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ORIGINAL ARTICLE

Male Fertility

The secretory products of *Trichomonas vaginalis* decrease fertilizing capacity of mice sperm *in vitro*

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Trichomonas vaginalis **infection is one of the most prevalent sexually transmitted infections in humans and is now recognized as an important cause of infertility in men. There is little information about the effect of extracellular polymeric substances (EPS) from** ***T. vaginalis*** **on sperm, but previous reports do not provide a conclusive description of the functional integrity of the sperm. To investigate the impact of EPS on the fertilizing capacity of sperm, we assessed sperm motility, acrosomal status, hypo-osmotic swelling, and** ***in vitro*** **fertilization rate after incubating the sperm with EPS** ***in vitro*** **using mice. The incubation of sperm with EPS significantly decreased sperm motility, viability, and functional integrity in a concentration and time-dependent manner. These effects on sperm quality also resulted in a decreased fertilization rate** ***in vitro.*** **This is the first report that demonstrates the direct negative impact of the EPS of** ***T. vaginalis*** **on the fertilization rate of sperm** ***in vitro.*** **However, further study should be performed using human sperm to determine if EPS has similar negative impact on human sperm fertilizing capacity** ***in vitro.***

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INTRODUCTION

Sexual and reproductive tract infections other than HIV are important global health priorities in their own right, not merely because they can facilitate HIV transmission. Depending on the disease, some untreated genital tract infections can lead to infertility, chronic pain or even death.[1] Of the protozoans *Trichomonas vaginalis* (*T. vaginalis*) infection has been associated with a 4.7-fold increase in the risk of pelvic inflammatory disease and over half the 248 million new TV infections each year occur in men.[2]

T. vaginalis, which is responsible for one of the most prevalent sexually transmitted infections in humans, is restricted to the genito-urinary tract. The global incidence of trichomoniasis has been continuously increasing and has exceeded the combined rates of gonorrhea and chlamydia infections.[2] Underestimates of trichomoniasis may be related to the asymptomatic nature of the infection and the lack of sensitive and specific diagnostic methods, especially for men.

T. vaginalis infection in women and men, regardless of the presence of symptoms, is now recognized as an important cause of infertility.[3,4] In fact, *T. vaginalis* was found 5 times more often in infertile women than in control subjects[3,4] and is probably associated with cervical and tubal factor in female infertility. On the other hand, among asymptomatic infertile men, approximately 4%–8% was found to be infected with *T. vaginalis*.[5] Furthermore, several investigators have noted abnormalities in the sperm parameters of men infected with *T. vaginalis*, such as a decrease in sperm motility and viability,[5–9] and a decline in the percentage of sperm cells with normal morphology.[5] Subsequent studies have attempted to establish a mechanism by which these abnormalities arise.[7,10] Almost all of the previous studies have concentrated on contact-dependent adhesion of *T. vaginalis* on sperm cells, whereas the possible consequences of substances secreted by *T. vaginalis* remain largely unexamined. Little information is available on the effect of the extracellular polymeric substances (EPS) of *T. vaginalis* on the functional integrity and fertilizing capacity of sperm.

Therefore, we evaluated whether the EPS of *T. vaginalis* impairs various aspects of fertilization and sperm physiology. To investigate the impact of EPS on the fertilizing capacity of the sperm *in vitro*, we incubated the sperm with EPS and assessed sperm viability, motility, functional integrity (by means of acrosomal status and the hypo-osmotic swelling [HOS] test) and the *in vitro* fertilization (IVF) rate.

MATERIALS AND METHODS

Animals

Male and female ICR mice, 8–10 weeks old (male = 30, female =25), were obtained from Samtako Biokorea (Kyunggi, South Korea). The weight range of the mice is 35–40 g for males and 30–35 g for females. All animals were kept under controlled humidity, temperature, and light conditions and were fed standard mouse chow *ad libitum*. Animal care followed institutional guidelines, and the Hanyang University IACUC approved all procedures involving the animals (HY-IACUC-09-043).

