

Developmental Changes in Inhibitory Effects of Arsenic and Heat Shock on Growth of Pre-Implantation Bovine Embryos

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ABSTRACT Although sensitive to various disrupters, pre-implantation embryos possess some cellular cytoprotective mechanisms that allow continued survival in the face of a deleterious environment. For stresses such as heat shock, embryonic resistance increases as development proceeds. Present objectives were to determine whether (1) arsenic compromises development of pre-implantation bovine embryos, (2) developmental changes in embryonic resistance to arsenic mimic those seen for resistance to heat shock, and (3) developmental patterns in induction of apoptosis by arsenic are correlated with similar changes in resistance of embryos to inhibitory effects of arsenic on development. Bovine embryos produced by in vitro fertilization were exposed at the two-cell stage or at day 5 after insemination (embryos \geq 16-cells in number) to either sodium arsenite (0, 1, 5, or 10 μ M) or heat shock (exposure to 41°C for 0, 3, 4.5, 6, or 9 hr). Arsenic induced apoptosis and increased group 2 caspase activity for embryos at the \geq 16-cell stage, but not for embryos at the two-cell stage. In contrast to these developmental changes in apoptosis responses, exposure to arsenic reduced cell number 24 hr after exposure for both two-cell embryos and embryos \geq 16-cells. Similarly, the percentage of embryos that developed to the blastocyst stage at day 8 after fertilization was reduced by arsenic exposure at both stages of development. Heat shock, conversely, reduced development to the blastocyst stage when applied at the two-cell stage, but not when applied to embryos \geq 16-cells at day 5 after insemination. In conclusion, arsenic can compromise development of bovine pre-implantation embryos, the temporal window of sensitivity of embryos to arsenic is wider than for heat shock, and cellular cytoprotective responses that embryos acquire for thermal resistance are not sufficient to cause increased embryonic resistance to arsenic exposure. It is likely that despite common cellular pathologies caused by arsenic and heat shock, arsenic acts to reduce development in part through biochemical pathways not activated by heat shock. Moreover, the embryo does not acquire significant resistance to these perturbations within the time frame in development examined. *Mol. Reprod. Dev.* 63: 335–340, 2002.

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INTRODUCTION

Successful development of the pre-implantation embryo is dependent on maintenance of cellular function in the face of adverse conditions in the embryonic microenvironment. Embryonic resistance to certain disrupters increases as the embryo advances in development. For example, more advanced pre-implantation embryos are more resistant to heat shock (Edwards and Hansen, 1997; Ju et al., 1999), cyanide (Donnay et al., 2000), and bichlorinated biphenyls (Küchenhoff et al., 1999) than are less advanced embryos. Such a finding suggests that embryos acquire additional or more effective cytoprotective mechanisms as they proceed through development. In contrast, sensitivity of embryos to hydrogen peroxide changes little during the period of pre-implantation development (Morales et al., 1999) and embryonic sensitivity to cadmium (De et al., 1993) and chlorambucil (Giavini et al., 1984) increases as embryos advance through pre-implantation development.

One developmentally-regulated process that may be involved in determining embryonic resistance to stress is apoptosis. Apoptosis may play an important role in protecting the embryo from embryotoxic conditions by removing damaged cells that would otherwise become necrotic or lead to the formation of dysfunctional daughter cells. Indeed, cells exposed to stress that cannot undergo apoptosis frequently undergo cell death more reminiscent of necrosis (Xiang et al., 1996; Woo et al., 1998). Evidence from bovine embryos exposed to

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heat shock indicates that stress-induced apoptosis is a developmentally-regulated phenomenon. In particular, heat shock was not effective in induced apoptosis in bovine pre-implantation embryos until day 4 of development (Paula-Lopes and Hansen, 2002a). Acquisition of heat-induced apoptosis is temporally associated with developmental resistance of bovine embryos to heat shock (Edwards and Hansen, 1997; Ju et al., 1999) suggesting that apoptosis may be one of the mechanisms by which the embryo gains thermal tolerance. Moreover, inhibition of apoptosis responses increased sensitivity of bovine embryos to heat shock (Paula-Lopes and Hansen, 2002b).

