To be or not to be—Determinants of embryonic survival following heat shock

P.J. Hansen *

Department of Animal Sciences, University of Florida, PO Box 110910, Gainesville, FL 32611-0910, USA

Abstract

Elevated temperature can reduce developmental competence of the preimplantation embryo. Whether an embryo survives elevated temperature depends on its genotype, stage of development, exposure to regulatory molecules and redox status. Following fertilization, the embryo is very sensitive to heat shock. By Days 4–5 after insemination, however, the embryo has acquired increased resistance to elevated temperature. One system that may potentiate embryonic survival at later stages of embryonic development is the apoptosis response—inhbition of apoptosis responses at Day 4 exacerbated effects of heat shock on development. Embryo responses to heat shock at Days 4–5 also depend upon genotype because Bos indicus embryos are more resistant than embryos from non-adapted B. taurus. Some experiments (although not all) indicate that survival following heat shock can be increased by reducing oxygen tension, suggesting involvement of reactive oxygen species or hypoxia-induced factors. Embryonic responses to heat shock are also affected by regulatory molecules that act to modify cellular physiology and improve cell survival. The best characterized of these is insulin-like growth factor-1 (IGF-1). Actions of IGF-1 to allow development following heat shock are independent of its anti-apoptotic actions because inhibition of the phosphatidylinositol-3 kinase pathway through which IGF-1 blocks apoptosis does not prevent thermoprotective effects of IGF-1 on development. Identification of specific determinants of embryonic survival creates the opportunity for new strategies to improve pregnancy rates in animals exposed to heat stress. Many environmental perturbations activate similar cellular responses. Therefore, molecular and cellular systems that improve embryonic survival to heat shock may confer protection from other embryotoxic conditions.

#2007 Elsevier Inc. All rights reserved.

Keywords: Embryo; Heat shock; Apoptosis; Insulin-like growth factor-1; Bovine

1. Introduction

A key determinant of embryonic development is the microenvironment formed by the oviduct and uterus. Perturbations in that environment can lead to altered cellular function and errors in development. Adverse environmental conditions do not necessarily lead to embryonic death because mechanisms exist within the embryo to preserve key components of cellular function in the face of stress. Whether or not an embryo survives a stressful environment depends upon its genetic and non-genetic inheritance, its internal state (including stage of development and presence or absence of biochemical pathways promoting survival), and the presence of cytoprotective molecules in the microenvironment that alter cellular function to provide protection from adverse stimuli. Thus, the embryo exposed to stress may either adjust successfully to the adverse environment and continue development or fail to adjust and die as a result of extensive necrosis or apoptosis.

One of the most well-characterized stresses affecting embryonic development is exposure to elevated...
temperature (i.e., heat shock). Interest in this phenomenon has been driven in large part by the large reduction in female fertility in hot environments. In lactating dairy cattle, where the metabolic demands of lactation exacerbate the decline in fertility caused by hot environments [1], heat stress arrests embryonic development in vivo [2,3]. There are probably a multitude of causes for reduced embryonic survival during heat stress including alterations in hormone secretion and uterine blood flow [4]. Importantly, elevated temperature can act directly on the embryo to compromise development [5–7]. The importance of disruption of the embryo as compared to the uterine environment in which the embryo resides was highlighted by an experiment using reciprocal embryo transfer in sheep [8]. Three days of heat stress compromised the embryo itself and, only to a lesser extent, the capacity of the uterus to support embryonic development. In contrast, recent studies in mice suggest that maternal heat stress compromises embryonic survival primarily because of effects on the oviduct rather than on the embryo directly [9].

The main purpose of this review is to describe factors intrinsic and extrinsic to the embryo that determine whether it survives heat shock. A second purpose is to delineate what is known about the mechanisms through which these factors control thermotolerance. Primary focus will be on domestic cattle because they have been studied most extensively but reference will be made to other species when pertinent.