Semen collection and preparation

After exposure of the peritoneal cavity, the cauda epididymis of male mice (n = 30) of proven fertility was removed from each testis and the fat was removed. Then, the cauda epididymes were washed immediately in prewarmed 1 ml of collection medium

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(Whitten's HEPES-buffered medium), and transferred to a 200 μl drop of human tubal fluid medium (Quinn's Advantage® Fertilization, *In vitro* Fertilization Inc., Trumbull, Connecticut, USA) containing 10% fetal bovine serum in a 35 mm culture dish equilibrated overnight under embryo-tested mineral oil in a humidified atmosphere with 5% CO_2 at 37°C. Sperm were gently squeezed out of the epididymis using a 26-gauge needle, and the residual caudal tissue was discarded. The sperm were then allowed to disperse for 15 min and large aggregations of immotile sperm in the culture drops were removed using a Pasteur pipette under a dissecting microscope.[11] Sperm concentration was assessed with a Neubauer-improved counting chamber (Marienfeld Superior, Lauda-Konigshofen, Germany) and aliquots of the sperm suspension were diluted under oil to achieve a specific concentration necessary for the experiment.

***Trichomonas vaginalis* culture and preparation of the extracellular polymeric substances**

Trichomonas vaginalis (T016 isolate; provided by Prof. John F. Alderete [Department of Microbiology, University of Texas Health Science Center at San Antonio, TX, USA]) was grown in trypticase-yeast extract-maltose medium supplemented with 10% heat-inactivated horse serum at 37°C.[12] EPS was prepared as follows: *T. vaginalis* was harvested in log-phase, washed 3 times with cold phosphate buffered saline (PBS, pH 7.4), suspended (10^7 trichomonads) in 1 ml of Hank's balanced salt solution (HBSS, pH 7.2) (Gibco, NY, USA) and incubated for 1 h at 37°C. Previous reports found that *T. vaginalis* secreted high concentrations of inflammatory factors after 1 h of incubation,[13] and hence we applied the same method to obtain EPS. Following incubation, parasites were pelleted in a microcentrifuge at 10,000 g for 30 min, and the supernatants were passed through a 0.22 μm filter. The supernatants were collected as EPS of *T. vaginalis* and were either frozen at − 20°C or used directly to assess the impact on sperm.

Sperm viability

Sperm viability was measured to determine the appropriate incubation time and concentrations of EPS to be used in further experiments. To investigate the effects of EPS on sperm, sperm (2×10^6 ml^{-1}) were incubated for 1–3 h at 37°C with various concentrations of EPS (10%, 25%, 50%, 75%, and 100%) diluted in HBSS. Viability tests were based on visual morphology under ×400 magnification, using vital staining with trypan blue; total or partly stained cells were considered dead, and the percentages of live sperm were evaluated. At least 200 sperm cells/smear were counted per group, and at least two smears per aliquot were assessed. The controls consisted of sperm incubated in HBSS without EPS and maintained at 37°C for 1–3 h.

Motility analysis

Based on the results of the analysis of viability, sperm motility was evaluated after the incubation of sperm (2×10^6 ml^{-1}) in 25%, 50%, or 75% of EPS for 2 h. The motility of at least 200 sperm per group was evaluated subjectively using a phase contrast microscope, across five fields at ×400 magnification. A drop of sperm was kept on a prewarmed slide and motility was graded in four categories (Grade A: rapid progressive motility; Grade B: slow or sluggish progressive motility; Grade C: nonprogressive motility; and Grade D: immotility) (**Figure 1**).[14] The assessment was then repeated in a separate 10 μl drop and the proportions of motility grades from those two independent counts were calculated.

