

Differential growth of human embryos in vitro: role of reactive oxygen species

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Objective: To examine the relationship of early human embryonic development with the level of reactive oxygen species (ROS) in the culture media on the first day (day 1 ROS) after insemination.

Design: A prospective study.

Setting: Patients undergoing assisted reproduction in a teaching hospital.

Patient(s): Patients undergoing conventional IVF (n = 104; 115 cycles) and intracytoplasmic sperm injection (ICSI) (n = 91; 96 cycles) were included. Both fertilization and early cultures were performed in human tubal fluid with 5% serum substitute supplement.

Intervention(s): Day 1 ROS levels in the central well (sample) and the outer well (control) of each embryo culture dish were measured after overnight incubation by chemiluminescence assay using luminol as the probe.

Main Outcome Measure(s): Fertilization rate and embryo quality at day 3 and 5 were recorded for each cycle. Age, parity, and demographic features were also compared.

Result(s): High day 1 ROS levels in culture media were associated with low blastocyst rate, low fertilization rate, low cleavage rate, and high embryonic fragmentation with ICSI but not with conventional IVF. High day 1 ROS levels in culture media were associated with lower pregnancy rates in both IVF and ICSI cycles.

Conclusion(s): Reactive oxygen species generated in culture media by day 1 may be an important biochemical marker for early embryonic growth. Increased embryonic fragmentation and slow cleavage rate may be partially attributed to early exposure of embryos to high ROS levels in ICSI cycles. Differential growth of ICSI embryos incubated under identical conditions may be in part due to differences in ROS levels of the culture medium surrounding these embryos. (Fertil Steril® 2004;82:593–600. ©2004 by American Society for Reproductive Medicine.)

Key Words: Reactive oxygen species, fertilization, embryo cleavage, fragmentation

The metabolic activity of growing preimplantation embryos remains poorly understood despite the efforts of many investigators (1–3). Currently, embryos are evaluated and selected for transfer based largely on their gross morphology. Ideally, selection of normal and viable embryos with high implantation potential would be based on both the chromosomal integrity and expression of the appropriate developmental genes. Aneuploidy testing can reduce embryonic loss after implantation (4). However, molecular markers indicative of optimal development are yet to be identified (5).

The presence of oxidative and antioxidant systems in various reproductive tissues has evoked interest in the role of oxidative stress in reproductive diseases (6). Oxidative stress has

been defined as an elevation in the steady state concentration of various reactive oxygen species (ROS) on a cellular level. It has been suggested that this is important in different reproductive scenarios such as endometriosis (7, 8), follicular fluid (9–11), and hydrosalpingeal fluid (12). Oxidative stress may also be involved in the etiology of defective embryo development.

Reactive oxygen species may originate from embryo metabolism and/or embryo surroundings (13). Reactive oxygen species can not only alter most types of cellular molecules, but also induce early embryonic developmental block and retardation (14). Multiple mechanisms of embryo protection against ROS exist (15). The exact role of oxidative stress in early embry-

onic development has yet to be identified. This study was designed to quantify ROS in the culture media the morning after oocyte retrieval (day 1 media) in conventional IVF and intracytoplasmic sperm injection (ICSI) cycles and to assess the relationship of day 1 ROS levels to fertilization, embryo quality on day 3 and 5, and pregnancy rates.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. A total of 211 IVF cycles in 184 patients between 2000 and 2001 were included. One hundred four patients underwent 115 conventional IVF cycles. Ninety-one patients were treated with ICSI in 96 cycles.

Controlled Ovarian Stimulation

All patients underwent pituitary down-regulation with the GnRH agonist leuprolide acetate (Lupron; Tap Pharmaceutical, Deerfield, IL) at a daily SC dose of 10 U (0.5 mg) initiated on cycle day 21. The dose was subsequently reduced to 5 U (0.25 mg) once serum E₂ was suppressed to ≤50 pg/mL and continued until the day hCG was administered. Controlled ovarian hyperstimulation with SC recombinant FSH (Gonal F; Serono, Randolph, MA) or Follistim (Organon, West Orange, NJ) was begun after pituitary down-regulation. The standard initial dose was 300 IU.

