BULL SPERMATOZOA EXPRESS RECEPTORS FOR PLATELET-ACTIVATING FACTOR

Los Espermatozoides Bovinos Expresan Receptores para el Factor Activador de Plaquetas

Pablo Bosch [1]***, William E. Roudebush*** [2]***, Royal A. McGraw*** [1]***, J. Mel DeJarnette*** [3]***, Clifton E. Marshall*** [3]***, Joe B. Massey*** [2] ***and Benjamin G. Brackett*** [1]

[1] *Department of Physiology and Pharmacology, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602, USA.* [2] *Reproductive Biology Associates, Atlanta, GA 30342, USA.* [3] *Select Sires Inc., Plain City, OH 43064, USA. E-mail: boschp@gmail.com Investigador Adjunto CONICET. Current address: Departamento de Biología Molecular, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nac. 36 - Km. 601- Código Postal X5804BYA, Río Cuarto, Córdoba, Argentina Tel: +54-0358-4676425, FAX: +54-0358-4676232.*

ABSTRACT

Platelet-activating factor (PAF; 1-*O*-Alkyl-2-acetyl-*sn*-glycero--3-phosphorylcholine) is a ubiquitous phospholipid that is implicated in the mediation of a wide variety of reproductive processes. To better understand the role of PAF in bovine reproduction, it was designed experiments to: (a) determine whether bull spermatozoa express receptors for PAF and (b) study the effect of exogenous PAF on in vitro sperm physiology (i.e., capacitation, acrosome reaction, motility, and fertilizing ability). Bull sperm express PAF receptor as determined by two approaches: RT-PCR and immunofluorescence. However, exposure of spermatozoa to different concentrations of exogenous PAF (10^{-11}-10^{-6} M) did not affect capacitation, acrosome reaction or motility. Consistent with these findings, coculture of gametes in medium containing increasing concentrations of PAF (1 x 10^{-8}-8 x 10^{-6} M) did not improve in vitro fertilization outcome as measured by percentage of inseminated oocytes reaching 2-cell stage 48 h after fertilization. In contrast, PAF at 8 x 10^{-6} M concentration significantly inhibited IVF. In conclusion, although bull sperm have PAF receptors, exposure of bull spermatozoa to exogenous PAF failed to enhance the sperm function parameters measured in this study. Additional studies are warranted to elucidate the biological role of PAF on bull spermatozoa.

Key words: Bull, spermatozoa, PAF, PAF receptor.

RESUMEN

El factor activador de plaquetas (PAF; del inglés Platelete Activating Factor; 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) es un fosfolípido ampliamente distribuido que participa como mensajero mediador en diferentes procesos reproductivos. Para comprender mejor la participación del PAF en la fisiología espermática bovina se diseñaron experimentos para: (a) determinar si los espermatozoides de toro expresan receptores para PAF y (b) estudiar el efecto del PAF sobre el comportamiento de los espermatozoides bovinos in vitro (capacitación, reacción acrosomal y capacidad fertilizante). De acuerdo a los resultados obtenidos por RT-PCR e inmunofluorescencia, los espermatozoides de toro expresan receptores para PAF. Sin embargo, la exposición de los espermatozoides a concentraciones crecientes de PAF exógeno (10^{-11}-10^{-6} M) no afectó la capacitación, reacción de acrosoma ni la motilidad. En concordancia con estos hallazgos, el cocultivo de gametas (ovocitos y espermatozoides) en medio al cual se le había adicionado PAF (1 x 10^{-8}-8 x 10^{-6} M) no mejoró la tasa de fertilización medida como el porcentaje de ovocitos inseminados que alcanzaron el estadio de 2 células 48 hs después de la inseminación. Por el contrario, PAF a una concentración de 8 x 10^{-6} M inhibió significativamente la tasa de fertilización. En conclusión, a pesar de que los espermatozoides bovinos poseen receptores para PAF, el agregado de PAF al medio de cultivo no mejora las funciones espermáticas examinadas en el presente trabajo. Otros estudios serán necesarios para dilucidar la participación del PAF en la fisiología espermática del toro.

Palabras clave: Toro, espermatozoide, receptor, factor activador de plaquetas.

