Journal of Reproduction and Development, Vol. 59, No 2, 2013

—Original Article—

In Vitro Culture of Feline Embryos Increases Stress-induced Heat Shock Protein 70 and Apoptotic Related Genes

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Abstract. Developmental competence and quality of *in vitro* produced embryos has been demonstrated to be lower than *in vivo* derived embryos. This study aimed specifically to determine the effects of *in vitro* culture of feline embryos using various culture densities on developmental competence and expression of stress- and apoptotic-related genes in terms of heat shock protein 70 (*HSP70*) and apoptotic-related (*BAX* and *BCL-2*) gene expressions. In experiment 1, we characterized the inducible form of a feline-specific *HSP70* mRNA sequence, as it has not been previously reported. The primers for feline *HSP70* mRNA were synthesized and tested on heat-treated cat fibroblasts. In experiment 2, feline embryos were cultured at different culture densities (embryo:culture volume; 1:1.25, 1:5 and 1:20). The developmental competence was determined along with *HSP70*, *BAX* and *BCL-2* transcript abundances using quantitative RT-PCR. *In vivo* derived embryos were used as a control group. A partial cat *HSP70* mRNA sequence (190 bp) was characterized and exhibited high nucleotide identity (93 to 96%) with other species. Cleaved embryos cultured at high density (1:1.25) developed to blastocysts at a lower rate than those generated from lower densities. Irrespective of the culture densities used, *in vitro* cultured blastocysts showed increased levels of *HSP70* and *BAX* transcripts compared with *in vivo* counterparts. Blastocysts derived from the highest culture density (1:1.25) showed higher levels of upregulation of *HSP70* and *BAX* transcripts than those cultured at lower culture densities (1:5 and 1:20). In conclusion, increased levels of pro-apoptotic (*BAX*) and stress-response (*HSP70*) transcripts correlated with developmental incompetence of embryos cultured at high embryonic density, indicating that stress accumulated during *in vitro* embryo culture affected the fate for embryo development and quality.

Key words: Apoptosis, Culture density, Embryo, Heat shock protein 70

(**J. Reprod. Dev. 59:** 180–188, 2013)

Currently, the *in vitro* culture system is still suboptimal as a result of lower developmental competence and quality of *in vitro* produced embryos compared with *in vivo* counterparts [1]. Embryo density (embryo number: medium volume ratio) is one of many factors affecting the developmental competence of *in vitro* produced embryos in several species [2–6] including the domestic cat [6]. Although "group" embryonic culture supports development better than culture individually, an excessive culture density could negatively affect embryo development. This is suggested to be caused by increasing cell stress resulting in an aberration of gene expression of embryos [2]. Normally, most cellular stresses contribute to free radical formation, membrane destabilization, protein denaturation, DNA damage and apoptosis. To protect the cell from these stress factors, cytoprotective mechanisms including heat shock protein (HSP) response, DNA repair cycle checkpoint regulation and the antioxidant system are essentially activated [7]. Among the mechanisms associated with the stress responses, heat shock 70 kDa proteins (HSP70s) have been used as the most potential tool for assessing stress response in mammalian embryos during culture [8]. Inducible HSP70 (HSP70) plays essential roles in both normal cell development and protection against stress-induced damage [9–11]. This protein is actively involved in protein folding, assembly and translocation processes of several cytosolic proteins [12]. Under stress conditions, inducible HSP70 is markedly produced to stabilize damaged proteins, allowing them to be consequently repaired or degraded, hence minimizing cell damage [12, 13]. HSP70's upregulation in *in vitro* produced embryos has therefore been used to indicate the embryonic stress induced by an inappropriate culture condition [2, 14].

In addition to heat shock protein expression, apoptosis is also a common mechanism used to eliminate mutated, damaged and healthy unwanted cells [15]. Differences in apoptotic rate between *in vitro* cultured and *in vivo* produced embryos have been demonstrated in several species [15–17]. Basically, increasing incidence of apoptosis in cultured embryonic cells indicates a suboptimal culture condition [18]. To balance the homeostasis between life and death of cells, pro-apoptotic BAX (Bcl-2 associated X) and anti-apoptotic BCL-2 (B-cell lymphoma protein 2) proteins globally play an important role [19], and the ratio between BAX and BCL-2 (BAX/BCL-2

Received: July 16, 2012
Accepted: December 17, 2012
Published online in J-STAGE: January 25, 2013

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ratio) has commonly been used to predict cell fate [20, 21]. In fact, HSP70 also plays a role in anti-apoptotic function [22, 23]; it interacts with intrinsic and extrinsic pathways of apoptosis at a number of steps. These include the inhibition of translocation of BAX into mitochondria, release of cytochrome c from mitochondria, formation of the apoptosome and inhibition of activation of caspase activity. It also modulates JNK, NK-kB and Akt signaling pathways in the apoptotic cascade [23].

In feline species, expression analysis of genes associated with embryo development has become an important tool to understand the physiological response of embryos to their *in vitro* culture environment [24–26]. Though information for several genes has been provided, the change in gene expressions associated with stress-induced apoptosis is not entirely known in this species. To demonstrate the effects of culture density on stress responses, this study determined the expressions of heat shock protein 70 (*HSP70*) and apoptotic-related (*BAX* and *BCL-2*) genes in feline embryos cultured at various culture densities.

