Attemps at applying cloning to the conservation of species in danger of extinction

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Attempts at Applying Cloning to the Conservation of Species in Danger of Extinction

Intentos de Aplicar la Clonación en la Conservación de Especies en Riesgo de Extinción

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SUMMARY: The somatic cloning by transfer of the nuclei of differentiated adult cells to previously enucleated oocytes is a promising technique for the production of embryos of high genetic value. The better mastering of somatic cloning gives us the possibility to produce embryos from endangered species.

The huemul is an Andean native deer, that has been declared an endangered species, it holds a great patrimonial value and it is a Chilean national emblem.

In Chile the huemul has the status of protected animal on thirteen Parks and National Reserves managed by Corporacion Nacional Forestal (CONAF). This protection, however, is considered insufficient due to the few geographical connections between the different protected areas. Furthermore, a great proportion of these areas are not subjected to use or they do not constitute adequate habitats.

Many authors have proposed that the use of biotechnological methods in reproduction and assisted procreation may help conservational programs orientated to the protection of deer species threatened by extinction. All the anterior prompted us to initiate this study concerning the production of cloned huemul embryos.

KEY WORDS: Huemul; Interspecific cloning; Endangered species.

The cloning of a sheep was achieved by Campbell *et al*. (1996) by transferring the nuclei from cells of the mammal gland in to an enucleated ovocyte of another sheep. Shortly after that, the same authors reported the birth of the first living cloned sheep (Wilmut *et al.,* 1997), named Dolly after the actress Dolly Parton. This cloned sheep evidenced normal reproduction capability, giving birth to six lambs. Later, it presented articulation problems and premature ageing and as a result, it was culled when it reached six years of age (Shields *et al.,* 199 and Lanza *et al*., 2000a). In this respect, it was reported that the shortening of the telomeres could be related to the ageing problems presented by cloned animals (Giles & Knight, 2003; Jie & Xiangzhong, 2003).

In spite of the inefficiency of this process, the technique has been improved and there are reports of 10 animal species that have been cloned successfully, giving birth to normal living offspring. Wells *et al*. (1998) and Wells (2000) reported the birth of Holstein calves of normal weight without any malformations, after the cloning using somatic cells. As opposed to Dolly, Cumulina, the first cloned mouse obtained from genetic material from the cumulus oophorus (Wakayama *et al.*, 1998, 2000 and Wakayama & Yanagimachi, 2001) died at the age of two years and seven months, which is considered to be very old for this species.

Bulls and goats have also been cloned by somatic nuclear transference. (Baguisi *et al.*, 1999; Shiga *et al.*, 1999). The first cloning of this kind in pigs was accomplished by Polejaeva *et al.* (2000) who reported the birth of five healthy piglets. Almost at the same time, Onishi *et al.* (2000) published the birth of a living clone by direct transference of a nucleus derived from pig fetal fibroblasts into enucleated ovocytes. Chesne *et al.* (2002) and Challah-Jacques *et al.* (2003) developed a method for producing rabbit clones. Furthermore, a mule (Woods *et al*., 2003), a colt (Galli *et al*., 2003) and even a domestic cat has been cloned which has been alive for 3 years now (Shin *et al.*, 2002).

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Attempts at applying cloning to the conservation of species in danger of extinction. Interspecific cloning. The applications of cloning procedures extend also to the conservation of endangered species and races (Wells *et al.,* 1998; Wells, 2000; Palma 2001). There are many examples in the literature, among others, *Bos gaurus*, (Lanza *et al.,* 2000a) *Bos javanicus* and Bucardo (*Capra pyrenaica pyrenaica*). What is special about these examples is that the authors have had to resort to interspecific cloning. In these cases, the cytoplasm of the ovocyte used in order to form the embryo, derives from common domestic species such as *Bos taurus* (cow) and *Capra hircus* (goat), whereas the nucleus are provided for the endangered species. The major problem that arises in this kind of interspecific cloning is the fate of the mitochondria from the receptive ovocyte (Da-Yuan Chen *et al.*, 2002), due to the fact that the embryo reconstructed in this way may contain mitochondria from both species (Holt *et al*., 2004).

Progress has also been made in the cloning of the giant panda bear (*Ailuropoda melanoleuca*) (Saegusa, 1998). According to Da-Yuan *et al.*, the interspecific cloning of this animal is practically impossible because of an insufficient number of ovocytes, as the female Panda produces only one or two ovocytes a year. The only possibility to clone these animals is the technique of interspecific cloning involving the transference of nuclei from cells of the giant panda into enucleated ovocytes from another bear species, the gestation being established in a species different to the nuclei donor species.

