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Original Article

Obstetrics and Gynaecology Section

Evaluation of Protein Carbonyl and Vitamin C in Seminal Plasma of Infertile Male: A Hospital-based Study in Bengali Population

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ABSTRACT

Introduction: Male infertility has been coupled with the imbalance between production of Reactive Oxygen Species (ROS) and antioxidant (e.g., vitamin C) level. Elevated concentrations of ROS in the semen can lead to oxidative protein damage as they counter with the amino acids' side chains in the protein, leading to the production of carbonyl groups.

Aim: To assess if there is any difference of seminal plasma Protein Carbonyl (PC) and vitamin C level in male infertile and fertile subjects in the midst of their correlation with other relevant seminal parameters.

Materials and Methods: This was a hospital-based case-control study of a Bengali population. Semen samples of 124 males (group A; 68 infertile males, group B; 56 fertile males) were tested. Seminal fluid analysis was done with Makler counting chamber. PC and vitamin C were measured by Levin's and Roe's photometric methods respectively. To evaluate the differences in the mean ranks of these parameters Mann-Whitney U test was used.

Results: Out of 124 male subjects, 68 infertile (55%) termed as cases and 56 fertile (45%) were termed as controls with mean age of cases (32.81±5.02) and controls (33.29±5.53). Both in group A and group B sperm count was positively correlated with motility and vitamin C but negatively correlated with PC at significant level $p<0.05$. Statistically significant differences of mean ranks of these parameters (sperm count: 52.82 and 74.26, motility: 52.10 Vs 75.13, PC: 76.57 and 45.41, vitamin C:55.99 and 70.40, Mann-Whitney U:1245, 1197, 947 and 1461, respectively) between the two groups were found. Hence, indicate that in infertile subjects the balance between PC and vitamin C is disturbed.

Conclusion: Assessment of oxidative status may serve the clinician in additional management of idiopathic male infertility.

Keywords: Antioxidant, Ascorbic acid, Reactive oxygen species, Sperm count

INTRODUCTION

Infertility is defined as incapability to conceive after one year of unprotected intercourse and it affects 7% of male population and 8-10% of couples [1]. The evaluation of male infertility is frequently underestimated or delayed. A synchronised assessment of the infertile male using standardised measures improves both diagnostic exactness and the results of subsequent management in terms of efficiency, risk and costs [2]. A 30-80% of infertile male produce excessive ROS in their ejaculate. Takeshima T et al., proposed the term "Male Oxidative Stress Infertility (MOSI)" to describe Oxidative Stress (OS) associated male infertility for this strong connection between OS and male infertility [3]. OS is defined as an inequity between levels of ROS and Total Antioxidant Capacity (TAC). If the balance is in favour of ROS generating system compared to ROS scavenging, the resulting condition is known as OS [4]. Elevated concentrations of ROS in the semen can lead to oxidative protein damage, as they react with the amino acids' side chains in the protein, leading to the production of carbonyl groups [4]. The toxic effects of ROS on sperm function can be estimated by lipid peroxidation, protein carbonylation and nuclear Deoxyribonucleic Acid (DNA) damage estimation [4,5].

Stress and decrease in antioxidants was found to play a significant part in reducing the fertilising potential of male infertile subjects [6]. When regulated suitably, ROS functions effectively; however, when uninhibited, they signify key players in male factor infertility. Mechanisms accountable for this comprise oxidative damage of sperm lipid membranes, damage to gamete DNA both by gene mutation and by direct breakdown of the DNA backbone, mitochondrial dysfunction and apoptotic cell death [7]. The spermatozoon is an extremely dedicated cell, whose main function is to carry the intact male genetic substance into the oocyte. During its production and transit all through male and female reproductive tracts, sperm cells are internally and externally bounded by ROS, which are formed from both endogenous and exogenous sources. While low amounts of ROS are recognised to be necessary for vital physiological sperm processes, such as acrosome reaction and sperm-oocyte interaction, high levels of those species cause misbalanced antioxidant: oxidant molecules, generating OS, which is one of the most destructive factors that affect sperm function and lower male fertility potential [8].

