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Comparison of the TBARS Assay and BODIPY C_{11} Probes for Assessing Lipid Peroxidation in Red Deer Spermatozoa

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Contents

Several methods are used to measure lipid peroxidation (LPO) in spermatozoa. The objective of this study was comparing the thiobarbituric acid reactive species (TBARS) method and the BODIPY 581/591 C_{11} (B581) and BODIPY 665/676 C_{11} (B665) fluorescent probes to measure induced peroxidative damage in thawed epididymal spermatozoa from Iberian red deer. Samples from three males were thawed, pooled, diluted in PBS, incubated at room temperature and assessed at 0, 3, 6 and 24 h under different experimental conditions: Control, hydrogen peroxide (H_2O_2) 0.1 mM or 1 mM, or *tert*-butyl hydroperoxide (TBH) 0.1 mM or 1 mM. LPO was assessed by the TBARS assay [malondialdehyde (MDA) detection] and by the fluorescence probes B581 and B665 (microplate fluorimeter and flow cytometry). Increasing MDA levels were only detectable at 1 mM of TBH or H_2O_2. Both fluorescence probes, measured with fluorometer, detected significant increases of LPO with time in all treatments, except Control. Flow cytometry allowed for higher sensitivity, with both probes showing a significant linear relationship of increasing LPO with time for all oxidizing treatments ($p < 0.001$). All methods showed a good agreement, except TBARS, and flow cytometry showed the highest repeatability. Our results show that both B581 and B665 might be used for LPO analysis in Iberian red deer epididymal spermatozoa, together with fluorometry or flow cytometry. Yet, the TBARS method offered comparatively limited sensitivity, and further research must determine the source of that limitation.

Introduction

The processes of cooling, freezing, and thawing produce biophysical and biochemical changes in the membrane of spermatozoa that might be detrimental for their viability and fertilizing ability (Chatterjee and Gagnon 2001). Part of these deleterious effects is due to the damage caused by reactive oxygen species (ROS), which lead to the event called 'oxidative stress'. Oxidative stress causes structural damage to biomolecules, DNA, lipids, carbohydrates and proteins (Niki 1991; Baumber et al. 2003; Agarwal et al. 2004), as well as other cellular components. The high polyunsaturated fatty acid content of sperm membrane (Lenzi et al. 1996) and the depletion of the antioxidant system after cryopreservation increase the susceptibility of spermatozoa to peroxidative damage (Bilodeau et al. 2000; Moore et al. 2005). In view of the importance of lipid peroxidation in defective sperm function, quantification of this process is of great interest.

To assess *in vitro* oxidant activity, several assays have been developed. The most widely used method for monitoring lipid peroxidation in mammalian spermatozoa is the thiobarbituric acid reactive species (TBARS) method, and involves the use of the thiobarbituric acid to measure the concentration of malondialdehyde (MDA), an end-point reaction product of lipid peroxidation (Aitken et al. 1993a; Baumber et al. 2000). In this process, two molecules of thiobarbituric acid react with one molecule of MDA, at low pH and elevated temperatures, resulting in a pink chromogen that can be quantified with a spectrophotometer (Janero 1990; Nichi et al. 2007). This method is cheap and practical. Yet, it only provides an indirect measure of lipid peroxidation, without subcellular resolution of the membrane changes (Pap et al. 1999).

As an alternative to the detection of LPO products such as malondialdehyde, several lipophilic fluorescent probes have been used to assess lipid peroxidation in mammalian spermatozoa. The fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591 C_{11}) is a fluorescent fatty acid analogue with fluorescent properties in the red and green ranges of the visible spectrum (emission maximum at 595 nm). Upon free radical-induced oxidation, its fluorescence shift from red to green. This characteristic is highly advantageous, since detection of LPO becomes feasible, either using fluorescence microscopy (Pap et al. 1999), confocal laser scanning microscopy (Neild et al. 2005), fluorometry (Drummen et al. 2002) or flow cytometry (Silva et al. 2007). Up to now, it has been used successfully in different experiments for assessing LPO in stallion (Ball and Vo 2002; Almeida and Ball 2005; Neild et al. 2005; Ortega-Ferrusola et al. 2009), bovine sperm (Brouwers et al. 2005; Silva et al. 2007), ram (Christova et al. 2004; Lymberopoulos et al. 2008), boar (Brouwers et al. 2005; Guthrie and Welch 2007) and human spermatozoa (Aitken et al. 2007; Koppers et al. 2008).

Another member of this class of dyes is the 4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene (BODIPY 665/676 C_{11}), with fluorescent properties in the red range of the visible spectrum. It has been used to assess antioxidant activity in liposomes by fluorometry, because its conjugated polyene system makes it easily susceptible to oxidation by peroxyl radicals Naguib (2000). Up to date, it seems to have been employed to assess lipoperoxidation in spermatozoa only once (Martinez-Pastor et al. 2009a), although it might have practical value due to its fluorescent properties, allowing a higher flexibility when combining LPO detection probes with other fluorochromes. Yet, a

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validation of this methodology seems to be warranted in order to exclude fluorescence-associated artefacts and misinterpretation of results Drummen et al. (2002).

