

## BIOMIMETIC TECHNOLOGIES - USEFULL TOOL IN CONSERVATION OF ENDAGERED SPECIES

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### Abstract

*It has been suggested that artificial insemination (AI) and other forms of artificial reproductive technologies (ART) might be useful for genetic conservation of endangered breeds, the most widely applied ART being the cryopreservation of semen. However, the storage of frozen gametes of unknown fertility for long term is surely not a sustainable policy for the conservation programs of endangered species. The development of in vitro methods of testing sperm fertility would contribute considerably to conservation efforts. Sperm selection prior to AI could bring significant benefits in conservation breeding by improving sperm quality. Biomimetic technologies such as SLC (single layer centrifugation) have proven efficient in reducing viral titre and bacterial load in semen while improving routine and functional parameters such as motility, DNA integrity and acrosome integrity. Sperm selection has been applied successfully in conservation programs of different species such as Iberian Red Deer and grey wolves. We may conclude that biomimetic technologies represent a huge breakthrough in the sperm cryopreservation programs that will enable to obtain sperm cells with improved quality and higher longevity, therefore increasing the chances of obtaining off-springs from endangered species.*

**Keywords:** Biomimetic technologies; Sperm selection; Endangered species

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### Introduction

According to IUCN Red List Criteria (IUCN 2001), which is the world's most widely accepted system for measuring the extinction risk, Europe has a population of 231<sup>3</sup> mammal species, from which nearly 15% are threatened, another 9% being close to qualifying for threatened status. A declining trend was observed in more than a quarter (27%) of the populations, while 32% registered a stability trend. Furthermore, only 8% of species populations increased, mainly due to successful species-specific conservation action that were implemented by the European Commission.

The major threats for European mammals are represented by the destruction and/or partially fragmentation of habitats due to urban sprawl [1], introduction of alien species into ecosystems, hunting and exploitation pressures [2-3], intoxications by pollutants, hydroelectric developments and water pollution [5-6], diseases [7], artificial hybridization [8-9], global warming, especially for mountain species [10].

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Although extinction of some species is part of a natural process of evolution and is irreversible, this phenomenon is now occurring at a much higher rate mainly due to human activities. In the case of domestic species, extinction has been imposed by the management techniques and market demands for certain breeds. On the other hand, the aim of wild animal conservation is to maintain biodiversity because the removal of a single species can affect the functioning of entire ecosystems. Decisive conservation measures are needed to prevent such imminent extinction, the most pursued conservation measure alongside habitat protection, being the genetic resource banking [11].

### **Challenges that arise in artificial reproductive technologies (ART) in endangered species**

It has been suggested that artificial insemination (AI) and other forms of artificial reproductive technologies (ART) might be useful for genetic conservation endangered breeds. These technologies had a successful outcome only in research setting, but none have retrieved results sufficient to produce improvements in the genetic management of a wide population [12].

#### *Sources of spermatozoa*

The main problem in preserving male gametes is the availability of sperm cells, which are very difficult to obtain, as is the case for most wild endangered species. The epididymides of dead animals such as animals that have been found dead, shot by hunters or poachers, or that require euthanasia in zoological collections, can be used as a source of sperm. Unfortunately, such epididymal sperm samples are often contaminated with cellular debris, erythrocytes, leukocytes, and sometimes bacteria or viruses. These contaminants may be sources of reactive oxygen species, thus determining damage to the sperm cells during freezing. Semen may also be collected by electroejaculation such is the case of big cats [13], transrectal massage, a method used for elephants, buffalos [14].

#### *Cryopreservation of semen from wildlife species*

Cryopreservation of semen has been the most widely applied ART in farm animals, the years of research and field trials resulting in remarkable success in the cattle industry. For many non-domestic species, semen freezing techniques are similar to those used for closely related domestic species. For example, studies regarding the cryopreservation of semen from antelope or gazelle were based on the protocols developed for domestic cattle, while for the semen of wild equids, the protocol for frozen stallion semen was used. However, species like the cheetah, which are taxonomically unique may require extensive research in order to develop an appropriate protocol for sperm freezing.

