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Expression and Localization of Heat Shock Protein 70 in Frozen-thawed IVF and Nuclear Transferred Bovine Embryos

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ABSTRACT

The objective of this study was to assess the developmental potential *in vitro* produced embryos frozen-thawed with the various containers, and also examined expression and localization of heat shock protein 70 at these embryos. For the vitrification, 2-cell, 8-cell and blastocyst stage embryos produced by *in vitro* fertilization (IVF) and nuclear transfer (NT) were exposed the ethylene glycol 5.5 M freezing solution (EG 5.5) for 30 sec, loaded on each containers such EM grid, straw and cryo-loop, and then immediately plunged into liquid nitrogen. Thawed embryos were serially diluted in sucrose solution, each for 1 min, and cultured in CRI-aa medium. Survival rates of the vitrification production were assessed by re-expanded, hatched blastocysts. There were no differences in the survival rates of IVF using EM grid and cryo-loop. However, survival rates by straw were relatively lower than other containers. The use of cryo-loop resulted in only survival of nuclear transferred embryos (43.7%). Also, there embryos after IVF or NT were analysed by semi-quantitive reverse transcription-polymerase chain reaction (RT-PCR) methods for hsp 70 mRNA expression. Results revealed the expression of hsp 70 mRNA were higher thawed embryos than control embryos. Immunocytochemistry used to localize the hsp70 protein in embryos. Two and 8-cell embryos derived under control condition was evenly distributed in the cytoplasm but appeared as aggregates in some frozen-thawed embryos. However, in the control, blastocysts displayed aggregate signal while Hsp70 in frozen-thawed blastocysts appeared to be more uniform in distribution. Therefore, this result suggests that the exploiting Hsp 70 in the early embryos may be role for protection of stress condition for increase viability of embryos within IVF, NT and there frozen-thawed embryos.

(Key words : Vitrification, Nuclear transfer, Hsp 70, Immnocytochmistry, RT-PCR)

Ⅰ. INTRODUCTION

Embryonic loss through death of the conceptus is the most important cause of reproductive failure in domestic animals and humans. The most of embryo spend part of their life *in vitro*, culture conditions may be important the embryo's developmental and survival rates. Unfortunately, today is relatively low developmental rates *in vitro* (approximately 50% of

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fertilized embryos fail to develop to the blastocyst stage) and suboptimal survival rates following sexing and freezing (30%), prohibit the single biggest limitation to the application of embryo biotechnology. Embryos undergo stress when they are cultured, biopsied, frozen or otherwise manipulated in conjunction with transfer, and the survival rates of these embryos lower than unmanipulated controls. Reproductive performance in domestic animals can be affected by various environmental stresses.

Environmental stresses including temperature, toxic and disease can induce a structurally similar set of heat shock proteins in a wide range of organisms. Heat shock proteins produced by most cells are belong to Hsp70 family (Parsell and Lindquist, 1993). Hsp70 proteins protect cells against adverse effects of stress (Welch, 1987) and function in the absence of stress as a molecular chaperone (Lindquist and Craig, 1988; Ellis and van der Vies, 1991). Members of this family appear in two isoforms, a constitutively synthesized variant (Hsc 70) and highly inducible form (Hsp 70). The major difference these two proteins is that the gene encoding Hsp 70 lacks introns and thus mRNA processing. (Welch and Feramisco, 1982). One of these is 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, 1982). Recently, the ability to inhibit apoptosis has become widely recognized as a function of heat shock proteins since this may contribute to their protective affect on cells (Samali and Orrenius, 1998). It is hypothesized that since mRNA activities is effected in cells after shocks (Welch, 1987; Hendry and Kola, 1991), the production of Hsp70 may be to rapidly increase the level of Hsp70 (cytosolic and nuclear) by bypassing the requirement for mRNA processing activities. Although the mechanisms are unclear, recent evidence also suggests an important role for heat shock proteins in fertilization and early development of mammalian embryos (Anderson, 1998; Dix et al., 1998; Neuer et al., 1999).

Therefore, this study was to assess the developmental potential *in vitro* produced embryos with using of the various containers and examined expression and localization of heat shock protein 70 after it's frozen-thawed. First, a semi-quantitative reverse transcription polymerase chain reaction (RT -PCR) procedure was used to test whether heat shock of *in vitro* produced embryos cause relative changes in the steady-state amounts of hsp70 mRNA. Secondly, produced embryos were examined by immunocytochemistry to characterize changes in the specific location of Hsp70 imposed by heat stress.