It is not known whether developmental acquisition of tolerance to other adverse conditions that can compromise embryonic survival is related to induction of apoptosis. One molecule that can compromise pre-implantation embryonic development, at least in the mouse, is arsenic (Müller et al., 1986; Dix et al., 1998). The objectives of the present study were to determine whether (1) arsenic compromises development of pre-implantation bovine embryos, (2) developmental changes in embryonic resistance to arsenic mimic those seen for resistance to heat shock, and (3) developmental patterns in induction of apoptosis by arsenic are correlated with similar changes in resistance of embryos to inhibitory effects of arsenic on development.

MATERIALS AND METHODS

Materials

Sodium arsenite was obtained from Sigma (St. Louis, MO). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed with the In Situ Cell Death Detection Kit with fluorescein label purchased from Roche (Indianapolis, IN). Propidium iodide was obtained from Sigma. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY), Prolong Antifade Kit was obtained from Molecular Probes (Eugene, Oregon), RQ1 RNA-free DNase was from Promega (Madison, WI) and RNase A was from Qiagen (Valencia, CA). The fluorogenic caspase substrate PhiPhiLux-G₁D₂ was obtained from OncoImmunin, Inc. (Gaithersburg, MD). Materials for production and culture of embryos were obtained as described by Rivera and Hansen (2001).

Oocyte Maturation, Fertilization, and Embryo Culture

Embryos were produced using oocytes recovered from ovaries obtained at a local abattoir. Procedures for in vitro maturation of oocytes, in vitro fertilization, and embryo culture were performed as described by Rivera and Hansen (2001). After fertilization, putative zygotes were cultured in 50- μ l drops of modified Potassium Simplex Optimized Medium (KSOM) overlaid with mineral oil. The KSOM, which contains 1 mg/ml BSA, was modified on the day of use by adding 2–3 mg/ml essentially fatty acid-free BSA, 2.5 μ g/ml gentamicin, 1 \times essential amino acids (Basal Medium Eagle), and 1 \times non-essential amino acids (Minimal Essential Medium).

Embryos were harvested from culture drops at the two-cell stage (26–34 hr after insemination of cumulus-oocyte complexes) or at the \geq 16-cell stage (day 5 after insemination). Embryos were placed into fresh microdrops of modified KSOM. Embryos were randomly distributed in approximately equal numbers to drops for each treatment. Thus, while the number of embryos varied from 6 to 19 embryos per drop for two-cell embryos and 2–9 embryos per drop for \geq 16-cell embryos, the number of embryos per drop was similar for each treatment within a replicate. Variations in the number of embryos per treatment reflect variation in the number of drops per replicate rather than in embryo number per drop. Drop volume was 50 μ l for experiments on apoptosis and 25 μ l for experiments where development was the endpoint.

Effect of Arsenic on Apoptosis and Development 24 hr After Exposure to Arsenic

Embryos were harvested at the two- and \geq 16-cell stage and placed in fresh microdrops of modified KSOM medium containing 0, 1, 5, or 10 μ M sodium arsenite. After 12 hr at 38.5°C, embryos were washed in HEPES-TALP and returned to culture in fresh microdrops of modified KSOM for 12 hr at 38.5°C. Embryos were then washed three times in 100- μ l drops of 10 mM potassium phosphate, pH 7.4 containing 0.9% (w/v) NaCl, and 1 mg/ml PVP (PBS/PVP), and fixed for 1 hr at room temperature in a 100- μ l drops of 4% (w/v) paraformaldehyde and 0.9% (w/v) NaCl in 100-mM potassium phosphate, pH 7.4. Embryos were then washed three times in PBS/PVP and stored in this buffer at 4°C for up to 30 days prior to evaluation for fragmented DNA using the TUNEL procedure.

