

Evaluation of The Role of Seminal Plasma Clusterin in Infertility Associated with Leukocytospermia

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ABSTRACT

Background: Many factors can contribute to male infertility and one of them is presence of high concentration of WBCs in semen which is known as leukocytospermia. Clusterin is a seminal plasma protein with proposed cytoprotective role against oxidative stress and other harmful effect of leukocytospermia.

Objective: This study aims to investigate the role of seminal plasma clusterin in infertility associated with leukocytospermia.

Patients and methods: A prospective case-control study was conducted on 96 men recruited from Mansoura University Hospitals Andrology outpatient clinic. We divide them into four groups: fertile without leukocytospermia (control group), fertile with leukocytospermia group, infertile without leukocytospermia group and infertile with leukocytospermia group.

Results: The seminal plasma levels of clusterin concentration were found to be significantly reduced in all groups in comparison to the control group ($P < 0.0001$). Also comparing both infertile groups showed significant reduction in clusterin in the group associated with leukocytospermia ($P = 0.0034$). Moreover, clusterin showed high significant positive relationship with semen parameters, alpha-glucosidase, acrosin activity index and total antioxidant capacity. However, it showed significant negative relationship with MDA ($p < 0.0001$). These results support the protective role of clusterin against the oxidative stress in semen, which represents one of the most important mechanisms by which leukocytospermia could affect semen quality and interfere with fertility function.

Conclusions: Clusterin has an important sperm protective role against oxidative stress and seminal plasma clusterin level is significantly reduced in infertile patients with leukocytospermia, which may explain one of the mechanisms of how leukocytospermia interferes with the fertility function.

Keywords: Infertility, Leukocytospermia, Seminal plasma clusterin.

INTRODUCTION

Infertility means failure to achieve a clinical pregnancy after 12 months of regular unprotected sexual intercourse⁽¹⁾. About 50% of the infertile couples are related to male factors of infertility⁽²⁾. Multiple factors can lead to a reduction in fertility, including lifestyle, environmental and genetic factors, that can act alone or in combination. In most cases, these factors lead to infertility related to low sperm count, reduced motility or abnormal sperm size and morphology⁽³⁾. Leukocytospermia is defined by the World Health Organization (WHO) as $\geq 1 \times 10^6$ WBC/ml of semen⁽⁴⁾. It has been demonstrated that leukocytospermia has negative impacts on sperm function and integrity⁽⁴⁻⁶⁾.

Aziz *et al.*⁽⁴⁾ reported a positive correlation between leukocytospermia and high sperm deformity index scores, acrosomal damage, midpiece defects, and tail deformities. Also the presence of oxidative stress in an individual with leukocytospermia is associated with impaired sperm function as measured by its acrosin activity⁽⁷⁾.

Clusterin (CLU) is a heterodimeric highly conserved disulphide-linked glycoprotein, which is expressed in a wide variety of tissues and secreted in all human fluids⁽⁸⁾. In the testis, secretory form of clusterin is regarded as the main protein synthesized by Sertoli cells and is secreted into the fluid of the seminiferous epithelium and deposited onto the membranes of elongating spermatids and mature spermatozoa⁽⁹⁾. Similar to other organs, an anti-apoptotic effect from

clusterin on germ cells has been reported. It is also demonstrated that clusterin produced by Sertoli cells effectively protects the rat testes from heat stress-induced apoptosis⁽¹⁰⁾. However, there has been little information with respect to the functional roles of clusterin in the male reproductive tract under physiological conditions. In particular, the exact function of seminal CLU is not known well yet⁽¹¹⁾.

The aim of the present study was to investigate the role of seminal plasma clusterin in infertility associated with leukocytospermia.

PATIENTS AND METHODS

The present study included male subjects attended the Andrology Outpatient Clinic of Dermatology, Andrology and STDs Department, Mansoura University Hospital from April 2020 to August 2021 for infertility. Laboratory investigations were conducted at the Molecular Biology Unit of the Medical Biochemistry Department, Faculty of Medicine, Mansoura University.

In this case-control study, the subjects were grouped into four categories:

1- Control group (n=24) (group1): Fertile men with normal seminogram, normal WBCs count and normal sex hormones levels were used as control group. Fertile men were those who had no history of fertility problem and those whose partners

became pregnant spontaneously within one year of regular unprotected intercourse.

