



Utilization of Fluorescent Probe Association for Simultaneous Assessment of Plasmatic, Acrosomal, and Mitochondrial Membranes of Rooster Spermatozoa

■ Author(s)

Celeghini ECC¹
Arruda RP^{1*}
Albuquerque R²
Silva FHA³
Faria DE³
Andrade AFC¹
Nascimento J¹
Raphael CF¹

¹ Laboratório de Biotecnologia do Sêmen e Andrologia, Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade de São Paulo (USP).

² Departamento de Nutrição e Produção Animal, FMVZ, USP.

³ Departamento de Zootecnia, Faculdade de Zootecnia e Engenharia de Alimentos (FZEA), USP.

■ Mail Address

RP Arruda
Av. Duque de Caxias Norte, 225
Post-box 23
13.635-900. Pirassununga, SP, Brazil.
Telephone: +55 019 3565-4221
Fax: +55 019 3565-4060

E-mail: arrudarp@usp.br

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ABSTRACT

This experiment was designed with the objective of developing a simple, practical, and high repeatability technique for the simultaneous evaluation of the integrity of the plasmatic and acrosomal membranes, as well as functional mitochondria of domestic fowl spermatozoa using an association of fluorescent probes. Four ejaculates (motility $\geq 80\%$ and abnormal morphology $\leq 10\%$) from each of six Ross male broiler breeder ($n=24$) were diluted in TALP sperm medium (25×10^6 spermatozoa/mL) and split into two aliquots, and one of these aliquots was flash frozen in liquid nitrogen and thawed to damage all cellular membranes. Three treatments were prepared from these aliquots, with the following ratios of Fresh semen:Flash frozen semen: 100:0 (T100), 50:50 (T50), and 0:100 (T0). A 150- μ L aliquot of diluted semen was placed in a microcentrifuge tube with the addition of 2- μ L PI, 2- μ L MITO, and 50- μ L FITC-PSA, and incubated at 38.5° C/8 min in the dark. An 8- μ L sample was placed on a slide, coverslipped, and examined by epifluorescence microscopy. Each sample had 200 cells counted and classified based on the fluorescence emitted by each probe. By regression analysis, plasma membrane integrity, as detected by PI, was determined as: $v=4.17+0.82X$ ($R^2=0.95$). Acrosome integrity, as detected by FITC-PSA, generated the equation: $v=4.19+0.84X$ ($R^2=0.96$). Functional mitochondria was estimated by the equation $v=3.20+0.83X$ ($R^2=0.96$). This is an efficient technique to simultaneously evaluate plasmatic, acrosomal, and mitochondrial membranes in fowl sperm. It is suggested that its application in flow cytometry systems allows this methodology to be applied in large scale.

INTRODUCTION

Fertility is one of the most important economic traits in poultry production, together with egg hatchability. Male fertility potential may be defined as the capability to produce and to ejaculate spermatozoa that are able to fertilize eggs, which includes accomplishment of all steps of the fertilization process: sperm moving across the female reproductive tract and reaching the sperm storage tubule, binding and penetration into the perivitelline layer, and fertilization (Celeghini *et al.*, 2001).

Sire selection in most commercial poultry breeds is performed on a subjective basis, determined by secondary sexual traits, such as the comb development (Celeghini *et al.*, 2001). Given the importance of fertility rate in a breeder flock, studies have investigated more precise evaluations of male fertility capacity, since one male is responsible for fertilizing several females. Thus, the analysis of fowl semen characteristics can be used as a tool for the selection of sires kept either under natural mating, or as semen donors in artificial insemination



systems (Wilson *et al.*, 1979). Some researchers found significant correlations between semen characteristics and egg fertility (Harris Jr. *et al.*, 1984), sustaining that male fertilizing potential is dependent on semen quality. It is known that, in order to ensure high egg fertility rate, the semen needs to present some characteristics, which evaluation is based on physical and morphological examination. More objective techniques to evaluate semen-fertilizing potential have been proposed, such as tests of sperm penetration in the blastodisc region (Bramwell *et al.*, 1995; Barbarato *et al.*, 1998), and sperm membrane evaluations (Bilgili & Renden, 1984; Chalah & Brillard, 1998).

