Free Rad. Res., Vol. 24, No. 4, pp. 261–268
Reprints available directly from the publisher
Photocopying permitted by license only

Promotion of Human Sperm Capacitation by Superoxide Anion

HONG ZHANG and RONG-LIANG ZHENG*

Department of Biology, Lanzhou University, Lanzhou, 730000 P.R. China

Accepted by Professor Ed Janzen

(Received February 6th, 1995; in revised form, May 15th, 1995)

Capacitation of spermatozoa is an essential procedure for fertilization. Capacitated spermatozoa have an increase in the intracellular cAMP and acrosome reaction (AR) occurs immediately. The effect of exogenous superoxide anion (O^-_2) on the level of intracellular cAMP and the percentages of both spontaneous AR and lysophosphatidylcholine-induced AR (LPC-AR) were studied using semen samples collected from 10 healthy and fertile volunteers working or studying in Lanzhou Medical College. Spermatozoa were separated by Percoll and incubated at 37°C in Ham's F-10 medium with O^-_2 generation system: xanthine + xanthine oxidase + catalase + diethylenetriaminepentaacetic acid + sodium formate. The intracellular cAMP was determined by (^{3}H) – cAMP radioimmunoassay at 3 h of incubation, and the percentages of AR and LPC-AR were evaluated by the triple-stain technique at 3.5 h of incubation. The effects of SOD with different concentration were also determined. The results showed: the level of intracellular cAMP (pmol/10^8 spermatozoa) of spermatozoa increased from 14.0 ± 1.3 to 23.2 ± 2.5 ($P<0.01$), and the percentages of AR and LPC-AR increased from 4.5 ± 1.1% and 14 ± 1.9% to 16 ± 2.0% and 32.5 ± 1.7%, respectively ($P<0.01$ in both comparisons). SOD inhibited these processes concentration dependently. To investigate the source of O^-_2 during *in vivo* sperm capacitation, female genital tract fluids collected from 6 healthy nonpregnant donors of reproductive age, and seminal plasma, capacitated and noncapacitated spermatozoa from 10 fertile volunteers were investigated by spin trapping method. The results showed: A typical electron paramagnetic resonance spectrum for O^-_2 spin adduct was exhibited only in capacitated spermatozoa but not in vaginal or cervical secretions, uterine and fallopian tubal fluids, nor in seminal plasma and noncapacitated spermatozoa. These results suggested that only capacitated spermatozoa themselves are able to generate O^-_2 which stimulated their capacitation in turn. Furthermore, on the basis of these data, we propose that it may be possible to utilize the inhibitory effect of SOD on sperm capacitation so as to regulate fertilization.

Key words: Human spermatozoa, capacitation, acrosome reaction, superoxide anion, superoxide dimutase, free radicals

INTRODUCTION

Spermatozoa present in freshly ejaculated semen of mammals lack the capacity to fertilize the oocyte immediately.[1] Prior to acquisition of the ability to fertilize the oocyte, spermatozoa undergo a series of membrane and metabolic changes

*Please send all correspondence to: Professor R.L. Zheng, Department of Biology, Lanzhou University, Lanzhou, 730000 P.R. China T. 0086–0931–8843000 X3163(0), X8136(H), FAX: 0086–0931–8885076

referred to as capacitation.[1] These changes occur in female genital tract and must be completed before fertilization of the oocyte.[1] Sperm capacitation can also be initiated under proper conditions *in vitro*, such as in physiological solutions containing serum albumin or follicular fluid. Capacitated spermatozoa have an increase in the intracellular cAMP,[2–5] and exhibit the characteristic of hyperactivation.[6] Once capacitation is completed, the acrosome reaction (AR) can be initiated immediately under certain conditions. AR is an exocytotic process involving fusion of outer acrosomal membrane with the overlying sperm head plasma membrane, followed by fenestration and vesiculation of the fused membrane, and eventually leading to dispersion of the acrosomal matrix.[4] The percentages of spontaneous AR of human spermatozoa *in vitro* is less than 20%. It can be significantly increased when some inducers are supplemented. Lysophosphatidylcholine (LPC) is a physiological agent known to induce AR in capacitated but not in noncapacitated spermatozoa.[7] So that percentage of LPC-AR can also reflect the level of sperm capacitation. Acrosomal enzymes, mostly acrosin and hyaluronidase, enable spermatozoa to penetrate the cumulus ophorus, corona radiate and zona pellucid which cover the oocytes. Contact with the oocyte plasma membranes and fusion with oocytes are also facilitated by acrosomal enzymes.

