Keywords: Heat stress; Reactive oxygen species; Ram; Male; Infertility; Oxidative stress induction

Introduction

e global environment temperature is growing and it is already established that it prejudices the male reproduction function. High temperature leads increase of reactive oxygen species (ROS) and, consequently, oxidative stress [1]. Sperm cells are highly susceptible to oxidative stress, which results in fewer ROS neutralizing enzymes in their characteristic small cytoplasm. is leads to a diminished cellular protection. In addition, sperm contain large amounts of polyunsaturated fatty acids, proteins and DNA, which are substrates for ROS [2,3]. Besides, sperm mitochondria produces the most ROS [4], which trigger lipid peroxidation of polyunsaturated fatty acids in the plasma membrane [3,5]. Lipid peroxidation, in turn, reduces plasma membrane uidity and, as a result, it prejudices sperm function, culminating in decreased sperm motility [6-8] and increased rates of sperm DNA fragmentation [9], cellular apoptosis [2,8,10,11] and morphologically abnormal sperm [2,6].

e detection of ROS in sperm samples is critical for diagnosing infertility. Chemiluminescence is the most commonly used technique for this purpose, but it does not di erentiate between ROS produced in sperm and ROS produced in leucocytes. Additionally, results with chemiluminescence have been inconsistent [12]. A widely [2,8,10,11] an5narw T[(in5(o)10.a)3(s)-7.9(510(p)6(g in dexid)-2.9(a3(t)-5(in)8(g)]T b)-8.)-5(ra)19(s)5(u)-5(5(pn o)12(h)4(e)0.5(t)-4.9(i(e)0.5r)9(ra

cytometry. In addition, it is used in samples preserved in formaldehyde [20] and in combination with other probes [21]. CellROX can be used to measure cellular ROS in the cytoplasm because it detects hydroxyl better than superoxide [22]. It is oxidized during cellular oxidative stress [23] and shows maximum excitation/emission at 640/655 nm. In the absence of ROS, it remains in its reduced state and emits no uorescence. CellROX is not commonly used in cell biology, and to the best of our knowledge, there are no published studies reporting its use in sperm.

On this way, this study evaluated the e ciency of the use of the CellROX Deep Red Reagent® uorescent probe to detect ROS production in ram sperm. e use of the probe was evaluated in two steps. e rst step was performed in experiment 1 that evaluated the e ciency of CellROX to detect sperm oxidative stress induced in vitro. In the second experiment, it was evaluated the e ciency of CellROX to detect sperm oxidative stress induced in vivo. Both of experiments were done to evaluate the e ciency of CellROX Deep Red Reagent® uorescent probe to detect ROS in ram sperm.

Materials and Methods

Experiment 1: Evaluation of CellROX Deep Red Reagent® uorescent probe e ciency in detect sperm

Oxidative stress induced in vitro: e experiment was conducted with three semen treatments: T0 (semen sample that was not 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 mild or no oxidative stress and sperm under moderate or intense oxidative stress.

Semen collection and preparation: ree White Dorper rams (Ovis aries

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Semen preparation and evaluation of testicular degeneration: For examination of the testicular degeneration induction was performed evaluation of total and progressive motility, sperm defects and sperm DNA fragmentation. e total and progressive motility was assessed by computer analyses system. e so ware was the Sperm Class Analyzer (SCA, Microptics, Spain) and the set up was adjusted by ram sperm. It was chosen ve elds for the analyses and it was used the Makler® chamber to perform the evaluation. e sperm defects were evaluated by di erential interference contrast microscopy (DIC). It was counted 200 cells that were classi ed in major and minor sperm defects. DNA fragmentation was determined by commercial kit Halomax® (Halotech, Spain) and it was determined the fragmentation in 1,000 cells. All evaluation was done by competent techniques.

Semen preparation and evaluation of oxidative stress: Two hundred microliters of semen samples diluted in TALP media [25] (25×10^6 sperm/mL) were added to 0.5 µL of CellROX (1 mM in DMSO) and 2 µL of Hoescht 33342 (2.5 mg/mL; in dPBS), and incubated for 30 minutes at 37ºC. A er incubation, the solution was centrifuged for 5 minutes at 2,000 g, the supernatant was removed, and the pellet resuspended in 200 µL of TALP. An aliquot of 5 µL was placed between a6(, a)9(n)4(d t)-6(h)4(e p)-8. BT/T1 9 0 0eended in 200 µLD 381((es)0.6(an 200 fs)5(

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