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Hydrogen Peroxide Is Involved in Hamster Sperm Capacitation In Vitro[1]

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ABSTRACT

We have investigated the possibility that the generation of hydrogen peroxide (H_2O_2) by spermatozoa plays a physiological role during capacitation. Capacitation is defined as the incubation period required for fertilization in mammals. Capacitation culminates in an exocytotic event, the acrosome reaction (AR). Mammalian sperm generate H_2O_2 during aerobic incubation and do not contain catalase, the enzyme that promotes scavenging of H_2O_2. In the present work we show that added catalase inhibited the AR, while glucose oxidase (GO), an enzyme that generates H_2O_2, accelerated the onset of the AR. Direct addition of H_2O_2 also stimulated the AR; catalase inhibited both the stimulation by GO and by H_2O_2. The onset of the AR was always preceded by the appearance of hyperactivated motility. The stimulation of the AR by H_2O_2 was manifest 1–2 h after the addition of H_2O_2. Catalase added at 3 h of incubation was less effective in inhibiting the AR than catalase added at the beginning. Incubation of sperm with catalase prevented the induction of the AR by the membrane-perturbing lipid, lysophosphatidyl choline. Taken together, these results suggest that H_2O_2 produced by hamster sperm plays a significant role during capacitation, possibly in membrane reorganization to facilitate the fusion that takes place during exocytosis of the acrosomal contents.

INTRODUCTION

Capacitation is a term used for the series of preparatory changes that occur during the period of mammalian sperm incubation required for successful triggering of the acrosome reaction (AR) by egg-associated factors. During the AR, the overlying plasma membrane fuses at multiple sites with the outer acrosomal membrane [1]. Capacitation is accompanied by changes in membrane lipid and protein composition and localization [2–4] and changes in ion fluxes, including calcium [5], sodium, and potassium [6, 7], and protons [8]. These subcellular changes regulate changes in motility patterns and the onset of the fusion that must occur in the vicinity of the egg for successful gamete interaction [9].

Rabbit, mouse, and human sperm generate superoxide (O_2-) and hydrogen peroxide (H_2O_2) during aerobic incubation [10–13]. Most of the H_2O_2 appears to be generated by the action of sperm superoxide dismutase on the superoxide radical produced by the sperm [14]. Under normal conditions, the H_2O_2 generated by nonphagocytic cells represents only 1–2% of the total oxygen consumption in animal tissues; in most tissues, the concentrations of catalase and peroxidases are well in excess to prevent the toxic effects of H_2O_2 [15]. Nevertheless, the apparent lack of catalase activity in sperm has made possible the detection of H_2O_2 outside the intact rabbit, mouse, and human sperm [10, 12, 13]. Mouse, rabbit, and human spermatozoa react with superoxide and H_2O_2 produced intracellularly; the result of this reactivity is spontaneous membrane lipid peroxidation and motility loss; superoxide, rather than H_2O_2, appears to be the main inducer of lipid peroxidation [11–14, 16, 17].

Our preliminary findings—including inhibition of in vitro fertilization by catalase and the growing evidence that H_2O_2 is involved in metabolic control [18] and in regulating cell division, chemotaxis, and the action of insulin and other hormones [19–21]—prompted us to examine the role of this metabolite in vitro sperm capacitation.

The AR occurs spontaneously in sperm capacitated in vitro in the presence of serum albumin [22]. This morphological modification of the sperm head at the end of capacitation has been used extensively to study capacitation. In this work, we report the effects of catalase, an H_2O_2 scavenger; glucose oxidase (GO), and enzyme that generates H_2O_2; and H_2O_2 on the spontaneous AR of cauda epididymal hamster sperm. The effect of lysophosphatidyl choline (LPC) in sperm incubated in the presence of catalase was also examined. The results allow us to speculate that the generation and release of H_2O_2 is not merely a means of discarding toxic waste products, but instead that H_2O_2 plays a significant role during sperm capacitation. Support for this hypothesis has been recently provided by work showing increased sperm-zona interaction following induction of limited peroxidation in human sperm [23] and activation of protein kinase C by mild oxidation of its regulatory domain by H_2O_2 [24].

