

Correlation of reactive oxygen species levels with the fertilization rate after in vitro fertilization: a qualified meta-analysis

Our meta-analysis analyzed the relationship between levels of reactive oxygen species (ROS) and fertilization rate after in vitro fertilization (IVF), and showed a statistically significant correlation between the ROS levels and the IVF fertilization rate (estimated overall correlation -0.374 [95% CI, $-0.520, -0.205$]). We conclude that ROS has a statistically significant effect on the fertilization rate after IVF, and that the measurement of ROS levels in semen specimens before IVF may be useful in predicting the IVF outcome and in counseling patients. (*Fertil Steril*® 2005;84:228–31. ©2005 by American Society for Reproductive Medicine.)

Because of their adverse effects on the sperm plasma membrane, DNA, and other physiologic processes, reactive oxygen species (ROS) affect the quality of spermatozoa. The assumption that ROS can influence male fertility has received substantial scientific support (1–10). The fertilizing potential of human spermatozoa depends on several factors such as motility, normal morphology, and the ability to undergo the acrosome reaction and bind to the zona pellucida. Studies have shown that all or most of these parameters are affected by ROS (11–16).

Investigators have used the amount of ROS in semen specimens prepared for in vitro fertilization (IVF) to assess the degree of damage to the spermatozoa (17–21). With the greater understanding of the critical role played by the ROS in the fertilization process, researchers have attempted to associate and thus predict IVF success using ROS levels. However, there is no clear consensus because some studies have shown no relationship between ROS levels and the fertilization rate after IVF (22–24) while others have shown a relationship (17–20).

Considering this lack of clear evidence, we conducted a meta-analysis of studies correlating ROS levels with the fertilization rate following IVF. Meta-analysis provides an overall consensus from studies with varying results, giving a more precise estimate than any one of the individual studies. Our objectives were to identify all of the studies in the literature and to collate the results of published studies using meta-analysis to determine the association, if any, between ROS levels and the fertilization rate following IVF.

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LITERATURE SEARCH STRATEGY

Using the PubMed and Ovid Web search engines, two of the study authors independently and meticulously searched the literature. The following key words were used: reactive oxygen species, oxidative stress, free radicals, fertilization rate, in vitro fertilization, and assisted reproductive techniques. We performed a hand search of reference citations from reports from the primary search as well as review articles. Abstract books (1993 to 2004) of the annual meetings of American Society of Andrology (ASA), American Urological Association (AUA), and American Society for Reproductive Medicine (ASRM) were searched for relevant information. The literature search was restricted to human studies written in the English language and no cut-off year was set.

All the published studies analyzing the relationship between ROS levels and IVF success rate, defined as the percentage of oocytes fertilized, were considered for inclusion. The meta-analysis was conducted using a correlation coefficient and weighing each study by its sample size. Correlation data from the included studies was combined using the random effects method (25). This method assumes that the included studies are a random sample of the possible ones and thus includes extra variability to account for that fact. Furthermore, the random effects method assumes that some of the differences in the studies' results may be related to unique features of each study's site or population or to subtle differences in design.

DESCRIPTIVE INFORMATION ABOUT STUDIES

A total of nine studies were examined for the role of ROS in IVF outcome (Table 1) (17–24, 26). These studies had different experimental designs and assessed the seminal ROS levels using different assays. The abstract of one study was discovered from a meeting's abstract book, but it did not provide the correlation value (27).

Eight studies measured ROS levels using the chemiluminescence method; the remaining study used flow cytom-

TABLE 1

Characteristics of identified studies (n = 9) with data on association between reactive oxygen species levels (ROS) and in vitro fertilization rate.

Study	Sample size	Assay for ROS	Results
Sukcharoen et al., 1995 (19) ^a	41	L + FMLP	$r = -0.459, P = .002$;
		L + PMA	$r = -.350, P = .02$
Sukcharoen et al., 1996 (18) ^a	73	Luminol	$r = -.221, P < .001$;
		L + FMLP	$r = .304, P < .001$;
		L + PMA	$r = .350, P < .02$
	8	L + FMLP	$r = -.609, P < .001$
Krausz et al., 1994 (17) ^a	20	L + PMA	No correlation
Saleh et al., 2003 (21)	10	Luminol	$r = -.59, P = .03$ (r value is for both IVF and ICSI)
Moilanen et al., 1998 (22)	72	Luminol	No correlation
		L + FMLP	
		L + PMA	
Moilanen et al., 1999 (24)	76	L + FMLP	ROS levels were NS between >25% and <25% IVF rate
Zorn et al., 2003 (20)	41	Luminol	$P = .031$ for ROS levels between >25% and <25% IVF rate
Yeung et al., 1996 (23)	75	Luminol	No correlation
Marchetti et al., 2002 (26)	45	Flow cytometry	$r = -.11, P = NS$

FMLP = *N*-formylmethionyl-leucyl-phenylalanine; IVF = in vitro fertilization; ICSI = intracytoplasmic sperm injection; L = luminol; PMA = 12-myristate, 13-acetate phorbol ester; NS = not statistically significant.

