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Supplementation of fructose in chemically defined protein-free medium enhances the *in vitro* development of bovine transgenic cloned embryos

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Summary

The present study evaluated the possible embryotrophic role of fructose supplementation in chemically defined protein-free KSOM on *in vitro* development of bovine transgenic cloned embryos. Bovine fetal fibroblasts transfected with expression plasmids for bovine prion protein (*PrP*) mutant gene with GFP marker gene were used as donor nuclei for reconstruction of slaughterhouse-derived *in vitro* matured oocytes. The reconstructed oocytes were cultured in KSOM supplemented with 0.01% PVA (KSOM–PVA) at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 192 h. In Experiment 1, when reconstructed oocytes were cultured in KSOM–PVA supplemented with glucose (0.2 mM), fructose (1.5 mM) or combined glucose and fructose (0.2 and 1.5 mM, respectively), significantly ($p < 0.05$) higher blastocyst (19.2%) and hatching/hatched blastocyst (13.1%) formation rates were obtained in combined fructose and glucose supplemented medium than glucose supplemented counterpart (10.0% and 5.7%, respectively). In Experiment 2, when reconstructed oocytes were cultured in KSOM–PVA supplemented with 0.0, 0.2, 1.5, 3.0 and 5.6 mM fructose in combination with 0.2 mM glucose, the blastocyst formation rate was significantly higher (17.6%) in 1.5 mM fructose supplemented group than that of no fructose supplemented counterpart (9.7%; $p > 0.05$). In conclusion, supplementation of combined fructose (1.5 mM) and glucose (0.2 mM) in chemically defined protein-free KSOM enhances the *in vitro* development of bovine transgenic cloned embryos.

Keywords: Bovine embryos, Fructose, KSOM, Nuclear transfer, Transgenic

Introduction

Production of prion protein (causal agent for bovine spongiform encephalopathy or mad cow disease) gene mutant cloned cattle by somatic cell nuclear transfer (SCNT) will contribute a lot in the field of medicine, because these cloned cattle can be better models for studying mad cow disease than mice. Moreover, it is important that transgenic cloned cattle for the purpose of therapeutic protein should be prion disease free. Until now, several transgenic models for studying transmissible spongiform encephalopathies (TSEs) have been generated by microinjection of DNA into the male pronucleus of a fertilized mouse egg. This approach generates transgenic mice in which the transgene is integrated randomly into the murine genome. In an alternative approach, transgenic mice carrying modifications of the endogenous murine genome have been produced by gene targeting (Manson & Tuzi, 2001). This method can be used to introduce mutations into the prion protein (*PrP*) gene or to delete or replace parts of the gene. Accordingly, *PrP* gene-deleted cloned sheep have been generated by SCNT using targeted gene-deleted donor nuclei (Denning *et al*., 2001). However, at this time there is no report on the production of cloned cattle using

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PrP gene mutant donor cells. Therefore, we used PrP mutant donor cells for generation of transgenic cloned embryos that might lead to production of viable mad cow disease resistant offspring (Anon, 2003).

SCNT is considered an efficient method for the production of transgenic animals (Niemann & Kues, 2000). Although production of live transgenic cloned offspring by SCNT has been reported in cows (Cibelli *et al*., 1998; Zakhartchenko *et al*., 2001; Chen *et al*., 2002; Forsberg *et al*., 2002; Bordignon *et al*., 2003), the efficiency of viable cloned animal production is still low (Renard *et al*., 2002). Many factors are involved in efficient *in vitro* production of cloned embryos. Among them, sub-optimal culture conditions for cloned embryos are known to cause incomplete or inadequate reprogramming of donor nuclei, resulting in low rates of blastocyst formation and pregnancy (Han *et al*., 2003). Differences in developmental competence in response to various culture media have been demonstrated between IVF and cloned embryos due to their different origins (Chung *et al*., 2002). These results suggest the importance of culture conditions for *in vitro* embryo development. Moreover, culture media for IVF embryos have long been investigated and improved. However, very little effort has been given to improve culture conditios for the production of cloned embryos in cattle (Choi *et al*., 2002; Jang *et al*., 2003; Kwun *et al*., 2003).

