Release of superoxide dismutase-1 by day 3 embryos of varying quality and implantation potential

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Abstract

Purpose To determine if the antioxidant superoxide dismutase-1 (SOD1 or Cu,Zn-SOD) is released by cultured human cleavage-stage embryos and to assess any link between SOD1 and implantation potential.

Methods Women ($n=91$; ≤ 40 years old) undergoing IVF treatment with transfer of one or two 8-cell embryos that resulted in 0 or 100% implantation were included. Following individual embryo culture, spent medium samples ($n=122$) were collected and levels of SOD1 protein were measured by an enzyme-linked immunosorbent assay. SOD1 detection and concentration in embryo spent medium were analyzed in relation to embryo fragmentation and symmetry scores, and implantation (viable fetus at >12 weeks).

Results Cleavage-stage embryos release SOD1 protein into the spent culture medium. Neither detection nor concentration of SOD1 was related to implantation. There was a positive relationship between increased embryo fragmentation scores and SOD1 release, with no apparent association with symmetry. In non-pregnant cycles, the release of SOD1 decreased with increasing maternal age.

Capsule Release of the antioxidant superoxide dismutase-1 by human 8-cell embryos bears no relationship to blastomere symmetry or implantation potential, but increases with increased embryo fragmentation.

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Conclusions While SOD1 does not predict implantation potential of select good-quality embryos, our data support the need to evaluate the biological significance of released SOD1 by embryos of varying quality and from patients of varying age.

Keywords Embryo culture medium · Secreted protein · Superoxide dismutase-1 · Cu,Zn-superoxide dismutase · Pregnancy outcome

Introduction

The persistent challenge in clinical IVF is to establish embryo selection methods which enable cohort embryos to be ranked according to their implantation potential. Although morphological evaluations remain the most common method for embryo assessment, with several morphology scoring systems having good predictive value [1–5], such evaluations have recognized limitations [6]. Thus, considerable research is focused on alternative methods for embryo assessment, including the noninvasive monitoring of culture media, with a particular focus on assessment of protein products released by embryos [7, 8]. One such approach has used sensitive proteomic array platforms; however, a set of profiles that enables ranking of embryos according to implantation potential has yet to be identified.

Beyond profiling an entire secretome, insightful knowledge can be gained from the assessment of relevant candidate molecules. Promising results include documented correlations between pregnancy success and secreted HLA-G [9]. However, some of the findings remain controversial, and the current inability to validate the use of secreted candidates may stem from limitations in experimental design. Many studies have analyzed culture media

samples that were either pooled or obtained from embryos cultured together, making it difficult to ascertain the true predictive power of any measured marker in relation to the developmental competency of a single embryo. Another major drawback relates to outcome data that may not always be complete and informative; for instance, transfers involving several embryos (rather than single or double embryo transfers with unequivocal outcome for each embryo) are significant limitations.

Embryos appear to secrete proteins in a stage-specific fashion during pre-implantation development [10]; while several proteins have been described extensively many, including antioxidants, remain unexplored. Antioxidant proteins help cells maintain a balance of oxidants, thereby preventing harmful effects associated with oxidative stress [11]. One enzymatic antioxidant is Cu,Zn-superoxide dismutase (SOD1) that may remain cytosolic or be secreted into the culture medium as previously demonstrated in human fibroblast, hepatocyte, neuroblastoma, and thymic-derived cell lines [12–14]. To our knowledge, no reports have yet investigated the expression and release of SOD1 by human embryos; yet recently, metabolic parameters that broadly reflect oxidative stress in culture medium were correlated to pregnancy outcome [15, 16]. Prior studies also indicate potential associations between oxidants or antioxidants produced by human embryos and developmental outcomes [17–21]. Our study aimed to determine whether SOD1 is released by embryos during culture from days 1 to 3 and, if so, whether a specific concentration is associated with implantation potential. Our primary hypothesis was that SOD1 could serve as a non-invasive predictor of human embryo quality.