The ovarian response was monitored by serial serum E₂ levels and transvaginal ultrasounds beginning on the fifth day of stimulation. The FSH dose and subsequent monitoring was individualized based on these results. Stimulation was continued until at least two follicles reached a mean diameter of ≥18 mm, at which time hCG 10,000 IU SC was administered 36 hours before oocyte recovery.

Oocyte Retrieval

Oocytes were collected by transvaginal ultrasound-guided needle aspiration of the follicles. The procedure was performed under deep conscious sedation. The retrieved oocytes were rinsed, graded, and placed in HEPES buffered human tubal fluid (HTF) (Irvine Scientific, Santa Ana, CA) at 37°C under 5% CO₂, 5% O₂, and 90% N₂.

Sperm Collection and Preparation for ICSI

After 2 days of abstinence, the male partner was asked to provide a semen sample by masturbation in a sterile container. The sperm were prepared by a modification of the ISolate (Irvine Scientific) density gradient centrifugation technique. A single 2-mL layer of 90% ISolate was used for most specimens. After the ISolate preparation, sperm were maintained in HTF medium with 5% synthetic serum substitute at room temperature until the time of IVF or ICSI. In conventional IVF, 150 × 10³ to 200 × 10³ sperm were added to each culture dish containing 4–5 oocytes. The single best looking spermatozoon was selected for ICSI.

Gamete and Embryo Culture

After cumulus dissection and wash, the oocytes were placed in 1 mL of HTF supplemented with 6% synthetic serum substitute (SSS; Irvine Scientific). Fertilization was confirmed 14–16 hours after insemination. Normal fertilization was defined as the presence of 2 pronuclei and the extrusion of the second polar body 14–16 hours after insemination or injection. Normally fertilized oocytes were cultured in groups of 4–5 oocytes in 1 mL of HTF with SSS until the early afternoon of day 3. They were then placed in 1 mL of blastocyst media (Irvine Scientific) after a five-drop rinse in the same medium. A second change over to fresh blastocyst medium was done on the morning of day 5 after embryo evaluation and before ET. Embryo transfers were scheduled between noon and 2:00 P.M. on day 3 or 5.

Embryo Evaluation

On days 3 and 5 of development, embryos were evaluated with an Olympus ×70 inverted microscope (Olympus America, Melville, NY), which was equipped with Hoffman Modulation Optics (Narishige, Tokyo). Total magnification was ×600. Cell number and degree and pattern of fragmentation were recorded on day 3 of development. The degree of fragmentation was expressed as a percentage and defined as the embryonic volume occupied by denuded cytoplasmic fragments.

Development on day 5 was recorded as well. A distinct inner cell mass (ICM), organized as a compacted mass of cells, was considered normal. A cohesive layer of numerous tightly packed cells in the trophectoderm (TE) was normal as well. Highly irregularly arranged ICM and/or TE cells were considered abnormal. All abnormal embryos on day 5 and day 6 were considered arrested.

ROS Measurement

After evaluating the oocytes for fertilization 14–16 hours after insemination or ICSI, the insemination media in the central well of the culture dish were collected as a sample. The media in the outer well of the culture dish served as a control. Both were immediately centrifuged for 7 minutes at 600 × g, and the supernatant was removed.

Aliquots of the supernatant were prepared for ROS measurement. The ROS production was measured by the chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma, St. Louis, MO) as the probe. Ten microliters of 5 mM luminol prepared in dimethyl sulfoxide (Sigma) was added to 400 μL of the day 1 media. The ROS levels were determined by measuring chemiluminescence with a luminometer (LKB 953, Wallac, Gaithersburg, MD) in the integrated mode for 15 minutes, and results were expressed as 104 cpm.

Statistical Methods

Descriptive statistics are presented as frequency (percent) or median and interquartile (25th and 75th percentiles). Because multiple cycles from the same patients were in-

TABLE 1

Demographic features and indications for assisted reproduction technologies.

