

Role of total antioxidant capacity in the differential growth of human embryos in vitro

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Objective: The objective of this study was to examine the relationship of early human embryonic development parameters with day 1 culture media total antioxidant levels (day 1 TAC).

Design: Prospective study.

Setting: Patients undergoing assisted reproduction (ART) in a teaching hospital.

Patient(s): Patients undergoing conventional IVF (n = 153; 167 cycles) and intracytoplasmic sperm injection (ICSI; n = 105; 116 cycles) were included. Both fertilization and early cultures were performed in human tubal fluid (HTF) with 5% serum substitute supplement.

Intervention(s): Levels of total antioxidants in the central well (sample) and the outer well (control) of each embryo culture dish were measured.

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

Result(s): After controlling for all demographic and clinical variables, day 1 TAC was related to fertilization rates in both groups of patients. Day 1 TAC was significantly related to high (>7) day 3 cell number, low (<10%) fragmentation rate, and blastocyst development rate in both conventional IVF and ICSI cycles. Day 1 TAC was related to pregnancy in ICSI but not conventional IVF cycles.

Conclusion(s): Day 1 TAC appears to be an important biochemical marker for early embryonic growth. Decreased embryonic fragmentation, enhanced cleavage rate, and increased blastocyst development rate may be partially related to day 1 TAC in the culture media. Whether this relationship is a cause or effect needs further assessment. (Fertil Steril® 2006;86:304–9. ©2006 by American Society for Reproductive Medicine.)

Key Words: Total antioxidant capacity, fertilization, embryo cleavage, fragmentation

Reactive oxygen species (ROS) have been reported in day 1 IVF culture media, where day number refers to the days since oocyte retrieval. Elevated day 1 ROS levels in culture media were associated with low blastocyst rate, low fertilization rate, low cleavage rate, and high embryonic fragmentation with intracytoplasmic sperm injection (ICSI) but not in conventional IVF. They were also associated with lower

pregnancy rates (PRs) in both IVF and ICSI cycles (1). Normally, there is equilibrium between ROS production and the ability of the antioxidants to scavenge the excessive ROS production. Antioxidants provide defense mechanisms through three levels of protection: [1] prevention, [2] interception, and [3] repair (2). Oxidative stress occurs when the scavenging ability of the antioxidants are overwhelmed due to excessive ROS formation (3). The ROS levels are usually measured by the chemiluminescence method (4), whereas total antioxidant capacity (TAC) can be measured by an enhanced chemiluminescence assay or a colorimetric assay (5).

Developing embryo metabolism or surrounding embryo environment can result in oxidative stress (6). The ROS can alter most types of cellular molecules during early embryonic development (7). In addition, embryos may have different sensitivities to ROS at different stages. Bovine embryos at the 9- to 12-cell stage are more resistant to

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exogenous H₂O₂ compared with zygotes and blastocysts. These differences in sensitivities are related to variations in thresholds of the defense mechanism (8). The two-cell embryo block is associated with an oxidative burst, suggesting that the embryo may be particularly vulnerable to ROS at this stage. At this time, the embryo in vivo is present in the oviduct, which provides various radical scavengers (9, 10). Female reproductive tract fluid contains high concentrations of certain amino acids suggesting that they may play a role in preimplantation development (11).

Although multiple mechanisms exist for protecting the embryos against ROS that include a variety of antioxidants, the exact nature of how these antioxidants affect early embryonic development is not clear. Our study was designed to quantify TAC in the culture media the morning after oocyte retrieval (day 1 media) during conventional IVF and ICSI cycles and assess the relationship of day 1 TAC with [1] fertilization rates, [2] day 3 and day 5 embryo quality, and [3] PRs.

MATERIALS AND METHODS

The Institutional Review Board of the Cleveland Clinic Foundation approved the study. A total of 283 IVF cycles in 258 patients were included between 2000 and 2001. One hundred fifty-five patients underwent 167 conventional IVF cycles and 105 patients underwent ICSI in 116 cycles.

