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# Comparison of Open Pulled Straw (OPS) vs Glass Micropipette (GMP) Vitrification in IVP Bovine Blastocysts

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## ABSTRACT

The purpose of these study was to investigate the use of a glass micropipette (GMP) as a vessel for vitrification of bovine IVP blastocysts, to compare the post-thaw survival rates of bovine blastocysts frozen in GMP with those frozen in OPS that have been previously investigated, and to improve the hatching rate following vitrification with GMP method. The GMP vessel permits higher freezing and warming rate than the OPS due to the higher heat conductivity of the glass and lower mass of the solution that contains the embryos. Groups of three bovine IVP blastocysts were sequentially placed into vitrification solution before being loaded into either the OPS or GMP vessels and immersed into $LN_2$ within 20 to 25 sec. Post-thaw blastocysts were serially washed in 0.25 and 0.15 M sucrose in HM and TCM-199 for each 5 min, respectively, and then cultured in TCM 199 supplemented with 10% FCS for 24 h. The rate of blastocyst re-expanding did not significantly different for OPS (75.9%) and GMP (80.0%) methods ($P>0.05$). The hatching rates in OPS (34.1%) and GMP (37.5%) methods were significantly lower than that in control group (54.3%) ($P>0.05$). In addition, the rate of blastocyst re-expanding was significantly lower if blastocysts were vitrified in the wide portion of the micropipette rather than the narrow portion of the micropipette (83.3 vs 56.7%) ($P>0.05$), even though three blastocysts were loaded per vessel. The hatching rate in 0.05% pronase solution treatment for 30, 60 and 90 sec (45.9, 54.7 and 57.5%) were significantly higher than that in control (35.0%), even though there was not significantly different between 30 sec and control.

These results indicate that both vitrification vessels can provide high survival rates of bovine IVP blastocysts. However, the GMP vessel has the advantage over the OPS, in that the former does not need a cap to protect the vessel from floating after immersion in $LN_2$. The location of the embryos (narrow or wide portion of immersion) were considered to be limiting factors to the viability of bovine IVP embryos. The exposing in 0.05% pronase solution for 60 or 90 sec can increase hatching rates of post-thaw bovine IVP blastocysts.

(Key words: OPP, GMP, Bovine, Vitrification)

## I. INTRODUCTION

Cryopreservation provides a means by which biological materials may be stored indefinitely without loss of functional activity and without genetic alteration (Mazur, 1984; Whittingham, 1980). The first successful cryopreservation of

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mammalian zygotes and embryos resulting in live births was achieved with mice by Whittingham et al. (1972). In the past decade, various new methods for embryo cryopreservation have been published (Niemann, 1991; Rall, 1992). Among these methods, vitrification has been widely used and is now regarded as a good alternative to transitional slow-rate freezing. There have been reports of successful embryo vitrification with various cryoprotectants in several species using the VS3a method (Dinnyes et al., 1995; Rall and Wood, 1994), the EFS method (Kasai et al., 1990; Tachikawa et al., 1993), the ethylene glycol/PVP method (Leibo and Oda, 1993) and the ethylene glycol/DMSO method (Vajta et al., 1998a). When embryos are cryopreserved by vitrification, ice crystal formation is prevented by use of high concentration of cryoprotectants and high cooling and warming rates. Acceleration of the speed of temperature changes may offer two advantages: permit use of lower concentrations of cryoprotectants with consequent reduction in toxicity and less severe chilling injury as a result of the rapid passage through the "dangerous" temperature zone (Vajta et al., 1998a).

The efficiency of vitrifying embryos has been markedly improved by increasing the speed of cooling and warming. Three techniques have been established for this purpose: direct immersing into liquid nitrogen (Landa and Tepla, 1990; Riha et al., 1991), using an electron microscopy grid to provide a support (Martino et al., 1996) and the OPS method (Hurtt et al., 1999; Lewis et al., 1999; Vajta et al., 1997, 1998b). Vajta et al. (1998a) demonstrated that the OPS method can increase the cooling and warming rates (over 20,000℃/min) and decrease toxic and osmotic damage. Although the OPS method is very useful and easy, it has the disadvantage of floating of the straw following immersion into $LN_2$ which leads to decreased freezing speed and possible damage to the embryo.

