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Peritoneal fluid from women with endometriosis impairs human spermatozoa functionality

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ABSTRACT

The success of mammalian fertilization depends largely on spermatozoa physiological events. However, the manner in which endometriosis influence morpho-functional spermatozoa biomarkers is poorly defined. Here, we studied *in vitro* the effect of peritoneal fluid (PF) from women with endometriosis (PFE) and non-endometriosis (PFNE) on spermatozoa parameters. This research was prospective and double-blind. A total of 45 PF samples were collected from women with (n = 25) and without endometriosis (n = 20). Semen samples were obtained from normozoospermic donors (n = 15) and cultured with 20 % (*v/v*) of PF or commercial culture medium (controls) during 0, 24, and 48 h. The outcome measures were spermatozoa/viability, motility, tyrosine phosphorylation (TP) and spontaneous acrosomal reaction. In addition, plasma membrane sugars were characterized by lectins [*Aleuria aurantia agglutinin* (AAA), *Concanavalin A* (ConA), *Peanut agglutinin* (PNA), and *Wheat germ agglutinin* (WGA)]. After a 24 -h culture, results reported a significant decrease in motility in cells cultured with PFE compared to the control, together with differences in the AAA and WGA-binding sites. Moreover, spermatozoa in contact with PFNE presented a significantly lower level of acrosome spontaneous reaction. At 48 h, no differences were observed in the biomarkers studied between the PFNE and the control, excluding ConA-binding sites. On the other hand, cells cultured with PFE exhibited significantly less motility, TP, and differences in the relocation of spermatozoa surface sugars. Viability was not affected in any culture condition. Overall, the effects of PFE could negatively affect spermatozoa quality, contributing to explain and diagnose the infertility associated to male partners of women with endometriosis.

1. Introduction

Endometriosis affects 6–10% of women in reproductive age and is largely associated to sterility problems (30–50%) [1]. This gynecological disease involves ovulatory dysfunctions such as alterations in folliculogenesis, peritoneal fluid (PF) or inflammatory responses [2]. Regarding the PF, women affected by endometriosis present a greater number of macrophages [3], resulting in a higher concentration of cytokines, growth factors, and reactive oxygen species [4,5]. Specifically, levels of vascular endothelial growth factor, interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis α-factor (TNF-α) are increased in the follicular fluids of endometriotic patients [6,7]. In this case, no physical barriers exist between the lumen of the fallopian tube and peritoneal cavity. Thus, *in vivo* interactions between the spermatozoa and PF may be prolonged for days until ovulation occurs [8], affecting the functionality of male and female gametes [9].

Fertilization success depends largely on spermatozoa physiological events such as the hyperactivation, capacitation, acrosome reaction, and the union to the zona pellucida [10]. Overall, several morpho-functional spermatozoa biomarkers have been previously identified in relation to endometriosis. However, most data obtained are controversial. For instance, previous reports found that endometriosis adversely affects spermatozoa motility [11], acrosome reaction [12], DNA damage [13], and oocyte binding capacity [14]. On the contrary, others

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did not find differences in these biomarkers [15,16]. Furthermore, *in vitro* studies reported that cytokines stimulate the lipid peroxidation of spermatozoa plasma membrane, affecting fertility capacity [17,18]. The redistribution of plasma membrane surface sugars is a major feature of spermatozoa capacitation and develops a prominent role in spermatozoa-zona pellucida binding [19]. However, to the best of our knowledge, there are no previous studies that analyze the implication of PFE in the redistribution of glycoconjugates during spermatozoa capacitation.

Here, we conducted, for the first time, a long-term culture (24 and 48 h) to analyze the influence of PFE and PFNE on physiological and structural biomarkers that reflect the human spermatozoa functionality. This methodology could be useful to understand the idiopathic infertility in the male partners of women with endometriosis and help to find the most adequate therapeutic approach to solve it.

2. Material and methods

2.1. Patients and peritoneal fluid sampling

Participants were 25 women (25–47 age range) with endometriomas who underwent conservative or total surgery for the treatment of moderate-to-severe endometriosis without any medical or surgical treatment for at least three months before surgery. Of the total, 15 and 10 patients were identified as presenting moderate or severe endometriosis, respectively, as according to the American Society for Reproductive Medicine guidelines (ASRM) [20]. The non-endometriosis group (with other ovarian pathologies) included 20 women (15–44 age range) undergoing surgery for benign non-hormone-dependent ovarian tumors (Fig. 1). Informed consent was obtained from each woman, and the study was approved by the Ethical Committee of San Juan University Hospital (Alicante, Spain). During surgery, the first course of action was the aspiration of 5 − 10 mL of blood-free PF from the pouch of Douglas into a sterile heparinized tube. Fluid was centrifuged at 300 *g* for ten minutes at 4 °C, the supernatant was aliquoted and stored at − 80 °C until use, in accordance with previous studies by our group [9].

