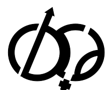

DOI: 10.21451/1984-3143-AR842

Anim. Reprod., v.14, n.2, p.437-441, Apr./Jun. 2017

Validation of the CellRox Deep Red® fluorescent probe to oxidative stress assessment in equine spermatozoa

Renata Lançoni[1], Rubens Paes de Arruda[1], Maíra Bianchi Rodrigues Alves[2], Letícia Zoccolaro Oliveira[1], Gabriel De Carli dos Santos, Kleber Menegon Lemes[3], Shirley Andréa Florez-Rodriguez, Eneiva Carla Carvalho Celeghini[2,4]

[1]Laboratory of Semen Biotechnology and Andrology, School of Veterinary Medicine and Animal Science, University of Sao Paulo (USP), Pirassununga, SP, Brazil.
[2]Laboratory of Research in Pathology of Reproduction, School of Veterinary Medicine and Animal Science, University of Sao Paulo (USP), Pirassununga, SP, Brazil.
[3]Laboratory of Pharmacology and Endocrinology of Reproduction, School of Veterinary Medicine and Animal Science, University of Sao Paulo (USP), Pirassununga, SP, Brazil.

Abstract

Considering the importance of ROS influence on sperm functionality and some limitations in sperm oxidative stress assessment methods, a field to studies of new techniques are still open. In this sense, the aim of this study is to validate the ROS detection technique through the CellRox Deep Red Reagent® probe in stallion sperm. Four stallions were used and the analyses were conducted on four replicates of semen samples from each of stallion (n = 16). The results of the polynomial regression presented a quadratic effect, high determination coefficient value ($R^2 = 0.88$) and high significant P value ($P < 0.0001$). The CellRox Deep Red® fluorescent probe is able to detect reactive oxygen species in equine sperm, indicating accurately the occurrence of oxidative stress in stallion semen.

Keywords: Cellrox, fluorescente probes, oxidative stress, peroxidative damage, stallion.

Introduction

Spermatozoa are constantly exposed to oxidizing environments during production in the testicles until fertilization in the female reproductive tract. Furthermore, sperm cells are very susceptible to oxidative stress because they have high amount of polyunsaturated fatty acids in their cellular and intracellular membranes. In addition, low concentrations of cytoplasm and cytoplasmic enzymes limits their ability to eliminate reactive oxygen species (ROS) and to repair DNA damage (Lenzi *et al*., 1996).

Mitochondrial metabolism is the major sperm source of ROS generation (Gibb *et al*., 2014). The induction of ROS generation by mitochondria is the first step of apoptosis cascade which is associated with several changes that prevent sperm to participate of fertilization process. ROS are able to attack sperm DNA and mitochondria, reducing mitochondrial membrane potential and sperm motility. Furthermore, they can also reach sperm plasma membrane, reducing its fluidity and integrity (Aitken *et al*., 2012).

Since ROS may interfere on sperm functionality, its identification during semen analysis is also important. Some oxidative stress measuring techniques are well know. In presence of ROS, lipid peroxidation occurs and with the C11-BODIPY probe a change from red to green fluorescence is detected, which can be read by flow cytometer or laser confocal microscopy (Ball and Vo, 2002; Brouwers *et al*., 2005; Raphael *et al*., 2008). In addition, the MitoSOX RED® probe only detects the superoxide anion mitochondrial, the H_2DCFDA only detects the hydrogen peroxide (Aitken *et al*., 2013) and the probe crosses the cell membrane in a non-fluorescent form, but when in contact with reactive species within sperm cells, it will emit detectable fluorescence (Wang and Joseph, 1999). The H_2DCFH-DA has the advantage of allowing quantification of oxidative stress by flow cytometry or fluorometry, however, has the disadvantage of only identifying the hydrogen peroxide (Wang and Joseph, 1999).

Considering the importance of ROS influence on sperm functionality and some limitations in sperm oxidative stress assessment methods, a field to studies of new techniques are still open. In this sense, the CellRox Deep Red Reagent® (CAT 10422, Molecular Probes) can detect ROS presence by emitting red fluorescence when reactive species are detected and not emitting any fluorescence if no oxidative stress is detected in the cell. This probe is able to detect the hydroxyl radical and the superoxide anion, but presenting more sensitivity to hydroxyl radical detection. Furthermore, it can be read in epifluorescence microscopy, flow cytometry and spectrophotometry. Still, it can be associated with other probes and can be fixed to read a posteriori (Grinberg *et al*., 2013; Alves *et al*., 2015).