Ⅱ. MATERIALS AND METHODS

1. *In Vitro* Maturation (IVM)

Embryos were produced by standard *in vitro* procedure (Xu et al., 1992). Bovine cumulus-oocyte -complexes (COCs) were isolated from the ovaries of slaughtered cow by aspiration using 18 gauge needles. Selection of COCs were based on their morphology and washed in TALP-hepes containing 1 mg/ml bovine serum albumim (BSA: Fraction V). The COCs were then matured in TCM-199 (Gibco BRL, Tokyo, Japan) medium containing 10% (v/v) heat-treated fetal bovine serum (FBS), 25 mM sodium bicarbonate, 0.2 mM sodium pyruvate, 1 μg /ml follicle stimulating hormone (FSH), 1 μg/μl estradiol-17β and 25 μg/ml gentamycin for 22~24 hours at 39℃, 5% CO_2 in humidified air.

2. *In Vitro* Fertilization (IVF)

After maturation, the oocytes were partially separated from cumulus cells by pipetting and washed and transferred in each drop (50 μl droplets) containing fertilization-TALP supplemented with PHE

mixture (2 mM penicillamine, 1 mM hypotaurine and 0.1 mM epinephrine) and 2 μg/ml heparin. Frozen semen thawed in a 37℃ water bath for 15 sec were loaded on top of a discontinuous percoll gradient consisting of 2 ml of 45% and 2 ml of 90% percoll dissolved in sperm-TALP containing 6 mg/ml BSA (fatty acid free) in 15 ml conical tube and centrifuged 2000 rpm for 15 min. The pellet was resuspended in sperm-TALP. After the initial culture period, presumptive zygotes were rinsed and placed in CR1-aa medium supplemented with 3 mg /ml fatty acid free BSA then transferred into 10% FBS added CR1-aa medium with bovine oviductal epithelial cells (BOEC) for culture until the time of each respective experiment (2-cell, 8-cell, and blastocysts). All embryos were cultured at 39℃, 5% CO_2 in humidified air.

3. Nucler Transfer (NT)

Oocytes with a first polar body were enucleated mechanically at 18 to 20h after maturation and chromosome removal was confirmed by staining with Hoechst 33342 dye under ultraviolet light using the methods previously reported (Collas and Barnes, 1994). A single ear cell was introduced into an enucleated oocyte through a slit in zona pellucida and then electrically fused by a double DC pulse (2.63 kv/cm for 30 μsec) delivered by a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA). The fusion was verified by observation 40 min later (0 h for development), and fused oocyte were activated by a 5 min. Oocytes exposed to 5 μM ionomycin for 5 min at 39℃, then exposed to 1.97 mM DMAP for 4 h (Susko-Parrish et al., 1994). They were then co-cultured with bovine oviductal epithelial cell (BOEC) in CR1-aa medium supplemented with 10% FBS at 39℃, 5% CO_2 in humidified air for a period of 2-cell, 8-cell and blastocysts.

4. Freezing Procedures

The vitrification procedure was based on the method originally designed for *in vitro* produced embryos by Martino (1996a,b), Hochi (1996) and Lane (1999) with significant modifications (Hong et al., 1999). In brief, cryoprotectant was on warm plate at 37℃ for 20 min equilibrated. And then, each stage (2, 8 and Blastocysts after IVF or NT) was placed in cryoprotectant of Dulbecco's phosphate-buffered saline (Gibco BRL, Grand Island, NY) supplemented with 5.5 M ethylene glycol (EG 5.5), 1.0 M sucrose and 10% FBS for 30 sec. They were loaded on each container such EM grid, straw and cryo -loop and then immediately plunged into liquid nitrogen.

