

## ROOSTER SPERMATOZOA CRYOPRESERVATION AND QUALITY ASSESSMENT

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### Abstract

Unsuccessful rooster fertility following cryopreservation may be linked to specific changes in spermatozoa quality, which can be determined using various methods. These determinations also facilitate the design of improved freezing and thawing processes. Here, we update the current state of methodologies available for the assessment of rooster semen quality after cryopreservation. Computer-assisted sperm analyses (CASA) is one of the main systems used to analyse motion parameters of spermatozoa (total motility, progressive motility and motion parameters). Moreover, fluorescent techniques and flow cytometry can improve the assessment of various aspects of semen quality (viability, acrosome status, mitochondrial potential, lipid peroxidation, DNA damage, lipid peroxidation and cell debris removal) using specific fluorescent markers such as ethidium bromide, Yo-Pro-1, Annexin V, propidium iodide, SYBR-14, PNA, JC-1, BODIPY, acridine orange and DRAQ5. Transmission electron microscopy also yields valuable information on spermatozoa ultrastructure. The application of these techniques to rooster spermatozoa is reviewed in relation to specific freezing techniques, the effects of cryoprotective agents (CPAs) and extenders, and the determination of spermatozoa quality after cryopreservation.

**Keywords:** cryopreservation; quality evaluation; rooster; semen.

### INTRODUCTION

Cryopreservation of spermatozoa is an effective approach to the conservation of domestic and farm animals. Artificial insemination (AI) is a technique that is often used to manage or accelerate the rate of genetic improvement; frozen-thawed spermatozoa (1) are used to inseminate multiple females or

semen obtained from a male of desired quality (2). Many protocols have been designed to create a suitable process for cryopreservation of rooster semen, but a successful cryopreservation procedure has not been yet developed (3, 4). Nowadays, cryopreservation of reproductive cells is an effective means of maintaining male genetic material within an ex situ cryobank (5). It is generally known that cryopreserved rooster

semen has some limitations resulting from low sperm motility traits and decreased fertilizing ability (6).

Semen freezing in poultry is the only method available to use in ex situ cryopreservation programmes, because the avian eggs contain high yolk content which prevents their successful cryopreservation (Blesbois, 2007). Nevertheless, this process still cannot be used routinely due to the high incidence of cryoinjuries (7, 8).

Quality of poultry semen is a proper predictor of fertility potential and subsequent hatchability of eggs. Since the total and progressive motility influences the fertility rate, parameters of semen motility and viability may indicate future rooster fertilization ability (9). Light microscopic techniques are commonly used analytic tools; however more precise and objective options are available, such as the computer-assisted spermatozoa analysis (CASA) assay that determinates spermatozoa concentration and motility parameters (10). For a more detailed determination of semen quality, flow cytometry allows the examination of 10,000 spermatozoa in a very short time, which makes this assay very objective, precise and sensitive (11).

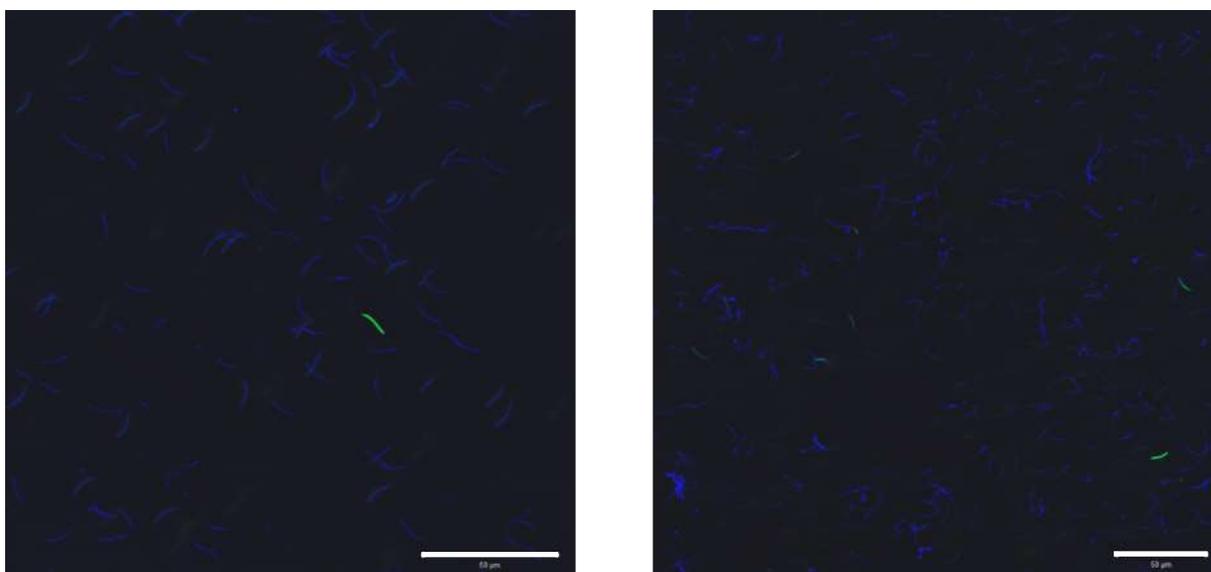
The increasing availability of fluorescent markers has resulted in the development of methods for the evaluation of many spermatozoa quality traits. Several staining techniques are

