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Conservation of genetic resources of subfamily Bovinae by the establishment of a cryo-bank

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Abstract: Conservation of genetic resources is connected with numerous problems which solution is crucial for the success of the entire program. At present the only effective method of long-term conservation of genetic material is its deep freezing in liquid nitrogen at -196°C. Some positive results were also obtained with the use of liquid oxygen which allows the use of lower temperatures. Cryo-conservation of sex cells of representatives of the Bovinae subfamily is widely applied in breeding practices. Experiences in cryo-preservation of sex cells of the European bison (*Bison bonasus)* and yak's (*Bos mutus)* are discussed.

Key words: Bovinae, European bison, yak, sperm, semen cryopreservation, electro--ejaculation, male sex cells, cryo-banks

Introduction

The solution of problems connected with conservation of genetic resources is crucial for the success of the entire program. Presently the only effective method of long-term conservation of genetic material is its deep freezing in liquid nitrogen at -196 C. Some positive results were also obtained with liquid oxygen which allows the use of even lower temperatures.

While selecting objects for sampling genetic material for cryo-banks, preference should be given to animal species threatened with extinction and highly important for people (from the economic, social etc. points of view).

Of various types of genetic material, such as female and male sex cells, embryos and cultures of somatic cells suitable for cryo-conservation, an optimal choice is the sperm, which freezing is relatively simple and efficient. Opinions diverge widely as regards the species representation in cryo-collections, and general consensus is still to be reached on this matter. For the time being, we accepted temporary regulations stipulating that each species should be represented in a cryo-bank by genetic material from all subspecies (or populations), whereas each taxon – by material from no less than seven specimens (based on the assumption that seven out-bred specimens are representative for about 75% of the population gene pool. Still, it appears advisable to take samples from 50 specimens, i.e. the number of animals ensuring a short-term survival of populations (Soule, 1987). For the species difficult to access, all their available genetic material should be conserved in a cryo-bank.

An important aspect of the establishment and operation of cryo-banks is the system of registration of conserved objects based on a standard, commonly accepted procedures, ensuring its continuity, determination of the required (minimal and maximal) quantity of sperm doses, and a program for the cryo-collection replenishment (dynamics).

Various sperm sampling techniques presently used may be grouped into the vaginal (vaginal proper and sponge-based), urethral (masturbation, use of fistulae, sperm collector, massage of the deferent duct ampullae, electro--ejaculation and simulated vagina) and surgical methods. In the group of vaginal methods the sperm is taken from the vagina of a female in oestrus, after coupling with a male, whereas in the urethral methods the sperm is sampled directly from the male deferent duct.

Naturally, investigators should have adequate equipment and experience allowing for the use of all techniques, though collection of genetic resources of wild animals involves certain peculiarities. To collect semen by the vaginal techniques as well as through masturbation and use of artificial vagina, it is necessary to have specially tamed and easily controlled animals. Organization of this work with domestic animals is not problematic, whereas in wild animal species such individuals are always rare, and application of the latter techniques will be insufficient for establishing a collection representative for the entire species gene pool.

Electro-ejaculation and surgical extraction of sperm are the best suited techniques to meet the requirements for the collection of genetic resources for cryo-conservation. In the former Soviet Union the first experiments based on the use of electricity for collecting sperm were carried out in 1933 by Serebrovsky and Sokolovskaya who demonstrated the possibility of obtaining bird sperm by electro-ejaculation. Application of this technique enables investigators to obtain semen from virtually any sexually mature animal with multiple repetition of this operation. This method requires a reliable fixation of experimental animals, and in work with wild animals this is possible only after their immobilization. Immobilization of animals is important however often requiring a complicated course of action, which needs a detailed description. The experience on this subject is presented most exhaustively in the manual written by Chizhov (1992). A disadvantage of this method is possible excretion of urea together with the ejaculate that deteriorates considerably the semen quality.

A method for surgical extraction of sperm from the testis appendage of a live animal, or more commonly after its death, was worked out by Ivanov (1907). He proposed to carry out artificial insemination with the sperm obtained by this method from high quality males, which lost their ability to cover females due to injuries or those which perished in accidents. This technique was also proposed for collecting material for hybridization experiments. The major obstacle restricting the usage of this method in the work

with rare animal species is the necessity to preserve each individual. However in many species the participation of males in reproduction is restricted by their age. For example, 10-year and older American bison males are usually ousted from the reproduction process by younger males, whereas normal spermatogenesis in this species continues up to the age of 18 years. Therefore, the removal of such animals from a population with an aim to preserve their genetic material seems well justified.

