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Direct transfer of frozen-thawed bovine embryos and its application in cattle reproduction management

Osamu DOCHI[1)]

[1)]*Rakuno Gakuen University, Hokkaido 069-0851, Japan*

Abstract. Embryo transfer entails many procedures and techniques, of which embryo freezing is an important component in bovine embryo transfer. Embryo freezing techniques have been developed over the last 40 years, allowing practical availability, and have become essential for cattle reproduction management under field conditions. The direct transfer methods of frozen-thawed, *in vivo*-derived, and *in vitro*-produced (IVF) bovine embryos using 1.5 M ethylene glycol (EG) with or without sucrose (SUC) are used widely under on-farm conditions, not only in Japan but also globally. The direct transfer method using 1.5 M glycerol (GLY) and 0.25 M SUC (GLY-SUC) is used mainly in Japan. The pregnancy rate with direct transfer of frozen-thawed bovine embryos in either EG or GLY-SUC has been found to not differ from conventional freezing with GLY and traditional dilution techniques. Pregnancy rates following direct transfer of frozen-thawed bovine embryos were affected by the developmental stage of the embryos and the parity of the recipients. The use of ultrasound-guided on-farm ovum pickup is ushering in a new revolution for the commercial application of IVF embryos. Globally, for the first time more IVF bovine embryos were transferred in 2017 than produced *in vivo*. More than 60% of IVF embryos were transferred fresh due to a low pregnancy rate of frozen-thawed IVF embryos. Many factors seemed to be involved in improving the survival rate of frozen-thawed IVF embryos. Therefore, further research is needed to improve the freezing tolerance of IVF embryos to develop efficient direct transfer methods analogous to those used for *in vivo* embryos.
Key words: Bovine embryo, Direct transfer, Ethylene glycol, Freezing, Pregnancy rate
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Introduction

During the 20th century, various breeding techniques, including artificial insemination (AI), were developed [1]. The development and widespread use of AI technology significantly advanced the genetic improvement of cattle. In particular, the success in developing cryopreservation technology for bovine semen [2] accelerated the spread of AI and contributed greatly to the development of the livestock industry. Today, AI of cattle has developed into a technology that can be used to control the sex of offspring with a 90% success rate [3–5].

The development of embryo transfer technology began when Walter Heape (1890) transferred Angora rabbit embryos to inseminated Belgian does and succeeded in the production of offspring of both breeds [6]. The first success in bovine embryo transfer was reported by Willett *et al*. [7] in 1951, and in the 1970s, commercial embryo transfer technology became established in North America with the import of continental breeds of cattle from Europe [1, 8, 9]. These breeds were valuable and in short supply, and embryo transfer offered a means to rapidly increase their numbers.

The study of freezing techniques for bovine embryos began in 1973 when Willmut and Rowson first successfully produced calves from frozen-thawed embryos [10]. Today, embryo transfer is an important technology used for bovine genetic improvement and breeding programs [11]. Embryo cryopreservation technology is one of the most important advancements in bovine embryo transfer [8, 9]. According to a survey by the International Embryo Technology Society [12], approximately 400,000 *in vivo*-derived bovine embryos were transferred worldwide in 2017, of which frozen embryos accounted for approximately 60% (240,000 embryos). In addition, approximately 750,000 *in vitro*-produced (IVF) bovine embryos were transferred, of which approximately 34% (260,000 embryos) were frozen-thawed. However, these results do not include data from Japan.

According to the 2015 report from the Ministry of Agriculture, Forestry and Fisheries of Japan, there were 14,777 bovine donors, 76,745 transfers, and 15,621 calves born from *in vivo*-derived bovine embryos, 23,567 IVF bovine embryo transfers, and 3,715 calves born, with a total of 19,336 calves born following bovine embryo transfer in Japan that year [13].

