

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Cryopreservation of Epididymal Sperm

Melina A.F. Bertol

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/65010>

Abstract

The recovery and cryopreservation of epididymal sperm are essential tools to preserve genetic stocks of valuable domestic or wild animals under adverse conditions and also as an alternative source of gametes in cases of human infertility. This technique is recommended after death or when it is not possible to recover semen by usual methods. Sperm from the epididymis has been studied by many authors in different species. Cryopreservation is the most effective method for long-term preservation of genetic material from valuable breeding individuals. The protocols and diluents used for cryopreservation of conventional semen are well established, but many challenges remain working with sperm extracted directly from the epididymis. The spermatozoa retrieved from the cauda epididymis have special features, such as the absence of seminal plasma and large numbers of distal cytoplasmic droplets, which necessitate special handling, both for cryopreservation and for fertilization. For these reasons, it is important to describe in detail the features needed to cryopreserve sperm from the epididymis.

Keywords: cryopreservation, epididymis, fertility, reproduction, sperm

1. Introduction

The cryopreservation technique is widely used in reproductive biology because it facilitates the application of assisted reproduction techniques such as artificial insemination and *in vitro* fertilization (IVF). Besides that it allows long-term storage of gametes, facilitating transport over long distances and the formation of a genetic bank, essential for the conservation of species. It is an important complementary tool for conservation to avoid excessive losses of genetic variation or races of extinction. Cryopreservation is considered to be the most effective way for long-term preservation of genetic materials, cells, tissues and microorganisms. It

allows to maintain biological material at very low temperatures, indefinitely. The expansion of genetic material from high-value breeders became possible with the advent of semen cryopreservation. The main advantages of the technique are: portability for world trade because it is not necessary to purchase or move the males, and it also acts in the prevention of diseases capable of transmission through natural breeding.

Semen constitutes the union of sperm with seminal plasma, secreted by the male accessory glands. Sperms are produced in the testicles (gametogenesis), and maturation occurs in the epididymis when cells enter in the caput of epididymis, progress to the corpus, and finally reach the cauda region, where they are stored until the moment of ejaculation. Spermatozoa stored in the cauda region are generally of good quality and have a high level of maturity, being able to fertilize oocytes. The epididymis provides a favorable environment to retain the sperm with fertilizing capacity for several weeks. Therefore, the cauda epididymis is a major source of gametes of a breeder and in special cases they can be retrieved directly from it.

The sperm recovery from the epididymis is the last chance to use gametes from dead breeders, in addition to enabling the maintenance of a germplasm bank of animals of commercial interest or threatened with extinction [1]. A factor to be considered is the difficulty in collecting semen from wild species and the unexpected death of animals of zoological interest. In this case, the technic can be used to ensure the rescue of male gametes and the preservation by cryopreservation process for maintaining germplasm banks [2]. For animal production as livestock, the sperm recovery directly from the epididymis is a viable alternative when there is a sudden death of breeder of high commercial value, to increase their genetic stock or in cases of bulls unable to ejaculate for some reason [3]. It is the last alternative for future use of gametes from a breeder in assisted reproduction programs. In equine, this technique is important because there is a high incidence of accidental and unexpected death due to high incidence of colic or severe traumatic accidents that compromise the reproductive life of the stallion.

The first important point is the recovery of still viable sperm with good parameters of motility, concentration, and morphological defects as soon as possible after the male death. There is a time limit for this to successfully occur, usually dependent on temperature. The first step is getting the epididymis in the field, after the death of the animal. For handling ease, due to their anatomical location, the testes can be removed with them. Then both should be sent to the laboratory in suitable containers, with or without cooling. In the lab, the trained technicians perform gonads cleaning, epididymis isolation and spermatozoa recovery. Then it can be used in three different ways: first, shortly after harvesting; second, chilled, and third, after cryopreservation process. The last increases the availability time of gametes for application of assisted reproduction techniques. Next we will focus on the key topics relevant to the understanding of the epididymis spermatozoa cryopreservation technique.

2. Epididymal spermatozoa

When the spermatozoon leaves the testicle to the epididymis, it is a non-functional and infertile gamete. Only after the passage through the epididymis, it becomes mature and acquires

progressive motility to become able to fertilize the oocyte. This maturation is complex and involves several factors, including the interaction of sperm with proteins that are synthesized in each epithelium region of the epididymis. Sperm passively migrates through the epididymis and after contact with the epididymal secretions get low-molecular weight and water-soluble compounds by an isovolumetric regulatory process. These compounds can be spent together with the cell water when the cells come into contact with hypo-osmotic fluids from accessory glands or genital female tract. The volume adjustment process serves to maintain the volume pattern into the sperm cell and to prevent angulation of the flagellum, which prevents the sperm to migrate efficiently in the female reproductive tract, being unable to fertilize oocytes. The channels responsible for this regulation are located in cytoplasmic droplets. The spermatozoa located in the cranial portion of the epididymis (caput) are considered immature to present an osmolyte content reduced insufficient for complete regulation of cell volume when exposed to hypotonic solutions [4]. The movement of the sperm through the epididymis is performed mainly by the contraction of smooth muscles of the wall of the Caput and corpus region. On the cauda, the smooth muscles of the epididymal duct is generally at rest until it is stimulated to contract at the time of ejaculation for the release of sperm, thus it is responsible for the protection and storage of sperm until ejaculation.

