

Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress

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Objective: To investigate sperm viability, incidence of apoptosis, and intracellular basal and induced reactive oxygen species (ROS) in sperm fractions.

Design: Prospective controlled study.

Setting: Center for Reproductive Medicine at a tertiary care hospital.

Method(s): Liquefied seminal ejaculates (n = 12) prepared by density gradient centrifugation were reconstituted to 2 mL with phosphate-buffered saline. Oxidative stress was induced by hydrogen peroxide (H₂O₂, 100 μM). Sperm viability, intracellular ROS, and incidence of apoptosis/necrosis in neat, immature, and mature sperm fractions were assessed.

Result(s): Before H₂O₂ exposure, mature spermatozoa fractions showed a significantly lower incidence of apoptotic sperm and intracellular O₂^{-•} levels but higher amounts of intracellular H₂O₂ compared with neat semen. Higher levels of intracellular H₂O₂ were demonstrated in immature sperm fractions compared with neat or mature fractions. In all sperm fractions, intracellular H₂O₂ levels correlated with the intracellular concentration of O₂^{-•}. After H₂O₂ exposure, neat semen showed a significantly higher percentage of apoptosis compared with the prepared mature spermatozoa. However, no differences were observed in the incidence of apoptosis between immature and mature sperm fractions.

Conclusion(s): There is a differential shift of both intracellular H₂O₂ and O₂^{-•} in each sperm fraction that may affect sperm quality. Sperm apoptosis is related to intracellular H₂O₂ levels, which in turn are affected by intracellular O₂^{-•} levels. Oxidative stress was not associated with an increased incidence of apoptosis in immature or mature sperm fractions. (Fertil Steril® 2008; ■: ■–■. ©2008 by American Society for Reproductive Medicine.)

Key Words: Reactive oxygen species, flow cytometry, apoptosis, human spermatozoa, intracellular staining

Oxidative stress has been implicated in male factor infertility (1–5). Superoxide (O₂^{-•}) and hydrogen peroxide (H₂O₂) are common reactive oxygen species (ROS), which are highly reactive and can interact with nearby molecules, inducing oxidative stress damage in cellular organelles and molecules (6–8).

Physiological levels of ROS are required for normal sperm functions such as hyperactivation, capacitation, and acrosome reaction (9–11). Oxidative stress occurs in spermatozoa when global levels of ROS (both extra- and intracellular) exceed the available total antioxidant capacity. Sperm have

a limited amount of cellular cytoplasm in which scavenging enzymes are found, making sperm highly susceptible to ROS damage (10, 12, 13). As ROS are able to readily permeate the membranes, they can cause DNA, proteins, and lipid molecules peroxidative damage within the cell (7, 8, 14). Motile sperm have been shown to be activated by excessive ROS formation and undergo apoptosis-like changes (15–17). This insult has been linked to sperm apoptosis and male factor infertility (18, 19).

Sperm preparation plays an important role in a successful outcome in assisted reproductive techniques (ART). Double-density gradient centrifugation is a standard sperm selection method for ART to separate mature motile sperm with superior morphology. A mature sperm fraction shows less incidence of apoptotic sperm compared with ejaculated unprocessed sperm (20, 21). Oxidative sperm damage can occur during sperm preparation and processing for ART (22–24).

Oxidative stress-induced sperm damage and apoptosis-like changes may occur when the intracellular ROS levels are in excess of the cells' scavenging capacity (20, 25). In addition, H₂O₂ has been recommended as a local vaginal contraceptive/spermicidal agent (26, 27). Measurement of the

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intracellular ROS would, therefore, be more beneficial than global measurement of the seminal ROS (6, 8, 28). On the other hand, simultaneous selective measurement of intracellular H_2O_2 or $O_2^{\bullet-}$ levels may be important in understanding how sperm preparation affects ART success rates.

Dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) are used for measurement of intracellular H_2O_2 and $O_2^{\bullet-}$, respectively, by flow cytometry. The advantages of using flow cytometry for the measurement of intracellular ROS in ejaculated human spermatozoa have been reported recently (29–31).

In this study, oxidative stress was induced after exposure to exogenous hydrogen peroxide (H_2O_2). Sperm viability and intracellular ROS levels were evaluated in seminal ejaculates (unprocessed) and in immature and mature sperm fractions to determine the basal and stimulated intracellular ROS levels and examine their relationship with viability and apoptosis.