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INTRODUCTION

Platelet activating factor (PAF,1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) is a signaling phospholipid with a broad range of physiological effects on different processes such as circulation [9], inflammation [42], development [21] and reproduction [14]. Abundant evidence indicates that PAF plays a preponderant role in many reproductive processes such as ovulation, fertilization, embryo development, implantation and parturition [10]. Mouse (*Mus musculus*) and human sperm cells possess receptors for PAF [29, 41] and can respond with enhanced motility when exposed to this phospholipid [11, 15], particularly sperm samples with poor initial motility [3, 4, 30]. Conversely, others were unable to demonstrate any effect of exogenous PAF on sperm motility parameters as assayed by a computer-assisted sperm analysis system [2, 23]. Improvements in fertilization [19] and embryonic development after addition of PAF to the culture media have been reported [20, 37]. Several studies showed that PAF can induce sperm capacitation [12] and/or the acrosome reaction AR [3, 11] and these effects explain, at least partially, the observed beneficial effect of PAF on in vitro fertilization [23]. A differential response to PAF in terms of motility and AR has been reported for bull (*Bos taurus*) and buffalo (*Bubalus bubalis*) sperm depending on the origin, i.e., fresh or frozen [3, 4]. In these studies [3, 4] frozen sperm responded to PAF with decreased motility and increased ARs, but a poor response in the same variables was observed in frozen-thawed sperm samples similarly treated. In one study spermatozoa responded to exogenous PAF with increased ARs and fertilization rates [23] equivalent to sperm treated with heparin, a widely used effector of bull sperm capacitation. However, the effect of PAF on capacitation of bull spermatozoa in vitro has not been investigated.

Wu et al. [41] have proposed an interesting model for mouse sperm capacitation in which PAF plays a pivotal role as an autocrine mediator. In this model, PAF released by sperm cells binds to specific receptors on the plasma membrane promoting capacitation; a PAF-acetylhydrolase (enzyme present in the seminal plasma) acts as a decapacitating factor since it degrades PAF to an inactive form [36]. Since bull spermatozoa contain PAF [22] and PAF-acetylhydrolase is present in seminal plasma [24], the mouse model might also apply to bovine *Bos taurus-indicus* species.

The aims of this study were: (a) to determine whether bull spermatozoa express receptors for PAF, (b) to study the effect of PAF at different concentrations and exposure times on bull sperm capacitation, AR, motility and *in vitro* fertilizing ability.

MATERIALS AND METHODS

Determination of transcripts and immunoreactive protein for PAF receptor in sperm

Indirect immunocytochemistry

To determine the presence of PAF receptors in bull spermatozoa, frozen semen was thawed and washed 3X in PBS pH 7.4. An aliquot of this suspension was dried on a microscope slide at room temperature. Sperm cells were fixed/permeabilized with 100% methanol for 30 min at –20°C followed by washing (3x) in PBS. Antigen retrieval was induced by microwaving the slides submerged in a solution of 0.01 M citric acid for 15 min. Subsequently, sperm cells were incubated with either a mouse monoclonal antibody specific for human PAF receptor (1:50 dilution; Cayman Chemical, Ann Arbor, MI, USA), or isotype control (Mouse IgG2a, κ; Sigma Chemical Co., Saint Louis, MI, USA), or PBS (negative control) for 3 h followed by washing in D-PBS. Specimens were then incubated with a FITC-conjugated secondary antibody (anti-mouse IgG FITC conjugate, 1:100 dilution; Sigma Chemical Co. Saint Louis, MI, USA) for 90 min at 37°C. After washing twice in D-PBS, samples were mounted with 1,4-diazabicycol-(2.2.2) octane (DABCO), covered with a coverslip and sealed. Specimens were examined under an epifluorescent microscope at 400 x magnification.

RT-PCR

RNA was extracted utilizing the Micro RNA Isolation Kit (Stratagene Cloning Systems, La Jolla, CA, USA) per manufacturer's instructions. Total RNA recovered was determined by spectrophotometry. Spermatozoal RNA was placed into chilled RT buffer [4dNTP chase solution (10mM each dNTP), RNase inhibitor (40 units/µL), RNA (1 µg), M-MuLV RT (200 units/µL); as control for genomic DNA contamination a RT- reaction in which M-MuLV RT was replaced by the same amount of water was simultaneously run with random primers. RT was carried out at 42°C, 60 min, then the reaction was stopped by heating at 95°C, 5 min. The RT products were amplified in PCR reaction buffer: PAF-receptor specific primer pairs (0.4 µM each; forward, 5'-GTGGATTCTGAGTTTCGATACAC-3'; reverse, 3'-CAGTAGTAGGTGTGCGACGAG-5', from GeneBank accession number: XM_357441), 10X PCR buffer, dNTP mix (0.4 µM each), Taq polymerase (0.04 µM), and $MgCl_2$ (2 µM). The reaction mixtures were amplified for 40 cycles at: 94°C, 30 sec; 60°C, 1 min; 72°C, 1 min. Following the final PCR round, the samples were heated for 5 min at 72°C, and then held at 4°C. The RT-PCR products were visualized following 1.5% agarose gel electrophoresis with ethidium bromide and UV transillumination.

