

Original Article

Evaluation of rooster semen quality using CBB dye based staining method

Sung Woo Kim^{1,*}, Jae-Yeong Lee¹, Chan-Lan Kim¹, Yeong Gyu Ko¹ and Bongki Kim^{2,*}

¹Animal Genetic Resources Research Center, National Institute of Animal Science, RDA, Hamyang 50000, Korea

²Department of Animal Resources Science, Kongju National University, Yesan 23439, Korea

Received March 21, 2022

Revised March 23, 2022

Accepted March 23, 2022

*Correspondence

Sung Woo Kim

E-mail: sungwoo@korea.kr

*Correspondence

Bongki Kim

E-mail: bkkim@kongju.ac.kr

Author's Position and Orcid no.

Kim SW, Research scientist,

<https://orcid.org/0000-0001-8521-3010>

Lee JY, Research scientist,

<https://orcid.org/0000-0003-2965-7826>

Kim CL, Research scientist,

<https://orcid.org/0000-0003-2826-3171>

Ko YG, Research scientist

<https://orcid.org/0000-0002-6108-6325>

Kim B, Professor,

<https://orcid.org/0000-0002-5229-7294>

ABSTRACT The acrosome cap allows sperm to penetrate the egg membrane and produce male pronuclei within female chicken eggs, facilitating successful fertilization. Given this, it is important to establish practical methods for evaluating the integrity of the acrosome cap and thus the quality of the rooster's sperm. There are several established methods for evaluating the acrosomes of mammalian sperm, but none of these methods are suitable for evaluating the acrosome status of rooster spermatozoa. Therefore, a simplified method for evaluating the rooster acrosome is needed. Here we evaluated the usefulness of CBB (coomassie brilliant blue) staining of the acrosome at concentrations of 0.04%, 0.08%, and 0.3% CBB solutions. Our data revealed a clear staining pattern for intact acrosome caps at 0.04% and 0.08% CBB but not at 0.3% CBB. This protocol revealed differences in acrosome integrity between fresh and frozen rooster sperm smears suggesting that CBB staining may facilitate easier semen evaluation in roosters. This protocol allows for the accurate differential staining of acrosome cap in rooster spermatozoa.

Keywords: acrosome, CBB, rooster, spermatozoa, staining

INTRODUCTION

The ability of avian spermatozoa capable of fenestration reaction in head membrane, acrosome reaction, is essential for the fertilization process and embryo development. Many diverse methods have been developed to evaluate the acrosome status and estimate the sperm quality of mammalian samples, but relatively few studies have focused on avian applications. These studies have been defined various staining techniques for the evaluation of the acrosome status in mammalian sperm samples (Köhn et al., 1997; Larson and Miller, 1999; Zhang et al., 2012), using a wide variety of materials including anti-glycoprotein agents such as enzyme-binding chemicals,

CBB (coomassie brilliant blue) dyes, triple staining kits, chlortetracycline, spermack kits, or the application of immune fluorescent antibodies (Talbot and Chacon, 1980; Wolf et al., 1985; Lee et al., 1987; Chan et al., 1999; Larson and Miller, 1999; Menkveld et al., 2011; Villaverde-Morcillo et al. 2015; Santiago-Moreno et al., 2016). In poultry breeding, artificial insemination is followed by the administration of fresh semen in the semen storage tubules (SST) of the female reproductive organs. This sperm preservation *in vivo* is unique to avian species and requires a far higher concentration of spermatozoa. Therefore, the evaluation of the acrosome cap is a critical step in semen quality evaluation and for the selection of avian breeding individuals. In addition, frozen rooster

semen is also one of the essential genetic materials in the development of avian cryo-banking systems, but to date, the fertilizing efficiency of these frozen samples is inefficient when compared with those of fresh semen. In general, membrane damage lowers sperm motility, fertility, and hatchability (Pommer et al, 2002; Correa et al., 2007; Peña et al., 2011). Therefore, the evaluation of frozen rooster semen is one of the most essential techniques in increasing fertility and hatchability in these applications. However, these techniques have not been evaluated in any detail in rooster samples, and their applicability in these settings is not well established

