Effect of Trypsin Treatment of In Vitro Fertilized Bovine Embryos on Their Subsequent Survival and Development

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ABSTRACT. The object of this study was to determine whether several washings with trypsin affected the survival and development of in vitro fertilized (IVF) bovine embryos. The embryos developed to blastocysts 7–8 days after insemination, were washed 12 times in washing medium (modified Dulbecco's phosphate-buffered saline (mPBS) containing 20% fetal bovine serum (FBS): 25 mM Hepes TCM199 with Earle's salts=1:1), or in a series of three washes in the washing medium, two in mPBS containing 0.3% bovine serum albumin (BSA), two in 0.25% trypsin in Hank's solution (without Ca\(^++\) and Mg\(^++\)) for a total time in the trypsin of 60 to 90 sec and five in the washing medium. After washing, the embryos were either cultured in vitro or cryopreserved. The fresh and frozen-thawed embryos were either cultured for 72 hr in vitro for evaluating development or transferred nonsurgically to recipient cows. Development of fresh and frozen-thawed embryos in vitro in control-washed and trypsin-washed embryos did not differ. Pregnancy rates did not differ (P>0.05) among recipient cows receiving control-washed or trypsin-washed embryos, and transferring fresh or frozen-thawed embryos. These results indicate that the treatment of bovine IVF embryos with trypsin during washing did not have a positive effect on embryonic development.

KEY WORDS: cattle, embryonic development, in vitro fertilization, trypsin.


With the advance of successful in vitro fertilization techniques and cryopreservation methods for bovine IVF embryos, it is possible for frozen early embryos to be utilized for international trade. However, concern has arisen regarding the possible transfer of infectious disease concomitant with the transfer of IVF embryos. Studies have been carried out to elucidate mechanisms by which embryos could transmit infectious disease\([8]\). Preventive techniques for transfer included a 10–12 step wash procedure to remove many adhering viruses and a few bacterial species. These pathogenic agents have been used in studies to assess the effectiveness of the technique for in vivo bovine embryos\([4, 6, 7, 9, 10, 13]\). Most pathogenic agents can be washed from the surface of zona pellucida-intact bovine embryos by subjecting the embryos to the 10 to 12 changes of medium\([5, 8]\). However, some viruses adhere firmly to the zona pellucida and are not removed in that way\([6, 7, 9]\). Exposure of the embryos to the proteolytic enzyme trypsin, in the middle of the washing procedure, has been found to inactivate or remove these infectious viruses\([6, 7, 9]\). Concern has arisen about the potential detrimental effects of the trypsin treatment on the integrity of the zona pellucida and subsequent viability of fresh or frozen-thawed embryos. Echtenkamp et al.\([1]\) reported that exposure of in vivo bovine embryos to trypsin for 1 to 2 min during washing did not have a detrimental effect on embryonic development. However, the effect of trypsin treatment during washing on the development of IVF embryos has not yet been reported. For that reason, the present study was performed to determine whether the brief treatment of bovine IVF embryos with trypsin during embryo washing has a positive effect on their subsequent survival and development.

MATERIALS AND METHODS

**IVF embryos**: The ovaries were collected from cows at a local slaughterhouse and brought to the laboratory in sterile saline (0.85% (w/v)NaCl) supplemented with antibiotics (penicillin G (100 IU/ml, Meiji Co., Tokyo) and streptomycin (100 µg/ml, Meiji)) at 25–30°C within 1 hr. Oocytes were collected by puncturing the follicles at a diameter of 1–7 mm with a needle. After washing once with mPBS (Embyroteck; Nihon Zenyaku Co., Fukushima, Japan), the oocytes were cultured in the medium (25 mM-Hepes TCM199 with Earle's salts; Gibco, NY, U.S.A.) supplemented with 5% fetal bovine serum (FBS: Gibco) for 20–22 hr at 38.5°C in 5% CO\(_2\) in air, subsequently fertilized in vitro with frozen-thawed semen, and the embryos were cultured on the cumulus cell layers according to the method previously reported\([2]\). The embryos 7–8 days after insemination developed to blastocysts, which were assigned randomly within a group to either the control-wash or trypsin-wash procedure described below. After washing, the embryos were either transferred nonsurgically to recipient cows or cryopreserved and transferred to recipients later.

