

—Research Note—

Relationship between Group II Caspase Activity of Bovine Preimplantation Embryos and Capacity for Hatching

Frank Dean JOUSAN¹#, Luiz Augusto DE CASTRO E PAULA¹, Amber Marie BRAD¹, Zvi ROTH¹## and Peter James HANSEN¹

¹Department of Animal Sciences, University of Florida, Gainesville, FL 32611-0910, USA

#Present: Department of Animal and Dairy Sciences, Mississippi State University, MS 39759, USA

##Present: Department of Animal Science, Faculty of Agriculture, Hebrew University, Israel 76100, Israel

Abstract. The occurrence of apoptosis in a fraction of blastomeres in the preimplantation embryo is well known but the consequences of this phenomenon for the developmental potential of the blastocyst has not been well established. Here we demonstrate that blastocysts with low amounts of activated group II caspase activity have increased potential for development to the hatched blastocyst stage. Bovine blastocysts produced *in vitro* were assayed using a non-invasive fluoregenic substrate that is cleaved by activated group II caspases (i.e., caspase-2, -3 and -7). Subsequently, blastocysts were cultured until Day 10 post-insemination and the proportion undergoing hatching determined. In Experiment 1, blastocysts were cultured without respect to stage of development (expanded or non-expanded); blastocysts classified as having low caspase activity had higher hatching rates than blastocysts with medium or high caspase activity. In Experiment 2, embryos were categorized as nonexpanded or expanded blastocysts. Caspase activity was lower and hatching rate higher for expanded blastocysts than for nonexpanded blastocysts. For nonexpanded blastocysts, embryos classified as having low caspase activity had higher hatching rates as compared to embryos with medium or high caspase activity. In conclusion, the capacity for blastocysts to undergo further development is related to degree of group II caspase activity. Conditions that enhance the incidence of apoptosis in blastocysts may reduce developmental competence. In addition, determination of caspase activity may be useful for selection of embryos for transfer into recipients.

Key words: Blastocyst, Bovine, Embryo, Group II caspase, Hatching

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Like most cells, blastomeres of the bovine preimplantation embryo can undergo apoptosis in response to various stimuli including heat shock, arsenic and tumor necrosis factor- α [1–4]. Incidence of apoptosis in the embryo is also affected by the growth characteristics of the oocyte from which it was derived [5]. The apoptosis response is developmentally acquired and first occurs between the 8–16 cell stage at Day 4 after insemination [1]. It has been demonstrated using heat shock that apoptosis can represent a survival strategy for the embryo to continue development after exposure to stress [6, 7].

While apoptosis may protect the embryo from stress, there may be long-term adverse consequences for sustained development. Activity of group II caspases (i.e., those caspases that recognize the peptide motif DEXD; includes caspase-2, -3 and -7) was greater for embryos that were retarded in development at 45, 80 and 117 h after insemination as compared to embryos that developed more rapidly [5]. At 168 h after insemination, embryos with a combination of slow prior development and poor morphology had higher caspase activity than other types of embryos [5].

The impact of apoptosis on further development of the blastocyst-stage embryo is not known. This is an important question because embryos are usually transferred to recipients as blastocysts. Establishing a relationship between apoptosis and

subsequent development could lead to new endpoints for evaluating improvements in culture medium and new criteria for selecting embryos for transfer. The hypothesis tested in this study was that the degree of apoptosis at the blastocyst stage affects competence of the embryo for subsequent development. This hypothesis was tested by assessing apoptosis using a non-invasive assay for group II caspase activity. This class of caspase is important for apoptosis in bovine embryos: induction of apoptosis by heat shock and tumor necrosis factor- α was blocked by inhibitors of group II caspases. Developmental capacity of the blastocyst was assessed *in vitro* by determining the ability to undergo hatching from the zona pellucida.

Materials and Methods

In vitro production of embryos

Embryo production using oocytes obtained from ovaries collected at an abattoir was performed as previously described [7]. Briefly, cumulus oocyte complexes (COCs) were matured for 20–22 h at 38.5 C in an atmosphere of 5% CO₂ and then fertilized with $\sim 1 \times 10^6$ Percoll-purified spermatozoa from a pool of frozen-thawed semen from three bulls for 20–22 h at 38.5 C in an atmosphere of 5% CO₂ in humidified air. Putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1,000 U/ml hyaluronidase and placed in groups of 30 in 50- μ l drops of KSOM-BE2 culture medium [3]

overlaid with mineral oil. Blastocysts classified as quality grade 1 (those with excellent or good morphology) [9] were selected from drops at Day 7 (Experiment 2) or 8 (Experiment 1) after insemination and used for the experiment.