^a Studies used in the meta-analysis.

Agarwal. Levels of ROS and IVF outcome. *Fertil Steril* 2005.

etry. Five studies correlated FMLP (*N*-formylmethionyl-leucyl-phenylalanine)-stimulated ROS levels with the fertilization rate. Two studies provided correlation coefficients with PMA (12-myristate, 13-acetate phorbol ester)-stimulated ROS levels, and two other studies correlated IVF rate with basal ROS levels.

Five studies consisted of infertile couples with male factor, female factor, or idiopathic infertility. Two studies failed to mention the female fertility status, and three studies mentioned that the female partner was healthy. Semen samples in eight studies were prepared using the density gradient technique, whereas the ninth study used the swim-up technique. Statistical methods used in the primary studies were regression analysis and Spearman rank correlation test.

RANDOM-EFFECTS META-ANALYSIS

Meta-analysis was conducted using the five studies that correlated FMLP-stimulated ROS levels with the IVF rate. We considered this analysis because there were a large number of studies correlating FMLP-stimulated ROS levels with the IVF rate. Two studies associating FMLP-stimulated ROS levels with the IVF rate were excluded

from the meta-analysis because they did not provide the actual correlation values.

A total of three studies were selected for the final meta-analysis with a total study population of 122 ($n = 41, n = 73, n = 8$). The study population consisted of couples undergoing IVF for various causes of infertility (male factor, female factor, and idiopathic). The density gradient technique was used for sperm preparation in all three studies. The estimated overall correlation by random effects analysis was -0.374 (95% CI, -0.205 to -0.520).

Many aspects of sperm quality, including motility, morphology, zona pellucida binding, and sperm-oocyte fusion, are considered major determinants of fertilization potential. According to De Geyter et al. (28), "at present, none of the available sperm function tests can reliably predict the absence of fertilizing ability." Production of ROS appears to be a measurable constituent that may predict the fertilization rate, and, further, the IVF outcome (17–21). There are several studies that provide indirect evidence demonstrating that ROS affects fertilization. Selective removal of ROS-producing leukocytes from sperm suspensions has been shown to increase the rate of sperm-oocyte fusion (22, 29).

Our meta-analysis suggests that ROS levels are negatively correlated with fertilization rates following IVF. The meta-analytical approach is used as a precise investigation for those studies that could fit into the specific criteria such as measuring ROS levels by chemiluminescence method, stimulation by FMLP, and providing the actual correlation value between ROS levels and fertilization rate. Information from the studies that failed to fit into these criteria was excluded; for example, we did not include the studies investigating the relationship between PMA-stimulated or basal ROS levels and fertilization rate.

A lack of statistically useful data in some studies, especially those that did not reveal any correlation between ROS levels and fertilization rate (22–24), was another major limiting factor in our qualified meta-analysis. Another study found a statistically significant negative association, but the results were not expressed using a correlation value (20).

We noted several inconsistencies between the published studies investigating the relationship between the ROS levels in semen samples and the fertilization rates after IVF, which may explain why the study results differed. For example, two of the studies considered couples with the male partner producing semen with normal sperm motility ($\geq 50\%$) and count (≥ 20 million/mL), which may have influenced the results (22, 24).

Most of the studies included couples undergoing IVF because of either female factor or male factor infertility or infertility of unknown etiology. However, it is possible that the correlation would have been stronger if the studies had used a pure population of male factor or idiopathic infertility patients. In the future, the effect of ROS levels on fertilization rate will become clear if only patients with male factor infertility or idiopathic infertility are enrolled.

The discrepancy in the results may also be due to the physiologic role of ROS in capacitation and fertilization (9, 10, 23, 30). Thus, defining the physiologic levels of ROS and studying the effect of ROS levels on fertilization only in patients with pathological levels of ROS (above the physiologic levels) may be necessary to accurately characterize the relationship between levels of ROS and IVF.

Why should we measure ROS? It is known to affect routine semen parameters, but this is not always the case. It is possible that patients with normal semen parameters may have high levels of ROS that may affect the fertilizing capacity of spermatozoa (12, 31). It is possible that the levels of ROS needed to impair sperm–oocyte fusion events are lower than those required to affect sperm motility. Therefore, estimation of ROS levels is important to assess the adverse effect on fertilization not identified by the measurement of routine semen parameters.

