
Study of semen parameters in captive brown bears (*Ursus arctos*)

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Abstract

As the Carpathian brown bear still represents one of the biggest populations of bears across Europe, the study of its semen parameters as well as cryopreservation can help advancing the research regarding conservation of genetic diversity for the endangered sub-species of European brown bear. The aim of this paper was to study the parameters of the Carpathian brown bear semen and to attempt its liquid storage at 4°C. The study was performed on two bears, one belonging to the Turda zoo and another to the Libearty bear sanctuary in Zarnesti. We obtained one sample of semen by electro-ejaculation from an 18 years old captive brown bear from the Turda zoo, while the Libearty bear sanctuary provided us with a pair of testicles from a 2.2 years old male, from which we obtained semen by retrograde flushing of the epididymis. We analyzed those samples in 2 different extenders, before and after 24h refrigeration, using the CASA system for concentration and motility and the flow-cytometer for viability. The results showed that semen obtained by electro-ejaculation had good motility, which decreased after 24h of refrigeration, while viability was adequately maintained. The epididymal extraction also provided semen with high motility and viability, but liquid storage at 4°C was unsuccessful. This study is just a beginning, as it needs to be further continued, in order to reach the goal of creating a sperm bank for the Carpathian brown bear and offer new data that could help the preservation of endangered populations of other European brown bears.

Key words: brown bear, semen collection, CASA, flow cytometry

Introduction

Bears are a large-bodied mammal of the order Carnivora of the family Ursidae. There are 8 species of bears around the world, living in 4 continents, in a variety of habitats from the tropical rainforest, to the arctic ice. The present research is focused on the brown bear (*Ursus arctos*), more specifically the Eurasian sub-specie (*Ursus arctos arctos*), which is one of the largest mammals of Europe, together with the lynx, the wolf and the wolverine (2). Studies showed that brown bears were an essential part of the nitrogen cycle in their ecosystem, spreading nitrogen around with salmon's carcass they had eaten, or by their urination and defecation in the environment (6). Moreover, studies by Gittleman in 2001 (4) insist on showing the consequence of predator's disappearance on the prey population. The non-regulated increase of prey populations has a direct impact on the balance of flora, which then lead to an ill equilibrium of the ecosystem. Romania still has one of the largest brown bear population of Europe, with approximately 7,000 individuals in it. The Romanian bear population even outnumbers the maximal capacity of their habitat and causes problems with the people surrounding it (3). When the drastic diminution in bear populations amongst others all over Europe was noticed in the 1980s, the European commission decided to put in place a juridical tool with the goal to protect the wild fauna and flora of Europe as well as its natural habitats. At the level of the bear populations, they decided to track them, study them in order to prevent future needs. They also decided to translocate bears of the same clade from northern region of Europe to add females and allow the population to grow faster, as a single male is capable of mating with multiple females (7,8). The establishment of a specific genetic resource bank requires the development of sperm cryopreservation procedures in brown bears (1,6). The sperm cryopreservation development is complicated by many issues like the lack of specific studies on the subject and problems on the quality of sperm during harvesting. The large

population of brown bears located in Romania could be used as a surrogate for the European brown bear, in order to study the sperm specific parameters and find protocols for successful cryopreservation and insemination. The aim of this study was to learn more about the Romanian brown bear semen parameters and the effect of two extenders, Triladyl and TRIS-based, on the quality of samples, before and after liquid storage at 4°C.

Materials and methods

The first semen sample was collected on a captive brown bear from the Turda Zoo, when it was transferred to Târgu-Mureş Zoo. The male was eighteen years old and weighed around 350 kilograms. The whole protocol included general anesthesia, monitoring and electro-ejaculation.

The anesthetic protocol: the bear was anesthetized with a cocktail of Zoletil (Tiletamine-Zolazepam) and Xylazine. It received four vials of Zoletil 50, each vial containing 5 ml with 25mg/ml of tiletamine and 25mg/ml of zolazepam, so a total of 500 mg of tiletamine and 500 mg of zolazepam. It also received 16.5 ml of 2% xylazine, so a total of 330 mg of xylazine. The total dose was divided in four separate anesthetic darts which were shot on four different spots, while the bear was still in its isolation cage. Although the bear managed to remove some of them after they were shot, the fluid had already been discharged and the general anesthesia settled very quickly, in 2-3 minutes. The male was then removed from his cage and pulled next to the transportation wagon that was already prepared. The vitals were checked, and all parameters were within normal limits (pulse, respiration rate), while the animal was completely numb, in lateral recumbency. Ophthalmic ointment was applied on the cornea so it would not become dry as the eyes were opened, and the bear could not blink.