Human and animal spermatozoa can generate reactive oxygen species (ROS), for instance superoxide anion (O^-_2) and hydrogen peroxide, and are especially sensitive to oxidative damage because of the high content of polyunsaturated fatty acids and the relatively low levels of antioxidant enzymes in spermatozoa.[8–10] ROS are able to initiate lipid peroxidation of polyunsaturated fatty acids in sperm membranes. The lipid peroxides, products of lipid peroxidation, cause irreversible damages of sperm integrity, motility and other sperm functions.[9–14] Until now, sperm ROS were regarded as the principal cause leading to oligospermia, azoospermia, sperm abnormalities such as altered motility and shape, and idiopathic male infertility.[13–15] However, recent studies by Lamirande and Gagnon[16,17] were contrary to previous reports: when spermatozoa were treated with an appropriate amount O^-_2, there was not only a complete prevention of any loss of motility but also a significantly high level induction of capacitation. Superoxide dismutase (SOD) can prevent this process.

The aim of the present study is to investigate the role of O^-_2 on human sperm capacitation and AR, and the source of O^-_2 in the genital system under the physiological conditions so as to evaluate the possibility of inhibiting sperm capacitation by interfering with the generation of O^-_2.

MATERIALS AND METHODS

Materials

Ham's F-10 culture medium, xanthine, xanthine oxidase, catalase, 5,5-dimethyl pyrroline N-oxide (DMPO) and lysophosphatidylcholine (LPC) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Superoxide dismutase was purchased from Xiahe Biological Products Factory (Xiahe, Gansu, China). Diethyldithiocarbamate (DDC), diethylenetriaminepentaacetic acid (DETAPA), sodium formate, trypan blue, Bismark brown Y, Rose Bengal and Percoll were purchased from Shanghai Biochemical Co. (Shanghai, China) and were reagent grade. [^{3}H]–cAMP medical kit (specific radio-activity>740 GBq/mmol, radioactive purity>98%) was offered by China Atomic Energy Scientific Institute (Beijing, China).

Methods

Collection and preparation of semen samples

Semen samples were collected into sterile containers by masturbation, after 5–7 days of sexual abstinence, from fertile volunteers (25–35 years old) who had no past or present history of

systemic diseases and working or studying in Lanzhou Medical College. The samples liquefied spontaneously from the freshly gel state for 30 min at room temperature to liquid state, and conventional semen analyses were performed. Only normozoospermic samples (vital percentages>60%, motility II–III grades) were used. The spermatozoa separated from the seminal plasma by Percoll (65–95%) density gradient centrifugation were washed and resuspended in Hepes – Ham's F-10 culture medium (pH 8). The sperm concentration in the suspension was adjusted to 20×10^6 spermatozoa/ml.

Collection of female genital tract fluids

Vaginal, cervical secretions and uterine, fallopian tubal fluids were obtained from 6 nonpregnant donors of reproductive age with no history of systemic disorders, who asked for female sterilization at the Department of Obstetrics and Gynecology of the First Hospital, Lanzhou Medical College. The samples were tested within 15 min.

Generation of O^-_2

O^-_2 was generated in Hepes-Ham's F-10 medium containing xanthine (0.5 mmol/L), catalase (426 unit/ml), DETAPA (0.1 mmol/L), sodium formate (0.4 mmol/L) and xanthine oxidase (0.05 unit/ml)[18]. All expressed as final concentrations.

Groupings and treatments

(a) control group: sperm suspension alone; (b) O^-_2 group: sperm + O^-_2; (C) SOD group: sperm + SOD (1600 unit/ml); (d) O^-_2 + SOD group: sperm + O^-_2 + SOD (400–3200 unit/ml); (e) O^-_2 + inactivated SOD (SOD_i) group: sperm + O^-_2 + SOD_i; (f) Whole semen + DDC (1 mmol/L) (DDC is the inhibitor of SOD). (A) non-incubated group: precapacitated sperm suspension alone; all expressed as final concentrations. All groups except A were incubated at 37°C for 3.5 h in 5% CO_2 to lead the spermatozoa capacitation.