Antioxidants are substances which at a low concentration can significantly delay or prevent oxidative damage of an oxidizable substrate. Though weak, there are antioxidants in seminal plasma which protect the sperm against the damaging effects of ROS [9-12].

After doing a tedious literature search, authors found several data related to PC and vitamin C levels of seminal plasma in different groups of population globally. This type of finding may be due to different socio-economic status and ethnicity. Considering this the present study was being proposed involving hospital-based male infertile Bengali population.

MATERIALS AND METHODS

This was a hospital-based, non interventional case-control study conducted in the Department of Physiology and Biochemistry of R G Kar Medical College and Hospital, Kolkata, West Bengal, India. Samples were collected from the Infertility clinic of the same institution. The study was conducted for 18 months (January 2014-June 2015) after getting the clearance from the Institutional Ethics Committee (IEC) (IEC: 17/12/13). The study followed the guidelines of the Helsinki declaration of 2009 in every aspects of the study.

Male infertile subjects who gave their informed written consent voluntarily at the infertility clinic after fulfilling the inclusion criteria were enrolled. Sample size was decided depending upon the previous studies [1,13].

Inclusion criteria: A total of 124 men (group A: 68 infertile and group B: 56 fertile) between the age group of 20-45 years were taken into the study, who had a history of infertility persisting longer than one year.

Exclusion criteria: History of smoking habits. Consumption of alcohol and tobacco chewing. Subjects suffering from diabetes mellitus, hypertension, various infectious diseases including Acquired Immunodeficiency Syndrome (AIDS), cryptorchidism, varicocele. Taking drugs like vitamin C, vitamin E and glutathione supplementation. Semen samples having more than one million leucocytes per mL.

Procedure

Semen samples were collected by masturbation into a sterile, wide mouthed container after 72 hours of sexual abstinence. It was then allowed to liquefy at room temperature approx. 37°C for at least 30-60 minutes. After liquefaction samples were analysed for the following:

A) Physical characters- volume, liquefaction time and pH.

B) Microscopic characters- sperm count (million/mL), motility (%)- progressive/non progressive/non motile, morphology (%)- normal/abnormal.

C) Biochemical characters- measurement of PC by Levine's method [14,15] and Measurement of vitamin C by Roe's photometric method [16].

Measurement of Protein Carbonyl (PC) content: Photometric Method [14,15]-

A 25 µL of seminal plasma were taken in two (control (C)/fertile and test (T)/infertile) tubes. To which 100 µL of 20% of Tri-Chloro Acetic Acid (TCA) solution was added. It was mixed and then centrifuged at 5000 rpm (revolution per minute) for 10 minutes. To the pellets separated after centrifugation in both the tubes 500 µL 2(N) Hydrochloric acid (HCL) and 500 µL of DNPH (Dinitrophenylhydrazine) was added to C and T tube, respectively. They were mixed thoroughly, waited for 15 minutes and this part is repeated for five times. Again, 100 µL of 20% TCA solution were added to the respective C and T tubes. They were mixed and centrifuged to get the pellet again and the supernatants were discarded now. A 500 µL each of 99% ethyl alcohol and ethyl acetate were added to each tube. They were mixed thoroughly and centrifuged, followed by discarding the supernatant. This procedure was repeated thrice. After that 1.5 mL protein dissolving solution was mixed thoroughly with 1 mL micro-pipette and kept for 20 minutes for better dissolution. Subsequently, the absorbence of both the test and control tubes was read at 370 nm in the spectrophotometer with 2(N) HCl as blank. The carbonyl content was calculated depending on the molar extinction coefficient of DNPH (ε=2.2×104 cm M^{-1}) and expressed as nanomoles per mg of protein. Seminal protein estimation was done using Lowry's method [17,18].

