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The effects of antibodies to heat shock protein 70 in fertilization and embryo development

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The role of heat shock proteins in shielding organisms from environmental stress is illustrated by the large-scale synthesis of these proteins by the organisms studied to date. However, recent evidence also suggests an important role for heat shock proteins in fertilization and early development of mammalian embryos. We found that the presence of anti-HSP70 antibody significantly reduced tight binding of spermatozoa to the zona pellucida of bovine oocytes and interrupted completion of meiosis II and pronuclear formation. Furthermore, the presence of anti-HSP70 in culture medium from day 3 to day 9 of development increased apoptosis and significantly reduced the number of embryos reaching the blastocyst stage. We further observed that the proportion of apoptotic cells in bovine blastocysts was significantly lower after in-vitro culture with a prior exposure to increased temperature. However, nuclear localization of the p53 protein, which is thought to be essential for the up-regulation of genes involved in apoptosis and cell cycle arrest, was detected in the majority of nuclei in blastocysts exposed to increased temperature, whereas in their untreated (control) counterparts, p53 protein was only detected in the cytoplasm. The decrease in apoptosis after exposure of blastocysts to increased temperature may be attributed to cell cycle arrest resulting from nuclear localization of the p53 protein and/or to an increase in heat shock protein synthesis. We propose that HSP70 plays a critical role in fertilization and early embryonic development.

Key words: apoptosis//blastocyst/fertilization/HSP70/p53

Introduction

To date, all organisms studied respond to environmental stresses by commencing the large-scale synthesis of a set of highly conserved proteins called heat shock proteins (Becker and Craig, 1994). They are said to mount a heat shock response, a stress response leading to the induction of heat shock proteins that confers resistance to subsequent cellular stress (Li and Werb, 1982). Heat shock proteins are classified into families according to their molecular weight and serve two major functions. One of these is as a molecular chaperone involved in facilitating protein folding and assembly, while the other is as a stabilizer of damaged proteins involved in the prevention of aggregation, allowing an opportunity for repair or degradation in cells experiencing cellular stress (Welch, 1992). Recently, the ability to inhibit apoptosis has become widely recognized as a function of heat shock proteins since this may contribute to their protective effect on cells (Samali and Orrenius, 1998). Although the mechanisms are unclear, recent evidence suggests an important role for heat shock proteins in fertilization and early embryonic development (Anderson, 1998; Dix *et al*., 1998; Neuer *et al*., 1998, 1999).

The sperm–oocyte interaction involves several events that culminate in fertilization. These include recognition of acrosome-intact spermatozoa, attachment to the zona pellucida, induction of the acrosome reaction, exocytosis of the outer acrosomal membrane, penetration of the zona pellucida, binding to the oocyte plasma membrane (oolemma), fusion with the oolemma, and finally, activation of nuclear events leading to zygote formation (Yanagimachi, 1994). Many of these events are mediated by protein molecules found on the sperm plasma membrane and zona pellucida (Tulsiani *et al*., 1997). A recent study has shown that HSP70 is present on the acrosome in ejaculated bovine spermatozoa and undergoes re-localization to the equatorial segment during induced capacitation and acrosome reaction (Kamaruddin, 1998). This finding is of particular significance since other surface proteins that are redistributed during membrane remodelling, such as PH-20, fertilin, CE9, SP-10 and 2B1, play important roles in the sperm–oocyte interaction during IVF (Myles *et al*., 1984; Primakoff *et al*., 1987; Petruszak *et al*., 1991; Coonrod *et al*., 1996; Jones *et al*., 1996). Although it is possible that HSP70 serves as a stress protector for spermatozoa prior to fertilization (Miller *et al*., 1992), the distribution and re-localization of HSP70 on bovine spermatozoa suggest that HSP70 plays a role in gamete interaction.

It is also likely that HSP70 plays an important role during

embryo development since the presence of antibodies to HSP70 significantly decreases progression to the hatched blastocyst stage in murine embryos (Neuer *et al*., 1998). Correspondingly, antisense oligonucleotides complementary to *HSP70* mRNA had a similar effect on embryo development which was amplified 9-fold by arsenic exposure at a subtoxic dose (Dix *et al*., 1998). In the latter study, the few embryos that did reach the blastocyst stage despite the inhibition of HSP70 expression, with and without the presence of arsenic, were characterized as degenerate with cell death accompanied by membrane blebbing. The authors suggested that the requirement for HSP70 during embryo development is amplified by exposure to adverse environmental conditions. Neuer *et al*. (1999) have further shown that murine blastocysts cultured in the presence of HSP antibodies show DNA fragmentation more frequently than unexposed embryos, suggesting that apoptosis may be a consequence of embryo toxicity associated with heat shock protein inhibition. Other studies have shown that developmental expression of HSP70 is correlated with resistance to apoptosis in response to stress in avian blastoderm (Bloom *et al*., 1998; Muscarella *et al*., 1998). All of these studies suggest that HSP70 functions in the prevention of apoptosis during early development.

Apoptosis is characterized by the loss of phospholipid symmetry in the plasma membrane, chromatin condensation, internucleosomal DNA fragmentation, separation of the nucleus into discrete masses, and blebbing of the plasma membrane to form apoptotic bodies that are phagocytized or extruded without causing damage to surrounding cells (Wyllie *et al*., 1980). It is known that apoptosis occurs as a normal feature of preimplantation development (Hardy, 1997; Matwee *et al*., 2000) probably in response to environmental stressors and gross chromosome abnormalities (Schimke *et al*., 1994; Ko and Prives, 1996; Edwards, 1998). The apoptotic process can be divided into an upstream signalling phase, and a downstream execution phase followed by the cell degradation phase (Kroemer *et al*., 1995). Regulation of apoptosis can occur at any of these phases and at multiple levels.