Further work with the extracted sperm includes following steps: preparation of sperm for artificial insemination (dilution, development of a cryo-protection protocol), storage (including freezing and thawing procedures), introduction of artificial semen into the cervical canal of female(s) and control of the sperm quality. Preparation of artificial sperm, protocols specifying usage of cryo--protective agents and working conditions are usually based on techniques, well-established for farm animals. To determine optimal conditions for handling the sperm from individual animal species, it is advisable to conduct experiments with the semen obtained in required quantity from specially tamed animals, and to take into account morphological features of their spermatozoa, because species peculiarities of individual specimens are also manifested in morphology of spermatozoa, and in each specimen they display a set of indices characteristic solely for a given species. The sperm morphology has been described for numerous ungulate species by Steklenev (1971).

Techniques of the sperm quality control are standard, and widely used in practical work with domestic animals. However, the most objective criterion for the sperm quality is the production of a healthy offspring after artificial insemination. The process of sperm introduction into the genital tracts of an immobilized female in oestrus is not highly complicated. First it requires a group of females with restricted freedom of movement. The best solution is a nursery for breeding a particular species where it is possible to select a female in heat or to bring a female in oestrus through treatment with appropriate drugs. In nurseries it is possible to breed descendants from the animals which are carriers of a preserved genotype and to prepare them for the release into wild conditions. In such nurseries investigators may also use a frozen sperm in 2–3 generations after its collection for maintaining the level of genetic variability, indispensable for sustaining a viability of a small population during long-term breeding.

We believe that genetic studies should be regarded as an integral component of the entire complex of measures aimed at long-term conservation of genetic resources of the wild fauna as male sex cells in cryo-banks. Each species, for which it was decided to conserve its gene pool in a cryo-bank in a form of sex cells, should undergo genetic examination aimed at determination of its genetic structure. In our opinion, the genetic inventory of particular species should involve various genetic techniques covering several levels of life organization. Following this principle, the work on conservation of the

European bison gene pool discussed in the present paper included analysis of DNA polymorphism, cytogenetic studies, evaluation of biochemical polymorphism as well as immuno-genetic analysis aimed at determination of blood groups. A knowledge of genetic structure (gene pool) will allow for ensuring maximal representation of actual genetic polymorphism of the species, available during the collection period of material for a cryo-bank. Such an approach would permit conservation of the species gene pool without considerable losses. Another situation requiring application of genetic techniques is certification (issuing passports) for animals which are donors of the sperm. The availability of information on the genotype of the sperm donors would surely be of great help especially when coupling such donors with selecting recipient females for artificial insemination.

The establishment of an international network of genetic cryo-banks would require earlier genetic inventory and certification based on a commonly agreed scheme and use of standard techniques.

Attempts to conserve genetic resources of wild animals in form of sex cells stored in cryo-banks has rather long historic record, and there are reviews covering activities in this area over a considerable period (Graham *et.al.*, 1978; Seager *et.al.*, 1988). The aim of the present review is to draw the attention of experts to the results of studies conducted in the Russia, since their results may be unfamiliar to many specialists interested in this problem, but working outside of Russia.

A review of studies on various ungulate species of Russia allowed for the collection and systematisation of their results. In the majority of cases, these works do not describe the complete sequence, starting from the extraction of sperm, its freezing, artificial insemination, offspring generation, genetic inventory of population and finally the certification of donors. So far, no sufficiently representative cryo-collection has been established yet for any studied species. Described experiments were basically aimed at isolation of animal sperm for economic purposes and inter-species hybridization.

Results and Discussion

Cryo-conservation of sex cells of representatives of the Bovinae subfamily is widely practiced in breeding of cattle (*Bos taurus*), zebu (*Bos indicus*), domestic buffalo (*Bubalus bubalus*). Techniques for isolation and cryo-conservation of sperm from wild populations of this subfamily are very similar to those used in work with farm animals. So, electro-ejaculation was successfully used by Junior et al. (1990) to obtain sperm of gaur (*Bos gaurus*) for its subsequent freezing and use in artificial insemination, whereas Seifert *et al.* (1974) succeeded in getting the sperm of anoa (*Bubalus depressicornis*) with the help of a phantom. Dorn *et al.* (1990) reported a study on artificial insemination of American bison (*Bison bison*) females with a frozen-thawed sperm.

Experiments aimed at collecting sperm from yak (*Bos mutus*) were conducted by Usupov (1981) in the Tyan-Shan mountains area. A study concerning the same species was also reported by Graham *et al*. (1978). Three yak males and one female were brought for experiments from a highland area (3000 m above sea level) to a valley (670 m above sea level). After a 3-month adaptation to new conditions the oestrus was induced in the female to stimulate sexual activity in the yak males. For this purpose, the yak female received five successive 50 mg doses of progesterone within two days followed by an injection of CGS (Cowper's gland secretion) at a dose of 2000 U. One day later the female achieved the oestrus that lasted 24 h. During the copulation of a tamed yak male with this female, the sperm was collected in a simulated vagina devised for bulls. The sperm quality was determined by standard techniques, and the results obtained are presented in Table 1. In experiments on yak sperm freezing in granules the best results were obtained by a fast freezing method based on the use of the medium composed of:

- twice distilled water, 100 ml;
- lactose, 11.5 g;
- egg yolk, 20 ml;
- glycerol, 5 ml.