Measurement of ascorbic acid (Vitamin C): Photometric method [16,19]- A 0.5 mL of seminal plasma from each samples were added to 2 mL of meta-phosphoric acid (freshly prepared: 6 gm/dL) in a test tube, mixed well and centrifuged (2500 g-10 min). From each tube 1.2 mL of clear supernatant was pipetted into Teflon lined, screw cap test tube. A 1.2 mL of each concentration of working calibrators (prepared daily, stock is 50 mg/dL) were then added to above test tubes (made in duplicate). A 1.2 mL of metaphosphoric acid as was used for this estimation. Next to this 0.4 mL of dinitrophenylhydrazine-thiourea-copper sulfate reagent (5 mL 5 gm/L thiourea and 5 mL 0.6 gm/dL copper sulphate solution mixed with 100 mL of 2,4-dinitrophenylhydrazine solution: 2 gm/L DNPH in 4.5 mol/L sulphuric acid and diluted upto final volume of 500 mL) were added to all tubes. All tubes were then mixed and incubated in a water bath at 37°C for three hours. Then the tubes were removed from water bath and chilled for 10 minutes in an ice bath. While mixing, 2.0 mL of cold sulfuric acid (12 mol/L) were added and mixed in a vortex mixture. Spectrophotometer was adjusted with the blank to read zero absorbance at 520 nm and all the readings were noted. The concentration of every working calibrators were plotted versus absorbance values.

STATISTICAL ANALYSIS

All statistical analysis were done by windows based Statistical Package for the Social Sciences (SPSS) version 16.0 (inc. Chicago Il, USA) and MedCalc version 11.3 softwares. Goodness of fit measure also done by D'Agostino Pearson test to find out the normality of data. Correlation of data within a group was performed by doing Spearman correlation test. Sample's t-test and Mann-Whitney U test was performed appropriately to find out the statistical significance of mean and mean ranks respectively between groups. A p-value of <0.05 (confidence interval=95%) was considered as statistically significant.

RESULTS

Out of 124 male subjects, 68 infertile (55%) termed as cases and 56 fertile (45%) were termed as controls. No statistically significant difference between the age distribution of cases (32.81±5.02) and controls (33.29±5.53) groups (t=0.503, p=0.616) was observed. Hence, all the male subjects being selected for this study were age matched. The other parameters are shown in [Table/Fig-1-3].

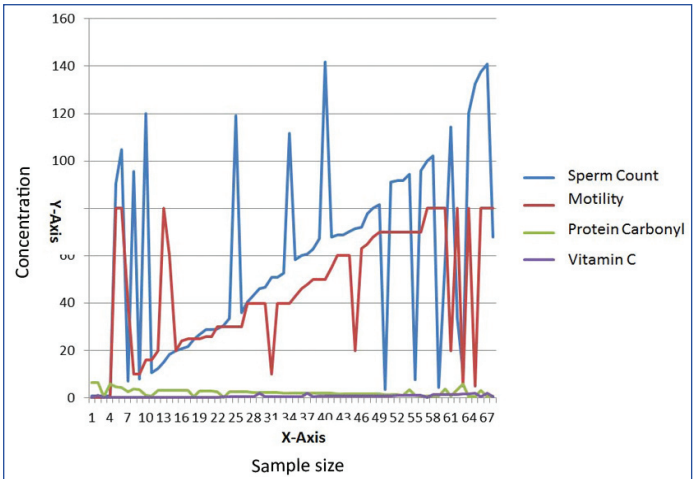
The data were not in the same agreement of the Normality. Hence, the authors have done non parametric analysis to evaluate their statistical significance further.

To find out the non parametric correlation of different parameters with sperm count in cases and controls authors performed Spearman's rho correlation. In both cases and controls group, it was found that sperm count is positively correlated with motility and vitamin C but negatively correlated with PC at significant level [Table/Fig-4].

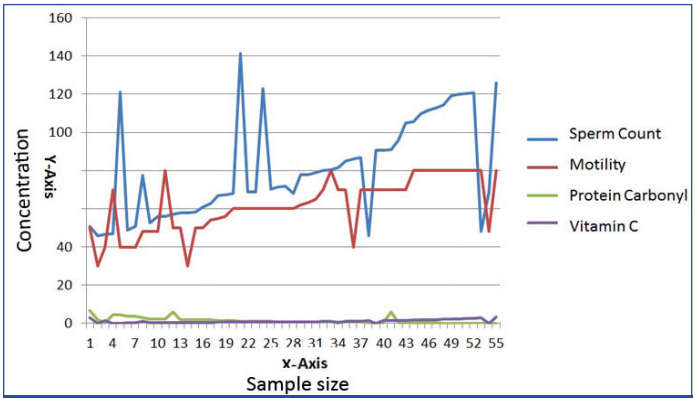
There was significant difference in between cases and controls of sperm count (1245.5, p<0.001), motility (1197, p<0.001), PC (947, p<0.001) and vitamin C (1461.5, p<0.05) [Table/Fig-5].

