

Nitric oxide metabolite production in the human preimplantation embryo and successful blastocyst formation

Eleven patients underwent controlled ovarian hyperstimulation yielding 72 embryos for evaluation. Mean nitric oxide metabolite levels in the insemination media were 2.6 times higher in embryos that progressed to blastocysts by culture day 5 than in those that did not. A comparison of the receiver operating characteristic curves between morphological predictors and nitric oxide metabolite levels revealed a trend toward a stronger association of insemination media nitric oxide metabolite with blastocyst formation. (*Fertil Steril*® 2009;91:1316–8. ©2009 by American Society for Reproductive Medicine.)

Improvements in IVF outcomes have been due largely to the generation of supernumerary embryos allowing for multiple ET. The development of extended embryo culture has resulted in increased implantation rates and the potential for reductions in multiple births through single ET (1, 2). One of the major disadvantages, however, has been the worry that in approximately 40% of patients, embryos would not be available for transfer (3).

Unfortunately, culture day 3 morphological assessment is not optimal for the prediction of blastocyst progression on culture day 5 (4, 5). Investigators have therefore sought quantifiable objective measures of embryonic health with a focus on metabolic activity (6–11). One such metabolic factor, nitric oxide (NO), has been implicated in a variety of biologic and reproductive processes including oocyte maturation, fertilization, and embryonic progression (12). Gouge et al. (13) demonstrated that NO production is essential for embryonic progression in the murine model. Chen et al. showed that blastocyst development in culture is inhibited by *N*-nitro-*L*-arginine methyl ester, an NO inhibitor, in a concentration-dependent manner and sodium nitroprusside, an NO donor, effectively can reverse this effect (14). These studies highlight the potential importance of NO in embryogenesis.

The objective of this study was to investigate the potential relationship of NO metabolite (NO_x) levels generated by human preimplantation embryos in culture with developmental capacity. Our goal was to compare the prediction of blastocyst formation on the basis of the standard subjective

morphological assessment on culture day 3 with an objective, quantifiable measure of metabolic function.

Eleven subfertile women between 27 and 44 years of age undergoing sonographic-guided transvaginal oocyte aspiration at Johns Hopkins Hospital between March 31 and June 26, 2006, were included in the study. This study was approved by the Johns Hopkins Institutional Review Board. Patients were treated with either a GnRH agonist or antagonist protocol. All patients received a single dose of 10,000 IU of hCG once three follicles achieved a diameter of at least 18 mm. Ultrasound-guided oocyte retrieval followed 36 hours later.

After oocyte retrieval, metaphase II oocytes were placed in individual 80 μ L droplets of insemination medium. Intracytoplasmic sperm injection was performed on 20 of the retrieved oocytes as described by Palermo et al. (15). Once the zygotes were transferred to cleavage media, the insemination medium was collected, placed in separate microcentrifuge tubes, and stored at -80°C until processing. Embryo-free media droplets were cultured under identical conditions and used as controls. Embryos were assessed morphologically on culture day 3 and either prepared for ET or placed in blastocyst media. Morphological assessment of the cleavage stage embryo on culture day 3 was performed through the use of a modified grading system as proposed by Veeck (16).

Media isolated from cultured embryos or control plates were thawed to room temperature. Because of the short half-life of NO, the stable metabolic products (nitrite and nitrate), referred to as NO_x, were measured as described by the QuantiChrom Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA), which is an assay based on a modified Griess reaction with a detection range of 0.1 to 50 $\mu\text{mol/L}$. Measurements of NO_x in duplicates from each sample were performed as described in the kit protocol. The NO_x (micromoles per liter) was calculated for each of the samples and controls. To account for nonindependence of insemination media NO_x levels in oocytes derived from the same donor, all analyses with insemination media

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NOx as the dependent variable used generalized estimating equations regression methods with an exchangeable correlation structure. The average levels of insemination media NOx in the analysis groups were calculated as linear combinations of the regression coefficients. We examined whether insemination media NOx, blastomere number, or subjective grade were effective in predicting survival of the embryo to culture day 5 with use of simple logistic regression adjusting for clustering of data by donor. Receiver operating characteristic (ROC) curve analysis was performed for predicting survival of the embryo to culture day 5 with use of insemination media NOx, blastomere number, and subjective grade as classifying variables. Eleven patients with a mean age of 34.3 years (range: 27–44 years) underwent oocyte aspiration. The level of NOx was assessed in the insemination media of 72 individually cultured embryos with 27 and 45 cultured for 3 or 5 days, respectively. The 5-day culture resulted in 27 blastocysts, 26 of which had media analyzed after insemination/injection.

