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Cryopreservation of Spermatozoa in Veterinary Medicine

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Cryopreservation of Spermatozoa in Veterinary Medicine

S. Pesch, B. Hoffmann

Semen cryopreservation and artificial insemination (AI) are the most important biotechnological techniques presently applied in animal breeding. Its large-scale introduction in cattle breeding some 60 years ago aimed at the prevention of genital infections transmittable via natural mating as well as breeding progress and success by rapid spreading of valuable genes. Relating to non-food (pet) animals, the avoidance of travel and quarantine restrictions and the conservation of genetic resources are additional advantages. A strict legal framework guarantees sire identity and health innocuousness. Similarly strict guidelines regulate the quality of fresh semen, its processing for cryopreservation and its quality after freezing and thawing.

Successful application of AI, particularly when using frozen and thawed semen, requires a proven breeding soundness of both, the semen donor and the semen recipient. Satisfactory results matching those of natural mating can then be obtained. It can be expected that the combined use of AI and sexed spermatozoa in distinct breeding programs will further boost breeding progress. **J Reproduktionsmed Endokrinol 2007; 4 (2): 101–5.**

Key words: semen cryopreservation, veterinary medicine, animal breeding, artificial insemination

Historical Background

In veterinary medicine, preservation of semen is closely connected with the development of artificial insemination (AI). The first report on attempted semen cryopreservation dates back to the Italian priest and physiologist Spallanzani in 1776 who tried to freeze semen with the help of snow [1], and who performed the first successful insemination in a dog in 1785 [2]. About 100 years later, Repiquet (1885) published his opus "About the theoretical possibilities of the practical use of artificial insemination" at the Académie Vétérinaire Française and postulated the successful application of AI as a tool for breeding. Development of AI and preservation of semen was pushed ahead in the 20th century. In Moscow, Ivanov [3, 4] initiated investigations on AI of sheep, and Milovanov (1927, cited by Salamon & Maxwell [5]) continued this work. As early as 1927, AI was used in large breeding programs in cattle. The detection of egg yolk to avoid or minimize cold shock effects was a milestone in the large-scale use of chilled semen (Milovanov & Selivanova 1932, cited by Salamon & Maxwell [5]). Bernstein and Petropavlovsky [6] were the first to describe the use of a 9.2 % glycerol solution for cryopreservation of mammalian (bull, boar, guinea pig, rabbit, ram, stallion) and avian (fowl, duck) spermatozoa. The cryoprotective properties of glycerol were focused on by Polge et al [7] and Polge and Rowson [8].

Advantages of AI and Semen Cryopreservation in Animal Breeding and the Legal Situation

The breeding progress brought upon by the rapid multiplication of valuable genes, thus leading to the establishment of first-class breeding populations today, is the main reason for the use of AI. However, when AI was introduced into animal production and veterinary medicine, another main goal was to combat venereal infections,

Table 1. Number of AI performed and % livestock inseminated (whereas in cattle only frozen semen is used, for insemination in pigs virtually only extended semen is used).

	Cattle [10]	Pig [11]	Horse [12]
Number of insemination stations	23	23	29
Number of first inseminations (Mio)	4.61	5.441	0.039
% deep-frozen semen	100	0	2.4
% inseminated females	82	> 90	82

with *Brucella bovis* and *Tritrichomonas fetus* for example, pathogens inducing epidemic abortions [9]. While this goal had already largely been achieved with the use of extended and chilled semen, it was the cryopreservation of semen which allowed for the long-term storage of semen and hence its virtually unlimited availability. Only after introduction of this technique into routine practice, the large scale provision of highly valuable genetic material became possible. As indicated in Table 1, annually almost 5 million inseminations using frozen semen are performed in cattle in Germany. At present, AI and semen cryopreservation can be considered the two major biotechnological methods applied in animal production. Breeding of farm animals is subject to strict international and national regulations. Within the EU, respective directives were transposed into national law, the "Tierzuchtgesetz" in Germany, last amended in 2006. These regulations are intended to insure sire identity, compliance with strict standards in hygiene, incontestable health conditions of sires and the adherence to breeding guidelines. These regulations also allow for international trade of farm animal semen. Since they also apply to canine semen, they thus help to avoid submitting stud-dogs to the stress of (intercontinental) travelling and quarantine restrictions [13]. In addition, long-term storage facilitates conservation of genetic resources of endangered species or breeds.

