Influence of Intergeneric/Interspecies Mitochondrial Injection; Parthenogenetic Development of Bovine Oocytes after Injection of Mitochondria Derived from Somatic Cells

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Abstract. Interspecies/intergeneric mitochondrial heteroplasmy can occur in interspecies/intergeneric hybrid embryos or following nuclear transfer. In the present study, intergeneric buffalo (*Bubalus bubalis*) mitochondria (WB-mt) or interspecies murine (*Mus spretus*) mitochondria (M-mt) were injected into bovine (*Bos taurus*) oocytes, and the subsequent embryonic development was characterized. Fibroblast mitochondria (WB-mt or M-mt) were microinjected into *in vitro* matured bovine oocytes followed by oocyte activation by a combination of electrical stimulation and 6-dimethylaminopurine treatment. After seven days of culture, embryo development was evaluated. The copy number of specific mtDNA populations (introduced and native mtDNA) from heteroplasmic oocytes was estimated using real-time PCR. The results illustrated that oocytes injected with either WB-mt or M-mt can develop to the blastocyst stage (20.6% and 19.6%). Cleavage division rates and development to the morula stage in oocytes injected with WB-mt were lower (76.2% and 45.9%, respectively) in comparison with uninjected oocytes (89.2% and 59.1%, respectively) ($P < 0.05$). However, no differences were found in comparing M-mt injected oocytes and controls ($P > 0.05$). An increase in bovine mtDNA copy number was observed at the expanded blastocyst stage of injected embryos ($P < 0.01$), while the number of injected mtDNA was stable throughout development. This study demonstrates that interspecies/intergeneric mitochondrial injected bovine oocytes have the ability to develop to the blastocyst stage after parthenogenetic activation and that injected mtDNA was neither selectively destroyed nor enhanced through development. Moreover, injected intergeneric mitochondria had a demonstrated influence on bovine parthenogenetic development and mtDNA replication.

Key words: Bovine oocyte, Mitochondria, Mouse, mtDNA, Water buffalo

(J. Reprod. Dev. 58: 323–329, 2012)

Somatic cell nuclear transfer (SCNT) offers the possibility of preserving endangered species, and SCNT has been applied to many mammalian species [1]. However, owing to the limited availability of oocytes from wild animals, the cloning of endangered species benefits from the use of recipient oocytes from a related domestic species. However, this methodology results in the production of nuclear-cytoplasmic hybrids [2, 3]. Despite numerous attempts in a wide variety of species, the number of live births of cloned offspring is still limited in instances that combine genetic compartments of closely related species [4, 5]. The generation of animals through interspecies/intergeneric SCNT (iSCNT) poses several problems including mitochondrial/genomic DNA incompatibility, embryonic

genome activation of the donor nucleus by the recipient oocyte, and availability of suitable foster mothers for iSCNT embryos. The species-specific nature of mitochondrial biogenesis and function makes this modeling particularly significant for iSCNT.

F1 hybrids and iSCNT live offspring obtained using river buffalo (*Bubalus bubalis bubalis*, $2n = 50$) and swamp buffalo (*Bubalus bubalis carabanesis*, $2n = 48$) have been reported [6, 7]. Although 25.9% of hybrid embryos in bovine (*Bos taurus*, $2n = 60$) oocytes exposed to water buffalo (*Bubalus bubalis*) sperm reached the blastocyst stage [8], attempts failed to obtain hybrid offspring [9, 10] with the exception of one rare case [11]. In a similar vein, buffalo-bovine iSCNT embryos demonstrated development to the blastocyst stage [12–14]; however, neither pregnancy nor natural delivery was reported.

In the process of SCNT, the transfer of donor cell mitochondria with nuclei into recipient oocytes resulted in mitochondrial heteroplasmy. Moreover, mitochondrial DNA (mtDNA) accompanying the donor cell was transmitted to offspring at varying but low

Received: October 21, 2011

Accepted: January 11, 2012

Published online in J-STAGE: March 9, 2012

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abundance in comparison with recipient oocyte mtDNA [15–17]. The donor cell mtDNA copy numbers in buffalo-bovine iSCNT embryos were constant throughout the iSCNT process until arrest at the 8- to 16-cell stage [18]. Using other genetic combinations in creation of iSCNT embryos, varying levels of mtDNA heteroplasmy were observed [19–22]. In addition to heteroplasmy identification, mtDNA homoplasmy of recipient oocyte mtDNA was also found in a host of analyzed iSCNT offspring [23–27]. In these instances, the host oocyte nuclear backgrounds did not interfere with subsequent oocyte mtDNA replication.

