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Rheology of Human Seminal Fluid: Role of Lysozyme

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SUMMARY. The aim of this work was to evaluate the role of lysozyme in the phenomenon of seminal hyperviscosity. The enzyme was determined in 142 samples of seminal plasma either leucospermic or not, with or without active macrophages, and classified according to their consistency (normal or high). Rheologic parameters determined with a Wells-Brookfield viscosimeter at 20 °C showed significant difference between the normal and high consistency batches ($p < 0.01$). The kinetic method with *Micrococcus lysodeikticus* as substrate was used, and working conditions were established for the determination of lysozyme in semen. No difference was found in enzymatic concentration on comparing normal and high seminal consistency groups while differences proved highly significant in batches either leucospermic or not ($\bar{X} \pm 2$ SEM nmol/l; n: 44, 197.2 ± 51.3, *vs.* n: 98, 108.3 ± 12.8; $p < 0.0005$). *In vitro* lysozyme addition showed no significant effect on samples with high consistency. Seminal vesicle and prostate functional indicators failed to correlate with lysozyme concen-tration.It is concluded that lysozyme plays no direct role in the phenomenon of seminal hyperviscosity, although its deficiency in cases of chronic infections could be a factor aggravating the clinical overview.

RESUMEN. "Reología del Semen Humano: Compromiso de la Lisozima". El objetivo del presente trabajo fue evaluar el compromiso de la lisozima en el fenómeno de hiperviscosidad seminal. La enzima fue determinada en 142 muestras de plasma seminal clasificadas de acuerdo a su consistencia (normal o aumentada) y a la presencia o no de leucospermia y macrófagos activos. Los parámetros reológicos determinados con un viscosímetro Wells-Brookfield a 20 °C mostraron diferencia significativa entre los lotes de consistencia normal y aumentada ($p < 0,01$). Se empleó el método cinético que utiliza *Micococcus lysodeikticus* como sustrato, estableciéndose las condiciones de trabajo para la determinación de la lisozima en semen. No se halló diferencia significativa en la concentración enzimática al comparar los grupos de consistencia normal y aumentada, siendo la diferencia altamente significativa entre los lotes que presentaron o no leucospermia ($\bar{X} \pm 2$ SEM nmol/l; n: 44, 197,2 ± 51,3, vs. n: 98, 108,3 ± 12.8; $p < 0,0005$). El agregado de la lisozima *in vitro* no disminuyó la consistencia de las muestras hiperviscosas. Los marcadores de funcionalidad de próstata y vesícula no se correlacionaron con la concentración de la lisozima. Se concluye que la lisozima no tiene un rol directo en el fenómeno de hiperviscosidad seminal, aunque su deficencia en casos de infecciones crónicas podría ser un factor agravante de la patología en estudio.

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KEY WORDS: Leucospermia, Lysozyme, Rheology, Semen.
PALABRAS CLAVE: Leucospermia, Lisozima, Reología, Semen.

INTRODUCTION

Biorheological studies have shown that the energy required by spermatozoa to achieve translational velocity is strongly influenced by the elastic properties of the fluid in which motion takes place [1]. Seminal plasma with high consistency [2] may cause asthenozoospermia [3,4]. This encouraged diverse teams to search for agents capable of reducing viscosity. Gersh [5] proposed the use of pancreatic dornase, while Hirschhauser and Eliasson [6] described a case of chronic prostatitis where lysozyme addition resolved the difficulty. Upadhyaya *et al.* [7] compared the effect of three mucolytic agents, sputolysin, alevaire and α-amylase, concluding that sputolysin was the one of choice. However, objections were raised to its use in artificial insemination due to the direct action of dithiotreitol on the human spermatozoon, and as a result plasmine was proposed instead [8]. Lysozyme, also termed muramidase, is an ubiquitous enzyme discovered by Fleming [9], which acts on β-1-4 glucosidic bonds in peptidoglycans belonging to the bacterial wall. It is believe to participate in a primitive non-specific defense mechanism associated to the monocytic-macrophagic system [10,11]. The goal of this study was to evaluate the role of lysozyme in the phenomenon of seminal hyperviscosity. Thus, lysozyme was measured in semen samples which were leucospermic or not having normal or high consistency. *In vitro* lysozyme addition on seminal viscosity was then evaluated in order to determine its effect.

MATERIAL AND METHODS

Material

A total of 142 semen samples from patients attending the Seminological Laboratory, Clinical University Hospital, Buenos Aires, Argentina, were studied. Only liquefied samples were employed. They were analysed following W.H.O. criteria [2] and classified according to their consistency as normal (NC) or high (HC), to the count of polymorphonuclear leucocyte (PMN) and the presence of spermiophages.