Despite being highly correlated to fertility, the sperm penetration test is labor-intensive, and difficult to apply in large breeding stocks (Bramwell *et al.*, 1995; Barbarato *et al.*, 1998). However, as membranes play an essential role in maintaining sperm ability to fertilize, they have been evaluated by more objective techniques, such as the use of fluorescent probes (Bilgili & Renden, 1984; Graham *et al.*, 1990; Chalah & Brillard, 1998; Celeghini *et al.*, 2005).

Sperm plasma membrane is responsible for establishing a barrier between intracellular and extracellular environments, which is important to maintain osmotic equilibrium and cellular homeostasis. Damages in this structure lead to cellular instability caused by homeostasis loss, resulting in cellular death. Therefore, plasma membrane integrity exerts a crucial role on sperm survival in the female reproductive tract and its fertilizing ability (Parks & Graham, 1992).

Earlier studies evaluating the integrity of the sperm plasma membrane in fowl, utilizing fluorescent probes, mention the use of ethidium bromide (Bilgili & Renden, 1984). Nevertheless, due to its high toxicity, ethidium bromide application is limited. Propidium iodide (PI), a fluorescent dye with properties similar to ethidium bromide, but less toxic, has DNA affinity, and stains damaged plasma membrane cell nucleus in red (Bayyari *et al.*, 1990; Graham *et al.*, 1990; Chalah & Brillard, 1998). Because it is a stable fluorescent stain, PI has been the most frequently utilized probe in research, with successful results in several species, both by fluorescent microscopy (Garner *et al.*, 1997; Sukardi *et al.*, 1997; Thomas *et al.*, 1997; Chalah & Brillard, 1998) and by flow cytometry system (Bayyari *et al.*, 1990; Graham *et al.*, 1990; Pintado *et al.*, 2000; Gillan *et al.*, 2005).

In addition to plasmatic membrane integrity, it is important to consider acrosomal membrane integrity and the maintenance of its enzymes, as acrosomal

reaction, characterized by the release of the acrosomal enzymes, is essential for the sperm to penetrate the blastodisc region, and to egg fertilization (Bakst & Howarth, 1977). Acrosome integrity can be checked by different fluorescent techniques. Among them, the use of marked lectins is emphasized (Graham *et al.*, 1990), such as *Pisum sativum* agglutinin (PSA), *Ricinus communis* agglutinin (RCA), *Arachis hypogea* agglutinin (PNA) (Cross & Meizel, 1989), and *Concanavalia ensiformis* (ConA), which are fluorescein isothiocyanate-conjugates (FITC). PSA is an agglutinin from edible peas, and binds to the glycoconjugate of the acrosomal matrix (Cross & Meizel, 1989). It has affinity for terminal α -D-glucosyl and α -D-mannosyl residues of glycoproteins, and binds specifically to the sugar α -mannoside found in acrosomal contents (Sukardi *et al.*, 1997). This agglutinin, when conjugated to FITC, marks the sperm acrosome in yellow-green, and identifies acrosome damage. It can be applied to spermatozoa of different species (Graham *et al.*, 1990; Arruda *et al.*, 2002).

