Advances in avian semen cryopreservation

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Abstract: Semen cryopreservation in domestic birds has been studied extensively in the past fifty years. However, efficient methods to freeze semen of chicken of different breeds have emerged only in the last decade of the 20th century. Methods using cryoprotectants Glycerol, Dimethyl Acetamide and Dimethyl sulfoxide, slow or rapid freezing-thawing procedures, pellets or ampoules or straws packaging have been developed. Other methods have been studied in turkeys, guinea fowl, ducks, geese and some wild species. They are often less successful than chicken freezing methods with wide intra-species variability.
Reliable predictors of suitability of semen for cryopreservation are also needed to improve freezing methods and to optimize the management of frozen semen. The quality of whole fresh semen measured by classical morphologic, metabolic and mobility tests is an indicator. More specialized tests such as membrane fluidity of fresh spermatozoa are indicative of the freezability of the gametes.
The main use of semen cryopreservation in birds is the *ex situ* management of genetic resources in the context of decreasing in avian genetic biodiversity. This is achieved through germplasm cryobanks. There are three main national avian germplasm cryobank programs in 2006, operating in North America, The Netherlands and France respectively. They mainly include semen and blood samples issued from Public Research Lines, indigenous breeds and individual specific genotypes.
The future need for semen cryopreservation programs will focus mainly on three objectives 1) improvement of predictors of suitability of males for semen freezing, 2) emergence of standardized methods of semen freezing in species other than chicken and 3) increasing development of avian cryobanks.

Keywords: poultry, sperm, freezing, cryopreservation

Introduction

Semen cryopreservation in domestic animal species is a biotechnology of reproduction that is used to increase the diffusion and measurement of genetic progress, sustain the conservation of genetic biodiversity and improve the management of artificial insemination.
The development of gamete cryopreservation in poultry has been one focus of the scientific community for almost 100 years. In their classical monograph summarizing low temperature biology from 1736 to 1936, Luyet and Gehenio (1940) cited observations of Atkins (1909) and of Moran (1925) on freezing points and the effects of low temperatures on hen's eggs. After that, however, little if any research on avian cells appeared to be conducted until Shaffner et al. (1941) showed that fertile eggs could be obtained from hens inseminated with frozen chicken semen, although no live chicks were produced. Then in 1949, Polge et al. reported their "chance observation" that glycerol protected spermatozoa against the effects of low temperatures. They found that fowl spermatozoa that were suspended in Ringer's solution containing 20% glycerol, frozen to -79ºC and then thawed rapidly exhibited motility indistinguishable from their unfrozen controls. The ramifications of that serendipitous finding cannot be exaggerated.

Since 1949, as summarized above, innumerable studies have been performed to improve and standardize methods for the long term preservation of spermatozoa of many animal species. These have included spermatozoa of domestic birds (see reviews by Lake, 1986; Bellagamba et al, 1993; Hammerstedt, 1995; Suraï and Wishart, 1996; Donoghe et Wishart, 2000; Blesbois and Labbé, 2003; Massip et al, 2004). These methods were first developed in the chicken and then more recently applied

to spermatozoa of turkeys, ducks, geese and guinea fowl. Despite the relatively intense investment of the scientific community in research on semen cryopreservation, however, these methods have been relatively unused in the actual breeding of poultry. One of the reasons is that artificial insemination is not widely used with many domestic species of birds. Although insemination with fresh semen is extensively used in the breeding of a few species, e.g. turkeys, guinea fowl and mule ducks, this method is less often employed with chickens, muscovy and pekin ducks, ganders, quails, ostriches and emus. In addition, the success of freezing procedures applicable to poultry is highly variable and depends on the species and the specific lines being bred. In addition, the costs of the various stages in the preparation, storage and use of frozen ejaculates in poultry species remain relatively high compared to the market price of day-old chicks.

Regardless of the above constraints, however, ratification of the international agreement on biodiversity in Rio de Janeiro, Brazil in 1992 provoked new interest in development of methods of semen freezing for domestic birds. Research into cryopreservation of avian cells, including spermatozoa, has received renewed attention in order to develop the capability of: [1] preservation of rare lines, [2] maintenance of acceptable genetic variability in parental lines selected by primary breeders, and [3] long-term availability of the genetic potential from exceptional animals. Recent progress in cryopreservation of avian semen, mainly of the chicken, has emphasized the demand for *ex-situ* management of gene banking both in Europe and in North America (Pisenti al, 1999; Blackburn, 2006; Fulton; 2006; Woelders et al, 2006) in addition to *in-situ* management of these species. This is very important for domestic bird species that include a very high number of rare lines. For example, 154 rare lines of the species *Gallus gallus* exist only in France. With present methods and understanding of low temperature biology, cryopreservation of embryos of domestic birds is not possible, undoubtedly because of the very large size and high vitellus content of eggs. The cryopreservation of blastodermal cells or primordial germ cells might offer a means to preserve the entire genome (reviewed by Tajima, 2002). However the low efficiency rate of reconstitution and the high costs associated with current technologies makes these last approaches prohibitive (Petite, 2006). Cryopreservation of avian semen is, therefore, the only efficient method of *ex-situ* management available for avian species.