There are two moments prior to recovery of sperm that interferes directly in the success of the process. The first is the time from the death of the animal until the necropsy, and the second is the period of recovery of the gonads to obtain the gametes in the laboratory [5]. The higher the time of the gametes permanence in the cauda epididymis after death or after orchiectomy, the greater the damage to the sperm cell. At room temperature, motility is the first parameter affected. With the increase in the hours before recovery, there is a reduction in the percentage of moving spermatozoa, worsening from 24 hours [6, 7].

There are two moments for the recovery of spermatozoa from the epididymis:

- a. After death
- b. After orchiectomy

The most common form of spermatozoa recovery from the epididymis is after death. It is indicated in cases of use of last spermatoc reserve after breeder death. It is usually recommended when the breeder is found after dead in the field in cases of sudden death from a serious illness, accidents, poisoning and stress problems. It is worth mentioning that infectious diseases can contaminate tissues with pathogenic microorganisms, and in such cases the technique is not recommended to avoid contamination. Moreover, in some infectious diseases, there is an acceleration of tissue degradation, affecting the preservation of tissues and reducing the time for manipulation. One example is infection by *Clostridium chauvoei*, a bacterium when multiplied produces a toxin that causes injury to the host body as well as muscles and other tissues. The acute disease is considered highly lethal.

After death there is limited time to work before the occurrence of the degeneration of tissues (postmortem autolysis), damaging the quality of sperm. This time must be sufficient for catching the gonads in the field and transport to the laboratory. Trained technicians are

required to perform rapidly obtaining of the spermatozoa, and then adding the medium to provide substrates necessary for maintenance of gametes.

The recovery after orchiectomy is usually performed in experimental works. Once is not common execute euthanasia in animals for research, the orchiectomy is an option to simulate the death for testis and epididymis. The interruption of blood supply to the testis and epididymis causes the same degenerative changes which occur after death. The orchiectomy is indicated in any situation where you do not want to eliminate the animal. In cases of unilateral testicular involvement, contralateral testicle can be tapped.

For wildlife, the difficulty of semen collection by conventional methods such as electroejaculation and artificial vagina turns recovery from epididymis interesting. Sperm can be obtained after orchiectomy, and the animal will stay alive, but will be unable to reproduce on their own. Both techniques are considered the last chance to use male gametes. The concentration of spermatozoa recovered is limited by storage capacity in the cauda epididymis of each species. The number of insemination doses is directly proportional to this concentration.

After recovery, the epididymis can be handled in two ways:

- a. Room temperature (about 19°C)
- b. Chilled (4–5°C)

When an animal is found dead in the field, it was exposed to climatic conditions for hours. This ambient temperature accelerates tissue degeneration with loss of sperm viability in a given time period. This condition has been represented in previous studies to be closer to reality, to verify the time available and establish a window of opportunity to work. A large variation in ambient temperatures was reported successfully, between 18 and 24°C [2, 7–9]. At a temperature of 18–20°C, it is possible to recover viable gametes, With 41.25% of progressive motility by up to 30 hours after orchiectomy [7]. Thus, after the death of bulls exposed at Ambient temperature, the ideal time until sperm recovery is up to 30 hours. With the increase of the residence time of the gametes in the cauda epididymis after death or after orchiectomy, there is greater damage to the sperm cell. In general, motility is the first parameter affected.

On the other hand, in most studies the epididymis are kept at a refrigeration temperature of 5°C before recovery, which slows down the process of cell degradation increasing the time for collecting viable gametes. For bulls, the maintenance of refrigerated epididymis enables the achievement of viable sperm for up to 72 hours after the death [10]. By comparing the refrigerating temperature (4.9–6°C) with room temperature (21.5–17.9°C) for maintenance of the epididymis before harvesting in sheep, the highest temperature affects earlier some spermatoc parameters such as the acrosome integrity, motility, concentration and morphology [11]. If possible, the testicles and epididymis should be transported to the laboratory chilled to increase the time for processing.

For spermatozoa recovery, The main techniques that can be used are:

a. Retrograde flow

b. Flotation

The methods to obtain gametes depend on the animal species, size of the epididymis and the experience of the laboratorist. The retrograde flow consists of cannulation to perfuse the lumen of epididymis with diluent, promoting the backflow of the cauda epididymis content [12]. This technique is used for large animals, which have bigger testicular size and larger epididymis. It has been used successfully in horses and cattle [6, 7]. Being a detailed technique, the time for extracting spermatozoa is wide. In general, the sperm collection technique by retrograde flow results in less contaminated samples and better sperm quality than other methods, and is more suitable [13].