2.2. Semen sample preparation

Semen samples were obtained by masturbation from 15 normozoospermic donors after three to five days of sexual abstinence. Basic semen analysis was performed in accordance with the World Health Organization (WHO) [21] guidelines. Spermatozoa capacitation was performed by a double-layer density gradient centrifugation (45 % and 90 %, PureSperm, Nidacon, Mölndal, Sweden) for 20 min at 250 *g*. The supernatant was removed and the pellet was washed (300 *g* for ten minutes) with 3 mL of culture medium (G-IVF PLUS, Vitrolife, Göteborg, Sweden). Then, the cells were resuspended in the medium at a concentration of 2×10^6/mL and cultured for 24 and 48 h (Fig. 1).

2.3. Spermatozoa culture

Cells were cultured for 24 and 48 h exposed to 20 % (*v/v*) of PFE and PFNE at 37 °C and 5.5 % (*v/v*) CO_2. Controls were performed only with commercial culture medium (G-IVF Plus, Vitrolife). An average of three PFs was tested for each seminal sample. A total of seven physiological conditions were analyzed with the spermatozoa: 0, 24, and 48 h controls, 24 and 48 h cells cultured with PFNE, and 24 and 48 h cells cultured with PFE (Fig. 1). The cell cultures were fixed in 2 % (*v/v*) paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) for 45 min at 4 °C. After fixation, paraformaldehyde was replaced with phosphate buffered saline without calcium and magnesium, pH 7.4 (PBS, Biowest, Nuaillé, France), and finally samples were stored at 4 °C to assess TP and membrane surface sugars. The evaluation of cell cultures was performed blindly, unknowing if the PF came from women with or without endometriosis.

2.4. Assessment of spermatozoa motility and viability

In each culture time (0, 24, and 48 h) motility and spermatozoa viability were evaluated. Motility was examined by depositing 10 µL of sample in a Makler® (BioCare Europe, Rome, Italy) counting chamber and evaluating the percentage of progressive, non-progressive and immobile spermatozoa. Viability was analyzed by flow cytometry using Annexin V/Propidium iodide (PI) Apoptosis Detection Kit (eBioscience™, San Diego, California, USA). An aliquot (50 µL, around 100.000 cells) of each sample was added to 1000 µL of binding buffer prepared according to the manufacturer's instructions and centrifuged at 800 *g* for five minutes. Then, 5 µL of Annexin V–FITC was added to the pellet and incubated in the dark at room temperature for 15 min. Cells were washed again with 1000 µL of binding buffer and finally, the pellets were resuspended in 500 µL of binding buffer containing 3 µL of PI. Additionally, negative controls were performed (without Annexin-V and propidium iodide) to set the working threshold. Data were acquired using a FC 500 MPL cytometer (Beckman Coulter®, Brea, California, USA). FL1 (FITC) signals were detected through a 530/30 nm band pass filter and FL2 (PI) through a 585/42 nm band pass filter. The spermatozoa population was gated on the basis of the linear orward (FSC) and side-scatter (SSC) properties. A total of 10,000 events were recorded and analyzed using the CXP software.

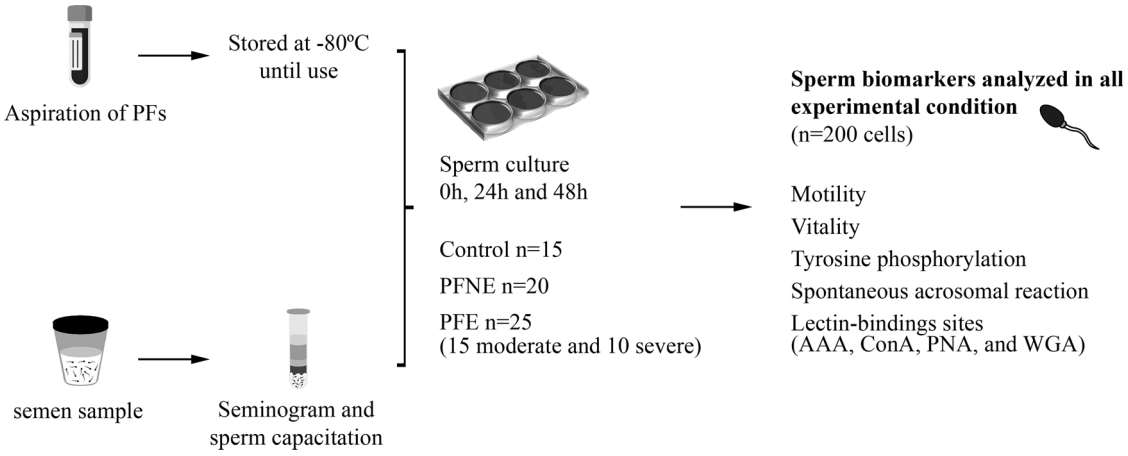

Fig. 1. Experimental design performed.

