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IMPORTANCE OF ASSISTED REPRODUCTIVE TECHNOLOGIES IN THE CONSERVATION OF WILD, RARE OR INDIGENOUS UNGULATES: REVIEW ARTICLE

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Biodiversity is increasingly threatened by intensive agriculture, environmental pollution, extinction of natural habitats and several other factors. Several mammalian species including ungulates have disappeared or are threatened by extinction. However, ungulates play an important role both in the ecosystem and in the economy. In general, species or breeds are considered endangered if their population does not exceed 1,000 individuals. In these cases conservation programmes should be initiated in order to maintain or even increase their number. This review deals with the possibilities and limitations of assisted reproductive technologies (ART) in the conservation of ecologically valuable wild, rare and indigenous ungulates. The methods discussed here are artificial insemination, cryopreservation of semen and embryos, embryo recovery and transfer, *in vitro* production of embryos, as well as micromanipulation techniques including sperm injection, assisted hatching and cloning. Some of these procedures are already being exploited in the breeding of farm ungulates, but more basic information about the reproductive patterns of wild, rare and indigenous animal species is needed before the routine use of ARTs.

Key words: Rare and endangered ungulates, assisted reproductive technologies, conservation programs

In many countries the development of agricultural technologies and the spread of intensive production systems including breeding programmes and forest management have resulted in serious changes in rare or ecologically valuable species. In the past two centuries 46 mammalian species have disappeared and many others are endangered, having a population size below 1,000 individuals (Loskutoff et al., 1995). However, due to their cultural, historical and genetic importance, attempts are being made to conserve genomes and/or individual genes by employing biotechnological and/or assisted reproductive technologies (ART). The conservation would allow, if required, reactivation in their original form that might have become extinct. It has been demonstrated that genetic ho-

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mogenization has negative effects on domestic and wild animal species, such as increased juvenile mortality, poor reproductive performance, and susceptibility to disease (Brem et al., 1989; Lasley et al., 1994). *In situ* conservation of threatened animals means a population saved and kept in national parks; they are visible and not to be forgotten but very susceptible to infectious or other lethal diseases. *Ex situ* conservation is a stock of genetic material (semen, embryos or ova) deep frozen in liquid nitrogen which is relatively safe and inexpensive, but over decades the physical appearance of the animals can be forgotten by the public. Biotechnology and ART have clear benefits for conservation biology and are very promising in the preservation of both the ecologically valuable, wild, rare and indigenous species and the genetic variability within such species (Raven et al., 1992; Lasley et al., 1994; Loskutoff et al., 1995; Loskutoff and Betteridge, 1993; Solti et al., 2000). ART have been widely and successfully used in both humans and domestic animals for many years. However, the application of such technologies in less or nondomesticated, native, rare or endangered animals proves to be very difficult. It still remains to be demonstrated that biotechnology and ART can be used routinely to actually conserve a rare or endangered species.

Among domestic animals, ungulates play an important economic role. However, intensive breeding of high-yielding farm animals seemed to make indigenous breeds dispensable for owners. In fact, indigenous breeds may possess several advantageous qualities (resistance to diseases, endurance, solid hoof structure, low nutritional demand, etc.) that must not become extinct in the future. Recently, attention has been paid to milk protein genetic variants with special regard to κ-casein gene of indigenous bovine breeds like Barrosa and Hungarian Grey (Baranyi et al., 1993; Bastos et al., 1999). These genes may be of economic importance for increasing the quality of milk for cheese production. Another example for this in Hungary is the change from the dominant traditional dual-purpose cattle breed to the high-yielding dairy Holstein-Friesian. Although this change has significantly increased the national milk production, the meat quality of the Holstein is inferior to that of dual-purpose or beef breeds, thus the ancient breeds should partly be re-introduced. Another example is the indigenous Mangalica swine which, compared to Landrace-type pigs, has a slower growth rate and significantly more fat and, therefore, almost became extinct in the last decades. Today it is bred again for valuable food products such as dried ham and salami.

For this overview, biotechnology and ART are considered as procedures used to conserve and/or assist the reproduction of ungulates. The methods discussed here range from practical, applied techniques [e.g. artificial insemination (AI), embryo transfer (ET)] to more complex methods [e.g. *in vitro* fertilisation (IVF), *in vitro* production of embryos (IVP), gamete or embryo micromanipulation]. Each of these techniques has been used to assist mammalian reproduction and improve the genetic diversity and reproductive efficiency of domestic livestock.