MATERIALS AND METHODS

Sample Collection and Preparation

This study was approved by the Cleveland Clinic Institutional Review Board. Semen samples were collected from 12 healthy male volunteers of unproven fertility status at the Cleveland Clinic andrology laboratory. All samples were collected by masturbation after 2–3 days of sexual abstinence.

After liquefaction, sperm count, percentage motility, viability, and presence of round cells were examined on an aliquot of the neat semen sample. The remaining aliquot was prepared for separating mature and immature fractions by double-density gradient centrifugation (PureCeption, SAGE BioPHARMA, Bedminster, NJ). Samples were centrifuged at 300 *g* for 20 minutes, and the resulting interface between the 40% and 80% layers (immature spermatozoa) was aspirated. Highly motile mature spermatozoa were obtained in an 80% pellet. Both fractions were resuspended in human tubal fluid media (HTF; Irvine Scientific, Santa Ana, CA).

Induction of Oxidative Stress

Oxidative stress was induced by exposing the sperm to H_2O_2 . A 30% stock solution of H_2O_2 (Sigma Chemical Co., St Louis) was diluted to 100 μ M and added to 1 mL of sperm suspension (stimulated ROS) and incubated for 15 minutes at 37°C. Another aliquot from the same fraction containing an equal volume of HTF served as a control (basal ROS).

Determination of ROS by Flow Cytometry

DCFH-DA, a specific probe for H_2O_2 , and DHE, a specific probe for $O_2^{\bullet-}$, are cell-permeable stains. DCFH is oxidized selectively by the free intracellular H_2O_2 into DCF that binds to DNA and emits green fluorescence. DHE is oxidized by the free intracellular $O_2^{\bullet-}$ into ethidium bromide that binds to the DNA and emits red fluorescence (31–33). DCFH-DA (25 μ M) and DHE (1.25 μ M; Sigma)

were added to the sperm suspension and incubated at 25°C for 40 minutes (DCFH-DA) and 20 minutes (DHE), respectively. Aliquots were subsequently analyzed using a flow cytometer. Green fluorescence (DCF) was evaluated between 500 and 530 nm, while red fluorescence (HE) was evaluated between 590 and 700 nm (excitation, 488 nm; emission, 525–625 nm in the FL-2 channel). Data were expressed as the percentage of fluorescent spermatozoa. Apoptotic spermatozoa were excluded by using counter nucleic acid stains. Propidium iodide (PI) was used as a counterstain dye for DCFH-DA; YO-PRO-1 was used as a counterstain dye for the HE (29).

Apoptosis Detection

To measure the apoptotic status of the spermatozoa, the Vybrant Apoptosis Assay (Invitrogen, Carlsbad, CA) was used. All samples were washed in cold phosphate-buffered saline (PBS), and the cell density was adjusted to $\sim 1 \times 10^6$ cells/mL in PBS. One microliter of the YO-PRO-1 solution (10 μ M) and 1 μ L of the PI solution (50 μ g/mL) were added to 1 mL of cell suspension and incubated for 20–30 minutes. Flow cytometric analysis of the stained cells was done within 1–2 hours using 488 nm excitation with green fluorescence emission for YO-PRO-1 (i.e., 530/30 band pass) and red fluorescence emission for PI (i.e., 610/20 band pass). Gating was performed to exclude any debris. Standard compensation was done using single-color stained controls. Three different populations can be identified by using this assay (Figure 1): viable sperm are negative for both PI and YO-Pro-1, apoptotic sperm are positive for Yo-pro-1 but negative for PI, and dead sperm show positivity for both PI and YoPro-1.

Flow Cytometry Analysis

All fluorescence signals of labeled spermatozoa were analyzed by a Becton Dickinson flow cytometer FACScan (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon laser as a light source. A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second.