available for evaluating cell survivability and can be used alone or in combination with other dyes for the assessment of spermatozoa quality (12, 13). Here we discuss the cryopreservation process, as well as the evaluation methods of fresh and frozen/thawed spermatozoa quality.

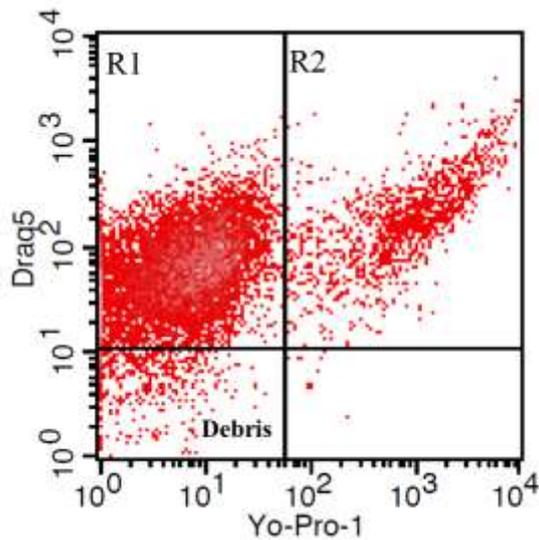
#### ***Methods for spermatozoa quality assessment using CASA***

The most important indicators associated with the fertilization potential and expression of spermatozoa viability and structural integrity are parameters of concentration, total motility and different motion characteristics. The CASA - computer-assisted sperm analysis - assay ensures precise and rapid evaluation of various semen parameters, such as total and progressive motility, patterns of spermatozoa movement, linearity, the beat cross frequency, the amplitude of the lateral head displacement and several velocity parameters (14, 15, 16, 17).

Previous studies have indicated that reduced spermatozoa concentration and semen volume in ageing broiler males contributed to reduction of fertility (18). The differences in rooster semen volume were usually affected by the type of breed, age, body size, nutrient feed and also by genetic factors, environmental factors and the interaction between them (19). A high degree of variation has been demonstrated in the semen quality parameters for various breeds, such as White Leghorn (20), Plymouth



**Figure 1.** Representative fluorescent staining of rooster spermatozoa using (A) DAPI and (B) Yo-Pro-1 dye under a fluorescent microscope. Scale bar: 50  $\mu\text{m}$



**Figure 2.** Representative dot plot of debris separation using DRAQ5 dye. R1 region represents DRAQ5+ (nucleated) cells and R2 region represents DRAQ5+/Yo-Pro-1+ (nucleated/apoptotic) cells.

Rock (21), Rhode Island Red (RIR) (22) and indigenous roosters (23). Moreover, differences in the motion parameters amongst individuals within one Oravka breed are known (15).

Differences in the semen quality parameters have been observed in broiler breeders (24), hybrid (25) indigenous (23) and other several breeds (26). The viability of cryopreserved spermatozoa and fertilizing ability differs between chicken breeds. The recorded difference between breeds or lines may be due to genetically-determined differences in the spermatozoa cryotolerance to the treatments. Composition of seminal plasma proteins and spermatozoa oxygen consumption rate can explain the greater resistance to cryo-damage of some selected lines (27).