The TUNEL reaction was performed at room temperature using embryos placed in groups in individual wells of polystyrene, flat-bottom 96-well microtiter plate (Falcon, Becton Dickinson, Lincoln Park, NJ). After warming embryos for 30 min at room temperature, embryos were washed two times by transferring to wells containing 75- μ l PBS/PVP for 6 min. Permeabilization of cellular membranes was accomplished by transfer of embryos to wells containing 75- μ l permeabilization solution (0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate) for 30 min at room temperature. Embryos were washed twice and positive and negative control embryos were then incubated with DNase (50 U/ml) for 1 hr and washed twice in PBS/PVP. All embryos were transferred to wells containing 50 μ l of TUNEL reaction mixture (prepared as provided by the manufacturer) and incubated for 1 hr in the dark. Negative controls were incubated in TUNEL reaction mixture in which the terminal deoxynucleotidyl transferase was excluded. After washing, embryos were incubated with RNase A (50 μ g/ml in PBS/PVP) for 1 hr at room temperature in the dark, washed again and then transferred to wells containing propidium iodide (0.5 μ g/ml in PBS/PVP) for 25 min at room temperature in the dark. Embryos were washed two more times in PBS/PVP. Embryos were transferred in a minimal amount of PBS/PVP to a clean

microscope slide, covered with 15 μl Prolong[®] Antifade mounting medium, dried for 10–15 min at 39°C in a drying oven and then examined for total cell number (red and yellow nuclei) and apoptotic nuclei (yellow nuclei) using a Zeiss Axioplan 2 fluorescence microscope with dual filter. The experiment was replicated on three occasions with a total of 19–27 embryos per group.

Effect of Arsenic on Caspase Activity

Caspase activity was measured using the fluorogenic substrate PhiPhiLux-G₁D₂. This molecule, which produces increased green fluorescence upon cleavage, is specific for group II caspases, including caspase 3, 2, and 7. Embryos were harvested from microdrops at the two- and ≥ 16 -cell stages. Embryos were placed in fresh microdrops of modified KSOM or modified KSOM containing 10 μM sodium arsenite. After 12 hr at 38.5°C, embryos were washed three times in 50- μl drops of HEPES-TALP and transferred to 25- μl microdrops containing modified KSOM (negative control) or modified KSOM containing 5 μM PhiPhiLux-G₁D₂ (PhiPhi; OncoImmunin). Embryos were incubated in a humidified box in an oven at 39°C for 30–40 min, removed from drops, washed once in 50 μl HEPES-TALP and then transferred in a small volume to slide glass microscope slides. Embryos were covered with a cover slip and examined within 30 min for fluorescence using a Zeiss Axioplan 2 fluorescence microscope. Digital images were obtained using a Spot camera (Diagnostic Instruments, Sterling Heights, MI) and software and stored as .tiff files. A total of 10 embryos per group collected in one replicate were examined.

Effect of Arsenic on Development to the Blastocyst Stage

Embryos were harvested at the two- and ≥ 16 -cell stage and placed in fresh microdrops of modified KSOM medium containing 0, 1, 5, or 10 μM sodium arsenite. After 12 hr at 38.5°C, embryos were washed in HEPES-TALP and returned to culture in fresh microdrops of modified KSOM. The proportion of embryos developing to blastocyst was measured on day 8 after insemination. The experiment was replicated on a total of five occasions (two-cell; 59–86 embryos per group) or nine occasions (≥ 16 -cell; 54–62 embryos per group).

Effect of Heat Shock on Development to the Blastocyst Stage

Embryos were harvested at the two- and ≥ 16 -cell stage, placed in fresh microdrops of modified KSOM medium, and then either cultured at 38.5°C and 5% (v/v) CO₂ continuously or placed in an incubator at 41°C and 7% (v/v) CO₂ for 3, 4.5, 6, or 9 hr. The increase in CO₂ content at 41°C was made to account for the lower solubility of CO₂ at higher temperature and to assure that pH remained constant in the face of changing temperature (Rivera and Hansen, 2001). At the end of the heat shock period, embryos were returned to an incubator at 38.5°C and 5% (v/v) CO₂. The proportion of embryos developing to blastocyst was measured on

day 8 after insemination. The experiment was replicated on a total of eight occasions (two-cell; 61–86 embryos per group) or 10 occasions (≥ 16 -cell; 58–61 embryos per group).