Parameter	IVF	ICSI	P ^a
No. of patients	104	91	—
No. of cycles	115	96	—
Age (years)	33 (30, 36)	33 (31, 36)	.68
Parity	—	—	.60
0	74 (71)	65 (75)	—
1 or more	31 (30)	22 (25)	—
Infertility	—	—	.31
Primary	52 (50)	52 (60)	—
Secondary	53 (50)	35 (40)	—
Infertility factors	—	—	—
Male	24 (26)	37 (48)	.004
Anovulation	10 (9)	12 (13)	.65
Endometriosis	20 (17)	11 (12)	.57
Tubal	25 (22)	17 (18)	.64
Unexplained	26 (23)	12 (13)	.44
Other	18 (16)	19 (20)	.45

Note: Data are n (%) or median (25th and 75th percentiles), where n = no. of cycles.

^a Logistic regression using generalized estimating equations.

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cluded, repeated-measures models were employed for all analyses. IVF and ICSI groups were compared on binary outcomes with logistic regression using generalized estimating equations (GEE) and on continuous variables using repeated measures mixed models. Groups were compared on rates of outcomes with events/trials logistic regression using GEE.

The association between day 1 ROS and outcomes was assessed separately for IVF and ICSI groups using repeated

measures mixed models and GEE logistic regression models. The base 2 logarithm of ROS+1 was used to better meet distributional assumptions. To assess whether the relationships between day 1 ROS and outcomes were linear, $\log_2(\text{ROS}+1)^2$ and $\log_2(\text{ROS}+1)^3$ were included in the models where significant. The percent change (with 95% confidence interval) in odds of outcome associated with a doubling of ROS level is given. All tests were two-tailed with a significance level of .05. Analysis was done with SAS 8.2 (SAS Institute, Cary, NC), and graphics were produced with S-plus 6.1 (Insightful Corp., Seattle, WA).

RESULTS

Demographic Variables and Assessment of Cycle Outcome (Tables 1 and 2)

The demographic features of the study population and the indications for assisted reproduction are presented in Table 1. One hundred eighty-four patients underwent 211 cycles of IVF throughout the duration of the study. Of those, 115 were conventional IVF cycles and 96 were ICSI cycles. The patients' ages, parity, and percentage of primary versus secondary infertility were similar in the IVF and ICSI groups.

There were no significant differences in the number of days of stimulation, amount of FSH used, cycle day 3, or peak E₂ levels or number of oocytes retrieved between the groups (Table 2). Pregnancy, multiple pregnancy, and implantation rates were similar between conventional IVF and ICSI, although the fertilization rate was higher among ICSI patients. The clinical pregnancy rate represents the number of cycles with fetal cardiac activity on ultrasound divided by the number of cycles initiated. The implantation rate was calculated by dividing the number of gestational sacs on ultrasound by the number of embryos transferred.

TABLE 2

Parameters of controlled ovarian hyperstimulation pregnancy rates.

Parameter	IVF	ICSI	P ^a
Days of stimulation	9 (8, 10)	9 (8, 10)	.13
Amount of FSH used (IU)	2,400 (1,800, 3,150)	2,700 (1,875, 3,300)	.99
Day 3 E ₂ (pg/mL)	38 (30, 54)	35 (21, 47)	.18
E ₂ on day of hCG administration (pg/mL)	1,711 (1,158, 2,292)	1,869 (1,339, 2,411)	.17
No. of oocytes retrieved	12 (8, 17)	13 (8, 18)	.96
Fertilization rate (%)	63 (43, 80)	69 (57, 83)	.001
Reactive oxygen species (cpm) ^b	22 (0, 63)	28 (0, 49)	.86
High (>7) day 3 cell number rate (%)	40 (11, 60)	40 (0, 67)	.98
Low fragmentation rate (<10%)	60 (40, 85)	73 (43, 100)	.035
Blastocyst rate (%)	22 (0, 50)	20 (0, 40)	.44
Implantation rate (%)	56/275 (20)	41/241 (17)	.41
Clinical pregnancy (%)	42/91 (40)	32/87 (37)	.54
Multiple pregnancy (%)	15/91 (15)	9/87 (10)	.32

Note: Data are n (%) or median (25th and 75th percentiles), where n = no. of cycles.

