

## Heat-shock proteins modulate the incidence of apoptosis and oxidative stress in preimplantation mouse embryos

We investigated the effect of antibodies to heat-shock proteins 60 and 70 on development, reactive oxygen species production, and incidence of apoptosis in preimplantation mouse embryos. Supplementation of culture media with antibodies to heat-shock proteins 60 and 70 resulted in significantly lower blastocyst development, hatching rate, reduced total cell number, and trophectoderm cell number, and increased the incidence of apoptosis in blastocysts compared to the control group. (*Fertil Steril*® 2007;87:1214–7. ©2007 by American Society for Reproductive Medicine.)

Heat-shock proteins (HSPs) are a group of constitutively expressed inducible proteins (1, 2) that are produced in response to a wide range of environmental stresses such as rapid cell growth and differentiation, inflammation and infection, toxic chemicals, and oxidative stress (3). HSPs are among the first proteins produced during embryogenesis (4, 5). Several studies have shown that Hsp70, Hsp90, and Hsp27 protect against cell death through apoptosis by a variety of stressors including oxidative stress (6).

Oxidative stress is a result of the relatively high oxygen concentrations in the *in vitro* microenvironment surrounding preimplantation embryos that may trigger apoptosis (7). Although apoptosis takes place as a normal part of embryonic development to eliminate defectively differentiated cells, it is more likely to occur during *in vitro* embryo culture because of suboptimal conditions (8). The higher incidence of apoptosis is detrimental to blastocyst formation, and leads to preimplantation embryo death (9). The aim of the present study was to determine the effect of antibodies to HSP 60 and 70 on preimplantation mouse embryo development, reactive oxygen species (ROS) production, and apoptosis.

A total of 222 normal appearing two-cell (B6C3F1 mouse crossed with B6D2F1) cryopreserved embryos were used (Embryotech Laboratories, Inc. Wilmington, MA). The embryos were transferred to 1 mL of human tubal fluid (HTF, Irvine Scientific, Santa Ana, CA) medium containing 10% serum substitute supplement (SSS, Irvine Scientific) and 100  $\mu\text{g}/\text{mL}$  of mouse monoclonal antibodies was

added to either HSP60 (SPA-806, isotype IgG1;  $n = 51$ ) or HSP70 (SPA-820, isotype IgG1;  $n = 46$ ). Because the monoclonal anti-HSP antibodies belong to immunoglobulin G1 (IgG1) subclass, HTF–SSS supplemented with purified monoclonal mouse IgG1 was used as a control immunoglobulin ( $n = 54$ ). Embryos simultaneously cultured in HTF–SSS were used as control ( $n = 71$ ). Groups of 8 to 10 embryos were randomly added to 1 mL of culture medium in center-well culture dishes and incubated at 37°C under a humidified atmosphere of 5%  $\text{CO}_2$  in air. Embryos were monitored daily for cleavage, fragmentation, regularity of blastomeres, compaction, and cavitation for 72 hours.

Levels of ROS in culture media were measured after 24 hours, 48 hours, and 72 hours by a Berthold luminometer (Autolumat LB 953, Wallace Inc., Gaithersburg, MD) (10). Levels of ROS were recorded for 15 minutes, expressed as  $\times 10^4$  counted photons per minute (cpm). To detect apoptosis, individual embryos were stained by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique (*in situ* cell death detection system; Roche Diagnostic GmbH, Germany). To distinguish apoptosis from necrosis, embryos were stained with 20  $\mu\text{g}/\text{mL}$  propidium iodide, a membrane-impermeable stain that is excluded from viable cells. Embryos were washed three times in phosphate-buffered saline containing 0.3% polyvinyl pyrrolidone, and permeabilized in 0.5% Triton X-100 on ice for 2 minutes. The embryos were washed and incubated in TUNEL reaction cocktail at 37°C for 1 hour in the dark. Total blastomere count per embryo was determined by staining with bisbenzimidazole (Hoechst dye 33258).

Images were collected with a Leica TCS-SP2 laser scanning spectral confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany). Each optical section of the blastocyst was analyzed for total number of nuclei, necrotic

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TABLE 1

The allocation of inner cell mass (ICM) and trophoectoderm (TE) and incidence of apoptosis in blastocysts cultured in HTF–SSS alone and in the presence of antibodies.