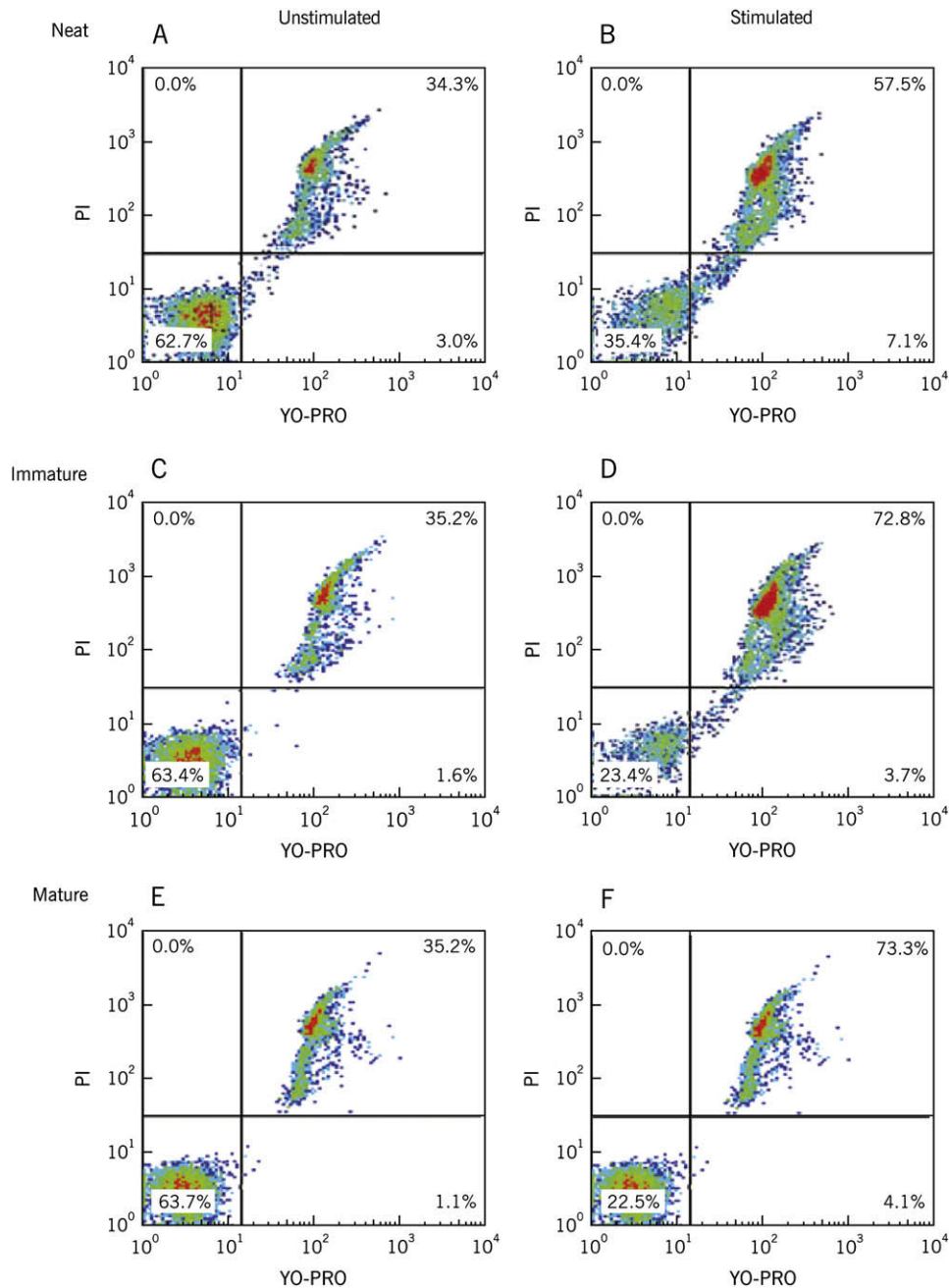
The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. DCF/ YO-PRO-1 emitting green fluorescence and PI/ HE emitting red fluorescence (580–630 nm) were recorded in the FL-1 and FL-2 channels, respectively. The percentage of HE-/PI-positive cells and the mean fluorescence were calculated on a 1023-channel scale and analyzed using the flow cytometer software FlowJo version 7.2.2 (FlowJo, Ashland, OR).

Statistical Methods

For all quantitative measured parameters, comparisons between stimulated and nonstimulated neat, mature, and immature spermatozoa were performed using the Wilcoxon

FIGURE 1

Representative flow cytometry pseudo-colored dot plots for unstimulated (left) and stimulated (right) neat (A, B), immature (C, D), and mature sperm fractions (E, F). Each quadrant is shown as follows: lower left: viable, nonstained sperm; lower right: apoptotic sperm (Yo-Pro positive only); upper right: dead spermatozoa (positive for Yo-Pro and PI). The numbers in parentheses represent the percentage of sperm population in each quadrant. Only three sperm populations could be identified using Yo-Pro-1/PI for apoptosis.