Semen Collection

In AI stations, bulls, stallions, and boars are generally deseminated by using a phantom for sexual stimulation. In case semen evaluation has to be performed on a stud used to natural mating, a teasing female might become necessary. With bulls and stallions, the reflex of ejaculation is provoked by using a properly prepared artificial

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vagina [9, 14, 15], with boar and dog, ejaculation can generally be provoked by manual stimulation/masturbation [9], however, particularly in the case of dogs, semen collection might only be possible when the stud is exposed to the olfactory stimulus of a bitch in standing heat [16, 17]. In special situations, electro ejaculation might be applied [18]. When using modern equipment, this procedure is rather well tolerated in ruminants (cattle, sheep, goat) and – in contrast to other species – no anaesthesia is required [19, 20].

Quality Requirements and Insemination Doses

AI is a developing process and the demands on the quality of fresh and frozen semen and the dose of insemination have evolved over time. The situation is best defined in cattle due to the large number of inseminations performed and the resulting solid statistical data.

Table 2 shows mean values for the most important semen parameters and – where appropriate – the critical values for the use of fresh semen for AI. Obviously, there are distinct differences between species; in addition, in the dog, a breed or “size” dependency must be considered: for small breeds, like the Teckel, a total sperm number of about 300×10^6 spermatozoa per ejaculate is satisfactory, whereas about 800×10^6 spermatozoa are requested for e. g. German Shepherds [25, 26].

Apart from the major microscopic parameters characterizing semen quality ([forward] motility, pathomorphology, sperm count) a whole array of other assays has been developed to further assess semen quality also of domestic animals, e.g. the HOS (hypoosmotic swelling) test, the hemizona assay, the swim-up technique, the test for chromatin condensation or post-thaw longevity [23, 27–29]. Valuable scientific data have been obtained. While estimates on a reduced fertility are possible when reference values are not met, until now the fertilizing capacity and the resulting breeding success can not be predicted, however. Here, the inevitable loss of motility associated with freezing and thawing, which should not exceed 20 %, is an important parameter and is tested with each batch of frozen semen [30–32].

The insemination dose (number of spermatozoa) routinely used in AI is species dependent and should allow for an optimal breeding success close or equivalent to natural mating.

According to recent recommendations for insemination of cattle, a minimum of 50 % of the spermatozoa should show a progressive forward motility following freezing and thawing, the minimum dose is 16×10^6 spermatozoa with progressive forward motility [10]. In the mare, the respective dose varies between 300 [33] and $> 800 \times 10^6$ spermatozoa [12]; following freezing and thawing, motility should be around 50 %, the lower level set by the “Deutsche Reiterliche Vereinigung” [12] is 35 %. In the dog about 150×10^6 forward motile spermatozoa [25, 34] are suggested as an insemination dose. In the sow only extended liquid semen is routinely used and an ejaculate meeting the criteria listed in Table 2 is extended to $2\text{--}3 \times 10^9$ spermatozoa per dose of insemination. This yields about 20–30 doses per ejaculate while, for example, in the bull more than 500 doses may be obtained from one ejaculate.

Table 2. Mean values (a) of some semen parameters and minimum requirements (b) for use in AI. According to [21–25].