Determination of cAMP in spermatozoa

After incubation for 3.5 h, 1 N $HClO_4$ 0.5 ml was added into 1 ml sperm suspension from each group. Spermatozoa were intermittently homogenized in the ice-water bath for 3 min with ultrasonic homogenizer (Jc-2 model, China), and then homogenate were centrifugated (1200 × g for 20 min, 4°C). The supernatant was adjusted to pH 7.0 with 2 N KOH, and centrifugated (1200 × g for 20 min, 4°C) once again. The supernatant was used to determine the intracellular cAMP with [^{3}H]–cAMP radioimmunoassay. [^{3}H]–cAMP 30 µl (0.37 KBq/30 µl) and combined – protein 60 µl (20 µg/60 µl) were successively added to a tube containing 40 µl of the testing supernatant, shaked gently. After reacting for 2 h, 50 µl activated charcoal was supplemented to the reactive system, then centrifugated 1000 × g for 6 min immediately. The supernatant 120 µl was put into a vial, 100% ethanol 1.5 ml and 3.5 ml scintillation liquid were successively added, evenly mixed, and then radioactivity was measured with liquid scintillation counter (FJ-2115 model, China). All reactions were performed in ice-water bath. The concentration of cAMP was calculated according to the standard curve.

Determination of AR

After 3.5 h of incubation, an aliquot of the sperm suspension was used to determine the percentage of spontaneous AR without any inducers. A second aliquot of the sperm suspension was treated with LPC (final concentration 100 µmol/L) for 30 min at 37°C, and then determined the percentage of inductive AR. The acrosomal status was evaluated by the triple-stain technique (TST).[19] Two hundred spermatozoa were counted for each sample by a bright-field microscope with 1000 multiple enlargement. There are the following four stainings in spermatozoa: (1) dark blue postacrosomal regions and pink acrosomes (dead spermatozoa in which the acrosome is still completely or partially intact); (2) dark blue postacrosomal regions with white 'acrosomal

regions' (dead spermatozoa lacking acrosome); (3) light brown postacrosomal regions and pink acrosomes (spermatozoa were alive at the time of fixation but had not undergone AR); (4) light brown postacrosomal regions and white 'acrosomal regions' (spermatozoa that were alive at the time of fixation and had undergone normal AR). The percentage of AR is calculated as numbers of (4)/(3) + (4).

Determination of O^-_2

DMPO 50 μl (final concentration 0.94 mmol/L) was added into 200 μl aliquots of vaginal and cervical secretions, uterine and fallopian tubal fluids, seminal plasma, capacitated spermatozoa and noncapacitated spermatozoa (adjusted sperm concentration to 20×10^8 spermatozoa/ml) respectively. After evenly mixed, electron paramagnetic resonance (EPR) signals were immediately measured with EPR instrument (BRUKER 200 D-SRC, Germany). Microwave frequency: 9.7 GHz; microwave power: 10 dB 16.7 mW; cavity: T.M; frequency: 100 KHz; field modulation intensity: 5 G_{PP}; gain: 5×10^3; time constant: 1000 sec; phase: 0; mid range: 3480 G; scan range: 500 G; scan time: 500 sec.

Statistical analysis

Each value is expressed as the mean and standard error of mean (SEM). The differences among data of individual groups were performed with the analysis of variance (ANOVA). $P<0.05$ was selected as a criterion for statistically significant difference.

RESULTS

Promotion of O^-_2 on Human Sperm Capacitation

The concentration of intracellular cAMP (pmol/10^8 spermatozoa) of spermatozoa treated with O^-_2 increased from 14.0 ± 1.3 to 23.2 ± 2.5 ($P<0.01$, Figure 1, groups a and c). The percentages

