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Effects of media and promoters on different lipid peroxidation assays in stallion sperm

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ABSTRACT

Effects of different media and promoters on lipid peroxidation (LPO) in viable stallion sperm have not been reported. Aims of this study were to determine effects of three media (INRA-96™, Equipro CoolGuard™, and Biggers, Whitten and Whittingham [BWW]), and promoters (iron sulfate-Fe; ultraviolet light-UV; or control-no exposure to promoters) on viable sperm LPO using four different flow cytometric assays (i.e., BODIPY, Liperfluo, 4-hydroxylnonenal [4HNE], malonaldehyde [MDA]). Significant media x promoter interactions were detected using the Liperfluo, 4HNE, and MDA assays ($P < 0.05$); therefore, data were sorted by media and by promoters. With inclusion of milk-based media, there were similar concentrations of LPO in control samples with use of all LPO assays. The effect of iron, as a promoter of LPO production, was media dependent, and milk-based media protected sperm from iron-induced LPO production when there were assessments with all assays. In contrast, iron promoted LPO in sperm diluted in BWW when there was use of in all assays, except BODIPY, probably because of the different target molecule with use of this assay. Ultraviolet light was the most potent LPO promoter with all media and assays evaluated. Data indicate milk-based extenders are generally more LPO-protective than BWW early in the LPO production pathway (based on BODIPY and Liperfluo assays), but are less protective during the later stages of LPO production (based on 4HNE and MDA assays). The use of different media and promoters of LPO allowed for determination of early and late stages of LPO in viable stallion sperm.

1. Introduction

Storage of stallion semen requires dilution with media that protect sperm during cooled or frozen conditions, and most of these media are milk-based, as these are more effective in preservation of sperm quality during storage. Dilution of semen with different media might affect sperm quality, including motility, viability, reactive oxygen species (ROS) production and lipid peroxidation (LPO). Sperm have a small content of antioxidant enzymes (superoxide dismutase, glutathione peroxide, catalase, and glutathione reductase), a large amount of polyunsaturated fatty acids (PUFA, Poulos et al., 1973; Wathes et al., 2007), and are capable of producing ROS (Almeida and Ball, 2005). The ROS are considered necessary for capacitation, acrosome reaction, hyperactivation and sperm-oocyte fusion, and PUFA are integral for membrane fusion functions associated with fertilization (Ball, 2008). The capacity of sperm to counteract excessive ROS is limited, thereby initiating oxidative stress and induction of the LPO production pathway, with

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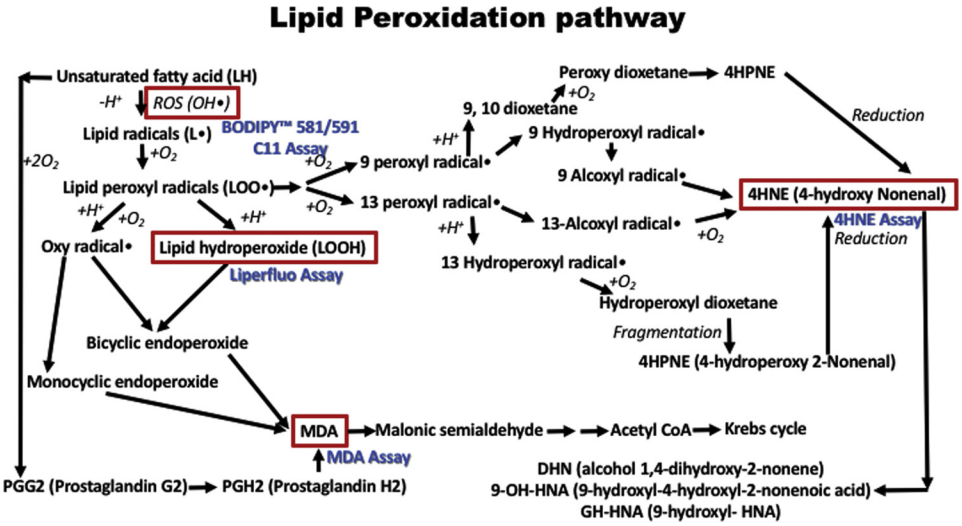

