

Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study

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Objective: To evaluate the mitochondrial membrane potential (MMP) of spermatozoa and its correlation with semen parameters and production of reactive oxygen species (ROS) in infertile men and healthy donors.

Design: Controlled prospective study.

Setting: Male infertility clinic, Glickman Urological Institute, The Cleveland Clinic Foundation, Cleveland, Ohio.

Patient(s): Nineteen infertile men and 7 healthy volunteers.

Intervention(s): Standard semen analysis, assessment of MMP and ROS production in spermatozoa. The MMP was assessed by flow cytometry using the probe carbocyanine DiOC₆(3) and ROS was measured with chemiluminescence assay using luminol.

Main Outcome Measure(s): The results of MMP are reported as the median interquartile range (IQR) number of cells counted in different areas of fluorescence. Results of ROS measurement are expressed as $\times 10^6$ counted photons per minute per 20 million sperm (cpm).

Results: The patients with abnormal semen parameters had a significantly lower MMP [1337.7 (1066.38, 1879.2)], and higher ROS [1.12 (0.26, 3.86)] than the donors [MMP: 2482.9 (2162.5, 3520.6)] and [ROS: 0.10 (0.01, 0.14)]. The MMP was positively correlated with sperm concentration ($r = 0.62$) and negatively correlated with the ROS produced ($r = -0.45$).

Conclusion(s): Measuring MMP in spermatozoa provides useful information about a man's fertility potential. Increased ROS production by spermatozoa is associated with a decreased MMP. (Fertil Steril® 2003;80(Suppl 2):844–50. ©2003 by American Society for Reproductive Medicine.)

Key Words: Spermatozoa, mitochondrial membrane potential, reactive oxygen species, male infertility, flow cytometry, chemiluminescence

Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants called reactive oxygen species (ROS). The susceptibility of human spermatozoa to oxidative stress has been suggested as a cause of male infertility (1, 2). At low levels, ROS mediates normal sperm functions such as capacitation, hyperactivation, acrosomal reaction, and sperm oocyte fusion, but at high levels, the increased production of ROS can cause oxidative stress and induce pathophysiological changes in the spermatozoa (3, 4). The principal means of ROS-mediated injury to spermatozoa is peroxidative damage to the cell membrane, impair-

ment of sperm motility, and oxidative damage to DNA (4–7). High levels of seminal ROS have been reported in up to 20%–40% of infertile men (8, 9). Increased understanding of the role of oxidative stress in the pathophysiology of human sperm function has therefore become imperative in the study of human fertility.

During the past few years, a number of tests based on flow cytometry have been proposed to explore the integrity of different cell compartments of spermatozoa such as acrosome reaction (10), expression of phosphatidyl serine (11), DNA damage (12, 13), and viability (14). Such assays have the advantage of being more

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statistically robust, accurate, and quicker. Evaluation of the mitochondrial membrane potential (MMP) is one such approach.

Mitochondria contain a double membrane. The outer membrane allows large molecules to flow into the mitochondrial intermembrane space, and the highly invaginated inner membrane, which has a large surface area, is responsible for oxidative phosphorylation. During the process of oxidative phosphorylation, the protons are pumped from inside the mitochondria to the outside, creating an electrochemical gradient called the inner MMP (14). The ability to discriminate between mitochondria exhibiting high membrane potential from those having low MMP provides a rigorous estimate of the metabolic function.

Several different fluorescent probes, such as rhodamine 123 (Rh-123) (14–17), lipophilic cationic probe JC1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide) (18), and carbocyanines dye DiOC₆(3) (3,3'-dihexyloxycarbocyanine iodide) (19) have been used to assess the MMP of spermatozoa. There is positive correlation between MMP and sperm motility and viability (14, 15, 18). Rh-123-positive spermatozoa sorted by flow cytometric cell sorting have significantly improved quality of movement (16). An increased proportion of spermatozoa with depolarized mitochondria has been reported in asthenozoospermic men (18). This suggests that MMP is an important indicator of functional integrity of the spermatozoa.

