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Research Article

Seminal Extracellular Traps in SARS-CoV-2 Infection

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Abstract

Introduction: Extracellular Traps (ETs) are fibers composed of chromatin and cytoplasmic proteins, which can trap and kill pathogens by the phenomenon called ETosis. They are released by neutrophils, macrophages, and monocytes, and can be found in semen. The aim of this presentation is to evidence of the indirect effect of SARS-CoV-2 in semen by ETs.

Patients and methods: Experimental design: retrospective descriptive observational study.

Semen samples from two groups were studied following WHO guidelines: 1) SARS-CoV-2 infected donors (n: 5; at 7, 15, 30, 60, and 90 days after PCR diagnosis); 2) COVID-19 positive patients assisted in our laboratory between 2021 and 2022 (n: 70). They were observed in fresh and in Papanicolaou-stained smears by CASA and light microscopy; the presence of macrophages, spermiophages, ETs and hyperviscosity were recorded while neutrophilic concentration was calculated. Two control groups were designed: a) Patients belonging to group 2, studied before de pandemia (n: 13); b) Culture-negative semen samples (n: 28).

Results: In the first group, ETs were observed in all the samples, while only 18% had leucospermia. Macrophages, spermiophages, and hyperviscosity were recorded in 68%, 27%, and 36% of the studied cases respectively.

In the second group, ETs were present 100% in the acute phase (< 90 days after diagnosis) and decreased to 71% in the later stage (90 to 270 days). The trapped sperm were non-progressive motile or immotile alive or dead.

No traps were found in either control group.

Conclusion: In our study ETs were the most sensitive seminal marker of SARS-CoV-2 infection.

More Information

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Keywords: Extracellular traps; SARS-CoV-2; Cytopathic effect; Semen analysis; Andrology; Male fertility

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Introduction

Two main documents for professionals working at the Andrology Laboratory were published in 2021. The first one is the 6th edition of the WHO Manual for the Examination of Human Semen [1] and the most novel form of a biochemical analysis guideline The ISO 23162:2021 [2]. Our group was strongly committed to its discussion all over the world for four years, and finally worked in its translation to Spanish. It is the first ISO Norm for a Laboratory Assay. In 2010, WHO distinguished between nonspecific aggregation and agglutinated sperm, being the presence of anti-sperm antibodies as the necessary test to confirm the etiology of the second feature. Although they describe the term "viscosity", they only define how to determine it in the lab. In our experience in some cases, there is heterogeneous hyperviscosity that may be confirmed while analyzing the smear under the microscope. Nothing was reported regarding extracellular traps in semen associated with innate immunity in both documents. Extracellular Traps (ETs) are fibers composed of chromatin and cytoplasmic proteins released by the immune cells in a proinflammatory microenvironment [3]. The activated cells immobilize and kill the pathogenic agents and during this process, they also die by a particular programmed cell death named "ETosis" [4]. They have been studied by electron microscopy (scanning and transmission) and immunofluorescence. They were described in 2004 by Volker Brinkmann of the Max Planck Institute (Berlin,

Germany) and published in the journal Science [5]. This phenomenon is observed in any infection causing epithelial damage in the tissues involved and was recently enhanced and evidenced in our laboratory during the last COVID-19, pandemic caused by the SARS-CoV-2 [6,7]. The key factors that mediate SARS-CoV-2 pathogenicity are highly expressed in urogenital organs. This virus invades a cell through binding to the ACE2 receptor and TMPRSS2 priming. ACE2, a negative regulator in the renin-angiotensin system, is highly expressed in renal tubules epithelial cells, testis seminiferous ducts, adult Leydig cells, adrenal gland, and prostate while TMPRSS2 was found in the kidney, prostate, seminal vesicles, and epididymis [8].

Our hypothesis is that extracellular traps are more sensitive to infection markers than the presence of either neutrophils or macrophages. The aim of this presentation is to evidence of the indirect effect of SARS-CoV-2 in semen by ETs' presence in routine semen analysis.

Materials and methods

Experimental design

Retrospective descriptive observational study.