In any culture medium, the energy substrate is one of the important ingredients for optimum *in vitro* development of embryos. Pyruvate and lactate, in the absence of glucose, are able to support *in vitro* development of bovine embryos (Kim *et al*., 1993), glucose, however, is widely used as a supplement and is the major energy substrate in most of the culture media. In contrast to the metabolism of pyruvate and lactate, glucose metabolism in bovine IVF embryos differs from its *in vivo* counterparts (Khurana & Niemann, 2000). Moreover, it has been demonstrated that inclusion of glucose in culture media affects negatively the development of early-stage embryos in hamsters (Schini & Bavister, 1988; Barnett & Bavister, 1996; Barnett *et al*., 1997), mice (Chatot *et al*., 1989; Lawitts and Biggers, 1991; Scott & Whittingham, 1996), rats (Kishi *et al*., 1991; Miyoshi *et al*., 1994), cattle (Kim *et al*., 1993), sheep (Thompson *et al*., 1992) and humans (Conaghan *et al*., 1993; Quinn, 1995). In contrast to the use of glucose by early-stage embryos, addition of glucose in culture media plays an important role in postcompaction bovine embryos for blastocyst formation (Rieger *et al*., 1992). As with glucose, fructose can be metabolized through the glycolytic pathway. It has been reported that fructose is present in the reproductive tract of cattle (Suga & Masaki, 1973) and is utilized by bovine embryos *in vitro* (Guyader-Joly *et al*., 1996). Moreover, expression of fructose transporter gene, glucose transporter-5, in bovine embryos emphasizes the embryotrophic role of fructose (Augustin *et al*., 2001). Consequently, it was hypothesized that replacement of glucose with fructose in culture medium may improve the *in vitro* development of bovine transgenic cloned embryos. Nevertheless, culture medium should be chemically defined for investigation for specific requirement of culture components by preimplantation embryos (Rosenkrans *et al*., 1993). Moreover, chemically defined medium is desired for avoiding variations among laboratories and chances of disease transmission. However, no study has yet been performed on the effect of fructose supplementation in protein-free culture media on *in vitro* development of bovine embryos under chemically defined condition. Therefore, the objective of the present study was to investigate the possible embryotrophic role of fructose supplementation in protein-free potassium simplex optimization medium (KSOM) on *in vitro* development of bovine transgenic cloned embryos under chemically defined conditions.

In the present study, we used KSOM as basal embryo culture medium because it supports bovine IVF embryo development, and the highest rate of hatched blastocyst formation has been observed in KSOM with amino acids (KSOMaa) compared to CR2 with amino acids (CR2aa) and SOF with amino acids (SOFaa) (Tavares *et al*., 2002). Moreover, recently KSOM has been demonstrated as a potential medium for *in vitro* production of IVF, non-transgenic and transgenic cloned embryos in bovine (Bhuiyan *et al*., 2004a). Further, mouse embryos cultured in KSOM were closer to *in vivo* embryos in terms of gene expression profiling (Ho *et al*., 1995).

Materials and methods

In vitro maturation (IVM) of oocytes

Bovine ovaries were collected from a local slaughterhouse and were transported to the laboratory within 2 h in 0.9% (w/v) NaCl solution at 35 °C. Cumulus–oocyte complexes (COCs) were retrieved from antral follicles 2 to 8 mm in diameter by aspiration with an 18-gauge hypodermic needle attached to a 10 ml disposable syringe. The COCs with evenly granulated cytoplasm and enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium (TCM)-199 (Life Technologies) (hTCM) supplemented with 0.5% (w/v) BSA (fatty acid free, fraction V, Sigma), 2 mM sodium bicarbonate and 10 mM HEPES (Sigma) and 1% (v/v) solution of penicillin and streptomycin (Sigma). For maturation, COCs were cultured in 4-well dishes

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(30–40 COCs per well, Nunclon) for 20 to 22 h in 500 μl bicarbonate-buffered TCM-199 supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies), 5 μg/ml bovine FSH (Antrin®, Denka) and 1 μg/ml estradiol (Sigma) at 39 °C in a humidified atmosphere of 5% CO_2 in air.