- 2- **Fertile with leukocytospermia (n=24) (group2):** This group included fertile men with leukocytospermia and normal sex hormones levels. They had no history of fertility problem and their partners became pregnant spontaneously within one year of regular unprotected intercourse.
- 3- **Infertile without leukocytospermia (n=24) (group3):** they were selected from men with normal leukocytic count in the semen whom their partners did not become pregnant spontaneously within one year of regular unprotected intercourse.
- 4- **Infertile with leukocytospermia (n=24) (group4):** They were selected from men with increased leukocytic count in semen whom their partners did not become pregnant spontaneously within one year of regular unprotected intercourse.

Ethical considerations:

This study was approved by Institutional Research Board (IRB) of Faculty of Medicine, Mansoura University. Every patient signed an informed written consent for acceptance of participation in the study. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans.

Inclusion criteria:

Age range from 20 to 50 years, continuous unprotected marital relationship through the last year, and control group: healthy age-matched men who have achieved pregnancy recently (within 12 months).

Exclusion criteria:

Patients with history of systemic diseases, local diseases, medical treatment or surgery with possible adverse effect on fertility. Patients with varicocele diagnosed by clinical examination. Patients with hypogonadism. Patients with azoospermia were excluded from this study, and patients younger than 20 years or older than 50 years.

All participants were subjected to:

- I. Thorough history taking.
- II. General physical examination: to detect possible abnormalities relevant to fertility in the following systems: endocrine, cardiovascular, respiratory, gastrointestinal and neurological.
- III. Local genital examination of the testis, epididymis, vas deferens and inguinal region: to exclude possible abnormalities.

Semen analysis:

Sample preparation:

Semen samples were collected from all the study subjects by masturbation after 2 to 5 days of abstinence, and then allowed to liquefy at 37°C for 15-30 min. After complete liquefaction, the liquefied semen was divided into 2 parts:

- a) One part used for semen analysis by Auto sperm analyzer according to the WHO recommendations⁽⁴⁾. Sperm morphology was evaluated by phase contrast microscope and Spermac stain (Fertipro, Belgium). WBCs were determined by peroxidase stain⁽¹²⁾.
- b) The other part was centrifuged at 10,000g for 10 minutes at 4°C to separate seminal plasma.

Techniques performed on human spermatozoa:

- **Computer-assisted method of semen analysis (auto sperm):** In this study concentration and sperm motility characteristics were assessed by means of a simple computer-assisted (Autosperm) method⁽¹³⁾.
- **Assay of acrosin activity (by gelatin-covered microslides and gelatinolysis) (Figure 1)**

Gelatin-covered slides were prepared by spreading 20 µL of 5 % gelatin (Merck, Darmstadt, Germany) in distilled water on the slides. The slides were then air-dried, stored at 4°C overnight and fixed and washed in phosphate-buffered saline⁽¹⁴⁾. Semen samples of 20 µL were diluted 1:10 in phosphate-buffered saline (PBS) containing 15.7 mmol/L α-D-glucose. Semen samples were smeared on prepared slides and incubated in a moist chamber at 37°C for 2 hours. The halo diameter around any 10 spermatozoa shown to be representative of sperm present in the ejaculate was measured in phase contrast with an eyepiece micrometer. The halo formation rate was calculated per slide as the percentage of spermatozoa showing a halo. One hundred spermatozoa were evaluated. An acrosin activity index was calculated by multiplying the halo diameter by the halo formation rate⁽⁷⁾.

Techniques performed on seminal plasma:

The supernatant (seminal plasma) was divided into 3 tubes and stored at -20°C until analysis:

1. First tube was analyzed for clusterin by ELISA technique.
2. Second tube was used for assessment of seminal alpha-glucosidase activity⁽¹⁵⁾ to evaluate epididymal function.
3. Third tube was used for malondialdehyde (MDA) by colorimetric method⁽¹⁶⁾ to assess lipid peroxidation.

