Survival and development of bovine blastocysts produced in vitro after assisted hatching, vitrification and in-straw direct rehydration

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The purpose of this study was to establish an efficient combination of assisted hatching and cryopreservation procedures for producing bovine embryos in vitro. A total of 1312 day 7 blastocysts were subjected randomly to 14 different combinations of three factors: osmotic stress, assisted hatching and vitrification. Re-expansion, initiation and completion of the hatching process, as well as attachment to the culture dish, were analysed by SAS Genmod procedure. Incubation with sucrose was found to decrease survival rates; among the assisted hatching procedures used, zona fenestration resulted in higher survival rates compared with partial zona dissection and controls; and vitrification decreased survival and further development. The combined effect of sucrose incubation and vitrification decreased further development markedly, as did partial zona dissection followed by vitrification. Partial zona dissection performed in medium containing sucrose severely lowered embryo survival. Zona fenestration without sucrose incubation followed by vitrification did not compromise further embryo development: 86%, 84% and 79% of the blastocysts initiated, completed hatching and attached to the bottom, respectively. These data were not different from the controls (80%, 76% and 63%, respectively; P > 0.05). Cell count analysis revealed a decrease in the total number of cells as a result of the assisted hatching and vitrification compared with controls (135 versus 202, respectively; P < 0.0001). Although embryo transfer results (36% pregnancy rate and 30% calving rate) require further improvement, this combination of methods may prove useful in the commercial production of bovine embryos in vitro.

Introduction

Bovine embryos produced in vitro differ considerably in structure and viability compared with their in vivo counterparts. These differences are considered to be the result of the inadequate in vitro environment and may be present at all stages of development from oocyte maturation to the birth of offspring (Greve et al., 1993; Leibo and Loskutoff, 1993; Walker et al., 1996).

Certain stages of development, including first cleavage, the process of compaction, blastocoel formation and the hatching of blastocysts, seem inherently sensitive to the in vitro conditions. Impaired hatching of mammalian embryos produced in vitro may be explained by the different composition of the zona pellucida and possible weakness of the embryos developing in vitro (Cohen et al., 1990; Alikani and Cohen, 1992; Antinori et al., 1996). One way to overcome this problem is to improve the in vitro culture conditions, but in spite of extensive work, this has so far resulted in only limited success. Another possibility is to promote hatching by modification of the zona pellucida, for example by circumferential or local digestion by acidic Tyrode’s solution or by partial zona dissection with mechanical tools or lasers. Such interventions have been reported to increase pregnancy rates for both human and bovine embryos (Cohen et al., 1990, 1992; Tucker et al., 1991; Loskutoff et al., 1993; Stein et al., 1995; Krüger et al., 1996; Pokorny and Pokorny, 1996).

In humans there are disadvantages to using the assisted hatching technology. Human embryos are transferred at an early stage of development (the 2–4-cell stage) and loss of blastomeres or premature hatching as a consequence of contractions of the uterus (Nichols and Gardner, 1989) as well as penetration of toxins, microorganisms or immune cells through the artificial gap can cause impaired development or even embryonic death. Therefore, the use of assisted hatching should be restricted to selected groups of patients in which it has been found to be most advantageous (that is, women over 38 years of age; Stein et al., 1995). Bovine embryos are transferred just before hatching, and so the same problems do not apply.

In spite of reasonable survival rates in vitro and in vivo using both traditional slow-rate freezing and vitrification methods, even when combined with in-straw dilution (Saha et al., 1994; Vajta et al., 1995; Palasz and Mapletoft, 1996; Saha et al., 1996), embryos produced in vitro have an increased sensitivity to cryoinjuries. From studies of embryos produced in vivo, these cryoinjuries have been shown to affect normal cell contacts and

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Revised manuscript received 7 April 1997.

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0022-4251/97 $10.00
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can, therefore, disturb the hatching process (Massip and Mulnard, 1980; Niemann et al., 1982).

Cryopreservation of embryos with either spontaneous or induced damage of the zona pellucida has resulted in impaired survival rates compared with intact cryopreserved controls (Kana-gawa et al., 1979; Lehn-Jensen and Rall, 1983; Thibier and Nibert, 1992; Gustafsson et al., 1994). However, the importance of an intact zona pellucida as a prerequisite for cryopreservation has been questioned (Picard et al., 1988; Garrisi et al., 1992; Schmidt et al., 1992; Loskutoff et al., 1993; Agca et al., 1995; Booth et al., 1996) and development in vitro after vitrification and in-straw direct rehydration has been found to be acceptable (Vajta et al., 1996a, c).