During normal fertilization, the oocyte contributes all mitochondria to the developing embryo, while the sperm mitochondria are destroyed shortly after fertilization [28]. Maternal inheritance of mtDNA in mammals is a developmental paradox because the fertilizing spermatozoon introduces up to 100 functional mitochondria into the oocyte cytoplasm at fertilization. Destruction of sperm mitochondria appears to be an evolutionary and developmental advantage because the paternal mitochondria and their DNA may be compromised by the deleterious action of reactive oxygen species (ROS) encountered by sperm during spermatogenesis, storage, migration, and fertilization [29, 30]. The ability to introduce exogenous mitochondria into mouse ova was initially used to study mitochondrial dynamics and mitochondrial heteroplasmy *in vivo* [31]. These models can be used to evaluate the biological significance and consequences of specific mitochondrial modifications and in various therapeutic modalities. Therefore, this modeling approach was employed to demonstrate that transferred somatic mitochondria dramatically influence parthenogenetic development of mammalian oocytes [32, 33].

To study the influence of intergeneric/interspecies mtDNA (i-mtDNA) transferred into oocytes, we investigated the developmental ability and mtDNA copy numbers during bovine (*Bos taurus*) parthenogenetic development using microinjected intergeneric (*Bubalus bubalis*) or interspecies (*Mus spretus*) mitochondria derived from somatic cells.

Materials and Methods

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

Preparation of oocytes

In vitro matured bovine oocytes were prepared as described previously [18, 33]. In brief, bovine (*Bos taurus*; Holstein or Japanese Black) oocytes collected from slaughterhouse-derived ovaries were cultured *in vitro* for 24 h at 38.5 °C under a humidified atmosphere of 5% CO₂ in air in Medium 199 (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). After maturation in culture, oocytes were treated with 0.2% bovine testicular hyaluronidase (Type I-S, 359 units/mg) in M2 medium to dissociate cumulus cells.

Donor mitochondrial preparation, injection and oocyte activation

Primary cultured fibroblasts were established from biopsied ear epithelium from a 5-year-old male water buffalo (swamp type;

Bubalus bubalis) as previously described [34] and frozen using a commercially available freezing solution (CELLBANKER, Nippon Zenyaku Kogyo, Fukushima, Japan).

Inbred *Mus spretus* mice (SPRET/Ei) purchased from The Jackson Laboratory were used as mitochondrial donors. Primary cell cultures were established from ear epithelium as previously described [32].

Frozen-thawed buffalo or murine fibroblast cells were prepared at confluency in D-MEM (Gibco, Invitrogen) supplemented with 10% FBS and 1X antibiotic-antimycotic solution (100 units/ml penicillin G, 0.1 mg/ml streptomycin and 0.25 µg/ml amphotericin B) (Fig. 1A). Mitochondria were isolated from cells by differential centrifugation. Isolated mitochondria (water buffalo mitochondria (Fig. 1B), WB-mt, or murine mitochondria, M-mt) were microinjected into oocytes as previously described [32, 33]. Briefly, mitochondrial pellets were resuspended in respiration buffer (225 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5 mM KH₂PO₄, pH 7.2), and, approximately 1–2 µl of mitochondrial suspensions were injected into oocytes using a Piezo micromanipulator (Prime-Tech, Tsuchiura, Japan) (Fig. 1C–D).

To examine the success of mitochondrial microinjection, confluent cultures were incubated in DMEM + 10% FBS containing 200 nM MitoTracker™ Green FM (Molecular Probes, Eugene, OR, USA) and 10 µg/ml cytochalasin B for 20 min at 37 °C. Fluorescence-labeled mitochondria were detected after injections using fluorescence microscopy (TE300; Nikon, Tokyo, Japan) with a B-2A filter set (excitation 450–490 nm, bandpass; emission 520 nm, longpass) (Fig. 1D').