Assay for group II caspase activity

Group II caspases are those caspases that have substrate specificity for the amino acid motif DEXD and include caspases 2, 3 and 7. Groups of equal numbers of blastocysts (not exceeding ten) were incubated in 25- μ l drops of Hepes-TALP containing 5 μ M PhiPhiLux-G₁D₂ (OncoImmunin, Gaithersburg, MD, USA) at 39 C on a stage warmer for 40 min in the dark. Following incubation, blastocysts were washed three times in 50- μ l drops of Hepes-TALP and placed individually in wells of two-well microscope slides (Fisher, Pittsburgh, PA, USA) in 125 μ l of prewarmed Hepes-Tyrode's albumin lactate pyruvate solution [8]. Caspase activity was measured using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) with a FITC filter and 10 \times objective. Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera set to an exposure length of 2,000 msec. Using the computer mouse, a circular draw function was manually performed around the internal side of the zona pellucida and intensity per unit area was determined. Embryos were separated on the basis of group II caspase activity into three categories (low: <15.0 pixels; medium: 15.0 to <30.0 pixels; high: \geq 30.0 pixels). Embryos were then returned to culture as described in the experiments below.

Experimental design

In Experiment 1, blastocysts cultured in KSOM-BE2 at 38.5 C in an atmosphere of 5% CO₂ in humidified air were removed from culture at Day 8 after insemination and subjected to the group II caspase activity assay. Embryos were classified according to caspase activity into low, medium and high groups and cultured within classification in groups of no more than 15 embryos in fresh 25- μ l microdrops of KSOM-BE2 at 38.5 C in an atmosphere of 5% CO₂ in humidified air. Embryos were evaluated at 24 h intervals and stage of development was recorded for each embryo until Day 10 post-insemination when the proportion of blastocysts that hatched (i.e. emerged from the zona pellucida) was assessed. A total of 264 blastocysts were used in this experiment.

Experiment 2 was conducted in a similar manner except that embryos were also grouped by blastocyst stage (non-expanded blastocysts vs expanded blastocysts) to avoid potential confounding effects of caspase activity with stage of development at time of collection. In addition, the atmosphere was changed to improve the yield of blastocysts. Embryos were cultured in KSOM-BE2 at 38.5 C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Those embryos identified as blastocysts at Day 7 after insemination were removed from culture and subjected to the group II caspase assay. Following determination of caspase activity, embryos were cultured individually in fresh 10- μ l drops of KSOM-BE2 at 38.5 C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Embryos were evaluated at 24 h intervals and stage of development was recorded for each embryo until Day 10 post-insemination when the propor-

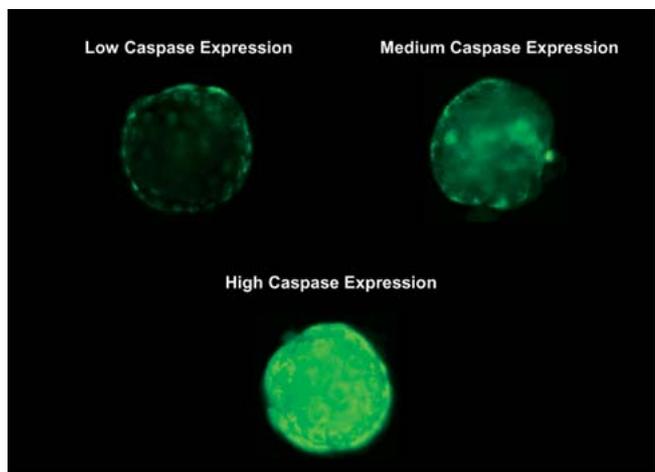


Fig. 1. Examples of grade 1 bovine blastocysts classified as having low, medium or high caspase expression based on group II caspase activity.

tion of blastocysts that hatched was assessed. A total of 243 blastocysts were used in this experiment.

Statistical analysis

Embryo hatching rates at Day 10 post-insemination were analyzed using the CATMOD and LOGISTIC procedures of SAS (SAS for Windows Version 8 1999-2001; Cary, NC, USA). Independent variables included caspase activity, blastocyst stage (Experiment 2 only), and replicate. Both statistical procedures were also used for Experiment 2 to determine effect of embryo stage (non-expanded vs expanded) on the distribution of embryos into caspase categories (low, medium, or high). Levels of significance were very similar for both analyses and statistical inferences reported are from the CATMOD procedure.

