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# Rabbit Oocyte Cytoplasm Supports Development of Nuclear Transfer Embryos Derived from the Somatic Cells of the Camel and Tibetan Antelope

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**Abstract.** This study was designed to examine the ability of rabbit metaphase II oocyte cytoplasm to support the development of interspecies nuclear transfer embryos reconstructed using donor nuclei from different species. Skin fibroblast cells from a camel and Tibetan antelope were used as donor nuclei. As a first step, we investigated the efficiency of different activation protocols by comparing the parthenogenetic development of rabbit oocytes. The protocol that yielded the highest blastocyst rate was used to activate the reconstructed embryos in nuclear transfer experiments. In addition, the effect of donor cell serum starvation on the development of the reconstructed embryo was also examined. More than half of the karyoplast–cytoplast couplets could be fused, and about one third of the reconstructed embryos were capable of completing first cleavage, regardless of the species of donor nuclei. Some of the cleaving reconstructed embryos were even capable of progressing further and developing to the blastocyst stage (1.4–8.7% for the Tibetan antelope and 0–7.5% for the camel, respectively). Our results suggest that the mechanisms regulating early embryo development may be conserved among mammalian species and some factors existing in rabbit oocyte cytoplasm for somatic nucleus reprogramming and dedifferentiation may not be species–specific. Rabbit oocyte cytoplasm can reprogram donor nuclei regardless of the origin of the nucleus and support in vitro development to an advanced stage.
**Key words:** Camel, Interspecies, Nuclear transfer, Tibetan antelope

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During the past several years, great achievements have been made in mammal clonging. Successful cloning by somatic nuclear transfer has been achieved in the sheep [1], mouse [2], bovine [3, 4], pig [5, 6], cat [7], rabbit [8], rat [9], mule [10], horse [11], and dog [12]. At the same time, interspecies somatic cell cloned gaur and mouflon [13, 14] have been obtained. Interspecies nuclear transfer, which involves transferring a donor cell from one species into a recipient oocyte of another species, is an invaluable tool for studying nucleus-cytoplasm interaction and may be an effective way to conserve endangered species whose oocytes are extremely difficult and even impossible to obtain. Moreover, when interspecies nuclear transfer is used for therapeutic research, ethical, legal, and experimental limitations encountered in clinical situations may be avoided. One of the problems of interspecies nuclear transfer is the unavailability of species-specific competent recipient cytoplasm. In particular, it is extremely difficult to get oocytes for

endangered species. This problem may be overcome if a type of well-understood and easy-to-obtain oocyte can be used successfully. Development of such a common model will also greatly benefit ongoing research efforts. Oocytes used for interspecies nuclear transfer should be easy to obtain, able to dedifferentiate the somatic cell nuclei of other species, and support development of the reconstructed embryo. In previous research, the oocytes of bovine, sheep, and rabbits have been used for interspecies nuclear transfer [15–18]. One of the first attempts at interspecies nuclear transfer using enucleated bovine oocytes as the recipient cytoplast was reported by Dominko *et al.* in 1999. Monkey, sheep, pig, and rat somatic cells were used as donor karyoplasts, resulting in various degrees of early in vitro development; however, no pregnancies were reported. Other nuclear transfer attempts have been conducted using bovine oocytes as the recipient and karyoplast from other species, including the pig [19], saola [20], eland [21], horse [22], bear [23], banteng [24], mountain bongo antelope [25], chicken [26], yak and dog [27], and human [28].

Sheep oocytes have also been used as recipients for interspecies nuclear transfer. In previous research, two pregnancies were established after interspecies nuclear transfer using domestic sheep cytoplast (*ovis aries*) as the recipient cytoplast and an exotic argali (*ovis ammon*) as the donor karyoplast, but both pregnancies were lost by day 59 of gestation [16]. In addition, another study using domestic sheep (*ovis aries*) oocytes as recipients and the mural granulose cells of a dead mouflon (*ovis orientalis musimon*) as donor nuclei resulted in one live offspring [14].

