Unfolded protein response prevents blastocyst formation during preimplantation embryo development in vitro

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Objective: To study the effect of increased endoplasmic reticulum (ER) stress as a major nongenomic mechanism for arrested blastocyst development.
Design: Cell and animal study.
Setting: The Ohio State University and Yale University.
Animal(s): Mice.
Intervention(s): Pregnant mare serum gonadotropin and hCG were administered IP; two cell embryos were collected 48 hours after hCG administration.
Main Outcome Measure(s): Blastocyst development rate.
Result(s): No morphological difference was detected in control versus tunicamycin- (TM) treated embryos until the blastocyst stage. On day 4 of embryonic development, TM treatment reduced blastocyst formation from 79% to 4% and induced nuclear fragmentation. TM treatment caused 2-fold and 2.6-fold increase in binding immunoglobulin protein and spliced-X-box binding protein 1 mRNA expression, respectively. By comparison, the tauroursodeoxycholic acid + TM combination reversed the effect of TM alone on blastocyst formation to near control levels.
Conclusion(s): These results indicate that increased ER stress during in vitro embryo development triggers an unfolded protein response (UPR) that negatively affects blastocyst formation and suggests that activation of UPR signaling may account for low rates of blastocyst development. (Fertil Steril® 2014;102: 1777–84. ©2014 by American Society for Reproductive Medicine.)
Key Words: Preimplantation embryo development, endoplasmic reticulum stress, unfolded protein response, apoptosis, developmental arrest

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The preimplantation embryo requires maternal RNA transition to embryonic RNA, which is followed by the extensive new protein syntheses (1) to maintain preimplantation development. These newly synthesized proteins must fold properly in the lumen of the endoplasmic reticulum (ER) to function properly. Various processes/factors that generate an imbalance between protein folding load and ER capacity can induce ER stress, which, in turn, activates the unfolded protein response (UPR). Initially, the UPR restores ER homeostasis by increasing production of the chaperone proteins involved in protein folding and degradation of unfolded/misfolded

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proteins. However, excess and prolonged accumulation of unfolded and misfolded proteins in the ER lumen redirects the UPR to activate apoptotic signaling. The UPR is controlled by several ER lumen protein sensors, including inositol-requiring kinase 1 (IRE1), a transmembrane kinase and endoribonuclease (2, 3), as well as protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6), which are present in the ER transmembrane (4). UPR signaling is initiated by binding immunoglobulin protein (BiP) also known as heat shock 70 kDa protein 5 (HSPA5) or 78 kDa glucose-regulated protein (GRP-78), a chaperone located in the ER lumen that binds newly synthesized proteins to maintain folding and oligomerization.

Under physiological conditions, binding of BiP to the luminal domain of IRE1 renders IRE1 inactive (5). However, stress conditions induce BiP to dissociate from IRE1 to support protein folding while activating IRE1 (5, 6). Activated IRE1 induces nonconventional splicing of Xbp-1 mRNA. This spliced Xbp-1 mRNA (sXbp-1) encodes a transcription activator that specifically drives transcription of ER chaperones to maintain cellular homeostasis by participating in ER protein folding (7). The underlying hypothesis of this study is that because of the high demand for protein synthesis and folding during blastocyst development, maintenance of ER homeostasis by UPR is vital for normal development of the preimplantation embryo and that excess UPR as a result of unfolded/misfolded protein overload blocks in vitro blastocyst development.

MATERIALS AND METHODS

Superovulation, Embryo Retrieval, and Culture

Mice were maintained according to the Yale University animal research requirements, and all procedures were approved by the Institutional Animal Care and Use Committee (protocol no. 2009-11300). Mouse embryos were collected by using standard protocols (8) under the guidelines approved by Yale Institutional Animal Care and Use Committee. Briefly, 5-week-old C57BL/6 female mice (Charles River Labs) were superovulated by IP injection of 5 units pregnant mare serum gonadotropin (PMSG; Folligon, Sigma-Aldrich). An additional injection of 5 units of hCG (Chorulon, Sigma-Aldrich) was given 48 hours after the PMSG injection. To obtain two-cell embryos, females were placed individually with 12-week-old C57BL/6 males immediately after the hCG injection. The following morning, the effectiveness of mating was confirmed by the presence of a vaginal plug (day 1; D1). Two-cell embryos were collected from the oviducts at 44–48 hours after hCG injection. Two-cell embryos were obtained by puncturing the ampulla portion of the oviduct with a needle in the HEPES-buffered media under the stereomicroscope.

All embryos were cultured in groups of 12 in 30-μL drops of medium at 37°C in 6% CO_2:5% O_2:89% N_2 till 96 hours with changeovers to fresh medium every 24 hours. Embryos were examined by an inverted microscope at $\times$200 magnification at the end of D2, D3, and D4. Embryos were evaluated by the Grillo et al. grading system; one grade was given for evenness of the blastomeres, and a separate grade for the degree of cellular fragmentation (9).

Experimental Design

Two sets of experiments were used to investigate the effects of long-term and short-term stimulation of ER stress on preimplantation embryo development. In experimental design 1, to determine the long-term effect of ER stress on in vitro mouse preimplantation embryo development, harvested embryos were divided into three groups that received continuous treatment for 3 days with [1] vehicle only (control), [2] the ER stress inducer, tunicamycin (TM; 0.5 μg/mL), and [3] the general protein biosynthesis inhibitor, cycloheximide (19 μg/ml; n = 50/group). In experimental design 2, to determine the short-term effect of ER stress on in vitro mouse preimplantation embryo development, harvested embryos were divided among four treatment groups: [1] a vehicle only (control); [2] to investigate the temporary effects of ER stress, a short term 2.5 μg/mL TM exposure for 4 hours was followed by vehicle-only treatment until termination of the experiments on day 4 of embryonic development; [3] to investigate the potential therapeutic effects of tauroursodeoxycholic acid (TUDCA), an ER stress inhibitor, short-term TM treatment was followed by vehicle treatment plus 200 μg/mL TUDCA until termination of the experiments on D4 of embryonic development; and [4] to investigate the potential protective effect of TUDCA, short-term TM + TUDCA combined treatment for 4 hours was followed by vehicle control only until termination of the experiments on D4 of embryonic development.

Handling and Embryo Culture Media

Quinn's Advantage Medium with HEPES (SAGE Inc.), supplemented with bovine serum albumin (BSA) was used for two-cell embryo collection and manipulation. Quinn's advantage cleavage medium (SAGE) was used for the first 48 hours of the culture and replaced with Quinn's advantage blastocyst medium (SAGE) for all subsequent culture stages. The embryos were washed 2 times in SAGE and cultured with the appropriate medium according to days of embryo stage for 3 days to monitor preimplantation development.

Immunofluorescence Analysis

D4 embryos were fixed in 4% paraformaldehyde for 10 minutes. Embryos were then washed with washing solution (WS; 500 mL PBS containing 1 g nonfat dry milk, 10 mL normal goat serum [Vector Laboratories, Inc.]), 5 g BSA [Sigma-Aldrich], 3.75 g glycine [Merck], and 0.1% triton $\times$100 [Sigma-Aldrich] twice for 5 minutes, followed by incubation in WS at 37°C for 30 minutes to block nonspecific binding. Thereafter, embryos were incubated with rabbit monoclonal anti-BiP and cleaved (active) caspase-3 antibody (Cell Signaling Technology) for 1 hour at room temperature (RT). After washing with WS 3 times, embryos were incubated with Texas Red labeled horse anti-mouse IgG secondary antibody (Vector Labs) for 1 hour at RT, followed by rinsing