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Source: *Biology of Reproduction*, 104(6) : 1271-1281

Published By: Society for the Study of Reproduction

URL: <https://doi.org/10.1093/biolre/ioab035>

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Research Article

HDL mediates reverse cholesterol transport from ram spermatozoa and induces hyperactivated motility

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Received 18 November 2020; Revised 5 February 2021; Accepted 21 February 2021

Abstract

Reverse cholesterol transport or cholesterol efflux is part of an extensive plasma membrane remodeling process in spermatozoa that is imperative for fertilization. For ram spermatozoa, sheep serum is well known to support in vitro fertilization (IVF), but knowledge of its explicit role is limited. Though, it is postulated to elicit cholesterol efflux owing to the presence of high-density lipoproteins (HDLs) that interact with transmembrane cholesterol transporters, such as adenosinetriphosphate (ATP)-binding cassette transporter A1 (ABCA1) and scavenger receptor class B, type I (SR-BI). In this study, we report that both sheep serum and HDLs were able to elicit cholesterol efflux alone by up to 20–40% (as measured by the boron dipyrromethene (BODIPY)-cholesterol assay). Furthermore, when the antagonists glibenclamide and valsopodar were used to inhibit the function of ABCA1 and SR-BI or ABCA1 alone, respectively, cholesterol efflux was only marginally reduced (8–15%). Nevertheless, it is likely that in ram spermatozoa, a specific facilitated pathway of cholesterol efflux is involved in the interaction between cholesterol acceptors and transporters. Interestingly, exposure to HDLs also induced hyperactivated motility, another critical event required for successful fertilization. Taken together, this study details the first report of the dual action of HDLs on ram spermatozoa, providing both an insight into the intricacy of events leading up to fertilization in vivo as well as demonstrating the possible application of HDL supplementation in media for IVF.

Summary sentence

First report of the dual action of high-density lipoproteins on ram spermatozoa in mediating critical fertilization-dependent processes.

Key words: sperm activation, cholesterol efflux, high-density lipoproteins, cAMP dependency, sperm motility, fertilization.

Introduction

As spermatozoa travel through the female reproductive tract to the site of fertilization in the oviduct, they are exposed to a complex and varied biochemical environment that stimulates capacitation [1, 2]; the final maturation event required for successful fertilization. During this process, spermatozoa undergo a series of functional modifications that collectively provide these cells with the capacity to hyperactivate (vigorous and propulsive motility), acrosome react, and fertilize [3, 4]. Notably during this transformation, along with the activation of signaling pathways [5], cellular ion fluxes [6], and sperm protein phosphorylation (serine, threonine, and tyrosine phosphorylation) [7, 8], the sperm plasma membrane is subjected to extensive remodeling [9]. This remodeling is comprised of successive changes in the plasma membrane, starting with an increase in fluidity (driven by bicarbonate-mediated cyclic adenosinemonophosphate - protein kinase A (cAMP-PKA) activity [10]), the relocation and removal of cholesterol by extracellular acceptors [11], and finally, the subsequent movement of lipid rafts containing requisite proteins for fertilization to the apical region of the sperm head [12, 13]. Although all these changes are necessary during capacitation, the loss of cholesterol through the regulated process of cholesterol efflux is a significant contributor to the alterations observed in the plasma membrane architecture. This is simply owing to the fact that this sterol is a stabilizing membrane lipid and a known decapacitation factor [14, 15]. Furthermore, the successful progression of cholesterol efflux has been shown to facilitate other events leading up to fertilization, including tyrosine phosphorylation and interaction with the oocyte [16–18].

Given the necessity of cholesterol efflux to adequately prepare spermatozoa for fertilization, the ability to support this capacitation-related process *in vitro* is imperative, not only for research purposes but also for the success of *in vitro* fertilization (IVF). This is typically achieved by the supplementation of serum, reproductive fluids, or derivatives of these fluids (like serum albumin), which have been shown to promote cholesterol efflux in different species [19–21]. For sheep, very few studies provide evidence that serum is able to elicit cholesterol efflux from ram spermatozoa. However, others have demonstrated that it is able to support events during or after capacitation, such as enhancing membrane fluidity and increasing acrosome reactivity [19, 22, 23]. Although the addition of serum albumin alone has been shown to elicit cholesterol efflux from ram spermatozoa that are exposed to upregulated cAMP conditions [24], there is substantial evidence that the presence of sheep serum in media is indispensable for successful ovine IVF [19, 25, 26]. Collectively, this suggests that there are derivatives of serum, other than albumin, that are actively participating in cholesterol efflux, and potentially other events, leading up to fertilization.

A likely candidate is high-density lipoproteins (HDL). HDLs are primarily known for their protective properties in atherosclerotic cardiovascular disease. Lipoproteins effectively remove cellular cholesterol from foam cells (lipid-loaded macrophages) that reside in atherosclerotic lesions within the arteries [27]. HDLs have also been isolated in reproductive fluids, including in the sheep [28, 29]. There are several reports illustrating the functionality of HDLs as cholesterol acceptors in human, mouse, and bull spermatozoa [16, 20, 21, 30, 31], but their role in the regulation of cholesterol efflux from ram spermatozoa are not described. HDLs have also shown to induce hyperactivated motility in human spermatozoa, which is an essential requisite for fertilizing the mammalian oocyte [32]. Endogenous HDLs are heterogeneous in nature; ranging from immature lipid-poor apolipoprotein A1 (apoA1), which is the major protein component of HDLs, to mature particles composed of a

core of neutral lipids surrounded by phospholipids, cholesterol, and apoA1 (Figure 1). One of the major differences between cholesterol efflux mediated by albumin, and that by HDLs or apoA1, is the pathway by which this sterol is prepared for removal. Extensive research in somatic cells has illustrated that cholesterol removed by albumin is a passive process, whereby contact with the plasma membrane allows for cholesterol desorption into the hydrophobic pocket of albumin's molecular structure [33]. In contrast, cholesterol efflux by HDLs and apoA1 is more likely to occur via a facilitated pathway that involves interaction with transmembrane proteins (collectively termed cholesterol transporters) [33]. Of these cholesterol transporters, two have established associations with HDLs and apoA1 that result in the efflux of cholesterol. These are scavenger receptor class B, type I (SR-BI) and ATP-binding cassette transporter A1 (ABCA1), respectively [34]. ABCA1 is an active carrier that controls the unidirectional movement of cholesterol to available acceptors in the surrounding environment [33]. There is currently no knowledge of this specific transporter in ram spermatozoa, but they have been identified in bull, canine, mouse, and human spermatozoa with proteomics or immunolocalization [35–38]. Furthermore, the inhibition of ABCA1 activity in mouse spermatozoa has shown to significantly reduce the capacity for fertilization [38], thus highlighting its functional importance. On the other hand, cholesterol transport from SR-BI to HDLs is predominantly a passive process and can be bidirectional in somatic cells [33, 39]. SR-BI has been localized in mouse and human spermatids [40, 41], as well as mature human spermatozoa [37]. Though at present, there is limited understanding of the role of this transporter during capacitation, specifically during cholesterol efflux from ram spermatozoa.