Owing to their small size, short reproductive life span, and easy manipulation and inducement of ovulation, rabbits have been one of the most popular animal models used for scientific research. Compared with the oocytes of the bovine and sheep, the rabbit oocyte offers greater advantages. The rabbit oocyte is easy to obtain, and as many as 30–40 oocytes can be obtained from one superovulated female rabbit. The rabbit oocyte has also been proven to be an ideal model for many types of studies due to its large size, elasticity, and easy handling. Furthermore, its cytoplasm is more transparent than that of most domestic animals, such as pig, cattle, and sheep [8, 29–33]. Our

laboratory is very intersested in interspecies nuclear transfer. Our previous studies have shown that rabbit oocyte cytoplasm is capable of dedifferentiating somatic cell nuclei from the giant panda, chicken, monkey, cat, and human and is capable of supporting the development of these interspecies nuclear transfer embryos to blastocysts, indicating that the rabbit oocyte is an ideal model for interspecies nuclear transfer [18, 34–37]. In order to extend our research, we selected camel and Tibetan antelope somatic cells to be used as nuclear donors for the present study and examined the ability of rabbit oocyte cytoplasm to reprogram the differentiated somatic nuclei of these two species. Cell lines have been established in our laboratory for these two species and primary research has been conducted on them. Furthermore, we wished to accumulate useful inormation for subsequent cloning research. The efficiency of different activation protocols and the effect of serum starvation on development of the nuclear transfer embryos were also examined.

## Materials and Methods

### Animals

Animal care and handling were in accordance with the policies on the care and use of animals promulgated by the ethical committee of the Institute of Zoology, Chinese Academy of Sciences (Beijing, P.R. China). Female Japanese big-eared white rabbits (purchased from Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences) were housed in stainless steal cages. Regular rabbit fodder and water were provided *ad libitum.*

### Oocyte collection

Mature female rabbits were superovulated by administering PMSG and hCG (Institute of Zoology, Chinese Academy of Sciences). Each rabbit was injected with 150 IU PMSG, and then 100 IU hCG 4 days after the PMSG injection. The rabbits were euthanized 14–15h after hCG injection. Mature MII oocytes and cumulus complexes were collected by flushing the separated oviducts with M199 medium (Sigma-Aldrich Co., St. Louis, MO, USA). After exposure to 300 IU/ml hyaluronidase (Sigma) in M199 for 3–5 min, cumulus cells were stripped from oocytes by repeated gentle pipetting.
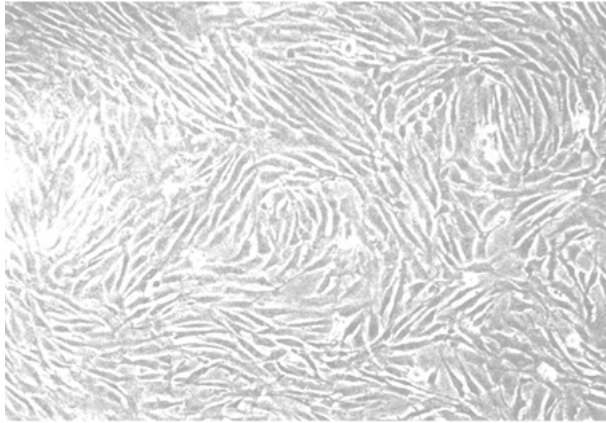
**Fig. 1.**   Monolayer of a camel somatic cell.

Cumulus-free oocytes were then subjected to parthenogenetic activation or nuclear transfer experiments as described below.

*Culture of donor cells*

The cell culture and assessment procedures have been described previously. Briefly, ear skin samples were obtained by biopsy from a 4-year-old camel and a 10-month-old Tibetan antelope. Tissues were manually cut into small pieces measuring about 1 mm$^2$ and digested with 0.25% (w/v) trypsin (Gibco BRL, Grand Island, NY, USA) for 12 h at 4 C. After that, they were digested for 30 min at 37 C, and then the digested cells and tissues were seeded into a 75-cm$^3$ cell culture flask containing Dulbecco's Modified Eagles, Medium/F12 (DMEM/F-12; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) and cultured in a 5% CO$_2$ incubator at 37 C. After attaining a 75–85% confluent monolayer, the primary cells were disaggregated for further culture (Fig. 1). Cells at passages 4–10 were used as donors. The serum concentration was decreased to 0.5% to starve the donor cells for 3–5 days prior to nuclear transfer. Both actively dividing cells and serum-starved cells were used as donor cells. Rabbit fibroblast cells were used as the control.

