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Article

Effect of viscosity on the medium for rooster (*Gallus gallus*) sperm cryopreservation

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Abstract:

In mammalian semen, viscosity has been shown to have a negative influence on its conservation. In bird semen, studies on the physical characteristics of ejaculates are limited, particularly viscosity has not been studied. The media for cryopreservation do not consider viscosity to maintain sperm viability. Therefore, the objective of this study was to evaluate the effect of viscosity on the medium to maintain its viability after thawing. The parameters of basic evaluation, maturation and acrosome reaction were determined, evaluating the presence and distribution of Ca^{2+} through co-incubation with

chlortetracycline. Twenty-five (25) evaluations of seminal pool were performed, cryopreserved in Lake medium supplemented with 6 % dimethylacetamide and with 0 % (Control), 10 %, 30 % and 45 % ficoll, to adjust the viscosity of the medium to conditions similar to those of semen and oviductal fluid and to a higher degree of viscosity. Sperm motility was lower ($P \leq 0.05$) in aliquots with a higher percentage of ficoll. The percentage of live spermatozoa was similar ($P > 0.05$) in the control and all aliquots with different percentage of ficoll. Sperm maturation presented a higher ($P \leq 0.05$) percentage of non-capacitated spermatozoa when 10 % ficoll was used. Conversely the percentage of spermatozoa with acrosome reaction was also lower ($P \leq 0.05$) when 10 % ficoll was used. The results of this study show that variations in the degree of viscosity of the medium can maintain or increase sperm viability after thawing.

Key words: Acrosome, Sperm capacitation, Freezing, *Gallus gallus*, Semen.

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Introduction

At present, poultry farming of domestic species cannot be conceived without the application of knowledge and innovative technologies of assisted reproduction; this includes cryopreservation of semen and artificial insemination (AI)[1]. One of the biggest difficulties in achieving this is maintaining the viscosity of the seminal fluid, which can decrease when mixing semen with a diluent for its preservation, which can contribute to the loss of spermatozoa in the oviduct during AI[2].

The viscosity of the seminal fluid is attributed to the presence of mucopolysaccharides, glycosaminoglycans and proteins, this has been studied in different animal species such as camelids, elephants and wild ungulates, where changes in semen viscosity represent a problem for its conservation and for AI[3-6].

From the anatomical and physiological point of view, within the oviduct of birds, there are sperm storage tubules, which provide a specific microenvironment to maintain sperm viability and their fertilizing capacity for a long time[7,8]. In birds, when spermatozoa are inside the storage tubules, they are immobile, so their metabolism is basal, resulting in a low consumption of ATP[9]. However, they have active mitochondria, which provide energy for the activation of their motility and sperm capacitation[10].

When the ejaculated spermatozoa carry out the acrosome reaction, they are able to penetrate the perivitelline membrane that surrounds the oocyte and carry out

fertilization[11]. It has been mentioned that bird spermatozoa "do not need" a sperm capacitation process in order to carry out fertilization[11]. During cryopreservation, cooling procedures, addition of cryoprotective agents, freezing and thawing contribute to a cryocapacitation process[12,13], which is evidenced by the continuity in sperm maturation, and reduces its fertilization capacity when used for artificial insemination.

Considering that in other species it is important to maintain the viscosity of the seminal fluid to maintain sperm viability after thawing during AI processes and that this has not been studied in roosters, the objective of this work was to evaluate the effect of viscosity in the cryopreservation medium, to maintain the sperm viability determined by its parameters of sperm maturation after thawing.

Material and methods

Use of animals

It was carried out in accordance with the Official Mexican Standard 062-ZOO-1999. "Official Mexican Standard NOM-062-ZOO-1999, Technical specifications for the production, care and use of laboratory animals"[14]. Five Lohmann Brown lite roosters (*Gallus gallus*) were used, which were provided with balanced feed with 18 % protein and water *ad libitum*, and individual housing in metal cages of 70 x 70 x 90 cm, provided with a drinker and feeder.

Semen collection

Twenty-five (25) samples of semen were obtained from each rooster by dorsoventral massage[15]. Semen was collected from the cloaca by aspiration with a SL10-1000 micropipette, (RANIN™, USA). The samples were mixed to obtain 25 groups, which were diluted in Lake medium composed of fructose 0.6 %, sodium glutamate 1.92 %, magnesium acetate 0.08 %, sodium acetate 0.51 %, potassium citrate 0.128 %, pH of 7.2 and osmolarity of 330 mOms (L)[16], and 6 % dimethylacetamide (DMA) was added as cryoprotectant.