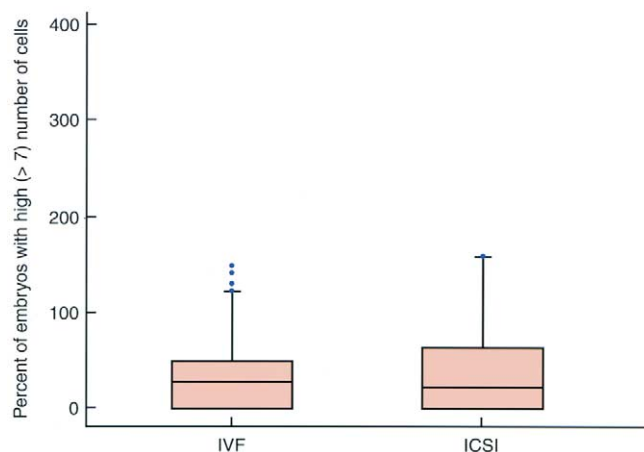
^a Logistic regression using generalized estimating equation, or mixed model.

^b P value from model using $\log_2(\text{ROS}+1)$.

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FIGURE 1

Day 1 ROS in IVF and ICSI cycles. *Box plot* represents the interquartile range (25th and 75th percentile) with a *line* indicating the median. *Whiskers* extend to 1.5 times the interquartile range. *Open circles* represent the outliers.



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The median and interquartile range (25th and 75th percentiles) of low (<10%) embryo fragmentation on day 3 was 60% (range, 40%–85%) in the IVF group and 73% (range, 43%–100%) in the ICSI group. Within the IVF group, 40% (range, 11%–60%) of the embryos had ≥ 7 blastomeres and this was comparable with ICSI. The main outcome measure for embryo quality at day 5 or day 6 is the blastocyst development rate. Similar rates of blastocyst formation were observed in the IVF group and the ICSI group, 22% (range, 0%–50%) versus 20% (range, 0%–40%), respectively. Re-

active oxygen species levels were comparable in both the IVF and ICSI groups (22 [range, 0–63] vs. 28 [range, 0–49]), respectively (Fig. 1).

Relationship of Day 1 ROS and the Cycle Outcome Parameters (Table 3)

Normal fertilization rate, number of embryos with ≥ 7 cells, <10% fragmentation on day 3, and blastocyst formation on day 5 or 6 were all considered to be favorable outcomes.

Fertilization Rate

Day 1 ROS levels were not significantly associated with the fertilization rate in conventional IVF cycles ($P=.89$). In ICSI cycles, our model indicated that fertilization rates increased slightly when day 1 ROS levels were low but then decreased as ROS levels increased ($P=.037$).

Cell Number on Day 3

In conventional IVF cycles, day 1 ROS levels were not significantly associated with the probability that more embryos contained at least 7 cells on day 3 ($P=.96$; Fig. 2A). In ICSI cycles, as day 1 ROS levels increased, the probability that an embryo contained at least 7 cells on day 3 decreased ($P=.014$; Fig. 2B).

Embryo Fragmentation

In conventional IVF cycles, there was no evidence that day 1 ROS levels in culture media were related to the probability that embryo fragmentation was <10% ($P=.54$; Fig. 3A). In ICSI cycles, with the increase in day 1 ROS levels, there was a concomitant increase in the embryo fragmentation rate (>10%) ($P=.037$; Fig. 3B). This indicated that the higher the levels of day 1 ROS, the greater the embryonic fragmentation at day 3. When both IVF and ICSI groups were combined, there was slight evidence

TABLE 3

Relationship of day 1 reactive oxygen species (ROS) with the main outcome measures of IVF and intracytoplasmic sperm injection cycles.

Factor	IVF		ICSI	
	% Change in odds of outcome when ROS doubles	P^a	% Change in odds of outcome when ROS doubles	P^b
Fertilization rate	-1 (-8, 8)	.89	— ^b	.037
High (>7) day 3 cell number rate	1 (-18, 23)	.96	-14 (-23, -3)	.014
Low (<10%) fragmentation rate	-3 (-12, 7)	.54	-12 (-22, -1)	.037
Blastocyst rate	-6 (-15, 5)	.28	-11 (-20, -1)	.027
Clinical pregnancy rate	-19 (-31, -5)	.010	-23 (-35, -9)	.002

Note: Data are percentage change with confidence interval in parentheses.