The tumour suppressor protein p53 is known for its role in signalling apoptosis and cell cycle arrest (Ko and Prives, 1996). Nuclear translocation is thought to be essential for p53 protein function as it acts as a transcription factor for genes including the gene for BAX which is involved in apoptosis and the gene for $p21^{WAF1}$, which is involved in cell cycle arrest (Ko and Prives, 1996). Although we have previously shown that the p53 protein exists in the cytoplasm in bovine blastocysts under normal culture conditions (Matwee *et al*., 2000) and that bovine blastocysts can up-regulate *HSP70* gene expression in response to increased temperature (Kawarsky and King, 2001), it is unknown whether nuclear translocation of p53 can be induced in embryos by exposure to increased temperature.

The aim of the present investigation was to explore the role of heat shock protein in bovine fertilization, embryo development, inhibition of apoptosis and translocation of the p53 protein. To accomplish this, two studies were conducted. In the first, HSP70 was inhibited during fertilization and embryo development by addition of anti-HSP70 monoclonal antibodies to the culture medium. Rates of fertilization and development to blastocyst stage as well as incidence of apoptosis in blastocysts were determined. In the second study, HSP70 synthesis was induced in blastocysts by brief exposure to elevated temperature and the incidence of apoptosis and location of p53 were determined. Here we report that anti-HSP70 prevents fertilization and significantly diminishes bovine embryo development *in vitro*. This reduced development may be attributed to the significant increase in apoptotic blastomeres observed. After a transient exposure to elevated culture temperature, bovine blastocysts displayed a significant reduction in apoptotic cells and nuclear localization of the tumour suppressor protein p53 was apparent.

Materials and methods

In-vitro embryo production

Bovine embryos were produced by in-vitro oocyte maturation, fertilization and culture as previously described (Xu *et al*., 1992). Briefly, cumulus–oocyte complexes (COC) were obtained by follicular aspiration and collected into HEPES-buffered Ham's F-10 (Gibco BRL, Burlington, ON, Canada) supplemented with 2.0% steer serum (v/v; Cocalico Biologicals, Inc., Reamstown, PA, USA), 72 IU penicillin–streptomycin (Gibco BRL), 2.0 IU heparin/ml (Organon Teknika, Toronto, ON, USA) and 1.0% $NaHCO_3$ (Fisher Scientific, Pittsburgh, PA, USA). The COC were matured in groups of 100 for 22–24 h in 750 µl of HEPES-buffered Tissue Culture Medium (TCM)-199 with 25 mmol/l HEPES (Gibco BRL) supplemented with 0.04 mmol/l L-glutamine (Fisher Scientific; Sigma, St Louis, MO, USA), 5.5 mmol/l sodium pyruvate (Gibco BRL; Sigma), 72 IU penicillin–streptomycin (Gibco BRL) and 10% steer serum (Cocalico Biologicals), at 39°C in a humidified atmosphere of 5% CO_2 in air. Motile spermatozoa were selected by the swim-up technique (Parrish *et al*., 1986) and mature oocytes were exposed to 1×10^6 spermatozoa per ml, for 18 h in 750 µl of Tyrode's albumin–lactate–pyruvate–IVF (TALP-IVF) medium (Greve *et al*., 1987). Cumulus cells were removed mechanically in HEPES-buffered TALP (TALP–HEPES) medium (Greve *et al*., 1987) and the presumptive zygotes were transferred to 50 µl microdrops (20–30 embryos/drop) of in-vitro culture (IVC) medium consisting of TCM-199 (Gibco BRL) supplemented with 10% steer serum, 5.5 mmol/l sodium pyruvate, 72 IU penicillin–streptomycin, and 0.35% BSA (Sigma) containing bovine oviductal epithelial cells (BOEC). Embryos were cultured in a humidified atmosphere of 5% CO_2 in air at 39°C and 50 µl of IVC medium was added to each culture drop on day 3 post-insemination.

Antibodies

Mouse monoclonal anti-human HSP70 (C92F3A-5 mAb; StressGen Biotechnologies Corp., Victoria, BC, Canada), which is specific for the induced form of HSP70, was used in each experiment. For a negative control, clarified mouse myeloma ascites IgG_1 (MOPC21; ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA), which has no identified hapten-binding specificity (Coonrod *et al*., 1996), was used at levels similar to the highest concentration of anti-HSP70 used in each experiment. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (BioCan Scientific, Mississauga, ON, Canada) was used for immunolabelling of HSP70. Sheep anti-human p53 and FITC-conjugated donkey anti-sheep IgG (Roche Diagnostics, Laval, QC, Canada) were used for the immunolabelling of the p53 protein.

Sperm–oocyte interaction and fertilization

The sperm–oocyte binding assay was performed *in vitro* using a modification of a published method (Tanphaichitr *et al*., 1992).

Oocytes were matured *in vitro* as described above. To ensure optimal contact between oocyte and antibody, cumulus cells were removed by gentle pipetting for 4 min in 0.1% hyaluronidase in TALP–HEPES supplemented with 0.32% (v/v) BSA (Sigma) and 0.005% (v/v) gentamycin (Gibco BRL). Denuded oocytes were washed three times in TALP–HEPES and once in TALP–IVF before they were transferred to TALP–IVF containing 0.0, 0.25, 0.5, 1.0 and 2.5 μg/ml of anti-HSP70 or 2.5 μg/ml MOPC21. Oocytes were co-incubated with 1×10^6 spermatozoa per ml at 38.5°C in a humidified atmosphere of 5.0% CO_2 in the presence of antibody for 5 h [the time required for bovine spermatozoa to bind to oocytes (Xu and Greve, 1988)].