Parameters	Cases			Controls		
	Mean±SD	Normal distribution (Significance)*	Mean ranks	Mean±SD	Normal distribution (Significance)*	Mean ranks
Sperm Count (SC) (million/mL)	58.21±40.28	0.1738	52.82	80.67±25.61	0.0609	74.26
Motility (MT) (%)	45.16±25.87	0.1766	52.10	62.08±14.23	0.1513	75.13
Protein Carbonyl (PC) (nmol/mg of protein)	2.37±1.39	0.0003	76. 57	1.40±1.59	<0.0001	45.41
Vitamin C (VC) (mg/mL)	0.76±0.51	0.0098	55.99	1.14±0.89	0.0026	70.40

[Table/Fig-1]: Mean±SD, mean ranks and normality of parameters in cases and controls.
*A p-value <0.05 was considered to be statistically significant



[Table/Fig-2]: Showing relation between studied parameters in cases.



[Table/Fig-3]: Showing relation between studied parameters in controls.

Parameters	Cases		Controls	
	rho-value	Significance	rho-value	Significance
Motility	0.428	<0.001	0.566	<0.001
Protein Carbonyl	(-) 0.601	<0.001	(-) 0.652	<0.001
Vitamin C	0.348	<0.001	0.504	<0.001

[Table/Fig-4]: Spearman's correlation of sperm count with other parameters in cases and controls.
A p-value <0.05 was considered as statistically significant

	Sperm count	Motility	Protein carbonyl	Vitamin C
Mann-Whitney U	1245.5	1197	947	1461.5
Significance	<0.001	<0.001	<0.001	<0.025

[Table/Fig-5]: Mann-Whitney U test between groups of different parameters.
A p-value <0.05 was considered as statistically significant

DISCUSSION

Infertility is a worldwide health problem concerning about 15% of couples. More or less half of the infertility cases are connected to male factors. The OS, which refers to an inequity in levels of ROS and antioxidants, is one of the main causes of infertility in men. ROS is necessary in a small concentration for the physiological role of sperm including the capacitation, hyperactivation and acrosomal reaction. Although, high levels of ROS can lead to infertility through not only by lipid peroxidation or DNA damage but inactivation of enzymes and oxidation of proteins in spermatozoa. OS is primarily caused by factors coupled with lifestyle however, immature spermatozoa, inflammatory factors, genetic mutations and altering levels of sex hormones are other major causes of ROS [20].

PC was selected in this study due to its early formation by ROS and stability compared to other biomarkers among oxidatively modified proteins [21] and vitamin C as the antioxidant of choice due to its accurate estimation [22].

In a study conducted by Saraniya A et al., involving subjects for microscopically abnormal (n=26) semen and normal semen (n=24) found significantly higher levels of PC in seminal plasma of abnormal semen [13]. They also found that the percentage of non motile spermatozoa had a significant (p<0.01) positive correlation with PC (r=0.49). Similarly, present study also found that in infertile subjects sperm count was positively correlated with motility (rho=0.428, p<0.001) and negatively correlated with PC (rho=-0.601, p<0.001). The mean±SD of seminal PC in infertile males were 2.37±1.39 nanomol/mg of total protein and in fertile males 1.40±1.59 nanomol/mg of total protein. The intergroup comparison of seminal PC levels have shown that the difference of the mean rank of PC (76.57 Vs 45.41) was statistically significant (p<0.001). Thus, infertile individuals have elevated PC levels.

In a study by Al Smadi MA et al., they found that sperm motility and laboratory Intracytoplasmic Sperm Injection (ICSI) outcomes were affected negatively by elevated concentrations of PC in the semen [4]. Hence, this finding also supports that semen quality is inversely related with the PC concentration as was found by Saraniya A et al., [Table/Fig-6] [13].