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* (Antinori et al., 1996; Vajta et al., 1997). 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 acid Tyroid's solution or by partial zona dissection with mechanical tools or lasers. Such intervenions have been reported to increase pregnancy rates for both human and bovine embryos (Cohen et al., 1992; Tucker et al., 1991; Loskutoff et al., 1993; Stein et al., 1995; Kruger et al., 1996).

To overcome this problem, we have investigated the use of a glass micropipette vessel (GMP) for vitrification, which may offer even faster cooling and warming rates than the OPS vessel, and also the 0.05% pronase solution to improve hatching rate in both intact and vitrified blastocyst embryos.

## Ⅱ. MATERIALS AND METHODS

### 1. Reagents and Culture Media

Inorganic salts were analytical grade from Mallinckrodt, Paris, KY. Fetal bovine serum (FBS) and packaged media were from HyClone Laboratories, Logan, UT. All other reagents were purchased from Sigma, St. Louis, MO, unless otherwise noted. "Embryo culture tested" grade was used whenever possible.

### 2. Oocyte Maturation and Fertilization

Bovine ovaries were obtained from a local abattoir. Cumulus-oocyte complexes were aspirated from 2 to 8 mm diameter antral follicles with an 18 gauge hypodermic needle, selected for an envelope of compact and complete cumulus cells, and then washed three times in HEPES-buffered Tyrode's medium (Fissore et al., 1992), supplemented with 3 mg/ml BSA, 0.2 mM pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (TL-HEPES). Oocytes were transferred into 500 μl TCM 199 maturation medium containing 10% FBS, 0.5 μg/ml bovine FSH, 5 μg/ml bovine LH, 100 IU/ml penicillin, 100 (μg/ml streptomycin (M199) in 4-well culture dishes and cultured at 39℃ in a humidified atmosphere of 5% $CO_2$ and air for 24 h. Fertilization was initiated 23 h after onset of maturation and was counted as Day 0. Spermatozoa were prepared for IVF as previously described (Parrish et al., 1988). Cryopreserved semen was thawed in 37℃ water and transferred into 10 mL PBS for washing by centrifugation, and then capacitated with 400 μl of 50 ng/ml heparin solution for 15 min at 39℃ The capacitated sperm were diluted with TL-FERT medium to approximately $1\sim2\times10^6$ sperms/ml in drops containing the oocytes.

### 3. *In Vitro* Development and Evaluation

Embryos were cultured in HECM-6 containing amino acids and 4 mg/ml BSA, 3 mg/ml PVA, 150 μg/ml sodium citrate and/or 500 μg/ml myo-inositol for 72 h after insemination. After 3 days in culture the cleaved embryos were counted. The embryos cleaved over 2-cell were cultured in TCM199 supplemented with 10% FCS on 30 mm dish with 50 μl volume and approximately 30 embryos with paraffin oil overlay. Embryos that reached the expanded blastocyst stage at Day 7 and 8 after insemination (initiation of insemination on Day 0) were recovered on those days and randomly assigned to either vitrification or assisted hatching.

### 4. Making of OPS and GMP Straw

OPS straws were made with French mini-straws (250 μl, IMV, L'Aigle, France) at our laboratory as described by Vajta et al. (1998a). Briefly, French mini-straws were heat-softened over a hot plate, and pulled manually until the inner diameter and the wall thickness of the central part decreased from 1.7 mm to approximately 0.8 mm. The straws were cooled in air then cut at the narrowest point with a razor blade. The GMP vessels were constructed from capillary glass pipette (outer/inner diameter (o.d./i.d.): 1.0/0.8 mm; Drummond Sci. Co., USA) by the method of Vajta et al. (1998a) with minor modifications. The capillary glass pipettes were pulled with pipette puller (Narishige, Japan) until the o.d. of central part decreased from 1.0 mm to approximately 0.3 mm. The GMP vessels were cooled in air then broken at the narrowest point after scribing with a diamond tip. The weight of OPS and GMP straw was approximately 0.070 vs 0.098 g, respectively, and the