Artificial insemination

Artificial insemination (AI) is the most widely applied ART. Offspring have been produced by AI in different animal species and in man (for a review see Wildt, 1991 and Wildt et al., 1992*a*). The experiences obtained in farm animals have demonstrated the value of AI combined with semen cryopreservation (CP). AI and semen CP can maximise the reproductive potential of valuable males, without limitations of time and distance. Moreover, genetic material of males can be preserved for a long time without taking up space in zoos or farms, thus genetic goals can be achieved even for small populations. Using a computer simulation model, Johnston and Lacy (1991) demonstrated that maximum genetic diversity can be maintained in a small gaur herd with as few as 40 individuals by AI combined with semen CP. Currently, frozen semen is being stored from 41 animal species, 15 of which no longer live. To date, AI with frozen-thawed sperm resulted in offspring from 28 mammalian species, 16 of which can be classified as nondomesticated (Wildt et al., 1992*a*; Wildt et al., 1992*b*).

Interest in applying AI to ecologically valuable wild, rare and indigenous ungulates has been preceded by comparative investigations of cattle and sheep. The results indicate that the efficiency of AI depends on the precise timing of insemination determined by the time of ovulation. Correct timing of AI can be achieved either by accurate identification of animals in natural heat or by using fixed-time AI of synchronised animals. Oestrus synchronisation has widely been used in domestic ungulates for 20 years. Standard synchronisation procedures developed for cattle (e.g. PGF_{2a}, norgestomet) and sheep (e.g. MAP-pessaries, CIDR intravaginal devices) have been used with acceptable success in nondomestic ungulates. However, the actual time of ovulation has been very difficult to assess, thus the routine use of AI and ET in these animals is quite limited (Schiewe et al., 1991; Wildt et al., 1992*a*). Unfortunately, behavioural cues are not reliable indicators for detecting oestrus in these animals. Measurement of blood hormone concentrations for this purpose can be confounded by the effects of physical restraint or anaesthesia that are often necessary for blood collection. Alternatively, methods for monitoring the ovarian cycle, characterising the endocrine patterns of the oestrous cycle and diagnosing reproductive failures have been performed using urine and/or faecal samples in nondomestic species (Loskutoff et al., 1983; Lasley and Kirkpatrick, 1991).

Noninvasive techniques of vaginal or transcervical semen application are generally ineffective in large nondomestic ungulates, particularly with frozen semen (Lasley et al., 1994). The pregnancy rate was higher when the semen was deposited directly into the uterine horn using a laparoscopic technique (Asher et al., 1990; Wildt et al., 1992*a*; Wildt et al., 1992*b*). Laparoscopic insemination is an alternative route involving direct deposition of very small quantities of semen into the uterine horns following visualisation of the abdominal and pelvic organs.

This technique, developed in ewes, has been used successfully with excellent conception rates in Hungarian Mangalica swine, fallow deer and red deer (Asher et al., 1988; Asher et al., 1990; Fenessy et al., 1990; Rátky et al., 2000, accepted for publication). Much research has been conducted in cervids on oestrus synchronisation and semen collection and conservation as well (Monfort et al., 1990; Garde et al., 1998).

Embryo transfer

Ovarian stimulation

Superovulation using cattle and sheep protocols has been performed on nondomestic ungulates such as antelopes, giraffe, deer, wild cattle, buffalo, camelids and bison (Elsden et al., 1978; Stover et al., 1981; Seidel and Seidel, 1981; Dresser et al., 1984; Dresser et al., 1985; Karaivanov, 1986; Pope et al., 1988; Loskutoff et al., 1988; Loskutoff et al., 1990; Dixon et al., 1991; Schiewe et al., 1991; Fenessy et al., 1994; McKinnon et al., 1994; Dorn, 1995; Del Campo et al., 1995). Ovarian response to traditional stimulation protocols in nondomestic ungulates is quite inconsistent. The limited success may be related, in part, to species resilience to the exogenous gonadotropins as well as to stress provoked by repeated restraining for treatments (Schiewe et al., 1991). In addition, the use of exogenous gonadotropins may cause the formation of antibodies and abnormal corpora lutea. Abnormal corpora lutea cause early luteal regression and loss of embryos (Loskutoff et al., 1990). However, recent progress provides encouraging evidence that bovine and ovine superovulatory protocols can be adapted successfully to nondomestic ungulates like deer (Dixon et al., 1991; Fenessy et al., 1994; Garde et al., 1998).