Therefore, in the present study, we investigated (i) whether sheep serum and the specific cholesterol acceptors, HDL and apoA1, were able to elicit cholesterol efflux from ram spermatozoa; (ii) whether cholesterol efflux mediated by certain cholesterol acceptors in ram spermatozoa is associated with the characteristic induction of hyperactivated motility; and (iii) whether the cholesterol transporters, ABCA1 and SR-BI, are involved in cholesterol efflux as assessed by the use of antagonists.

Materials and methods

Chemicals

Unless otherwise stated, products were sourced from Sigma-Aldrich and were of the highest reagent grade available. HDL isolated from human plasma was purchased from Lee Biosolutions (Maryland Heights, MO, USA) and apoA1 was kindly donated by the Heart Research Institute, NSW. Fatty acid-free bovine serum albumin fraction V (FAF-BSA) and complete ultra mini Ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor tablets were sourced from Roche (Schnelldorf, Germany). Boron dipyrromethene (BODIPY) cholesterol was purchased from Avanti Polar Lipids, Inc. (TopFluor Cholesterol; Product No. 810255; Abilast, AL, USA). Propidium iodide (PI), peanut agglutinin fluorescein isothiocyanate (PNA-FITC), and MagicMark XP Western Blot Standards were sourced from Invitrogen (NSW, Australia), and merocyanine 540 (M540) and YO-PRO-1- from Molecular Probes (Eugene, OR, USA). For density gradients, BoviPure and BoviDilute were purchased from NidaCon (Gothenberg, Sweden).

Incubation media

The basal medium used for all experiments was modified Tyrode medium supplemented with albumin, lactate, and pyruvate (TALP)

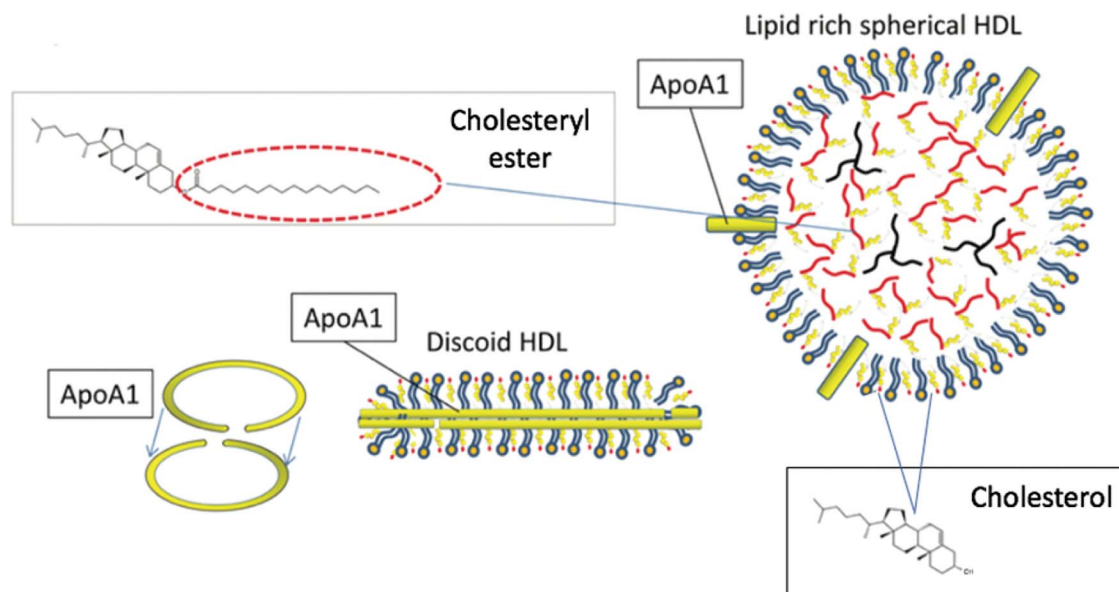


Figure 1. The high-density protein (HDL) subtypes used in the current study to elicit cholesterol efflux from ram spermatozoa. Apolipoprotein A1 is the predominant protein of HDLs that has the capacity to associate with and bind to lipids-like cholesterol. ApoA1 can exist as immature, lipid-poor particles or built into the surface of developed HDL particles. The developing HDL particle can import free cholesterol and phospholipids into its bilayer or esterify the free cholesterol to neutralize the lipids for intracellular storage. This figure has been adapted from that in [14].

[42]. TALP consisted of 2 mM CaCl_2 , 3 mM KCl, 0.4 mM MgCl_2 , 90 mM NaCl, 0.3 mM NaH_2PO_4 , 10 mM HEPES, 21.6 mM sodium lactate, 5 mM D-glucose, 2 mM sodium pyruvate, 25 mM NaHCO_3 , and 3 mg/mL FAF-BSA (0.3% FAF-BSA), which has been shown to be an effective cholesterol acceptor in ram spermatozoa [24]. To all TALP media, 1.5 mM D-penicillamine was supplemented to eliminate agglutination of ram spermatozoa during in vitro capacitation [43]. Where necessary, the pH of media was adjusted to 7.3 with NaOH, and the osmolarity was measured as 300 ± 10 mOsm by freezing point depression using an osmometer (Fiske 210 Micro Osmometer; Fiske Associates; Norwood, MA, USA). For semen preparation before in vitro capacitation, noncapacitating TALP was formulated by omitting 25 mM bicarbonate (NaCl concentration was adjusted from 90 to 115 mM to ensure the osmolarity of the media was maintained) as well as replacing 0.3% FAF-BSA with 1 mg/mL polyvinylalcohol.