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signed-rank test. Associations among quantitative variables were measured using Spearman's correlation coefficients both within and across sample groups. $P < .05$ was considered statistically significant.

RESULTS

Neat semen samples showed a mean (\pm SD) semen volume of $2.7 (\pm 1.3)$ mL, sperm concentration of $62.4 \pm 53.1 \times 10^6$ cells /mL, and motility of $66.6\% \pm 9.2\%$. Before H_2O_2

exposure, mature sperm showed a significantly higher motility ($72.7\% \pm 19.3\%$) when compared with the neat sample ($48.9\% \pm 19.7\%$, $P=.014$) and immature sperm ($41.7\% \pm 12.3\%$; $P<.001$), respectively.

Sperm Population Identification

When viable, apoptotic, and dead sperm were examined, three different sperm staining patterns were observed upon analyzing the sperm for apoptosis using the Yo-Pro-1/PI assay (Fig. 1A–F). The apoptotic sperm population stained only positive for Yo-Pro-1. Yo-Pro-1- and PI-positive cells represented the dead sperm population, while viable sperm showed negative Yo-Pro-1 and negative PI fluorescence. The percentages of each sperm population in neat, immature, and mature sperm fractions are shown in Figure 1 (A–F).

Sperm Apoptosis and Intracellular ROS before H₂O₂ Exposure

Density gradient centrifugation selected a population of mature cells with a significantly lower number of apoptotic cells than were present in neat semen ($P=.006$; Table 1). Mature sperm also displayed a higher percentage of DCF^{+ve} ($P<.001$) and a lower HE^{+ve} % ($P=.004$) fluorescence compared with sperm in the neat semen. Immature sperm showed a significantly higher percentage of DCF-stained sperm when compared with both neat ($P<.001$) and mature sperm ($P=.05$).

Sperm Viability, Apoptosis, and Intracellular ROS after H₂O₂ Exposure

Exposure to H₂O₂ significantly reduced viability and increased the percentage of dead sperm in neat and immature and mature groups as shown in Figure 1. However, exposure to H₂O₂ was associated with an increase in the mean percentage of apoptotic sperm in neat semen and in the mature and immature sperm fractions, but the difference was not significant (Table 1). After H₂O₂ exposure, a significantly lower percentage of apoptosis was seen in mature versus neat sperm fractions ($P=.024$). Other comparisons between immature versus mature and neat (stimulated or nonstimulated) showed no significant differences.

The percentage of sperm with DCF fluorescence (intracellular H₂O₂) increased in both neat and mature sperm, but the difference was not significant in the immature sperm fractions (Table 1; Fig. 2A). Moreover, neat, immature, and mature sperm fractions exhibited a significant increase in the percentage of sperm showing HE fluorescence (increase in intracellular O₂^{-•}; Table 1; Fig. 2B).

Correlation of Intracellular ROS with Other Sperm Parameters

Sperm viability was inversely related to the percentage of apoptotic spermatozoa (mature nonstimulated, $r = -0.76$, $P=.006$; mature stimulated, $r = -0.58$, $P=.047$; all fractions,

$r = -0.55$, $P<.001$). After H₂O₂ exposure, the percentage of viable spermatozoa was positively correlated with the intensity of DCF fluorescence in neat ($r = 0.63$, $P=.031$) and in mature sperm fractions ($r = 0.59$, $P=.04$).

The percentage of apoptotic sperm was positively correlated with DCF fluorescence (intracellular H₂O₂) in neat non-exposed fractions ($r = 0.60$, $P<.041$). We wanted to examine the relationship of apoptotic sperm with dead sperm. When the neat, immature, and mature fractions (overall) were examined before and after stimulation, the percentage of apoptotic sperm was positively correlated with the percentage of dead sperm in the overall group ($r = 0.52$; $P<.001$) and in the mature sperm fraction ($r = 0.78$; $P=.004$). After H₂O₂ exposure, the percentage of sperm positive for DCF showed a negative correlation with the percentage of dead sperm in neat ($r = -0.68$, $P=.01$) and in mature sperm fractions ($r = -0.59$; $P=.04$). Similarly, after exposure, the percentage of HE^{+ve} sperm (intracellular O₂^{-•}) was significantly correlated with the percentage of DCF^{+ve} sperm (intracellular H₂O₂) in the overall ($r = 0.52$; $P<.001$) and mature sperm fractions ($r = 0.78$; $P=.004$).