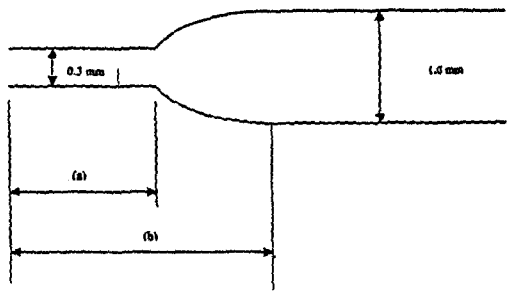


**Fig. 1. Scheme of OPP straw loading column by means of the capillary effect as (a) narrow loading column type (10 mm length) and (b) wide loading column type.**

volume loaded into narrow column was 2.68 vs 0.14 $mm^3$ per straw, respectively. The volume loaded into 10 mm length of narrow column was calculated by $4/3 \cdot \pi r^3 \cdot 10$ (Takahashi and Kanagawa , 1990) (Fig. 1). All straws were sterilized by flushing with 70% ethanol and dried in a clean bench.

**5. Vitrification Procedure**

The vitrification solution consisted of VS1 {10% ethylene glycol (EG), 10% DMSO in holding medium (D-PBS supplemented with 5% FCS: HM)} and VS2 {16.5% EG, 16.5% DMSO in HM; EDS}. The blastocysts collected were vitrified using EDS as reported previously (Vajta et al., 1998a). In brief, the embryos were first incubated in VS1 for 1 min, and then transferred within approximately 1 to 2 μl VS1 solution into a 20 μl droplet of VS2. Embryos were mixed quickly by pipetting then another drop containing approximately 1 to 2 μl VS2 solution and embryos was made using a 10 μl automatic pipette. Loading and cooling were performed as described by Vajta et al. (1998a). The time between the contact of the embryos with the concentrated cryoprotectant solution and cooling did not exceed 25 sec. In the GMP vitrification, the researcher has to control the capillary reaction with finger carefully because the GMP was very sensitive to capillary phenomenon. The loaded OPS or GMP straws were laying them down into $LN_2$ first almost horizontal and then immersed them in the $LN_2$ vertically.

**6. Warming and Cryoprotectant Dilution**

Warming was performed by immersing the end of the straw containing the embryos into 1.0 ml of 0.25 M sucrose in HM prepared in the 4-well dish. After 1 min, the embryos were transferred into 1.0 ml of 0.15 M sucrose in HM for another 5 min, and then washed with HM twice for 5 min each time. The temperature of the media used for warming was held to approximately 35℃.

**7. Assessment of Embryo Viability**

Survival of cryopreserved embryos was determined by development to re-expanding or hatching blastocysts during *in vitro* culture for 24 or 48 h, respectively. The embryos were cultured in TCM199 supplemented with 10% FCS in humidified atmosphere of 5% $CO_2$ at 39℃.

**8. Assisted Hatchiing**

Intact or vitrified blastocysts were exposed in 0.05% pronase solution in 39℃, 5% $CO_2$ incubator for 30, 60 or 90 sec, and then washed twice with TL-Hepes exch 5 min. After treatment of pronase solution, embryos were cultured in TCM 199 supplemented with 10% FCS for 24 or 48 h.

**9. Statistical Analysis**

Data were analyzed by chi-square tests with SAS program.

## Ⅲ. RESULTS

**1. Post-thaw Survival Rates according to OPS or GMP Methods**

To determine the optimal vitrification vessels,

**Table 1. Effect of OPS or GMP methods on post-thaw survival rates of vitrified mouse blastocyst**

| Vitrification vessels | No. of embryos (%) | | |
|---|---|---|---|
| | Vitrified | Re-expanding | Hatching |
| OPS | 54 | 41(75.9) | 14(34.1)[a] |
| GMP | 60 | 48(80.0) | 18(37.5)[a] |
| Control | 46 | | 25(54.3)[b] |

[a, b] Values with different superscripts in same column were significantly different ($P<0.05$).

the blastocysts were vitrified by OPS or GMP straw with EDS vitrification solution. As shown Table 1, the post-thaw survival rates at re-expanded stage were not significantly different between OPS and GMP vessels (75.9%: 41/54 vs 80%: 48/60), respectively. However, the hatching rates in control (54.3%: 25/46) was significantly higher than those in OPS and GMP (34.1%: 14/41 vs 37.5%: 18/48)($P<0.05$), respectively. All of the experiments were loaded under three blastocyst per straw.