Preparation of PAF and Lyso-PAF for in vitro studies

Synthetic PAF-16 (1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3--phosphocholine) and lyso-PAF-16 (1-*O*-Hexadecyl-*sn*- glycero-3-phosphocholine) were obtained from Calbiochem-Novabiochem Corporation, La Jolla, CA, USA. Both phospholipids were resuspended in absolute ethanol to obtain a 1 x 10^{-2} M stock solution [37] which was stored at -20°C and used within 2 wk. Working solutions were prepared immediately before use by serial dilutions of stocks with appropriate medium (Sperm TALP or IVF-TALP) in siliconized glass tubes.

The biological activity of the exogenous PAF was demonstrated in parallel studies involving human sperm motility and intracellular calcium assays as previously described [28, 31]. Exogenous PAF was found to increase track speed (11.4%), lateral head displacement (40%) and linearity (24.3%) and also, to cause a 2-fold rise in intracellular calcium.

Effects of PAF on sperm capacitation, acrosome reaction and motility

Frozen or fresh (refrigerated) bull semen was obtained from 2 Holstein bulls (Select Sires Inc., Plain City, OH, USA). The motile sperm fraction was separated by swim-up in sperm TALP medium (sp-TALP) [26]. In this procedure frozen-thawed or refrigerated semen (100 µL) is transferred to the bottom of a tube containing 1 mL of pre-equilibrated sp-TALP. After 1 h in the incubator (38.5°C, 5% CO_2 in air), the top 800 µL containing motile sperm were removed, centrifuged and the sperm concentration adjusted to 2 x 10^6 sperm/mL in sp-TALP. Alternatively, Percoll® separation was used to select the motile fraction from the sperm samples [25]. Experiment 1 was performed with frozen-thawed spermatozoa. Aliquots of sperm suspension were treated with PAF at the following final concentrations: 0, 1 x 10^{-6}, 1 x 10^{-7}, 1 x 10^{-8} and 1 x 10^{-9} M and Lyso-PAF (a biologically inactive metabolite of PAF) at 1 x 10^{-7} M. At 0, 1, 2 and 4 h after addition of treatments, an aliquot of sperm suspension was taken from each treatment to assess sperm capacitaton, AR and motility. Percentage of capacitated and AR spermatozoa were determined by chlortetracycline stain patterns as previously described [7]. Alternatively, to confirm results obtained with chlortetracycline procedure sperm samples were stained with Coomassie Blue following the method described elsewhere [5] to determine the percentage of AR spermatozoa. Sperm motility was assessed at 38°C in a hematocytometer chamber (BOECO, Neubaüer Improved Counting Chamber, Hamburg, Germany) loaded with the sperm suspension. Only the number of non-motile spermatozoa was recorded. Subsequently, all sperm cells (total number) were counted on a hematocytometer loaded with sperm rendered immotile by exposure to low temperature (4°C). The percentage of sperm that was motile was determined after subtracting the number of non-motile sperm from the total number determined in the sample rendered non-motile. The effect of PAF on sperm capacitation, AR and motility was assessed in experiment 2 in which refrigerated bull sperm was exposed to different concentrations of PAF (0, 1 x 10^{-11}, 1 x 10^{-9}, 1 x 10^{-7} or 1 x 10^{-6} M) or Lyso-PAF (1 x 10^{-7} M). Semen for experiment 2 was collected by artificial vagina, diluted 1:3 in 2.9% sodium citrate buffer containing 20% egg-yolk, and stored 24 h at 4°C until the study was conducted as described above for frozen-thawed spermatozoa.

Effect of PAF on bovine IVF

In vitro maturation, fertilization and embryo culture was performed as previously described [16]. Oocytes were collected from abattoir-derived ovaries and matured in TCM-199 (M-0650; Sigma Chemical Co., St Louis, MO, USA) supplemented with 0.1 IU/mL of recombinant human FSH (1.7 IU/µg; Ares Advanced Technology Inc., Randolph, MA, USA), 5 ng/mL of recombinant human IGF-I (Promega, Madison, WI, USA), and fetal calf serum (Atlanta Biologicals, Atlanta, GA, USA). Oocyte maturation was performed at 38.5°C under moist 5% CO_2, 5% O_2 and 90% N_2 for 22 h in a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA, USA).

