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Effects of lactoferrin, a protein present in the female reproductive tract, on parameters of human sperm capacitation and gamete interaction

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SUMMARY

In a recent study, lactoferrin (LF) was detected in human oviductal secretion. The protein was able to bind to oocytes and sperm, and modulated gamete interaction. The aim of the present study was to investigate the effect of LF on parameters related to human sperm capacitation and sperm–zona pellucida interaction. Semen samples were obtained from healthy normozoospermic donors (n = 7). Human follicular fluids and oocytes were collected from patients undergoing *in vitro* fertilization. Motile sperm obtained by swim-up were incubated for 6 or 22 h under capacitating conditions with LF (0–100 μg/mL). After incubations, viability, motility, presence of α-D-mannose receptors (using a fluorescent probe on mannose coupled to bovine serum albumin), spontaneous and induced acrosome reaction (assessed with *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate), and tyrosine phosphorylation of sperm proteins were evaluated. Sperm–zona pellucida interaction in the presence of LF was investigated using the hemizone assay. The presence of LF did not affect sperm viability or motility, but caused a dose-dependent significant decrease in sperm α-D-mannose-binding sites, and the effect was already significant with the lowest concentration of the protein used after 22 h incubation. Dose-dependent significant increases in both induced acrosome reaction and tyrosine phosphorylation of sperm proteins were observed in the presence of LF. The present data indicate that LF modulates parameters of sperm function. The inhibition of gamete interaction by LF could be partially explained by the decrease in sperm D-mannose-binding sites. The presence of the LF promoted sperm capacitation *in vitro*.

INTRODUCTION

Up to the present, numerous efforts have been made to understand the physiology of the fertilization process and to determine the intrinsic mechanism of the interaction between the male and female gametes. Data from different studies indicate that protein factors from the fertilization microenvironment could participate in the modulation of the reproductive process (Suarez & Pacey, 2006; Kölle *et al.*, 2009; Munuce *et al.*, 2009; Zumoffen *et al.*, 2010, 2013). We have recently shown that proteins from oviductal tissue secretion modulate parameters of human sperm capacitation (Zumoffen *et al.*, 2010). In a recent study, we have isolated one protein from human oviductal tissue secretion, and identified it as human lactoferrin (LF) (Zumoffen *et al.*, 2013). Sperm-binding sites for LF were shown to be related to capacitation and acrosome reaction (AR) and the oviductal expression of the protein appeared to be cycle dependent (Zumoffen *et al.*, 2013). Taking into account these results, in the present study the potential influence of LF on parameters of sperm function associated with capacitation was investigated. In addition, LF was shown to bind to zona pellucida (ZP) of human oocytes, and the presence of LF inhibited sperm–ZP interaction (Zumoffen *et al.*, 2013). Since sperm mannose receptors have been linked to gamete interaction as well as with the success of *in vitro* fertilization, the potential action of LF on sperm D-mannose-binding sites was also assessed in order to investigate the mechanism behind the inhibitory effect of LF on gamete interaction (Benoff *et al.*, 1993; Miranda *et al.*, 1997).

MATERIAL AND METHODS

The study was approved by the Institutional Bioethical Board of the Facultad de Ciencias Bioquímicas y Farmacéuticas (National University of Rosario, Resolution Number 033/2011), and written consent was obtained from all donors.

Chemicals and reagents

Unless otherwise mentioned, all the chemicals and reagents were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA) or from MP Biomedicals Inc. (Santa Ana, CA, USA), and were of the highest purity available.

Semen samples and sperm processing

Semen samples were obtained from normozoospermic donors (n = 7, age: 23 ± 1.6 years), and collected by masturbation after 3–5 days of sexual abstinence. After complete liquefaction, semen analysis was performed according to the World Health Organization guidelines for the examination and processing of human semen (WHO, 2010). Morphology was analysed by strict criteria (Kruger *et al.*, 1986). Sperm motility and viability were assessed as described in the WHO laboratory manual (WHO, 2010). Briefly, to assess viability, one drop of 0.5% w/v Eosin Y solution and one drop of the sperm suspension were mixed on a microscope slide, covered with a cover slip, and examined after 30 sec at ×400 with a light microscope. A total of 200 sperm were counted, differentiating live (unstained) from the dead (stained) sperm. The viability was reported as a percentage. For motility assessment, a 10 μL drop from a well-mixed sperm suspension was placed in a Makler chamber (Sefi Medical Instruments, Haifa, Israel) and examined at a magnification of ×200 under a light microscope. Sperm motility was classified according to the WHO criteria (WHO, 2010). A minimum of 200 sperm from multiple fields was assessed. The percentage of motile sperm with progressive motility was considered for the analysis. Motile sperm were selected by swim-up. In brief, 1 mL of Ham's F10 medium was layered directly onto 1 mL of liquefied semen and incubated for 1 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The uppermost aliquot (1 mL) containing the motile fraction was aspirated and sperm concentration and viability were assessed. The suspension was adjusted to 10×10^6 sperm/mL with Ham's F10 medium supplemented with BSA (35 mg/mL, fraction V, fatty acid-free), streptomycin (100 μg/mL), and penicillin (100 U/mL). Sperm suspensions were incubated for either 6 or 22 h in the presence of increasing concentrations of LF (human milk LF, Sigma-Aldrich Inc.; 0.0, 0.1, 1.0, 10.0, and 100.0 μg/mL) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After the incubations, sperm viability and motility, induced AR, and tyrosine phosphorylation in sperm proteins and D-mannose-binding sites were assessed.