Semen analysis was performed according to WHO guidelines [9]. Fresh samples were observed by light microscopy and phase contrast microscopy -CASA (Computer Assisted Semen Analysis) then eosin stain was employed to evaluate vitality. In fixed smears Papanicolaou stained, the presence of leukocytes, macrophages, and spermiophages was determined, then neutrophilic polymorphonuclear leukocytes concentration (PMN/ml) was calculated. The presence of ETs was evidenced, and the seminal consistency was recorded. The present study was approved by the Research Ethics Committee of the Buenos Aires University Clinical Hospital "José de San Martin" following the Helsinki Declaration Guidelines (1975; reviewed in 2013). Each patient signed a free informed consent to be included in the study which was also approved by the mentioned Committee.

Semen samples from two groups were studied following WHO guidelines: Group 1) SARS-CoV-2 infected donors diagnosed by PCR in our hospital at 7, 15, 30, 60, and 90 days after diagnosis; 5 patients and 25 samples in total (July 2021 – December 2021). Group 2: Patients who attended the Male Fertility Laboratory and who reported having had COVID-19; n: 70; 69 consulted for fertility and only one for post-vasectomy control (April 2021-January 2022). Two control groups were studied: a) Patients belonging to group 2, studied before de pandemia (n: 13); b) Culture-negative semen samples (n: 28)*.

*Standard bacterial cultures were performed in first-void urine and semen samples. All patients were screened for *Chlamydia trachomatis* and *Mycoplasma hominis* in urethral swabs.

Results

ETs were visualized as a viscous and transparent material by phase contrast microscopy (Figure 1a,b). Fresh observation showed that some were "mobile in situ" and others immobile (CASA).

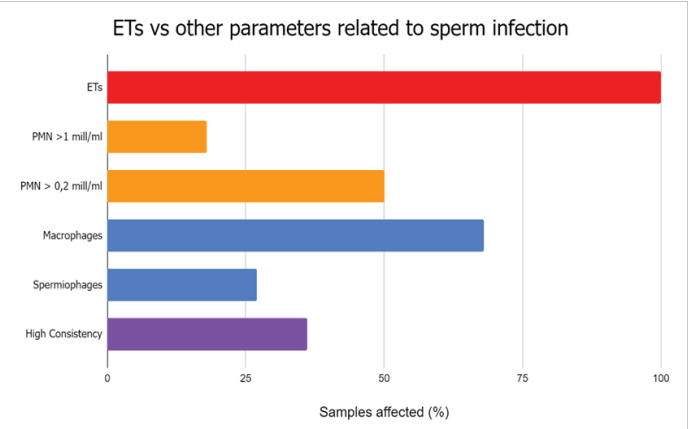
ETs stained were observed with light microscopy. Fresh observation with eosin showed alive or dead trapped sperm (Figure 2a). In smears fixed and stained with Papanicolaou, they are recognized morphologically as networks stained with affinity for hematoxylin (Figure 2b).

In Group 1, the parameters associated with sperm infection were recorded (Graph 1). Leucospermia referred to according to WHO guidelines was present in only 18% of the samples belonging to COVID-19 patients in the acute phase of the disease. If a more sensitive cut-off value for PMN concentration is employed according to Punab M [10] the number of samples affected improved (50%). Neither spermiophages, macrophages nor semen hyperviscosity resulted in better markers for the pathology under study. The unique and distinctive parameter that was present in all the samples studied was ETs. This graph clearly shows the sensitivity of ETs.

Having demonstrated in Group 1 that ETs are the best marker of acute SARS-CoV2 infection we stratified Group 2 according to the time elapsed since the clinical manifestation of COVID-19, less than 90 days (n: 7), and between 90 and 270 days (n: 63), namely acute and post-acute phase of the disease.

In group 2 sperm concentration varied from 0 to 344 mill/ml (Median: 27,45 (22,5 - 43,8) (IC 95%) That means that also azoospermias were included (n: 5), one belonging to a post vasectomy control. The latter was examined in the acute phase and presented ETs, the remaining four azoospermias were studied in the post-acute phase and only one showed ETs.

In the acute phase (<90 days after diagnosis) ETs were



Graph 1: Group 1, n: 25; Sperm concentration: 5,26 - 116,12 x 106 Ez/ml; Median: 39,17 (16,0 - 53,6) (IC 95%).

