Inhibition of human sperm respiration by 4-hydroperoxycyclophosphamide and protection by mesna and WR-1065

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Objective: To determine the effect of 4-hydroperoxycyclophosphamide (4OOH-CP) on the respiration of human sperm, and investigate the protective properties of mesna and WR-1065.
Setting: SUNY Upstate Medical University, Syracuse, NY.
Patient(s): Men (n = 12) visited the Andrology Department for fertility evaluation.
Intervention(s): None.
Main Outcome Measure(s): Sperm respiration.
Result(s): Immediate decline in the rate of respiration was observed when 4OOH-CP was added to washed sperm or semen. The inhibition was concentration dependent. The respiration was less affected when 4OOH-CP was added to semen, suggesting the presence of protective factors in the seminal plasma. Excess of mesna or WR-1065 ameliorated the effect of 4OOH-CP. Mesna was the more potent of the two compounds. 4OOH-CP also inhibited the respiration of mitochondria from beef heart.
Conclusion(s): These findings emphasize the adverse effects of alkylating agents on sperm function. The results also provide a framework for thiol drug administration with high-dose alkylating agents to protect male fertility. The protective capacity of seminal plasma deserves further testing. (Fertil Steril® 2009;91:173–8. ©2009 by American Society for Reproductive Medicine.)

Key Words: Alkylating agents, sterility, mitochondria, thiol drugs

Cyclophosphamide [2-(bis(2-chloroethyl)amino)tetrahydro-2-oxide-2H-1,3,2-oxazaphosphorine] is a leading anticancer drug (1). Its hydroxylation by the hepatic microsomal cytochrome P-450 system is required for activating. The resulting 4-hydroxylated metabolite spontaneously degrades to phosphoramide mustard (the active moiety) and acrolein (the highly toxic α,β-unsaturated aldehyde, CH_2=CH–CHO) (2, 3). Phosphoramide mustard (pK_a ~4.8) bears a negative charge at pH ~7.4, and thus it is much less membrane permeable than acrolein or other precursor metabolites (4). 4-Hydroperoxycyclophosphamide (4OOH-CP), a preoxidized analog of cyclophosphamide, is frequently used in vitro as an activated congener of cyclophosphamide, because it spontaneously degrades to phosphoramide mustard and acrolein (5).

The reaction of phosphoramide mustard [*N,N*-bis-2-(2-chloroethyl)phosphorodiamidic acid] involves generating the intermediate phosphoramide aziridinium ion through intramolecular nucleophilic attack (cyclization) of the nitrogen on the β-carbon of chloroethyl chain (2). Nucleophiles (e.g., glutathione, metallothionein, and drug thiols) react rapidly with the phosphoramide aziridinium ions, producing stable thioethers (6).

Clinically, plasma concentrations of cyclophosphamide vary with the dose (7). Peak phosphoramide mustard plasma levels of ~50 to 100 μM have been reported in patients receiving 60 to 75 mg/kg (~1.8-2.2 g/m^2) of the drug (3). The side effects of cyclophosphamide include gonadal atrophy, hematopoietic suppression, cardiac and lung toxicities, hemorrhagic cystitis, and induction of cancer.

The compounds mesna ($HS\text{-}CH_2\text{-}CH_2SO_3Na$) and WR-2721 [amifostine, S-2-(3-aminopropylamino)ethyl phosphorothioic acid, $^+H_3N\text{-}(CH_2)_3\text{-}NH_2^+\text{-}(CH_2)_2\text{-}S\text{-}PO_3H^-$] are used clinically to ameliorate the toxicity of cyclophosphamide (8–10). The protective mechanism of mesna and WR-1065—the active metabolite of WR-2721, $^+H_3N\text{-}(CH_2)_3\text{-}NH_2^+\text{-}(CH_2)_2\text{-}SH$—involves the thiols, which participate in chemical reactions similar to glutathione (11–16). WR-1065 distributes equally between the extra- and intracellular compartments, whereas mesna distributes mostly in the extracellular compartment (10, 11).