We have previously adapted different physiological probes in red deer (Fernandez-Santos et al. 2007, 2009; Martinez-Pastor et al. 2008, 2009a), due to the growing importance of this species in the animal production area, and to the subsequent interest in applying artificial reproductive techniques to this species Garde et al. (2006). Thus, the aim of the present work was to assess the sensitivity and reliability of the aforementioned techniques for measuring lipid peroxidation in red deer spermatozoa. Therefore, we have assayed the TBARS method and the BODIPY 581/591 C_{11} and BODIPY 665/676 C_{11} fluorescent probes for detecting LPO in red deer sperm samples incubated with peroxidative agents. Furthermore, we have measured BODIPY C_{11} fluorescence both by fluorometry and by flow cytometry, and fluorescence was confirmed by confocal laser scanning microscopy. The objective of the study was to evaluate not only the relative sensitivity of each method, but also the repeatability, correlation and agreement among them.

Materials and Methods

Chemicals and harvest regulation

Chemicals used in this study were at least of Reagent grade and obtained from Sigma (Madrid, Spain), except where otherwise stated.

Hunting activities were performed in accordance with the harvest plans of the game reserves, which followed Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, conforming to European Union Regulation. The ethical committee of the University of Castilla-La Mancha approved the procedures involving animals reported in this study.

Stags and testes collection

For this study, we used spermatozoa recovered from epididymis of three mature stags (age > 4.5 years, weight > 130 kg) that were legally culled and hunted in their natural habitat during the rutting season (September–October). Gamekeepers collected the complete male genitalia and provided the hour of the dead. Immediately upon removal, the testes with attached epididymes were placed into plastic bags and transported to the laboratory at room temperature (approximately 22°C) within 2 h after being removed.

Spermatozoa were collected by performing several incisions with a surgical blade always in the middle of each epididymis part, and collecting the oozing sperm mass emerging from the cut tubules and placing it in 1 ml of Tris-citrate-fructose medium supplemented with 20% egg yolk (TCFY). To diminish blood contamination, superficial blood vessels were cut previously, wiping their content and drying the epididymis surface thoroughly. Then, the sperm mass was again diluted at room temperature to a final concentration of 400×10^6 spermatozoa/ml with TCFY, and to 200×10^6 spermatozoa/ml with TCFY supplemented with 6% glycerol (final: 3% glycerol). The diluted samples were refrigerated approximately for 10 min to reach 5°C and then were held for equilibration at that temperature for 2 h.

After the equilibration of the diluted sperm samples, the extended sperm was loaded into 0.25 ml plastic straws (IMV, L'Aigle Cedex, France). They were frozen for 10 min in nitrogen vapors, 4 cm above the level of liquid nitrogen (−120°C). The straws were immediately immersed into liquid nitrogen for storage (at least one year).

Thawing and sample processing

Thawing was carried out by dropping straws from three different males into a water bath with saline solution at 37°C. After 30 s, straws were thoroughly wiped and its content was poured in a tube, forming a pool. Semen was diluted in PBS (10^7 spermatozoa/ml), from which we saved part as a control and divided the rest among four oxidant treatments. To the oxidant tubes, we applied 1 mM or 0.1 mM of the lipid peroxide analogue *tert*-butyl hydroperoxide (TBH), or 1 mM or 0.1 mM of hydrogen peroxide (H_2O_2). We did not added Fe^{2+}, which would have accelerated the effect, because we were more interested in studying a sustained effect for a long time than a faster effect (Brouwers and Gadella 2003; Neild et al. 2005).

For fluorometric evaluation of lipid peroxidation (fluorometer and flow cytometry), the samples were split previously to the oxidizing treatment, and loaded with either BODIPY 581/591 C_{11} or BODIPY 665/676 C_{11}, at a final concentration of 2 μM (from stocks at 0.2 mM in DMSO). After 30 min, spermatozoa were washed clear of dye by centrifuging with PBS (10 min at $300 \times g$) and resuspended in PBS (Brouwers and Gadella 2003). BODIPY C_{11} dyes incorporate in the cytoplasmic membrane, responding to eventual lipid peroxidation by changing their fluorescence properties.

All tubes were incubated 24 h at room temperature (22°C), assessing lipid peroxidation at 3, 6 and 24 h. Baseline data were collected from controls at 0 h.

TBARS assay for quantification of lipid peroxidation

The susceptibility of the spermatozoa to peroxidation was estimated by the thiobarbituric acid reactive substance (TBARS) method according to Ohkawa et al. (1979). Samples of 100 μL were thoroughly mixed 2 with 200 μL of a stock solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.25 N HCl. This mixture was heated at 90°C for 15 min, and then the reaction was stopped by placing the tubes in ice-cold water for 5 min. The tubes were centrifuged at $1500 \times g$ for 15 min to pellet the precipitate, and the clear supernatant was collected and transferred to wells (200 μL/well) in a 96-well flat bottom transparent plate (Nunc, Roskilde, Denmark). The plate was completed with a calibration curve prepared from a malondialdehyde (MDA) stock (1,1,3,3-tetramethoxypropane). Sample absorbance at 532 nm was read on a microplate reader (Synergy HT; BIO-TEK, Winooski, VT, USA). MDA concentration was calculated from a standard curve. The lipid peroxidation index was calculated as nmol of MDA

per 10^8 spermatozoa. This assay was duplicated for each sample.