Acrosome reaction assessment

After incubation for 6 or 22 h in the presence of increasing concentrations of LF, sperm samples were divided into two aliquots and exposed for 30 min to either control medium (spontaneous AR) or 20% v/v human follicular fluid (hFF induced AR), after which sperm AR was assessed. The rate of acrosome-reacted sperm was estimated by fluorescein isothiocyanate (FITC) labelled *Pisum sativum* agglutinin (PSA-FITC) according to the protocol of Cross *et al.* (1986). In brief, 50 μL of sperm suspension were smeared onto a slide. After air drying, the cells were fixed and permeabilized in methanol for 30 sec. The smears were then covered with PSA-FITC solution and placed in the dark at room temperature for 30 min in a moist chamber. After that they were rinsed with PBS twice, mounted with glycerol–PBS (9:1), and examined at ×1000 using an epifluorescence microscope (Nikon Eclipse-800 microscope, Nikon Instruments, Inc., Melville, NY, USA). Sperm were classified as (i) non-reacted, if the acrosomal cap was uniformly labelled; (ii) reacting, if the acrosomal cap was labelled in a patchy pattern; or (iii) reacted, if only the equatorial segment was labelled. At least 200 sperm were evaluated in each slide. The percentage of reacted cells was reported as (ii + iii). The inducible population (IP) was calculated as the difference between the percentage of reacted cells in the presence of hFF (induced AR) and the percentage of reacted cells in the absence of this fluid (spontaneous AR).

Analysis of tyrosine phosphorylation

For this analysis, at the end of the incubations in the presence or the absence of LF, sperm were washed in PBS by centrifugation and resuspended in sample buffer (0.2% w/v bromophenol blue; 20% v/v glycerol; 0.125 M Tris–HCl; 4% w/v SDS, pH 6.8) and boiled for 5 min; 2-mercaptoethanol was then added to the supernatant to a final concentration of 5% v/v (Laemmli, 1970). Proteins from 2×10^6 sperm were loaded on each lane of the gel. Samples were analyzed by an SDS-PAGE (7%) at 100 V in a Mini-protean II system (Biorad, Hercules, CA, USA), and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Germany) (Towbin *et al.*, 1979). Membranes were first incubated with 5% dry skimmed milk in TBS–0.1% Tween 20 (blocking solution). Tyrosine phosphorylated proteins were detected with a monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology, NY, USA). After washes with TBS–0.1% Tween 20, peroxidase-conjugated anti-mouse IgG antibody was added. Following 1 h of incubation, the membranes were washed and reactive bands were detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. A commercial protein marker was used as molecular weight standard (Kaleidoskope, Biorad). The membranes were washed and reprobed with a mouse monoclonal antibody (Sigma-Aldrich Inc.) against β-actin, in which signal was used as a loading control for western blot analysis. The immunoreactive bands were analyzed with the software Gel-Pro (Media Cybernetics, Silver Spring, MD, USA). The intensities of the main protein bands in controls (after 6 h or 22 h incubation, respectively) were arbitrarily considered as 100% (after correction with respect to β-actin signal) and the relative intensities of the bands on the other lanes were referred to those in the controls.

In addition, a western blotting of LF (1.5 μg/lane, 10% SDS-PAGE) with anti-human LF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with anti-phosphotyrosine antibody (following protocols describe above) was performed to investigate possible non-specific reactivity of the later antibody with LF.

Collection of human follicular fluid

Human follicular fluids (hFFs) were collected during oocyte retrieval from women (n = 5) participating in an *in vitro* fertilization (IVF) programme, as described in a previous report (Zumoffen *et al.*, 2010). Only hFF with no blood contamination and from follicles containing a mature oocyte (metaphase II) were collected. Follicular fluids from each sample were centrifuged (10 min, 600 ***g***) to remove cellular debris, filtered through a

0.22-μm membrane (Millipore Corp., Bedford, MA, USA) and mixed into a pool, before being stored at −20 °C.