Those specimens with PMN concentrations superior to 10^6/mL, were defined as leucospermic samples, while the presence of active macrophages in the ejaculate were called macrophagic reaction.

Methods

The consistency (often referred to as "viscosity") is estimated by WHO guidelines. A glass rod is introduced into the sample; on withdrawal of the rod the length of the thread that forms is observed. In normal consistency semen smears it should be less than 2 cm, while in high consistency samples this length is exceeded [2].

A Wells-Brookfield rotational viscosimeter which allows for eight shear rates was used. The readings were done every three minutes, starting from 1.15 sec^{-1} to 230 sec^{-1}, from that point the reverse procedure was followed. The corresponding rheograms were plotted (shear stress *vs.* shear rate) and from these graphs the apparent viscosity at maximum shear rate η_{am} (cp) as well as two other rheological variables were obtained: yield value τ_0(dyne/cm^2) and thixotropy area μ (dyne/cm^2 sec) (determined by the sum of trapezoids method).

Lysozyme dosage was performed in seminal plasma by the kinetic method using a suspension of *Micrococcus lysodeikticus* as substrate [12].

In previous studies [13],we have shown that the use of diluents alters the biophysical properties of sperm, the enzyme addition was carried out following lyophilization. A stock solution of lysozyme in saline buffer phosphate, pH = 6.2 was prepared (14.3 µmol/L) and dispensed in vials (0.5 mL/ vial) where the lyophilization was performed. The lyophilizates were reconstituted with 0.5 mL of semen. The enzyme concentration was determined before lyophilization and after the reconstitution of the lyophilizates with the samples. In these cases, it was important to consider the loss of activity caused by the lyophilization process. The effect of lysozyme on semen consistency was evaluated by adding an excess of lyophilizated enzyme (14.3 µmol/L to 14 hyperviscous sperm samples, 4 of which were leucospermic). The consistency of treated samples maintained at room temperature [5] was determined at different times(0,15,30,60,120 and 180 minutes). Fructose, citric acid and acid phosphatase determinations were performed by Roe, Chambon and Bessey-Lowry-Brock methods, respectively, modified to be used in seminal plasma [14].

Student's Test was used to evaluate differences between mean values and linear regression analysis to correlate the variables under study.

RESULTS

Semen is a non-newtonian, thixotropic fluid. Two different groups could be defined according to rheological parameters assessed (Table 1).

Rheological variables	Normal consistency (n: 42)	High consistency (n: 10)
η_{am}(cp)	3.9 ± 1.0	5.3 ± 2.0 *
τ_0 (dina / cm^2)	0.40 ± 0.06	1.00 ± 0.24 **
µ (dina / cm^{2}sec)	46.5 ± 8.4	285.1 ± 124.1 **

Table 1. Seminal consistency and rheological variables determined with a rotational viscosimeter at 20 °C. Data are expressed as means ± 2 SEM.
* $p < 0.01$, ** $p < 0.00005$.

Working conditions for the detection of lysozyme in semen were studied. Using 100, 200, 300, and 400 µL of seminal plasma it was established that the optimal volume added to assess lysozyme concentration was between 300 and 400 µl, thus the percentage of transmitance obtained is in the effective range of the calibration curve. The assays were performed in duplicate, resulting in high reproducibility. In any turbidimetric assay particularly designed to be used in a biological fluid, it is important to be aware of any kind of interference. So an internal standard of pure lysozyme was added (2 µg/mL) to each test mixture and the percentage of recovery was calculated.There was no significant difference neither in the reproducibility nor in the percentage of recovery obtained by the addition of either 300 or 400 µL of seminal plasma (Table 2).

Seminal plasma (μL)	Enzymatic concentration (nmol L-1)	Reproducibility	% Recovery
300	120.7 ± 45	123.4 ± 12.1	81.0 ± 10.6
400	124.3 ± 41.4 *	119.2 ± 19.1	79.6 ± 9.6 *

Table 2. Adjustment of seminal plasma volume (n:20). Values are expressed as means ± 2 SEM. * NS.

Preincubation time (min)	Enzyme concentration (nmol L-1)
120	15.7 ± 13.6
180	56.4 ± 31.4 *
240	85.0 ± 35.0 **
300	122.1 ± 39.3 **

Table 3. Lysozyme dosage in seminal plasma (n:13) at various incubation times (37 °C). Values are expressed as means ± 2 SEM (* $p < 0.05$, ** $p < 0.001$).