Fig. 1. Schematic representation of the lipid peroxidation pathway. Each lipid peroxidation assay is highlighted in blue and their respective targets are surrounded by red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

synthesis of several intermediate compounds, such as lipid radicals (L˙) and lipid-hydroperoxides (LOOH) during the early-stages of the LPO pathway, and lipid alcohols and lipid aldehydes during the later stages of LPO pathway (i.e., 4-hydroxylnonenal and malonaldehyde, 4HNE and MDA; Fig. 1; Ayala et al., 2014). This cascade of actions is characterized by repetitive hydrogen abstraction and is catalyzed by transition metal complexes (Halliwell and Gutteridge, 1984). Oxidative stress has been associated with loss of sperm function and increased apoptosis in humans (Aitken et al., 2012, 2013a; Aitken, 2013b) and stallions (Baumber et al., 2000; Neild et al., 2005; Ball, 2008).

Different assays have been developed to target the intermediate compounds generated during the early and later stages of the LPO production pathway (i.e., BODIPY, Liperfluo, 4HNE or MDA assays), and various promoters have been used to induce ROS production and LPO in sperm to evaluate these effects on sperm function. The effect of media type on early- and late-stage sperm LPO has not been reported. Use of both the BODIPY and Liperfluo assays allows for detection of early-stage LPO compounds because of the reaction with peroxyl radicals (Quinlan et al., 2013), but use of Liperfluo for this purpose has not been reported. The MDA and 4HNE assays can be used to detect late stages of LPO (Ayala et al., 2014). The MDA compound has been implicated as a cause of human sperm DNA fragmentation (Muratori et al., 2015). Sperm oxidative damage (as determined by 4HNE) is "paradoxically" greater in fertile than sub-fertile stallions (Gibb et al., 2014).

Ortega Ferrusola et al. (2009) reported that there were basal LPO concentrations in freshly ejaculated stallion sperm, and there was only a 2%–3% increase in LPO, as quantified using the BODIPY assay, following freezing/thawing using commercial extenders. Ball and Vo (2002), however, reported that there was an increase in LPO, when there was use of the BODIPY assay, following short-term treatment with a ferrous promoter (iron sulfate), and only a slight increase in LPO in the absence of the promoter following cooled storage in a milk-based extender. Results of research of the same group (Almeida and Ball, 2005) indicated there was no increase in sperm LPO in the absence of iron promoters following cooled storage, and attributed this lack of LPO to the lipoproteins or lipids contained in the milk-based extender.

Interpretation of results when there is use of the LPO assays might be confounded by the use of different media, and by the presence of dead cells. The use of ROS/LPO promoters, therefore, is of relevance to more accurately characterize the response of the viable sperm to the different media and different promoters.

In the current study, ultraviolet light (UV) was used as a novel stressor of ROS/LPO in stallion sperm. Dilution of stallion semen with different media might affect sperm motility, viability, and LPO production. It is of interest to evaluate LPO in viable sperm diluted in different media using probes that target early- and late-stage intermediate compounds of LPO production. The aims of the present study, therefore, were to evaluate the effects of different media (INRA-96™, EquiPRO CoolGuard™, and Biggers, Whitten, and Whittingham-BWW) and different promoters of ROS/LPO (iron-hydrogen peroxide, UV light) on sperm LPO, as quantified using four lipid peroxidation assays (BODIPY, Liperfluo, 4HNE and MDA assays).