Materials and Methods

All chemicals used in the experiments were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Fibroblast cell culture and heat activation

Cat skin fibroblasts were cultured as monolayer in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin in a sealed cap tissue culture flask (25 cm^2, BD Falcon™, Franklin Lakes, NJ, USA). The cells from passages 3 to 5 with 80% confluence were used for heat treatment by submerging the flask sealed with Parafilm under water in a water bath set at 45 C for 45 min (modified from Liu [27]). The cells from one culture flask were harvested immediately at the end of heat treatment (0 h). The other flasks were returned to the incubator and further cultured at 37 C in 5% CO_2 for 3, 6 and 12 h. Non-heated cat fibroblasts served as a control group.

RNA extraction from feline fibroblasts

Following heat treatment, cat fibroblasts were dissociated using 0.25% (wt/vol) trypsin EDTA (Gibco) and centrifuged, and the cell pellets were finally stored at –80 C until use. Total RNA was extracted from cat fibroblasts using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly, fibroblast pellets were lysed using RLT buffer and then vortexed for 1 min. An equal volume of 70% ethanol was added into the cell lysate. The mixture was then transferred to an RNA-binding column and centrifuged at ≥ 8,000 × *g* for 15 sec. The filtrate was discarded, and RW1 buffer was added into the reseated column. After centrifugation, the filtrate was discarded, and the reseated column was washed with RPE buffer twice and dried by centrifugation at ≥ 8,000 × *g* for 2 min. The RNA was eluted with 30 µl of RNase-free water. The purity and quantity of extracted RNA was assessed using a spectrophotometer (NanoDrop ND-2000, Wilmington, DE, USA), and only extracted RNA with an A260/A280 ratio ranging from 1.5 to 2.0 was immediately stored at –80 C for further analysis.

Reverse transcription polymerase chain reaction (RT-PCR) analysis of HSP70 transcript in feline fibroblasts

Reverse transcription (RT) was performed using a First-Strand cDNA Synthesis Kit (Omniscript Kit, Qiagen, CA, USA). The RT reaction (20 µl) was prepared following the kit manufacturer's protocol. Briefly, 8 µl of total RNA (1 µg) were mixed with 2 µl of random hexamers (100 µM), 2 µl of dNTP Mix (5 mM each dNTP), 2 µl of 10xRT buffer, 0.25 µl of RNaseOut (40 units/ µl), 1 µl of Omniscript RT and 4.65 µl of nuclease-free water. The reaction was incubated at 37 C for 60 min. Reverse transcription containing all RT-PCR reagents, except the reverse transcriptase, was run in parallel in RT-PCR as a minus-reverse transcriptase ("-RT") control. The product was stored at –20 C for further use in PCR. The conserved region of the *HSP70* mRNA sequence among the human, mouse, rat and canine was used to design the primers by using the rat *HSP70* mRNA sequence as the template. PCR primers were designed from *HSP70* mRNA, complete cDNA of the rat (accession number: L16764), using the LightCycler Probe Design Software Version 2.0 (Table 1). A primer of glyceraldehyde 3-phosphatedehydrogenase (*GAPDH*) transcripts designed from a previous study [28] was used in this study as the endogenous normalizer. The PCR was performed using the GoTaq Green Mastermix (Promega, Madison, WI, USA). Each PCR reaction (total volume of 25 µl) consisted of 2 µl of reverse transcription product and 23 µl of reaction mixture, which consisted of 12.5 µl of Gotaq Green Master Mix, 2 µl of both 10 µM forward and reverse primers and 6.5 µl of nuclease-free water. Thermal cycling conditions were as follows: 2 min at 95 C to activate Taq DNA polymerase; 30 cycles of 30 sec at 95 C for denaturing, 30 sec at 55 C for annealing and 30 sec at 72 C for extension; and 2 min at 72 C for the final extension. At the end of the program, PCR products were confirmed by electrophoresis. The amplified products were run in 2% (wt/vol) agarose gel (Bio-Rad, Hercules, CA, USA) prepared in 1 × TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8) containing 0.4 mg/ml ethidium bromide (Promega, Madison, WI, USA). The separated products in agarose gel were visualized under the UV light of a Gel Documentation system (Syngene, Cambridge, CB, UK). The band intensity of amplified RT-PCR products was converted into mean pixel per area using Scion Image Analysis software (Scion, Frederick, MD, USA). The PCR products of *HSP70* were normalized with *GAPDH* at each condition.

Sequencing of partial feline-specific HSP70 mRNA

The PCR products acquired from gel electrophoresis were collected for sequencing, and the sequence was confirmed in GenBank. The amplicons were purified from agarose gel using NucleoSpin® Extract II kits (Macherey-Nagel, Düren, Germany). The purity of the eluted products was confirmed by running in a 1.5% agarose gel. After confirming the eluted products, both strands of each amplicon were sequenced using the same *HSP70* primers. The sequences derived from both strands were assembled using BioEdit Version 7.0.8.0 (T.A. Hall Software, Raleigh, NC, USA). Assembled sequences were then blasted in GenBank to determine the nucleotide similarity to other species.

Oocyte recovery

The ovaries were obtained following ovariohysterectomy from

Table 1. Description of forward (FP) and reverse (RP) primers used to assess expression of target genes in blastocysts acquired from different culture conditions

Genes	Sequence (5′-3′ orientation)	Fragment length (bp)	GenBank accession number or Reference
HSP70[1]	FP: ATCCAGGTGTACGAGGG RP: TGGTGATCTTGTTGGCCT	190	L16764
BAX	FP: CCGATGGCAACTTCAACTGGG RP: GTCAGCACTCCCGCCACAAAG	244	[35]
BCL-2	FP: GGAGGATTGTGGCCTTCT RP: GTTATCCTGGATCCAGGTGT	143	[36]
GAPDH	FP: GGAGAAAGCTGCCAAATATG RP: CAGGAAATGAGCTTGACAAAGTGG	191	[28]

[1] *HSP70* primers were used for feline fibroblast (experiment 1) and blastocysts (experiment 2).

domestic cats with unknown reproductive status. They were collected in a 0.9% (wt/vol) saline solution supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. Within 2 h, they were washed and minced to obtain the cumulus-oocyte complexes (COCs) in holding medium (HM) consisting of Hepes-buffered M199, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 4 mg/ml bovine serum albumin (BSA, embryo tested) under a stereomicroscope (SMZ645 Nikon, Tokyo, Japan). Only COCs surrounded with compacted cumulus cells and containing with a homogeneous-dark ooplasm (Grades I and II) were used in this study [29].

