

Effects of the lipid peroxidation product (E)-4-hydroxy-2-nonenal on ram sperm function

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(E)-4-hydroxy-2-nonenal (HNE) is a lipid peroxide end-product which exerts powerful biological effects in a variety of cell and tissue systems. The effects of exogenous HNE on ram spermatozoa were examined *in vitro*. HNE inhibited the motility of diluted ram spermatozoa in a dose-dependent ($100\text{--}400\ \mu\text{mol l}^{-1}$) manner ($P < 0.05$). The extent of motility loss varied with sperm concentration as well as with HNE concentration, and was manifested as a progressive decrease in mean sperm velocity. The suppressive effect of $250\text{--}500\ \mu\text{mol HNE l}^{-1}$ on the motility of reactivated ram sperm models ($P < 0.05$) was prevented by the addition of $1\ \text{mmol reduced glutathione l}^{-1}$ to the reactivation medium, suggesting that HNE inhibits ram sperm motility via oxidation of sulfhydryl groups in the axoneme. Oxygen uptake by ram spermatozoa was inhibited ($P < 0.05$) by the addition of 100 or $200\ \mu\text{mol HNE l}^{-1}$. Glucose utilization was maintained in the presence of $200\ \mu\text{mol HNE l}^{-1}$, suggesting that fructolysis was unaffected by HNE. As was the case with motility, the inhibition of oxidative metabolism by HNE was not reversed by washing the spermatozoa. The activity of ram sperm acrosomal enzymes released by cold shock, as measured by hydrolysis of *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), was reduced in the presence of $100\ \mu\text{mol HNE l}^{-1}$ ($P < 0.05$). No evidence was found of disruption of the acrosomal outer membrane or the sperm plasma membrane as a result of exposure to HNE. The effects of HNE on the motility, oxidative metabolism and enzyme activity of ram spermatozoa are in keeping with the effects of lipid peroxides on spermatozoa, and of HNE in other biological systems, reported elsewhere. The lack of membrane disruption by HNE is surprising, given the ability of exogenous peroxide mixtures to disrupt sperm membranes, and the powerful haemolytic activity of HNE.

Introduction

Aerobic incubation of mammalian or avian spermatozoa is typically associated with a deterioration of motility and metabolic function (review by Mann and Lutwak-Mann, 1981). Jones and Mann (1973) pointed out that analogous changes could be induced in somatic cells by lipid peroxidation and demonstrated the accumulation of a peroxidation product (malonaldehyde) by ram spermatozoa *in vitro*.

The effects of endogenous or exogenous lipid peroxides on ram spermatozoa include irreversible loss of motility, inhibition of fructolysis and respiration, leakage of intracellular enzymes and structural damage to the plasma membrane (Jones and Mann, 1976, 1977a, b). Motility loss due to fatty acid peroxides has also been noted in spermatozoa of boars, rats, stallions (Jones and Mann, 1977a), humans (Jones and Mann, 1977a; Jones *et al.*, 1979; Diezel *et al.*, 1980), rabbits (Jones and Mann, 1977a; Alvarez and Storey, 1982), bulls (Jones and Mann, 1977a; Slaweta *et al.*, 1988), roosters (Fujihara and Howarth,

1978) and mice (Alvarez and Storey, 1984b). Severe plasma membrane damage has also been ascribed to peroxidation of membrane lipids in rabbit (Alvarez and Storey, 1984a) rooster (Fujihara and Koga, 1984) and bull (Slaweta *et al.*, 1988) spermatozoa. Exposure to peroxidized lipids has been associated with a decline in the fertilizing capacity of rooster spermatozoa, even at concentrations that are too low to impair motility (Wishart, 1984). Increased production of free radicals by human spermatozoa has been linked to decreased ability to penetrate oocytes, and peroxidation has been proposed as a likely mechanism (Aitken and Clarkson, 1987).

In light of the observation that peroxidation and free radical production are most active in damaged or less motile spermatozoa (Jones and Mann, 1973, 1977b; Jones *et al.*, 1978, 1979; Aitken and Clarkson, 1988; Slaweta *et al.*, 1988), it seems likely that peroxidation is a factor in the toxic effects that such spermatozoa have been shown to exert on healthier spermatozoa (Shannon, 1965; Shannon and Curson, 1972; Sexton, 1988). This contention and the role proposed for lipid peroxides in sperm senescence by Jones and Mann (1973) suggests that management of peroxidation is an important consideration when preparing spermatozoa for long term storage.