2. Materials and methods

2.1. Semen collection and general processing

Three ejaculates from each of five light-horse breed stallions (n = 15), aged 6 to 26 years, were used in this study. Semen was collected using a Missouri model artificial vagina (Nasco, Ft. Atkinson, WI, USA), lubricated with Therio-gel® (Agtech Inc.,

Manhattan, KS, USA) prior to semen collection. All animal procedures were conducted using procedures consistent with those of the Institutional Animal Ethics Committee guidelines (IACUC#- 2018-0032). Following semen collection, gel-free semen was weighed for determination of volume, and sperm concentration was determined using a fluorescence-based cell counter (NucleoCounter-SP100™, Chemometec, A/S, Allerød, Denmark).

2.2. Exposure to different media

Raw sperm samples were diluted to 2×10^6 sperm/mL in each of the following media: 1) INRA-96™ (IMV Technologies, L'Aigle, France; INRA); 2) EquiPRO CoolGuard™ (MOFA Global, Wisconsin, USA; CG); or 3) Biggers, Whitten and Whittingham (BWW; Biggers et al., 1971). Media were pre-warmed at 37 °C prior to semen collection. Diluted semen was aliquoted into conical-bottom microfuge tubes (1.5 mL, VWR International, LLC Radnor, PA, USA). Processed semen was evaluated using four different LPO assays (BODIPY, Liperfluo, 4HNE, and MDA).

2.3. Treatment with ROS/LPO promoters

Sperm samples were treated, or in the case of controls not treated, with use of each assay using the following promoters of ROS/LPO: 1) Control (sperm with no treatment with promoters); 2) UV light (UV; irradiance: 70.16 W/m^2, light source: Medical Illumination Century Diagnostic UV light or Woods light, San Fernando, CA, USA); or 3) $Fe_2(SO4)_3$-H_2O_2 (20 μM of ferric sulphate and 40 μM hydrogen peroxide; Fe; Sigma-Aldrich, St. Louis, Mo, USA). All sperm samples were incubated for 2 h at 37 °C in the dark.

2.4. Sperm LPO assays (BODIPY, Liperfluo, 4HNE, and MDA)

The protocol for the BODIPY was modified from that previously reported (Ortega Ferrusola et al., 2009) and there was use of the fluorescent probe BODIPY™ 581/591 C11 (Life Technologies, Invitrogen, Eugene, OR, USA) at a final concentration of 0.5 μM. BODIPY™ 581/591 C11. The BODIPY is the trade name for fatty acid 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid which oxidizes in the presence of ROS (peroxyl; $OH^{\cdot}$ or superoxide; O^{2-}) and emits red fluorescence at 510–665 nm (Naguib, 1998; Guthrie and Welch, 2010). A BODIPY stock solution (2 mM in DMSO) was stored frozen (-80 °C) in 10 μL aliquots and thawed immediately prior to use. A working solution (0.02 mM) was prepared by diluting the stock solution 1/100 (v/v) in BWW. Following dilution of sperm with different media (INRA, CG, or BWW) and exposure to ROS/LPO promoters (control, UV light or Fe), 25 μL of BODIPY working solution were added to sperm aliquots ($n = 9$ per ejaculate: one sample per each of three media, and one sample per each of three promoters; final concentration: 0.5 μM, 0.025% DMSO), and samples were then incubated in the dark at 37 °C for 30 min. The flow cytometer voltage settings were SSC-240, FL1-613, and FL2-476. The compensation was set using FL2 as 99.9% of FL1. The threshold FSC-H was set at 543.