Frozen semen from Holstein bulls (n=2), specially processed for the laboratory with 10^8 sperm/straw, was used in the IVF experiments. Swim-up selected spermatozoa were prepared in low bicarbonate-Tyrode-Albumin-Lactate-Pyruvate (TALP) [26] modified to contain 5.15 mM caffeine, 3.35 mM D-penicillamine and 1 mg/mL polyvinyl alcohol (PVA). Final sperm concentration for fertilization was adjusted to 2 x 10^6 motile sperm per mL in Fert-TALP containing 5 mM glucose, 5 mM HEPES, 3.35 mM D-penicillamine and 3 mg/mL BSA (experiment 1) or 1 mg/mL PVA (experiment 2). In experiment 1 spermatozoa were incubated for 10-15 min in medium containing PAF (0, 10^{-8}, 10^{-7}, or 10^{-6} M) or Lyso-PAF (10^{-7} M) before adding to the oocyte-containing drops (fertilization). In experiment 2, sperm was incubated for ~5 min with increasing concentrations of PAF (Control, 2 x 10^{-6} M, 4 x 10^{-6} M, and 8 x 10^{-6} M) and then added to fertilization drops. Therefore gametes were exposed during fertilization to 1/10^{th} of the corresponding PAF concentration in the sperm suspension. As positive control for fertilization, a subset of female and male gametes was exposed to 10 µg/mL of heparin during fertilization. Matured oocytes were cocultured with sperm for 18 h in Fert-TALP. After 18 h, presumptive zygotes were cultured in groups of 20 in 50 µL drops of synthetic oviductal fluid (SOF) supplemented with 0.1 mM non-essential amino acids (M7145; Sigma Chemical Co., St Louis, MO, USA), 0.5 mM glutamine, 0.4 mM threonine, and 3 mg/mL PVA. The end point for this Experiment was the percentage of oocytes reaching the 2-cell stage by 48 h post insemination.

Statistical analyses

The effects of PAF on the percentages of capacitated, acrosome reacted and motile sperm were analyzed as a repeated measures experiment using the MIXED procedure of the Statistical Analysis System [33]. Three different structures, i.e., compound symmetry, unstructured and autoregressive

were fitted to the sperm function data in order to model the covariance. These covariance structures were objectively compared using Schwarz Bayesian criterion values printed by PROC MIX. Based on this criterion the structure that best fit the sperm function data was chosen as covariance structure. The two-factor model included variation due to PAF treatment (control, Lyso-PAF and different PAF concentrations), time (0, 1, 2 and 4 h) and their interaction. IVF data consisting of proportions of 2-cell embryos were analyzed by Chi-square with $P < 0.05$ taken as significant.

RESULTS AND DISCUSSION

Determination of transcripts and immunoreactive protein for PAF receptor in sperm

PAF's action on target cells is mediated through a well characterized receptor [38] which belongs to the G protein-linked superfamily of plasma membrane proteins with seven transmembrane spanning domains. Following activation of PAF receptors, diverse intracellular signaling pathways can be activated [Reviewed in 6]. For instance, PAF receptor activation in sperm and embryos has been associated with an increase of intracellular calcium levels [8, 28] reflecting the sequential activation of phospholipase C and inositol 3 phosphate [8]. Less is known about a second class of PAF receptor with intracellular localization which has been associated with neural function [13]. The present RT-PCR results indicated that transcripts for PAF receptor are present in bull spermatozoa (FIG. 1). A PCR product with the right size (618 bp) was present (FIG. 1, lane 3) when reverse transcriptase was used (RT+), whereas no band was present in the RT- reaction in which reverse transcriptase was omitted (FIG. 1, lane 2).

In addition, bovine sperm cells express PAF receptor as evidenced by immunofluorescence analysis (FIG. 2). Distribution of PAF receptors on the sperm surface was not uniform (FIG. 2A); while in some sperm immunoreactive PAF receptors were distributed on almost the entire surface, in others only the head or the tail was stained. Acrosomal caps were either intensely fluorescent or not fluorescent at all. Isotype and no primary antibody control slides had only faint fluorescence (FIG. 2C).

In human and bovine spermatozoa, PAF receptor distribution was associated with sperm morphology [29, 32, 36, 39]. Abnormal human spermatozoa had reduced fluorescence on the neck region compared with the normal counterparts [32] and defective bull spermatozoa displayed reduced immunoreactivity to anti-PAF antibodies [39]. The centriole, a sperm structure that plays a critical role in early embryo development, is located in the neck region. It has been suggested that PAF may have a stimulatory effect on centriole-rich spermatozoa, accounting for the observed beneficial effect of sperm exposure to PAF on early embryonic development previously reported [19]. At this point, it is not clear whether the distribution characteristics of PAF receptor observed in bull spermatozoa in our study are of biological significance.