Establishment of a bovine fetal fibroblast (BFF) cell line

A day-40–50 fetus was surgically removed from the bovine uterus in a local slaughterhouse and washed several times in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies). Decapitated fetus was eviscerated and the remainder of the fetal tissue was minced with a surgical blade. The minced tissues were dissociated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 0.25% trypsin and 1 mM EDTA (Life Technologies) for 30 min. Trypsinized cells were washed twice in DPBS and once in DMEM by centrifugation at 300 *g* for 2 min before being seeded into plastic culture dishes. Then the pellet of cells was seeded and cultured for 6 to 8 days in DMEM supplemented with 10% FBS, 1% (v/v) non-essential amino acids (Life Technologies), and 1% solution of penicillin and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured until (100%) confluency, and then sub-cultured at intervals of 5 to 7 days. At two to three passages, cells were cryopreserved in DMEM containing 20% FBS and 10% (v/v) dimethyl sulfoxide (DMSO; Sigma) at –140 °C deep freezer for subsequent use.

Transfection of BFF

Expression plasmids for bovine prion protein (*PrP*) mutant gene with green fluorescent protein (GFP) marker (PrP–GFP) were constructed following standard protocol. The DNA for target gene was transfected into the donor cells by lipid-mediated method using FuGene6® (Roche Diagnostics Corporation) according to the manufacturer's instruction. Briefly, frozen-thawed donor cells were sub-cultured in 2 ml volume of DMEM with 10% FBS until 50–60% confluency in a 35 mm culture dish. Cells in 35 mm culture dish were transfected by addition of a 100 μl mixture of FBS-free DMEM containing 1 μg DNA and 3 μl transfection reagent. Two days after transfection, a culture dish containing PrP–GFP transfected BFF was examined for GFP expression under ultraviolet light (Fig. 1*a*) using a standard fluorescein isothiocyanate (FITC; excitation wavelength: 450–490 nm; B-mode filter, Nikon) filter set. The transfected cells were cultured for at least 3–4 days in order to induce firm chromosomal integration of transgene and 100% confluency of the cell. PrP–GFP transfected cultured BFF were used for nuclear transfer without freezing.

Enucleation of oocytes

At the end of IVM, denuding of matured oocytes were performed in handling medium (hCR2aa) (Rosenkrans *et al*., 1993) supplemented with 0.1% (w/v) hyaluronidase (Sigma) by gentle pipetting with a mouth controlled pipette. After denuding, oocytes were placed in a drop (3–4 μl) of handling medium with 10% FBS containing 7.5 μg/ml cytochalasin B (Sigma) covered with mineral oil in a micromanipulation dish (Falcons) under differential interference contrast (DIC) microscopy (Nikon) equipped with micromanipulation system (Narishige). Each oocyte was held with a holding micropipette and the zona pellucida was partially dissected with a fine glass needle to create a slit near the first polar body. The first polar body and adjacent cytoplasm, presumably containing the metaphase-II chromosomes, were extruded by squeezing with the needle. Oocytes were then stained with 5 μg/ml bisbenzimide (Hoechst 33342, Sigma) for 15 min and observed under an inverted microscope equipped with epifluorescence at ×200 magnification. Oocytes still containing DNA material were excluded from experiment. The enucleated oocytes were incubated in TCM-199 supplemented with 10% FBS at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air until used for nuclear transfer.

Preparation and transfer of donor cells

Cultured donor cells in 35 mm dish were washed three times with DPBS and trypsinized using 0.25% trypsin–EDTA. After trypsinization, single cell suspension was prepared with DPBS supplemented with 0.5% FBS. Donor cells were aspirated into the cell insertion pipette and single cell was deposited into the perivitelline space through the same slit in the zona pellucida that was made during enucleation. Only GFP expressing BFF under FITC filter was selected for transfer (Fig. 1*b*, c). The cell was wedged between the zona and the cytoplast membrane to facilitate close membrane contact and the couplets were incubated in TCM-199 supplemented with 10% FBS at 39 °C in a humidified atmosphere of 5% CO_2 and 95% air until fusion.

