Seminal plasma mitigates the adverse effect of uterine fluid on boar spermatozoa

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Abstract

Artificial insemination (AI) is widely used in farms with an intensive pig production. After natural or AI, spermatozoa start their journey within the uterus to reach the site of fertilization, but only few spermatozoa can do it. So, it is interesting to study spermatozoa-uterus interaction, trying to know how the spermatozoa selection is made in order to increase AI efficiency. Our aim was to analyze the effect of the UF on spermatozoa with or without SP. The experiments were performed with boar spermatozoa from ejaculate and epididymis. For each kind of spermatozoa, three experimental groups were used: 1) Control: spermatozoa with 20 % of SP; 2) UF group: spermatozoa with 20% UF; 3) UF-SP group: spermatozoa with 20% SP and 20% UF. Each group has been incubated for 180 min and total and progressive motility, kinetic parameters (VCL, VSL, VAP, LIN, STR, WOB, BCF), viability and acrosome integrity were analyzed over the time (15, 60, 120 and 180 min). Our results showed a lower total and progressive motility in ejaculated sperm incubated with UF than control and UF-SP. The VCL was greater in control and UF-SP than UF; the VSL was greater in UF-SP than control and UF; the VAP was greater in UF-SP than control and UF and it was greater in control than UF; the LIN was greater in UF-SP than control and UF. The STR was greater in UF-SP than UF. For BCF an WOB there was no significant differences between treatments. Regarding the viability, it did not show difference between the treatments. The acrosome damage was greater in UF compared with control and UF-SP. In epididymal spermatozoa it was no difference in total and progressive motility. They showed a greater WOB in UF-SP than control. There was no significant differences in the other kinetic parameters. The viability was higher in control and UF-SP than UF, and the acrosome damage was greater in UF than control and UF-SP. From these results, we can conclude that the presence of UF affects both ejaculated and epididymal sperm and SP seems to play a critical role on spermatozoa, preserving motility and acrosome integrity, mitigating by this way the negative effect of UF.

Keywords: artificial insemination, ejaculated sperm, epididymal sperm, porcine, reproductive fluids, sperm function

1. Introduction

After mating or artificial insemination (AI) in pigs, the spermatozoa are deposited in the female genital tract and they start its journey from the site of deposition to the site of fertilization, crossing the uterine horns and reaching the oviduct to meet and fertilize the egg (Hunter, 1981) (1). Although, a few minutes after insemination, the first spermatozoa have been observed in the oviduct, but those do not participate in the fertilization (Rodriguez-Martinez et al., 2010) (2). Normally, spermatozoa take between 1 to 2 h to reach and colonize the oviduct in swine (Rath et al., 2008) (3). During this journey from the uterus to the oviduct, a lot of spermatozoa are lost (Sumransap et al., 2007) (4), only reaching the site of fertilization less than 0.01% of inseminated spermatozoa (First et al., 1968; Viring and Enarsson, 1981) (5,6). The spermatozoa are exposed to different known mechanisms which reduce the population within the uterus such as: 1) backflow, where part of the semen is expelled through the vulva after insemination, decreasing up to 75% of the total number of spermatozoa deposited (Steverink et al., 1998; Hernandez-Caravaca et al., 2012) (7,8). This mechanism occurs in a high percentage of inseminated sows (Hernández-Caravaca et al. 2015) (9); 2) the influx of polymorphonuclear (PMN) granulocytes, from the endometrium into the uterus causing spermatozoa phagocytosis (Taylor et al., 2009; Matthijs et al., 2003) (10,11).

Moreover, other factors mostly unknown are involved in the decrease of spermatozoa population within the uterus (reviewed by Holt and Fazeli, 2015) (12). It is known that some own properties of the spermatozoa such as motility, morphology, DNA fragmentation status, sensitivity to signaling molecules and many other properties are important to reach the vicinity of the oviduct (reviewed by Garcia-Vazquez et al., 2016) (13) and also determine fertilization success (reviewed by Holt et al., 2004) (14). The spermatozoa are subjected to different environments in the male genital tract before be deposited in the female genital tracts. Once

78 leave the testicle, spermatozoa travel through the epididymis and finally they are stored in the
79 cauda immersed in the epididymal fluid (reviewed by Holland and Nixon, 1998) (15). The
80 epididymal fluid is a biological fluid containing substances (water, inorganic ions, proteins, …)
81 that contributes to create a suitable environment for sperm protection,maturation and storage
82 previous to contact with seminal plasma (SP) during ejaculation (Rodriguez-Martinez, 2007)
83 (16). The SP is a complex mixture of secretions originated from the testes, epididymis, vas
84 deferens and accessory sex glands that include seminal vesicles, prostate gland and
85 bulbourethral gland (Garner and Hafez, 2000) (17). The SP plays an important role during
86 fertilization, acting during spermatozoa capacitation, and modulating the female immune system
87 (Centurion et al., 2003) (18). In this sense, SP reduces the influxes of PMNs in the uterus
88 (Rozeboom et al., 1998) (19), and improves the spermatozoa transport and fertility after
89 insemination (Rozeboom, 2000) (20). Thus, SP presents protective effects on spermatozoa
90 during their journey through female genital tract (revised by Katila, 2012) (21). Subsequently,
91 once the spermatozoa are deposited in the uterus, the journey in search of the oocyte starts
92 contacting with uterine fluid (UF). The UF, is an intricate biological fluid which contains ions,
93 growth factors, cytokines and a multitude of proteins and proteolytic enzymes, as has been
94 shown in human (Gardner et al., 1996) (22), ewe (Iritani et al., 1969) (23), rabbit (Iritani et al.,
95 1971) (24) . These act as a line of defense against pathogens, aid spermatozoa migration and,
96 therefore, influence fertility (Casado-Vela et al., 2009) (25). Moreover, UF acts against
97 unprotected spermatozoa from SP, presenting a negative effect on spermatozoa motility,
98 viability and acrosome at least in mice (Kawano et al. 2014) (26). When spermatozoa take
99 contact with UF, a change in protein composition of UF occurs, leading to changes in
100 spermatozoa motility, viability and acrosome integrity (reviewed by Holt and Fazeli, 2015) (12).
101 Nowadays, AI is the most widespread reproductive technique in swine (Bortolozzo et al., 2015)
102 (27). AI requires seminal doses which are prepared diluting the ejaculate in an appropriate
103 extender (commonly composed by several nutrients: ions, monosaccharides, bovine serum
104 albumin, bicarbonate, antibiotics), used to preserve spermatozoa function and fertilizing ability

(Yeste M, 2017) (29). Consequently, seminal doses are highly diluted, containing less amount of SP which decreases the benefits aforementioned (Maxwell and Johnson, 1999) (30).

