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Intergenerics Nuclear Transfer Technology for Conservation of Endangered Species

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The International Union for Conservation of Nature and Natural Resources (IUCN) considers the western/lowland bongo *Tragelaphus eurycerus eurycerus* to be a threatened species, and the eastern/mountain bongo *Tragelaphus eurycerus isaaci* an endangered species [1]. Although extinction is considered by many biologists to be a natural process during evolution, the exponential growth of the human population has drastically and prematurely reduced the numbers and genetic diversity of many species [2]. Species have evolved to adapt to a specific habitat or environment that meet their survival needs. Alteration or destruction of their habitat results in a species becoming incapable of adapting and hence becoming threatened with extinction. A widespread scientific and public consensus has emerged suggesting that governments should assign high priority to the maintenance of biological diversity via habitat preservation and management for species conservation [3]. Unfortunately, the loss of biological diversity far surpasses the available conservation resources and species are lost forever on a daily basis [4]. Notwithstanding the focus on habitat preservation and wildlife management, conservation biologists have also become increasingly interested in using the technologies of reproductive and developmental biology to help manage or rescue endangered species [5].

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This is most commonly observed in the captive propagation programs established by most zoological organizations. Today, the zoo conservation field offers researchers the ability to study the genetics and demographics of small isolated populations and the information obtained can then be directly applied to small remnant populations in the wild.

However, these programs are not without limitations, which include limited physical space for animals, problems with animal husbandry and general reproductive failure of the animals [3]. In attempting to increase the reproductive potential of a given species, many zoos have evaluated assisted reproductive techniques (ART). To date, several ART procedures; such as artificial insemination, *in vitro* or *in vivo* embryo production, cryogenics of gametes/embryos, and intra- or inter-specific embryo transfer that are routinely used in laboratory and agricultural species have become employed as alternative methods to reproduce non-domestic and critically endangered species [6-10]. For those species that are critically endangered, the numbers of individuals remaining are so few that it may be very difficult to apply even some of the basic assisted reproductive technologies.

Recent advances in somatic cell nuclear transfer (SCNT) have resulted in the production of offspring from a variety of laboratory and domestic animals including mice [11], sheep [12], cattle [13], goat [14], pig [15], cat [16] and rabbit [17]. Efforts are currently underway in many laboratories to produce cloned embryos from other species such as rats, dogs, horses and non-human primates. The success of nuclear transfer technology with livestock species has fostered speculation that cloning technologies might be applied to increase population sizes of endangered species, or even restore them following extinction. Preliminary studies have shown the possibility of using SCNT for conservation of endangered animals [18;19] and even for rescuing their genetic potential post-mortem [20-23]. However, there are additional challenges when applying SCNT cloning to endangered species [24]. Unlike the cloning of domestic animals where there is a ready supply of oocytes for embryo production and of surrogate animals for embryo transfers, the cloning of highly endangered or extinct species will require the use of alternative methods of cloning, which may involve the use of domestic counterparts.

Previously, it was reported that bovine oocytes could support development of embryos produced by SCNT from various domestic mammalian species [25-27]. A technique termed interspecific nuclear transfer has recently been applied to some endangered species that are closely related to domestic sheep and cattle. Using ovine oocytes and somatic cells from the endangered argali sheep (*Ovis ammon*), researchers reported embryonic development following nuclear transfer, however no live offspring were produced following embryo transfer [28]. Domestic cow (*Bos taurus*) oocytes and recipients were used in an attempt to clone gaurs (*Bos gaurus*), resulting in both late fetal development and a live offspring, although the calf died two days later [20-22]. Recently a mouflon lamb (*Ovis orientalis*) was born using similar techniques, further verifying the application of interspecific nuclear transfer in conserving endangered ovine species [23]. In contrast to these successes with *intra*-generic nuclear transfer, there have few reports of *inter*-generic preimplantation embryo production with endangered species using SCNT [18;19].

In the current study we tested the hypothesis that embryonic development would occur following intergeneric nuclear transfer of mountain bongo (*Tragelaphus eurycerus Isaaci*) somatic cells into domestic cow (*Bos taurus*) oocytes. To determine if culture conditions for the nuclear donor cells affected overall developmental rates, we evaluated the effect of serum deprivation of the donor cells prior to nuclear transfer. Finally, to determine if embryo culture conditions would influence the efficiency of the intergeneric nuclear transfer procedure, three embryo culture media were evaluated: a modified synthetic oviductal fluid (mSOF; [30, 31]), and a two stage culture system consisting of hamster embryo culture medium-6 (HECM-6; [32]) or HECM-6 supplemented with pantothenic acid (HECM-9; [33]), followed by TCM199 supplemented with serum.

Bongo skin cells were cultured in DMEM supplemented with 10% FBS. After two to six passages, starved (1% FBS) or non-starved cells were used as donor nuclei. *In vitro* matured (1920 h) bovine oocytes were enucleated by squeezing the first polar body and surrounding cytoplasm through a slit in the zona pellucida. After injection of a somatic cell into the perivitelline space, couplets were fused electrically and activated chemically and then subjected to different embryo culture treatments. After culture for 7d, developmental stages

were examined morphologically. Serum starvation had no effect on the frequency of cleavage to two cells or on development to the blastocyst stage in either the HECM-6 system or the mSOF culture medium. When couplets from non-starved donor nuclei were cultured, the frequency of cleavage, development to 9 cells and formation of blastocysts were all significantly higher ($p<0.05$) in the HECM-6 media than in mSOF medium. There was no significant difference in cleavage and developmental frequencies between the two HECM media. These results indicate that non-starved somatic cells are suitable for intergeneric nuclear transfer into oocytes, and that the HECM-6 medium is better than mSOF for culture of these reconstructed embryos. In conclusion, bovine oocytes can support blastocyst development after intergeneric transfer of bongo fibroblasts. This technique could potentially be used as an alternative to using scarce bongo oocytes in attempts to propagate these endangered animals.