On the other hand, mitochondria, arranged helicoidally in the spermatozoa midpiece, are responsible for producing the energy necessary for flagellar beat. It warrants sperm motility, essential for sperm to cross the female reproductive tract, to be stored in the sperm storage tubule, and to reach the fertilizing site. The energy produced by mitochondria is ATP, generated by internal membrane potential in the respiratory chain (Bereiter-Hahn, 1990). The ability to monitor changes in mitochondrial membrane potential *in situ* inside of cells can be crucial to interpret cellular physiology changes in various experimental situations. Mitochondrial function can be evaluated by different fluorescent probes, as rhodamine 123; 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1); 8-(4'-chloromethyl) phenyl-2,3,5,6,11,12,14,15 octahydro-1H,4H,10H,13H-diquinolizino-8H-xanthylium chloride (CMXRos or Mitotracker red); and MitoTracker green FM (MITO). MITO is non-fluorescent in water solutions, and becomes fluorescent once it accumulates in the lipid environment of mitochondria (Haugland, 2005). This probe has been utilized in human sperm to visualize mitochondrial sheath dysplasia (Rawe *et al.*, 2001) and in bovine spermatozoa to verify cryopreservation effects (Garner *et al.*, 1997). MITO stain preferentially accumulates in the mitochondria, independent of mitochondrial potential, and therefore can be used a tool for mitochondrial mass determination (Haugland, 2001). Contradictorily, in bovine cryopreserved sperm,



a high correlation was observed between sperm stained by MITO and sperm motility ($r = 0.96$), as well as sperm viability, as detected by SYBR-14 ($r = 0.97$), indicating that this probe reflects the functional status of mitochondria (Garner *et al.*, 1997).

Considering that, in order for to fertilize the oocyte, sperm need to have all its membranes intact, it is then vital that sperm evaluation be simultaneous, supplying information on the number of spermatozoa with intact plasmatic and acrosomal membranes, as well as preserved mitochondrial function. The association of fluorescent probes allows the simultaneous evaluation of more than one sperm cell compartment, as well as simultaneous evaluation of plasmatic and acrosomal membranes, using Hoechst 33258 and FITC-PSA association (Arruda *et al.*, 2002), PI and LYSO-G or PI + SYTO-17 and FITC-PNA (Thomas *et al.*, 1997), phycoerythrin (PE)-conjugated PNA associated to probes SYBR-14 and PI (NAGY *et al.*, 2003), or PI and FITC-PSA association (Centola *et al.*, 1990; Graham *et al.*, 1990; Sukardi *et al.*, 1997; Arruda *et al.*, 2002).

Graham *et al.* (1990) demonstrated that at least three bovine sperm compartments can be simultaneously evaluated by addition of three probes. These authors utilized PI for plasmatic membrane integrity evaluation, PSA to determine acrosomal integrity, and R123 to verify mitochondrial function. For simultaneous evaluation, single flow cytometry apparatus was used, and the validation was performed separately for each probe.

The more sperm parameters are evaluated in a semen sample, the higher the value in the *in vitro* fertility prognostic. However, in commercial poultry production, a large number of samples must be processed and evaluated. Consequently, this technique may require to be faster and cheaper to be routinely applied.

This experiment was designed with the objective of developing a simple, practical, and highly repeatable technique for simultaneous integrity evaluation of plasmatic and acrosomal membranes, as well as of mitochondrial function in domestic fowl spermatozoa by the association of fluorescent probes.

MATERIALS AND METHODS

Semen Preparation

Four ejaculates from six Ross male broiler breeder ($n=24$) were utilized, all presenting motility $\geq 80\%$, and abnormal morphology $\leq 10\%$. Immediately upon collection, the semen was diluted in TALP sperm

medium (Bavister *et al.*, 1983) to a final concentration of 25×10^6 spermatozoa/mL. The sample of diluted semen was split into 2 aliquots, and 1 aliquot was flash frozen in liquid nitrogen and thawed in 3 continuous cycles in order to damage cellular membranes and to disturb mitochondrial function. Three treatments were prepared from these aliquots, with the following ratios of Fresh semen:Flash frozen semen: 100:0 (T100), 50:50 (T50) and 0:100 (T0).

Spermatozoa Stain

After the preparation, the three samples, T100, T50 and T0, were submitted to a stain technique adapted from Arruda & Celeghini (2003). A 150- μ L aliquot of diluted semen was placed in a microcentrifuge tube, and 2- μ L PI (3 mM, Sigma, 28,707-5, in DPBS), 2- μ L MITO (500 mM, Molecular Probes, M-7514, in DMSO), and 50- μ L FITC-PSA (100 μ g/mL, Sigma, L-0770, in DPBS) were added. The sample was incubated for 8 minutes at 38.5° C, in the dark.

