A comparative study of two methods to determine acrosome integrity of frozen-thawed boar sperm: FITC-PNA/EthD-1 versus Coomassie blue staining

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Abstract

Coomassie blue staining has been reported as an effective and inexpensive method for evaluating the acrosome integrity of spermatozoa, though to date its use to evaluate cryopreserved boar sperm has not been reported. Moreover, there is no information concerning the agreement between Coomassie blue staining and fluorescein isothiocyanate conjugated peanut agglutinin and ethidium homodimer (FITC-PNA/EthD-1) methods for assessing sperm acrosome integrity for any species. The current study was performed to determine the efficacy and agreement between Coomassie blue and FITC-PNA/EthD-1 staining methods for evaluating the acrosome integrity of frozen-thawed boar sperm. A total of 25 semen samples were cryopreserved using lactose-egg yolk-based extender and loaded into 0.5 PVC-French straws. Sperm motility and motion characteristics were determined using a computer-assisted sperm analysis system. Sperm viability and plasma membrane integrity were evaluated using the SYBR-14/EthD-1 and hypo-osmotic swelling test, respectively. Acrosome integrity of frozen-thawed boar sperm was evaluated using both FITC-PNA/EthD-1 and Coomassie blue staining to assess the association between sperm acrosome integrity and agreement between these two methods. The average percent acrosome integrity of frozen-thawed boar sperm as determined by FITC-PNA/EthD-1 and Coomassie blue staining was 48.8 ± 12.6% and 52.6 ± 13.6%, respectively (P>0.05). Interestingly, Coomassie blue staining found a correlation between sperm viability and

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acrosome integrity (r=0.609, *P*=0.002), while FITC-PNA/EthD-1 staining did not (*P*>0.05). However, the acrosome integrity of frozen-thawed boar sperm evaluated by FITC-PNA/EthD-1 and Coomassie blue staining was significantly correlated (r=0.448, *P*=0.025, *n*=25). The Bland-Altman plot determined that this agreement was acceptable. In conclusion, the acrosome integrity of the frozen-thawed boar sperm assessed via Coomassie blue staining was significantly correlated with that obtained via the FITC-PNA/EthD-1 staining method, and the two methods showed good agreement. Moreover, the significant association between the acrosome integrity of frozen-thawed boar sperm determined by Coomassie blue staining with other sperm quality parameters indicates that this is an effective method for assessing the acrosome integrity of frozen-thawed sperm in pigs.

Key words: *acrosome integrity; boar; Coomassie blue; cryopreservation; FITC-PNA*

Introduction

In general, viable and motile sperm obtained from ejaculated semen are not immediately ready for fertilization, as a period of physiological changes is required to develop fertilizing ability, which is known as 'capacitation' (Sirivaidyapong et al., 2000). The acrosomal status of spermatozoa needs to be safeguarded when sperm is preserved prior to artificial insemination, as acrosome-reacted sperm can no longer penetrate the zona pellucida. Therefore, to determine the efficacy of semen extender or any media used for sperm processing and storage, assessing the acrosomal status of sperm is of the utmost importance.

For the past 20 years, evaluation of the acrosome integrity of spermatozoa of many animal species has been based on the fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) and ethidium homodimer-1 (EthD-1) staining method (FITC-PNA/EthD-1) (Fazeli et al., 1997; Sirivaidyapong et al., 2000; Laura et al., 2008; Ponglowhapan and Chatdarong, 2008; Chanapiwat et al., 2009; Chanapiwat et al., 2010; Pearodwong et al., 2019). This method is complicated and requires expensive equipment. Acrosome integrity of spermatozoa can also be detected using epifluorescence microscopy or flow cytometry (Kowalczyk et al., 2020). Despite giving accurate acrosome integrity results, these methods require even more complex equipment including fluorescence, therefore making them unsuitable for field assessments.