A new species of Vietnamese ox, weighting approximately 100 kilos and known as Saola or Vu Quang Ox (*Pseudoryx Nghetinhensis*) was discovered in 1993 (Dung *et al.*, 1993 and 1994). Unfortunately, only about a hundred animals are alive today (Kemp *et al*., 1997), in consequence, it is considered an endangered species (Red List of Threatened Species). The functioning of the Saolas embryo genome is currently being characterized in France. These embryos have been obtained after the fusion of a Saolas cell nucleus to the cytoplasm from the ovocyte of a filogenetically close species, such as cattle. Studies are underway in order to establish how nuclear-cytoplasmic interactions occur between these two closely related species at early developmental stages (Service Presse INRA). Regarding this study, six-day embryos have been obtained by the cloning method and are currently kept frozen, waiting to be implanted into a surrogate mother.

In these three cases analyzed, interspecific cloning must be performed. The main problem of this procedure occurs in the first generation and it is due to the mitochondria issue previously described. However, it is worthwhile to point out that if the nucleus comes from the male individual of the species of interest the reconstructed embryo, and even more, the first generation may present mitochondria from both species. This problem can be overcome if the cloned male reproduces naturally with a female of the species of interest, because the paternal mitochondrias are not transmitted to the next generation. This argument cannot be applied to the female because the mitochondrias are inherented through the maternal cytoplasm (Sutovsky *et al*., 2000; Da-Yuan *et al*. and Holt *et al*., 2004).

Natural reproduction should be the preferred method to increase the number of individuals of a determined species. This is not the case, however, when the population of the wild species is in decline. Reproductive technology may represent an important support, forming genetic reserve banks of endangered animals (Holt *et al*., 1996; Holt & Pickard, 1999, Watson & Holt, 2001 and Holt *et al*., 2002) and the reproduction technology for the conservation of wildlife. Reproduction by cloning could be one of the methods used to increase the number of individuals in a population. It is well known that populations with low numbers of individuals posse a minimal genetic variation, but if some of the individuals fail to reproduce, a loss of genetic variation will occur. According to Holt *et al*. (2004) a possibility for small populations could be to clone each individual (not impossible if the population size is between 9 -18 animals), this allows the offspring to grow, mature and reproduce in a natural way. The probability of loss of genetic variation can be reduced, especially if every parent gives rise to two identical copies of itself. This is an interesting theoretical model for animal conservation.

Native Chilean mammals declared vulnerable or endangered species. There are many endangered mammal species in Chile. Among them there are some native carnivors such as the puma (*Puma concolor*), the andean mountain cat (*Oreailurus jacobita*), the guiña (*Oncifelis guigna*), the argentinian mountain cat (*Oncifelis geoffroyi*), the colocolo cat (*Lynchailurus colocolo*), grey or chilla fox (*Pseudalopex griseus*), Chiloé's fox (*Pseudalopex fulvipes*), red or culpeo fox (*Pseudalopex*), and the huillín (*Lontra provocax*). There are also grain eating species, such as the mountain chinchilla (*Chinchilla brevicaudata*) and the coast chinchilla (*Chinchilla manigera*) which are also threatened by extinction.

Among the native ruminants that have been considered as protected species there are three cervidae: the andean huemul or taruca (*Hippocamelus antisensis*), the pudu (*Pudu pudu*), a small deer native to Chile and Argentina, that has been considered one of the smallest deer in the world and has been declared a vulnerable species (Glade, 1993) and the southern huemul (*Hipocamelus bisulcus*). Other

ruminants are the vicuña (*Vicugna vicugna*) and the guanaco (*Lama guanicoe*). From now on we will refer to the southern huemul because out of all these endangered species, this is particularly the more emblematic in Chile.

Characteristics of the Southern Huemul (*Hipocamelus bisulcus*). The Southern Huemul (*Hippocamelus bisulcus*) Molina 1782, is a native deer that inhabits the southern Chilean and Argentine Andean Mountains. It belongs to the Mammal Class, Order Artiodactyla, Family Cervidae. Historically, it was distributed between Central Chile (34° S) and the Estrecho de Magallanes (54° S) but actually. it has been declared an endangered species that risks extinction. Its actual population is estimated in less than 1000 individuals gathered in isolated and fragmented groups (Aldridge, 1988; Saucedo & Gill a, b, c, 2004). It is included in the Red Book from the World Union for Nature (I.U.C.N.), besides the protection regulation performed for national and international private organisms such as the World Wild Fund (W.W.F.).

In Chile, the huemul is considered a protected animal on thirteen Parks and National Reserves managed by Corporacion Nacional Forestal (CONAF). This protection, however, is considered insufficient due to the few geographical connections presented between the different protected areas. Furthermore, a great proportion of these areas are not subjected to use or they do not constitute adequate habitats. The major threats for the species are the alteration of its natural habitat due to forest fire, forest cut down and stockbreeding, illness transmitted by domestic cattle (Povilitis, 1998; Diaz & Smith 2000; Serret, 2001; Saucedo, 2004a,b), illegal hunting, dog predation, and its natural predator the puma (*Puma concolor*).