Fluorimetric Assessment

An 8- μ L sample of stained spermatozoa suspension was placed on a slide, a coverslip added, and the examination was immediately performed under epifluorescence microscopy (Nikon, model Eclipse 80i) in a triple filter (D/F/R, C58420), with the set: UV-2E/C (340-380 nm excitation and 435-485 nm emission), B-2E/C (465-495 nm excitation and 515-555 nm emission), and G-2E/C (excitation 540-525 and emission 605-655), at 1.000 x magnification. Each sample had 200 cells counted and classified based on the fluorescence emitted by each probe.

Statistical Analysis

Data obtained from T0, T50, and T100 treatment groups were evaluated by analysis of variance (ANOVA) (SAS, 1998). Treatment means across all samples were compared by Fisher's LSD test. The data of plasmatic and acrosomal membranes integrity, and mitochondrial function (dependent variables) in the treatments T0, T50, and T100 (independent variables) were submitted to simple linear regression analysis by StatView software (SAS, 1998).

RESULTS

The association of PI, FITC-PSA and MITO probes resulted in eight cell categories, according to plasmatic and acrosomal integrity, and mitochondrial function, as shown in Table 1. The fluorescence standard presented



Table 1 - Classification of fowl sperm cells according to fluorescence emitted by PI, FITC-PSA, and MITO probes.

Sperm Cells	PI*	FITC-PSA**	MITO***
Intact plasmatic membrane, intact acrosome, and functional mitochondria	-	-	+
Intact plasmatic membrane, intact acrosome and non-functional mitochondria	-	-	-
Intact plasmatic membrane, damaged acrosome and functional mitochondria	-	+	+
Intact plasmatic membrane, damaged acrosome and non-functional mitochondria	-	+	-
Damaged plasmatic membrane, intact acrosome and functional mitochondria	+	-	+
Damaged plasmatic membrane, intact acrosome and non-functional mitochondria	+	-	-
Damaged plasmatic membrane, damaged acrosome and functional mitochondria	+	+	+
Damaged plasmatic membrane, damaged acrosome and non-functional mitochondria	+	+	-

*PI positive (+) = red-stained nucleus. **FITC-PSA positive (+) = yellow-green acrosome region. ***MITO positive (+) = bright green in midpiece region.

by sperm stained by the fluorescent probes association is shown in Figure 1.

ANOVA and Fisher tests detected statistical differences ($p < 0.0001$) between the T100, T50, and T0 treatment groups, as shown in Table 2, validating the submission of data to linear regression analysis.

Table 2 - Mean \pm standard deviation of intact plasma membrane (IPM), intact acrosome (IA) and functional mitochondria (MF) in fowl spermatozoa submitted to treatments 100, 50 and 0.

Characteristic	T100	T50	T0
IMP	86.29 \pm 8.4 ^c	46.40 \pm 8.6 ^b	3.71 \pm 2.8 ^a
IA	87.71 \pm 6.9 ^c	47.89 \pm 8.8 ^b	3.42 \pm 2.6 ^a
MF	86.06 \pm 8.1 ^c	46.81 \pm 8.3 ^b	2.33 \pm 2.4 ^a

Different superscript letters in the same row indicate statistical differences ($p < 0.0001$).

Regression analysis results for plasma membrane integrity, as detected by PI, are displayed in Figure 2. Acrosome integrity, as detected by FITC-PSA probe, generated the equation shown in Figure 3. Mitochondrial function estimated by MITO is displayed in Figure 4.

DISCUSSION

In this experiment, the association of fluorescent probes was tested and validated for the simultaneous evaluation of plasmatic (PI) and acrosomal (FITC-PSA) membranes integrity, as well as of mitochondrial function (MITO) in fowl sperm. These tests are important in order to obtain a highly repeatable technique that presents results reflecting the real status of each structure. It is still necessary to identify which probes associate better, as there may be different results with different associations due to changes in its characteristics and fluorescence standards.