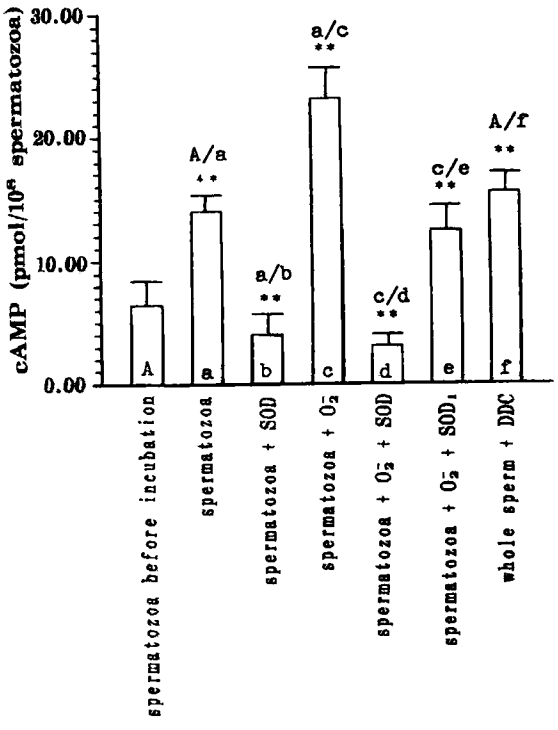

FIGURE 1 cAMP accumulation induced by O^-_2 in human spermatozoa and effects of SOD and inactivated SOD (SOD_i) on this process. Except the group A, all the other groups are incubated at 37°C for 3.5 h. Values represent mean ± SEM, n = 10, **: $P<0.01$.

of spontaneous and inductive AR were significantly higher than the control group, and increased from 4.5 ± 1.1% and 14 ± 1.9% to 16 ± 2.0% and 32.5 ± 1.7% respectively ($P<0.01$ in both comparisons, Figure 2, groups a and c).

Inhibition of SOD on Capacitation and AR

SOD (1600 unit/ml) prevented both spontaneous capacitation and that induced by exogenous O^-_2. Intracellular cAMP of spermatozoa significantly declined ($P<0.01$, Figure 1, groups b and d), and the percentages of spontaneous and inductive AR also declined significantly ($P<0.01$ in both comparisons Figure 2, groups b and d). The inhibitions of SOD were concentration dependent, and the lowest concentration needed for inhibition was 400 unit/ml. Within the extent of 400–1600

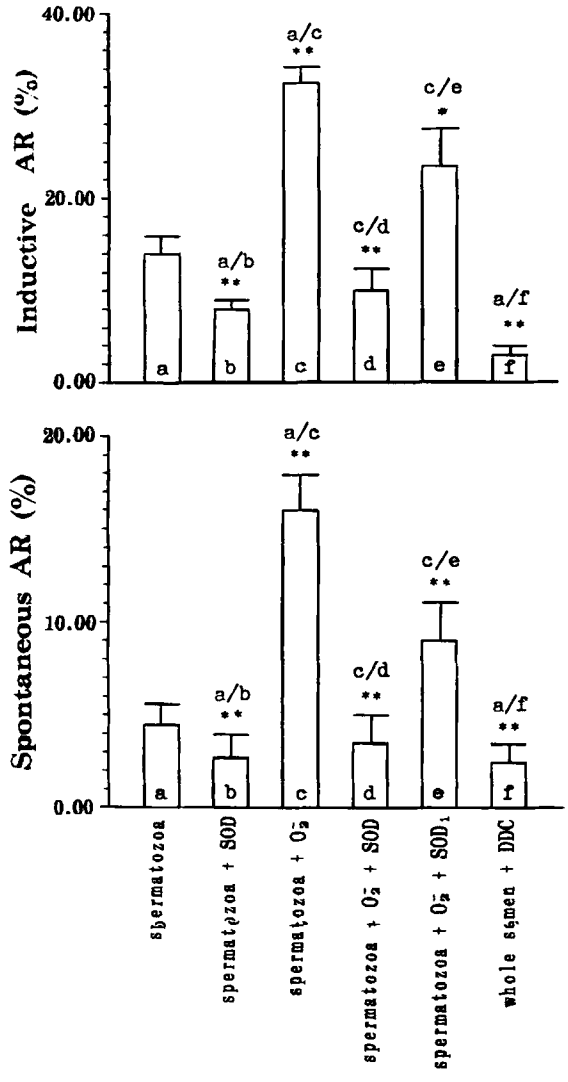

FIGURE 2 Levels of spontaneous AR and inductive AR induced by O^{-}_{2} in human spermatozoa and effect of SOD and inactivated SOD (SOD_i) on these processes. All groups are incubated at 37°C for 3.5 h. Values represent mean ± SEM, n = 10, *: P<0.05; **: P<0.01.

unit/ml, the inhibitions increased with concentrations. Maximum inhibition was achieved with a concentration of 1600 unit/ml, (Figure 3).