Oocyte maturation and fertilization

In vitro oocyte maturation (IVM) and fertilization (IVF) were essentially performed as previously described [30]. Groups of 20–30 COCs were cultured for 24 h in 500 µl of IVM medium ($NaHCO_3$-M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mg/ml bovine serum albumin (BSA) and 25 ng/ml epidermal growth factor (EGF)) containing with 0.05 IU/ml recombinant human follicle stimulating hormone (rhFSH, Organon, The Netherlands). After oocyte culture, cumulus cells were partially removed by gentle pipetting, and a group of 8 to 10 oocytes were cultured in a 50 µl droplet of IVF medium (Tyrode's balanced salt solution containing 1% MEM nonessential amino acid, 6 mg/ml BSA, 100 IU/ml penicillin, 30 µg/ml heparin and IVF×100) [31]. IVF×100 stock consisted of 100 mM L-glutamine, 36.3 mM sodium pyruvate and 110.9 mM calcium lactate in a Tyrode's balanced salt solution.

The semen used in this study was collected from two fertility proven tom cats and then frozen according to Andersen [32] with minor modifications. In brief, the cats were anesthetized with 0.04 mg/kg atropine sulphate (A.N.B. Laboratories, Bangkok, Thailand), 3 mg/kg xylazine hydrochloride (Laboratorios Calier, Barcelona, Spain) and 10 mg/kg ketamine hydrochloride (Gedeon Richter, Budapest, Hungary). The semen was collected by electro-ejaculation [33]. It was cryopreserved by placing the straws horizontally 4 cm above liquid nitrogen vapors for 10 min and then plunging them into liquid nitrogen. After thawing at 70 C for 6 sec, the sperm were subjectively evaluated, and only sperm that had more than 50% progressive motility were used for *in vitro* fertilization. The frozen-thawed sperm were washed twice in IVF medium, and the sperm concentration was determined. To perform IVF, the IVM oocytes were co-incubated with sperm at a final concentration of 0.5×10^6 sperm/ml for 18 h at 38.5 C in a humidified condition of 5% CO_2 in air.

In vitro embryo culture (IVC)

After co-incubation of the oocytes with sperm, cumulus cells were removed by gentle pipetting. Presumptive zygotes were then washed and cultured in a synthetic oviductal fluid (SOF) containing 4 mg/ml BSA, 100 µg/ml streptomycin and 100 IU/ml penicillin (IVC-1) [6]. After 24 h of culture, only cleaved embryos were washed and cultured in IVC-2 medium (SOF containing 10% (vol/vol) FCS (Gibco®, Invitrogen, Carlsbad, CA, USA)). Culture medium was changed every two days. In all cases, *in vitro* culture was performed at 38.5 C in a humidified condition of 5% CO_2 in air.

In vivo produced embryos

Surgical collection of feline embryos was performed according to the Committee of Animal Ethics, Faculty of Veterinary Science, Chulalongkorn University (accession no. 11310032). Four queens were intramuscularly injected with 150 IU equine chorionic gonadotropin (eCG, Intervet/Schering-Plough, Boxmeer, The Netherlands). After eCG injection for 96 h, the queens received 150 IU human chorionic gonadotropin (hCG, Intervet/Schering-Plough) to ensure ovulation and simultaneously allowed to mate with a tom cat. Natural mating was performed three times a day at 3-h intervals on the first and second days (day 0) after hCG injection. Ovulation was expected to occur 24 to 28 h after mating/hCG injection [34]. On day 7 post hCG injection, the queens were anesthetized as previously described, and ovariohysterectomy was performed. Embryos were recovered from the uterine horns by gentle flushing with HM. The embryos classified as expanded blastocysts were washed twice in phosphate buffered saline (PBS) containing with 0.1% (wt/vol) BSA and immediately stored at –80 C in a minimum volume (less than 2 µl) for further analysis.

Assessment of embryo development and embryo quality

The percentages of cleaved embryos (2 to 16 cells), morula (≥16 cells without blastocele) and blastocysts (≥50 cells with blastocele formation) were evaluated on days 2, 5 and 7 of IVC, respectively. Morula and blastocyst rates were calculated relative to the cleaved embryos, while hatching rates were calculated relative to the number of blastocysts. To count the number of embryonic cells, blastocysts

were first fixed in 4% (wt/vol) paraformaldehyde and kept at 4 C for 2 days before the staining procedure. They were then stained with 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS at 37 C for 10 min. Nuclear fragmentation of embryonic nuclei was calculated relative to the total embryonic cells under an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan).