DISCUSSION

Our study aim was to evaluate the basal and stimulated intracellular H₂O₂ and O₂^{-•} levels in different sperm fractions and to examine their relationship with sperm apoptosis. We have measured basal levels of both intracellular H₂O₂ and O₂^{-•} in neat, immature, and mature sperm fractions. Interestingly, both mature and immature sperm showed reduced intracellular levels of O₂^{-•} compared with the neat sperm. Higher levels of intracellular H₂O₂ (as represented by DCF^{+ve} fluorescence) were seen in immature compared with mature or neat sperm fractions (Table 1). We report for the first time the shift in intracellular H₂O₂ and O₂^{-•} levels. This shift may be explained by the fact that conventional centrifugation increases heat generation, which may affect sperm quality (17, 34, 35). Avoiding sperm centrifugation for longer times and/or higher speed(s) or its modification may be helpful in preserving sperm quality (36).

We hypothesize that conventional centrifugation may increase the activity of the superoxide dismutase enzyme that converts the generated superoxide ion during centrifugation into hydrogen peroxide, which may prove fatal to sperm cells (37). This conversion lowers the available intracellular superoxide levels in both immature and mature sperm when compared with the neat unprocessed spermatozoa. However, mature spermatozoa may have higher catalase activity/expression (38) when compared with immature spermatozoa, enabling them to scavenge the generated H₂O₂ more effectively. Interestingly, despite the higher levels of intracellular H₂O₂ seen in the mature sperm fraction, the percentage of dead sperm in this fraction did not increase significantly, even though a significant decrease was seen in the percentage of apoptotic sperm. This might be explained by the fact that H₂O₂ is not as lethal as O₂^{-•} for cell viability. Another, more

TABLE 1

Comparison of the measured parameters in different sperm fractions before and after H₂O₂ exposure.

Marker (ROS assessed)	Neat semen, mean ± SD (n = 12)			Mature sperm, mean ± SD (n = 12)			Immature sperm, mean ± SD (n = 12)		
	Nonstimulated	Stimulated	P	Nonstimulated	Stimulated	P	Nonstimulated	Stimulated	P
Viable sperm, %	59.4 ± 8.98	46.2 ± 11.9	.006	67.4 ± 13.4	52.1 ± 19.3	.08	65.3 ± 9.2	45.8 ± 16.9	.008
Apoptotic sperm, %	0.76 ± 0.77	1.7 ± 1.75	.2	0.25 ± 0.24 ^a (0.006)	0.73 ± 0.85 ^b (0.024)	.18	0.41 ± 0.41	1.0 ± 1.15	.11
Dead sperm, %	38.9 ± 10.5	50.3 ± 12.6	.035	30.9 ± 15.2	46.8 ± 19.0	.07	33.7 ± 9.2	52.3 ± 16.8	.009
DCFH-DA, % (H ₂ O ₂)	6.4 ± 8.7	38.2 ± 18.2	<.001	25.3 ± 14.2 ^a (<0.001)	50.4 ± 24.1	.015	36.6 ± 12.0 ^{c,d} (<0.001; 0.05)	50.3 ± 22.6	.16
DHE, % (O ₂ ^{-•})	3.5 ± 1.8	8.1 ± 5.7	.024	1.5 ± 1.2 ^a (0.004)	16.7 ± 13.1	<.001	2.1 ± 1.3	9.9 ± 7.3	.002

Note: Statistical comparison between stimulated and nonstimulated samples within neat, mature, and immature sperm were performed using the Wilcoxon signed-rank test. Pairwise comparisons of neat, mature, and immature within stimulated and nonstimulated samples were also performed using the Wilcoxon signed-rank test.

^a Significant difference between nonstimulated mature and neat sperm fractions.

^b Significant difference between stimulated mature and neat sperm fractions.