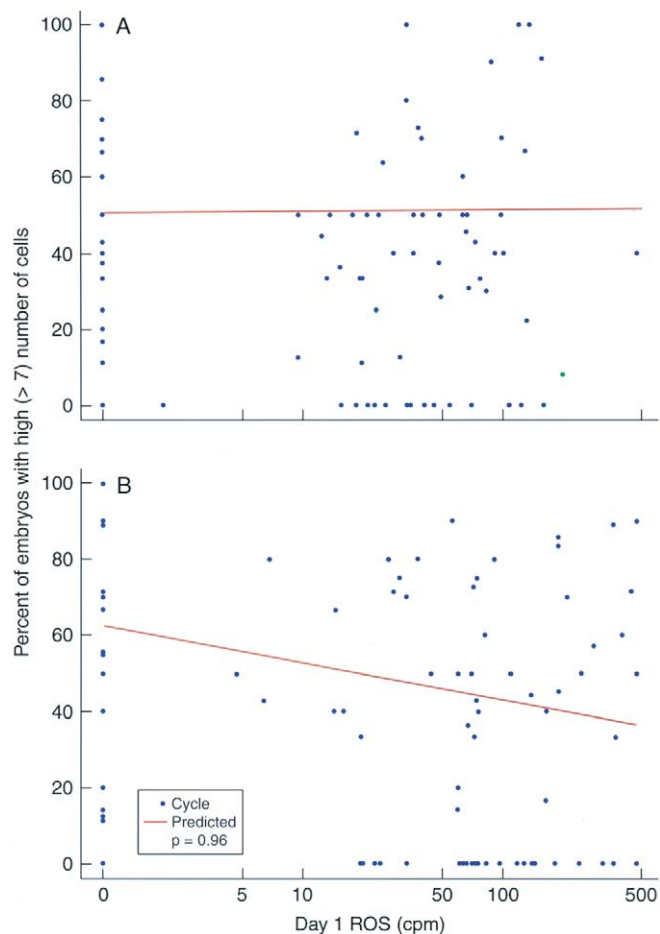
^a Logistic regression using generalized estimating equations.

^b Model included $\log_2(\text{ROS}+1)^2$ term, indicating that fertilization rates showed a slight initial increase as ROS increased and then decreased as ROS increased. The odds of fertilization increased by 20% (-2%, 45%) when ROS values increased from 1 to 2 and then decreased by -8% (-15%, -1%) when ROS values increased from 10 to 20.

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FIGURE 2

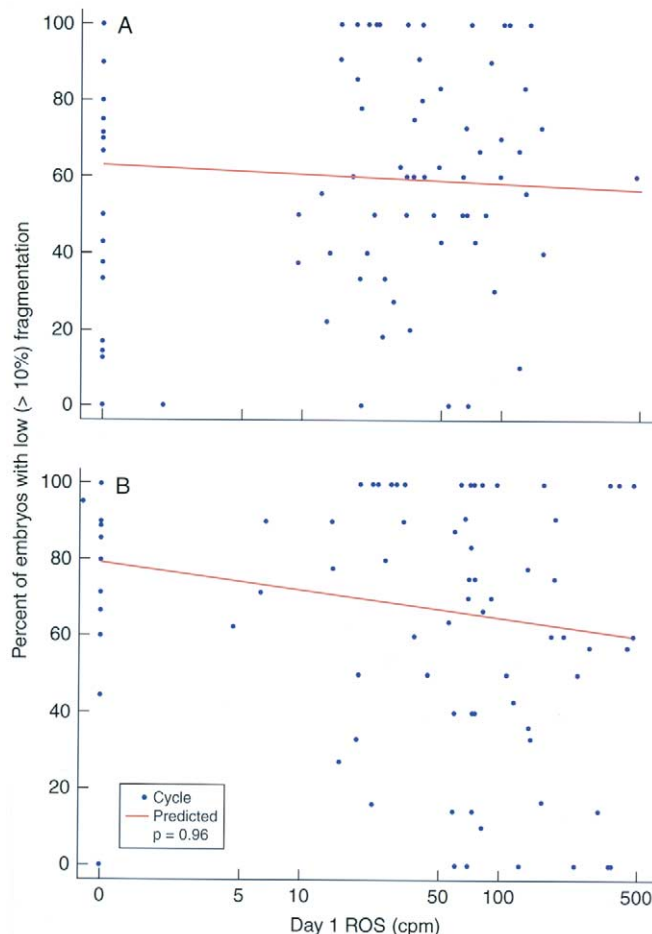
(A) Relationship of day 1 ROS with % embryos in IVF cycles with high (>7) cell number at day 3. (B) Relationship of day 1 ROS in ICSI cycles with % embryos with high cell (>7) number at day 3.