Fluorometric assay

Samples loaded with BODIPY 581/591 C_{11}, BODIPY 665/676 C_{11} (Invitrogen, Carlsbad, CA, USA) were transferred to wells (200 μL/well) in a 96-well flat bottom black opaque plate (Nunc, Roskilde, Denmark). Plates were read using a read on a multipurpose microplate reader (Synergy HT). BODIPY 581/591 C_{11} presents both red (no peroxidation) and green (peroxidation) fluorescence. Thus, these wells were read at 530/590 nm (excitation/emission) for red fluorescence and 485/528 nm for green fluorescence. BODIPY 665/676 C_{11} wells were read at 590/645 nm (red fluorescence). This assay was performed in duplicate for each sample.

Flow cytometry

Samples loaded with BODIPY C_{11} were added to PBS (final concentration 10^6 cells/ml) and analysed in a LSR-I flow cytometer (BD Biosciences, San José, CA, USA). BODIPY 581/591 C_{11} was excited using a 488 nm Ar-Ion laser, detecting their fluorescence with the FL1 photodetector (530/28BP filter). BODIPY 665/676 C_{11} was excited using a 633 nm He-Ne laser, and it was read with the FL6 photodetector (670/40BP filter). Event acquisition was controlled using the Cell Quest Pro 3.1 software (BD Biosciences, San Jose, CA, USA).

Confocal laser scanning microscopy

BODIPY C_{11} fluorescence was confirmed and located using confocal microscopy. Samples were mounted with conventional slides and 22 × 22 coverslips, adding 5 μl of sample and 10 μl of antifade solution (0.22 M of 1,4-diazabiciclo[2,2,2]octane in 10% PBS and 90% glycerol). Images were taken with a Leica TCSP2 confocal laser scanning system on an inverted microscope DMIRE2 (Leica Microsystems, GmbH, Heidelberg, Germany) with an Argon-Krypton laser as excitation source. The green and red fluorescence of BODIPY 581/591 C_{11} were acquired simultaneously using double wavelength excitation (laserlines 488 and 568 nm) and detection (emission bandpass filters 530/30 and 590/30), while red fluorescence of BODIPY 665/676 C_{11} was excited using the laserline at 645 nm and detected using the emission bandpass filter 676/30.

Statistical analysis

The experiment was replicated four times. Statistical analyses were performed using the R statistical environment (R Development Core Team. R 2007). The variation of lipid peroxidation, as given by each method, was analysed using linear mixed-effects models. p values less than 0.05 were considered as significant. Correlations (Pearson product–moment correlation coefficient) were used to estimate the relationship among methods. The repeatability of each method for assessing lipoperoxidation and the agreement between methods (TBARS, BODIPY 581/591 C_{11}, BODIPY 665/676 C_{11}) were determined by calculating repeatability and agreement coefficients following Bland and Altman (1986). Since methods differed on scale, means were averaged to 10, to allow the comparison of repeatability coefficients between methods, and to allow the calculation of the agreement coefficients. Whenever given, results are showed as mean ± SEM, unless otherwise stated.

Results

Lipid peroxidation assessment

Table 1 shows the average values of lipid peroxidation resulting from this study. The TBARS method exhibited low sensitivity to increasing levels of lipoperoxidation in

Table 1. Lipid peroxidation levels (mean ± SEM) measured by five different techniques: thiobarbituric acid reactive substances method (TBARS), BODIPY 581/591 C11 [B581 (fmt)] and BODIPY 665/676 C11 [B665 (fmt)] measured by fluorimetry, and BODIPY 581/591 C11 [B581 (fcm)] and BODIPY 665/676 C11 [B665 (fcm)] measured by flow cytometry. TBARS is expressed as nmol of malondialdehyde per 108 spermatozoa. BODIPY C11 results are expressed as fluorescence intensity (arbitrary units)

Time (h)	Treatment	TBARS	B581 (fmt)	B665 (fmt)	B581 (fcm)	B665 (fcm)
0	Baseline	1.0 ± 1.8	17 ± 5	17 ± 4	181 ± 1	110 ± 10
3	Control	0.4 ± 0.7	22 ± 6	18 ± 5	156 ± 5	86 ± 16
	H2O2 1 mM	5.6 ± 2.7***	36 ± 9*	36 ± 4	237 ± 13***	129 ± 20*
	H2O2 0.1 mM	2.1 ± 1.6	34 ± 9*	35 ± 3	228 ± 22**	127 ± 21*
	TBH 1 mM	7.7 ± 3.8***	47 ± 16***	49 ± 9**	243 ± 24***	130 ± 22*
	TBH 0.1 mM	0.7 ± 1.0	31 ± 7	40 ± 7*	211 ± 16*	133 ± 31*
6	Control	0.2 ± 0.4	18 ± 4	14 ± 4	190 ± 6	153 ± 10**
	H2O2 1 mM	4.5 ± 2.4**	38 ± 8*	39 ± 4*	269 ± 8***	186 ± 11***
	H2O2 0.1 mM	2.2 ± 1.5	37 ± 9*	39 ± 5*	262 ± 21***	190 ± 8***
	TBH 1 mM	7.2 ± 3.2***	53 ± 15***	64 ± 13***	287 ± 26***	211 ± 11***
	TBH 0.1 mM	0.2 ± 0.3	35 ± 6*	46 ± 9**	244 ± 8***	197 ± 13***
24	Control	1.8 ± 2.9	31 ± 10	23 ± 10	212 ± 8*	201 ± 38***
	H2O2 1 mM	6.1 ± 2.6***	44 ± 15**	49 ± 3**	279 ± 13***	246 ± 13***
	H2O2 0.1 mM	2.9 ± 3.1	40 ± 14*	51 ± 8**	283 ± 26***	248 ± 17***
	TBH 1 mM	11.9 ± 4.1***	92 ± 51***	116 ± 41***	332 ± 24***	272 ± 14***
	TBH 0.1 mM	1.7 ± 3.1	48 ± 11***	80 ± 13***	286 ± 6***	271 ± 11***