Currently, in other countries, the percentage of frozen-thawed bovine embryos that are transferred annually is approximately 60%, but the percentage of frozen-thawed embryos transferred annually in Japan is over 70%. These observations highlight that embryo freezing techniques are indispensable in bovine embryo transfer. A simple embryo freezing protocol with high conception rates is always a desired goal.

A Brief History of Freezing Techniques for Bovine Embryos

The summary of the historical development of cryopreserved bovine embryos is presented in Table 1. The first successful cryopreservation of mammalian embryos was the production of the offspring from cryopreserved mouse embryos by Whittingham *et al*. in 1972 [14]. The following year (1973), Willmut and Rowson reported the first calves produced from frozen-thawed embryos [10]. In the early days, embryos were cooled to –60°C at very slow rates (< 1°C/min) and then thawed slowly (4–20°C/min) to achieve adequate dehydration of cells and avoid the damaging effects of intracellular ice formation [15]. Soon thereafter, techniques were developed in which embryos were cooled to between –30°C and –40°C, and then plunged into liquid nitrogen; these embryos were subsequently thawed rapidly [15,16]. Embryos frozen this way may contain some intracellular ice, and should be thawed rapidly to prevent injury from recrystallization. Early reports of the cryopreservation of mammalian embryos utilized dimethyl sulfoxide (DMSO) as a cryoprotectant. It had been reported that glycerol (GLY) has higher permeation of bovine embryos than DMSO and that GLY can obtain a high survival rate by the fast cooling-rapid thawing method [15]. From these facts, GLY has come to be used as a cryoprotectant for freezing bovine embryos. At that time, the cryoprotectant was added and removed (diluted) from frozen-thawed embryos in several small steps to avoid osmotic shock to embryo cells. This method was called the stepwise method of cryoprotectant addition and removal. Thus, cryopreservation treatments of embryos prior to freezing and after thawing was carried out in a laboratory or indoors using a stereomicroscope. Since then, many studies regarding the improvement and simplification of the freezing of bovine embryos have been reported, and today embryo freezing has become an important technique, often conducted on farms, supporting modern animal husbandry [17–19]. On the other hand, in the late 1970s, the effectiveness of ethylene glycol (EG) as a cryoprotectant for mammalian embryos was reported. The development of freezing technology using EG was described later in this paper.

One-step Dilution of Cryoprotectant from Frozen-thawed Embryos

Between 1982 and 1984, the dilution of GLY following the thawing of frozen bovine embryos was simplified using sucrose (SUC) [20, 21] . The nonpermeating solute, such as SUC, maintains a constant external osmolality and has sufficient osmotic pressure to prevent swelling during the efflux of intracellular cryoprotectants [22]. In 1982, Leibo *et al*. [23] and Renard *et al*. [21] described a one-step *in situ* dilution method for frozen-thawed bovine embryos using SUC. The following year, Leibo [24, 25] conducted a large-scale field trial of the "one-step[TM]" method which facilitated the dilution of GLY within the straw in which it was frozen. Leibo showed the high practicality of the "one-step[TM]" method under field conditions which dramatically simplified the removal of cryoprotectant from frozen-thawed embryos and their subsequent transfer, similar to AI on a farm. In Japan, a modified "one-step [TM]" method was developed in which the SUC concentration was reduced to 0.37 molar (M) from

1.1 M by some groups [26, 27]. A method of diluting and removing GLY in straws using SUC was called the "one-step straw method". It was expected to be an improvement on the "one-step[TM]" method under field conditions. However, with the "one-step straw method", loading embryos and dilution solutions into the straw and handling straws for the dilution of GLY after thawing was complicated, making it easy to introduce human error during the process. The conception rate was found to be lower than that with the stepwise method of diluting GLY. Therefore, the development of a new embryo freezing method that could eliminate the dilution of cryoprotectant after thawing was required. The new method is no need of a microscope or dilution procedures and can eliminate technical errors. And, the new method can transfer embryos to recipients immediately after thawing. The new method is called direct transfer.