Clinically, WR-1065 is rapidly formed from WR-2721, peaking at 100 μM in the plasma and cells. WR-1065 decays in the plasma and cells with a half-life of ~16 minutes (17).

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Cellular mesna uptake occurs at a level much less than WR-1065. For both agents, the predominant intracellular form is the free thiol (9, 10).

The mitochondria are responsible for sperm bioenergetics. This vital organelle is commonly targeted by toxins, including chemotherapeutic agents. Using our home-made instrument (18), we recently used the phosphorescence of Pd (II)-meso-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin to monitor cellular respiration (mitochondrial oxygen consumption) under cytotoxic conditions (19). Oxygen concentration is calculated from the decay of a phosphorescence of the Pd phosphor in solution. The rate of respiration (μM O_2 min^{-1}) is calculated as the negative of the slope of a plot of [O_2] versus t in closed containers (20). In this study, we investigated the adverse reaction of 4OOH-CP on human sperm respiration, and the protection by mesna and WR-1065. For this purpose, mitochondrial oxygen consumption (cellular respiration) was monitored over long periods of time in sperm suspensions treated with 4OOH-CP (18–21).

MATERIALS AND METHODS

Study Population

Semen was obtained from 12 patients attending the Andrology Laboratory at State University of New York Upstate Medical University (21). The samples were evaluated according to the World Health Organization criteria (22). The study was approved by the institutional review board for protection of human subjects at State University of New York, Upstate Medical University. Informed consent was obtained from each patient.

Semen was liquefied at 37°C for 30 min. Each sample was then mixed, divided into equal aliquots (usually 1.0 mL each), and stored at 25°C until used (storage time, $\leq$1 hour). To measure respiration, an aliquot was diluted twofold in mHTF and centrifuged at 25°C (300 × g) for 10 min. The pellet was suspended in 1.0 mL mHTF plus 2 μM Pd phosphor and placed in our home-made oxygen analyzer for measurement of sperm respiration as previously described (18–21).

Chemicals and Solutions

4-Hydroperoxycyclophosphamide (D-18864, MW 293.09) was obtained from ASTA Medica AG (Frankfurt, Germany); 4OOH-CP solutions were made in dH_2O *immediately* before each addition. Mesna (MW 164.18; 100 mg/mL or 609 mM) was obtained from Bedford Laboratories (Bedford, OH) and stored at 25°C. WR-1065.2HCl (MW 207.16) was obtained from U.S. Bioscience (West Conshohocken, PA). The WR-1065 solution was prepared in dH_2O and stored at −70°C in small aliquots; its concentration was determined by titration with 5,5'-dithio-bis(2-nitrobenzoic acid) (9). Pd (II) complex of *meso*-tetra-(4-sulfonatophenyl)-tetrabenzoporphyrin sodium salt (Pd phosphor) was purchased from Porphyrin Products (Logan, UT). Pd phosphor solution (2.5 mg/mL or 2.0 mM) was made in dH_2O and stored at −20°C in small aliquots. Modified human tubal fluid (mHTF, containing 97.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 4.0 mM $NaHCO_3$, 21 mM HEPES, 2.78 mM glucose, 0.33 mM Na pyruvate, 21.4 mM Na lactate, 10 μg/mL gentamicin sulfate, 5 mg/L phenol red and 0.5% bovine serum albumin; pH 7.2) was purchased from Irvine Scientific. The remaining reagents were purchased from Sigma-Aldrich (St. Louis, MO). NaCN (1.0 M) was made fresh in dH_2O; the pH was adjusted to ~7.0 with 12 N HCl immediately before use (careful titration is necessary to avoid an acid solution that could produce hydrogen cyanide).