A huemul weights between 45 and 65 kilograms (100 – 140 pounds). Adult male specimens can reach 90 to 100 cm at the withers and have a body size and weight higher than those showed for the females. These have a height of 80 cm and an average weight of about 65 kg. The huemul can be considered a medium size animal, stockily built, with relatively short legs, characteristics that demonstrate its great adaptation to the steeped and rocky territory that constitute its natural habitat (Acosta-Jamett, 2004).

Its social organization has been studied during spring time, when it has been observed that they constitute small groups of individual from both sexes (Povilitis, 1983b, 1985; Frid, 1991, 1994,1999 y 2001). These groups occupy higher

places in summer; descending the mountains and staying in forested valleys during winter time. This altitudinal migratory behavior, described a long time ago, shows some differences with reports from Saucedo & Gill (2004a,b,c) whom, by using radiocollars, were able to follow the movements of the animals thus observing short displacements in a relatively small area of approximately 400 ha. The analysis of the selected habitat revealed a preference for the "lenga" (*Nothofagus pumilio*). The areas with freshwater sources were preferred. This deer feeds principally from grasses and bush vegetation (Saucedo & Gill, 2004c).

Reproduction: The huemul is an animal of seasonal reproduction, which implies an alternation between periods of sexual resting and sexual activity determined by the photoperiod (variations between the hours of light and darkness). It is characterized for presenting rutting periods between March to May and the gestational period spans over 7 months, with only one fawn being born. The birth season takes place on spring time, from the beginning of November until the first days of January, a period very favorable for the growing of the fawn. The fawn are born weighing approximately 3,5 kg. (Jara *et al.*, 2003)

There is no precise information on the literature about different reproductive parameters of male huemuls, such as ejaculated seminal volume (ml), sperm cells concentration per ml, morphology of the sperm cells in the ejaculated, testicular volume evolution, progression of the germinal line and interstitial (Leydig) cells during the annual reproductive cycle. There is also no detailed information available about the anatomy of the reproductive tract, cycle length, estrus duration or if delayed implantation exits or not. However, we can extrapolate a few facts from what it is known in other deer species: 1) Each year the males present a resting gonadal phase, recrudescency, activity and gonadal regression, 2) the animal is no fertile during the two first phases. During the active phase, the germinal line is completed and the interstitial cells differentiate, increasing in size and elaborating high concentrations of testosterone, which is related to the sexual drive and the sexual secondary characters, such as a greater development of the antlers (Lincoln & Short, 1980; Drion *et al.,* 2003 y Locatelli *et al*., 2005).

Embryonic Development. There are no thorough works in relation with the huemul embryonic development, however, extrapolating from what is known from other cervidae it is inferred that the uterus is bicorneal and that it presents endometrial carunculae. Following the fertilization and segmentation the embryo reach the blastocyst stage and after hatching its stay in spherical form for a few days and locates in the ipsilateral horn. Later, the trophoblast growth is directed towards the contralateral horn. The embryonic disk does not growth too much but the trophoblast elongates forming a filament of about 30 cm long. Placentation is of the synepitheliochorial and oligocotiledonical type. It is possible to observe six to eight trophoblastic cotyledons forming placentomes with uterine carunculae. Both the trophoblast and the allantois undergo a phase of elongation similar to what occurs in other ruminants (Drion *et al*.). In the majority of deer there also exists a reproductive strategy called embryonic diapause, Diapause is observed in the roe deer (*Capreolus capreolus*), only but it is not known if this also occurs in the huemul.

In its first month of life, the fawn is immature; its walking performance is still unstable and keeps hidden and quiet during most of the time, changing places after suckling. The weaning probably takes place after the fourth month of age, because some fawns have been observed still suckling at that age. At the second month of age they frequently feed on vegetation. The growth of juvenile individuals is relatively fast as a yearling male has already reached the size of an adult female.

At present, the scarce evidences of the presence of fawns or juveniles at the region of Nevados de Chillán, may indicate either a decrease in the birthrate or an increase in the natural mortality rate which, in both cases, may leads to the extinction of the population in a short period of time (Povilitis 1983a; 1998; 2002; Plan to the conservation of the southern Huemul). Studies performed between 1980 and 2003 have shown a 80% decrease in the huemul population at Reserva Nacional Ñuble (Acosta-Jamett, 2004). After 1995, a strong decrease in the number of huemul specimens was observed and this was probably related to the great snow storm of 1995, which may be responsible for the disappearance of many groups both inside and outside the protected area. The same author suggests following more intense activities in order to reestablish the habitat, otherwise the species will became definitely extinct in central Chile.