D-Mannose-binding sites

Capacitated sperm were incubated 6 or 22 h in the presence of increasing concentrations of LF, and then, the presence of mannose-binding sites was analyzed according to the protocol of Benoff *et al.* (1995). In brief, samples were washed three times with core buffer (30 mM HEPES, 0.5 M $MgCl_2$, 150 mM NaCl, 10 mg/mL BSA, pH 7.0) containing 20 mM [Ca^{2+}] and incubated 30 min with 100 μg/mL FITC-conjugated mannosylated BSA (Man-FITC-BSA) in calcium-supplemented core buffer at 37 °C in an atmosphere of 5% CO_2/95% air. After labelling, motile sperm were pelleted by centrifugation, washed three times with core buffer (without calcium), air dried onto glass slides, and mounted in a glycerol-based medium. Specimens were evaluated at ×1000 using a confocal microscope Nikon Eclipse TE-2000-E2 (Natick, MA, USA). At least 200 sperm were evaluated per slide. Each spermatozoon was classified according to the pattern of the fluorescent signal as unspecific pattern I (stain on tail and mid-piece, observed in all cells), specific pattern II (stain on entire acrosomal cap), or specific pattern III (stain on equatorial/post-acrosomal regions). The results were reported as the percentage of cells showing specific patterns II and III in each treatment group.

Hemizona assay

The hemizona assay (HZA) was performed as described elsewhere (Burkman *et al.*, 1988). Supernumerary human oocytes in metaphase II, donated by women undergoing IVF procedures in a reproductive clinic (PROAR, Rosario, Argentina), were stored in a salt solution containing 1.5 M $MgCl_2$, 0.1% w/v polyvinylpyrrolidone and 40 mM HEPES in PBS solution (pH 7.2) at 4 °C, until use. Before oocytes were used, excess salt was removed by washing them in Ham's F10 and incubating for 20 h at 37 °C in an atmosphere of 5% CO_2. After separation, one hemizonae was placed in 100 μL of Ham'F10 medium supplemented with antibiotics and 35 mg/mL BSA (control) and the counterpart hemizonae in another drop of 100 μL of the same medium and in the presence of any of the concentrations used of LF (0.1, 1.0, 10.0, or 100.0 μg/mL). The drops were covered with mineral oil to prevent evaporation, inseminated with motile sperm (10^5 sperm/mL), and incubated for 4 h at 37 °C and 5% CO_2. Finally, the hemizonas were washed by repeated vigorous pipetting in drops of medium and the number of sperm tightly bound to the outer surface of each hemizona was counted. The evaluation was carried out at ×400 magnification in an inverted microscope (Olympus, Japan). For each concentration of LF used, hemizona index (HZI) was calculated as follows: (number of sperm bound to the hemizona exposed to LF/number of sperm bound to the hemizona control) ×100.

Data analysis

Statistical analysis was performed using the GRAPH-PAD INSTAT program (GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) and the Tukey–Kramer test for multiple comparisons were used to compare the means of percentages of AR, specific D-mannose binding sites, and sperm viability and motility from different treatment groups. The Kruskal–Wallis nonparametric test was used to compare the mean values of sperm protein phosphorylation. Data were expressed as the mean ± SEM. A $p < 0.05$ was considered significant.

RESULTS

Sperm viability and motility

The assayed concentrations of LF did not affect sperm viability or progressive motility after 6 or 22 h of incubation, which were similar to the values in the respective controls (Table 1).

Effect of LF on induced AR

After 6 h of incubation, the values of spontaneous AR in control and in the presence of 0.1, 1.0, 10.0, and 100.0 μg/mL of LF were 5.2 ± 0.48, 4.2 ± 0.47, 6.0 ± 0.41, 6.0 ± 0.71, and 6.8 ± 0.85, respectively. After 24 h of incubation, the values of spontaneous AR in control and in the presence of LF (0.1, 1.0, 10.0, or 100.0 μg/mL) were 6.8 ± 0.75, 6.5 ± 0.29, 6.7 ± 0.25, 7.2 ± 0.8, and 8.2 ± 0.85, respectively. None of the LF concentrations significantly affected spontaneous AR at both incubation times assayed as compared with the values in the respective controls. The incubation with hFF for AR induction did not affect sperm viability. A dose-dependent increase in % IP was observed in sperm incubated in the presence of increasing concentrations of LF, at both incubation times used. After the 6-h incubation period, the increase in % IP was significant with the highest concentration of LF (IP control 6 h: 15.6 ± 1.4% vs. $IP_{100\ 6h}$: 26.0 ± 1.1%, $p < 0.05$) (Fig. 1A). After 22 h of incubation, a significant increase in % IP was already observed in the presence of 10 μg/mL of LF [$IP_{10\ 22h}$: 30.0 ± 1.8% ($p < 0.05$), and 100 μg/mL of LF [$IP_{100\ 22h}$: 35.3 ± 1.8% ($p < 0.01$)] vs. control (IP control 22 h: 17.6 ± 1.2%) (Fig. 1B).

