

—Original Article—

Prolonging hypothermic storage (4 C) of bovine embryos with fish antifreeze protein

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Abstract. Embryos obtained via superovulation are necessary for mammalian artificial reproduction, and viability is a key determinant of success. Nonfreezing storage at 4 C is possible, but currently used storage solutions can maintain embryo viability for only 24–48 h. Here we found that 10 mg/ml antifreeze protein (AFP) dissolved in culture medium 199 with 20% (v/v) fetal bovine serum and 25 mM HEPES could keep bovine embryos alive for 10 days at 4 C. We used a recombinant AFP isolated from the notched-fin eelpout (*Zoarces elongatus* Kner). Photomicroscopy indicated that the AFP–embryo interaction was enhanced at 37 C. Embryos pre-warmed with the AFP solution at 37 C for 60 min maintained high viability, whereas those that were not pre-warmed could live no longer than 7 days. Thus, short-term storage of bovine embryos was achieved by a combination of AFP-containing medium and controlled pre-warming.

Key words: Antifreeze protein, Bovine embryo, Nonfreezing preservation

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The technique for cryopreservation of bovine embryos has been developed since the 1970s [1], and frozen embryos accounted for 80% of bovine embryo transfer (ET) procedures in the 1990s. However, the pregnancy rate with this approach has been less than 50% for many years, and this is much lower than when utilizing fresh embryos for ET [2]. Damage to the embryos associated with freezing and thawing can be mitigated to some extent by the addition of cryoprotectants such as dimethyl sulfoxide and glycerol. Preservation in liquid nitrogen (LN₂) at –196 C gives better cryopreservation results and is now widely used to freeze embryos and other cells, but LN₂ needs to be replenished continuously during storage and requires inconvenient means of transportation. For example, the International Air Transport Association strictly regulates the carriage of LN₂ in aircraft [3], so embryos stored in LN₂ have to be transported worldwide by other means such as trains, trucks and ships. It is hence relevant to examine the feasibility of short-term nonfreezing preservation of embryos without LN₂, which enables transportation of them by air even between distant countries. Other obvious advantages of cold storage are that it is more simple, cheap and does not require special equipment, and therefore, it is applicable under farm conditions for short-term storage of embryos. Recently, we developed a medium that enabled bovine embryos to be held for up to 7 days at 4 C [4].

In the present study, we examined whether the performance of this medium could be improved further by the addition of a fish-derived antifreeze protein (AFP), which has a special ability to protect cells and their membranes from hypothermic damage [5].

AFPs were first identified from the blood of fish species that can survive in ice-laden environments. They are macromolecules that can bind to ice crystals and prevent the body from freezing [6]. The proteins were categorized into AFP types I–IV and as antifreeze glycoprotein (AFGP) according to amino acid sequence and tertiary structure [7]. Rubinsky *et al.* first reported that AFGP could protect pig oocytes from ion leakage across the oolemma for 24 h at 4 C [8]. AFP types I–III could also protect the membrane of bovine oocytes for 24 h at 4 C [5], and sheep embryos could be stored for up to 4 days at 4 C in a solution of AFP type I [9]. A dose-dependent preservation ability for human hepatoma G2 cells was identified for natural and recombinant AFP type III at concentrations between 2 and 10 mg/ml [10]. AFP type III-induced inhibition of swelling and rupturing of cells was assumed to be the protective mechanism [11]. AFP types I and III dissolved in Euro–Collins solution prolonged the lifetime of rat insulinoma cells to 5 days [12]. These data prompted us to examine the effect of an AFP when it was dissolved in our recently developed medium consisting of medium 199, fetal bovine serum (FBS) and HEPES, which by itself extended the viability of bovine embryos to 7 days [4].