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The hydroxyalkenals are among the most plentiful (Esterbauer *et al.*, 1982) and toxic products of biological peroxidation. Although these compounds have been examined for their effects on a variety of tissues, notably in the liver (reviewed by Comporti, 1985), their influence on spermatozoa has largely escaped scrutiny. A number of their effects have the potential to play a role in lipid peroxidation damage to spermatozoa. Membrane damage (Benedetti *et al.*, 1977, 1979; Roders *et al.*, 1978), for instance, leads to motility loss and impaired acrosomal function. Inhibition of adenylyl cyclase (Paradisi *et al.*, 1985) prevents synthesis of cyclic AMP, which plays an important role in flagellar motility (Mann and Lutwak-Mann, 1981). The reactivity of aldehydic peroxides with the free sulphhydryl groups of tubulin (Gabriel *et al.*, 1983, 1985) suggests that the axoneme is directly inhibited, via its microtubular components. Even if motility were maintained, and fertilization unimpaired by acrosomal lysis or inhibition of acrosomal enzymes, the spermatozoa could still be rendered ineffective by the direct effects of hydroxyalkenals on the genome (Brambilla *et al.*, 1986; Griffin and Segall, 1986; Winter *et al.*, 1986).

One of the most plentiful (Benedetti *et al.*, 1980; Poli *et al.*, 1985) and cytotoxic (Cadenas *et al.*, 1983) hydroxyalkenals is (*E*)-4-hydroxy-2-nonenal (HNE). The presence of HNE has been demonstrated in human semen, as has its ability to abolish motility in human spermatozoa (Selley *et al.*, 1991). The aim of the following experiments was to observe the effects of exogenous HNE on ram spermatozoa *in vitro*, and to examine these in the light of previous reports of lipid peroxide intoxication of spermatozoa.

Materials and Methods

Preparation and assessment of sperm suspensions

Semen was collected from mature Merino rams by electro-ejaculation, and its quality assessed on a scale from 0 to 4 as described by Emmens (1947). Precautions were taken to prevent cold shock to spermatozoa during collection or storage. Spermatozoa were counted in a haemocytometer (sperm concentrations typically ranging from 2×10^9 to 4×10^9 spermatozoa ml^{-1}). Semen was diluted to the desired sperm concentration by addition of warmed Krebs–Ringer phosphate solution (Umbreit *et al.*, 1959) containing 0.1% glucose (KRPBG).

Motility of experimental samples assessed under light microscopy was scored on a scale of 0 to 4, including half values. A drop of sperm suspension was placed on a microscope slide under a coverslip and examined on a warm stage at 37°C. A motility index (1–100) was produced by multiplying this score by the estimated percentage of motile spermatozoa present in the sample, and dividing the product by four.

Preparation of HNE

HNE was supplied as a stock solution (approximately 70 mmol l^{-1}) in chloroform. Aqueous solutions of HNE for experimental purposes were prepared as follows. A 50 μl aliquot of stock solution was transferred to a 10 ml glass centrifuge tube and gently agitated until the chloroform evaporated, leaving a few small droplets of HNE. These droplets were dissolved by the addition of 500 μl of the appropriate buffer,

covered and vortexed for 30 s. The HNE concentration of the resulting solution was determined by UV spectrophotometry as described by Selley *et al.* (1988).

Photographic measurement of velocity of ram spermatozoa

The swimming velocity of ram spermatozoa was determined by the stroboscopic technique described by Molinia and Swan (1991). Spermatozoa were diluted to a final concentration of $2 \times 10^7 \text{ ml}^{-1}$ in KRPBG, which contained 1% BSA to prevent agglutination of spermatozoa or adhesion of spermatozoa to glass. A droplet, 12.5 μl , of sperm suspension was mounted on a cleaned microscope slide, under a cleaned coverslip.

Sperm movement in control and treated samples was recorded on 35 mm Tri-X film (400 ASA) using 0.5 or 1 s exposures with a Wild MPS 12 camera. A stroboscope was matched to the tailwave frequency and mounted between a Xenon arc light source (Wild) and a Zeiss WL microscope with a 0.8/0.95 dark field condenser, 40 \times /0.65 objective and 16 \times Kpl ocular.

Motility observations were made at room temperature (23–25°C). All observations were made within 3 h of semen collection.

Velocity ($\mu\text{m s}^{-1}$) was calculated by measuring the net displacement of the sperm head (in μm) and dividing by the exposure time (in seconds) to give the point-to-point straight line velocity.

Reactivation of ram sperm models

The techniques for demembration and reactivation of ram sperm models were based on those of White and Voglmayr (1986) as modified by Vishwanath *et al.* (1986). Sperm membranes were extracted by pipetting a 100 μl aliquot of sperm suspension ($1 \times 10^8 \text{ ml}^{-1}$) into 0.9 ml of demembration medium, consisting of 25 mmol potassium glutamate l^{-1} , 0.2 mol sucrose l^{-1} , 1 mmol $\text{MgSO}_4 \text{ l}^{-1}$, 2 mmol dithiothreitol l^{-1} , 1 mmol EGTA l^{-1} , and 0.025% (w/v) Triton X-100. Spermatozoa were gently agitated for 15 s, and diluted tenfold into a reactivation medium, which had the same composition as the demembration medium except that Triton X-100 was replaced by 50 $\mu\text{mol cAMP l}^{-1}$ and 1 mmol ATP l^{-1} . Soybean trypsin inhibitor (40 $\mu\text{g ml}^{-1}$) was added to the reactivation medium to reduce ultrastructural damage to sperm models by the acrosomal proteases released at demembration (Vishwanath *et al.*, 1986).