Mean±SD of seminal vitamin C in infertile males is 0.768±0.51 mg/mL and in fertile males is 1.1421±0.89 mg/mL in the present study and are positively correlated with sperm count in both of the groups (ρ=0.348 and 0.504 respectively, p<0.001). The intergroup evaluation of seminal vitamin C levels have revealed that the disparity of the mean concentration of vitamin C between both the groups is statistically significant (p<0.001), i.e., the fertile individuals have elevated vitamin C levels. PC is a marker of oxidatively damaged

Studies	Sample size (N)	Findings
Saraniya A et al., (2008) [13]	Microscopically abnormal semen (n=26) and normal semen (n=24)	1. Significantly higher levels of Protein Carbonyl (PC) in seminal plasma of abnormal semen. 2. The percentage of non motile spermatozoa has a significant (p<0.01) positive correlation with protein carbonyl (r=0.49).
Al Smadi MA et al., (2021) [4]	A total of 150 couples from the laboratory Intracytoplasmic Sperm Injection (ICSI) cycle	Sperm motility and laboratory ICSI outcomes are affected negatively by higher concentrations of protein carbonyl in the semen.
The present study	Semen samples of 124 males (68 infertile males and 56 fertile males)	1. In infertile subjects, sperm count is positively correlated with motility (ρ=0.428, p<0.001) and negatively correlated with protein carbonyl (ρ=-0.601, p<0.001). The Mean±SD of seminal protein carbonyl in infertile males were 2.37±1.39 nanomol/mg of total protein and in fertile males 1.40±1.59 nanomol/mg of total protein. 2. The intergroup comparison of seminal protein carbonyl levels have shown that the difference of the mean rank of protein carbonyl (76.57 Vs 45.41) was statistically significant (p<0.001). 3. Mean±SD of seminal vitamin C in infertile males is 0.768±0.51 mg/mL and in fertile males is 1.1421±0.89 mg/mL and are positively correlated with sperm count in both of the groups (ρ=0.348 and 0.504 respectively, p<0.001). 4. The intergroup comparison of seminal vitamin C levels have shown that the difference of the mean concentration of vitamin C between both the groups was statistically significant (p<0.001), i.e., the fertile individuals have elevated vitamin C levels. 5. Protein carbonyl showed a negative correlation (ρ=-0.601, p<0.001 in cases and in controls ρ=-0.652, p<0.001) with sperm count.

[Table/Fig-6]: Comparison table between relevant studies with the present study.

protein and this damage might harm their role in maintaining the motility of spermatozoa and affect fertilisation. Cytotoxic PC may be the reason of poor motility and low sperm count. PC showed a negative correlation (ρ=-0.601, p<0.001 in cases and ρ=-0.652, p<0.001 in controls) with sperm count. This means in infertile subjects the balance between PC (indicator of oxidative damage) and vitamin C (antioxidant) is disturbed. The fall in vitamin C in infertile semen and its correlation with PC point towards that the rise in vitamin C might be a defensive response of body to abate the effect of ROS. In fact, vitamin C is a water soluble ROS scavenger with high potency and found in concentrations 10-fold in seminal plasma than serum [23], protecting human spermatozoa against endogenous oxidative damage by neutralising hydroxyl, superoxide and hydrogen peroxide radicals and preventing sperm agglutination [24]. Considerably reduced concentrations are seen in semen samples with excess ROS [9] these observations are shown in the present study.

By this study, the authors want to state that in cases where all the seminal parameters were normal but still the subject remains infertile (idiopathic male infertility) comes under the purview of functional assay. Amongst this estimation of OS status, pro-oxidants and antioxidant level is crucial. Though chemiluminescence assay is presently under discussion but, this study is more relevant in for the developing world as it is cost friendly with limited infrastructure. Last but not the least, it throws some light over individual oxidants in comparison to composite ROS-TAC score. Further studies will show the clinical importance of the findings of this present work in a resource poor set-up to a clinician.

Limitation(s)

After initiation of therapy, a more detailed follow-up was beyond the scope of the present study. A prospective study with more planning and greater control over confounding variables is needed to generate stronger evidences. Modern techniques may be applied.

CONCLUSION(S)

Antioxidant regime of vitamin C can be a helpful therapeutic approach to overcome the problem of OS associated with male infertility due to lack of antioxidants and its protective role in semen.

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