Controlled Ovarian Stimulation

All patients underwent pituitary down regulation with the GnRH agonist (GnRH-a) leuprolide acetate (LA; Lupron; Tap Pharmaceutical Inc., Deerfield, IL) administered as 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 levels were suppressed to ≤ 50 pg/mL and continued until the day of hCG administration. Controlled ovarian hyperstimulation (COH) with a daily SC dose of 300 IU recombinant FSH (Gonal F, Serono, Randolph, MA) or follistim (Organon, West Orange, NJ) was begun after pituitary down regulation.

The ovarian response was monitored by serial serum E₂ levels and transvaginal ultrasonograms beginning on day 5 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 10,000 IU SC of hCG 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

Semen samples were obtained by masturbation concurrently with the oocyte retrieval after a 3- to 5-day period of abstinence. Spermatozoa were prepared by a density gradient centrifugation technique. Samples were loaded onto a single 90% ISolate (Irvine Scientific, Santa Ana, CA) layer and centrifuged at 1600 rpm for 20 minutes at room temperature. The resulting pellet was washed by centrifugation for additional 7 minutes and resuspended in HTF media supplemented with 5% synthetic serum substitute (SSS; Irvine Scientific) at room temperature until the time of IVF or ICSI. In conventional IVF, 150–200 $\times 10^3$ sperm were added to each culture dish containing four to five oocytes. A single morphologically normal-appearing 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 5% SSS. Fertilization was confirmed 14–16 hours after insemination by the presence of two pronuclei and extrusion of the second polar body. Normally fertilized oocytes were cultured in groups of 4–5 in 1 mL of HTF with SSS until early afternoon of day 3. They were then placed in 1 mL of blastocyst media (Irvine Scientific) after a 5-drop rinse in the same medium. A second change to fresh blastocyst medium was done on the morning of day 5 after embryo evaluation and before embryo transfer. Embryo transfers were scheduled between noon and 2 PM 3 or 5 days after oocyte retrieval.

Embryo Evaluation

Embryos were evaluated on days 3 and 5 with an Olympus X70 inverted microscope (x600, Olympus America, Melville, NY), equipped with Hoffman Modulation Optics (Narishige, Tokyo, Japan). Cell number, degree, and pattern of fragmentation were recorded on day 3. The degree of fragmentation was defined as the embryonic volume occupied by enucleated cytoplasmic fragment and expressed as a percentage.

Development on day 5 was recorded as follows. 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 was also considered normal. Highly irregular ICM or trophectoderm cells were considered abnormal and arrested on days 5 and 6.

Colorimetric Assay for TAC Measurement

Total antioxidant capacity was measured using a colorimetric assay (Randox Laboratories, Oceanside, CA) as per the manufacturer's instructions. In brief, 20 μ L of day 1 culture media were added to 1 mL of the reconstituted chromogen, ABTS-metmyoglobin (10 mL vial with 10 mL of phosphate buffered saline [PBS0, pH 7.4]). Twenty microliters of Trolox (6-hydroxyl-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) at

TABLE 1**Demographic features and indications for assisted reproduction technologies.**

Factor Level	IVF		ICSI		P value
	N ^a	Median (Q1, Q3) or %	N ^a	Median (Q1, Q3) or %	
Number of patients	153		105		
Number of cycles	167		116		
Age (y)		33 (30, 36)		33 (31, 37)	.34
Parity					
0		46.6		50.0	.80
1 or more		53.4		50.0	
Infertility					.62
Secondary	73	47.7	51	48.6	
Primary	80	52.3	54	51.4	
Infertility factors					<.001
Male	13	7.8	59	50.9	
Tubal	41	24.6	11	9.5	
Ovarian	20	12.0	8	6.9	
Endometriosis	37	22.2	23	19.8	
Unexplained	56	33.5	15	12.9	

Note: ^a N = number of cycles for infertility and infertility factors.