Effect of LF on protein tyrosine phosphorylation

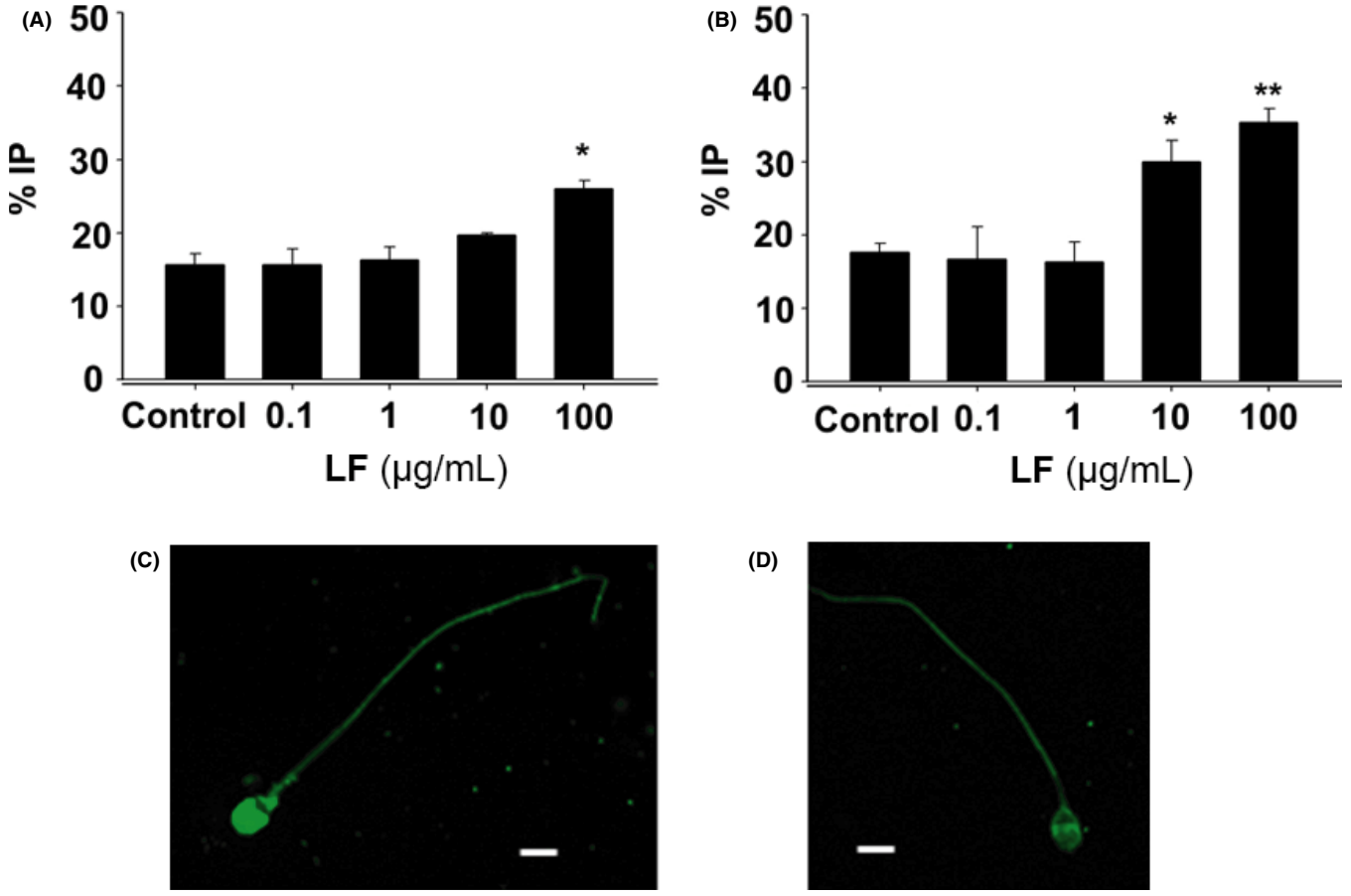
A dose-dependent increase in protein tyrosine phosphorylation (PTP) was detected in sperm incubated in the presence of LF for 6 h (Fig. 2A and C). The increase was significant with the highest concentration of LF used, with respect to the control [PTP_{100LF}: 258.3 ± 18.1% ($p < 0.05$) vs. control PTP].

Table 1 Viability and progressive motility of sperm incubated for 6 h or 22 h in the presence or absence of increasing concentrations of LF ($n = 4$)

	Control	0.1 μg/mL LF	1.0 μg/mL LF	10.0 μg/mL LF	100.0 μg/mL LF	*p*
6 h incubation						
Viability (%)	94.6 ± 1.3	94.0 ± 1.2	96.0 ± 0.6	94.5 ± 1.3	95.0 ± 1.3	N.S.
Progressive motility (%)	85.5 ± 2.7	86.0 ± 0.8	87.0 ± 3.0	84.0 ± 2.4	87.5 ± 1.7	N.S.
22 h incubation						
Viability (%)	90.4 ± 2.3	92.0 ± 3.0	93.0 ± 2.1	97.6 ± 0.2	92.8 ± 0.9	N.S.
Progressive motility (%)	72.0 ± 5.6	77.8 ± 5.8	82.6 ± 2.4	84.6 ± 1.8	81.0 ± 2.6	N.S.

N.S: no statistically significant.