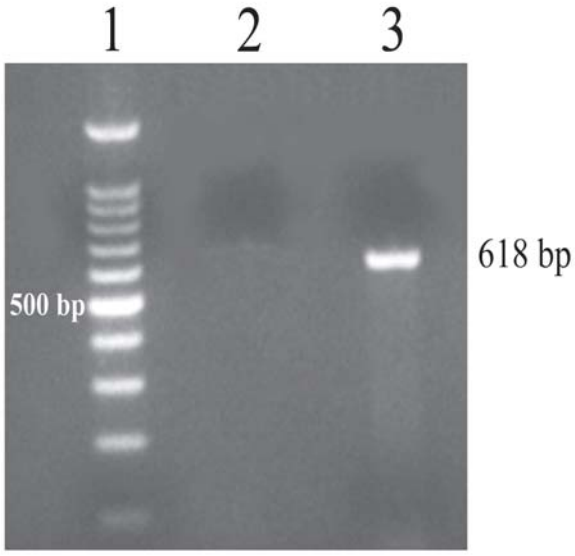

FIGURE 1. PHOTOGRAPH OF AGAROSE GEL SHOWING PCR PRODUCTS ORIGINATED BY REVERSE TRANSCRIPTION OF TOTAL SPERMATOZOAL RNA. LANE 1 WAS LOADED WITH MOLECULAR WEIGHT MARKERS (100 BP DNA LADDER). LANE 2 WAS LOADED WITH RT- REACTION. LANE 3 WAS LOADED WITH RT+ REACTION. A BAND OF THE CORRECT SIZE (618 BP) IS PRESENT ONLY IN LANE 3 / FOTOGRAFÍA DE UN GEL DE AGAROSA MOSTRANDO LOS PRODUCTOS ORIGINADOS POR TRANSCRIPCIÓN REVERSA DE ARN TOTAL DE ESPERMATOZOIDES / EL CARRIL 1 SE CARGÓ CON MARCADORES DE PESO MOLECULAR (100 PARES DE BASES). EL CARRIL 2 SE CARGÓ CON LA REACCIÓN DE TRANSCRIPTASA REVERSA NEGATIVA (RT-). EL CARRIL 3 SE CARGÓ CON LA REACCIÓN TRANSCRIPTASA REVERSA POSITIVA (RT). UNA BANDA CORRESPONDIENTE A UN PRODUCTO DE TAMAÑO ESPERADO (618 PB) SE OBSERVÓ SOLAMENTE EN EL CARRIL 3.

Another aspect that will require further clarification is the precise localization of PAF receptors in sperm cells. Since a fixation/permeabilization step was included in the immunocytochemistry protocol it cannot resolve whether these immunoreactive receptor sites are on the plasma membrane, localized to intracellular structures or both. In light of the putative existence of intracellular PAF receptors in neural cells [13], more studies are warranted to establish if such internal receptors are present in sperm cells.

Effects of PAF on sperm capacitation, acrosome reaction and motility

In the first experiment using frozen spermatozoa significant effects of time but not treatment were observed for percentages of capacitated (FIG. 3A), acrosome reacted (FIG. 3B) and motile sperm (FIG. 3C).