The protocol for the Liperfluo assay was performed according to manufacturer's recommendations with minor modifications (Dojindo Molecular Technologies, Inc., Rockville, Maryland, USA). Liperfluo is a polycyclic aromatic hydrocarbon [(perylene derivative), 2-(4-diphenylphosphanyl-phenyl)-9-(3,6,9,12-tetraoxatridecyl)-anthra[2,1,9-def:6,5,10-d9e9f9]diisoquinoline-1,3,8,10-tetraone)]. Treatment with this compound reduces lipid hydroperoxides (LOOH, first stable product in lipid peroxidation process) to the hydroxy-homologues and emits green fluorescence at 524–535 nm (Kagan et al., 2017). A Liperfluo stock solution (1 μg/μL in DMSO) was prepared adding 60 μL of 100% DMSO to the 50-μg vial of Liperfluo, and stored in an air-free container at 4 °C. Following dilution of sperm with different media (INRA, CG, or BWW), 1 μL of Liperfluo stock solution was added to each sperm aliquots ($n = 9$ per ejaculate) and samples were then incubated in the dark at 37 °C for 30 min (Liperfluo final concentration: 1 μM with 0.1% DMSO). Following 30 min, sperm were treated, or in the case of the control not treated, with ROS/LPO promoters (control, UV or Fe) for an additional 2 h at 37 °C. The flow cytometer voltage settings were SSC-240, FL1-721, and FL2-752. The compensation was set using FL2 as 99.9% of FL1. The threshold FSC-H was set at 525.

The protocol for the MDA assay was performed according to manufacturer's recommendations with minor modifications (Abcam, Life technologies Corporation, Cambridge, MA, USA). The primary antibody working solution (0.01 μg/μL concentration) for the MDA assay was prepared by diluting the stock solution (1 mg/mL) 1/100 (v/v) in BWW. Following dilution of sperm with different media (INRA, CG, or BWW) and treatment with, or in the case of the control not treatment with, ROS/LPO promoters (control, UV or Fe), 100 μL of MDA working solution were added to each sperm aliquots ($n = 9$ per ejaculate) and samples were, then, incubated in the dark at 37 °C for 1 h (final MDA concentration: 0.003 μg/μL). Following incubation, samples were centrifuged twice at 400*g* for 5 min, the supernatant was aspirated, and each sample was re-suspended with 100 μL of BWW. The secondary antibody (FITC-conjugated anti-mouse IgG; Abcam; working solution 0.02 μg/μL concentration) was prepared by diluting the stock solution (2 mg/mL; 1/100; v/v) in BWW. There were 100 μL of MDA working solution added to each sperm aliquot, and there was incubation of the samples in the dark at 37 °C for 1 h (final MDA concentration of 0.01 μg/μL). Following incubation, samples were centrifuged twice at 400 *g* for 5 min, the supernatant was aspirated, and each sample was re-suspended with 300 μL of BWW. The flow cytometer voltage settings were SSC-240, FL1-628, and FL2-752. The compensation was set using FL2 as 99.9% of FL1, and the threshold FSC-H at 525.

The protocol for 4HNE was performed according to manufacturer's recommendations with minor modifications (Alpha Diagnostic International, San Antonio, TX, USA). A primary antibody working solution was prepared by diluting the stock solution (1 μg/μL; 3/50; v/v) in BWW (final 4HNE concentration: 0.06 μg/μL). Following dilution of sperm with different media (INRA, CG, or BWW) and treatment with, or in the case of the control no treatment with, ROS/LPO promoters (control, UV, or Fe), 50 μL of 4HNE primary antibody working solution were added to each sperm aliquot ($n = 9$ per ejaculate), and samples were incubated in dark at 37 °C for