A drop of sperm suspension from the reactivation mixture was transferred to a 10 μm Makler chamber and examined under an Olympus BH2 light microscope. The proportion of spermatozoa exhibiting tail movement (percentage reactivation) was recorded at regular intervals.

Substances being tested for their effects on the axoneme were added to the reactivation medium before the addition of spermatozoa.

D-glucose and L-lactate analyses

Spermatozoa were washed twice in Krebs–Ringer phosphate solution containing 0.05% (w/v) glucose (to remove endogenous lactate and fructose) before counting and dilution to

1×10^8 spermatozoa ml^{-1} . The spermatozoa were then incubated at 30°C with HNE (0, 100 or $200 \mu\text{mol l}^{-1}$). Immediately, and at 1, 2 and 3 h after the addition of HNE, a 1 ml aliquot of sperm suspension was deproteinized with 1 ml 0.3 M Ba(OH)_2 and 1 ml 5% (w/v) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. The resulting suspension was centrifuged at 500 g for 15 min. The supernatant was removed, and stored at -20°C until required for glucose and lactate determination. D-Glucose and L-lactate concentrations were measured enzymically as described by Bergmeyer *et al.* (1974) and Gutman and Wahlefeld (1974).

Measurement of oxygen uptake by spermatozoa

Spermatozoa were diluted in KRPG to a concentration of 1×10^8 spermatozoa ml^{-1} , and their rate of respiration at 30°C was measured with an oxygen electrode (Rank Bros., Cambridge, UK). Oxygen uptake ($\mu\text{l } 10^{-8}$ spermatozoa h^{-1}) was determined from the slope of the chart recorder trace of the electrode.

Measurement of amidase activity of spermatozoa

The effect of HNE on acrosomal enzymes was compared by measurement of sperm-bound amidase activity. This technique monitors hydrolysis of *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), a synthetic chromogenic substrate of trypsin-like enzymes (Froman *et al.*, 1984, 1987). The activity of such enzymes in spermatozoa is restricted to the acrosome (Yanagimachi and Teichman, 1972). Lysis of the acrosomal membrane releases proteases which hydrolyse the amide bonds present in BAPNA, resulting in a colour change. The product of this reaction is detectable at 410 nm, and its concentration is directly proportional to the level of proteolytic enzyme activity in the suspending medium.

Ram spermatozoa were diluted to $1 \times 10^8 \text{ ml}^{-1}$ in KRPG, and incubated at 30°C for 90 min in the presence of 0, 100 or $200 \mu\text{mol HNE l}^{-1}$. Spermatozoa were then divided into 1.5 ml aliquots, half of which were cold shocked to disrupt the acrosomal plasma membrane.

Amidase activity was determined by the addition of 0.5 ml of $2.5 \text{ mmol BAPNA l}^{-1}$ in KRP containing 0.3% polyvinyl alcohol. After incubation for 30 min at 30°C , further amidase activity was inhibited by the addition of 0.5 ml of $50 \text{ mmol benzamidine l}^{-1}$ in KRP. Spermatozoa were pelleted by centrifugation (15 min, 500 g), and 1.5 ml of supernatant was diluted 1:1 in KRP before measurement of its optical density at 410 nm.

Supravital staining of ram spermatozoa

The proportion of spermatozoa with intact plasma membranes was judged on the basis of their ability to exclude eosin (Hancock, 1951). Sperm suspensions were diluted fivefold in eosin-nigrosin stain, and thin films prepared on precleaned slides. Smears were air dried, and examined by bright field microscopy. Two-hundred spermatozoa were examined on each slide, and the relative proportions of stained and unstained cells determined.

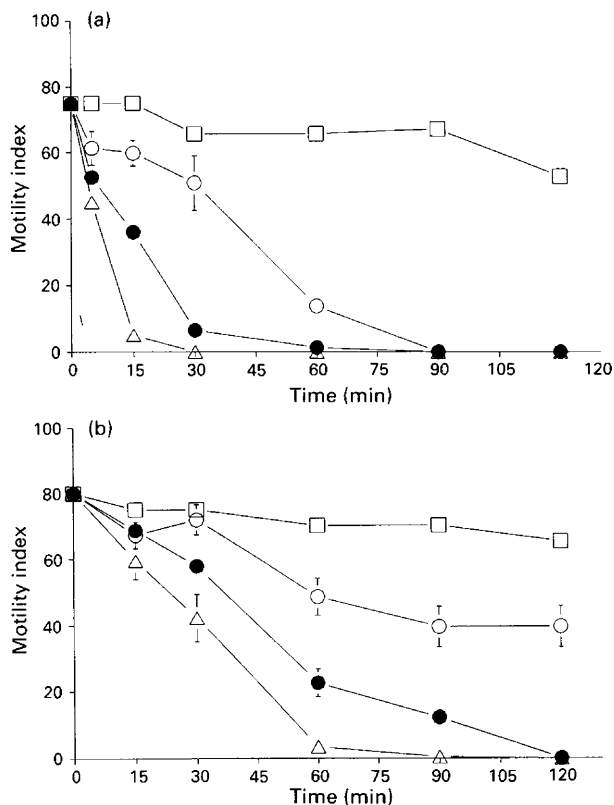


Fig. 1. The effect of incubation with (\square) 0, (\circ) 100, (\bullet) 200 or (Δ) $400 \mu\text{mol (E)-4-hydroxy-2-nonenal (HNE) l}^{-1}$ on the motility of fresh ram spermatozoa diluted to (a) 1×10^7 or (b) 1×10^8 spermatozoa ml^{-1} in Krebs-Ringer phosphate solution containing 1% glucose (KRPG). Points represent the mean (\pm SEM) of four observations.