Based on our qualified meta analysis, which included all of the available evidence from the literature, we can con-

clude that there is a significant correlation between ROS levels in spermatozoa and the fertilization rate after IVF. Measuring ROS levels in semen specimens before IVF may be useful in predicting the IVF outcome and in counseling selected patients with male factor or idiopathic infertility. Future studies should include the use of uniform study design (homogeneous patient population, similar sperm preparation methods, uniform reporting of fertilization results, etc.) and standardized ROS measurement protocols to conclusively prove the effect of seminal ROS on fertilization rate after IVF in infertile couples.

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REFERENCES

1. Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl* 2002;23:737–52.
2. Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. *Urol Clin North Am* 2002;29:817–27.
3. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003;79:829–43.
4. Aitken RJ. The Amoroso lecture. The human spermatozoon—a cell in crisis? *J Reprod Fertil* 1999;115:1–7.
5. Gagnon C, Iwasaki A, De Lamirande E, Kovalski N. Reactive oxygen species and human spermatozoa. *Ann NY Acad Sci* 1991;637:436–44.
6. Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. *Urology* 1996;48:835–50.
7. Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* 2003;7:65–70.
8. Sikka SC. Role of oxidative stress and antioxidants in andrology and assisted reproductive technology. *J Androl* 2004;25:5–18.
9. Aitken J, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* 1994;16:259–67.
10. de Lamirande E, Gagnon C. Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. *Hum Reprod* 1995;10(Suppl 1):15–21.
11. Agarwal A, Ikemoto I, Loughlin KR. Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. *J Urol* 1994;152:107–10.
12. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, et al. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998;59:1037–46.
13. 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.
14. Gil-Guzman E, Ollero M, Lopez MC, Sharma RK, Alvarez JG, Thomas AJ Jr, et al. Differential production of reactive oxygen

- species by subsets of human spermatozoa at different stages of maturation. *Hum Reprod* 2001;16:1922–30.
15. Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertil Steril* 2000;73:459–64.
 16. Whittington K, Harrison SC, Williams KM, Day JL, McLaughlin EA, Hull MG, et al. Reactive oxygen species (ROS) production and the outcome of diagnostic tests of sperm function. *Int J Androl* 1999;22:236–42.
 17. Krausz C, Mills C, Rogers S, Tan SL, Aitken RJ. Stimulation of oxidant generation by human sperm suspensions using phorbol esters and formyl peptides: relationships with motility and fertilization in vitro. *Fertil Steril* 1994;62:599–605.
 18. Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Prediction of the in-vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Hum Reprod* 1996;11:1030–4.
 19. Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Predicting the fertilizing potential of human sperm suspensions in vitro: importance of sperm morphology and leukocyte contamination. *Fertil Steril* 1995;63:1293–300.
 20. Zorn B, Vidmar G, Meden-Vrtovec H. Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection. *Int J Androl* 2003;26:279–85.
 21. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003;79(Suppl 3):79–87.
 22. Moilanen JM, Carpen O, Hovatta O. Flow cytometric light scattering analysis, acrosome reaction, reactive oxygen species production and leukocyte contamination of semen preparation in prediction of fertilization rate in vitro. *Hum Reprod* 1998;13:2568–74.
 23. Yeung CH, De Geyter C, De Geyter M, Nieschlag E. Production of reactive oxygen species by and hydrogen peroxide scavenging activity of spermatozoa in an IVF program. *J Assist Reprod Genet* 1996;13:495–500.
 24. Moilanen JM, Carpen O, Hovatta O. Flow cytometric analysis of semen preparation, and assessment of acrosome reaction, reactive oxygen species production and leukocyte contamination in subfertile men. *Andrologia* 1999;31:269–76.
 25. Hedges L. *OI Statistical methods for meta-analysis*. San Diego, CA: Academic Press, 1985.
 26. Marchetti C, Obert G, Deffosez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod* 2002;17:1257–65.
 27. Hammadeh ME, al-Hasani S, Spengler C, Rosenbaum P, Schmidt W. Comparison between total antioxidant status (TAS), reactive oxygen species (ROS) and DNA integrity of IVF and ICSI patients and their predictive value of IVF/ICSI outcome. *Fertil Steril* 2003;80(Suppl 3):S163.
 28. De Geyter C, De Geyter M, Meschede D, Behre HM Assisted fertilization. Heidelberg, Germany: Springer-Verlag, 2000.
 29. Aitken RJ, Buckingham DW, West K, Brindle J. On the use of paramagnetic beads and ferrofluids to assess and eliminate the leukocytic contribution to oxygen radical generation by human sperm suspensions. *Am J Reprod Immunol* 1996;35:541–51.
 30. Griveau JF, Le Lannou D. Reactive oxygen species and human spermatozoa: physiology and pathology. *Int J Androl* 1997;20:61–9.
 31. Pasqualotto FF, Sharma RK, Kobayashi H, Nelson DR, Thomas AJ Jr, Agarwal A. Oxidative stress in normospermic men undergoing infertility evaluation. *J Androl* 2001;22:316–22.