RNA extraction of feline embryos

An Absolutely RNA Nanoprep Kit (Stratagene, San Diego, CA, USA) was used to extract the total RNA from a pool of five expanded blastocysts following the manufacturer's instruction. Briefly, pooled blastocysts acquired from each experimental replicate were lysed using a lysis buffer containing with 0.7% (vol/vol) β-mercaptoethanol (β-ME). Cell lysate was added with an equal volume of 80% sulfolane. The mixture was then transferred to an RNA-binding nano-spin cup and was centrifuged at ≥ 12,000 × *g* for 60 sec. The filtrate was discarded, and a reseated spin cup was added with low-salt wash buffer. After centrifugation, the filtrate was discarded, and the cup was reseated and dried by centrifugation at ≥12,000 × *g* for 60 sec. DNase I (Stratagene, San Diego, CA, USA) was added onto the fiber matrix inside the cup, and the mixture was incubated at 37 C for 15 min. After incubation, the fiber matrix was washed once with a high-salt washing buffer and then twice with a low-salt washing buffer. RNA was eluted with 12 µl RNase-free water by centrifugation (≥12,000 × *g* for 5 min). The purity and quantity of extracted RNA was assessed using a spectrophotometer (NanoDrop ND-2000, Wilmington, DE, USA), and only extracted RNA with an A260/A280 ratio ranging from 1.5 to 2.0 was immediately stored at –80 C for further analysis.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analysis of HSP70, BAX and BCL-2 transcripts in embryos

RT reaction (20 µl) was performed using a First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA) according to the kit manufacturer's protocol. Initially, 8 µl of extracted RNA was incubated with 1 µl of random hexamers (50 ng/µl) and 1 µl of 10 mM dNTP mix at 65 C for 5 min, and then immediately placed on ice for at least 1 min. After chilling on ice, the reaction mixture was then added to master mix containing 2 µl of 10×RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOut (40 U/µl) and 1 µl of SuperScript III RT (200 U/µl). The reaction mixture was further incubated at 50 C for 50 min followed by 85 C for 5 min. RNA template from the cDNA:RNA hybrid molecule was removed by digestion with 1 µl of RNase H (2 U/µl) after first-strand synthesis at 37 C for 20 min. Reverse transcription containing all RT-PCR reagents, except the reverse transcriptase, was run in parallel in RT-PCR as a minus-reverse transcriptase ("-RT") control. The product was stored at –20 C for further use in PCR.

The mRNA transcript levels at steady state of individual target genes (*HSP70*, *BAX* and *BCL-2*) were normalized to the endogenous normalizer (*GAPDH*), and were run in separate wells. The PCR was performed using an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Each PCR reaction (total volume of 20 µl) consisted of 2 µl of reverse transcription product (equivalent to 0.5 blastocysts) and 18 µl of reaction mixture, which was consisted of 10 µl of SYBR Green Master Mix, 1 µl of both 5 µM forward and reverse primers (Table 1) and 6 µl of nuclease-free water. Thermal cycling conditions were as follows: 10 min at 95 C to activate Taq DNA polymerase, 45 cycles of 15 sec at 95 C for denaturing, 30 sec at 55 C for annealing and 60 sec at 72 C for extension. PCR products were confirmed by melting curve analysis and gel electrophoresis. The amplified products were confirmed by running in 2% agarose gel (Bio-Rad, Hercules, CA, USA) prepared in 1 × TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8) containing 0.4 mg/ml ethidium bromide (Promega, Madison, WI, USA). The separated products in agarose gel were visualized under the UV light of a Gel Documentation System (Syngene, Cambridge, CB, UK). Three biological replicates of pooled embryos were analyzed for each gene. All PCRs were performed in a technical replicate of 20 µl for every transcript of interest.

The Sequence Detection System (SDS) Software Version 1.4 (Applied Biosystems, Carlsbad, CA, USA) was used to analyze the relative quantitation (RQ) by comparative C_q method using the *in vivo* derived (IVV) blastocysts as a control group. RQ was reported as relative abundance in relation to the control sample. The ΔC_q was calculated from the target gene C_q (*HSP70*, *BAX* and *BCL-2*) by subtracting the endogenous normalizer (*GAPDH*) C_q. $\Delta\Delta C_q$ was formulated from $\Delta C_{q\ (IVP\ embryo)} - \Delta C_{q\ (IVV\ embryo)}$, and the RQ was calculated from $2^{-\Delta\Delta Cq}$.

Statistical analysis

Three to four biological replicates were performed in each experiment. The data were expressed as the mean ± standard error (SE). Values were analyzed using one-way ANOVA and Duncan's test for statistical differences among groups. Differences with $P<0.05$ were considered statistically significant.

Experimental design

Experiment 1–Identification of a partial feline HSP70 mRNA sequence in response to heat activation: This experiment was designated to identify feline *HSP70* mRNA in heat-treated cat fibroblasts because the sequence is lacking in GenBank. In each replicate, 5 culture flasks of cat skin fibroblasts were used. One vessel was kept at 37 C in 5% CO_2 incubator as a control group, and the others were heat treated (45 C) for 45 min. The heat-treated cells were harvested for RNA extraction at 0, 3, 6 and 12 h after heat treatment, while the control was harvested simultaneously with the cells heat-treated for 0 h. The cells were analyzed for expression of HSP70 using RT-PCR with designed primers. The acquired PCR products were sequenced and then blasted in GenBank in order to determine the nucleotide similarity to other species.