Earlier observations using transmission electron microscopy revealed that the sperm plasma membrane detaches from the sperm head upon exposure to severe osmotic stress (Sieme et al., 2015), which is easily detected in mammalian sperm via the application of live/dead staining dyes such as SYBR and PI (Chalah and Brillard, 1998; Mehaisen et al., 2020). The fenestrated membrane of the acrosome region releases acrosome-enzymes during the mammalian acrosome reaction (Partyka et al., 2010), allowing PNA molecules to easily discriminate between intact and damaged acrosome caps. Also, the binding property of agglutinins were not reported clearly with images of rooster spermatozoa. The acrosome reaction in chickens can be induced by physiological reactions such as ROS stress, increased calcium, or contact with the glycoprotein from the inner perivitelline layer of the egg yolk *in vivo*, making semen evaluation factors (Siudzińska and Łukaszewicz, 2008). The tendency of frozen semen on higher injuries, causing reduced fertility in chickens was reported by many researchers (Chalah and Brillard, 1998; Watson, 2000; Partyka et al., 2010; Gliozzi et al., 2011). Only 40–50% of the sperm survive freezing (Horrocks et al., 2000; Watson, 2000), and while this can be accommodated by increasing the freeze-thaw volume for larger mammals like cattle (Schenk, 2018). This strategy is not applicable in hens due to the small size of their reproductive tract and the likely loss of additional volume following the contractile reaction of the vagina (Wishart, 1985; Chalah et al. 1999; Thélie et al., 2019)

The morphological structure of rooster spermatozoa is an essential factor in the quality of the semen and unfortunately, rooster sperm are very easily damaged in response to mechanical stress, such as centrifugation (Mazur, 1984; Feyzi et al., 2018). This is largely because

of their long, slender, vermiform, or fusiform shape. In addition, detached heads, extended or swollen heads, coiled heads, swollen acrosomes, missing or detached acrosomes, mitochondrial detachment from the mid-piece, simple bending in the tail, and untiled tail ends are all common defects in frozen rooster semen samples (Siudzińska and Łukaszewicz, 2008; Santiago-Moreno et al., 2016). However, the intactness of the acrosome cap remains the most critical indicator for semen quality as the final step of fertilization allowing for the fusion of the membranes of egg yolk and spermatozoa (Ahammad et al., 2013). In addition, the acrosome cap is quite small when compared with the total size of spermatozoa, necessitating extremely careful observation of these samples using a microscope. Moreover, there is no standardized method for acrosome cap staining of rooster spermatozoa. Given this, it is critical to develop a simple, standardized method for the differential staining of rooster sperm head and cap which should facilitate the rapid evaluation of semen quality under these conditions.

This study was designed to evaluate the utility of CBB dyes for the differential staining of rooster spermatozoa and develop a simple standardized protocol for their evaluation. Previous reports have described the use of CBB dyes in similar mammalian applications but not in avian semen (Larson and Miller, 1999). Thus we optimized the concentration of CBB for effective histological staining of the rooster acrosome cap and developed a fast-staining technique that could be useful for semen evaluation in chicken breeding programs.

MATERIALS AND METHODS

Animals and ethics statement

We collected semen from 35–45 week-old leghorn or Ogye (Korean black chicken) roosters. All the roosters were housed in individual battery cages (35 × 40 × 59 cm, width × length × height) using a 14 h light/10 h dark photoperiod and fed a standard diet and water *ad libitum*.

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated.

Semen treatment for fresh, heat-stressed and cryo-damaged spermatozoa

Semen was collected from 6 to 10 roosters from each strain using the dorsal massage method. Semen was collected and pooled twice per week and immediately placed in an ice slurry at 5°C before being transported to the laboratory. Samples were placed in the laboratory within 15 min and then diluted in BPSE diluent containing 0.1% fatty-acid-free BSA (FAF-BSA) to assess motility. Heat stressed acrosome reaction was induced by incubation with BPSE diluent for 3 h at 37.5°C. The cryo-damaged sperm was prepared by freezing process with fresh semen. The fresh semen was dipped into ice slurry and dilution with 1:1 HS-1 medium (v/v) at 4°C for 15 min. The semen was then further diluted using the dropwise addition of an equal volume of media containing 18% methylacetamide (MA) to yield a final concentration of 9% MA. After 20 min of equilibration at 4°C, the semen was packed into straws and then placed in liquid nitrogen vapor above 5cm from the surface for 30 min, before being plunged in liquid nitrogen. After preservation for several weeks, the semen was thawed by immersion in a 5°C program freezer (ET-1N, Fujihira Inc., Japan) with 30% ethanol solution (v/v) for 2 min.