Assessment of acrosomal status

Acrosomal status was assessed after the incubating sperm (2×10^6 ml^{-1}) in 25%, 50% or 75% EPS for 2 h. Sperm were air-dried onto glass slides,

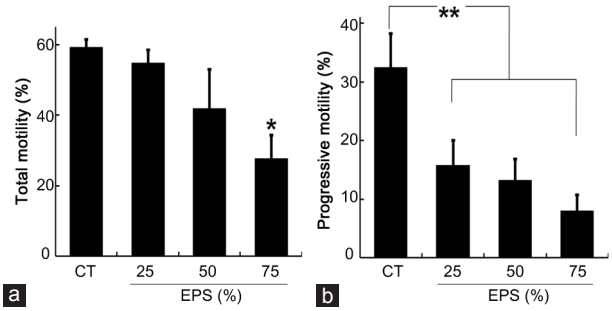

Figure 1: The effect of the EPS of *Trichomonas vaginalis* on the motility parameters of sperm. (**a**) The proportion of motile sperm with total motility after incubation for 2 h in 25%, 50%, or 75% of EPS of *T. vaginalis* diluted in Hank's balanced salt solution (HBSS). (**b**) The proportion of sperm with progressive motility after incubation for 2 h in 25%, 50%, or 75% EPS diluted in HBSS. Values are the mean ± standard deviation of three separate experiments; 200 cells were counted per treatment per experiment. Total motility: Grade A + B + C; Progressive motility: Grade A + B; Grade A: rapid progressive motility; Grade B: slow or sluggish progressive motility; Grade C: nonprogressive motility; Grade D: immotility; CT: control sperm cells were incubated at 37°C for 2 h in HBSS that did not contain EPS. EPS: extracellular polymeric substances prepared from *T. vaginalis*. $^*P < 0.02$ compared with CT; $^{**}P < 0.04$ compared with CT.

fixed with 5% paraformaldehyde in PBS (pH 7.4) for 15 min, and washed once with PBS. The slides were stained for 5 min with aqueous 0.25% Coomassie brilliant blue R-250 (Amresco Inc, Solon, Ohio) in 10% glacial acetic acid and 25% methanol. Sperm were then rinsed with water and covered with cover slips under mounting medium (90% glycogen). This method stains the acrosomal cap blue in acrosome-intact sperm, but does not stain the acrosome region in acrosome-reacted sperm.[15] Each acrosome status was assessed by counting 200 sperm across five fields twice.

Hypo-osmotic swelling test

Sperm (2×10^6 ml^{-1}) were incubated as above and the HOS test was performed as described by Jeyendran *et al.*[16]; the pretreated sperm were incubated in 2 ml of hypo-osmotic solution (7.35 g sodium citrate·$2H_2O$ and 13.51 g fructose mixed with 1 L distilled H_2O) for 1 h at 37°C. The percentage of sperm undergoing tail swelling was determined under high power magnification (×400) on a phase contrast microscope. A minimum of 200 sperm were counted twice across three to five slides.

In vitro fertilization

Because HOS was impaired by 75% EPS, the same concentration of EPS was applied to evaluate fertilization. Female mice ($n = 25$) were superovulated at 8–10 weeks of age by intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (PMSG; Sigma, St. Louise, MO, USA) followed 48 h later by an intraperitoneal injection of 5 IU of human chorionic gonadotropin (hCG; Sigma). The oviducts were removed at 12 h after hCG injection and placed in a prewarmed collection medium. Cumulus–oocyte complexes, found in the ampullar oviduct at the site of the cumulus bulge, were collected by tearing the oviduct using a 26-gauge needle. The IVF procedure was carried out in 100 μl drops of the insemination medium under mineral oil. Sperm, preincubated as described above, were gently added to the drops containing cumulus–oocyte complexes to yield a final motile sperm concentration of 1×10^5 per oocyte. After incubation with the sperm suspension at 37°C under 5% CO_2 for 8 h, the oocytes were transferred to fresh medium and cultured for 12–14 h. They were evaluated for fertility using an inverted phase contrast microscope counting two-cell embryos.