AFPs and AFGP purified from fish bodies are typically a mixture of 2–13 isoforms [13]. Among them, we chose an AFP type III isoform named nfeAFP11 because a preliminary study showed its superior protection ability for embryo storage. It is a 66-residue monomeric polypeptide consisting of the amino acid sequence NQESVVA AVL IPINTALTVG MMTTRVVSPT GIPAEDIPRL

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ISMQVNQVVP MGTTLMPDMV KGYAPA. It was discovered as the eleventh isoform of AFP type III from *Zoarces elongatus* Kner (the notched-fin eelpout), which lives off the northeast coast of Japan [14]. The structure of nfeAFP11 was determined by NMR spectroscopy [15], which revealed that it forms a flat-faced globular protein comprised of short β -strands and one helical turn.

Here we used a modified genetic engineering method to produce a recombinant protein sample of nfeAFP11 (400 mg/l of culture) and examined its effect on the hypothermic storage of bovine embryos. Special attention was paid to the temperature dependence of interactions between nfeAFP11 and the cell membrane.

Materials and Methods

Ethics statement and animal care

This study was approved by the Animal Care and Use Protocol Committee of the Research Animal Resources Center at Zen-noh ET Center (Approved Animal Study Proposal #H25-2-1). Donor cows (3–7 years old) and recipient heifers (14–18 months old) were fed the same diet, and water was supplied *ad libitum*. The compositions of the herds were set in consideration of body constitution and social hierarchy.

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Preparation of recombinant nfeAFP11

Our previously developed expression method using *E. coli* produced approximately 50 mg of nfeAFP11 per liter of culture on average [14]. Because a series of embryo storage experiments would require much more of the proteins, we modified the preparation method. First, we constructed two genes for expressing nfeAFP11 comprising a ribosomal binding site upstream by amplifying the following two DNA fragments: GCGGATCCGGCACCCCTCAGAACTACTAAGC (*Bam*HI site underlined) and CGGGATCCAATAATTTGTTTAACTTTAAG (*Bam*HI site underlined). They were digested with *Nde*I/*Bam*HI and *Bam*HI/*Xho*I, respectively, and then introduced into the corresponding cloning sites of a pET20b vector (Merck Millipore, Darmstadt, Germany). The modified expression vector was named pET20b–dual–nfeAFP11. Second, the host strain was changed to *E. coli* BL21 Star (DE3, Novagen, Madison, WI, USA), which carries a mutated *rne131* for increasing mRNA stability. The yield of nfeAFP11 was increased 2-fold (100 mg/l) by these two modifications. To further increase output, we performed a high cell density fermentation of the *E. coli* host strain BL21, which carries pET20b–dual–nfeAFP11, inoculated into LB medium with ampicillin at 100 μ g/ml at 28 C. Five milliliters of the overnight culture were transferred into 500 ml auto inducing Terrific Broth containing 97 mM Na₂HPO₄, 42 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄, 0.9% glycerol, 0.05% glucose and 0.2% α -lactose monohydrate and supplemented with 2 mM MgSO₄, 10 μ M FeCl₃, 4 μ M CaCl₂, 2 μ M each of MnCl₂ and ZnSO₄ and 0.4 μ M each of CoCl₂, CuCl₂, NiCl₂, Na₂MoO₄ and H₃BO₃ as described previously [16, 17]. Cell culture was then carried out in a 1-l BMJ–01PI fermenter (ABLE & Biott, Tokyo, Japan) at 25 C and agitated at 60 g for 24 h. The overnight culture was harvested

by centrifugation at 4,000 g for 15 min. The cells were resuspended in 250 ml of TE buffer supplemented with 0.1 mM PMSF and were homogenized using a high-pressure EmulsiFlex-C3 homogenizer with a heat exchanger (AVESTIN, Ottawa, Canada). Following the above procedures, nfeAFP11 purified from the lysate was lyophilized. These improvements significantly increased the yield of nfeAFP11 to 400 mg/l with high purity. The purity of nfeAFP11 was evaluated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and reverse–phase high–performance liquid chromatography analyses. The purified nfeAFP11 was dialyzed against pure water and then lyophilized again. The activity and structure of the purified nfeAFP11 have been reported elsewhere [15, 18].