Assisted hatching performed after cryopreservation has proved beneficial for the further development of human and bovine embryos in vitro or in vivo (Tucker et al., 1991; Leibo and Loskutoff, 1993; Hoover et al., 1995; Check et al., 1996). However, direct transfer of such embryos is not possible. The present study compares different assisted hatching technologies used before cryopreservation and in-straw dilution, after which direct transfer is possible.

The aim of this study was to establish a simple and efficient combination of assisted hatching and cryopreservation for producing bovine embryos in vitro. Survival and developmental rates in vitro after 14 combinations of the following: osmotic stress produced by sucrose incubation, two mechanical assisted hatching procedures and vitrification and in-straw direct rehydration were investigated. In addition, the numbers of cells of embryos in groups with the highest survival rates were determined. Finally, embryos from the group with the highest survival rates in vitro were transferred to recipient heifers to test their ability to survive in vivo.

Materials and Methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co., St Louis, MO.

Production of embryos in vitro

The method used for production of embryos is described in detail by Vajta et al. (1996b). Briefly, ovaries from cows of mixed breed were obtained from an abattoir and transported to the laboratory in physiological saline solution at a temperature of 30–35°C. Cumulus–oocyte complexes were aspirated within 2–4 h after slaughter using 19 gauge needles and a vacuum pump that produced a 10 ml min⁻¹ flow rate. Oocytes with intact compact cumulus cell layers were selected under a stereomicroscope and washed three times in TCM–Hepes (TCM-199 medium containing 2.5 mmol Hepes 1⁻¹ and 5 mmol sodium bicarbonate 1⁻¹, 0.2 mmol sodium pyruvate 1⁻¹, 2.5 mg amphotericin B ml⁻¹ and 15 i.u. heparin ml⁻¹, adjusted to pH 7.4 and 280 mOsm) supplemented with 1% (v/v) calf serum (National Veterinary Laboratory, Frederiksberg). Cumulus–oocyte complexes were then placed in four-well dishes (Nunc, Roskilde) with each well containing 400 µl TCM–bicarbonate (TCM-199 medium with 25 mmol sodium bicarbonate 1⁻¹, 0.2 mmol sodium pyruvate 1⁻¹, 0.4 mmol L-glutamine 1⁻¹, 50 µg gentamycin ml⁻¹, adjusted to pH 7.4 and 280 mOsm) supplemented with 15% (v/v) calf serum, 10 i.u. eCG ml⁻¹ and 5 i.u. hCG ml⁻¹ (Suigonan Vet, Intervet Scandinavia, Skovlunde) and incubated under paraffin oil for 24 h at 39°C in 5% (v/v) CO₂ in humidified air (hereafter referred to as standard culture conditions).

Fertilization was performed with the frozen–thawed spermatozoa of one Danish Holstein–Friesian bull (preselected for in vitro fertilization ability). Semen was subjected to a discontinuous Percoll (Pharmacia, Uppsala) gradient to separate live spermatozoa. Meanwhile, cumulus–oocyte complexes were transferred to another four-well dish containing 250 µl of IVF–TALP medium (Parrish et al., 1986) per well supplemented with 30 µg heparin ml⁻¹, 30 µg penicillinamine ml⁻¹, 15 µmol hypotaurine 1⁻¹ and 1 µmol adenine 1⁻¹ under paraffin oil. Insemination was performed by adding 0.02 ml volumes of 1.5 x 10⁶ spermatozoa ml⁻¹ to the oocytes. The dishes were incubated at 39°C in 5% (v/v) CO₂ in humidified air. At the time of insemination, the media in the dishes used for maturation were replaced by 400 µl TCM–bicarbonate supplemented with 5% (v/v) calf serum and these dishes were also incubated under standard culture conditions.

Presumptive zygotes were vortexed 20 h after insemination to remove cumulus cells, and then transferred into wells of the maturation dishes (50–60 zygotes per well) onto the monolayer of cumulus–granulosa cells attached during maturation. After 72 h of incubation under standard culture conditions, the embryos were transferred to other wells of the maturation dish. Before this transfer the serum concentration in the wells was adjusted to 10% (v/v) by adding extra serum.