Statistical Analysis

Data were analyzed by least-squares analysis of variance using the GLM procedure of SAS (SAS, 1989). For experiments evaluating development, the percentage of embryos that developed to the blastocyst was calculated for each replicate. Data on the percent of blastomeres positive for TUNEL reaction were calculated on a per embryo basis. Percentage data were subjected to an arcsin transformation before analysis to normalize data. Probability values reported in the article are based on analysis of transformed data, while least-squares means are from analysis of untransformed data. Mathematic models for analysis of variance included effects of treatment (e.g., arsenic concentration), replicate, and treatment \times replicate. Some analyses were also done to examine effects of stage of exposure and stage \times treatment on development to the blastocyst stage. Effects of arsenic concentration were separated into individual degree of freedom contrasts to determine linear, quadratic, and cubic effects of arsenic concentration. The polynomial coefficients to perform this analysis were calculated using the PROC IML procedure of SAS. In all analyses, replicate was considered a random effect, while other main effects were considered fixed. Therefore, treatment \times replicate was used as the error term to test effect of treatment.

RESULTS

Effects of Arsenic on Two-Cell Embryos

To determine whether arsenic exposure could induce apoptosis, two-cell embryos were cultured for 12 hr with various concentrations of arsenic and the percent of blastomeres positive for the TUNEL reaction determined at 24 hr after arsenic exposure (i.e., at 12 hr after the end of arsenic treatment). The proportion of two-cell embryos exhibiting apoptosis in the absence of arsenic exposure was very low—in fact, only 1 of 27 embryos exhibited apoptosis in the absence of arsenic exposure. Treatment of embryos with arsenic did not increase the percentage of cells labeled with the TUNEL reagent (Fig. 1). Arsenic also did not increase group 2 caspase activity of two-cell embryos as determined by cleavage of the substrate PhiPhiLux-G₁D₂ and generation of green fluorescence (Fig. 2C). There was little or no detectable caspase activity in control or arsenic-treated embryos.

Effects of arsenic on developmental competence of embryos were ascertained in two ways. First, cell number at 24 hr after initiation of a 12-hr arsenic exposure was determined. Cell number was affected by concentration of arsenic in a dose–response relationship best described as linear ($P < 0.001$) (Fig. 1). In the second experiment, development to blastocyst was determined for embryos exposed to arsenic for 12 hr at the two-cell stage. Again, the proportion of embryos developing to

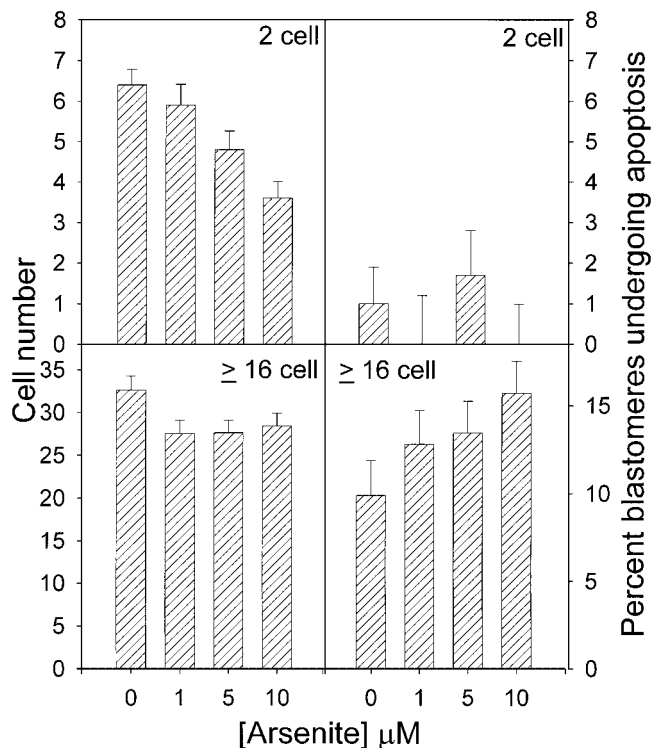


Fig. 1. Developmental changes in effects of arsenic on embryonic growth and induction of apoptosis. Embryos were collected at the two-cell stage or on day 5 after insemination (embryos ≥ 16 cells) and exposed to various concentrations of arsenic for 12 hr. At 24 hr after initiation of arsenic exposure, embryos were stained using propidium iodide and the TUNEL procedure to determine the cell number and degree of apoptosis. The experiment was replicated on three occasions with a total of 19–27 embryos per group. Results are least-squares means \pm SEM.

the blastocyst stage was reduced by arsenic treatment in a linear manner (linear contrast, $P < 0.01$) (Fig. 3).