Coomassie blue staining has been reported to be an effective and inexpensive method for evaluating acrosome integrity of spermatozoa in several species (Pukazhenthi et al., 2006; Arakaki et al., 2019). Recently, a study in pigs demonstrated the use of Coomassie blue staining for evaluating acrosome integrity in liquid-preserved boar sperm (Chen et al., 2021). However, its use for evaluating acrosome integrity in cryopreserved boar sperm has never been demonstrated. Moreover, information on the agreement between Coomassie blue staining and FITC-PNA/EthD-1 for assessing sperm acrosome integrity is not available for any species. Therefore, the current study was performed to compare the efficacy and agreement between the Coomassie blue and FITC-PNA/EthD-1 staining methods for evaluating the acrosome integrity of frozen-thawed boar sperm. The association between acrosome integrity of the frozen-thawed boar sperm as determined by the two methods and other sperm quality parameters was also investigated.

Materials and Methods

The experiment was conducted in compliance with The Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes by the National Research Council of Thailand (NRCT), and approved by the Institutional Animal Care and Use Committee (IACUC) in accordance with our university regulations (animal use protocol no. 1631032).

Animals

The experiment was conducted at the Swine Research and Development Centre, Nakhon Ratchasima, Thailand. A total of 25 semen samples were collected from 23 boars (9 Landrace, 7 Yorkshire, 5 Duroc and 2 Hybrid boars). The boars were proven sires used for semen collection. The ejaculates were selected based on their good semen quality, with ≥70% subjective motility and ≥75% normal morphology. All boars were housed in a conventional open-housing system and were allocated individual pens. The sperm-rich fraction of semen was collected using the gloved-hand technique. The interval between each semen collection was 5–7 days.

Semen cryopreservation

Semen was cryopreserved according to our previous protocol (Buranaamnuay et al., 2019). Briefly, the semen was diluted (1:1, v/v) in extender, then cooled to 15°C and held for 2 hours. Following this, the semen was centrifuged at 15°C, 800 x *g* for 10 min. The sperm pellet was diluted in lactose-egg yolk (LEY) extender (80 mL 11% lactose solution and 20 mL egg yolk) (extender II) to a concentration of 1.5 x 10^9 cells per mL, then cooled to 4°C for 2 hours. The diluted sperm were then added to extender III (LEY extender, 10% glycerol and 1.5% Equex STM Paste; Nova Chemical Sales Inc., MA, USA) to a final concentration of 1 x 10^9 sperm per mL and loaded into 0.5 PVC-French straws (IMV, IMV Technologies, France). To freeze the samples, the straws were placed approximately 4 cm above liquid nitrogen for 20 min, and then dropped into the liquid nitrogen. For thawing, straws were plunged into 50°C water for 12 sec.

Sperm motility

Sperm motility and motion characteristics were determined using the computer assisted sperm analysis (CASA) system (SCA® CASA System, MICROPTIC S.L., Barcelona, Spain). Images were taken under a phase contrast microscope with a green filter at 100x magnification. The frozen-thawed semen was diluted with phosphate buffer saline solution (PBS) at a ratio of 1:20. The diluted semen (8 μL) was dropped onto a warmed slide (37°C) and covered with a coverslip. A total of 1500 sperm cells from five different fields of each sample were randomly selected to determine sperm motility and motion characteristics. The motion characteristics of spermatozoa, including straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, %), straightness (STR, %), wobble coefficient (WOB, %), mean lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz), were recorded using the CASA software.

Sperm viability

Sperm viability was evaluated with SYBR-14/EthD-1 (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, Netherlands). Thawed semen (10 μL) was diluted with PBS (140 μL). Then, 50 μL diluted semen was gently mixed with fluorescence solution, composed of SYBR-14 (2.7 μL) and EthD-1 (10 μL). The mixed semen sample was incubated at 37°C for 20 min. Two hundred stained sperm were evaluated under a fluorescence microscope at 1000x

magnification with an oil objective lens. Under the fluorescence microscope, sperm heads with green luminescence were defined as having intact plasma membranes (live), while red luminescence was defined as damaged membranes (dead). Sperm viability was presented as the percentage of live sperm.

