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Analysis of the Relationship Between Defective Sperm Function and the Generation of Reactive Oxygen Species in Cases of Oligozoospermia

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The ability of human spermatozoa to exhibit sperm-oocyte fusion in response to the ionophore, A23187, was examined in relation to the capacity of these cells to generate reactive oxygen species. In 70 fertile control donors, there was an overwhelming pattern of high levels of sperm-oocyte fusion associated with low levels of reactive oxygen species production. By contrast, 88% of the 74 oligozoospermic patients exhibited < 25% oocyte penetration in response to A23187 and 58% exhibited no penetration whatsoever. Of the 40 oligozoospermic patients who failed to respond to A23187, nine had low levels of reactive oxygen species production in association with impaired liquefaction of seminal plasma. Of the remainder, 17 (55%) exhibited defective sperm function together with elevated production of reactive oxygen species. These observations, which are the first to describe a biochemical defect in the spermatozoa of oligozoospermic patients, may carry significant implications for the etiology and treatment of this condition.

Key words: Oligozoospermia, oxygen radicals, A23187, hamster oocyte penetration.

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Defective sperm function has been identified recently as the most frequent defined cause of human infertility, accounting for at least 24% of couples attending infertility clinics (Hull et al, 1985). Oligozoospermia (a sperm count of less than 20×10^6/ml; World Health Organization, 1987) is a particularly frequent attribute of male infertility that has been described in 16 to 41% of infertile couples (Belsey and Ware, 1986), the incidence varying with geographic location. Despite the prevalence of this condition, there are few, if any, treatments that materially affect the ability of such patients to initiate a pregnancy. Because of our lack of knowledge concerning the multifarious causes of oligozoospermia, a rational approach toward the development of appropriate therapies cannot be undertaken.

In response to this situation, studies have recently

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been initiated on the cell biology of defective sperm function to define at the biochemical level the nature of the lesions present in the spermatozoa of infertile patients (Aitken and Clarkson, 1987; Aitken et al, 1987; Huszar et al, 1988). Such information should be of value in tracing the development of a given syndrome back through epididymal maturation and spermatogenesis to its source.

Using calcium signals generated by the divalent cation ionophore, A23187, we have already shown that the defective sperm function encountered in most oligozoospermic patients involves lesions downstream from calcium influx, since the spermatozoa of such patients generally fail to show a biologic response to the ionophore, in marked contrast to the normal fertile population (Aitken et al, 1984). A possible reason for this refractoriness to calcium stimulation has been identified in recent investigations focusing on the production of reactive oxygen species by human spermatozoa (Aitken and Clarkson, 1987; Alvarez et al, 1987). In these studies an inverse relationship was observed between the ability of human spermatozoa to generate reactive oxygen species and their capacity to exhibit sperm-oocyte fusion in response to A23187 (Aitken and Clarkson, 1987). These findings, and those of other key papers (Jones and Mann, 1973; Jones et al, 1979; Alvarez et al, 1987; Kwenang et al, 1987), have led us to hypothesize that the excessive production of reactive oxygen species may play a causative role in the etiology of defective sperm function through the peroxidation of the unsaturated fatty acids in the human sperm plasma membrane. As a consequence of such damage, the latter loses its capacity to respond to the calcium signal that normally initiates the acrosome reaction (Aitken and Clarkson, 1987).

Since previous studies in this area have used unselected populations of donors, the frequency with which defective sperm function is associated with the excessive generation of reactive oxygen species is unknown. To address this problem, we have examined the relationship between sperm function and reactive oxygen species production in two defined cohorts of men—the normal fertile population and men exhibiting infertility associated with oligozoospermia.

Materials and Methods

Semen Samples

The fertile population (n = 70) had previously initiated at least one pregnancy and all subjects exhibited a normal semen profile (> 20×10^6 spermatozoa/ml; > 40% motility and > 40% normal morphology; Aitken et al, 1982), with the exception of one patient who provided a sample exhibiting a slightly diminished motility value of 36%.