For small animals, the flotation method is most appropriate because of the anatomical size of the epididymis. It consists of performing longitudinal and cross cuts in the cauda region of the epididymis, the fragments are deposited into a petri dish containing medium for release of sperm and later retrieval by filtration [14]. For the smallest mammal in the world, the shrew (*Tupaia belangeri*), this is the only technique applicable because of the epididymal size in this species [15]. The flotation method requires less technician experience, because of the facility of implementation it is also often used for large animals such as sheep [16] and bulls [5, 10]. One disadvantage of this practice is that the samples are usually contaminated with blood and cellular debris, as some blood vessels are also incised during the process. The recovered sperm concentration is lower, as some are stuck between the tissue fragments so they cannot swim through the diluent in the petri dish.

The spermatozoa collected from the epididymis are free of seminal fluid which is added by the accessory glands during ejaculation, and serves as a vehicle, stimulating sperm metabolism and provides the energy necessary for the spermatozoa to pass through the uterus [17]. For it is a gamete storage location, the concentration of spermatozoa in the epididymal tail is significantly higher than the concentration of semen after ejaculation. When sperms are retrieved from the epididymis, there is a lack of ejaculatory reflex, and thereby sperms have no contact with the plasma rich in electrolytes, fructose, ascorbic acid, various enzymes and vitamins. Because of this it is recommended to add seminal plasma or other medium that has the components necessary to maintain sperm viability after recovery. It is important that whatever the diluent, it must have composition substances favorable to sperm: macromolecules such as lipoproteins and phospholipids to act as stabilizers of the plasma membrane, nutrient source for sperm metabolism (sugars) and buffer to maintain the pH. For cryopreservation, it is indispensable to use a medium containing cryoprotectants.

Besides the absence of seminal plasma, another important characteristic of epididymal sperm is the presence of large amounts of medial and distal cytoplasmic droplets in the tail of the sperm [7, 10, 17, 18]. The cytoplasmic droplet is a small spherical mass of 2–3 μm in diameter found in low amounts in the ejaculated spermatozoa as it is released during ejaculation, as shown in **Figure 1**. During the transit through the epididymis, the caudal migration of the cytoplasmic droplet occurs. The presence of distal cytoplasmic droplets is not considered a severe alteration because it does not interfere with the fertilizing capacity of the sperm.

Although it represents an alteration in the normal morphology of the spermatozoa, the presence of cytoplasmic droplets in epididymal sperm is a physiological finding. Sperms with a large number of distal cytoplasmic droplets tend to lose them after 15–30 minutes of incubation in a water bath (35°C) or after the agitation [17]. Therefore, it is recommended to keep the sperm from the epididymis in a water bath at 29 to 35°C for a period of 30–60 minutes, so that the cytoplasmic droplets are released spontaneously. With the release of the drops, the sperm changes the circular movement pattern for rectilinear.



Figure 1. Phase contrast microscopy (1000×) of a medial cytoplasmic droplet in bull epididymal sperm. During the transit through the epididymis the cytoplasmic droplet migrates along the middle piece of the sperm from the proximal to the medial and distal region.

The pair of testicles (right and left sides) of a male breeder has similar characteristics of dimensions and gamete production and reserve. It has been reported for bull and stallion [6, 19]. Bulls at reproductive age (between 3 and 7 years) have epididymis (caput, corpus and cauda) with an average weight of 34.2 g. Moreover, sperm motility parameters, morphology and concentration do not differ when compared with the epididymal spermatozoa recovered from right and left. The information becomes useful in cases of unilateral testicular involvement, assisting in the reproductive male prognosis and also in clarifying the normal male genital tract physiology.

3. Cryopreservation of epididymal sperm

The first experiments arose with the need for establishment of a genetic bank for maintenance of the gametes for future use. Another reason for the cryopreservation of sperm from the

epididymis is because there is a possibility that a bull could die before their semen is deposited in semen banks or at a time when only a small amount is in storage. While the use of frozen bovine semen has favored the establishment of semen banks which prolongs the usefulness of a bull and acts as a safety procedure in the event of an unexpected illness or death, not always the existing reserve is sufficient. The semen conservation methods in liquid form allow maintenance for only a few days, but if frozen in liquid nitrogen (N_2L) it is possible to keep for years.

Sperm were recovered from the epididymis of a Jersey bull aged nine years after death and were frozen for the first time on November 26, 1953 [3]. The procedure was carried out as follows: The intact scrotum from the slaughtered bull was brought to the laboratory as soon after death as possible. The time for delivery varied between 1.5 and 3 hours. On arrival the scrotal skin and tunica vaginalis were incised and the testicle was handled as aseptically as possible after its removal. Spermatozoa were recovered from the epididymis by means of several incisions in each, following which the organ was squeezed, and the expressed fluid was scraped off with a scalpel and deposited in a small volume of diluent (pasteurized homogenized whole milk and antibiotics added). Epididymal fluid containing spermatozoa were added to milk diluent. An equal volume of 20% of glycerinated milk diluent was then added gradually in increasing amounts, over a period of 30 minutes, so that a final glycerol concentration of 10% by volume was present. The sample was then placed in a refrigerator for an equilibration period of 18 hours. Subsequently the sample was distributed into ampoules and frozen.