Electron microscope grid (EM grid; Gilder Co., West Chester, PA) consisted of a 400 mesh cooper in a 3.05 diameter and 0.037 mm thick. Embryos were mounted on EM grid using a fine glass pipette, and surplus cryoprotectant was removed using sterilized tissue. Straw (0.25 ml French mini straw; IMV, L'Aigle, France) was filled with 0.5 M sucrose solution (prepared in m-DPBS containing 10% FBS) followed by a 1.5 cm air bubble, 0.5 cm EG 5.5 solution, 0.5 cm air bubble, 0.5 cm EG 5.5 solution, 0.5 cm air bubble, 2 cm EG 5.5 solution, 1.5 cm air bubble. The remaining part of the straw was filled with 0.5 M sucrose. The straw was sealed with power and heat after embryo loading. To prevent cracking of the straw, the first part of the straw filled with 0.5 M sucrose solution (4 cm) was slowly immersed into liquid nitrogen. Cryo-loop used for vitrification consisted of a nylon loop (20 μm wide; 0.5~0.7 mm in diameter) mounted on a stainless pipe inserted into the lid of a cryovial. The loop was purchased, mounted and epoxied into vials (Hampton Research, Laguna Niguel, CA, USA). Embryos were transferred to the nylon loop that had previously been dipped into cryoprotectant solution to create a thin film. The cryo-loop containing the embryos was then plunged into the cryovial, which is submerged and filled with liquid

nitrogen. Then, vitrification containers containing the embryos were immediately plunged into liquid nitrogen.

5. Thawing Procedures

Cryoprotectant in vitrified-thawed embryos was removed by the 3-step as follows: After a few hours or days of storage in liquid nitrogen, embryos were thawed ultra-rapidly. Each vitrification containers were directly placed in 0.5 M sucrose solution (prepared in m-DPBS containing 10% FBS) as soon as possible. And then, embryos were exposed serially in 0.25 M, 0.125 M sucrose solution with 1 min interval at 37℃. Thawed embryos were washed in culture medium and culture in CR I -aa medium supplemented with 10% FBS at 39℃ in 5% CO_2 in humidified air. Survival rates of the vitrification production were assessed by re-expanded, hatched in culture at 24 h intervals for 3 days.

6. *In Vitro* Culture (IVC)

In vitro produced embryos (IVF, NT and it's frozen-thawed embryos) were cultured in 50 μl of CR1-aa medium supplemented with 3mg/ml fatty acid free BSA for 48 h. Embryos were co-cultured with bovine oviductal epithelial cells (BOEC) in 50 μl of CR1-aa medium containing 10% FBS until the time of each respective experiment. At hours 48, 72 or 168 of *in vitro* culture, visually nomal 2-cell, 8-cell and blastocysts were selected. All embryos were cultured at 39℃, 5% CO_2 in humidified air.

7. Analysis of Gene Expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

In vitro produced embryos (IVF, NT and it's frozen-thawed embryos) were collected at 2-cell, 8-cell and blastocyst stage, and their zona pellucida removed by brief exposure to TL-HEPES containing 0.5% protease. Single embryos were snap frozen in liquid nitrogen and stored at −70℃ prior to use. When required, samples were heated to 39℃ for 1 min, transferred directly to ice, and oligo dT was added. The mixed samples were incubated at 70℃ for 5 min and the chilled on ice. The incubated mixture were transferred to RT premix tube (Bionner) and DEPC-DW added to fill up the reaction volume. RT reaction was performed at 42 ℃, 60 min and 94℃, 5 min. And then RT reaction samples were stored at −20℃. PCR amplification was carried out on 10 μl of the RT product from embryos and PCR premix [1u Taq DNA polymerase, 250 μM dNTPs, 10 mM Tris-HCL, 40 mM KCl, 1.5 mM $MgCl_2$ (Bionner)]. PCR cycle for Hsp70 was 94℃ for 5 min followed by 33 cycles embryos of 94℃ for 30 sec, 59℃ for 30 sec and 72℃ for 30 sec. 5 μl the PCR products were visualized under ultraviolet light on 1.5% agarose gels. Primer sequences for Hsp70 were CAAGATCACCATCACCAACG (5'→3') and CAGCTCCTTCCTCTTGTG (3'→5'). DNA fragment sizes was produced 301 base pairs (bp).

8. Immunocytochemistry

The immunocytochemical protocol was used as previously-described (van Stekelenburg-Hammers et al., 1994) with minor modifications. Oocytes and embryos were rinsed twice in PBS supplemented with 0.5% BSA. Embryos were fixed with 4.0% paraformaldehyde in 0.1 M phosphate buffer for 15 min. The fixed embryos were rinsed in PBS and permeabilised by a 10 min incubation in 0.1% (v/v) Triton X-100 (Fisher Scientific) in PBS. Blocking was accomplished with an incubation in 2.0% (v/v) normal goat serum (NGS; Jackson Immuno Research, Jackson, MI, USA) in PBS for 1 h, followed by a 1 h incubation with the primary antibody against hsp70 (W-27: sc-24, Santa Cruz) diluted 1: 40 in 2.0% (v/v) NGS in PBS. Oocytes and embryos were rinsed in 0.05% (v/v) Tween-20 (Bio