In the current andrology field, the CellRox Deep Red® fluorescente probe has been tested in sheep sperm with induced oxidative stress to validate the technique (Alves *et al*., 2015). Due to the equal importance of oxidative stress on equine spermatozoa, the aim of this study is to validate the ROS detection technique through the CellRox Deep Red Reagent® probe in stallion sperm.

Materials and Methods

The experiment was conducted with three semen treatments: T0 (semen sample that was not

[4]Corresponding author: celeghin@usp.br
Phone:+55(19)3565 4242
Received: May 20, 2016
Accepted: March 22, 2017

submitted to oxidative stress induction), T50 (semen sample that was 50% induced to oxidative stress and 50% not induced) and T100 (semen sample that was entire submit to oxidative stress induction); and two variables: sperm under no oxidative stress and sperm under oxidative stress.

Semen collection and preparation:

Four stallions were used. Before starting the experiment, the animals underwent a biological leveling through a regular interval of semen collection, totalizing four semen collections in a week. Stallions were maintained under the same management conditions during the experiment, housed in pens and free access to appropriate individual pickets three times weekly. Hay, grass and concentrates were provided to attend the NRC, with water and mineral salt provided *ad libitum.*

The analyses were conducted on four replicates of semen samples from each of stallion (n = 16). Each of the four replicates was collected as two ejaculates from the same animal collected within a 90 min interval; therefore, a total of 32 ejaculates were collected.

Prior to each ejaculate collection, the stallion´s penis was washed with wet cotton at 35°C. The ejaculates were collected using an artificial vagina with temperature of internal mucosa at 42-45°C. The experiment agree with Ethical Principles in Animal Research adopted by Ethic Committee in the use of animals of the School of Veterinary Medicine and Animal Science of University of São Paulo, protocol number 6982031014.

The first ejaculate of each replicate was diluted in TALP media (Bavister and Lorraine, 1983) at a concentration of 25 x 10^6 sperm/ml for the preparation of two samples: control samples without oxidative stress induction (T0; semen *in natura*) and samples with induced oxidative stress (T100).

For the induction of oxidative stress, an aliquot of 1000 µl was centrifuged at 500 x *g* for 10 min to remove seminal plasma. Then, the supernatant was removed and the pellet resuspended in 200 µl of TALP, 50 µl of sodium ascorbate (20 mM), 50 µl of iron sulfate (4 mM) and 30 µl of t-butylhydroperoxide (15 mM) and incubated at 37°C for 90 min (adapted from Alves *et al*., 2015).

The second ejaculate, collected after past 90 min of oxidative stress induction, was used for the preparation of the third sample (T50), which contained 50% semen from the second ejaculate diluted in TALP (without oxidative stress induction) and 50% semen from the T100 sample (after oxidative stress induction).

Oxidative stress analysis:

In the present experiment, the CellROX Deep Red Reagent® fluorescent probe (2.5 mM; Life Technologies, New York, USA) was diluted in dimethyl sulfoxide (DMSO; 472301, Sigma-Aldrich, St. Louis, USA), as precise indication of the manufacturer, for a final concentration of 1 mM (working solution) and stored at -20°C in the dark. During use, this working solution was kept in the dark at 37°C.

For technique preparation, 200 µl (25 x 10^6 sperm/ml) from each semen sample (T0, T50, and T100) were added to 4.0 µl of CellROX® (1 mM, Invitrogen) and 2 µl of Hoescht 33342 (0.5 mg/ml, Molecular Probes) and incubated at 37ºC for 30 min. After incubation, each sample was centrifuged for 5 min at 2000 x *g*, the supernatant was removed, and the pellet was resuspended in 200 µl of TALP. An aliquot of 4 µl of the solution stained with CellRox® fluorescente probe was placed between a slide, coverslip and read under epifluorescence microscopy (Nikon, model Eclipse N*i*-U 80i, Tokyo, Japan) at 1000X magnification using a triple filter (D/F/R, C58420) featuring the UV-2E/C (340-380 nm excitation and 435-485 emission), B-2E/C (465-495 excitation and 515-555 emission), and G-2E/C (excitation 540-525 and 605-655 emission) sets.

A total of 200 cells were counted for each sample by the same technique. The cells were classified in two categories: sperm under no oxidative stress (unstained midpiece), sperm under detectable oxidative stress (midpiece stained red), as demonstrated in Fig. 1.