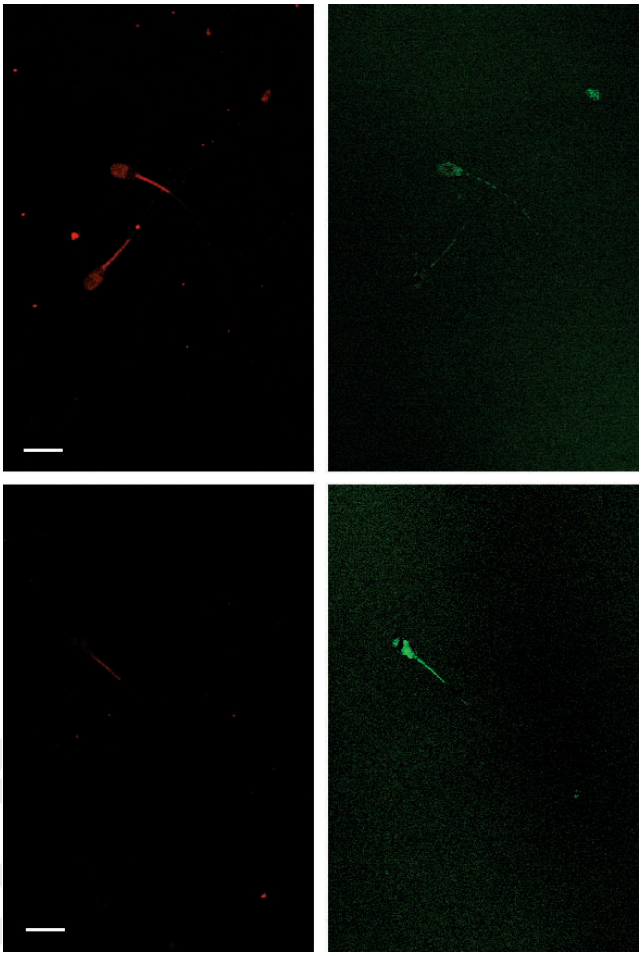
Asterisks show significant differences of each treatment with baseline levels: * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Control and H_2O_2 0.1 mM treatments, resulting in many of the measurements falling below the detection limit. The analysis of the statistical model showed that only the two 1 mM treatments increased MDA from the baseline levels (1.0 ± 1.8 nmol MDA per 10^8 spermatozoa) with time ($p < 0.001$), although TBH was more efficient producing MDA (slope for the lineal model: 0.46 ± 0.04 for TBH 1 mM vs 0.21 ± 0.04 for H_2O_2 1 mM). MDA levels did not increase significantly for 0.1 mM concentrations, even after 24 h.

Fluorometry assessment of both B581 and B665 allowed detecting increasing lipid peroxidation in all treatments ($p < 0.05$), except Control. The relative efficiency of the treatments for modifying BODIPY fluorescence (indicating increasing peroxidation) was in the order TBH 1 mM > TBH 0.1 ~~mm~~ > H_2O_2 1 mM ~ H_2O_2 0.1 mM. When compared to baseline levels, B581 and B665 behaved similarly, but they showed several differences at 3 h (Table 1). At that time, B581, but not B665, showed significant differences for both H_2O_2 treatments. Nonetheless, the effect size of B665 was very similar to that of B581, and it was very close to significance ($p \sim 0.05$).

When BODIPY C_{11} probes were measured by flow cytometry, both probes showed a positive linear relationship between lipoperoxidation and time for all oxidizing treatments ($p < 0.001$). Futhermore, B665 showed that the lipoperoxidation in the Control samples increased with time too ($p < 0.001$). Although B581 did not show such a significant relationship for Control, the median fluorescence of Control at 24 h was significantly higher than baseline levels (but not at other times). Treatment performance regarding to the relative efficiency of the oxidizing treatments were similar to those found in the fluorimetry study (TBH 1 mM > TBH 0.1 mM > H_2O_2 1 mM ~ H_2O_2 0.1 mM), although B665 showed a more pronounced change than B581 in all cases (slope values for B581 vs. B665 were: 5.88 ± 0.61 vs. 6.44 ± 0.48 for TBH 1 mM, 3.61 ± 0.57 vs. 6.35 ± 0.48 for TBH 0.1 mM, 3.43 ± 0.54 vs. 5.33 ± 0.51 for H_2O_2 1 mM, and 3.50 ± 0.57 vs. 5.38 ± 0.48 for H_2O_2 1 mM).