Effect of Arsenic on Embryos ≥ 16 Cells at Day 5 After Insemination

In contrast to two-cell embryos, apoptotic cells were observed in the absence of arsenic exposure (9.9% of

blastomeres were positive for the TUNEL reaction). Culture of embryos with arsenic increased the proportion of blastomeres that exhibited apoptosis in a concentration-dependent manner best described as a linear response ($P = 0.056$) (Fig. 1). Exposure to 10- μM arsenite also increased group 2 caspase activity as determined by cleavage of PhiPhiLux- G_1D_2 (compare Fig. 2A for embryo without arsenic with Fig. 2B for embryo with arsenic).

As shown in Figure 1, cell number at 24 hr after initiation of a 12-hr arsenic exposure was reduced by increasing concentrations of arsenic in a cubic manner ($P < 0.05$). The cubic nature of the response reflects the fact that the reduction in cell number caused by arsenic exposure was similar for all concentrations of arsenic. In the second experiment, development to blastocyst was determined for embryos exposed to arsenic for 12 hr. The proportion of embryos developing to the blastocyst stage was reduced by arsenic treatment in a linear manner ($P < 0.001$) (Fig. 3).

A combined set of blastocyst development data from exposure at the two- and ≥ 16 -cell stage was also analyzed. In this case, there was an effect of arsenic concentration on development ($P < 0.05$), but there was no concentration \times stage of development interaction. Such a result indicates arsenic exposure affected development to the blastocyst stage equally at both embryonic stages.

Heat Shock Effects on Embryonic Development

To compare developmental changes in resistance to arsenic to those with heat shock, an experiment was performed in which development to blastocyst was determined for embryos exposed to heat shock at the two-cell stage or for embryos ≥ 16 -cells (Fig. 4). Application of heat shock at the two-cell stage reduced the proportion of embryos that developed to the blastocyst stage ($P < 0.001$). In contrast, application of heat shock to embryos ≥ 16 -cells at day 5 after fertilization did not affect the proportion of embryos that became blastocysts. When a combined data set was analyzed, there was a stage of exposure \times temperature interaction ($P = 0.05$), indicating that effects of heat shock were

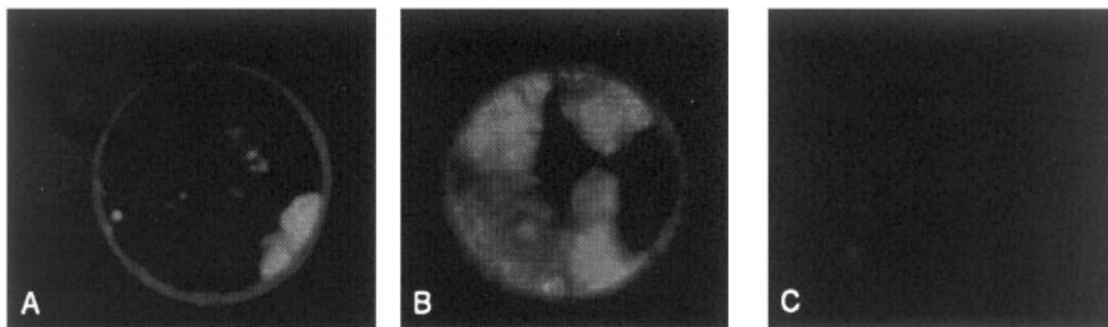


Fig. 2. Group 2 caspase activity in bovine embryos. Represented are an embryo ≥ 16 -cells collected at day 5 post IVF (A), an arsenic-treated embryo ≥ 16 -cells collected at day 5 (B) and a two-cell embryo treated with arsenic (C). Caspase activity was monitored by a fluorogenic substrate for group 2 caspases. Note that for the control day 5 embryo,

only a few cells stained intensely for caspase, while there was intense reaction product over more than 50% of the arsenic-treated day 5 embryo. There was no detectable caspase activity for the arsenic-treated two-cell embryo.