Staining procedures and microscope examination of semen smear

Both the fresh and frozen sperm were centrifuged at 500 × g for 10 min in 5 mL of PBSE diluent before a small amount (0.5 mL) of the sperm pellet was tapped for mixing. The 5-10 µL of diluted semen was smeared on a microscope slide as previously described (Mota and Ramalho-Santos, 2006). Briefly, the sperm suspension was dragged with a coverslip and dried using a slide warmer at 37.5°C. Sperm morphology was examined using histological smears stained by eosin in Diff Quik kits and dipped into 0.04%, 0.08%, and 0.3% CBB solution. The Diff Quik® staining kits (Dade Behring Inc., Newark, NJ, USA) included a methanol fixative, eosin dye, and a thiazine-like stain. The slides were sequentially immersed in each solution for 3-5 s without drying and then rapidly dipped in water to remove excess dye. The thiazine-like solution with a CBB solution was substituted by the CBB. The stock solution of 0.5% CBB dye stock (w/v) was prepared using CBB solvent, a mixture of ethanol, acetic acid, and water at a ratio of 9:2:9 (v/v/v). CBB working solutions of

0.04%, 0.08%, and 0.3% were freshly prepared using distilled water. The stained smears were then observed under a bright-field microscope (IX51, Olympus, Japan) using a 100× oil immersion objective, and images were taken using a digital camera (DP20, Olympus, Japan). These slides were then used to compare the acrosome integrity in the fresh and frozen-thawed semen.

Acrosome integrity analysis

We then examined three independent semen smears from each of the three biological replicates using a single direction of movement under the microscope. Acrosome cap count data were collected from at least 200 spermatozoa per slide per experiment.

Statistical analysis

We produced at least three slides from individual samples at each time point and the Student's t-test was applied to identify any significant differences between these groups. Inter group comparisons were completed using one-way analysis of variance (ANOVA) and we set a *p*-value of < 0.05 as the cut off for statistical significance.

RESULTS

Morphological examination of acrosome region by Diff Quik stains

The morphological differences in the acrosome cap between the fresh and heat-stressed rooster sperm were evaluated using Diff Quik staining kits which facilitate the differential staining of the acrosome region. The majority of the fresh sperm presented with undamaged acrosomes represented by their rigid, straight, rod-like structures (black arrows), but some sperm did demonstrate a twisted acrosome structure (white arrow; Fig. 1A). The activated fresh semen presented with an enlarged head with a shallow and shortened acrosome (white arrow). These sperm also presented without the acrosome cap (black arrow; Fig. 1B), with these images magnified in Fig. 1C and 1D, respectively. Unmodified Diff Quik stains stained the rooster sperm heads purple, which meant that we could not distinguish between the head and acrosome region clearly (hollow arrow in Fig. 1C).