Fusion and activation

Reconstructed couplets were electrically fused at 24 h post maturation (hpm) in a fusion medium comprising 0.28 M mannitol (Sigma), 0.5 mM HEPES, 0.1 mM magnesium sulphate and 0.05% fatty acid-free BSA. Fusion was performed at room temperature in a chamber with two stainless steel electrodes

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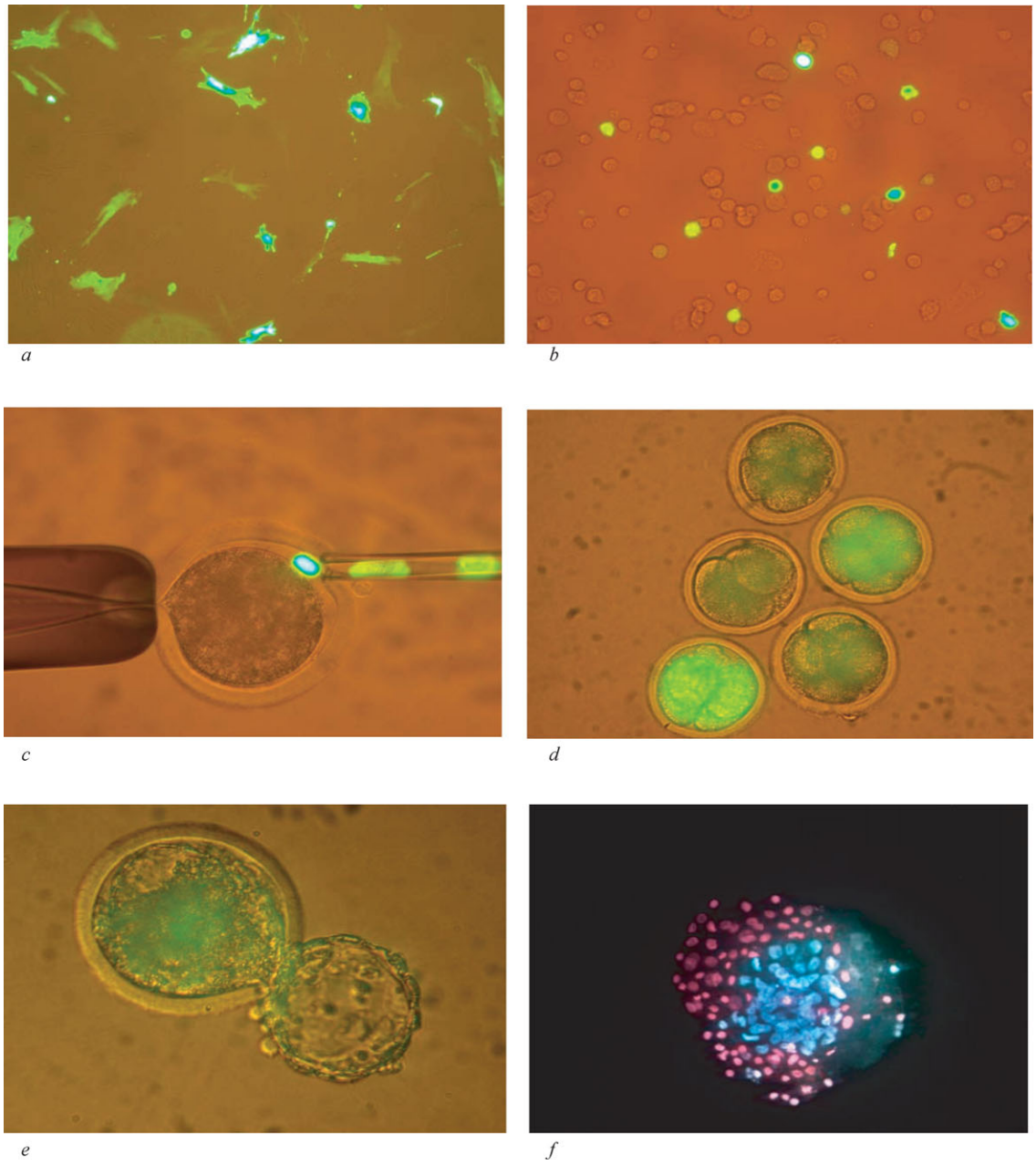

Figure 1 (*a*) GFP expression in cultured BFF. (*b*) GFP expression in BFF before injection. (*c*) Nuclear transfer of GFP expressed BFF. (*d*) GFP expression in 2–8-cell embryos. (*e*) GFP expression in a hatching blastocyst. (*f*) A differentially stained blastocyst.