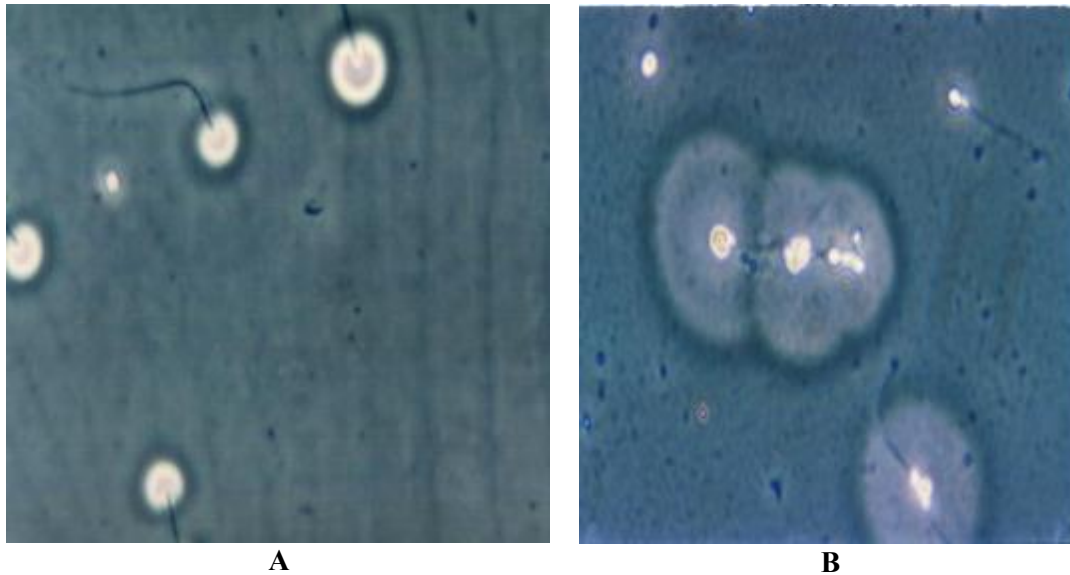


Figure (1): Photomicrograph of human spermatozoa after 2 hours incubation on gelatin slides showing good acrosine activity (**A**) and poor and no acrosin activity (**B**) (500X) phase contrast microscope.

Assay of Clusterin concentration in seminal plasma by ELISA:

Level of clusterin in sperm pellet was estimated by enzyme linked immune sorbent assay technique (ELISA) using SunRed human Clusterin ELISA kit (SunRed biotechnology Inc, China, Cat. no: 201-12-1190).

Assay of alpha- glucosidase activity:

Alpha glucosidase was measured using (Episcreen Kit; FertiPro, 32,8730 Lotenhulle, Belgium).

Statistical analysis

Statistical analysis was done by using MedCalc® program version 8.1. Quantitative data were expressed as median, and range. Mann-Whitney test was used as a test of significance for comparison of two groups. Spearman rank correlation coefficient was calculated to study the relation between variables. P value < 0.05 was considered significant.

RESULTS

In comparison between control group and fertile leukocytospermia group: grade A motility, grade B motility, grade A+B motility, velocity, linear velocity, linearity index and normal morphology were significantly decreased. However, concentration of sperms was not significantly different between the two groups.

In comparison between the control group and both infertile groups: Concentration, grade A motility, grade B motility, grade A+B motility, velocity, linear velocity, linearity index and normal morphology were significantly decreased in both infertile groups.

In comparison between both infertile groups: Concentration, grade A motility, grade B motility, grade A+B motility, velocity, linear velocity, linearity index and normal morphology were significantly decreased in leukocytospermic group (Table 1).