### Measurement of spermatozoa characteristics using flow cytometry

Many researchers have used flow cytometry to analyse specific features of spermatozoa, such as viability, acrosomal status, membrane mitochondrial potential, production of ROS or changes in DNA. The ethidium bromide (EtBr) exclusion procedure is often used to evaluate the viability of rooster semen (28). In roosters, the Annexin V kit is often used for the determination of membrane phosphatidylserine externalization as one of the earliest indicators of apoptotic changes in the semen sample (29). In addition, Yo-Pro-1 dye can also be used for the determination of apoptotic spermatozoa; whilst apoptotic cells are permeable to Yo-Pro-1, they remain impermeable to propidium iodide (PI), a marker of dead cells (30). However, SYBR-14 is also a widely used marker to determine the proportion of live cells (15, 31, 32) (Figure 1).

Another spermatozoa quality parameter is the mitochondrial potential. Mitochondria are responsible for producing the energy necessary for flagellar beat. Sperm motion enables sperm to cross the female reproductive tract to reach the fertilizing site. Mitochondrial function can be evaluated by different fluorescent probes, such as rhodamine 123 or JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide) (30).

Spermatozoa acrosomal status can be assessed by flow cytometry using fluorescence-labeled lectins, which are able to distinguish and bind to glucosidic residues in various parts of the acrosomal membrane. The most commonly used lectin *Arachis hypogaea*-agglutinin (PNA, or peanut agglutinin), derived from the peanut plant, is a marker of the acrosome status (33).

**Table 1.** Summary of fluorescence markers used for rooster spermatozoa evaluation.

Parameter	Stain	Reference
Viability	Annexin V Yo-Pro-1 Propidium iodide SYBR-14	(28), (30), (31), (32) (16)
Mitochondrial function	rhodamine 123 JC-1	(30)
Acrosomal status	PNA	(33), (34) (35)
DNA damage	Acridine orange	(30), (33)
Lipid peroxidation	BODIPY	(30)
Cell nucleation	DRAQ5	(16)

PNA coupled with fluorescein isothiocyanate (FITC) is used for the determination of acrosome integrity (33, 34, 35). An undamaged acrosome is needed for the acrosome reaction, which occurs at the time suitable to facilitate fertilization (36).

As a predictor of DNA damage in many cell types, acridine orange (AO) has been widely used. Because of variability of protocols used in previous studies, the AO staining technique has not been widely accepted as a screening test to predict DNA damage in poultry semen. However, AO has been used to successfully evaluate chromatin status (33, 30). Cells with double-stranded DNA configuration emit green fluorescence, whilst cells with denatured DNA fluoresce red.

The cell membrane of avian sperm contains high amounts of polyunsaturated fatty acids (PUFAs) that can easily undergo lipid peroxidation (LPO) in the presence of reactive

oxygen species (ROS) (37). In studies on oxidative stress, lipid peroxidation has been evaluated using a fluorescent lipid probe C11-BODIPY581/591 (30) (Table 1).

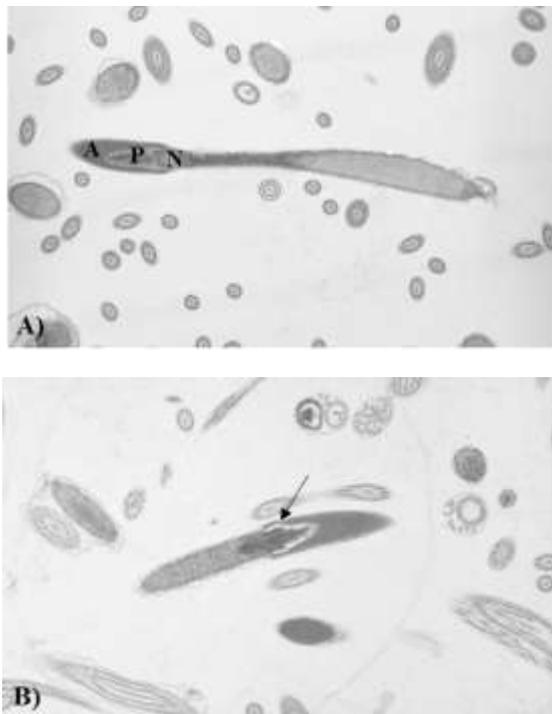
There is a risk that cell debris or other cells can be present in the seminal plasma. To assess this risk, DRAQ5 dye has been used as a suitable nuclear marker of rooster and gander spermatozoa. The dye enables the recognition of cells with a nucleus and allows their separation from surrounding debris or other cells (15, 17). This technique allows an accurate assessment of target cells by flow cytometry (Figure 2).