Materials and methods

Study design

This study was approved by the Partner's Institutional Review Board. The study employed a retrospective design that analyzed archived spent media microdrops in which individual embryos had been cultured from the time of fertilization check (Day 1) to embryo transfer (Day 3). To strengthen our ability to detect relationships between spent medium samples and pregnancy outcome, only patients ≤ 40 years old who had one or two 8-cell embryos transferred of known implantation fate were included. That is, transfer of a single embryo resulted in either no clinical sac ever on ultrasound ($n=36$), or a viable fetus to at least 12 weeks of pregnancy ($n=22$), while a double embryo transfer resulted either in no clinical sac ever on ultrasound ($n=34$) or the presence of viable dizygotic twins at ≥ 12 weeks of gestation ($n=30$). The final dataset included a total of 122 media samples used

to culture 122 embryos from 91 patients with a mean ($\pm$ SD) age of 32.5 ± 3.4 y (range: 24.7–40.3).

Oocyte retrieval, fertilization, embryo culture, and embryo evaluation

Oocytes were retrieved 36 h after hCG using routine ovarian stimulation protocols as previously described [22]. Following either standard insemination ($n=79$) or ICSI ($n=43$), each zygote with two pronuclei was cultured singly in embryo growth medium (Vitrolife Inc., Englewood, CO, U.S.A.; $n=53$ and 69 samples in G1.3 and G1.5, respectively) in 25 μ l microdrops under oil at 37°C, 5% CO₂ in humidified air. On the third day after retrieval, embryos were evaluated for cell numbers, fragmentation, and symmetry. Fragmentation was given a score of 0, 1, 2, 3 or 4 corresponding to 0, 1–9, 10–25, or >25 – $<50\%$ or $\geq 50\%$ of the total volume of the embryo occupied by fragments, respectively [23]. Symmetry scores of 1, 2, and 3 indicated no, moderate, and severe asymmetry, respectively.

Collection and processing of spent media from embryos cultured individually

After morphological evaluation and transfer of embryos, the medium used to culture each embryo was collected. When removing embryos in preparation for transfer, precaution was taken to gently push the embryo away from its original position before removal, thereby ensuring that if any gradients of secretory products existed in closest proximity to the embryo, such products would not be aspirated out along with the embryo. A volume of 20 μ l from the remainder of each 25 μ l microdrop was immediately frozen and stored at -80°C until later analysis. Control medium samples consisted of G1.3 or G1.5 medium cultured under identical conditions.

Measurement of SOD1 protein concentrations by ELISA

Spent medium samples were analyzed using an ultra-sensitive enzyme-linked immunosorbent assay (ELISA) for human Cu,Zn-SOD/SOD1 (Bender MedSystems, Vienna, Austria). Each sample was thawed on ice, centrifuged, and 9.5 μ l aliquots were run in duplicate. Control medium samples of each lot used during the study were run alongside corresponding experimental samples. Standards, ranging from 0.04 ng/ml to 5.0 ng/ml (8 in total), were run in each assay. The concentration of SOD1 in each sample was determined from the standard curve, with the final reported values obtained by subtracting the values measured in corresponding control samples. This calculation permitted the evaluation of SOD1 contributed by the embryo only.

The inter- and intra-assay coefficients of variation averaged 11.9% and 8.4%, respectively (for 9 assays).

Statistical analysis

Chi-square Fisher's Exact tests, One-Way ANOVA (Tukey's LSD post-hoc), and Pearson's analysis were used to compare proportions, to test SOD1 concentrations, and to determine correlations between SOD1 concentration and patient age. Patient and cycle characteristics were compared for samples with or without detectable SOD1 using Mann–Whitney non-parametric tests. Statistical significance was assumed for $p < 0.05$ (PASWStatistics 18.0, Chicago, IL, U.S.A.).

Results

Release of SOD1 by embryos

Of the 122 samples analyzed for SOD1, 78 had no detectable SOD1 (i.e. below the limit of sensitivity of the assay at 0.04 ng/ml) while 44 samples contained SOD1 at concentrations ranging from 0.055 to 1.179 ng/ml. Only a single sample had a concentration close to the lower limit of sensitivity of the assay (sample value of 0.055 ng/ml, versus assay limit of 0.04 ng/ml), while all others were ≥ 0.091 ng/ml; the mean value for all samples was 0.470 ± 0.045 ng/ml ($\pm$ SEM). There was no difference in SOD1 detection between samples that originated from either IVF or ICSI cycles. There were also no differences across patient diagnosis categories or stimulation protocols, and for embryos cultured in either culture medium (G1.3 or G1.5). Table 1 summarizes the patient and cycle characteristics according to SOD1 detection. There were no differences in any of the parameters

except for the total numbers of follicles and retrieved eggs; however, these numbers remain high in samples with or without SOD1 detection and the statistical difference was marginal.