Experiment 2–Effect of embryo density on the transcripts of *BAX*, *BCL-2* and *HSP70* in relation to developmental competence: Cumulus-oocyte complexes (COCs) were matured and fertilized *in vitro* (day 0 = day of *in vitro* fertilization). Ten cleaved embryos were cultured in different culture volumes (12.5, 25, 50, 100 and 200 µl) to acquire the embryonic densities of 1:1.25, 1:2.5, 1:5, 1:10 and 1:20, respectively. *In vitro* developmental competence of embryos cultured at each embryonic density was determined by means of morula (day 5) and blastocyst (day 7) formation rates in relation to

the number of cleaved embryos. On day 7, blastocysts were fixed and stained with a DNA labeling dye to count the total cell numbers and nuclear fragmentation. To examine the effect of embryonic culture density on *HSP70*, *BAX* and *BCL-2* expressions, the culture densities of 1:1.25, 1:5 and 1:20 were selected as the representative "high," "medium" and "low" densities. *In vivo* produced expanded blastocysts were included as a control group. Real-time PCR was used in this study due to the limited levels of gene expression in particular embryos. The mean values of *BAX* and *BCL-2* transcripts were used to calculate the *BAX/BCL-2* ratio**.**

Results

Experiment 1: Characterization of a partial feline HSP70 mRNA sequence in response to heat activation

The heat treatment significantly upregulated the *HSP70* transcript in cat skin fibroblasts. The acquired *HSP70* transcript (190 bp) was transiently expressed in response to heat, gradually increasing from 0 to 3 h after heat activation and then gradually decreasing afterward (Fig. 1a and 1b). *GAPDH* transcripts were constantly expressed (Fig. 1a). The heat-response profile of *HSP70* in this study was similar to that acquired from other species.

The acquired partial feline *HSP70* mRNA sequence was subsequently determined and compared with the sequences previously reported in other species (Fig. 2 and Table 2). High similarity of *HSP70* mRNA sequence between feline and other species was demonstrated (93 to 96% nucleotide identity) (Table 2). The designed *HSP70* primers were then used for quantification of *HSP70* transcripts of feline blastocysts in experiment 2.

Experiment 2: HSP70, BCL-2 and BAX expressions related with developmental competence of embryos cultured at various embryonic densities

Following IVF, approximately 50 to 70% of inseminated oocytes cleaved (data not shown). Although the numbers of cleaved embryos that developed to the morula stage did not significantly differ among the embryonic culture densities (morula rates: ~80 to 95%, P> 0.05), high embryonic density during culture adversely affected the blastocyst formation rates. High embryonic culture density (1:1.25) significantly decreased the blastocyst formation rate when compared with those cultured at 1:5, 1:10 and 1:20 culture densities (Fig. 3 and Table 3). While blastocysts culture at a 1:1.125 culture density had lower cell numbers compared with other embryo culture densities, hatching and nuclear fragmentation rates of blastocysts were not significantly different among embryonic culture densities (Table 3).

To examine the effect of embryonic culture density on *HSP70*, *BAX* and *BCL-2* expressions, culture densities of 1:1.25, 1:5 and 1:20 were selected as the representative "high," "medium" and "low" densities. *In vivo* produced blastocysts served as a control group. Overall, *in vitro* produced embryos, irrespective of the culture density employed, showed upregulated levels of HSP70 and *BAX* transcripts compared with *in vivo* derived feline embryos. However, only *HSP70* and *BAX* transcripts from blastocysts cultured at a culture density of 1:1.25 were significantly higher than in *in vivo* embryos (P<0.05, Fig. 4a and Fig. 4b). Although expression of *HSP70* was not significantly different among *in vitro* culture densities, *BAX* transcripts were

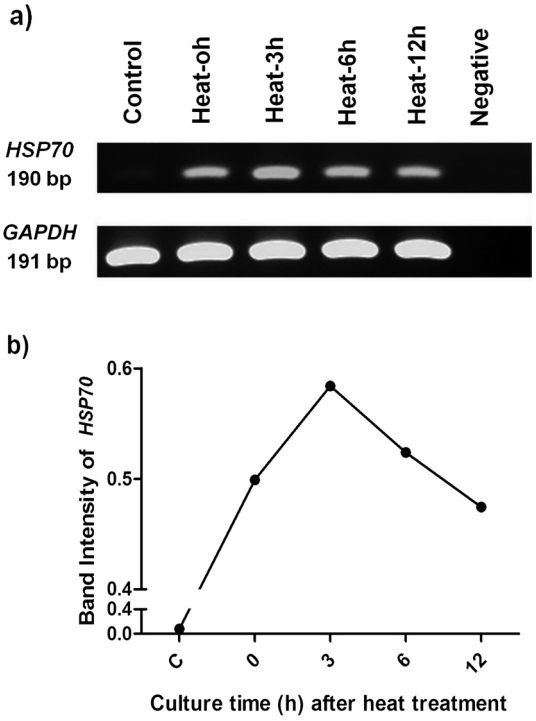

Fig. 1. HSP70 transcript profiles of cat fibroblasts heat-treated at 45 C for 45 min followed by 0, 3, 6 and 12 h of culture (0, 3, 6 and 12 h, respectively) compared with non-heated control fibroblasts (C). PCR reaction without template served as a negative control (N). The amplified RT-PCR products (1a) and semi-quantitative analysis of HSP70 mRNA (1b) indicate the heat stress response of HSP70 of cat fibroblasts.

significantly increased in embryos cultured at a culture density of 1:1.25 compared with a culture density of 1:5. Moreover, *BAX/BCL-2* ratios appeared to decrease when the culture volume per embryo was increased (Fig. 4c).

Discussion

In this study, the partial sequence of feline *HSP70* mRNA was firstly characterized and then further applied to evaluate the physiological response of embryos to the culture density. Embryos cultured at a high embryonic density showed upregulated levels of pro-apoptotic (*BAX*) and stress-response (*HSP70*) transcripts, and this adversely affected their developmental competence. This suggested possible stress accumulation that inhibited embryo development and probably induced apoptosis.