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MATERIALS AND METHODS

Media and Reagents

Modified Tyrode's medium containing taurine and bovine serum albumin (BSA) was used in all experiments. Media contained 117.5 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 10 mM Hepes (pH 7.4), 5 mM glucose, 0.09 mM sodium pyruvate, 9 mM lactate, 0.5 mM taurine and 5 or 20 mg/ml BSA (fraction V, Sigma A4503), where indicated. The pH of the incubation solution at room temperature was 7.4–7.5 in media with 20 mg/ml of BSA and 7.5–7.6 in media with 5 mg/ml of BSA. Osmolarity was 290–300 mOsm/kg. Thymol-free catalase from bovine liver, superoxide dismutase from bovine erythrocytes, glucose oxidase from *Aspergillus niger,* and all other reagents were obtained from Sigma Chemical Company (St. Louis, MO). Sperm capacitation in the presence of 50 μM H_2O_2 was performed in media containing 3 mM benzoic acid and 5 mg/ml BSA. Unless indicated, all enzymes were included in the media at the beginning of the incubation.

Spermatozoa

Sperm were recovered from the cauda epididymis of 4–6 mo-old virgin male hamsters. The excised epididymides were minced in 0.5–1 ml of medium and allowed to stand for 15 min at room temperature to allow motile sperm to escape into the medium before the pieces of tissue were removed. The spermatozoa were diluted to final concentration of 1–3 million/ml and incubated at 37°C in 100- or 200-μl drops under mineral oil. Animals that yielded less than 80 million sperm were not included in this study.

Glucose Oxidase Activity

The amount of H_2O_2 evolved by GO was determined using the scopoletin fluorescence assay originally described by Andreae [25] as modified by De la Harpe and Nathan [26]. Scopoletin concentration was 1 μM, and horseradish peroxidase was 0.25 units/ml in a total of 200 μl in each well. Fluorescence was determined using a filter fluorometer (Dynatech Microfluor, Alexandria, VA). The catalase-sensitive decrease in fluorescence induced by 15 μg/ml of GO after 1 h of incubation corresponded to that induced by 2.6 μM H_2O_2.

Synchronization

To investigate whether the observed effects of catalase were due to its action on capacitation or directly on the AR, we synchronized capacitation by delaying Ca^{2+} and K^+ addition after preincubating spermatozoa in Ca^{2+}-free, low K^+ medium. Spermatozoa were incubated in 200 μl of Ca^{2+}-free modified Tyrode's medium that contained 0.1 mM instead of 10 mM K^+, as described above. At 1 h after start of the incubation, 8 μl of 50 mM CaCl in K^+-free modified Tyrode's was added so that the concentration of Ca^{2+} in the medium was raised to 2 mM. One hour after the addition of Ca^{2+}, an aliquot of Na^+-free modified Tyrode's medium was added to give a final concentration of 10 mM K^+. Catalase was added at 0 and 3 h.

Observations

Samples of sperm suspension (10 μl) were observed by phase-contract microscopy to determine the percentage of ARs in at least 100 strongly motile sperm [27]. Determinations were made at 4, 5, 6, 7, and 8 h of incubation. The values shown are the mean percentages ± standard deviation.

Statistics

For statistical analysis, all percentages were transformed to arcsin angle values. The significance of the differences between the means in Table 1 was analyzed using the *t*-test for paired samples. Figure 1 and all other tables were analyzed using randomized block analysis of variance, within each time period, followed by multiple comparisons with the Student-Newman-Keuls test.

RESULTS

Effect of Activated Oxygen Scavengers on the AR

Table 1 shows the percentage of sperm that spontaneously lost the acrosomal cap after 5 h of incubation with two enzymes that scavenge reactive oxygen species. Catalase (10 μg/ml), an enzyme that scavenges H_2O_2, inhibited the AR of sperm treated with epinephrine (50 μM). Superoxide dismutase (SOD, 50 μg/ml), a superoxide scavenger, had no effect. Epinephrine, a commonly used stimulator of the AR [28], generates H_2O_2 during aerobic incubation; therefore, the inhibition of the AR by catalase could be due to inhibition of epinephrine stimulation rather than inhibition of the basal capacitation rate. Table 1 also shows the

TABLE 1. Catalase inhibits the AR.*

Enzyme†	Epinephrine (μM)	Acrosome-reacted sperm (% at 5 h) Control	Test	n
Catalase (10–20 μg/ml)	50	75 ± 17[a]	9 ± 6[a]	7
Superoxide dismutase (50–100 μg/ml)	50	75 ± 12[a]	81 ± 11[b]	4
		(% at 7–8 h)		
Catalase (10–20 μg/ml)	—	61 ± 15[a]	17 ± 12[a]	9

*Inhibition of the AR by catalase in the presence and absence of epinephrine. Values represent the mean ± SD.