Chemicals

HNE ((*E*)-4-hydroxy-2-nonenal) was the gift of H. Esterbauer (University of Graz, Austria). The purity of HNE was confirmed by high performance liquid chromatography. BAPNA, benzamidine, reduced glutathione, cyclic AMP, EGTA, potassium glutamate and soybean trypsin inhibitor were purchased from Sigma Chemicals (St Louis, MO). Hydrazine hydrate was supplied by Ajax Chemicals (Auburn, NSW), and dithiothreitol by Calbiochem (La Jolla, CA). ATP, NAD, NADPH, LDH, glucose-6-phosphate dehydrogenase and hexokinase were supplied by Boehringer-Mannheim (Sydney, NSW). All chemicals were of analytical reagent or the highest quality alternative grade.

Statistical analyses

Data were analysed by analysis of variance. Differences between specific means were determined using either least significant differences or orthogonal contrasts (Steel and Torrie, 1981).

Results

Effect of HNE on the motility of ram spermatozoa

The impact of HNE on ram sperm motility is demonstrated (Fig. 1). The reduction in sperm motility during incubation at

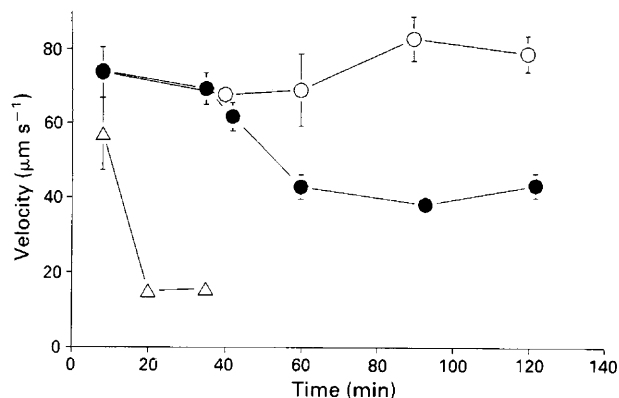


Fig. 2. The effect of (*E*)-4-hydroxy-2-nonenal (HNE) (\circ) 0, (\bullet) 250 or (\triangle) 500 $\mu\text{mol l}^{-1}$ on the velocity of ram spermatozoa (10^7 ml^{-1}) as estimated by strobographic photography. Points represent the mean (\pm SEM) of between three and ten observations.

30°C was clearly dose dependent, and the means corresponding to 0, 100, 200 and 400 $\mu\text{mol HNE l}^{-1}$ all differed significantly ($P < 0.01$). Both the overall effect of HNE ($P < 0.01$) and the time taken for motility to decline ($P < 0.01$) were also strongly influenced by the concentration of spermatozoa present in the incubation mixture. This was also the case for spermatozoa outside the concentration range (10^7 – 10^8 ml^{-1}) shown. Incubation of $1 \times 10^6 \text{ spermatozoa ml}^{-1}$ with 100 $\mu\text{mol HNE l}^{-1}$ normally resulted in their complete immobilization within 10 min. However, $1 \times 10^9 \text{ spermatozoa ml}^{-1}$ endured incubation with up to 200 $\mu\text{mol HNE l}^{-1}$ for 2 h with no apparent deterioration in their motility.

The motility score (Emmens, 1947) component of the motility index tended to deteriorate more rapidly than the percentage of motile spermatozoa. Substantial increases in the proportion of completely immotile cells did not normally occur until almost all spermatozoa were no longer exhibiting progressive motility.

Effect of HNE on the swimming velocity of ram spermatozoa

HNE caused a dose dependent decrease in progressive motility velocities of ram spermatozoa ($P < 0.05$) (Fig. 2). After incubation for 45 min with 500 $\mu\text{mol HNE l}^{-1}$, all spermatozoa had ceased progressive motility, but the proportion of completely immotile cells was no higher than in the control group.