### ***Transmission electron microscopy (TEM)***

Rooster spermatozoa of good quality have an intact acrosome membrane, well developed nucleus, mitochondria, axoneme, centriole and perforatorium. In contrast, post-freezing samples tend to have markedly swollen plasma membrane and damage around the perforatorium (16). Different classes of plasma membrane damage in cryopreserved chicken spermatozoa can also be identified using electron microscopy (38). It is already known that cryopreservation causes numerous negative impacts including damage to cell membranes (plasma and mitochondrial) and, in some cases, to the nucleus with devastating consequences for spermatozoa survival (39). Previous studies have demonstrated that cryopreservation and the thawing process impact the ultrastructure of poultry spermatozoa, especially the mitochondria, midpiece, and perforatorium (40). Ultrastructural studies on spermatozoa have shown that 60% of poultry spermatozoa organelles have underlying damage after cryopreservation (41).

### ***Spermatozoa cryopreservation***

Cryopreservation uses very low temperatures to preserve structurally intact living cells and tissues for a long period of time. Moreover, cryopreservation of semen is one of the most effective methods for the preservation of genetic resources of endangered animals. This method is non-invasive for donors and also for recipients and provides the option of collecting and storing a very large number of doses in a short period. The discovery of the cryoprotective effect of glycerol started with experiments on semen storage. Survivability of rooster spermatozoa was observed after exposure to  $-79^{\circ}\text{C}$  in the presence of 20% glycerol, with high total motility after thawing (42). Since



**Figure 3.** Electron micrograph of longitudinal and diagonal section of fresh and frozen/thawed spermatozoa obtained by ultrastructural analysis. A: axoneme, P: perforatorium; N: nucleus. Spermatozoa with intact plasma membrane of the head and intact acrosome are presented in Figures A and B. Swollen plasma membrane of head and acrosome in frozen sample are marked by an arrow. Magnification  $\times 7200$ .

**Table 2** Summary of CPAs used for rooster spermatozoa cryopreservation.

Permeability	CPA	Sperm motility (%)	Reference
Permeating CPAs	Glycerol	57.8;	(59)
		45.3	(16)
	DMSO	53.0;	(56)
		44.5	(57)
	EG	39.0;	(16)
		46.6;	(32)
	DMA	45.0	(58)
		21.3;	(59)
Non – permeating CPAs	DMF	15.2	(60)
		31.5	(61)
	Propanediol	21.8	(58)
		48.8;	(32)
	Ficoll	44.7;	(7)
		30.7;	(32)

then, many laboratories have focused on the storage of avian spermatozoa. In roosters, it was not until the end of the 20th century that consistently high fertility levels were achieved, indicating a long path to developing an effective and reliable procedure.

Freezing and thawing rates are important factors that can affect the process of cryopreservation. However, the type of CPA and its concentration, diluents and dilution, and semen packaging can all influence the success of freezing (43). Also, as spermatozoa heads have less cytoplasmic mass, they have less ability to move in the cryoprotective agents (CPAs) (44).

There are several ways to control semen cooling. The use of programmable freezers enables a controlled cooling rate and a precise decrease of temperature. Alternatively, vitrification by very rapid cooling can be used. The major differences between these two types of cryopreservation are the concentrations of CPAs needed and the cooling rates used. Slow freezing first enables the (partial) substitution of water in the cytoplasm with CPAs. This process is less detrimental to the cell when the cooling rate is slow enough to allow the equilibrium flow of water and CPAs across the cell membrane. However, slow freezing has a high risk of freeze cryoinjury due to the formation of extracellular ice. On the other hand, vitrification is the process of liquid solidification without the formation and growth of ice crystal after the exposure of biological material to high concentrations of CPA (in the ratio of 40–60%,

weight/volume), with subsequent rapid cooling to avoid ice nucleation. The disadvantage of vitrification is the high concentration of CPAs that could be toxic to cells (45).