After co-incubation, sperm–oocyte complexes (SOC) were removed and the spermatozoa loosely bound to the zona pellucida were removed by washing four times in TALP–HEPES. Sperm–oocyte complexes were placed on slides to which 20–30 μl of Hoechst 33342 (10 μg/ml in PBS) was added, followed by a 10 min incubation in a humid chamber to stain the DNA of live spermatozoa. Excess stain was removed and a drop of Dabco (Sigma) in glycerol and PBS (9:1) was added to prevent fading. After coverslip addition, the number of spermatozoa bound to the zona pellucida of each oocyte was counted using a Leitz Aristoplan (Leica, Toronto, ON, Canada) fluorescence microscope.

To study the effect of anti-HSP on fertilization, oocytes were matured *in vitro*, washed and subsequently co-incubated at 38.5°C for 18 h [the time required for bovine pronuclei formation (Xu and Greve, 1988)] with 1×10^6 spermatozoa per ml in TALP–IVF containing 0.0, 1.0 and 2.5 μg/ml of anti-HSP70 or 2.5 μg/ml MOPC21. Cumulus cells were removed from presumptive zygotes using 0.1% hyaluronidase in TALP–HEPES followed by fixation in acetic acid–methanol (1:3) at 4°C for 24 h. Fixed zygotes were mounted and stained with 1.0% aceto-orcein (Gibco BRL) and observed for pronuclear development under phase contrast using a Leitz Aristoplan microscope. Oocytes arrested at metaphase II (MII) or with one pronucleus (1PN) were considered unfertilized, and those with two or three pronuclei (2PN or 3PN) were considered fertilized (Coonrod *et al*., 1994).

Embryo development and apoptosis

To determine the effect of HSP70 inhibition on in-vitro development, bovine embryos were produced as described above ('In-vitro embryo production') with a few modifications. On day 3 post-insemination, embryos were removed from culture drops and transferred to 25 μl drops of IVC containing anti-HSP70 at 0, 0.1 or 50 μg/ml, or MOPC21 at 50 μg/ml followed by incubation at 38.5°C. Embryos were fed on day 5 of development with 12 μl of media containing the appropriate concentration of anti-HSP70 or MOPC21. Development to the blastocyst stage was assessed as a percentage of cleaved oocytes on day 9 of development.

Blastocysts with and without exposure to anti-HSP70 were analysed for apoptosis using terminal 5′-deoxynucleotidyl nick end labelling (TUNEL) which labels fragmented DNA characteristic of this form of cell death. The zona pellucida was removed using 0.1% pronase, followed by fixation in 4% paraformaldehyde (in PBS, pH 7.4) for 45 min at room temperature. Embryos were washed three times in PBS/PVP, permeabilized for 2 min on ice using 0.1% Triton X-100 (Bio-Rad), and washed again. Embryos were transferred into microdrops of terminal deoxynucleotidyl transferase enzyme and fluorescein-conjugated dUTP (TUNEL reagents; Roche Diagnostics) in a 1:9 ratio under silicone oil, and incubated in a humidified atmosphere at 39°C for 1 h. After incubation, embryos were washed three times in PBS/PVP, counterstained with 5 μg/ml propidium iodide (PI), and whole-mounted onto slides using Fluoro-guard antifade reagent (BioRad), before adding a coverslip and applying gentle pressure to compress the embryos to facilitate cell counting. The total cell number and the number of TUNEL positive cells were counted using a Leitz Aristoplan fluorescence microscope.

In order to establish whether the antibody is able to penetrate the zona pellucida and cell membrane of live embryos, embryos were removed after 3 and 66 h of incubation in culture medium with 50 μg/ml anti-HSP70 and washed briefly in PBS. The zona pellucida was removed by a 3 min incubation in 0.1% pronase, and embryos were washed again in PBS and fixed in 4.0% paraformaldehyde for 15 min before being rinsed and permeabilized in 0.1% Triton X-100 (BioRad) for 10 min. Non-specific binding was blocked using 2.0% normal goat serum (NGS; Jackson Immuno Research, Jackson, MI, USA) in PBS for 1 h, followed by a 1 h incubation with FITC-conjugated goat anti-mouse IgG diluted 1:100 in PBS. Embryos were washed in 0.05% Tween-20 in PBS, prior to counterstaining in 5 μg/ml PI, followed by washing again in 0.05% Tween-20. Embryos were mounted and analysed by laser scanning confocal microscopy.

Assessment of temperature effect on apoptosis and localization of p53 protein

To determine the effect of increased temperature, day 8 blastocysts were divided and placed in two Petri dishes each containing 3 ml of media preheated to 38.5°C. The first group was placed in an incubator at 42.0°C for 6 h, a treatment previously shown in our laboratory to increase *HSP70* mRNA 5-fold (Kawarsky and King, 2001); the second group (control) was left at 38.5°C. After 6 h, both groups were transferred back to IVC drops for an additional 18 h incubation at 38.5°C. On day 9 of development, the embryos were assessed for apoptosis by TUNEL, as described above, and for immunofluorescent localization of the p53 protein.

