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Research Article

Effect of seminal or plasma antioxidant levels on seminal parameters in selected sub-fertile male

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Abstract: Antioxidants play a significant role in neutralizing oxidative stress, which could cause impairments to the spermatozoa. Various studies have pointed to various conclusions about the effect of antioxidants on seminal quality. Thus, this cross-sectional study was set up to analyze the relationship between the antioxidants and seminal parameters in Sri Lanka from August 2014 to April 2016. The selected sub-fertile population was investigated to gather demographic data and food habits. Eventually, blood and seminal sample (after three days of abstinent from ejaculation) were collected from them. The blood and semen of each subject were analyzed for total antioxidant concentrations, and the semen was further undergone for the seminal analysis. Normal healthy males were selected for the control group. The average total antioxidant levels of the semen (892.49 ± 187.49) and plasma (808.39 ± 423.88) of the test group ($n = 75$) were compared with the same of the control group (semen; 929.58 ± 190.70, plasma; 878.73 ± 426.28) separately and found to have no statistically significant difference in each ($p > 0.05$). Spearman correlation and linear regression analysis were carried out to determine the relationship between plasma and semen antioxidant levels with each seminal parameter of the test group. It was evident that no statistically significant relationship was found in either test for tested variables. Thus, the quality of semen is independent of the seminal or blood level of antioxidants in the experimental group. This could be due to a sufficient level of antioxidants in the body fluids of the study group.

Keywords: Antioxidant, Oxidative stress, Semen parameters, Semen quality, Body fluids.

1. Introduction

On the new findings, the contribution of the male party to infertility is considerable, and as a percentage, it is around 30 - 40% (Thonneau et al., 1998). Male factor infertility could be due to various biological and biochemical factors, most of which are idiopathic. Identification of those is vital to controlling male infertility—free radicals, which develop the oxidative stress in an individual, one of such causes. Free radicals are elements or compounds with unpaired electrons; thus, they are highly reactive in action. Free radicals always try to attack other molecules to get electrons to stabilize them (Horward, 2014). This could lead to chain reactions where the undergone molecules become free radicals and get damaged. So, the free radicals are liable to attack macro-molecules such as proteins, enzymes, DNA, carbohydrates, lipids, and other molecules. Damaged DNA, as well as dammed molecules of cellular membranes, ultimately could carry the cell-specific impairments. Thus, free radical attack on spermatozoa may lead to infertility.

An antioxidant system which includes Vitamins (A, C, E), certain antioxidant enzymes, and minerals (zinc, selenium) may control the level of free radicals in body fluid up to a certain extent to control the level of oxidative stress (oxidation caused by an excessive level of free radicals). In several studies, it has been pointed out that the antioxidants, which can control the extra level of free radical (oxidative stress), have a favourable positive effect on seminal parameters hence the quality of semen. However, the level of antioxidants in body fluid may depend on the individual's food habits, environmental exposures (Mathies, 2014), and other metabolic factors. The finding of the relationship between antioxidant levels in body fluid (semen, plasma) and seminal parameters such as semen volume, count, motility, and morphology helps control male infertility indirectly. The study was set up to check the effect of antioxidants on seminal parameters, hence the quality of semen.

2. Methodology

Procedures of the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

2.1. Design and Method

This study was a prospective cross-sectional study. The sample size (n) was determined according to the follow-

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ing equation (Levin, 2006; Patra, 2012):

$$n = \frac{4Z_{\alpha}^{2}P(1-P)}{D^{2}} \quad (1)$$

where Z_{α} is the standard normal deviate, P is the prevalence of male infertility and D is the total width of confidence. In this study, P=8% (Khani et al., 2013) and D=0.125; thus, the sample size at a 95% confidence level was calculated as n=72.

The control group: The age matching and number equal (as much as possible) healthy men were selected for the control group. These individuals had normal semen profiles.