Although some characteristics are already previously mentioned, the epididymal spermatozoa resemble the ejaculated and may be successfully cryopreserved similarly. The protocols used for freezing and thawing interfere with sperm fertility rate, so different situations are tested to identify the best protocol and the best diluent used in this process. The absence of seminal plasma in epididymal spermatozoa seems to be a positive factor in the maintenance of membrane integrity during the cryopreservation process. Furthermore, the sperm membrane composition between individual breeders can affect the resistance to cryopreservation [20]. The post-thaw incubation of the spermatozoa from epididymis of cats with seminal plasma resulted in a lower total and progressive motility, and plasma membrane integrity than control [21]. Thus, if the objective is the cryopreservation, it is recommended to add a diluent itself instead of seminal plasma.

The protocols used for cryopreservation of conventional semen with the same diluents seem to be effective for epididymal spermatozoa. What differs is the pre-preparation of sperm samples. If there is a high percentage of cytoplasmic droplets in the tail of the spermatozoa after recovery, it must remain for a period of about 30–60 minutes in a water bath, for drops release. For this first step, the sperm must be diluted in a medium without cryoprotectants that present some toxicity when stay long in contact with the sperm cells. At the end of the process, a sperm sample should be evaluated under light microscope to see if there was the release of most of the cytoplasmic droplets. After this step, the sample must be centrifuged to remove any trace of the previous medium and other dirt, and then the cryopreservation diluent with cryoprotectants is added. The amount of diluent is based on the concentration of spermatozoa and the number of insemination doses.

The epididymal spermatozoa seem to be more resistant to the cryopreservation process than the ejaculated. In sheep, the sperms from the epididymis were more resistant to the stresses caused by freezing (osmotic variations, cryoprotectants toxicity and temperature variation) compared to the ejaculate [22].

The cryopreservation protocol in liquid nitrogen and the dilution with TRIS base-egg yolk are effective [8, 23]. For bulls, the process consists of first sample centrifugation to separate the spermatozoa from other contaminants and the recovery medium. The supernatant needs to be discarded, and the pellet resuspended with an extender consisting of Tris-egg yolk and 7% glycerol as cryoprotectant. The pre-freezing parameters of motility and the total cell number should be assessed to verify the effects of centrifugation and diluent. The straws are filled with a concentration of 20 million viable spermatozoa and sealed. For temperature stabilization, the doses were maintained for 3 hours in a semen cooling container (5°C), and then placed horizontally on a 6-cm high support in an expanded polystyrene box containing liquid nitrogen for 20 minutes. Finally, straws they are immersed in liquid nitrogen at a storage temperature of -196°C. Cryopreservation can also be performed in automatic machines with controlled temperature drop.

There is not yet a specific commercial diluent for epididymal sperm in any species. Thus, recent research was conducted in order to test and identify the best diluent in this case. Some results are mentioned below.

In sheep, the results of post-thaw viability and fertility after artificial insemination with Cryopreserved successfully epididymal spermatozoa show that the diluent with 20% egg yolk and 8% glycerol and base TES-Tris-fructose (TTF) was significantly more effective in maintaining the sperm viability [24]. Egg yolk base medium with 4% glycerol was used for epididymal sperm cryopreservation of domestic and wild cats (*Panthera tigris*) successfully, providing the application of this technology in genetic resources of banks of wild species of cats [25]. Also with the aim of maintaining a genetic resource bank of wild species, Cuvier's gazelle (*Gazella cuvieri*) spermatozoa can be cryopreserved using a diluent containing 18.5% raffinose with 20% egg yolk and 6% glycerol [26]. The concentration of glycerol varies among species and must be previously tested in each case.

The concentration of sugars and the type of carbohydrate added to the medium also affect the quality of post-thaw spermatozoa from the epididymis. In tests with epididymal sperm from wild deer (*Cervus elaphus hispanicus*), the use of monosaccharides in diluents for freezing, especially fructose, improves the maintenance of post-thaw viability compared to trisaccharides [27]. Furthermore, the addition of antioxidants such as cysteine, water-soluble vitamin analog or enzymes [28, 29] leads to improvement in total motility and post-thaw integrity in sperm plasma membranes of cat epididymal sperm.

Commercial extenders for Conventional bull semen (Botu-Bov® and Bovimix®) are viable options for cryopreservation of epididymal bull sperm. Both are effective in maintaining sufficient amounts of post-thaw viable spermatozoa for use in artificial insemination. When compared, these show no difference in sperm viability (movement, morphology and integrity

of plasma and acrosomal membrane) after thawing [23]. These two extenders have egg yolk in its formulation and glycerol as a cryoprotectant.