To establish the intracellular localization of the p53 protein in control embryos and those exposed to increased temperature, the zona pellucida was removed by the method used in the TUNEL protocol. Embryos were fixed for 1 min in cold 1:2 PBS: methanol (–10°C) first and then for 2 min in 100% methanol followed by three washes in PBS/PVP. Embryos were treated with 2% horse serum in 0.03% Triton X-100 for 30 min at room temperature to simultaneously permeabilize cell membranes and to block non-specific binding. They were then incubated in a 1:50 dilution of sheep anti-human p53 overnight at 4°C, followed by rinsing in 0.05% Tween-20 and exposure to a 1:160 dilution of FITC-conjugated donkey anti-sheep IgG for 4–6 h. Embryos were rinsed, counterstained with PI (5 μg/ml) and analysed by laser scanning confocal microscopy.

Image acquisition and processing of immunolabelled embryos

Immunolabelled embryos were observed using a Bio-Rad MRC-600 laser scanning confocal microscope. For fluorescein detection, an argon ion laser adjusted to $<$560 nm was used, and for propidium iodide, a helium–neon laser adjusted to $>$560 nm was used. Images were obtained using a $\times$60 objective. Optical sections of each fluorochrome were acquired individually and stored digitally. These were then recombined digitally using Confocal Assistant software and processed using Corel Photo-Paint software.

Statistical analysis

Analysis of variance using the SAS General Linear Model procedure (Statistical Analysis System Inc., Carey, NC, USA) was carried out on the mean number of spermatozoa bound to each oocyte and on the arcsine transformed percentages of presumptive zygotes at different stages of meiosis and pronuclear development. Duncan's multiple range test was used to determine differences between treatment means. Development rates were assessed using Fisher's exact test, and proportions of TUNEL positive cells were analysed by Mantel–

Haenszel χ^2-analysis with a Rao/Scott adjustment to account for clustering of apoptotic cells within embryos and the odds ratio was determined (SAS). All experiments were performed on two to five separate occasions and $P < 0.05$ was considered statistically significant.

Results

Effect of anti-HSP70 on sperm–oocyte interaction

Following co-incubation of spermatozoa and oocytes in the presence of various concentrations of anti-HSP70 or MOPC21, and subsequent washing, the mean number of spermatozoa tightly bound to the zona pellucida (i.e. those remaining after washing) in each treatment group, was determined (Table I). The mean number of spermatozoa tightly bound to zona pellucida was 70.7 when oocytes and spermatozoa were co-incubated without any antibody, and was 70.8 in sperm–oocyte complexes exposed to 2.5 μg/ml of MOPC21. However, when oocytes were exposed to 0.25 μg/ml anti-HSP70, the mean number of tightly bound spermatozoa dropped significantly to 33.7 which is approximately half that of controls. When sperm–oocyte complexes were exposed to higher levels of anti-HSP70 (0.5 and 1.0 μg/ml), the mean number of zona-bound spermatozoa was significantly lower and the reduction in sperm–zona binding was the greatest in the group exposed to the highest concentration of anti-HSP70 used in the present study (2.5 μg/ml).

Effect of anti-HSP70 on IVF

The nuclear status of oocytes co-incubated with spermatozoa in the presence of various concentrations of anti-HSP70 or MOPC21 is summarized in Table II. The percentage of oocytes arrested at MII after exposure to 2.5 μg/ml of MOPC21 and 1.0 μg/ml of anti-HSP70 did not differ significantly from that of the control group. However, oocytes exposed to 2.5 μg/ml of anti-HSP70 antibody showed a significantly higher percentage of oocytes arrested at MII. Similarly, no differences were observed in the percentage of oocytes with 1PN in the former three groups [those not exposed to any antibody (control), 2.5 μg/ml of MOPC21, or 1.0 μg/ml of anti-HSP70], whereas a significantly higher percentage of oocytes with 1PN were observed in oocytes exposed to 2.5 μg/ml of anti-HSP70. The percentage of presumptive zygotes with 2PN was highest in the control group (oocytes not exposed to any antibody) and 2.5 μg/ml of MOPC21 group, while co-incubation with 1.0 and 2.5 μg/ml of anti-HSP70 caused a dose-dependent reduction. The percentage of presumptive zygotes with 3PN was low in all groups, although slightly higher after exposure to 1.0 μg/ml of anti-HSP70.

Table I. Sperm–zona binding in the presence of anti-HSP70 or MOPC21 at the indicated concentrations

Group	No. of SOC examined	No. of spermatozoa attached (mean ± SE)
Control	441	70.7 ± 1.97[a]
MOPC21 (2.5 μg/ml)	426	70.8 ± 1.71[a]
anti-HSP70 (0.25 μg/ml)	421	33.7 ± 0.89[b]
anti-HSP70 (0.5 μg/ml)	456	16.5 ± 0.43[c]
anti-HSP70 (1.0 μg/ml)	419	19.0 ± 0.50[c]
anti-HSP70 (2.5 μg/ml)	419	8.6 ± 0.29[d]

[a–d]Values with different superscripts differ significantly ($P < 0.05$).
SOC = sperm–oocyte complexes.