Table (1): Semen parameters of the studied groups

Parameters	Fertile without leukocytospermia	Fertile with Leukocytospermia	Infertile without Leukocytospermia	Infertile with Leukocytospermia	Significance
Concentration of sperms	66.2±14.51	56.1±13.31	26.8±6.31	14.8±3.43	P2<0.0001 P3<0.0001 P4<0.0001
White blood cells (WBC)	0.35±0.035	2.1±0.41	0.7±0.11	4.0±0.91	P1 < 0.0001 P2 = 0.0015 P3 < 0.0001 P4 < 0.0001
Grade A Motility	40±8.91	33±7.82	23.5±5.12	16±3.62	P1<0.0001 P2<0.0001 P3<0.0001 P4<0.0001
Grade B Motility	31±7.11	19.5±4.28	13±3.31	8.5±2.13	P1<0.0001 P2<0.0001 P3<0.0001 P4<0.0001
A+B	69.5±15.67	53±12.3	36.5±8.61	23.5±4.81	P1<0.0001 P2<0.0001 P3<0.0001 P4<0.0001
Velocity	40.7±9.22	35.7±7.73	22.9±5.61	15±3.45	P1=0.011 P2<0.0001 P3<0.0001 P4<0.0001
Linear velocity	36.2±7.63	28.3±6.78	11.9±2.56	7.3±1.48	P1=0.0002 P2<0.0001 P3<0.0001 P4<0.0001
Linear velocity index	87.7±18.21	80.8±14.5	55.9±12.83	47.9±9.84	P1<0.0001 P2<0.0001 P3<0.0001 P4=0.018
Morphology	42.5±9.21	32±7.51	23±5.13	18±4.51	P1<0.0001 P2<0.0001 P3<0.0001 P4=0.0007

Data are expressed as median and range, Control group is the fertile without leukocytospermia group

P1= control versus fertile leukocytospermic groups

P2=control versus infertile without leukocytospermia groups

P3= control versus infertile leukocytospermic groups

P4= infertile without leukocytospermia versus infertile leukocytospermic groups

In comparison between control group and other groups, CLU, acrosin activity index, α - glucosidase (mU/ ml), total antioxidant capacity were significantly decreased, while malondialdehyde was significantly increased in the last three groups (Table 2).

Table (2): CLU, acrosin activity index, α - glucosidase, malondialdehyde, total antioxidant capacity, in all studied groups

Parameters	Fertile without leukocytospermia	Fertile with Leukocytospermia	Infertile without Leukocytospermia	Infertile with Leukocytospermia	Significance
Clusterin (CLU) (ng/ml)	55.4±12.61	41.7± 9.31	18.9±4.51	14.5±3.31	P1<0.0001 P2<0.0001 P3<0.0001 P4=0.0034
Alpha glucosidase (mu/ml)	63.6±13.64	50.0±11.11	33.3±8.21	24.0±5.46	P1<0.0001 P2<0.0001 P3<0.0001 P4<0.0001
Acrosin activity index	14.6±3.32	12.9±2.51 11.0 – 15.0	8.2±1.25	5.6±1.31	P1<0.0001 P2<0.0001 P3<0.0001 P4<0.0001
Total antioxidant capacity	14.1±3.14	10.2±2.34	7.2±1.37	4.3±0.97	P1<0.0001 P2<0.0001 P3<0.0001 P4<0.0001
Malondialdehyde (nmol)	2.4±0.51	3.4±0.91	5.5±1.12	8.3±1.82	P1<0.0001 P2<0.0001 P3<0.0001 P4<0.0001

Data are expressed as median and range, Control group is the fertile without leukocytospermia group

P1= control versus fertile leukocytospermic groups

P2=control versus infertile without leukocytospermia groups

P3= control versus infertile leukocytospermic groups

P4= infertile without leukocytospermia versus infertile leukocytospermic groups

In comparison between the control group and the fertile with leukocytospermia group there was no significant change in hormonal levels. However, when we compared the control group with the infertile with no leukocytospermia group we found significant increase in the level of both FSH and prolactin while other hormones levels remain unchanged. Comparing the control group with the infertile leukocytospermia group showed significant increase in the levels of all hormones except for total testosterone, which remained unchanged. The comparison between the two infertile groups showed only mild increase in the LH level in the leukocytospermia group, but other hormones remained unchanged (Table 3).

Table (3): Blood hormone levels of all studied groups

Parameters	Fertile without leukocytospermia	Fertile with Leukocytospermia	Infertile without Leukocytospermia	Infertile with Leukocytospermia	Significance
FSH (IU/mL)	7.3±1.82	7.2±1.52	8.9±1.88	9.4±2.11	P2 < 0.0001 P3 < 0.0001
LH (IU/L)	8.0±1.61	8.7±1.92	8.5±1.11	9.4±2.34	P3 < 0.007 P4 < 0.02
Total testosterone (nmol/L)	5.3±1.33	6.3±1.54	5.7±1.61	5.6±1.22	
Prolactin (ng/mL)	9.7±2.33	10.4±2.47	11.5±2.87	11.3±2.61	P2 < 0.003 P3 < 0.01
Estradiol (pg/mL)	30.8±6.81	29.6±5.91	30.8±4.88	35.9±6.97	P3 < 0.03