The patient population was recruited on the basis of at least 12 months infertility associated with persistent (at least three semen samples) oligozoospermia, defined as a sperm count of < 20×10^6/ml. The mean ± SEM count for this cohort of patients (N = 74) was 15.2 ± 0.8 × 10^6 sperm cells/ml. Among these patients, 19 were found on examination to possess a clinical varicocele, Grade I to III (Comhaire, 1983), associated with the active refluxing of blood down the left internal spermatic vein, and 12 (nine with idiopathic oligozoospermia and three with oligozoospermia in association with a varicocele) exhibited impaired liquefaction, i.e. a thread of more than 2 cm in length was formed when a pipette was inserted into the semen sample and gently withdrawn (World Health Organization, 1987).

None of the samples exhibited a white blood cell count in excess of 1×10^6 cells/ml (World Health Organization, 1987). This is an important factor, since leukocytes, particularly macrophages and neutrophils, are known to generate reactive oxygen species during phagocytosis, and semen specimens exhibiting significant leukocytic infiltration (more than 1×10^6/ml) are associated with high levels of reactive oxygen species production (R.J. Aitken, unpublished observations). Finally, five of the patients had oligozoospermia in association with the administration of sulphasalazine for ulcerative colitis.

Analyses of Sperm Function

The semen samples were washed free of seminal plasma by three cycles of centrifugation (500 × g for 5 minutes) and resuspension in 8-ml volumes of medium BWW (Biggers et al, 1971). The spermatozoa were adjusted to a final concentration of 20×10^6 cells/ml and then diluted with an equal volume of medium BWW containing an aqueous suspension of the Ca^{2+}/Mg^{2+} salt of A23187 (0.05 mg/ml) (Aitken et al, 1984). After a 3-h incubation at 37 C in an atmosphere of 5% CO_2 in air, the spermatozoa were centrifuged at 500 × g for 5 minutes, resuspended in normal medium BWW and dispersed as 50-μl droplets under liquid paraffin. Zona-free hamster oocytes were then added to the sperm suspensions and incubated at 37 C in 5% CO_2 in air for 3 h before being examined by phase contrast microscopy for signs of penetration. The results were expressed in terms of the percentage of hamster oocytes penetrated at a motile sperm concentration of 5×10^6 spermatozoa/ml (Aitken and Elton, 1986).

Reactive Oxygen Species

Washed populations of human spermatozoa were resuspended at a concentration of 20×10^6/ml, and 1 μl of luminol (5-amino 2,3,-dihydro-1,4-phthalazinedione), stored as a 100-mM stock solution in dimethyl sulfoxide (DMSO), was added to 500 μl of the sperm suspension. This luminol-supplemented suspension was then diluted with 500 μl medium BWW (control) or 500 μl of medium BWW containing the Ca^{2+}/Mg^{2+} salt of A23187 (0.05 mg/

ml). After 3 minutes, the degree of luminescence was recorded on a Berthold, Biolumat, LB 9500T luminometer, in the integration mode. Luminol is an extremely sensitive chemiluminescent probe that will react with a variety of reactive oxygen species (H_2O_2; $.O_2-$, .OH, $^1O_2$ with the emission of light (Aitken and Clarkson, 1987). At neutral pH, luminol exhibits a tendency toward hydrophobic binding and will permeate cells, giving an indication of the reactive oxygen species generated within the cell, as well as activity released into the ambient medium (Allen, 1982). Background luminescence before luminol addition was negligible and was not influenced by exposure to A23187 (Aitken and Clarkson, 1987). The latter induces a rapid increase in the generation of reactive oxygen species in a calcium-dependent manner (Aitken and Clarkson, 1987).

Chemically induced oxidation of luminol with potassium permanganate was used to determine the consistency of the luminometers' photomultiplier response. This analysis gave an interassay coefficient of variation of 16.5% (n = 29). All statistical comparisons were made using nonparametric statistics, particularly, the Wilcoxon matched pairs signed ranks- and Mann Whitney U tests.