Mitochondrial DNA damage and altered membrane potential by ROS have been reported in pancreatic acinar cells (20). Artificially induced oxidative stress by incubation with H₂O₂ has been shown to inhibit sperm motility, decrease ATP levels, and dissipate the MMP (19). Spermatozoa are the major source of ROS generation in oligozoospermic patients (21). It has been suggested that in infertile patients, there is increased G6PD-mediated NADPH generation, which in turn fuels the ROS generation by the NADPH oxidase activity of spermatozoa (8, 9). However, studies on other cell types raise a possibility that ROS generation by these spermatozoa may be due to damaged mitochondria (22–24).

Reports evaluating the relationship between ROS production and the MMP in human spermatozoa are lacking. The purpose of our study was to examine the MMP of spermatozoa from infertile patients and healthy donors. We also evaluated the correlation of MMP with conventional semen parameters and ROS production.

MATERIALS AND METHODS

Sample Collection and Preparation

The Institutional Review Board of the Cleveland Clinic Foundation approved this study, and written consent was obtained from all subjects. The infertile patient population

consisted of 19 patients attending the male infertility clinic in the Glickman Urological Institute. All patients had at least one semen parameter that was abnormal for sperm concentration, motility, or morphology, based on the World Health Organization (WHO) criteria (25).

The study also consisted of 7 healthy donors. All donors had an ejaculate volume of at least 2 mL and a sperm concentration of at least 20×10^6 /mL of which at least 50% were motile and 30% had normal sperm morphology, based on WHO criteria (25). All semen samples were obtained by masturbation after at least 48 hours of sexual abstinence. Samples were collected into sterile containers, allowed to liquefy at room temperature, and analyzed for sperm concentration, percent motility, and morphology according to WHO criteria (25). Both ROS and MMP measurements were done in washed semen specimens. Each sample was centrifuged to remove seminal plasma and washed (wash and resuspend technique). An aliquot of sample was used for ROS measurement in duplicate. Similarly, another aliquot was used for MMP measurement.

Semen Analysis

All sperm specimens were evaluated with computer-assisted semen analysis (CASA) using a Motion Analysis VP 50 semen analyzer (Motion Analysis Corporation Technologies, Santa Rosa, CA). For each measurement, a 5- μ L aliquot was loaded onto a counting chamber (MicroCell; Conception Technologies, La Jolla, CA). Four to eight representative fields containing 200 or more spermatozoa were examined. Samples were analyzed for concentration, percent motility, and complex motion characteristics. To ensure the accuracy of the CASA results, sperm count and motility were also manually assessed.

ROS Measurement

Aliquots of liquefied semen were centrifuged at $300 \times g$ for 7 minutes. The seminal plasma was discarded, and the sperm pellet was washed twice with Dulbecco's phosphate buffer saline (PBS, pH 7.4) and resuspended in the PBS at a concentration of 20×10^6 sperm/mL. Levels of ROS were determined by chemiluminescence assay using luminol (5-amino-2,3, dihydro-1,4 phthalazinedione; Sigma Chemical Co., St. Louis, MO) as the probe (26). Measurements were made using a Berthold luminometer (Autolumat LB 953, Wallac Inc., Gaithersburg, MD). Aliquots of 400 μ L of processed specimens were prepared in duplicate. Five millimoles of luminol prepared in dimethyl sulfoxide (DMSO; Sigma Chemical Co.) was added to 400 μ L of sperm suspension. Levels of ROS were determined by measuring chemiluminescence for 15 minutes; the results were expressed as $\times 10^6$ counted photons per minute (cpm) per 20×10^6 sperm.

Spermatozoa MMP Measurement

The spermatozoal MMP was measured by flow cytometric analysis performed on a FACScan (Becton Dick-

TABLE 1

Semen characteristics of patients with abnormal parameters and healthy donors.