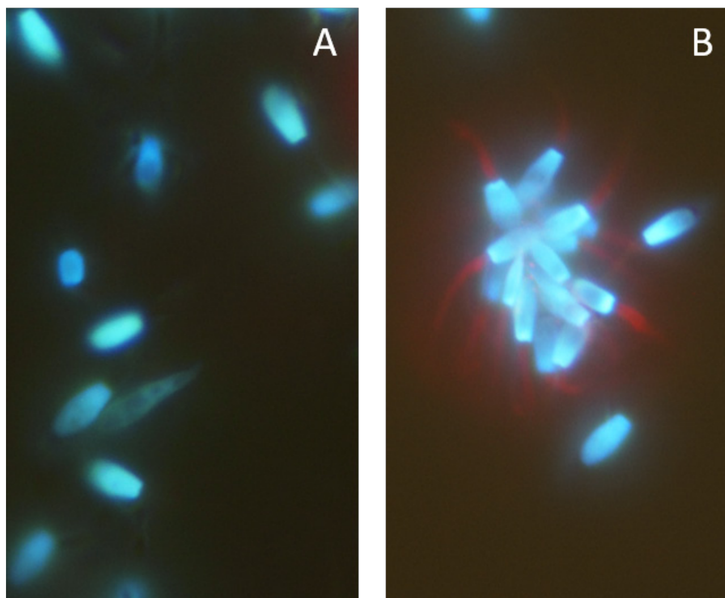

Figure 1. Epifluorescence photomicrography of stallion sperm cells stained with CellROX Deep Red® and Hoescht 33342 fluorescent probes at 1,000X magnification. A: sperm under no oxidative stress (middle peace no stained). B: sperm under detectable oxidative stress (middle peace stained in red).

Statistical analysis

Data were evaluated for normality (Shapiro-Wilk test) and homogeneity of variances. When normality and/or homogeneity was significant ($P < 0.05$), data were transformed and reassessed. An analysis of variance (ANOVA) was conducted to detect differences between treatments. Means were separated by the Tukey test. Percentage of positive cells (with oxidative stress) were submitted to polynomial regression analysis (PROC GLM) of SAS (version 9.3; SAS Institute, Inc., Cary, NC, USA) to determine the model, regression equation and the coefficient of determination value (R^2), when $P \leq 0.05$. Statistical differences were considered at $P \leq 0.05$.

Results

The Tukey test was conducted to prove that there were differences between treatments T0, T50 and T100, demonstrating that oxidative stress induction method was efficient, as shown in Table 1.

Subsequently the data were submitted to polynomial regression. The results of this analysis presented a quadratic effect, high determination coefficient value (R^2 = 0.88) and high significant P value ($P < 0.0001$), as demonstrated in Fig. 2.

Table 1. Mean ± standard error of oxidative stress (%) using CellRox Deep Red in equine sperm with treatments T0 = without oxidative stress induction (n = 16); T50 = 50% of the sample without and 50% of the sample with oxidative stress induction (n = 16); T100 = with oxidative stress induction (n = 16).

Degree of oxidative stress	Treatments			P
	T0	T50	T100	
Oxidative stress (%)	8.15 ± 3.03[c]	58.37 ± 4.33[b]	81.75 ± 3,16[a]	<0.0001
Absent oxidative stress (%)	91.84 ± 3.03[a]	41.62 ± 4.33[b]	17.62 ± 3,28[c]	<0.0001

[a,b,c]Lines with superscript letters differ statistically ($P < 0.05$) each other by the Tukey test.