Embryos produced in 21 in vitro fertilization trials were evaluated on day 7 and day 8 (day 0 = day of insemination) under a stereomicroscope. The average blastocyst per oocyte rate of these trials was 44% (range 33–53%). Only day 7, grade 1 (according to the International Embryo Transfer Society standards) expanded blastocysts (about 60% of the total blastocysts) were selected and subjected randomly to a combination of three treatments: incubation in sucrose, assisted hatching and vitrification. The number of selected blastocysts produced in one trial was between 33 and 130, and these blastocysts were distributed randomly into one of four groups each consisting of 8–50 blastocysts. Each group was then subjected to one of the 14 different treatments (Table 1), of which one consisted of further incubation in the original dish and served as control.

Short time incubation in medium with or without sucrose

Blastocysts were transferred for 15 min at 22–24°C to TCM–Hepes supplemented with 20% (v/v) calf serum and 0.25 mol sucrose 1⁻¹. Subsequently, blastocysts were transferred back to the original culture dishes and incubated under standard culture conditions.

Assisted hatching

Two mechanical forms of assisted hatching were applied:

(1) Partial zona dissolution involved incision of blastocysts in TCM–Hepes supplemented with 20% (v/v) calf serum with
or without sucrose for 5 min at 22—24°C. A Leitz micro-manipulator and stereomicroscope were used to make slits approximately 30% of the circumference of the blastocysts in the zona pellucida using a glass holder pipette and a micro-needle (Cohen et al., 1989). This manipulation was performed with groups consisting of 20–25 blastocysts and took approximately 8–10 min. (2) Manual zona fenestration involved incubation of blastocysts for 5 min as described above, before approximately 20–25% of the zona pellucida was cut manually under a stereomicroscope with vertical movement of a razor blade glued to a glass tube (Bredbacka et al., 1995). In both partial zona dissection and manual zona fenestration, blastocysts were oriented to make the cut on the zona away from the inner cell mass. After the manipulations, the blastocysts were transferred back to the original culture dishes to be incubated under standard culture conditions.

Vitrification and thawing

Blastocysts were vitrified according to the method described by Vajta et al. (1996b). They were placed for 5 min in four-well dishes containing 500 µl holding medium (TCM–Hepes supplemented with 20% (v/v) calf serum) per well, and then transferred to 500 µl VS50 (12.5% (v/v) ethylene glycol and 12.5% (v/v) dimethyl sulfoxide in holding medium) for 60 s at 20—22°C. Subsequently, blastocysts were transferred three times between 8 µl droplets of 4°C VS100 (25% (v/v) ethylene glycol and 25% (v/v) dimethyl sulfoxide in holding medium) for 10–15 s each. French Mini straws (250 µl, IMV, L'aigle) were loaded first with 180 µl of 4°C holding medium, and then with three 5 µl droplets of VS100 solution at 4°C separated by air bubbles from each other and the holding medium. The second droplet of VS100 solution contained the blastocysts (1–3 per straw). The total time of exposure to VS100 including the actual loading did not exceed 60 s. Straws were immediately heat-sealed, placed horizontally on a styrofoam boat floating on nitrogen liquid for 2 min, and then submerged into liquid nitrogen. The cooling rate was approximately 200°C min⁻¹ (Ishimori et al., 1993).

Thawing was performed by immersing the straws horizontally in a water bath at 22°C for 8 s until the diluent was melted (the warming rate was between −196 and −130°C in air for 3 s (1320°C min⁻¹); between −130 and −40°C for 1.3 s in 22°C water (4150°C min⁻¹); and between −40 and 0°C for 2.3 s in 22°C water (1040°C min⁻¹); M. Kuwayama, unpublished). The straws were then held at the sealed end and shaken three times to unify the liquid columns. Subsequently, straws were placed horizontally on the surface of the same water bath for 25–30 min before the content was expelled. The blastocysts were washed in holding medium at 22°C and cultured in TCM–bicarbonate and 10% (v/v) calf serum on a granulosa cell monolayer at standard culture conditions.