The hypothesis of study is that SP protects boar spermatozoa from negative effects of UF, avoiding the decreasing of spermatozoa quality in presence of this reproductive female fluid. Moreover, although SP is removed from ejaculated, the previous contact may modulate the plasma membrane of spermatozoa, changing receptors and adding molecules than change their physiology (Tapia et al., 2012) (31). Therefore, the aim of the present study was to evaluate the effect of UF on the quality of ejaculated spermatozoa (previously contacted with SP) and epididymal spermatozoa (without previous contact with SP) analyzing motility, kinetic parameters, viability and acrosome integrity in presence or absence of SP over the time (15, 60, 120 and180 min).

2. Material and methods

2.1. Ethics

The study was carried out following the Spanish Policy for Animal Protection RD 53/2013, which meets European Union Directive 2010/63/UE on animal protection. All the procedures carried out in this work were approved by the Ethical Committee of Animal Experimentation of the University of Murcia and by the Animal Production Service of the Agriculture Department of the Region of Murcia (Spain) (ref. Nº A13160609).

2.2. Spermatozoa collection (epididymal and ejaculate)

Epididymis from 6 different mature boars were obtained from a slaughterhouse (El Pozo S.A., Alhama de Murcia, Murcia, Spain). The caudal portion from epididymis was dissected. Then, a 24G BD Insyte™ catheter (381212, Becton Dickinson Infusion Therapy Systems, Inc., Sandy, Utah, USA) adapted to a syringe full of air was introduced into deferent duct, and the epididymal fluid containing spermatozoa was collected by retrograde airflow.

Ejaculated spermatozoa were collected by manual technique from 9 boars with proved fertility (CEFU S.A., Murcia, Spain). All boars were maintained in abstinence during 3-4 days before

ejaculate collection. The spermatozoa samples had a minimal of quality criteria before use (rich fraction volume ≥ 75 ml, concentration ≥ $200x10^6$ sperm/ml, total motility ≥ 70% and viability ≥ 85%). Spermatozoa concentration was calculated by a SpermaCue photometer (Minitüb, Germany) or by hemocytometer (Neubauer counting chamber; VWR International, Haasrode, Belgium).

2.3 Collection and preparation of biological fluids (SP and UF)

In order to obtain the SP, immediately after collection, the ejaculate was centrifuged at 13800*g* (Model 5418 R, Eppendorf®, Germany) for 10 min at 4ºC. Then, the supernatant was collected and centrifuged again under the same conditions to remove cell debris and any remaining spermatozoa (microscopically verified). The SP samples were then separated into aliquots and stored at -80ºC (New Brunswick Premium u570 ULT Freezer) until use. Boar SP from 3 different males were mixed in a single pool to perform the experiments.

The UF was obtained from genital tracts at the slaughterhouse (El Pozo S.A., Alhama de Murcia, Murcia, Spain). The oestrus cycle stage corresponded with the late follicular phase [periovulatory follicles (8-11 mm Ø)] based on the appearance of the ovary (Carrasco et al., 2008) (32). The female genital tracts were transported to the laboratory within 30 min after collection and the UF was extracted through a mechanical pressure from the uterine tubal junction to the end of the horns. It was centrifuged twice at 7200 x *g* for 10 min at 4ºC to remove debris, and finally the samples were stored in aliquots at -80ºC until use. For experiments a pool of UF from 3 different females were used.

2.4 Evaluation of spermatozoa motility

The Computer Assisted Semen Analysis (CASA) was used for the evaluation of spermatozoa motility by ISAS® software (PROiSER R+D S.L., Valencia, Spain) connected to a phase-contrast microscope (negative-pH 10x objective; Leica DMR, Wetzlar, Germany) and a digital camera (Basler Vision, Ahrensburg, Germany). A 4 µl drop of the sample was placed in a prewarmed (38ºC) chamber (20 micron Spermtrack® chamber, Proiser R+D, SL; Paterna,

157 Spain) and at least three fields per sample were recorded. Total motility (%), progressive
158 motility (%), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), straight line
159 velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm), linearity of the
160 curvilinear path (LIN, ratio of VSL/VCL, %), straightness of the average path (STR, ratio of
161 VSL/VAP, %), wobble coefficient (WOB, %) and beat-cross frequency (BCF, Hz) were
162 analyzed.

163 *2.5 Analysis of spermatozoa acrosome status*

164 The spermatozoa acrosome status was analyzed by epifluorescence microscopy using a
165 fluorescein isothiocyanate-conjugated peanut agglutinin from *Arachis hypogea* lectin (PNA-
166 FITC) (Sigma-Aldrich®, Madrid, Spain), following the procedure by Kawano et al., 2007 (33).
167 A solution of PNA-FITC was diluted in PBS (Phosphate Buffer Solution, Sigma-Aldrich®,
168 Madrid, Spain) free of Ca^{2+} and Mg^{2+} to reach a concentration of 200 μg/ml and stored at -20°C
169 until use. A 10-μl drop of each spermatozoa sample was placed and smeared on the slides, air-
170 dried, fixed and permeabilized with 100% methanol. Then, spermatozoa were washed with PBS
171 three times during 5 min each and incubated with PNA-FITC (stock solution of 200 μg/ml) for
172 10 min in darkness. Finally, spermatozoa were washed again in PBS. The fluorescent images
173 were captured by fluorescence microscope (blue filter, BP 480/40; emission BP 527/30; Leica
174 DM4000 B LED), and at least 200 spermatozoa per sample were counted. Spermatozoa
175 exhibited a green fluorescence had intact acrosome.