Effect of HNE on duration of reactivation of ram sperm models

The influence of 250 or 500 $\mu\text{mol HNE l}^{-1}$ on the proportion of demembrated ram sperm models showing tail movement (percentage reactivation) after the addition of 1 mmol ATP l^{-1} is shown (Fig. 3a). Both HNE concentrations reduced the duration of reactivation ($P < 0.05$). While addition of 500 $\mu\text{mol HNE l}^{-1}$ decreased the initial percentage of sperm models reactivating ($P < 0.05$) in the initial experiment (Fig. 3a), this effect was not apparent in the subsequent experiment (Fig. 3b). Duration of reactivation appears to be longer for control

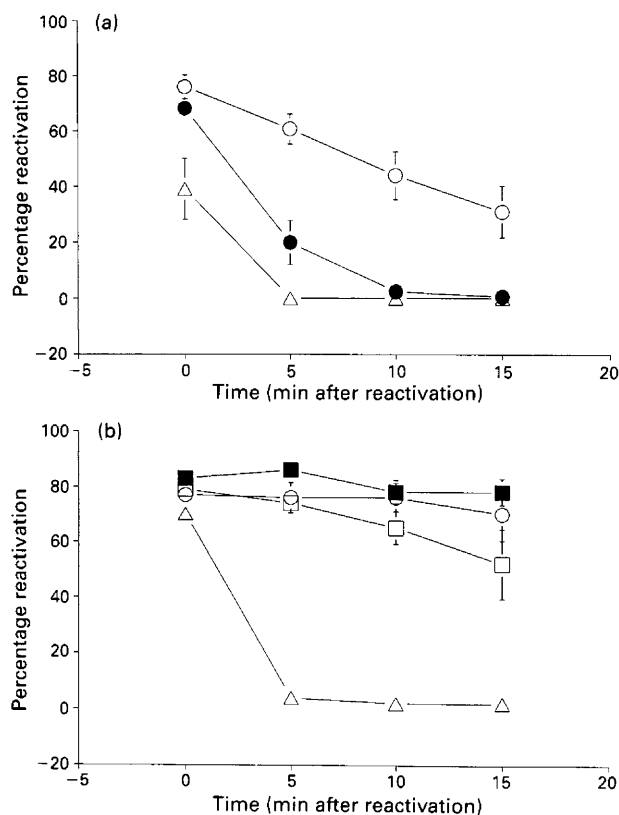


Fig. 3. (a) The effect of (*E*)-4-hydroxy-2-nonenal (HNE) (\circ) 0, (\bullet) 250 or (\triangle) 500 $\mu\text{mol l}^{-1}$ on the duration of reactivation of ram sperm models and (b) the protective effect of 1 mmol reduced glutathione l^{-1} (\blacksquare) control plus glutathione; (\square) 500 $\mu\text{mol HNE l}^{-1}$ plus glutathione. Points represent means (\pm SEM) of four observations.

spermatozoa in Fig. 3b than Fig. 3a, possibly owing to variation between the pooled ejaculates used in the two experiments.

Addition of 1 mmol reduced glutathione l^{-1} (GSH) to the reactivation medium resulted in a substantial increase in the duration of reactivation of sperm models exposed to 500 $\mu\text{mol HNE l}^{-1}$ (Fig. 3b). The proportion of reactivated spermatozoa in HNE plus GSH treated spermatozoa did not differ from that of controls.

Effects of HNE on the glycolytic and oxidative metabolism of ram spermatozoa

The influence of 100 or 200 $\mu\text{mol HNE l}^{-1}$ on oxygen uptake of ram spermatozoa is shown (Fig. 4). Both concentrations of HNE produced a substantial decrease in oxygen uptake ($P < 0.05$) within 30 min. The overall suppression of oxygen uptake was greater after addition of 200 $\mu\text{mol HNE l}^{-1}$.

The quantity of glucose present in the 100 $\mu\text{mol HNE l}^{-1}$ samples was consistently lower than in the controls ($P < 0.05$; Fig. 5a). This was probably due to lower initial concentrations of glucose, as there was no interaction effect between treatments and time to suggest any difference in the rate of glucose utilization.

Overall lactate accumulation was lower in control spermatozoa than in those exposed to 100 or 200 $\mu\text{mol HNE l}^{-1}$

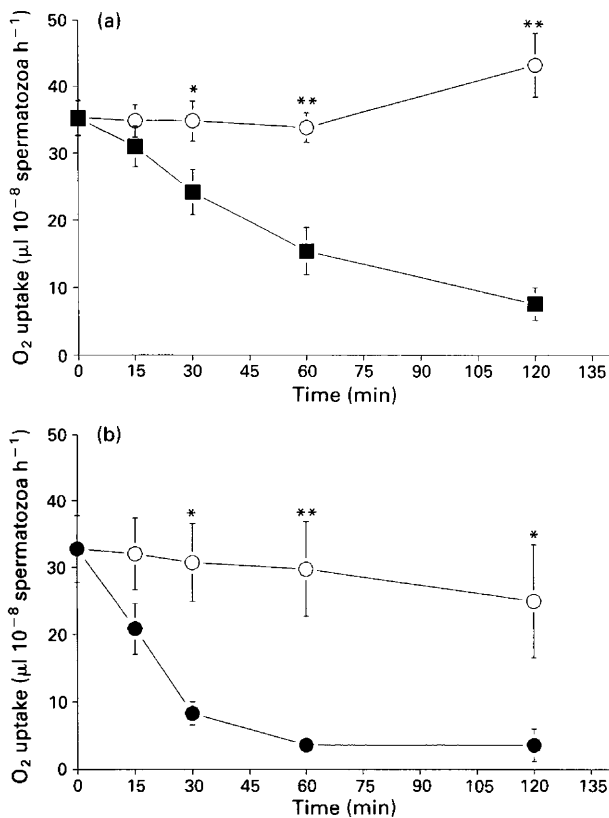


Fig. 4. Inhibition of oxygen uptake of ram spermatozoa by (a) (■) 100 or (b) (●) 200 $\mu\text{mol (E)-4-hydroxy-2-nonenal (HNE) l}^{-1}$ compared with (○) controls. Points represent means (\pm SEM) of four observations (* $P < 0.05$; ** $P < 0.01$).

