Short Communication

A Correlative Study of Antioxidants Present in Human Seminal Plasma with Sperm Count

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Abstract

Background: The production of reactive oxygen species (ROS) in human semen is a normal process, but the overproduction may lead to determined effect on human sperm. Further in recent times, over production of ROS and less production of antioxidants correlated to male infertility.

Aim and objective: To evaluate various antioxidants in human seminal plasma and correlates to the number of sperms present.

Methods:

Inclusion criteria: Males leading normal life and without conception for one year without any contraceptive with regular sexual intercourse are included in this study.

Exclusion criteria: Males with sexually transmitted infections, cardiac diseases, and so on were excluded from this study. Semen collection: Samples were collected from Andrology department and processed for semen analysis as per WHO, 2010 protocols. 116 semen samples were collected, semen categorized into different groups as per WHO, 2010. Then for this study, semen samples were grouped based on sperm count in millions per ml, 0-20 (n=20), 20-40 (n=18), 40-60 (n=12), 60-80 (n=18), 80-100 (n=32), >100 (n=16).

Statistical analysis: Antioxidant activity for SOD, catalase and glutathione were evaluated and correlated with sperm count by using statistical software graphpad prism version 9.1. P value calculated and represented as P<0.001 (***Significance), P<0.01 (**Significance), P<0.05 (*Significance) and P>0.5 (Not significance).

Results: The antioxidant activity was found to be increased as increase in sperm count. Sperm count between 0-20 millions/ml was found to be not significance with respect to all the antioxidant activity measured or evaluated. Sperm count with above 100 millions/ml was found to be with three star level significance (P<0.001) with respect to SOD, catalase and glutathione.

Conclusion: The sperm is more susceptible to ROS, because it is rich in polyunsaturated fatty acids, so it is hypothesized that the antioxidant mechanism is working good, the number of live sperm could be more and it helps in proper fertilization.

INTRODUCTION

Oxidative stress (OS) at the cellular level turns out to be apparent, when the oxidants present in the semen or spermatozoa devastate the overall antioxidant defence arrangement in spermatozoa. The remaining oxidants play a major role in unspecific chemical reactions with in close proximity of cells in particular with DNA, protein and some lipids especially unsaturated. Oxidative stress to spermatozoa may occur due to the presence of enzymatic and non-specific enzymatic antioxidant defence molecules present in seminal plasma. Oxidative stress leads to many major diseases, which remains untreatable by any research, particularly cancer, diabetes, heart diseases, male and female infertility. It has been reported that spermatozoa were vulnerable to oxidative stress by many researchers. Authors established the spermatozoa oxidative damage and loss of motility when the semen sample was preserved with the oxygen rich atmosphere. When the same sample was preserved with the antioxidant catalase, there was able to recover the motility of spermatozoa, this was the first hypothesis proved, later many researches has been carried out to establish further in the antioxidant capacity of spermatozoa in both preservation and insemination steps.

There exists a pathophysiological consequence in the nature of various type of male infertility, when the reactive oxygen species

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(ROS) present in semen has been recognized as significant [1]. Peroxidation of many unsaturated fatty acids occurs due to the oxidants i.e. free radicals interference in the seminal plasma and it plays negative role in normal functioning of the sperm especially in the sperm plasma membrane [2]. This unsaturated fatty acids having the capacity to engender the ROS into the seminal plasma and crating the oxidative stress, which cannot be solved by the sperm on its own, if antioxidant defensive system not working well. The chemical and structural modifications in the sperm nuclear DNA, and the modifications in the lipid, protein structure and functions were mainly due to increase in the content of the free radicals in seminal plasma [3]. Many antioxidant defensive mechanism were available for spermatozoa, which is due to presence of catalase (CAT), glutathione (GSH), and superoxide dismutase (SOD) in human seminal plasma, helps in protecting the spermatozoa from cellular oxidative damage and stress related damage [4].

Prominent ROS level were detected in 20-45% of male infertile patients, and in case of spinal cord injured male patients, 98% of samples showed elevated levels of ROS [5]. The main cellular source of ROS in the semen is immature sperm cells and white blood cells. The increase in the number of leukocytes may be due to infection and inflammation, but can also be secondary for harmful environmental factors, long sexual abstinence, or varicocele [6]. The mechanism of loss of sperm function by ROS appears to be multifold. ROS may affect the quality and number of spermatozoa reaching the ovum in female reproductive tract. Increased ROS in reproductive system can decrease the effective concentration of essential antioxidants, increasing the harmful effects on spermatozoa that are associated with abnormal sperm parameters [7]. In the ejaculated volume of semen, there exists some 50000 leukocytes on an average and leukocytes are having the capacity to induce and activate the free radicals the so called ROS. The level of antioxidants present in human seminal plasma varies from infertile, fertile and patients with varicocle [8]. Even, some researchers argued that the trace amount of free radicals generated initially by the respiratory mechanism is always necessary for the normal functioning of the sperm includes capacitation, acrosomal reaction, and ejaculation [9].