Bedaiwy. Total antioxidant capacity in day 1 culture media. *Fertil Steril* 2006.

a concentration of 1.73 mmol/L were used as the standard, and 20 μ L of deionized water were used as a blank. One milliliter of chromogen was added to the standard, blank, and the sample. With spectrophotometer adjusted at a wavelength of 600 nm and with the temperature at 37°C, the initial absorbance (A_1) was measured. Two hundred microliters of H_2O_2 (250 μ mol/L) were then added to all tubes (standard, blank, and sample), and absorbance (A_2) was measured after 3 minutes. The difference between A_2 and A_1 (Δ) was calculated. Total antioxidant capacity was calculated as the Concentration of the standard \times (ΔA Blank - ΔA Sample)/(ΔA Blank - ΔA standard). The results were expressed as micromoles of Trolox equivalents.

Statistical Methods

Descriptive statistics are presented as frequency (percent) or median and interquartile (25th and 75th percentile). Because multiple cycles from the same patients were included, a repeated measures model was used for all analyses. To account for the correlation between cycles of the same patient or oocytes of the same patient, a mixed model was used to describe continuous outcomes using repeated measures mixed models. The generalized linear mixed model was used to describe the binary outcomes such as IVF and ICSI outcome with logistic regression using generalized estimating equations. The IVF and ICSI groups were also compared on rates of outcomes using events/trials logistic regression by the generalized estimating equations.

The association between day 1 TAC and outcome was assessed separately for IVF and ICSI groups using repeated measures mixed models and generalized estimating equations logistic regression models. All tests were two-tailed with a significance level of .05. Analysis was done with SAS 8.2 (SAS Institute Inc., Cary, NC), and graphics were produced with S-plus 6.1 (Insightful Corp., Seattle, WA).

RESULTS

Demographic Variables and Assessment of Cycle Outcome

The demographic features of the study population and the indications for assisted reproduction (ART) are presented in Table 1. Two hundred fifty-eight patients underwent 283 cycles of IVF throughout the duration of the study. The patients' ages, parity, and percentage of primary vs. secondary infertility were similar in the IVF and ICSI groups. A significantly higher number of patients with male factor infertility underwent ICSI rather than conventional IVF ($P < .001$).

There were no significant differences in the number of days of stimulation, amount of FSH used, cycle day 3 or peak E_2 levels, or number of oocytes retrieved between the groups (Table 2). Clinical PRs, implantation rates, and blastocyst development rates were similar between conventional IVF and ICSI, although the fertilization rate and multiple pregnancies were higher among ICSI patients ($P = .003$ and $.005$, respectively). The clinical PR represents the number of cycles with fetal cardiac activity on ultrasonography divided

TABLE 2

Parameters of controlled ovarian hyperstimulation pregnancy rates.

Factor	IVF		ICSI		P value
	N ^a	Median (Q1, Q3) or Events/Trials (%)	N ^a	Median (Q1, Q3) or Events/Trials (%)	
Days of stimulation		9 (8, 10)		9 (8, 10)	.46
Amount of FSH used (IU)		2,550 (1,800, 3,150)		2,700 (1,950, 3,450)	.28
Day 3 E ₂ (pg/mL)		34.5 (20, 50)		36 (28, 47)	.45
E ₂ on day of hCG administration (pg/mL)		1,846.5 (1,313, 2,435)		1,805 (1,280, 2,459)	.46
Number of oocytes retrieved		12 (8, 17)		13 (8, 16.5)	.67
Fertilization rate (%)		65.0 (50.0, 77.8)		53.8 (41.3, 70.3)	.003
TAC		1.4 (0.6, 3.0)		1.3 (0.7, 3.0)	.41
High (>7) day 3-cell number rate (%)		38.8 (0.0, 60.0)		33.3 (0.0, 60.0)	.72
Low fragmentation rate (%)		60.0 (33.3, 81.7)		60.0 (33.3, 90.9)	.34
Blastocyst rate (%)	128	11.3 (0.0, 44.2)	77	10.0 (0.0, 40.0)	.64
Implantation rate (%)		107/471 (22.7)		55/330 (16.7)	.16
Clinical pregnancy (%)		71/167 (42.5)		48/116 (41.4)	.96
Multiple pregnancy (%)		34/166 (20.5)		8/115 (7.0)	.005

Note: ^a N = number of cycles.