Variable	Patients (n = 19)	Donors (n = 7)	P value ^a
Volume (mL)	4 (2.65, 4.75)	2.5 (1.3, 5.45)	.79
Motility (%)	53 (34, 59)	69 (52, 72)	.02
Velocity (μ /sec)	43.2 (35.9, 54.3)	41.9 (40.2, 47.1)	.73
Linearity (μ /s)	51 (49, 53)	42 (41, 53)	.3
Concentration ($\times 10^6$ /mL)	15.7 (12.8, 44.6)	56.4 (40.1, 131.8)	.02
Normal morphology-WHO (%)	20 (13.5, 25.5)	39 (37, 52)	.0001
Round cells ($\times 10^6$ /mL)	1 (.6, 1.65)	1 (.6, 1.14)	.82
Endtz test ($\times 10^6$ /mL)	0 (0, .2)	0 (0, .41)	.16

^a $P < .05$ was significant. The values are presented as median and interquartile range. Mann-Whitney test was used for comparison.

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inson, San Jose, CA) fitted with an argon laser operated at 15 mW and excitation/emission setting of 488 nm/525 nm. The carbocyanines dye DiOC₆(3) was used to detect changes in the MMP. Because DiOC₆(3) has a single wavelength emission, a high MMP was attributed to cells with a high fluorescence signal. Spermatozoa (0.5×10^6) from each fresh sample were incubated with DiOC₆(3) (0.1 nM) at 37°C water bath for 5 minutes in PBS (pH 7.4). Ten thousand cells were analyzed by flow cytometry. As a negative control, sperm sample was also incubated with 1 mM uncoupler carbamoyl cyanide *m*-chlorophenylhydrazone (CCCIP). Data were acquired in the list mode, and the relative proportions of the cells within different areas of the fluorescence profiles were quantified using the LYSYS II software program (Becton Dickin-

son). The MMP assay used in our study has been validated by Marchetti et al. (27).

Statistical Analysis

The Mann-Whitney test was performed to compare the results of the healthy donors with those of the patients. A P value of $<.05$ was considered statistically significant. All summary statistics are presented as median and interquartile range (25 and 75 percentiles). All statistical analyses were performed with the SAS statistical software package (SAS Institute, Cary, NC).

RESULTS

Semen Analysis

The results of the classic semen analysis from 19 infertile patients and 7 donors are shown in Table 1. The pathological patterns were defined according to the criteria of WHO guidelines (25). The patients had significantly poorer concentration [15.7 (12.8, 44.6)], motility [53 (34, 59)], and morphology [20 (13.5, 25.5)] than the donors [concentration: 56.4 (40.1, 131.8) $P < .05$]; [motility: 69 (52, 72) $P < .05$], and [morphology: 39 (37, 52) $P < .01$].

ROS Measurement

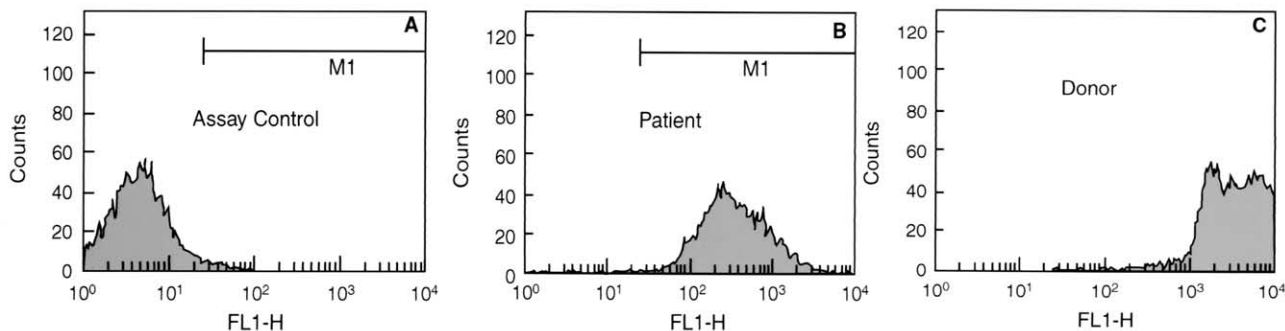
High levels of ROS were seen in patients with abnormal semen parameters [1.12 (0.26, 3.86)] compared to donors [0.10 (0.01, 0.14) $P < .05$].