Statistical analysis

Data are presented as the mean ± standard deviation of duplicate measurements, and each experiment was repeated 3–5 times. Significant differences between data sets were assessed by *t*-tests and one-way analyses of variance using the Statistical Package for Social Sciences 13.5 (SPSS Inc., Chicago, IL, USA). Significance was accepted at the level of $P < 0.05$.

RESULTS

The effect of the extracellular polymeric substances of Trichomonas vaginalis on sperm viability

Fresh sperm were approximately 52% viable before interaction (**Figure 2**). The proportion of viable sperm *in vitro* decreased over time, and the percentage of viable sperm was reduced by half in the control samples after 3 h of incubation, indicating that this incubation time was too long. In addition, the significant decrease in the viable sperm percentage after 2 h of incubation with EPS-10 (36.6% ±4.96% *vs* 51.5% ±7.56% before incubation, $P < 0.002$), or EPS-25 (30.8% ±9.46% *vs* 55.3% ±9.61% before incubation, $P < 0.005$) were observed. As shown in **Figure 2**, a profound effect of EPS on sperm viability was observed after 1 h of incubation in EPS-50 (37.4% ±5.90% *vs* 47.9% ±8.66% for the control, $P < 0.05$), whereas a significant difference between EPS-25 and the control was noted after 2 h of incubation (30.8% ±9.46% *vs* 42.0% ±7.91%, $P < 0.05$). Incubation in EPS-100 markedly decreased the percentage of viable sperm after 1 h of incubation (14.6% ±3.06% *vs* 47.9% ±8.66% for the control) and seemed to be too cytotoxic. We decided to adopt 2 h of incubation and 25%, 50% or 75% of EPS for the following experiments investigating the effect of EPS on sperm fertilizing capacity.

The effect of the extracellular polymeric substances of Trichomonas vaginalis on sperm motility

Sperm motility was evaluated after 2 h of incubation with 25%, 50% or 75% of EPS. No significant differences were observed between the control and EPS-25 or EPS-50 with respect to total motility, which includes fast progressive motility, slow progressive motility and nonprogressive motility (**Figure 1a**). However, incubation with 75% EPS led to a decrease in the proportion of motile sperm (27.7% ±6.66% *vs* 59.2% ±2.25% in the control, $P = 0.0148$) (**Figure 1a**). The percentage of progressive motile sperm (Grade A + B) declined in EPS-25 (15.8% ±4.25% *vs* 32.5% ±5.77% in the control, $P = 0.0326$) and was reduced further at EPS-50 and EPS-75 (13.2% ±3.65% and 8.0% ±2.68%, respectively) (**Figure 1b**). These results indicate that progressive motility is impaired even by a low concentration of EPS.

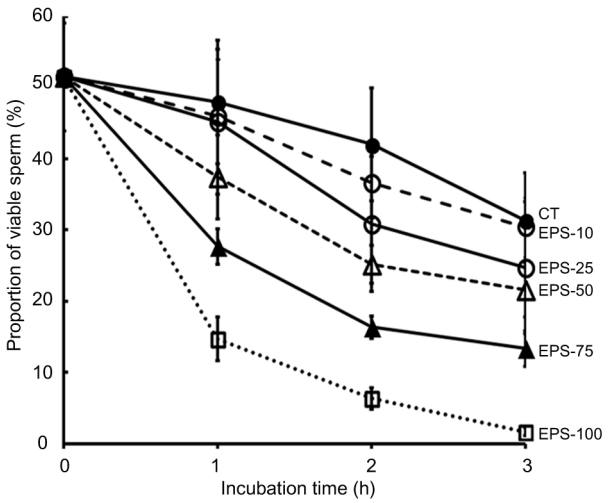

Figure 2: The effect of extracellular polymeric substances (EPS) of *Trichomonas vaginalis* on the viability of sperm. Viability was analyzed based on sperm morphology and vital staining with trypan blue. Data are presented at the mean ± standard deviation percent of viable sperm (relative to the total sperm numbers set at 100%) in three separate experiments, in which 200 cells were counted per treatment per experiment. EPS-10, -25, -50, -75, -100: sperm cells were incubated for 1, 2, or 3 h in 10%, 25%, 50%, 75%, or 100% of EPS prepared from *T. vaginalis* were diluted in Hank's balanced salt solution (HBSS). CT: control sperm cells were incubated at 37°C for 1, 2, or 3 h in HBSS that did not contain EPS.