**Embryo transfer**: Both fresh and frozen-thawed embryos were transferred nonsurgically to recipients on day 7 or 8 of the estrous cycle. Embryo transfers were performed by three technicians. Pregnancy was diagnosed by rectal palpation at 60 days of gestation.

**Control washing procedure**: Twelve 35 mm sterile plastic dishes containing 2 ml of washing medium (mPBS supplemented with 20% FBS: 25 mM Hepes TCM199 =
1:1) containing antibiotics were used for the washing of the embryos. Sterile micropipettes were used to place the embryos into another dish. The ratio of the volume of medium containing the embryos in the pipette to each washing medium was at least 1:100. The total washing procedure was completed within 30 min at room temperature.

**Trypsin washing:** The embryos were transferred through three washing mediums and then through two washes of mPBS supplemented with 0.3% BSA, followed by two washes in 0.25% trypsin (Difco, Detroit, Mich, U.S.A.) in Hank's balanced salt solution without Ca++ and Mg++ (pH 7.6, Gibco) for a total time in the trypsin of 60 to 90 sec. After trypsin treatment, the embryos were transferred through five lots of washing medium. Some embryos were placed in the trypsin solution for a total time of 5 min or 10 min during washing to evaluate the effect of the longer exposure time on *in vitro* embryonic development.

**Cryopreservation:** After washing, control-washed and trypsin-washed embryos were cryopreserved by a modified method of Suzuki et al. [11]. The embryos were washed twice in mPBS supplemented with 0.3% BSA and were suspended in mPBS supplemented with 0.3% BSA and 1.6 M of 1, 2-propanediol. Cryoprotectants were added at room temperature in one step. After 15 min for equilibration, the embryos were loaded into 0.25 ml plastic straws in mPBS containing 0.3 M of sucrose. The straws were placed directly into a cooling chamber and cooled from 0°C to −5.5°C at −1°C/min, seeded at −5.5°C and cooled at the rate of −0.4°C/min to −30°C, then plunged and stored in liquid nitrogen.

The straws were thawed in air for 5 sec and then plunged into a 37°C water bath for 30 sec. After thawing, the contents of each straw were drained into a sterile petri dish. Within 1 min, thawed embryos were placed in mPBS supplemented with 0.3% BSA and were washed twice. After washing, the embryos were transferred to a cumulus cell layer in the dish containing culture medium (25 mM Hepes TCM199 supplemented with 1% FBS, insulin (5 μg/ml, Sigma, St. Louis, Mo. U.S.A.) and antibiotics). After 20 hr in culture, the viable embryos were transferred to recipient cows. And some embryos were placed in the culture medium and cultured for 72 hr.

**In vitro embryo culture:** After washing and cryopreservation, fresh and frozen-thawed embryos were placed in 25 mM Hepes TCM199 supplemented with 1% FBS, insulin (5 μg/ml) and antibiotics, and incubated for 72 hr at 38.5°C in 5% CO₂ in air. The aspects of each embryo were examined under a microscope at the initiation of culture and at 24 hr intervals. After 72 hr in culture, the numbers of hatched blastocysts were recorded.

The statistical significance of differences between groups was analyzed by Chi-square test (p<0.05).

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**RESULTS**

When bovine IVF embryos were exposed to the trypsin solution during washing for a total time of 5 min or 10 min, 91.7% (11/12) of the embryos exposed for 5 min hatched from the zona pellucida after 72 hr in culture, but the hatching rate of the embryos exposed for 10 min was reduced significantly (p<0.05) (53.8%, 7/13). However, treatment of the embryos with trypsin for a total time of 60–90 sec during washing did not have a positive effect on development of the *in vitro* culture or on pregnancy rates in recipient cows for fresh or frozen-thawed embryos (Tables 1, 2). And the percentage of hatched embryos after 72 hr in culture for fresh and frozen-thawed embryos did not differ (p>0.05) between the control- and trypsin-wash procedure (fresh: 76.7 vs 82.2%, frozen-thawed: 46.7 vs 43.9%, respectively). Likewise, pregnancy rates for fresh and frozen-thawed embryos did not differ (p>0.05) between the control- and trypsin-wash procedures (fresh: 66.7 vs 30.0%, frozen-thawed: 27.3 vs 27.3%, respectively). However, pregnancy rates tended to be lower for fresh embryos washed by the trypsin solution than those washed by the mPBS without trypsin.