Figure 1 Effect of lactoferrin (LF) on acrosome reaction (AR). Motile sperm were incubated in the absence (control) or the presence of increasing concentrations of LF (0.1, 1.0, 10.0, and 100.0 μg/mL) under capacitating conditions for 6 h (A) or 22 h (B). AR was then induced with follicular fluid. The results were reported as percentage of inducible population (% inducible population) and were expressed as mean ± SEM ($n = 4$). *$p < 0.05$ vs. respective control; **$p < 0.01$ vs. respective control. (C) Fluorescent micrograph of a spermatozoon with intact acrosome stained with *Pisum sativum* agglutinin (PSA-FITC). (D) Fluorescent micrograph of an acrosome-reacted sperm. Bar: 5 μm.



After 22 h of incubation, the presence of LF also caused a dose-dependent increase in sperm PTP level (Fig. 2B and D). The incubation in the presence of 10 μg/mL or 100 μg/mL of LF produced significant increases in PTP with respect to the controls [PTP_{10hLF}: 197.3 ± 20.0% ($p < 0.05$), PTP_{100hLF}: 270.6 ± 30.7% ($p < 0.01$)]. No cross-reactivity of the anti-phosphotyrosine antibody with LF was detected (Fig. 2E).

Effect of LF on D-mannose-binding sites and HZA

To evaluate the effect of the presence of LF on sperm α-D-mannose-binding sites, sperm were incubated under capacitating conditions for 6 and 22 h in the absence or the presence of increasing concentrations of the protein. At the end of each incubation period an aliquot of every sample was recovered for the detection of sperm α-D-mannose receptors, according to the protocol described in Material and Methods section.

The assay for α-D-mannose-binding sites did not affect sperm viability (data not shown). In all evaluated samples, the analyzed cells exhibited one of the three staining patterns for α-D-mannose-binding sites described in Material and Methods section. The incubation in the presence of LF for 6 or 22 h under capacitating conditions caused a dose-dependent decrease in (II + III) α-D-mannose-binding patterns (Fig. 3A and B). It is interesting to note that after 22 h of incubation, even the lowest concentration of LF caused a significant decrease ($p < 0.01$) in the % of sperm that expressed specific sites for α-D-mannose.

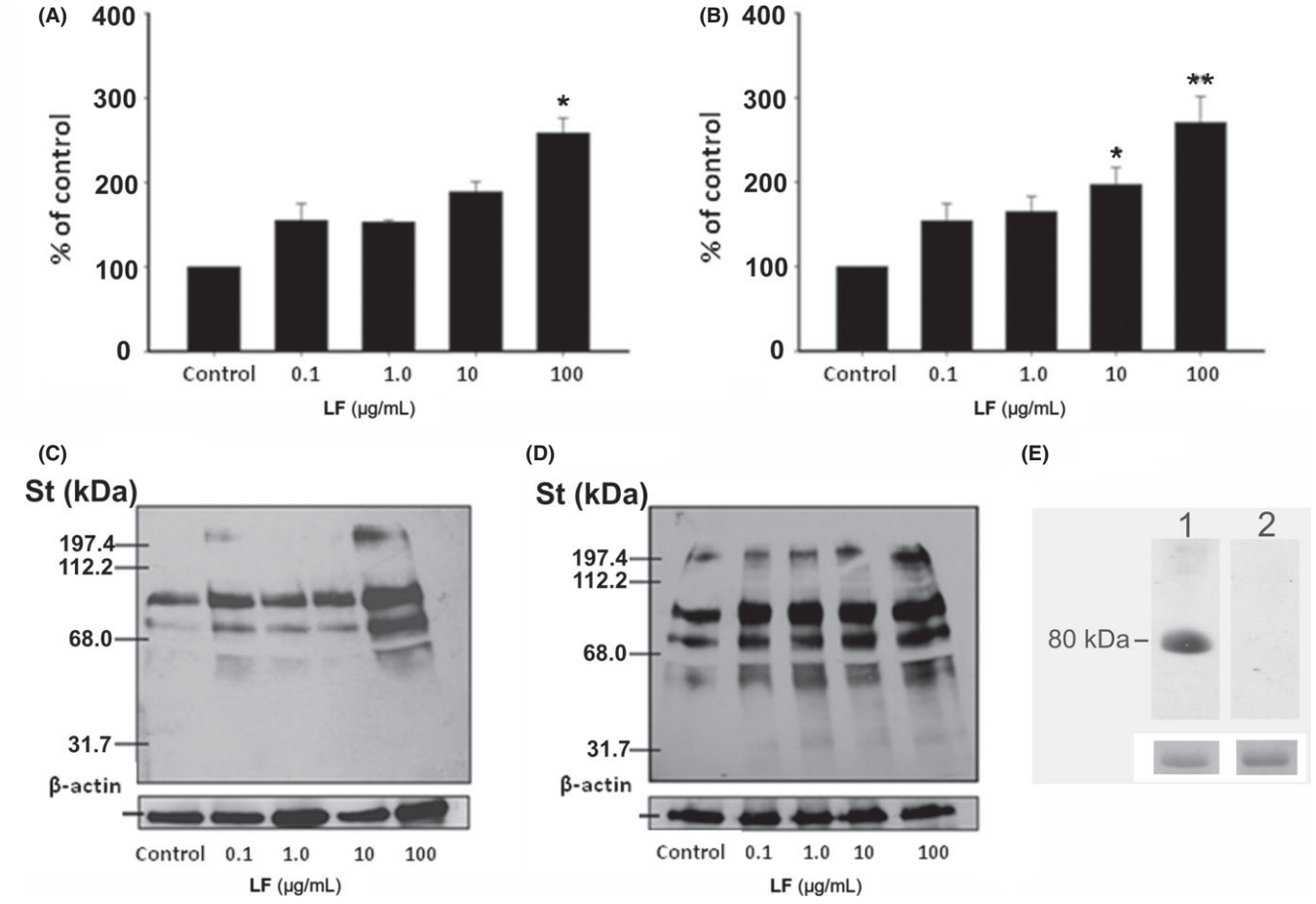
The HZA showed a dose-dependent decrease in the number of sperm bound to hemizonas after the incubation in the presence of LF. The results showed that LF caused a significant dose-dependent decrease in the HZI, except between the doses 0.1 μg/mL (91.9 ± 1.9%) and 1.0 μg/mL (87.0 ± 2.7%) of LF (Fig. 4).