Sperm plasma membrane integrity

Sperm plasma membrane integrity was determined using the hypoosmotic swelling test (sHOST). The hypoosmotic solution was prepared with fructose and Na-citrate in distilled water until final osmolality of 75.0 mOsm⁄ kg. Solution osmolality was measured by freezing point depression. Each frozen-thawed semen sample (100 μL) was mixed with 1000 μL hypoosmotic solution and incubated at 38°C for 30 min. Sperm were then fixed with 1000 μL hypoosmotic solution with 5% formaldehyde (Merck, Darmstadt, Germany). A well-mixed sample (10 μL) was placed on a glass slide with a coverslip. A total of 200 sperm were evaluated under a light microscope at 400x magnification. Coiled tail sperm were defined as sperm with functional sperm membranes. The proportion of sperm with functional sperm membranes was presented as a percentage.

Sperm acrosome integrity

FITC-PNA/EthD-1

The acrosome integrity of frozen-thawed boar sperm was evaluated using FITC-PNA/EthD-1 staining. FITC-PNA/EthD-1 was performed according to a previously described protocol (Chanapiwat et al., 2012) with some modifications. Briefly, 10 μL diluted semen was mixed with 10 μL EthD-1 and incubated at 37°C for 15 min. Thereafter, 5 μL mixture was smeared on a slide and fixed with 95% ethanol for 30 sec. Each sample was smeared with 15 μL FITC-PNA (FITC-PNA: PBS = 1:10, v: v). The sample was incubated in a moist chamber at 4°C for 30 min, then rinsed with PBS and dried. For the assessment, 200 sperm were viewed under a fluorescence microscope at 1000x magnification, and the sperm acrosome was classified as intact or reacted (Figure 1). The proportion of intact acrosomes was presented as a percentage.

Coomassie blue staining

Coomassie blue staining was performed according to a previous report (Larson and Miller, 1999) with some modifications. Briefly, sperm were fixed with 4% paraformaldehyde solution (110 mM Na_2HPO_4, 2.5 mM NaH_2PO_4, 4% paraformaldehyde pH 7.4) for 10 min. Sperm were centrifuged at 450 x *g* for 5 min twice using 500 μL 100 mM ammonium acetate (pH 9.0). The final sperm pellet was re-suspended in 1 mL 100 mM ammonium acetate, and 50 μL sperm suspension was smeared on glass microscope slides and air dried, then incubated in freshly prepared Coomassie blue stain (0.22% Coomassie Blue G-250, 50% methanol, 10% glacial acetic acid, 40% water) for 2 min. Slides were washed with distilled water to remove excess stain and air-dried, and then covered with coverslips. For the assessment, a total of 200 sperm were viewed (1000x) using bright field microscopy. The spermatozoa with an intact acrosome stained purple-blue and the posterior part of the acrosome was either not stained or stained a light purple (Figure 1). The proportion of intact acrosomes was presented as a percentage.

Statistical analysis

Statistical analysis was carried out using SAS version 9.4 (SAS Inst. Inc., Cary, NC, USA). Descriptive statistics on the sperm characteristics post-thawing were calculated using the MEANS procedure of SAS (PROC MEANS). The difference in acrosome integrity of the frozen-thawed boar sperm obtained

via FITC-PNA/EthD-1 and Coomassie blue staining was compared using a paired *t*-test. Pearson's correlation was performed to determine the association between acrosome integrity determined by FITC-PNA/EthD-1 and Coomassie blue staining. Agreement between the two methods of measurement was assessed using a Bland-Altman plot (Bland and Altman 1986). For all analyses, differences of $P<0.05$ were regarded as statistically significant.

