

Effects of Timing on Cell Biopsy from Pre-compacted Morula Stage Bovine Embryos on Subsequent Embryonic Development

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Abstract

Introduction: Embryo biopsy has potential applications in molecular research processes in domestic animals, besides its application in sex determination in embryo transfer programs. The objective of the present study was to assess the in vitro development of bovine embryos biopsied on different days of precompacted morula stage.

Materials and Methods: Slaughterhouse-derived oocytes were matured in vitro, fertilized (Day-0) by frozen-thawed, Percoll-separated spermatozoa and cultured on oviductal cell monolayer. The embryos were subjected to cell biopsy on Days 2, 3, and 4 postinsemination at 4-16-cell stages. The data were analyzed using ANOVA and Chi-squared tests (SigmaStat, version 2). A p-value < 0.05 was considered significant.

Results: Biopsies carried out at 16-cell stage (Day-4) resulted in 94% of embryos developing to the blastocyst stage, which was significantly higher ($p < 0.05$) than the ones biopsied at 8-cell stage on Day-4 (64%), and those undergoing the procedure on Day-3 (49% and 46% at 4-cell and 8-cell stages, respectively) and Day-2 (39% and 33% at 4-cell and 8-cell stages, respectively). No significant differences were observed between biopsied and non-biopsied embryos on a given day. The total cell number in biopsy-derived blastocysts ranged between 103 and 135. The difference in the number of total cells, dead cells and cell allocation to trophectoderm and inner cell mass between non-biopsied and biopsy-derived blastocysts was insignificant.

Conclusion: Biopsy of bovine embryos at 4-16-cell stages had no adverse effects on in vitro developmental potentials and the 16-cell stage embryos, biopsied on Day-4 was the best stage for blastomere removal.

Keywords: Biopsy, Bovine, Embryo, Fertilization, In vitro, Precompacted morula.

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Introduction

Nowadays, improvements in embryo micromanipulation techniques have led clinicians to the use of embryo bisection technology in the majority of preimplantation genetic diagnosis (PGD) centers and in commercial embryo transfer programs in domestic animals.

Genetic analysis, including the assessment of the genetic profile of the originating embryo can be performed on a single or two blastomeres obtain-

ed from an early preimplantation embryo.

In an animal model, embryo biopsy has potential applications in sex determination, identification of genetic markers linked to economic trait loci, mitochondrial genome analysis, paternity identification, and prenatal diagnosis of genetic abnormalities and diseases (1-3). Moreover, in transgenic technology, animals harboring exogenous DNA have been successfully produced by the

transfer of transgenic positive embryos screened by molecular approaches (4, 5).

Embryo damage, upon biopsy is of a very low prevalence (6-8). In humans, the procedure is usually carried out 62 - 64 hrs after fertilization, when compaction begins but it has not been completely settled yet, since there can be damage if the junctions between neighboring cells have tightened (8).

Blastocyst biopsy has also been suggested in this regard, but since the blastocyst has a higher degree of chromosomal mosaicism and since trophectodermic cells could be mistakenly taken out (they can differ chromosomally from the inner mass cells), Day-3 biopsies prove more accurate (9). Therefore, the timing of embryo biopsy is an important factor, influencing subsequent embryonic development, as well as reliability of the results.

Genotyping by molecular approach for sex determination has recently become a practical technique in bovine embryos.

Since, developing in-vitro embryos are more sensitive to biopsy than in vivo ones; this technology should be carefully selected, performed and coordinated. There have been few studies on the sensitivity of embryos to manipulation and cell sampling which can result in cumulative negative effects leading to a decrease in embryo viability (10). However, there seems to be no data available on the survival of in vitro produced bovine embryos that are biopsied at different times of pre-compacted morula stage, considering embryonic cellularity.

The objective of the present study was to determine the best time for cell sampling, considering the age and embryonic cell numbers, from pre-compacted in vitro produced bovine embryos.

Materials and Methods

Except where otherwise stated, all the chemicals used in this research were obtained from Sigma (St. Louis, MO, USA).

In vitro embryo production: The ovaries (n = 190) were collected at a local slaughterhouse and transported to the laboratory within 2 to 3 h in normal saline at a temperature between 30 and 35° C. Ovaries were washed three times with prewarmed fresh saline (37° C), and all visible

follicles with a diameter of 2 - 8 mm were aspirated using gentle vacuum (30 mm Hg) via an 18-gauge short beveled needle connected to a 10 ml syringe. The follicle content was released in pre-incubated hepes-TCM, supplemented by penicillin (100 IU/ml), streptomycin (100 µg/ml) and heparin, 50 IU/ml.