†Enzymes were added at the beginning of the incubation; media contained 20 mg/ml BSA.

[a,b]Within each row, treatments with different superscripts differ significantly ($p < 0.001$).

results in the absence of epinephrine. In these experiments the AR was determined at 7–8 h. Catalase inhibited the AR of sperm in the absence of stimulation by epinephrine, indicating that catalase inhibition is independent of epinephrine stimulation. Boiled catalase had no effect (not shown). The inhibition of the AR by catalase but not by SOD suggests that H_2O_2, rather than superoxide, plays a role during capacitation. Long-term (8 h) sperm motility was similar in all treatments, even though an apparent decrease in motility was observed at 3–5 h of incubation in the samples treated with catalase. This phenomenon was not observed when epinephrine was included in the media. The inhibition by catalase was observed under a variety of incubation conditions. The experiments shown were performed in media buffered by 11.9 mM bicarbonate and 10 mM Hepes and incubated in room air atmosphere, but similar results were obtained later in medium buffered with 25 mM bicarbonate and 5.5% CO_2. Similarly, decreasing the BSA concentration from 20 mg/ml to 5 mg/ml did not affect the inhibition by catalase.

Effect of GO on the AR

Corroboration of the involvement of H_2O_2 in capacitation was possible with H_2O_2 generated in the medium by GO and glucose, a situation that resembles H_2O_2 production by sperm. Figure 1 shows that incubation of sperm in the presence of 30 ng/ml of GO stimulated the AR, whereas catalase (15 μg/ml) inhibited this stimulation. The stimulation by GO was significant at 4, 5, and 6 h. Boiled GO had no effect. GO stimulation was not observed in sperm capacitated in the presence of epinephrine. GO had no deleterious effect on sperm motility at the concentration used. Hyperactivated motility was first observed at 4 h of incubation in GO-treated sperm and at 5 h in control samples. The experiments shown were performed in media containing 5 mg/ml of BSA and 3 mM benzoic acid; similar results were obtained with 20 mg/ml of BSA in the absence of benzoic acid.

Direct Effect of H_2O_2

A bolus addition of 50 μM H_2O_2 also stimulated the AR. Table 2 shows percentage of sperm undergoing the AR in samples treated with 50 μM H_2O_2. Addition of H_2O_2 at the beginning of the incubation resulted in a significant increase in AR at 4 h of incubation. Addition of H_2O_2 at 3 h resulted in stimulation 2 h later, although significance was not achieved until 3 h later. Catalase (15 μg/ml) inhibited the stimulation by H_2O_2. These results are consistent with the concept that H_2O_2 stimulates the AR indirectly through stimulation of capacitation. All samples were incubated in