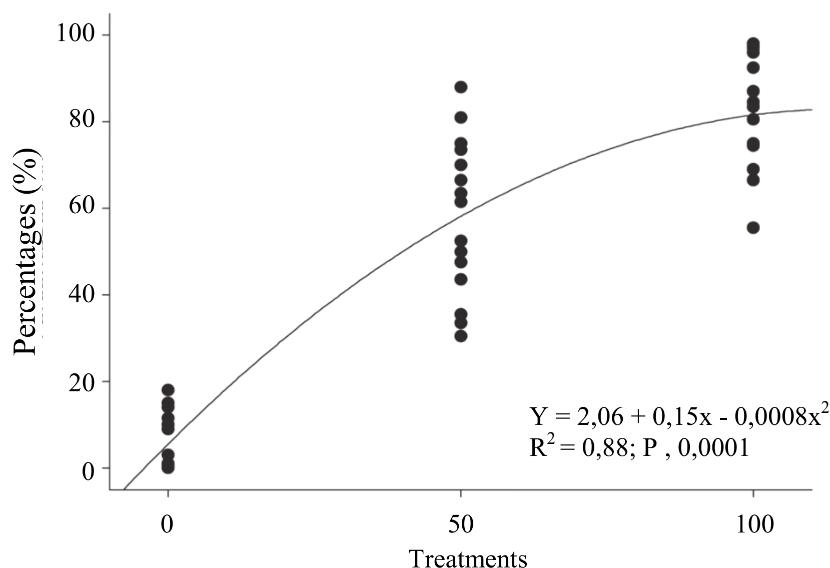

Figure 2. Polynomial regression analysis for the percentage of positive cells (with detectable oxidative stress) detected in stallion sperm with the CellRox Deep Red® fluorescent probe submitted to the experimental treatments (T0 = without oxidative stress induction; T50 = 50% of the sample without and 50% of the sample with oxidative stress induction; T100 = with oxidative stress induction). Data are presented as second-order polynomial regression, with the corresponding model equation, coefficient of determination (R^2) and probability value (P).

Discussion

Two systems are involved in sperm production of ROS. One is the NADPH oxidase at plasma membrane and the other is NADH-dependent oxidoreductase at mitochondrial levels (Aitken and Baker, 2004). In horses, oxidative stress is particularly important because semen cooling and transportation for subsequent artificial insemination are routine practices in equine industry. Previous studies suggests that the decrease in equine sperm functionality after storage at 5°C may be due to oxidative damage caused by an increase in ROS generation during cooling (Ortega-Ferrusola *et al*., 2011).

For the induction of oxidative stress in the present experiment, ascorbic acid, iron sulfate and t-Butylhydroperoxide were utilized (Ball and Vo, 2002). Those substances were chosen due to Fenton reaction, which is responsible for turning the hydrogen peroxide into hydroxyl radical, through iron oxidation. It occurs primarily by the reaction between one molecule of Fe^{2+} and another molecule of H_2O_2, resulting in the

production of OH^{-}, OH and Fe^{3+}. Among the added substances, ascorbic acid has the function of reducing iron and t-Butylhydroperoxide (a hydroperoxide) has the function of catalyzing the reaction. In summary, this technique is based on the formation of iron ions which will catalyze hydroperoxides breakdown, initiating the chain reaction of lipid peroxidation (Aitken *et al*., 1993; Nichi *et al*., 2006).

In general, equine ejaculate is composed by high amount of seminal plasma, mainly when compared to other species. High concentrations of ascorbate, urate and thiol groups are present in mammals' semen. In addition, in lower concentration, glutathione and α-tocopherol are also present (Li, 1975) and several antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase (Kankofer *et al*., 2005). These components promote antioxidant capacity to the seminal plasma. For this reason, the sample that underwent induction of oxidation in this experiment was centrifuged in order to remove the seminal plasma.

Using the procedures mentioned above, the present experiment succeeded in both, induction of oxidative stress and its detection by the tested probe, as demonstrated in Table 1 and Fig. 2.

The linear equation demonstrated a quadratic deviation due to T50 group, which demonstrated better results than expected since more than 50% of cells presented detectable oxidative stress in this group. This result can be explained by the applied methodology, because the T50 sample consisted of 100 ul of fresh diluted semen (newly collected) and 100 ul of the semen sample with induced oxidative stress (after incubation). However, the Cell ROX Deep Red® fluorescent probe needs a high incubation time (30 min) which may have allowed a slight effect of oxidation inductors from the T100 sample during this period. In order to avoid this effect, preliminary experiments were conducted and other incubation periods were tested, however, for a correct interaction of the probe with ROS this minimum incubation time of 30 min was necessary.

The time of the fluorescent probe incubation and induction method, were based on the study of Alves *et al*. (2015) with ram sperm cells. In this paper the technique was described and was successful in detecting oxidative stress *in vivo*, through the induction of testicular degeneration, and *in vitro*.

A direct comparison of the results obtained by CellRox® with other probes as C11-BOBIPY, MitoSOX Red® and H_2DCFDA is not feasible. The C11-BOBIPY evaluates lipid peroxidation (Ball and Vo, 2002) and not ROS directly. MitoSOX Red® probe detect only superoxide anion (Aitken *et al*., 2013) and H_2DCFDA detect specifically hydrogen peroxide (Wang and Joseph, 1999; Aitken *et al*., 2013). Even being more sensitive in the detection of the hydroxyl radical, the CellRox® probe can detect both, the hydroxyl radical and the superoxide anion (Alves *et al*., 2015).