Confocal imaging (Fig. 1) confirmed the shift of fluorescence on BODIPY C_{11} labelled samples. The labelling of Control samples at early times was characteristic of lack of lipid peroxidation, with spermatozoa incubated with BODIPY 581/591 C_{11} showing a moderate or intense red fluorescence (specially over the acrosomal cap and midpiece), while green fluorescence was faint (either patchy or uniform). At longer times, samples submitted to oxidative stress showed many spermatozoa with decreased red fluorescence (although still noticeable on the midpiece), and increasing green fluorescence (mainly on the head and midpiece). Incubating with BODIPY 665/676 C_{11} showed a similar pattern than early BODIPY 581/591 C_{11} red labelling. Yet, in this case, we could not notice clear changes on the labelling pattern among treatments by using confocal microscopy.



LOWRESOLUTION COLOUR FIG

Fig. 1. Confocal fluorescent microscopic images of sperm cells labelled with BODIPY 581/591 C_{11}. Each pair of images shows the same spermatozoa, using a different excitation/emission configuration. The right side of each pair was acquired with the red fluorescence combination (ex. 568 nm, em. 590/30 nm), showing the red fluorescence of BODIPY 581/591 C_{11} (lack of lipoperoxidation), while the right side of each pair was acquired with the green fluorescence combination (ex. 488 nm, em. 530/30 nm), showing the green fluorescence of peroxidized BODIPY 581/591 C_{11}). Spermatozoa not submitted to oxidative stress (top) showed bright red labeling, especially on the acrosomal cap and midpiece, and the green labeling was generally faint. After incubating with the oxidants (here, TBH 1 mM, 6 h), red fluorescence decreased considerably, while the green labelling increased (bottom). The bar represents a distance of 10 μm

Repeatability of techniques for assessing LPO

As indicated in the Statistics subsection, different techniques yielded results in different scales and units (TBARS: nmol MDA per 10^8 sperm; fluorimetry: arbitrary fluorescence units; cytometry: median of fluorescence intensity), thus, results were normalized to a mean of 10 before attempting the evaluation of repeatability or agreement. Repeatability coefficients (2SD) were lower for the samples stained with BODIPY dyes. Fluorimetry measurements rendered a 2SD of 2.65 for B581 green fluorescence, and of 2.12 for B665. When flow cytometry was used, 2SD were lower (1.45 for B581 and 1.98 for B665). TBARS measurements had a much higher 2SD of 9.33 (lower repeatability). Several repeatability plots are shown in Fig. 2.

Correlation and agreement between methods

Table 2 shows that fluorescence methods correlated significantly, although B665 had a weaker linear relation

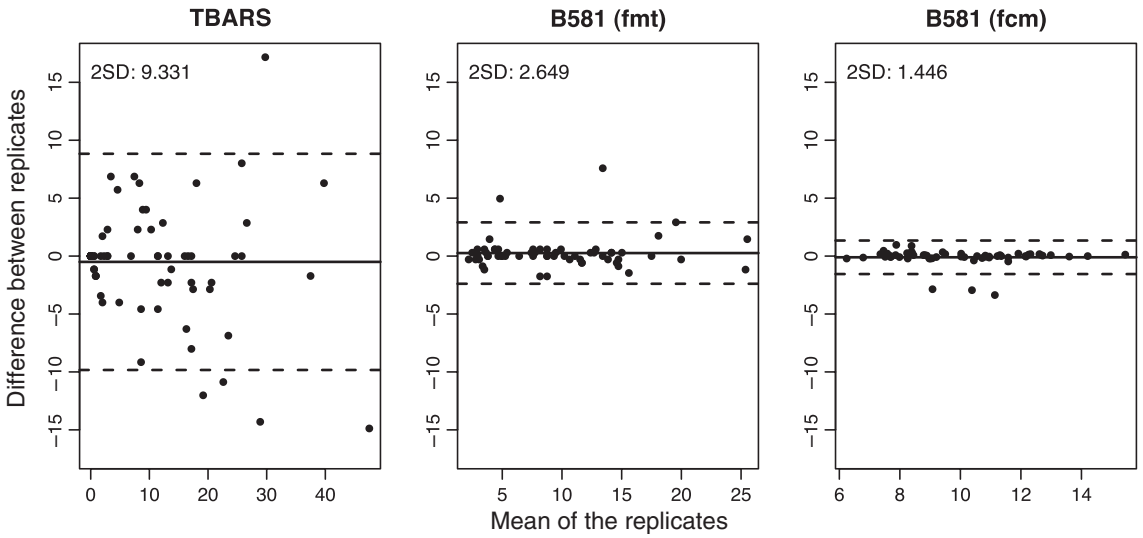


Fig. 2. Repeatability of the TBARS and BODIPY C_{11} techniques (fmt: fluorimeter; fcm: flow cytometer); BODIPY 665/676 C_{11} (B665) plots were similar to BODIPY 581/591 C_{11} (B581) plots. The differences between replicates were plotted against the corresponding means. The repeatability coefficient (twice the standard deviation of the differences, 2SD) is showed for each plot. The solid line shows the mean difference (nearly 0 in all cases), and the dotted lines mark the limits of repeatability (mean ± 2SD)