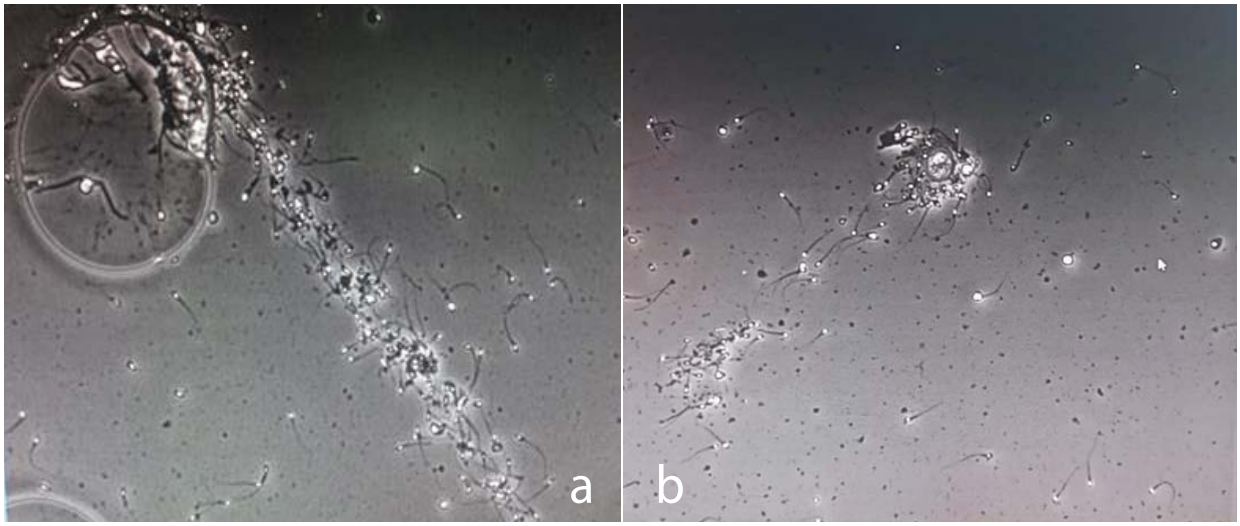


Figure 1: a,b: Extracellular traps in COVID-19 patient´s semen – phase contrast microscopy at 200x. Note the presence of trapped sperm.

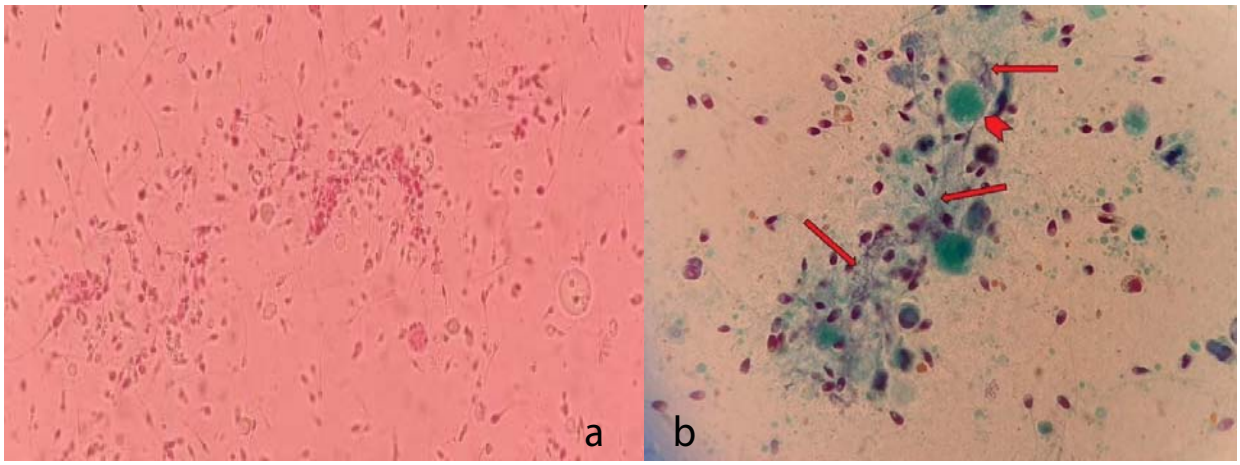


Figure 2: Extracellular traps in COVID-19 patient's semen - light microscopy. (a) Fresh observation with eosin (100X). (b) Note the presence of trapped sperm in the extracellular material stained by hematoxylin (1000X). Arrowhead shows the anucleated cell. The thin arrow shows the extracellular chromatin (Papanicolaou stain).