The role of antioxidants was studied in epididyamal fluid where the sperm is already mature enough for capacitation and acrosome reaction. Authors studied about various antioxidants and its activity and correlates with the sperm quality [10]. Epididymis also an important organ which generates the antioxidant defence mechanism molecules [11,12]. GPX epididymal mRNA were also detected in the epididymis in a trace amount, which acts as an antioxidative protective mechanism [13]. Epididymis provides a best possible atmosphere for the sperm storage and later on the maturation. During this process the role of the epididymis in protecting the spermatozoa from the oxidative stress were not clearly studied so far [11]. Even spermatozoa itself is able to produce and protect themselves from the free radicals, by its own thiol groups, uric acid and α tocopherol. Epididymis posses many site specific antioxidants that helps in protecting the spermatozoa from the oxidative stress [11]. In this research, the major objective is to find the correlation of antioxidants/antioxidative enzymes present in human seminal plasma with the increase in sperm count and not with the category of semen samples.

MATERIALS AND METHODS

Sample collection and categorization

For this experiment, 116 samples were collected from the outpatients who attended the Andrology lab, The Milann Fertility center, for semen analysis. For comparing the semen quality with seminal antioxidant markers, the samples collected were categorized by the number (sperm count in millions/ml). The different categories by evaluating the sperm count were cut off with 0 to >100 millions/ml, 0-20 (n=20), 20-40 (n=18), 40-60 (n=12), 60-80 (n=18), 80-100 (n=32), >100 (n=16).

Research ethics

Ethical approval and clearance to work on human semen samples was obtained from VIT Human Ethical Committee (Ref. No. VIT/UHEC-3/NO.11).

All the semen samples were subjected to centrifugation at 1500 rpm and -4° C for 10 min. The spermatozoa free seminal plasma was lay up in a fresh tube and preserved in -22° C until further assay of all antioxidant markers.

Statistical analysis

All the statistical analysis for this research was done by using graph pad prism version 9.01. Grouping semen samples, mean and standard error of mean were calculated for each group by this software. Grouping of sperm count in millions per ml was also evaluated by using this software. The graph was plotted by using mean and standard error of mean for each antioxidant activity against sperm count in millions per ml. P value for the correlation between sperm count and various antioxidant activities was evaluated by graph pad prism. P<0.001 (***Significance), P<0.01 (**Significance), P<0.05 (*Significance) and P>0.5 (Not significance).

Superoxide dismutase (SOD) assays (Marklund and Marklund, 1974)

SOD assay was carried out with the protocol Marklund and Marklund, 1974 [14]. The principle of this assay is based on the capability of the SOD to inhibit the auto-oxidant of the standard pyrogallol. 200 µl of seminal plasma sample was mixed with 3 ml of phosphate buffer (0.25 M with pH 8.4). The standard pyrogallol was purchased and 9 mM was prepared for this sensitive assay. With the 200 μl of sample, 100 μl of pyrogallol was added and was made miscellaneous. Instantaneously, the absorbance for all these solution mixture was taken (recorded) for 5 min at the wavelength of 450 nm. For the standard curve, auto-oxidation of pyrogallol was deliberated by adding 3 ml of phosphate buffer (pH 8.4) and 100 µl of standard pyrogallol. The readings were documented for 3 min at 450 nm. The early absorbance (A_1) at Zeroth min and the absolute (final) absorbance (A_2) at third min were deliberated for both the standard and the samples. The activity of SOD was deliberated by using the calibration curve (percentage of inhibition of each standard against log₁₀ Ultimately, the activity of SOD was measured and expressed as U/ml.

Catalase assay (Aebi, 1984)

Catalse assay was conceded with the standardized protocol Aebi, 1984 [15], with slight adaption according to our lab conditions (which was standardized with almost 10 test samples). 2 ml of 110 mM phosphate buffer (pH 7.0) was prepared only just each time previous to starting the assay. 1 ml of H_2O_2 (50 mm) was taken and mixed well with the phosphate buffer. 50 µl of seminal plasma was mixed with the solution and stirred it well. The decomposition of H_2O_2 was pursued frankly by the decline in extinction at 240 nm which was recorded after every 50 s for 5 min. The divergence in disappearance per unit time was quantified as the catalase activity. Catalase activity (one unit) was defined as the amount of catalase obligatory to decompose 1 M H_2O_2 per 1 min. Finally, catalase activity was expressed in specific activity, U/ml.