The fertilizing capacity of spermatozoa should be taken into consideration when a cryopreservation protocol is developed, since there are involved several cellular properties and factors such as motility, morphologic integrity as well as properties indicative for maturation, capacitation, and acrosome integrity [11; 15].

Cryopreservation is supposed to extend the longevity of the sperm cells, by reduction of their metabolic activity through a controlled chilling/freezing procedure. Nevertheless, cold shocks represent a severe stress for sperm cells often determining cryodamage, at structural and functional level and low sperm survival rates. The degree of the cryo-injuries depends on the sperm physiology of the species [16]. For improving the success of cryopreservation techniques there are several cytological and cryobiological approaches which are taken into consideration such as comparative trials with semen extenders of different medium types (organic buffers, skimmed milk, egg yolk, and coconut water) and permeable cryoprotectants (DMSO, glycerol) [17-18], comparative chilling/ thawing regimes, sperm sorting (sexing) [19-20].

Cryopreservation protocols for wildlife mammalian spermatozoa have been developed for an increasing number of species, the sperm cells being stored in the genomic bank. The foundations of the genomic bank from Leibniz Institute for Zoo and Wildlife Research (IZW) were laid out in 1998 and since then, the collection has grown considerably, over 3500 samples from more than 500 species being stored. The collection includes gametes, blood, embryos, testicular and ovarian tissues as well as other body tissues from various wildlife species (Table 1). In 2007 the IZW initiated the “Felid Gametes Rescue Project” which included a collection of about 80 cryopreserved samples of spermatozoa, oocytes, embryos, testicular and ovarian tissues from 20 felid species. The aim of the project was to build up a European network for the extraction and storage of feline gametes, which are made available to breeding programs of zoos. The studies are now directed towards the establishment of AI, IVF, or intracytoplasmic sperm injection with cryopreserved gametes in species which are highly endangered such is the case of Iberian lynx [21].

**Table 1.** Cryopreserved semen from non-domestic mammals stored in the IZW spermbank and references documenting the cryopreservation of spermatozoa in this mammals