The Direct Transfer of Frozen-thawed Bovine Embryos

In 1978, a method of direct transfer was described by Willadsen *et al*. [28], in which embryos were frozen in a solution containing DMSO. However, only one of 20 recipients were reported to have become pregnant in this study. In 1984, another method of direct transfer was described by Massip and Van der Zwalmen in Belgium [29], in which embryos were frozen in a solution containing 1.4 M GLY and 0.25 M SUC (GLY-SUC); embryos were transferred directly to recipients after thawing without diluting the cryoprotectant. Subsequently, Massip's group [30] reported conception rates of 51.8% using their direct transfer method with GLY-SUC (Table 2). Massip's method was the first report of the direct transfer of frozen-thawed bovine embryos that was practical under field conditions. Massip's method was followed by a couple of Japanese groups [31, 32], and acceptable results were obtained. However, almost no reports were found from countries other than Japan. Although the reason is unknown, research on one-step *in situ* dilution techniques was attracting attention.

In 1990, Suzuki *et al*. [33] reported pregnancy rates (60.0%) following the direct transfer of bovine embryos frozen-thawed in 1.6 M propylene glycol (PG) and 0.2 M SUC. This reported pregnancy rate was comparable to that following step-wise dilution of GLY. In the next year, Dochi *et al*. reported at the annual conference of the Japanese Society of Animal Sciences that EG could be used effectively for direct transfer of bovine embryos [34]. The same year, Kocoskie *et al.* [35] also reported that EG could be used effectively to freeze bovine embryos and could be diluted and transferred to recipients after thawing (The seventh annual conference of the Association of Embryo Transfer in Europe). Kocoskie studied the direct transfer of frozen-thawed bovine embryos with EG used by Dochi's group following a visit to the National Livestock Breeding Hidaka Station in Hokkaido as a trainer for the Japan International Cooperative Association. Shortly thereafter, Voelkel and Hu [36] reported that 1.5 M EG could be used effectively for the direct transfer of bovine embryos. Dochi *et al*. [32] subsequently reported that 1.8 M EG could also be used effectively for direct transfer of bovine embryos with the birth of normal calves (Tables 3 and 4).

Table 1. Summary of historical development of embryo cryopreservation in cattle

Year	Cryoprotectant	Event	Authors
1972	DMSO	First offspring from frozen mammalian embryos (mouse)	Whittingham *et al*. [14]
1973	DMSO	First calf from frozen embryos	Wilmut and Rowson [10]
1977	EG	First report of EG as a cryoprotectant for mammalian embryos (mouse and rat).	Miyamoto and Ishibashi [39]
1978	DMSO	Direct transfer using DMSO	Willadsen *et al*. [28]
1982	GLY	One step dilution of glycerol using SUC.	Leibo *et al*. [23]
1983	GLY	One-step *in situ* dilution of glycerol using SUC.	Leibo [24]
1986	GLY	Field trials of transfer of frozen thawed bovine embryos by improved one step straw method.	Suzuki *et al*. [26]
1984	GLY + SUC	Direct transfer using GLY and SUC	Massip and Van der Zwalmen [29]
1990	PG + SUC	Direct transfer using PG and SUC	Suzuki *et al*. [33]
1991	EG	Direct transfer using EG (1.8 M)	Dochi *et al*. [34]
1992	EG	Direct transfer using EG (1.5 M)	Voelkel and Hu [36]

DMSO: dimethyl sulfoxide, EG: ethylene glycol, GLY: glycerol, SUC: sucrose, PG: propylene glycol.