The cumulus-oocyte complexes (COCs) with at least three layers of cumulus cells, oocytes with a uniform granulated cytoplasm and a homogenous distribution of lipid droplets in the cytoplasm were selected for the experiments (n = 1150). The selected COCs were matured in vitro in TCM199, supplemented by 10% Fetal Bovine Serum (FBS), (Gibco 10270), 0.02 mg/ml cysteamine and 0.1 IU/ml FSH. Ten to 15 COCs were transferred into 50 µl of the aforementioned maturation medium in a 60 mm Petri dish (Falcon 3004; Becton & Dickinson, Franklin Lakes, NJ, USA) layered with sterile mineral oil and cultured for 24 h in 5% CO₂ in air at 39° C. The matured oocytes were exposed to motile spermatozoa obtained by centrifugation of frozen-thawed semen on a discontinuous Percoll density gradient (1 ml of 40% over 1 ml of 90% Percoll) at 700 g for 20 min. Oocytes were cultured in TALP medium supplemented by 6 mg/ml BSA, 10 µg/ml heparin, and 0.3 mM sodium pyruvate and were later incubated with motile spermatozoa at 1 × 10⁶ spermatozoa/ml concentration layered with mineral oil, for 22 - 24 h at 39° C in an atmosphere containing 5% CO₂ in air.

After fertilization, presumptive zygotes were mechanically denuded of their cumulus cells and cultured in synthetic oviductal fluid-amino acids-BSA (SOFaaBSA), co-cultured with oviduct cells-monolayer (SOF-OCM) under mineral oil in maximum humidified atmosphere with 5% CO₂. The composition of SOFaaBSA was the one proposed by Tervit et al. (11) with minor modifications containing SOF supplemented by 2% (v/v) basal medium eagle (BME)-essential amino acids, 1% (v/v) MEM nonessential amino acids, 1 mM glutamine and 8 mg/ml fatty acid free BSA. Cell sampling of cleaved embryos was performed on Days 2, 3, and 4 postinsemination.

Embryo biopsy: In vitro fertilized embryos on Days 2, 3, and 4 with different cell numbers (4 to 16), in batches of 12, were transferred post-insemination into drops of HEPES-SOF in a Petri

dish under mineral oil irrespective of their grades. Biopsy was performed using micromanipulators (Narishige, Japan) in conjunction with an inverted microscope with Nomarsky optics (IX71 Olympus, Tokyo, Japan). While the embryos were being immobilized by suction using a holding pipette, a drilling pipette (internal diameter 22 μm) was placed in close contact with the zona pellucida and a hole was made with a controlled stream of pronase (P8811, Sigma, UK) solution (5 mg/ml ; 28 IU/ml prepared in H-SOF). Immediately after penetration of zona pellucid, an embryo was transferred into the next H-SOF drop in the same Petri dish. Following embryo penetration, the sampling pipette was pushed through the hole and one or two cells judged to be equal to the 8-cell stage were removed by gentle suction.

Culture of biopsied embryos: The biopsied embryos were cultured (1 embryo in 20 μl) in drops of SOF-OCM at 39° C under a gas phase of 5% CO₂ in air. The embryos were assessed for morphological development to blastocysts and hatched blastocysts until Day 9. The total cell number, as well as differential cell numbers representing the inner cell mass (ICM) and trophectoderm were determined by a differential staining technique for blastocysts.

Staining: Blastocysts were incubated for 15 min at 39° C in the basic medium (H-SOF containing 5 mg/ml BSA) supplemented by 10 mg/ml propidium iodide (PI). They were then transferred to a drop of basic medium on a glass slide and examined under an epifluorescent microscope (IX71 Olympus, Tokyo, Japan). The trophectoderm (TE) cells with membrane injury (dead cells) were stained with PI. Indeed, PI only enters cells with altered membrane integrity illuminating red color following UV excitation.

For differential staining of inner cell mass (ICM) and TE cell compartments, the blastocysts which had been stained by PI were incubated in Triton X-100 prepared in the base medium for 20 seconds. The blastocysts were then stained in the base medium containing 30 $\mu\text{g/ml}$ PI for 1 min. After washing in the base medium twice, the blastocysts were transferred into ice-cold ethanol containing 10 mg/ml Hoechst 33342 for 15 min. The blastocysts were directly mounted onto a glass slide and examined under an epifluorescent microscope. ICM nuclei appeared blue, caused by

DNA labeling with the membrane permeable Hoechst 33342, and TE cells appeared red due to staining of nuclear DNA with the membrane impermeable PI.