3.2 mm apart (BTX), overlaid with fusion medium. The reconstructed embryos were manually aligned with a fine mouth-controlled pipette, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. Cell fusion was induced with two DC pulses of 1.75 kV/cm for 15 μs, delivered by an Electro-cell Manipulator (BTX 2001). After the electrical stimulus, the reconstructed embryos were cultured in modified KSOM (Biggers *et al.*, 2000) supplemented with 0.8% BSA (KSOM–BSA) at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for reprogramming. At 4 h post fusion, activation was induced by incubation of reconstructed embryos in hTCM containing 5 μM ionomycin (Sigma) for 5 min. Embryos were then extensively washed in ionomycin-free hTCM and cultured in 1.9 mM 6-dimethylaminopurine (Sigma) in KSOM–BSA for 4 h for postactivation. During washing of reconstructed embryos, fusion rates were recorded under stereomicroscope at ×40 magnification. Fusion was confirmed by observing GFP expression in recipient cytoplasm under DIC microscopy equipped with FITC filter.

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In vitro culture (IVC)

A group of five to 10 fused oocytes were cultured in a 25 μl microdrops of KSOM supplemented with 0.01% PVA (KSOM–PVA) overlaid with mineral oil at 39 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 192 h. The KSOM–PVA was supplemented with different concentrations of fructose in different experiments. The embryos were rinsed three times in specific group of culture medium before being cultured finally to avoid presence of residue of BSA or FBS. Care was also taken to avoid cross contamination among the culture media by changing embryo handling pipette. The development of embryos was monitored at 48, 96, 144 and 192 h after activation. The expression of GFP in PrP–GFP transgenic embryos was determined under a FITC filter (Fig. 1*d*, *e*).

Evaluation of blastocyst quality

The quality of blastocysts was assessed by differential staining of the inner cell mass (ICM) and the trophectoderm (TE) according to the method described by Thouas *et al*. (2001) after modification (Bhuiyan *et al*., 2004a). Briefly, hatched blastocysts were used as such and non-hatched blastocysts were treated with 0.25% pronase (w/v, Sigma) for 5 min to remove the zonae pellucidae. After rinsing in hCR2aa medium, zona free blastocysts were stained with 0.01% (w/v) bisbenzimide for 1 h. After rinsing in hCR2aa medium, the blastocysts were treated with 0.04% (v/v) Triton X-100 (Sigma) for 3 min followed by treatment with 0.005% (w/v) propidium iodide (Sigma) for 10 min. After rinsing in hCR2aa medium, stained blastocysts were mounted on glass slides under a coverslip and examined under an inverted microscope (Nikon Corp.) equipped with epifluorescence. The ICM nuclei labelled with bisbenzimide appeared blue and TE cell nuclei labelled with both bisbenzimide and propidium iodide appeared pink (Fig. 1*f*).

Experimental design

Experiment 1

To determine whether replacement of or combination of glucose with fructose in KSOM–PVA improved the *in vitro* development of bovine transgenic cloned embryos or not, fused oocytes were randomly distributed for culture in 0.2 mM glucose, 1.5 mM fructose, and combination of 0.2 mM glucose and 1.5 mM fructose. Addition of neither glucose nor fructose in KSOM–PVA was used as control medium. The concentration (0.2 mM) of glucose was selected for using similar concentration of glucose in KSOM formulation (Biggers *et al*., 2000) and 1.5 mM concentration of fructose supplementation was selected due to its stimulatory effect on cloned embryo development over other concentrations (Kwun *et al*., 2003). Transgenic embryos were reconstructed using *PrP–GFP* genes transfected into cultured BFF.

Experiment 2

To determine the optimum concentration of fructose supplementation in combination with 0.2 mM glucose in KSOM–PVA, fused oocytes were randomly distributed for culture in 0.0, 0.2, 1.5, 3.0 and 5.6 mM fructose supplemented media and *in vitro* development of transgenic embryos to blastocysts and their qualities were evaluated. Transgenic embryos were reconstructed using *PrP–GFP* genes transfected into BFF.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) and protected least significant different (LSD) test using general linear models (PROC-GLM) in a statistical analysis system (SAS) program to determine differences among experimental groups. When a significant treatment effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was considered where the p value was less than 0.05.