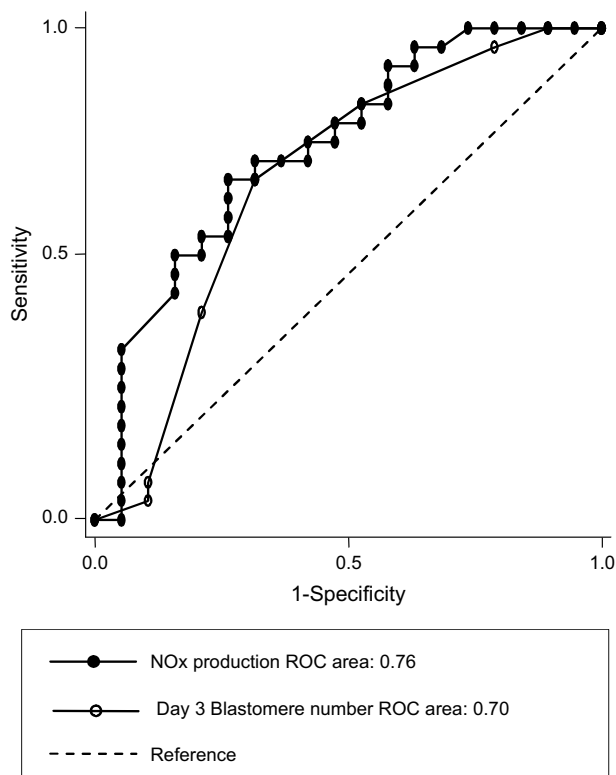
No significant differences were noted between insemination media NOx of embryos fertilized via ICSI ($1.00 \pm 1.22 \mu\text{mol/L}$) or IVF ($0.73 \pm 0.72 \mu\text{mol/L}$) ($P=.849$). There was no statistically significant difference in mean insemination media NOx when considering culture day 3 blastomere number or morphological grade. The insemination media NOx produced from embryos that were eventually transferred or cryopreserved was $0.630 \mu\text{mol/L}$, compared with embryos that were ultimately discarded, $-0.204 \mu\text{mol/L}$ ($P=.053$). The mean NOx level in the insemination media was 2.6 times higher in embryos that progressed to blastocysts by culture day 5 than in those that did not (1.33 ± 0.51 vs. $0.52 \pm 0.84 \mu\text{mol/L}$, $P=.009$).

Although we did not find differences between insemination media NOx levels, morphological grade, and blastomere number at culture day 3, others have shown that embryonic morphology can be of assistance in predicting blastocyst progression on culture day 5 (17). Therefore, given that we found that embryos with higher insemination media NOx levels progressed compared with embryos with lower insemination media NOx levels, we used logistic regression and ROC to compare insemination media NOx levels, morphological grade, and blastomere number as predictors for blastocyst progression. Analysis revealed that only the insemination media NOx level and culture day 3 blastomere number were significantly associated with progression to the blastocyst stage by culture day 5 ($P<.02$).

Because culture day 3 morphological grade was found to have little predictive value, this parameter was omitted from the model used to generate the final ROC. Figure 1 displays the ROC generated from insemination media NOx and culture day 3 blastomere number, highlighting the predictive nature of the two parameters. Insemination media NOx alone revealed a sensitivity of 73.1% with associated spec-

FIGURE 1

Receiver operating characteristic analysis: insemination medium NOx, culture day 3 blastomere number, and progression to the blastocyst stage by culture day 5.



Lipari. Nitric oxide and blastocyst formation. *Fertil Steril* 2009.

ificity of 68.4%, with use of a cutoff of insemination media NOx $\geq 0.136 \mu\text{mol/L}$. A culture day 3 blastomere number of at least six yielded a sensitivity of 69.2% with associated specificity of 68.4%. Statistical analysis revealed that the decision for extended culture to day 5 was not dependent on culture day 3 morphology. Although there was no statistically significant difference between the predictive potential of insemination media NOx and blastomere number on culture day 3, a comparison of the curves revealed a trend toward a stronger association of insemination media NOx with blastocyst formation by culture day 5.

To the best of our knowledge, this is the first study to examine the relationship between NOx levels in the insemination media of individually cultured human preimplantation embryos and blastocyst progression. It appears that when considering culture day 3 morphology and NOx, NOx was the stronger predictor of blastocyst formation and is an assessment made approximately 48 hours earlier. The level of NOx did not differ according to fertilization technique and therefore may be a reflection of the metabolic activity of the oocyte or zygote.

The study has several limitations that deserve mention. The sample population limits our ability to see significant differences with regard to final embryo disposition or pregnancy. Furthermore, although the NOx levels did not differ with regard to fertilization technique, one cannot assume that gamete manipulation does not result potentially in increases in NOx. Furthermore, the study uses blastocyst progression as a measure of embryonic health, whereas live birth would be optimal.

Battaglia et al. were the first to demonstrate secretion of NOx in human preimplantation embryos. They showed that embryonic mean NOx concentrations were significantly higher in embryos transferred that resulted in pregnancy when compared with nonpregnant patients, $P=.02$ (18). This study differs from ours in that all embryos in the former were transferred at the two- to four-cell stage, hence limiting the ability to comment on sequential morphology or blastocyst progression over time.

The presence of NO synthase in each reproductive compartment has been demonstrated in the literature (19–21). Chen et al. proposed a biphasic relationship of NO, with apoptosis induced through a cyclic guanosine monophosphate independent pathway at high NO levels (14).

Although the reasoning behind a beneficial role of higher levels of NOx in the media of human preimplantation embryos remains unclear, this study has demonstrated that the production of NOx does correlate with blastocyst progression, even more so than the standard morphological criteria. This objective, quantitative measurement obtained early in the developmental process potentially can have an impact on embryo selection at an early stage.

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