176 *2.6 Analysis of spermatozoa viability*

177 The percentage of viable spermatozoa were determined evaluating membrane integrity by
178 eosin/nigrosin staining (Campbell RC et al., 1956) (34). The staining solution was prepared with
179 the following reagents: hydrosoluble nigrosin (Merck®, Darmstadt, Germany), yellow eosin
180 (Merck®, Darmstadt, Germany) and trisodium citrate (Sigma-Aldrich®, Madrid, Spain).
181 Trisodium citrate was diluted at 3.98% (w/v) in water and the pH was adjusted at 6.9. Later,
182 yellow eosin (2.5 g) and nigrosin (5 g) were mixed in 100 ml of citrate solution and then

filtrated. For the evaluation, 10 µl of sample was added in equal proportion to the stain. Immediately, a brightfield microscope (40x objective; Nikon® Model YS100, Tokyo, Japan) was used for evaluation counting at least 200 spermatozoa per sample. Spermatozoa with damage membrane (dead) showed rose color and spermatozoa with intact membrane showed colorless (alive).

2.7 Analysis of spermatozoa viscosity

Considering that variable viscosity of the different biological fluids (UF, SP) and PBS could influence on spermatozoa motility (Ishimoto et al., 2018) (35), this was measured by using the Anton Paar DMA 5000 M density-meter that includes the module Lovis 2000 ME rolling ball micro-viscometer. For the measurements it was used a steel ball of diameter 1.50 mm and a capillary of diameter 1.59 mm. The samples were introduced in the capillary and the viscosity at 38°C was supplied automatically by the instrument. Three replicates were performed and the viscosity was expressed in millipascal-second (mPa-s). The viscosity was higher when spermatozoa were incubated in UF and UF mixed with SP than in control and SP groups ($p < 0.05$) (PBS: 0.808 ± 0.003 mPas; SP: 0.843 ± 0.115 mPas; UF: 1.370 ± 0.135 mPas and UF-SP: 1.157 ± 0.105 mPas), without differences between them.

2.8 Experimental design

The effect of SP and UF on boar spermatozoa functionality (motility parameters, viability and acrosome status) was analyzed. In total three different experimental groups were evaluated: 1) control group: spermatozoa with 20% of SP; 2) UF group: spermatozoa incubated with 20% of UF; 3) UF-SP group: spermatozoa incubated with 20% of UF and 20% of SP. Two different spermatozoa conditions [ejaculate (n= 6 replicates) and epididymal spermatozoa (n= 6)] were analyzed in each experimental group. Ejaculate spermatozoa (but not epididymal) were carefully centrifuged at 500 *g* for 10 min at room temperature to eliminate SP. Samples were adjusted to $20x10^6$ sperm/ml in PBS and incubated during 3h at 38ºC and evaluated at different times (15, 60, 120 and 180 min).

209 *2.9 Statistical analysis*

210 The statistical analysis was performed using the free statistical software, SAS University
211 Edition (SAS, 2016). All the motion parameters (total motility, progressive motility, VCL,
212 VAP, VSL, ALH, LIN, STR, BCF, WOB), the percentage of alive spermatozoa and the
213 percentage of spermatozoa with acrosome damage were compared with the mixed model of
214 SAS. The model included procedures (control, UF, UF-SP), the time related to procedures and
215 their interaction as main effects, and spermatozoa as random effect. A first order autoregressive
216 covariance structure (AR1) was used to adjust the difference on data according to the
217 differences over time. The viscosity of PBS, SP, UF and UF-SP were compared by ANOVA.
218 Data are expressed as the mean ± SEM. Differences were considered statistically significant
219 when $p \leq 0.05$.

220 **3. Results**

221 *3.1 Assessment of UF and/or SP effect on ejaculated spermatozoa function (motility parameters,*
222 *viability and acrosome status)*

223 Ejaculated spermatozoa from different experimental groups (control, UF and UF-SP) were
224 incubated at 38ºC during 180 min. Table 1 shows the results analyzed by repeated
225 measurements. Thereby, the total motility decreased when spermatozoa were incubated in UF in
226 absence of SP (UF group) compared with control and UF-SP groups (both $p < 0.0001$), without
227 differences between those (Table 1). There was an interaction between time and treatment in
228 total motility of spermatozoa (Fig. 1a: $p = 0.02$). The percentage of total motility was lower in
229 spermatozoa incubated in UF at 120 and 180 min than control ($p = 0.0005$ and $p < 0.0001$,
230 respectively) and UF-SP group ($p = 0.0004$ and $p = 0.0009$).
231 The progressive motility was lower in UF group than when spermatozoa were incubated in
232 control and UF-SP ($p = 0.003$ and $p = 0.0003$, respectively), without difference between those
233 (Table 1). The progressive motility did not show any interaction between time and treatment
234 (Figure 1b).

The viability did not differ between incubations (Table 1) and there was no interaction between time and treatment (Figure 1c). Regarding the acrosome damage, it was greater in spermatozoa incubated with UF than control and UF-SP incubations ($p< 0.0001$ both of them) (Table 1), without significant differences between time and treatment (Figure 1d).

Related to motion kinetic parameters, the VCL was greater in control and UF-SP group than when spermatozoa were incubated with UF ($p= 0.01$ and $p< 0.0001$, respectively) (Table 1).

The VSL was greater when spermatozoa were incubated in UF with SP than control and UF group ($p= 0.02$ and $p< 0.001$, respectively) (Table 1). The VAP was greater in spermatozoa incubated with UF and SP than control and UF incubations ($p= 0.02$ and $p< 0.0001$, respectively) and in control group than when spermatozoa were incubated in UF ($p= 0.05$) (Table 1). The LIN was greater in spermatozoa incubated with UF and SP than control and UF incubations ($p= 0.03$ and $p= 0.009$, respectively), without differences between control and UF groups (Table 1). The STR was greater when spermatozoa were incubated in UF with SP than UF without SP ($p= 0.005$), without differences with the control group (Table 1). For the parameters BCF and WOB there were no significant differences between treatments (Table 1). There were not differences between time and treatment in VAL, VSL, VCL, LIN, STR, BCF and WOB (Supplemental Figure 1).

3.2 Assessment of UF and/or SP effect on epididymal spermatozoa function (motility parameters, viability and acrosome status)

When total and progressive motility of epididymal spermatozoa were analyzed by repeated measurements no differences were found between experimental groups (Table 2) and there were no interactions between time and treatments (Figure 2a and b).