Species		Volume (ml)	Total sperm number ($\times 10^9$)	Motility (%)	Pathomorphology (%)
Bull	a	5	6	$> 70^{(1)}$	< 20
	b			65	
Boar	a	200	60	$> 70^{(2)}$	$< 20^{(3)}/< 25^{(4)}$
	b			60	
Stallion	a	60	9	$50^{(1)}$	< 30
	b			$> 70^{(5)}$	
Dog	a	$1^{(6)}$	$0.3\text{--}1.0^{(7)}$	$> 75^{(1)}$	< 20
	b				

¹⁾ progressive forward motility; ²⁾ total motility; ³⁾ without plasma droplets; ⁴⁾ plasma droplets; ⁵⁾ total motility, depending on AI station; ⁶⁾ sperm rich fraction; ⁷⁾ depending on breed

Problems of Cryopreservation

Inevitably, semen cryopreservation results in a reduction of semen quality, mostly due to the exposure to cold shock occurring when the temperature is decreased from 15 °C to 4 °C, as well as freezing damage [35]. Among other effects, this results in a loss of forward motility and membrane integrity [36]. Functionally, the time necessary for sperm capacitation in the female genital tract is reduced [37].

There are distinct species variations in the reaction to cold shock, with boar spermatozoa being much more sensitive than human spermatozoa, for example. This has been attributed to the difference in size of the sperm head; which is $9.0 \times 5.0 \mu\text{m}$ in the boar and $4.6 \times 3.2 \mu\text{m}$ in the human; however, no functional proof has been obtained yet. In general, presently available extenders allow the routine use of extended and chilled ($\sim 5^\circ\text{C}$) semen for AI in cattle, horses, and dogs. In addition to securing an isoosmotic/isotonic condition, an adequate buffering capacity and the necessary energy supply (e.g. addition of glucose), the provision of a plasma-membrane protecting agent is of vital importance. Egg yolk has been shown to act in this manner (see above) and is still widely used. As neither the semen nor the egg yolk can be considered free of bacterial contaminations, even when hens are held under specific pathogen free conditions, generally an antibiotic is added to the extender (procain penicilline, ampicillin, gentamicin sulphate, lincomycin hydrochloride) to prevent further bacterial growth [9, 38–40]. The high prevalence of salmonella infections in laying hens and the resulting possibility of also the egg yolk getting contaminated during the handling procedure, is a specific problem associated with the use of egg yolk. Hence, the observation of strict hygiene measures is general practice (e.g., rinsing of the shell with alcohol, deflagration of the shell). Furthermore, extenders like AndroMed® (Minitüb, Landshut, Germany) have been developed in which egg yolk has been replaced by a substrate devoid of this inherent problem [41].

For cryopreservation glycerol is added. In general, the final concentration in the extended semen ranges from 4 to 6 %, leading to a decrease of the freezing point to about -30°C . A recent study has confirmed that higher concentrations are detrimental to semen quality [28]. Freezing poses several obstacles that need to be overcome or their effect kept at a minimum by observing adequate freezing protocols: the formation of intracellular

ice crystals, leading to a destruction of the cytoskeleton; the extracellular ice formation (pure H₂O crystals), resulting in an increase of solutes and hence an increased osmolarity, leading to a loss of cell water (exosmosis). The adaption period to 5 °C should be kept as short as possible, -196 °C should be reached stepwise with rapid temperature changes from one step to the next (see Table 3). With the arrival at eutectic temperatures, no further damage should occur [36, 43].

The process of thawing also affects semen quality [44] and adequate protocols have to be followed. Thawing at 70 °C for 5 seconds has been shown to be superior to thawing at 37 °C for 20 seconds [28, 36]. However, for practical reasons the latter procedure is commonly applied. Under no circumstances, however, should frozen and thawed semen again be exposed to cold stress (temperatures below 15 °C).

The above mentioned and accepted loss of about 20 % in motility as a result of freezing and thawing indicates that – despite considerable progress in recent years – cryopreservation still imposes great stress on spermatozoa. Factors leading to sperm damage are oxidation of sperm membrane lipids and a damage of selective permeability mechanisms of the membrane [45–47]. The percentage of spermatozoa in frozen and thawed semen samples exhibiting a decreased fertilizing capacity is strongly increased due to the decreased life-span in the female genital tract and a decreased ability to interact with the oviductal epithelium [48–51].