Collection of in vivo fertilized embryos

Embryos produced *in vivo* were collected from superovulated Japanese black beef cattle [19]. Briefly, the cattle (n = 24) were administered decreasing doses of follicle-stimulating hormone (FSH, total 20 Armour units, Antrin R-10, Kawasaki-Mitaka, Kanagawa, Japan) for 3 days, twice a day, between days 8 and 13 of their estrus cycles (day of estrus = day 0). An analogue of PGF_{2 α} (2 ml Resipron-C containing 0.25 mg/ml cloprostenol, ASKA Pharmaceutical, Tokyo, Japan) was injected at the fifth FSH treatment. The cattle were inseminated with one dose of frozen-thawed semen on the morning after the day of estrus induced by superovulation treatment. Seven days after estrus, embryos were recovered by uterine flushing. Embryos of grades 1 to 2 (high quality) embryos according to the International Embryo Transfer Society classification (IETS manual) were used in this study [20].

Photomicroscopy of nfeAFP11–embryo interactions

Bovine embryos were incubated with nfeAFP11 labelled with the fluorescent probe Alexa 488 (Invitrogen, Carlsbad, CA, USA) in phosphate buffered saline (PBS) at 4–5 C (Medicool, Sanyo, Osaka, Japan), 19–23 C (room temperature, RT) or 37 C (MIR–162 incubator, Sanyo) for 10, 20, 30 and 60 min. After incubation, the embryos were washed several times in PBS supplemented with 5% FBS (MP Biomedicals, Illkirch, France) and mounted under cover slips without compression in medium containing 50% glycerol and 25 mg/ml of sodium azide. Interaction between bovine embryos and nfeAFP11 was observed using a Leica DM LD fluorescence microscope (Leica, Wetzlar, Germany) at an excitation/emission wavelength of 485/530 nm. The morphology of the chilled embryos was evaluated using light microscopy (Leica Wild M3Z, Leica), and all the photomicroscope images were recorded using an Olympus DP21 microscope digital camera (Olympus, Tokyo, Japan). Individual mean pixel intensity was measured on a per embryo basis using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Furthermore, a Leica DM IRE2 confocal microscope system (Leica) was also used to observe nfeAFP11–embryo interactions. The embryos were incubated with nfeAFP11 labelled with Alexa 488 in PBS at RT or 37 C for 30 min. All the confocal microscope images were recorded using a 3CCD color video camera (Sony, Tokyo, Japan).

Hypothermic storage experiments

As photomicroscopy showed that heating at 37 C enabled us to attach nfeAFP11 molecules onto the surface of the embryos,

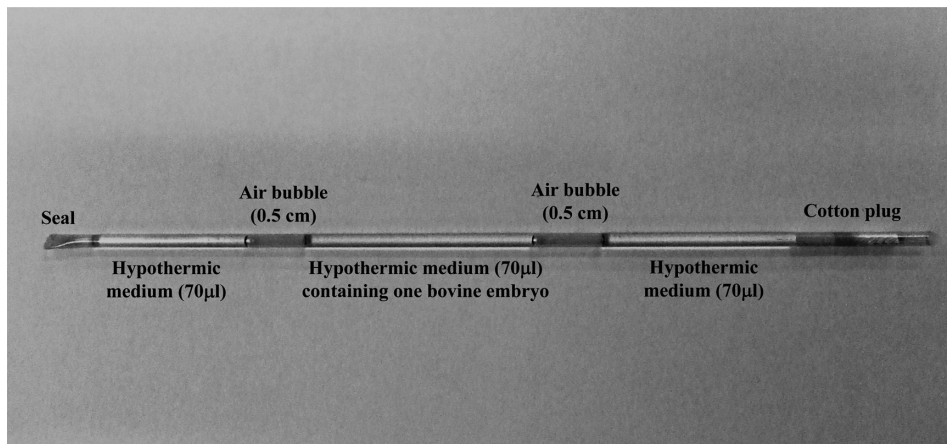


Fig. 1. Volume of each component in the loading procedure.