Bedaiwy. Total antioxidant capacity in day 1 culture media. *Fertil Steril* 2006.

by the number of cycles initiated. The implantation rate was calculated by dividing the number of gestational sacs on ultrasonography by the number of embryos transferred.

The median and interquartile range (25th and 75th percentiles) of low (<10%) embryo fragmentation on day 3 was 60% (33.3%, 81.7%) in the IVF group and 60% (33.3%, 90.9%) in the ICSI group. Within the IVF group, 38.8% (0.0%, 60%) of the embryos had ≥7 blastomeres compared with 33.3% (0%, 60%) with ICSI. The main outcome measure for embryo quality on days 5 or 6 was the blastocyst development rate. Similar rates of blastocyst development were observed in the IVF and ICSI groups, 11.3% (0%, 44.2%) vs. 10% (0%, 40%), respectively. Levels of TAC

were comparable in both IVF and ICSI groups, 1.4 (0.6, 3) vs. 1.3 (0.7, 3), respectively.

Relationship of Day 1 TAC and Cycle Outcome Parameters

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

Fertilization Rate. Day 1 TAC levels were significantly associated with the fertilization rate in conventional IVF cycles ($P < .001$). In ICSI cycles, our model indicated that

TABLE 3

Relationship of TAC with main outcomes of IVF and ICSI.

Factor	IVF		ICSI	
	Odds ratio ^a (95% CI)	P value	Odds ratio (95% CI) ^a	P value
Fertilization rate (%)	1.2 (1.1, 1.2)	<.001	1.2 (1.1, 1.3)	<.001
High (>7) day 3-cell number rate (%)	1.5 (1.3, 1.7)	<.001	1.7 (1.5, 2.0)	<.001
Low fragmentation rate (%)	1.2 (1.0, 1.4)	.011	1.5 (1.2, 1.7)	<.001
Blastocyst rate (%)	1.8 (1.5, 2.0)	<.001	1.7 (1.4, 2.1)	<.001
Clinical pregnancy (%)	1.2 (1.0, 1.4)	.098	1.3 (1.1, 1.7)	.019

Note: ^a Odds ratios > 1 indicate higher TAC is associated with increased probability of outcome.

Bedaiwy. Total antioxidant capacity in day 1 culture media. *Fertil Steril* 2006.

fertilization rates also increased significantly when day 1 TAC levels were high ($P < .001$).

Cell Number on Day 3. In conventional IVF cycles, day 1 TAC levels were significantly associated with the probability that more embryos contained at least 7 cells on day 3 ($P < .001$). In ICSI cycles, as day 1 TAC levels increased, the probability that an embryo contained at least 7 cells on day 3 also increased ($P < .001$).

Embryo Fragmentation. In conventional IVF cycles, day 1 TAC levels in culture media appeared to be related to less than 10% embryo fragmentation ($P = .01$). In ICSI cycles, there was a concomitant decrease in the embryo fragmentation rate with the increase in day 1 TAC levels ($P < .001$). This indicated that higher levels of day 1 TAC were associated with lesser rates of embryonic fragmentation on day 3.

Blastocyst Development and Clinical PR A significant association was seen in ICSI cycles between elevated day 1 TAC levels and elevated blastocyst development rate ($P < .001$). This association was also observed in conventional IVF cycles ($P < .001$). Elevated day 1 TAC levels in culture media were associated with higher PRs in ICSI ($P = .01$) cycles but not in conventional IVF cycles ($P = .09$).

DISCUSSION

Assisted reproductive techniques have become the treatment of choice in many cases of male and female infertility. Despite recent methodological advances, the current PRs of these procedures remains unsatisfactory (12). This is probably due in large part to the current suboptimal embryonic evaluation and selection process before embryo transfer. Selection is mainly based on the gross morphology of the embryos. Ideally, selection of normal viable embryos with high implantation potential should be based on both the chromosomal integrity and expression of the appropriate developmental genes. This is supported by the fact that aneuploidy testing can reduce embryonic loss after implantation (13). Identification of reliable molecular and metabolic markers will further refine the selection process. However, molecular and metabolic markers indicative of optimal development have yet to be identified.