Results

Images of blastocysts exhibiting low, medium, and high group II caspase activity are depicted in Fig. 1. In Experiment 1, using grade 1 blastocysts collected at Day 8 after insemination, 88 of 264 (33.3%) were classified as having low caspase activity, 123 of 264 (46.6%) had medium caspase activity, and 53 of 264 (20.1%) had high caspase activity. Caspase classification had a significant effect ($P < 0.05$) on the proportion of blastocysts that hatched from the zona pellucida by Day 10 post-insemination. As shown in Table 1, rate of hatching was highest for blastocysts with low caspase activity (40 of 88; 45.5%), intermediate for blastocysts with medium caspase activity (45 of 123; 36.6%), and lowest for blastocysts with high caspase activity (13 of 53; 24.5%).

One possible explanation for the results of Experiment 1 was that blastocysts with low caspase activity were more likely to be expanded when selected at Day 8 and therefore more likely to hatch from the zona pellucida. Accordingly, Experiment 2 was conducted with Day 7 blastocysts to determine whether the caspase assay could distinguish embryo competence for hatching among a

Table 1. Hatching rates of grade 1 blastocysts selected on Day 8 post-insemination as affected by group II caspase activity ^a

Caspase activity	Hatching rate (n)	Percent hatched
Low	40/88	45.5
Medium	45/123	36.6
High	13/53	24.5

^a Percent hatched was affected by caspase activity classification (P<0.05).

Table 2. Hatching rates of grade 1 blastocysts selected on Day 7 post-insemination as affected by group II caspase activity and embryo stage of development at the initiation of the caspase assay

Caspase activity	Stage of development at time of caspase assay ^a			
	Nonexpanded blastocysts ^b		Expanded blastocysts ^c	
	Hatching rate (n)	Percent hatched	Hatching rate (n)	Percent hatched
Low	39/52	75.0	36/40	90.0
Medium	8/21	38.1	7/8	87.5
High	23/37	62.2	7/9	77.8

^a Stage of development affected the distribution of embryos into caspase activity classes (P<0.05) and the percent hatched (P<0.01). ^b For non-expanded blastocysts, percent hatched was affected by caspase activity classification (P<0.01). ^c For expanded blastocysts, there was no effect of caspase activity classification on percent hatched.

group of grade 1 blastocysts at the same stage of development. Results are presented in Table 2. Stage of development affected caspase classification (P<0.05); a greater proportion of expanded blastocysts were classified as having low caspase activity (40 of 57; 70.2%) as compared to non-expanded blastocysts (52 of 110; 47.3%). Also, hatching rate was greater (P<0.01) for expanded blastocysts (50 of 57; 87.7%) as compared to non-expanded blastocysts (70 of 110; 63.6%). For nonexpanded blastocysts, caspase classification affected hatching rate (P<0.01). Low-caspase embryos had the highest hatching rate, medium-caspase embryos had the lowest hatching rate and high-caspase embryos were intermediate. There was no effect of caspase classification on hatching rate among expanded blastocysts. Few expanded blastocysts were classified as having medium or high caspase activity and the rate of hatching was high in all groups.

A group of expanded blastocysts and non-expanded blastocysts not subjected to the caspase assay were also evaluated for hatching rate. The percent hatched was 89.7% (26/29) for expanded blastocysts and 66.0% (31/47) for non-expanded blastocysts.

Discussion

The results reported here indicate that blastocysts with low caspase activity are more likely to undergo hatching than blastocysts with higher caspase activity. Group II caspases include two caspases, caspase-3 and -7, that serve as execution caspases during apoptosis [10], as well as caspase-2, an upstream initiator of mitochondrial permeability [11]. Group II caspase was measured as an indication of apoptosis because the assay is a non-invasive one that allows monitoring of development after the assay is performed and because group II caspases are required for apoptosis induced by heat shock and tumor necrosis factor- α [4, 6].

In both experiments, the probability that a blastocyst could hatch

was highest in embryos with low caspase activity. This effect did not occur simply because blastocysts with low caspase activity were more advanced in development. The relationship between caspase activity and subsequent hatching was observed in Experiment 2 for a group of blastocysts that were all at the same stage of development (non-expanded).