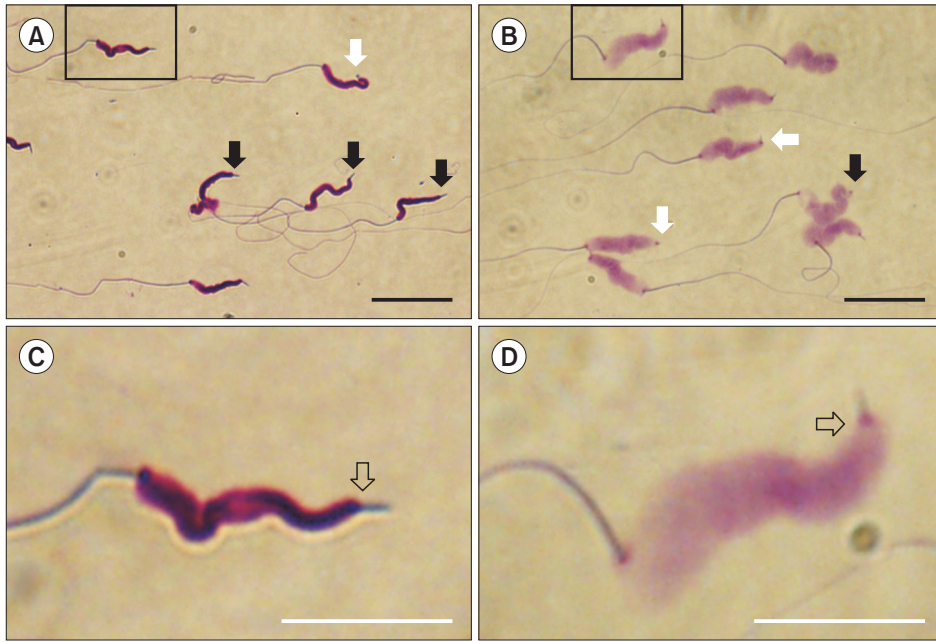


Fig. 1. The morphological differences in heat stressed rooster spermatozoa with shortened acrosomes. The intact acrosomes from fresh semen are indicated by black arrows, and the damaged acrosomes are indicated by white arrows (A) which mark the shortened or missing acrosomes (black arrow) (B). Each sperm (black rectangular box in A and B) was magnified to reveal the connected region between the sperm heads at (C) and (D). The linking area between head and acrosome cap is indicated by the hollow arrows. The scale bars in (A) and (B) represent 20 μM . The scale bars in (C) and (D) represent 10 μM .

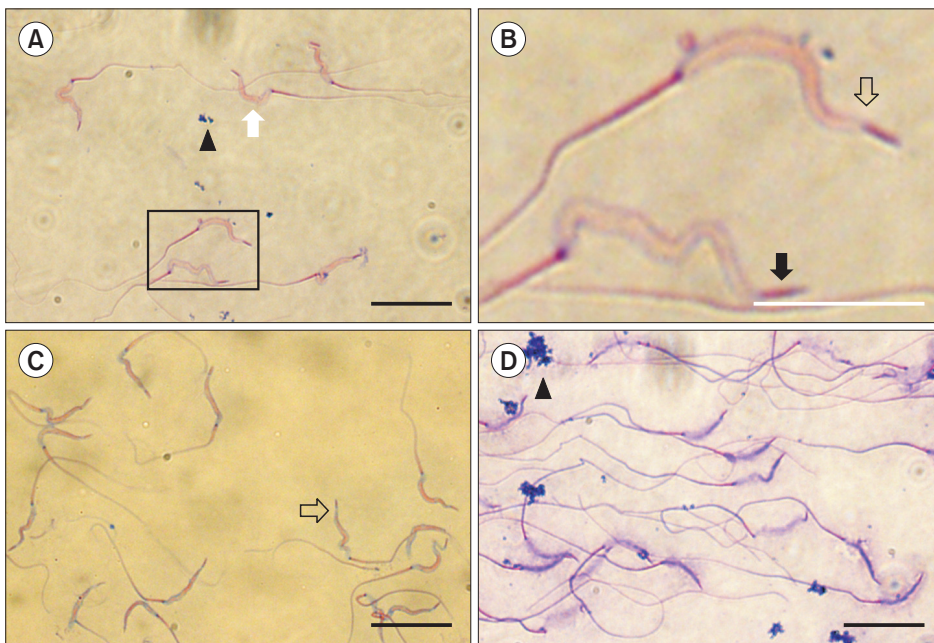


Fig. 2. Modified Diff Quik stained images of fresh rooster spermatozoa with CBB. After staining with the eosin solution from the Diff Quik stain kits, the smears were dipped into 0.04% (A), 0.08% (C), or 0.3% (D) CBB. The light purple tints of the heads (white arrow in A) and dark purple acrosomes (black arrow in B) showed a more distinct differentiation staining pattern than the classic Diff Quik stains. Black arrowheads in A and D identify unresolved CBB staining with the scale bars respectively (20 μM in A, C, and D; 10 μM in B).

Effects of CBB concentration on differential staining between head region and acrosome cap

We determined the optimal concentration of CBB in rooster sperm staining by comparing the staining effects of 0.02%, 0.08%, and 0.3% CBB on the differential staining of the acrosome and sperm head in these samples. We first applied the eosin solution from the Diff Quik which stained the positively charged proteins in the acrosome cap and tail red while leaving the head reasonably

unstained. We then added CBB solution at various concentrations and observed the differential staining of the head (purple) and acrosome cap (Red) at both 0.04% and 0.08% CBB (Fig. 2A and 2C). However, 0.3% CBB overstained these samples imparting a blue and red-tinted background to the smears, resulting in less obvious differentiation (Fig. 2D).