Results

Comparison of the levels of sperm-oocyte fusion observed in response to A23187 revealed a marked difference in the functional competence of the motile spermatozoa obtained from normal fertile and oligozoospermic men. Reference to Fig. 1, therefore, indicates that 58% of the oligozoospermic population exhibited < 10% sperm-oocyte fusion in response to the calcium signal generated by A23187, compared with only 3% of the fertile group (Figs. 1a, b). Conversely, while 96% of the fertile men penetrated more than 26% of the oocytes following exposure to A23187, the equivalent figure for the oligozoospermic population was only 18% (Figs. 1a, b).

The relationship between the biologic responses of the cells and the capacity for generating reactive oxygen species is indicated in Figs. 1 to 3. In the normal fertile population (Figs. 1b, 3), the overwhelming pattern was one of high levels of sperm-oocyte fusion in association with low levels of reactive oxygen species production. Only two specimens (2.8%) exhibited a steady state luminol signal in excess of 10×10^3 cpm and one of these samples was atypical in that it gave a hamster oocyte penetration score of 0%. Addition of A23187 to these populations of normal, functionally competent cells resulted in a significant, yet limited, increase in the levels of reactive oxygen species production ($P < 0.001$; Wilcoxon matched-pairs signed-ranks test). In 95% of these samples, the luminol signal was not in excess of 30×10^3 cpm (Figs. 1b, 3).

In the oligozoospermic group, addition of A23187 induced a very marked, statistically significant increase in the level of reactive oxygen species production (Fig. 1a). Furthermore, both the basal level of reactive oxygen species production and the magnitude of the response to A23187 was greater ($P < 0.02$; Mann Whitney U) for specimens exhibiting seriously defective sperm function (0 to 10% oocyte penetration) than for samples in which sperm function was normal (26 to 100% oocyte penetration; Fig. 1a).

Detailed analysis of the responses given by individual specimens (Fig. 2) revealed the clear presence of a cohort of samples in which defective sperm function was associated with the hyperactive generation of reactive oxygen species. Of the 40 oligozoospermic specimens exhibiting 0% oocyte fusion, 42.5% exhibited luminol signals in excess of 20×10^3 cpm after addition of A23187. Sufficient spermatozoa were also available in 33 oligozoospermic specimens exhibiting 0% penetration to assess the basal level of reactive oxygen species production in the absence of A23187. In 12 such cases (36%), an abnormally high luminol signal of $> 10 \times 10^3$ cpm was observed. None of the oligozoospermic samples exhibiting oocyte penetration rates in excess of 25% exhibited luminol signals of more than 0.4×10^3 cpm or 15×10^3 cpm before and after exposure to A23187, respectively (Fig. 2).

The presence or absence of a varicocele or the induction of oligozoospermia with sulphasalazine did not appear to influence the distribution of the data (Fig. 2). All three groups included examples of specimens exhibiting high levels of reactive oxygen species production in association with defective sperm function. Furthermore, within the oligozoospermic group as a whole, the level of reactive oxygen species produced was unrelated to any aspect of the conventional semen profile (data not shown).

Clearly, oligozoospermia is a condition with multiple etiologies, and while a significant proportion (36 to 42.5%) of the samples failing to achieve oocyte fusion exhibited elevated levels of reactive oxygen species production, the remaining specimens were normal in this respect. In several such cases, the co-existence of impaired sperm function and low levels of reactive oxygen species production was associated with impaired liquefaction of the seminal plasma. Of the 23 cases exhibiting 0% oocyte penetration and low levels of reactive oxygen species production, impaired semen liquefaction was noted on nine occasions (39%). The mean (± SE) levels