Cellular Respiration

O_2 concentrations in sperm suspensions were determined as a function of time. The phosphorescence decay ($1/\tau$) of the Pd phosphor probe was exponential. The values of τ were linear in [O_2]: $\tau^o/\tau = 1 + \tau^o k_q$ [O_2]; τ, lifetime in the presence of O_2; τ^o, lifetime in the absence of O_2; k_q, second-order O_2 quenching constant. Samples were exposed to 10 light flashes per second from a pulsed light-emitting diode array with peak output at 625 nm (OTL630A-5-10-66-E, Opto Technology, Inc., Wheeling, IL). Emitted light was detected by a Hamamatsu photomultiplier tube after passing through a wide-band interference filter centered at 800 nm. The amplified phosphorescence decay was digitized at 1 MHz by an A/D converter (Computer Boards, Inc., Norton, MA). The values of τ were determined in a series of ascorbate plus ascorbate oxidase solutions, simultaneously with electrochemical measurements of [O_2]. A plot of $1/\tau$ vs. [O_2] was linear; the value of k_q (the slope) was 96.1 ± 1.2 μM^{-1} s^{-1} and $1/\tau^o$ (the intercept) 10,087 ± 156 s^{-1} (19, 20).

Sperm respiration was measured at 37°C in sealed vials (8-mm clear vials, Krackler Scientific, Albany, NY). For each run, 1.0 mL of washed sperm suspension was placed in 1.0-mL glass vials. The vials were sealed with a crimp-top aluminum seal (using a Wheaton hand crimper; Fisher Scientific, Fairlane, NJ). Mixing was with the use of a parylene-coated stirring bar (1.67 × 2.01 × 4.80 mm; V&P Scientific, Inc., San Diego, CA). Rate of respiration (k, in μM O_2 min^{-1}) was the negative of the slope of [O_2] versus t. The addition of 10 mM NaCN (final concentration) resulted in inhibition of oxygen uptake, confirming the decline in [O_2] with t was mostly because of mitochondrial consumption (21, 23, 24). Other additions included glucose oxidase (5 μL = 7.0 units), which catalyzed the reaction: β-Glucose + H_2O + ½O_2 → Glucono-1,5-lactone + H_2O_2.

Mitochondrial Respiration

Measurements with mitochondria were included to determine any direct effect of 4OOH-CP on the mitochondrial respiratory chain. The mitochondria were prepared from beef heart (25) and suspended in 1.0 mL of 10 mM Tris-Cl (pH 8.2), 250 mM sucrose, 2 μM Pd phosphor and 0.5% fat-free albumin. The mixture was then placed in our home-made oxygen analyzer for measurement of mitochondrial respiration as previously described (18–20).

RESULTS

4-Hydroperoxycyclophosphamide Added to Washed Sperm

We first investigated the effect of 4OOH-CP (34 μM) when injected into washed sperm suspensions during the oxygen measurement. Samples from two patients are shown in Figure 1. For patient A (0.19×10^8 sperm per run), the addition of 34 μM 4OOH-CP decreased the rate of respiration (k) from 0.46 μM O_2/min to 0.27 μM O_2/min (41% inhibition). For patient B (0.55×10^8 sperm per run), the addition of 34 μM 4OOH-CP decreased the value of k from 1.27 μM O_2/min to 0.84 μM O_2/min (34% inhibition). Thus, immediate inhibition of respiration was observed with therapeutic concentrations of 4OOH-CP.

We next investigated the protection by mesna and WR-1065. Sample from one patient is shown in Figure 2 (1.88×10^8 sperm per run). The addition of 200 μM 4OOH-CP decreased the value of k from 1.33 μM O_2/min to 0.51 μM O_2/min (62% inhibition). In the presence of 2.0 mM mesna, the addition of 200 μM 4OOH-CP increased the value of k from 1.92 μM O_2/min to 2.24 μM O_2/min (17% increment). In the presence of 2.0 mM WR-1065, the addition of 200 μM 4OOH-CP decreased the value of k from 1.65 μM O_2/min to 1.52 μM O_2/min (8% inhibition). The addition of 10 mM NaCN resulted in about 90% inhibition of respiration. The remaining oxygen in the solutions was rapidly depleted with the addition of glucose oxidase.