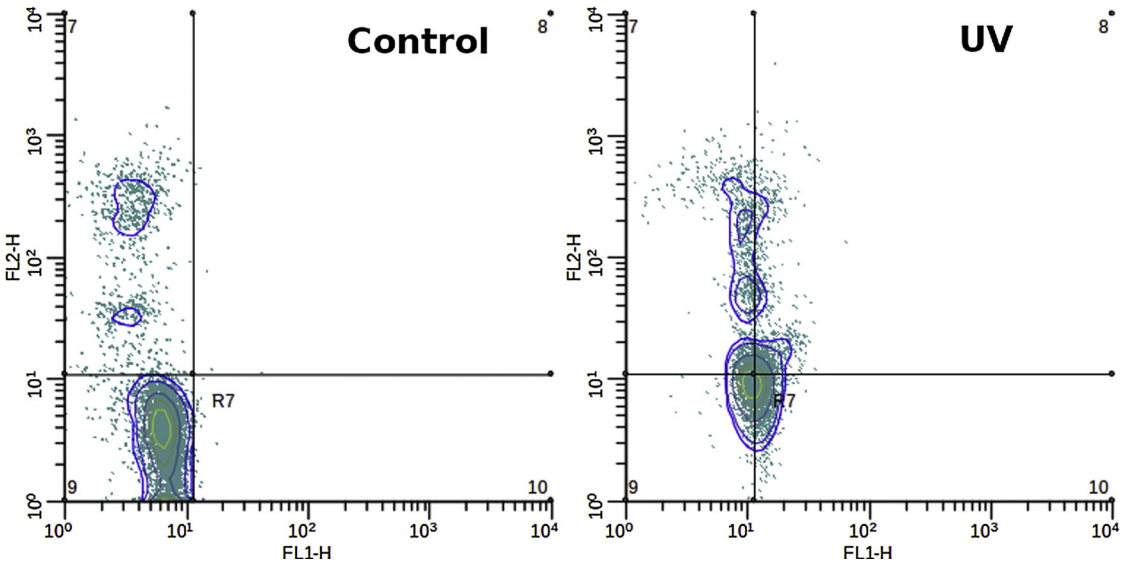

Fig. 2. Scattergrams of sperm lipid peroxidation assays (i.e., BODIPY, Liperfluo, 4HNE or MDA assays). The left scattergram represents the viable-lipid peroxidation negative sperm (VLPN, lower left quadrant) and the non-viable lipid-peroxidation negative sperm in the control sample (left). The right scattergram is obtained following 2 h of ultraviolet (UV) light exposure, in which the sperm population that shifts to the right is considered viable lipid-peroxidation positive (VLPP, lower right quadrant) or non-viable lipid-peroxidation positive (NVPP, upper right quadrant).

1 h (final concentration in each aliquot was 0.03 μg/μL). Following incubation, samples were centrifuged at 400 *g* for 5 min, the supernatant was aspirated, and each sample was re-suspended with 100 μL of BWW. A working solution of 4HNE secondary antibody (Goat anti-Rabbit IgG, Alexa Flour 488, ThermoFisher Scientific, Waltham, MA, USA) was prepared by diluting the stock solution (2 mg/mL; 3/100; v/v) in BWW (final concentration: 0.06 μg/μL). There were 100 μL of 4HNE secondary antibody added to each sperm aliquot and samples were subsequently incubated at 37 °C for 30 min (final secondary antibody concentration: 0.03 μg/μL). Following incubation, samples were centrifuged at 400 *g* for 5 min, the supernatant was aspirated, and each sample was re-suspended with 300 μL of BWW. The flow cytometer voltage settings were SSC-240, FL1-489, and FL2-628. The compensation was set using FL2 as 94.4% of FL1. The threshold FSC-H was set at 543.

For each LPO assay, after incubation with the respective fluorescent probe, samples were centrifuged at 400 *g* for 5 min, the supernatant was aspirated, and 40 μL of sperm suspension were transferred to a tube (Falcon® Round-Bottom Tubes, Disposable, Polystyrene, Corning®, VWR) containing 200 μL of BWW media, then stained with propidium iodide (PI, Invitrogen Molecular Probes, Eugene, OR, USA; final concentration 9.6 μM) prior to analysis using a flow cytometer equipped with a 488 nm argon laser (FACScan, Becton Dickinson, Mountain View, CA, USA). A minimum of 5000 cellular events was evaluated per sample. Data were stored in List-Mode, and subsequently analyzed using WinList™ software (Verity Software House, Topsham, ME, USA). The experimental endpoint was the percentage of the sperm population that was both viable and lipid-peroxidation negative (VLPN; Fig. 2).

2.5. Statistical analysis

Data were subjected to rank transformation and analyzed using ANOVA procedures (PROC ANOVA; SAS Version 9.4; SAS Institute, Inc. Corp., Cary, NC, USA). Means were compared using the Tukey's studentized range test with significance set at $P < 0.05$.