Table 1
Influence of peritoneal fluid on spermatozoa motility and membrane integrity.

Parameter (%)	0 h	24 h	48 h
Total motility (P + NP)			
Control	96.13 ± 2.58	70.75 ± 3.88[a]	35.38 ± 4.64[a,b]
PFNE		49.33 ± 4.66[a,b]	36.44 ± 5.86[a,b]
PFE		40.00 ± 5.24[a,b]	23.08 ± 4.05[a,b,c,d]
Membrane integrity			
Control	94.52 ± 3.94	69.47 ± 4.93[a]	65.56 ± 5.54[a]
PFNE		77.01 ± 3.81	73.86 ± 3.88
PFE		80.70 ± 3.59[b]	70. 85 ± 5.09

Progressives and non-progressives (P + NP); Peritoneal fluid from non-endometriosis women (PFNE); peritoneal fluid from endometriosis women (PFE). Data are represented as mean and standard error (SE).
Significant differences (Mann–Whitney U test at $P < 0.05$): to 0 -h control (a); to 24 -h control (b); to 48 -h control (c); to 24 -h PFE (d).

2.5. Immunolocalization of tyrosine phosphorylation (TP)

A total of 5 µL of sperm suspension from each paraformaldehyde-fixed condition was deposited on a coverslip. When dry, the cells were washed and rehydrated three times in PBS during five minutes. Spermatozoa were permeabilized by incubation in 0.1 % (*v/v*) Triton X-100 (Sigma-Aldrich®, Saint Louis, MO, USA) for ten minutes. In an attempt to prevent unspecific binding, spermatozoa were blocked with 2 % (*w/v*) BSA-PBS for 30 min. TP was detected using an anti-phosphotyrosine primary antibody produced in mice (PY20, Sigma-Aldrich) at 1:500 for one hour and a secondary mouse IgG (H + L) antibody conjugated to Cyanine ™3 (Jackson ImmunoResearch, Ely, UK) at 1:300 for one hour in the dark, taking previous protocols as reference [22]. Slides were rinsed with PBS between both incubations. Finally, coverslips were washed again three times in PBS and subsequently mounted with Vectashield® and 4′,6-Diamidino-2′-phenylindole dihydrochloride (DAPI, Vector Laboratories, Burlingame, CA, USA), the latter to stain the cell nucleus. The whole process was conducted at room temperature.

2.6. Assessment of the spermatozoa acrosomal status

The assessment of the spontaneous acrosome reaction was performed placing 5 µL of each physiological condition on coverslips and fixing in methanol for 30 min. After drying, the spermatozoa were washed with PBS three times for five minutes and unspecific bindings were blocked with 2 % (*w/v*) BSA-PBS for 30 min. Smears were then incubated in the dark with *Pisum sativum agglutinin* lectin conjugated to fluorescein-5-isothiocyanate (PSA-FITC, Sigma-Aldrich) 50 µg mL^{-1} for 30 min, as previously reported [23]. After three washes in PBS, the samples were mounted using Vectashield antifade medium and DAPI.

2.7. Lectin cytochemistry

As lectins bind to the carbohydrate group of glycoconjugates, several lectins were used to characterize the spermatozoa surface carbohydrate distribution by means of cytofluorescence. More specifically, four lectins were conjugated with FITC (Vector Laboratories): *Aleuria aurantia agglutinin* (AAA), *Concanavalin A* (ConA), *Peanut agglutinin* (PNA), and *Wheat germ agglutinin* (WGA). Each lectin presents a high specificity for a different carbohydrate. AAA recognizes fucose residues [24]; ConA has a high affinity for mannose [25]; PNA for galactose [26], and WGA for sialic acid [27]. A total of 5 µL sperm suspension from each paraformaldehyde-fixed condition was deposited on a coverslip. Once dry, the smear was rehydrated three times with PBS for five minutes and incubated in 2 % (*w/v*) BSA-PBS blocking solution for one hour. Next, the cells were incubated with each FITC-conjugated lectin at a final concentration of 20 µg mL^{-1} for one hour at room temperature in a dark humid chamber [28]. Finally, spermatozoa were rinsed three times in PBS for five minutes and assembled with Vectashield antifade medium with DAPI.

2.8. Fluorescence microscopy

The data related to TP, spontaneous acrosome reaction, and lectin biding sites were examined using a Leica TCS-SP2 (Leica Microsystems GmBH, Wetzlar, Germany) confocal microscope, and Leica Confocal Software was used to obtain the images (1024 × 1024 pixels). At least 200 cells were evaluated for each biomarker and physiological condition. In addition, the appropriate negative controls served to corroborate the specificity of each biomarker.