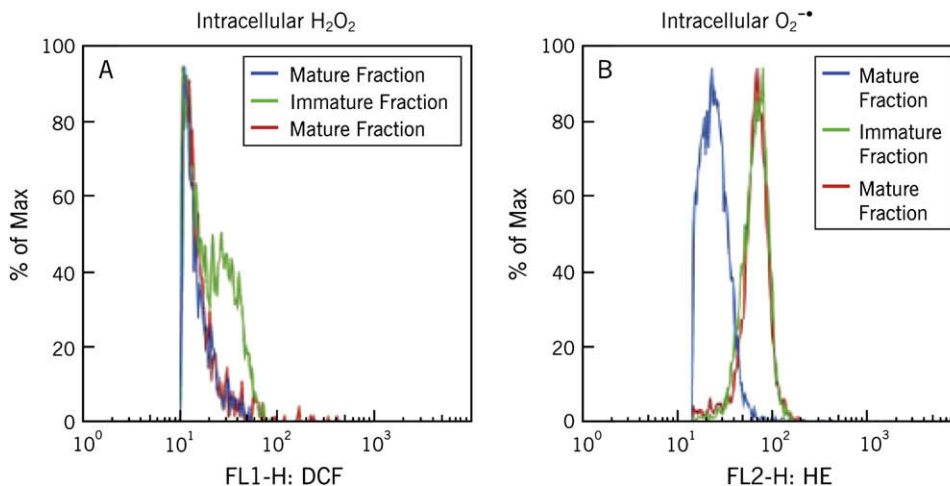
^c Significant difference between nonstimulated immature and neat sperm fractions.

^d Significant difference between nonstimulated mature and immature sperm fractions.

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FIGURE 2

Representative flow cytometry marker histogram for stimulated neat, immature, and mature sperm fractions. (A) positive DCF fluorescence (represents intracellular H_2O_2); (B) positive fluorescence for HE fluorescence (represents intracellular $\text{O}_2^{\bullet-}$). The histograms show the differential shift of the intracellular ROS in sperm fractions.



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likely, explanation is the fact that mature sperm have the ability to protect themselves against the harmful effects of H_2O_2 exposure (39, 40). On the other hand, our finding confirms the reports of Donnelly et al. (41) that supplementation with ascorbate and alpha-tocopherol in combination protects the sperm from H_2O_2 -induced sperm DNA damage by scavenging the ROS generated during sperm preparation.

Our study data demonstrated that mature spermatozoa fractions exhibited lower levels of both apoptotic and dead sperm and displayed higher percentages of viable sperm compared with neat semen (Table 1). These findings were in agreement with earlier studies from our group (30, 42), as well as with other studies in which superoxide anions were shown to induce both caspase activation and apoptosis. From these results, it is also worth mentioning that acute exposure of spermatozoa to H_2O_2 results in decreased sperm viability and increased percentage of dead spermatozoa. Apoptosis and necrosis are different death pathways, and we examined their relations to sperm viability. This is in agreement with the findings of Conde de la Rosa et al. (6) who reported that H_2O_2 induced cell death. It also agrees with other investigators who evaluated the efficacy of H_2O_2 as a local vaginal contraceptive/spermicidal agent with a short time exposure (21, 43–45).

We have demonstrated that intracellular H_2O_2 levels are related to the intracellular $\text{O}_2^{\bullet-}$ levels as both are end products of one reaction. Sperm motility is positively related to the percentage of viable sperm. When all the fractions were considered together, motility was inversely related to the percentage of dead or apoptotic sperm. This may explain why sperm motility decreases in pathologically high-ROS condi-

tions in which there is an increase in the percentage of dead sperm. It may also explain why sperm preparation by density gradient separation shows higher motility due to reducing the percentage of apoptotic spermatozoa. As shown earlier, removal of apoptotic sperm improves the quality of the prepared sperm (42).

Our study findings show that the percentage of apoptotic sperm was positively related to the basal intracellular levels of H_2O_2 in the neat sperm fraction. However, under high-ROS conditions (pathological or induced), intracellular H_2O_2 level was positively related to the percentage of viable spermatozoa in stimulated as well as in neat sperm. This may be explained by the fact that induced high-ROS conditions will increase the percentage of apoptotic/dead sperm. In high-ROS conditions (pathological or induced), viable spermatozoa may have the ability to adapt to increased H_2O_2 as a result of their high defense status (Fig. 1). This observation may be important and is supported by the report that shows 100% efficacy (sperm immobilization and loss of viability) in mating studies not earlier than at 2 hours of H_2O_2 exposure (43). This may explain the poor efficacy of H_2O_2 as a local vaginal contraceptive for short-time usage (<1 hour) and is in accordance with a report (43) that 2 hours of exposure may be appropriate to improve the H_2O_2 contraceptive efficiency.