Statistical analysis: The data were collected upon at least five replicates. The blastocyst cell numbers were analyzed using one-way ANOVA. When ANOVA revealed a significant effect, comparison of means among the study groups was performed using the Tukey test. When the normality test failed the Kruskal-Wallis One Way Analysis of Variance on Ranks was applied. Comparisons of post-biopsy embryonic development between the groups were carried out by the Chi-square test. A p-value smaller than 0.05 was considered significant (SigmaStat, version 2). The data were expressed as means \pm SEM.

Results

The subsequent embryonic development of the biopsied embryos was significantly influenced by the age and cell number of the embryos at the time of biopsy. The embryonic development to blastocyst stage in embryos biopsied on Day-4 (fertilization on Day-0) at 16-cell stage was significantly higher than those biopsied on Days 2 and 3 and at 8-cell stage on Day-4 ($p < 0.05$). The blastocyst formation for embryos biopsied at 8-cell stage on Day-4 was significantly higher than those biopsied on Day-2 ($p < 0.05$). Among the biopsied embryos, the lowest blastocyst rate was obtained in embryos biopsied on Day-2. The difference in the proportion of hatched blastocysts was insignificant among the groups (Table 1). In non-biopsied embryos, there was a trend of increasing rate in the number of blastocysts as the developmental stage of embryos increased. No significant differences were observed in the rates of blastocysts and hatched blastocysts between biopsied and corresponding non-biopsied embryos in different experimental groups.

As shown in Table 2, the differences in the number of total cells, cell allocation to trophectoderm, the inner cell mass or the number of dead cells between the biopsy-derived blastocysts at different developmental stages were insignificant. The difference in the proportion of ICM to the total cell numbers (ranging from 21 to 29%) was insignificant. There were no significant differences in the end points between biopsied and non-

Table 1. Effects of age and cell numbers of biopsied embryos on subsequent embryonic development

Biopsy (+/-)	Embryo		Blastocyst		Hatched blastocyst**	
	Age (Day)	Cell No.	No.		n (%)	
			+	-	+	-
-*	--	--	105		40 (38%) ^a	
+/-	2	4	59	36	23 (39%) ^a	20 (56%) ^{a,b}
		8	69	38	23 (33%) ^a	18 (47.3%) ^{a,b}
+/-	3	4	41	40	20 (49%) ^{a,b}	22 (55%) ^{a,b}
		8	84	40	39 (46%) ^{a,b}	24 (60%) ^b
+/-	4	8	56	36	36 (64%) ^b	24 (66.7%) ^{b,c}
		16	33	36	31 (94%) ^c	30 (83.3%) ^c

a,b,c: Data with different superscripts in the same column differ significantly ($p < 0.05$).

*: The proportion of blastocysts expressed on the basis of the total number of presumptive zygotes.

**: The proportion of hatched blastocysts expressed on the basis of the total number of blastocysts.

biopsy derived blastocysts.

Discussion

Despite the invasiveness of embryo biopsy, the early embryos' plasticity is such that the procedure does not seem to adversely affect the capacity of the embryo to develop or to implant normally (8). However, there is evidence, indicating the importance of timing and method of biopsy in influencing the subsequent embryonic development and survival after cryopreservation (10). In mouse, biopsy at 2-cell stage using a non-contact infrared laser for zona drilling was superior to biopsy at 8-cell stage. Indeed, at 8-cell stage it was not possible to drill the zona while maintaining a safe distance (generally $> 8 \mu m$) and as a consequence almost all embryos had at least one damaged blastomere which could influence the subsequent embryonic development. In chem-

ical zona drilling, using acid Tyrode's solution, the substance frequently lysed an adjacent blastomere which in turn could decrease the number of cells at late morula/ early blastocyst stage (12). Moreover, in the majority of studies which have compared the effect of acidified Tyrode's solution and laser zona drillings on subsequent embryonic development, there has been no significant difference between the two methods (13 - 15). Some parameters, however, have been adversely affected by embryo biopsy (12, 15).

In the current study, the subsequent embryonic development was not affected by cell sampling, using pronase, compared to that of the non-biopsied embryos. The proportion of blastocysts, however, was slightly higher, though insignificant, in non-biopsied than biopsied embryos on Days 2 and 3.