Effect of anti-HSP70 on embryo development and apoptosis

To determine the effect of HSP70 inhibition on in-vitro bovine embryo development, embryos were cultured from day 3 of development in the presence of various concentrations of anti-HSP70 or MOPC21. On day 9, blastocyst development was assessed as a proportion of cleaved oocytes and the results are summarized in Figure 1. The presence of large quantities of anti-HSP70 in the culture medium caused a reduction in the number of embryos reaching the blastocyst stage. In contrast to 16.1% of embryos cultured without any antibody and 17.3% cultured in 50 μg/ml of MOPC21, only 7.7% of the embryos cultured in the presence of 50 μg/ml of anti-HSP70 reached the blastocyst stage. The percentage of embryos (14.9) to reach the blastocyst stage when cultured with 0.1 μg/ml of anti-HSP70 was not significantly reduced compared to those cultured without any antibody and those cultured with 50 μg/ml of MOPC21. Similarly, preliminary studies with the concentration used in the fertilization studies (2.5 μg) showed no significant effects on embryo development.

Control blastocysts and those cultured in the presence of anti-HSP70 were analysed for apoptosis using TUNEL (Table III). A significant increase in the proportions of TUNEL positive cells was detected after culture in the presence of 50 μg/ml of anti-HSP70 ($P < 0.05$) relative to the proportion of TUNEL positive cells in embryos cultured without any antibody. The odds ratio was 0.68 with a 95% confidence interval of 0.47–0.96. The HSP70 antibody appeared to be able to

Table II. Nuclear status of oocytes co-incubated with spermatozoa in the presence of anti-HSP70 at the indicated concentrations

Group	No. of SOC examined	Nuclear status (mean % ± SE)			
		Oocytes at MII	Oocytes with 1PN	Zygotes with 2PN	Zygotes with 3PN
Control	419	11.1 ± 2.5[a]	1.8 ± 0.7[a]	79.8 ± 2.3[a]	7.3 ± 1.9[ab]
MOPC21 (2.5 μg/ml)	423	14.5 ± 3.0[a]	2.8 ± 0.8[a]	74.5 ± 5.1[a]	7.8 ± 1.8[ab]
anti-HSP70 (1.0 μg/ml)	430	20.7 ± 4.8[a]	5.3 ± 2.0[a]	63.1 ± 2.8[b]	10.8 ± 2.1[a]
anti-HSP70 (2.5 μg/ml)	436	49.2 ± 4.4[b]	13.3 ± 3.5[b]	34.8 ± 3.6[c]	2.6 ± 1.0[b]

Values with different superscripts within a column differ significantly ($P < 0.05$).
SOC = sperm–oocyte complexes; MII = metaphase II; PN = pronucleus.

penetrate the zona pellucida and cell membrane of live embryos as shown by immunofluorescent labelling of embryos cultured in the presence of 50 μg/ml anti-HSP70. Although few embryos showed any labelling after 3 h of culture with anti-HSP70, there was a dramatic increase in labelled embryos after 66 h of culture. Morphologically good quality embryos (Figure 2A and B) appeared not to incorporate the antibody and it is not clear whether poor quality embryos (Figure 2C) were a result or a cause of antibody incorporation (Figure 2D).

Effect of increased temperature on apoptosis and localization of p53 protein

Bovine blastocysts exposed to a temperature of 42°C for 6 h on day 8 of development were harvested for TUNEL after an additional 16 h at 38.5°C (day 9). The results are summarized in Table IV. The proportion of TUNEL positive cells was significantly lower in blastocysts exposed to increased temperature compared to those in the control group ($P < 0.05$). The odds ratio was 1.64 with a 95% confidence interval of 1.11–2.55.

To determine the effect of increased temperature on nuclear localization of the p53 protein, day 8 blastocysts were subjected to the same increase in temperature as for TUNEL study and immunolabelled on day 9 of development (Figure 2E–J). In contrast to the intense punctate cytoplasmic staining of the p53 protein in control blastocysts ($n = 7$; Figure 2E–G), p53 nuclear localization was detected in the majority of cells in all blastocysts in the heat-treated group ($n = 8$; Figure 2H–J). The distribution of p53 protein was less intense and more uniform in blastocysts exposed to increased temperature compared to that in the control group.

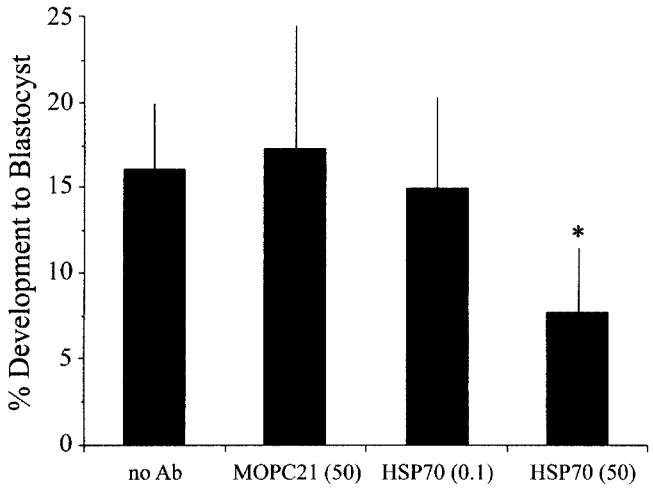

Figure 1. Development to the blastocyst stage expressed as a percentage of cleaved oocytes in the presence of anti-HSP70 or MOPC21 at the indicated concentrations (μg/ml). 95% confidence intervals are shown. *Significant difference compared to controls ($P < 0.05$).

Table III. Terminal 5′-deoxynucleotidyl nick end labelling (TUNEL) in blastocysts cultured with or without anti-HSP70

Group	No. analysed	TUNEL positive (mean ± SD)	Total cells (mean ± SD)	% TUNEL positive
Control	38	5.3 ± 3.4	93.0 ± 15.6	5.7
Heat stress	21	7.1 ± 3.9	88.3 ± 13.8	8.0*

*$P < 0.05$.