The effect of the extracellular polymeric substances of Trichomonas vaginalis on acrosome status

Fertility is closely related to acrosomal status, and the loss of the acrosomal content by either premature sperm activation or the breakdown of membranes would prevent normal sperm-egg interactions during fertilization.[17] Acrosomal status may, therefore, be a significant factor in infertility, and we assessed the acrosomal status of sperm as a contributor to fertilization. We noted that the sperm incubated with EPS was less likely to have an intact acrosome than the controls (**Table 1**). After incubation with 25% EPS, an average of 38% of the sperm had an intact acrosome, and this percentage fell to 30.1% and 21.3% after incubation with 50% and 75% EPS, respectively, compared with an average of 54.0% in the control samples. The percentage of sperm with an intact acrosome at EPS-75 was significantly lower than in the control samples ($P = 0.01$).

The proportion of sperm positive for the HOS test decreased as the concentration of EPS increased, as shown in **Figure 3**. When incubated with 25% and 50% EPS, the percentage of swollen sperm decreased to 33.5% ±9.26% and 31.5% ±9.76%, respectively. Although these values were not significantly different from the control samples (44.2% ±11.77%, $P > 0.1$) (**Figure 3**), the decline with 75% EPS was significantly different (23.7% ±8.08%, $P = 0.0338$). In addition, the proportion of sperm positive for the HOS test was similar to the proportion of sperm cells with intact acrosomes (**Table 1**).

The effect of the extracellular polymeric substances of Trichomonas vaginalis on the in vitro fertilization rate of sperm

We also assessed fertilization 18 h after incubation of the ova with sperm suspensions. As both the acrosomal status and HOS test, which are highly correlated with fertilization capacity, were significantly compromised by incubation with 75% EPS, we used this concentration to evaluate the fertilization rate. Oocytes with two-cells were considered normally fertilized. The results of the IVF procedures in **Table 2** show that the percentage of fertilized eggs was significantly lower in the EPS group than in the control group (40.9% ±15.64% *vs* 62.9% ±13.81%, $P < 0.05$).

Table 1: The effect of the EPS of *Trichomonas vaginalis* on acrosome status

Group	*Acrosome-intact sperm (%)*	*Acrosome-reacted sperm (%)*	*P versus the control*
Control	54±9.53	46±9.53	
EPS-25	38±7.54	62±7.54	0.0849
EPS-50	30.1±12.06	69.8±12.06	0.0550
EPS-75	21.3±10.11	78.6±10.11	0.0152[a]

[a]Significant difference compared with the control. Mean±s.d. of three separate experiments. EPS-25, -50, -75: sperm incubated for 2 h in 25%, 50% or 75% EPS of *Trichomonas vaginalis* diluted in HBSS. Control sperm cells were incubated at 37°C for 2 h in HBSS that did not contain EPS. s.d.: standard deviation; EPS: extracellular polymeric substances; HBSS: Hank's balanced salt solution