-Rad) in PBS, followed by a 1 h incubation with the secondary antibody (fluorescein isothiocyanate (FITC) conjugated with mouse monoclonal IgG2a (Santa Cruz) diluted 1 : 100 in 0.5% (v/v) BSA in PBS. Embryos were further rinsed in 0.05% (v/v) Tween-20 in PBS, prior to counterstaining the nuclei by a 10 min incubation with propidium iodide (Sigma) diluted 1 : 100 in PBS, and a final rinse in 0.05% (v/v) Tween-20 in PBS. All incubations were performed at room temperature. For mounting, embryos were placed on ethanol pre-cleaned microscope slides in Fluoroguard (Bio-Rad). One gram of glass beads (90 ~ 120 μm in diameter) was thoroughly mixed into 50 ml of medical grade petroleum jelly (Vaseline) and the mixture applied sparingly to the four corners of a 22 × 30 mm glass coverslip. The prepared coverslip was mounted over the embryos and gently pressed into place so as to compress but not crush the embryos. Rubber cement glue was applied to all sides of the coverslip to affix it permanently to the slide.

9. Statistical Analysis

The significant difference among treatment group in each experiment was determined by the t-test and ANOVA analysis (Abacus, Berkeley, CA).

Ⅲ. RESULTS

1. Developmental Rates following IVF and NT Embryos

Developmental compentence of bovine oocytes reconstructed with somatic cells (ear cells) was compared to IVF-derived embryos (Table 1). The cleavage rate of NT embryos (76.3%, 90/118) was not significant difference IVF embryo (84.4%, 689/816). However, developmental rates to blastocysts stage of NT embryos (27.7%, 25/118) were relatively lower than IVF embryos (42.2%, 345/816). Also, total cell numbers of expanded blastocysts after IVF embryos were about 181.1 ± 9.3 per embryo and those of NT embryos were about 168.4 ± 7.5 per embryo.

2. Developmental Rates of Frozen-thawed using Various Containers following IVF and NT Embryos

In this study, three types of vitrification containers, EM grid, straw, cryo-loop were used to cryopreservation of the bovine blastocysts. All blastocysts were belong to Grade 1 according to the International Embryo Transfer Society standards. As shown in Table 2, mean rates of blastocyst re-expanded and hatched after IVF were 87.8 and 71.9 %, 52.5 and 45.0%, and 82.3 and 76.4%, respectively. There were no differences in the survival rates of IVF using EM grid and cryo-loop. However, survival rates by straw were relatively lower than the other containers. The use of cryo-loop resulted in only survival of nuclear transferred embryos (43.7%).

3. Developmental Rates of Frozen-thawed using Various Containers Each Stage Embryo following IVF and NT Embryos

Table 1. *In vitro* development of bovine embryos following IVF and NT

	No. of oocyte	No. (%) of			Mean No. of cell ± SD[2]
		Fused	Cleavaged	Blastcysts[1]	
IVF	816	-	689 (84.4)	345 (42.2)	181.1 ± 9.3
NT	118	90 (76.3)	69 (76.6)	25 (27.7)	168.4 ± 7.5

[1,2] No significant difference was observed.

Table 2. Effect of container on the *in vitro* development of frozen-thawed embryos following IVF and NT

Container type		No. of blastocysts	No. (%) of blastocysts	
			Re-expanded	Hatched
EM-grid	IVF	82	72 (87.8)	59 (71.9)
	NT	37	-	-
Straw	IVF	40	21 (52.5)	18 (45.0)
	NT	11	-	-
Cryo-loop	IVF	34	28 (82.3)[a]	26 (76.4)
	NT	16	7 (43.7)[b]	-

[a,b] Values are significantly different ($P<0.05$).

According to the pre-experiment (Table 2), it was carried out frozen-thawed each stage embryo using cryo-loop. After IVF and NT, two cell embryo at 48h, 8-cell embryo at 72h and blastocysts at 168h were selected for vitrification. Table 3 show survival rates of 2-cell (81.3, 67.4 and 60.4%), 8-cell embryos (75.0, 59.3 and 53.1%) and blastocysts (86.2, 81.0 and 79.3%) after IVF. After NT were 2-cell (43.7, 31.2 and 31.2%), 8-cell (38.8, 22.2 and 11.1%) and blastocysts (60.0, 53.3 and 53.3%).