The estimated activity of thawed sperm was 4.0–4.5 units. Comparison of the parameters of yak sperm with those of the Alatau race bull sperm, cryo--conserved with the same procedure, did not reveal any significant differences.

Table 1. Characteristics of yak (*Bos mutus*) sperm (n = 25) Usupov (1981)

Characteristics	Minimal	Maximal	Mean
Ejaculate volume (ml)	2.00	4.50	3.25
Concentration (10^9/ml)	1.20	1.80	1.50
Activity (units)	8.00	9.50	8.75
Resistivity (10^3)	20.00	40.00	30.00
Dehydrogenase activity(min)	6.00	11.00	8.50
Pathologic spermia (%)	12.50	20.00	16.25
Absolute survival index (h)	135.00	190.30	163.65
Viability reserve (h)	6.50	8.50	7.50

Electro-ejaculation and massage of the duct's deferent ampullae were used to obtain 18 normal 1.0–2.0 ml ejaculates. Concentration of sperm reached 0.18–2.77 x 10^9/ml with an activity of 7–8 units. The sperm thus collected proved to be well-suited regarding its physiological parameters for insemination in a concentrated form after storage at 0–2°C, as well as when diluted with a synthetic medium after deep freezing.

According to Steklenev (1987), relatively small ejaculate volumes and considerable variation of sperm concentration in individual ejaculates were basically due to an inhibition of sexual reflexes in bison bulls during experimental manipulations and ejaculation of only the portion of sperm which was contained in the deferent duct ampullae at that moment.

Extensive experimental work with European bison from the Prioksoko--Terrasny Biospheric Reserve, Moscow Zoo, and the Nadvirnyansky protected natural area (Ivano-Frankovsk region, Ukraine) was reported by Sipko *et al.* (1994; 1997).

Conventional techniques of sperm collection (simulated vagina, massage--based stimulation) are effective solely with especially tamed donor animals which are not sufficiently numerous to ensure representativeness of genome collections in cryo-banks. Therefore, we used the techniques of ejaculation by electro-stimulation and *post mortem* sperm isolation from the epididymis. In the first case the source of the electric current was a dynamo for a bicycle headlight, allowing for the regulation of the frequency and voltage of the current pulses with pedalling. In the second case the sperm was isolated either in field conditions immediately after shooting of an animal or in the laboratory after transporting there the animal's genitals. In both versions the sperm was isolated from fragmented epididymis tissues. When the sperm was extracted immediately after animal death, it was placed into a thermos with synthetic medium at 4°C for 4 h; within this time the sperm was transported to laboratory for filtration before freezing. When the donor testes were brought to the laboratory within 1 h after animal's death, filtration was also performed prior to a 4-h equilibration at 4°C. The sperm was diluted with the lactose-yolk-glycerol medium (pH 6.8). After equilibration the semen was frozen in two steps: the sperm was transferred by 0.2 ml aliquots into wells on solid carbon dioxide plates. The granules formed there were kept for 5 min at the plate surface and then the plate was placed into a tray filled with liquid nitrogen to carry out the second freezing step.

The sperm obtained by electro-ejaculation was processed using a different procedure. An ejaculate was diluted with a cryo-protective mixture and frozen in liquid nitrogen vapour as granules on a ftoroplast plate with subsequent immersion into liquid nitrogen for storage until its usage. Biological evaluation of the sperm quality was carried out immediately after freezing and after few years of storage in liquid nitrogen. For this purpose, the sperm was thawed by placing granules into a test-tube with 2.9% sodium citrate solution (1 ml) which was then placed into a water bath maintained at 39–40°C. Immediately after thawing, the sperm quality was estimated on the basis of sperm mobility in unity fractions, extent of acrosomes preservation by the technique reported by Sokolovskaya *et.al.* (1982) and live sperm colouring using the procedure developed by Morozov (1938). The dynamics of sperm survival was studied by incubating the sperm in a thermostat at 37°C.

Samples of blood and muscle tissue were taken from all bull donors for a dual purpose: cryo-banks of genetic resources and genetic studies. Immunogenetic analysis was used to determine phenotypes on the basis of blood groups, electrophoresis allowed for the acquisition of the spectra of enzyme systems, whereas the technique of isoelectric focusing provided the spectra of proteins in blood serum and muscle tissues. The fertilizing capacity of cryo-conserved sperm was determined by the way of insemination of physiologically normal cows of the black-white breed, weighing 450–500 kg, and not having visible abnormalities after three calvings.