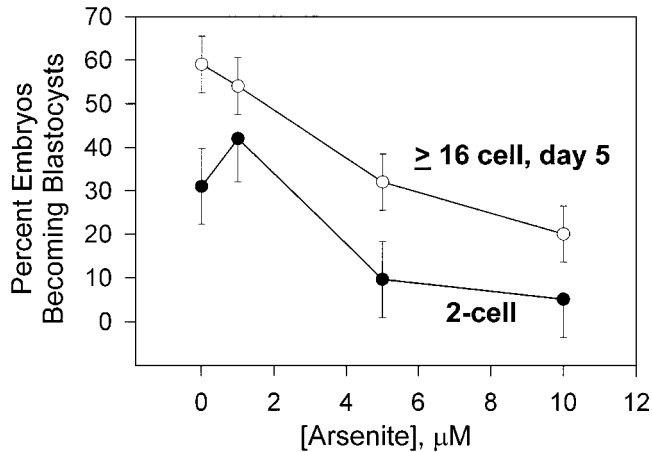


Fig. 3. Developmental changes in effects of arsenic on progression to the blastocyst stage of development. Embryos were collected at the two-cell stage or on day 5 after insemination (embryos ≥ 16 -cells) and exposed to various concentrations of arsenic for 12 hr. The proportion of embryos developing to the blastocyst stage was determined at day 8 after insemination. The experiment was replicated on a total of five occasions (two-cell; 59–86 embryos per group) or nine occasions (≥ 16 -cell; 54–62 embryos per group). Results are least-squares means \pm SEM.

less when applied at the ≥ 16 -cell stage than when applied at the two-cell stage.

DISCUSSION

Previous research has shown that pre-implantation embryos can acquire increased resistance to certain cellular stresses as they advance in development. For bovine embryos, this has been shown to be the case for cyanide toxicity (Donnay et al., 2000) and heat shock

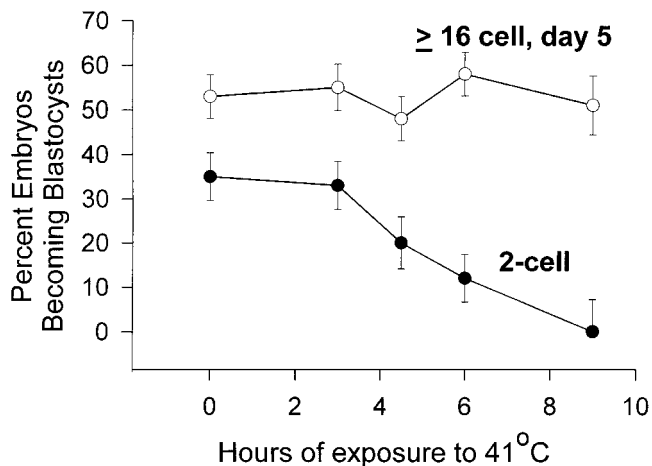


Fig. 4. Developmental changes in effects of heat shock on progression to the blastocyst stage of development. Embryos were collected at the two-cell stage or on day 5 after insemination (embryos ≥ 16 cells) and exposed to 41°C for 3, 4.5, 6, or 9 hr. The proportion of embryos developing to the blastocyst stage was determined at day 8 after insemination. The experiment was replicated on a total of eight occasions (two-cell; 61–86 embryos per group) or 10 occasions (≥ 16 -cell; 58–61 embryos per group). Results are least-squares means \pm SEM.

(Edwards and Hansen, 1997; Ju et al., 1999). Present results confirm that embryos that are ≥ 16 -cells in size at day 5 after insemination are better able to continue development to the blastocyst stage following heat shock than if heat shock occurs at the two-cell stage. Heat shock and arsenic cause some of the same detrimental actions on cells including free radical production (Burdon et al., 1987; Wang et al., 1996; Chen et al., 1998), alterations in protein structure (Oehme, 1972; Kampinga, 1993) and increased apoptosis (Fuse et al., 1998; Wang et al., 1996; Boreham et al., 1997; Chen et al., 1998). Thus, it was hypothesized that embryos would undergo developmental changes in resistance to arsenic that patterned the developmental pattern for resistance to heat shock. This was not the case, however. Rather, the developmental acquisition of cytotoxicity that occurs for heat shock does not occur for resistance to arsenic, at least within the range of developmental stages examined.