2. Determinants of embryonic resistance to heat shock

2.1. Stage of development

Preimplantation bovine embryos become more resistant to elevated temperature as they advance in development. Data illustrating this concept are presented in Fig. 1. Heat-shock conditions that caused a large reduction in the proportion of two-cell embryos becoming blastocysts had intermediate effects when applied to four to eight-cell embryos and little or no effect on development of morulae [5,10,11]. Similarly, heat shock on Days 0 and 2 relative to fertilization was more detrimental to subsequent development to the blastocyst stage than heat shock on Day 4 or 6 [7] and heat shock was more detrimental when applied at Day 3 after insemination than at Day 4 [12]. Developmental acquisition of thermotolerance is also apparent in vivo. Exposure of cows to heat stress reduced development and viability of embryos on Day 8 after estrus if superovulated cows were exposed to heat stress at Day 1 after estrus but not if heat stress was imposed on Day 3, 5 or 7 [3]. Heat stress is also more likely to cause embryonic mortality when applied sooner after estrus in sheep [13] and pigs [14]. In the mouse, in contrast, there was little difference in resistance to heat shock between embryos at the two-cell, four-cell and morula stage of development [15].

The cellular consequences of heat shock have been best described for the bovine two-cell embryo. At that stage, exposure to 41 °C causes disruption of the microfilament and microtubule network which in turn results in a redistribution of organelles into the interior of the cell [16,17]. There is also an increase in the proportion of mitochondria which have a swollen phenotype indicative of depolarization [16,17] and, consistent with the idea of reduced oxidative phosphorylation, a tendency for reduced oxygen consumption [18]. Despite these apparently serious changes in cellular function, the 2-cell embryo exposed to 41 °C is capable of development until the 8–16-cell stage, when development becomes blocked [18]. Given that this is also the stage when the embryo first becomes capable of transcription on a major scale [19], it may be that heat shock reduces ability of the embryo to become transcriptionally competent.

2.2. Genotype

There is evidence that cattle which have evolved in hot climates have acquired genes that protect embryos from elevated temperature (Fig. 2). When heat shock was applied at Day 4 or 5 after fertilization, the
reduction in development was less for Brahman and Nelore embryos (Bos indicus breeds) and Romosinuano embryos (a thermally adapted, Criollo-type B. taurus) than for Angus or Holstein embryos [20–22]. Differences also exist in survival following transfer into recipients [22]. For recipients receiving Nelore embryos, however, there were no differences in pregnancy rate between those receiving heat-shocked embryos and those receiving control embryos.

These observations make cattle the first example of an endothermic species having genetic adaptations in cellular resistance to elevated temperature. It is possible that the same genes conferring cellular thermotolerance are present in Brahman, Nelore, and Romosinuano, because of the contribution of B. indicus genotypes to Criollo cattle breeds and of B. taurus genotypes to New World B. indicus breeds [23,24]. Another tropically adapted B. taurus, the Senepol, also shows evidence for increased cellular resistance using lymphocytes as a model [20]. It is also possible that distinct thermotolerance genes are present in different breeds of cattle.

It is not clear whether breed differences exist before the embryonic genome is fully activated at the 8–16-cell stage. In a study with small number of embryos, there were no differences between Brahman and Holstein embryos in development to the blastocyst stage when a heat shock of 41 °C was applied at the two- to eight-cell stages [25]. In this same study, however, there was a tendency for more Brahman embryos exposed to heat shock to advance past the eight-cell stage compared to Holstein embryos.

Fig. 2. Effect of genotype on embryonic survival after heat shock. Embryos were produced in vitro and those ≥9 cells harvested at Day 4 after insemination, placed in new drops and cultured at either 38.5 °C continuously (black bars) or to 41 °C for 6 h and 38.5 °C thereafter (open bars). Development was poor in Senepol. When data from this breed were excluded, there were breed \times temperature interactions for each trait (P < 0.05). The figure is reproduced from Reproduction [20] with permission (© Society for Reproduction and Fertility, 2003).

There are conflicting data regarding whether genotype effects on cellular thermotolerance are conferred equally by the oocyte and spermatozoa. Experiments by Block et al. [26] with embryos at Day 4 after insemination indicated that oocyte genotype but not sperm genotype is a determinant of embryonic resistance to heat shock. Embryos produced by insemination of Brahman oocytes with Angus spermatozoa were more thermotolerant than embryos produced by insemination of Holstein oocytes with Angus spermatozoa. In contrast, there were no differences in thermotolerance between embryos produced by insemination of Holstein oocytes with either Brahman or Angus spermatozoa. These results could be interpreted to indicate that either genes conferring thermotolerance are paternally imprinted or that embryonic thermotolerance depends upon some genetically controlled factor produced in the oocyte and still active in the embryo. Results of Block et al. [26] are contradicted by experiments by Barros et al. [22] who compared the effects of heat shock on Day 4 embryos produced by either Nelore or Angus sires. In some breed combinations, Nelore-sired embryos were more resistant to heat shock than Angus-sired embryos.
2.3. Insulin-like growth factor-1