*Karyotype analysis of Tibetan antelope somatic cells*

There are no reports in the literatures concerning the karyotype of Tibetan antelope somatic cells. Therefore, the karyotype of Tibetan antelope somatic cells was analyzed before the nuclear transfer procedure. Briefly, cells at 80% confluency were treated with 0.3 $\mu$g/ml demecolcine and then exposed to KCl (0.075 M) for 20 min at 37 C. The cells were then fixed in methanol:acetic acid [3:1 (V:V)] and drops of cells suspension were spread on clean and pre-cooled microscpe slides. The air-or fire-dried slides were then stained with 5% Giemsa's solution for 10–15 min. The numer of chromosomes was counted under a light microscope at 1,000 × magnification.

*MII oocyte activation*

Cumulus-free MII oocytes (16–17 h after HCG injection) were randomly assigned to one of the following treatments:

(1) two sets of double DC pulses: electroporation by double DC pulses of 1.4 kv/cm for 80 $\mu$s spaced 1 sec apart in fusion medium, followed by another double DC pulse of 1.2 kv/cm for 20 $\mu$s, 30 min apart;

(2) electroporation by double DC pulses of 1.4 kv/cm for 80 $\mu$s spaced 1 sec apart in fusion medium, followed 30 min later by another double DC pulse of 1.2 kv/cm for 20 $\mu$s, 1 sec apart, and then incubation in 2.0 mM 6-DMAP for 3 h;

(3) Incubation for 7 min with 7% ethanol, followed by incubation with 2.0 mM 6-DMAP for 3 h;

(4) Incubation for 4 min with 5 $\mu$M ionomycin, followed by incubation with 2.0 mM 6-DMAP for 3 h.

The fusion medium consisted of 0.25 M sorbitol, 0.5 mM magnesium acetate, 0.1 mM calcium acetate, 0.5 mM HEPES, and 1 mg/ml BSA.

*In vitro culture of activated oocytes*

Following activation treatment, the oocytes were washed several times and cultured in 100 $\mu$l drops of M199+10% FCS. All embryos were cultured for 6 days in a humidified atmosphere of 5% CO$_2$ in air at 38 C. On Day 1 and Day 6 (Day 0=day of activation), cleavage and development to the blastocyst stage were determined and recorded, respectively.

*Nuclear transfer procedure*

Nuclear transfer was conducted as described previously [17, 18]. Cumulus-free oocytes were incubated for 15 min in M199+10% FCS containing 7.5 $\mu$g/ml cytochalasin B (CB; Sigma) and 10 $\mu$g/ml Hoechst 33342 (Sigma) before enucleation. For enucleation, the first polar body and a small

**Table 1.** Effect of different activation protocols on parthenogenetic development of rabbit oocytes

| Activation | Culture medium | Number of oocytes | Number (%) cleaved | Number (%) blastocyst |
|---|---|---|---|---|
| Pulse+6–DMAP | M199+10% FCS | 87 | 68 (78.2)[a] | 43 (49.4)[a] |
| Electrical pulses | M199+10% FCS | 89 | 50 (56.2)[b] | 26 (29.2)[b] |
| Ionomycin+6–DMAP | M199+10% FCS | 80 | 43 (53.8)[b] | 7 (8.8)[c] |
| Ethanol+6–DMAP | M199+10% FCS | 86 | 32 (37.2)[c] | 14 (16.3)[c] |

Values with different superscripts within each column are significantly different (P<0.05).