($P < 0.05$; Fig. 5b). The interaction between HNE concentration and time was also significant ($P < 0.05$), suggesting that lactate concentrations increased more rapidly in the presence of HNE.

Effect of washing on ram spermatozoa pretreated with HNE

After addition of 200 $\mu\text{mol HNE l}^{-1}$ to sperm suspensions ($1 \times 10^8 \text{ ml}^{-1}$), incubation for 60 min and washing spermatozoa twice in ten volumes of KRPG to reduce the concentration of HNE, the inhibitory effects of HNE on either motility or oxygen uptake were not reversed (Fig. 6).

When a sperm suspension ($1 \times 10^9 \text{ sperm ml}^{-1}$) was centrifuged (15 min, 500 g) after incubation for 2 h with 250 $\mu\text{mol HNE l}^{-1}$, the resulting supernatant supported $1 \times 10^6 \text{ spermatozoa ml}^{-1}$ with no observable effect on motility over 30 min. Dilution of spermatozoa ($1 \times 10^6 \text{ ml}^{-1}$) with a 250 $\mu\text{mol l}^{-1}$ solution of the same batch of HNE abolished all motility within 5 min ($n = 3$).

Effect of HNE on amidase activity of ram spermatozoa

The amidase activity detectable in intact ram spermatozoa after 90 min exposure to HNE was lower ($P < 0.01$) than that of cold shocked spermatozoa, and was not altered by HNE (Fig. 7). The amidase activity in cold shocked spermatozoa was

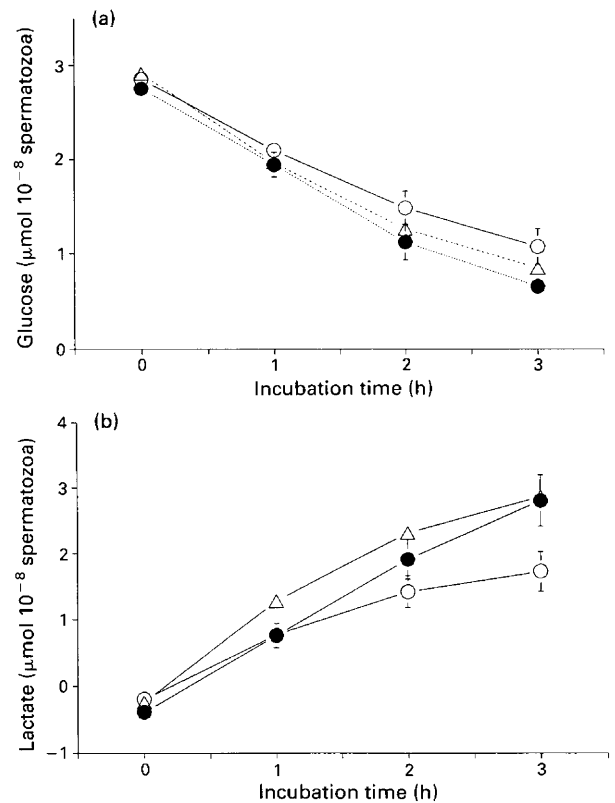


Fig. 5. The effect of (○) 0, (●) 100 or (△) 200 $\mu\text{mol (E)-4-hydroxy-2-nonenal (HNE) l}^{-1}$ on (a) glucose utilization and (b) lactic acid accumulation by ram spermatozoa (10^8 ml^{-1}). Points represent means (\pm SEM) of four observations.

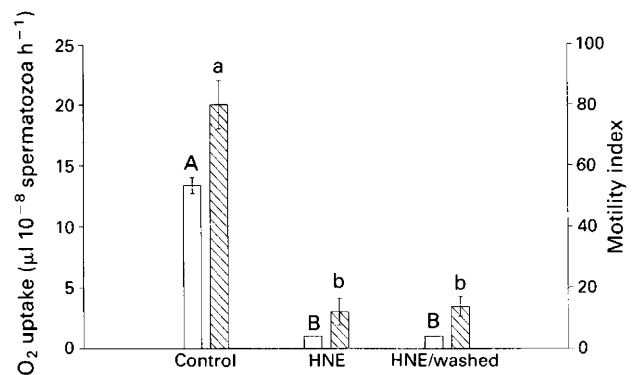


Fig. 6. The effect of removal of (E)-4-hydroxy-2-nonenal (HNE) (200 $\mu\text{mol l}^{-1}$) by washing twice in Krebs–Ringer phosphate solution containing 1% glucose (KRPG) after 60 min exposure on (□) the motility and (▨) oxygen uptake of ram spermatozoa (10^8 ml^{-1}). Bars represent means (\pm SEM) of three observations. Bars with different superscripts are significantly different ($P < 0.01$).

reduced by either 100 or 200 $\mu\text{mol HNE l}^{-1}$ ($P < 0.05$). No difference in sperm bound amidase activity was detected between spermatozoa subjected to the two concentrations of HNE.