present in 100% of samples. In the post-acute phase (90 to 270 days) 71% of the samples showed ETs (n: 45), in the remaining 29% (n: 18), these were not found. The trapped sperm were non-progressively motile or immotile alive or dead. We wonder which were the parameters affected in the minor part of the samples studied (29%) in the second phase (Table 1).

Table 1: Group 2 – Samples in post-acute phase without ETs.

Parameter Related to Sperm Infection	Samples Affected
PMN/ml > 1 10^6 (OMS)	11%(n: 2)
PMN/ml > 0,2 10^6PMN/ml*	28%(n: 5)
Presence of macrophages	67%(n:12)
Presence of spermiophages	22%(n: 4)

*Punab M, et al. The limit of leucocytospermia from the microbiological viewpoint. Andrologia 2003.

ETs were present in all the semen samples from infected patients studied in the acute phase of SARS-CoV-2. However, neither leucospermia nor macrophages were found in all of them. In our study ETs were the most sensitive marker of infection as leucocytes cannot be completely quantified because in their fight against the pathogenic agent, they release their viscous chromatin and cytoplasmic microbicidal proteins and they finally die. More beyond that we cannot affirm that ETs are a marker of the acute phase as they were also found in the post-acute phase. Our results are according to those reported by Pisareva and col. who revealed the persistence of ETs in post-acute phase COVID-19 patients (six months or more post-acute infection) [11].

Hyperviscosity is associated with seminal infection and/or inflammation of the male urogenital tract [12]. Nevertheless, high consistency was not observed in all cases. Due to the rheologic thixotropic properties of these samples, this macroscopic characteristic may be underestimated [13].

Discussion

According to our recently published papers in mild SARS-

CoV-2 infected patients its effects on male fertility may be considered in the acute phase of the disease as fortunately, sperm parameters normalize in time [14,15].

ETs are not exclusive to SARS-CoV-2, they are part of the innate immunity being enrolled in other infections. In our opinion and according to our results ETs' presence should be informed and included in the international guidelines for semen analysis. Special interest may be focused on reporting their presence, they may be an alarm in asymptomatic infection, a main cause of infertility by affecting the sperm characteristics directly or indirectly by regulatory systems [16].

Schultz, et al. demonstrated that ETs can be activated by spermatozoa in contact with leucocyte polymorphonuclear (*in vitro*), inducing sperm entrapment and decreased motility in seminal fluids [17]. Sperm coincubated either with monocytes or uropathogenic *Escherichia coli* release extracellular trap (MET) [18]. Thus, it was demonstrated that ETosis does not depend exclusively on bacterial contamination. Note that we also found ETs in azoospermic samples either vasectomized or not explaining that their occurrence is independent of sperm presence. ETs can be present in both aseptic inflammatory processes and autoimmune diseases [19].

Although ETs are part of innate immunity, the effect is not always beneficial. They have a deleterious effect on the urogenital tract cells of infected patients [20]. In semen, they trap sperm, which by losing progressive motility are unable to fulfill their main function in natural fertilization. Neutrophil Extracellular Traps (NETs) have been implicated in mammalian reproduction. After insemination either naturally or artificially, an immune reaction is triggered in the vagina and uterus, neutrophils release their content and trap the dead sperm (natural selection process) [21]. On the other hand, it was reported that NETs may have a deleterious effect causing infertility, eclampsia, and fetal loss in humans [22]. We agree with Texeira and col. who stated that "the pathophysiological understanding of the male genital tract is essential to assess its clinical impact on male reproductive health and guide future research" [23].

In our study, the best seminal marker of SARS-CoV-2 infection was extracellular traps. All the data reported in this manuscript came out from our daily work in the frame of a comprehensive reading of the available publications. However, more studies are required to confirm these results, basically including a higher number of patients.

Conclusion

We would strongly remark on the relevance of cytologic feature interpretation in routine semen analyses.

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