Regarding the viability, it was greater in control group and in spermatozoa incubated with UF and SP than UF ($p= 0.01$ and $p= 0.002$, respectively), without differences between control and UF-SP incubations (Table 2). Epididymal spermatozoa from control had a greater viability than UF from 120 min of incubation ($p= 0.02$) onwards (180 min, $p< 0.0001$). Moreover,

spermatozoa incubated with UF showed a lower viability than when they were incubated in UF with SP at 120 min ($p= 0.001$) and 180 min ($p< 0.0001$) (Figure 2c). The acrosome damage was greater in spermatozoa incubated with UF than control and UF with SP ($p< 0.0001$ and $p= 0.0005$, respectively) (Table 2), without differences between control and UF-SP incubations. Additionally, there was an interaction between time and treatment (Figure 2d; $p< 0.0001$), increasing the acrosome damage in spermatozoa incubated with UF over time beginning at 60 min ($p= 0.009$ respect to control group) and continues after 120 min ($p< 0.0001$, respect to control and UF-SP groups) and 180 min ($p< 0.0001$, respect to control and UF-SP groups).

Regarding kinetic parameters only significant differences were found in WOB (Table 2). The WOB was greater when spermatozoa were incubated in UF with SP than the control incubation ($p= 0.01$) (Table 2), without differences between them and UF incubation.

The rest of the parameters (VAP, VSL, VCL, LIN, STR, BCF) were not different between experimental groups (Table 2) and there were no interactions between time and treatments in none of them (Supplemental Figure 2).

4. Discussion

Spermatozoa, through the female reproductive tract, are subjected to a complex mechanism of transport and selection, being exposed to different environments before their encounter with the oocyte. This study aimed to demonstrate that UF, which is the first milieu that encounters the sperm once deposited in the female genital tract, exerts an adverse effect on spermatozoa. However, the presence of SP minimizes the effect of UF protecting spermatozoa function, in particular motion parameters, viability and acrosome integrity.

Previous studies in mice (Kawano et al., 2014) (26), and according with our results, showed that spermatozoa function is reduced in presence of UF. This effect was identified so far only in UF, but not in OF. In fact, porcine OF from the follicular phase of the oestrus cycle induces biochemical, biophysical and functional modifications in spermatozoa after relatively short exposure time, protecting spermatozoa viability and improving the maintenance of acrosome integrity in different species (Coy et al., 2010; Kawano et al., 2014) (36,37). Thus, UF and OF,

289 although they are both reproductive fluids, differently contribute to modulate spermatozoa
290 functions. The UF may be important selecting spermatozoa after they enter the uterus, while OF
291 protects suitable spermatozoa that have passed the previous selection. Although not in porcine
292 species, the proteome of ewe reproductive fluids differs between oestrus stage (estrus *vs.* luteal
293 phase) and female reproductive tract sections (cervix *vs.* uterus *vs.* oviduct) (Soleilhavoup et al.
294 2016) (38). Interestingly, some of the uterus proteins detected in a higher abundance in estrus
295 compared with luteal phase are related with the complement cascade (Soleilhavoup et al. 2016)
296 (38). In some species, such as stallion, it has been shown that this complement cascade is
297 activated after the entry of spermatozoa and exerts an immune response inducing the migration
298 of PMN granulocytes (Katila et al., 2001) (39).
299 This harmful effect exerted on the spermatozoa in the uterus by the UF action, may indicate two
300 theories not mutually exclusive: first, that spermatozoa, such as pathogens are recognized as
301 foreigners within the uterus; and second, the UF exerts a spermatozoa selection based on
302 extrinsic or intrinsic properties due to the fact that some of them are able to pass the uterus and
303 survive this hostile environment. Actually, SP is one of the protective strategies against the UF.
304 In mice, a specific protein of SP, the SVS2 protein, protects the spermatozoa helping them to
305 survive in the uterus and reach the oviduct (Kawano et al. 2014) (26). In the case of porcine, this
306 specific SVS2 protein has not been identified in SP proteome (Perez-Patiño et al. 2016) (40),
307 although our study has demonstrated that SP has a protective effect when spermatozoa are in
308 presence of UF. A large number of proteins identified in boar SP has binding activity (Perez-
309 Patiño et al. 2016) (40), which may modify the sperm functionality. This statement is
310 corroborated with our results, because an adverse effect of UF on the acrosome spermatozoa in
311 absence of SP has been observed. For instance, as SVS2 in mice could protect spermatozoa
312 from the uterine attack by coating the spermatozoa surface, in the same way in porcine species
313 there might be some protein with analogous function. Spermadhesins has been identified as a
314 family of proteins with a protective role towards spermatozoa (Calvete et al., 1995) (41). These
315 proteins, secreted by the seminal vesicle epithelium, play critical roles in various aspects of
316 porcine fertilization (Assreuy et al., 2003) (42) such as, binding to sperm membrane, they

317 aggregate to porcine B1 protein (Jonakova et al., 2000) (43) forming a protein layer that
318 stabilizes the acrosome (Topfer-Petersen et al., 1998; Dostalova et al., 1995) (44,45).
319 In another instance, spermatozoa in absence of SP had a greater amount of protein tyrosine
320 phosphorylated than spermatozoa incubated with SP (Okazaki et al., 2012) (56). This
321 mechanism, by adding phosphate groups to protein tyrosine residues, is involved in the
322 regulation of different cellular processes and it is related to the acquisition of hyperactive
323 motility necessary for sperm-oocyte interaction (Hunter, 1996, Visconti 1998) (48,49). It can
324 induce a greater acrosome damage in absence of SP according to the evidence that SP may
325 suppress phosphorylation of tyrosine residues (Okazaki et al., 2012) (47), inducing an early
326 acrosome reaction, a fusion between the spermatozoa acrosome membrane and spermatozoa
327 plasma membrane (Hunter and Rodriguez-Martinez (2004) (50). Other authors (Fukami et al.,
328 2001; Suarez and Pacey, 2006) (51,52) and according with our results, speculate that the
329 maintenance of spermatozoa plasma membrane integrity may depend on SP components. Thus,
330 the presence of SP, by coating the spermatozoa surface, may be a defensive barrier protecting
331 them avoiding the decrease of motility, kinetic parameters and viability.
332 In our study, the results showed that UF in absence of SP negatively influenced the sperm
333 function either in ejaculated or epididymal spermatozoa, although in some cases the functional
334 parameters affected were different depending on the spermatozoa source. It was observed that
335 total and progressive motility were higher in presence of SP than incubated with UF when using
336 ejaculated spermatozoa but not when epididymal spermatozoa were used – the latter had no
337 significant differences in the same parameters. Rickard et al. (2014) (53) showed that when ram
338 epididymal spermatozoa were incubated in presence or absence of SP no changes in motility
339 and velocity were found but the presence of SP improved the ability to cross cervical mucus;
340 Harkema et al. (2004) (54) also found that when epididymal spermatozoa were incubated with
341 SP, membrane stability was not affected.Actually, ejaculated and epididymal spermatozoa show
342 different behavior either *in vivo* (Rickard et al. 2014; Okazaki et al. 2012) (39,40) or *in vitro*
343 (Matás et al. 2010; García-Vázquez et al. 2015 JRD) (37,41) conditions. In our case, it is likely
344 that epididymal spermatozoa show a different modulation towards the negative effect of UF