Packing and the Technique of Cryopreservation

Various forms of packing have been applied during the development of AI and cryopreservation of domestic animal semen. Frozen bovine semen samples have been made available as an uncoated pellet (drop of extended semen in a well, drilled in dry ice, -73 °C, followed by storage at -196 °C), packed in an ampulla and packed in “straws”. For practical reasons and for the required labelling of each individual semen dose, ampullas and pellets are no longer in use. Presently, straws with 0.25 ml volume (length 130 mm, diameter 1.9 mm: Minipaillette) are the most common form of confection, followed by straws with 0.5 ml volume (length 133 mm, diameter 2.8 mm: Midipaillette). A special form of straw is the “Minitüb” (volume 0.3 ml, length 65 mm).

Midipaillettes are generally used for the packing of dog and horse semen as well. Other than with cattle, one insemination dose is packed in more than one paillette, requiring pooling prior to insemination. The formerly rather common packing of stallion semen in “Makrotübs” (4 ml volume, length 24 cm, diameter 50 mm) is no longer internationally accepted, possibly due to the risk of semen loss associated with this type of packing and the superior thawing conditions under the use of Midipaillettes. According to our own experiences, the thawing of “Makrotübs” has also the inherent risk of loosing the sample (explosion due to rapid volume extension).

To establish a semen bank, extended boar semen is cryopreserved in 0.5 ml straws. For this purpose, 2 ml “flat straws” [52] and 5 ml plastic bags [53] have also been developed.

Table 3. Procedures for deep-freezing of dog semen (Gießen procedure, modified according to Günzel-Apel, 1994) [42]

- 1) Preparation of a tris-egg yolk diluent. Microbiological examination before use.
- 2) Labelling of straws (name of the dog, stud book number, date of collection, owner's name, identification of the clinic)
- 3) Semen examination (volume, pH, motility, % live spermatozoa, % pathomorphology, sperm concentration)
- 4) Dilution of the semen depending on sperm concentration:

concentration	semen (ml) + diluent (ml)
0.3–0.5 × 10 ⁶ sperm/μl:	1 + 3
0.5–0.8 × 10 ⁶ sperm/μl:	1 + 4
> 0.8 × 10 ⁶ sperm/μl:	1 + 5
- 5) Careful mixing of the semen with first 50 % of diluent, not yet containing glycerol
- 6) Careful mixing of diluted semen with second 50 % of diluent, supplied with glycerol to yield a final concentration of 6 %
- 7) Examination of motility of the diluted semen
- 8) Packing in straws (0.5 ml) with an air bubble in the middle, sealed with a ball
- 9) Adaptation in a styrofoam box at 4 °C for 2–3 hours
- 10) Cooling at –140 °C for 10 minutes
- 11) Transfer into liquid nitrogen (–196 °C)
- 12) Thawing and semen examination (pH, motility, % live sperm, % pathomorphology, sperm concentration) after 24 hours in liquid nitrogen

At AI stations dealing with bulls, packing and freezing in labelled straws is fully automated. According to present legal regulations, labelling must include identity of the sire (name, stud book number), breed, date of semen collection, and the identity of the AI station. With an automatic filling machine, up to 4 straws can be simultaneously filled, resulting in a capacity of 15.000 straws per hour (e.g. MPP Quatro, Minitüb, Landshut, Germany). After filling, straws are heat- or ultrasound-sealed.

Freezing is also automatic and computer-controlled (e.g. Icecube, Minitüb, Landshut, Germany). 4140 Minipaillettes or 2760 Midipaillettes are frozen simultaneously. A typical freezing protocol for cryopreservation of bovine spermatozoa requires an apparatus for controlled temperature lowering. However, efficient cryopreservation can be performed without such an apparatus. Table 3 shows the applied program for the freezing of dog spermatozoa in our laboratory. Semen is stored in large liquid nitrogen tanks.

Artificial Insemination

General Aspects

Since AI in farm animals is not suited to overcome breeding problems, it should only be performed with sexually healthy male and female animals. Semen should be deposited into the corpus uteri; however, pregnancy rates in cattle and horses may be increased by semen deposition in the uterine horn, ipsilateral to the ovary carrying the preovulatory follicle. This does not apply to polytalous species like pig and dog.