Results

Descriptive statistics of the sperm quality parameters of the frozen-thawed boar semen are presented in Table 1. The average acrosome integrity of the frozen-thawed boar sperm determined by FITC-PNA/EthD-1 and Coomassie blue staining was 48.0 ± 12.1 and 52.8 ± 12.9%, respectively ($P>0.05$). Although the percentage of acrosome integrity assessed by FITC-PNA/EthD-1 was 4.8% lower than that assessed by Coomassie blue staining, the difference was not statistically significance ($P=0.093$). The correlations between sperm quality parameters and acrosome integrity of frozen-thawed boar sperm determined using the FITC-PNA/EthD-1 and Coomassie blue staining method are presented in Table 2. Interestingly, most sperm quality

Table 1. Descriptive statistics of sperm quality parameters of frozen-thawed boar semen ($n=25$)

Variable (%)	mean ± SD	range
Fresh semen		
Sperm motility (%)	71.4 ± 4.5	70.0 – 90.0
Sperm concentration (x 10^6 sperm/mL)	817 ± 224	448 – 1287
Frozen-thawed semen		
Sperm motility (%)	41.9 ± 17.2	12.5 – 69.9
Progressive motility (%)	18.8 ± 11.1	2.0 – 39.2
Sperm viability (%)	46.0 ± 11.8	23.0 – 70.0
Plasma membrane integrity (%)	37.6 ± 12.8	15.0 – 62.5
Acrosome integrity		
FITC-PNA/EthD-1	48.0 ± 12.1	26.0 – 74.0
Coomassie blue	52.8 ± 13.9	25.0 – 74.0
Sperm motion characteristics		
VCL	37.6 ± 8.1	21.7 – 49.1
VSL	13.7 ± 5.0	5.5 – 21.2
VAP	23.1 ± 7.5	10.1 – 35.8
LIN	30.0 ± 6.6	16.0 – 39.5
STR	49.3 ± 5.3	35.9 – 57.7
WOB	54.1 ± 8.5	36.8 – 68.2
ALH	1.9 ± 0.3	1.3 – 2.2
BCF	3.3 ± 1.0	1.4 – 4.9

VCL (μm/s): velocity curved line, VSL (μm/s): velocity straight line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR (%): straightness (VSL divided by VAP), WOB (μm/s): wobble ALH (μm): Amplitude of lateral head displacement BCF (Hz): beat-cross frequency

Table 2. Pearson's correlation coefficient (r) between sperm quality parameters and acrosome integrity of frozen-thawed boar sperm determined using the FITC-PNA/EthD-1 and Coomassie blue staining methods

Sperm quality parameters	Acrosome integrity (%)	
	FITC-PNA/EthD-1	Coomassie blue
Sperm motility (%)	NS	NS
Progressive motility (%)	NS	0.478*
Sperm viability (%)	NS	0.609**
Plasma membrane integrity (%)	NS	NS
Sperm motion characteristics		
VCL	NS	0.502**
VSL	NS	0.551**
VAP	NS	0.538**
LIN	NS	0.487**
STR	NS	0.447*
WOB	NS	0.509**
ALH	NS	NS
BCF	NS	0.517**

Significant levels were indicated as * $P<0.05$, ** $0.05<P<0.01$, *** $P<0.001$ and NS = $P>0.05$; VCL (μm/s): velocity curved line, VSL (μm/s): velocity straight line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR (%): straightness (VSL divided by VAP), WOB (μm/s): wobble ALH (μm): Amplitude of lateral head displacement BCF (Hz): beat-cross frequency