Data are expressed as median and range, Control group is the fertile without leukocytospermia group

P1= control versus fertile leukocytospermic groups

P2=control versus infertile without leukocytospermia groups

P3= control versus infertile leukocytospermic groups

P4= infertile without leukocytospermia versus infertile leukocytospermic groups

In table 4, CLU showed highly significant positive correlation with sperm concentration, grade A motility, grade B motility, grade (A+B) motility, velocity, linear velocity, linearity index and normal morphology. However, it showed highly significant negative correlation with WBCs.

Table (4): Correlation of CLU with sperm parameters of all studied groups

Parameters	CLU (ng/ml)	
	R	p
Concentration (mill/ml)	0.80	<0.0001
Grade a (%)	0.80	<0.0001
Grade B (%)	0.86	<0.0001
Grade A+B (%)	0.87	<0.0001
Morphology (%)	0.81	<0.0001
Velocity (µm/sec)	0.73	<0.0001
Linear velocity (µm/sec)	0.80	<0.0001
Linearity Index	0.79	<0.0001
WBCs (million/ml)	-0.50	<0.0001

R=correlation coefficient

It was found that CLU was highly significantly positive correlated to α - glucosidase, total antioxidant capacity and acrosin activity index. On the other hand, there was highly significant negative correlation between CLU and malondialdehyde (MDA) as shown in table 5.

Table (5): correlation of seminal plasma CLU with alpha glucosidase, malondialdehyde, total antioxidant capacity and acrosin activity index of all studied groups

Parameters	CLU (ng/ml)	
	R	P
Alpha- glucosidase (mu/ml)	0.85	<0.0001
Acrosin activity index	0.80	<0.0001
MDA (nmol)	-0.84	<0.0001
Total antioxidant capacity (TAC)	0.81	<0.0001

R=correlation coefficient

Table 6 shows that CLU had significantly negative correlation with FSH, prolactin and estradiol (E₂). However it had no significant correlation with testosterone and LH.

Table (6): Correlation of seminal plasma CLU with hormonal profile of all studied groups

Parameters	CLU	
	R	p
Testosterone (ng/dl)	0.062	0.55
Estradiol (pg/ml)	-0.24	0.019
Prolactin (ng/ml)	-0.31	0.002
FSH (µIU /ml)	-0.69	<0.0001
LH (µIU /ml)	-0.20	0.06

R=correlation coefficient

DISCUSSION

To the best of our knowledge, among the studies done to demonstrate seminal plasma CLU concentration in infertile men, this is the first study to focus on infertile patients who have associated leukocytospermia. In the current study, CLU concentration was investigated in seminal plasma of 96 men divided into four groups each contains 24 men as following: Group 1: fertile with no leukocytospermia (as a control group). Group 2: fertile with leukocytospermia. Group 3: infertile with no leukocytospermia, and Group 4: infertile with leukocytospermia.

In the present work, CLU concentration in seminal plasma in the last three groups was significantly lower in comparison to the control group. On the same line, **Fernandez-Encinas *et al.*** ⁽¹⁷⁾ found that CLU was one of 17 proteins, which were under expressed in seminal plasma of infertile patients in comparison with healthy fertile donors. These results correspond with the data of **Salehi *et al.*** ⁽¹⁸⁾ who found that CLU concentration in infertile patients was significantly lower in infertile males in comparison to fertile group. However, **Zalata *et al.*** ⁽¹⁹⁾ contradict the ones mentioned above. As they found that the expression of CLU RNA and the CLU gene was higher in the seminal plasma of infertile men. They showed that it is correlated negatively with, sperm count, motility, linear velocity, and significantly correlated positively with the percentage of abnormal forms of spermatozoa and sperm DNA fragmentation. This contradiction can be explained as they didn't measure the concentration of the protein itself in the seminal plasma but the expression of the CLU RNA.