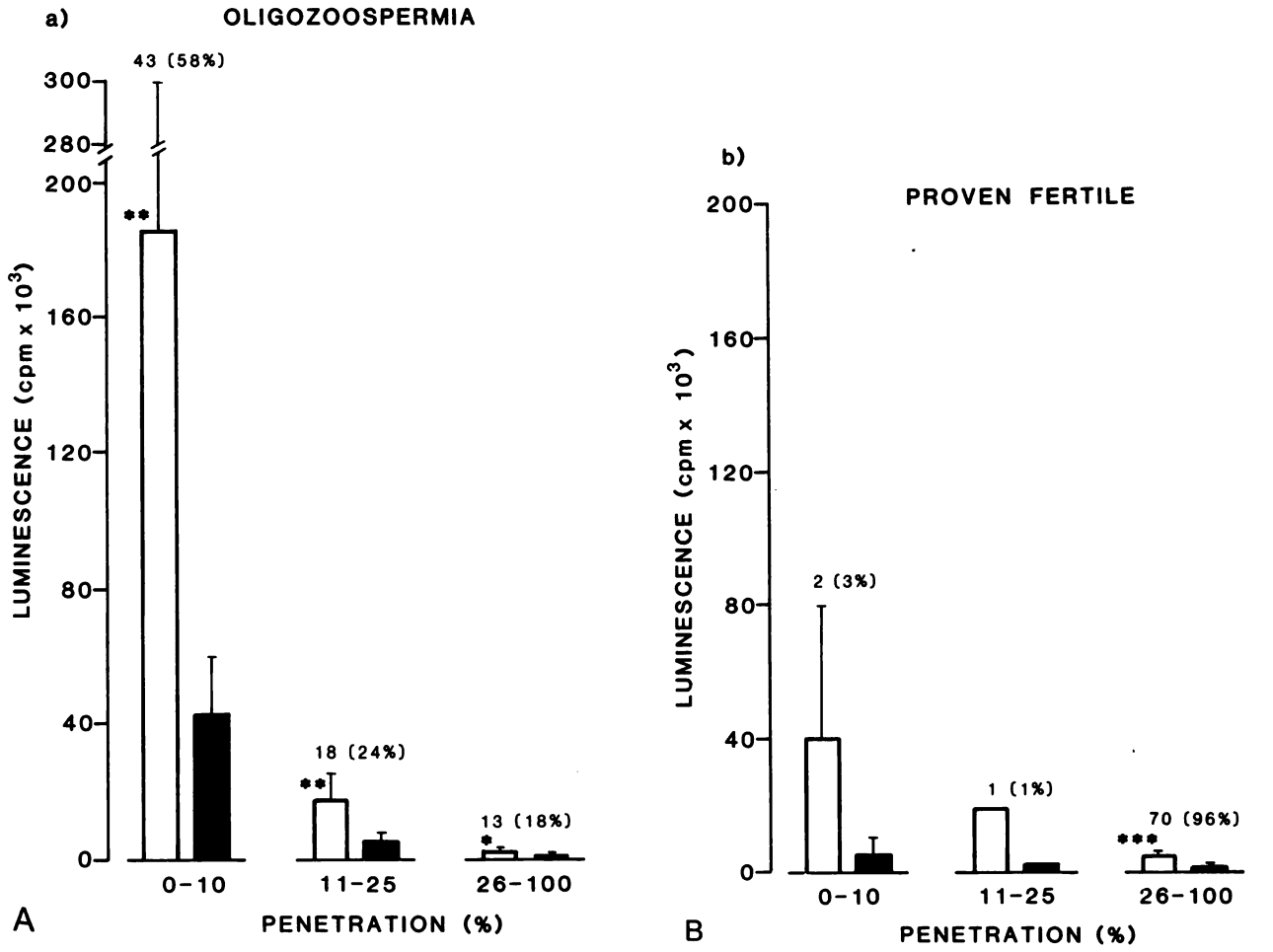

Fig. 1. Relationship between the frequency distribution of hamster oocyte penetration scores and the production of reactive oxygen species in **A)** oligozoospermic patients; and **B)** normal fertile donors. Open bars = response measured 3 min after the addition of A23187, closed bars = basal control level. Numbers at the head of each pair of columns = number of patients (5) in each section of the frequency distribution. *** = $P < 0.01$ ** $P = < 0.01$ * $P < 0.05$ for difference between control and A23187-treated samples.

of reactive oxygen species produced by samples with such poor liquefaction were 846 ± 523 cpm and 2015 ± 1030 cpm before and after A23187 treatment, respectively. These values were significantly ($P < 0.01$; Mann Whitney U) lower than those recorded for the remaining samples with normal liquefaction (59,302 ± 23,772 cpm and 253,830 ± 161,554 cpm).