Surviving oocytes, 15–30 min after mitochondrial microinjection, were activated using a double DC pulse of 57 V/mm for 50 µsec in Zimmerman's mammalian cell fusion medium and cultured in Medium 199 containing 0.4 mg/ml 6DMAP and 10% FBS for 5 h. Oocytes were washed and cultured in IVD101 medium (Research Institute for Functional Peptides, Yamagata, Japan) [35] for 7 days at 38.5 °C under 5% O₂, 5% CO₂ and 90% N₂. Differences in developmental rates were calculated using Chi-squared analysis. The critical threshold value was derived by application of a Bonferroni correction. Differences were defined as P < 0.05.

Quantification of bovine mtDNA and buffalo mtDNA in bovine oocytes and embryos

Oocytes were randomly selected at various stages: after injection of mitochondria, electrical stimulation, chemical activation, and 7 days of culture. Post culture, oocytes were washed six times in PBS supplemented with 1% polyvinylpyrrolidone. Individual embryos were placed into 1.5 ml siliconized microcentrifuge tubes (Assist, Tokyo, Japan) containing 10 µl of sterile distilled water and frozen at –80 °C until DNA extraction. Total DNA was extracted from individual embryos using a QIAamp DNA Micro Kit (Qiagen, Mannheim, Germany) [36]. The species-specific quantification of bovine (BO) and water buffalo (WB) mtDNA was as previously described [18]. Briefly, quantification analysis was performed using a LightCycler system (Roche Diagnostics GmbH, Mannheim, Germany) and a FastStart DNA Master^{plus} SYBR Green 1 kit (Roche). Species-specific primers were designed from the cytochrome b (*Cyt b*) region of bovine and buffalo mitochondrial genomes [18, 33].

Fig. 1. Microinjection of buffalo mitochondria (WB-mt, B) obtained from buffalo cells (A) into bovine oocytes (C–D) and the parthenogenetic development. Fluorescence-labeled mitochondria were detected after injections using fluorescence microscopy (TE300) with a B-2A filter (D'). Some of the WB-mt-injected bovine oocytes could develop to the blastocyst stage (E).

Specific amplification was carried out with initial denaturation at 95 C for 7 min followed by 45 (WB) or 40 (BO) cycles at 95 C for 1 sec, 60 C (WB) or 55 C (BO) for 5 sec and 72 C for 13 sec. Each mtDNA sample was analyzed twice to obtain a mean mtDNA copy number. Differences in mtDNA copy number per embryo during development was analyzed by one-way ANOVA, and then differences in mtDNA copy numbers between treatments or at different developmental stages were compared using the Student's *t*-test with Bonferroni correction ($P < 0.05$).

Quantification of bovine mtDNA and murine mtDNA in bovine oocytes and embryos

The oocytes were randomly selected after injection of M-mt and 7 days of culture. Total DNA was extracted from individual embryos as described for WB-mt injected embryos. Species-specific real-time PCR was performed as previously described [33]. Briefly,

quantification analysis was performed using a LightCycler system and a FastStart DNA Master^{plus} SYBR Green 1 kit (Roche). Specific amplification was carried out with initial denaturation at 95 C for 7 min followed by 45 cycles of at 95 C for 1 sec, 61 C (M) or 55 C (BO) for 5 sec and 72 C for 13 sec. Each mtDNA sample was analyzed in duplicate to obtain an average number of mtDNA molecules. mtDNA copy numbers per embryo during development were analyzed by one-way ANOVA ($P < 0.05$).

Animal use

All procedures and experiments were performed according to protocols approved by the NILGS Laboratory Animal Experimental Guide and the Japanese Prime Minister's Office of Laboratory Animal Welfare (No. 6, 1980).