Results

Experiment 1

The effect of fructose (1.5 mM) supplementation in protein-free KSOM (KSOM–PVA) in presence or absence of glucose (0.2 mM) on *in vitro* development of PrP–GFP transgenic cloned embryos is shown in Table 1. The fusion rate was 74.8% (525/702). There were no significant differences in rates of cleavage (76.2 to 79.1%), 8–16-cell (45.4 to 49.6%) and morula (19.3 to 23.8%) formation among the culture groups. Moreover, the differences in rates of GFP expressions in blastocysts among the culture groups were not significant (68.0 to 78.6%). However, there were significantly ($p < 0.05$) higher rates of blastocyst (19.2%) and hatching/hatched blastocyst (13.1%) formation when fructose was supplemented in presence of glucose than only glucose supplementation (10.0% and 5.7%, respectively).

Experiment 2

The effect of fructose supplementation at different concentrations in KSOM–PVA in presence of glucose (0.2 mM) on *in vitro* development of PrP–GFP transgenic cloned embryos is shown in Table 2. The rates of cleavage (75.3 to 82.8%), 8–16-cell (47.6 to 53.8%), morula (18.6 to 29.0%) and hatching/hatched blastocyst (8.3 to 11.0%) formation, and GFP expression

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Table 1 Effect of supplementation of fructose in presence or absence of glucose in protein-free KSOM on *in vitro* development of bovine transgenic cloned embryos[a]

			No. (%) of embryos					
Glucose	Fructose	No. of fused oocytes cultured (no. of replicates)	Cleaved	8–16-cell	Morula	Blastocyst	Hatching/hatched blastocyst	GFP-expressing blastocyst[b]
–	–	126 (11)	99 (78.6)	59 (46.8)	27 (21.4)	15 (11.9)[c,d]	9 (7.1)[c,d]	11 (73.3)
+	–	140 (11)	107 (76.4)	65 (46.4)	27 (19.3)	14 (10.0)[c]	8 (5.7)[c]	11 (78.6)
–	+	129 (12)	102 (79.1)	64 (49.6)	28 (21.7)	17 (13.2)[c,d]	8 (6.2)[c]	13 (76.5)
+	+	130 (12)	99 (76.2)	59 (45.4)	31 (23.8)	25 (19.2)[d]	17 (13.1)[d]	17 (68.0)

[a]Fusion rate was 74.8 (525/702). [b]Percentage of the number of blastocysts expressing GFP. [c,d]Values with superscripts within same column differed significantly ($p < 0.05$).

Table 2 Effect of different concentrations of fructose supplementation in presence of glucose in protein-free KSOM on *in vitro* development of bovine transgenic cloned embryos

		No. (%) of embryos developed to					
Concentration (mM) of fructose (no. of replicates)	No. of fused oocytes cultured	Cleaved	8–16-cell	Morula	Blastocyst	Hatching/hatched blastocyst	GFP-expressing blastocyst[c]
0.0 (17)	145	120 (82.8)	69 (47.6)	27 (18.6)	14 (9.7)[a]	12 (8.3)	10 (71.4)
0.2 (17)	141	116 (82.3)	72 (51.1)	31 (22.0)	21 (14.9)[a,b]	13 (9.2)	15 (71.4)
1.5 (18)	142	107 (75.3)	69 (48.6)	41 (28.9)	25 (17.6)[b]	14 (9.9)	16 (64.0)
3.0 (18)	145	112 (77.2)	78 (53.8)	42 (29.0)	19 (13.1)[a,b]	16 (11.0)	12 (63.2)
5.6 (18)	144	116 (80.6)	73 (50.7)	36 (25.0)	19 (13.2)[a,b]	14 (9.7)	16 (84.2)

Fructose 0.0 mM (containing 0.2 mM glucose) was used as control. Fusion rate was 66.5% (572/860). [a,b]Values within same column differed significantly.[c]Percentage of the number of blastocysts expressing GFP.

Table 3 Effect of different concentrations of fructose supplementation in presence of glucose in protein-free KSOM on number of total cells, ICM and TE in transgenic cloned blastocysts

		No. of cells (mean ± SD)			
Concentration (mM) of fructose	No. of blastocysts examined	ICM	TE	Total	ICM: Total (%)
0.0	13	39.0 ± 14.9	69.2 ± 13.4	109 ± 17.3	35.6 ± 10.7
0.2	13	42.4 ± 15.8	72.2 ± 19.5	110.4 ± 29.7	28.0 ± 17.5
1.5	13	36.8 ± 11.3	67.9 ± 10.0	105.0 ± 11.6	34.9 ± 8.4
3.0	16	39.0 ± 12.7	73.0 ± 16.9	111.2 ± 23.9	34.6 ± 7.0
5.6	13	40.3 ± 19.7	64.0 ± 15.6	103.2 ± 26.9	37.4 ± 8.7