The male partners of infertile couples who visited the fertility clinic of an institute in Sri Lanka between August 2014 and April 2016 were involved in the study. The individuals who wished to participate in the study were evaluated on exclusive and inclusive criteria on their consent.

Inclusive criteria: Male persons, over 18 years old.

Exclusive criteria:

1. Male persons who were suffering from diseases such as hypertension, diabetes, arthritis cancer, during the period.
2. Male persons who were on drugs relevant to the above disease conditions.
3. Male persons who had been addicted to recreational drugs (e.g. marijuana, abin and ganja.
4. Male persons who had been on anti-gastric drugs such as cimetidine or any steroidal drugs.
5. Male persons who were with pathological diseases in the reproductive system (varicocele, testicular problems).
6. Male persons who were dumb, deaf and mentally disabled (as they are unable to communicate)
7. Male persons who were on fertility treatment during the time

The subjects who were satisfactory according to the criteria were selected randomly on a lottery-based method for the study and gathered demographic data (age, residence) and lifestyle data (food habits, exposures). The interview was followed by the collection of blood from the subject under aseptic methods by a trained technician. Eventually, the subject was given a clean, dry wide-mouth glass container with advice to collect the semen by ejaculation (after three days of abstinence). The body fluids were considered as infective and universal precautions were followed when handling and analyzing.

Analysis of volume of the semen: After the liquefaction was reached, the semen volume was measured with 10 ml of a measuring cylinder (Engbaek et al., 2003).

Analysis of sperm count :Using a Sahli pipette well mixed, liquified semen sample was drawn up to the 0.5 microliter mark. Then, the semen diluting fluid was drawn up to the 11 microliter mark, and interior content was mixed well by keeping the pipette on a working rotator. The mixture was loaded into the Improved Neubauer counting chamber and allowed the sperm to settle in it. Eventually, the number of sperms in four corner squares was counted.

$$\text{Number of sperm}/ml = \frac{n_s \times 10 \times 20 \times 1000}{4} \quad (2)$$

where n_s is the number of sperm counted in all four corner squares.

Analysis of sperm motility: A clean glass slide was taken, and a drop of liquefied semen (10 μl) was placed on it. A cover slip was placed on the specimen and rimed the edge with petroleum jelly to prevent evaporation. By observing the several fields of the specimen through the high power of a microscope ($\times 40$), the proportion of motile to non-motile sperms was obtained. With the finding, the percentage of motile sperm was calculated.

Analysis of sperm morphology: A clean glass slide was taken, and a drop of liquefied semen (10 μl) was introduced to make a thin smear. The smear was air-dried. Then, the dried smear was washed thoroughly with semen diluting fluid to remove the mucous. The smear was covered for around 8 minutes with the diluted Leishman stain. The stain was prepared by mixing 10 ml of stain and 20 ml of distilled water. After that, the stain was washed thoroughly well with buffered distilled water. Finally, the slide with stained smear was dried. The slide was observed for morphology under a high power field, and the normal to abnormal spermatozoa ratio was observed in different microscopic fields to have the final average percentage of normal spermatozoa.

Analysis of plasma total antioxidant level: The ferric reducing ability of plasma (FRAP assay) was selected as the assay to measure the total antioxidant capacity of the blood as well as seminal plasma of the subjects. This was because the assay was reproducible, inexpensive, and regents were simple to prepare, straightforward and speedy (Benzie and Strain, 1996).

Preparation of final test solution: Following reagents were prepared freshly (Benzie and Strain, 1996).

(A) 1 liter of 300 mM acetate buffer solution.

(B) 5 ml of 2, 4, 6 - tripyridyl -5- triazine (TPTZ) solution.

(C) Ferric chloride solution (mol).

(D) Preparation of standard solution (1 mol of ascorbic acid).

