EXPOSURE OF BOVINE EMBRYOS TO TRYPSIN DURING WASHING DOES NOT DECREASE EMBRYONIC SURVIVAL

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ABSTRACT

The objective of this study was to assess whether the exposure of zona pellucida-intact bovine embryos to the proteolytic enzyme, trypsin, during embryo washing has a detrimental effect on their subsequent survival and development. Embryos were collected nonsurgically from superovulated cows (n = 19) 7.5 d after insemination. Grade 1 and Grade 2 embryos were washed 12 times in modified Dulbecco's phosphate buffered saline (PBS) containing 0.4% bovine serum albumin (BSA), or in a series of five washes in BSA-PBS (without Ca++ and Mg++), two in 0.25% trypsin in Hank's solution (without Ca++ and Mg++) and five in PBS-BSA medium. Within 30 min after washing, embryos were either transferred nonsurgically into recipient cows, 7 to 8 d post estrus, or cryopreserved and transferred later. Frozen-thawed embryos from five of the donors were cultured for 72 h in vitro and their development was evaluated.

Pregnancy rates did not differ ($P>0.1$) between recipient cows receiving control-washed and trypsin-washed embryos transferred fresh (51.0 vs 56.3%). However, pregnancy rates were higher ($P<0.05$) for frozen-thawed embryos treated with trypsin before cryopreservation than for frozen-thawed, control-washed embryos (68.2 vs 38.5%). Survival and development of embryos in vitro after cryopreservation did not differ between embryos subjected to the control- and trypsin-wash procedures. These results suggest that exposure of bovine embryos to trypsin for 2 to 3 min during washing did not have a detrimental effect on embryonic development, but may have enhanced cryopreservation of the embryos.

Key words: embryo washing, trypsin treatment, cryopreservation, bovine embryos

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INTRODUCTION

The recent development of methodology for the successful cryopreservation of bovine embryos has expanded the opportunities for international movement of embryos and has facilitated distribution of germ plasm as well as pathogenic agents. Most pathogens can be washed from the surface of zona pellucida-intact bovine embryos by subjecting the embryos to 10 to 12 changes of medium, each change of medium resulting in a 100-fold dilution of the medium (1, 2). However, some viruses (e.g., infectious bovine rhinotracheitis virus and vesicular stomatitis virus) adhere firmly to the zona pellucida and are not removed by the 10 to 12 changes of medium (3-5). Exposure of the embryos to the proteolytic enzyme, trypsin, in the middle of the wash procedure has been found to inactivate or remove these infectious viruses (4, 5). Consequently, several countries have incorporated the trypsin-wash procedure into their health regulations for importation of either fresh or cryopreserved (deep-frozen) embryos. Because of the proteolytic activity of the trypsin enzyme, concern arose as to potential detrimental effects of the trypsin treatment on the integrity of the zona pellucida and subsequent viability of fresh or frozen-thawed embryos. Availability of such information is limited; thus, the objective of our study was to determine whether a brief treatment of bovine embryos with trypsin affects survival of washed embryos evaluated either fresh or after cryopreservation.

MATERIALS AND METHODS

Embryos to be washed were obtained from 19 mature cyclic beef cows superovulated with a total dosage of 34 mg of follicle stimulating hormone (FSH)[a]. The FSH was administered at 12-h intervals in decreasing dosages (5, 5, 4 and 3 mg twice daily, i.m.) for 4 d beginning 9 to 14 d after estrus. Prostaglandin $F_{2\alpha}$[b] was administered i.m. on the morning of the fourth day of FSH treatments. Donor cows were artificially inseminated 48 and 60 h after prostaglandin $F_{2\alpha}$ or 12 h after onset of estrus, or both.

Embryos were collected from the donor cows nonsurgically 7.5 d after insemination; flushing medium was 5.56 mM D-glucose, 0.3 mM Na pyruvate PBS supplemented with 2% BSA, fraction V. Embryos were recovered from the flushing medium within 1 to 1.5 h after flushing, transferred to a holding medium of 0.4% BSA in PBS (BSA-PBS) and held at 24°C (maintained room temperature) until all embryos were recovered and evaluated microscopically (75x) for stage of development, embryo quality and integrity of the zona pellucida. Grade 1 (excellent) and Grade 2 (good) quality morulae, early blastocysts and blastocysts with an intact zona pellucida were grouped within donor by stage of development and quality, and were assigned randomly within group to either the control-wash or trypsin-wash procedure described below. After washing, embryos were either

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[a]FSH-P®; Burns-Biotec, Omaha, NE.
[b]Lutalyse®, Upjohn Co., Kalamazoo, MI.

transferred nonsurgically into recipient cows or cryopreserved and transferred into recipients at a later date. Control-washed and trypsin-washed embryos from five of the donor cows were evaluated in vitro after cryopreservation rather than transferred to recipient cows. The cryopreservation and culture procedures are described below.