Further modifications to TALP media were also made depending on the experiment. For Experiment 1, TALP was modified to include 2% sheep serum (v/v) and 1 mM dibutyl cyclic AMP, caffeine, and theophylline (cAMP upregulators). In some conditions, cAMP upregulators were omitted, and these samples served as controls. In Experiment 2, TALP was modified to include cAMP upregulators and either supplemented with 250 $\mu\text{g}/\text{mL}$ HDL, 25 $\mu\text{g}/\text{mL}$ reconstituted apoA1, or both. ApoA1 was isolated from human plasma by ultracentrifugation and anion-exchange chromatography, as described previously [44, 45], and stored at -20°C until use. Prior to experimentation, purified apoA1 protein was reconstituted in guanidine hydrochloride buffer (10 mM Tris, 3 M guanidine HCl, pH 8.2) for 30 min and was followed by dialysis over 5–7 days at 4°C to allow for the removal of buffer constituents. The protein concentration of apoA1 was estimated using the Quant-iT Protein Assay (Invitrogen; NSW, Australia). For both Experiments 1 and 2, additional 0.3% FAF-BSA was omitted from some conditions with sheep serum, HDLs, and/or apoA1 in order to elucidate the role of these components alone. Finally, for Experiment 3, TALP was modified to include cAMP

upregulators and supplemented either with 2% sheep serum (v/v) or 250 $\mu\text{g}/\text{mL}$ HDL as cholesterol acceptors (0.3% FAF-BSA present in both conditions). To inhibit both ABCA1 and SR-BI activity, both of which are involved in cholesterol efflux, glibenclamide was selected as an appropriate antagonist. This compound is known to inhibit ABCA1, as well as other members of the ABC (ATP-binding cassette) superfamily in addition to SR-BI [46]. Previous work has shown that the concentration of glibenclamide that is capable of inhibiting ABCA1 activity in somatic cells is between 100 and 1000 μM [46, 47], and that the potency of this inhibition is virtually identical to SR-BI when within this range [46]. For the purpose of this preliminary experiment, we selected a concentration of 100 μM glibenclamide, which is on the lowest end of this range, in order to examine the potential antagonistic effects of this compound on ABCA1- and SR-BI-mediated cholesterol efflux in ram spermatozoa. With respect to valsopodar, this compound is reported to inhibit ABCA1 activity by directly binding to this protein in a concentration-dependent manner [48]. To explore the importance of ABCA1 activity alone in this study, we selected a concentration of 2 μM valsopodar. This concentration was selected on this basis that it can induce a half-maximal inhibition of ABCA1-mediated cholesterol efflux (IC_{50}) in somatic cells without being toxic [48].

Sperm preparation

For each experiment, ram semen was collected from three Merino rams using an artificial vagina with project approval from the University of Sydney's animal ethics committee (Project No: 2016/1106). Only ejaculates that had a wave motion score of 4 (out of 5) or higher were used in the study [49]. Rams were housed at the animal house at the Faculty of Science, The University of Sydney, Camperdown, NSW, Australia. Each experiment was performed nine times, using three separate ejaculates from each ram. For all assessments (excluding the BODIPY-cholesterol assay), seminal plasma-free spermatozoa were obtained by a swim-up procedure by layering 200 μL of raw semen

below a 2 mL layer of noncapacitating TALP followed by incubation for 60 min at 37°C. After incubation, the top 1 mL of medium was carefully removed, and the concentration was determined using a Neubauer Improved hemocytometer (Marienfeld Superior; Lauda-Königshofen, Germany). For the BODIPY-cholesterol assay, raw semen was diluted in noncapacitating TALP to 250×10^6 sperm/mL prior to labeling. Subjective motility was evaluated with light microscopy following dilution. Only those samples with 70% total motility and higher were used for the BODIPY-cholesterol assay.

For *in vitro* capacitation, washed spermatozoa collected from the swim-up were diluted to a final concentration of 20×10^6 sperm/mL in the respective experimental media conditions (see section BODIPY-cholesterol assay for preparation of labeled spermatozoa). All samples were assessed for capacitation-related changes immediately after exposure to the various conditions (referred to as “10 min” of incubation; with the exception of tyrosine phosphorylation) and again after 180 min (3 h) of incubation at 37°C.

Flow cytometric analysis of sperm function

The assessment of various sperm functions during *in vitro* capacitation, including cholesterol efflux, were performed using a CytoFLEX flow cytometer (Beckman Coulter, Lane Cove, Australia). Prior to each assessment, a sperm cell-specific population was gated based on forward and sideward light scatter profiles. Laser and band-pass filter specifications for the excitation and detection of fluorophores are detailed in the relevant methods. For each sample, 10 000 events were recorded for later analysis in CytExpert software, and no compensation was applied for the various assessments.

BODIPY-cholesterol assay

For the quantification of cholesterol efflux from ram spermatozoa, we utilized the BODIPY-cholesterol assay, a flow cytometric-based assessment that has been previously validated by our group [50]. Ram spermatozoa were labeled with BODIPY-cholesterol and prepared for *in vitro* capacitation as previously described [24]. Briefly, following dilution to 250×10^6 sperm/mL with noncapacitating TALP, spermatozoa were labeled with 1 μ M BODIPY-cholesterol (1 mM stock solution dissolved in dimethylsulfoxide (DMSO)) for 10 min at 37°C. Excess BODIPY-cholesterol was removed via centrifugation ($300 \times g$, 20 min, 25°C) through a two-way step discontinuous gradient of 40 and 80% isotonic Bovipure diluted with Bovidilute. A gradient for each ram was performed in duplicate, and the sperm pellets for a single ram were pooled and then washed of density gradient medium with noncapacitating TALP via centrifugation ($300 \times g$, 10 min, 25°C). The concentration of the resulting sperm pellet was determined using a hemocytometer and was diluted to a final concentration of 10×10^6 sperm/mL with the respective media conditions for each experiment.

To identify the membrane intact population for assessment of cholesterol efflux (in order to eliminate cells with potential intracellular labeling), suspensions were counterstained with 6 μ M PI for 10 min prior to analysis [50]. BODIPY-cholesterol and PI fluorescence were excited by a 50 mW 488 nm laser and detected with either a 525/40 nm or 585/42 nm band-pass filter, respectively. BODIPY-cholesterol fluorescence was only examined in the membrane intact (PI-) population as recommended, and the percentage change in BODIPY-cholesterol fluorescence from 10 to 180 min (3 h) was calculated in order to determine cholesterol efflux induced by the various media conditions.

Changes in membrane fluidity and integrity

Membrane fluidity and integrity were assessed through dual fluorescent staining with 0.83 μ M M540 and 25 nm YO-PRO-1 for 10 min at 37°C, as described previously [43, 51]. M540 and YO-PRO-1 were excited by a 50 mW 488 nm laser and detected with either a 585/40 nm or 525/40 nm band-pass filter, respectively. When analyzing this data, only membrane intact (YO-PRO-1-) spermatozoa with either low or high M540 fluorescence (corresponding to low and high membrane fluidity, respectively) were assessed and presented.

Acrosome and membrane integrity

Acrosome and membrane integrity were also examined by dual fluorescence staining with 0.4 μ g/mL PNA-FITC and 6 μ M PI for 10 min at 37°C, as described previously [43, 51]. PNA-FITC and PI were both excited by a 50 mW 488 nm laser and detected with a 525/40 nm or 585/40 nm band-pass filter, respectively. These two parameters were measured to monitor sperm quality over the course of the incubation period, and the results were presented where appropriate.