Among the various metabolic factors, oxidative stress has recently emerged as one of the potential causes for low embryonic quality in IVF and ICSI cycles (1). Moreover, women who became pregnant after IVF therapy had a tendency toward higher levels of TAC in their follicular fluid (FF) compared to those who do not (14, 15). We previously 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. In this study we reported that slow development (<7 cells on day 3), high fragmentation (>10%), and reduced formation of morphologically normal blastocysts may be associated with increased levels of ROS on day 1. Although, there was

no apparent relationship between day 1 ROS levels and fertilization rate in conventional IVF cycles, a significant relationship was seen with fertilization rate in ICSI cycles (1). In addition, although day 1 ROS levels were not significantly related to the blastocyst development rate with conventional IVF, higher levels of day 1 ROS were associated with lower blastocyst development rates in the ICSI cycles.

Current reports indicate that oxidative stress is involved in the etiology of defective embryo development (7). The imbalance that leads to oxidative stress may be due to decreased TAC as well as to increased ROS levels. The environment surrounding the oocyte and embryo contains nonenzymatic antioxidants such as vitamin C, glutathione, hypotaurine, and taurine, which protect the embryo from external sources of ROS (16). The levels of these antioxidants (called as total antioxidant activity or TAC as measured by us) may be indicative of the extent of oxidative stress (OS). In conventional IVF, TAC 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 TAC in ICSI as the incubation is initiated after denuding the oocytes from all their cumulus cells. The potential cellular sources of TAC in ICSI setting are the spermatozoa and the injected oocytes.

In the present study, we have shown that TAC levels in day 1 culture media appear to be an additional biochemical marker reflecting the oxidative stress status during early embryonic growth. We measured the total antioxidant activity or TAC in day 1 culture media. This is a standard approach to evaluate all the antioxidant constituents. Because the environment surrounding the oocyte and embryo contains mainly nonenzymatic antioxidants such as vitamin C, glutathione, hypotaurine, and taurine, which protect the embryo from external sources of ROS, we presume that all TAC levels measured in the sample well are of intracellular origin. Fertilization rate, embryo development, and fragmentation, as well as blastocyst development rates were all impacted by TAC levels during both IVF and ICSI. In agreement with our results, incubation of poor quality embryos was associated with a decline in TAC (17). The findings suggest that impaired embryo development may be associated with an increased generation of ROS by the embryo neutralizing as much of the available antioxidants leading to reduction in TAC levels.

Clinical PRs were associated with day 1 TAC levels only during ICSI cycles but not during conventional IVF. There are two possible explanations for such a difference. First, the lack of significance for clinical PR in Table 3 for the relationship between day 1 TAC and the PR in the conventional IVF cycles could be because of small sample size as there is a trend toward significance with a P value of .098. This assumption could be further reconfirmed with the significant ($P = .011$) relationship with low fragmentation rate, where the confidence interval (1.0–1.4) is exactly the same for

clinical PR, but obviously there are more embryos than the patients (Table 3).

Second, this discrepancy may also be attributed to the technical nature of ICSI, which provides lesser opportunities for the development of oxidative stress. During ICSI, the incubation time is much shorter, which decreases the exposure to external environment and, in turn, high oxygen tension. Moreover, the contact time between the sperm and oocyte is abolished, which minimizes the possibility of ROS induction by spermatozoa. Finally, it is important to note that during ICSI, a motile and a morphologically normal-appearing sperm is selected for injection. Therefore, abnormal spermatozoa characterized by ROS production are mostly excluded.

In conclusion, TAC levels in day 1 culture media are related with many in vitro embryonic growth parameters. Whether this relationship is a cause or effect needs further assessment. Differential growth of ICSI embryos incubated under the same conditions may be due to differences in TAC levels surrounding them. In the IVF setting, strategies to reduce oxidative stress by increasing the presence of free radical scavengers would be of importance for improving ART outcomes.

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