In each seminal group, its sperm concentration was determined by microscopy and with the use of a Neubauer chamber[1], to make six aliquots of 100 µL, with 100 x 10^6 spermatozoa, to which ficoll (F) was added to achieve different levels of semen viscosity. An aliquot of ejaculated semen (S) was considered as a control group. The group (S+L+ ficoll 10% +DMA) was conventionally diluted and 10 % F was added to achieve lower

viscosity compared to the control group. The group (S+L+ ficoll 30% +DMA) was added 30 % F to achieve similar viscosity compared to the control group and the group (S+L+ ficoll 45% +DMA) was added 45 % F to achieve greater viscosity compared to the control group.

Obtaining oviductal fluid

A polypropylene probe (5 FR) of 1 cm was introduced into the cloaca of five Lohmann Brown lite hens, 0.5 ml of sterile PSS were administered, which were aspirated with a 3 ml syringe, the samples were mixed to make a group.

Determination of viscosity

Fifty (50) microliters of semen from each group or oviductal fluid were deposited in a refractometer to observe the degree of density (°Brix), to convert them to g/ml, the °Brix to specific gravity conversion table of the National Institute of Standards and Technology (NIST) was used. With a Cannon-Manning Semi-Micro viscometer (Size 50, Cannon Instrument, PA), the kinematic viscosity of each sample in mm^2/s (cSt) was determined by multiplying the flow time in seconds by the viscometer constant (Co= 0.003812). To obtain the viscosity in mPa*s (cP), the result was multiplied by the kinematic viscosity in mm^2/s (cSt) and by the density in g/ml.

Seminal cryopreservation

Subsequently, aliquots diluted and added with the different percentages of ficoll were frozen in straws of 0.25 ml with 100 x 10^6 spermatozoa. It started with aliquots at 25 °C that were cooled to a curve of 1.6 °C per min, then they were kept in nitrogen vapor (-70 °C) for 10 min; finally, they were immersed in liquid nitrogen (-196 °C) to be cryopreserved for 30 d. The thawing of each straw was at 37.5 °C for 30 sec[17].

Basic sperm evaluation

In fresh and post-thawed semen, progressive motility was determined by optical microscopy (400X) to estimate the percentage of spermatozoa with a vigorous progressive movement in an aliquot of 15 µL at 37.5 °C. Sperm viability and morphology

were determined in a preparation on a slide, with 10 µL of the aliquot with spermatozoa and 3 µL of eosin blue vital stain (1 % of eosin and 5 % of nigrosin), two hundred cells were evaluated in each preparation, with a phase-contrast microscope (400X). Morphology was evaluated with a 100X magnification[1,18].

Sperm maturation and acrosome reaction

Aliquots of 50 µL of semen belonging to all groups with a concentration of 5 x 10^6 spermatozoa were incubated with 0.9 M chlortetracycline (CTC) in darkness at 38 °C[19,20]. By means of fluorescence microscopy (495 nm excitation and 520 nm emission), the spermatozoa were evaluated to determine their level of maturation, determining the proportion of non-capacitated, capacitated and with acrosome reaction spermatozoa[17].

Statistical analysis

The normality of the data was verified using a Jack-Vera test; subsequently, a Kruskal-Wallis analysis was performed for the different treatments and variables. A Tukey test was performed to identify the difference in means, all statistical tests were performed with a significance level of $P<0.05$. The statistical program PAST[21] was used.

Results

Density and viscosity

The density and viscosity parameters that were determined in the cryopreservation media showed different values, which are shown in Table 1.

Table 1: Seminal density and viscosity and cryopreservation media (n=25)

	Density °Brix	**Specific gravity**	**Viscosity (mm^2/s)**	**Viscosity *mPa*s**
Semen (S)	7.0	1.028	3.1440	3.2320
S+L+ ficoll (10%) +DMA	17.0	1.070	2.0279	2.1698
S+L+ ficoll (30%) +DMA	28.0	1.120	3.1601	3.5393
S+L+ ficoll (45%) +DMA	30.2	1.130	5.4092	6.1123

S= Semen; L= Lake; DMA= dimethylacetamide

Post-thawing basic sperm evaluation

Thawed sperm with different percentages of ficoll showed changes in motility; the less viscosity in the medium, the greater the motility, approaching that of the ejaculated semen ($P \leq 0.05$). The percentages of live spermatozoa showed no significant changes between ejaculated semen and thawed sperm ($P > 0.05$). On the other hand, as the viscosity of the medium increases, the normal morphology of thawed spermatozoa improves ($P \leq 0.05$) (Table 2).