Detection of hyperactivation

Sperm motility and kinematics were objectively measured by computer-assisted semen analysis (CASA; HT CASA IVOS II (Animal Breeder) Version 1.4; Hamilton-Thorne, Beverly, MA, USA) using factory CASA ram settings. For the initial assessment of motility, semen samples (5.5 μ L) were placed on prewarmed slides (Cell Vu; Millenium Sciences, Mulgrave, VIC, Australia) and enclosed using a 22 \times 22 mm coverslip before immediate transfer to the system. For each sample, video recordings of eight fields were taken (frame rate of 60 Hz; minimum of 200 spermatozoa total), and the following parameters were assessed, including but not limited to total motility (%), progressive motility (%), curvilinear velocity (VCL; μ m/s), amplitude of lateral head displacement (ALH; μ m), and linearity (LIN; %). For the assessment of hyperactivity, scatterplots of VCL, ALH, and LIN were first produced for each treatment group, and suitable thresholds were determined based on whether ALH and VCL increased and LIN decreased in samples. These scatterplots were then compared across all treatment groups to produce a final set of thresholds for each of the parameters plotted. Each video recording was assessed again using these thresholds, and the percentage of spermatozoa displaying hyperactivity was calculated for each sample.

Extraction of ram sperm proteins

For the analysis of tyrosine phosphorylation during the late stages of capacitation, aliquots of $8\text{--}10 \times 10^6$ sperm were taken for protein extraction at 180 min of incubation only. Spermatozoa were first washed ($600 \times g$, 10 min) in phosphate-buffered saline in order to remove media-derived protein and the resulting pellet was suspended 1:1 in lysis buffer (62.6 mM Tris, 1 mM sodium orthovanadate, 2% w/v SDS, complete ultra mini EDTA-free protease inhibitor tablet) before being kept at room temperature for 1 h with frequent vortexing. Following this lysis period, samples were then centrifuged ($7500 \times g$, 15 min) and the lysate was retained. The protein concentration of lysates was estimated using Quant-iT Protein Assay (Invitrogen; NSW, Australia) and standardized with Milli-Q water to 1 mg/mL before further dilution with loading buffer (final concentration of 62.5 mM Tris, pH 6.8; 5% (v/v) 2-mercaptoethanol; 2% (v/v) SDS; 10% glycerol (v/v); 0.2% (w/v) bromophenol blue). Samples were

then incubated for 5 min at 95°C and then stored at -80°C until required.

SDS-PAGE and tyrosine phosphorylation western blot

All reagents and equipment used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis were purchased from Bio-Rad (Gladesville, NSW, Australia), unless otherwise specified. Precision Plus Protein Western C standards and processed sperm protein lysates (10 µg) were loaded into 10% Mini-PROTEAN TGX stain-free gels and electrophoresis was carried out initially at 75 V for 10 min and then increased to 200 V for a further 30 min. To validate gel loading, quantification of protein bands was achieved using a stain-free analysis protocol on a Chemi-Doc MP Imaging System. Proteins were then transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane at 100 V for 1 h in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol (v/v)). Nonspecific sites on the membrane were blocked for 1 h in Tris-buffered saline (20 mM Tris, 150 mM NaCl, pH 7.6) supplemented with 0.1% Tween-20 (TBS-T) containing 5% (w/v) BSA. Blots were incubated with anti-phosphotyrosine horseradish peroxidase (HRP)-conjugated antibody (monoclonal antibody, clone 4G10, Product No. 16-105; Merck; Billerica, MA, USA) diluted 1:2000 and StrepTactin-HRP conjugate diluted 1:10 000 in TBS-T + 0.1% BSA (w/v) with agitation for 1 h at room temperature. After washing three times for 15 min in TBS-T, tyrosine phosphorylated proteins were visualized on a Chemi-Doc system using enhanced chemiluminescence (Clarity) as per manufacturer's instructions.

Statistical analysis

All experiments and the respective functional assays were analyzed using linear mixed model regression in R [52]. For all in vitro capacitation measures, the media condition and incubation time were set as fixed effects in the model, replicate and ram were included as nested random effects. The same was applied for the BODIPY-cholesterol assay results, excluding the individual effect of time. Interactions between the fixed effects were assessed where appropriate. Pairwise comparisons between the levels of a fixed effect were determined using the predictmeans function with a Holm adjustment. Normality and homoscedasticity of the residuals were assessed for all models by use of Shapiro-Wilkins test and Bartlett test, respectively. Manipulation of the model to reduce heteroscedasticity of the residuals was performed if necessary and, in some cases, this required the application of a log transformation. If a log transformation was performed, the results were back-transformed and presented as the geometric mean ± 95% confidence intervals (CIs). Otherwise, data are presented as the mean ± SEM and results that are $P < 0.05$ are considered significant.

Results

Sheep serum stimulates cholesterol efflux alone, and in combination with FAF-BSA, only in high-cAMP conditions

Sheep serum is a common additive to media to support IVF in sheep, but the effect of this complex mixture on ram spermatozoa during capacitation is largely unknown, particularly for cholesterol efflux. As such, we examined the capacity for 2% sheep serum to elicit cholesterol efflux and support other capacitation-related processes, including changes in membrane fluidity and tyrosine phosphorylation. This study was able to show, with the use of the

BODIPY-cholesterol assay, that sheep serum elicits cholesterol efflux from ram spermatozoa under capacitating conditions in likeness to 0.3% FAF-BSA alone [24] (Figure 2A). When compared with TALP alone, the supplementation of 2% sheep serum with cAMP upregulators was able to induce an ~40% loss in cholesterol ($86.9 \pm 2.0\%$ vs $44.7 \pm 1.9\%$ BODIPY-cholesterol remaining, respectively) by 180 min of incubation. In contrast, a lack of cholesterol efflux was observed when spermatozoa were incubated in TALP with 2% sheep serum devoid of cAMP upregulators, where similar levels of BODIPY-cholesterol labeling remained in the spermatozoa when compared with TALP without sheep serum ($82.9 \pm 1.5\%$ vs $86.9 \pm 2.0\%$, respectively; Figure 2A). This observation further confirms the necessity of high-cAMP conditions alongside the presence of cholesterol acceptors to support cholesterol efflux from ram spermatozoa [24]. There was a small, yet significant additive effect of 2% sheep serum with 0.3% FAF-BSA as observed by the increase in cholesterol lost from spermatozoa incubated in this condition, which was greater than when these components were supplemented alone ($P < 0.05$; Figure 2A).

To confirm that the loss of BODIPY-cholesterol corresponded with the presence of other capacitation-related processes, membrane fluidity (early capacitation event [10, 53]) and tyrosine phosphorylation (late capacitation event [54, 55]) were also assessed. In this experiment, exposure to conditions with cAMP upregulators stimulated a significant increase in the percentage of spermatozoa with high membrane fluidity over the incubation period (Figure 2B; $P < 0.001$). A similar finding was observed with respect to the presence of tyrosine phosphorylation, where only conditions with cAMP upregulators were able to support phosphorylation of high-molecular weight proteins (75–250 kDa) (Figure 2C). The percentage of acrosome reacted, membrane-damaged cells did not greatly differ depending on the media condition, and the percentage of membrane and acrosome intact spermatozoa remained consistent across all conditions (data not shown).