we suspended high-quality bovine embryos (morula to blastocyst stages) for 60 min at 37 C (MIR-162 incubator) in 50 µl of droplet of medium 199 containing 25 mM HEPES, 20–50% FBS and 10 mg/ml of nfeAFP11 under sterile paraffin oil (Nacalai Tesque, Kyoto, Japan). The embryos were then washed three times with the same hypothermic medium containing nfeAFP11. For evaluating the viability of the chilled embryos, the following components were loaded into a plastic ministray (0.25 cm³ clear straw, Pets, Canton, TX, USA) from left to right: hypothermic medium, an air bubble, hypothermic medium containing one bovine embryo, another air bubble and more hypothermic medium. The straw was sealed hermetically (Fig. 1). The loaded straws were placed in a refrigerator set to 4–5 C for 10 days. The stored embryos were flushed out from the straws, put into PBS supplemented with 5% FBS and washed three times in the same medium at RT. The embryos were transferred into CR1aa medium [21] supplemented with 5% FBS for 48 h at 38.5 C under 5% CO₂ in air with high humidity. The viability and hatching rate of embryos were assessed at the end of the culture period. Briefly, embryos that appeared dark and shrunken with no cell proliferation or cellular integrity were considered to have degenerated. Viable embryos with a clear breach in the zona pellucida made by the trophectoderm were classified as hatching blastocysts.

Embryo transfer to recipient female

The bovine embryos were loaded into a plastic ministray and used for 10-day hypothermic (4–5 C) preservation experiments. After 10 days, the stored embryos were transferred into PBS with 5% FBS and washed several times in the same medium at RT. The chilled embryos were reloaded into plastic straws (0.25 cm³ clear straw) with the washing medium and transferred nonsurgically into Holstein heifers (one embryo per recipient) to the uterine horn ipsilateral to the existing corpus luteum using an ET device (mo-No.1, Misawa Medical Industry, Tokyo, Japan) on days 6–8 of the estrous cycle (day of estrus = day 0). Pregnancy was determined by real-time B-mode ultrasonography (HS-1500 convex scanner, Honda Electronics, Toyohashi, Japan) on days 30 and 90 of gestation. The decision to induce parturition and deliver the calves by caesarean section was

based on the days of gestation, calf viability and observed changes in the appearance of the placenta seen by ultrasonography.

Statistical analysis

The data were analysed using the StatView software package (SAS Institute, Cary, NC, USA). The statistical significance of differences between treatments was assessed by analysis of variance followed by a Fisher's protected least-significant difference procedure as a multiple comparison test and Chi-squared test. A probability of $P < 0.05$ indicated that a difference was significant.

Results

Photomicroscopic observation of bovine embryos

Bovine embryo-nfeAFP11 interaction was observed using fluorescence microscopy. As shown in Fig. 2, the embryos incubated at 4 C did not show green fluorescence, while all the other embryos exhibited green fluorescence within their zona pellucida and perivitelline space. The most intense fluorescence was observed for the embryos preincubated at 37 C. Here the nfeAFP11 was seen to be tightly attached to the surface of embryos following removal of the zona pellucida with 5 mg/ml pronase (Fig. 3). Very weak fluorescence was observed in embryos incubated at RT, especially within 20 min (Fig. 2). These fluorescent data are presented as a bar graph generated using the imageJ software (Fig. 4). This result showed that the pixel intensity of the embryos preincubated at 37 C was significantly high in each preincubation period ($P < 0.05$). Furthermore, the photos obtained using a confocal microscope system also showed that more bright fluorescence was exhibited in embryo preincubated at 37 C within the zona pellucida and perivitelline space than in embryo preincubated at RT (Fig. 5).