The possibility of using the association of fluorescent probe for simultaneous assessment of plasmatic and acrosomal membranes, has already been well described in literature relative to mammalian sperm

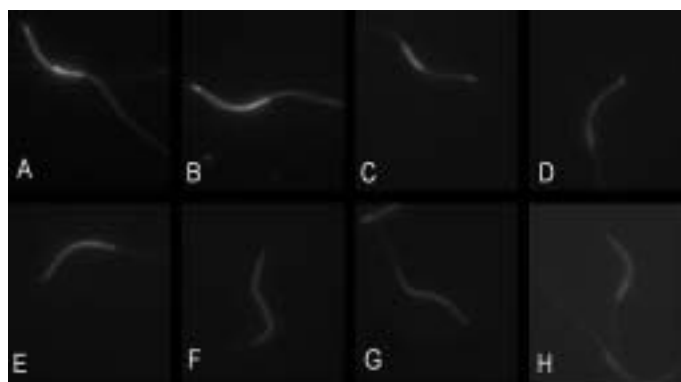


Figure 1 - Photomicrography of fowl sperm stained with PI, FITC-PSA, and MITO association (1,000x magnification). A. intact plasma membrane, intact acrosome and functional mitochondria sperm. B. intact plasma membrane, damaged acrosome and functional mitochondria sperm. C. intact plasma membrane, intact acrosome and non-functional mitochondria sperm. D. intact plasma membrane, damaged acrosome and non-functional mitochondria sperm. E. damaged plasma membrane, damaged acrosome and mitochondrial function sperm. F. and G. damaged plasma membrane, intact acrosome and non-functional mitochondria sperm. H. damaged plasma membrane, damaged acrosome and non-functional mitochondria sperm.

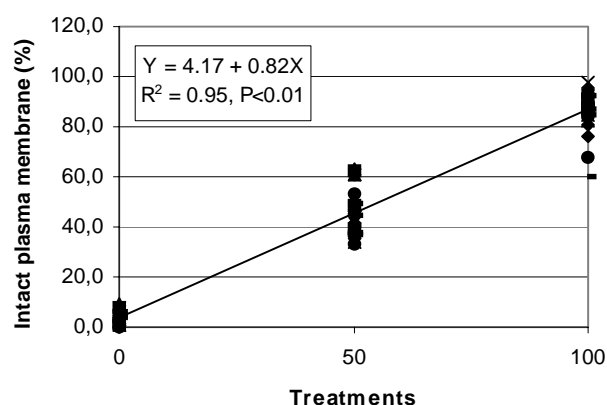


Figure 2 - Linear regression of plasma membrane integrity, as verified by exclusion of propidium iodide in PI/FITC-PSA/MITO association, in fowl spermatozoa submitted to treatments T0 (Fresh semen:Flash frozen semen, 0:100), T50 (Fresh semen:Flash frozen semen, 50:50) and T100 (Fresh semen:Flash frozen semen, 100:0).

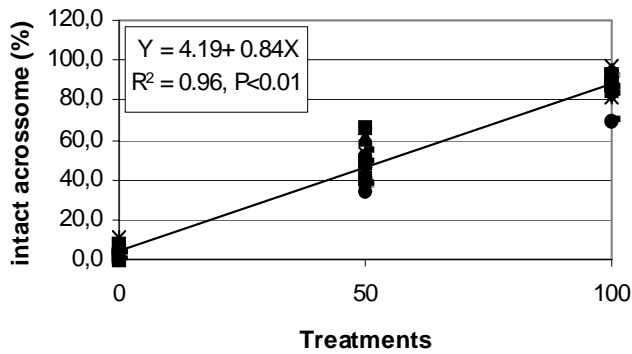


Figure 3 - Linear regression of intact acrosome, detected by FITC-PSA in PI/FITC-PSA/MITO association, in fowl spermatozoa submitted to treatments T0 (Fresh semen:Flash frozen semen, 0:100), T50 (Fresh semen:Flash frozen semen, 50:50) e T100 (Fresh semen:Flash frozen semen, 100:0).