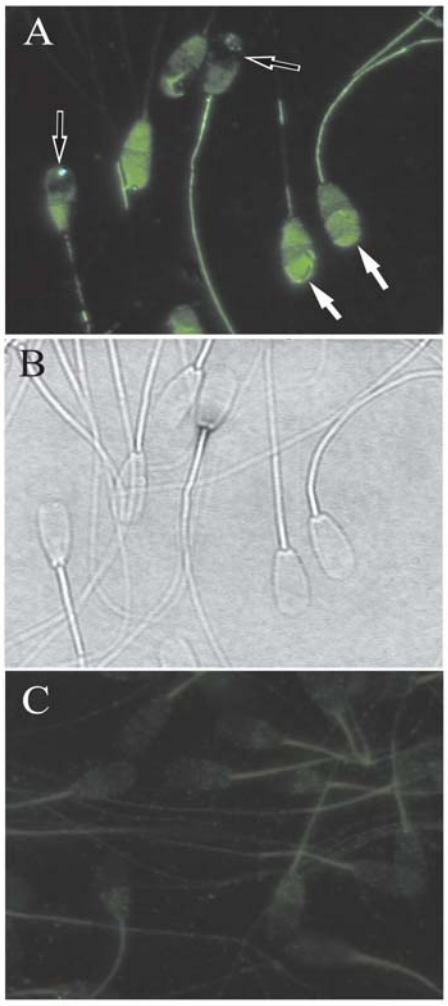

FIGURE 2. PAF-RECEPTOR IMMUNOFLUORESCENCE OF BULL SPERMATOZOA. (A) PAF RECEPTOR IN SPERMATOZOA DETECTED BY INDIRECT IMMUNOFLORESCENCE USING A MONOCLONAL ANTIBODY AGAINST PAF RECEPTOR. SOME SPERMATOZOA WERE INTENSELY LABELED ON THE ACROSOME CAP (WHITE ARROWS) WHILST OTHERS SHOWED ONLY FAINT FLUORESCENCE ON THE ACROSOME (OPEN ARROWS). (B) PHOTOMICROGRAPH OF THE SAME MICROSCOPIC FIELD AS IN (A) SHOWING DETAILED SPERM MORPHOLOGY BY PHASE CONTRAST MICROSCOPY. (C) NEGATIVE CONTROL IN WHICH PAF-RECEPTOR SPECIFIC PRIMARY ANTIBODY WAS REPLACED BY THE SAME CONCENTRATION OF AN ISOTYPE ANTIBODY WITH UNKNOWN SPECIFICITY. (ORIGINAL MAGNIFICATION 400 X). / INMUNOFLUORESCENCIA PARA RECEPTOR DE PAF EN ESPERMATOZOIDES DE TORO. (A) RECEPTOR PARA PAF DETECTADO POR INMUNOFLUORESCENCIA INDIRECTA MEDIANTE EL USO DE UN ANTICUERPO MONOCLONAL ESPECÍFICO CONTRA EL RECEPTOR PARA EL PAF. ALGUNOS ESPERMATOZOIDES PRESENTARON INTENSA FLUORESCENCIA SOBRE EL CAPUCHÓN AROSOMAL (FLECHA BLANCA) MIENTRAS QUE OTROS MOSTRARON ESCASA FLUORESCENCIA EN DICHA REGIÓN. (B) MICROFOTOGRAFÍA DEL MISMO CAMPO MICROSCÓPICO QUE EN (A) MOSTRANDO EL DETALLE MORFOLÓGICO DE LOS ESPERMIOS MEDIANTE MICROSCOPIA DE CONTRASTE DE FASE. (C) CONTROL NEGATIVO EN EL CUAL EL ANTICUERPO PRIMARIO ESPECÍFICO PARA EL RECEPTOR PARA EL PAF SE REEMPLAZÓ POR UN ANTICUERPO DEL MISMO ISOTIPO CON ESPECIFICIDAD DESCONOCIDA. (MAGNIFICACIÓN ORIGINAL 400 X).**

Similar results were obtained in the second experiment with fresh semen (refrigerated) when specimens were exposed to increasing concentrations of PAF or Lyso-PAF. Neither proportions of capacitated sperm determined by chlortetracycline (FIG. 3D), nor acrosome reacted sperm determined by chlortetracyclin stain (FIG. 3E) and motility (FIG. 3F) were affected by PAF treatment; however an effect of time was observed for all three variables (FIGS. 3D, E, and F). Despite the apparent positive effect of PAF 10^{-6} M treatment on percentage of capacitated spermatozoa (FIG. 3D), it was not statistically different (P=0.21).

The experiment was unable to demonstrate any effect of PAF supplementation on different physiological sperm parameters. Fresh or frozen bull sperm were exposed to a broad range of PAF concentrations over a period of 4 h, and capacitation, AR and motility were determined in aliquots collected at discrete intervals. The inability of PAF to promote ARs contrasts with previous studies in which PAF supplementation in the culture media enhanced the AR of bull [23] and buffalo [3, 4] spermatozoa. This discrepancy in terms of AR response between this study and the previous reports may be accounted for by differences in PAF formulations. In this study it was used a synthetic hexadecyl (C_{16}) PAF whereas in Parks' [23] and Aravindakshan and Sharma's [3, 4] studies a natural purified PAF (a mixture of hexadecyl and octadecyl PAF isoforms) was evaluated in its capacity to induce ARs. An isoform-specific stimulatory effect of PAF on mouse embryo development has been reported [37]. Hexadecyl PAF produced a significant stimulation of early embryo development and hatchability, whilst octadecyl PAF had no effect on embryonic development [37]. It is plausible that sperm response to exogenous PAF is also isoform-specific. The present results may suggest that bull sperm is refractory to hexadecyl PAF rather than octadecyl PAF as reported for mouse embryos [37]. Alternatively, PAF may have caused changes in sperm function (such as respiratory rate) not measured in the present study.

The potential effect of PAF on bull sperm function in vitro was tested over a broad range of PAF concentrations from 10^{-6} to 10^{-11} M. In preliminary experiments it was observed that bull sperm exposed to PAF at a final concentration of 10^{-5} M resulted in loss of cell viability (data not shown), results that coincide with a previous report [30]. This phenomenon can be interpreted as a disruptive effect of high PAF concentration on the sperm plasma membrane as suggested by Pike et al. [27]. PAF concentrations tested in the present study and others [2, 34, 35] contrasted with those used in experiments performed with bull and buffalo [3, 4, 23] in which spermatozoa were incubated in micromolar PAF concentrations. This further supports the idea that the different PAF isoforms may elicit diverse responses on spermatozoa as discussed above.