345 than ejaculated spermatozoa maybe because epididymal sperm were immersed in epididymal
346 fluid that could have a protective role (Dacheux and Dacheux, 2014) (55) different than when
347 spermatozoa are ejaculated and surrounded of SP. In fact, the protein composition in male
348 reproductive fluids (epididymal fluid *vs.* SP) and spermatozoa source (epididymal *vs.*
349 ejaculated) has important differences. Epididymal fluid is poor in proteins (Rodriguez-Martinez
350 2007) (16) compared to the SP that surrounds ejaculated spermatozoa that show several SP-
351 proteins on their extracellular surface (Perez-Patiño et al., 2018) (56). Moreover, some proteins
352 found in epididymal spermatozoa are overexpressed compared to ejaculated spermatozoa
353 collected from rich sperm fraction (Perez-Patiño et al., 2018) (56). Both different contributions
354 (fluid and sperm source) may explain some of the differences found in the effect of UF when
355 epididymal or ejaculated sperm were used. Moreover, significant different levels of miRNA
356 expression between boar cauda epididymal spermatozoa and fresh ejaculated has been observed.
357 Concretely, five target genes involved in spermatozoa apoptosis were expressed in both
358 epididymal and ejaculate spermatozoa, 3 of them up-regulated in the cauda epididymal
359 spermatozoa (Chang et al. 2016) (57). These differences found mainly in metabolic processes
360 could be involved in the different behavior observed between epididymal and ejaculated sperm
361 in the present study.
362 What is clear from our results is that SP plays a pivotal role when spermatozoa are incubated in
363 UF. The AI is one of the most used artificial reproductive techniques widely spread in the
364 porcine industry. In order to optimize the pig production, the ejaculate is diluted in commercial
365 extender, and consequently SP concentration is reduced (Kirkwood et al., 2008) (58). The SP
366 has a positive effect either in the male or female. In the case of the male, and based on results
367 from this study, adding SP to seminal doses could improve seminal quality, increasing motility
368 and decreasing acrosome damage. It is according to previous investigations showing that use of
369 SP in seminal dose may improve spermatozoa transport and fertilizing ability within the hostile
370 uterine environment (Rozeboom et al., 2000) (20). In the case of female, the SP may not be
371 necessary for pregnancy but it is required to avoid pathologies during pregnancy and,
372 consequently, to have a greater pregnancy outcome, while in absence of SP it was observed a

reduced embryo development (reviewed by Bromfield, 2018) (59). For this reason SP could be not only required in AI but also in the subsequent phases (such as embryo implantation) (Robertson, 2016) (60) and no exposure to SP could result in a reduced fertilization as evidenced in other species (reviewed by Robertson, 2007) (61). However how the SP or some components affect the fertility in porcine has not totally elucidated yet.

5. Conclusions

In conclusion, this study shows that both ejaculated and epididymal spermatozoa are affected by UF, exerting a negative effect on the spermatozoa quality. This negative effect of UF on the spermatozoa quality may be reduced by the presence of SP, improving the spermatozoa functionality, preserving motility and acrosome integrity. It would be interesting to characterize SP and UF proteins that, after incubation with the mentioned fluids, adhere to the spermatozoa playing a critical role in the reproductive processes to better understand how they can affect the spermatozoa functions during the journey through the uterus.

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Author´s contribution

C Luongo and S Abril-Sánchez contributed to the design of the study, performed the experiments, analyzed the data and wrote the draft of the paper. JG Hernández performed viscosity experiment and analyzed the data. FA García-Vázquez is the advisor of the project, conceived and designed the experiment, analyzed the data and wrote the manuscript. All the authors have revised the final version of the manuscript.

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Table 1. Parameters of ejaculated spermatozoa, incubated with seminal plasma (Control), with uterine fluid (UF), with uterine fluid and seminal plasma (UF-SP).

	Incubation treatment				
Parameters	**Control**	**UF**	**UF-SP**	**p-value**	**Pooled SEM**
Ejaculated spermatozoa					
Total motility (%)	86.7[a]	81.2[b]	86.6[a]	<0.0001	1.5
Progressive motility (%)	68.8[a]	57.9[b]	71.7[a]	0.0007	2.7
VCL (µm/s)	84.0[a]	70.2[b]	94.6[a]	0.0002	4.6
VSL (µm/s)	52.1[a]	42.5[a]	65.3[b]	0.0002	3.6
VAP (µm/s)	64.6[a]	54.9[b]	76.6[c]	0.0002	4.0
LIN (%)	62.0[a]	60.5[a]	68.8[b]	0.02	2.5
STR (%)	80.5[ab]	77.3[a]	85.0[b]	0.02	1.9
WOB (%)	76.5	77.8	80.2	0.2	2.1
BCF (Hz)	7.8	7.7	8.1	0.3	0.2
Viability (%)	84.6	83.1	84.4	0.4	1.5
Acrosome damage (%)	4.1[a]	13.1[b]	5.7[a]	<0.0001	1.0

[a,b,c] Values within a row with different superscripts differ significantly between procedures (control, UF, UF-SP) at $p<0.05$.

435 **Table 2.** Parameters of epididymal spermatozoa, incubated with seminal plasma (Control), with
436 uterine fluid (UF), with uterine fluid and seminal plasma (UF-SP).