Fructose 0.0 mM (containing 0.2 mM glucose) was used as control. Values on different cell parameters did not differ significantly.

in blastocyst (63.2 to 84.2%) did not differ significantly among the concentrations of fructose supplementations. However, the rate of blastocyst formation was significantly higher (17.6%) in 1.5 mM fructose supplemented group than that of 0.0 mM fructose supplemented counter part (9.7%; $p < 0.05$). The number of ICM (36.8 to 42.4), TE (64.4 to 73.0) and total cells (103.2 to 111.2), and percentage of ICM to total cells (28.0 to 37.4%) in blastocysts derived from transgenic embryos did not differ significantly among the concentrations of fructose supplementations (Table 3).

Discussion

The aim of the present study was to investigate whether addition of fructose as energy substrate in medium improved the developmental rate and quality of transgenic SCNT embryos or not? To avoid variation in results due to presence of undefined (serum) or semi-defined (BSA) protein in KSOM, we used chemically defined protein-free KSOM as basic medium for embryo culture. Moreover, in our previous study, although we demonstrated numerically higher

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blastocyst formation rate in bovine transgenic SCNT embryos cultured in protein supplemented KSOM than that in protein-free counterpart, the difference in embryo development was not significant (Bhuiyan *et al*., 2004a)

The present study clearly demonstrated that combined supplementation of fructose with glucose in protein-free KSOM–PVA significantly ($p < 0.05$) improved the blastocyst and hatching/hatched blastocyst formation rates in bovine PrP mutant transgenic cloned embryos (Experiment 1; Table 1). Although no additional experiment was carried out to specify the metabolism of exogenous energy substrates by the embryos in the present study, it is obvious that fructose in combination with glucose may be a good energy supplementation for *in vitro* development of bovine transgenic NT embryos. This can be explained by the fact that bovine transgenic NT embryos may utilize fructose without altering the activity of rate-limiting enzymes for glycolysis (isomerase or phosphofructokinase). Glucose oxidation is known as glycolysis. During glycolysis glucose is converted into fructose-1,6-biphosphate (F1, 6BP) followed by degradation into lactate with the production of energy and NADH. However, fructose can enter the glycolytic pathway directly after conversion into F1,6BP with less energy requirement. Moreover, fructose is present in reproductive tract of cattle (Suga & Masaki, 1973) and expression of glucose transporter-5 (*Glut5*) gene in 8–16-cell bovine embryos emphasizes the embryotrophic role of fructose (Augustin *et al*., 2001). The *Glut5* gene has a higher affinity for fructose than glucose. It is localized in the apical brush border membranes of the small intestine but has also been found in kidney, muscle, brain and adipose tissue of human (Davidson *et al*., 1992).

On the basis of the finding in Experiment 1, fructose supplementations at 0.2 to 5.6 mM concentrations in combination with glucose in protein-free KSOM–PVA were tested to determine the optimum concentration for *in vitro* development of PrP mutant transgenic cloned embryos. The present study demonstrated that supplementation of fructose at 1.5 mM concentration with glucose numerically improved the *in vitro* development of PrP mutant transgenic cloned embryos (Experiment 2; Table 2). Contrasting to the present finding in transgenic cloned embryos, earlier study did not observe any improvement in embryo development after combined supplementation of fructose and glucose in semi-defined protein containing mSOF in bovine non-transgenic cloned embryos (Kwun *et al*., 2003). This study also demonstrated that replacement of glucose with fructose in KSOM–PVA did not improve the development of transgenic cloned embryos. Similarly, earlier study did not observe any difference in embryo development after replacing glucose with fructose in chemically defined culture media in hamsters (Ludwig *et al*., 2001). In contrast, Kwun *et al*. (2003) obtained significantly higher blastocyst formation rate in bovine non-transgenic cloned embryos cultured in 1.5 mM fructose supplemented semi-defined protein containing mSOF than that in 0.75 mM fructose or non-supplemented control. This variation in embryo development among studies may be due to differences in basal embryo culture media (mSOF versus KSOM), nature of the media (semi-defined protein-containing versus chemically defined protein-free media (Vanroose *et al*., 2001), concentration of glucose (1.5 versus 0.2 mM) and types of donor cells (non-transfected ear fibroblast versus transfected fetal fibroblast) used.