**2. Effect of Type of Loading Column on Post-thaw Survival Rates**

The type of loading column containing embryos and vitrification solution was very important to post-thaw survival of bovine IVP blastocysts. That is, the narrow column means that medium and embryos fills only the narrow column and then fills both narrow and wide in the wide column. The blastocyst re-expansion rates were significantly different for narrow and wide column types (83.3 vs 56.7%, $P>0.05$), although the embryos loaded per micropipette were limited to three blastocysts (Table 2).

**Table 2. Effect of type of loading column on post-thaw survival rate following vitrification in GMP vessles**

| Types of loading column | No. of embryos (%) | | |
|---|---|---|---|
| | Loaded | Replicated | Re-expanding |
| Narrow column only | 30 | 10 | 25(83.3)[a] |
| Wide column | 30 | 10 | 17(56.7)[b] |

* Medium fills only the narrow column in the narrow column and then fills both narrow and wide in the wide column.

[a, b] Values with different superscripts were significantly different ($P<0.05$).

**3. Hatching Rate of Vitrified IVP Blastocysts Following Assisted Treatment**

To improve the hatching rate of IVP bovine blastocysts, the vitrified blastocysts were exposed in 0.05% pronase solution for 30, 60 and 90 seconds. As shown in Table 3, the hatching rates in 0.05% pronase solution for 30, 60 and 90 sec (45.9, 54.7 and 57.5%) were significantly higher than that in control group (35%), even though there was no significantly different between 30 sec exposed and control.

## Ⅳ. DISCUSSION

The OPS and GMP vitrification vessels with EDS vitrification solution were very successful for cryopreservation of IVP bovine blastocysts. Either the OPS or the GMP methods can result in over 75.9% re-expanding or 34.1% hatching rates. However, the OPS method has a problem with the straw floating in $LN_2$ necessitating a

**Table 3. Effect of exposed time in pronase on assisted hatching rates of IVP blastocyst cryopreserved by GMP vitrifcation**

| Exposure time in pronase solution (sec) | No. of blastocyst exposed | No. of blastocysts at 24 or 48 h | |
|---|---|---|---|
| | | Hatched | Collapseor unhatched |
| 30 | 37 | 17(45.9)ab | 20(54.0) |
| 60 | 42 | 23(54.7)a | 19(45.2) |
| 90 | 40 | 23(57.5)a | 17(42.5) |
| Control | 40 | 14(35.0)b | 17(42.5) |

* Values with different superscpits were significantly different.

cap to protect the floating of OPS straw. Because of higher density of the glass straw, the problem is overcome and cap is not necessary. We concluded that the OPS straw has real disadvantages compared to the GMP straw for the straw floating to be truly useful. However, we did not met any worry for this working that a glass micropipette is more likely to break and therefore would result in greater loss of frozen embryos. In addition, the GMP straw may increase the freezing speed due to diminished pipette size and loading column, if we only can fill the narrow part of the pipette. If the freezing and warming speed can be increased, the post-thaw survival rates might be improved following vitrification of embryos of species that are far more difficult to cryopreserve.

Because of the type of material that the OPS straw is constructed from there is a limitation on how small a diameter can be formed. If OPS straw are pulled to under 0.8 mm o.d., they become fragile and often tear or break near the narrowest point. However, the size of glass pipette can be decreased to approximately 0.3 mm o.d. and 0.26 mm i.d. easily, that is, approximately 0.02 mm wall thickness, which is three to four diameters larger than mouse embryos and approximately two diameters larger than cattle embryos. The weight of GMP straw also was a little heavier rather than the OPS straw (0.098 vs 0.070 g). When pulled as indicated, the volume of GMP was 19 times less than the OPS straw (0.14 vs 2.68 $mm^3$), when the volume loaded was calculated for the 10 mm length of narrow column. Although the smaller size of GMP could increase the freezing speed, it was more sensitive to the loading of embryos and vitrification solution by capillary reaction. If we use GMP method, we have to load the embryo and vitrification solution carefully by controlling capillarity with a finger. As shown in Table 2, the loading column is very important to viability of vitrified blastocyst. Careful loading of the GMP straw might increase the speed of freezing or warming and decrease the loading volume and the damage of embryos. It is important in the GMP method to assure that the embryos are loading in the narrow column only.