MMP

Decreased labeling of DiOC₆(3) indicates a decrease in sperm MMP and a loss of mitochondrial membrane integrity. Representative FACScan results from the patients and donors are shown in Figure 1. Levels of MMP were significantly lower in the patients with abnormal semen parameters

FIGURE 1

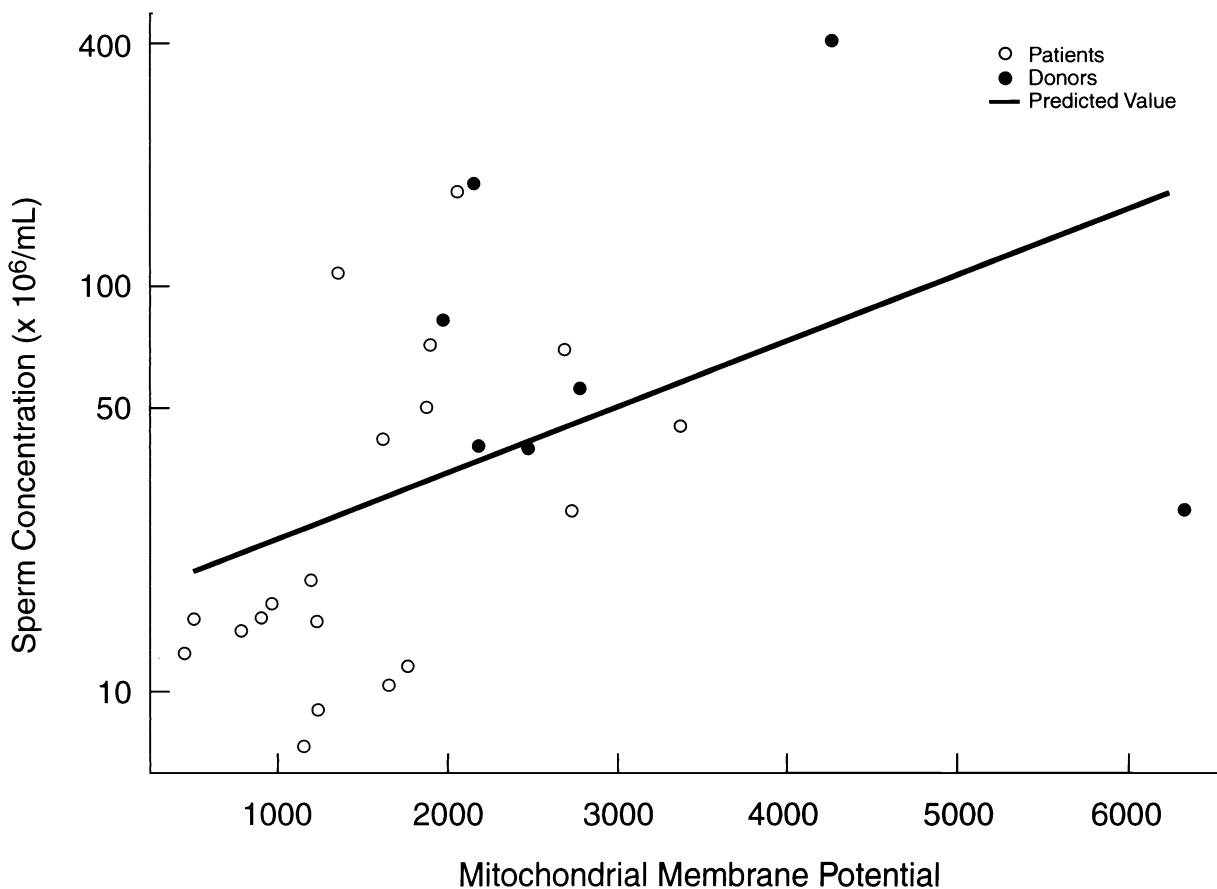
Representative results for (A) sperm sample incubated with carbamoyl cyanide *m*-chlorophenylhydrazone (1 mM, CCCIP) as a negative control for MMP, (B) patients (n = 19) with abnormal semen parameter, and (C) donors (n = 7). The assay control cells show a low intensity of fluorescence indicating a disrupted MMP. The donors have a higher intensity of fluorescence than the patients. FL1 = fluorescent channel designed to detect green fluorescence; M1 = spermatozoa displaying positive fluorescence for MMP.