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FIGURE 3

(A) Relationship of day 1 ROS in IVF cycles with % embryos with low (<10%) fragmentation rate at day 3. (B) Relationship of day 1 ROS in ICSI with % embryos with low (<10%) fragmentation rate at day 3.



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that high day 1 ROS levels were associated with lower probability that the embryo fragmentation rate was <10% ($P = .067$).

Blastocyst Rate

A significant association was seen in ICSI cycles between high day 1 ROS levels and low blastocyst development rate ($P = .027$). This association was not observed in conventional IVF cycles ($P = .28$).

Clinical Pregnancy Rate

High day 1 ROS levels in culture media were associated with higher pregnancy rates in both IVF ($P = .01$) and ICSI ($P = .002$) cycles.

DISCUSSION

A majority of the retrieved mature oocytes fertilize, and up to 70% of these undergo the first three cleavage divisions during the first 3 days in culture. However, less than half of the cleaved embryos undergo cavitation and proceed to blastocyst formation by day 5 in culture (16). Similarly, only about one-third of day 3 embryos form morphologically optimal blastocysts (17).

A number of intrinsic and extrinsic factors have been shown to influence in vitro survival of the embryos to the blastocyst stage in extended culture, including sperm quality (18), etiology of infertility (19), and maternal age (20). Cleavage patterns have also been linked to blastocyst for-

mation (21). There is a strong association between chromosomal abnormalities and aberrant early embryo morphology, as well as with a high implantation rate with in vitro grown blastocysts (22). However, the relationship among embryo morphology, chromosomal integrity, embryo development in vitro, and implantation is much more complex (23).

These observations suggest that in vitro extrinsic factors such as prolonged culture conditions and the autocrine and paracrine activities of the embryos may also contribute to the failure of optimal embryo development. It is unclear as to which embryos may be adversely affected and to what extent. Among the factors that might affect in vitro development of embryos is the balance between oxidative stress, as early as day 1 in culture, and the ability of the embryos to neutralize their effects. Free radicals are generated from leakage of high-energy electrons as they proceed down the electron transport chain. The free radicals have many harmful effects including DNA damage (14). The potential cellular sources of ROS with conventional IVF are different from those with ICSI.

In conventional IVF, ROS in the culture media may originate from the oocytes (4–5 per dish), cumulus cell mass (thousands of cells), and spermatozoa used for insemination (150×10^3 to 200×10^3 per dish). The cumulus cells are not a potential source of ROS in ICSI since the incubation is initiated after denuding the oocytes from all their cumulus cells. The potential cellular sources of ROS in the ICSI setting are the spermatozoa and the injected oocytes.

We assessed the impact of day 1 ROS in culture media on the fertilization rate, cleavage rate, fragmentation, and blastocyst formation after prolonged culture based on whether the embryos were generated by conventional IVF or ICSI. This study suggested that slow development (<7 cells on day 3), high fragmentation (>10%), and reduced formation of morphologically normal blastocysts are associated with increased levels of day 1 ROS. Our culture system yielded an overall blastocyst formation rate similar to those reported by other investigators (24–26). Although there was no apparent relationship between day 1 ROS levels and fertilization rate in conventional IVF cycles, there was a significant relationship with the fertilization rate in ICSI cycles ($P=.037$). In the ICSI model, fertilization rates increased when ROS levels were low then decreased as ROS increased further.