Evaluation of blastocysts

Two types of evaluation were used: (1) Observation under a stereomicroscope of embryo development after thawing: at 24 h for re-expansion; between 24 and 72 h for initiation and completion of the process of hatching (that is, hatching and hatched rate, respectively); and between 72 h and 144 h for attachment to the bottom of the dish. (2) Determination of the total number of cells: blastocysts were fixed on day 8 in methanol containing 25% (v/v) acetic acid, mounted on a glass slide and stained by orcein (King et al., 1979). The number of cells of 20 blastocysts from each group was determined using light microscopy.

Statistical analysis

Data were analysed by the Genmod procedure (SAS Technical Report, 1993) using a generalized linear model based on the logit transformation and binomial distribution of data. The number of blastocysts developing further per number of treated blastocysts was analysed using a model that examined the effects of the main factors: medium (with or without sucrose), assisted hatching (partial zona dissection or fenestration) and cryopreservation (with or without vitrification) as well as the interactions of assisted hatching × cryopreservation, and medium × cryopreservation.

The contrast function was used to evaluate differences between individual treatments. The LSM function of the general linear model procedure was used to elucidate the significant interactions.

The mean numbers of cells in the blastocysts were expressed as means ± SEM. Data were compared using Student’s t test and differences were considered significant where P ≤ 0.05.

Embryo transfer experiments

Twenty day 7, grade 1 expanded blastocysts produced in vitro were subjected to manual zona fenestration without previous incubation in sucrose and then vitrified as described above. The blastocysts were transferred directly to synchronized Holstein–Friesian heifers. Non-return, early and late pregnancy rates on days 21, 42 and 250 after transfer as well as calving rates were recorded.

Results

Survival and developmental rates of 1312 day 7 expanded blastocysts distributed among 14 treatment groups are shown (Table 1).

Effects of main factors

Overall the type of incubation (with or without sucrose), assisted hatching procedures and vitrification treatments influenced all dependent variables (P ≤ 0.05), namely, the percentages of re-expanded, hatching, hatched and attached blastocysts.

Incubation with sucrose (groups 1–2, 5–6, 9–10) decreased the rates of re-expanded, hatching and hatched blastocysts compared with controls (groups 3–4, 7–8, 11–14; P ≤ 0.05). However, differences were not found between blastocysts incubated at standard culture conditions and those incubated in TCM–Hepes (groups 13–14 versus groups 3–4, 7–8 and 11–12, respectively) nor were differences found between
Table 1. Survival and development in vitro of day 7 expanded bovine blastocysts subjected to medium with sucrose, assisted hatching, vitrification treatments, or combinations of treatments

<table>
<thead>
<tr>
<th>Group</th>
<th>Medium</th>
<th>Assisted hatching</th>
<th>Vitrified</th>
<th>Number of blastocysts</th>
<th>Number vitrified or cultured (%)</th>
<th>Number re-expanded (%)</th>
<th>Number hatching (%)</th>
<th>Number hatched (%)</th>
<th>Number attached (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>110</td>
<td>108 (98)</td>
<td>78 (71)</td>
<td>66 (60)</td>
<td>44 (40)</td>
<td>32 (29)</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>91</td>
<td>90 (99)</td>
<td>83 (91)</td>
<td>81 (89)</td>
<td>78 (85)</td>
<td>64 (70)</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>77</td>
<td>71 (92)</td>
<td>58 (75)</td>
<td>54 (70)</td>
<td>50 (65)</td>
<td>44 (57)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>80</td>
<td>74 (93)</td>
<td>69 (86)</td>
<td>64 (80)</td>
<td>57 (71)</td>
<td>52 (65)</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>74</td>
<td>71 (96)</td>
<td>57 (76)</td>
<td>54 (73)</td>
<td>50 (68)</td>
<td>41 (55)</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>70</td>
<td>69 (99)</td>
<td>67 (96)</td>
<td>63 (90)</td>
<td>58 (83)</td>
<td>52 (74)</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>83</td>
<td>80 (96)</td>
<td>73 (88)</td>
<td>71 (86)</td>
<td>70 (84)</td>
<td>66 (79)</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>88</td>
<td>82 (93)</td>
<td>81 (92)</td>
<td>80 (91)</td>
<td>80 (91)</td>
<td>75 (85)</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>83</td>
<td>83 (100)</td>
<td>64 (77)</td>
<td>53 (64)</td>
<td>49 (59)</td>
<td>43 (52)</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>107</td>
<td>107 (100)</td>
<td>95 (89)</td>
<td>78 (73)</td>
<td>74 (69)</td>
<td>66 (62)</td>
</tr>
<tr>
<td>11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>107</td>
<td>107 (100)</td>
<td>95 (89)</td>
<td>78 (73)</td>
<td>74 (69)</td>
<td>66 (62)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>78</td>
<td>78 (100)</td>
<td>70 (89)</td>
<td>56 (72)</td>
<td>54 (69)</td>
<td>50 (64)</td>
</tr>
<tr>
<td>13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>153</td>
<td>153 (100)</td>
<td>135 (88)</td>
<td>117 (76)</td>
<td>101 (66)</td>
<td>90 (58)</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>111</td>
<td>111 (100)</td>
<td>111 (100)</td>
<td>89 (80)</td>
<td>84 (76)</td>
<td>70 (63)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>1312</td>
<td>1284 (98)</td>
<td>1136 (87)</td>
<td>1004 (77)</td>
<td>923 (70)</td>
<td>811 (62)</td>
</tr>
</tbody>
</table>