amount of the adjacent cytoplasm containing the meiotic spindle were aspirated using a 20–25 $\mu$m glass pipette. The aspirated karyoplast was exposed to UV light and examined to confirm the presence of the removed polar body and metaphase II chromosome. Only oocytes from which all chromosomes were removed were used for nuclear transfer. A single donor cell was selected and transferred into the perivitelline space of an enucleated oocyte in close contact with the plasma membrane of the enucleated oocyte. The karyoplast–cytoplast couplets were transferred to a fusion chamber containing 100 $\mu$l fusion medium. Fusion was induced by double 80 $\mu$s DC pulses of 1.4 kv/cm with an Electro Cell Manipulator (ECM2001 Electro Cell Manipulator, BTX, Inc., San Diego, CA, USA). Couplets were then washed in M199+10% FCS several times and incubated in the same medium for 30 min at 38 C in a humidified air containing 5% $CO_2$. They were then checked for fusion with an inverted microscope. Nonfused pairs were induced to fusion again.

*Activation and in vitro culture of reconstructed embryos*

Fused couplets were activated by double DC pulses of 1.2 kv/cm for 20 $\mu$s spaced 1 sec apart and then incubated in M199+10% FCS containing 2 mM 6-DMAP for 3 h. After activation, the nuclear transfer units were washed and placed into drops of three types of culture media, M199+10% FCS, SOF, and mCR1aa. The media were composed of the following: M199+10% FCS, M199 supplemented with 10% FBS; SOF, SOF medium supplemented with 1% minimal essential medium (MEM) nonessential amino acids, 2% essential amino acids (both Gibco BRL), and 10% FBS; and mCR1aa, mCR1aa medium plus 10% FBS. The embryos were examined 24 h after activation for initial cleavage and monitored every 24 h for

progression of development through Day 6.

*Chromosome analysis*

Reconstructed embryos were assessed for karyotype at the blastocyst stage. Briefly, blastocysts were incubated in 5 $\mu$g/ml colchicines (Sigma) for 5–6 h to maximize the number of cells in metaphase. The embryos were then exposed to a hypotonic solution consisting of 0.075M KCl for 40 min, and their chromosomes were examined by the method described above.

*Statistical analysis*

The percentages of embryos at different stages were compared between groups using Chi-square analysis. Significant differences were determined at P<0.05.

**Results**

We investigated the parthenogenetic development of rabbit oocytes induced by various activation treatments. Oocytes were activated by various protocols and cultured in M199+10% FCS culture medium. Generally, five types of parthenogenetic oocytes were produced. The first type had one pronucleus plus the first polar body and second polar body and further cleavage that contributed to formation of a haploid embryo. The second type had two or more pronuclei plus the first polar body and cleavage that usually contributed to formation of a diploid embryo. The third type had the first and second polar bodies, but no subsequent cleavage. The fourth type was not activated and was arrested at the MII stage. The fifth type had cytoplasm fragmentation. About 60% of the ativated oocytes treated by electrical pulses alone had one pronulus plus the first and second polar bodies and the other 40% had two or

**Table 2.**  Effect of serum starvation treatment of camel donor cells on the development of camel-rabbit reconstructed embryos in M199+10% FCS

| Donor cell treatment | Number of NT | Number (%) fused | Number (%) cleaved | Number (%) blastocyst |
|---|---|---|---|---|
| Serum–starved | 223 | 143 (64.1 )[a] | 50 (35.0)[a] | 9  (6.3)[a] |
| Serum–fed | 421 | 253 (60.1)[a] | 82 (32.4)[a] | 17 (6.7)[a] |

Values with different superscripts within each column are significantly different (P<0.05).