Table 1. Parthenogenetic development of bovine oocytes injected with water buffalo mitochondria (WB-mt)

Source of injection (No. of replicates)	Survival post activation ^a	Development (%)		
		Cleaved	Morula	Blastocyst
WB-mt (11)	281	214 (76.2) ^b	129 (45.9) ^b	58 (20.6)
Respiration buffer (11)	174	148 (85.1) ^c	88 (50.6) ^{bc}	46 (26.4)
Uninjected (12)	186	166 (89.2) ^c	110 (59.1) ^c	50 (26.9)

^a The total number of oocytes incubated after activation. ^{b,c} Within a parameter, values are significantly different ($P < 0.05$).

Table 2. Bovine and buffalo mtDNA copy number in bovine oocytes injected with water buffalo mitochondria (WB-mt)

Timing and stage of embryos for examination	No. of embryos analyzed	Average ($\pm$ SD) copy number of mtDNA		WB mtDNA %
		BO (10^5 copies)	WB (10^2 copies)	
After injection	26	8.4 ± 3.4 ^{ac}	5.8 ± 4.3	0.08 ^a
After pulse	10	5.1 ± 1.0 ^b	4.3 ± 2.2	0.09 ^a
After 6DMP	15	6.2 ± 0.8 ^c	4.3 ± 6.5	0.07 ^{ab}
Arrested at morula	20	8.9 ± 3.4 ^{ad}	4.0 ± 3.2	0.06 ^{ab}
Blastocyst	21	9.4 ± 3.3 ^{ad}	2.7 ± 2.4	0.03 ^b
Expanded blastocyst	19	10.8 ± 4.0 ^d	6.2 ± 6.3	0.06 ^{ab}

^{a,b,c,d} Within a parameter, values are significantly different ($P < 0.05$). After injection: after WB-mt injection. After pulse: after electric stimulation. After 6DMP: after chemical activation by 6DMP. BO: bovine. WB: water buffalo.

Results

Development of bovine oocytes microinjected with water buffalo mitochondria

To study influences of intergeneric exogenous mitochondria on parthenogenetic development of bovine oocytes, oocytes were microinjected with mitochondrial fractions derived from water buffalo ear propagated cells. Fluorescence-labeled mitochondria were observed in the oocytes after mitochondrial injection (Fig. 1D').

WB-mt were injected into bovine oocytes ($n=281$), which showed a lower rate of cleavage (76.2%) when compared with oocytes injected with buffer or uninjected controls (85.1% and 89.2%, respectively, $P < 0.05$, Table 1). Some WB-mt injected oocytes developed to the blastocyst stage (Fig. 1E). The developmental rate of blastocyst formation was not different between oocytes injected with WB-mt (20.6%) and oocytes injected with buffer or uninjected oocytes (26.4% and 26.9%, respectively, $P > 0.05$). However, the developmental rate of morula formation was lower in oocytes injected with WB-mt (45.9%) when compared with uninjected oocytes (59.1%, $P < 0.05$).

Quantification of bovine mtDNA and exogenous buffalo mtDNA in bovine oocytes

The bovine and buffalo mtDNA content in WB-mt injected oocytes was analyzed after mitochondrial injection, after electric stimulation, after chemical activation, and after 7 days of culture. The oocyte (BO) mtDNA copy numbers in WB-mt injected oocytes were significantly decreased after electric stimulation and then increased following 7 days of incubation ($P < 0.05$; Table 2). After electric stimulation and at the expanded blastocyst stage, the BO mtDNA copy number in WB-mt injected oocytes was lower than in uninjected controls ($P < 0.05$, Fig. 2). The copy numbers of bovine

Fig. 2. Bovine (BO) mtDNA copy number per bovine oocyte/embryo injected with water buffalo (WB) mitochondria. Oocytes/embryos injected with WB-mt or buffer or that were uninjected (after injection) were analyzed after electric stimulation (after pulse), chemical activation by 6DMP (after 6DMP) and 7 days of culture (arrested at the morula, blastocyst, expanded (Ex) blastocyst stage). ^{a,b} Superscripts represent differences among treatments, at each individual stage, analyzed by the Student's *t*-test ($P < 0.05$).

mtDNA in WB-mt injected blastocysts ($9.4 \pm 3.3 \times 10^5$) or expanded blastocysts ($10.8 \pm 4.0 \times 10^5$) were not different from those in oocytes arrested at the morula stage ($8.9 \pm 3.4 \times 10^5$, $P > 0.05$, Table 2). In contrast, exogenous (WB) mtDNA copy numbers in WB-mt injected oocytes were constant throughout development until the expanded blastocyst stage (Fig. 3).