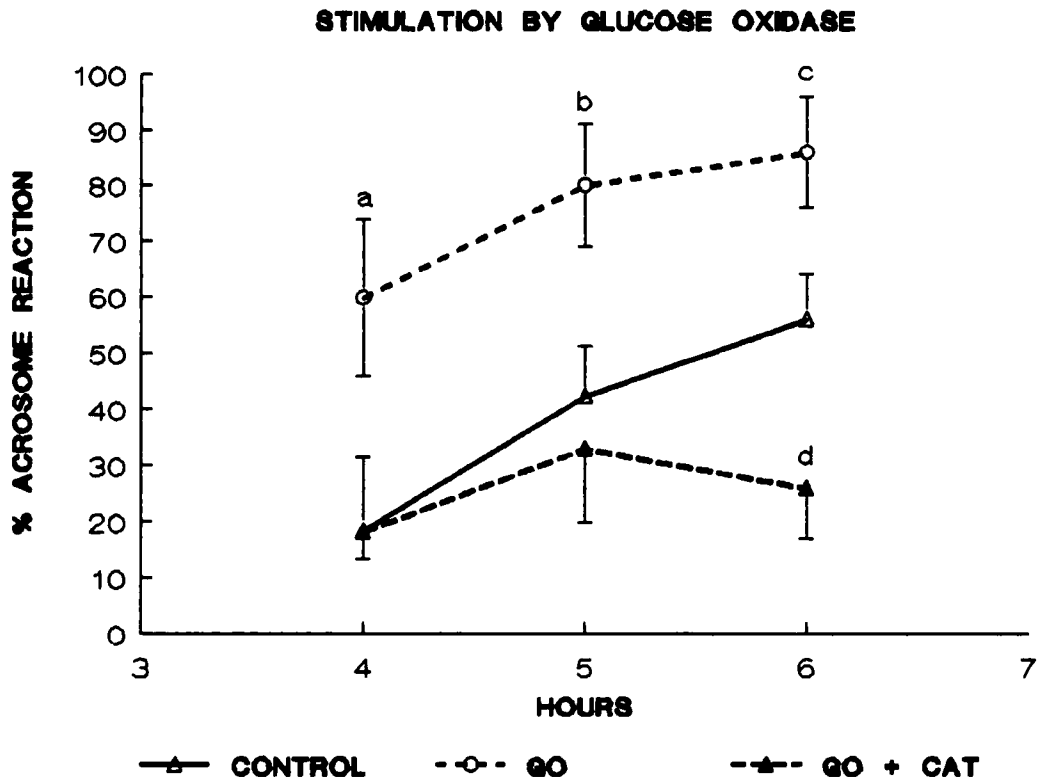

FIG. 1. Stimulation of the AR by GO and inhibition of the stimulation by catalase. GO and catalase were added at the beginning of the incubation. The error bars represent the standard deviation; four experiments were performed. [a]Different from control and from GO + catalase, $p \leq 0.01$. [b]Different from control, $p \leq 0.05$, and from GO + catalase $p \leq 0.01$. [c]Different from control and from GO + catalase, $p \leq 0.01$. [d]Different from control, $p \leq 0.05$.

TABLE 2. Stimulation of the AR by 50 μM H_2O_2.*

Treatment†	Time of addition	Acrosome-reacted sperm (%) 4 h	5 h	6 h
Control	—	21 ± 14[a,cd]	39 ± 8[ab,cd]	53 ± 8[a,c]
H_2O_2	0 h	53 ± 15[b,d]	72 ± 17[b,d]	84 ± 7[b,d]
H_2O_2	3 h	32 ± 4[ab,cd]	63 ± 12[b,cd]	80 ± 2[b,d]
H_2O_2 + catalase	0 h	13 ± 12[a,c]	21 ± 13[a,c]	45 ± 13[a,c]

*Values represent the mean ± SD, n = 4.
†Media contained 5 mg/ml BSA and 3 mM benzoic acid.
[a–d]Within each time, treatments with different superscripts differ significantly: [a,b]$p \leq 0.05$; [c,d]$p \leq 0.01$.

medium with 5 mg/ml of BSA and 3 mM benzoic acid. Under these conditions, sperm displayed vigorous motility in the presence of H_2O_2. Initial experiments performed without benzoic acid resulted in decreased motility and yielded erratic results for the AR.

Effect of Delayed Addition of Catalase

In an effort to separate the involvement of H_2O_2 in capacitation from the AR, we studied the effects of catalase, which scavenges H_2O_2, in a synchronized sperm population. In these experiments, the sperm population had been synchronized so that most sperm underwent the AR between 4 and 5 h in the control condition. Synchronization by withholding Ca^{2+} and K^+ is detailed in *Materials and Methods*. The inhibition of the AR by catalase added at 0 and 3 h of incubation is shown in Table 3. Catalase added at 3 h of incubation inhibited the AR in populations due to react 2 h later less effectively than catalase added from the start of the incubation. Because the AR was not abolished in the presence of catalase at 6 h, the effects of H_2O_2 probably were not directly on the AR, but rather on some later capacitation event.