Table 2. Correlation matrix of the methods for detecting lipid peroxidation. Pearson moment–roduct correlation coefficients are showed

Time (h)	TBARS	B581 (fmt)	B665 (fmt)	B581 (fmc)	B665 (fcm)
TBARS	1				
B581 (fmt)	0.12	1			
B665 (fmt)	0.16	0.78***	1		
B581 (fcm)	0.20	0.68***	0.76***	1	
B665 (fcm)	0.02	0.30*	0.57***	0.77***	1

*p < 0.05, ** p < 0.01, *** p < 0.001.

Table 3. Agreement coefficients among the methods for detecting lipid peroxidation. A higher coefficient (2SD approximately the mean of the differences) indicates a lower agreement. Numbers within parentheses are correlation coefficients (all significant at p < 0.05 except B665-fmt vs. B581-fmt) between the difference and the mean for each combination (see Fig. 3)

Time (h)	TBARS	B581 (fmt)	B665 (fmt)	B581 (fmc)
B581 (fmt)	27.4 (0.47)			
B665 (fmt)	26.6 (0.51)	9.8 (0.08)		
B581 (fcm)	24.5 (0.94)	13.1 (0.93)	12.0 (0.93)	
B665 (fcm)	25.7 (0.84)	14.9 (0.72)	12.2 (0.74)	4.7 (−0.65)

with the other methods ($r < 0.6$). Interestingly, TBARS results did not correlate with any of the fluorescence-based methods.

Agreement coefficients (Table 3) indicated that the highest agreement (lower agreement coefficient) was found between flow cytometry measurements, whereas TBARS showed a relatively low agreement with the other methods. This confirmed that TBARS did not render the same information than BODIPY C_{11} probes. Furthermore, we detected significant correlations between the differences and the means in all comparison pairs, except for the agreement between B581 and B665 when measured by fluorometry (Fig. 3). These correlations indicate that the differences between methods varied depending on the size of the measurements (one of the methods gave higher results than the other when values were low, but the situation inverted when values were high).

Discussion

The purpose of this study was to assess the efficiency of several methods for measuring the level of oxidative stress in red deer spermatozoa. We used cryopreserved spermatozoa for convenience, assuming that the cryopreservation process causes some degree of lipoperoxidation, but that it should not interfere with the purpose of our study (comparing analytical techniques). The results obtained with fluorimeter (fluorescence microplate assay) and the fluorescence BODIPY C_{11} probes showed an increase in lipid peroxidation in all treatments with time (excepting control). Similar techniques have been successfully used for measuring LPO in stallion, combining BODIPY 581/591 C_{11} with a microplate fluorescence reader (Ball and Vo 2002; Almeida and Ball 2005). Although not allowing reaching the fine discrimination of a flow cytometer, fluorometry using a microplate reader can be an affordable and quick method for many kind of analyses. In fact, we have reported here similar results using a fluorimeter and flow cytometry, including acceptable repeatability and agreement between methods. Nevertheless, the most precise and informative results were rendered after flow cytometry analysis. This was expected, since flow cytometry allows for the individual analysis of thousand of spermatozoa in a very short time. Furthermore, the results obtained with the fluorescence probe BODIPY 665/676 C_{11} were similar to those obtained with BODIPY 581/591 C_{11}, only with minor differences, which should be explored in future studies.

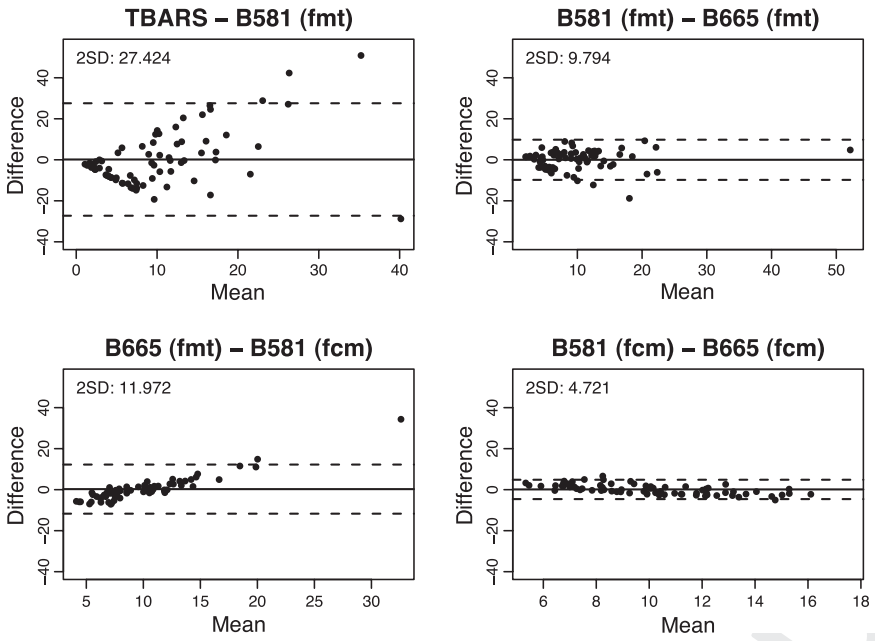


Fig. 3. Examples of the between-method agreement analysis (fmt: fluorimeter; fcm: flow cytometer). Between-method differences were plotted against the corresponding means. The agreement coefficient (twice the standard deviation of the differences, 2SD) is showed for each plot. The solid line shows the mean difference (nearly 0 in all cases), and the dotted lines mark the limits of agreement (mean ± 2SD). The correlations of the differences with the means (Table 3 can be readily appreciated in the plots [correlations were significant, except for the B581 (fmt)–665 (fmt) analysis]