Table 2. Development of bovine blastocysts (re-expanded, hatching, hatched, attached; values are least square means) after the three main treatments used in this study: medium with sucrose, assisted hatching or vitrification, or assisted hatching and vitrification

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation</th>
<th>Assisted hatching</th>
<th>Cryopreservation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sucrose (535)</td>
<td>Medium (513)</td>
<td>Control (264)</td>
</tr>
</tbody>
</table>

Group number: 1–2, 5–6, 9–10; 3–4, 7–8, 11–12; 13–14
Re-expanded: 0.83\(^a\) 0.87\(^a\); 0.93\(^a\); 0.83\(^d\) 0.91\(^d\) 0.89\(^e\), 0.94\(^f\), 0.81\(^e\)
Hatching: 0.75\(^b\) 0.76\(^b\); 0.83\(^b\); 0.77\(^b\) 0.88\(^b\) 0.73\(^b\); 0.85\(^b\) 0.74\(^b\)
Hatched: 0.67\(^b\) 0.75\(^b\); 0.75\(^b\); 0.69\(^e\) 0.64\(^e\); 0.57\(^e\) 0.74\(^e\) 0.60\(^e\); 0.70\(^e\) 0.56\(^e\)
Attached: 0.57\(^b\) 0.69\(^b\); 0.64\(^b\); 0.57\(^c\) 0.74\(^c\) 0.60\(^c\); 0.70\(^c\) 0.56\(^c\)

Effects of interactions between main factors

Incubations combined with vitrification resulted in a decrease \((P \leq 0.05)\) in all survival rates. This influence was particularly marked in the group of blastocysts incubated in sucrose before vitrification.

Assisted hatching combined with vitrification decreased the percentages of hatching, hatched and attached blastocysts \((P < 0.05)\). The interaction was a result of the low survival rates of vitrified, partially zona dissected blastocysts. However, manual fenestration without sucrose followed by vitrification did not reduce the rates of hatching, hatched and attached blastocysts compared with controls.

No significant interaction was observed between incubations and manipulations.
Determination of number of cells

Groups 7 and 14 were selected for determination of number of cells. In blastocysts subjected to manual fenestration without sucrose followed by vitrification and 24 h culture, the mean number of cells ± SEM was 135 ± 6. Control and non-vitrified day 8 blastocysts contained a mean ± SEM of 202 ± 9 cells. The difference between the two groups was significant (P < 0.0001).

Embryo transfer

Ten of twenty heifers (50%) did not return to the normal cycle 2 weeks after embryo transfer. Pregnancy rates on day 42 and day 250 were 7/20 (35%) and 6/20 (30%), respectively. Six offspring were born (30%) but one calf died at birth from dystocia.

Discussion

After years of development, systems for producing bovine embryos in vitro have reached a level of stability that facilitates controlled experiments with sufficient numbers of embryos to allow full statistical analyses. The large number and high quality of day 7 expanded blastocysts produced in vitro in our laboratory allowed us to perform a comparative investigation of different forms of assisted hatching combined with vitrification on 14 groups including controls.