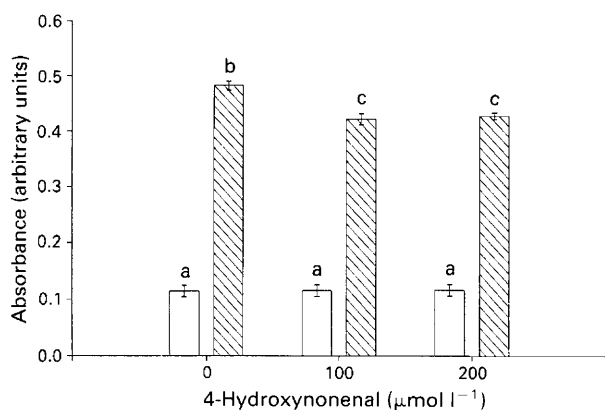


Fig. 7. Amidase activity associated with (□) fresh and (▨) cold shocked ram spermatozoa (10^6 ml^{-1}) pretreated for 90 min with (*E*)-4-hydroxy-2-nonenal (HNE). Bars show means (\pm SEM) of three observations. Bars with different superscripts are significantly different (a–b: $P < 0.05$; b–c: $P < 0.01$).

Effect of HNE on the ability of ram spermatozoa to exclude eosin

Ram spermatozoa (10^8 ml^{-1} ; $n = 3$) were incubated for 1 h in the presence or absence of $400 \mu\text{mol HNE l}^{-1}$. The substantial decline in mean (\pm SEM) sperm motility index over this period, from 65.6 ± 2.5 to 8.3 ± 3.1 , was not accompanied by a decrease in the proportion of unstained spermatozoa. The mean proportions of unstained spermatozoa before and after incubation with HNE were $80.4 \pm 1.6\%$ and $83.7 \pm 3.3\%$, respectively.

Discussion

The motility of both intact and demembrated ram spermatozoa and of washed human spermatozoa was inhibited by (*E*)-4-hydroxy-2-nonenal in a dose-dependent manner. Oxidation of sulfhydryl (-SH) groups by HNE and related compounds has been demonstrated in microtubular protein (Gabriel *et al.*, 1983, 1985) and low density lipoproteins (Jürgens *et al.*, 1986). HNE also binds the thiol groups of sulfur-bearing amino acids (Esterbauer *et al.*, 1976), which suggests that HNE inhibits sperm motility by reacting with the free sulfhydryl groups of the axonemal microtubules. The observation that motility loss was characterized by a steady decline in the vigour and velocity of motility throughout the population of spermatozoa, rather than abrupt cessation of movement by individual spermatozoa, is consistent with a steadily increasing inhibition of the axoneme. Further evidence for this hypothesis is provided by the observation that providing an alternative source of -SH groups (in this case reduced glutathione) reduces the severity of HNE-induced motility loss. HNE-induced chemiluminescence in hepatocytes depends on the quantity of HNE present exceeding that of glutathione, as the two compounds react in a 1:1 ratio (Cadenas *et al.*, 1983).

The presence of 1% serum albumin in the suspending medium (compare the effects of HNE on motility in Fig. 2 and Fig. 1a) also appeared to provide some protection against HNE. This may well be because the propensity of HNE for reacting

with the thiol (Esterbauer *et al.*, 1976) or amino (Jürgens *et al.*, 1986) groups of amino acids, thereby reducing the quantity of HNE available to react with axoneme. The inverse relationship between sperm concentration and the rapidity and severity of HNE induced motility loss is presumably due to the reduced ratio of HNE to microtubular protein. Residual seminal plasma may also provide more protection at lower dilution rates, although Jones and Mann (1977b) found that ram seminal plasma offered little defence against peroxide toxicity. The observation that an HNE solution exposed to spermatozoa is subsequently incapable of immobilizing a second sperm sample, in a similar manner to peroxidized arachidonic acid (Jones and Mann, 1977a), suggests that the reaction of HNE with sperm tubulin is, like that from other tissues, largely irreversible (Gabriel *et al.*, 1985).

Repeated washing with fresh buffer failed to reverse the effects of HNE on either the motility or metabolism of ram spermatozoa. This finding is consistent with the observation that hydroxyalkenals react with thiols to form cyclic hemiacetals, the stability of which render the reaction largely irreversible (Esterbauer *et al.*, 1975), and the high affinity of HNE for membrane lipids. Jones and Mann (1977a, b) also observed that the disruption of ram sperm function by lipid peroxides could not be reversed by washing.