Analysis of SOD1 release in relation to pregnancy outcome

Spent media samples were just as likely to contain SOD1 levels above control culture media when the corresponding embryos resulted in either a viable pregnancy (fetal heart at ≥ 12 weeks) or no implantation (Table 2). There was also no relationship between detectable SOD1 concentrations and pregnancy outcome (Fig. 1a).

SOD1 release according to patient age and embryo quality parameters

No relationship was observed between SOD1 concentration in spent media samples and patient age. When stratifying the data based on clinical outcome, there was a negative correlation between patient age and SOD1 concentration in samples that did not result in pregnancy ($r = -0.293$, $p = 0.014$; Fig. 2a), but no such association existed for samples associated with viable fetuses (Fig. 2b).

To increase the dataset for analyzing a potential association between SOD1 and embryo morphology, an additional 25 samples from non-transferred embryos were analyzed, also having 8-cell embryos and from ≤ 40 yo patients (to result in a total of 147 samples). Only samples from embryos with fragmentation scores 0–2 were included as there were just 4 samples with embryos having a score of 3, and none with a score of 4. Although not significant, there was an increase in the proportion of spent medium samples with detectable SOD1 for embryos with a fragmentation score of 2 when compared to a score of 0 (Table 3; $p = 0.069$). When

Table 1 Patient demographics and cycle characteristics for spent medium samples with or without detectable SOD1

	SOD1 Detection	No SOD1 Detection	p value
Attempt #	1.4 $\pm$ 0.1	1.6 $\pm$ 0.1	0.535
Age	32.2 $\pm$ 0.5	32.9 $\pm$ 0.4	0.321
Day 3 FSH	7.3 $\pm$ 0.4	6.9 $\pm$ 0.2	0.499
Peak E ₂	2100.0 $\pm$ 129.5	2202.3 $\pm$ 93.5	0.519
# Follicles	13.9 $\pm$ 1.0	17.4 $\pm$ 0.9	0.019
hCG dose	10.9 $\pm$ 0.3	10.9 $\pm$ 0.2	0.740
# Eggs	14.0 $\pm$ 1.0	17.8 $\pm$ 1.0	0.025
# Mature metaphase-II eggs	11.9 $\pm$ 1.0	15.2 $\pm$ 0.9	0.021
% Mature/total retrieved	84.2 $\pm$ 1.6	86.0 $\pm$ 0.7	0.353
# Fertilized	10.0 $\pm$ 1.0	11.8 $\pm$ 0.8	0.182
% Fertilized/total metaphase-II	80.9 $\pm$ 2.4	76.0 $\pm$ 1.8	0.056
# Frozen	9.9 $\pm$ 1.0	11.3 $\pm$ 0.7	0.196
# High quality embryos ^a	5.7 $\pm$ 0.8	6.6 $\pm$ 0.5	0.059
% High quality embryos ^a /fertilized	60.1 $\pm$ 4.2	57.4 $\pm$ 2.3	0.743

^a ≥ 8 cells, with fragmentation scores of 0 or 1. Values represent mean $\pm$ SD

Table 2 Release of SOD1 by 8-cell embryos as related to pregnancy outcome

	SOD1 detection (<i>n</i> =43)	No SOD1 detection (<i>n</i> =79)
Pregnancy	18 (41.9)	34 (43.0)
No pregnancy	25 (58.1)	45 (57.0)

Values in parentheses are the percentages of the total number of samples within each column; no differences were observed in the percentages of samples with and without SOD detection for either those associated with pregnancy outcome or not; $p>0.05$

detectable, there was a trending difference in SOD1 concentrations between fragmentation scores 0 and 2 ($p=0.102$); SOD1 concentration was also higher in spent media from embryos with a fragmentation score of 2 when compared to a score of 1 (Fig. 1b; $p=0.018$). There were no differences in SOD1 detection (Table 2) or SOD1 concentration (Fig. 1b) between fragmentation scores 1 and 2 ($p>0.1$). There was no relationship between the blastomere symmetry of 8-cell embryos and their SOD1 release (Table 3), with no difference in SOD1 concentrations across symmetry scores.