The variations in response to cooling protocols and semen freezing must be attended. Individual male effects affect directly the longevity of spermatozoa during preservation. The mammals may be regarded as “good” or “bad” freezers, according to the characteristics of the sperm plasma membrane structure which is genetically determined and the survival predisposes to thermal stress. This fact allows researchers to sort the spermatozoa as resistant or susceptible to cryopreservation. The proportion of cells, which survive the cryopreservation protocol, is determined by the sensitivity to osmotic stress during cryopreservative addition and removal, and during cooling and rewarming. While there may be species differences in overall sperm sensitivity to cryopreservation, the ejaculate is heterogeneous with a variable resistance to osmotic stress among the cells. Nevertheless, even if we optimize the process and minimize the cell death, there will still be a proportion of cells which fail to survive. We need, therefore, to concentrate on the function of the surviving population [30].

After cryopreservation identified doses containing the spermatozoa can be deposited in canisters of cryogenic cylinder, indefinitely. Inside the cylinder, the semen is kept in liquid nitrogen (N_2L), which preserves the frozen doses at a temperature of $-196^\circ C$. The storage time is indefinite as long as the same is supplied periodically in order to maintain the N_2L above the minimum required level, which should never be below 15 cm height. The cylinder should be stored and handled with utmost care, avoiding possible damage.

4. Epididymal sperm fertility

It is already proven the fertility potential of spermatozoa retrieved directly from the Epididymis in some species. The epididymal sperm, as well as the ejaculated, can be used in assisted reproductive technology (ART) as conventional artificial insemination, fixed time artificial insemination (FTAI), *in vitro* fertilization of oocytes (IVF) and intracytoplasmic sperm injection (ICSI). The viability of spermatozoa after cryopreservation depends on how well the quality is preserved throughout processing, storage and thawing procedures. High-quality semen is the one that will initiate a high percentage of pregnancies when properly used. The longest time to sperm recovery after death or after orchiectomy reduces the motility and other sperm parameters before freezing. Thus, this results in worse performance for fertilization and in consequence worse pregnancy outcomes.

In vitro fertilization is the most appropriate technique to use this material because it requires a lower concentration of viable sperm for embryo production, when compared to artificial insemination. The ability to *in vitro* and *in vivo* fertilization has been proven. Sperms collected from the epididymis tail of bulls and kept at $5^\circ C$ have been used for *in vitro* production of embryos [10]. Under these conditions, it was viable to produce embryos *in vitro*, with blastocyst formation up to 9 days of development using Cryopreserved sperm from epididymides refrigerated for 24, 48 and 72 hours at $5^\circ C$. When the sperm was recovery up to 30 hours at ambient temperature ($18-20^\circ C$) and then cryopreserved in liquid nitrogen successfully, it was

also possible to produce viable embryos (blastocysts) of eight days from *in vitro* development. The total number of blastocysts and hatching rates were lower when the recovery of spermatozoa was performed 24–30 hours after orchiectomy. When the collection was performed 6–18 hours after orchiectomy the embryo production rate was approximately 30% [31]. This means that sooner the recovery and cryopreservation are performed, the better the results. The individual bull effect on embryo production is a relevant factor that can influence the success of IVF in those cases. Great individual variation in both post-thaw sperm parameters and embryo production between bulls can be observed. It is important to know the genetic background and the fertilization potential of sperm donors to maximize the success of IVF.

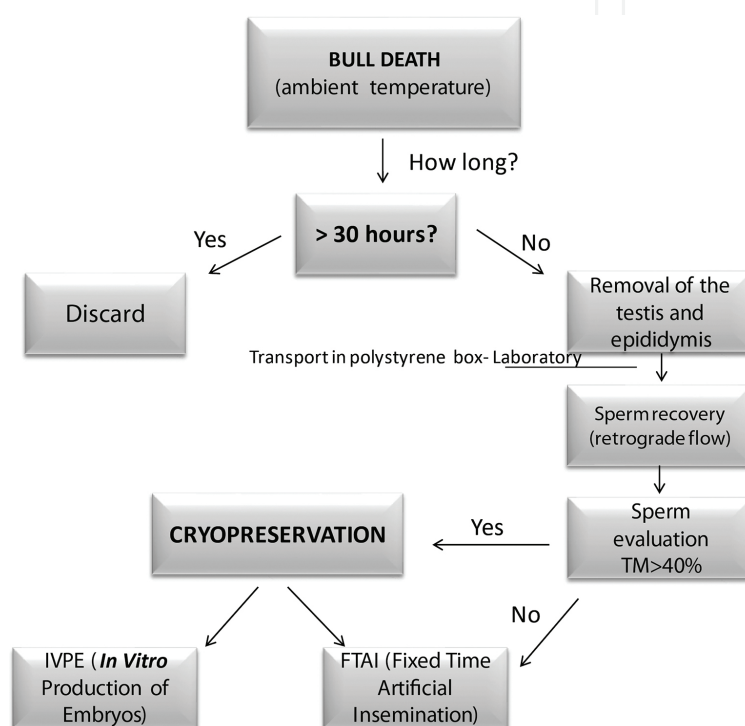


Figure 2. Flowchart of the recovery technique and sperm utilization from bulls epididymides, post-orchiectomy or postmortem when kept at ambient temperature; TM, total motility (adapted from: Bertol [34]).