2.9. Statistical analyses

The Shapiro-Wilk (W) test showed significant spermatozoa biomarker differences in distribution and variance ($W = 0.753$ to 0.964; $P < 0.01$). The non-parametric Mann–Whitney U test was used for bivariate associations between the effects of different physiological conditions within each biomarker analyzed. Descriptive and statistical results were obtained by means of IBM SPSS Statistics 22.0 (IBM, Armonk, NY, USA). Results are presented as mean and standard error (ER). Two-sided *P*-values < 0.05 were deemed statistically significant.

3. Results

3.1. Effect of peritoneal fluid on spermatozoa motility and membrane integrity

After 24 h, results showed significant differences (Mann–Whitney U test; $P < 0.05$) in motility percentage, ranging from 40.00 % in spermatozoa incubated with PFE ($U = 96.50$; $P = 0.005$), 49.33 % in PFNE ($U = 82.50$; $P = 0.033$), to 70.75 % in the control (see Table 1). In the 48 -h culture, only the cells in contact with PFE (23.08 %; $U = 118.00$; $P = 0.028$) presented a significantly lower percentage of motility compared to the control (35.38 %). Conversely, no significant results were observed in the motility between the spermatozoa cultured with PFNE (36.44 %; $U = 141.50$; $P = 0.932$) and the controls (35.38 %) after 48 h of incubation.

The viability data indicated different patterns in the bivariate Annexin V/PI analysis. Results classified spermatozoa as normal (negative Annexin V and negative PI), early apoptotic (positive Annexin V and negative PI), late apoptotic (positive Annexin V and positive PI), and necrotic (negative Annexin V and positive PI). Normal and early apoptotic spermatozoa were considered as intact membrane (viable). The exposure of the spermatozoa to PFE and PFNE did not affect the percentage of viability in any studied culture time compared to 0 h (Table 1). In fact, the PFE recorded greater spermatozoa survival after 24 h (80.70 %; $U = 117.00$; $P = 0.050$) of culture compared to the control (69.47 %). On the other hand, a significant decrease was detected in spermatozoa viability in the controls from 94.52 % at 0 h to 69.47 % at 24 h ($U = 52.00$; $P = 0.011$) and 65.56 % at 48 h ($U = 65.50$; $P = 0.050$).

3.2. Effect of peritoneal fluid on spermatozoa tyrosine phosphorylation

The state of TP in the spermatozoa flagellum was analyzed in order to confirm if the PFs from women with and without endometriosis influence in the spermatozoa capacitation (Fig. 2A). As a result, a significant increase was detected (Mann–Whitney U test; $P < 0.05$) in the percentage of cells with TP from 18.50 % at 0 h to approximately 60.00 % at 24 h in all culture conditions (PFE, PFNE, and control). However, in the 48-h culture, only the cells in contact with PFE presented a significant decrease in phosphorylation, being 52.92 % ($U = 99.00$; $P = 0.006$) compared to 69.75 % in the control (Fig. 2B).

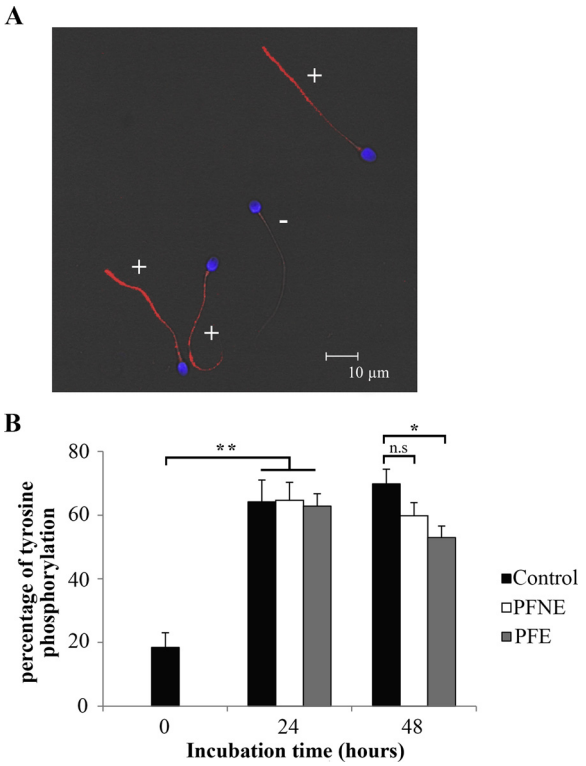

Fig. 2. Influence of peritoneal fluid on spermatozoa tyrosine phosphorylation. (A) Immunofluorescence of tyrosine phosphorylation. Positive label (+); negative label (-). (B) Percentage of spermatozoa positive tyrosine phosphorylation. Bars represent standard error. Significant differences (Mann–Whitney U test); $P < 0.05$ (*); $P < 0.001$ (**); not significant (n.s).