Discussion

The purpose of this study was to compare the functional competence of spermatozoa from oligozoospermic and normal fertile men and to relate any differences observed to the capacity of these cells to generate reactive oxygen species. As a measure of functional competence, we examined the responses of human spermatozoa to the divalent cation ionophore, A23187, in the zona-free hamster oocyte penetration test (Aitken et al, 1984). This system specifically generates information on the capacity of human spermatozoa to respond to a calcium signal by engaging in the membrane-fusion events associated with fertilization, namely the acrosome reaction and sperm-oocyte fusion (Aitken et al, 1984). The properties measured by this test, while they do not encompass every aspect of sperm function, have been shown to reflect the fertilizing potential of human spermatozoa *in vivo* (Irvine and Aitken, 1986).

Previous studies with this system have revealed

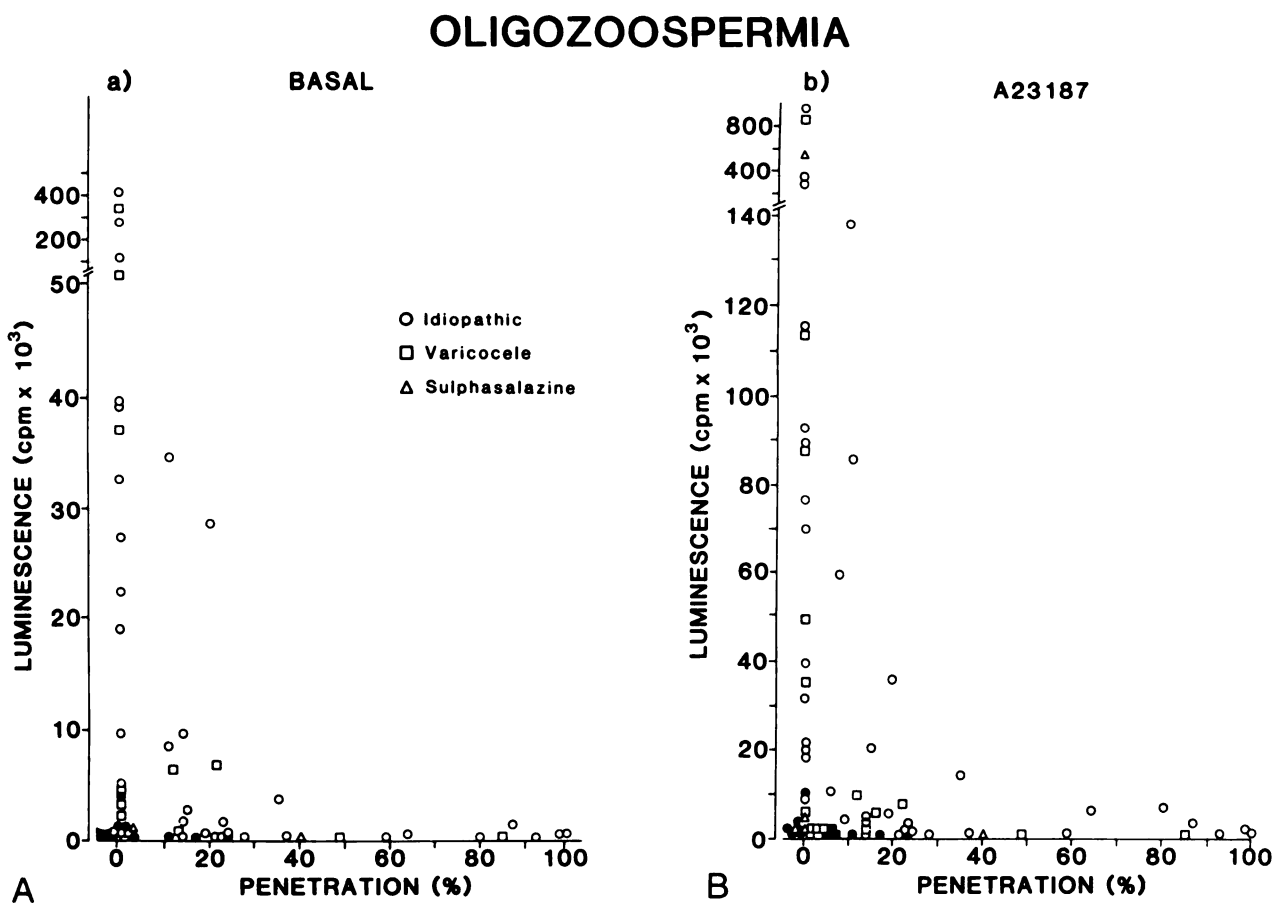

Fig. 2. Scattergram plots for individual samples describing the relationship between reactive oxygen species production and hamster oocyte fusion in the oligozoospermic population. **A)** Basal control level; **B)** Response measured 3 min after the addition of A23187. Solid points indicate samples with abnormal liquefaction.