DISCUSSION

This is the first study showing the effects of LF, a glycoprotein present in the oviductal secretion, on parameters of human sperm function.

The results of the HZA indicated that LF decreased sperm–ZP interaction in a dose-dependent manner, in accordance with results of our recent study (Zumoffen *et al.*, 2013). The highest concentration of LF caused approximately a 60% reduction in the HZI with respect to the value in the presence of the lowest dose used of the protein. Since sperm α-D-mannose receptors had been related to gamete interaction, in the present report the LF effect on the presence of these receptors was investigated. Increasing concentrations of LF caused a dose-dependent reduction in the percentage of cells with α-D-mannose receptors on the head. This effect was significant for all the concentrations of LF tested, except with the lowest one after 6 h of incubation.

Figure 2 Effect of lactoferrin (LF) on sperm protein tyrosine phosphorylation. The figure shows the densitometric analysis of the pattern of tyrosine-phosphorylated proteins of sperm incubated in the absence (control) or the presence of increasing concentrations of LF (0.1, 1.0, 10.0, or 100.0 μg/mL) under capacitating conditions for 6 h (A, $n = 4$) or 22 h (B, $n = 4$). The intensities of the main bands of the control group after 6 or 22 h incubation were arbitrarily considered as 100%. The results were expressed as mean ± SEM. *$p < 0.05$ vs. respective control; **$p < 0.01$ vs. respective control. Representative result of immunodetection of sperm protein tyrosine phosphorylation after (C) 6 h and (D) 22 h of incubation. β-actin detection was used as loading control. (E) Western blot of LF (1.5 μg/lane) using anti-LF antibody (lane 1) and a monoclonal anti-phosphotyrosine antibody (lane 2). The bands below correspond to loading control staining with Ponceau S. No cross-reactivity of the anti-phosphotyrosine antibody with LF was observed.



It has been shown that the subpopulation of sperm with staining pattern II of α-D-mannose would be able to recognize and bind to the ligand on the ZP, while pattern III is associated with sperm that have undergone AR or are in the process of reaction (Hershlag *et al.*, 1998). Thus, the decrease in α-D-mannose-binding sites in the presence of LF could contribute to the decrease in sperm–ZP binding observed in the presence of the protein. It could be speculated that this effect of LF could be due to a masking/blocking action on α-D-mannose-binding sites, which could interfere with the binding to α-D-mannose residues. As reported in our recent study, the localization of LF sperm-binding sites was similar to the patterns II and III of α-D-mannose-binding sites, supporting the hypothesis mentioned earlier (Zumoffen *et al.*, 2013). On the other hand, since LF was shown to bind to ZP, it could be thought that the protein could also block some ZP sites involved in the interaction between gametes. Yao *et al.* suggested the existence of glycosylated factors in the oviductal fluid whose sugar residues could bind to receptor molecules on the ZP, blocking the sperm binding (Yao *et al.*, 1998). In many species, it was proposed that gamete binding depends on interactions between lectin-like proteins in the sperm and glycoconjugates residues of the ZP (Clark, 2010). Evidence from different studies support the ability of LF to block molecules involved in cell interaction (Ji & Mahley, 1994; van Berkel *et al.*, 1997; Sakamoto *et al.*, 2006; Ando *et al.*, 2010).