In conclusion, both immature and mature sperm produce higher intracellular H_2O_2 levels compared with neat spermatozoa. This probably is attributable to sperm processing with centrifugation. Mature spermatozoa may adapt to H_2O_2 generated during sperm preparation involving centrifugation. This may explain the presence of the higher percentage of

viable and the lower percentage of dead/apoptotic sperm in the prepared mature spermatozoa fraction. Sperm preparation may be associated with a differential shift of both intracellular H_2O_2 and $O_2^{\bullet-}$ that may affect the sperm quality. Apoptotic changes in sperm are attributed largely to the intracellular H_2O_2 levels, while dead sperm are related to intracellular $O_2^{\bullet-}$ levels. Finally, intracellular ROS levels may affect sperm quality through their effects on sperm viability.

REFERENCES

- Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol* 2008;59:2–11.
- Pasqualotto FF, Sundaram A, Sharma RK, Borges E Jr., Pasqualotto EB, Agarwal A. Semen quality and oxidative stress scores in fertile and infertile patients with varicocele. *Fertil Steril* 2008;89:602–7.
- Agarwal A, Saleh RA. [Utility of oxidative stress test in the male infertility clinic]. *Zhonghua Nan Ke Xue* 2002;8:1–9.
- Aydemir B, Onaran I, Kiziler AR, Alici B, Akyolcu MC. The influence of oxidative damage on viscosity of seminal fluid in infertile men. *J Androl* 2008;29:41–6.
- Hurtado de Catalfo GE, Ranieri-Casilla A, Marra FA, de Alaniz MJ, Marra CA. Oxidative stress biomarkers and hormonal profile in human patients undergoing varicocelectomy. *Int J Androl* 2007;30:519–30.
- Conde de la Rosa L, Schoemaker MH, Vrenken TE, Buist-Homan M, Havinga R, Jansen PL, et al. Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. *J Hepatol* 2006;44:918–29.
- Coyle CH, Kader KN. Mechanisms of H_2O_2 -induced oxidative stress in endothelial cells exposed to physiologic shear stress. *ASAIO J* 2007;53:17–22.
- Molina-Heredia FP, Houee-Levin C, Berthomieu C, Touati D, Tremey E, Favaudon V, et al. Detoxification of superoxide without production of H_2O_2 : antioxidant activity of superoxide reductase complexed with ferrocyanide. *Proc Nat Acad Sci U S A* 2006;103:14750–5.
- Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Indian J Exp Biol* 2005;43:963–74.
- Henkel R. The impact of oxidants on sperm function. *Andrologia* 2005;37:205–6.
- Sikka SC. Relative impact of oxidative stress on male reproductive function. *Curr Med Chem* 2001;8:851–62.
- Khosrowbeygi A, Zarghami N. Fatty acid composition of human spermatozoa and seminal plasma levels of oxidative stress biomarkers in subfertile males. *Prostaglandins Leukot Essent Fatty Acids* 2007;77:117–21.
- Bansal AK, Bilaspuri GS. Oxidative stress alters membrane sulfhydryl status, lipid and phospholipid contents of crossbred cattle bull spermatozoa. *Anim Reprod Sci* 2008;104:398–404.
- Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr., Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum Reprod* 1999;14:2801–7.
- Levy R, Seifer-Akkin I. [Apoptosis during spermatogenesis and in ejaculated spermatozoa: importance for fertilization]. *Ann Biol Clin (Paris)* 2001;59:531–45.
- Lysiak JJ, Zheng S, Woodson R, Turner TT. Caspase-9-dependent pathway to murine germ cell apoptosis: mediation by oxidative stress, BAX, and caspase 2. *Cell Tissue Res* 2007;328:411–9.
- Stevanato J, Bertolla RP, Barradas V, Spaine DM, Cedenho AP, Ortiz V. Semen processing by density gradient centrifugation does not improve sperm apoptotic deoxyribonucleic acid fragmentation rates. *Fertil Steril* 2008;90:889–90.
- Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod* 2002;66:1061–7.
- Huyghe E, Izard V, Rigot JM, Pariente JL, Tostain J. [Optimal evaluation of the infertile male. 2007 French urological association guidelines.]. *Prog Urol* 2008;18:95–101.
- Hammoud AO, Gibson M, Peterson MC, Carrell DT. Effect of sperm preparation techniques by density gradient on intra-individual variation of sperm motility. *Arch Androl* 2007;53:349–51.