For the electro-ejaculation protocol: the hair around the preputial opening was clipped and the area was cleaned with dry paper towel. The probe of the electro-stimulator (Premier 1) was inserted into the rectum and stimuli were gradually applied. Erection was initially very weak, and several electric stimuli were applied until a slight erection was obtained. The penis was manually manipulated in order to remove it out of the prepuce. Initially a small amount of watery liquid was obtained and in order to avoid urine contamination, the collection bottle was changed multiple times. After several more stimuli, a semen sample was obtained. The sample's motility and density were subjectively evaluated under the microscope and then diluted.

One ml of the semen sample was diluted using a TRIS-based extender. Another ml was diluted with the commercial extender Triladyl, while a third ml was left raw. The dilution in all those tubes was performed in a 1:1 ratio with the extenders at 37°C, allowed to cool to environmental temperature, which was around 27°C and then another ml of extender was added. The diluted samples were transported to the laboratory within 45 min, where they were analyzed using the CASA system and flow-cytometry and then submitted to refrigeration for 24 hours.

The second semen sample was acquired from the testicles of a 2.2 years old male, thanks to a collaboration with the Libearty Bear Sanctuary, where they castrate their male bears in order to avoid reproduction.

Anesthesia was induced with a detomidine and ketamine cocktail, using a hypodermic gun. As soon as the male was asleep, the surgical procedure took around ten minutes, and then anesthesia was reversed with Atipamezole. After castration, the testicles were kept on ice for 24h before they could be processed in the laboratory, due to transportation and organizational issues.

The protocol of semen collection from the testicles involved their dissection in order to retrieve the epididymis. For each testicle, the connective tissue and the tunica vaginalis was removed and an epididymo-deferentectomy was performed in order to isolate the cauda epididymis and vas deferens from the testicle. The standard technique was used, consisting of catheterizing the

vas deferens with an 18-gauge needle and retrograde flushing of the vas deferens and epididymis with 5 ml of warmed (37°C) commercial Triladyl extender for one testicle and TRIS-based extender for the other testicle.

Preparation of the sample for CASA was done by diluting 1µl of raw semen with the respective extender in a 1:300 ratio. It was then placed on a prewarmed Leja slide, while analysis was performed using the appropriate CASA modules.

Sperm viability was assessed using a BD FACS Canto II flow. Staining was performed using SYBR-14/PI according to the following protocol: 10µl sperm was diluted at 1:100 ratio and 4µl SYBR-14 as well as 1µl PI were added. The three components were mixed and incubated for 20 minutes at 37°C. Examination was performed right after the incubation. Fluorescence compensation was performed, as SYBR-14 and PI spectra slightly overlap. Detection of fluorescence was made using the 488nm, blue, air-cooled, 20 mW solid state excitation laser, as well as the 530/30 filter for SYBR-14 and 575/26 filter for PI. FACSDiva 6.1.2 was the software used for analysis.

Results

The volume of the sample obtained by electroejaculation was about 3 ml, and had a creamy white color, without any abnormal smell. A droplet was put under the microscope for a subjective evaluation of motility, which was about 80%, and density, which was rare (below 0.5 billion/ml).

The results obtained for CASA evaluation of fresh bear semen obtained by electroejaculation and diluted with TRIS-based and Triladyl extenders are shown in table 1.

Table 1 CASA evaluation fresh bear semen obtained by electroejaculation, diluted with TRIS-based and Triladyl extenders

	Concentration (million per ml)	Concentration (million per sample)	Motility (%)	Motile spz. (million per ml)	Motile spz. (million per sample)	Progressive motility (%)
Sample 1 TRIS	28,40	85,20	82,42	23,41	70,42	36,91
Sample 1 Triladyl	28,61	85,83	81,27	23,25	69,75	31,15

Results of for CASA motility after 24 h of refrigeration of bear semen obtained by electroejaculation diluted with TRIS-based and Triladyl extenders are shown in table 2:

Table 2 CASA motility after 24 h of refrigeration of bear semen obtained by electroejaculation, diluted with TRIS-based and Triladyl extenders

	Motility (%)	Progressive motility (%)
Sample 1 TRIS 24 h	77,87	21,15
Sample 1 Triladyl 24 h	71,09	17,47

Results obtained for sperm viability by flow cytometry on fresh bear semen obtained by electroejaculation, diluted with TRIS-based and Triladyl extenders are shown in table 3:

Table 3 Sperm viability of fresh bear semen obtained by electroejaculation

	Triladyl	TRIS
Viability (%)	92,0	99,7

Results obtained for sperm viability by flow cytometry on bear semen obtained by electroejaculation, after 24h of refrigeration in TRIS-based and Triladyl extenders, are shown in table 4:

Table 4 Sperm viability of fresh bear semen obtained by electroejaculation

	Triladyl	TRIS
Viability (%)	91,2	99,1

For the second sample, flushing of semen from the epididymis was successful for both testicles, while the microscopic examination showed a lot of immature spermatozoa, which is normal, as the bear was only 2.2 years old. Nevertheless, subjective motility was 85%.