The rate of acrosome cap intactness in fresh, heat-stressed, and cryopreserved semen

The intactness of acrosome cap was measured in 3 different rooster semen. The rate was higher in fresh semen ($87.5\% \pm 3.8\%$) and slightly lower when placed at 5°C for 3 h ($78.6 \pm 3.3\%$, $p < 0.05$). However, the heat-stressed semen with spontaneous induction of the acrosome reaction at 37.5°C for 3 h significantly reduced the integrity of the acrosome cap producing a proportional increase in significantly damaged sperm ($82.0 \pm 2.3\%$ vs. $50.4 \pm 7.1\%$, $p < 0.05$). The percentage of the intact acrosome caps in the cryopreserved semen was reduced to $40.3 \pm 2.4\%$. The treatment of spontaneous acrosome reaction of frozen-thaw sperm at in 5°C for 3 h also reduced the intactness of acrosome ($20.9 \pm 3.1\%$). Therefore, the heat-stressed fresh semen and cryo-damaged semen also shown a lowered percentage of intact acrosome caps (Fig. 3).

DISCUSSION

Many of acrosome staining protocols are inappropriate for rooster sperm because their delicate properties of membranes. Also the head of rooster sperm is easily damaged by mechanical stress and strong organic solvents, and the accuracy of most of these assessments depends on careful preparation during fixation, dipping, and washing of the sperm samples (Menkveld et al., 2011).

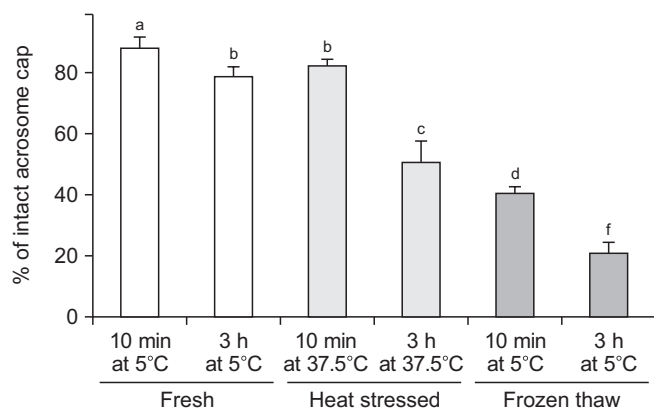


Fig. 3. The percentage of intact acrosome cap in rooster spermatozoa. Three different semen samples were stained using the CBB solution and then compared. The semen smears were prepared within 2 min after centrifugation. The hollow bar was fresh, gray bar was heat stressed, and dark gray was cryopreserved semen. Values represent the mean of three samples \pm standard deviation. Superscript indicate significant difference ($p < 0.05$).

Thus the standard protocol for this assay should include a minimal number of experimental steps and use a mild solvent that does not affect the membrane integrity of the rooster sperm. Unlike mammalian sperm, rooster sperm membranes are not resistant to organic solvents such as acetone or higher acetic acid content solvents. So, the CBB dye was dissolved firstly in ethanol and an acetic acid-based solvent to prepare the stock solution. We further diluted the working solution using only distilled water to minimize the solvent content in the dye. We also used a 3.7% paraformaldehyde fixative to preserve these weak membranes and limited fixation to only 2 min. The resulting staining pattern of acrosome using 0.04% CBB showed clear blue staining of the acrosome cap but although this procedure could be simplified, the CBB-stained membranes tended to lose their blue color during the final wash step.

Evaluating chicken sperm quality is one of the most challenging tasks in the laboratory because the long tail and head region cannot be easily distinguished from the tiny acrosome caps (Gliozzi et al., 2011; Rui et al., 2017). However, our results show that the acrosome status of these samples can be determined quickly when using (0.04%–0.08%) CBB dye, with these evaluations taking only a few minutes to complete. These findings are supported by the fact that 0.2% CBB staining is commonly applied for the rapid evaluation of mammalian acrosome (Larson and Miller, 1999). Our data also shows that a combination of CBB dye and the eosin stain from the Diff Quick kit can be used to intensify the differential staining of the sperm and aid in the clear evaluation of their acrosome status. The optimal concentration of CBB in rooster samples is likely to be 0.04%. Acrosome-damaged sperm present with reduced or missing acrosome caps, or with only the perforatorium, was identified via CBB mediated staining of the remaining proteins in the detached area. The intact acrosome cap demonstrated a clear differential staining pattern with a small rod or spike-like structure. Given this, we were able to confirm that the simple application of CBB dye allowed for a clearer evaluation of the acrosome cap in these specimens when compared with other more conventional methods. Given the clear efficacy of this method, we went on to evaluate 0.08% CBB staining, which produced a slightly blue tint to the head which produced a mixed blue/red pattern when combined with the eosin from the Diff Quik stain kit (red), re-