a marked difference in the functional competence of motile spermatozoa from oligozoospermic and normal fertile men (Aitken et al, 1984). The present study has provided confirmatory evidence showing that the motile spermatozoa produced by a high proportion of oligozoospermic patients apparently lack the ability to respond to the calcium signal generated by A23187. This contrasts with the normal fertile population, a great majority of which exhibit high levels of sperm-oocyte fusion in response to the sudden influx of calcium induced by the ionophore.

To determine why the spermatozoa of oligozoospermic men should exhibit such refractoriness, we have examined the generation of reactive oxygen species by these cells. In previous studies (Aitken and Clarkson, 1987) we have shown that there is a general inverse relationship between the capacity of human spermatozoa to generate reactive oxygen species and their ability to exhibit sperm-oocyte fusion in response to A23187. Furthermore, we have postulated that the mechanism underlying this relationship involves the peroxidation of unsaturated fatty acids in the sperm plasma membrane as a result of which the latter loses its integrity and function.

The major reactive oxygen species generated by human spermatozoa appears to be superoxide anion (R.J. Aitken, unpublished observations; Alvarez et al, 1987), which secondarily generates hydrogen peroxide through the action of superoxide dismutase (Menella and Jones, 1980; Alvarez et al, 1987). The combination of hydrogen peroxide and superoxide anion would be expected to generate the pernicious hydroxyl radical if sufficient quantities of iron or copper are available to catalyze the Haber-Weiss