It is generally agreed that the best time to

Table 2. Effects of age and cell number of biopsied embryos on cell allocation of biopsy-derived blastocysts

Groups	Embryo			Embryonic cells (M \pm SEM) (%)			
	Age (Day)	Cell No.	No.	Total	ICM	Trophectoderm	Dead cells
Non-biopsied	--	--	22	137.9 \pm 10.6	33.6 \pm 3.1 (24%)	104.3 \pm 9 (76%)	5.9 \pm 0.8 (4%)
Biopsied	2	4	8	102.5 \pm 11.4	21.8 \pm 4.8 (21%)	80.8 \pm 7.1 (79%)	8.8 \pm 0.9 (8%)
		8	8	135.2 \pm 30.3	31.5 \pm 3.7 (23%)	103.8 \pm 27.5 (77%)	7.3 \pm 1.3 (5%)
	3	4	10	119.5 \pm 19.9	34.0 \pm 7.8 (28%)	85.5 \pm 15.8 (72%)	5.2 \pm 1.0 (4%)
		8	34	112.9 \pm 6.7	32.9 \pm 3.0 (29%)	80.0 \pm 6.6 (71%)	5.2 \pm 0.6 (5%)
	4	8	12	109.8 \pm 14.6	28.3 \pm 3.2 (24%)	81.4 \pm 13.3 (76%)	6.4 \pm 1.3 (6%)
		16	10	119.2 \pm 18.4	26.2 \pm 2.8 (22%)	93.0 \pm 20.4 (78%)	7.0 \pm 2.8 (6%)

remove one or two cells from the human embryo is between the six- and ten-cell stages (16). The efficiency of producing bovine *in vitro* produced (IVP) blastocysts transferable after vitrification on Day-7 was higher when biopsy was performed on Day-4 rather than Day-7.5 (10). In contrast, the *in vitro* viability of manipulated or vitrified ovine embryos was significantly lower at precompacted morula (Day-4) and compacted morula (Day-5) stages than blastocyst (Day-6) stage (17).

In the current study removal of blastomeres on Day-4 (16-cell stage) resulted in a higher proportion of embryos reaching the blastocyst stage ($p < 0.05$) compared with embryo biopsy on Days 2 and 3 at both 4-cell and 8-cell, as well as the Day-4, 8-cell stages. In this context, biopsy at the 8-cell stage in mouse had the lowest detrimental effect on subsequent embryonic development compared with 4-cell and morula stages (18).

A reduced viability following blastomere removal at 4-cell compared with 8-cell stage (18) was inconsistent with the results of the current study. On the other hand, in the present study when embryo biopsy was performed on Days 2 and 3, there was no significant difference in post-biopsy development between the 4-cell and 8-cell stage biopsied embryos. However, for the 8-cell stage embryos biopsied on Day-4, the rate of blastocyst formation was higher compared with embryos biopsied on Day-2. Moreover, there was a trend for increased developmental potential as the day of biopsy increased. Since this trend was similarly observed in non-biopsied embryos (the controls), it could be concluded that the differences in blastocyst rate were due to the dissimilar comparison among the groups. Therefore, such comparison undermine the conclusion that biopsy in early developmental stages impair the subsequent embryonic development. On the other hand, the higher blastocyst rates in biopsied and non-biopsied embryos on Day-4 than corresponding rates in Days 2 and 3 could be due to the fact that the calculation of blastocyst rate on Day-4 has been based on the total number of 8-cell and 16-cell stage embryos on Day-4, which was smaller than the total number of 4-cell and 8-cell stage embryos on Days 2 or 3. The developmental stage at the time of biopsy, however, is a more reliable predictor of post-biopsy development and hence

the embryos with more cells have a higher chance of developing after biopsy. Accordingly, the highest post-biopsy blastocyst formation rate was observed in those biopsied on Day-4 at the 16-cell stage.

It is generally believed that tighter intercellular adhesions are formed alongside embryonic development, especially after compaction; therefore, it can be speculated that the intercellular adhesions on Day-4, the 16-cell stage embryos or the precompacted morula stage, may have not been tight enough to interfere with embryo biopsy and subsequent embryonic development.

In other trials, biopsies at earlier stages had resulted in reduced development to the blastocyst stage or lowered post-transfer survival in mouse (18-20) or reduced cell numbers in human blastocysts (21), although others have not demonstrated such differences (22).