4. Analysis of Bovine Embryos Derived from Control and Thawed

Hsp70 mRNA was detected in embryo stage (2, 8-cell, and blastocysts) after IVF and NT or it's thawed embryo, and the amount of message relative to GAPDH mRNA was unaffected by treatment. Fig. 1 show hsp70 : GAPDH signal strength ratios in control and frozen-thawed embryos. There was no differences in hsp70 mRNA was between IVF and NT. However, hsp level of control was relatively lower than that of frozen-thawed embryo.

Ⅳ. DISCUSSION

The role of heat shock protein in shielding organism from environmetal stress is illustrated by the large-scale synthesis of these protein by the

Table 3. Effect of embryo stage on the developmental of frozen-thawed using cryo-loop

Embryo stage		No. of embryos	No. of recovered	No. (%) of blastocysts	
				Re-expanded	Hatched
2-cell	IVF	43	35 (81.3)	29 (67.4)[a]	26 (60.4)[a]
	NT	16	7 (43.7)	5 (31.2)[b]	5 (31.2)[b]
8-cell	IVF	32	24 (75.0)	19 (59.3)[a]	17 (53.1)[a]
	NT	18	7 (38.8)	4 (22.2)[b]	2 (11.1)[b]
Blastocysts	IVF	58	50 (86.2)	47 (81.0)[a]	46 (79.3)[a]
	NT	15	9 (60.0)	8 (53.3)[b]	8 (53.3)[b]

[a,b] Values are significantly different ($P<0.05$).

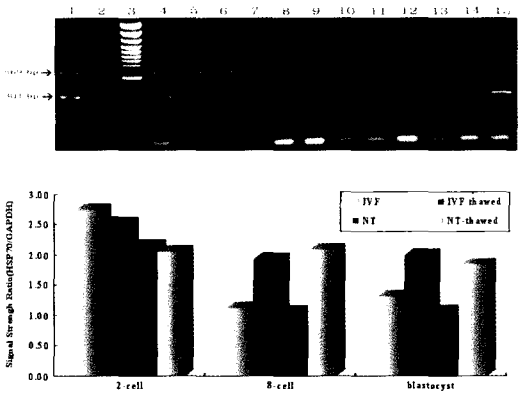

Fig. 1. Quantitative analysis of Hsp70: bar graph for signal strength ratios of Hsp70 (301 bp) to GAPDH (569 bp) amplicon for control and frozen-thawed group from *in vitro* bovine embryos. Lane 1 : positive control, Lane 2 : negative control, Lane 3 : 100 bp molecular ladder, Lane 4, 5 and 6 : IVF control (2, 8-cell, and blastocyst), Lane 7, 8 and 9 : frozen-thawed embryos, respectively. Lane 10, 11 and 12 : NT control (2, 8-cell, and blastocyst), Lane 13, 14 and 15 : frozen-thawed embryos, respectively.

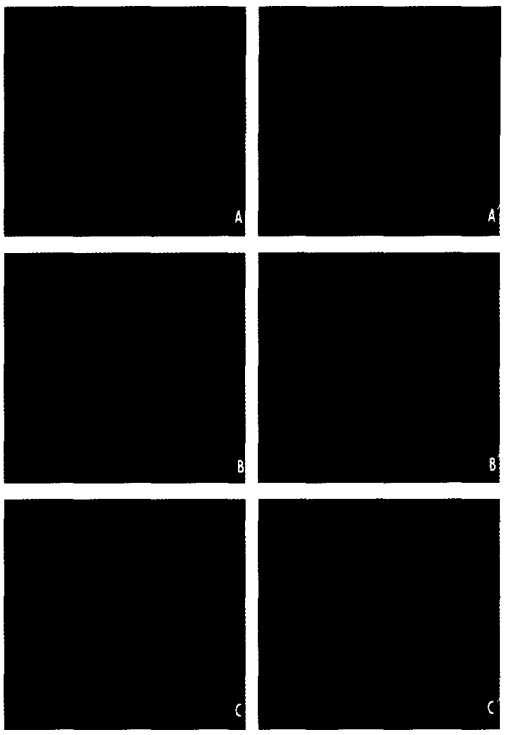

Fig. 2. Immunocytochemistry of bovine embryos. A, B, C : control 2-, 8-cell and blastocyst. A', B', C' : vitrified 2-, 8-cell and blastocyst.