Embryo recovery and transfer

The nonsurgical bovine embryo recovery method is effective in most large ungulate species where per rectum palpation is possible, except in giraffe and okapi in which the cervices are impenetrable (Dresser et al., 1984; Dresser et al., 1985; Loskutoff et al., 1988; Schiewe et al., 1991*a*; Fenessy et al., 1994). The laparoscopic technique developed for sheep and goats could be a very valuable procedure to retrieve embryos in small ungulates (Kraemer, 1989; Loskutoff et al., 1991; Besenfelder et al., 1994; Kuhholzer et al., 1997).

Nonsurgical ET through the cervix has also been possible in the large antelope, wild cattle, buffalo and camelid species. Birth of live offspring following transcervical ET has been achieved in the eland, bongo, oryx, gaur, dromedary camel and llama (Dresser et al., 1984; Dresser et al., 1985; Pope et al., 1988; Pope et al., 1991; McKinnon et al., 1994; Del Campo et al., 1995). Transabdominal laparoscopic ET technique developed on sheep has been successfully

applied to the suni antelope (Schiewe et al., 1984; Kraemer, 1989; Loskutoff et al., 1990).

In some species in which the number of available individuals is very limited, interspecies ET might be the only choice to keep acceptable genetic variability within the population and in this way to avoid inbreeding. Successful interspecies ETs have been performed from gaur to domestic Holstein cattle, from exotic wild horses to domestic ones, from Indian desert cat to domestic strains (from wild cats to domestic ones), from bongo antelope to eland (Stover et al., 1981; Dresser et al., 1985; Summers et al., 1987; Pope et al., 1989). However, interspecies ET is frequently accompanied by side effects like early resorption and late abortion, malformations, reduced number of placentomes, abnormal histological development, etc. (Stover et al., 1981; Summers et al., 1987; Hradecky et al., 1988; Buckrell et al., 1990).

In vitro embryo production (IVP)

In vitro embryo production (*in vitro* maturation, fertilisation and culture; IVM, IVF, IVC; IVMFC) offers several advantages over the collection of *in vivo* derived embryos including: *(1)* circumvention of the problem of timing ovulation for AI; *(2)* the potential for producing more embryos than can usually be collected from hormonally-stimulated donors; *(3)* the ability to use animals with certain types of infertility, such as endometritis or tubal obstruction; *(4)* a reduction in the number of viable spermatozoa as compared to AI or natural breeding; *(5)* using sperm microinjection techniques, the potential of using nonmotile or nonviable sperm and testicular- or epididymal-derived sperm for assisted fertilisation; *(6)* the potential of salvaging genetic material from female animals after death; and *(7)* the possible inclusion of prepuberal or pregnant animals as oocyte donors (Loskutoff et al., 1995).

Although offspring have been produced from *in vitro* produced embryos in a variety of ungulates, primates and carnivores, the IVM of immature oocytes proves to be very difficult in certain species such as felids and equids (Loskutoff and Betteridge, 1993; Li et al., 1994; Gordon, 1994; Lasley et al., 1994; Meintjes et al., 1994; Meintjes et al., 1995; Pope et al., 1995). Recently, an Indian gaur calf has been obtained after *in vitro* maturation, fertilisation and culture of an oocyte (Johnston et al., 1993). Elephant oocytes may be capable to undergo maturation *in vitro*, however this ability of elephant oocytes depends upon the age and parity of the oocyte donor (Christensen et al., 1993).

Transvaginal ultrasound-guided oocyte retrieval has been proven to be safe and efficient for collecting oocytes from cows, mares, and goats as well as from pregnant cows and mares (Pieterse et al., 1988; Gordon, 1994; Meintjes et al., 1994; Meintjes et al., 1995). These ovum pick up procedures have been suc-

cessfully applied, with minor modification, to different nondomestic zoo ungulates housed in captivity (Meintjes et al., 1994; Meintjes et al., 1995; Loskutoff et al., 1995; Armstrong et al., 1995). In the future, an alternative technique could be laparoscopy developed for the repeated aspiration of bovine follicular oocytes (Reichenbach et al., 1994).

Sperm quality and *in vitro* sperm capacitation are the most important factors determining the success of *in vitro* embryo production (Bárándi et al., 1993). Embryos have been recently produced from IVM-IVF oocytes in domestic horses (Li et al., 1994; Meintjes et al., 1994). In certain species (Burchell's zebra stallion) and circumstances (epididymal sperm) the successful induction of acrosome reaction and capacitation are limiting factors of IVM-IVF (Schiewe et al., 1984). An alternative to cryopreserving ejaculatory semen may be the direct refrigeration of testicles and freezing of epididymal sperm (Bezuidenhout et al., 1995). Ejaculated semen can be routinely collected and frozen in domestic and nondomestic ungulate species; however, optimal cryoprotective extenders are species dependent (Howard et al., 1986; Schiewe, 1991; Schiewe et al., 1991*a*, 1991*b*).