In response to stress, induced *HSP70* protects essential cell components against heat damage and also allows a rapid resumption of normal cellular activities during the recovery period. *HSP70* is rapidly and transiently induced in response to heat shock (around 40 to 50 C for mammalian cells) [37]. Because the structure and function of HSP70 proteins are highly conserved across species [38], conserved rat-specific *HSP70* primers designed from human-mouse-rat-canine *HSP70* mRNA alignments were used to amplify feline cDNA in the present study. In order to determine the inducible

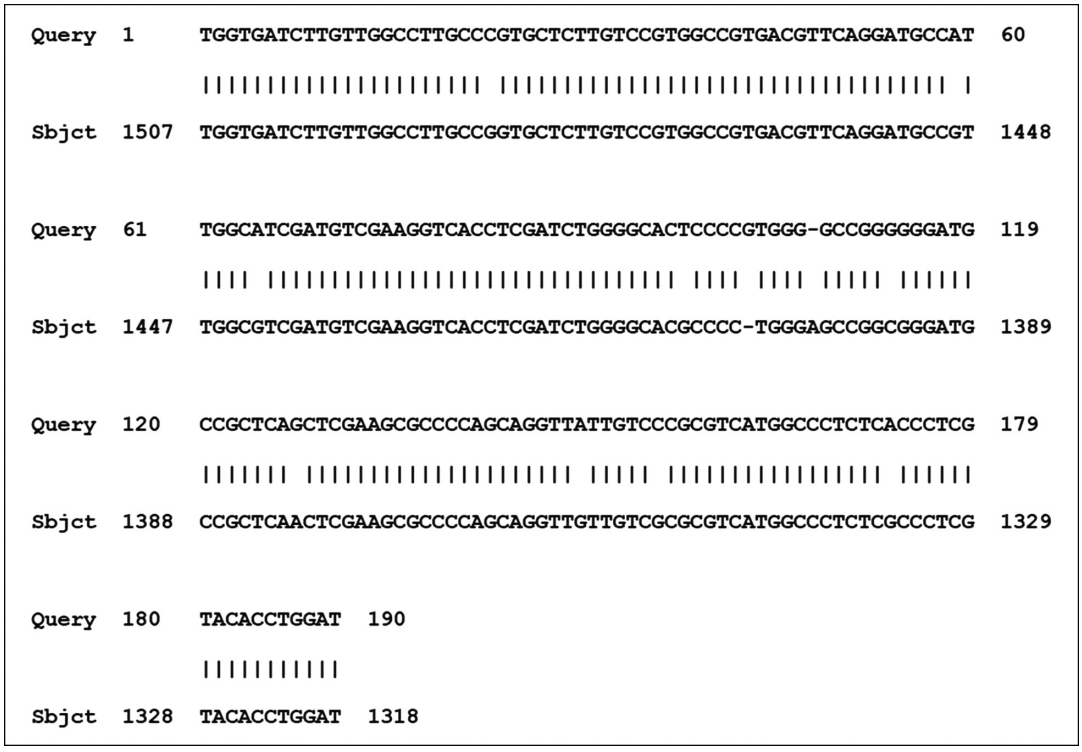

Fig. 2. The alignment between the acquired partial cat (Query) and rat (L16764.1) (Sbjct) heat shock protein 70 (*HSP70*) mRNA. The size of the feline mRNA product was 190 bp, and the position of the rat mRNA shown was from 1318 to 1507 bp. The vertical bar indicates the similarity of nucleotides between the sequences.

Table 2. Homology of *HSPA1A* mRNA between domestic cat and other species

Species	GenBank accession number	mRNA identity (%)
Rat *(Rattus norvegicus)*	L16764.1	94
Mouse *(Mus Musculus)*	M35021.1	93
Human *(Homo sapiens)*	DQ451402.1	93
Canine *(Canis lupus familiaris)*	AB114672.1	95
Bovine *(Bos Taurus)*	AY662497.1	96
Swine *(Sus scrofa)*	M69100.1	95
Buffalo *(Bubalus bubalis)*	EU099315.1	95

expression properties of *HSP70*, examination of heat response was firstly studied. Similar to previous studies [39,40], *HSP70* response in cat fibroblasts in the current study was transiently increased during 0 to 3 h after heat treatment, and this was followed by a progressive decline (6 to12 h). However, the heat-response time of cat *HSP70* in this study was different from that in a previous report in humans [39]. This appeared to be caused by the temperature- and cell-type specific response of *HSP70 per se* [41,42]. Of note, this study only evaluated *HSP70* transcription, and evaluation of the protein translation was not performed because of a limitation of the feline embryos produced.

According to our previous study [6], suboptimal *in vitro* culture density detrimentally affected the developmental competence of feline embryos. While paracrine and autocrine embryo-derived factors are critically important for their development [43–45], excessive embryo density could also, in turn, inhibit embryo development probably due to accumulation of stress from particular culture conditions (nutritional inadequacy, pH imbalance, etc.) [2]. Reduction of blastocyst formation rates was observed in embryos cultured at a high embryonic density (1:1.25). However, morphological study only is inadequate to clearly understand embryonic stress during *in vitro* culture. We therefore examined the *HSP70*, *BAX* and *BCL-2* genes of embryos cultured at various densities in correlation with their morphology.

Similar to our previous report [6], high *in vitro* culture density (1:1.25) reduced developmental competence and quality of feline embryos in terms of blastocyst formation rates and quality. Interestingly, the low blastocyst development and quality of feline embryos derived from this high embryonic culture density coincided with an increase in *HSP70* and pro-apoptotic (*BAX*) transcripts and *BAX/BCL-2* ratio. The upregulation of these transcripts during embryo culture may indicate a stress response in the embryos when they were cultured at a high density. More importantly, it is clearly evident that the levels of the transcripts of *in vitro* produced embryos also increased to be higher than those obtained from *in vivo* embryos, even though the embryo density was optimal (density 1:20) in terms of the high blastocyst rates obtained (approximately 40 to 50%). The results thus suggested the detrimental effect of high embryonic culture density on developmental competence, probably via excessive cellular stresses.