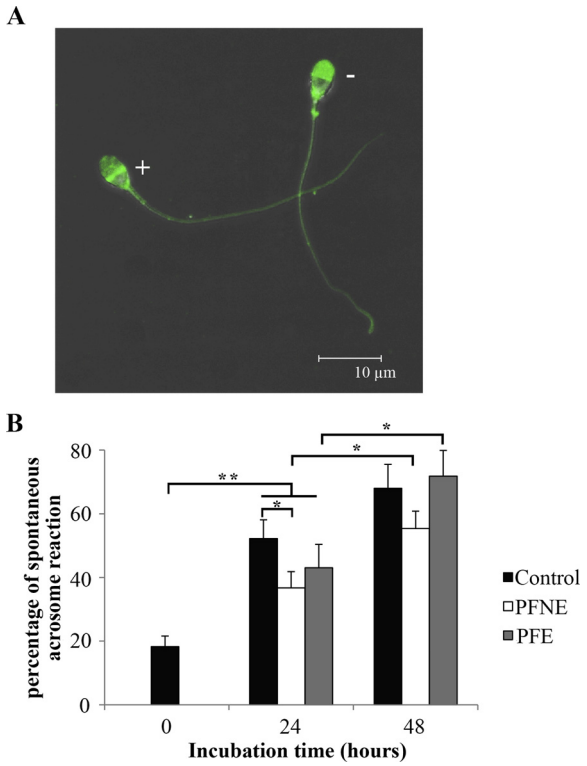

Fig. 3. Influence of peritoneal fluid on spontaneous acrosome reaction. (A) Staining with PSA lectin: acrosome reacted spermatozoa (+); non-acrosome reacted spermatozoa (-). (B) Percentage of spontaneous acrosome reaction. Bars represent standard error. Significant differences (Mann–Whitney U test); $P < 0.05$ (*); $P < 0.001$ (**).

3.3. Effect of peritoneal fluid on spontaneous acrosome reaction

To study whether the PFE and PFNE influence in the spermatozoa acrosome state, the percentage of spontaneous acrosome-reacted cells were evaluated using PSA binding (Fig. 3A). Results reported a significant increase (Mann–Whitney U test; $P < 0.05$) in the percentage of cells with acrosome reaction from 9.18 % at 0 h to ~20.00 % in all conditions (PFE, PFNE, and control) after 24 h (see Fig. 3B). Specifically, at 24 h of culture, the cells cultured with PFNE (18.41 %; U = 71.00; $P = 0.033$) presented a significantly lower spermatozoa rate with reacted acrosome compared to the control (26.07 %). On the contrary, no significant differences were recorded between PFE (21.54 %; U = 118.50; $P = 0.076$) and control (26.07 %) at 24 h. On the other hand, after 48 h of culture, the percentage of reacted spermatozoa in contact with PFE (35.41 %; U = 131.50; $P = 0.004$) and PFNE (27.65 %; U = 79.50; $P = 0.024$) increased significantly compared to the 24 h culture, being 21.54 % and 18.41 %, respectively. However, no significant differences were detected in PFE and PFNE acrosomal data compared to the control (34.08 %) at 48 h of culture (Fig. 3B). Overall, the cells cultured with PFNE exhibited higher rates of acrosomal integrity at 24 and 48 h.

3.4. Effect of peritoneal fluid on plasma membrane sugar-binding sites

To discern whether PF from women with or without endometriosis influences in the location of fucose, mannose, galactose, and sialic acid membrane sugars, fixed cells were incubated with AAA, ConA, PNA and WGA lectins, respectively. The results revealed a high degree of carbohydrate surface heterogeneity, since spermatozoa subpopulations with different location patterns appeared in all physiological conditions. This allowed to characterize five clearly distinct patterns (see Fig. 4A, for further details). Pattern 1 (P1) presented a highly stained acrosomal region; Pattern 2 (P2) comprised of a highly stained acrosomal and post-acrosomal region; Pattern 3 (P3) included labelling in the equatorial segment; Pattern 4 (P4) presented a dotted label in the whole head; and Pattern 5 (P5) was identified by a faint labeling in the whole head. All patterns were counted for both the AAA and ConA lectins. However, only three patterns (P1, P3, and P5) were used to measure PNA and four for WGA (P1, P3, P4, and P5).