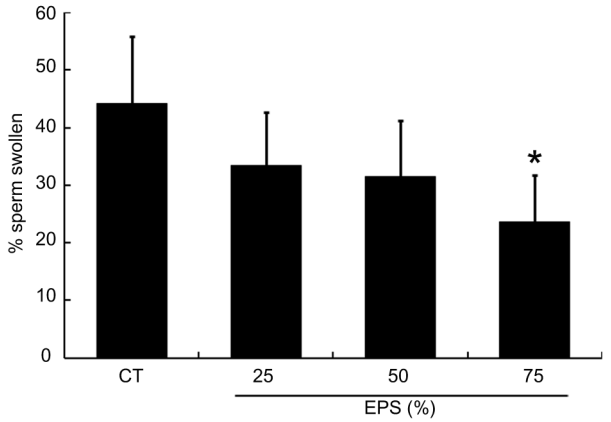

Figure 3: The effect of extracellular polymeric substances (EPS) of *Trichomonas vaginalis* on the proportion of sperm reacting positively to the hypo-osmotic swelling test. The percentage of swollen sperm was greatly reduced in sperm incubated with 75% EPS prepared from *T. vaginalis*. Sperm were incubated for 2 h in 25%, 50%, or 75% of EPS diluted in Hank's balanced salt solution (HBSS). CT: control sperm were incubated at 37°C for 2 h in HBSS that did not contain EPS. Values are the mean ± standard deviation of three separate experiments; 200 cells were counted per treatment per experiment. $^*P = 0.033$ compared with the control.

Table 2: *In vitro* fertilization of mouse oocytes with sperm preincubated with or without *Trichomonas vaginalis* EPS

	Number of oocytes	*Number of two-cell*	*Fertilization rate (%)*
Control	81	51	62.9±13.81
EPS-75	88	36	40.9±15.64[a]

[a]*P*=0.049 compared with the control. Mean±s.d. percentage of five separate experiments. EPS-75: sperm incubated for 2 h in 75% EPS of *Trichomonas vaginalis* diluted in HBSS. Control sperm were incubated at 37°C for 2 h in HBSS that did not contain EPS. EPS: extracellular polymeric substances; HBSS: Hank's balanced salt solution; s.d.: standard deviation

DISCUSSION

Here, we have shown that, *in vitro*, the EPS produced from *T. vaginalis* has a negative impact on sperm fertilizing capacity. If this effect also occurs *in vivo*, it would lead to a decline in fertility. This result may help to clarify the mechanism that causes infertility or subfertility in women and men infected by *T. vaginalis*. Infection of the male accessory glands may compromise sperm quality, depending on the underlying inflammation and affected site. In fact, infections of the genito-urinary tract account for up to 15% of male infertility.[18] *T. vaginalis* has also been found to infect the urethra and accessory glands,[8,9] as well as the testis, in which case it can induce azoospermia or hypogonadism.[19] In addition, chronic urethritis has also been shown to have a negative impact on semen quality. Although the semen have a short exposure time to trichomonads in the male urethra after ejaculation, sperm can be exposed to *T. vaginalis* or EPS contained in the ejaculates for several days through the female tract.[20]

There is conflicting information on the effects of the interaction between *T. vaginalis* and sperm cells, but the majority of *in vitro* studies have demonstrated a detrimental effect of *T. vaginalis* on sperm motility.[6,21] This could be due to the direct binding of *T. vaginalis* to the sperm,[22] to the circular whirling movement of *T. vaginalis* interrupting the normal horizontal movement of sperm within the vagina,[23] or to the contact-independent cytopathic mechanisms induced by substances secreted by *T. vaginalis*.[24] Our results show that incubation of sperm with EPS markedly decreases sperm motility (**Figure 1**), confirming that, *in vitro*, *T. vaginalis* adherence is not the sole factor affecting sperm motility. In fact, *T. vaginalis* has been shown to release molecules that are capable of acidifying the growth medium,[25] or lysing host cells[26] under triggering conditions. A number of biological agents have also been isolated from *T. vaginalis*; for example proteinases,[27] phospholipases,[28] acid phosphatase,[29] and peroxide[10] have been identified and are involved in cytotoxicity,[30] hemolysis,[28] and cytoadherence.[30]