Table 2. The quality of sperm in E. bison (*Bison bonasus*) before and after cryopreservation

EBPB No and name	Sperm mobility (unity fractions)			Number of sperm (10^6/ml) before cryo-conservation			
	Before freezing	Immediately after thawing	4 hours after thawing	total	Live and full-fledged		
					By mobility	Acrosome preservation	Live staining*
5213 Morus	0.6	0.3	0.4	16.4	5.50	8.60	8.60
4069 Mont	0.7	0.4	0.2	7.8	3.15	4.88	3.36
3337 Meron	0.2	0.1	0.0	0.3	0.00	0.00	0.00

* Live staining according to Morozov (1938)

In Table 2 given are the parameters of the sperm obtained by electro-ejaculation (Meron), isolated from testes of shot animal (Morus) and in laboratory (Mont). The adult bull shot in the Ivano-Frankovsk region was found to have underdeveloped genitals. Such facts as well as instances of cryptorchism are rather frequent in this species. The lowest parameters were found for the electro-ejaculated sperm, and this might be due to a high stress of the sperm donor and imperfection of the protocol. In two other cases such characteristics were much similar before and after cryo-conservation (Tabl. 2, 3).

Table 3. Effect of cryo-conservation on the preservation of sperm membranes in E. bison (*Bison bonasus*)

EBPB No and name	Preservation of acrosome (Sokolovskaya)			Live staining (Morozov)		
	Before freezing	Immediately after thawing	4 hours after thawing	Before freezing	Immediately after thawing	4 hours after thawing
5213 Morus	89.0±1.6	54.5±2.5	44.0±2.4	54.0±2.5	46.0±2.4	28.0±2.2
4069 Mont	82.5±1.9	67.0±2.3	42.0±2.5	58.0±2.4	43.0±2.4	26.0±2.0
3337 Meron	0	0	0	0	0	0

Table 4. Effect of the duration of cryo-conservation on sperm quality in E. bison (*Bison bonasus*)

EBPB No and name	Number of sperm counted	Sperm mobility (unity fractions)			Preservation of acrosomes [%]		
		Before freezing	After storage		Before freezing	After storage	
			1 month	3 years		1 month	3 years
5213 Morus	200	0.6	0.3	0.3	80.0±2.0	54.5±2.5	55.0±2.5
4069 Mont	200	0.7	0.4	0.4	82.5±1.9	67.0±2.3	64.5±2.3
3337 Meron	200	0.2	0.1	0.1	0	0	0

After 3-year storage in liquid nitrogen the mobility of sperm and the extent of acrosomes preservation remained virtually unchanged (Tabl. 4).

To check the fertilizing capacity of sperm after cryo-conservation, 8 cows (*Bos taurus*) were inseminated with a thawed sperm. None of the cows calved after the insemination during the first heat period, the second insemination resulted in the birth of 3 hybrid calves, but the only male calf died after three days. After the third insemination another male calf lived only four days (Sipko *et al.* 1997; 1998; Ernst *et al.* 1993).

According to the electrophoresis-based spectra of 12 systems of enzymes encoding 18 loci, all bison bulls were monomorphous, and only in Morus the polymorphism was revealed in the phosphoglucomutase (Pgm) *locus*. Isoelectric focusing (ISF) revealed spectral variability of muscle tissue proteins only in the alkaline spectral zone, and variants with regard to this index were again found solely in Morus. ISF analysis of the spectra of blood serum proteins did not reveal any variability in the studied animals.

The above genetic analysis of the sperm of European bison has shown that tested specimens were not unique regarding their gene set and the level of their polymorphism corresponds to average values for the population. But it is noteworthy that the antigen H' revealed in Mont is rare and characteristic for no more than 0.8% of the entire E. bison population, whereas the polymorphism of two systems revealed in Morus indicates for a high value of this genotype as an object for cryo-banking. It should be emphasized that genetic certification of sperm donors should be an indispensable condition for the establishment of genome cryo-banks.

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Ochrona zasobów genetycznych podrodziny Bovinae poprzez utworzenie krio-banku

Streszczenie: Ochrona *ex situ* zasobów genetycznych jest powiązana z szeregiem problemów, których rozwiązanie jest kluczowe dla sukcesu całego programu ochrony. Aktualnie jedyną efektywną metodą długotrwałego mrożenia materiału genetycznego jest przetrzymywanie w ciekłym azocie, w temperaturze –196°C. Uzyskuje się czasami pozytywne efekty przy zastosowaniu ciekłego tlenu, który umożliwia przechowywanie w niższych temperaturach. Krio-konserwacja komórek rozrodczych pozyskanych od przedstawicieli gatunków należących do Bovinae jest szeroko stosowana w praktyce hodowlanej. W pracy omawiane są wyniki zamrażania komórek rozrodczych żubra (*Bison bonasus*) oraz jaka (*Bos mutus*).