Development of bovine oocytes microinjected with murine mitochondria

After M-mt injection into bovine oocytes ($n=102$), developmental rates were not different between oocytes injected with M-mt and oocytes injected with buffer or uninjected oocytes (Table 3).

Quantification of bovine mtDNA and exogenous murine mtDNA in bovine oocytes

Bovine and murine mtDNA content in oocytes injected with M-mt was $8.1 \pm 2.6 \times 10^5$ and $6.1 \pm 11.1 \times 10^2$, respectively (Table 4). There was no difference in bovine and murine mtDNA copy numbers in arrested morulae ($7.8 \pm 0.7 \times 10^5$ and $6.3 \pm 3.9 \times 10^2$, respectively) and blastocysts ($9.3 \pm 2.6 \times 10^5$ and $3.8 \pm 4.7 \times 10^2$, respectively) when compared with those of injected oocytes.

Discussion

Previously, injection of foreign (exogenous) mitochondria into pronuclear zygotes resulted in the production of heteroplasmic mice [31, 37, 38]. Mitochondrial injection models can be used to evaluate the biological significance and consequences of specific mitochondrial modifications. These models are important in our understanding of the complex interactions between intergeneric/interspecies exogenous mitochondria and recipient nuclei and mitochondria in early development. Previous reports demonstrated that exogenous mitochondria originating from donor cells directly affected murine parthenogenetic development [32, 33]. Similarly, this study found that intergeneric somatic mitochondria influenced

Fig. 3. Water buffalo (WB) mtDNA copy number per oocyte/embryo injected with water buffalo (WB) mitochondria. Oocytes/embryos were analyzed after WB-mt injection (after injection), electric stimulation (after pulse), chemical activation by 6DMP (after 6DMP) and after 7 days of culture (arrested at morula, blastocyst, expanded (Ex) blastocyst stage). Bars represent the average mtDNA copy number. MtDNA copy number per embryo showed no difference during development analyzed by one-way ANOVA ($P=0.11$).

the rate and success of bovine parthenogenetic development. A decreased developmental rate through the morula stage was shown in WB-mt injected oocytes, although no differences in developmental rate were found between M-mt injected oocytes. These results raise the possibility that the interspecies mitochondria also affected bovine parthenogenetic development.

Alteration of mtDNA copy numbers has been observed during bovine early development [15, 39]. Interestingly, the increase in

Table 3. Parthenogenetic development of bovine oocytes injected with murine mitochondria (M-mt)

Source for injection (No. of replicates)	Survival post activation ^a	Development (%)		
		Cleaved	Morula	Blastocyst
M-mt (5)	102	80 (78.4)	45 (44.1)	20 (19.6)
Respiration buffer (5)	94	77 (81.9)	52 (55.3)	23 (24.5)
Uninjected (3)	44	39 (88.6)	22 (50.0)	10 (22.7)

^a The total number of oocytes incubated after activation. There are no significant differences within a parameter ($P > 0.05$).

Table 4. Bovine and murine mtDNA copy number in bovine oocytes injected with murine mitochondria (M-mt)

Timing and stage of embryos for examination	No. of embryos analyzed	Average ($\pm$ SD) copy number of mtDNA		M mtDNA%
		BO (10^5 copies)	M (10^2 copies)	
After injection	11	8.1 ± 2.6	6.1 ± 11.1	0.08
Arrested at morula	10	7.8 ± 0.7	6.3 ± 3.9	0.08
Blastocyst	8	9.3 ± 2.6	3.8 ± 4.7	0.04

There are no significant differences within a parameter ($P > 0.05$). After injection: after M-mt injection. BO: bovine. M: murine.