Since assisted hatching by means of chemical agents or laser beams may hamper normal embryonic development (Stein et al., 1995) and requires extreme care or expensive equipment, two mechanical methods of assisted hatching were selected. Both manipulation methods were simple and lasted approximately 20 s per blastocyst. One advantage of the manual fenestration over partial zona dissection is that it does not require micromanipulation. In addition, survival rates increased after manual fenestration compared with partial zona dissection and controls, and were only slightly decreased after vitrification. Therefore, manual fenestration can be regarded as a successful assisted hatching procedure.

At the expanded blastocyst stage, the perivitelline space is only virtual, and there is no way of opening the zona pellucida without penetrating the trophoblast cell layer. Therefore, some groups of blastocysts were incubated and manipulated in sucrose to widen the perivitelline space. Sucrose incubation is often used in manipulation and cryopreservation procedures, and even as a means of quality assessment, and is usually regarded as harmless (Herr et al., 1990; Hasler et al., 1995; Van Soom et al., 1996). However, some workers have described decreased viability or an increase in the number of lysed cells when micromanipulation was performed in medium with similar sucrose concentrations (Széll and Hudson, 1991; Bredbacka, 1995). The results of the present study show that sucrose incubation decreased survival rates of blastocysts, an effect that was not accentuated by the assisted hatching procedures. Contrary to this, the combination of sucrose incubation with subsequent vitrification reduced survival even further than sucrose alone. These observations indicate a potential damaging effect of sucrose, especially in combination with chemical rather than mechanical procedures.

The size of the hole or slit in the zona pellucida made by certain biopsy, cloning or assisted hatching methods is small and may result in a higher incidence of incomplete hatching (also known as blastocyst entrapment), which is characterized by an ‘8’ form shape of the blastocyst (Cohen, 1991; Cohen and Feldberg, 1991; Germond et al., 1995). This problem can be minimized by increasing the size of the gap (Stein et al., 1995).

In the present study, both partial zona dissection and zona fenestration created large slits or holes, and the proportion of entrapped blastocysts did not exceed 10% overall. However, of blastocysts subjected to sucrose incubation, partial zona dissection and vitrification, 20% were unable to complete the hatching process (Group 1, Table 1). This decreased viability is likely to be a cumulative result of injuries caused by the three consecutive interventions. Such marked decreases in the survival rate of embryos biopsied and vitrified using a similar technology were not found in earlier experiments in which sucrose incubation and biopsy were performed on day 4 blastocysts and embryos were vitrified 3.5 days later (Vajta et al., 1997). This 3.5 day interval seemed to be sufficient to allow recovery from the injuries caused by the sucrose incubation and manipulation.

The vitrification procedure applied in this study is suitable for direct in-straw rehydration, as has been described by Vajta et al. (1995, 1996c). One of the two cryoprotectants used, ethylene glycol, is very permeable, particularly in combination with dimethyl sulfoxide (Vicente and Garcia-Ximénez, 1994). Therefore, neither an osmotic buffer nor a large volume of diluent is required.

The number of cells in the blastocysts subjected to the most efficient method of assisted hatching (manual fenestration in medium) and vitrification was decreased by approximately 30% compared with untreated and non-cryopreserved control blastocysts culture for the same time in vitro. Future investigations are required to determine which part of the blastocyst (the inner cell mass or trophoblastic layer) suffered the more severe loss, and whether manipulation or vitrification produced the greater decrease.

The results of this study can be summarized thus: (1) Incubation with sucrose decreased the proportion of re-expanding, hatching and hatched blastocysts. (2) Manual zona fenestration resulted in higher survival rates compared with partial zona dissection and controls. (3) Vitrification decreased the survival rate of blastocysts. (4) Sucrose incubation followed by vitrification resulted in accumulated damage to blastocysts. (5) Partial zona dissection followed by vitrification markedly decreased the survival rates of blastocysts.

Thus, manual zona fenestration performed in medium without sucrose seems to be an efficient method of assisted hatching for bovine blastocysts produced in vitro. This method can be followed by vitrification and in-straw direct rehydration without compromising further development in vitro. Further improvement of the pregnancy and calving rates achieved in this study that involve a limited number of direct transfers are required. However, considering that manual fenestration also allows genotyping of the embryo, this combination of methods may have significant value for the commercial production of bovine embryos in vitro.

The authors thank A. Kovács for the suggestions connected to the fixation and staining of the blastocysts, J. Jensen for useful advice with the statistical analyses and M. Kuwayama for measuring the warming rates of the vitrification solution.