When SOD was inactivated by boiling for 20 min, the inhibition decreased extremely, but still retained part of its activity. (Figure 1, group e, Figure 2, group e). This result probably reflects the

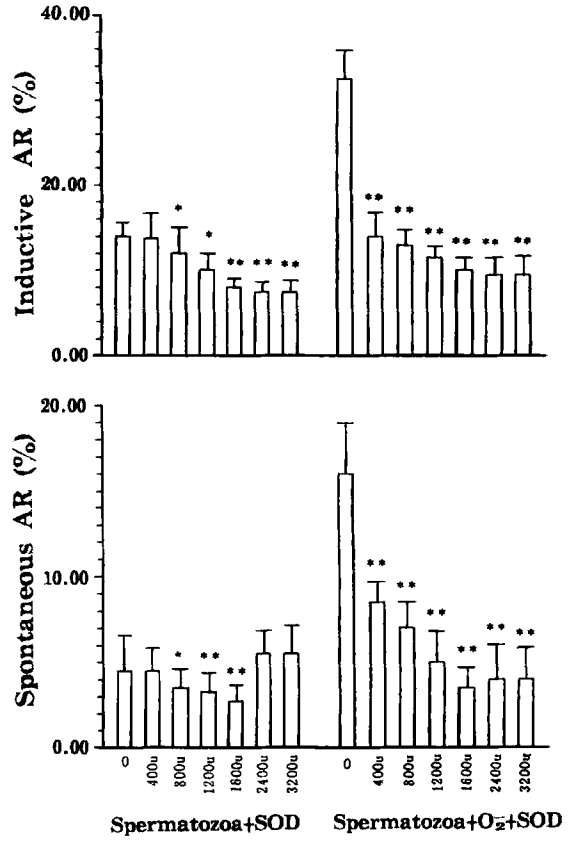

FIGURE 3 Effects of different concentrations of SOD on human sperm spontaneous and inductive AR treated with or without O^{-}_{2}. All groups are incubated at 37°C for 3.5 h. Values represent mean ± SEM, n = 10; *: P<0.05; **: P<0.01.

O^{-}_{2} scavenging effect of proteins and amino acids contained in SOD besides its enzymatic activity.

Effects of DDC on Capacitation and AR in Whole Semen

The sperm capacitating process in the female genital tract is actually metabolic activating process. Seminal plasma (the fluid surrounding spermatozoa after ejaculation) plays a metabolic conserving role to spermatozoa and inhibits sperm capacitation and thus the sperm capability to penetrate the zona-free hamster oocytes.[20] Therefore, spermatozoa must be free of seminal plasma before capacitation is possible. Human seminal

plasma exhibits SOD activity,[10,21] and DDC is an effective inhibitor of Cu Zn SOD.[22] After treatment with DDC 3 mmol/L for 1 h, the SOD activity in fibroblast cells BP6T declined significantly.[23] In order to examine whether the capacitating inhibition of seminal plasma is at least partly, if not all, due to SOD, whole semen treated with DDC (1 mmol/L) for 3.5 h. The level of sperm intracellular cAMP (Figure 1, group f) was significantly higher than pre-incubation level ($P<0.01$, Figure 1, group A), and close to the level of the incubated group (Figure 1, group a). But, both percentages of spontaneous and inductive AR were far lower than the percentages of the control group ($P<0.01$, Figure 2, groups a and f).

The Source of O^{-}_2 During Internal Sperm Capacitation

EPR signal obtained by the spin trapping method was detected only in capacitated spermatozoa. This signal was identified as a DMPO-OO (H) spectrum with 12 peaks hyperfine splitting contributed by O^{-}_2 (Figure 4). After SOD (1600 unit/ml) was added to capacitated spermatozoa, the signal disappeared, which further confirmed that the spectrum was contributed by O^{-}_2. After SOD was replaced with equal quantity of SODi, the signal declined significantly, but still retained trace (spectrum not shown). The EPR signal was obtained neither in vaginal and cervical secretions, uterine and fallopian tubal fluids, nor in seminal plasma and noncapacitated spermatozoa.