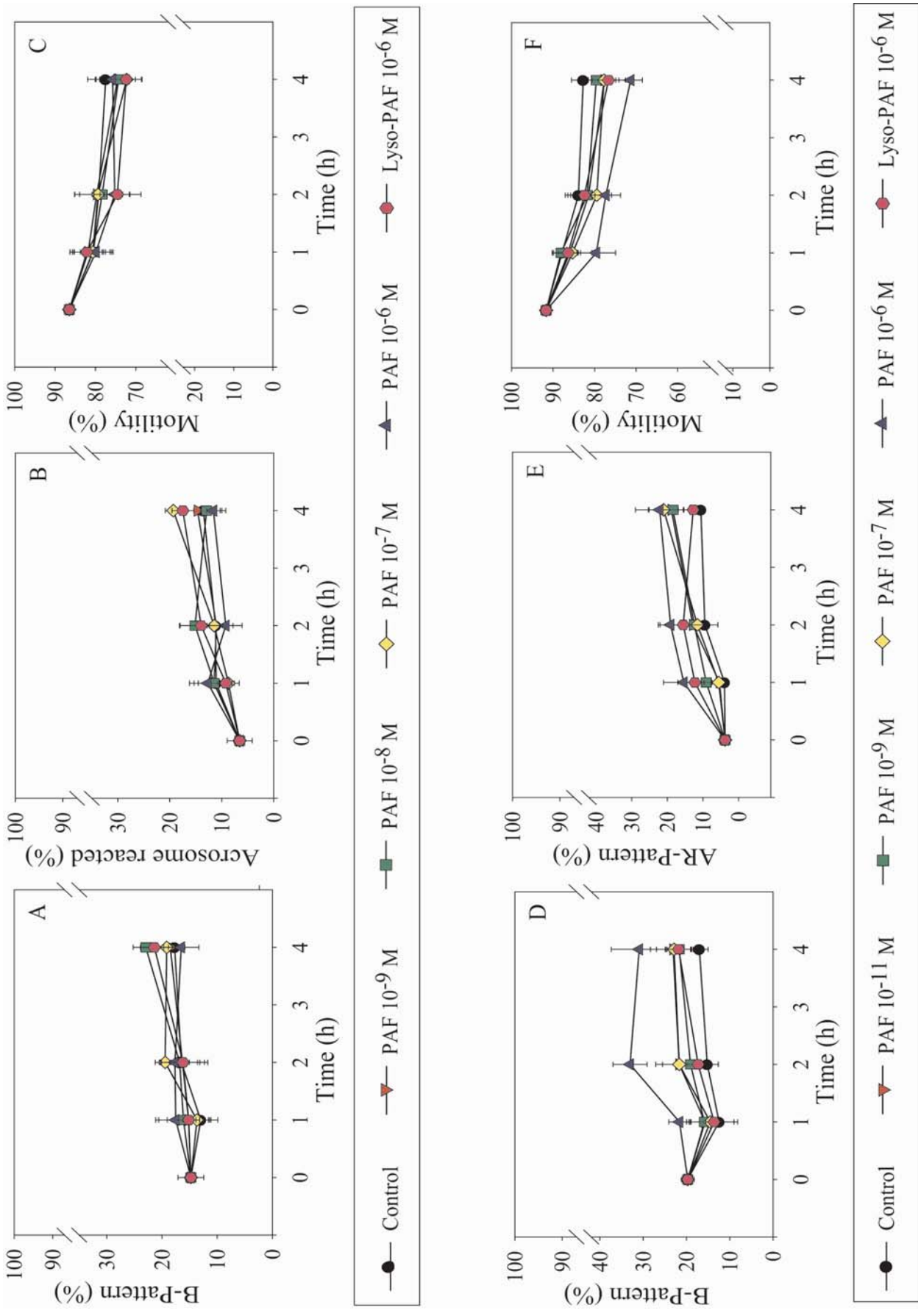

FIGURE 3. EFFECTS OF TIME AND EXPOSURE TO INCREASING CONCENTRATIONS OF PAF OR LYSO-PAF ON CAPACITATION (B-PATTERN), ACROSOME REACTION AND MOTILITY OF FROZEN-THAWED (A, B, AND C) AND REFRIGERATED BULL SPERMATOZOA (D, E, AND F). DATA REPRESENT MEDIA ± SEM FROM 3 REPLICATES / EFECTOS DEL TIEMPO Y EXPOSICIÓN A CONCENTRACIONES CRECIENTES DE PAF O LYSO-PAF SOBRE LA CAPACITACIÓN (PATRÓN B), REACCIÓN DE ACROSOMA Y MOTILIDAD DE ESPERMATOZOIDES BOVINOS CONGELADOS/DESCONGELADOS (A, B Y C) O REFRIGERADO (D, E, Y F). SE PRESENTAN MEDIA ± ESM OBTENIDOS A PARTIR DE 3 RÉPLICAS DEL EXPERIMENTO.