In another patient (data not shown; 1.38×10^8 sperm per run), the baseline value of k was ~0.70 μM O_2/min. The value of k was 0.15 μM O_2/min in the presence of 200 μM 4OOH-CP alone (79% inhibition), 0.97 μM O_2/min in the presence of 2.0 mM mesna plus 200 μM 4OOH-CP (39% increment), and 0.67 μM O_2/min in the presence of 2.0 mM WR-1065 plus 200 μM 4OOH-CP. Thus, 10-fold excess of mesna or WR-1065 protected sperm respiration from the effect of 4OOH-CP. Fivefold excess of mesna or WR-1065 provided ≤53% protection (data not shown).

FIGURE 1

Sperm respiration with additions of 34 μM 4OOH-CP. Washed sperm from two patients are shown. The inhibition in patient A (circles, 0.19×10^8 sperm per run) was ~41% and in patient B (diamonds, 0.55×10^8 sperm per run) ~34%.

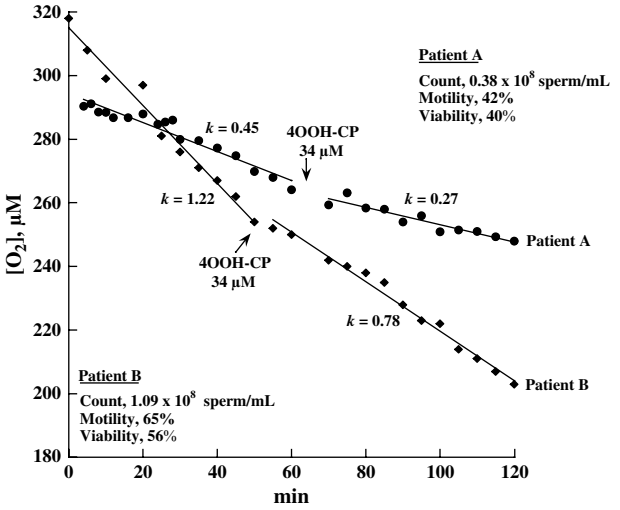

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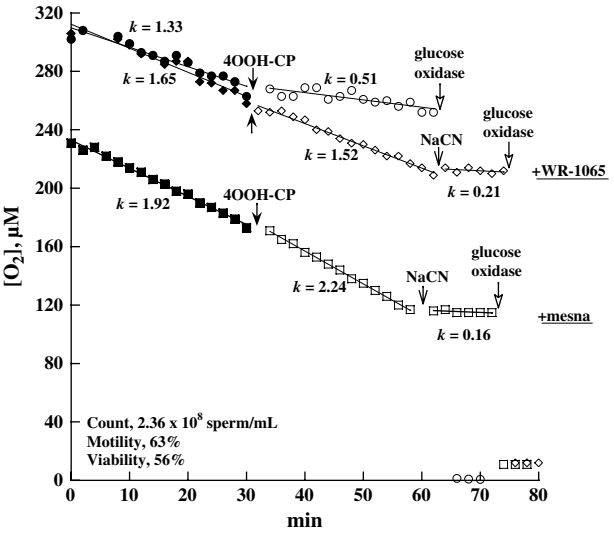
FIGURE 2

Sperm respiration with additions of 200 μM 4OOH-CP, with and without 2.0 mM mesna or WR-1065. Washed sperm (1.89×10^8 sperm per run) from one patient is shown. Circles, with no added thiol drugs; diamonds, with added WR-1065; squares, with added mesna. The addition of 10 mM NaCN and seven units of glucose oxidase are also shown.