The results obtained for CASA evaluation of fresh bear semen obtained by retrograde flushing of the epididymis and diluted with TRIS-based and Triladyl extenders are shown in table 5. After 24h of refrigeration, the subjective analysis showed that all spermatozoa had died.

Table 5 CASA evaluation fresh bear semen obtained by retrograde flushing of the epididymis, diluted with the TRIS-based and Triladyl extenders

	Concentration (million per ml)	Concentration (million per sample)	Motility (%)	Motile spz. (million per ml)	Motile spz. (million per sample)	Progressive motility (%)
Sample 2 TRIS	11,85	8,30	84,65	10,03	7,02	79,61
Sample 2 Triladyl	42,0	42,0	89,19	37,46	37,46	75,14

The results of viability for sample 2 using flow-cytometry was above 99% for both extenders, with slightly better results for the TRIS-based extender (table 6).

Table 6 Sperm viability of fresh bear semen obtained by retrograde flushing of the epididymis

	Triladyl	TRIS
Viability (%)	99.2	99.9

This study is just a timid beginning of what we desire it to be, an ampler research on the conservation of genetic material in brown bear. We did not have enough samples for conclusive results, but we still considered them worth presenting, as they represent the first such attempts in Romania and we hope to raise awareness and open new opportunities for further research and collaboration.

The collaboration with the bear sanctuary Libearty is a real opportunity to study the semen from epididymal extraction, which is understudied worldwide in brown bears, and real source of

genetic diversity for conservation, once the ideal protocol for cryopreservation of brown bear semen is found. This ampler research could help find the ideal protocol for the other sub-species of brown bears endangered, as the Romanian brown bear could be studied as a surrogate. The biological material collected with the collaboration of the Libearty bear sanctuary could become a bank of great genetic diversity and resolve present and future inbreeding problems in zoos and in the wild, as it could give more time for long term solutions, like management of corridors against fragmentation of the habitat, resolution of conflicts, management of anti-poacher strategies, etc.

Moreover, those results add elements to the understanding of the sperm cells in this species and help to further push the studies in order to find the best protocol for the creation of a sperm bank.

The results obtained for the sample 1 make us confident that in the future, we will be able to establish a stable protocol for bear semen refrigeration, and allow us to hope that bear semen freezing will be possible with a high success rate, as it is already possible and done but with a low success rate. Therefore, I will allow artificial insemination, for a greater genetic diversity as resolution of inbreeding problems in bear populations.

The fact that sample 2 did not survive the 24h refrigeration shows that the male needs to be sexually mature for semen conservation, as most of the sample 2 spermatozoa were immature, with the droplet still present in the middle of their tail.

Although, the motility decreases after 24h refrigeration, the viability is adequate, so semen with a low motility but high viability could be used for in vitro fertilization by ICSI, as the head of the spermatozoon is still in good condition, with intact genetic information, but not being able to move, and therefore unable to normally fertilize an oocyte.

Conclusions

The protocol for tele-anesthesia involving tiletamine/zolazepam + xylazine proved to be extremely efficient, as all maneuvers could be performed safely, and the recovery of the bear thereafter was without any negative incidents.

The main drawback of semen collection using an electro-stimulator is the possible urine contamination of the sample, but this can be avoided by carefully observing the viscosity of the ejaculate and frequently changing the collection bottles.

During general anesthesia for semen collection, various other procedures can be performed, like general and specific examination of the animal, blood sampling, treating various other conditions (like wounds, infections, etc.).

Semen collected by electroejaculation proved to have adequate parameters, with a slightly decreased progressive motility. Motility decreased after 24h of refrigeration, but coupled with the viability results, the semen can still be used for in vitro fertilization by ICSI.

Semen collection by retrograde flushing from the epididymis proved to be an efficient technique, with great potential regarding sperm collection from bears after castration.

Taking into consideration the young age of the bear and the 24h that passed from castration until the testicles were processed, we consider the results obtained for sample 2 as being very positive, showing that technique can be further used for sexually mature bears. CASA and flow-cytometry are powerful tools of sperm analysis and are mandatory for such biodiversity conservation studies, as they provide accurate and objective results.

Our research needs to be continued in order to achieve the goal of repopulating the areas where the brown bear used to live in the past and where it became extinct.

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