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**DISCUSSION**

Pregnancy rates for fresh embryos without trypsin treatment obtained in our study were similar to that in the report by Kajihara et al. [3]. Echternkamp et al. [1] reported that pregnancy rates of fresh *in vivo* embryos were significantly higher than the percentage of hatched blastocysts. However, pregnancy rates for frozen-thawed embryos treated with trypsin were lower than the percentage of hatched blastocysts.

**Table 1.** Comparison of developmental rates for control-washed and trypsin-washed bovine IVF embryos

<table>
<thead>
<tr>
<th>Washing procedure</th>
<th>Fresh (%)</th>
<th>Frozen-thawed (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>33/43 (76.7)</td>
<td>21/45 (46.7)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>37/45 (82.2)</td>
<td>18/41 (43.9)</td>
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a) Embryonic aspects were determined after 72 hr of culture in TCM199 supplemented with 1% FBS and 5 μg/ml insulin at 38.5°C in 5% CO₂ in air.
b) Frozen-thawed embryos were washed before cryopreservation.
c) Number of hatched blastocysts/Number of washed blastocysts (percentage).

**Table 2.** Comparison of pregnancy rates for control-washed and trypsin-washed bovine IVF embryos

<table>
<thead>
<tr>
<th>Washing procedure</th>
<th>Fresh (%)</th>
<th>Frozen-thawed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14/21 (66.7)</td>
<td>3/11 (27.3)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>3/10 (30.0)</td>
<td>3/11 (27.3)</td>
</tr>
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a) Frozen-thawed embryos were washed before cryopreservation.
b) Number of recipients pregnant/Number of transfers (percentage).
were similar when washed with and without trypsin. In our study, the percentages of hatched blastocysts after 72 hr in culture for fresh embryos were similar in the control- and trypsin-wash procedures. Moreover, when the length of exposure of the embryos to the trypsin solution was extended to 5 min, the percentage of hatched embryos did not decrease. These results showed that a brief exposure of IVF embryos to trypsin did not have a positive effect on in vitro embryonic development. On the other hand, the pregnancy rate for fresh embryos after washing by the trypsin procedure tended to be lower than that of fresh embryos without trypsin treatment. Most fresh embryos treated with trypsin were transferred to recipients during June. In this season, the pregnancy rates obtained with the transfer of bovine embryos that were collected from donor cows tended to decrease (unpublished data). These results indicate that the causes of the lower pregnancy rates obtained with fresh embryos treated with trypsin may be the seasonal effects. Echterkamp et al. [1] reported that pregnancy rates were higher for frozen-thawed embryos treated with trypsin before cryopreservation than for frozen-thawed, control-washed embryos (68.2 vs. 38.5%). Also, he stated that the causes of the higher pregnancy rates in recipients receiving frozen-thawed in vivo embryos treated with trypsin before cryopreservation might lie in enhanced permeation of the cryoprotectant into embryonic cells before freezing, and removal of the cryoprotectant from the cells after thawing. In our study, however, the pregnancy rates were similar in frozen-thawed embryos treated with trypsin and frozen-thawed control-washed embryos. Takai et al. [12] reported that the number of cells in bovine IVF embryos was smaller than that in bovine embryos collected from donor cows. And IVF embryos have lower resistance to colcemid than embryos collected from donor cows [14]. The causes of the difference between IVF embryos and embryos derived from donor cows in the effectiveness of trypsin treatment may lie in the quality of each bovine embryo.

In summary, the brief exposure of bovine IVF embryos to trypsin during washing did not have a positive effect on embryonic survival when the embryos were cultured for 72 hr or when the embryos were transferred into recipients after washing and cryopreservation.

REFERENCES