In relation to the disposition of sugars recognized by AAA lectin (Fig. 4B) in the 24- and 48 -h cultures, no significant differences (Mann–Whitney U test; $P < 0.05$) were identified between controls and PFNE, since the majority of the sugar residues were located in the acrosomal region (P1: > 30.00 %). Conversely, the cells in contact with PFE presented a significantly lower percentage of P1 after 24 h of culture (15.27 %; U = 57.00; $P = 0.001$) than the control (36.21 %). On the other hand, after 48 h, the results reported a significantly lower percentage of cells in the PFE cultures with the equatorial segment labelled (35.36 %; U = 101.50; $P = 0.030$) compared to the control (20.21 %; Fig. 4B).

Regarding the localization of the ConA-binding sugars (Fig. 4B), at 24 h, a significant decrease in P4 (dotted label over the head) was detected between the control and the PFs (PFE and PFNE). At this time, P2 (label at the acrosomal and post-acrosomal regions) was the most abundant pattern in all conditions (> 30.00 %). After 48 h in the PFE cultures, a non-significant increase in cells with equatorial region staining (P3) was detected, up to 36.35 % (U = 121.50; $P = 0.117$), which was the most numerous pattern detected in this condition, with a significant decrease in the percentage of P2 (14.50 %, U = 103.50; $P = 0.035$) compared to control (27.21 %). On the contrary, after 48 h, P2 continued to be the predominant pattern in the control and PFNE culture.

The PNA-label sites revealed that, at both 24 and 48 h, there were no differences in the sugar distribution of the surface between the