**Table 3.**  Effect of serum starvation treatment of Tibetan antelope donor cells on the development of Tibetan antelope-rabbit reconstructed embryos in M199+10% FCS

| Donor cell treatment | Number of NT | Number (%) fused | Number (%) cleaved | Number (%) blastocyst |
|---|---|---|---|---|
| Serum–starved | 121 | 85 (70.2 )[a] | 40 (47.1)[a] | 5  (5.9)[a] |
| Serum–fed | 155 | 79 (51.0)[b] | 32 (40.5)[a] | 6  (7.6)[a] |

Values with different superscripts within each column are significantly different (P<0.05).

more pronuclei plus the first polar body.  The activated oocytes in the three groups, Pulse+6-DMAP, Ionomycin+6-DMAP and Ethanol+6-DMAP, rarely extruded the second polar body, and most of the activated oocytes had two or more pronuclei plus the first polar body.  Cleavage of parthenotes was recorded at 24 h of culture, and development to the blastocyst stage was recorded at 6 days of culture.  These results are summarized in Table 1.  The cleavage and blastocyst rates of the Pulse+6-DMAP group (78.2% and 49.4%, respectively) were both significantly higher than those of the other groups.  Pulse+6-DMAP proved to be the most efficient activation protocol in this study.  The cleavage and blastocyst rates were significantly different between the multi-pulse and ethanol +6-DMAP groups.  Although the cleavage rates were comparable among the multi-pulse and ionomycin +6-DMAP groups, the blastocyst rates were significantly different.  The blastocyst rates were comparable between the Ionomycin+6-DMAP and Ethanol+6-DMAP groups, however, the cleavage rates differed significantly.

The effect of serum starvation treatment of donor cells on the development of camel-rabbit and Tibetan antelope-rabbit reconstructed embryos is summarized in Table 2 and Table 3, respectively. Although both the fusion and cleavage rates of the serum-starved groups were higher than those of the serum-fed groups, blastocyst development was lower in the serum-starved groups compared with those of the serum-fed groups for both the camel-
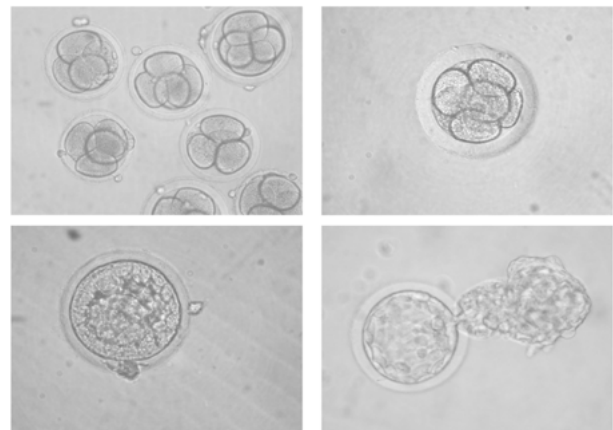


**Fig. 2.**  Representative *in vitro* development of reconstructed camel–rabbit embryos.

rabbit and Tibetan antelope-rabbit embryos.  Based on these results, serum-fed donor cells were used in subsequent experiments.

We also compared the developmental capacity of nuclear transfer embryos cultured in different culture media.  On average, more than half of the fibroblast-oocyte pairs could be fused for both camel-rabbit and Tibetan antelope-rabbit embryos. After 24 h culture, about one-third cleaved, and after culture for 6 days, some of the cleaving reconstructed embryos developed further to the blastocyst stage, regardless of culture medium type and donor cell species (Fig. 2).

The development of the Tibetan antelope-rabbit

**Table 4.**   Development of Tibetan antelope-rabbit embryos in different culture systems

| Culture media | Number of NT | Number (%) fused | Number (%) cleaved | Number (%) blastocyst |
|---|---|---|---|---|
| M199+10% FCS | 350 | 242 (69.1)[a] | 103 (42.6)[a] | 21  (8.7)[a] |
| SOF | 246 | 166 (67.5)[a] | 65 (39.2)[a] | 13 ( 7.8 )[a] |
| mCR1aa | 225 | 141 (62.7)[a] | 36 (25.5)[b] | 2  (1.4 )[b] |

Values with different superscripts within each column are significantly different (P<0.05).

**Table 5.**   Development of camel-rabbit embryos in different culture systems

| Culture media | Number of NT | Number (%) fused | Number (%) cleaved | Number (%) blastocyst |
|---|---|---|---|---|
| M199+10% FCS | 452 | 293 (64.8)[a] | 120  (41)[a] | 22  (7.5)[a] |
| SOF | 326 | 185 (56.7)[b] | 66  (35.7)[a] | 11  (6.0)[a] |
| mCR1aa | 207 | 142 (68.6)[a] | 29  (20.4)[b] | 0[b] |

Values with different superscripts within each column are significantly different (P<0.05).