TABLE I
EFFECT OF PAF ON BOVINE IVF / EFECTO DEL PAF SOBRE LA FERTILIZACIÓN IN VITRO.

Treatment	No of oocytes	No 2-cell-embryos	Fertilization
Control	163	69	42.3%[a]
PAF 1 x 10^{-8} M	174	67	38.5%[a]
PAF 1 x 10^{-7} M	165	74	44.8%[a]
PAF 1 x 10^{-6} M	180	71	39.4%[a]
Lyso-PAF 1 x 10^{-7} M	167	68	40.7%[a]
Heparin 10 µg/mL	318	191	60.1%[b]

[a,b,] Different superscripts denote significant differences (P<0.05; Chi square test; Data from 4 replicates).

TABLE II
EFFECT OF PAF AND LYSO-PAF ON BOVINE IVF / EFECTO DEL PAF Y LYSO-PAF SOBRE LA FERTILIZACIÓN IN VITRO

Treatment	No of oocytes	No 2-cell-embryos	Fertilization
Control	98	28	28.6%[a]
PAF 2 x 10^{-6} M	98	29	29.6%[a]
PAF 4 x 10^{-6} M	91	22	24.1%[a]
PAF 8 x 10^{-6} M	94	14	14.9%[b]
Heparin 10 µg/mL	101	68	67.3%[c]

[a,b,c] Different superscripts denote significant differences (P<0.05; Chi square test).

Since sperm cells not only contain PAF [17, 18, 22] but also release PAF to the culture media [41] it cannot be ruled out that sperm-derived PAF may have masked the effects of exogenous PAF in the present experiments. It can be hypothesized that production and release of spermatozoal PAF was even enhanced in the *in vitro* system used due to the presence of ether-linked phospholipids in the semen extender utilized (containing egg yolk). Conversion of these PAF precursors in sperm could augment the sperm-derived PAF present in the culture media by several orders of magnitude, thus reaching a threshold that would cloud the effect of any exogenous PAF added. Moreover, peroxidation of phosphatidylcholines contained in hen egg yolk can originate biologically active PAF-like lipids [40] which could potentially confound the effects of exogenous PAF on sperm function. The reduced effect of PAF on the motility and ARs of frozen-thawed bull and buffalo spermatozoa compared with that in the fresh counterparts [3, 4] supports these hypotheses.

Effect of PAF on bovine IVF

In experiment 1, fertilization rate in PAF-treated and Lyso-PAF treated groups were similar to the control group in which PAF was omitted. On the other hand, addition of heparin to the fertilization drops enhanced fertilization rate by ~20% compared with the control group (60.1 vs 42.3%; P<0.05; TABLE I).

Results from experiment 1, prompted to perform experiment 2 to investigate the effect of higher PAF concentrations on fertilization rate. Since there is evidence that BSA can bind PAF molecules and therefore reduce PAF-induced responses in a dose dependent manner [1], in experiment 2 BSA was omitted from IVF-TALP medium. Fertilization rates in PAF 2 x 10^{-6} M and 4 x 10^{-6} M were not different from the controls in which PAF was absent. However, addition of PAF at a concentration of 8 x 10^{-6} M significantly impaired in vitro fertilization (TABLE II). Fertilization rate of gametes cocultured in presence of heparin at a concentration of 10 µg/mL, was significantly higher (P<0.05) than those in the control group and PAF treated sperm groups (TABLE II).

Despite higher PAF concentration and exclusion of BSA from the media, fertilization rate in PAF treated oocytes in experiment 2 was not different from that of the control group. These results contrast with the beneficial effect of PAF on mouse IVF [19]. A significant reduction in the percentage of fertilized oocytes was observed with PAF at 8 x 10^{-6} M which might reflect a membrane-perturbing effect of PAF on the spermatozoa or oocytes at this concentration.

CONCLUSION

In summary, bull spermatozoa express receptors for PAF, as indicated by presence of transcripts and immunoreactive sites in sperm cells. However, addition of PAF to the culture media apparently did not enhance capacitation, AR, motility or the in vitro fertilizing ability of the spermatozoa. Further studies are needed to elucidate the biological role of PAF-receptor activation in bull spermatozoa and the discrete effects of different PAF isoforms on sperm function.

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