Discussion

With the continuing need to improve identification of the single most developmentally competent embryo in each cohort, arises the potential use of monitoring the secretome of embryos cultured individually. Our study documents release of the antioxidant protein SOD1 by cleavage-stage human embryos between 24 and 72 h of culture. To control for developmental stage and maternal age, our analysis was restricted to transferred embryos at the 8-cell stage, and only included embryos from women ≤ 40 years old. Among these

highly selected samples, the amount of secreted SOD1 varied, with significant relationships only found with fragmentation scores, and with patient age but only in the non-pregnant group. Secreted SOD1 was not associated with clinical outcome. To our knowledge, this is the first documentation of the extracellular release of SOD1 by human embryos or, indeed, by embryos of any other mammalian species. This documentation is consistent with previous studies showing expression of SOD1 transcript and protein in pre-implantation bovine embryos [24–26].

There is literary support for the extracellular presence of an isoform of SOD that is typically cytosolic. Indeed, SOD1 is secreted by a variety of somatic cells cultured in vitro [12–14], with both constitutive and regulated exocytosis [27]. In neuronal cell types, there is an association between SOD1 release and augmented cellular stress [13, 28] or neurotoxicity [28, 29], and SOD1 secretion may also protect the external surfaces of plasma membranes [30]. SOD1 secretion may thus confer heightened protection to cells that are subject to oxidative insults, a situation relevant to cultured human embryos. Yet, our findings indicate that not all embryos release the same amount of SOD1.

Given the previously established involvement of oxidants and antioxidants in the developmental progression of mammalian embryos [31–35], SOD1 release could conceivably correlate with cleavage stage. The homogeneity of our analyzed samples is relevant to note; we analyzed spent medium samples exclusively from 8-cell embryos on the day of transfer, thereby narrowing the possible range of SOD1 variation. Nonetheless, a sub-analysis by morphological parameters and patient age provided further insight. Even among 8-cell embryos, the more fragmentation, the more SOD1 was released in the culture medium. Of relevance is an increase in cellular hydrogen peroxide with fragmentation of human embryos [36]; further, reactive oxygen

Fig. 1 Spent medium SOD1 concentrations according to pregnancy outcome (**a**; no pregnancy, $n=25$; pregnancy, $n=18$) and fragmentation scores (**b**; 0, $n=12$; 1, $n=25$; 2, $n=11$) of hyc=10?>8-cell embryos. Boxes show the 25th and 75th percentile of the data, with the 50th

percentile (median) depicted by the central line. Bars extending from the box indicate the full range of the data, and circles represent data outliers. In (B), the asterisk denotes a significant difference ($p=0.018$, One-Way ANOVA)

Fig. 2 Spent medium SOD1 concentrations in relation to patient age for non-pregnant (a, $n=70$) and pregnant (b, $n=52$) cycles. In (A), there is a significant negative correlation between SOD1 and patient age (Pearson's $r=-0.293$, $p=0.014$). Shown are the best fitting lines for the data. Note that a single circle may reflect the SOD1 concentration for more than one sample; there was no difference in the distribution of samples across the range of patient ages

species modulate programmed cell death [37], itself a mechanism involved in embryo fragmentation [38, 39]. In pre-implantation embryos of SOD1 knockout mice, developmental abnormalities including degeneration were reported [40], also buttressing a likely involvement of SOD1 in the viability and health of pre-implantation embryos. Future studies remain to examine SOD1 release by embryos of morphological quality other than ones analyzed herein.