Table 2. Development after transfer of day 6.5–8 bovine embryos frozen in 1.36 M glycerol or 1.36 M glycerol –1.25 M sucrose in phosphate buffer saline

Temperature [a] (°C)	Cryoprotectant			
	1.36 M glycerol in PBS (diluted before transfer)		1.36 M glycerol –0.25 M sucrose in PBS (transferred without dilution)	
	No. of frozen-thawed embryos	No. of pregnancies (%)	No. of frozen-thawed embryos	No. of pregnancies (%)
–25	16	2 (12.5)	27	14 (51.8)
–35	24	10 (41.7)	11	2 (18.2)
Total	40	12 (30.0)	38	16 (42.1)

[a] Temperature at which samples were plunged into liquid nitrogen. PBS: phosphate buffer saline. From Massip *et al*. (1987) [30].

Table 3. Pregnancy results after direct transfer of frozen-thawed bovine embryos without dilution of the cryoprotectant

	Cryoprotectant [a]		
	EG	EG + SUC	GLY + SUC
Temperature [b]			
–25°C	71.4 (5/7)	50.0 (7/14)	60.0 (15/25)
–30°C	68.2 (15/22)	54.5 (6/11)	
Stage of embryos			
Morula	60.0 (9/15)	63.6 (7/11)	41.7 (5/12)
Blastocyst	78.6 (11/14)	42.9 (6/14)	76.9 (10/13)
Pregnancy rate	69.0 (20/29)	52.0 (13/25)	60.0 (15/25)

[a] EG, 1.8 M ethylene glycol; EG + SUC, 1.8 M ethylene glycol + 0.25 M sucrose ; GLY + SUC, 1.4 M glycerol + 0.25 M sucrose. [b] Temperature at which samples were plunged into liquid nitrogen. Values are pregnancy rates (no. of pregnant heifers/no. of recipient heifers) [32].

Table 4. Calving results after direct transfer of frozen-thawed bovine embryos without dilution of the cryoprotectant

	Cryoprotectant [a]		
	EG	EG + SUC	GLY + SUC
No. of pregnancies	20	13	15
No. of abortions	1	1	3
No. of calves	19	12	14 [b]

[a] EG, 1.8 M ethylene glycol; EG + SUC, 1.8 M ethylene glycol + 0.25 M sucrose; GLY + SUC, 1.4 M glycerol + 0.25 M sucrose. [b] Includes two sets of twins [32].

The Conditions of Cryoprotectants Used for Direct Transfer

The embryos are slowly cooled when cryoprotectant equilibration is complete under ambient temperature. After seeding, slow cooling continues, extracellular ice crystal formation occurs, and dehydration from embryonic cells proceeds gradually. As a result, intracellular and extracellular concentrations of salts and cryoprotectants increase. Then, the embryos are rapidly cooled and vitrified by plunging into liquid nitrogen.

In general, if frozen-thawed embryos are placed directly into an isotonic solution, embryonic cells will swell, as water flows in more quickly than cryoprotectants diffuse out. Consequently, the embryos expand excessively. An important issue in the direct transfer of frozen-thawed embryos without dilution of cryoprotectants is that a differential rate of permeation between water and cryoprotectants (which permeates embryo cells more slowly) results in osmotic

shock. Osmotic shock (and even cell membrane rupture) occurs when embryos are frozen using a cryoprotectant with a low cell membrane permeability coefficient such as GLY [37]. In the direct transfer method, cryoprotectants with high cell membrane permeability (such as PG or EG) are used resulting in very much less osmotic shock. Alternatively, it is necessary to add non-permeating substances as an osmotic buffer (or counter-force) to the cryoprotectant such as SUC. EG has high cell membrane permeability in bovine embryos [37] and is suitable as a cryoprotectant for direct transfer without the use of SUC. Conversely, GLY cannot be used alone as a cryoprotectant for direct transfer because it permeates cells more slowly, but it can be used along with SUC as an osmotic counter-force.

