of lipid peroxidation are not an acquired property of the spermatozoa.

CONCLUSIONS

Lipid peroxidation in the semen of normal men is not involved in the membrane damage that occurs during cryopreservation. Sperm membrane damage induced during cryopreservation in normal semen specimens may be linked to membrane stress induced by the freeze-thaw process. The lipid peroxidation level increases with an increase in the sperm concentration. Lipid peroxidation

levels are not related to sperm motility or normal morphology.

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