This conclusion is made despite the fact that embryos ≥ 16 -cells may be slightly more resistant to arsenic than two-cell embryos. Whereas increasing concentrations of arsenic caused an increasing magnitude in reduction of cell number when applied at the two-cell stage, the reduction in cell number 24 hr after arsenic exposure at the ≥ 16 -cell stage was similar at concentrations of 1, 5, and 10 μM arsenic. If real, this subtle difference in resistance to arsenic is of minimal significance for the overall survival of the embryo, however, since arsenic was equally effective at blocking development to the blastocyst stage regardless of whether exposure occurred at the two-cell or ≥ 16 -cell stage.

There are two implications to the fact that developmental acquisition of resistance to arsenic toxicity is minimal during the stages of embryonic development examined. First, pregnant females are likely to be susceptible to embryonic loss induced by arsenic toxicity during a relatively wide window in early pregnancy. In contrast, effects of maternal heat stress on embryonic survival are greatest when occurring the day after insemination and decline at later stages of early pregnancy (Ealy et al., 1993). The second implication is that the mechanisms that the pre-implantation bovine embryo acquire to counteract effects of heat shock are not sufficient to counteract arsenic toxicity. Perhaps, the degree to which arsenic causes pathological changes in the embryo, such as increased free radical production and protein denaturation, are of greater magnitude than is the case for a 41°C heat shock. Another possibility is that arsenic causes decreased development through cellular pathways that are not involved in heat shock and that advanced embryos do not possess mechanisms to prevent these types of arsenic-induced cellular pathologies. For example, arsenic alters protein structure through a different mechanism than heat shock. Whereas heat shock disrupts hydrogen bonding, arsenic is a sulphhydryl reagent (Léonard and Lauwerys, 1980).

Heat shock can induce apoptosis in a limited number of blastomeres (Paula-Lopes and Hansen, 2002a).

Heat-induced apoptosis first occurs at the 8–16 cell stage at day 4 after insemination. There is thus a temporal relationship between ability of embryos to undergo apoptosis and resistance to heat shock. It has been suggested that a limited amount of heat-induced apoptosis may be beneficial to the survival of the heat-shocked embryo by removing damaged blastomeres (Paula-Lopes and Hansen, 2002a). In fact, it has been recently shown that inhibition of heat-induced apoptosis exacerbates the inhibitory effect of heat shock on embryonic development (Paula-Lopes and Hansen, 2002b). The present results demonstrate that arsenic can also activate group 2 caspases and cause apoptosis in the pre-implantation bovine embryo. Like for heat shock, arsenic-induced apoptosis is limited in scope (the average percent of apoptotic cells after exposure of ≥ 16 -cell embryos to 10- μ M arsenic was 15.7%) and developmentally-regulated (i.e., did not occur at the two-cell stage). However, development of the capacity for arsenic-induced apoptosis was not sufficient to prevent effects of arsenic on development to the blastocyst stage.

In conclusion, present results indicate that both heat shock and arsenic can compromise development of pre-implantation bovine embryos. However, while embryos become more resistant to heat shock as they reach the ≥ 16 -cell stage, acquisition of tolerance to arsenic does not occur by this stage. Thus, the embryo is likely to be sensitive to arsenic toxicity during a wider period of time in development than is the case for heat shock. In addition, mechanisms that the embryo acquires to allow for increased thermal resistance are not sufficient to protect the embryo from deleterious actions of arsenic. These results highlight the fact that despite shared pathological effects and cellular cytoprotective mechanisms, the ontogeny of cellular resistance to embryotoxic conditions can vary widely between specific embryo stressors.