The Direct Transfer Method Using Glycerol with Sucrose

As indicated earlier, Massip and Van der Zwalmen [29] reported that a solution containing GLY-SUC could be used for direct transfer of bovine embryos. In this method, embryos are placed in a freezing medium containing 1.4 M GLY and 0.25 M SUC and are equilibrated for 10 to 20 min. After equilibration, embryos are loaded into a 0.25 ml straw, transferred to a cooling bath at –7°C for two min before they are seeded and held for an additional 8 min at that temperature, and then cooled to –25°C at 0.3°C/min before being plunged into liquid nitrogen. Massip et al. reported that when embryos frozen in 1.4 M GLY are plunged into liquid nitrogen at –25°C, recrystallization occurs which adversely affects the viability of embryos during warming because of insufficient dehydration [30].

Conversely, when 0.25 M SUC is added to 1.4 M GLY, embryos shrink, and the volume of embryos after equilibrium is lower by 30 to 40% than that of embryos in 1.4 M GLY. Therefore, the embryos in GLY-SUC are already dehydrated before cooling and can be plunged into liquid nitrogen at a higher subzero temperature than GLY alone. They reported that the pregnancy rate of embryos frozen with GLY-SUC was higher than that of GLY when plunged into liquid nitrogen at –25°C. Also, they reported that the pregnancy rate of GLY frozen embryos is higher when plunged into liquid nitrogen at –35°C than when frozen with GLY-SUC (see Table 2). These results indicated that when plunged into liquid nitrogen at –35°C using GLY-SUC, an embryo is too dehydrated and osmotic injuries occur during thawing.

Massip provided a recommendation regarding seeding and the plunging temperature during the freezing process by his method of direct transfer with GUL-SUC to the editor of the International Embryo Transfer Society newsletter [38]. He recommended that the seeding point should be at –7.5°C instead of –7°C, and that the straw should be plunged into liquid nitrogen at –25°C, not –35°C.

The Cryopreservation of Embryos Using Ethylene Glycol

Miyamoto and Ishibashi [39] first reported in 1977 that EG was effective as a cryoprotectant for mammalian embryos (mouse and rat, see Table 1) . In 1982, Elsden et al. [40] reported the freezing of bovine embryos in EG. EG was then used to freeze rabbit [41] and mouse [42] embryos.

Methods of direct transfer using EG for bovine embryos was first described by Dochi et al. in 1991 at the annual conference of the Japanese Society of Animal Sciences as indicated earlier [34]. In 1992, Voelkel and Hu [36] also described the direct transfer of bovine embryos using EG at the annual meeting of the IETS. After this, the direct transfer of bovine embryos using EG spread throughout the world. It has been reported that the conception rate following the direct transfer of bovine embryos in EG is not different from the step-wise dilution of GLY [32]. Similar results were obtained in field trials carried out in Japan [43], Europe [44], and North America [45].

Factors Affecting the Pregnancy Rate after the Direct Transfer of Frozen-thawed Bovine Embryos

Dochi et al. [43] investigated the factors affecting pregnancy rate after direct transfer of in vivo-derived frozen-thawed embryos in an integrated embryo transfer program (Table 5). The embryos were frozen in 1.6 M PG, 1.8 M EG, or 1.4 M GLY. Embryos frozen-thawed in 1.6 M PG or 1.8 M EG were transferred directly to recipient animals without diluting the cryoprotectants. Embryos frozen-thawed in 1.4 M GLY were transferred after removing GLY either by the stepwise method (GLY-1) or by in situ dilution with 0.3 M SUC (GLY-2). A total of 1,237 (PG: 400, EG: 418, GLY-1: 177, GLY-2: 278) frozen-thawed embryos were transferred, yielding 545 pregnancies (overall pregnancy rate: 42.8%, PG: 36.0%, EG: 44.7%, GLY-1: 48.6%, GLY-2: 46.0%). The pregnancy rate with PG was significantly lower than with EG or GLY-2 (P < 0.05). The pregnancy rate was affected by the type of cryoprotectant, the region where embryo transfers were carried out, the developmental stage of the embryos (compacted morulae, early blastocysts > blastocysts, expanded blastocysts), the parity of the recipients (nulliparous heifers > parous), and the corpus luteum quality in the recipients (excellent, good, poor > fair). Leibo and Mapletoft [45] reported that there was no difference in pregnancy rate between GLY (dilution) and EG (direct transfer). Dochi et al. [32] also reported that there was no difference in the pregnancy rate between the two methods of direct transfer (EG and GLY-SUC). These results indicate that the pregnancy rate following the direct transfer of frozen-thawed bovine embryos in EG and GLY-SUC is equivalent to conventional freezing and dilution methods with GLY.