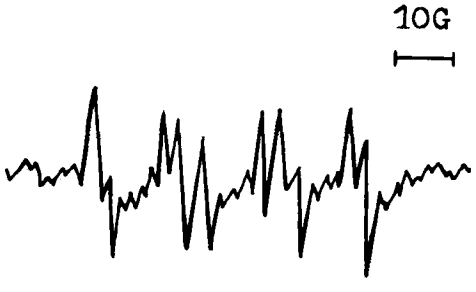

FIGURE 4 Representative EPR spectrum of capacitated human spermatozoa.

DISCUSSION

The present study directly demonstrates that human sperm capacitation can be induced by exogenous O^{-}_2, and the percentage of AR can be enhanced by exogenous O^{-}_2 as well. Our results also show that SOD significantly inhibits these processes, which supported further the conclusion that O^{-}_2 could induce human sperm capacitation. The trigger mechanism of O^{-}_2 on human sperm capacitation is unknown yet. For the first time, the EPR signal of O^{-}_2 was detected in capacitated spermatozoa and disappeared when SOD was added. Furthermore, no EPR signal was detected whatever in female genital tract fluids, seminal plasma and noncapacitated spermatozoa. These findings indicate that capacitated spermatozoa themselves can generate endogenous O^{-}_2. This result supports Lamirande and Gagnon's[16,17] hypothesis that capacitated spermatozoa generate O^{-}_2. Since many of the changes in the sperm capacitating process, including influx of calcium, synthesis of inositol triphosphate and diacylglycerol, activation of phospholipase and protein kinases *etc.* are associated with activation of the NADPH oxidase,[3–5] we suggested that sperm membrane may contain a NADPH oxidase system for generating O^{-}_2. In the presence of seminal plasma, this system would be dormant or inhibited by SOD, so that O^{-}_2 can not be accumulated to a concentration sufficient to induce capacitation. Therefore, sperm capacitation can not occur in seminal plasma. After spermatozoa were separated from seminal plasma and incubated under the capacitating conditions in the present studies, we propose that NADPH oxidase would become activated and consequently generates O^{-}_2 which induced and promoted sperm capacitation in turn.

It is known that seminal plasma has a very high capacity for scavenging ROS. The content of SOD in the human testicle is very high, but lower than that of liver and cerebral cortex.[24] Human seminal plasma also has the activity of SOD.[10,21] Thus SOD can not only protect spermatozoa against ROS but

also inhibit sperm metabolism and prevent against premature capacitation.[17,25] Inhibition of SOD in seminal plasma by DDC, significantly increased the concentration of sperm intracellular cAMP. This result indicates that O^{-}_{2} surely plays a role in sperm capacitation. However, in the same experiment, the levels of AR and LPC-AR were lower than that of the control group. These results suggested that capacitation and AR are quite different mechanisms at the molecular level. Capacitation only shows physiological and biochemical changes of spermatozoa before AR, and these changes are reversible. Besides the physiological changes including sperm acrosomal splitting and dispersion of the acrosomal matrix, the AR exhibits morphological changes of spermatozoa, which are irreversible. Reports[20,26] have shown that seminal plasma contained a high molecular weight glycoprotein called antifertility factor-1 (AF-1), which could block sperm capacitation without affecting the AR. AF-1 did not inhibit the activity of acrosin that participated in AR. These results also can be explained as capacitation and AR are different mechanisms. We suggested that AF-1 probably is but one of the inhibitors of capacitation, and distinct inhibitors for AR must also exist.

In summary, our results suggest that O^{-}_{2} can induce and promote human sperm capacitation, while SOD can prevent this process. O^{-}_{2} generates in spermatozoa under capacitating conditions. It is known that spermatozoa cannot perform AR unless capacitation is complete, and AR must occur before penetrating the zona pellucid for fusion with the oocyte. Since SOD is able to inhibit sperm capacitation, it would be useful to interfere with the essential capacitating process in reproduction so as to regulate fertilization.