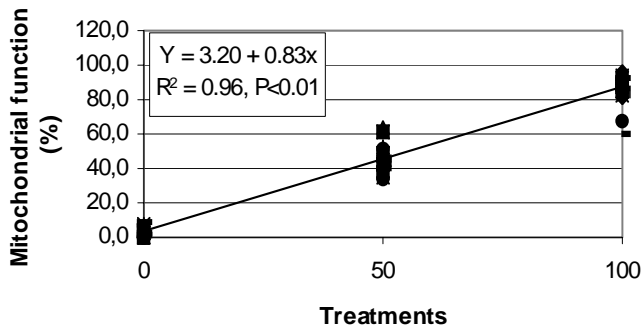


Figure 4 - Linear regression of mitochondrial function, verified by MitoTracker Green FM, in PI/FITC-PSA/MITO association in fowl spermatozoa, submitted to treatments T0 (Fresh semen:Flash frozen semen, 0:100), T50 (Fresh semen:Flash frozen semen, 50:50) and T100 (Fresh semen:Flash frozen semen, 100:0).

(Centola *et al.*, 1990; Sukardi *et al.*, 1997; Arruda *et al.*, 2002; Nagy *et al.*, 2003) and poultry sperm (Chalah & Brillard, 1998). The PI and FITC-PSA probe association to assess plasmatic and acrosome membranes integrity, respectively, as utilized in this experiment, was reported in sheep (Sukardi *et al.*, 1997), human (Centola *et al.*, 1990), horse (Arruda *et al.*, 2002), and cattle (Graham *et al.*, 1990). PI was also associated with probes to evaluate mitochondrial function as R123, MITO and JC-1; these probes showed high positive correlation ($r > 0.96$) with sperm motility (Garner *et al.*, 1997). However, this experiment validated the association of PI and FITC-PSA with an additional probe for mitochondrial function evaluation in fowl sperm.

PI, FITC-PSA and MITO association resulted in very consistent marking of spermatozoa, making it easy to identify the evaluated structures. The association of PI and MITO probes was mentioned by Garner *et al.* (1997) in bovine spermatozoa assessment. T100, T50

and T0 treatment groups were prepared to validate the technique, according to the methodology described by Thomas *et al.* (1997). The results obtained were submitted to ANOVA, and means were compared using the Fisher test. The comparison of the results and the confirmation of differences among treatments were important to determine the efficacy of each treatment for technique validation before submitting them to linear regression analysis.

As confirmed by regression analysis results, this technique presented good results and high repeatability. Some MITO characteristics were observed: when it binds to mitochondria that have membrane potential, it emits bright green fluorescence. However, it is important to stress that MITO also binds to regions of the membrane of the head and tail of the sperm in a non-specific fashion, emitting less intense fluorescence, as observed by Garner *et al.* (1997). This unspecific binding of MITO is, in a way, beneficial to the evaluation, because it makes it possible to visualize the shape of cells with intact plasma membrane. However, it is important to differentiate midpiece stain nuances in functional and non-functional mitochondria. In an attempt to investigate more objectively these associations in bovine sperm, Celeghini *et al.* (2005) validated two fluorescent-probe techniques for simultaneous evaluation of plasmatic, acrosomal, and mitochondrial membranes, using PI, Hoechst 33342, FITC-PSA, and CMXRos or JC-1, and obtained very consistent results. Nevertheless, it must be noted that the addition of one additional probe (H342) increases the technique cost.