Since combined fructose and glucose supplementation improved the blastocyst formation rate in the present study, one may raise question whether this positive effect is specific to transgenic NT embryos or not? However, in the present study, although the effect of fructose supplementation was not investigated on non-transgenic NT embryos simultaneously, fructose supplementation in protein-free KSOM did not improve the IVF embryo development rate in another investigation (Bhuiyan *et al*., 2007). This may be due to differences in origin of embryos (Chung *et al*., 2002). Nevertheless, unlike other studies, the present investigation used chemically defined protein-free medium as it is essential to use this condition to determine the specific requirement of culture components by preimplantation embryos (Rosenkrans *et al*., 1993).

To determine whether supplementation of fructose affected the quality of blastocyst or not, cell numbers in blastocysts were determined by differential staining technique in Experiment 2. The quality of blastocysts derived from transgenic embryos with respect to cell numbers were within normal range for *in vivo*-derived blastocysts (Koo *et al*., 2002). However, supplementation of fructose in combination with glucose did not affect the cell numbers in transgenic blastocysts at any concentrations tested (Table 3). Contrasting to the present finding, Kwun *et al*. (2003) obtained higher number of ICM cells in blastocyst derived from combined fructose and glucose supplemented medium. This may be due to variations in nature of embryos (transgenic versus non-transgenic) and culture medium (semidefined versus defined and mSOF versus KSOM).

Although the supplementation of fructose in medium did not clearly improve the developmental competences of embryos, the present study indicates that supplementation of fructose up to 5.6 mM concentration in protein-free KSOM in presence of glucose (0.2 mM) has no detrimental effect on *in vitro* development of transgenic SCNT embryos. Similarly,

replacement of glucose with fructose in culture media at high concentrations (around 5 mM) did not affect the embryo development in earlier studies in hamster (Ludwig *et al*., 2001) and bovine (Kwun *et al*., 2003). In contrast to the effect of fructose supplementation, glucose supplementation in medium at high (5.0 to 5.6 mM) concentration significantly inhibited the embryo development in hamster (Ludwig *et al*., 2001) and bovine (Kwun *et al*., 2003; Kim *et al*., 1993).

One may raise question about the average lower blastocyst formation rate in the present study with comparison to other researches using SCNT embryos reconstructed with non-transfected cells. This can be explained by the fact that in the present study, we used transfected cells for reconstruction of SCNT embryos resulting in average lower blastocyst formation rate than that of non-transfected counterparts which has already been documented elsewhere (Zakhartchenko *et al*., 2001; Arat *et al*., 2002). Similarly, in an earlier study, when we compared the developmental rate of embryo derived from either transfected or non-transfected cells, embryos reconstructed with transfected donor cells developed to blastocyst at significantly ($p < 0.05$) lower rate than that of non-transfected counterpart (Bhuiyan *et al*., 2004b). This indicates the negative effect of transfection on embryo development *in vitro*. Moreover, low rate of blastocyst formation in the present study may be due to using protein-free and cell co-culture-free medium for embryo culture. In contrast to the present study, most of the transgenic and non-transgenic SCNT embryos have been produced after culturing in media containing protein or cell co-culture. Obtaining lower blastocyst formation rate in embryos cultured in protein free medium than that of protein supplemented counterpart has already been demonstrated elsewhere (Wrenzycki *et al*., 1999). Nevertheless, the present study has demonstrated that chemically defined protein-free KSOM without cell co-culture is enough to support the *in vitro* development of PrP mutant cloned embryos.

In conclusion, fructose up to 5.6 mM concentration can be used as an alternative for energy substrate in culture media without any detrimental effect on *in vitro* development of bovine transgenic cloned embryos. Moreover, 1.5 mM fructose in combination with 0.2 mM glucose can be supplemented in chemically defined protein-free KSOM–PVA to improve the *in vitro* development of bovine PrP mutant transgenic cloned embryos. This study will lead to possible production of viable mad cow disease resistant cloned cattle using chemically defined medium avoiding variations among laboratories and chances of disease transmission. Further studies are needed to determine whether the positive effect of fructose supplementation in medium correlates with *in vivo* embryo development or not.

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