The results presented above offer no evidence for the inhibition of ram sperm fructolysis by HNE. The rate of glucose utilisation in HNE-treated spermatozoa did not differ from that of controls. Further evidence of continued glycolytic activity is provided by the observation that spermatozoa maintained progressive motility under conditions in which oxygen uptake was reduced by over 90% (compare the effects of $200 \mu\text{mol HNE l}^{-1}$ in Figs 1b and 4b). This result is in contrast to the observation that exposure to both exogenous and endogenous lipid peroxide products irreversibly abolished fructolytic activity in ram spermatozoa (Jones and Mann, 1977a, b). This may be due to the fact that peroxidation induced by UV light or iron catalysis (as in the earlier studies) produces many toxic substances other than 4-hydroxyalkenals. Glycolytic inhibition may therefore be due to peroxidation products other than HNE, or require a higher concentration of total inhibitory substances than were used in the study reported here.

Oxygen uptake by ram spermatozoa was reduced in a dose-dependent manner in the presence of HNE. The increased rate of accumulation of lactic acid by HNE-treated spermatozoa indicates a decrease in activity of the TCA cycle. Whether this is the result of direct TCA cycle inhibition, or a consequence of end-product accumulation resulting from inhibition of the respiratory chain cannot be determined from the current data. The observed inhibition of respiration is concurrent with the effect of lipid peroxides on ram spermatozoa (Jones and Mann, 1977a, b) and other mammalian tissues (reviewed by Barber and Bernheim, 1967).

The amidase activity associated with cold shocked ram spermatozoa was reduced by the addition of HNE. While an inhibition of acrosin by HNE would parallel the inhibition of membrane bound enzymes by peroxidized lipids (Roubal and Tappel, 1965; Wills, 1971) or HNE in a number of other tissues (Ferrali *et al.*, 1980; Paradisi *et al.*, 1985; Lame and Segall, 1987), the small decrease in amidase activity observed that was not dose dependent may not be due to direct effects on the enzyme.

High concentrations of HNE may affect the enzyme via indirect mechanisms. A possible alternative explanation is that HNE provided a degree of protection to spermatozoa against cold shock. Although the acrosomal plasma membrane has been shown to be particularly susceptible to peroxidation damage (Jones and Mann, 1977a, b), there was no evidence to suggest that incubation with HNE increased the release of acrosomal enzymes from spermatozoa that had not been cold shocked.

Similarly, treatment of spermatozoa with 400 $\mu\text{mol l}^{-1}$ for 1 h, sufficient to completely abolish motility and respiration, did not increase the proportion of ram spermatozoa stained by eosin. These results together with those for amidase activity (indicating intact acrosomal membranes) suggest that disruption of the sperm plasma membrane by HNE is minimal. This is surprising in light of the widespread effects of peroxidized lipids on a variety of membranes, including those of spermatozoa (Jones and Mann, 1973, 1976, 1977a, b; Alvarez and Storey, 1984a; Fujihara and Koga, 1984; Slaweta *et al.*, 1988). Hydroxyalkenals also produce a number of membrane related phenomena, including lysis of erythrocytes (Benedetti *et al.*, 1977, 1979; Roders *et al.*, 1978). It is notable, however, that the haemolytic activity of synthetic HNE is less than that of a mixture of the aldehydes produced by peroxidation, although a 450 $\mu\text{mol l}^{-1}$ solution was sufficient to lyse 50% of erythrocytes present (Comporti *et al.*, 1985). The mechanism by which ram spermatozoa are better able to resist membrane disruption by HNE than are erythrocytes is unknown.

In conclusion, the data presented here indicate that HNE exerts powerful inhibitory effects on the motility and oxidative metabolism of ram spermatozoa. The reactivation studies suggest that the reaction of HNE with thiol groups in the axoneme is an important factor in the inhibition of motility. The nature of the interaction between HNE and sperm respiration has not been determined, but the lack of effect of HNE on the plasma membrane systems examined suggests that enzyme inhibition is a likely cause, possibly via modification of enzymatic thiol or amino groups.

Although it is evident that HNE is toxic to ram spermatozoa *in vitro*, the concentrations used in this study were 40–200 times the maximum HNE concentrations (approximately 2.7 $\mu\text{mol l}^{-1}$) recorded in human semen by Selley *et al.* (1991). Unless HNE concentrations in ram semen are substantially higher than those in humans, the results above suggest that HNE occurring naturally in seminal plasma is unlikely to have a deleterious effect on the motility or metabolism of ram spermatozoa at the sperm concentrations present in semen when ejaculated ($1\text{--}5 \times 10^9$ spermatozoa ml^{-1}), or diluted for artificial insemination ($10^8\text{--}10^9$ spermatozoa ml^{-1}). The lipophilic nature of HNE means, however, that locally produced HNE may remain within the sperm membrane at much higher concentrations than those diffusing into seminal plasma; and there is little information currently available on the production of hydroxyalkenals by ram spermatozoa after stressful events such as freeze–thawing. The potential effects of lower concentrations of hydroxyalkenals on the fertilizing capacity or genome of ram spermatozoa also remain to be investigated.

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