Among its many actions, insulin-like growth factor-1 (IGF-1) is a survival factor that protects cells from a variety of stresses. In the preimplantation mouse embryo, IGF-1 has been reported to block effects of hydrogen peroxide on development [27] as well as apoptosis induced by camptothecin [28], actinomycin D [28] and tumor necrosis factor-α [29,30]. Insulin-like growth factor-1 also blocked apoptosis in rabbit embryos caused by ultraviolet radiation [31]. In the preimplantation bovine embryo ≥16 cells, IGF-1 diminishes the reduction in development and the increase in apoptotic cells caused by heat shock [32,33]. Effects on development are illustrated in Fig. 3. An important, yet unanswered question is whether protective effects of IGF-1 also occur at early stages of development when the embryonic genome is not fully activated.

Insulin-like growth factor-1 can activate several signaling pathways in cells [34–37]. The anti-apoptotic actions of IGF-1 are mediated through activation of phosphatidylinositol-3 kinase (PI3K) and protein kinase B (PKB) (otherwise called Akt). Protein kinase B/Akt in turn phosphorylates and inactivates pro-apoptotic proteins such as Bad, caspase-9, and forkhead transcription factors and induces transcription of antiapoptotic proteins Bcl-2, Bcl-xL and NFκB. In addition, IGF-1 can promote proliferation through at least two different pathways including insulin receptor substrate-mediated activation of PI3K and Grb2-mediated activation of the guanine nucleotide exchange factor SOS, Ras, Raf, and mitogen activated protein kinase (MAPKinase), with the MAPKinase being the most important.

Anti-apoptotic actions of IGF-1 in the heat-shocked bovine morula were blocked by the PI3K inhibitor LY 294002 while actions of IGF-1 to increase cell number were not affected by LY 294002 but were inhibited by the MAPKinase inhibitor PD 98059 [33]. Thus, IGF-1 acts in the bovine morula to block apoptosis through the PI3K/PKB pathway and stimulates cell proliferation through a pathway involving MAPKinase. Interestingly, the ability of IGF-1 to prevent a reduction in development to the blastocyst stage by heat shock occurred even when the anti-apoptotic actions of IGF-1 were blocked by LY 294002 [33]. Thus, the mechanism by which IGF-1 preserves developmental competence following heat shock does not depend upon regulation of apoptosis responses.

2.4. Oxygen

There is conflicting evidence whether inhibition of development of the preimplantation embryo by elevated temperature is dependent upon the concentration of oxygen in which embryos are cultured. The heat-shock experiments with bovine embryos discussed to date were performed under conditions in which culture was performed in an atmosphere of 5% CO₂ in air (i.e., about 20.95% oxygen by volume). Two studies from our laboratory suggest that similar effects of heat shock on development of two-cell embryos are seen when embryos are cultured in 5% O₂, either continuously [18] or during heat shock [38]. In contrast, more recent results, also from our laboratory, indicate that effects of heat shock on development and apoptosis do not occur when embryos are cultured in 5% O₂ [39]. The reason for this discrepancy is not clear and additional research is required to characterize the role of oxygen concentration in determining embryonic responses to heat shock and identify determinants that modify oxygen effects.