Table 2: Percentages of basic evaluation in spermatozoa cryopreserved with different viscosity conditions (n=10)

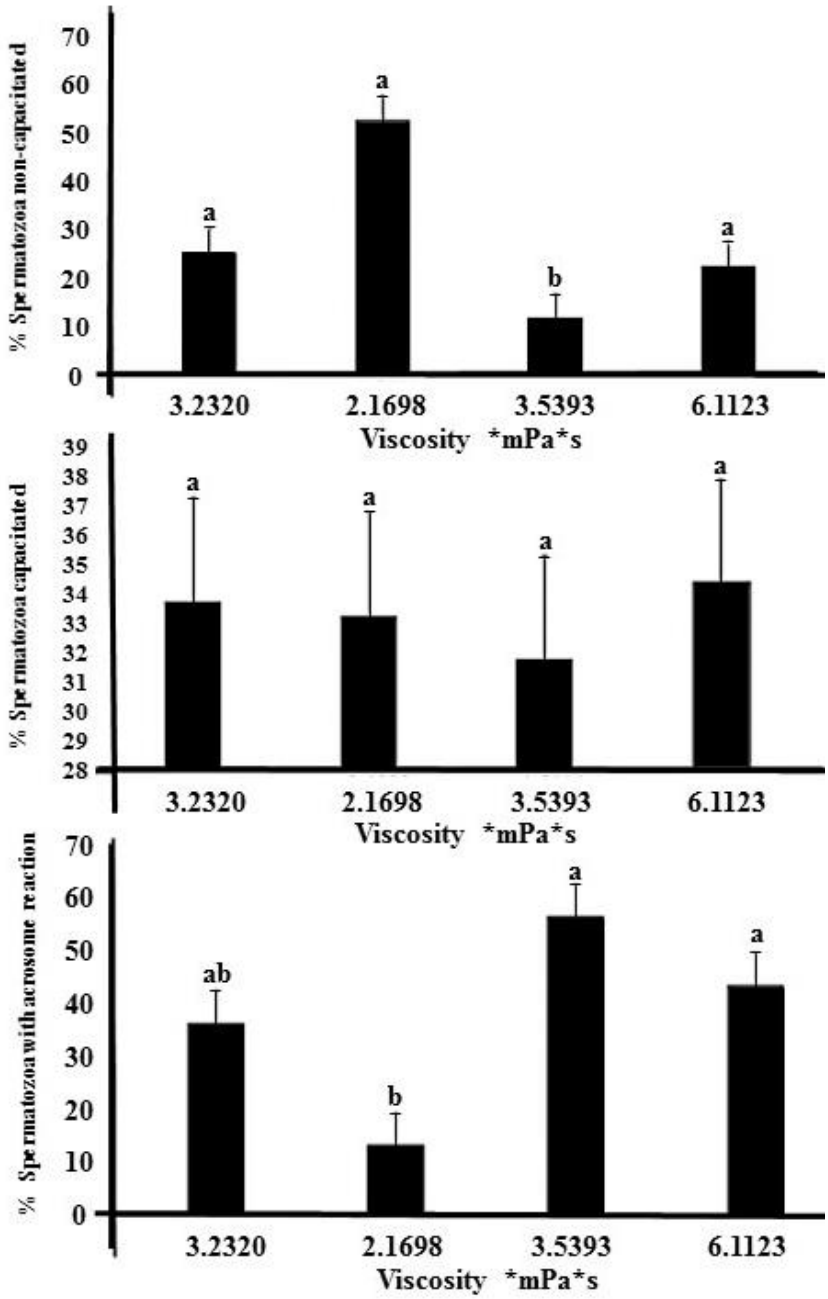
Percentage of spermatozoa (X±SE)			
Viscosity *mPa*s	**Motility**	**Viability**	**Morphology**
3.2320	46.1 ± 0.7[a]	74.2 ± 2.9 [a]	86.1 ± 1.0 [a]
2.1698	32.8 ± 0.7[b]	72.4 ± 2.9 [a]	86.7 ± 1.0 [a]
3.5393	10.9 ± 0.7 [c]	70.5 ± 2.9 [a]	93 ± 1.0 [b]
6.1123	5.1 ± 0.7 [d]	70 ± 2.9 [a]	91.2 ± 1.0 [b]

[abcd] Values with different literal in columns differ ($P < 0.05$).