| Variable                      | Control                        | IgG1              | anti-HSP60        | anti-HSP70        | P value <sup>c</sup> |       |       |       |       |      |
|-------------------------------|--------------------------------|-------------------|-------------------|-------------------|----------------------|-------|-------|-------|-------|------|
|                               | (n = 71)                       | (n = 54)          | (n = 51)          | (n = 46)          | A                    | B     | C     | D     | E     | F    |
| TCN <sup>a</sup>              | 54.5 (41.0, 65.0) <sup>b</sup> | 57.0 (51.0, 59.0) | 41.0 (37.0, 48.0) | 32.0 (29.0, 35.0) | NS                   | .0001 | .0001 | .0001 | .0001 | .03  |
| ICM <sup>a</sup>              | 13.0 (11.0, 16.0)              | 15.0 (13.0, 16.0) | 12.0 (11.0, 13.0) | 10.0 (9.0, 10.0)  | NS                   | NS    | NS    | .045  | .11   | .99  |
| TE <sup>a</sup>               | 40.0 (33.0, 44.0)              | 43.0 (38.5, 45.0) | 29.0 (26.0, 36.0) | 23.0 (20.0, 26.0) | .02                  | .001  | .0001 | .0001 | .0001 | .002 |
| ICM/TE                        | 0.4 (0.3, 0.4)                 | 0.4 (0.3, 0.4)    | 0.4 (0.4, 0.4)    | 0.4 (0.3, 0.5)    | NS                   | NS    | .004  | NS    | .003  | .002 |
| Apoptotic cell/blastocyst (%) | 4.9                            | 10.6              | 12.8              | 13.4              | .0001                | .0001 | .0001 | .4    | .004  | .08  |
| Apoptosis in TCN <sup>a</sup> | 2.3 (2, 3.3)                   | 5 (4, 7)          | 4.5 (3, 6)        | 3.5 (2, 5)        | .0001                | .002  | .04   | .2    | .02   | .21  |
| Apoptosis in ICM <sup>a</sup> | 1.4 (1.2, 1.6)                 | 3.5 (2, 6)        | 3 (2, 4)          | 3 (2, 4)          | .001                 | .08   | .2    | .18   | .23   | .9   |
| Apoptosis in TE <sup>a</sup>  | 1 (0.95, 1.4)                  | 1.5 (1, 3)        | 2 (1, 2)          | 1 (1, 2)          | .0001                | .0002 | .007  | .74   | .84   | .70  |

Note: HTF = Human tubal fluid; SSS = Serum substitute supplement.

<sup>a</sup> TCN = Total cell number, ICM = Inner cell mass, TE = Trophoectoderm.

<sup>b</sup> Values are median and interquartile range.

A = Comparison between control group and IgG1.

B = Comparison between control group and anti-HSP60.

C = Comparison between control group and anti-HSP70.

D = Comparison between group IgG1 and anti-HSP60.

E = Comparison between group IgG1 and anti-HSP70.

F = Comparison between anti-HSP60 and group anti-HSP70.

<sup>c</sup>  $P < 0.05$  was considered significant using mixed model analysis of variance.

Esfandiari. Heat shock proteins and preimplantation embryos. *Fertil Steril* 2007.

blastomers, and number of TUNEL-labeled nuclei in inner cell mass (ICM) and trophoectoderm (TE), and identified based on their size and intensity of stain (11).

Per-embryo findings were clustered with other embryos during each trial. Therefore, comparisons between treatments utilized treatment as the sole independent variable, with embryos treated as clustered observations within each experiment. Statistical significance was assessed with two-tailed tests, using  $P < .05$  as Type I error rates. The sample size had 90% power to detect a difference of 1.5 apoptotic cells per blastocyst between groups, and 90% power to detect a  $0.17 \times 10^4$  cpm difference in ROS levels between groups. Calculations were performed with SAS version 8.1 software (SAS Institute Inc., Cary, NC).

The blastocyst formation rate after 48 hours and 72 hours was not significant for embryos cultured in HTF–SSS compared to those cultured in HTF–SSS-supplemented IgG1 after 48 hours (32% and 22.2%) and 72 hours (92% and 88.9% consecutively). However, the percentage of embryos undergoing blastocyst formation was significantly reduced by anti-HSP 60 and 70 supplementation after 48 hours (13.7% and 0%) and 72 hours (39.2% and 15.2% consecutively). The hatching rate was significantly lower in the presence of antibodies compared to the embryos that were cultured in the HTF–SSS ( $P < .05$ ). Growth-retarded/arrested embryos before the morula stage were observed in IgG group (6 of 54, 9%), HSP60 (9 of 51, 17.6%) and HSP70 (19 of 46, 41.3%). However, the effect of HSP antibodies are more evident at the morula–blastocyst tran-

sition where 63% of morula stage embryos failed to develop to blastocyst in HSP60 and 74% in HSP70 groups.

The total cell number (TCN) for the embryos that were cultured in the four groups are shown in Table 1. The median and interquartile (IQR) range for TCN in the HTF–SSS group was similar to that of the blastocysts that were cultured in the presence of IgG1. The ICM number in the HTF–SSS group was similar to other groups, and the ICM/TE ratio in IgG1 and HSP60 groups was comparable with the HTF–SSS group. The number of TE cells was significantly lower in HSP60 and 70 groups compared to the control group ( $P = .001$ , and  $.0001$  consecutively).

The median (IQR) level ( $10^4$  cpm) of ROS for IgG1, HSP 60, and HSP 70 groups at 24 hours, 48 hours, and 72 hours were comparable to those observed in the HTF–SSS group.