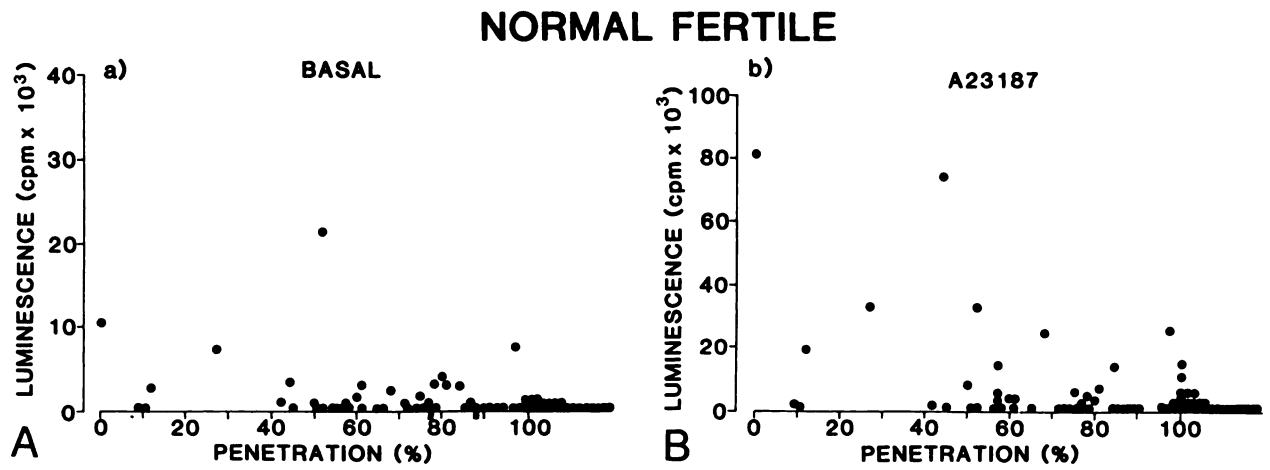

Fig. 3. Scattergram plots for individual samples describing the relationship between reactive oxygen species production and hamster oocyte fusion in the normal fertile population. **A)** Basal control level; **B)** Response measured 3 min after the addition of A23187.

reaction. The conditions for this reaction certainly appear to prevail when the spermatozoa are present in seminal plasma, since this fluid contains iron that is bound neither to transferrin nor ferritin and is therefore theoretically available to promote hydroxyl radical formation (Kwenang et al, 1987). In addition, spermatozoa that are excessively active in generating superoxide anion should be able to promote the local availability of iron by stimulating its release from intracellular ferritin (Biemond et al, 1984).

The hydroxyl radicals generated by the Haber-Weiss reaction are particularly significant since these agents are powerful initiators of lipid peroxidation. The human sperm plasma membrane is very susceptible to such peroxidative damage because of its high content of unsaturated fatty acids (Jones et al, 1979). The biologic significance of lipid peroxidation is indicated by the fact that the induction of this process by the addition of iron to the culture medium results in a loss of human sperm function (Jones et al, 1979), including a failure to respond to A23187 with sperm-oocyte fusion (R.J. Aitken, unpublished observations).

Peroxidative damage to human spermatozoa will result when the generation of reactive oxygen species exceeds the defense mechanisms of the cell, chiefly superoxide dismutase (Alvarez et al, 1987). Occasional samples from the fertile population, exhibiting significant levels of sperm-oocyte fusion despite a slightly elevated level of reactive oxygen species production, may have been protected from lipid peroxidation by virtue of an intracellular deficiency in available iron or a particularly efficient superoxide dismutase system. For a great majority of the normal fertile population, however, the normal pattern observed was of high levels of oocyte fusion in association with low levels of reactive oxygen species production (Fig. 3).

In the oligozoospermic population, the distribution of data was quite different. About 60% of these samples exhibited oocyte penetration rates of less than 10% and in about 40% of such cases high levels of reactive oxygen species production were observed. In view of the foregoing discussion, it is possible that this association is causative, the excessive generation of reactive oxygen species overwhelming the defense mechanisms of the cell and leading to a loss of sperm function through the peroxidation of unsaturated fatty acids in the sperm plasma membrane. Alternatively, the primary lesion in the spermatozoa of such patients may be a defect in the organization of the plasma membrane. In these cells, the mechanisms normally controlling the superoxide generating system are ineffective and, as a consequence, the production of reactive oxygen species is elevated. Given the high frequency of this lesion in the oligozoospermic population, assessment of which of these alternatives is correct is clearly a high priority area for the future, with important therapeutic implications.

In terms of routine diagnostics it should be possible to improve upon the luminol assay as a means of identifying patients exhibiting peroxidative damage to the sperm plasma membrane. The luminol

assay is sensitive but lacks specificity and is difficult to standardize. A sensitive method is required for directly measuring the products of lipid peroxidation, targeting, for example, conjugated dienes or lipid peroxides.

The loss of sperm function observed in cases of varicocele or following exposure to sulphasalazine was not consistently associated with the excessive generation of reactive oxygen species. Oligozoospermia is evidently a condition with multiple etiologies, several of which do not involve the peroxidative mechanisms discussed above. As a consequence, several samples exhibiting defective sperm function were analyzed that did not show elevated levels of reactive oxygen species production. In about 40% of the samples exhibiting defective sperm function in the *absence* of elevated levels of reactive oxygen species production, impaired liquefaction of the seminal plasma was noted during the routine semen analysis. The reason for this association is, as yet, unclear.