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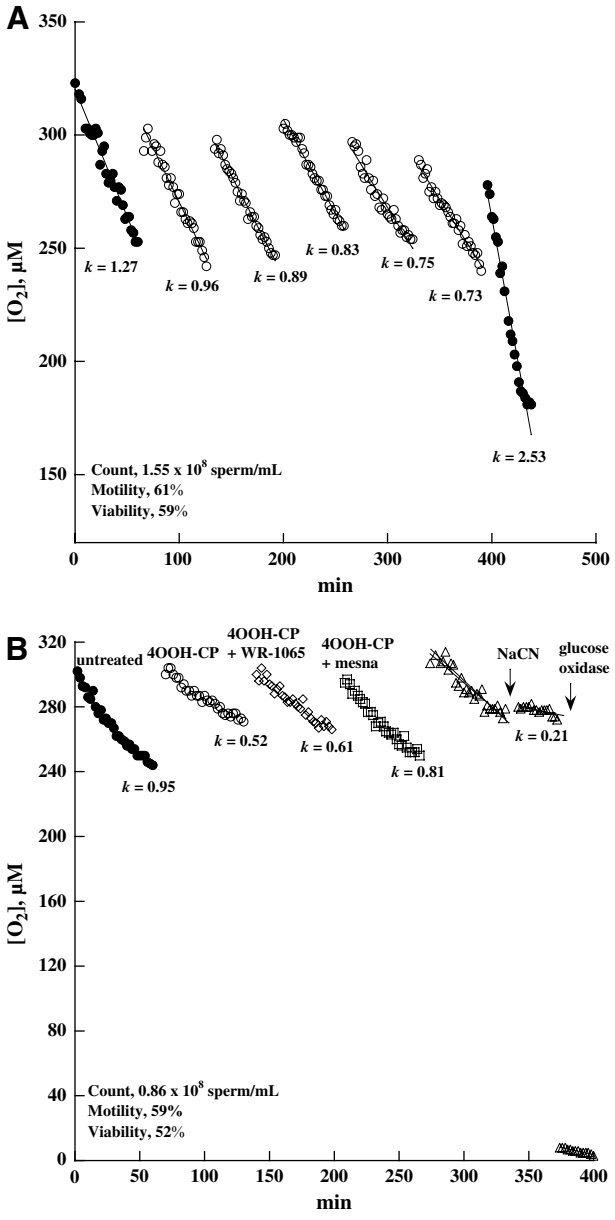
4-Hydroperoxycyclophosphamide Added to Semen

We then studied the effect of 4OOH-CP when added to semen. The semen sample (1.55×10^8 sperm per mL) was divided into seven equal aliquots (1.0 mL each). The aliquots were incubated at 25°C for the indicated periods of time with and without 100 μM 4OOH-CP (Fig. 3A; minute zero corresponds to the addition of 4OOH-CP). At the end of each incubation period (Fig. 3A), the samples were washed and analyzed for oxygen consumption. For untreated semen, the value of k increased from 1.27 μM O_2/min at min zero to 2.53 μM O_2/min at minute 360; this ~twofold increment was likely because of sperm capacitating. For 4OOH-CP-treated semen, the values of k decreased exponentially with time ($r > 0.996$ for $60 \leq t \leq 240$ min). The degree of inhibition at 60 minutes was ~15% and at 300 minutes ~35%.

The protection by mesna and WR-1065 is shown in Figure 3B. The semen sample (0.86×10^8 sperm per mL) was

FIGURE 3

Inhibition of sperm respiration by 4OOH-CP in semen and protection by 2.0 mM mesna or WR-1065. (**A**) The semen sample (1.55×10^8 sperm per mL) was divided to seven equal aliquots (1.0 mL each). The aliquots were then incubated at 25°C with (open circles) and without (closed circle) 100 μM 4OOH-CP for indicated periods of time; minute zero corresponds to the addition of 4OOH-CP. At the end of each incubation period, the sample was washed and analyzed for oxygen consumption. (**B**) The semen sample (0.86×10^8 sperm per mL) was divided into five equal aliquots (1.0 mL each). The aliquots were incubated at 25°C for 60 minutes without addition (closed circles) or with the addition of 4OOH-CP alone (open circles), WR-1065 plus 4OOH-CP (diamonds) or mesna plus 4OOH-CP (squares). At the end of the incubation period, each sample was washed and analyzed for oxygen consumption. The value of *k* decreased by 78% with addition of 10 mM NaCN (triangles). The remaining oxygen in the solution was rapidly consumed addition of 7.0 units of glucose oxidase (triangles).