3. Cellular mechanisms controlling embryonic resistance to heat shock

3.1. Heat-shock proteins and induced thermotolerance responses

Cells exposed to heat shock can undergo an array of biochemical responses for protection from elevated temperature. This response is often documented experimentally as the induced thermotolerance
response from where exposure to a mild heat shock protects cells from a subsequent, more severe heat shock [40]. One group of molecules that play a role in induced thermotolerance are the heat-shock proteins and the most well characterized of these are proteins of the heat-shock protein 70 (HSP70) family. Some hsp70 genes are highly inducible by heat shock, some are induced by heat shock but are also expressed constitutively (heat-shock cognate 70 or hsc70) and some are regulated by glucose concentration (glucose regulated protein 78). The HSP70 proteins are molecular chaperones that protect cells from elevated temperature by stabilizing intracellular proteins and organelles and by inhibition of apoptosis [41]. The phenomenon of induced thermotolerance has been described in preimplantation bovine and mouse embryos [15,42–45]. Acquisition of the response is developmentally regulated. In the cow, induced thermotolerance has been observed at the blastocyst stage [43] but not at the two-cell stage [46] nor at Day 3 after insemination [12]. One report, using development as the endpoint, indicates induced thermotolerance does not occur at Day 4 [12]. In contrast, using apoptosis as the endpoint, results from another study indicates induced thermotolerance is possible in embryos at Day 4 after insemination [45]. In mice, induced thermotolerance has been observed at the eight-cell, morula and blastocyst stages of development but not at the one- or two-cell stage [15,42–44].

Developmental acquisition of the induced thermotolerance response involves more than capacity for HSP70 synthesis. Indeed, preimplantation embryos gain the ability for heat-shock induced synthesis of the heat-inducible HSP70 before induced thermotolerance develops—as early as the two-cell stage in both cattle [47] and mice [48]. The increase in HSP70 synthesis following heat shock in the two-cell bovine embryo involves increased transcription [49] even though widespread embryonic genome activation does not occur until the 8–16-cell stages [19]. One possibility is that it is the pattern of synthesis (i.e., amount and duration) of HSP70 rather than the acquisition of heat inducibility itself that determines timing of induced thermotolerance. In the mouse, there are developmental effects on steady-state levels of mRNA for HSP70 with respect to magnitude and duration of increases following heat shock [48]. Other molecules may also play a role in timing of induced thermotolerance responses. One molecule required for induced thermotolerance in the mouse morula is glutathione: addition of the glutathione synthesis inhibitor buthionine sulfoxomine inhibited induced thermotolerance [44].

Genetic effects on resistance of embryos to heat shock probably are not caused by differential HSP70 synthesis. In lymphocytes, there were no significant differences between Brahman, Senepol and Angus in the increase in amounts of the heat-shock inducible HSP70 caused by culture at elevated temperature [50]. There are also no reports that IGF-1 regulates HSP70 synthesis. In addition, it is unlikely that any increase in embryonic resistance to heat shock caused by culture in a low oxygen environment is due to changes in heat-shock protein synthesis. Porcine blastocysts produced by culture in a 5% O2 atmosphere had lower amounts of HSC70 than blastocysts produced in air [51].

3.2. Redox status

Free radicals have been implicated in effects of heat shock on mouse embryos [9,44,52] but the situation is not clear for the preimplantation bovine embryo. One report indicates that exposure of embryos to 41 °C increased free radical production at Days 0 and 2 relative to insemination but not at Days 4 and 6 [7]. Moreover, heat shock only reduced development to the blastocyst stage when applied at Day 0 or 2. This finding could indicate that the developmental acquisition of thermotolerance involves either a reduction in heat-shock induced synthesis of free radicals or an increase in antioxidant status of the embryo. Interestingly, recent evidence suggests that mitochondria are more active in the early stages of bovine embryonic development [53] and presence of active mitochondria could enhance the free radical response to heat shock. In addition, intracellular concentrations of the cytosolic antioxidant glutathione are lowest in bovine embryos during the two- to eight-cell stages [54].

Data also exist that are not supportive of a major role for free radicals in actions of heat shock on the bovine embryo. Culture at 41 °C did not cause a reduction in glutathione concentrations in two-cell bovine embryos [18]. In addition, effects of heat shock on development were not reversed by treatment with the antioxidants vitamin E [11], glutathione [55], and glutathione ester [55] and were only partially alleviated by dithiothreitol (LA de Castro e Paula and Hansen, unpublished).