African elephant ( <i>Loxodonta africana</i> )	Howard et al. 1986 [22]
African buffalo ( <i>Syncerus caffer</i> )	Dankoff et al. 2001 [23]
African wild dog ( <i>Lycaon pictus</i> )	Hermes et al. 2001 [24]
Amur leopard ( <i>Panthera pardus orientalis</i> )	
Asian elephant ( <i>Elephas maximus</i> )	Saragusty et al. 2005 [25];
Black rhinoceros ( <i>Diceros bicornis</i> )	O'Brien and Roth, 2000 [26]
Brown bear ( <i>Ursus arctos</i> )	Anel et al. 1999 [27]
Cheeta ( <i>Acinonyx jubatus</i> )	Swanson et al. 1996 [28]
Chital deer ( <i>Axis axis</i> )	Haigh et al. 1993 [29]
Clouded leopard ( <i>Neofelis nebulosa</i> )	Pukazhenthil et al. 2006 [30]
China leopard ( <i>Panthera pardus japonensis</i> )	
Degu ( <i>Octodon degus</i> )	
American mink ( <i>Mustela vison</i> )	
Fossa ( <i>Cryptoprocta ferax</i> )	
Giant panda ( <i>Ailuropoda melanoleuca</i> )	Spindler et al. 2006 [31]
European brown hare ( <i>Lepus europaeus</i> )	
Iberian red deer ( <i>Cervus elaphus hispanicus</i> )	Martinez-Pastor et al. 2006 [32]
Impala ( <i>Aepyceros melampus</i> )	Loskutoff et al. 1996 [33]
Jaguar ( <i>Panthera onca</i> )	Swanson et al., 2003 [34]
Indian rhinoceros ( <i>Rhinoceros unicornis</i> )	
Leopard ( <i>Panthera pardus</i> )	
Lion ( <i>Panthera leo</i> )	
Eurasian lynx ( <i>Lynx lynx</i> )	
Swamp (or marsh) deer ( <i>Blastocerus dichotomus</i> )	
Mesopotamian fallow deer ( <i>Dama dama mesopotamica</i> )	
Musk ox ( <i>Ovibos moschatus</i> )	
Okapi ( <i>Okapia johnstoni</i> )	
Orang utan ( <i>Pongo pygmaeus</i> )	
Pallas cat ( <i>Felis manul</i> )	
Pig-deer ( <i>Babyrousa babyrussa</i> )	
Mountain lion ( <i>Puma concolor</i> )	
Rabbit ( <i>Oryctolagus cuniculus</i> )	Si et al. 2006 [35]
Red fox ( <i>Vulpes vulpes</i> )	
Roe deer ( <i>Capreolus capreolus</i> )	
Rusa deer ( <i>Cervus timorensis</i> )	
Saiga antelope ( <i>Saiga tatarica</i> )	
Snow leopard ( <i>Uncia uncia</i> )	
Sun bear ( <i>Ursus malayanus</i> )	
Asian tapir ( <i>Tapirus indicus</i> )	
Tiger ( <i>Panthera tigris</i> )	
White rhinoceros (southern: <i>Ceratotherium simum simum</i> ; northern: <i>C. simum cottoni</i> )	Hermes et al. 2005 [36]
Pannonian screw-horn sheep ( <i>Ovis aries</i> )	

## **Biomimetics technologies in action**

Biomimetics is the use of technologies and/or processes that mimic a naturally occurring event that takes place in the female reproductive tract. These methods can be divided into 2 categories: removal of seminal plasma only (sperm washing) and selection of spermatozoa with specific characteristics, such as motility (sperm migration), membrane integrity (filtration). Colloid centrifugation, a method which is also included in the second category selects spermatozoa that present all the characteristic mentioned above: sperm motility, morphology, viability and chromatin integrity. This method can be subdivided into density gradient centrifugation (DGC) and Single Layer Centrifugation (SLC) [37]

### *Sperm washing*

This method separated the spermatozoa from most of the seminal plasma from [38], but the sperm cells are not selected therefore, dead, abnormal cells [39] as well as sources of reactive oxygen species (ROS), which are detrimental for sperm survival may remain in the washed sample. Many of the commercial extenders due contain antioxidants which may counteract ROS released during centrifugation.

### *Migration of sperm (Swim-up)*

This method relies on the ability of motile spermatozoa to move in the suspension. The original sperm population may be placed underneath, on top of, or on one side of the migration medium [40]. Since the seminal plasma (SP) has no motility, the spermatozoa will move away from the SP. Unfortunately, this method does not provide any selection based on chromatin integrity, viability, or acrosome integrity [41], but spermatozoa with tail abnormalities are prevented from migrating into the swim-up medium. Comparative studies on washing and swim-up showed significantly better midpiece- and tail-morphology after swim-up [39]. By adding hyaluronic acid to the migration medium a higher percentage of with intact membranes was obtained [42]. Unfortunately, the low-recovery sperm rate, for example, 10%–20% [39], makes this method impractical for AI in most mammal species.

### *Filtration of sperm*

This technique uses different filter substance, such as, glass fibers, Sephadex beads, or membrane pores, but the sperm selection is also based on sperm motility [43]. Non-viable spermatozoa will adhere to the matrix [44], although the mechanism of action is uncertain [45]. Due to the changes in surface charges [46], immotile and dead spermatozoa may also form agglomerates that will bind to the sephadex particles [47]. Filtered bovine spermatozoa showed improved post-thaw viability compared to unfiltered spermatozoa [48]. Since a certain amount of SP and cellular debris may remain in the sample, the sperm yielded through filtration is not as clean compared to the separation method [49]. Still, fewer spermatozoa are lost compared to the other methods, a sperm recovery rate of approximately 63% being reported [48].