Glutathione assay

Glutathione assay was conceded with the standardized protocol Aebi, 1984, [15]. Volume of 50 μ l of trichloro-acetic acid (22%) was very well mixed with 5 ml of phosphate buffer (0.5 M). 1000 μ l of DTNB (5-5 dithio bis 2-nitrobenzoic acid, 0.1% w/v) was added and made miscellaneous. 200 μ l of plasma (seminal) was further added to the tube. A chain of known concentrations of glutathione were prepared and the standard curve was developed at 420 nm with ELISA plate. Finally, the blank was prepared by adding TCA, DTNB, and phosphate buffer and made prepared. The readings were recorded at 420 nm for all the test as well as standard samples.

RESULTS

For this study, 116 semen samples were collected and used. The samples were than analyzed for the preparation of semen analysis report as per World Health Organization, WHO, 2010 protocols [16]. The major semen parameters analyzed were sperm concentration, sperm total motility in %, number of rapid progressive motile sperms present in % and the number of morphologically normal sperms present in the ejaculate in %. All the semen parameters were analyzed and for all the samples which collected on the day.

The samples were categorized and the semen parameters were tabulated for each category in Table 1. After the categorization of samples, the remaining samples were aliquot into two vials and stored until for the further processing. The samples were then transferred to our laboratory and processed immediately for the antioxidant assays.

Initially, the samples were grouped based on the sperm count in to different categories by evaluating the sperm count based on cut off with 0 to >100 millions/ml, 0-20 (n=20), 20-40 (n=18), 40-60 (n=12), 60-80 (n=18), 80-100 (n=32), >100 (n=16). After segregation of group, the samples were processed for centrifugation to acquire seminal plasma.

The activity of various categories of samples based on the sperm count from 0 to > 100 millions/ml was tabulated (Table 2). Sperm count between 0-20 millions/ml was found to be not significance with respect to all the antioxidant activity measured or evaluated. Sperm count with above 100 millions/ml was found

to be with three star level significance (P<0.001) with respect to SOD, catalase and glutathione. The activity was found to be increasing with increase in sperm count for all the antioxidant markers as shown (2).

The major antioxidants present in human seminal plasma like SOD and catalase were compared (Figure 1). The antioxidant activity was represented as Units/ml and then the activity of these antioxidants was compared with each other with increase in sperm count. The activity of SOD was slightly increasing from 20 millions/ml to 60 millions/ml. Later the activity of SOD was suddenly showing an increasing trend from 60 millions/ml of sperm count to > 100 millions/ml. This shows that the activity of the antioxidant marker SOD was increasing with increase in sperm count, but after 80 millions/ml the activity was very much increasing. The catalase activity was also increasing with increase in sperm count form 1 to >100 millions/ml of sperm count. The activity of catalase was seems to be stationary between 60 to 100 millions/ml of sperm count. Later the activity of catalase was found to very much increased after >100 millions/ml (Figure 1).

The trend shows an increasing manner for antioxidant marker GSH from 0-20 millions/ml to >100 millions/ml, between 100 to >100 millions/ml of sperm count, the antioxidant activity of GSH was suddenly increasing in a great manner as shown in Figure 2.

DISCUSSIONS

Antonio Patricio and his researchers studied about the relation between seminal quality and oxidative balance in sperm cells in 2016. According to his results, lipid peroxidation leads to a reduction in sperm concentration; antioxidant proteins protect the spermatozoa against protein oxidation and contribute to an increased sperm motility and normal semen viscosity. Thus, evaluation of oxidative parameters may be a useful tool for male infertility diagnosis and follow-up of antioxidant treatments [17]. Reactive oxygen species (ROS) were found to be the



Figure 1 The curve shows the comparison of the two major seminal antioxidant markers SOD and catalase in human seminal plasma. The trend shows an increasing manner for both the antioxidant markers SOD and catalase from 0-20 millions/ml to >100 millions/ml. SOD activity was suddenly increasing in a great manner for the sperm count with >100 millions/ml, whereas the catalase activity was gradually increasing from low to high sperm count groups. The catalase activity was found to be in stationary between 60-100 millions/ml of sperm count. All the values were represented with mean ± SEM.