	Incubation treatment				
Parameters	**Control**	**UF**	**UF-SP**	**p-value**	**Pooled SEM**
Epididymal spermatozoa					
Total motility (%)	85.8	83.4	84.5	0.6	5.4
Progressive motility (%)	69.4	67.2	67.3	0.8	6.7
VCL (µm/s)	90.0	85.9	90.0	0.7	10.5
VSL (µm/s)	49.9	47.8	53.0	0.6	7.4
VAP (µm/s)	61.9	60.5	65.9	0.6	8.3
LIN (%)	55.3	57.5	60.2	0.2	7.3
STR (%)	78.6	78.7	79.7	0.8	4.9
WOB (%)	68.5[a]	71.3[ab]	73.6[b]	0.03	5.6
BCF (Hz)	7.3	7.4	7.3	0.9	0.4
Viability (%)	89.3[a]	85.7[b]	90.2[a]	0.004	3.3
Acrosome damage (%)	2.1[a]	9.0[b]	3.4[a]	<0.0001	1.5

437 [a,b] Values within a row with different superscripts differ significantly between procedures
438 (control, UF, UF-SP) at $p<0.05$.

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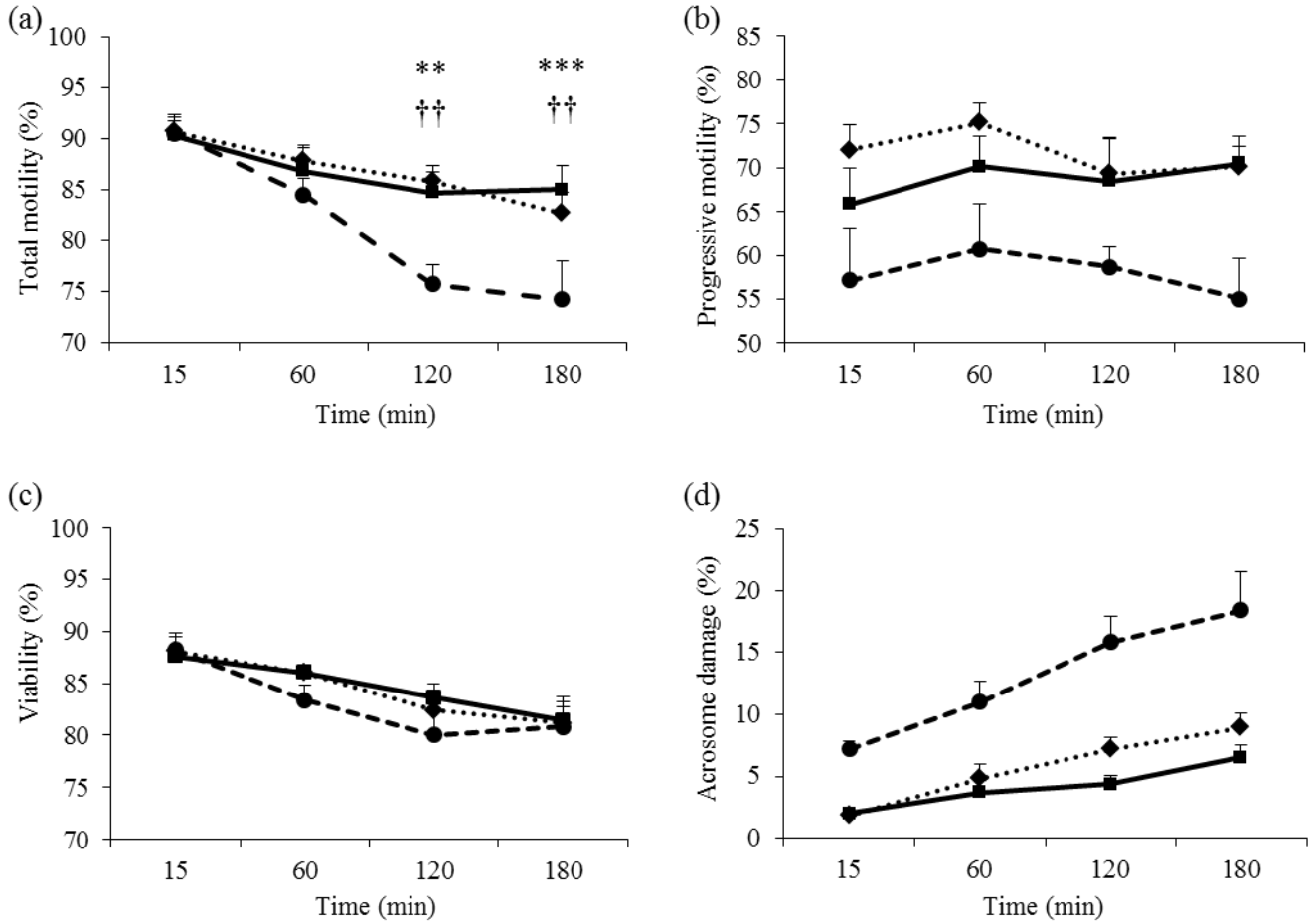


Figure 1. Average total motility percentage (a), average progressive motility percentage (b), viability (c) and acrosomal dam
(d) in ejaculated spermatozoa incubated over the time (15, 60, 120 and 180 min) with seminal plasma (–■–), incubated
uterine fluid (--●--) and incubated with uterine fluid and seminal plasma (· · ·♦· · ·) (mean ± SEM). Different symbols indi
differences between treatments in each time point: * * means difference between control and UF, $p<0.001$; * * * m
difference between control and UF, $p<0.0001$; †† means difference between UF and UF-SP, $p<0.001$.