It is accepted that sperm ability to undergo induced AR is an indirect indicator of capacitation (De Jonge, 2005; Ickowicz *et al.*, 2012). In addition, Varano *et al.* showed that occurrence of AR requires phosphorylation of sperm proteins, since they reported that inhibition of tyrosine phosphorylation suppressed AR (Varano *et al.*, 2008). The results of the present study showed that the presence of LF did not induce basal AR. However, it stimulated in a dose-dependent manner both induced AR and tyrosine phosphorylation of sperm proteins. Since both parameters are associated with capacitation, these results support that LF could promote this process *in vitro*. Other oviductal proteins, such as oviductins and osteopontin, were also shown to increase *in vitro* sperm capacitation (Abe *et al.*, 1995; Monaco *et al.*, 2009; Saccary *et al.*, 2013). In contrast, other proteins, such as

Figure 3 Sperm α-D-mannose receptors in the presence of lactoferrin (LF). Motile sperm were incubated under capacitating conditions for 6 h (A) and 22 h (B), in the presence or absence of different concentrations of LF (0.1, 1.0, 10.0, and 100.0 μg/mL). Detection of α-D-mannose receptors was performed in an aliquot of each sample at the end of the respective incubation periods. The results were expressed as mean ± SEM ($n = 4$). *$p < 0.05$ vs. respective control; **$p < 0.01$ vs. respective control; ***$p < 0.001$ vs. respective control. (C) Fluorescence micrographs showing the localization patterns for sperm α-D-mannose-binding sites. Pattern I: fluorescent signal localized at the neck, mid-piece, and tail; Pattern II: fluorescent signal includes pattern I and sperm head; Pattern III: stain localized at the equatorial segment and/or post-acrosome region. Bar: 10 μm.

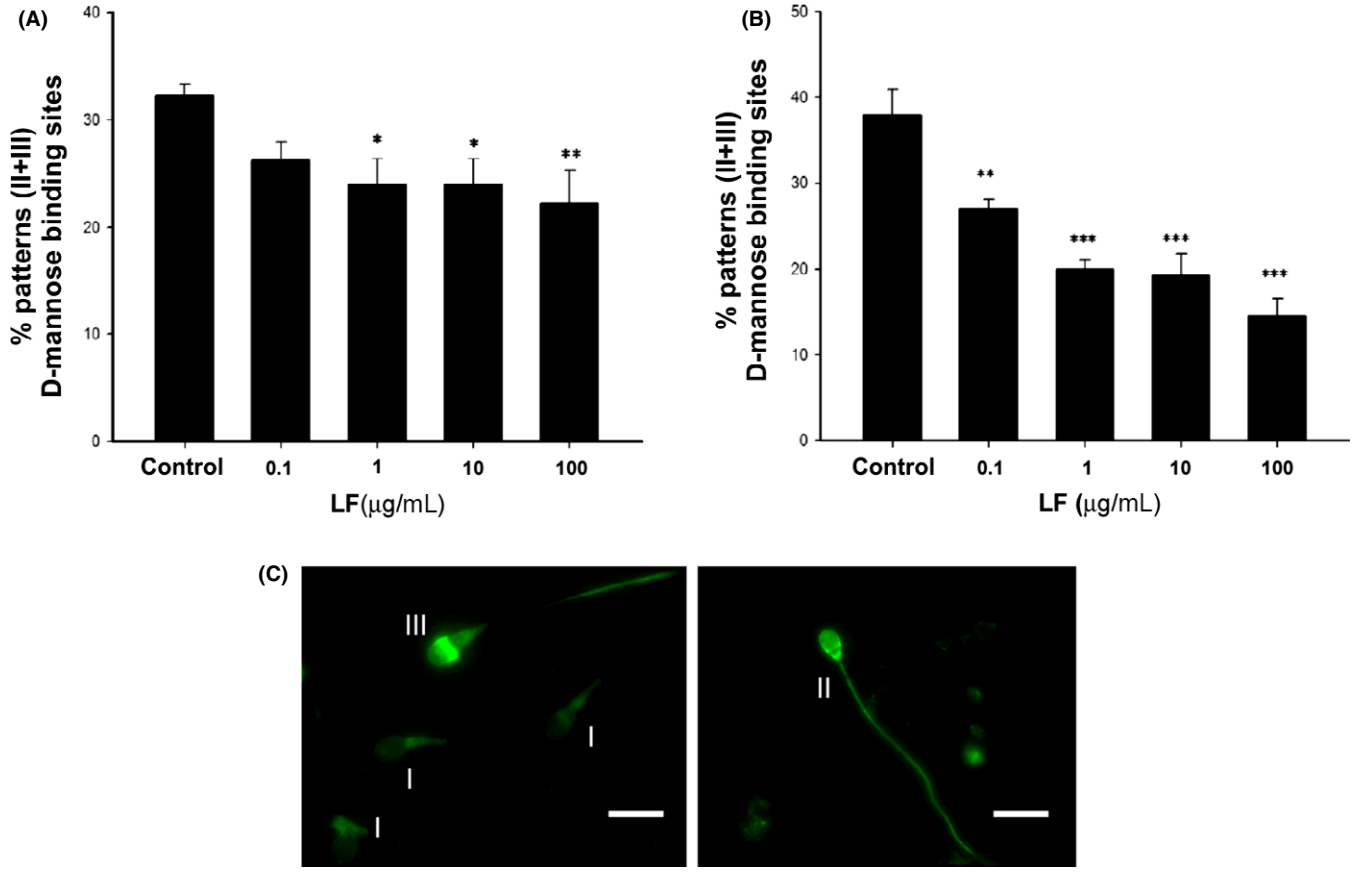
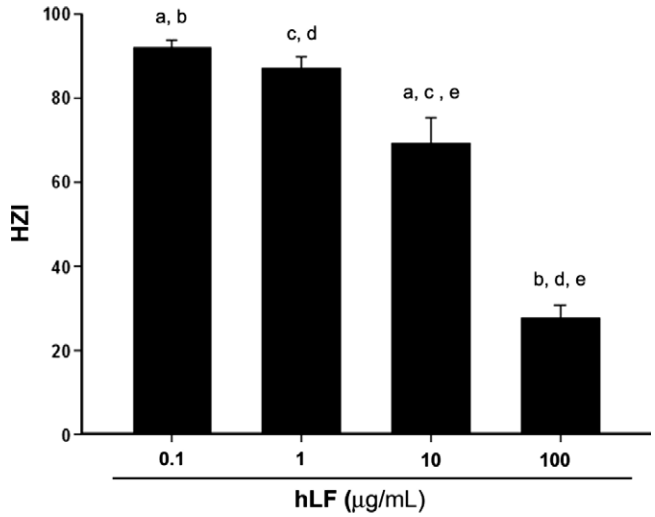