In the regression analysis equation obtained for plasmatic membrane integrity (Figure 2), evaluated by the exclusion of PI from the nucleus, it is possible to verify that the value of point “a” (where the line crosses the Y axis, i.e., showing the value of Y when X = 0) is next to zero ($a = 4.17$), as would be expected from a sample that was submitted to flash freezing with the objective of damaging all membranes. The value of “b” (regression coefficient, i. e., how much X varies in relation to Y) is also near the expected ($b = 0.82$) for T100, representing a sample with at least 80% of motility. The regression coefficient of 95% confirms these observations. Similar results were found by Graham *et al.* (1990), who compared the efficiency of PI with the stain technique by eosin/nigrosin, and found a positive correlation ($r = 0.78$) between techniques, and confidence interval of 95%. Positive correlations between PI and eosin/nigrosin were also observed in dogs ($r = 0.88$) (Peña *et al.*, 1998), boars ($r = 0.71$) and



bulls ($r = 0.83$) (Pintado *et al.*, 2000). Pintado *et al.* (2000) also observed high positive correlations between PI and H258 in swine ($r = 0.96$) and bovine ($r = 0.94$) sperm.

Similar coefficients were found for the same probe association by regression equations that calculated for acrosome integrity (Figure 3), as verified by the FITC-PSA probe. The value of "a" (4.19) is close to the expected. The value of "b" (0.84) also reflects the desired value and the determination coefficient of 95% demonstrates the efficiency of the technique. The FITC-PSA efficiency was evaluated by Graham *et al.* (1990), comparing it to naphthol yellow/erythrosin b, finding a confidence interval of 95%.

The equation obtained for mitochondrial function, with MITO associated to PI and FITC-PSA, showed a similar characteristics to plasmatic membrane integrity and acrosomal integrity ($a = 3.20$, and $b = 0.83$). Nevertheless, Arruda & Celeghini (2003) obtained a regression equation, for the same association of probes in bovine sperm, ($Y = 35.0 + 0.55X$) different than expected, in spite of finding a high determination coefficient ($R^2 = 0.84$). This same technique, with minor changes, was validated in equine sperm (Celeghini *et al.*, 2004), using the same methodology, which yielded an equation ($v = 9.57 + 0.78 X$) with results similar to those obtained in the present experiment, with a determination coefficient of 93%. These differences could be explained by differences among species or by the greater ability of the technique to differentiate stain nuances of MITO when bound to mitochondrial membranes presenting potential or not.

This is an efficient and easy technique to simultaneously evaluate plasmatic, acrosomal, and mitochondrial membranes of fowl sperm. It is possible to suggest that the application of this methodology in large scale can be maximized by the use of flow cytometry systems, providing higher accuracy and swiftness, as it is to read approximately 10,000 cells in a few seconds.