Procedures used for IVC of embryos have been developed in humans, nonhuman primates, laboratory or domestic animals. However, the results indicate differences in the requirements for embryo development *in vitro* even between closely related species. Culture methods for bovine embryos appear to be effective to ensure embryo development to the blastocyst stage in wild cattle, water buffalo, giraffe and South American camelids, as well as in certain antelope species such as the greater kudu and impala (Johnston et al., 1993; Monfort et al., 1993; Del Campo et al., 1995; Loskutoff et al., 1995). However, using the same procedure no embryo development beyond the morula stage can be obtained in the African buffalo (Shaw et al., 1995). It has been shown that IVP ungulate embryos can be successfully cryopreserved but they are more sensitive to low temperature (Leibo and Loskutoff, 1993; Cseh et al., 1995). IVF has been used successfully for embryo production from the endangered Hungarian Grey cattle breed (Solti et al., 1992).

Gamete and embryo micromanipulation

Micromanipulation has become increasingly common in mammalian embryology. Bovine proteins have been injected into mouse embryos, two-cell mouse embryos were separated into constituent blastomeres, tissue was excised from rabbit blastocysts for genetic typing, a donor cell was injected into the blastocoelic cavity of mouse blastocysts, and cloning (even from somatic cells) began in mammals (Wilmut et al., 1997). Recently, adult somatic cell nucleus transfer was used successfully to preserve the last surviving cow of the Enderby

Island cattle breed. The technology may be used to propagate other endangered indigenous breeds in the future (Wells et al., 1999).

Pioneering fertilisation experiments concerning with microinjection of hamster and human sperm into the cytoplasm of hamster oocytes led to the development of a clinical application called 'assisted fertilisation' (Uehara and Yanagimachi, 1976). Three micromanipulative methods of assisting fertilisation are already in practice (Edwards and Brody, 1995). The first involves making small holes or slits in the zona pellucida by methods known as zona drilling, cracking, or cutting, or by using acidified medium or a sharp glass needle permitting weakly motile spermatozoa to gain access to the oolemma (Loskutoff et al., 1993; Edwards and Brody, 1995). Second, one or more spermatozoa are inserted into the perivitelline space (subzonal insemination, SUZI; Mann, 1988). Third, a spermatozoa or sperm head is injected directly into the ooplasm called intracytoplasmic sperm injection (ICSI; Kimura and Yanagimachi, 1995; Edwards and Brody, 1995). More recently, the development of ICSI has resulted in a marked increase in the fertilisation rates achieved by microinjection as well as in improved pregnancy rates after transfer. A fourth method has been added recently when a spermatid or spermatocyte nucleus was injected into the oocyte (Kimura and Yanagimachi, 1995).

The importance of ICSI to wildlife or rare animal preservation appears limitless, as even testicular-and epididymal-derived or freeze-dried spermatozoa are capable of decondensing in the ooplasm and producing viable embryos and offspring (Kimura and Yanagimachi, 1995; Wakayama and Yanagimachi, 1998). ICSI of nonviable sperm has even resulted in the birth of live calves (Goto et al., 1991). However, the results of sperm injection indicate that the procedure may be species specific. Results indicate that for cat and mouse oocytes ICSI is less effective than SUZI which produced embryos capable of full-term development (Mann, 1988; Pope et al., 1995; Yanagimachi, 1998). The different procedures used for sperm insertion will have an important role to play in future conservation efforts, particularly for endangered species in which males have a high proportion of abnormal sperm and/or no method available for successful IVF or cryopreservation of sperm (Gordon and Talansky, 1986; Monfort et al., 1993; Li et al., 1994; Meintjes et al., 1994; Burruel et al., 1996; Wakayama and Yanagimachi, 1998).

Treatments on embryos such as zona drilling or partial zona dissection could raise their chances of implantation (Gordon and Talansky, 1986; Edwards and Brody, 1995). Drilling holes on the zona pellucida might facilitate embryos to hatch earlier from the zona pellucida hardened by ovarian stimulation and/or embryo culture. Results obtained by Loskutoff et al. (1993) indicate that partial zona dissection improves the hatching frequencies of CP bovine blastocysts produced *in vitro* and co-culture conditions can affect survival after thawing. It is probable that IVP wildlife, rare and indigenous animal embryos will also benefit from assisted hatching techniques.

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