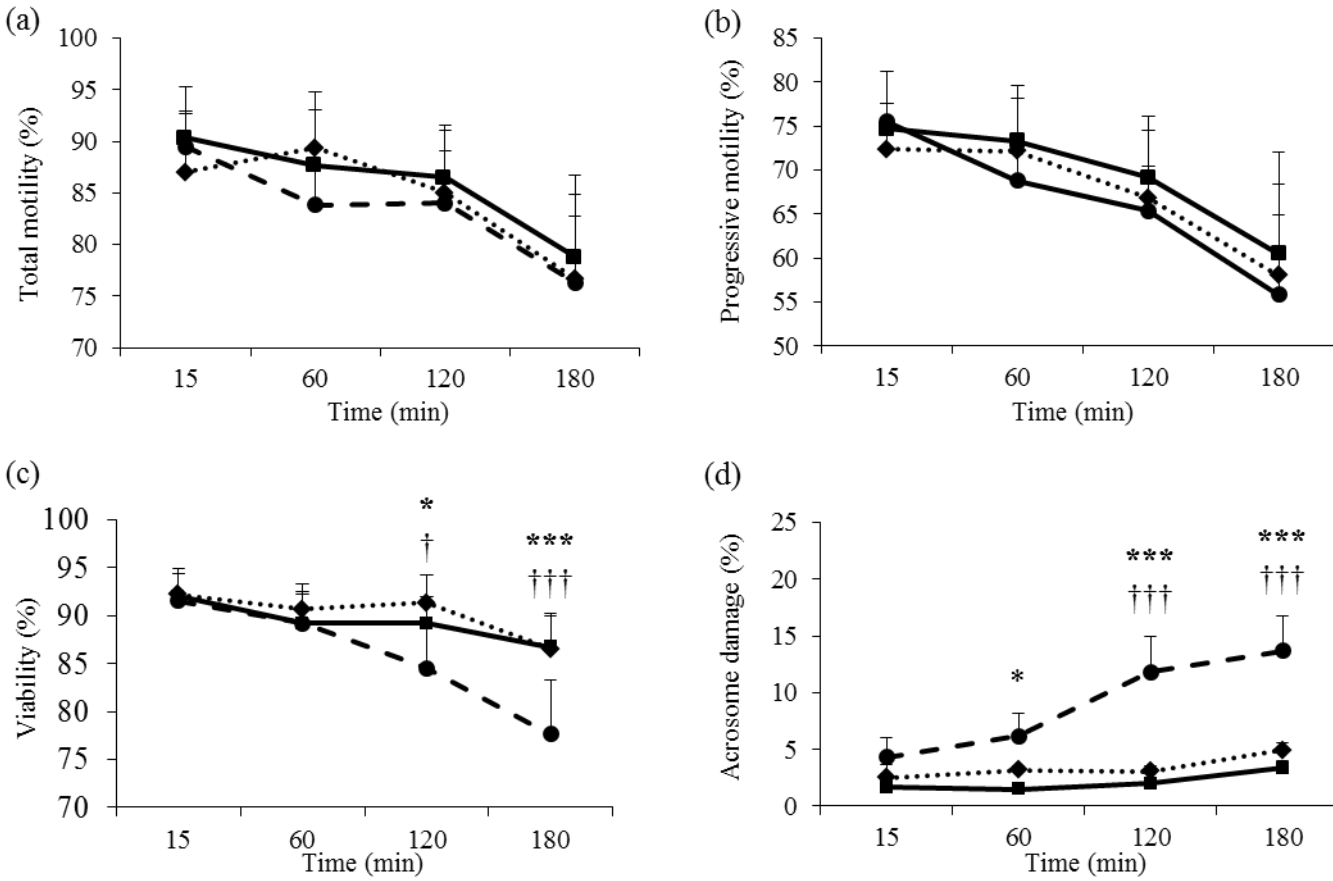
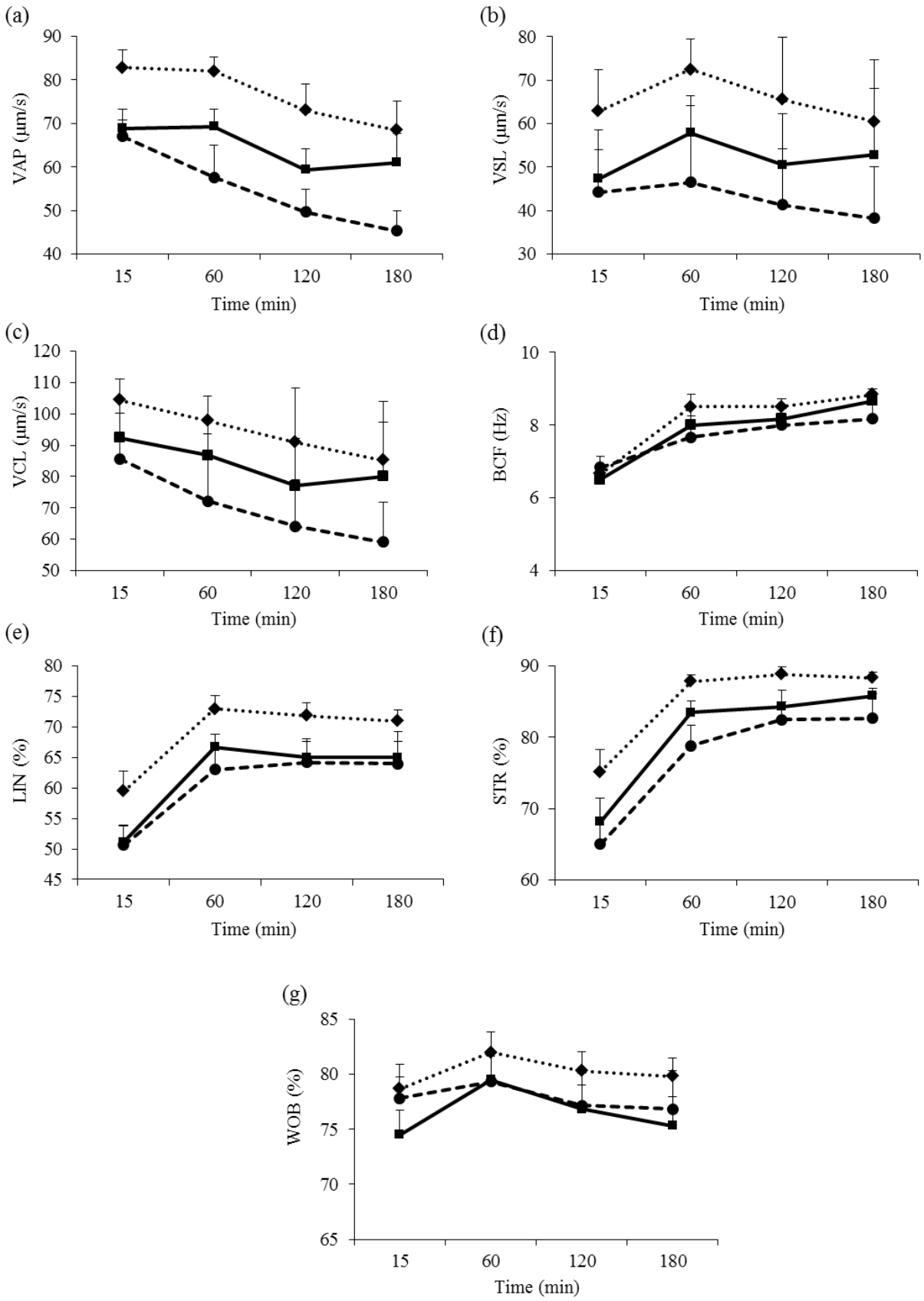
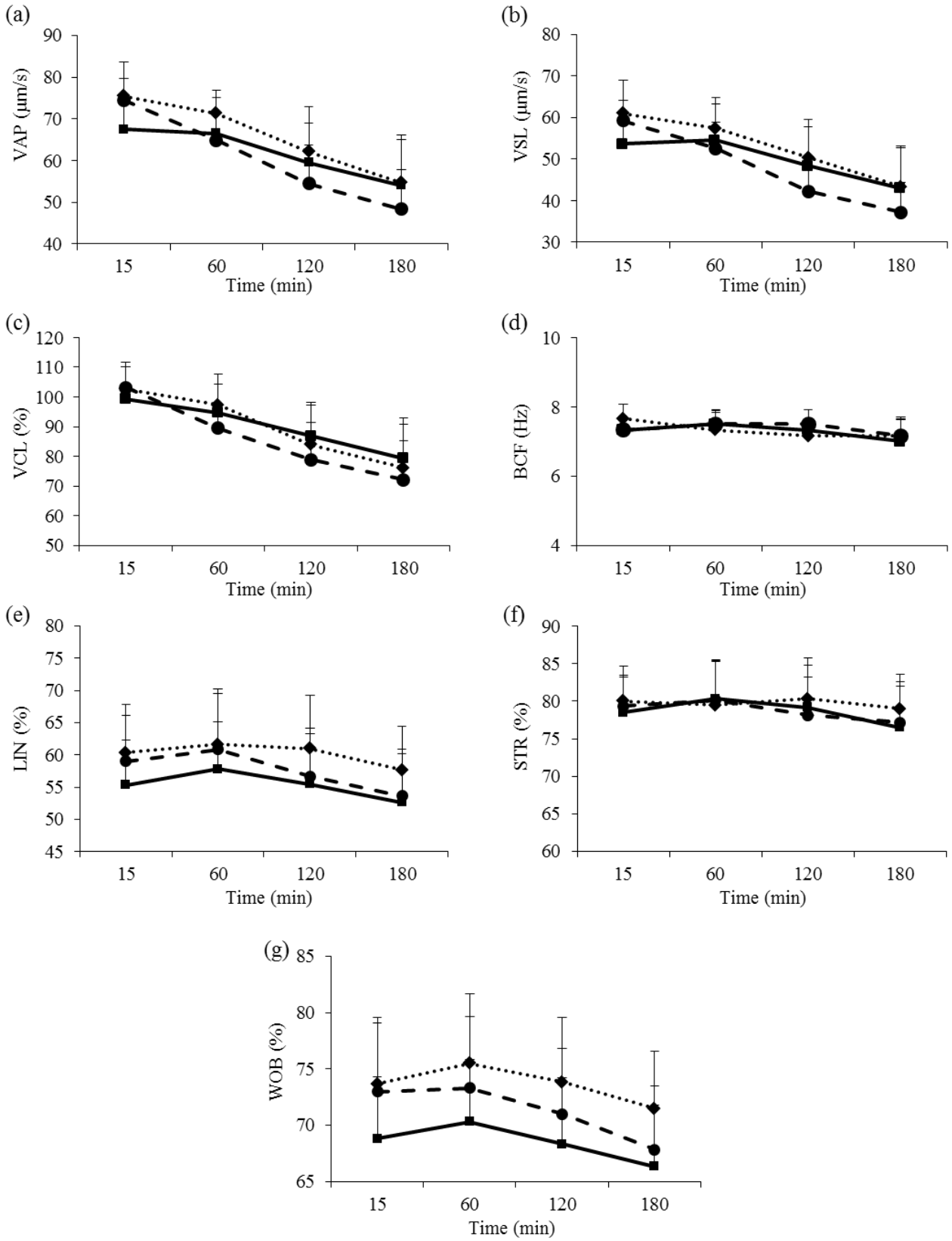