	Normal consistency	High consistency	Total according to leucospermia with or without marcophagic response
PNM < 10^6/mL	112.3 ± 22.9 (n:56)	94.9 ± 20.6 (n:42)	108.3 ± 12.8 (n:98)
PNM > 10^6/mL	200.2 ± 61.6 (n:32)	189.3 ± 70.4 (n:12)	197.2 ± 51.3 (n:44)
Total according to consistency	147.2 ± 28.9 (n:88) (n:54)	121.5 ± 26.7	

Table 4. Lysozyme dosage in seminal plasma according to consistency and concentration of PMN. Values are expressed as means ± 2 SEM nmol/L.

Enzyme concentration may be evaluated either in fresh samples or in specimens preserved by freezing at -15 °C, as no significant difference was found in the enzymatic activity determined neither in fresh samples nor in the same ones preserved by freezing at -15°C for 30 days (n: 34, $\overline{X}$ ± 2 SEM, 137.5 ± 59.1 y 136.7 ± 61.5, respectively).

The preincubation (37° C) time suggested for the measurement of the enzymatic activity is four hours. It was observed at that time that lysozyme activity reached levels which were mathematically highly significant ($p < 0.001$) in respect to the controls (Table 3).

Table 4 summarizes data obtained from lysozyme dosage. No significant difference was found while comparing groups with NC and HC. However, the difference between batches (leucospermic or not) proved to be highly significant at $p< 0.0005$ level. Likewise, on subdividing NC and HC groups according to presence or absence of leucospermia, differences were significant *inter se* ($p < 0.005$). In

contrast, differences between batches leucospermic or not, and NC and HC subatches all proved negligible.

The addition of the enzyme in semen was carried out to establish its action *in vitro* and to find out the percentage of recovery of additional enzymatic activity. It was performed by employing lyophilized lysozyme. It could not be added in solution, because in that case we would not have had the possibility to discriminate the effect of the enzyme on the viscosity from the one of the diluent. On dissolving the enzyme lyophilizated in semen, 73% of enzymatic concentration was recovered and in a single sample with high consistency (one of the four leucospermics samples) a slight decrease in viscosity was observed 60 minutes after addition.

Lysozyme concentration failed to correlate with fructose or citric acid concentrations, or with acid phosphatase activity (Table 5).

	Correlation coefficient	
	Lysozyme *vs.* Fructose	**Lysozyme *vs.* Citric Acid**
Normal consistency without leucospermic reaction (n:41)	0.3379	0.0538
High consistency without leucospermic reaction (n:30)	0.3779	0.2792
Samples with leucospermic reaction (n:36)	0.3411	0.2493

Table 5. Correlation coefficients of lysozyme *vs.* indicators of seminal vesicle and prostate.

DISCUSSION AND CONCLUSIONS

The kinetic method employed is widely accepted for lysozyme dosage [15]. Another recognized technique is that of lysoplate in semen [16-18], which renders values higher than the former, but results are not comparable [19]. The kinetic method employing Micrococcus lysodeikticus as substrate was modified for its use in semen.

In order to eliminate possible changes induced by factors apart from viscosity, we only used liquefied samples [3], as liquefaction disorders and the phenomenon of seminal hyperviscosity express distinct and mutually independent pathologies.

Working conditions set for the choosen kinetic method were studied establishing the incubation time with Micrococcus lysodeikticus substrate, following preincubation at 37 °C for a period of four hours using 300 µL of seminal plasma, either fresh or preserved by freezing at -15 °C.

Data recorded infers that lysozyme plays no major role in the phenomenon of seminal hyperviscosity.

Lysozyme concentration increases in the leucospermic samples, which recalls its role in defense against infection. On subdividing the groups under study

according to such reaction, no differences could be found leading to new speculations. We may not infer that a deficiency in this enzyme could affect viscosity despite having achieved some improvement in one of the leucospermic samples. This single case brings to mind the similar finding, described by Hirschhauser and Eliasson [10], although these authors failed to report how the enzyme was added, a crucial fact for proper interpretation since the mere presence of an aqueous medium is enough to decrease viscosity [13]. Seminal vesicle and prostate functional indicators, fructose for the former and citric acid and acid phosphatase for the latter, failed to correlate with enzyme concentration regardless of using samples having either normal or high consistency, or inflammatory reaction, so that it may be inferred that lysozyme concentration is unrelated to routinely employed indicators. Mardh and Colleen [12] studied seminal lysozyme in prostatitis and reported that patients who increased or maintained lysozyme levels after antibiotic treatment suffered no recurrences, in contrast to those cases in which enzyme level remained low. The results indicated that individual glandular response varies considerably and thus directly affects the andrological pathology.

Nevertheless, it shoud be stressed that not all semen samples with inflammatory reaction were hyperviscous and that within the batch of those with high consistency there were samples without leucospermic reaction. Therefore, although infection seems a likely cause of hyperviscosity, other so-far-undiscovered factors may exert greater influence on this phenomenon.

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