**Table 6.**   Development of rabbit-rabbit embryos in different culture systems

| Culture media | Number (%) fused | Number (%) cleaved | Number (%) blastocyst |
|---|---|---|---|
| M199+10% FCS | 104 | 84 (80.8)[a] | 26 (25.0)[a] |
| SOF | 98 | 74 (75.5)[a] | 19 (19.4)[a] |
| mCR1aa | 93 | 61 (65.6)[b] | 5 (5.4)[b] |

Values with different superscripts within each column are significantly different (P<0.05).

embryos in different culture systems is presented in Table 4.  Although no significant differences were found in the cleavage and blastocyst rates when cultured in M199+10% FCS or SOF, slightly higher cleavage and blastocyst rates were obtained in M199+10% FCS.  The cleavage and blastocyst rates in M199+10% FCS and SOF were significantly higher than those in mCR1aa.

The development of the camel-rabbit embryos in different culture systems is presented in Table 5. The cleavage and blastocyst rates in M199+10% FCS and SOF were not significantly different, but they were significantly higher than those in mCR1aa.  No blastocysts were obtained when cultured in mCR1aa.  Slightly higher cleavage and blastocyst rates were obtained in M199+10% FCS than in SOF.

A total of 388 oocytes were used as a control, among which 295 were fused with rabbit fibroblast cells.  Reconstructed oocytes were activated and randomly assigned to three different culture systems.  The development of rabbit-rabbit embryos in different culture systems is presented in Table 6.  Although no significant differences were found in the cleavage and blastocyst rates when cultured in M199+10% FCS or SOF, slightly higher cleavage and blastocyst rates were obtained in M199+10% FCS.  The cleavage and blastocyst rates in M199+10% FCS and SOF were significantly higher than those in mCR1aa.

Karyotype analysis of Tibetan antelope somatic cells demonstrated that the karyotype of Tibetan antelope was 2n=60 (Fig. 3) Reconstructed blastocysts were randomly selected for chromosome examination.  The number of chromosomes in reconstructed blastocysts was 74 or 60, which was the same as the camel karyotype (2n=74) or Tibetan antelope karyotype (2n=60), respectively, but different from that of rabbit somatic cells (2n=44), indicating that the origin of the genetic material of the reconstructed embryos was the donor cells (Fig. 4 and Fig. 5).
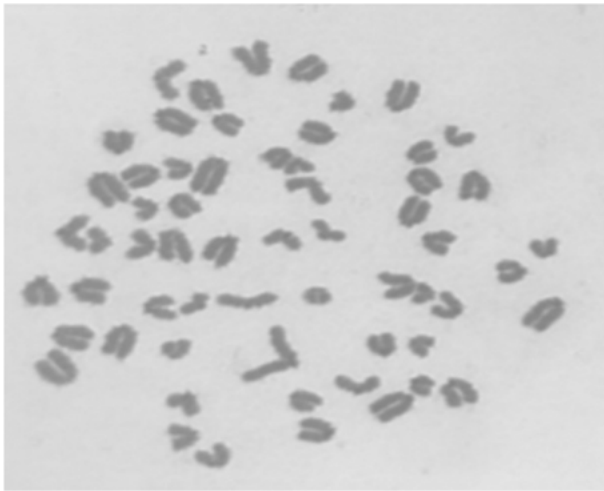
**Fig. 3.** Karyotype of the Tibetan antelope somatic cell (2n= 60).