HDLs are able to support cholesterol efflux

Sheep serum was able to successfully elicit cholesterol efflux from ram spermatozoa. It was presumed that this was owing to the activity of cholesterol acceptors, such as HDL and apoA1, which are abundant in serum. For this reason, we specifically examined the role of HDLs and apoA1 as cholesterol acceptors in ram spermatozoa and whether these components were able to support other capacitation-related processes, including changes in membrane fluidity and tyrosine phosphorylation. When HDLs were supplemented with 0.3% FAF-BSA under high-cAMP conditions for 180 min, there was a significant ~30% loss of cholesterol, compared with media devoid of cholesterol acceptors ($46.1 \pm 3.0\%$ vs $79.1 \pm 2.4\%$ BODIPY-cholesterol remaining) and approximately an extra 8% loss compared with when FAF-BSA was the sole cholesterol acceptor present ($46.1 \pm 3.0\%$ vs $54.0 \pm 3.6\%$ BODIPY-cholesterol remaining; Figure 3A; $P < 0.05$). Upon examining the capacity of HDLs alone under high-cAMP conditions, the addition of this lipoprotein was able to support cholesterol efflux at a similar rate to 0.3% FAF-BSA ($59.3 \pm 1.6\%$ vs $54.0 \pm 3.6\%$ BODIPY-cholesterol remaining; Figure 3A). The purified apoA1 protein did not appear to induce cholesterol efflux even under high-cAMP conditions. This was illustrated by the apparent lack of additional cholesterol efflux when purified apoA1 was combined with FAF-BSA, HDLs, or in combination with both (Figure 3A).

With evidence of cholesterol efflux in the vast majority of conditions, it was important to assess other indicators of capacitation in

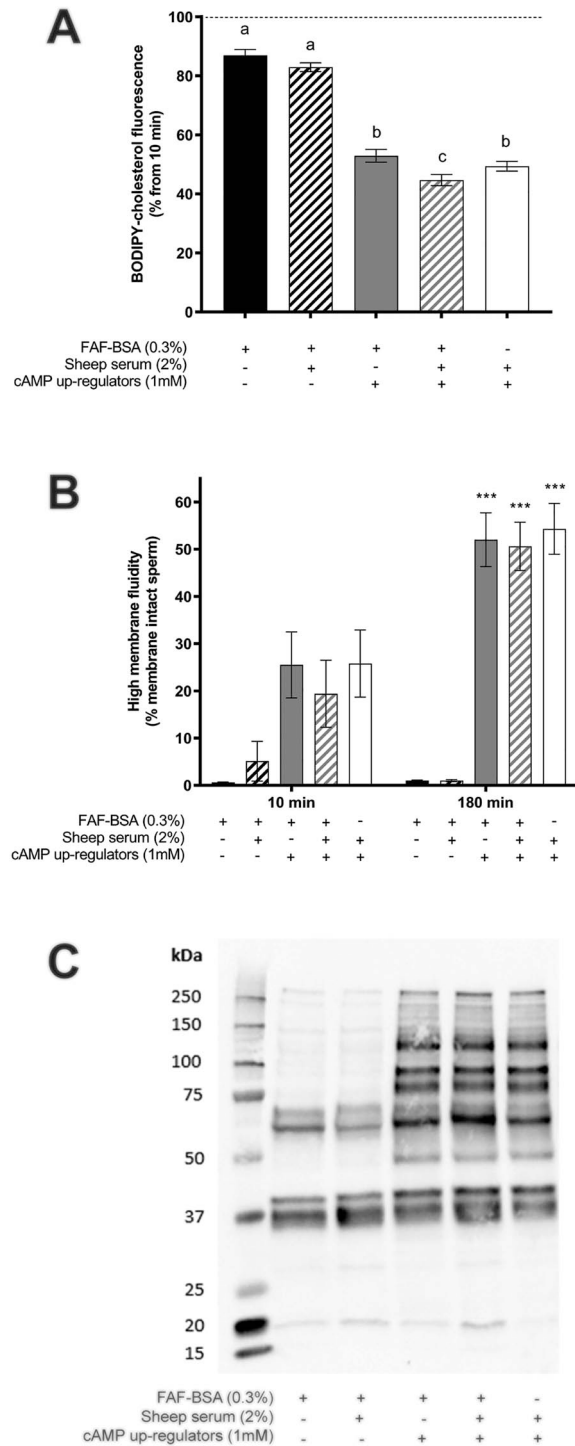


Figure 2. The percentage of BODIPY-cholesterol fluorescence remaining (A), changes in membrane fluidity (B), and the presence of tyrosine phosphorylation (C) in ram spermatozoa following exposure to various combinations of FAF-BSA, sheep serum, and cAMP up-regulators for up to 180 min of incubation. Tyrosine phosphorylation was examined under these conditions at 180 min only. Data were analyzed with linear mixed model regression. Results are based on nine independent samples ($n = 9$) and are presented as mean \pm SEM. Letters denote significant differences between treatments ($P < 0.05$) or $***P < 0.001$ indicates differences across incubation time within a treatment. Western blots were replicated three times and a representative blot is shown.