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FIGURE 2

Relationship between MMP (number of cells positive for MMP) and sperm concentration ($\times 10^6/\text{mL}$) showed a significant positive correlation ($r = 0.62, P < .001$). Nineteen patients with abnormal semen parameters and 7 donors with normal semen parameters were analyzed.



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[1,337.7 (1,066.38, 1,879.2)] than in the donors [2,482.9 (2,162.5, 3,520.6) $P < .02$].

Correlation Between MMP and Conventional Sperm Concentration and ROS Production

A significantly positive correlation was seen between MMP and sperm concentration ($r = 0.62, P < .001$) (Fig. 2). The MMP was inversely correlated with ROS levels ($r = -0.45, P < .05$) (Fig. 3).

DISCUSSION

In the recent past there has been a growing interest in the molecular mechanisms that affect the fertility potential of an individual (4, 6, 9, 21). In our present study, we examined the integrity of the mitochondrial membrane in spermatozoa of infertile patients and its relationship with conventional semen parameters and ROS generation. Our results indicate

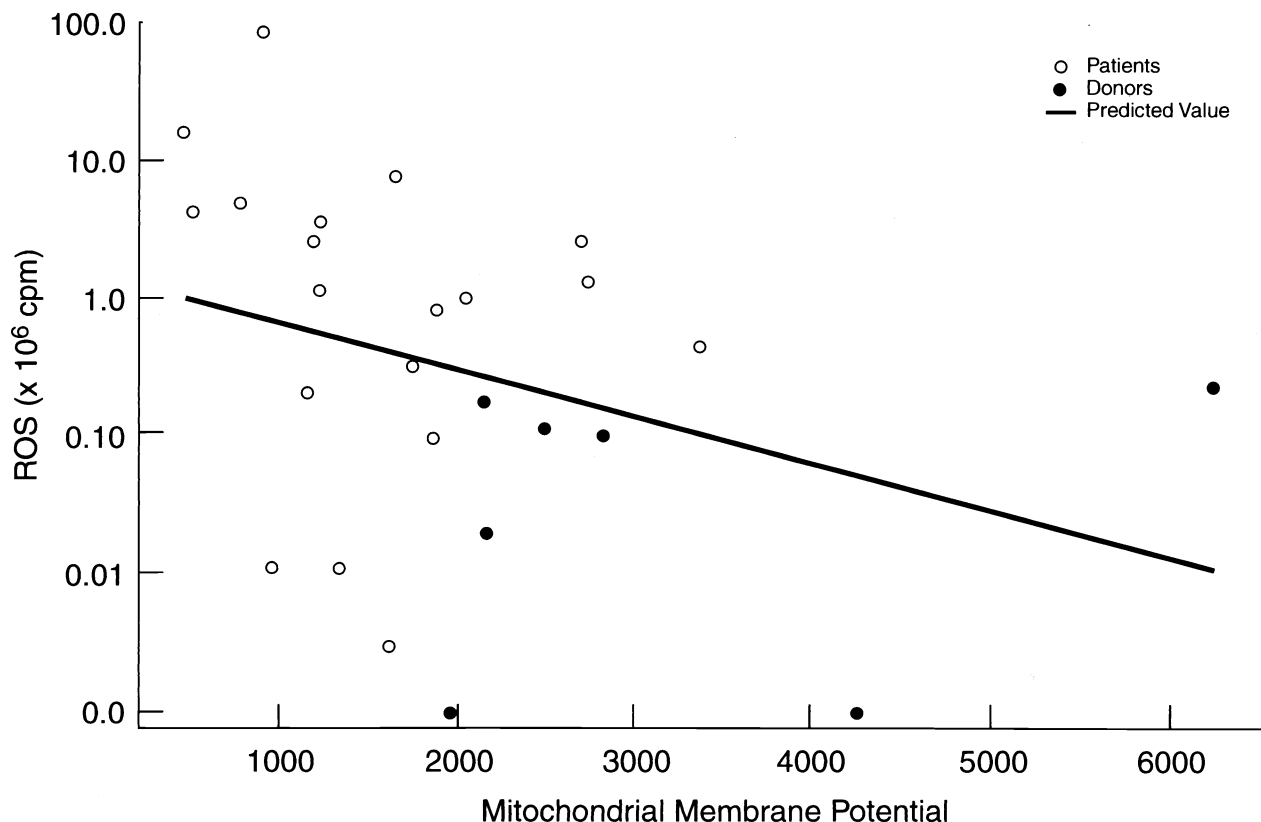
that mitochondrial function is inhibited in spermatozoa from infertile men and is significantly correlated with the sperm concentration and the level of ROS production.