Both fresh and frozen-thawed embryos were transferred nonsurgically into recipient cows on Day 7 or 8 of a natural estrous cycle (estrus = Day 0). All embryos were transferred by the same technician. Recipient cows received 3 x 10^6 units of benzathine penicillin G[c] and 3 x 10^6 units of procaine penicillin G[c] at the time of transfer. Pregnancy was determined at 75 d of gestation by palpation per rectum. Effects of wash procedure (control vs trypsin), type of embryo (fresh vs frozen-thawed) and wash procedure by type of embryo on pregnancy rate were evaluated using the SAS GLM procedure (6); donor within wash by type of embryo was the error term to test main effects. Differences in embryonic survival and development in vitro between control-washed and trypsin-washed embryos were assessed by Chi-square analysis (7).

Wash Procedures

Control wash. The control-wash procedure consisted of pipetting embryos in groups of 10 or less, using a micropipettor, through 12 sequential changes of sterile BSA-PBS medium (2). Washes were performed in disposable microtiter plates (12 wells) using 2 ml of BSA-PBS medium per well. Embryos were pipetted between wells in a total volume of 20 µl of medium to produce a 1:100 dilution of the medium from the preceding well; a fresh sterile pipette was used for each well. Embryos were agitated gently in each well. Embryos from each donor were washed separately using a new plate and medium for each donor. The total wash procedure was completed within 40 min at 24°C.

Trypsin wash. Embryos treated with trypsin were pipetted similarly through five changes, or washes, of 0.4% BSA-PBS medium (2 ml) without Ca++ and Mg++, two washes in 0.25% trypsin (1:250) in Hank's balanced salt solution without Ca++ and Mg++ (pH 7.6) for 60 to 90 sec each, followed by five washes in 0.4% BSA-PBS complete medium with 2% fetal calf serum.

Cryopreservation

Subsequent to washing, control-washed and trypsin-washed embryos of Grade 1 and 2 quality were pipetted into the cryoprotective medium of 1.5 M glycerol (11% v/v) in 0.4% BSA-PBS and allowed to equilibrate for 5 min at 24°C; then, each embryo was aspirated individually into 0.25-cc plastic French straw (8), and the open end was heat sealed.

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[c]Flo-Cillin, Bristol Lab., Syracuse, NY.

The straws were loaded vertically in a mechanically refrigerated alcohol freezer[d], cooled to -4.5°C at 1.5°C/min, seeded manually and held for 5 min, cooled to -36.0°C at -0.45°C/min and then plunged into liquid nitrogen for long-term storage. The ice crystallization temperature of the cryoprotective medium was measured and the seeding temperature was adjusted for each batch of medium.

Straws were thawed in air (24°C) for 15 sec and 37°C water for 30 sec. The cryoprotectant was removed by placing the recovered embryos for 5 min each in 0.14 M glycerol, 0.67 M sucrose in 0.4% BSA-PBS, 0.4 M sucrose in BSA-PBS and twice in BSA-PBS. Embryos were either transferred into recipient cows immediately without further evaluation, or placed in modified Ham's F-10 supplemented with 1.5% BSA and cultured for 72 h.

In Vitro Embryo Culture

The in vitro culture procedure and synthetic culture medium have been described previously for rabbit embryos (9). Frozen-thawed embryos were placed in 1 ml of modified Ham's F-10 supplemented with 1.5% BSA, and incubated for 72 h at 38.5°C in a water-saturated atmosphere of 5% CO_2 in air at a pressure of 30 mm mercury. The status of each embryo was evaluated microscopically at initiation of culture and at 24-h intervals. Culture vessels were sterile open-well microtiter trays (six wells) contained within 10 x 100 mm petri dishes containing 2 ml of sterile double distilled water to ensure adequate humidity. Petri dishes were stacked in cylindrical holding jars contained within an incubator.

RESULTS AND DISCUSSION

Exposure of bovine embryos to the proteolytic enzyme, trypsin, for 2 to 3 min during washing did not have a detrimental effect on pregnancy rate in recipient cows receiving fresh or frozen-thawed embryos (Table 1), or on the rate of embryonic development in vitro (Table 2). Pregnancy rates (Table 1) did not differ ($P>0.1$) between the control- and trypsin-wash procedures (51.0 vs 56.3%, respectively) when the embryos were transferred into recipient cows within 30 min after washing and without cryopreservation. These pregnancy rates were similar to pregnancy rates (57.7%) obtained with unwashed embryos ($n = 104$) of similar quality and stage of development that were collected from contemporary donor cows[e]. Likewise, microscopic examination of the embryos did not reveal any changes or deterioration in the integrity or quality of the zona pellucida or embryonic cells. However, when the embryos were washed, cryopreserved and thawed before transfer into recipients, the pregnancy rate was significantly higher ($P<0.05$) for recipient cows that received embryos washed by the trypsin procedure (68.2%) than for embryos washed by the control

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[d]Bio Cool II Freezer, FTS Systems, Inc., Stone Ridge, NY.
[e]Echternkamp, S. E. and Kappes, S. M., unpublished data.