The one surprising result was observed for nonexpanded blastocysts in Experiment 2. For these blastocysts, hatching rate was numerically greater for embryos with high caspase activity than for embryos classified as having medium caspase activity. This finding is probably a reflection of the fact that the assay is an imperfect one for assessing apoptotic status. In particular, distribution of caspase activity is often non-uniform with some blastomeres showing intense fluorescence and some blastomeres being negative for caspase activity. Additional heterogeneity in fluorescence pattern is induced by the presence and size of the blastocoele. A quantitative estimate of caspase activity based on pixel intensity for the entire blastocyst could result in some blastocysts having more or less apoptotic nuclei than indicated by the average fluorescence.

There are at least two possible implications of the present findings that could form the basis for future research. The first is that conditions that lead to an increased incidence of apoptosis in a blastocyst may compromise its ability to establish pregnancy after transfer into recipients. Such a phenomenon could contribute to the poor post-transfer survival of embryos produced *in vitro* [12] because the incidence of apoptosis in blastocysts can be greater for embryos produced *in vitro* [13]. The second implication is that determination of group II caspase activity may be useful as a tool for selecting embryos for transfer into recipients. All of the embryos used in the current study were classified as grade 1 (i.e. had morphological characteristics of an excellent or good embryo according to the IETS guidelines) and represent the type of blastocyst routinely transferred in commercial bovine embryo transfer

systems. Even among these “excellent or good embryos”, however, there were clear differences in developmental competence as revealed by the caspase assay.

The present findings on caspase activity are consistent with the idea that blastocyst survival after transfer is related to caspase activity. In particular, a higher percent of expanded blastocysts had low caspase activity as compared to non-expanded blastocysts in Experiment 2 (70.2 vs. 47.3%) and pregnancy rates in cattle were higher following transfer of expanded blastocysts as compared to transfer of non-expanded blastocysts [14].

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References

1. **Paula-Lopes FF, Hansen PJ.** Heat-shock induced apoptosis in preimplantation bovine embryos is a developmentally-regulated phenomenon. *Biol Reprod* 2002; 66: 169–177.
2. **Krinninger CE III, Stephens SH, Hansen PJ.** Developmental changes in inhibitory effects of arsenic and heat shock on growth of preimplantation bovine embryos. *Mol Reprod Dev* 2002; 63: 335–340.
3. **Soto P, Natzke RP, Hansen PJ.** Actions of tumor necrosis factor- α on oocyte maturation and embryonic development in cattle. *Am J Reprod Immunol* 2003; 50: 380–388.
4. **Loureiro B, Brad AM, Hansen PJ.** Heat shock and tumor necrosis factor- α induce apoptosis in bovine preimplantation embryos through a caspase-9 dependent mechanism. *Reproduction* 2007; 133: 1129–1137.
5. **Vandaele L, Mateusen B, Maes DG, de Kruif A, Van Soom A.** Temporal detection of caspase-3 and -7 in bovine *in vitro* produced embryos of different developmental capacity. *Reproduction* 2007; 133: 709–718.
6. **Paula-Lopes FF, Hansen PJ.** Apoptosis is an adaptive response in bovine preimplantation embryos that facilitates survival after heat shock. *Biochem Biophys Res Commun* 2002; 295: 37–42.
7. **Jousan FD, Hansen PJ.** Insulin-like growth factor-I promotes resistance of bovine preimplantation embryos to heat shock through actions independent of its anti-apoptotic actions requiring PI3K signaling. *Mol Reprod Dev* 2007; 74: 189–196.
8. **Parrish JJ, Susko-Parrish JL, Critser ES, Eyestone WH, First NL.** Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology* 1986; 25: 591–600.
9. **International Embryo Transfer Society.** Manual of the International Embryo Transfer Society. 3rd ed. Savoy:1998.
10. **Chang HY, Yang X.** Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev* 2000; 64: 821–846.
11. **Troy CM, Shelanski ML.** Caspase-2 redux. *Apoptosis* 2002; 7: 173–177.
12. **Hansen PJ, Block J.** Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. *Reprod Fertil Dev* 2004; 16: 1–14.
13. **Knijn HM, Gjørret JO, Vos PL, Hendriksen PJ, van der Weijden BC, Maddox-Hyttel P, Dieleman SJ.** Consequences of *in vivo* development and subsequent culture on apoptosis, cell number, and blastocyst formation in bovine embryos. *Biol Reprod* 2003; 69: 1371–1378.
14. **Kubisch HM, Sirisathien S, Bosch P, Hernandez-Fonseca HJ, Clements G, Liukkonen JR, Brackett BG.** Effects of developmental stage, embryonic interferon-tau secretion and recipient synchrony on pregnancy rate after transfer of *in vitro* produced bovine blastocysts. *Reprod Domest Anim* 2004; 39: 120–124.