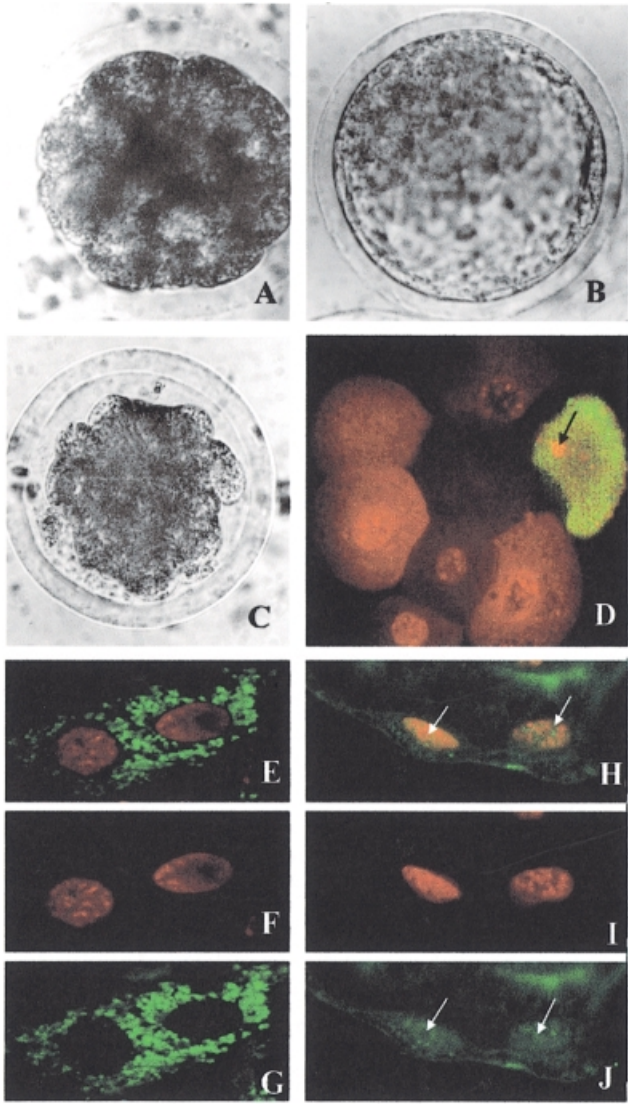

Figure 2. Immunofluorescent labelling of bovine embryos. (**A**) Morula stage bovine embryo, (**B**) blastocyst stage bovine embryo, (**C**) morula stage embryo with poor morphology, (**D**) late cleavage stage embryo showing intracellular localization of anti-HSP70 (green fluorescence) in a cell that appears to be undergoing apoptosis as evidenced by condensed chromatin. Arrows show condensed chromatin stained with propidium iodide (PI). (**E–J**) Immunofluorescent localization of p53 protein in bovine blastocysts. High power magnification of two trophectoderm cells of control blastocysts (**E**, **F**, **G**) and those exposed to elevated temperature (**H**, **I**, **J**). Nuclei were counterstained with PI (red). Fluoroscein isothiocyanate (FITC) and PI channels merged (**E**, **H**). Single channel only showing PI counterstain (**F**, **I**). Single channel only showing FITC-labelled p53 protein (green **G**, **J**). Nuclear and cytoplasmic localization of p53 protein in control blastocysts (**E**, **G**). Cytoplasmic and nuclear localization of FITC-labelled p53 protein (green) in blastocysts exposed to increased temperature (arrows **H**, **J**). Original magnifications: **A–C**, ×300; **D**, ×600; **E–J**, ×1200.

Table IV. Terminal 5′-deoxynucleotidyl nick end labelling (TUNEL) in heat-stressed and control blastocysts

Group	No. analysed	TUNEL positive (mean ± SD)	Total cells (mean ± SD)	% TUNEL positive
Control	21	6.7 ± 4.8	87.9 ± 21.0	7.6
Heat stress	17	4.1 ± 2.8	85.4 ± 22.5	4.8*

*$P < 0.05$.

Discussion

This study has shown that exposure to HSP70 protein monoclonal antibodies is sufficient to disrupt sperm–oocyte interaction, fertilization and embryo development. It has further shown that the reduction in embryo development in response to HSP70 inhibition is associated with an increase in apoptosis, and that increased temperature, in turn, is associated with a decrease in apoptosis and the nuclear translocation of p53 protein. The inhibitory effect of anti-HSP70 on sperm–oocyte interaction may occur at any stage of fertilization, from initial recognition to the fusion of spermatozoa plasma membrane with the oolema. Although fertilization is thought to require an interaction between the zona pellucida antigen 3 (ZP3) (Florman and Wassarman, 1985) and β1,4-galactosyltransferase located on the anterior portion of the sperm head (Miller *et al*., 1992), recent evidence suggests that β1,4-galactosyltransferase is not the only sperm protein involved in oocyte binding since β1,4-galactosyltransferase knockout mice are fertile (Lu and Shur, 1997). While antibodies binding to the ZP3 have previously been shown to inhibit sperm binding (Florman and Wassarman, 1985), our study clearly demonstrates that anti-HSP70 also exerts a negative influence on fertilization possibly by interfering with other steps of fertilization.