According to our results, the higher blastocyst rate in Day-4 biopsied embryos _ especially at the 16-cell stage_ compared to Day-2 and 3 embryos, was in agreement with several reports which have similarly shown that biopsies at later stages do not influence development to the blastocyst stage (23-25).

For bovine embryos, the blastomere removal 48 hours postinsemination, resulted in 17.2% of the embryos proceeding to the blastocyst stage, which was lower than when biopsies were performed 72 h after insemination (37.5%), (26), although there is evidence that neither *in-vitro* (27) nor *in-vivo* development (28) is impaired after aspiration of up to two blastomeres from 8-cell human embryos.

One possible explanation for the observed differences in post-biopsy developmental behavior between Day-4 and Day-2 or Day-3 embryos may be related to the higher number of surviving blastomeres after the biopsy, while the earlier stage embryos lack a sufficient number of blastomeres to develop to the blastocyst stage. It seems that with the increased number of blastomeres at the morula stage, the chances of biopsied embryos to develop to the blastocyst stage is increased. It has been confirmed that removal of 10 to 20% of the cells at postcompaction stage has no detrimental effect on the survival rate of bovine embryos (29).

The prominent finding of the current study was the higher post-biopsy developmental potential of 8-cell stage, Day-4 embryos compared with the corresponding developmental potential of 8-cell stage embryos biopsied on Days 2 or 3. This finding was contrary to the general knowledge indicating that zygotes which initiate the first mitotic cleavages faster have a better chance of reaching the blastocyst stage (30). Hypothetically, this might be related to the lower sensitivity of Day-4 embryos to the manipulations compared with embryos manipulated on Days 2 or 3.

There is evidence indicating that embryo biopsy could exert detrimental effects on embryo quality and development (31) through cell damage and inflammatory response induced by formation of cyclooxygenase and lipoxygenase metabolites such as prostaglandins (PGF 2α) (32-34). Moreover, apoptosis has been shown to be inducible by environmental factors, such as cytotoxically high levels of glucose (35) and heat stress (36). Whether and to what extent the blastomere removal on Days 2 or 3 is more stressful for embryos to induce apoptosis compared with the action on Day-4 is to be elucidated.

In ovine embryos, both biopsy and vitrification could influence the hatching rate at earlier stages (Days 4 and 5 vs. Day 6 postinsemination) as the hatching rates of precompacted and compacted morula are lower after the manipulation than that of the intact embryos (17). No such deleterious effects, however, were observed in the current study as no significant differences were observed between the biopsied and non-biopsied embryos in terms of survival and hatching rates. It seems that, at least in terms of hatching process, the bovine embryos are more resistant to such micromanipulations compared with those of ovine. However, in the majority of biopsied embryos, the hatching process occurred without thinning and expansion of the zona pellucida, as compared with the intact embryos. Moreover, in some blastocysts the hatching process took place incompletely. These conditions have been previously reported in biopsied ovine embryos by Naitana et al. (17). Since the difference in the number of total cells in biopsied and non-biopsied embryos was insignificant, the lack of zona pellucida thinning and expansion in biopsied embryos could not be related to the number of

total cells. Instead, it might be related to the fact the ZP did not narrow, probably due to the hole created in the ZP, helping embryos start hatching without exerting pressure on the ZP.

In the current study, no significant differences were found in the number of ICM, trophoblast, and total cells in biopsy-derived blastocysts compared to non-biopsied ones. These results were in contrast to those reported by Hardy et al. (27) and Tarin et al. (21), who observed that removal of one or two blastomeres from 4 to 8-cell human embryos produced blastocysts with fewer cells. The present results were also in contrast to what was reported by Jimenez-Macedo et al. (37), indicating a decreased blastocyst cell number of biopsied compared with intact embryos in goats. It has also been shown that biopsy-derived bovine blastocysts have fewer total cells than intact blastocysts (26). In the current study, the proportion of dead cells at the blastocyst stage was neither influenced by the day nor by the number of cells at the time of biopsy. Moreover, the proportion of dead cells in biopsy-derived blastocyst was not significantly different with the corresponding rate in non-biopsy derived blastocysts.

Conclusion

This study has provided evidence that biopsy by the aspiration method does not compromise the in vitro developmental potential of bovine pre-compacted embryos and that the 16-cell stage embryos biopsied on Day-4 are the best stage for blastomere removal. Moreover, pronase zona drilling has no adverse effects on subsequent embryonic development and it may be an alternative to acid Tyrode's solution or laser zona drilling.

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