BODIPY 665/676 C_{11} has been previously used for assessing LPO in several systems, such as liposomes (Naguib 2000) or cat and cattle skin (Meyer et al. 2009), but, to our knowledge, it has only been used in spermatozoa only once, by own our group (Martinez-Pastor et al 2009a). The main advantage of validating this new probe, which showed a good agreement coefficient with BODIPY 581/591 C_{11}, is that one can count on another probe working in a different fluorescence range. This can be especially useful in dual-laser flow cytometry, allowing combining BODIPY C_{11} with other probes working in the green to orange-red emission ranges.

Contrarily, the TBARS results have showed little agreement with the BODIPY C_{11} probes. Our results showed differences among the TBARS method (based in measuring lipid peroxidation end-products, namely MDA) and the reporter probes BODIPY 581/591 C_{11} and BODIPY 665/676 C_{11}. Nevertheless, it must be highlighted that this comparison have to be considered in context, and taking into account that both kind of techniques differ on its mechanism (MDA production vs. probe oxidation) and measured units (nmol MDA vs. fluorescence units). Therefore, the correlations showed among TBARS and fluorescence measurements are little representative, and, due to the aforementioned reasons, may be perfectly normal. MDA production during oxidative stress depends strongly on membrane composition, and other authors have showed a high

Yet, only the two 1 ~~mm~~ treatments, either with TBH or H_2O_2, increased the detection of MDA levels with time, whereas BODIPY C_{11} probes were capable to detect LPO increase in the lower oxidant concentrations. Similar results regarding MDA detection were reported by Peris et al. (2007), using fresh and thawed ram semen. These authors could not detect increasing MDA levels with H_2O_2 concentrations up to 300 μM, even after incubating the samples 24 h with the oxidizing treatments.

These observations for red deer and for ram spermatozoa differ from other studies using human (Potts et al. 2000; Williams and Ford 2004), equine (Fatma et al. 2009; Ortega-Ferrusola et al. 2009) and buffalo (Garg et al. 2008) spermatozoa, in which H_2O_2 concentrations, ranging from 10 to 100 μM, could increase MDA levels. Furthermore, other authors (Alvarez and Storey 1984, 1985); reported spontaneous MDA production in mouse and rabbit epididymal spermatozoa, even though these studies did not include LPO inducers. The same authors (Alvarez and Storey 1982, 1985) reported a high correlation between MDA production and loss of motility, which has been confirmed in other studies inducing LPO in human spermatozoa (Aitken et al. 1993b; Gomez and Aitken 1996; Twigg et al. 1998). Although we did not analysed sperm motility in the present study, we previously showed that loss of sperm motility in thawed red deer samples seemed not to be connected with LPO (using BODIPY 665/676 C_{11}), which was also relatively low (Martinez-Pastor et al. 2009a). Considering these previous studies, one likely explanation is that the spermatozoa from small ruminants (deer and ram) could be especially resilient to LPO, at least expressed as MDA production. Indeed, Alvarez and Storey (Alvarez and Storey 1989) found that millimolar concentrations of H_2O_2 are required to induce lipid peroxidation (LPO) in human and mouse sperm, whereas these concentrations were insufficient to induce LPO in rabbit spermatozoa, highlighting possible differences, not only between species (Holt 2000), but perhaps among methods. In fact, Jones and Mann (1997) were able to detect a considerable increase of MDA after incubating ram spermatozoa for only 1 h, but these authors used Fe^{2+} as peroxidation promoter, which is reputedly considered a very good inductor of MDA generation. Nevertheless, the Fe^{2+}/ascorbate system in red deer spermatozoa neither induced a dramatic LPO increase when we tested it on red deer spermatozoa (Martinez-Pastor et al. 2009a).

It is interesting to consider the findings of Brouwers and Gadella (2003) in bull sperm, who detected the presence of hydroxilated lipids resulting from the enzymatic reduction of peroxidated polyunsaturated phospholipids. Since hydroxylated lipids do not result in TBARS production, this technique would be of lesser utility in those species with a tendency for yielding a significant amount of hydroxylated lipids upon sperm LPO. If this is the ultimate cause of our results regarding TBARS is still to be confirmed. Indeed, other factors could be interfering with the analysis, such as unwanted precipitates, sample turbidity, MDA loss, coloured by-products. Nevertheless, care was taken to avoid these unwanted factors (use of blanks and internal controls, replicates, variable wavelength scanning of random samples), therefore we consider their influence being marginal.