Different antibodies have been shown to block different facets of the murine fertilization process implying that several molecules are important for successful sperm–oocyte recognition, binding and fusion (Saling and Lakoski, 1985). Furthermore, a cocktail of these antibodies at a concentration lower than that at which each is effective individually, yields a synergistic effect suggesting that their inhibitory activities are cumulative and complementary to one another (Saling, 1986). It would appear that the initial sperm–oocyte recognition was not compromised by exposure to anti-HSP70 in the present study, since microscopic evaluation of loose attachment to the zona pellucida demonstrated no difference in this parameter between the treated and control groups. As in other antibody inhibition studies involving sperm surface antigens including SP-10 mAb in bovine (Coonrod *et al*., 1996) and M42 mAb in the mouse (Saling, 1986), HSP70 antigens may not play an active role at this early stage of sperm–oocyte recognition. However, the dose-dependent reduction in the mean number of spermatozoa that were tightly attached to the zona pellucida following co-incubation with anti-HSP70 suggests that HSP70 may be involved at the sperm binding stage that follows initial recognition. It is not likely that the inhibition of sperm–oocyte binding is due to non-specific binding because exposure to MOPC21 did not show a similar effect.

If it is assumed that zygotes with more than 1PN are the result of fertilization, then the fertilization rate of oocytes exposed to anti-HSP70 was significantly lower than among oocytes not exposed to any antibody, or those exposed to MOPC21. Furthermore, the percentage of oocytes arrested at MII or presumptive zygotes with 1PN, representing failed fertilization with anti-HSP70, was inversely proportional to the percentage of zygotes with two or more pronuclei. Together these observations show that anti-HSP70 has a negative impact on the fertilization of bovine oocytes. It is not likely that fertilization failure in the presence of anti-HSP70 is attributable to a change in the rate of parthenogenic activation of mature oocytes since in the absence of spermatozoa, activation was low in the presence of 2.5 μg/ml of anti-HSP70 and did not differ from oocytes that were not exposed to the antibody (data not shown).

There is a clear indication that the inhibition of fertilization in the presence of anti-HSP70 is a direct result of the reduction in sperm-binding to the zona pellucida also caused by anti-HSP70. Other studies have reported changes in sperm–oocyte interaction and a reduction in fertilization after exposure to antibodies to other sperm surface proteins in species including the cow and mouse (Primakoff *et al*., 1987; Tanphaichitr *et al*., 1992; Coonrod *et al*., 1994, 1996). It was suggested that SP-10 is involved in tight binding of spermatozoa to the zona pellucida (Coonrod *et al*., 1996), while it was demonstrated that M29 and M37 antibodies prevent fertilization with or without the zona pellucida, presumably by interfering with sperm–oocyte membrane fusion (Saling *et al*., 1985). Our results suggest that HSP70 may be involved either in the tight binding of spermatozoa to the zona pellucida or in a later stage of the sperm–oocyte interaction cascade such as sperm membrane fusion with the oolemma. The presence of HSP70 on the equatorial segment of acrosome-reacted spermatozoa (Kamaruddin, 1998) and the fact that the final stage of sperm–oocyte interaction involves fusion of the equatorial segment of a spermatozoon with the oolemma lend credence to the latter suggestion.

HSP70 present in bovine spermatozoa may also play a role in post-fertilization events. It may be required as a stress protector during early cleavage prior to the activation of the embryonic genome. However, bovine oocytes and 2-cell embryos are known to possess their own stores of heat shock proteins (Edwards and Hansen, 1996; Kawarsky and King, 1998) and synthesis in 2-cell embryos increases upon heat shock (Edwards and Hansen, 1997). However, heat-induced synthesis of heat shock proteins is probably not regulated at the level of transcription until the 4-cell stage since α-amanitin was able to block this response in 4-cell embryos, but not in 2-cell embryos (Edwards *et al*., 1997). In the mouse, spontaneous HSP70 synthesis occurs with the activation of the embryonic genome (Christians *et al*., 1995, 1997a), but induction of heat shock protein synthesis may not be optimal until the blastocyst stage (Muller *et al*., 1985; Hahnel, 1986). Furthermore, HSP70 expression has been found to be 5–15-fold higher in cultured murine embryos (Christians *et al*., 1995, 1997b). It is likely that the increase in heat shock protein synthesis allows embryo development to continue in suboptimal conditions. We have

shown that inhibiting heat shock protein function by exposure of bovine embryos to anti-HSP70 decreased the rate of development to the blastocyst stage. However, exposure to MOPC21 had no deleterious effect on development. In the mouse it has also been shown that embryos exposed to heat shock protein antibodies (Neuer *et al*., 1988, 1999) or transfection of preimplantation embryos with antisense oligonucleotides complementary to *HSP70* mRNA (Dix *et al*., 1998) significantly decreases development to the hatched blastocyst stage. Thus, functional HSP70 appears to be essential for optimal embryo development.

The precise mechanism of heat shock protein antibody inhibition of development is unclear at present. It is possible that anti-HSP binds receptors on the zona pellucida, embryo plasma membrane, oviductal cell membrane, or some other necessary factor in the culture media. However, immunolabelling of embryos cultured in the presence of anti-HSP has shown that the antibodies can penetrate the zona pellucida and cell membranes of live embryos and, therefore, may produce an intracellular effect. This result is not surprising since murine embryos cultured in the presence of a primary antibody for 1–3 days, followed by washing and incubation in a secondary antibody for 1 h, have demonstrated strong immunofluorescence (Kaider *et al*., 1999). Indeed, the idea that antibodies are unable to penetrate living cells has been challenged for some time, particularly since IgG anti-ribonucleoprotein and IgG anti-DNA have been shown to enter living cells, alter their function and cause apoptosis (Alarcón-Segovia *et al*., 1996).