### *Colloid Centrifugation*

Extended semen is centrifuged through a colloid, spermatozoa with specific characteristic (motile, viable, chromatin integrity) being separated from the SP and debris. The method is based on the ability of sperm cells to move in the gradient to a point that matches

their own density—the isopycnic point [50]. By modifying the centrifugation force and time, as well as the physical properties of the colloid, the good quality spermatozoa will form a pellet in the bottom of the centrifugation tube. The DGC, uses several layers of colloid of different density (hence “density gradient”), while SLC uses a single layer species-specific colloid (Androcoll) [37], thus, being easier to used and less time consuming [51]. Species-specific formulations of this new developed colloid (with a suffix to denote the species) have been developed for stallion [37], boar [52], bull [53], dog [54], and cat [55]. The SLC-selected samples showed both better sperm motility and chromatin integrity, characteristics which were maintained during a 72 hours survival test.

The colloid centrifugation removes the ROS sources (cell debris, leukocytes, epithelial cells and dead or dying spermatozoa), thus improving the sperm survival during frozen or chilled storage. Another advantage given by this method is the bacterial contamination control without antibiotics supplementation. SLC was successfully associated with swim up method to reduce viral infectivity from boar semen spiked with porcine circovirus type 2 [56]. Studies performed by Morrell et al., 2012 [57] on horse semen showed that SLC significantly reduces the equine arteritis virus titer in fresh and stored semen but did not completely eliminate the virus. Since spermatozoa may function as vectors for viruses [58], further work is required to investigate how closely different viral particles are associated with the sperm membrane. The potential application of this method would be the improvement of sperm quality in AI doses [59]; the removal of pathogens [60-61]; improvement of cryo-survival by removing dead and dying spermatozoa [62-63]; Selection of spermatozoa with normal morphology and good chromatin integrity for AI [64] or IVF [53];

Biomimetic sperm selection using colloid centrifugation has been applied in reproductive programs especially designed for the conservation of certain species such as Iberian primitive ruminant breeds [65], Iberian red deer wild mountain ruminants [66-67] gray wolves [68]. These studies may be the ground base for investigations in other endangered species such as the big cats, which, accordingly to previous studies, are producing extraordinarily poor quality ejaculates, therefore the use of traditional AI with thawed sperm as an adjunct management tool would be particularly challenging.

## **Conclusions**

In species with only very few individuals remaining, such is the case of Iberian Lynx, which may not mate naturally due to various reasons such as sibling relationship, major histocompatibility complex incompatibility, artificial reproduction based on cryopreserved germ cells may provide a significant contribution to the survival of species on the board of extinction. Cryopreservation of semen offers can reduce the potential influence of inbreeding by offering the possibility to determine the mating partners (Ludwig 2006). If the cryopreservation protocols are adapted to species-specific conditions, the preservation of sperm cells may become a valuable tool in conservation programs for rare and endangered species (Holt and Pickard 1999).

On the other hand, cryopreservation may induce cryo-injuries to the sperm cells, thus future projects of conservation must include research regarding the longevity and nonetheless, the fertility potential of the cryopreserved gametes since the storage of frozen gametes of unknown fertility for long term is surely not a sustainable policy. Biomimetic selection of the best quality spermatozoa for AI or for cryopreservation could improve pregnancy rates

especially in species with poor quality sperm [69]. Furthermore, the development of in vitro methods of testing potential fertility would contribute considerably to conservation efforts.

We may conclude that the use of biomimetic technologies for sperm selection represent a huge breakthrough for sperm cryopreservation programs, enabling to obtain sperm cells of improved quality and higher longevity, therefore increasing the chances of obtaining off-springs from endangered species.

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