ducing the clarity in these stains, and suggesting that the lower concentration provides clearer differentiation under these conditions. We then used this CBB staining method to evaluate rooster acrosome cap integrity in fresh, heat-stressed, and cryopreserved semen samples and revealed that the integrity of these samples tended to correlate with the fertility and hatchability rates from specific samples and may be useful in the routine application of artificial insemination with further research.

CBB-stained sperm smears have additional value, with these slides being compatible with room temperature preservation for further examination. This allowed us to confirm our evaluations using an additional investigator who used the same slides to evaluate and score the quality of our semen samples. These samples are also helpful in the education of trainee researchers who need to learn rooster sperm morphology.

CONCLUSION

The CBB staining method using the eosin solution from the Diff Quik kit is beneficial for the precise imaging and evaluation of the rooster acrosome cap and may have different stainability for avian spermatozoa compared to mammalian species. So, the overall semen evaluation in rooster for artificial insemination could be examined easily.

Author Contributions: Conceptualization, S.W.K.; methodology, S.W.K., J.Y.L., and C.L.K.; investigation, S.W.K., J.Y.L., and C.L.K.; data curation, S.W.K., and B.K.; writing-original draft preparation, S.W.K.; writing-review and editing, S.W.K., and B.K.; supervision, S.W.K., and Y.G.K.; project administration S.W.K.; funding acquisition, S.W.K.

Funding: This work was supported by the Cooperative Research Program for Agriculture Science and Technology Development (Project No. PJ01558302) from the Rural Development Administration.

Ethical Approval: This study was conducted in strict accordance with the recommendations in the Guide for the Care and Management of Experimental Animals as described by the National Institute of Animal Science (NIAS) and our study protocol was approved by the NIAS Com-

mittee on the Ethics of Animal Use in Experiments (protocol approval number: 2021-511).

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: None.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

REFERENCES

- Ahammad MU, Nishino C, Tatemoto H, Okura N, Okamoto S, Kawamoto Y, Nakada T. 2013. Acrosome reaction of fowl sperm: evidence for shedding of the acrosomal cap in intact form to release acrosomal enzyme. *Poult. Sci.* 92:798-803.
- Chalah T and Brillard JP. 1998. Comparison of assessment of fowl sperm viability by eosin-nigrosin and dual fluorescence (SYBR-14/PI). *Theriogenology* 50:487-493.
- Chalah T, Seigneurin F, Blesbois E, Brillard JP. 1999. In vitro comparison of fowl sperm viability in ejaculates frozen by three different techniques and relationship with subsequent fertility in vivo. *Cryobiology* 39:185-191.
- Chan PJ, Corselli JU, Jacobson JD, Patton WC, King A. 1999. Spermac stain analysis of human sperm acrosomes. *Fertil. Steril.* 72:124-128.
- Correa LM, Thomas A, Meyers SA. 2007. The macaque sperm actin cytoskeleton reorganizes in response to osmotic stress and contributes to morphological defects and decreased motility. *Biol. Reprod.* 77:942-953.
- Feyzi S, Sharafi M, Rahimi S. 2018. Stress preconditioning of rooster semen before cryopreservation improves fertility potential of thawed sperm. *Poult. Sci.* 97:2582-2590.
- Gliozzi TM, Zaniboni L, Cerolini S. 2011. DNA fragmentation in chicken spermatozoa during cryopreservation. *Theriogenology* 75:1613-1622.
- Horrocks AJ, Stewart S, Jackson L, Wishart GJ. 2000. Induction of acrosomal exocytosis in chicken spermatozoa by inner perivitelline-derived N-linked glycans. *Biochem. Biophys. Res. Commun.* 278:84-89.
- Köhn FM, Mack SR, Schill WB, Zaneveld LJ. 1997. Detection of human sperm acrosome reaction: comparison between methods using double staining, Pisum sativum agglutinin, concanavalin A and transmission electron microscopy. *Hum. Reprod.* 12:714-721.
- Larson JL and Miller DJ. 1999. Simple histochemical stain for acrosomes on sperm from several species. *Mol. Reprod. Dev.* 52:445-449.