Apart from the stress response of *HSP70*, apoptotic-related genes

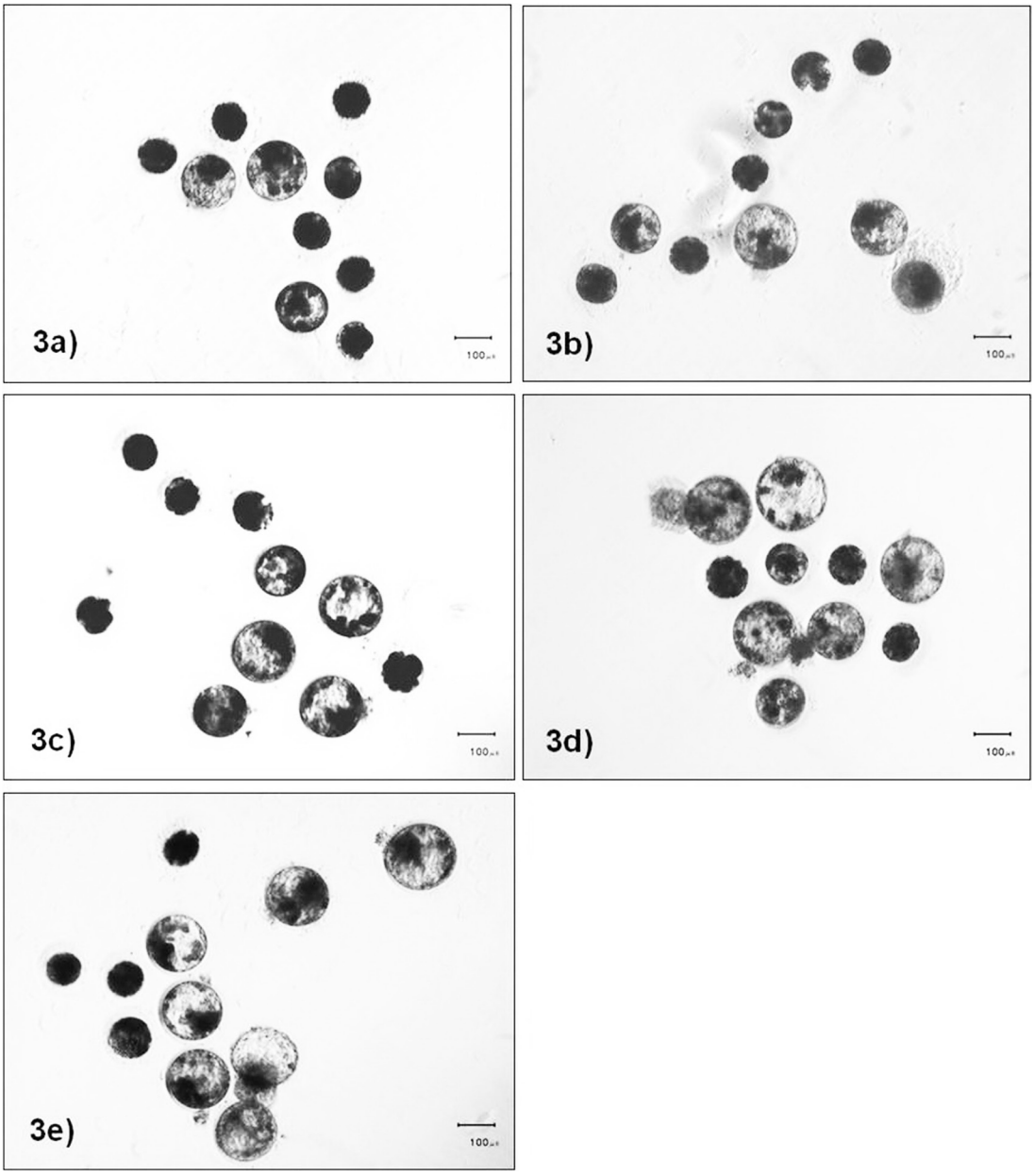

Fig. 3. Bright field images of day 7 cat embryos cultured at different densities (10 embryos per media volume). They were cultured at densities of 1:1.25 (3a), 1:2.5 (3b), 1:5 (3c), 1:10 (3d) and 1:20 (3e).

Table 3. Mean percent ± SE of developmental competence of cat embryos (3 replicates) cultured in groups of ten in different volume of medium to acquire different culture densities

Culture volume (µl)	Culture density	N	Cleaved embryos developed to		Hatching	Cell no.	Fragmentation
			Morula	Blastocyst			
12.5	1:1.25	50	81.7 ± 7.3^{a}	15.0 ± 10.4^{a}	0.0 ± 0.0^{a}	69.1 ± 42.7^{a}	19.5 ± 12.1^{a}
25	1:2.5	50	80.0 ± 7.6^{a}	20.0 ± 5.0^{ab}	13.3 ± 13.3^{a}	180.1 ± 41.8^{b}	22.5 ± 6.6^{a}
50	1:5	60	97.2 ± 1.5^{a}	39.4 ± 7.5^{bc}	21.0 ± 1.0^{a}	140.5 ± 15.4^{ab}	14.3 ± 1.4^{a}
100	1:10	50	86.7 ± 3.3^{a}	48.3 ± 1.7^{c}	14.1 ± 7.1^{a}	149.2 ± 14.9^{ab}	14.4 ± 1.5^{a}
200	1:20	60	90.6 ± 4.7^{a}	43.3 ± 3.3^{c}	19.4 ± 10.0^{a}	152.2 ± 28.0^{ab}	14.3 ± 1.2^{a}

$^{a, b, c}$ Within a column, values with different superscripts were significantly different ($P<0.05$). N = Total number of cleaved embryos used. Culture density = 10 embryos: medium volume (µl).