copy number at the blastocyst stage was similar to that in bovine embryos derived from *in vitro* fertilization [15, 40]. The mtDNA copy number can provide one index for normal embryonic development; therefore, one could surmise that nuclear function was affected in these embryos. Additionally, our results illustrated that exogenous interspecies/intergeneric mtDNA injected into oocytes were not selectively destroyed; indeed, they were maintained throughout the early development. In iSCNT embryos, constant copy numbers of donor somatic cell mtDNA from the 1-cell to the 8-cell stage were also found in water buffalo–bovine [18], sheep–bovine [20], cat–bovine [21], goat–sheep [22], and macaque–rabbit [17] arrested embryos. Sperm mitochondria destruction is induced by a process related to the ubiquitin-proteasome pathway in fertilization [30, 41]. The dramatic reduction of mtDNA content argues in favor of active destruction rather than a reduced turnover of mtDNA molecules [40]. Moreover, paternal mtDNA was detected only through the early pronucleus stage in intraspecific hybrids of *Mus musculus*. In contrast, paternal mtDNA was detected throughout development, from the pronucleus stage through to neonates, in interspecific hybrids between *Mus musculus* and *Mus spretus* [42]. It is doubtful that exogenous intergeneric or somatic cell mitochondria would not be recognized by recipient cellular surveillance from mitophagy to the ubiquitin-proteasome pathway observed in sperm mitochondria. Interestingly, buffalo ooplasm transfer into bovine zygotes by fusion methodology introduced 8.3% of buffalo mtDNA and maintained a comparable ratio through to the blastocyst stage. However, no vestiges of buffalo mtDNA were found in subsequent offspring [43]. This would support an inability of buffalo mtDNA to establish a functional interaction with the bovine nucleus. In another report, donor embryonic cell mtDNA was markedly decreased during embryo development and eliminated in the majority of offspring derived from bovine–bovine embryonic nuclear transfer technology [44]. Sheep–goat iSCNT embryos also showed a loss of donor mtDNA during development [22]. From our results, injected WB-mtDNA copy numbers were stable, while the bovine mtDNA increased after the morula stage (Table 2). The i-mtDNA was not selectively destroyed following either WB-mt or M-mt injection. In one other study, gaur–bovine iSCNT embryos showed an increase in donor (gaur) mtDNA copy number at the blastocyst stage [45]. Indeed, the need to exclude defective exogenous mtDNA from the developing embryo does represent a major biological selective pressure against survival of exogenous mtDNA.

An attempt at iSCNT using gaur (*Bos gaurus*) donor cell and domestic bovine (*Bos taurus*) recipient oocytes produced several pregnancies and a single offspring [23]. In addition, reconstructed embryos resulting from iSCNT between water buffalo (*Bubalus bubalis*) and domestic bovine developed to the blastocyst stage [14] but did not result in production of term fetuses or offspring. As epigenetic reprogramming of the donor nucleus from its differentiated status to a totipotent state in the presence of interspecies ooplasm requires an elaborate interplay between the nucleus and the ooplasm [45]. This orchestrated interaction requires the involvement of many nuclear-encoded proteins with the displacement loop (D-loop) of the mtDNA genome to mediate replication and transcription efficiently [46], regulation of total mtDNA copy numbers [47] and supply of ATP for energy requiring activities

[48, 49]. The results of this study illustrate that characteristic and complex mitochondrial-nuclear interactions are affected during early parthenogenetic development.

In conclusion, intergeneric/interspecies mitochondria injected into bovine oocytes provide oocytes the ability to develop to the blastocyst stage after parthenogenetic activation. Injected i-mtDNA was not selectively destroyed or increased throughout development. Moreover, injected intergeneric mitochondria influence bovine parthenogenetic development and mtDNA replication- illustrating an important consideration in developing these and related reproductive technologies.

Acknowledgments

We thank Prof Y Kanai (University of Tsukuba), Dr M Iwamoto (Prime Tech) and Dr Y Kameyama (Tokyo University of Agriculture) for their assistance and consideration. This work was supported by a grant from the National Agriculture and Food Research Organization of Japan, the Thailand Research Fund, Suranaree University of Technology and the Japan Society for the Promotion of Science – National Research Council of Thailand (JSPS-NRCT) scientific cooperation program.

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