- Lee MA, Trucco GS, Bechtol KB, Wummer N, Kopf GS, Blasco L, Storey BT. 1987. Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay. *Fertil. Steril.* 48:649-658.
- Mazur P. 1984. Freezing of living cells: mechanisms and implications. *Am. J. Physiol.* 247(3 Pt 1):C125-C142.
- Mehaisen GMK, Partyka A, Ligocka Z, Nizański W. 2020. Cryoprotective effect of melatonin supplementation on post-thawed rooster sperm quality. *Anim. Reprod. Sci.* 212: 106238.
- Menkveld R, Holleboom CA, Rhemrev JP. 2011. Measurement and significance of sperm morphology. *Asian J. Androl.* 13: 59-68.
- Mota PC and Ramalho-Santos J. 2006. Comparison between different markers for sperm quality in the cat: Diff-Quik as a simple optical technique to assess change in the DNA of feline epididymal sperm. *Theriogenology* 65:1360-1375.
- Partyka A, Nizański W, Łukaszewicz E. 2010. Evaluation of fresh and frozen-thawed fowl semen by flow cytometry. *Theriogenology* 74:1019-1027.
- Peña FJ, García BM, Samper JC, Aparicio IM, Tapia JA, Ferrusola CO. 2011. Dissecting the molecular damage to stallion spermatozoa: the way to improve current cryopreservation protocols? *Theriogenology* 76:1177-1186.
- Pommer AC, Rutllant J, Meyers SA. 2002. The role of osmotic resistance on equine spermatozoal function. *Theriogenology* 58:1373-1384.
- Rui BR, Angrimani DSR, Losano JDA, Bicudo LC, Nichi M, Pereira RJG. 2017. Validation of simple and cost-effective stains to assess acrosomal status, DNA damage and mitochondrial activity in rooster spermatozoa. *Anim. Reprod. Sci.* 187:133-140.
- Santiago-Moreno J, Estes MC, Villaverde-Morcillo S, Toledano-Déaz A, Castaño C, Velázquez R, López-Sebastián A, Goya AL, Martínez JG. 2016. Recent advances in bird sperm morphometric analysis and its role in male gamete characterization and reproduction technologies. *Asian J. Androl.* 18:882-888.
- Schenk JL. 2018. Review: principles of maximizing bull semen production at genetic centers. *Animal* 12(s1):s142-s147.
- Sieme H, Oldenhof H, Wolkers WF. 2015. Sperm membrane behaviour during cooling and cryopreservation. *Reprod. Domest. Anim.* 50 Suppl 3:20-26.
- Siudzińska A and Łukaszewicz E. 2008. Effect of semen extenders and storage time on sperm morphology of four chicken breeds. *J. Appl. Poult. Res.* 17:101-108.
- Talbot P and Chacon R. 1980. A new procedure for rapidly scoring acrosome reactions of human sperm. *Gamete Res.* 3:211-216.
- Thélie A, Bailliard A, Seigneurin F, Zerjal T, Tixier-Boichard M, Blesbois E. 2019. Chicken semen cryopreservation and use for the restoration of rare genetic resources. *Poult. Sci.* 98: 447-455.
- Villaverde-Morcillo S, Estes MC, Castaño C, Toledano Díaz A, López-Sebastián A, Campo JL, Santiago-Moreno J. 2015. Influence of staining method on the values of avian sperm head morphometric variables. *Reprod. Domest. Anim.* 50: 750-755.
- Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. *Anim. Reprod. Sci.* 60-61:481-492.
- Wishart GJ. 1985. Quantitation of the fertilising ability of fresh compared with frozen and thawed fowl spermatozoa. *Br. Poult. Sci.* 26:375-380.
- Wolf DP, Boldt J, Byrd W, Bechtol KB. 1985. Acrosomal status evaluation in human ejaculated sperm with monoclonal antibodies. *Biol. Reprod.* 32:1157-1162.
- Zhang YX, Ping SH, Yang SH. 2012. [Morphological characteristics and cryodamage of Chinese tree shrew (*Tupaia belangeri chinensis*) sperm]. *Dongwuxue Yanjiu* 33:29-36. Chinese.