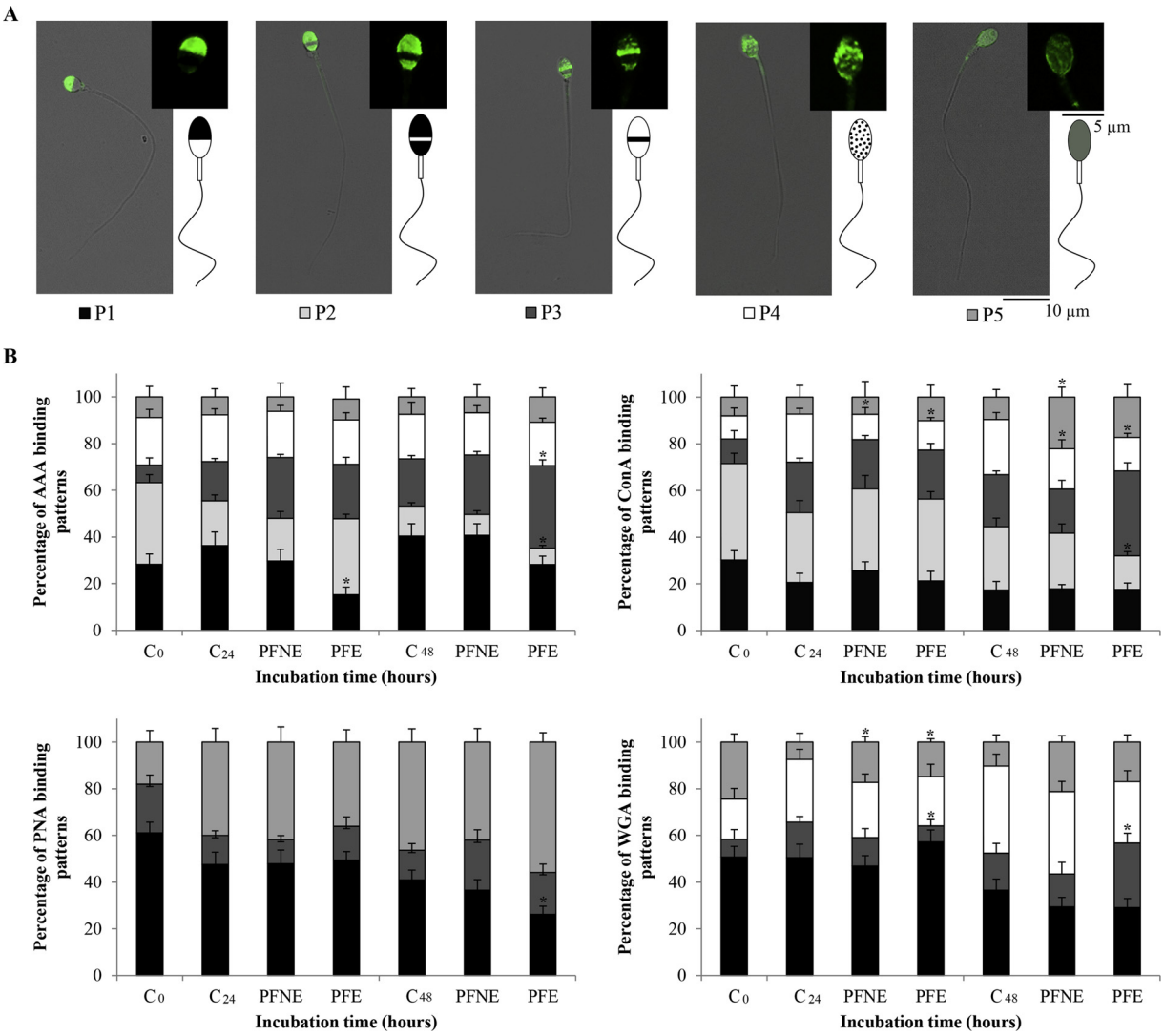

Fig. 4. Influence of peritoneal fluid on plasma membrane sugars binding sites. (A) Location of spermatozoa membrane sugars patterns using fluorescent lectins (AAA, ConA, PNA, and WGA). Acrosomal region (P1); acrosomal and post-acrosomal region (P2); equatorial segment (P3); dotted label in the whole head (P4); faint label in the whole head (P5). (B) Percentage of different fluorescent patterns in each lectin. Bars represent standard error. Significant differences (Mann–Whitney U test) refer to the control of the respective time $P < 0.05$ (*).

spermatozoa cultured in the controls and with PFNE (see Fig. 4B). The majority of the spermatozoa population distributed the sugars in the acrosomal region (P1) or stained weakly throughout the head (P5). Nonetheless, after 48 h of incubation, a lower percentage of cells of the PFE cultures had the acrosome stained (P1: 26.05 %; U = 90.50; $P = 0.012$) compared to its control (40.94 %).

Finally, WGA-binding sites reported significant differences in the distribution of sugar residues of the gametes cultured with PFNE compared to controls only in P5 (faint label head) in the 24 -h culture (see Fig. 4B). Conversely, the cultures with PFE, at both 24 and 48 h, presented significant differences in the head-spatial organization of the sugars compared to the controls. Particularly, significant differences were observed at 24 h regarding the number of cells with the equatorial band stained (P3: 6.91 %; U = 91.50; $P = 0.028$) and over the head (P5: 14.75 %; U = 88.50; $P = 0.022$) compared to the control (15.29 % and 7.50 % respectively). Similarly, after 48 h the percentage of spermatozoa with P3 increased relevantly (27.68 %; U = 99.00; $P = 0.032$) in relation to its control (15.85 %). The most abundant WGA-binding pattern was P1 (> 50.00 %) in all the culture conditions (control, PFNE, and PFE) after 24 h and in cells cultured with PFE after 48 h of incubation (30.01 %). However, spermatozoa from control and PFNE presented P4 (dotted fluorescence; > 35.00 %) as principal pattern after 48 h.

4. Discussion

The findings of this study showed that the PFE caused a significant deleterious effect in spermatozoa quality and functionality in comparison with controls. In particular, a longer exposure time (48 h) of cells in the presence of PFE caused significant alterations in spermatozoa motility, tyrosine protein phosphorylation, and the relocation of glycocalyx sugars. Therefore, this could be interpreted as that the PF cytotoxicity towards the spermatozoa may be due to the presence of soluble factors associated specifically with endometriosis (PFE) and not with other ovarian pathologies (PFNE). There are potential clinical implications in this study, since spermatozoa cultured in presence of PF could be useful to assess, *in vitro,* the male partner functionality of women with endometriosis, helping to diagnose the infertility. Overall, this report provides novel insights regarding spermatozoa physiology and endometriosis, and highlights the role of spermatozoa cultures in assessing the toxicity of the peritoneal cavity environment.

Many points related to endometriosis and oocyte quality have been

explored, including the status of granulosa cells [29], nitrate and cytokines levels in follicular fluid [30,31], and oocyte mitochondrial structure [32]. In addition, previous studies assessed oocyte morphology, maturation, and fertilization rates [33,34]. Therefore, endometriosis affects both the development of the oocyte and the embryo [9]. However, few reports have focused on male gamete functionality during PFE interaction. In this context, PF has been regarded as an endometriosis potent biomarker [35,36] and the abundant cytokines/growth factors present in PFE could cause detrimental effects on the reproductive process [37,38].

Previous reports revealed that PFE could inhibit spermatozoa motility [11,39], while others report that this effect does not occur [15,16]. Possibly this controversy is due to the differences in factors such as the preparation of the gametes, the incubation time or the spermatozoa motility analysis method. In fact, reports that did not find differences in motility only maintain the exposure of spermatozoa with PFE for up to six hours [15,16]. However, this time interval differs from those observed in physiological conditions, since spermatozoa can remain viable for a total of five days in the oviduct reservoir [8]. Our findings revealed a significant decreased motility in spermatozoa cultured with PFE after 24 and 48 h compared to their respective controls. This fact reaffirmed that the PFE impairs spermatozoa motility [11,40], especially in long-term incubation.