Another option for use of gametes is fixed time artificial insemination (FTAI). This technique offers the following advantages: (1) does not require detection of estrus, (2) there is induction of the estrus and ovulation, (3) synchronizes births and (4) reduces the calving interval. It is already reported in the literature a pregnancy after FTAI with spermatozoa that remained for 30 hours in the epididymis at room temperature [31]. As already mentioned, recovery at room temperature is an important condition in gamete handling of epididymal reserve. After the death of a breeder, the first question we must ask is how long the animal is exposed to ambient temperature. If it was for a period of 30 hours, it would be possible to perform the retrograde flow technique for spermatozoa recovery from the cauda epididymis. If the evaluation of the total motility parameter (TM) in sperm after retrieval is greater than or equal to 40% of motile cells, it can be cryopreserved. This is important for future use without time limit in techniques of *in vitro* produce of embryos and artificial insemination. If it was less than 40% sperm

must be used immediately after recovery. The flowchart of how to act in this situation is shown in **Figure 2**.

Regarding insemination with spermatozoa from the epididymis, the literature is scarce. The ability of frozen-thawed boar spermatozoa obtained from epididymides stored at 4°C for 1 day to produce piglets (three males and two females) after fallopian insemination or *in vitro* fertilization (IVF) of *in vitro* matured (IVM) oocytes was described [32]. This demonstrates the potential for *in vivo* fertilization of gametes. In sheep, insemination with the frozen/thawed epididymal sperm resulted in a lambing rate of 87.5%. Fourteen lambs (10 males, four females) were born from seven ewes. The average gestation length was 145.6 ± 1.1 days. Birth weights were 4.4 ± 0.4 , 3.2 ± 0.7 and 3.6 ± 0.7 kg for single lambs, twins and triplets, respectively [18].

The technique of intracytoplasmic sperm injection to achieve fertilization, especially using retrieved epididymal spermatozoa from men with obstructive or non-obstructive azoospermia, has revolutionized the field of assisted reproduction in humans. The characteristics of postpartum development of children born after fertilization with spermatozoa from the epididymis did not differ from those from conventional methods. Children born from the intracytoplasmic injection of spermatozoa method (ICSI) with gametes from the epididymis were monitored and compared to those born from ICSI with spermatozoa from the ejaculated and by *in vitro* fertilization of embryos. The results showed that the method was successful, not to cause any fetal malformations, stillbirth or problems in the development of children [33]. These scientific findings have grounded the progress of biotechnology of reproduction. The possibility of recovery sperm from epididymis without ejaculation of animals and man, for later cryopreservation, has ensured the maintenance of important genetic source and the maintenance of compromised male in reproduction.

5. Conclusion

It is very important to have knowledge that it is possible to use the genetic reserve of a breeder even after his death. This avoids gamete wastage and an early loss of reproductive potential of a male of important genetic value. The application of this biotechnology should be proposed by the veterinarian at the time of the death of a high-value breeder. The owner of the animal should be aware that it is still possible to obtain the last descendants of the breeder. A basic protocol for the cryopreservation of epididymal sperm can be suggested as follows:

1. Recovery of gonads as soon as possible.
2. Transport to the laboratory (preferably refrigerated at 5°C).
3. Manipulation of the epididymis and the spermatozoa recovery by retrograde flow or flotation methods.
4. After recovery, spermatozoa kept in recovery medium—without cryoprotectant—in a water bath (29–35°C) for at least 30 minutes.

5. Centrifugation at least $600 \times g$ for 10 minutes.
6. Resuspension with an extender of Tris-egg yolk base, and glycerol as cryoprotectant, in appropriate concentrations for each species and preparation of the straws.
7. First temperature drop to 5°C for 3 hours for stabilization, preferably in a semen cooling container.
8. Cryopreservation in liquid nitrogen by a 6-cm high support in an expanded polystyrene box or automatic cryopreservation systems.
9. Immersing straws in liquid nitrogen at a storage temperature of -196°C .
10. Storage in a cryogenic container for an indeterminate period.

It is important to note that in all of these steps (1–10), an aliquot should be removed for sperm evaluation of some parameters such as motility, concentration and morphology. This is essential for identifying flaws in the sperm handling or viability changes by temperature drop during processing, osmotic stress and the formation and dissolution of extracellular ice crystals, which can impair the fertility of sperm. The success of epididymal sperm cryopreservation depends on the effectiveness of the process, including cautious handling of the sperm cells and the technical skills in the laboratory.