Hypothermic storage of bovine embryos

To evaluate the effect of nfeAFP11, hypothermic preservation of embryos was performed in medium 199 plus 20–50% FBS supplemented with or without 10 mg/ml nfeAFP11. We used 160 high-quality Japanese black beef cattle embryos produced *in vivo* and

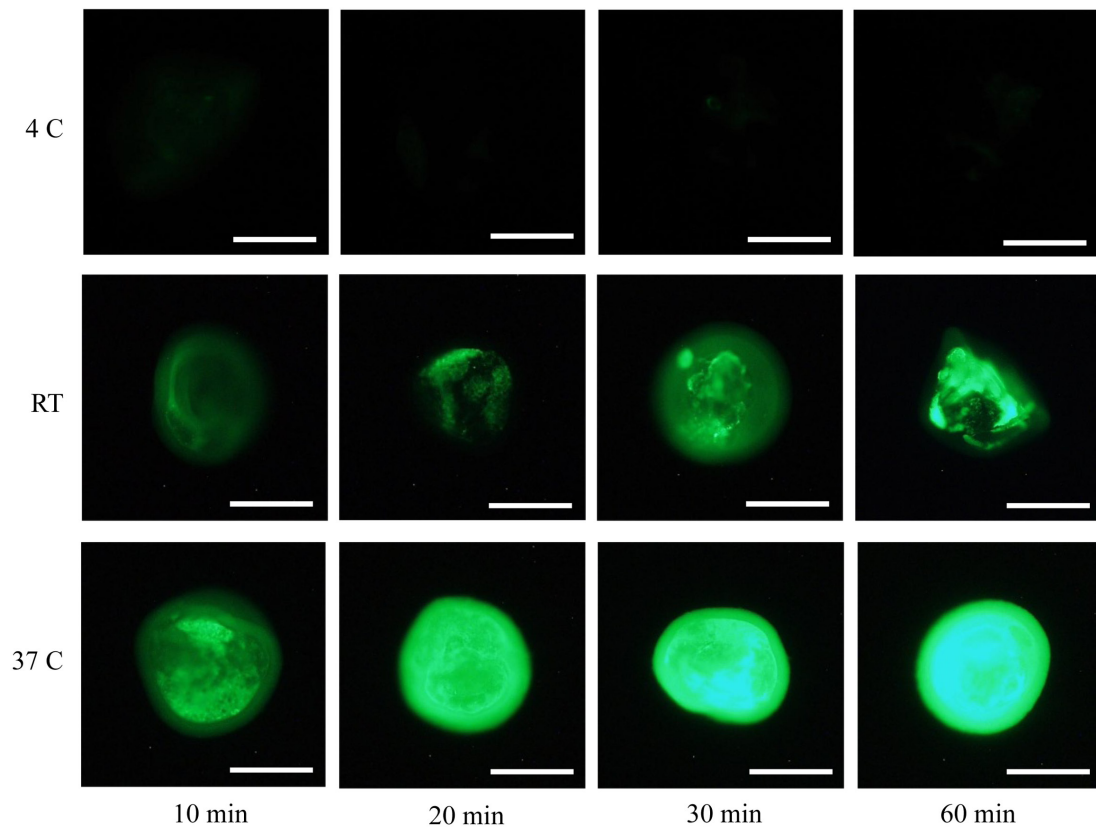


Fig. 2. Photomicroscope images of AFP-embryo interaction. Bovine embryos were incubated with nfeAFP11 labelled with the fluorescent probe Alexa 488 at 4 C, room temperature (RT; 19–23 C) or 37 C for 10, 20, 30 or 60 min. Embryos preincubated at 4 C did not show green fluorescence. Very weak fluorescence was observed in embryos preincubated at RT, especially within 20 min. Intense fluorescence was observed in embryos preincubated at 37 C. Scale bars = 100 μ m.

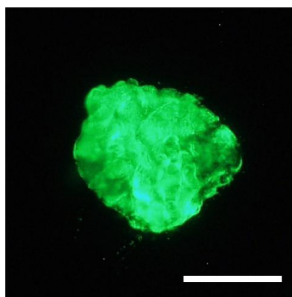


Fig. 3. AFP labelled with Alexa 488 attached tightly to the surface of zone pellucida-free embryos. Scale bar = 100 μ m.