Table 1: Comparison of semen parameter analysis for various categories.								
Semen category	Volume (ml)	рН	Sperm concentration (millions/ml)	Total motility (%)	Rapid progressive motility (%)	Normal morphology (%)		
Oligoasthenospermia (N=20)	3.5±0.5	7.8±0.0	3.2±0.3	6.2±1.5	1.9±0.7	15.3±1.6		
Asthenospermia (N=32)	3.6±0.6	7.7±0.0	28.4±3.9	7.2±1.9	4.6±0.2	20.9±1.7		
Normospermia (N=34)	3.9±0.9	7.6±0.3	72.6±10.2	55.8±6.3	30.2±6.5	28.7±3.8		
Oligospermia (N=12)	3.1±0.5	7.6±0.1	10.2±1.9	21.8±1.5	22.8±2.6	24.8±8.9		
Control (N=18)	4.2±0.9	7.7±0.0	101.1±16.2	48.6±6.7	32.0±6.6	48.2±6.5		
All the parameters were expressed as mean ± standard error of mean								

Table 1: Comparison of semen parameter analysis for various categories.

 Table 2: Comparison of antioxidant markers with increase in sperm count.

Sperm Count (millions/ml)	Seminal SOD (U/ml)	Seminal GSH (µmol/ml)	Seminal Catalase (U/ ml)			
0-20	1.92±0.6 ^{NS}	4.34±0.9NS	3.52±0.4 ^{5N} S			
21-40	2.35±0.8**	4.78±0.9*	3.98±0.67 ^{NS}			
41-60	2.73±0.9**	5.01±0.7**	4.67±1.10**			
61-80	3.56±1.1***	6.34±0.6***	7.39±0.70**			
81-100	3.57±0.8***	8.94±0.8***	9.82±0.80***			
>100	4.98±0.5***	16.78±2.2***	11.34±0.80***			
All the values were represented as mean ± standard error of mean						

P<0.001 (***Significance), P<0.01 (**Significance), P<0.05 (*Significance) and P>0.5 (Not significance), [With respect to Sperm count (millions/ml)].

existing arbitrator that causes damage to the spermatozoa. In order to retard and counterattacking of the ROS effects, human seminal plasma and spermatozoa, prostasomes have their own antioxidant system. In addition to many antioxidant enzymes present in the seminal plasma and spermatozoa, semen contains thiol groups and vitamin E and Vitamin C (ascorbic acid) in higher concentrations that will help in protecting the spermatozoa from the damage and heat shock [18]. The antioxidant enzymes were present in the higher concentration in human seminal plasma, our result clearly indicate that all the antioxidants were present in higher concentration when the sperm count increases from 1 to > 100 millions/ml (Table 2). It is in literature that human spermatozoa itself having the capacity of generating ROS like superoxide anion which drastically damage the other spermatozoa and subsequently turns into the hydrogen peroxidise under the influence of superoxide dismutase [19].

HU Ting-xi and his researchers studied the sperm pretreatment with GSH during IVF can maintain better the viability and fertility of sperm through reducing apoptosis and increasing the antioxidant capacity, which improves the IVF embryos development [19]. Our results correlates that there is an increase in seminal GSH when there is an increase in sperm number per ejaculation. The ability of generating the ROS in higher level into the seminal plasma by defective spermatozoa is so far a controversial study [2]. Our results proved that even with the defective spermatozoa (asthenospermia), there is an increase in all the antioxidant markers, particularly glutathione. Oxidative damage and injury to the spermatozoa present in the total ejaculate was the major cause of sperm non-capacitating and sperm dysfunction. The estimation of non-enzymatic antioxidants present in the seminal plasma was mostly inversely correlated to the lipid peroxidation present in the seminal plasma [21]. Follicular fluid which is very rich in antioxidants and to some extent ROS, which is believed to have positive correlation with pregnancy outcomes of IVF patients. The quality of oocyte, sperm mediated oocyte activation; sperm to oocyte interaction purely depends upon the quality of follicular fluid. Again, antioxidants play a major role in follicular fluid quality and for successful pregnency outcome in IVF patients [22].

CONCLUSIONS AND FUTURE PROSPECTIVE

This research concludes that there is a positive correlation with sperm count and the antioxidant markers present in seminal plasma. In future, the herbal products with more antioxidants could be in practice for gaining the reproductive functions. Further, we believe that good lifestyle management, exercise and yoga will pave the way for reproductive function attainment and quality sperm. This will lead to postulate a hypothesis about miRNA expression studies on each antioxidants present in human seminal plasma.



Figure 2 The curve shows the comparison of a major seminal antioxidant marker GSH in human seminal plasma. X axis represents sperm count in millions/ml; Y axis represents antioxidant activity of GSH. The trend shows an increasing manner for antioxidant marker GSH from 0-20 millions/ml to >100 millions/ml. between 100 to >100 millions/ml of sperm count, the antioxidant activity of GSH was suddenly increasing in a great manner. All the values were represented with mean and standard error of mean.

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