Table 1: The way of mixing each reagent and samples

Solution	Test	Standard	Blank
Plasma/semen	100 μl	–	–
Ascorbic acid solution	–	100 μl	–
Freshly prepared FRAP reagents	3000 μl	3000 μl	3000 μl

Table 2: The average total antioxidant values of the individuals in each group of normal and abnormal seminal parameters

Type of body fluid	Control group (n = 75) (µmol/l)	Test group (n = 75) (µmol/l)	p-value (Wilcoxon signed rank test)
Plasma	878.73 ± 426.28	808.39 ± 423.88	$p > 0.05$
Semen	929.58 ± 190.70	892.49 ± 187.49	$p > 0.05$

The first three reagents were mixed in a beaker according to the ratio (A: B: C = 10:1:1) to have the final working solution for the assay. The final reagent was incubated at 37 ^oC for ten minutes before use.

Ascorbic acid (vitamin C), 1.76 g, was measured and dissolved with distilled water in 10 ml of the volumetric flask to obtain the final 10 ml solution.

Measurement of semen/plasma antioxidant level: Standard and blank samples were prepared by mixing the solution and plasma/semen as in Table 1.

All were mixed well separately in a vortex mixture, and absorption of each was obtained at room temperature. Then, the samples were kept at 37 ^oC for four minutes, and the second absorption was measured separately. The antioxidant level was obtained finally according to the following equation.

$$\text{Antioxidant level} = \frac{2 \times D_{ts}}{D_{ss}} \tag{3}$$

where D_{ts} is the difference of the absorption of test sample and D_{ss} is the difference of the absorption of standard sample.

Quality control of reagent: A quality control sample of known antioxidant concentration was run 15 times during the test procedure, and a Leavey Jenning chart was drawn. The data was scattered between the mean 2 Standard Deviation margins of the chart; further, the values' coefficient variance was less than 5%. Thus, it is clear that the accuracy of the test procedure was up to the level.

Limitations of the procedure: The quality of chemicals, the reaction time, and the level of mixing may affect the outcome.

2.2. Data processing and statistical methods

Results were subjected to a normality test, indicating a non-normal distribution. Thus, a non-parametric test was used in the analysis of the results. Two groups, such as test (sub-fertile males) and control (normal healthy males), were selected, and average antioxidant levels of body fluids (semen and plasma) of each group were compared comparatively under Wilcoxon signed-rank test. Further, the antioxidants level of each fluid were run against the respective seminal parameters (volume, count, motility, morphology) to find out the relationship of antioxidants in each fluid with their respective seminal parameters. We used IBM SPSS 20 to carry out the analysis.

3. Results and Discussions

The measured antioxidant level of plasma and semen of both groups (control and test) were summarised in Table 2.

The normospermic group (control) of the study was distinctive in every aspect from the abnormospermic group (test) as there were significantly higher seminal parameter values ($p < 0.05$) compared to the abnormospermic group.

There is no cut-off value generally for the antioxidant level of normal healthy human beings. However, the average plasma antioxidant value of healthy Sri Lankans with normal seminal parameters was 878.73 ($\pm$ 426.28) μmol/l, which was slightly lower than that of the healthy Chinese men (1017 $\pm$ 206 μmol/l) (Benzie and Strain, 1996). Both analyses were carried out with a FRAP assay. This difference in values could be due to food habits, genetic factors, environmental factors, and lifestyle factors, which are dependent on race.

However, the blood and seminal plasma of the normospermic group of the present study had a higher level of antioxidant capacity than the abnormospermic group. The difference of each fluid in the respective groups was compared statistically ($p = 0.07$, $p = 0.09$, respectively) under the Wilcoxon Signed Rank Test and found to have statistically insignificant ($p > 0.05$). Additionally, the semen of both groups had a higher level of antioxidants than that of plasma. As the difference was statistically insignificant, it cannot be expressed that it had happened causatively.