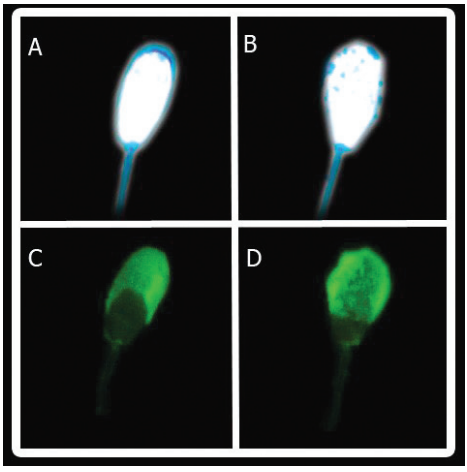

Figure 1. (A and B) Frozen-thawed boar spermatozoa with Coomassie blue staining demonstrating spermatozoa with intact acrosome (A) and reacted acrosome (B). (C and D) Frozen-thawed boar spermatozoa with fluorescein isothiocyanate conjugated peanut agglutinin and ethidium homodimer-1 (FITC-PNA/EthD-1) staining demonstrating spermatozoa with intact acrosome (C) and reacted acrosome (D)

parameters were significantly correlated with the acrosome integrity of frozen-thawed boar sperm as determined using the Coomassie blue staining method, but not the FITC-PNA/EthD-1 staining method. For instance, sperm viability as determined using SYBR-14/EthD-1 was correlated with the Coomassie blue acrosome integrity (r =0.609, P=0.002), but not with FITC-PNA/EthD-1 staining acrosome integrity (P>0.05). However, the acrosome integrity of frozen-thawed boar sperm evaluated by FITC-PNA/EthD-1 and Coomassie blue staining was significantly correlated (r=0.448, P=0.025, n=25) (Figure 2). This agreement was determined to be acceptable based on the Bland-Altman plot (Figure 3).

Discussion

In the present study, acrosome integrity of frozen-thawed boar

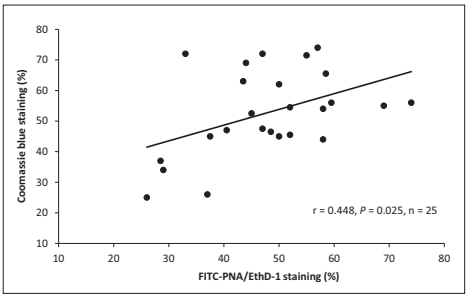

Figure 2. Pearson's correlation between acrosome integrity of frozen-thawed boar sperm determined by Coomassie blue and FITC-PNA/EthD-1 staining

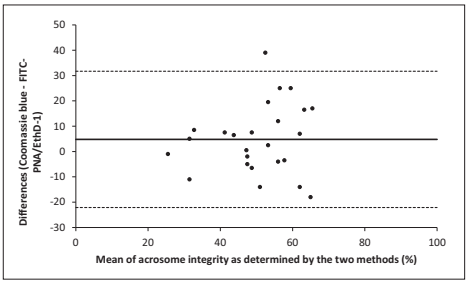

Figure 3. Bland-Altman plot illustrating agreement between Coomassie blue staining and FITC-PNA/EthD-1 for evaluating acrosome integrity of frozen-thawed boar semen

spermatozoa determined by FITC-PNA/EthD-1 averaged 48.8% and ranged between 26.0 and 74.0%. This data is consistent with previous studies (Chanapiwat et al., 2009; Pearodwong et al., 2019). Chanapiwat et al. (2009) demonstrated that the average acrosome integrity of extended fresh boar sperm was 83.7%, while the average acrosome integrity of frozen-thawed boar sperm was 31.9–49.1%. This indicates that the cryopreservation process used in the current study dramatically reduced the percentage of sperm with an intact acrosome. Nevertheless, the percentage of intact acrosomes in some ejaculates remained high (up to 74%), indicating that the freezability of boar sperm as indicated by acrosome defects is highly variable. Therefore, assessing the acrosome integrity of frozen-thawed boar sperm is essential to confirm the fertilizing ability of this semen.