There were no differences between Brahman, Senepol, Angus, and Holstein lymphocytes in the magnitude of reduction of glutathione following heat shock [20]. Effects of IGF-1 on free radical metabolism have not been evaluated in embryos but IGF-1 has been reported to block effects of hydrogen peroxide on development [27].
3.3. Apoptosis

One of the consequences of exposure to elevated temperature in the preimplantation bovine embryo is induction of apoptosis among a fraction of the blastomeres. The extent of apoptosis after exposure to 41 °C is not large. In one experiment using embryos ≥16 cells collected at Day 5 after insemination, the proportion of blastomeres that were positive for the TUNEL reaction was 15.8% for embryos exposed to 41 °C for 9 h versus 8.0% for embryos cultured at 38.5 °C [45]. Thus, embryos experiencing apoptosis are not necessarily prevented from further development as long as apoptosis is limited. Induction of apoptosis is mediated through activation of group II caspases because addition of z-DEVD-fmk, a specific inhibitor of group II caspases, blocks induction of apoptosis by heat shock [56]. Group II caspases are those caspases that have substrate specificity for DEXD [57] and include the execution caspases 3 and 7 that are responsible for destruction of structural and regulatory proteins that lead to DNA damage and cell demise [58]. The other group II caspase is caspase 2, which has been implicated as an upstream initiator of mitochondrial permeability [59].

In the bovine embryo, capacity for apoptosis is a developmentally acquired phenomenon that becomes apparent between the eight and sixteen cell stage. When 8–16-cell embryos are collected at Day 3 after insemination, there is no induction of apoptosis by heat shock [45]. However, 8–16-cell embryos at Day 4 after insemination were capable of apoptosis [45] as are embryos at later stages of development [32,33,60]. At the two- or four-cell stage, heat shock does not induce apoptosis (as determined by TUNEL assay or group II caspase assay) [45]. Similarly, the two-cell embryo is also refractory to apoptosis induced by arsenic [10] and tumor necrosis factor-α [61].

Recently, it was shown that the block to heat-shock induced apoptosis in the two-cell embryo is the result of a failure of mitochondria-mediated caspase-9 activation and caspase-3 mediated cleavage of DNA [62]. These conclusions are based on findings that heat shock did not induce caspase-9 activation at the two-cell stage and that artificial depolarization of mitochondria resulted in activation of caspase-9 and -3 but without DNA degradation as measured by TUNEL assay.

It is likely that as the embryo advances through development and acquires a large number of cells, limited apoptosis is not detrimental to development. Indeed, apoptosis can serve a protective function by eliminating damaged cells from the embryo. Experimental evidence for the concept of apoptosis as a protective response comes from experiments in which z-DEVD-fmk was used to inhibit group II caspase activity [55]. For embryos ≥16 cells at Day 4 after fertilization, z-DEVD-fmk blocked the effects of exposure to 41 °C for 9 h on group II caspase activity and the proportion of TUNEL positive cells. Moreover, the reduction in development of embryos exposed to heat shock for 6–9 h was magnified in the presence of z-DEVD-fmk (temperature x treatment, \( P < 0.05 \)). The figure is reprinted from Biochemical and Biophysical Research Communications [56] with permission from Elsevier.

4. Relevance of the heat-shock model for understanding embryo responses to other stresses

Many forms of cellular stress activate similar endpoints—free radical formation, membrane destabilization, protein denaturation, DNA damage, and apoptosis. Similarly, a common set of cytoprotective mechanisms (including the heat-shock protein response, DNA repair, cell cycle checkpoint regulation, and antioxidant systems) are engaged to protect cells from a variety of adverse environments. It is reasonable to suppose, therefore, that determinants of embryonic response to heat shock may also be important for
embryo survival in response to other stresses. One example supporting this idea is found in the mouse. Experiments with antisense oligonucleotides have shown that the inducible form of HSP70 protects preimplantation mouse embryos from arsenic [63]. Nonetheless, there are specific features of the actions of individual stresses on cellular function so that factors that determine resistance of embryos to heat shock may not have the same action for other adverse environments. For example, the developmental pattern in sensitivity of bovine preimplantation embryos to inhibition in development caused by gossypol [64] and arsenic [10] is different than developmental patterns in sensitivity to heat shock.

Acknowledgements

The author thanks all his students and colleagues, past and present, for helping develop the ideas in this paper. Original recent research from the author’s laboratory was supported in part by Research Grant Award No. US-3551-04 from BARD, The United States–Israel Binational Agricultural Research and Development Fund, Grant No. 2004-34135-14715 from the U.S. Department of Agriculture T-STAR program, and by Funds from the Southeast Milk Producers Milk Checkoff Program.

References


[62] Brad AM, Hansen PJ. The block to apoptosis in bovine two-cell embryos involves inhibition of events leading to caspase 9 activation and to group II caspase-mediated DNA damage. Biol Reprod 2006;93 [Abstract].