#### ***Rooster cryopreservation as a model system***

During the freezing-thawing process avian spermatozoa may be irreversibly damaged, resulting in a decrease in spermatozoa motility, viability and fertility (46). The rooster spermatozoa membrane contains a large amount of phospholipids that could be a cause of their damage. Rigidity of spermatozoa membrane can be caused by the decline in phospholipids (during in vitro storage) leading to changes in the proportion of cholesterol to phospholipids (47). Normal integrity of the avian spermatozoa membranes is needed to protect the spermatozoa functions during storage in the female's reproductive tract (48). Moreover, a limiting factor in successful rooster semen cryopreservation is the filiform shape of the spermatozoa head, which is not much wider in diameter than the tail (between 90-100  $\mu\text{m}$ ) (44) compared to bull spermatozoa (45). The spermatozoa head has a small portion of cytoplasm that reduces its ability to move in the cryoprotectants. Moreover, long-tailed spermatozoa are easily damaged by freezing/thawing.

Semen extenders are enriched with protective supplements that promote survivability of spermatozoa outside the reproductive tract. Lipoproteins from milk or

egg yolk have a protective and stabilizing effect. Monosaccharides, such as glucose are a plentiful source of energy for spermatozoa. Addition of antibiotics to extenders can eliminate growth of bacterial organisms. The osmotic pressure and pH of extenders can be adjusted to maximize spermatozoa survival. There are various types of extenders for avian semen, such as Beltsville (37, 50), Nabi (51), Kobidil<sup>+</sup> (32, 16), Lake (52, 53), and (54). Nabi extender has been demonstrated to maintain frozen-thawed spermatozoa motility parameters better than the Beltsville extender (37). Similarly, the percentages of live and early-apoptotic spermatozoa were higher in the Nabi extender compared to the Beltsville extender.

Successful rooster semen cryopreservation also depends on the choice of a suitable cryoprotectant. Many commonly used cryoprotectants have been tested, such as glycerol (55, 37, 16), dimethylsulphoxide (56, 57), ethylene glycol (16, 37, 58), dimethylacetamide (59, 60), dimethylformamide (61) and propanediol (58). Moreover, the addition of non-permeating substances such as trehalose, sucrose or Ficoll can improve the cryosurvival of rooster spermatozoa (32, 7) (Table 2).

There are many differences in size, shape and lipid composition of spermatozoa with respect to species, and this can potentially affect the occurrence of cryoinjuries (62). The initial quality of native semen, such as total motility, and the abstinence period of spermatozoa donors, can also influence the cryosurvival rate of post-thawed spermatozoa. Rooster's semen is generally cryopreserved using the conventional slow freezing (63) or cooling in the vapour phase) (64).

Taking into account the various factors that affect rooster cryopreservation, a common protocol for slow freezing includes semen dilution using specific extenders, addition of CPAs in a suitable concentration, an equilibration period at 4°C for 30 min and packing semen into the 0.25- or 0.5-mL plastic straws. The straws are suspended horizontally in liquid nitrogen vapour 5 cm above the liquid nitrogen level for 15 min (-125 to -130°C) and subsequently plunged into the liquid phase (-196°C) for long storage. During protocol development, the straws are often removed after 2–3 days, then thawed at +4°C for 2 min and analysed for spermatozoa quality parameters using CASA and flow cytometry (16, 17, 37).

Other freezing methods, such as programmable freezing (59) and vitrification (8), with spermatozoa cryopreserved by slow freezing tending to have a higher motility rates compared to preservation by the vitrification method. Improvements in the freezing and thawing process, osmotic pressure, choice and concentration of suitable CPAs, and equilibration times in the CPAs might result in better survival and functionality of animal quality of frozen/thawed spermatozoa, permitting their successful future practical application.

## CONCLUSION

Spermatozoa cryopreservation is an important tool for conservation of genetic resources (breeds in danger or high-value males), timely access to semen from selected lines and for research studies. However, cryodamage may have detrimental effects on spermatozoa function and quality. There are several main points that can influence semen freezing, such as the concentration of CPA, the use of an appropriate extender, the system of the packaging, and cooling rate. These factors can influence greatly spermatozoa motility, viability, membrane mitochondrial potential and acrosome status; and also cause lipid peroxidation and DNA damage. DRAQ5 dye is a suitable nuclear marker of spermatozoa, as it enables detection of cells with a nucleus and their separation from surrounding debris or other cells; this makes it possible to accurately assess target cells by flow cytometry. Here we have provided an overview of the markers that can be used to help improve semen quality assessment relating to motion parameters that vary with type of breed, age, body size, nutrient feed and also genetic factors, environmental factors and the interaction between them. However, further research could be aimed at expanding the portfolio of suitable CPAs and markers for a more detailed evaluation of rooster spermatozoa quality prior to and after cryopreservation.

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