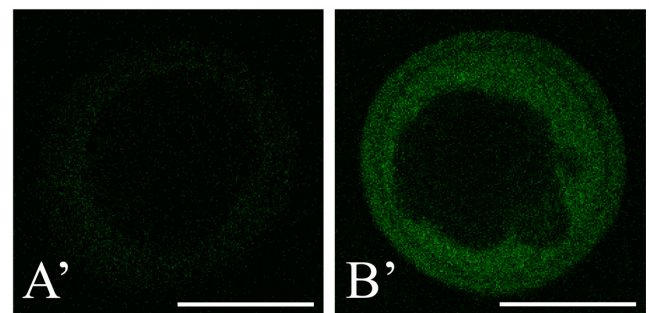
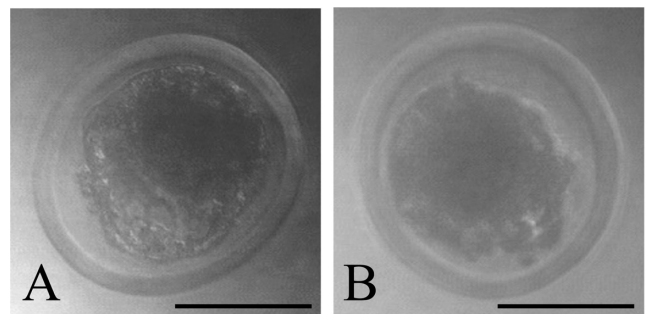


Fig. 5. Confocal photomicroscope images of AFP-embryo interaction. Bovine embryos were incubated with nfeAFP11 labelled with the fluorescent probe Alexa 488 at RT (A and A') or 37 C (B and B') for 30 min. Images A and B, light images; images A' and B' show the slice data of 1 μ m-wide slices of bovine embryos. Very weak fluorescence was exhibited in embryos preincubated at RT (A'). Intense fluorescence was exhibited in embryo preincubated at 37 C (B') within the zona pellucida and perivitelline space. Scale bars = 50 μ m.

Fig. 5.

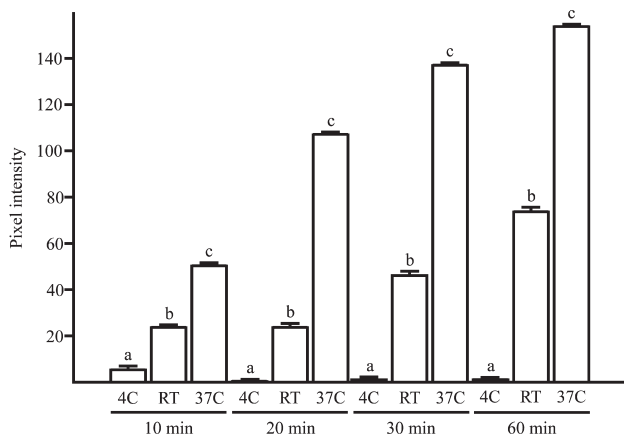


Fig. 4. Pixel intensity of AFP-embryo interaction. Bovine embryos were incubated with nfeAFP11 labelled with the fluorescent probe Alexa 488 at 4 C, room temperature (RT; 19–23 C) or 37 C for 10, 20, 30 or 60 min. Individual mean pixel intensity was measured on a per embryo basis using the ImageJ software. Bars show the mean \pm SEM pixel intensities for 3 times measurement. Different letters (a, b, c) indicate significant differences for each preincubation time ($P < 0.05$).

assigned them randomly to four experimental groups. The viability and hatching rates of the embryos after hypothermic storage for 240 h were higher for hypothermic medium supplemented with nfeAFP11 than those of without nfeAFP11 (Table 1). Notably, the hatching rate of the embryos was significantly increased for medium 199 plus 20% FBS supplemented with nfeAFP11 compared with the other experimental groups ($P < 0.05$).

To examine the pregnancy rate of chilled embryos, 4 embryos produced *in vivo* were stored at 4–5 C for 240 h in 25 mM HEPES medium 199 plus 20% FBS supplemented with 10 mg/ml nfeAFP11. The chilled embryos were transferred to recipient heifers. The pregnancy rate of the chilled embryos was 50%: no embryonic death was observed between pregnancy days 30 and 90. One live and one stillborn calf were delivered by two recipient heifers at around 285 days of gestation. The birth weights of the calves were 38.0 kg (Fig. 6, live) and 36.6 kg (stillborn). We did not observe malformation or large offspring syndrome in the dead calf.

