The study was conducted to evaluate the efficiency of vitrification for in vitro produced and splitted bovine blastocysts. In vitro produced bovine embryos with good or fair morphology were bisected using a simplified splitting technique without the aid of a holding pipette or other microinstruments. Zona-free demi-blastocysts were vitrified with 20% glycerol, 20% ethylene glycol, 0.5 M sucrose and dextrose. The viability of fresh and vitrified splitted and intact blastocysts was evaluated after in vitro culture by means of their re-expansion. The survival rates after in vitro culture of fresh intact and splitted blastocysts, and of vitrified intact and splitted blastocysts were not significantly different (p>0.01).

INTRODUCTION

The ability to bisect morulae and blastocysts and produce offspring from each of the halves is being used increasing in bovine embryo transfer. Pregnancy rates are slightly lower than that can be obtained from intact embryos (1). Splitting followed by cryopreservation and subsequent non-surgical transfer will facilitate the commercial application of in vitro production (IVP) of bovine embryos. As in IVP embryos are sensitive to micromanipulation and cryopreservation, these technologies must be carefully performed and evaluated. Reports on conventional freezing of biopsied in vivo fertilized embryos are available (2-3). However, data on survival of splitting IVP embryos following vitrification is very limited. Moreover, the overall efficiency of the IVP splitting cryopreservation technology i.e. the percentage of oocyte developing to blastocyst and surviving splitting and cryopreservation has not been studied. The aim of the present study was, therefore, to evaluate the efficiency measured by embryonic in vitro survival of IVP embryos following splitting and cryopreservation.
MATERIALS AND METHODS

In Vitro Embryo Production (IVP)

Collection of oocytes, maturation (IVM), fertilization (IVF) and embryo culture (IVC) were performed according to procedures described previously (4). The ability of inseminated oocytes to accomplish fertilization was judged by their development to the 2-cell stage. Grade 1 blastocysts on d 7 or d 8 after insemination were used for bisection and vitrification.

Bisection of d 7 to d 8 Blastocysts

Biopsies of d 7 to d 8 blastocysts were performed according to the procedure of Bredbacka et al. (5). Excellent or good blastocysts were selected and incubated in TCM-Hepes without Ca++ and Mg++ for 5 min at 25 °C. Embryos were transferred into a 500 µl droplet of TCM-Hepes with 10% FCS in a 35 mm plastic petri dish. Bisection was made under stereomicroscope by hand with a slice of a razor blade glued to a glass tube. Avoid to make too hard cut, otherwise the embryos will remain attached to the bottom. Ideally, both demi-embryos will float in the medium when the blade is lifted. Careful movements are needed to separate them.

Vitrification of Demi-Embryos

Vitrification and warming were performed as described earlier (6). Briefly, embryos were washed in PBS with 20% FCS and incubated for 5 min in a mixture of 10% glycerol (G), 10% ethylene glycol (EG), 0.25 M sucrose (S) and dextrose (D) (VS1), then 5 min in a mixture of 20% G, 20% EG, 0.25 M S and D (VS2). Finally, they were briefly exposed to VS3 which consists of 40% G, 40% EG, 0.375 M S and D. Straws (0.25 ml) were loaded with 2-3 embryos and plunged immediately into the liquid nitrogen. At warming, straws were immersed horizontally in 20 °C water-bath for 8 sec before the contents were expelled. The embryos were put in TCM-Hepes with 20%FCS and 0.5 M S for 5 min and 0.25 M S for another 5 min. The embryos were washed in PBS with 20% FCS and cultured under TCM 199 with 10% FCS. At 24 h re-expansion rates were evaluated.

Effect of bisection and vitrification on blastocyst re-expansion rates with five replicates were compared to the control group by Chi-square test.
RESULTS

Table 1. Cumulative efficiency of d 7-8 blastocysts after splitting and vitrification; (%)* refer to number of oocytes matured and (%)+ refer to number of blastocysts manipulated.

<table>
<thead>
<tr>
<th>No. oocyte Matured</th>
<th>No. of Blastocyst (%) *</th>
<th>Splitting</th>
<th>Vitrification</th>
<th>No. Re-expanded (%) +</th>
</tr>
</thead>
<tbody>
<tr>
<td>92</td>
<td>41 (45)</td>
<td>-</td>
<td>-</td>
<td>37 (90)</td>
</tr>
<tr>
<td>85</td>
<td>33 (39)</td>
<td>-</td>
<td>+</td>
<td>23 (70)</td>
</tr>
<tr>
<td>78</td>
<td>33 (42)</td>
<td>+</td>
<td>-</td>
<td>54 (82)</td>
</tr>
<tr>
<td>86</td>
<td>37 (43)</td>
<td>+</td>
<td>+</td>
<td>46 (62)</td>
</tr>
</tbody>
</table>

The survival rates of bovine blastocysts after splitting and/or vitrification were summarized in Table 1. The viability after in vitro culture of fresh intact and splitted blastocysts, and of vitrified intact and splitted blastocysts was not significantly different (90, 82, 70 and 62%, respectively).

Fig. 1. A) Representative of d 7 to d 8 bovine blastocysts  
B) A bovine blastocyst was bisected by a razor blade.  
C) Demi-blastocysts after cutting.  
D) Re-expansion of demi-blastocysts.
DISCUSSION

In the present study, we have shown that bovine blastocysts bisected by a simple manual method survive after cryopreservation by vitrification. Various methods have been used to biopsy preimplantation embryo, including aspirating several blastomeres through a hole in the zona made with glass capillary (7), acid solution (8) or bevelled pipette (9). In vitro produced embryos are more sensitive to environmental effect, therefore, the simple and quick methods are preferred. In this study, the manual technique was fast and all high quality blastocysts survived by this procedure.

Stage of embryonic development at bisection was the most critical factor affecting survival rate of semi-embryos. Blastocysts resulted the best following bisection (10). There are several possible explanations, (1) the morphology of the blastocyst allows for visualization of the inner cell mass and polarity of the embryo, and, therefore, blastocysts are more amenable to symmetrical bisection, (2) blastocysts may be more tolerant to the bisection procedure and they may also be less affected by cracked zonae pellucidae and (3) the need for retention of the zona pellucida is not essential for blastocysts.

The ability of embryos to survive splitting also makes it possible to expand other related technologies for being used in the field of commercial embryo transfer i.e. embryo cryopreservation or sexing. The survival rates and pregnancy results after transfer of biopsied frozen-thawed embryos were reported to be significantly lower compared to fresh biopsied embryos (11,12). However, the present study indicated that IVP splitted blastocysts can survive by vitrification. Re-expansion rates did not differ from those achieved with intact blastocysts. The present study has high practical value in embryo transfer because a few cells from demi-embryos can be sexed utilizing the PCR before transferring to the recipients.

CONCLUSION

These results indicated that bisected in vitro produced bovine blastocysts can be cryopreserved without any reduction of their viability. The vitrification method was suitable to cryopreserve blastocysts after bisection.

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REFERENCES