Recently, Chen et al. (2021) used Coomassie blue to determine the acrosome integrity of extended fresh boar semen kept at 17°C for 3 days. They demonstrated that the proportion of sperm with an intact acrosome in semen extender with a low concentration of glucose (30.6 mM) was significantly higher than in semen extender containing a high concentration of glucose (153.0 mM) after incubation at 17°C for 3 days (92.5% versus 89.4%). As indicated in previous studies (Chanapiwat et al., 2009; Chen et al., 2021), the acrosome integrity of extended fresh semen is relatively high and is still used as an important fertilizing capacity parameter for boar semen preservation. In the current study, the acrosome integrity of frozen-thawed boar sperm determined by Coomassie blue staining was significantly associated with most sperm quality parameters, e.g., sperm viability and sperm motion characteristics post-thawing. On the other hand, the acrosome integrity of frozen-thawed boar sperm determined by the FITC-PNA/EthD-1 method was not significantly correlated with other sperm quality parameters. This could be explained by the relatively low number of observations used in the present study (*i.e.*, *n*=26 samples) and the high variation in the results obtained with the FITC-PNA/EthD-1 method. Nevertheless, the significant association between acrosome integrity of frozen-thawed boar sperm determined by Coomassie blue staining and other sperm quality parameters indicates that Coomassie blue staining is an effective method for assessing the acrosome integrity of frozen-thawed boar sperm.

In dog sperm, acrosomal status can be visualized using FITC-PNA, with binding limited to the outer acrosomal membrane and no staining on any other

part of the sperm membrane, as the outer acrosomal membrane is the specific binding site for PNA (Sirivaidyapong et al., 2000). In boar spermatozoa, during the acrosomal reaction process, the sperm plasma membrane fuses with the outer acrosomal membrane and results in the release of the acrosomal contents. This process enables detection of the acrosome reaction using PNA and fluorescence microscopy (Fazeli et al., 1997). For this reason, FITC-PNA is commonly used as a reliable probe for detection of the acrosome reaction in boar spermatozoa. This technique allows for distinctions between stages of the acrosome reaction, i.e., intact, reacting and reacted acrosome (Fazeli et al., 1997). Larson and Miller (1997) demonstrated that acrosomal status can also be determined quickly using the Coomassie blue G-250 staining procedure. Acrosome-intact sperm stained darkly near the apical portion of the sperm head (Figure 1A) in the sperm acrosome, while acrosome-reacted sperm exhibited very faint or no staining in the acrosome region (Figure 1B). The difference between spermatozoa with intact and reacted acrosomes is clear and the percentage of intact or reacted acrosomes can be confirmed by bright field microscopy (Larson and Miller, 1997). These studies confirm the feasibility of determining acrosome integrity of boar sperm using either the FITC-PNA or Coomassie blue staining procedure. Both procedures are useful for male fertility evaluations. In the present study, both techniques were applied to determine the percentage of frozen-thawed boar sperm with an intact or reacted acrosome. The agreement between the acrosome integrity results obtained from each technique was acceptable.

In conclusion, the acrosome integrity of the frozen-thawed boar sperm assessed with Coomassie blue staining was significantly correlated with that obtained via the FITC-PNA/EthD-1 staining method, and the acrosome integrity obtained using these two methods showed good agreement. Moreover, the significant association between acrosome integrity of frozen-thawed boar sperm determined by Coomassie blue staining and other sperm quality parameters indicates that Coomassie blue staining is an effective method for assessing the acrosome integrity of frozen-thawed sperm in boars.

Acknowledgement

Financial support for this study was provided by the 90th Anniversary of Chulalongkorn University Fund (Grant number GCUGR1125604078M). P. Tummaruk was awarded a Thailand Science Research and Innovation Senior Researcher Scholar grant (RTA6280013). J Rungruangsak is a grantee of the Research and Researcher for Industry RRI Master of Science Degree Programme, TSRI (MSD59I0067). Chulalongkorn University supported the One Health Research Cluster. The authors are grateful to the embryo transfer technology and germplasm research centre and their staff for help with boar semen processing. All boar semen samples were provided by the Swine Research and Development Centre, Department of Livestock Development, Nakhon Ratchasima, Thailand.