1. IUCN. 1996 red list of threatened animals. Gland: Switzerland and Cambridge; 1996: 448.
2. Tilman D. Causes, consequences and ethics of biodiversity. Nature 2000; 405: 208-211.
3. Lasley BL, Loskutoff NM, Anderson GB. The limitation of conventional breeding programs and the need and promise of assisted reproduction in nondomestic species. Theriogenology 1994; 41:119-132.
4. Porter S. Biodiversity projections look grim. World Watch 2000; 13:8.
5. Corley-Smith GE, Brandhorst BP. Preservation of endangered species and populations: a role for genome banking, somatic cell cloning, and androgenesis? Mol Reprod Dev 1999; 53:363-367.
6. Hradecky P, Stover J, Stott T. Histology of a heifer placentome after interspecies transfer of a Gaur embryo. Theriogenology 1988; 30:593-604.
7. Hammer CJ, Tyler HD, Loskutoff NM, Armstrong DL, Funk DJ, Lindsey BR, Simmons LG. Compromised development of calves (Bos gaurus) derived from *in vitro-* generated embryos and transferred interspecifically into domestic cattle (Bos taurus). Theriogenology 2001; 55:1447-1455.
8. Durrant B, Benirschke K. Embryo transfer in exotic animals. Theriogenology 1981; 15:77-83.

9. Loskutoff N, Bartels P, Meintjes M, Godke R, Schiewe M. Assisted reproductive technology in nondomestic ungulates: a model approach to preserving and managing genetic diversity. Theriogenology 1995; 43:3-12.
10. Solti L, Crichton EG, Loskutoff NM, Cseh S. Economical and ecological importance of indigenous livestock and the application of assisted reroduction to their preservation. Theriogenology 2000; 53:149-162.
11. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394:369-374.
12. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385:810-813.
13. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM. Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat Biotechnol 1998; 16:642-646.
14. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y. Production of goats by somatic cell nuclear transfer. Nat Biotechnol 1999; 17:456-461.
15. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 2000; 407:86-90.
16. Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L, Westhusin M. Cell biology: A cat cloned by nuclear transplantation. Nature 2002; 415:859.
17. Chesn P, Adenot P, Viglietta C, Baratte M, Boulanger L, Renard J. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat Biotechnol 2002; 20:366-369.
18. Hwang W, Kim K, Kim G, Jin Y, Kim Y, Chung H, Yoon T, Han C, Eo Y, Lee B. Interspecies somatic cell nuclear transfer for the production of endangered Korean tiger (*Panthera tigris altaica*). Theriogenology 2001; 55:271.
19. Bui L, Vignon X, Campion E, Laloy E, Lavergne Y, Ty L, Nguyen B,

Renard J. Use of interspecies nuclear transfer to study the early embryonic development and nuclear activities of the endangered species *Pseudoryx nghetinhensis* (Saola). Theriogenology 2002; 57: 427.

20. Lanza RP, Dresser BL, Damiani P. Cloning Noah's ark. Sci Amer 2000; 283:84-89.
21. Vogel G. Endangered species. Cloned gaur a short-lived success. Science 2001; 291: 409.
22. Lanza RP, Cibelli JB, Diaz F, Moraes CT, Farin PW, Farin CE, Hammer CJ, West MD, Damiani P. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. Cloning 2001; 2:79-90.
23. Loi P, Ptak G, Barboni B, Fulka J, Jr., Cappai P, Clinton M. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat Biotechnol 2001; 19:962-964.
24. Ryder OA. Cloning advances and challenges for conservation. Trends Biotechnol 2002; 20:231-2.
25. Dominko T, Mitalipova M, Haley B, Beyhan Z, Memili E, McKusick B, First NL. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. Biol Reprod 1999; 60:1496-1502.
26. Meirelles FV, Bordignon V, Watanabe Y, Watanabe M, Dayan A, Lobo RB, Garcia JM, Smith LC. Complete replacement of the mitochondrial genotype in a Bos indicus calf reconstructed by nuclear transfer to a Bos taurus oocyte. Genetics 2001; 158:351-356.
27. Kitiyanant Y, Saikhun J, Chaisalee B, White K, Pavasuthipaisit K. Somatic cell cloning in buffalo (*Bubalus bubalis*): Effects of interspecies cytoplasmic recipients and activation procedures. Cloning and Stem Cells 2001; 3:97-104.
28. White K, Bunch T, Mitalipov S, Reed W. Establishment of pregnancy after the transfer of nuclear transfer embryos produced from the fusion of atgali (*Ovis ammon*) nuclei into domestic sheep (*Ovis aries*) enucleated oocytes. Cloning 1999; 1:47-54.
29. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 1998; 280:1256-1258.

30. Lee ES, Fukui Y. Synergistic effect of alanine and glycine on bovine embryos cultured in a chemically defined medium and amino acid uptake by vitro-produced bovine morulae and blastocysts. Biol Reprod 1996; 55:1383-1389.
31. Crosier AE, Farin PW, Dykstra MJ, Alexander JE, Farin CE. Ultrastructural morphometry of bovine compact morulae produced in vivo or *in vitro*. Biol Reprod 2000; 62:1459-1465.
32. McKiernan SH, Clayton MK, Bavister BD. Analysis of stimulatory and inhibitory amino acids for development of hamster one-cell embryos *in vitro*. Mol Reprod Dev 1995; 42:188-199.
33. McKiernan SH, Bavister BD. Culture of one-cell hamster embryos with water soluble vitamins: pantothenate stimulates blastocyst production. Hum Reprod 2000; 15:157-164.