Acknowledgements

The author thanks all the researchers and scientists from different institutions involved in this work, and the financial support granted by the National Counsel of Technological and Scientific Development (CNPQ) for the development of the mentioned researches from Brazil.

Author details

Melina A.F. Bertol

Address all correspondence to: melbertol@hotmail.com

Department of Technology, Postgraduate studies in Bioprocess Engineering and Biotechnology, Human and Animal Health, Federal University of Paraná, Curitiba, Brazil

References

- [1] Tittarelli C, Savignone CA, Arnaudín E, Stornelli MC, Stornelli MA, de la Sota RL. Effect of storage media and storage time on survival of spermatozoa recovered from canine

- and feline epididymides. *Theriogenology* 2006;66:1637–40. doi:10.1016/j.theriogenology.2006.01.021.
- [2] Kaabi M, Paz P, Alvarez M, Anel E, Boixo JC, Rouissi H, et al. Effect of epididymis handling conditions on the quality of ram spermatozoa recovered post-mortem. *Theriogenology* 2003;60:1249–59. doi:10.3923/ajava.2008.400.408.
 - [3] Barker CAV. Low temperature preservation of bovine epididymal spermatozoa. *Can J Comp Med* 1954;18:390–3.
 - [4] Cooper TG. Sperm maturation in the epididymis: a new look at an old problem. *Asian J Androl* 2007;9:533–9. doi:10.1111/j.1745-7262.2007.00285.x.
 - [5] Martins CF, Rumpf R, Pereira DC, Dode MN. Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses *in vitro* embryo production. *Anim Reprod Sci* 2007;101:326–31. doi:10.1016/j.anireprosci.2007.01.018.
 - [6] Weiss RR, Muradas PR, Graneman LC, Meira C. Freezing sperm from cauda epididymis of castrated stallions. *Anim Reprod Sci* 2008;107:356. doi:10.1016/j.anireprosci.2008.05.133.
 - [7] Bertol MAF, Romualdo Weiss R, Tomaz-Soccol V, Ernandes Kozicki L, Silva Fujita A, Azevedo de Abreu R, et al. Viability of bull spermatozoa collected from the epididymis stored at 18–20°C. *Brazilian Arch Biol Technol* 2013;56:777–83. doi:10.1590/S1516-89132013000500008.
 - [8] Turri F, Madeddu M, Gliozzi TM, Gandini G, Pizzi F. Influence of recovery methods and extenders on bull epididymal spermatozoa quality. *Reprod Domest Anim* 2012;47:712–7. doi:10.1111/j.1439-0531.2011.01948.x.
 - [9] Lone FA, Islam R, Khan MZ, Sofi KA. Effect of transportation temperature on the quality of cauda epididymal spermatozoa of ram. *Anim Reprod Sci* 2011;123:54–9. doi:10.1016/j.anireprosci.2010.10.012.
 - [10] Martins CF, Driessen K, Costa PM, Carvalho-Neto JO, de Sousa RV, Rumpf R, et al. Recovery, cryopreservation and fertilization potential of bovine spermatozoa obtained from epididymides stored at 5°C by different periods of time. *Anim Reprod Sci* 2009;116:50–7. doi:10.1016/j.anireprosci.2008.12.018.
 - [11] Lone FA, Islam R, Khan MZ, Sofi KA. Effect of transportation temperature on the quality of cauda epididymal spermatozoa of ram. *Anim Reprod Sci* 2011;123:54–9. doi:10.1016/j.anireprosci.2010.10.012.
 - [12] Garde J, Aguado M, Perez S, Garrido D, Perez-guzman M, Montoro V. Physiological characteristics of epididymal spermatozoa from postmortem rams. *Theriogenology* 1994;41:2003.
 - [13] Martinez-Pastor F, Garcia-Macias V, Alvarez M, Chamorro C, Herraes P, De Paz P, et al. Comparison of two methods for obtaining spermatozoa from the cauda epididymis