Loading in excess of four embryos loaded in an GMP straw was detrimental to survival. Although the number of embryos loaded per straw may effect of type of loading column, the loading of fewer than three blastocysts per straw resulted in 83.3% survival rates in this study. It is possible that increased numbers of embryos in the straw spaced them outside of the narrowest portion of the fluid column resulting in slower freezing and cooling and decreased embryo survival. Vajta et al. (1998a) demonstrated that the OPS method, which renders very high cooling and warming rates (over −20,000℃/min) and short contact with concentrated cryoprotective additives before freezing (less than 30 sec) offers a possibility to circumvent chilling injury and to decrease toxic and osmotic damage. The GMP method can achieve even more rapid freezing speed and heat conductivity, because it made of the capillary glass pipette. A disadvantage of both system as well as the electron microscopy grid system is that there is the potential hazard of contamination as the embryo holding medium is directly in contact with liquid nitrogen (Tedder et al., 1995; Vajta et al., 1998b).

Vajta et al (1997) reported that among the assisted hatching procedures used, zona fenestration as zona drilling resulted in higher survival rates compared with partial zona dissection and 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 ex-

pensive equipment, two mechanical methods of assisted hatching were selected. 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 after partial zona dissection and controls, and were only slightly decreased after vitrification.

In conclusion, the GMP method has been established to increase the speed of freezing and warming, heat conductivity, post-thaw survival rates, and decrease the loading volume and embryo damage by reducing of straw size and loading column. The GMP method attained almost 100% post-thaw survival rates in mouse blastocyst is a potentially valuable technique applicable to other species. The assisted hatching treatment could be increased hatching rates in vitrified bovine IVP blastocyst and also might be improved implantation or pregnancy rate following embryo transfer for application of ET.

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## 요 약

# 소 체외수정란의 배반포기배의 OPS 대 GMP Vitrification의 비교

공 일 근 · 조 성 균

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본 연구는 체외생산된 배반포기배의 vitrification을 위한 용기로서 glass micropipette(GMP)을 이용할 수 있는지, GMP와 OPS로 동결융해 후 생존율의 비교 및 GMP vitrification 후 hatching 율의 향상을 위하여 실시하였다. GMP vessel은 열전도율과 수정란을 포함하는 적은 질량 때문에 OPS보다 동결 및 융해 속도를 높일 수 있다. 3개의 체외수정란을 vitrification 용액에 노출시키고 OPS 또는 GMP vessel에 loading시킨 후 액체질소에 침적하는데까지 20~25초 이내에 실시하였다. 동결 · 융해한 배반포기배는 0.25와 15 M sucrose solution 및 TCM 1999에 각각 5분씩 차례로 희석한 후 10% FCS가 첨가된 TCM 199에 24시간동안 배양하였다. OPS(75.9%)와 GMP(80.0%) 방법간의 re-expanding율은 유의적($P<0.05$)인 차이가 없었다. OPS(34.1%)와 GMP(37.5%)방법에서 hatching율은 intact group(54.3%)보다도 유의적($P<0.05$)으로 낮았다. 비록 GMP straw 당 3개 이하의 blastocysts를 loading 하였더라도 narrow portion(83.3%)보다도 wide portion(56.7%)에서 vitrified 되었다면 re-expanding 율이 유의적($P<0.05$)으로 낮았다. 비록 30초 처리군과 무처리군 간에는 유의적인 차이가 없었지만 0.05% pronase 용액에 30, 60 및 90초간 처리군(45.9, 54.7 및 57.5%)의 hatching 율은 무처리구(35.0%)보다 유의적($P<0.05$)으로 높았다.

이러한 결과들은 OPS와 GMP vitrification vessel은 체외생산된 배반포기배의 높은 생존율을 얻을 수 있다. 그러나 GMP vessel은 $LN_2$ 침적 후 vessel의 floating을 방지하기 위한 또다른 cap이 필요하지 않다는 유리한 점을 가지고 있다. 수정란의 loading 위치, 즉 narrow 또는 wide portion에 따라 소 체외생산된 배반포기배의 생존력에 제한적인 요인으로 고려된다. 0.5% pronase 용액에 60 또는 90초간의 노출은 융해 후 hatching율을 향상시킬 수 있었다.

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