Figure 4 Effect of increasing concentrations of lactoferrin (LF) on hemizona index (HZI). The presence of LF caused a dose-dependent decrease in the HZI. The results are expressed as mean ± SEM of three independent experiments. a: $p < 0.01$; b: $p < 0.001$; c: $p < 0.05$; d: $p < 0.001$; e: $p < 0.001$. The same letters indicate the columns that were compared statistically.



glycodelins, were reported to inhibit capacitation (Dutta *et al.*, 2001). It could be speculated that sperm that reach the oviduct will contact modulatory factors, whose action could stimulate or inhibit sperm function and gamete interaction (Ghersevich *et al.*, 2015). Thus, the modulation of the expression of these factors would contribute to regulate the reproductive process, affecting the number of male gametes that initiate the capacitation process and the gamete interaction.

Since both the presence of LF sperm-binding sites and a LF sperm receptor have been reported, it could be thought that the protein exerted its effects through the interaction with specific receptors on the sperm membrane (Wang *et al.*, 2011; Zumoffen *et al.*, 2013). The reported sperm LF receptor, known as intelectin-1, is also expressed in other cell types such as enterocytes, liver cells, and lymphocytes (Suzuki & Lönnerdal, 2002; Wang *et al.*, 2011). It was shown that intelectin-1 activation involved signals that would trigger the pathways of PI3K/Akt and ERK1/2 kinases (Liao *et al.*, 2012). It is interesting to notice that both PI3K/Akt and MAPK/ERK signalling pathways have been involved in human sperm capacitation and AR (Luconi *et al.*, 1998; Lu *et al.*, 1999; Nauc *et al.*, 2004; Liguori *et al.*, 2005). Therefore, it could be

speculated that LF effects on sperm protein phosphorylation and on induced AR could be mediated by the activation of sperm LF receptors. In addition, different types of cells and tissues have been shown to express distinct LF receptors (Takayama, 2012). It has been suggested that the glycans attached to the globular structure of LF may participate in the recognition of its receptors on the cell surface and this would help to explain the specificity of its multiple biological actions (Spik *et al.*, 1982; Yu *et al.*, 2011; Takayama, 2012). Thus, the presence of other types of receptor molecules for LF on sperm should not be ruled out.

In a recent study, we demonstrated that pre-treatment of LF with anti-LF antibody avoid the effect of the protein on sperm–ZP interaction, supporting the specificity of the LF effect (Zumoffen *et al.*, 2013). Nevertheless, since the purity of LF used in the present study was around 90%, other components present as impurities might also contribute to some of the reported effects.

The accumulated experimental evidence supports that the oviduct is actively involved in the reproductive process, considering that its secretion contains molecules capable of modulating gamete functions and interaction. The present data together with our previous results suggest that LF could contribute to the modulation of the reproductive process, stimulating sperm capacitation, regulating the sperm subpopulation with the ability to interact and fertilize the oocyte, helping to avoid polyspermy.

In order to investigate the mechanisms behind the LF effects on tyrosine phosphorylation, induced AR, and gamete interaction, further studies are directed to assess the potential involvement of different transduction pathways (such as PKA and PKC pathways, usually associated with the regulation of sperm functions) in the reported effects of LF as well as to determine whether the carbohydrate residues of the protein could be implicated in the reported actions.

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AUTHOR'S CONTRIBUTIONS

CMZ, EM, AMC, MJM, and SAG contributed substantially to the acquisition and analysis of data, drafting the article, and final approval of the version to be published. AMC, MJM, and SAG substantially contributed to the study design and revising critically the article for important intellectual content. SAG managed the project.

CONFLICT OF INTEREST

The authors do not have any conflicts of interest to declare.

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