Previous studies have demonstrated an association of MMP with sperm motility and viability (14–16, 18). Recent reports showed a positive correlation between the percentage of sperm with high MMP and standard semen parameters like sperm concentration and motility (27). High correlation of MMP with forward motility confirms the strong link between functional status of mitochondria and sperm quality (27). Correlation of MMP results with sperm morphology may provide interesting information as morphologically abnormal spermatozoa with midpiece defects have been linked with excessive production of ROS (9).

Our results suggest that higher sperm concentration is associated with higher MMP. The fact that the spermato-

FIGURE 3

Relationship between MMP (number of cells positive for MMP) and ROS production (10^6 cpm) showed a significant negative correlation ($r = -0.45$, $P < .05$). Nineteen patients with abnormal semen parameters and 7 donors with normal semen parameters were analyzed.



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zoa from the infertile patients had lower MMP than the spermatozoa of the healthy donors suggests that MMP is an indicator of the fertility potential of an individual. Assessment of MMP by flow cytometric analysis may provide reliable data that can be used in the diagnosis of male infertility and to advance our understanding of the molecular basis of this disorder. We found a significant inverse correlation between MMP and ROS levels in spermatozoa. Semen samples from patients with abnormal semen parameters had higher levels of ROS and significantly lower MMP than the donors. Such a relationship could be due to two mutually interconnected phenomena: ROS causing damage to the mitochondrial membrane, and the damaged mitochondrial membrane causing increased ROS production. It has been demonstrated previously (19) that H_2O_2 inhibits sperm motility, ATP levels, and MMP. However, it was noted that low levels of H_2O_2 (that inhibit the sperm motility and ATP levels) do not affect the spermatozoal MMP (19). This may indicate that physiological

levels of ROS do not alter the integrity of the mitochondrial membrane. Mitochondrial dysfunction has been shown to increase production of ROS (22–24). Alterations in MMP may occur during the early stages of apoptosis (27–31). It has been suggested that mitochondrial membrane damage is an important initiator of apoptosis in germ cells in the human testis (32). This means that those germ cells in which the process of apoptosis is initiated but is not completed will produce spermatozoa with damaged mitochondria. Such an apoptotic mechanism, known as abortive apoptosis, is known to occur during spermatogenesis, more so in men with abnormal semen parameters (33). Therefore, the interrelationship can be due to a self-perpetuating cycle of damaged mitochondria producing ROS, which in turn damages the mitochondrial membrane.

High levels of ROS result in oxidative stress and are associated with DNA damage in men with the male infertility factor (34–36). DNA fragmentation is strong evidence

that this damage is free radical mediated and is induced by oxidative stress (6). In a previous study, the stainability of sperm cells with DNA intercalating dyes was reported to be elevated and hence appeared to be a useful marker related to fertility potential (16, 17). Our study provides another marker to evaluate the fertility potential using DIOC₆(3) dye to measure the mitochondrial membrane potential. The decrease in MMP is correlated with ROS production and may be one of the earlier steps occurring before sperm nuclear DNA damage.

In conclusion, our study suggests that measurement of MMP can provide useful information about the fertility potential of an individual. However, future studies are needed to better define the clinical implications of mitochondrial injury demonstrated in our *in vitro* studies. Increased ROS produced by the spermatozoa is associated with mitochondrial injury with a marked decrease in MMP. This is the first report to our knowledge that shows the negative association of MMP with the amount of ROS produced. Measuring the sperm DNA damage and correlating the damage with the MMP can provide much stronger evidence for the relationship between nuclear DNA damage and MMP. Identifying the molecular and cellular mechanisms of oxidative stress will provide a better understanding of pathophysiological features of the spermatozoa from infertile men and may help identify more effective therapeutic modalities for male infertility.

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