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REFERENCES

Boreham DR, Doling J-A, Maves SR, Miller S, Morrison DP, Mitchel REJ. 1997. Heat-induced thermal tolerance and radiation

- resistance to apoptosis in human lymphocytes. *Biochem Cell Biol* 75:393–397.
- Burdon RH, Gill VM, Rice-Evans C. 1987. Oxidative stress and heat shock protein induction in human cells. *Free Radic Res Commun* 3:129–139.
- Chen YC, Lin-sbiau SY, Lin JK. 1998. Involvement of reactive oxygen species and caspase-3 activation in arsenite-induced apoptosis. *J Cell Physiol* 177:324–333.
- De SK, Paria BC, Dey SK, Andrews GK. 1993. Stage-specific effects of cadmium on pre-implantation embryo development and implantation in the mouse. *Toxicology* 80:13–25.
- Dix DJ, Garges JB, Hong RL. 1998. Inhibition of hsp70-1 and hsp70-3 expression disrupts pre-implantation embryogenesis and heightens embryo sensitivity to arsenic. *Mol Reprod Dev* 51:373–380.
- Donnay I, Bernard S, Feugang JM, Kaidi S, Moens A. 2000. Effect of cyanide (KCN) on bovine embryo development and apoptosis. *Theriogenology* (abstract) 53:350.
- Ealy AD, Drost M, Hansen PJ. 1993. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. *J Dairy Sci* 76:2899–2905.
- Edwards JL, Hansen PJ. 1997. Differential responses of bovine oocytes and pre-implantation embryos to heat shock. *Mol Reprod Dev* 46:138–145.
- Fuse T, Yoon K-W, Kato T, Yamada K. 1998. Heat-induced apoptosis in human glioblastoma cell line A172. *Neurosurgery* 42:843–849.
- Giavini E, Bonanomi L, Ornaghi F. 1984. Developmental toxicity during the pre-implantation period: Embryotoxicity and clastogenic effects of chlorambucil in the rat. *Teratog Carcinogen Mutagen* 4:341–348.
- Ju JC, Parks JE, Yang X. 1999. Thermotolerance of IVM-derived bovine oocytes and embryos after short-term heat shock. *Mol Reprod Dev* 53:336–340.
- Kampinga HH. 1993. Thermotolerance in mammalian cells. Protein denaturation and aggregation, and stress proteins. *Cell Sci* 104:11–17.
- Küchenhoff AR, Eckard R, Buff K, Fischer B. 1999. Stage-specific effects of defined mixtures of polychlorinated biphenyls on in vitro development of rabbit pre-implantation embryos. *Mol Reprod Dev* 54:126–134.
- Léonard A, Lauwerys RR. 1980. Carcinogenicity, teratogenicity and mutagenicity of arsenic. *Mutat Res* 75:49–62.
- Morales H, Tilquin P, Rees JF, Massip A, Dessy F, Van Langendonck A. 1999. Pyruvate prevents peroxide-induced injury of in vitro pre-implantation bovine embryos. *Mol Reprod Dev* 52:149–157.
- Müller WU, Streffer C, Fisher-Lahdo C. 1986. Toxicity of sodium arsenite in mouse embryos in vitro and its influence on radiation risk. *Arch Toxicol* 59:172–175.
- Oehme FW. 1972. Mechanisms of heavy metal toxicities. *Clin Toxicol* 5:151–167.
- Paula-Lopes FF, Hansen PJ. 2002a. Heat-shock induced apoptosis in pre-implantation bovine embryos is a developmentally-regulated phenomenon. *Biol Reprod* 66:1169–1177.
- Paula-Lopes FF, Hansen PJ. 2002b. Apoptosis is an adaptive response in bovine pre-implantation embryos that facilitates survival after heat shock. *Biochem Biophys Res Commun* 295:37–42.
- Rivera RM, Hansen PJ. 2001. Development of cultured bovine embryos after exposure to increased temperatures in the physiological range. *Reproduction* 121:107–115.
- SAS. 1989. SAS User's Guide Version 6. Statistical Analysis System Institute, Inc., Cary, NC.
- Wang TS, Kuo CF, Jan KY, Huang H. 1996. Arsenite induces apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species. *J Cell Physiol* 169:256–268.
- Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem DA, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. 1998. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. *Genes Dev* 12:806–819.
- Xiang J, Chao DT, Korsmeyer SJ. 1996. BAX-induced cell death may not require interleukin-1 β -converting enzyme-like proteases. *Proc Natl Acad Sci USA* 93:14559–14563.