BODIPY 581/591 C_{11} fluorescence was detected and visualized by confocal microscopy in the midpiece (mitochondria) and in the head. Similar results were obtained in human sperm incubated with 100 μm of H_2O_2 at 37°C for 5 min (Kao et al. 2008) and stallion sperm incubated with 0.1 μM of THB at 38°C for 17 h (Neild et al. 2005), or not submitted to oxidative stress (Ortega-Ferrusola et al. 2009). Interestingly, Brouwers and Gadella (2003) reported that the distribution of the green fluorescence of BODIPY 581/591 C_{11} over bovine spermatozoa incubated for 12 h at 38°C with 25 μM of TBH for 12 h occurred mainly in the midpiece and tail, being the head apparently free of lipid peroxidation (only red fluorescence). Furthermore, these authors found that lipid peroxidation hardly occurred in fresh bovine sperm cells, but that frozen/thawed sperm cells underwent extensive peroxidation when they were separated from the egg yolk extender they were frozen in; yet, BODIPY 581/591 C_{11} green fluorescence seemed to be restricted to the midpiece too. This is a very interesting difference with our observations, since cattle are more closely related to red deer than the human or equine species. Brouwers and Gadella (2003) suggested the presence of a potent antioxidant system in the head of the bull spermatozoa, which might be absent from red deer spermatozoa. Brouwers and Gadella (2003) used ejaculated spermatozoa, which had contacted with seminal plasma, whereas we used epididymal spermatozoa. Thus, that could be the cause of the differences found between our observations, being a hypothesis to test through the comparison of ejaculated and epididymal red deer spermatozoa while seeking for membrane differences (specially the regarding antioxidant enzymes associated to the plasma membrane). Indeed, we have previously reported several differences between these two sources of spermatozoa in red deer (García-Macías et al. 2006; Martinez-Pastor et al. 2006; Martinez-Pastor et al. 2009b).

Concerning the BODIPY 665/676 C_{11} probe, the observation with confocal microscopy allowed us to find several similarities of the staining pattern with that observed for BODIPY 581/591 C_{11}. Yet, visual changes between treatments seemed to be more subtle when using BODIPY 665/676 C_{11}, perhaps because the fluorescence shift did not involve a change in cell colour, just as it happens when visualizing BODIPY 581/591 C_{11} stained samples. Whereas such changes would be detectable by resorting to image analysis, it was not an objective of this part of the study (which had only the purpose of locating the fluorescence on the sperm surface).

Therefore, in the present study, LPO results obtained either using BODIPY 581/591 C_{11} or BODIPY 665/676 C_{11} agreed well after testing with different oxidizing treatments and incubation times. Results were consistent and similar to those obtained in equine (Neild et al. 2005; Ball and Vo 2002), bovine (Brouwers and Gadella 2003) or boar (Brouwers et al. 2005) species. Measurements performed by fluorimetry with either probe agreed with those performed using flow cytometry, and repeatability was reasonably high with either method (although it was higher when using flow cytometry). The fluorimeter approach is well suited for the evaluation of the effects of multiple promoters in dose-response studies as presented here, and might be a low cost and practical method.

This study has shown that the use of the BODIPY C_{11} probes might be a more sensitive analysis than MDA assessment (at least, by the TBARS method) for measuring lipid peroxidation in red deer spermatozoa. Detection of lipid peroxidation with BODIPY C_{11} labelling thereby differs fundamentally from TBARS, and the reasons for the lower TBARS performance should be reviewed in small ruminants. Furthermore, BODIPY C_{11} allows for a per-cell analysis of lipid peroxidation, either by using confocal fluorescent microscopy or flow cytometry. Furthermore, one of the many advantages of using fluorescent probes is that they could be combined with other fluorochromes to detect different features in spermatozoa, therefore distinguishing different subpopulations (for instance, allowing to differentiate LPO in live or dead spermatozoa (Brouwers and Gadella 2003)). This is precisely one of the outcomes of this study, showing that the BODIPY 665/676 C_{11} probe works in a similar manner than the validated BODIPY 581/591 C_{11}, therefore contributing with a LPO marker with different fluorescent characteristics.

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Author contributions

Felipe Martínez-Pastor, Julián Garde and M. Rocío Fernández-Santos designed the experiments. Julián Garde, Álvaro Domínguez-Rebolledo, M. Rocío Fernández-Santos and Felipe Martínez-Pastor provided the samples. Álvaro Domínguez-Rebolledo, M. Rocío Fernández-Santos, Alfonso Bisbal and J. Luis Ros-Santaella prepared the reagents and carried out the experiment. Enrique del Olmo and J. Luis Ros-Santaella carried out the flow cytometry and fluorometry analyses. Álvaro Domínguez-Rebolledo, Felipe Martínez-Pastor and Alfonso Bisbal performed the TBARS analyses. Juián Garde, Enrique del Olmo and Felipe Martínez-Pastor checked the samples by confocal

microscopy and prepared the images. Álvaro Domínguez-Rebolledo and Julián Garde analysed the data. Felipe Martínez-Pastor and M. Rocío Fernández-Santos wrote the manuscript. All the authors revised critically the manuscript.

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