The initial rise in the fertilization rate when ROS values were low may signify that low ROS levels are physiological and perhaps needed for normal fertilization. The lack of a detrimental relationship between higher day 1 ROS levels and fertilization in conventional IVF cycles could be explained by the potential antioxidant activity of the cumulus cell mass and normal spermatozoa present in culture. Antioxidant activity neutralizes the ROS and negates its effect on fertilization and subsequent embryonic development. This antioxidant function is not present in ICSI cycles because the oocytes were completely denuded

of the cumulus cell mass and only one spermatozoa was injected inside the oocyte (27).

The role of ROS in human fertilization has been studied extensively. It was demonstrated that a certain level of ROS is necessary for normal sperm function (6, 28–30), whereas an excessive level of ROS may have a detrimental effect on sperm DNA and subsequent fertilization (31). The concern about sperm DNA damage by ROS is especially relevant during the ICSI procedure (33).

Lower blastocyst development rates have been observed in ICSI cycles (32). In our study, day 1 ROS levels were not significantly related to blastocyst development rate in conventional IVF ($P=.28$). However, in the ICSI cycles, higher levels of day 1 ROS were associated with lower blastocyst development rates ($P=.027$). This may help explain the reduced blastocyst development rates observed by Menezo and Barak (33). The reduced ability to form normal blastocysts was, in many instances, observed at compaction, which occurs on day 4 in humans (34). Reactive oxygen species is also known to induce zona hardening, tanning, and DNA damage (33).

Lower clinical pregnancy rates were also associated with higher day 1 ROS levels in both conventional IVF ($P=.01$) and ICSI cycles ($P=.002$). Day 3 embryos with reduced blastomere numbers and higher fragmentation due to the negative association with day 1 ROS would be predicted to develop into blastocysts at a lower rate. Such embryos have been shown to yield higher pregnancy rates after assisted hatching, fragment removal, and ET on day 3 rather than by extending culture to day 5 (35).

Perhaps one of the most obvious ways of reducing oxidative damage in IVF clinics is to reduce exposure of the gametes and embryos to environments that contain free radicals or that allow their generation. This can be partially achieved by reducing or eliminating exposure of gametes and embryos to air during handling. Although the single greatest source of exogenous ROS is likely to be derived from spermatozoa that are used to inseminate oocytes in vitro, our findings also show that ROS can be hazardous with ICSI where there is only one spermatozoon per oocyte. It is a routine in most IVF laboratories to use relatively long insemination times (14–16 hours). Such long exposure of oocytes to spermatozoa can increase oxidative damage (36), and therefore a number of laboratories have investigated reducing the exposure time. The results have been conflicting. Several investigators claimed beneficial effects of short co-incubation of gametes in IVF (37, 38), and others noted the opposite (39).

There were several limitations of our study. We could not evaluate the relationship to ROS levels of each embryo as they were cultured in groups (4–5 embryos in each group). Also, we could not determine the relative contribution of ROS from the oocytes, spermatozoa, or cumulus cell mass in culture or their potential antioxidant effects in conventional IVF cycles. However, evaluating each embryo alone may negate the potentially

beneficial embryonic paracrine functions including antioxidants as proposed by some investigators (14, 40). We did not account for the spatial orientation of fragmentation in the embryos. A very large number of embryos would have been required to stratify by this factor. To overcome this issue, we selected the 10% fragmentation rate as a threshold value between potentially good and bad embryos. This rate is halfway between the previously proposed rate of 5%–15% (17, 35). Other indicators of oxidative stress such as lipid peroxides and total antioxidant capacity could have provided a more comprehensive picture of oxidative stress in culture.

In conclusion, low fertilization rates and cleavage abnormalities are evident only in ICSI embryos exposed to ROS early in culture. We propose measurement of day 1 ROS levels in the medium as a potential marker for poor embryo quality, particularly in ICSI cycles. We demonstrated that the addition of antioxidants (vitamins C and E), especially vitamin C, can improve blastocyst development rates in mouse embryo models (41). Therefore, short co-incubation time of the gametes in conventional IVF cycles and supplementing the culture media with vitamin C or E to help scavenge excessive production of ROS in the culture media may further improve these results.

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