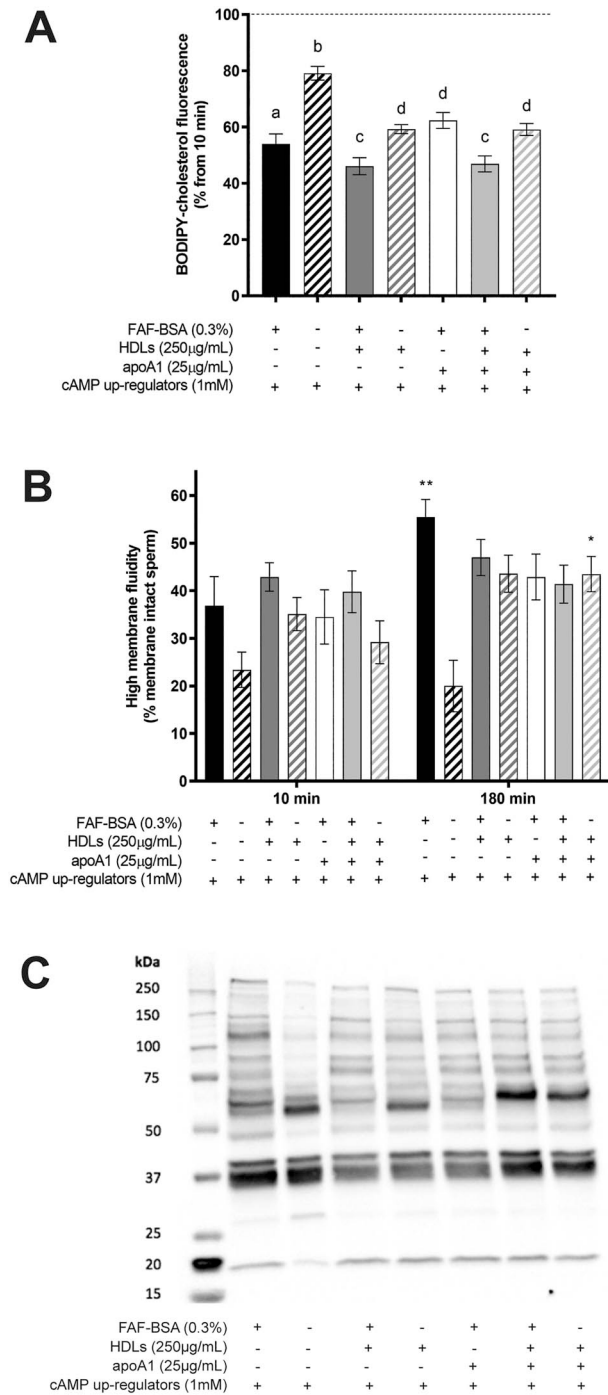


Figure 3. The percentage of BODIPY-cholesterol fluorescence remaining (A), changes in membrane fluidity (B), and the presence of tyrosine phosphorylation (C) in ram spermatozoa following exposure to cAMP up-regulated conditions and various combinations of FAF-BSA, HDLs, and apoA1 for up to 180 min. Tyrosine phosphorylation was examined under these conditions at 180 min only (C). Data were analyzed with linear mixed model regression. Results are based on nine independent samples ($n = 9$) and are presented as mean \pm SEM. Letters denote significant differences between treatments ($P < 0.05$) or $*P < 0.05$ and $**P < 0.01$ indicates differences across incubation time within a treatment. Western blots were replicated three times and a representative blot is shown.

order to verify these results. High membrane fluidity was observed in at least 30% of membrane intact cells across all conditions and for the duration of the incubation period (Figure 3B). The exception to this was when 0.3% FAF-BSA and HDLs were omitted from media. This is likely attributable to the increase in the percentage of spermatozoa with a loss of membrane integrity (data not shown; 10 min: $54.2 \pm 20.7\%$; 180 min: $79.9 \pm 16.5\%$). Since cAMP upregulators were present in all conditions, tyrosine phosphorylation of high-molecular weight proteins was evident after incubation. An increased level of tyrosine phosphorylation, compared with TALP with cAMP upregulators alone, was noted for any combination of supplemented FAF-BSA, HDL, or apoA1 (Figure 3C).

Finally, the addition of HDLs was able to maintain a higher level of membrane and acrosome integrity (as assessed by PI and PNA-FITC, respectively) when compared with those conditions devoid of this cholesterol acceptor, with the lowest integrity observed in media without any cholesterol acceptors (Supplementary Figure S1). Furthermore, acrosome reactivity in membrane-damaged spermatozoa was also found to differ after exposure to the various conditions, but this did not exceed $\sim 10\%$ (Supplementary Figure S1).

Exposure to HDLs stimulates hyperactivation in ram spermatozoa

In addition to investigating the role of HDLs and apoA1 as cholesterol acceptors from ram spermatozoa, we were interested in their ability to stimulate hyperactivation. Remarkably, a consequence of supplementing HDLs to media, whether alone or in combination with other cholesterol acceptors, was the apparent induction of hyperactivated motility. According to the final set of thresholds determined for the kinematic parameters of interest, spermatozoa presenting with an ALH $>9 \mu\text{m}$, VCL $>250 \mu\text{m/s}$, and LIN $<65\%$ were identified as hyperactive (Figure 4A and B). These cells also exhibited a whiplash-like flagella motion that is consistent with this type of motility [56] and a star-shaped or tight, circular trajectory (Figure 4C). The thresholds set in this study were within the range of those that have been previously used to detect hyperactivation in ram spermatozoa [57]. When spermatozoa were incubated in media supplemented with HDLs, the percentage of hyperactive spermatozoa was significantly increased when compared with media with or without FAF-BSA or with purified apoA1 included (Figure 5; $P < 0.05$). Of all HDL-based conditions, hyperactivation was greatest in ram spermatozoa that were only exposed to HDL as the primary cholesterol acceptor (36.7% [CI 95%: 23.9–47.4%]).

Cholesterol efflux from ram spermatozoa is marginally affected following inhibition of ABCA1 and SR-BI

With evidence of sheep serum and HDL-mediated cholesterol efflux present in ram spermatozoa, the next step was to attempt to identify mechanisms that may prepare cholesterol for removal. For this preliminary experiment, glibenclamide and valsopodar were selected to inhibit cholesterol efflux via the activity of both ABCA1 and SR-BI or ABCA1 alone, respectively. Upon the addition of glibenclamide or valsopodar with 2% sheep serum or HDLs, cholesterol efflux was reduced by ~ 8 –15% compared with the absence of these antagonists ($P < 0.05$). There was no apparent effect of the inclusion of these antagonists on capacitation, as observed by the increase in tyrosine phosphorylation across all conditions (data not shown). The percentage of spermatozoa with an intact plasma membrane and acrosome was consistent across all conditions and was between 50 and 55% (data not shown).

Discussion

Sheep serum is imperative for successful ovine IVF, though there are very few studies that have elucidated the exact role of this biological fluid, especially in the context of ram sperm capacitation. It is theorized that serum-derived components like HDLs are functioning alongside serum albumin as cholesterol acceptors during cholesterol efflux, yet there is currently no solid evidence to support this or knowledge of how HDL-mediated cholesterol efflux from ram spermatozoa is regulated. In this study, we not only confirm that sheep serum has cholesterol efflux capabilities, but reveal that HDLs have a dual action in ram spermatozoa, promoting both cholesterol efflux and hyperactivation. In addition, our results showed a marginal but significant decrease in sheep serum or HDL-mediated cholesterol efflux when SR-BI and/or ABCA1 inhibitors were present. The specific cholesterol transporters are thus still to be determined in ram spermatozoa. In this light, a recent analysis of the ram sperm plasma membrane proteome is of interest as it reported on the presence of the transporters, ABCA3 and ABCA3L [58]. Given the structural homology between transporters of the ABC A-subfamily and the reported function in lipid (as well as cholesterol) transport [59–61], it is well possible these transporters may function in cholesterol efflux from ram sperm to cholesterol acceptors, like HDLs.