Discussion

Notably, little is known about the hypothermic preservation of mammalian embryos, particularly bovine embryos, because LN₂ cryopreservation has generally been adopted for storage in cattle production. Several studies have indicated that AFPs could improve the viability of cryopreserved or hypothermically preserved mammalian cells [22–25], whereas others have shown no appreciable preservation effect of AFPs or even controversial results [26, 27]. One of the reasons for such variation might be the lack of pre-warming to 37 C before hypothermic preservation. We observed interaction between the bovine embryo and nfeAFP11 labelled with fluorescent probe at 4 C, at RT and at 37 C. Significantly, intense fluorescence emission was observed for those embryos preincubated at 37 C, whereas the

Table 1. Viability and hatching rates of high-quality bovine embryos produced *in vivo* by artificial insemination following storage at 4 C for 240 h in hypothermic medium supplemented with nfeAFP11

Concentration of FBS (%)	Concentration of nfeAFP11 (mg/ml)	No. of embryos	No. (%) of viable embryos	No. (%) of hatching embryos
20	0	40	11 (27.5) ^a	0 (0.0) ^a
20	10	40	23 (57.5) ^b	12 (30.0) ^b
50	0	40	15 (37.5) ^{ab}	2 (5.0) ^a
50	10	40	24 (60.0) ^b	2 (5.0) ^a

^{a, b} Data with different superscript letters differ significantly ($P < 0.05$). Experiments were repeated 4 to 5 times. FBS, fetal bovine serum.



Fig. 6. A 1-day-old calf produced from a chilled embryo stored for 240 h.

emission became weaker with lower preincubation temperatures. These data suggest that the interaction between nfeAFP11 and the cell membrane changes as a function of temperature. We also loaded the embryos into a plastic ministraw with hypothermic fluid containing nfeAFP11 and 20–50% FBS, pre-warmed them to RT for 20 min and stored them at 4 C for 10 days. In this case, we observed no dramatic increase in the developmental capacity *in vitro* of the embryos, and their viabilities were low (25–35%). This suggests that treatment at RT is not sufficient to adhere AFP molecules to the plasma membrane for protection. Furthermore, our preliminary test revealed that not only nfeAFP11 but also natural AFP type I purified from *Liopsetta pinnifasciata* showed temperature dependence in mammalian cells. We believe that these findings will contribute significantly to the advancement of research into AFPs.

Serum contains a wide variety of substances, including biological energy substrates, growth factors, cytokines and hormones [28]. It also contains amino acids that play important roles as osmolytes and pH buffers [29]. In our previous study, the concentration of

FBS in hypothermic medium affected the survival rate of bovine embryos after chilled preservation: the optimal concentration was 50% [4]. However, the present study showed that the hatching rate of embryos decreased for those cultured in medium 199 plus 50% FBS supplemented with nfeAFP11 compared with 20% FBS supplemented with nfeAFP11. The serum concentration changes the viscosity of the medium. Although we needed to attach nfeAFP11 tightly to the embryo surface, the hypothermic medium containing 50% FBS and nfeAFP11 after pre-warming at 37 C showed high viscosity, but this was not the case for the nfeAFP11 supplemented in medium containing 20% FBS. Thus, this viscosity might impair the development of chilled bovine embryos. Alternatively, it might cause hardening of the zona pellucida and inhibit blastocyst hatching. Further experimentation is required to determine which components of FBS have a detrimental role on the development of bovine embryo after hypothermic preservation.

In conclusion, hypothermic storage of bovine embryos for up to 10 days using an AFP-containing medium appears to be feasible, and this might be useful for ET when a recipient animal is a few days before the optimal time in its estrous cycle. To be of practical value, cells that can remain viable for longer than 10 days must be provided in the future for mammalian embryo preservation at hypothermic temperatures, and we believe that other types of AFPs (e.g., type I or type II AFP) might maintain the viability of mammalian embryos for longer periods.

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