The Addition of Sucrose to Freezing Medium with Ethylene Glycol

In a North American survey involving nearly 27,000 embryos cryopreserved in 1.5 M EG [45], there was no improvement in conception rates even when SUC was added to EG. However, in Japan, it has been shown that the survival rate of in vitro-produced (IVF) embryos after thawing is improved by adding 0.1 M SUC to the 1.5 M EG freezing medium [46]. Embryos at advanced stages such as blastocysts and expanded blastocysts are more susceptible to osmotic shock than late morula and early blastocyst stages, as they have formed a blastocoele and have a low survival rate after thawing [43]. Some researcher believes that blastocysts have two fluid chambers (the cell and the blastocoel) that may perhaps lead to lower survival (Mapletoft, personal communication); therefore,

Table 5. Least-squares means and standard errors of pregnancy rate (%) after transfer of embryos frozen-thawed in propylene glycol, ethylene glycol or glycerol

Effect	Probability	No. of transfers (No. of pregnancies)	Least-squares mean	Standard error
Overall mean		1273 (545)	33.3	2.39
Cryopreservation	P < 0.05			
Propylene glycol		400 (144)	27.2 [b]	3.09
Ethylene glycol		418 (187)	34.5 [a]	3.14
Glycerol-I[e]		177 (86)	33.5 [ba]	4.52
Glycerol-II[f]		278 (128)	37.7 [a]	3.08
Region	P < 0.0001			
1		324 (137)	34.8 [bc]	3.87
2		228 (112)	42.8 [ab]	4.05
3		298 (164)	46.7 [a]	3.89
4		194 (50)	10.4 [d]	4.59
5		229 (82)	31.6 [cd]	4.00
Developmental stage	P < 0.001			
Compacted morulae		278 (131)	40.8 [a]	3.42
Early blastocysts		377 (187)	38.8 [a]	3.15
Blastocysts		425 (160)	28.3 [b]	3.08
Expanded blastocysts		193 (67)	25.1 [b]	4.28
Parity	P < 0.0001			
Heifer		791 (355)	46.2 [a]	2.41
1		207 (83)	33.6 [b]	3.72
2		139 (47)	23.8 [b]	4.47
3≤		136 (60)	29.4 [b]	4.60
Corpus luteum quality[g]	P < 0.001			
Excellent		477 (219)	36.3 [a]	2.73
Good		509 (222)	36.9 [a]	2.53
Fair		176 (50)	22.8 [b]	4.15
Poor		111 (54)	36.9 [a]	5.78

[a, b, c, d] Values within the same column with different superscripts differ significantly (P < 0.05). [e] Glycerol-I: glycerol-step wise dilution, [f] Glycerol-II: glycerol-*in situ* dilution, [g] Corpus luteum quality was classified into four categories based on size [43].

improvement in survival occurs by collapsing the blastocoel before freezing. Likewise, the addition of SUC effectively dehydrates embryos before freezing, and excessive flows of water into embryonic cells can be suppressed during thawing. Thus, it is recommended to add 0.1 M SUC to 1.5 M EG for direct transfer.