Depending on the species, monitoring of oestrus behavior, oestrus-related morphological and hormonal changes, and follicular development is generally indicated to determine the optimum insemination time. In cattle and formerly also in pigs, the number of animals not scheduled for a second insemination within a defined period of time yields the so-called non-return rate (NRR), a statistical parameter for grading the success of AI.

Other than in cattle and pig breeding, in horse and dog breeding individual pregnancies are regarded the benchmark of AI success. In polytocous species, the number of newborns is additionally evaluated for the "fertility of the semen resp. semen donor". Following an adequate training, veterinarians or other persons are authorized for semen transfer in farm animals. In pig production, for example, almost 99 % of inseminations are performed by farm personnel. Apart from the mere training in semen transfer techniques, the maintenance of and adherence to utmost hygienic conditions for the insemination procedure itself are part of every training program since the animals are left in their normal farm environment when an insemination is performed.

Special Aspects

Cow

Oestrus length varies between 18 to 24 hours, ovulation occurs 8 to 12 hours after the end of oestrus. When using frozen-thawed semen exhibiting a reduced capacitation period and life-span, the optimum time for insemination is during the last third of oestrus until ovulation. The expected 90-day-NRR should be around 65 % [54]. This parameter also serves to test for the fertility of a bull; a farm-based decrease in the NRR may be indicative for management problems or an insufficient insemination technique.

Mare

Oestrus length is 5 to 7 days and ovulation occurs approximately 1 to 2 days before the end of standing oestrus [55]. Follicular maturation exhibits distinct characteristic features and can be monitored by rectal palpation and ultrasound. As indicated above, following thawing, semen must be pooled prior to insemination. Other than in cattle, this procedure offers the opportunity to conduct a brief semen check prior to insemination.

Pig

Oestrus length varies between 40 to 60 hours the ovulations occurring about 38 to 42 hours after the onset of oestrus [55]. The use of cryopreserved semen is the exception and for more than 99 % of the inseminations, extended liquid semen is used [56]. The insemination dose of $2-3 \times 10^9$ spermatozoa is suspended in 80–100 ml in order to stimulate uterine contractility.

Bitch

Average standing heat in the bitch lasts for 5 to 9 days. In general, ovulation occurs on days 2 to 3 after onset of standing heat; opposed to other domestic animals, the second meiotic division occurs only after ovulation and requires a period of 2 to 4 days. When using cryopreserved semen with a reduced life span, the determination of the appropriate time of insemination is essential for achieving acceptable pregnancy rates. Insemination should occur when oocytes have become fertilizable, i.e. after the second meiotic division; vaginal cytology, vaginoscopy and – most reliable – quantitative determination of blood progesterone concentrations, which start to rise prior to ovulation, serve as indicators. Progesterone levels between 10 and 12 ng/ml are generally considered optimal for insemination with cryopreserved semen [25, 57].

Transcervical/intrauterine deposition of the frozen-thawed semen is inevitable for acceptable pregnancy

rates and litter sizes [58]. Insemination can be performed with a rigid Norwegian catheter with transabdominal fixation of the cervix [13] or an endoscope under visual control [59]. Intrauterine insemination following laparoscopy is for ethical reasons less accepted; however, in some rare cases it is the only possibility to achieve intrauterine semen deposition. In general, repeating AI after 24 hours results in significantly higher pregnancy rates and litter sizes [60].

Recent Developments

After several decades of experimentation, recent progress in separating y- and x-spermatozoa finally allows for selected breeding of male or female progeny in distinct cattle and swine breeding programs. This progress is largely due to the development of high-speed sorting machines, yielding the separation of up to 15 millions x- and y-spermatozoa per hour [61] as well as methods of "low-dose" inseminations deeply into the uterine horn, ipsilateral to the ovulatory follicle at an optimal time [62–65]. Great potential is also seen in the combined use of sexed-spermatozoa, in vitro maturation of oocytes and ICSI [61].

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