According to the current study, there was a positive correlation between CLU concentration in seminal plasma and different sperm parameters. This is in agreement with the finding of **Fukuda *et al.*** ⁽⁹⁾ who found that the semen samples of patients with oligospermia and azoospermia show significant decreased CLU concentration in comparison of semen samples of normospermia. Additionally, **Salehi *et al.*** ⁽¹⁸⁾ noted that there was a negative correlation between seminal plasma CLU and abnormal morphology, specifically head and neck abnormalities. However and on the contrary of our study, they found that no significant relationship was present between CLU and sperm motilities. Those findings can be explained by the protective anti-apoptotic effect of CLU on human germ cells as CLU shows anti-apoptotic behavior ⁽²⁰⁾, consistent with the presence of borane trihydridoboron (BH₃) domains within CLUs ⁽²¹⁾. These types of domains are usually found on the Bcl-2 family of proteins, which are known to act as central regulators of apoptotic pathways ⁽²²⁾. Most members of the Bcl-2 family interact with each other or with other proteins through these domains ⁽²³⁾. Moreover, CLU is related to the prevention of protein precipitation, agglutination of

abnormal spermatozoa and control of complement induced sperm lysis⁽¹⁹⁾.

Leukocytospermia is defined as the presence of more than 1×10^6 WBCs per milliliter of semen⁽⁴⁾ and there are numerous reports indicating that leukocytospermia has a negative impact on male fertility and associated with high levels of ROS which in turn may cause a decline in sperm parameters including sperm concentration and motility⁽²⁴⁾. In male infertility, oxidative stress seems to stand out as one of the underlying mechanisms. ROS excessive generation leads to loss of sperm integrity and function⁽²⁵⁾. Plasma membranes that contain a high content of polyunsaturated fatty acids enable ROS to damage membranes and DNA through oxidation and peroxidation reactions⁽²⁶⁾.

In the present work there was significant decrease in seminal plasma concentration of CLU in the two groups associated with leukocytospermia. Also, a significant decrease of the total antioxidant capacity of semen in the two groups associated with leukocytospermia was noticed in the current study.

In addition to that, the current work showed that there was highly significant negative correlation between seminal plasma CLU and MDA, which is a byproduct of lipid peroxidation, and which has been used in biochemical assays to monitor the degree of seminal oxidative stress and peroxidative damage in spermatozoa⁽²⁷⁾. Our data come in harmony with the data of **Sharma et al.**⁽²⁸⁾ who identified CLU as one of proteins under regulated in seminal plasma of ROS +ve semen samples in comparison to ROS -ve samples. These data are also in agreement with the work of **Saleh et al.**⁽²⁹⁾ who found statistically significant difference in seminal CLU level between infertile patients with varicocele and controls. They explained the results of lower levels of seminal CLU in men with infertility and varicocele by the presence of oxidative stress interfering with processing, level and function of the mature CLU form⁽²⁸⁾ and the negative correlation between seminal CLU level and DNA fragmentation⁽¹⁸⁾.

Conversely, these results are contradictory with results of **Zalata et al.**⁽³⁰⁾ who used cell phone radiation as in vitro source of oxidative stress and tested the expression of seminal CLU gene and protein concentration under normal conditions, and after 1 hour of exposure to cell phone radiation. Sperm motility, acrosin activity and sperm DNA fragmentation have been also assessed before and after the exposure. Cell phone radiation significantly increased not only the concentration of CLU, but also the expression of the CLU gene. This contradiction may be partially explained by the in vitro nature of the study and the limited exposure time of the semen to oxidative stress.

In the present work a strong significant positive correlation was found between seminal plasma CLU concentration and alpha-glucosidase, which is considered a sensitive indicator of epididymal function

^(31, 32). Not only the alpha-glucosidase but also the acrosin activity index, which is considered as important indicator of the fertilizing capacity of human spermatozoa⁽⁷⁾ and was found to have strong positively significant correlation with seminal plasma CLU concentration.

These results support the important role of seminal plasma CLU as sperm protecting agent and its capability as indicator for conception ability of sperm.

CONCLUSIONS

Clusterin has an important sperm protective role against oxidative stress and seminal plasma clusterin level is significantly reduced in infertile patients with leukocytospermia, which may explain one of the mechanisms of how leukocytospermia interferes with the fertility function.

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Author contribution: Authors contributed equally in the study.

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