According to the results, sheep serum was able to effectively support cholesterol efflux under high-cAMP conditions at a rate that was similar to 0.3% FAF-BSA alone. This finding now clarifies one of the vital functions of sheep serum when supplemented to ovine IVF medium. A similar rate of cholesterol efflux was observed with HDLs alone, whereas a combination of HDLs and 0.3% FAF-BSA caused additional cholesterol efflux. Our group has previously reported on the capacity for cholesterol efflux from ram spermatozoa while in the presence of high-cAMP conditions and a suitable cholesterol acceptor [24], and this is only further confirmed by the current study. It is important to recognize that the addition of FAF-BSA in this study is not meant to represent the action of native serum albumin during cholesterol efflux from ram spermatozoa. In fact, albumin derived from serum or reproductive fluids has been shown to have a lower capacity for cholesterol efflux from spermatozoa in comparison with native HDLs [20, 21]. In parallel with observed cholesterol efflux, the percentage of spermatozoa with high membrane fluidity was increased across all conditions where there was evidence of sheep serum or HDL-mediated cholesterol efflux. It is well established that an increase in membrane fluidity is vital to prime the plasma membrane for cholesterol efflux and that this process is driven by the cAMP-PKA pathway [11, 53]. When comparing this study with others in the literature, HDLs have already been shown to function as effective cholesterol acceptors following exposure to spermatozoa during capacitation [16, 21, 30, 31], though this is the first report in sheep. Unlike HDLs, the purified apoA1 protein used in this study had no obvious effect on cholesterol efflux despite several reports that lipid-poor apoA1 has been shown to support this process in somatic cells [46, 62, 63]. It is possible that this protein alone is ineffective at removing cholesterol from the plasma membrane of ram spermatozoa, compared with more developed HDL subtypes. Although, this idea remains to be confirmed since there is a lack of studies that have investigated the effect of apoA1 on spermatozoa, but in those that have, apoA1 is usually included within prepared liposomes prior to incubation [64]. In any case, the fact that HDLs were able to stimulate cholesterol efflux under conditions that support capacitation verifies the function of these lipoproteins as a cholesterol acceptor for this species.

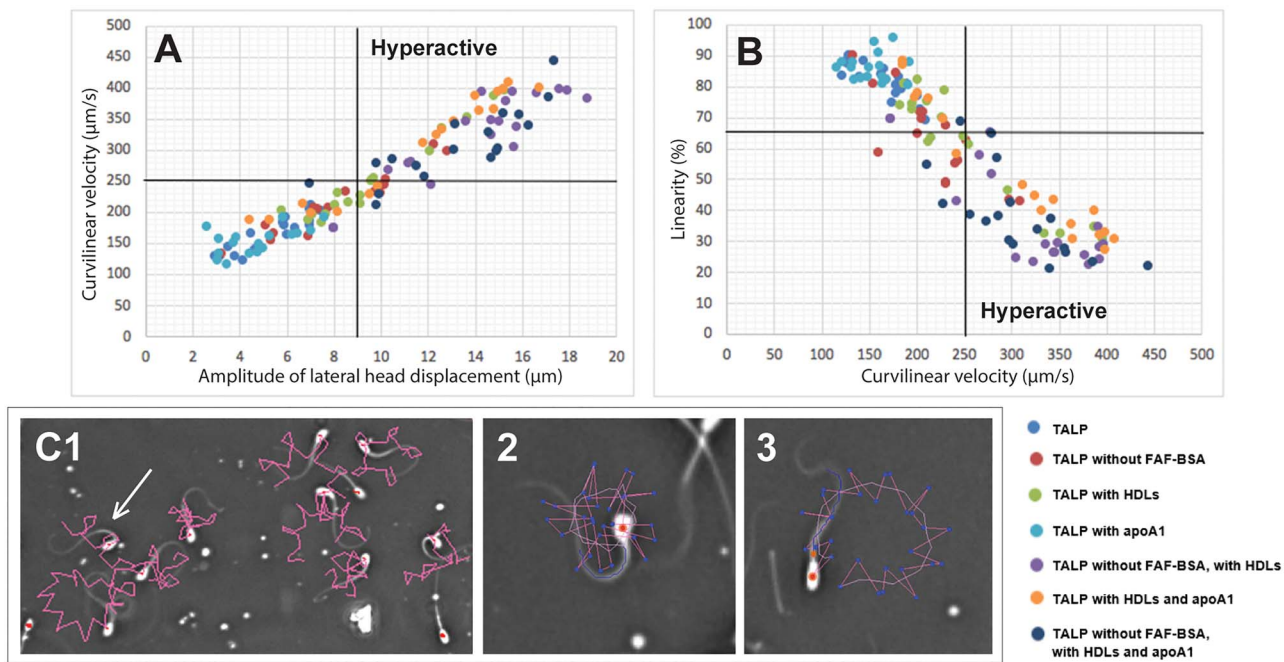


Figure 4. Complete scatterplots of curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), and linearity (LIN) used to determine thresholds (A and B) that identified hyperactive spermatozoa using CASA (C). Spermatozoa were identified as hyperactive when ALH >9 µm, VCL >250 µm/s, and LIN <65%. All conditions containing HDLs (green, purple, orange, and navy dots) elicited hyperactivity in a proportion of ram spermatozoa as shown by the increase in ALH, VCL, and decrease in LIN (A: upper right quadrant; B: lower right quadrant). Note that cAMP upregulators are present in all conditions and 0.3% FAF-BSA is supplemented, unless otherwise stated. The trajectories shown in pink are representative of hyperactivated ram spermatozoa as detected with CASA (C1). These spermatozoa presented the classic whiplash-like flagella motion of hyperactive spermatozoa (shown by arrow), and in most cases, illustrated a representative star-shaped (2) or tight, circular trajectory (3).

Aside from the capacity for HDL to be an effective cholesterol acceptor in ram spermatozoa, incubation with HDLs and cAMP upregulators also resulted in the stimulation of hyperactivated motility. This marks the first report of HDL-induced hyperactivation in ram spermatozoa. In fact, the only evidence of this phenomenon is documented in humans, where HDLs (derived from follicular fluid) both mediated cholesterol efflux and stimulated hyperactivated motility in ~15% of spermatozoa upon contact with these lipoproteins [30]. The significance of HDL-induced hyperactivation is twofold. First, hyperactivated motility can only be stimulated during successful progression through capacitation. Second, it acts as the propellant spermatozoa require to efficiently penetrate the cumulus matrix and zona pellucida of the mature oocyte in order to fuse with the oolemma [32, 56], an event that concerns both *in vivo* and IVF. Bearing this in mind, it only seems plausible that the necessity of sheep serum for successful ovine IVF is likely associated with the presence of HDLs, which is simply owing to the fact that this serum-derived component is able to support more than one event leading up to fertilization. Although further research is required to verify the ability for sheep serum alone to induce hyperactivation in this species, the results of the study clearly demonstrate the potential of utilizing isolated HDLs as a supplement for *in vitro* media that is formulated for capacitation and/or IVF. These lipoproteins may not only act as a standardized alternative for variable, biological mixtures like serum, but it could also be one of the key components required to support IVF in species where this technology is not currently successful, such as in the horse [65]. In further support of this idea, the presence of HDLs was able to maintain a high level of sperm viability and acrosome integrity over the incubation period,

thus indicating the positive effect of these lipoproteins on overall cell function. However, specific investigation of the effect of HDLs on IVF success in a range of species is necessary before HDLs can be widely applied in a practical setting.