Effect of Lysophosphatidyl Choline (LPC) on Sperm Treated with Catalase

The membrane-perturbing properties of LPC have been useful to the study of capacitation. LPC induces the AR in capacitated sperm but not in noncapacitated sperm [29, 30]. We probed capacitation with this compound in order to further define the involvement of released H_2O_2 on capacitation or the AR. Table 4 shows that 15 min after the addition of LPC (200 μM), sperm preincubated for 3 h in the absence of catalase displayed 56% AR, whereas only 14% of sperm preincubated for 3 h in the presence of catalase (50 μM) were AR. Therefore, removal of H_2O_2 with catalase reduced the number of sperm that were sufficiently capacitated for LPC to induce the AR. These results again suggest that H_2O_2 effects on the AR are indirect through stimulation of capacitation.

TABLE 3. Effect of delayed addition of catalase to synchronized sperm.*

Treatment†	Time of addition	Acrosome-reacted sperm (%) 4 h	5 h	6 h
Control	—	5 ± 1[a]	64 ± 21[a]	86 ± 15[a,c]
Catalase (50 μg/ml)	3 h	6 ± 5[a]	33 ± 23[a]	53 ± 24[b,c]
	0 h	2 ± 1[a]	12 ± 6[c]	39 ± 15[b,d]

*Diminished inhibition of the AR by catalase added at 3 h of incubation.
†Media contained 20 mg/ml BSA and 50 μM epinephrine.
[a–d]Within each time, treatments with different superscripts differ significantly: [a,b]$p \leq 0.05$; [c,d]$p \leq 0.01$.

TABLE 4. Effect of LPC on sperm treated with catalase.*

Preincubation†	LPC (μM)	Acrosome-reacted sperm (%) 3 h	3 h 15 min
Control	—	6 ± 4	18 ± 10
	200	4 ± 2	56 ± 15[a]
Catalase (50 μg/ml)	—	1 ± 1	5 ± 2
	200	3 ± 3	14 ± 5

*Absence of stimulation of the acrosome reaction by LPC on sperm treated with catalase. Values represent the mean ± SD; n = 3.
†Sperm were preincubated for 3 h in the presence and absence of catalase before the addition of LPC. Media contained 20 mg/ml BSA and 50 μM epinephrine.
[a]Within time, different from all other treatments, $p \leq 0.01$.

DISCUSSION

The ability of catalase to inhibit the AR in the absence of an exogenous H_2O_2 source indicates that the generation of H_2O_2 by the sperm during capacitation has an effect on a subsequent stage of the process. The absence of catalase in sperm is consistent with the concept that oxidation reactions that utilize H_2O_2 are integral to the capacitation process. Considering the high molecular activity of catalase, the concentration needed to inhibit sperm capacitation is relatively high. Nevertheless, if one considers that the enzyme does not penetrate the sperm membrane [10], one would expect to find that the effectiveness of catalase is limited by the diffusion of H_2O_2 to its active site.

The inhibition of the AR by external catalase indicates that H_2O_2 exported to the medium exerts its effects on the outside of the cell. This idea has already been put forward by Holland et al. [11], whose studies suggested reactivity of the plasma membrane of rabbit and mouse epididymal sperm with H_2O_2 [11, 17]. An alternative explanation is that H_2O_2-reactive sites are located inside the cell, and that external catalase effectively lowers the intracellular concentration of H_2O_2 by increasing the magnitude of the inside-outside concentration gradient.

The evidence that external catalase inhibits the AR reaction could be taken as a suggestion that H_2O_2 is produced at the plasma membrane by an enzymatic system analogous to the NADH oxidoreductase of plasma membranes [18]. The absence of stimulation by GO in samples incubated with epinephrine could also be taken as indicative of an

external source of H_2O_2 analogous to the plasma membrane oxidase that is stimulated by catecholamines [18]; i.e., the sperm component that reacts with H_2O_2 could already be saturated by H_2O_2 generated in response to epinephrine. Nevertheless, preliminary work designed to measure the rate of H_2O_2 generation by hamster sperm do not support this conclusion: sperm demembranated by hypotonic treatment [31] appear to generate H_2O_2 at the same rate that intact motile sperm do [32].

The design of the experiments described in this work does not permit us to rule out the possible contribution of epididymal fluid components to H_2O_2 generation. However, the recent data from demembranated hamster sperm mentioned above [32] make the contribution of these components unlikely. Demembranated sperm are obtained after dilution of the sperm suspension in hypotonic buffer and centrifugation [31]. The supernatant, where most epididymal components are expected to be found, is discarded. This tentative conclusion is also supported by data showing that washed human sperm generate H_2O_2 [13].