The Toxicity of Ethylene Glycol in Frozen and Thawed Bovine IVF Embryos During Direct Transfer

Dochi *et al*. [47] reported the toxicity of EG. As indicated earlier, EG is a highly permeable cryoprotectant in bovine embryos [37]. However, EG is thought to be more toxic than GLY in bovine embryos. Dochi *et al*. [43] reported that lower pregnancy rates were obtained in a large scale on-farm experiment when more than 11 min were required to complete the embryo transfer after thawing. It seems likely that keeping frozen-thawed embryos in the freezing medium for a long period after thawing may reduce embryonic viability. However, it was reported that there was no difference in survival when IVF embryos were exposed to 1.5 M EG for 10 or 40 min before freezing

[48]. As far as we know, the toxicity of EG in bovine embryos after thawing has not been tested directly in embryos produced *in vivo*. Matoba *et al*. [49] studied the toxicity of EG in frozen and thawed IVF embryos in the direct transfer method; Day 7 blastocysts (n = 529) of grade 1 quality were frozen in 1.5 M EG using a 0.25 ml straw. After thawing, the straws were held for 0, 10, 20, 30, and 60 min at either 26.0 or 38.5°C. Then, EG was removed from the embryos by placing them in PBS supplemented with 20% calf serum (CS), warmed to 38.5°C for more than 20 min [50]. The holding temperature for the same holding time did not affect the survival of frozen-thawed embryos. However, the survival rate of the frozen-thawed embryos decreased significantly when the holding time exceeded 30 min at 38.5°C. The survival rate of the frozen-thawed embryos decreased significantly when the holding time was 60 min at 26.0°C. Thus, the toxicity of EG as a cryoprotectant for frozen-thawed embryos was only apparent after a holding time of 30 min at 38.5°C or after 60 min at 26.0°C. According to these results, the toxicity of EG is low and can be safely used for direct transfer in bovine embryos.

The Effects of Handling Straws on the Survival of Frozen-thawed IVF Embryos

Under on-farm conditions, frozen bovine embryos are frequently thawed under varying environmental conditions (temperature, place, season, area). There is some doubt whether the same thawing procedure can be used consistently under all environmental conditions. Two experiments on the effect of thawing and maintenance conditions on the survival of frozen-thawed IVF embryos in GLY-SUC for direct transfer were conducted. Japanese Black IVF embryos were produced as previously described [51]. On Day 7 and 8, expanded blastocysts were frozen in TCM-199 supplemented with 1.4 M GLY, 20% CS, and 0.25 M SUC (GLY-SUC). The embryos were loaded into 0.25 ml straws (one embryo per straw) and frozen as previously described. Embryos were thawed by holding the straws at room temperature air for 10 sec, and then immersing them in a 35°C water bath for 10 sec. We investigated the exposure temperature and the time in the air after thawing on the survival of frozen-thawed IVF bovine embryos [52]. After thawing, the straws were maintained in the freezing medium for 0, 5, 10, 15, 30, and 45 min at –15, –10, –5, 4, 10, 15, 23, 30, and 35°C, respectively (a total of 54 groups, with 30 embryos in each group). Then embryos were placed directly in PBS + 5% CS [50]. Embryos were cultured in TCM199 supplemented with fetal calf serum (FCS) for 72 h to assess post-thawing embryo survival based on their hatching ability. There was no difference among any holding times or temperatures on the survival or hatching rates of frozen-thawed embryos (70 to 100%). These results suggest that exposure temperature and time in straws after thawing do not affect the survival of frozen-thawed IVF bovine embryos cryopreserved in GLY-SUC.

We also investigated the effects of rapid temperature changes on the viability of frozen IVF bovine embryos after thawing [53]. The thawed straws were randomly assigned to one of two groups (a total of 11 groups, with 40 embryos per group). Some thawed straws were held for 5 min at either –15, –5, 0, 5, or 15°C, and were then transferred directly into a water bath at 35°C for 5 min (Group 1). The remaining straws were subjected to the same post-thawing cooling procedure and repeated twice (Group 2). The embryos were then placed directly into PBS supplemented with 5% CS at 35°C, and then cultured as described above [50]. The morphology and hatching of embryos were assessed 72 h later. There were no significant differences in the hatching rate (85 to 97.5%) among the five temperatures tested in Group 1. Although, there were no differences in the hatching rate of embryos held at –5, 0, 5, or 15°C (85.0% to 100%) after thawing in Group 2, only embryos held at –15°C (57.5%) had a significantly lower hatching rate (P < 0.05). The straws held at –15°C twice (Group 2), apparently recrystallized. These results suggest that exposing thawed straws to a broad range of environmental temperatures (–5 to 15°C) did not affect the viability of frozen-thawed IVF bovine embryos. However, embryos might be irreversibly damaged when held at lower temperatures after thawing.