Sperm maturation and acrosome reaction

The percentage of non-capacitated spermatozoa was higher in the group added with 10 % ficoll compared to the rest of the treatments ($P \leq 0.05$) as shown in Figure 1. The percentages of spermatozoa with sperm capacitation showed no statistical differences between the treatments and the ejaculated semen ($P > 0.05$). The percentage of spermatozoa with acrosome reaction found was higher in the treatments added with 30 % and 45 % ficoll, compared to the treatment with 10 % ficoll ($P \leq 0.05$) and similar with ejaculated semen ($P > 0.05$).

Figure 1: Post-thawing sperm maturation parameters in media with different viscosity



Discussion

The content and characteristics of seminal plasma are different in each species; one of these physical differences is viscosity, which is produced from proteins that help maintain sperm viability for several days, until the sperm reaches the fertilization site[22]. In camelid semen, the high viscosity is attributed to Mucin 5B[23,24]. On the other hand, in roosters and turkeys, there are no studies related to the viscosity of semen, but it has been shown

that, in the seminal plasma of birds, up to 822 proteins are found in the case of the rooster and 607 in the turkey[9,22]. In this work, it was determined that the viscosity of rooster semen is 3.23 mPa*s.

The mechanisms that prolong sperm life in sperm storage tubules are unknown, but are thought to include reversible suppression of sperm respiration and motility, as well as membrane stabilization and acrosome maintenance[25].

Although there is no demonstrable statistical difference in this work, the percentage of live spermatozoa was higher in semen cryopreserved with lower viscosity. The increase in viscosity decreased sperm motility; however, they kept their viability. In another study[26], different concentrations of Arabic gum were used to increase the viscosity of the medium for cryopreserving horse semen, and a better sperm viability after thawing was reported.

Sperm capacitation is necessary to initiate the acrosome reaction, which occurs *in vivo* in the genital tract of the female, where different signals that cause a destabilization of the membrane participate, sperm hyper activation that facilitates the acrosome reaction[27].

DMA at 5 % and glycerol have been used to freeze rooster spermatozoa, finding in both treatments, a reduction in the motility of cryopreserved spermatozoa, as well as their fertilizing capacity; however, this effect was lower in the group treated with DMA[28]. Although a slightly higher concentration of 6 % of DMA was used in this work, and the cryopreserved spermatozoa reduced their motility, this effect is attributed to the increase in the viscosity of the medium, finding sperm viability greater than 70 %.

It was also observed that in the parameters of incapacitation of thawed spermatozoa, they are greater than 30 %, remaining statistically the same ($P>0.05$), unlike spermatozoa with inclusion of 10 % ficoll, where there was a significant increase, reaching percentages higher than 50 %. Thus, the percentage of capacitated spermatozoa remains homogeneously above 30 %, regardless of the inclusion of ficoll.

In thawed spermatozoa, the percentages of acrosome reaction, it was observed that the inclusion of 10 % ficoll decreased the percentage below 20 %, comparing them with the other inclusions (30 % and 45 %) where the percentages were greater than 40 %. It must be considered that cryopreservation is a process of thermal and osmotic stress that causes the viability and motility of the sperm to decrease. In different species, the process of sperm freezing makes the stimulus to induce the acrosome reaction greater[29], when comparing the process of acrosome reaction through cryopreservation with different cryoprotectants, they suggest in their results that the process for the acrosome reaction to take place is very sensitive to osmotic stress and rapid water/solute changes in the rooster, demonstrating that, using cryoprotectants such as DMA, there is less induction of the acrosome reaction.

Conclusions and implications

In the results of the present work, it is evident that viscosity is a physical factor that influences sperm conservation in rooster semen; the appropriate viscosity parameters were determined to be 2.1698 mPa*s and 3.2320 mPa*s. The viscosity preserves the parameters of sperm viability and morphology in post cryopreservation, preventing energy expenditure in the sperm through the decrease of its motility. It was shown to reduce the spontaneous acrosome reaction in thawed spermatozoa and increase sperm incapacitation, which showed that it is a reversible physiological state, with which the sperm can be preserved intact until it reaches the fertilization site.

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Conflict of interest

It is declared that there is no type of conflict of interest.

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