3. Results

A significant media x promoter interaction was detected for three of four assays (Liperfluo, 4HNE and MDA); therefore, data were sorted by media and by promoter prior to analysis (Tables 1 and 2).

When sorting data by media, the number of sperm that were VLPN was greater in Control and Fe-treated than UV-treated group in both milk-based extenders and when all assays were used ($P < 0.05$). In BWW media, the number of sperm that were VLPN was also greater in Control than both the Fe- and UV-treated groups when assessed using three of the four assays utilized in this study (Liperfluo, 4HNE and MDA; $P < 0.05$), and the number of sperm that were VLPN was greater in control than UV-treated group with use of all assays ($P < 0.05$). This difference was most pronounced with use of the BODIPY and Liperfluo assays.

When sorting data by promoters, the number of sperm that were VLPN was greater in the CG- and INRA- than BWW-treated semen sample with use of both the BODIPY and Liperfluo assays, regardless of promoter type ($P < 0.05$). This effect was most pronounced when UV was used as a promoter. With use of the 4HNE assay, the number of sperm that were VLPN in the control group was similar when the different media were used ($P > 0.05$); whereas with use of the Fe assay, the number of VLPN sperm was greater when the

Table 1
Percent viable lipid peroxidation negative sperm (VLPN; Mean ± SEM) following 2 h of treatment with ROS/LPO promoters (n = 9: one sample per each of three media, and one sample per each of three promoters).

Promoter[†]	Media[‡]	LPO Assay[*]			
		BODIPY	Liperfluo	4HNE	MDA
Control	BWW	67 ± 3[a]	69 ± 3[a]	72 ± 3[a]	61 ± 3[a]
Fe	BWW	63 ± 2[a]	62 ± 2[b]	54 ± 3[b]	44 ± 3[b]
UV	BWW	4 ± 1[b]	10 ± 3[c]	57 ± 3[b]	47 ± 4[b]
Control	CG	75 ± 2[a]	74 ± 2[a]	69 ± 3[a]	54 ± 2[a]
Fe	CG	75 ± 2[a]	75 ± 2[a]	67 ± 3[a]	53 ± 1[a]
UV	CG	27 ± 7[b]	23 ± 4[b]	54 ± 3[b]	34 ± 4[b]
Control	INRA	76 ± 2[a]	75 ± 2[a]	66 ± 3[a]	56 ± 3[a]
Fe	INRA	75 ± 2[a]	75 ± 2[a]	66 ± 4[a]	56 ± 2[a]
UV	INRA	23 ± 5[b]	33 ± 6[b]	46 ± 4[b]	32 ± 5[b]

[a, b, c]Within media and promoters, means with different superscripts differ ($P < 0.05$).
[*] Lipid peroxidation assay (LPO) included: BODIPY (BODIPY™ 581/591 C11), Liperfluo, 4HNE, and MDA.
[†] Promoters: control = no exposure to promoters; Fe = 20 μM of ferric sulphate and 40 μM hydrogen peroxide; UV = ultraviolet light (irradiance: 70.16 W/m^2).
[‡] Media: BWW = Biggers, Whitten, and Whittingham, CG = EquiPRO CoolGuard™, INRA = INRA-96™.

Table 2
Percent viable lipid peroxidation negative sperm (VLPN; Mean ± SEM) following 2 h of treatment with ROS/LPO promoters (n = 9: one sample per each of three media, and one sample per each of three promoters).