Table 1. Comparison of pregnancy rates for control-washed and trypsin-washed bovine embryos[a]

Wash procedure	Type of embryo	
	Fresh	Frozen-thawed
Control	25/49 (51.0)[b]	10/26 (38.5)[c]
Trypsin	27/48 (56.3)	15/22 (68.2)[d]

[a]One embryo was transferred nonsurgically per recipient. Frozen-thawed embryos were washed before cryopreservation.
[b]Number of recipients pregnant/Number of transfers (percentage).
[c,d]Pregnancy rates differ ($P<0.05$).

procedure (38.5%) before cryopreservation (Table 1). Pregnancy rates for fresh and frozen-thawed embryos obtained in our study were similar to the pregnancy rates of 50 to 60% for fresh embryos and 40 to 50% for frozen-thawed embryos reported by other investigators (10-13). Consistent with other studies (14), the pregnancy rate was about 12% lower for frozen-thawed than for fresh control-washed embryos (38.5 vs 51.0%), whereas, the pregnancy rate was about 12% higher for frozen-thawed than for fresh trypsin-washed embryos (68.2 vs 56.3%). Because of the high pregnancy rate obtained with frozen-thawed, trypsin-washed embryos, pregnancy rates did not differ ($P>0.1$) between recipients receiving fresh or frozen-thawed embryos (53.6 vs 52.1%, respectively).

The cause(s) for the higher pregnancy rate obtained with embryos treated with trypsin and subsequently frozen-thawed is not known. Trypsin is a digestive enzyme whose role is to hydrolyze polypeptide chains. Presumably, it is this property of trypsin that removes or inactivates pathogens adhered to the surface of the zona pellucida of infected embryos. Likewise, the exposure of zona-intact embryos to trypsin before cryopreservation in our study may have digested or denatured proteinaceous substances on the surface of and within the zona pellucida, thus increasing the permeability of the zona pellucida to the cryoprotectant. The higher pregnancy rate for recipients

Table 2. Effect of trypsin washing on survival and development of bovine embryos in culture

Treatment	N	Embryonic status after cryopreservation and culture[a]		
		No. of expanded hatched blastocysts (%)	No. of hatching or expanded blastocysts (%)	No. of degenerated embryos (%)
Control	18	9 (50.0)	5 (27.8)	4 (22.2)
Trypsin	18	8 (44.4)	4 (22.2)	6 (33.3)

[a]Embryonic status was determined after 72 h of culture in modified Ham's F-10, 1.5% BSA medium and in a humidified atmosphere of 5% CO_2 in air.

receiving frozen-thawed embryos treated with trypsin before cryopreservation may thus result from enhanced permeation of embryonic cells by the cryoprotectant before freezing, and removal of the cryoprotectant from the cells after thawing. Alternatively, trypsin converts several enzymes from the inactive state (zymogen precursor) to the active state and, thus, may act indirectly to enhance cryopreservation of embryos through activation of other enzymes (e.g., phospholipases). Also, the authors acknowledge that the media used in the first seven washes of the trypsin procedure did not contain Ca++ and Mg++, but are unaware of evidence that Ca++ and Mg++ are detrimental to embryonic development at the described concentrations, which are contained in most culture media.

Although treatment of bovine embryos with trypsin before cryopreservation increased embryonic survival in utero, the treatment did not have a beneficial effect on embryonic development or survival in vitro (Table 2). The percentage of embryos surviving (77.8 vs 66.7%) and hatching (66.7 vs 61.1%) after 72 h in culture did not differ ($P>0.1$) between embryos subjected to the control- vs trypsin-wash procedure. Because of substantial differences between the in vitro and in vivo culture systems, direct comparisons of their results are limited, but the implications are that the beneficial effect(s) of trypsin on survival of cryopreserved embryos occurred during or after hatching.

In summary, a brief exposure of bovine embryos to trypsin during washing did not have a detrimental effect on embryonic survival when the embryos were transferred into recipient cows shortly after washing, or when embryos were frozen, thawed and cultured for 72 h. Conversely, a brief exposure of bovine embryos to trypsin before cryopreservation may increase embryonic survival in utero for the frozen-thawed embryos. The cause(s) for the increased fertility for frozen-thawed embryos exposed to proteolytic enzymatic activity before cryopreservation is being investigated.

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