Scoring spontaneous ARs in a sperm population is a measure of the degree of capacitation of that sperm population. While there is a distinction between sperm that have undergone membrane fusion but retain the acrosomal vesicle and sperm that have reacted more completely and lost them, the term "acrosome-reacted" has been used to denote both conditions [33]. In our experiments, we score the loss of the acrosomal cap; therefore, it is not clear whether we are stimulating capacitation in general, membrane fusion, or acrosomal matrix dispersal; nevertheless, our observations that decreased flagellar beat frequency—a measure of hyperactivation [34]—appears earlier in the GO-treated sperm support the idea that we are stimulating capacitation and not the AR (membrane fusion or matrix dispersal).

There is reasonably good agreement between the concentration of H_2O_2 and of GO used to achieve stimulation of the AR in these experiments. Direct addition of 50 μM H_2O_2 to the capacitation media accelerated the onset of the AR to the same extent that 30 ng/ml of GO did. In the presence of 5 mg/ml of BSA, 3 mM benzoic acid, and 10 mM Hepes, the rate of H_2O_2 production by 30 ng/ml of GO was close to 5 nmol as determined by the scopoletin fluorescence assay. Therefore, the amount of H_2O_2 generated by 30 ng/ml of GO is estimated to reach 25 μM after 5 h of incubation.

The inhibition of motility loss by benzoic acid is not surprising as this antioxidant would be expected to prevent lipid peroxidation induced by H_2O_2. It is of interest to note that when H_2O_2 is produced at a low constant rate, as in incubations with GO, there is no motility loss in the absence of benzoic acid. On the other hand, when sperm are challenged by a bolus addition of H_2O_2, motility decreases—probably due to membrane lipid peroxidation. In this case, motility loss is prevented by 3 mM benzoic acid.

The molecular targets of H_2O_2 in sperm are unknown. Whether the effects of H_2O_2 are due to nonspecific lipid peroxidations, membrane protein sulphydryl group oxidation, or a lowering of the redox potential is a matter for further study. Inhibition of lipid peroxidation by epinephrine and albumin [16], two agents that accelerate capacitation, does not lend support to the concept that nonspecific lipid peroxidation is the mechanism of action of H_2O_2. Reduction of membrane thiols by dithiothreitol has been shown to inhibit the AR of guinea pig sperm [35] and hamster sperm (Bize, unpublished observations). It is possible that controlled oxidation of membrane thiol groups by H_2O_2 produced by the sperm is an integral part of sperm capacitation. However, the stimulation of capacitation by micromolar concentrations of H_2O_2 suggests that its effects could be more specific. Reportedly, specific effects of H_2O_2 in enzymatic systems include activation of soluble guanylate cyclase [36], activation of cycloxygenase [37], stimulation of carrier-mediated glucose transport, stimulation of glucose oxidation [38], and, more recently, activation of protein kinase C [24].

Our observations suggest that all events of capacitation are shortened by incubation with GO or H_2O_2, and are lengthened by incubation with catalase, including the appearance of low-frequency sperm beating and the appearance of the AR. These observations, together with studies suggesting that the source of sperm H_2O_2 is partly mitochondrial [10], allow us to speculate that sperm mitochondrial activity may have a cumulative effect resulting in the membrane destabilization that accompanies capacitation. In this context, sperm mitochondria could act as a biological clock in a manner analogous to the proposed role of mitochondria as a biological clock timing cell aging as presented in the free radical theory of aging [39].

The absence of stimulation of the AR by LPC in sperm populations preincubated with catalase could be an indication that the primary effect of H_2O_2 is on membrane lipid organization. It has been observed that the fraction of lipids that diffuses within the membrane bilayer decreases during capacitation [40]. The mechanism of lipid immobilization in sperm has been sought in oxidative reactions. Nevertheless, incubation in oxygen-free media does not seem to affect the fraction of diffusing lipids in ejaculated ram sperm [4].

In summary, even though the mechanism of action of H_2O_2 in stimulating the AR is unknown, our results show that the generation of this metabolite in the sperm is not merely a by-product of other reactions. The relevance of our findings to the in vivo situation is difficult to assess, because it is likely that the generation of H_2O_2 is highly dependent on the local concentration of oxygen.

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