We further showed that SOD1 release decreased with increasing patient age among 8-cell embryos that do not result in a pregnancy. There is prior evidence for a decline in antioxidant expression with advanced maternal age, whether in mouse oocytes [41], human cumulus cells [42], human granulosa cells [43], or human follicular fluid [44]. In light of the free radical theory of reproductive aging [45, 46], our reported correlation between patient age and SOD1 release is not surprising. The lack of association in embryos resulting in pregnancy may stem from the superior quality of all the embryos in our cohort, irrespective of maternal age and SOD1 release. Of relevance is also the age cut-off in our study (≤ 40 year), and although supporting evidence is needed, a decline in SOD1 release may be more pronounced in older patients.

Prior results involving supplementation of SOD to mammalian embryo cultures have provided benefit [47–49], no effect [50, 51], or a detrimental effect [52] on embryonic

development. These apparently conflicting results may perhaps be explained by a window within which SOD may not influence cellular function, while effects may become negative or positive at concentrations at either end of a spectrum. Such thresholds would relate to the benefits and detriments of ROS at physiologically low and excessively high levels, respectively [37]. Mouse and cow embryos produce and release reactive oxygen species, including superoxide and hydrogen peroxide, in levels that vary during in vitro development [53–55]. A need to maintain an appropriate balance of antioxidants is thus likely during early development.

Whether SOD1 release by the embryos even occurs in vivo, or whether it is a sole response of exposure to in vitro conditions remains to be clarified. The bovine oviduct is a known source of secreted active SOD [56], and human oviduct epithelial cells express SOD1 [57]; in vivo cleavage-stage embryos may thus rely on antioxidants provided externally by the oviductal fluid [58]. While in vivo studies may carry relevance, the oxidative environment that the embryo faces in vitro is entirely different; future studies should thus extend our pilot evaluation of the antioxidant secretome of the cultured human embryo.

SOD1 secretion beyond the 8-cell transition also merits consideration. Indeed, antioxidant expression changes dynamically between cleavage- and blastocyst-stage bovine embryos [26, 59], and there is a transition in metabolism and thus redox potential at around the 8-cell stage [60, 61]. It is also not known whether released SOD1 originates from maternal stores accumulated in the oocyte or a newly expressed product upon activation of the embryonic genome between the 4- and 8-cell stages in human [62]. The latter possibility could explain variations in SOD1 detection in the spent medium, thus perhaps merely reflecting differing onsets of embryonic genome activation across embryos. Such conjecture merits direct assessment. In studies of the human embryo secretome, antioxidants other than SOD1 also ought to be considered, including non-enzymatic antioxidants and enzymes acting in concert with SOD and further neutralizing hydrogen peroxide into harmless species. Antioxidants other

Table 3 Release of SOD1 according to fragmentation and symmetry scores in 8-cell embryos

Parameter	Scores	SOD1 detection	No SOD1 detection
Fragmentation	0 ($n=43$)	12 (27.9)	31 (72.1)
	1 ($n=78$)	25 (32.1)	53 (67.9)
	2 ($n=22$)	11 (50)	11 (50.0)
Symmetry	1 ($n=85$)	28 (32.9)	57 (67.1)
	2 ($n=55$)	19 (34.5)	36 (65.5)
	3 ($n=7$)	3 (43.9)	4 (57.1)

The percentage of the total number of embryos within each fragmentation or symmetry score are shown by the numbers in parentheses

than SOD1 may represent a superior biological metric of embryo quality, while it is also likely that additive or synergistic relationships exist among antioxidants, thereby perhaps supporting the use of a combinatorial approach when evaluating the antioxidant secretome of the embryo.

One must also consider the possibility that our lack of any demonstrated association between secreted SOD1 and implantation potential may be confounded by compromised endometrial receptivity or other implantation-related failure; any samples in the non-pregnancy group may not necessarily equate to embryos not capable of sustaining a pregnancy. This is, indeed, an important limitation of all studies aimed at establishing a reliable marker of embryo quality, at a time when we still cannot ascertain the true developmental potential of each embryo [8].

In conclusion, human embryos that are 8-cells by Day 3 of culture release SOD1 enzyme in their extracellular milieu. Relationships with patient age and embryo fragmentation buttress the need for future studies analyzing the antioxidant secretome of human embryos. Together with other markers, antioxidants may thus prove informative in the identification of a profile predictive of the viability and developmental potential of embryos.

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