**Fig. 4.** Karyotype of the camel-rabbit reconstructed blastocyst (2n=74).
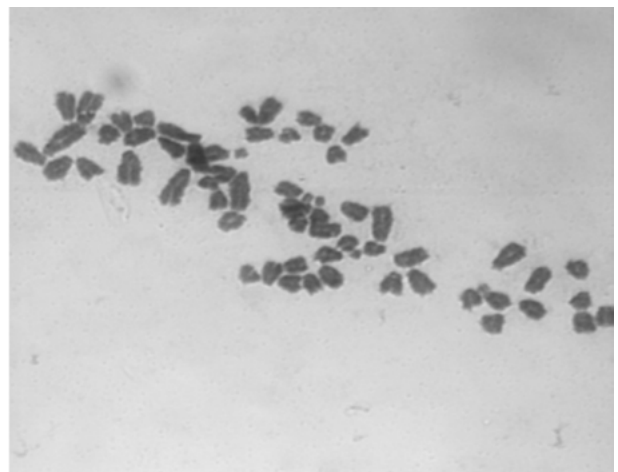


**Fig. 5.** Karyotype of the Tibetan antelope-rabbit reconstructed blastocyst (2n=60).

## Discussion

Oocyte activation is a critical step for improving the efficiency of nuclear transfer. In rabbits, Stice and Roble reported that the least efficient step of the nuclear transfer procedure was oocyte activation (46%) [39]. Activation of the rabbit oocyte has been induced by various types of stimuli, such as cooling [40], electrical pulses [41], and chemical reagents [8, 31, 32]. However, many agents that are used to induce activation of oocytes cause a monotonic rise in the level of free intracellular calcium and do not completely mimic sperm-induced intracellular calcium transients. A periodic intracellular increase in calcium during fertilization is critical for oocyte activation. In our study, the efficiency of different combined activation protocols was examined. The cleavage and blastocyst rates in the Pulse+6-DMAP group were significantly higher than those of the other groups. It is apparent that parthenogenetic activation of oocytes could be improved by a protocol that combines both electrical pulses and chemicals. Electrical pulses could induce obvious ionic calcium influx, and 6-DMAP, a kinase inhibitor, is assumed to promote pronuclear formation. Sequential treatment with pulses and 6-DMAP resulted in one diploid pronucleus without second polar body extrusion, ultimately producing diploid parthenotes. It is generally accepted that activated haploid oocytes are less competent than dipl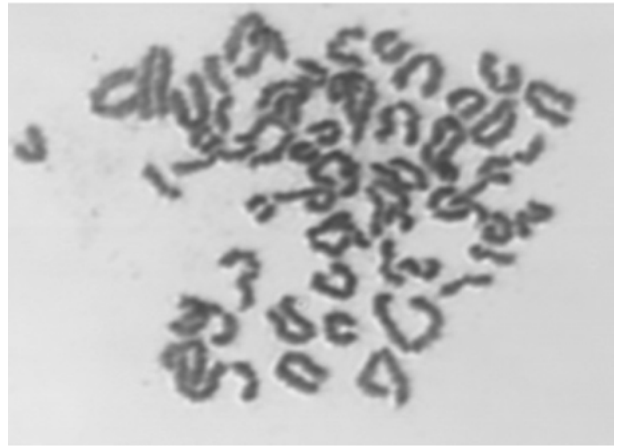oid parthenotes in bovine. Thus, the larger number of diploid parthenotes in the pulse and 6-DMAP group may in part account for the further development. However, previous reports [42] suggest that the presumed diploidization of parthenotes by 6-DMAP treatment was not the major cause of the observed increase in blastocyst rate since cytochalasin B, which also inhibited second polar body extrusion, failed to enhance parthenogenetic development. Therefore, the increase in blastocyst rate may be attributed to the inhibitory effect of 6-DMAP on MPF activity or possibly on another protein kinase involved in the regulation of parthenogenetic development.