Figure 2. Average total motility percentage (a), average progressive motility percentage (b), viability (c) and acrosomal dar (d) in epididymal spermatozoa incubated over the time (15, 60, 120 and 180 min) with seminal plasma (–■–), incubated uterine fluid (--●--) and incubated with uterine fluid and seminal plasma (· · ·♦· · ·) (mean ± SEM). * means difference bet control and UF, $p<0.05$; * * * means difference between control and UF, $p<0.0001$; † means difference between UF and UI $p<0.05$; ††† means difference between UF and UF-SP, $p<0.0001$.



Supplementary Figure 1. Average path velocity (VAP) (a), straight line velocity (VSL) (b), curvilinear velocity (VCL) (c), beat cross frequency (BCF) (d), linearity of the curvilinear path (LIN) (e), straightness of the average path (STR) (f), wobble coefficient (WOB) (g), determined by CASA for ejaculated spermatozoa incubated over the time (15, 60, 120 and 180 min) with seminal plasma (–■–), incubated with uterine fluid (--●--) and incubated with uterine fluid and seminal plasma (· · ·♦· · ·) (mean ± SEM).



Supplementary Figure 2. Average path velocity (VAP) (a), straight line velocity (VSL) (b), curvilinear velocity (VCL) (c), beat cross frequency (BCF) (d), linearity of the curvilinear path (LIN) (e), straightness of the average path (STR) (f), wobble coefficient (WOB) (g), determined by CASA for epididymal spermatozoa incubated over the time (15, 60, 120 and 180 min) with seminal plasma (–■–), incubated with uterine fluid (--●--) and incubated with uterine fluid and seminal plasma (· · ·♦· · ·) (mean ± SEM).

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Highlights

- Uterine fluid affects ejaculated and epididymal spermatozoa functionality (motility, kinetic parameters, viability, acrosome integrity).
- Seminal plasma can mitigate the negative effect of uterine fluid, preserving the spermatozoa quality.