- of Iberian red deer. *Theriogenology* 2006;65:471–85. doi:10.1016/j.theriogenology.2005.05.045.
- [14] Yu I, Leibo SP. Recovery of motile, membrane-intact spermatozoa from canine epididymides stored for 8 days at 4°C. *Theriogenology* 2002;57:1179–90. doi:10.1016/S0093-691X(01)00711-7.
- [15] Ping S, Wang F, Zhang Y, Wu C, Tang W, Luo Y, et al. Cryopreservation of epididymal sperm in tree shrews (*Tupaia belangeri*). *Theriogenology* 2011;76:39–46. doi:10.1016/j.theriogenology.2011.01.011.
- [16] Tamayo-Canul J, Álvarez M, Mata-Campuzano M, Álvarez-Rodríguez M, de Paz P, Anel L, et al. Effect of storage method and extender osmolality in the quality of cryopreserved epididymal ram spermatozoa. *Anim Reprod Sci* 2011;129:188–99. doi:10.1016/j.anireprosci.2011.11.003.
- [17] Barth AD, OKO RJ. *Abnormal Morphology of Bovine Spermatozoa*. Ames: Iowa State University Press, 1989. 285 p.
- [18] Ehling C, Rath D, Struckmann C, Frenzel A, Schindler L, Niemann H. Utilization of frozen-thawed epididymal ram semen to preserve genetic diversity in Scrapie susceptible sheep breeds. *Theriogenology* 2006;66:2160–4. doi:10.1016/j.theriogenology.2006.07.003.
- [19] Bertol MAF, Weiss RR, Kozicki LE, de Abreu ACMR. Biometrics and sperm parameters between right and left testes and epididymis of zebu bulls. *Arch Vet Sci* 2015;20:156–63.
- [20] Aurich JE, Kühne A, Hoppe H, Aurich C. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. *Theriogenology* 1996;46:791–7. doi:10.1016/S0093-691X(96)00237-3.
- [21] Thuwanut P, Chatdarong K. Incubation of post-thaw epididymal cat spermatozoa with seminal plasma. *Reprod Domest Anim* 2009;44:381–4. doi:10.1111/j.1439-0531.2009.01436.x.
- [22] Varisli O, Uguz C, Agca C, Agca Y. Motility and acrosomal integrity comparisons between electro-ejaculated and epididymal ram sperm after exposure to a range of anisotonic solutions, cryoprotective agents and low temperatures. *Anim Reprod Sci* 2009;110:256–68. doi:10.1016/j.anireprosci.2008.01.012.
- [23] Bertol MAF, Weiss RR, Fujita AS, Kozicki LE, De Abreu ACMR, Pereira JFS. Two commercial extenders for cryopreservation of epididymal bull sperm. *Ciência Rural* 2014;44:1658–63. doi:10.1590/0103-8478cr20130747.
- [24] Álvarez M, Tamayo-Canul J, Martínez-Rodríguez C, López-Urueña E, Gomes-Alves S, Anel L, et al. Specificity of the extender used for freezing ram sperm depends of the spermatozoa source (ejaculate, electroejaculate or epididymis). *Anim Reprod Sci* 2012;132:145–54. doi:10.1016/j.anireprosci.2012.05.006.

- [25] Cocchia N, Ciani F, El-Rass R, Russo M, Borzacchiello G, Esposito V, et al. Cryopreservation of feline epididymal spermatozoa from dead and alive animals and its use in assisted reproduction. *Zygote* 2010;18:1–8. doi:10.1017/S0967199409990256.
- [26] Martínez AF, Martínez-Pastor F, Álvarez M, Fernández-Santos MR, Estes MC, de Paz P, et al. Sperm parameters on Iberian red deer: electroejaculation and post-mortem collection. *Theriogenology* 2008;70:216–26. doi:10.1016/j.theriogenology.2008.04.001.
- [27] Fernández-Santos MR, Martínez-Pastor F, García-Macías V, Estes MC, Soler AJ, de Paz P, et al. Extender osmolality and sugar supplementation exert a complex effect on the cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa. *Theriogenology* 2007;67:738–53. doi:10.1016/j.theriogenology.2006.10.005.
- [28] Thuwanut P, Chatdarong K, Techakumphu M, Axnér E. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. *Theriogenology* 2008;70:233–40. doi:10.1016/j.theriogenology.2008.04.005.
- [29] Chatdarong K, Thuwanut P, Morrell JM. Single-layer centrifugation through colloid selects improved quality of epididymal cat sperm. *Theriogenology* 2010;73:1284–92. doi:10.1016/j.theriogenology.2009.12.009.
- [30] Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci* 2000;60–61:481–92. doi:10.1016/S0378-4320(00)00099-3.
- [31] Bertol MAF, Weiss RR, Kozicki LE, De Abreu ACMR, Pereira JFS, da Silva JJ. *In vitro* and *in vivo* fertilization potential of cryopreserved spermatozoa from bull epididymides stored for up to 30 hours at ambient temperature (18°C–20°C). *Theriogenology* 2016;86:1014–21. doi:10.1016/j.theriogenology.2016.03.030.
- [32] Kikuchi K, Kashiwazaki N, Nagai T, Noguchi J, Shimada A, Takahashi R, et al. Reproduction in pigs using frozen-thawed spermatozoa from epididymis stored at 4°C. *J Reprod Dev* 1999;45:345–50. doi:10.1262/jrd.45.345.
- [33] Woldringh GH, Horvers M, Janssen AJWM, Reuser JJCM, De Groot SAF, Steiner K, et al. Follow-up of children born after ICSI with epididymal spermatozoa. *Hum Reprod* 2011;26:1759–67. doi:10.1093/humrep/der136.
- [34] Bertol, M.A.F. *In vitro* and *in vivo* fertilization potential of cryopreserved spermatozoa from bull epididymides stored for up to 30 hours at ambient temperature (18°C–20°C) [thesis]. Brazil: Federal University of Paraná; 2016.