Media[‡]	Promoter[†]	LPO Assay[*]			
		BODIPY	Liperfluo	4HNE	MDA
BWW	Control	67 ± 3[b]	69 ± 3[b]	72 ± 3[a]	61 ± 3[a]
CG	Control	75 ± 2[a]	74 ± 2[a]	69 ± 3[a]	54 ± 2[b]
INRA	Control	76 ± 2[a]	75 ± 2[a]	66 ± 3[a]	56 ± 3[ab]
BWW	Fe	63 ± 2[b]	62 ± 2[b]	54 ± 3[b]	44 ± 3[b]
CG	Fe	75 ± 2[a]	75 ± 2[a]	67 ± 3[a]	53 ± 1[a]
INRA	Fe	75 ± 2[a]	75 ± 2[a]	66 ± 3[a]	56 ± 2[a]
BWW	UV	4 ± 1[b]	10 ± 3[b]	57 ± 3[a]	47 ± 4[a]
CG	UV	27 ± 7[a]	23 ± 4[a]	54 ± 3[ab]	34 ± 4[b]
INRA	UV	23 ± 5[a]	33 ± 6[a]	46 ± 4[b]	32 ± 5[b]

[a, b, c] Within promoters and media, means with different superscripts differ ($P < 0.05$).
[*] Lipid peroxidation assay (LPO) included: BODIPY (BODIPY™ 581/591 C11), Liperfluo, 4HNE, and MDA.
[†] Promoters: control = no exposure to promoters; Fe = 20 μM of ferric sulphate and 40 μM hydrogen peroxide; UV = ultraviolet light (irradiance: 70.16 W/m^2).
[‡] Media: BWW = Biggers, Whitten, and Whittingham, CG = EquiPRO CoolGuard™, INRA = INRA-96™.

CG and INRA than BWW media were used ($P < 0.05$). With use of the UV-light treatment, the number of sperm with VLPN was greater when the BWW than INRA media were used ($P < 0.05$). Regarding the MDA assay, the number of sperm that were VLPN was greater with use of the BWW than CG media in the absence of promoter ($P < 0.05$). The number of sperm that were VLPN was also greater with use of the BWW media than milk-based extenders when UV light was used as a promoter ($P < 0.05$), but less with use of the BWW media with milk-based extenders when Fe was used as a promoter ($P < 0.05$).

4. Discussion

This study was conducted to evaluate the effects of various media and promoters on ROS/LPO production in stallion sperm, as determined using four different LPO assays. The LPO pathway involves the production of multiple intermediate compounds generated during early and late stages of the LPO synthetic biosynthetic pathway, and each assay targets quantitation of specific intermediate compounds. Media used to dilute stallion sperm vary considerably, and include both milk-based extenders and defined culture media. The BWW media was originally developed for *in-vitro* culture of mice embryos (Biggers et al., 1971) but is also widely used as a stallion sperm diluent. The INRA96™ compound (Batellier et al., 1998) contains native phosphocaseinate, a fractionated component of milk, and is commonly used in commercial horse breeding programs. Because INRA96™ is opaque and might contain components that interfere with fluorochrome binding, there was also assessment of another skim milk-based extender (Equipro CoolGuard™, Mofa America) that has a more transparent appearance to determine whether results with use of this media would be similar to those with use of INRA96™. Diluent type and composition may affect the results with use of ROS/LPO-quantitation assays. The present study is the first in which there is comparison of the effects of different media (INRA, CG and BWW) and different promoters (Fe, UV) on sperm LPO production using four different flow cytometric assays. The results of the present study confirmed, that with use of

CoolGuard™ and INRA96™, there are similar VLPN sperm numbers with use of all four LPO assays evaluated.