orgnism studied to date. However, recent evidence also suggests an important role for heat shock protein in fertilization and early development of mammalian embryos. Effects of elevated *in vitro* temperature on *in vitro* produced bovine embryos were analysed in order to determine its impact on the expression of heat shock protein 70 (Hsp 70) by control and frozen-thawed after IVF or NT. Semi-quantitative RT-PCR, based on the ratio of co-amplified Hsp70 and GAPDH, revealed that there was an increase in the level of expression of Hsp70 as *in vitro* produced bovine embryos (control and frozen-thawed). In addition, heat inducibility of Hsp70 mRNA was clearly evident by the 8-cell stage and onwards. Immnocytochemical localization of Hsp70 protein in bovine embryos from the same pools as those used for RNA extraction revealed the presence of Hsp70 at all stage examined. After being frozen-thawed, exposure to elevated temperatures caused a redistribution of Hsp70 in 8-cell embryos and blastocysts, but not in 2-cell embryos. In the previous studies, levels of both Hsp70 mRNA (Wrenzycki et al., 1998) and protein (Edwards et al., 1997) were incresed the bovine embryo develops *in vitro*. Also, bovine embryos can produce increased amounts of Hsp70 protein in response to heat shock as early as the 2-cell stage, although the mechanism for that increase was unclear (Edwards & Hansen, 1996).

While α-amanitin blocks the response at the 4-cell stage, it had no effect on Hsp70 response to heat at the 2-cell stage (Edwards et al., 1997). Present results indicate that, at least by the 8-cell

stage, the increase in Hsp70 synthesis in response to heat shock caused by a change in Hsp70 mRNA. The most likely cause for this increase is transcription, since α-amanitin blocks Hsp70 synthesis in response to heat at this stage (Edwards et al., 1997). Consequently, change in amounts of hsp70 mRNA with respect to GAPDH mRNA, particularly from the 8-cell stage onward, may be responsible for the acquisition of thermotolerance after frozen -thawed. The situation with respect to the synthesis of Hsp70 mRNA at 2-cell stage is less clear. Only, it is conceivable that 2-cell bovine embryos are spontaneous transcription or near the maximum level. This possibility is supported by the observation that spontaneous activation of the Hsp70 gene in 2-cell mouse embryos is already at its maximal rate of transcription and cannot be increased by alterations in the environment (Christians et al., 1997).

The present study revealed that Hsp70 was evenly distributed in the cytoplasm of the blastomeres of 8-cell embryos of control. The presence of Hsp 70 within cytoplasm may indicate a role for this protein *in vitro* 8-cell embryo as a molecular chaperone and/or in evoking anti-apoptotic activity; only 5% of bovine embryos at the 8-cell and 16-cell stage have been shown to display evidence of apoptosis (Matwee et al., 1999). Among the frozen -thawed 8-cell embryo blastomeres, aggregate signal for Hsp70 were detected in their cytoplasm. These results provided visual evidence in which *in vitro* 8-cell bovine embryos are able to respond to heat stress by augmentation and/or translocation of the hsp70 protein. In an 8-cell stage bovine embryo, the small nucleus precursor body undergoes a change to form a functional nucleus (Kopecny et al., 1989; Plante and King, 1994), which corresponds with the onset of embryonic genome activation at this stage of development (Frei et al., 1989). Therefore, it is possible that a translocation of Hsp70 into the nucleus of 8-cell bovine embryos occurs upon heat stress to promote the recovery of their nucleolar morphology and, possibly, to protect the nucleus from further damage.

Also in this study, the presence of Hsp70 aggregates in blastocysts was the opposite of that seen for 8-cell embryos. In the control condition, Hsp70 displayed aggregate signal, while the distribution of Hsp70 in frozen-thawed embryos appeared to be more uniform in distribution. The even distribution of Hsp70 in the blastocysts exposed to the heat stress may represent the ability of these embryos to respond to their environment by increasing the amount of Hsp70 (Muller et al., 1985; Hahnel et al., 1986; Edwards et al., 1997). Therefore, this result strongly indicates that the transcription of the Hsp70 gene and the specific location of this protein within the embryos may be play a vital role in the survival and fitness of early bovine embryos challenged with heat stress. Morever, the study of Hsp70 may be provide a means of protecting the embryo from the stress of manipulation, culture and frozen subsequently increse productivity.

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