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Komparativna studija dviju različitih metoda određivanja integriteta akrosome smrznute pa odmrznute sperme nerasta: bojanje s FITC-PNA/EthD-1 u usporedbi s bojanjem Coomassie plavom

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Postoje izvješća da je bojanje Coomassie plavom učinkovita i jeftina metoda procjene integriteta akrosome spermija. Međutim, ne postoje izvješća o bojanju Coomassie plavom za procjenu integriteta akrosome krioprezervirane sperme nerasta. Nadalje, informacije u svezi podudarnosti između bojanja Coomassie plavom i fluorescein izotiocijanatom konjugiranim s aglutininom kikirikija i etidij homodimerom (FITC-PNA/EthD-1) za procjenu integriteta akrosome spermija nisu dostupne niti za jednu vrstu. Stoga je ova studija provedena za određivanje učinkovitosti i podudarnosti između metoda bojanja Coomassie plavom i bojanja FITC-PNA/EthD-1 za procjenu integriteta akrosome smrznute pa odmrznute sperme nerasta. U eksperiment je uključeno ukupno 25 uzoraka sjemena. Sjeme je krioprezervirano uporabom razrjeđivača na bazi laktoze i žumanjka i pohranjeno u 0,5 PVC-pajete. Svojstva pokretljivosti i gibanja spermija ustvrđena su uporabom sustava za računalno potpomognutu analizu

spermija. Vijabilnost spermija i integritet njihove stanične membrane procijenjeni su uporabom SYBR-14/EthD-1, odnosno hipoosmotskim testom bubrenja. Integritet akrosome smrtznute pa odmrznute sperme nerasta procijenjen je uporabom bojanja FITC-PNA/EthD-1 i Coomassie plavom. Procijenjena je povezanost između integriteta akrosome sperme ustvrđene pomoću bojanja FITC-PNA/EthD-1 i Coomassie plavom te podudarnost između dviju metoda mjerenja. Prosječni postotak integriteta akrosome smrznute pa odmrznute sperme nerasta ustvrđene bojanjem FITC-PNA/EthD-1 i Coomassie plavom bio je 48,8 ± 12,6 %, odnosno 52,6 ± 13,6% (P>0,05). Zanimljivo, vijabilnost spermija korelirala je s integritetom akrosome procijenjenom uporabom Coomassie plavom (r=0,609, P=0,002), ali nije korelirala kada je procjenjivana uporabom FITC-PNA/EthD-1 bojanja (P>0,05). Međutim, integritet akrosome smrznute pa odmrznute sperme nerasta procijenjen bojanjem FITC-PNA/EthD-1 i Coomassie plavom značajno je korelirao (r=0,448, P=0,025, n=25). Uz to, podudarnost između bojanja Coomassie plavom i FITC-PNA za procjenu integriteta akrosome smrznute pa odmrznute sperme nerasta ustvrđena Bland-Altmanovim grafikonom bila je prihvatljiva. Zaključno, integritet akrosome smrznute pa odmrznute sperme nerasta procijenjena bojanjem Coomassie plavom značajno je korelirao s onim dobivenim FITC-PNA/EthD-1 metodom bojanja te su te dvije metode pokazale dobru podudarnost. Uz to, značajna povezanost između integriteta akrosoma smrznute pa odmrznute sperme nerasta koja je ustvrđena bojanjem Coomassie plavom i drugih parametara kakvoće sperme ukazuju na to da je bojanje Coomassie plavom učinkovita metoda procjene integriteta akrosome smrznute pa odmrznute sperme u svinja.

Ključne riječi: *integritet akrosome, nerast, Coomassie plava, krioprezervacija, FITC-PNA*