Methods of semen cryopreservation

Cryopreservation is a non physiological method that involves a high level of adaptation of biological cells to the osmotic and thermic shocks that occur both during the cooling-freezing procedure and during the thawing procedure. Bird spermatozoa are cells that contain very little cytoplasm and very large relative surface of plasmatic membranes. The only cytoplasmic organites are mitochondria. The nucleus contains very condensed chromatin.

Damages occurring during the freezing-thawing procedures affect mainly cellular membranes (plasmatic and mitochondrial) and in the worth case the nucleus. Such damages affect fertility and its duration. The duration of fertility is a very important factor in birds due to the long term storage of semen in the utero-vaginal glands of the female.

The main critical points that affect cell structures and metabolism during cryopreservation are 1) the interactions between the spermatozoa and the internal cryoprotectant agent added to the sperm to limit temperature stress, 2) the temperature curve and 3) the type of semen packaging. In consequence, many studies has been performed to find the best cryoprotectant agent, the highest combination of freezing and thawing temperature curves and the least deleterious system of semen packaging for spermatozoa that permits traceability and respect of healthy and sanitary conditions. Their results have given rise to different procedures depending on the species and sometimes on different breeds of the same species.

Chicken

Despite early attempts to freeze chicken spermatozoa, the first methods of successful semen cryopreservation in chickens were published by Lake and Stewart (1978) and by Sexton (1980) more

than thirty years after the report by Shaffner et al. (1941). Lake and Stewart used low cooling rates, glycerol as a cryoprotective agent, and glass ampoules to package the semen, whereas Sexton also cooled the samples slowly, but used dimethyl sulfoxide (DMSO) as the cryoprotectant and straws for packaging. Both methods were later optimized by Seigneurin and Blesbois (1995) and by Van Voorst and Leenstra (1995), respectively. Two other methods issued from the former USSR used rapid cooling by freezing the spermatozoa as pellets with either dimethyl formamide (DMF, Schramm 1991) or dimethyl acetamide (DMA, Tselutin et al. 1995). Comparison of cryoprotectants and methods of cryopreservation under standardized conditions (Tselutin et al., 1999; Challah et al., 1999) showed that the highest fertility rates after artificial insemination with frozen semen were obtained either with semen frozen slowly as straws with glycerol or semen frozen rapidly as pellets with DMA. This last method, optimized and combined with packaging of frozen pellets in straws (to optimize identification and safety) has been chosen as a reference for gene banking of local breeds of chickens in The Netherlands (Woelders, 2006), while in France, the method with glycerol is still considered to be the best for gene banking because it is also effective with low fertility lines (Blesbois et al, 2006). Glycerol is probably the best cryoprotectant for bird spermatozoa. However, it needs to be removed from semen at thawing. This may be done by successive centrifugations and dilution or dialysis, percoll or Accudenz gradient centrifugation (Lake and Ravie, 1978; Buss, 1993; Long and Kulkarni, 2004).

Turkey semen

Several authors (Sexton, 1981; Zavos and Graham, 1983; Lake, 1986; Tselutin et al., 1995) have attempted to freeze turkey semen using various cryoprotectants (glycerol, DMSO, ethylene glycol, DMA), freezing the specimens either as pellets or in straws. To date, none of the above studies has yielded reproducible results of fertility, indicating that turkey spermatozoa are much more sensitive to damage caused by cooling/freezing procedures than chicken semen (Blanco et al, 2000). A study by Blesbois and Grasseau (2001) suggested that the variability of fertility results may be partially reduced by removing seminal plasma before freezing. Current techniques used to freeze and thaw turkey semen do not result in fertility levels comparable to those obtained in the chicken (Table 1), but, with minor modifications they are sufficient to allow development of sperm banks in this species.

Semen of other bird species

Other species in which significant research efforts have been conducted to develop freezing procedures for semen preservation include drakes, ganders and guinea fowl (Tselutin et al, 1995; see review by Suraï and Wishart, 1996; Seigneurin et Blesbois, 2006). As with semen of the turkey and chicken, several cryoprotectants and freezing methods have been tested. It appears from these studies that spermatozoa of some lines of ganders are fairly resistant to freezing/thawing procedures, exhibiting acceptable and reproducible rates of fertility (>60%, Lukaszwicz, 2001; Tai et al., 2001), while other lines gave far lower results (Lukaszwickz et al, 2004). For duck species, it appears that the spermatozoa of muscovy ducks are more resistant to freezing than those of Pekin ducks. Guinea fowl sperm seem very sensitive to cryopreservation damages (Seigneurin and Blesbois, 2006).

Attempts have also been made to freeze semen of different wild avian species and some of these were successful (Blanco et al, 2000; Penfold et al, 2001; St James et al, 2003). However these studies were dispersed and sometimes performed with already inbred males of endangered species. A real policy of genetic conservation to sustain their development is currentlylacking.