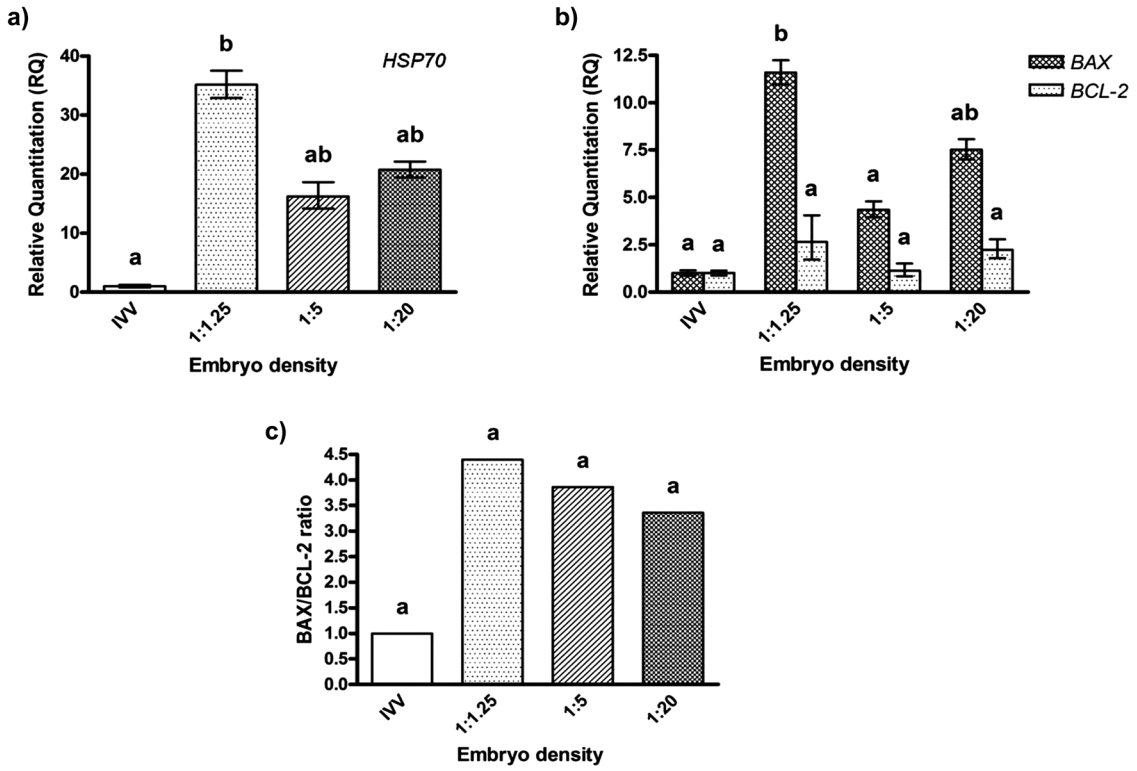

Fig. 4. Comparison of specific transcripts analyzed by real-time PCR in feline blastocysts produced *in vivo* (IVV) and *in vitro* at densities of 1:1.25, 1:5 and 1:20. Mean values were obtained from three independent replicates. Bars indicate the upper and lower bounds of values. [a, b] Different superscripts denote values that differ significantly ($P<0.05$).

(*BAX* and *BCL-2*) also play an important role in adaptive response to stress condition by removal of damaged cells in embryos [16, 46]. Pro-apoptotic (*BAX*) expression and the *BAX/BCL-2* ratio were increased in cat embryos cultured at high culture density (1:1.25) in the same manner as *HSP70* expression. As previously described, upregulation of *HSP70* appears to reduce pro-apoptotic activity of *BAX*, depending on the severity of cell damage [47]. In this study, high culture density may have severely induced embryonic stress, resulting in *HSP70* not being able to overcome the BAX activation. This could contribute to the positive correlation between *BAX* and *HSP70* expressions levels in densely cultured embryos (Fig. 4a and 4b). Although other apoptotic pathways may negatively contribute to embryo development, our results suggested the possible pathway of embryonic cell stress induced apoptosis from high embryonic culture density. Other pathways that trigger apoptosis in feline embryos still need to be further examined, since several types of stress from high embryo density involve the fate of embryo development. For example, oxidative stress in *in vitro* cultured embryos is commonly caused by excessive accumulation of embryotoxic elements secreted from crowded embryos such as radical oxygen species [48] and ammonium [2, 4, 49], an inadequacy of nutritional requirement and pH imbalance [50]. A large amount of reactive oxygen species (ROS) generated by this condition could possibly trigger *HSP70* expression [51] and could also initiate apoptosis of cells [52]. In order to understand the importance of oxidative stress at a high culture density, further determination of its effects should be performed.

From our study, embryonic stress from high culture density results in developmental incompetency and affects gene expression patterns. The combination of morphological and molecular observations helps us to identify the probable mechanisms responsible for suboptimal culture conditions for feline embryos.

Acknowledgments

This work was financially supported by an RGJ-PhD grant, Thailand Research Fund, the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (HR1166IB-55); French Embassy; and "Integrated Innovation Academic Center: IIAC" Chulalongkorn University Centenary Academic Development Project (CU56-HR07). The authors would like to thank the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Thailand, for sample collection and the Office of Academic Affairs, Chulalongkorn University, for linguistic editing of the manuscript.

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