- Ricci G, Peticarari S, Boscolo R, Montico M, Guaschino S, Presani G. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. *Fertil Steril*. Published online 16 January, 2008 [Epub ahead of print].
- Agarwal A, Allamaneni SS. The effect of sperm DNA damage on assisted reproduction outcomes. A review. *Minerva Ginecol* 2004;56:235–45.
- Bedaiwy MA, Sharma RK, Alhussaini TK, Mohamed MS, Abdel-Alem AM, Nelson DR, et al. The use of novel semen quality scores to predict pregnancy in couples with male-factor infertility undergoing intrauterine insemination. *J Androl* 2003;24:353–60.
- Sinclair KD. Assisted reproductive technologies and pregnancy outcomes: mechanistic insights from animal studies. *Seminars Reprod Med* 2008;26:153–61.
- Hammadeh ME, Zavos PM, Rosenbaum P, Schmidt W. Comparison between the quality and function of sperm after semen processing with two different methods. *Asian J Androl* 2001;3:125–30.
- Oehninger S, Blackmore P, Mahony M, Hodgen G. Effects of hydrogen peroxide on human spermatozoa. *J Assist Reprod Genet* 1995;12:41–7.
- Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med* 1999;26:869–80.
- Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ Jr., et al. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod* 2004;19:129–38.
- Guthrie HD, Welch GR. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J Anim Sci* 2006;84:2089–100.
- Mahfouz RZ, Sharma R, Lackner J, Aziz N, Agarwal A. Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa. *Fertil Steril*. Published online 19 August, 2008 [Epub ahead of print].
- Carter WO, Narayanan PK, Robinson JP. Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. *J Leuko Biol* 1994;55:253–8.
- Qin Y, Lu M, Gong X. Dihydrorhodamine 123 is superior to 2,7-dichlorodihydrofluorescein diacetate and dihydrorhodamine 6G in detecting intracellular hydrogen peroxide in tumor cells. *Cell Biol Int* 2008;32:224–8.
- Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, et al. Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. *Proc Nat Acad Sci U S A* 2006;103:15038–43.
- Knop K, Hoffmann N, Rath D, Sieme H. Effects of cushioned centrifugation technique on sperm recovery and sperm quality in stallions with good and poor semen freezability. *Anim Reprod Sci* 2005;89:294–7.
- Fischer C, Scherfer-Brahler V, Muller-Schlosser F, Schroder-Printzen I, Weidner W. [A thermodynamic study on bovine spermatozoa by microcalorimetry after Percoll density-gradient centrifugation—experimental probe of its utility in andrology]. *Aktuelle Urol* 2007;38:237–42.
- Carvajal G, Cuello C, Ruiz M, Vazquez JM, Martinez EA, Roca J. Effects of centrifugation before freezing on boar sperm cryosurvival. *J Androl* 2004;25:389–96.
- Burnaugh L, Sabeur K, Ball BA. Generation of superoxide anion by equine spermatozoa as detected by dihydroethidium. *Theriogenology* 2007;67:580–9.
- Sahoo DK, Roy A, Bhanja S, Chainy GB. Hypothyroidism impairs antioxidant defence system and testicular physiology during development and maturation. *Gen Comp Endocrinol* 2008;156:63–70.

39. Kumar PG, Laloraya M, Laloraya MM. Superoxide radical level and superoxide dismutase activity changes in maturing mammalian spermatozoa. *Andrologia* 1991;23:171–5.
40. Said TM, Agarwal A, Sharma RK, Mascha E, Sikka SC, Thomas AJ Jr. Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. *Fertil Steril* 2004;82:871–7.
41. Donnelly ET, McClure N, Lewis SE. The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis* 1999;14:505–12.
42. Said TM, Agarwal A, Grunewald S, Rasch M, Glander HJ, Paasch U. Evaluation of sperm recovery following annexin V magnetic-activated cell sorting separation. *Reprod Biomed Online* 2006;13:336–9.
43. Chaki SP, Misro MM. Assessment of human sperm function after hydrogen peroxide exposure. development of a vaginal contraceptive. *Contraception* 2002;66:187–92.
44. Said TM, Grunewald S, Paasch U, Glander HJ, Baumann T, Kriegel C, et al. Advantage of combining magnetic cell separation with sperm preparation techniques. *Reprod Biomed Online* 2005;10:740–6.
45. Singh NP. Apoptosis assessment by the DNA diffusion assay. *Methods Mol Med* 2005;111:55–67.