Table 1. Reproductive fertility performance with frozen semen in poultry species

	Chickens	**Turkeys**	**Ganders**
Mean fertility (% fertile/incubated eggs) Range (min-max)	60 [0-90]	30 [0-65]	60 [0-70]
Mean number of chicks hatched per frozen ejaculate	2	1	1.5

It can be concluded that recent progress in freezing/thawing procedures applicable to chickens and ganders is now adequate to allow gene banking and *ex-situ* management for these species. Significant progress can be expected in the long term storage of turkey, duck and guinea fowl semen within the next few years.

Predictors of semen suitability for cryopreservation

It is generally accepted, and confirmed in practice, that one criterion to predict the suitability of semen for freezing is the quality of the fresh semen. Cryopreservation leads to damage to cells and systematically results in lower quality than fresh sperm. This decrease in semen quality due to freezing also increases the damages that were previously present in fresh semen. A poor fresh semen quality still gives very poor frozen semen.

In addition to these general factors, specific biological and biophysical factors affect the ability of sperm to prevent the damage caused by the cryopreservation procedure. These factors include membrane permeability, lipid composition and fluidity, and their damage has consequences on motility and different metabolic factors including ATP concentration of spermatozoa (Ansah and Buckland, 1982; Blanco et al, 2000; Blesbois et al, 2005; Long, 2006). Among all these factors, the membrane fluidity of fresh semen has been shown to be proportional to the cholesterol/phospholipids ratio of spermatozoa and to behave as an indicator of inter species sperm freezabililty. Experiments currently being conducted in our laboratory confirm this predictive capacity, but within a specific species (Blesbois al., 2006b)

Semen cryopreservation for the development of animal germ plasm cryobanks

The main use of semen cryopreservation in birds is the management of genetic diversity. This diversity is rapidly decreasing due mainly to the progressive disappearance of many small populations and to the very high specificity of selected commercial lines. In addition, the increasing risks of epidemic avian influenza in the past few years have emphasized the need for acceleration of *ex situ* conservation of genetic resources. In North America, the policy of *ex situ* management of poultry resources is included in the National Animal Germplasm Program. In Europe there are two main national germplasm cryobanks: one in the Netherlands managed by The Netherlands Centre of Genetic Resources and one in France managed by the French National Cryobank of Domestic Animals. The three programs currently contain semen and blood from the species *Gallus gallus*.

In 2006, the North America Cryobank contains semen and blood originating from 59 lines, representing a total of 451 animals and 2132 semen straws (Blackburn, 2006) mainly originating from the collection of Public Research Lines. In The Netherlands, the collection of semen stored in the cryobank mainly originates from rare indigenous breeds. It contains 11 breeds representing a total of 121 males and 7762 insemination doses (Woelders et al, 2006). In France, the collection of semen stored in the avian cryobank originates mainly from public experimental lines but also contains the oldest rare indigenous breed, the “gauloise dorée” and semen of males with specific particular genotypes. The French cryobank includes the semen originating from18 breeds and 64 particular genotypes, representing at total of 391 males and more than 11000 straws (Table 2).

Conclusion - Future needs

The development of methods of avian semen cryopreservation during the last 50 years has been slow. The emergence of effective methods for chickens during the last ten years of the 20th century, in conjunction with the observation of dramatic decrease in genetic variability of avian resources, gave rise to the emergence of cryobanks comprising mainly semen to prevent genetic degeneration of livestocks. The main future need for semen cryopreservation in birds will take place in the development of cryobanks to complete the *in situ* management of avian genetic resources by *ex situ* management.

In the species *Gallus gallus* (but also in other species), this will involve more basic understanding of the variability of suitability for freezing of semen of different origins (individual variations, breed or line effect) to reach the number of frozen samples needed for further restoration of the main genetic characteristics of the animals.
Improvements in cryopreservation methods specific for each species will also be necessary to include the semen of species others than chicken in cryobanks. For example, in France, Pekin duck and gander semen will soon be added to the avian cryobank.
Finally, better coordination of national policies for the management of genetic resources, industrial commercial needs and international networks will be also in the interests of each partner.

Table 2 Numbers and origins of the straws stored in the French avian cryobank

Line or strain	Number of males	Number of straws/male	Number of straws/line or strain
"Gauloise dorée"	20	8-40	491
Exp line R+	22	30-55	994
Y33	22	21-61	982
…….B4	20	7-41	474
…….B13	2	20	40
…….B 21	2	19-20	39
Fayoumi Epileptique	15 4	44-60 23-40	759 140
Exp Line DPF + ……...DPF -	9 14	56-65 36-67	552 777
……D+	21	32-40	825
……D-	22	22-40	803
……Thin …… Fat ……...X 33 ……...X 44 ……...S 6 ……...S N ……...S 15	22 22 22 22 22 22 22	35-40 6-40 29-32 27-32 in due course in due course in due course	859 835 700 692 about 700 about 700 about 700
Males with particular Genotypes	64	16	1024

Exp line : experimental line

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