In conclusion, this study has demonstrated that a high frequency of oligozoospermic samples exhibit defective sperm function in association with the excessive generation of reactive oxygen species. Of the 31 samples that were totally unresponsive to activation by A23187 (0% oocyte fusion) and in which semen liquefaction was normal, 17 (55%) were found to exhibit elevated levels of reactive oxygen species production. This is the first description of a biochemical defect in the spermatozoa of oligozoospermic patients and may contribute to our understanding of the impaired fertilizing capacity of these cells. Elucidation of the mechanisms involved in regulating the activity of the superoxide generating system and assessment of its role in the genesis of the defective sperm function are the subject of our current research programme.

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References

Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fertil 1987; 81:459–469.

Aitken RJ, Elton RA. Quantitative aspects of sperm-oocyte interaction. Int J Androl 1986; Suppl 6:14–30.

Aitken RJ, Best FSM, Richardson DW, Djahanbakhch O, Lees MM. The correlates of fertilizing capacity in normal fertile men. Fertil Steril 1982; 38:68–76.

Aitken RJ, Clarkson JS, Huang G-F, Irvine DS. Cell biology of defective sperm function. In: Mohri H, ed. New horizons in sperm cell research. New York: Gordon and Breach Science Publishers, 1987: pp. 75–90.

Aitken RJ, Ross A, Hargreave T, Richardson D, Best F. Analysis of human sperm function following exposure to the ionophore A23187: comparison of normospermic and oligozoospermic men. J Androl 1984; 5:321–329.

Allen RC. Biochemical excitation: chemiluminescence and the study of biological oxygenation reactions. In: Adam W and Cilento G, eds. Chemical and biological generation of excited states. London: Academic Press, 1982: 310–341.

Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. J Androl 1987; 8:338–348.

Belsey MA, Ware H. Epidemiological, social and psychosocial aspects of infertility. In: Insler V and Lunenfeld B, eds. Infertility: male and female. London: Churchill Livingston, 1986: 631–647.

Biemond P, van Eijk HG, Swaak AJG, Koster JF. Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes: possible mechanism in inflammation diseases. J Clin Invest 1984; 73:1576–1579.

Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: Daniel JC ed. Methods in mammalian embryology. San Francisco: Freeman, 1971: 86–116.

Comhaire FH. Varicocele infertility: an enigma. Int J Fertil 1983; 6:401–404.

Hull MGR, Glazener CMA, Kelly NJ, Conway DI, Foster PA, Hunton RA, Coulson C, Lambert PA, Watt EM, Desai KM. Population study of causes, treatment and outcome of infertility. Br Med J 1985; 291:1693–1697.

Huszar G, Corrales M. Vigue L. Correlation between sperm creatine phosphokinase activity and sperm concentrations in normospermic and oligozoospermic men. Gamete Res 1988; 19:67–75.

Irvine DS, Aitken RJ. Predictive value of *in vitro* sperm function tests in the context of an AID service. Hum Reprod 1986; 1:539–545.

Jones R, Mann T. Lipid peroxidation in spermatozoa. Proc R Soc Lond 1973; 108:103–107.

Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids by human spermatozoa, spermicidal properties of fatty acid peroxides and protective action of seminal plasma. Fertil Steril 1979; 31:531–537.

Kwenang A, Krous MJ, Koster JF, van Eijk HG. Iron, ferritin and copper in seminal plasma. Hum Reprod 1987; 2:387–388.

Menella MRT, Jones R. Properties of spermatozoal superoxide dismutase and lack of involvement of superoxides in metal ion-catalysed lipid-peroxidation reactions in semen. Biochem J 1980; 191:289–297.

World Health Organization. WHO Laboratory manual for the examination of human semen and semen-cervical mucus interaction. Cambridge University Press, Cambridge, 1987.