The effect of PFE and PFNE on spermatozoa membrane integrity was also studied. Our results revealed that, after 24 and 48 h of cell culture, both PFs were favorable environments for spermatozoa survival, since no significant differences in viability were observed in relation to controls. In fact, the results showed better viability rates in the cultures with PFs (PFE and PFNE) than in the controls. Other studies have observed similar effects of PFs on spermatozoa vitality in short-term incubation [15,35]. On the other hand, a previous report in which spermatozoa were incubated in commercial culture medium for 22 h showed a significant increase in spermatozoa redox activity and in lipid peroxidation, but not in cell viability [41]. This information could explain why spermatozoa in contact with PFE maintain viability while a detrimental effect on motility and membrane sugars occurs.

It is known that spermatozoa hyperactivation during capacitation is associated with an increase in TP [22]. Therefore, the current study evaluates for the first time the influence of PFs from women with and without endometriosis in this biological process. Our results revealed that 24 h of incubation did not affect the percentage of cells with TP protein. Similar results have been observed using PF from women with unexplained infertility after six hours of exposure [35]. Nevertheless, our experiments indicated that longer exposure times (48 h) had a significantly negative effect on the cells incubated with PFE, since the percentage of spermatozoa with TP decreased compared to the control. Previous studies observed that high concentrations of IL-6, as well as macrophage migration inhibitory factor, had a deleterious effect on protein TP [42,43]. Therefore, the decrease in our study of flagellar TP in PFE after 48 h of culture could be related with the high concentration of cytokines present. In addition, since TP is associated with spermatozoa hypermotility [22], this reduction in phosphorylation after 48 h could be one of the factors involved in the low percentage of motility observed in cells cultured with PFE.

The effect of PFs (PFE and PFNE) on spermatozoa spontaneous acrosome reaction was analyzed, since an intact acrosome is necessary for fertilization and to pass through the zona pellucida [44]. Our findings revealed that, in the 24 -h culture, both PFs were non-inducers of acrosome reaction. However, the reacted spermatozoa rate was significantly lower in the cultures with PFNE compared to the control. Previous studies have also observed the lack of effect of PFs as acrosome reaction inducers during short incubation times [12,35]. In fact, the detriment of spermatozoa acrosome reaction has been associated with an increase in iron concentration in this fluid [45]. Similarly, after 48 h of culture, we recorded similar results between the rates of spermatozoa spontaneous acrosome reaction of the cultures with PFs and the control. Therefore, this demonstrated that the PF continues to be a non-inducer of the acrosomal reaction after long exposure times (48 h).

An essential requirement for spermatozoa capacitation and oocyte's zona pellucida binding is the correct redistribution of plasma membrane glycoconjugates [19,46]. Our results indicated that PFE significantly impacted the location of carbohydrates on the spermatozoa surface, especially after 48 h of culture. In particular, we observed a relocation of the sugars, identified as lectins AAA and ConA, moving from the acrosomal region (P1 and P2) to the equatorial segment (P3) as the cultivation time increased. Similarly, we recorded a decrease of spermatozoa with the PNA-binding sites in the acrosomal region (P1) and an increase of the residues bound to WGA in the equatorial region (P3). Previous studies have described that cytokines, such as IL-6, IL-8, and TNF-α, seems to be involved in the increase of lipid peroxidation in spermatozoa membranes [17,18]. Therefore, this generalized relocation of surface sugars from the acrosomal region to the equatorial segment could be closely linked to the presence of pro-inflammatory cytokines in the PFE [6,7]. Overall, the increase in fluidity and instability in the membrane of spermatozoa cultured with PFE could be the cause of the improper lectins-binding sites redistribution, affecting the spermatozoa fertilizing capacity [11,47].

To our knowledge, this is the first study to have examined and compared spermatozoa functionality after 24 and 48 h of incubation with PF from women with endometriosis or with other non-endometriosis ovarian pathologies. Here, we reported that PFE affects spermatozoa functionality *in vitro*. Specifically, PFE impairs spermatozoa motility, tyrosine protein phosphorylation, and the redistribution of membrane sugars after 48 h of exposure. Therefore, taking into account the detriment of sperm and oocyte quality, specialists should recommend to the endometriosis patients to adjust the timing of intercourse for minimum exposure of gametes to PF. Overall, our results contribute to explain the physiological and molecular basis associated with the male infertility in endometriosis.

Author contribution

P Sáez-Espinosa: Data collection, Data analysis, Manuscript preparation

I Velasco: Project development, Data collection, Data analysis, Manuscript preparation

P Lorca: Data collection, Manuscript preparation

MI Acién: Project development, Data collection, Manuscript preparation

A Romero: Data analysis, Manuscript preparation

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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