Positive TUNEL labeling in apoptotic cells was observed in the blastocysts of all groups. However, a positive labeling could not be identified in the growth-retarded or arrested embryos before the morula stage. Supplementation of culture media with antibodies increased the incidence of apoptosis in the cultured embryos as evidenced by the significantly higher percentage of apoptosis in the blastocysts and in the higher median and IQR values of apoptotic cells per blastocyst (Table 1). Positive TUNEL labeling was seen in ICM and TE in both groups. Cell death in the blastocysts occurred primarily in the ICM in both groups.

No significant correlation was seen between the levels of ROS at 24 hours, 48 hours, and 72 hours, and embryo

parameters in the control (HTF–SSS) and HSP 60 group. Levels of ROS at 24 hours and 48 hours were negatively correlated with the number of ICM in IgG1 group ( $r = -0.8$  and  $-0.81$ ,  $P = .02$  and  $.02$  respectively). However, in the HSP 70 group, a positive correlation was seen between the levels of ROS at 24 hours and number of cells in ICM, the ICM/TE ratio, and the incidence of apoptosis ( $r = 1$ ,  $P < .0001$ ). The level of ROS at 48 hours was negatively correlated with blastocyst formation ( $r = -1$ ,  $P < .0001$ ).

In the present study, we have shown that supplementation of embryo culture media with anti-HSP antibodies adversely affects embryo development in different transition stages including cleavage-morula, morula-blastocyst, and hatching. It also increases the incidence of apoptosis in the cultured embryos as evidenced by the significantly higher percentage of apoptosis in the blastocysts and in the higher apoptotic cells per blastocyst. However, there was no significant effect on the level of ROS production, and the higher amount of ROS in the control and IgG1 groups may be largely because of the number of blastocysts in the culture.

The molecular mechanism(s) involved in inhibition of apoptosis by HSPs is still undefined. It is proposed that HSPs following stress may move from the cytoplasm into the nucleus and confer some protective effects against oxidant-induced DNA damage (12). HSP70 can prevent mitochondrial membrane depolarization, although it does not exert its protective effect by scavenging ROS or by preventing cell damage. Rather, it appears to modulate the cellular response to the toxic insult (13). The beneficial effects of HSP in embryo development have been shown in previous studies (14, 15). By limiting the expression of HSP70 in four-cell embryos, *in vitro* blastocyst development was reduced with increased embryo sensitivity to toxic agents. Significantly diminished development of hatched blastocysts was reported in two-cell mouse embryos cocultured with monoclonal antibodies against HSP 60 and 70.

Upon differentiation at the early blastocyst stage, trophoblast secretes zona lysis that is necessary for zona hatching (16). However, continued exposure to unbalanced culture environment, the presence of IgG or anti-HSP, as in our study, and the signals produced by some embryos that are arrested at the cellular or morula stage could affect normal cell function. This results in a decrease in the total lysis production by TE cells below the threshold level necessary to promote zona thinning and subsequently inhibiting the blastocyst hatching (17).

Although HSPs were, until recently, assumed to localize exclusively at various intracellular sites, it has been recently reported that several HSP molecules are expressed on the outer cellular membranes and show cell surface binding and/or cellular uptake (18, 19). The zona pellucida of mouse oocytes and zygotes is permeable to macromol-

ecules up to 170 kDa (20), that is, larger than IgG (150 kDa) (21). Therefore, it is plausible to assume that anti-HSP antibodies can pass through the zona and attach to HSPs on the surface of blastomeres to exert their effect. These molecules are now recognized as participants in signal transduction pathways, and some studies show active signaling by HSP for induction of cytokine secretion by various cell types (22).

Apoptosis is more likely to occur to a greater degree during *in vitro* embryo culture due to suboptimal conditions (23). Nutrient imbalance, oxygen tension, variation of temperature, altered cellular environment, manipulation of embryos in culture, extra-uterine environment, and the nutrients for six to eight cell-stage embryos may not be optimal, and all these may cause additional stress. All these conditions cause apoptosis in cultured embryos and the HSP family; Hsp70 especially may protect against apoptosis. Overexpression of HSP, up to 15-fold higher, in cultured embryos is reported to have beneficial effects on the developing embryos (24).

Women undergoing *in vitro* fertilization who have local and systemic immunity to human HSP60 following infections, show an increased prevalence of *in vitro* fertilization failure (25). During an infection, enhanced microbial HSP synthesis may be part of the protective response of the pathogen to host defenses, and can contribute to microbial virulence. This is important for reproduction because many couples seeking infertility treatment have had a previous exposure to microbial pathogens. Several studies have revealed that sensitizing HSP60 to *Chlamydia trachomatis* and subsequent expression of the highly homologous human HSP60 can lead to unsuspected infertility problems (25).

In conclusion, this study provides evidence that HSPs play an essential role in mammalian embryo development. Inhibition of HSP function by antibodies causes a significant reduction in blastocyst development and an increase in cell death. HSP-mediated regulation of the apoptotic pathways probably constitutes a fundamental protective mechanism that decreases cellular sensitivity to damaging events to allow cells to escape the otherwise inevitable engagement of apoptosis

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