The culture system is also a very important factor affecting the efficiency of the nuclear transfer procedure. It is not clear whether the culture system is dependent on the donor or the recipient for interspecies nuclear transfer embryos. In this study, three different culture systems were used. They were referred to as M199+10% FCS, SOF, and mCR1aa. It is well established that embryos from different mammalian species require species-specific embryo culture conditions. However, in our studies, relatively high proportions of reconstructed embryos, regardless of the donor nucleus species, developed to advanced stages in M199+10% FCS medium, indicating that M199+10% FCS may be a suitable culture medium for interspecies NT embryos using rabbit oocytes as the recipient cytoplasm. Previous reports have also shown that M199+10% FCS supports in vitro development of panda-rabbit, cat-rabbit, and chicken-rabbit embryos to the blastocyst stage [17, 18, 34, 36]. SOF, a chemically defined medium, has been widely used to culture embryos from various mammalian species. In the present study, SOF was also fairly capable of supporting development of reconstructed embryos and there were no significant differences in terms of ability to support development of the reconstructed embryos. The reason for this may be good compatibility between SOF and reconstructed embryos. Furthermore, most of Eagle's 20 amino acids are present in rabbit uterine fluids and blastocysts. Inclusion of most or all of both EAA and NEAA in the culture media improved development in the rabbit. In the present study, SOF was supplemented with EAA and NEAA, which are also beneficial to the development of reconstructed embryos. The mCR1aa medium, desidned for culture of bovine embryos, can well support the development of interspecies reconstructed embryos using bovine oocytes as the recipient. However, in the present study, mCR1aa poorly supported development of the interspecies reconstructed embryos. This may be attributed to incompatibility between mCR1aa and the reconsrtucted embryos using rabbit oocytes as recepients.

The cell cycle stage is another important factor affecting nuclear transfer. But, which combination is optimal remains to be determined. Since the landmark study of Wilmut *et al.* [1] describing the birth of Dolly, a cloned lamb from a somatic cell nucleus, there has been debate concerning the nucleus cell cycle stage required for somatic cell nuclear transfer. Wilmut *et al.* suggested that induction of quiescence by serum starvation was critical for allowing donor somatic cells to support development of cloned embryos. However, there are contradicting reports. A subsequent report suggested that G0 was unnecessary and that calves could be produced from actively dividing fibroblasts [3]. So far, progeny has been successfully obtained by nuclear transfer using either serum-starved fibroblast cells as donor cells in cattle, sheep, and goats or non-starved fibroblasts as door cells in cattle. In this study, the effect of serum starvation of donor cells was examined. Slightly higher fusion and cleavage rates were observed using serum-starved fibroblast cells as donor nuclei, but the blastocyst rate did not differ. Therefore, in our subsequent experiments, actively dividing cells were used as the donor cells, not only because they are convenient for this procedure, but also because serum deprivation for at least 48 h can induce apoptosis in murine and human cell lines [43–46] as well as in pig fibroblasts [47, 48]. Serum starvation of bovine and porcine cultured cells for 48 h causes reduced cell survival and increased DNA fragmentation [49]. Following nuclear transfer, greater embryonic loss has been observed in serum-starved cells compared with serum-fed granulose cells. The high rates of embryonic loss and abortion/fetal loss in cloned cattle may be related to serum starvation of the donor cells [50–52].

In this study, the in vivo development potential of the reconstructed embryos was not investigated. Embryo transfer was not conducted because an appropriate foster mother was not easy to obtain. The percentage of hybrid embryos developing to blastocysts in this study was slightly lower than those of previous reports [34, 36]. There seems to be two possible explanations for this. The micromanipulation skills of different researchers may be one reason. A lesser skilled operator would require more micromanipulation time. Rabbit oocytes are known to be sensitive to mechanical manipulation and temperature fluctuations, and prolonged exposure to room temperature during micromanipulation would be detrimental to further development of the cloned embryos [53]. Alternatively, this difference may be related to donor species. In this study, animals of different ages were used to provide the donor cells. Somatic

cells from a ten-month-old Tibetan antelope and a 4-year-old camel were used as donor karyoplasts, respectively, and there were no significant differences in the in vitro development potential of the reconstructed embryos, indicating that the age of the donor animal may not be a factor affecting the efficiency of nuclear transfer.

In conclusion the results of this study, together with previous reports, demonstrate that rabbit oocyte cytoplasm may be a suitable common host for dedifferentiation of the somatic cell nuclei of different species. Establishment of such a model would be beneficial for ongoing nuclear transfer research, in particular for interspecies nuclear transfer research.

## Acknowledgements

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