The increase in the proportion of cells undergoing apoptosis in blastocysts exposed to anti-HSP70 suggests that cell death may be causally related to the observed reduction in developmental potential. This has been suggested in previous studies in the mouse where the presence of antibodies to heat shock protein not only significantly decreased development to the hatched blastocyst stage but also increased the frequency of TUNEL staining (Neuer *et al*., 1999), and exposure to antisense oligonucleotides complementary to *HSP70* mRNA caused a reduction in embryo development accompanied by cell death with membrane blebbing (Dix *et al*., 1998). Heat shock proteins or heat shock protein overexpression may also protect cells from necrosis (Guénal *et al*., 1997; Vayssier *et al*., 1998), presumably through protein chaperoning. This may also have contributed to the negative impact of heat shock protein inhibition on embryo development observed in the present study.

The protective effect of heat shock proteins against apoptosis is supported by the decrease in apoptosis observed after in-vitro culture with a prior exposure to increased temperature. It is well established that although heat stress has a significant detrimental effect during the early stages of bovine embryo development, morulae and blastocysts are relatively resistant (Ealy *et al*., 1995). Significantly higher numbers of viable nuclei were noted in a study on porcine embryos 24 h after exposure to increased temperature on day 6 of development compared to those in control embryos (Kojima *et al*., 1996). Furthermore, an acute rise in temperature at the morula stage has been shown to increase the rate of embryo development in the cow (Ryan *et al*., 1992). The ability of later stage embryos to resist the damaging effects of increased temperature is probably due to the ability to up-regulate the *HSP70* gene as in other cell types, since a 5-fold increase in *HSP70* gene expression has been noted in in-vitro bovine blastocysts exposed to 42°C for 4 h on day 3 of development when compared to control embryos (Kawarsky and King, 2001). Furthermore, as the period of exposure to increased temperature is extended, it is accompanied by a progressive increase in the level of HSP70 expression, which in turn correlates with the degree of stress protection conferred (Mirkes *et al*., 1999). Stress protection may also be associated with the length of the recovery period after exposure to increased temperature since umbilical vein endothelial cells have been noted to display a significant protection from oxidative stress 20 h after exposure to increased temperature but not after 2 h (Gill *et al*., 1998). The half-life of HSP70 induced by elevated temperature in mammalian cells under in-vitro conditions is thought to be around 48 h (Mizzen and Welch, 1988). It has been shown that a previous exposure to elevated temperature protects cells from apoptosis following subsequent transfer to serum-free media and the degree of protection correlates with the extent of heat shock protein synthesis (Mailhos *et al*., 1993). In the present study, the medium used to expose blastocysts to increased temperature was not preheated to 42°C but to 38.5°C. The slow warming of the culture medium probably allowed cells to adapt more successfully by up-regulating heat shock protein synthesis without inducing damage. An additional 18 h culture under conditions known to be suboptimal for bovine embryo growth, as evidenced by a reduction in reproductive efficiency (Thompson, 1997) and increase in apoptosis compared to in-vivo embryos (Brison and Shultz, 1997; Hardy, 1997), resulted in increased cell survival in embryos with a prior exposure to increased temperature.

While the induction of heat shock protein synthesis is a possible explanation for reduced levels of apoptosis seen in blastocysts with a prior exposure to increased temperature, other protective mechanisms exist and may also play a role in reducing the rate of cell death. For instance, heat shock has been shown to induce a 24 h cell cycle arrest in normal human fibroblasts and this was associated with nuclear accumulation of p53 protein and an increase in the amount of p21/WAF1/CIP1 (Nitta *et al*., 1997). Other studies also report nuclear translocation of the p53 protein (Sugano *et al*., 1995; Yin *et al*., 1997) and the p53-mediated induction of WAF1 in response to increased temperature (Ohnishi *et al*., 1996). Cell cycle arrest is thought to be a mechanism by which cells are protected from apoptosis by allowing time for repair of damage incurred by cellular stress. The decision between arrest and apoptosis probably depends on the cell type and extent of damage (Ko and Prives, 1996). It has been proposed that the effect of thermal stress is mediated both by increasing HSP70 and modulating intracellular p53 function (Chen *et al*., 1999). However, the potential role played by alterations in ionic permeability of membranes and changes in pH associated with temperature fluctuations is unknown.

The ability of p53 protein to enter the nucleus may be affected by its ability to complex with other proteins expressed in the cell (Shaulsky *et al*., 1990). Heat shock proteins could

be potential regulators of p53 protein accumulation in the nucleus. In human glioblastoma cells, p53 protein accumulated by heat stress is associated with HSP70 family members (Matsumoto *et al*., 1994) but, after nuclear translocation, p53 proteins are freed from heat shock proteins for binding to DNA (Selkirk *et al*., 1994). Furthermore, HSP70 was found to be associated with the nucleus after heat stress in the bovine embryo (Kawarsky and King, 1998, 2001). All of these findings suggest that the nuclear localization of p53 protein after exposure to increased temperature observed in the present study may be associated with heat shock proteins. Hence, the possibility that heat shock proteins and p53 work in concert to prevent apoptosis in bovine embryos is not excluded.

In conclusion, this study provides strong evidence to suggest that heat shock proteins play an essential role in mammalian fertilization and embryo development. Inhibition of heat shock protein function has shown that HSP70 is one of the sperm antigens that play an important role during sperm–oocyte interaction and fertilization. Furthermore, heat shock proteins may also serve a protective role in embryo development since inhibition of HSP70 caused a reduction in blastocyst development that may be mediated by an increase in cell death.

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