Taking into account the results above mentioned, it was concluded that the CellRox Deep Red® fluorescent probe is able to detect reactive oxygen species in equine sperm, indicating accurately the occurrence of oxidative stress in stallion semen.

Acknowledgments

This study was funded by CAPES. We are grateful to Professor Marcilio Nichi for help in preliminary tests and to Professor Andre Furugen Cesar de Andrade for the use of Laboratory of Andrology and Embryo Technology of Swine.

References

Aitken RJ, Harkiss D, Buckingham D. 1993. Relationship between iron-catalysed lipid peroxidation potencial and human sperm function. *J Reprod Fertil*, 98:257-265.

Aitken RJ, Baker MA. 2004. Oxidative stress and male reproductive biology. *Reprod Fertil Dev*, 16:581-588.

Aitken RJ, De Iuliis GN, Gibb Z, Baker MA. 2012. The simmet lecture: new horizons on an old landscape – oxidative stress, DNA damage and apoptosis in the male germ line. *Reprod Domest Anim*, 47(suppl. 4):7-14.

Aitken RJ, Smith TB, Lord T, Kuczera L, Koppers AJ, Naumovski N, Connaughton H, Baker MA, De Iuliis GN. 2013. On methods for the detection of reactive oxygen species generation by human spermatozoa: analysis of the cellular responses to catechol oestrogen, lipid aldehyde, menadione and arachidonic acid. *Andrology*, 1:192-205.

Alves MBR, De Andrade AFC, Arruda RP, Batissaco L, Florez-Rodriguez SA, Lançoni R, Oliveira BMM, Torres MA, Ravagnani GM, Almeida TG, Vellone VS, Celeghini ECC. 2015. An efficient technique to detect sperm reactive oxygen species: the CellRox Deep Red® fluorescente probe. Biochem Physiol, 4:1-5.

Ball BA, Vo A. 2002. Detection of lipid peroxidation in equine spermatozoa based upon the lipophilic fluorescent dye C11-BODIPY581/591. *J Androl*, 23:259-269.

Bavister D, Lorraine M. 1983. Development of preimplantation in a defined embryos of the Golden culture medium. *Biol Trace Elem Res*, 28:235-247.

Brouwers JF, Silva PFN, Gadella BM. 2005. New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. *Theriogenology*, 63:458-469.

Gibb Z, Lambourne SR, Aitken RJ. 2014. The paradoxical relationship between stallion fertility and oxidative stress. *Biol Reprod*, 91:1-10.

Grinberg YY, Dibbern ME, Levasseur VA, Kraig RP. 2013. Insulin-like growth factor-1 abrogates microglial oxidative stress and TNF-a responses to spreading depression. *J Neurochem*, 126:662-672.

Kankofer M, Kolm G, Aurich J, Aurich C. 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion sêmen during storage at 5°C. *Theriogenology*, 63:1354-1365.

Lenzi A, Picardo M, Gandini L, Dondero F. 1996.

Lipids of the sperm plasma membrane: From polyunsaturated fatty acids considered as markers of sperm function to possible scavenger therapy. *Hum Reprod Update*, 2:246-256.

Li TK. 1975. The glutathione and thiol contente of mammalian spermatozoa and seminal plasma. *Biol Reprod*, 12:641-646.

Nichi M, Bols PEJ, Züge RM, Barnabe VH, Goovaerts IGF, Barnabe RC, Cortada CNM. 2006. Seasonal variation in sêmen quality in *Bos indicus* and *Bos taurus* bulls raised under tropical conditions. *Theriogenology*, 66:822-828.

Ortega-Ferrusola C, Johannisson A, Peña FJV, Tapia JA, Rodriguez-Martinez H, Dalin AM, Morrell JM. 2011. Effect of diferente extenders and seminal plasma on the susceptibility of equine spermatozoa to lipid peroxidation after single-layer centrifugation, through androcoll-E. *J Equine Vet Sci*, 31:411-416.

Raphael CF, Andrade AFC, Nascimento J, Arruda RP. 2008. Effects of centrifugation on membrane integrity and lipid peroxidation of equine cooled spermatozoa. *Anim Reprod Sci*, 107:344-345.

Wang H, Joseph J. 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader11mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee by the United States Department of Agriculture and does not imp. *Free Radic Biol Med*, 27:612-616.