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divided into five equal aliquots (1.0 mL each). The aliquots were preincubated at 25°C with and without 2.0 mM mesna or WR-1065 for 5 minutes. 4-Hydroperoxycyclophosphamide (200 μM) was then added and the mixtures were incubated at 25°C for 60 minutes. At the end of the incubation period, the samples were washed and analyzed for oxygen consumption. The value of k decreased by ~45% with 4OOH-CP alone, by ~36% with WR-1065 plus 4OOH-CP, by ~15% with mesna plus 4OOH-CP, and by 78% with 10 mM NaCN. The remaining oxygen in the solution was rapidly consumed with the addition of glucose oxidase (Fig. 3B). Thus, respiration was less affected when 4OOH-CP was added to semen, suggesting the presence of protective factors in the seminal plasma. Moreover, mesna and WR-1065 protected sperm from the effect of 4OOH-CP.

Mitochondrial Respiration

The effect of 4OOH-CP on the respiration of mitochondria from beef heart was also studied. In the presence of 140 μM 4OOH-CP, the value of k decreased by about 65% (data not shown).

DISCUSSION

The alkylating agents are a well-known cause of male sterility. To further investigate this serious adverse effect, we measured sperm respiration in the presence of 4OOH-CP. We also studied the protection by mesna and WR-1065. Immediate inhibition of sperm respiration was noted in the presence of therapeutic phosphoramide mustard concentrations (35–200 μM) (Figs. 1–2) (3). An exponential inhibition of sperm respiration with time was observed when 100 μM 4OOH-CP was added to semen (Fig. 3A). In washed sperm, the 10-fold excess of mesna or WR-1065 provided a nearly full protection (Fig. 2), whereas the five-fold excess provided partial protection. In semen, the protection was less prominent (Fig. 3B). 4OOH-CP depletes cellular glutathione, and excess of WR-1065 or mesna prevents the depletion (5, 24, 26). Moreover, the binding of cyclophosphamide metabolites to cell components decreases by about 50% in the presence of equal molar concentration of mesna or glutathione (16).

Available thiols are determined by the pK_a (~9.1 for mesna and ~7.7 for WR-1065) (12–15). At pH 7.2 (pH of mHTF), the equation: $pH = pK_a + \log (RS^-/RSH)$ shows that the thiolate anion represents about 1% of the total mesna and about 32% of the total WR-1065. Thus, protection by WR-1065 is expected to be about 30 times more than mesna. The finding that mesna is more effective than WR-1065 (Figs. 2–3) implies that considerations other than pK_a contribute to sperm protection (e.g., the primarily extracellular distribution of mesna) (10, 11).

The second-order rate constants for reactions of mesna and WR-1065 with 4OOH-CP are 25 ± 5 and 880 ± 50 M^{-1} s^{-1}, respectively. The corresponding rate constants for reactions of mesna and WR-1065 with acrolein are 700 ± 150 and >2000 M^{-1} s^{-1}, respectively (26). The rate limiting step of the alkylation reaction involves only formation of the aziridinium ion. Because most drug thiols react rapidly with the aziridinium ion, differences in the "protection levels" are likely related to multiple factors other than the rate constants alone.

Various metabolites (acrolein, phosphoramide mustard, and possibly H_2O_2) may be responsible for the inhibitory effect of 4OOH-CP on sperm respiration. High thiol concentrations may reversibly bind to the 4-hydroxycyclophosphamide and aldophosphamide metabolites, slowing phosphoramide mustard formation. It should also be noted that similar binding could occur between the two metabolites and the HEPES buffer (21 mM in mHTF) (27). However, very similar results were obtained when 4OOH-CP was added to the semen samples (Fig. 3A–B), which did not contain HEPES.

The fact that the drug immediately inhibits respiration suggests a direct injury to sperm components (e.g., membrane, proteins, mitochondria, etc.). 4-Hydroperoxycyclophosphamide also inhibits the respiration of isolated beef heart mitochondria (see Results), supporting a direct toxicity on the mitochondrial respiratory chain. Clinical studies are necessary to determine whether thiol drugs can improve the fertilizing capacity of human sperm in patients receiving high-dose alkylating agents.

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