In agreement with Locatelly & Mermillod, the use of reproductive biotechnology and methods of assisted procreation may help conservational programs orientated to the protection of deer species threatened by extinction. The implementation of classical techniques of production of embryos based on super ovulation, artificial insemination and embryonic transfer, have resulted extremely difficult for deer species. In addition to these, the characteristics of the huemul do not allow its successful husbandry in captivity. All the anterior prompted us to initiate this study concerning the production of cloned huemul embryos. From an ethical point of view, we must emphasize that our best interest is to

help the conservational programs by optimizing the process of clone reproduction. The techniques to be used do not posse any danger or suffering to the animals. On the other hand, the genome of the species will be conserved with no introduction of other genes; neither genetics manipulation procedures will be performed. The techniques to be used will not induce any alterations on the ecosystem.

The huemul an animal species seriously threatened by extinction (Povilitis, 1983a; Frid, 1991, 1994, 1999, 2001). It holds a great patrimonial value and it is one of the Chilean national emblems. The anterior constitute enough reason to justify the obtaining of a blastocyst embryo constituted by the inner cell mass from the huemul and the trophoblastic cells (future placenta) from another deer and to follow its subsequent development after the *in vivo* transference into a surrogate female.

As in the cases of the panda bear (Da-Yuan *et al*.), and the Saola ox, the lack of huemul females donors of ovocytes, receptive females and the interspecific barriers largely compromise the success of the cloning technique. In fact, embryos obtained by somatic cloning from a determined species, such as the huemul, can not be implanted into the uterus of a receptive female from a different species. In the implantation, only the extra-embryonic trophoblast is involved in the interactions associated with maternal tissues. To deal with this problem it will be necessary to construct a chimeric embryo constituted by trophoblast from the same species of the surrogated female and the inner cell mass from the huemul. This approach may allow the clone to develop successfully, and the trophoblast from a ruminant species (compatible with the receptive female) may allow a better implantation and the subsequent successful development of the clone. It must be kept in mind that the inner cell mass will originate the embryo and embryonic annexed and the trophoblast will produce the placenta.

The development of chimerical embryos will allow the role played by the trophoblast of the surrogate species in the growing of the experimental embryo to be established, and also to analyze some abnormalities in the embryonic development, comparing them with those found in the somatic cloning of some bovine species (Heyman *et al.* (1995). Therefore, the hypothesis of our lab is that "the development of embryos constituted by embryonic cells from the huemul and trophoblast cells from a different origin, such as the red or dama deer, will allow a better implantation and subsequent development of the embryo in a foster mother from different specie".

Several arguments in favor of the cloning programs of endangered species have been proposed by authors such as Ryder & Benirschke, 1997; Ryder, 2002; Lanza *et al*., 2000b; Wells; Ryder *et al*.; Ryder, Critser *et al*., 2003 and Rojas *et al*., 2004). However, once the clone birth as been achieved it is important to consider a care and observation program for the first generation of newborns. Holt *et al*. (2004) have indicated that the first generation of the cloned descendants must be kept in a proper place for observation and veterinary handling. An inadequate nuclear re-programming and phenotype abnormalities can compromise the success of the total population, however in some species these abnormal phenotypes are not inherited for the next generation. This has been observed in the mouse (Shimozawa *et al*., 2002) and the pig (Prather *et al*., 2003). These studies show that cloning may became a potential tool in conservationism, taking into account the restrictions of the effects on the first generation, on the one hand, and the possibility to obtain a second generation of healthy individual, on the other.

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RESUMEN: El clonaje somático por transferencia del núcleo de células diferenciadas adultas a un ovocito, al que se le ha extraído el núcleo (enucleado), es una técnica prometedora para la producción de embriones de alto valor genético. El mejor dominio del clonaje somático da la posibilidad de producir embriones de especies amenazadas de extinción.

El huemul es un ciervo andino autóctono, declarado como especie en peligro de extinción. tiene un gran valor patrimonial, y es emblema de la nación chilena. En este país, el huemul se encuentra protegido en trece Parques y Reservas Nacionales, manejadas por la Corporación Nacional Forestal (CONAF). Sin embargo, su protección se considera insuficiente debido a la baja conectividad entre las áreas protegidas y además, una gran proporción de estas áreas no son utilizadas o no constituyen un hábitat adecuado.

Para las especies de cérvidos en vías de extinción el uso de biotecnología reproductiva y métodos de procreación asistida, según varios autores, pueden ayudar a los programas de conservación. Las técnicas clásicas de producción de embriones basados en superovulación, inseminación artificial y transferencia embrionaria,en los cérvidos, han resultado muy dificultosa. Esto, sumado a las características del huemul, que no permite su estabulación en cautiverio, nos ha movido a iniciar un estudio tendiente a la producción de embriones clonados de esta especie.

PALABRAS CLAVE: Huemul; Clonación interespecífica; Riesgo de extinción.

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