Table 3: Spearman correlation analysis between plasma antioxidant level and seminal parameters

Variable	Correlation coefficient	Significance
Plasma antioxidant level, semen volume	-0.192	p >0.05
Plasma antioxidant level, sperm count	-0.240	p >0.05
Plasma antioxidant level, sperm motility	-0.327	p >0.05
Plasma antioxidant level, sperm morphology	-0.290	p >0.05

Table 4: Spearman correlation analysis between seminal antioxidant level and seminal parameters

Variable	Correlation coefficient	Significance
Semen antioxidant level, semen volume	0.136	p >0.05
Semen antioxidant level, sperm count	0.031	p >0.05
Semen antioxidant level, sperm motility	-0.018	p >0.05
semen antioxidant level, sperm morphology	0.041	p >0.05

Table 5: Linear regression analysis between plasma antioxidant level and seminal parameters

Variable	R-value	R square
Plasma antioxidant level, semen volume	0.063	0.004
Plasma antioxidant level, sperm count	0.209	0.044
Plasma antioxidant level, sperm motility	0.189	0.036
Plasma antioxidant level, sperm morphology	0.078	0.006

Table 6: Linear regression analysis between semen antioxidant level and seminal parameters

Variable	R-value	R square
Semen antioxidant level, semen volume	0.125	0.016
Semen antioxidant level, sperm count	0.009	0.001
Semen antioxidant level, sperm motility	0.125	0.016
Semen antioxidant level, sperm morphology	0.047	0.002

Table 7: The average total antioxidant capacities of the individuals in the normospermic and oligoasthenoteratozoospermic groups

Type of body fluid	Average total antioxidant capacity (μmol/l)		p-value
	Group with normospermic parameters (n = 75)	Group with oligoasthenozoospermic individuals (n = 13)	(Wilcoxon signed-rank test)
Plasma	878.73 $\pm$ 426.28	632.27 $\pm$ 530.87	p > 0.05
Semen	929.58 $\pm$ 190.70	820.80 $\pm$ 319.09	p > 0.05

Spearman correlation test: The test was run to determine the strength of the relationship between each antioxidant level of plasma and semen with the seminal parameters, respectively, and found no significant relationship (refer Table 3 and Table 4).

Linear regression analysis: The relationship of each semen parameter was investigated against the concentration of antioxidant level of plasma and semen, and neither semen nor plasma antioxidant correlated with particular seminal parameters (refer Table 5 and Table 6).

However, under the Spearman correlation test, an interesting relationship was seen between the level of seminal pus cells (over 10 per high power field) and the level of seminal antioxidant for the whole study population. Further, a moderate positive correlation was also found between the two according to the linear regression analysis, ($r = 0.42$, $r^2 = 0.176$, $p < 0.05$).

Thus, as mentioned previously, the free radicals generated by higher-level pus cells might stimulate all the sources (accessory glands of the reproductive system) to secrete antioxidants toward the semen. However, because the difference of antioxidant level between the semen and plasma was insignificant, the mentioned conclusion would be abolished.

Out of the total abnormospermic individuals (test), the most important and the second highest group was the oligoasthenozoospermic group (individuals whose three important seminal parameters are count, motility, morphology have been affected simultaneously). Additionally, the antioxidant level of the individuals in the group was compared with the normospermic group for both

body fluids and found to have no significant difference (refer Table 7).

The average values of the total antioxidant capacities of the two body fluids (semen and blood) of oligoasthenozoospermic group were lesser than that of the normospermic group (control) and were statistically insignificant ($p > 0.05$). Meanwhile, the seminal plasma of both groups possessed a higher antioxidant capacity than that of the blood. The gap was highest in the oligoasthenozoospermic group (188 mol/l) and was statistically insignificant ($p > 0.05$).

Moreover, the concept of having a high antioxidant level in semen more than the other body fluids could be an added advantage for the semen to be protected from free radical attacks mentioned previously. This concept becomes prominent further with the high seminal antioxidant value of the oligoasthenoteratozoospermic group. This massive increase could be due to the group finding individuals with three defective seminal parameters. It is obvious that the morphologically defective cells are more prone to generate free radicals it selves and to attract neutrophils (white blood cells) toward the premise. It has been known that neutrophils are good sources of free radicals. Thus, in the particular group, the relative level of higher free radicals might stimulate the body to concentrate antioxidants toward semen to minimize the free radical effect on semen.