An inhibitory role of EPS on sperm motility, similar to the effect of live *T. vaginalis*, has been reported in previous studies,[6,7,10] although incubation medium and time, and the protocol for preparing EPS, differed between studies. Before analyzing sperm motility, we evaluated sperm viability after 1, 2, and 3 h of incubation with different concentrations of EPS. Our results indicate that longer incubation times and higher concentrations of EPS have an increasing detrimental impact on sperm viability. We also observed a profound decrease in sperm motility after 2 h of incubation with EPS, which is consistent with a previous *in vitro* study showing that sperm motility decreased after 2 h of incubation with trichomonads.[6]

The impact on sperm quality may be dependent on the protocol used to prepare EPS. Sperm exposed to seminal plasma or vaginal secretions harboring *T. vaginalis* are exposed to the harsh environment within the vagina. For *in vitro* studies it, therefore, seemed reasonable to incubate the sperm in HBSS instead of *T. vaginalis* culture medium. Furthermore, EPS composition within the vagina may be different from that in infected semen, as the secretory activity from *T. vaginalis* could be pH-dependent.[28] To maximize the secretory activity of *T. vaginalis* for preparing EPS we used HBSS and a short incubation time.

Most studies limit semen evaluation to the observation of sperm motility, viability, and morphology. Thus, the putative detrimental effect of EPS on the fertilizing capacity of the sperm is unknown. Although sperm motility reflects a large number of biochemical functions such as sperm metabolism and microtubular action in the tail fibers,[16] it does not provide any indication of the fertilizing capacity of the sperm. In fact, 30% of all patients with normal semen analyses have abnormal sperm function.[31] Among the functional tests of sperm, the evaluation of acrosomal status and the HOS test are indicative of normal membrane integrity and function.[32] Membrane and acrosome integrity are strongly associated with fertilization rate;[16] hence we decided to look at those three parameters *in vitro*. As shown in **Table 1**, the incubation of sperm with EPS reduced the proportion of sperm cells with an intact acrosome. Reactive oxygen species such as peroxide have been identified in EPS,[10] and sperm membrane peroxidation could be responsible for the breakdown of the acrosomal membrane.

Furthermore, it has been reported that the proportion of sperm showing HOS was markedly reduced in infected semen.[5] Consistent with this decrease, we also observed a reduction in HOS, indicating that the integrity of the sperm membrane is affected by EPS. A previous study showed that the percentage of sperm cells positive for the HOS test was positively correlated with the percentage of intact acrosomes.[32] Consistent with this, our results in 75% EPS yielded a similar proportion of sperm cells positive in the HOS test and with intact acrosomes (23.6% ±8.08% and 21.3% ±10.11%, respectively).

Because the HOS test reflects the functional integrity of the sperm cell membrane and is closely related to the IVF ability of sperm,[16] our results indicate that EPS contributes to the ultimate loss of fertilizing capacity. As predicted, incubation with EPS significantly reduced the sperms' fertilization rates. Some clinical cases suggest that the sperm-mucus interaction in men infected with *T. vaginalis*[4] may not impair the sperms' ability to travel and fertilize egg. However, our study suggests that only 2 h of incubation of sperm in the semen or a vagina harboring *T. vaginalis* could impair the fertilizing capacity of sperm.

This is the first report demonstrating a direct negative impact of the EPS from *T. vaginalis* on the fertilization rate of the sperm *in vitro*. However, further study should be performed using human sperm to determine if EPS has similar negative impact on sperm fertilizing capacity *in vitro*. Sperm fertilizing capacity may however not be affected by a single component of EPS and the secretions of *T. vaginalis* could be affected by its microenvironment. Thus, further research is needed to determine the molecular mechanisms responsible for the reduction of sperm fertilizing capacity by *T. vaginalis*.

AUTHOR CONTRIBUTIONS

JR participated in design, data analysis, and development of the manuscript; YSL, MYS and YC participated in the experiments and data analysis; JSR participated in the design of the study, data analysis, and supervision. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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