Freezing of Bovine IVF Embryos for Direct Transfer

The survival of bovine IVF embryos after freezing has been reported to be affected by culture conditions [54, 55]. IVF embryos have an apparent chilling and freezing sensitivity associated with their lipid content when cultured in a medium supplemented with serum [54–57]. The composition of the culture medium has been reported to affect the membrane composition of bovine embryos and also affect freezing sensitivity [58]. Membrane fatty acids affect the fluidity of membrane lipid bilayers and also may affect the freezing sensitivity of bovine IVF embryos [59, 60]. Linoleic acid-albumin (LAA) is one of the substances expected to improve the fluidity of membrane lipid bilayers and improve the viability after freezing and thawing of IVF embryos. Therefore, we investigated the effect of calf serum and LAA in the *in-vitro* culture (IVC) medium on *in vitro* development and survival after the freezing of bovine IVF embryos [57]. Presumed zygotes were cultured in modified CR1aa medium [61] supplemented with 5% CS and 0, 0.25, 0.5 or 1 mg/ml LAA for 9 days. Blastocysts were frozen in 1.5 M EG. The hatching rate after freezing of embryos produced in medium supplemented with LAA (63 to 72%) was significantly higher than those produced without LAA (44%) (P < 0.01). These results indicated that although the addition of LAA to the medium for *in vitro* culture of bovine IVF embryos had no apparent effect on embryo development, it enhanced their survival after freezing.

Thereafter, we investigated the effect of CS and LAA in the IVC medium on the *in vitro* development and survival after freezing of bovine IVF embryos [62]. Presumed zygotes were cultured in mSOF supplemented with 0, 0.15, or 0.3% of LAA in either the presence or absence of 5% CS at 38.5°C under 5% CO_2, 5% O_2, and 90% N_2 for 8 days. Embryo development was evaluated for cleavage and blastocyst rate on Days 2, and 7 to 8, after IVF. Blastocysts were frozen in PBS + 20% CS containing 1.5 M EG and 0.1 M trehalose. The blastocyst rate in media containing CS (24.6 to 29.6%) was higher than in media without CS (11.2 to 16.1%) (P < 0.05). The hatching rate after freeze-thawing of blastocysts produced in medium containing CS alone (18.9%) was significantly lower than those of all other treatments (55.6 to 74.5%) (P < 0.01). These results demonstrate that the addition of CS in the medium for *in vitro* culture of IVF bovine embryos affected both their ability to develop into blastocysts and their hatching rate after freezing.

Moreover, the survival rate of frozen-thawed embryos was significantly improved by the addition of LAA to the medium containing CS. In summary, the freezing tolerance of IVF bovine embryos can be increased by adding LAA to *in vitro* culture media containing serum. It remains to be determined whether the addition of LAA to serum-containing media will increase pregnancy rates after direct transfer of frozen-thawed IVF embryos.

Conclusions

Direct transfer of bovine embryos with EG or GLY-SUC has become a widely used technique under farm conditions throughout Japan. It is possible to obtain a high and consistent conception rate using the direct transfer method because technical errors associated with the use of dilutions and microscopy can be eliminated. Since, Miyamoto and Ishibashi [39] first reported the effectiveness of EG as a cryoprotectant in 1977, the freezing of bovine embryos has become a widely accepted and important technology for the livestock industry, and Japan has played an essential role in its achievement.

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