However, in particular studies, a relationship was found between the mentioned variables. A study (Das et al., 2009) it had found a significantly low level of plasma ascorbic acid (an antioxidant) in an Indian oligoasthenoteratozoospermic group ($n = 34$) compared to the control ($n = 48$). In an Indian study (Kothari and Chaudhari, 2016), it was found that the seminal zinc value (an antioxidant) in the oligoasthenoteratozoospermic group ($n = 24$) had been lesser than that of the control. In addition, a positive correlation was seen between the zinc and semen parameters such as sperm motility, sperm concentration, and sperm morphology. Fazeli et al. (2016) also concluded that the antioxidant capacity was significantly low in abnormospermic individuals ($n = 35$). There was a positive correlation between the seminal antioxidant and sperm motility and morphology.

Moreover, even in the two previous Sri Lankan studies, it had been found that the occurrence of antioxidants was lesser in the males with sub-fertility and fertility issues due to unknown aetiology (Amarasekar et al., 2014). However, the outcome was true in the present study. In the study carried out by Dissanyaka et al. (2010), it was found that the level of Zinc in semen had a positive relationship with sperm count but a negative relationship with their viability. However, the study's objective differed from the present one, which was not on Zinc.

Under all these outcomes, it was clear that the level of seminal antioxidants was significantly low in men with abnormal semen qualities. There was a positive relationship between the seminal parameters and seminal antioxidants, which was not seen in the present study.

However, in the studies mentioned above, the gap of antioxidants in the abnormospermic and normospermic groups might have been higher than that of the present study, which was less such as 70 mol/l and 37 mol/l for the plasma and serum, respectively. Due to the low gap, it could be unable to show a significant difference between the two groups.

Thus, it implies that all individuals in the current Sri Lankan study have sufficient levels of antioxidants in both groups, which can neutralize the effect of reactive oxygen species (free radicals).

The mean plasma antioxidant level of all subjects of the present study was 853.47 ($\pm$ 421.19) mol/l, and everyone was within the average $\pm$ 2SD range. The same result was found regarding the semen antioxidant level; the mean value was 905.50 ($\pm$ 187.55) mol/l. Further, an antioxidant such as vitamins A, C, and E, minerals (selenium, Zinc), and specific proteins directly depend on the nutritional intake. In the current study, the individuals' nutrition was satisfactory under both plasma albumin and mid-arm circumference. So, it is evident that the concentration of antioxidants in the body fluid of present individuals is good enough.

However, a similar outcome had also been found in certain foreign studies. In a study carried out with an oligoasthenoteratozoospermic group ($n = 5$), it was disclosed that though the antioxidant level of the group was lesser than the control ($n = 20$), the figure of the reduction was not significant, which was similar to the outcome of the present Sri Lankan study.

Further, Eroglu et al. (2014) had disclosed in their study ($n = 44$) that there was no significant difference in serum antioxidant levels between normal and abnormal seminal groups. Further, the study showed that the seminal parameters were independent of the serum antioxidant level.

4. Conclusion

In the study's results, no significant effect was found from seminal and plasma antioxidants on seminal parameters. This could be due to the adequate level of antioxidants in the body fluid of the individuals. Anyway, it is better to repeat the study in various populations and categories (e.g. ones with high exposures to environmental pollution, ones with high exposure to mobile rays, and subjects with low intake of fruit and vegetables) to check the effect further.

5. Limitations

This study was limited due to individuals' reluctance to participate and disclose valid details in this type of study. This can affect achieving a higher sample size and data validity. Further, instrumental/technical errors, the lack

of previous research and time constraints were the limitations of this study.

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