The focus in the present study was to assess ROS/LPO production in viable sperm populations only; hence, there was incorporation of propidium iodide with use of all assays to allow for identification of the viable sperm subset in the semen samples. For each LPO assay, two promoters of ROS/LPO production were assessed: iron sulfate in combination with hydrogen peroxide and ultraviolet light. Iron sulfate, in combination with hydrogen peroxide, initiates and propagates the lipid peroxidation production pathway (Repetto et al., 2010). Of importance, in the present study the effect of iron as a promoter of ROS/LPO production was media dependent and iron was ineffective in inducing ROS/LPO production when there was assessment using all the LPO assays evaluated when semen was diluted in INRA96™ or CoolGuard™. In essence, milk-derived media appeared to protect stallion sperm from the iron-induced ROS/LPO production during all stages of the lipid peroxidation biosynthetic pathway. In contrast, the treatment with iron promoted ROS/LPO production when used in BWW media when there was assessment with three of four assays (exception was BODIPY). Both the BODIPY and Liperfluo assays can be used to detect early intermediate compounds of LPO biosynthetic pathway, but bind to different compounds of the LPO production pathway. Liperfluo binds to lipid hydroperoxides (Kagan et al., 2017), whereas BODIPY binds to hydroxyl and superoxide radicals (Naguib, 1998; Guthrie and Welch, 2010). The difference in intracellular target molecules may have affected assay results. To the best of our knowledge, the present study was the first where there was evaluation of stallion semen with Liperfluo. The increase in LPO in stallion sperm diluted in BWW media, following treatment with iron sulfate, is similar to that reported by Baumber et al. (2000), using MDA and use of spectrophotometry LPO quantitation; however, the present study was the first where there was use of flow cytometry for LPO quantitation utilizing the MDA assay, allowing the differentiation between viable and non-viable sperm.

In the present study, there was use of a novel promoter for ROS/LPO production in stallion sperm, i.e., ultraviolet light, to examine its effects on both early and late stages of ROS/LPO production. The results of the present study indicate that 2-h of treatment with UV light decreased the number of sperm that were VLPN regardless of the media evaluated. The extent of this decrease was greater during the early stages of the ROS/LPO biosynthetic pathway, resulting overall in lesser numbers of sperm that were VLPN when using the BODIPY and Liperfluo assays. In contrast to iron as a promoter, the use of milk-derived media did not prevent ROS/LPO production generated by UV. Previous investigators have reported that there was an increase in ROS in human sperm, as measured using the BODIPY assay, following only 3 min of treatment with UVB irradiation (Amaral et al., 2013). Other investigators imposed UVA and UVB treatments on sea urchin sperm for 30 min and reported there was an impairment of fertility with use of UVB than UVA treatments, as quantified using the MDA assay (Lu and Wu, 2005). In the present study, the ultraviolet component was UVA. This form of UV irradiation was found to be more potent than iron/hydrogen peroxide as a promoter of ROS/LPO production in stallion sperm. The effect was detected when using any of the four ROS/LPO assays, and its effect was greater with use of BWW than milk-derived media when there was use of the BODIPY and Liperfluo assays. The fact that more sperm treated with UV had a lesser VLPN with use of BWW than milk-based media when there was utilization of the BODIPY and Liperfluo assays indicates that this media does not promote sperm viability in the *in-vitro* conditions imposed in the present study. Of note, this effect of media was not apparent with use of the assays where there was detection of late-stage intermediates in the LPO biosynthetic pathway (i.e., 4HNE and MDA assays).

In conclusion, there was an effect of media on ROS/LPO production in viable sperm during early and late stages of the biosynthetic pathway for lipid peroxidation, and the effect of promoters on the numbers of sperm that were VLPN is media dependent. The assessment of ROS/LPO production pathway may have clinical relevance, because the results of the present study can be utilized to develop procedures to more effectively evaluate the lipid peroxidation pathway of viable sperm when various cooling or freezing conditions are imposed. Based on results of the present study, the Liperfluo assay may have advantages as compared with the BODIPY assay to assess early stage of ROS/LPO production if iron-hydrogen peroxide is used as a promoter in media containing no milk products. Results of the present study also indicate 4HNE and MDA can be used interchangeably to assess late-stage intermediates in the LPO biosynthetic pathway.

Authors' contributions

S.-G. contributed to experimental procedures and manuscript preparation. R.-S. contributed to manuscript preparation. C.C.-L. contributed to experimental design and manuscript revision. S.R.-T, C.H.-A., and K.A.-LaC contributed to experimental procedures, D.D.-V. contributed to manuscript revision, experimental design and statistical analysis.

Declaration of Competing Interest

The authors declare no conflict of interest.

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