REFERENCES

- Arruda RP, Celeghini ECC. Validation of a technique to evaluation simultaneous of the plasmatic, acrosomal and mitochondrial membranes in bovine spermatozoa. *Acta Scientiae Veterinariae* 2003; 31:230-231.
- Arruda RP, Souza NL, Marques A, Celeghini ECC, Gobesso AAO, Meirelles FV, Binelli M, Blasques FJH. Evaluation of techniques using CFDA/PI, H258/FITC-PSA and Trypan Blue/Giemsa for assessment of the viability and acrosomal Integrity of cryopreserved equine spermatozoa. *Theriogenology* 2002; 57(1):477.
- Bakst MR, Howarth B. Hydrolysis of hen's perivitelline layer by cock sperm. *Biology of Reproduction* 1977; 17:370-379.
- Barbarato GF, Cramer PG, Hammstedt RH. A practical in vitro sperm-egg binding assay that detects subfertile males. *Biology of Reproduction* 1998; 58:686-699.
- Bavister BD, Leibfried ML, Lieberman G. Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biology of Reproduction* 1983; 28(1):235-47.
- Bayyari GR, Cook JR, Harris Jr GC, Macy LB, Slavick MF, Skeeles JK. Research note: The evaluation of chicken spermatozoa using fluorescent staining in a 96-well format. *Poultry Science* 1990; 69: 1602-1605.
- Bereiter-Hahn J. Behavior of mitochondria in the living cell. *International Review of Cytology* 1990; 122:1-63.
- Bilgili SF, Renden JA. Fluorimetric determination of avian sperm viability and concentration. *Poultry Science* 1984; 63(11):2275-77.
- Bramwell RK, Marks HL, Howarth B. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. *Poultry Science* 1995; 74(11):1875-1883.
- Celeghini ECC, Albuquerque R, Arruda RP, Lima CG. Seminal characteristics evaluation of the male broiler breeder selected by comb development to reproduction. *Brazilian Journal of Veterinary Research and Animal Science* 2001; 38(4):177-183.
- Celeghini ECC, Arruda RP, Andrade AFC, Raphael CF, Nascimento J. Simultaneous evaluation of the plasmatic, acrosomal, and mitochondrial membranes in equine spermatozoa. *Proceedings of the 15th Internacional Congress Of Animal Reproduction*; 2004; p. 511. Porto Seguro, BA-Brazil.
- Celeghini ECC, Nascimento J, Andrade AFC, Raphael CF, Souza LWO, Arruda RP. Use of CMXRos and JC-1 on mitochondrial function evaluation, associated to fluorescent probes to plasmatic and acrosomal membranes evaluation in bovine spermatozoa. *Acta Scientiae Veterinariae* 2005; 33:321.
- Centola GM, Mattox JH, Burde S, Leary JF. Assessment of the viability and acrosome status of fresh and frozen-thawed human spermatozoa using single-wavelength fluorescence microscopy. *Molecular of Reproduction and Development* 1990; 27(2):130-135.
- Chalah T, Brillard JP. Comparison of assessment of fowl sperm viability by eosin-nigrosin and dual fluorescence (SYBR-14/PI). *Theriogenology* 1998; 50:487-493.
- Cross NL, Meizel S. Methods for evaluating the acrosomal status of mammalian sperm. *Biology of Reproduction* 1989; 41:635-641.
- Garner DL, Thomas AC, Joerg HW, Dejarnette JM, Marshall CE. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biology of Reproduction* 1997; 57:1401-06.



Gillan L, Evans G, Maxwell WMC. Flow cytometric evaluation of sperm parameters in relation to fertility potential. *Theriogenology* 2005; 63:445-457.

Graham JK, Kunze E, Hammerstedt RH. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biology of Reproduction* 1990; 43:55-64.

Harris Junior GC, Benson JA, Sellers RS. The influence of daylength, body weight, and age on the reproductive ability of broiler breeder cockerels. *Poultry Science* 1984; 63(9):1705-1710.

Haugland, RP. Introduction to fluorescence techniques. In: Spence, MTZ. *The handbook: a guide to fluorescent probes and labeling technologies*. 10th ed. Invitrogen Corp.; 2005. p.1-6.

Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma -and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. *Biology of Reproduction* 2003; 68:1828-1835.

Parks JE, Graham JK. Effect of cryopreservation procedures on sperm membranes. *Theriogenology* 1992; 38:209-222.

Pintado B, De La Fuente J, Roldan ERS. Permeability of boar and bull spermatozoa to the nucleic acid stains propidium iodide or Hoechst 33258, or the eosin: accuracy in the assessment of cell viability. *Journal of Reproduction and Fertility* 2000; 118:145-152.

Rawe VY, Galaverna GD, Acosta AA, Olmedo SB, Chemes HE. Incidence of tail structure distortions associated with dysplasia of the fibrous sheath in human spermatozoa. *Human Reproduction* 2001; 16(5):879-886.

SAS Institute. *User's guide: statistic*. version 6.11. 2nd ed. Cary; 1998.
Sukardi S, Curry MR, Watson PF. Simultaneous detection of the acrosomal status and viability of incubated ram spermatozoa using fluorescent markers. *Animal Reproduction Science* 1997; 46:89-96.

Thomas CA, Garner DL, DeJarnette JM, Marshall CE. Fluorometric assessments of acrosomal integrity and viability in Cryopreserved bovine spermatozoa. *Biology of Reproduction* 1997; 56:991-998.

Wilson HR, Piesco NP, Miller ER, Nesbeth WG. Prediction of the fertility potential of broiler breeder males. *World's Poultry Science Journal* 1979; 35(2):95-118.