References

1. M.C. Chang (1984) The meaning of sperm capacitation. *Journal of Andrology*, **5**, 45–50.
2. R.V. Hyne and D.L. Garbers (1979) Calcium-dependent increase in adenosine 3′,5′-monophosphate and induction of the acrosome reaction in guinea pig spermatozoa. *Proceedings of the National Academy of Sciences of the United States of America*, **76**, 5699–5703.
3. P.E. Visconti, J.P. Muschietti, M.M. Flawia and J.G. Tezon (1990) Bicarbonate dependence of cAMP accumulation induced by phorbol esters in hamster spermatozoa. *Biochimica et Biophysia Acta*, **1054** (2), 231–236.
4. R. Yanagimachi and N. Usui (1974) Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. *Experimental Cell Research*, **89**, 161–174.
5. N.J. Monks, D.M. Stein and L.R. Fraser (1986) Adenylate cyclase activity of mouse sperm during capacitation *in vitro*: Effect of calcium and a GTP analogue. *International Journal of Andrology*, **9** (1), 67–76.
6. D.F. Katz and R. Yanagimachi (1980) Movements characteristics of hamster spermatozoa within the oviduct. *Biology of Reproduction*, **22**, 759–764.
7. R. Yanagimachi and F. Suzuki (1985) A further study of lysolecthinmediated acrosome reaction of guinea pig spermatozoa. *Gamete Research*, **11**, 29–40.
8. M.K. Holland, J.G. Alvarez and B.T. Storey (1982) Production of superoxide and activity of superoxide dismutase in rabbit epididymal spermatozoa. *Biology Reproduction*, **27**, 1109–1118.
9. M.K. Holland and B.T. Storey (1981) Oxygen metabolism of mammalian spermatozoa. Generation of hydrogen peroxide by rabbit epididymal spermatozoa. *Biochemical Journal*, **98**, 273–280.
10. J.G. Alvarez, J.C. Touchstone, L. Blasco and B.T. Storey (1987) Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. *Journal of Andrology*, **8**, 338–348.
11. R.J. Aitken and J.S. Clarkson (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *Journal of Reproduction and Fertility*, **81**, 459–469.
12. R.J. Aitken, D. Harkiss and D.W. Buckingham (1993) Analysis of lipid peroxidation mechanism in human spermatozoa. *Molecular Reproduction Development*, **35**, 302–315.
13. A. Iwaski and C. Gagnon (1992) Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertility and Sterility*, **57**, 409–416.
14. R.J. Aitken (1994) A free radical theory of male infertility. *Reproduction Fertility and Development*, **6**, 19–24.
15. G. Huszar and L. Vigue (1994) Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. *Journal of Andrology*, **5** (1), 71–77.
16. E. de Lamirade and C. Gagnon (1993) A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *International Journal of Andrology*, **16** (1), 21–25.
17. E. de Lamirande and C. Gagnon (1993) Human sperm hyperactivation and capacitation as part of an oxidative process. *Free Radical Biology and Medicine*, **14**, 157–166.
18. I. Fridorich (1985) Xanthine oxidase, in *Handbook of Methods for Oxygen Radical Research*, ed. R.A. Greenwald, CKC Press, Boca Raton, Florida, pp. 15–53.
19. P. Talbot and R.S. Chacon (1981) A triple-stain technique for evaluating normal acrosome reaction of human sperm. *Journal of Experiment Zoology*, **215**, 201–208.
20. Van der Ven H. Asok, K. Bhattacharyya, Zvi Binor, S. Leto and L.J.D. Zaneveld (1982) Inhibition of human sperm capacitation by a high-molecular-weight factor from human seminal plasma. *Fertility and Sterility*, **38**, 753–755.
21. M.R.F. Mennella and R. Jones (1980) Properties of

spermatozoal superoxide dismutase and lack of involvement of superoxide in metal-ion-catalyzed lipid-peroxidation reaction in semen. *Biochemical Journal*, **191,** 289–297.

22. C. Michels and J. Remacle (1988) Use of the inhibition of enzymatic antioxidant system in order to reveal its physiological importance. *European Journal of Biochemistry*, **177,** 435–441.
23. R.L. Zheng, S.A. Lesko and P.O.P. Ts'o (1984) Toxicity of Diethyldithiocarbamate on tumor cells with reference of superoxide dismutase. *Acta Biochem Biophys Sinica*, **6,** 675–678.
24. B.H. Halliwell and J.M.C. Gutteridge (1985) *Free Radicals in Biology and Medicine*. Clarendon Press, Oxford, p. 94.
25. R. Jones, T. Mann and R. Scherins (1979) Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertility and Sterility*, **31,** 531–537.
26. J.M. Reddy, J.C. Audhya, Goodpasture and L.J.D. Zaneveld (1982) Properties of a highly purified antifertility factor from human seminal plasma. *Biology of Reproduction*, **27** (5), 1076–1083.