As this study is only one of two that have observed HDL-mediated cholesterol efflux and hyperactivation, the exact underlying mechanisms that are responsible for modulating this interaction in spermatozoa are currently unknown. One conceivable explanation may be that HDLs are able to evoke hyperactivated motility in the sperm flagellum simply by modifying the lipid composition following cholesterol efflux. Lipids have recently emerged as critical regulators of flagella or cilia function, whereby the distinct lipid composition and compartmentalization of the ciliary membrane is known to be essential for ion channel activity and transduction of cilia-based signaling cascades [66]. Since the trigger and maintenance of hyperactivation is dependent on the influx of Ca²⁺ through designated channels, like CatSper [67], the relationship between lipid composition and ion channel activity could potentially explain the dual action of HDLs in ram spermatozoa. From a broader cell biological perspective, the effect of HDLs as observed in the current study may also be relevant in ciliated epithelial cells and thus in fluid movement dynamics in the oviduct or even in cells outside the reproductive tract, such as in the gut or lung. Taking this theory into consideration, it is intriguing that FAF-BSA could not induce an analogous hyperactivated response in ram spermatozoa exposed to high-cAMP conditions. This is in spite of the fact that both HDLs and FAF-BSA had a comparable effect on capacitation-related processes, such as supporting an increase in membrane fluidity and tyrosine phosphorylation as well as mediating cholesterol efflux at

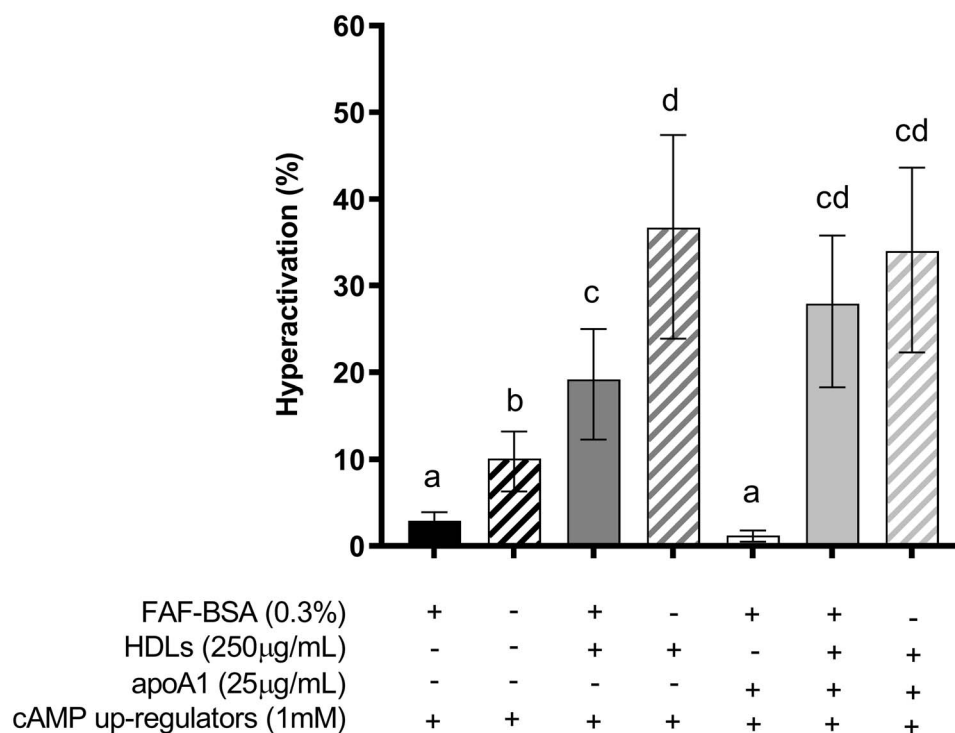


Figure 5. The percentage of ram spermatozoa presenting hyperactivated motility following exposure to cAMP upregulated conditions and various combinations of FAF-BSA, HDLs, and apoA1 for 180 min. The supplementation of HDLs was able to support a significantly increased proportion of cells with hyperactivation, particularly when ram spermatozoa were incubated with HDLs as the sole cholesterol acceptor (dark gray hatched bar). Data were analyzed with linear mixed model regression. Results are based on nine independent samples ($n = 9$) and are presented as the geometric mean \pm 95% CI. Letters denote significant differences between treatments ($P < 0.05$).

a similar rate. It is possible that the mode of cholesterol transport and removal from the plasma membrane could play a role in this disparity, though a targeted assessment is required to elucidate the mechanistic association between HDL-mediated cholesterol efflux and hyperactivation.

In summary, by exploring the role of sheep serum during in vitro capacitation of ram spermatozoa, we were able to confirm that this complex mixture has the capacity to support cholesterol efflux, and that HDLs are likely to be one of the components present in sheep serum that is mediating this regulated process. Of considerable interest is the observation that HDLs, unlike FAF-BSA or apoA1, could induce hyperactivated motility, an essential feature that spermatozoa must attain to fertilize the oocyte. This finding also has potential practical implications, where HDLs may be an optimal replacement for complex supplements, like serum, in IVF systems. From the data presented in the current study, it is clear that future research ought to address the interaction between HDLs and spermatozoa with respect to both inducing the cholesterol efflux and how this may cause the activation of signaling pathways involved in the stimulation of hyperactivation. Taking the collective findings of this study together, it brings us one step closer to understanding the complexities of cholesterol efflux from ram spermatozoa both in vitro and vivo.

Supplementary material

Supplementary material is available at *BIOLRE* online.

Author' Contributions

B.M.G and T.L. conceptualized the study. N.C.B. designed, performed, and analyzed all experiments, with assistance from T.L. The manuscript and figures were prepared by N.C.B. and critically reviewed by S.P. de G., T.L., and B.M.G. Funding was acquired by S.P. de G., T.L., and B.M.G. All authors reviewed the manuscript and approved the final version of the manuscript.

Acknowledgment

The authors would like to thank Mr. Cameron Sharpe, Mr. Keith Tribe, and Mr. Manuel Menouhos for their assistance with the rams as well as Kelsey Pool and Emmah Tumeth for their technical assistance.

Conflict of interest

The authors have declared that no conflict of interest exists.

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