

RESEARCH ARTICLE

Effect of Continuous 6 Months Oral Antioxidant Combination with Universally recommended Dosage in Idiopathic Male Infertility

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ABSTRACT

The aim of the present study was to observe the adverse effects of high level of reactive oxygen species (ROS) in idiopathic male infertility and the role of a combined antioxidant therapy for six months to overcome the detrimental effects of ROS.

A prospective study including 185 infertile male was conducted at Institute of Reproductive Medicine (IRM) from January 2014 to April 2015. All the major sperm parameters as well as ROS, antioxidant level and the effect of high ROS level were evaluated before and after the therapy.

A significant improvement in sperm motility and concentration were observed after 6 months of therapy. Antioxidant level had increased and ROS level had decreased significantly after the antioxidant treatment. Improvement in morphology and leukocyte concentration were observed though not clinically significant.

Conclusion: This combined antioxidant therapy may improve sperm quality after continuous 6 months of treatment. However, further study is needed regarding this experiment for validating the trend.

Keywords: Antioxidant, Idiopathic male infertility, Oxidative stress, Reactive oxygen species.

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INTRODUCTION

Nowadays, infertility has become an important medical and social problem which has a major impact on well-being of the couple. Eighty million people worldwide are affected by the inability to have children. Male factor infertility accounts for up to half of all cases of infertility

and affects one man in 20 in the general population. However, World Health Organization (WHO) parameters of semen characteristics address only few strictures of sperm quality and function (WHO guidelines, 2010). Infertility of unknown origin or idiopathic male infertility is responsible in 37 to 40% of overall infertile males. Recent evidence suggests that semen of infertile men, especially infertile male of unknown origin, has a high amount of reactive oxygen species (ROS, 30–80%), which adversely affects the plasma membrane integrity and the genomic material of sperm cells.^{1,2} A fine equilibrium is always maintained between ROS level and total antioxidant capacity (TAC) of seminal plasma. When it is disturbed by either excessive generation of ROS or a weak defence mechanism of antioxidants, a condition known as oxidative stress (OS) is developed which is characterized by different toxic effects on spermatozoa. Oxidative stress in sperm cells is known to cause peroxidative damage to the sperm plasma membrane and subsequent loss of its DNA integrity. Reactive oxygen species includes oxygen ions, free radicals and peroxides, both inorganic and organic. They are generally highly reactive small molecules with presence of unpaired valence shell electrons, trying to attain stable configuration by binding with other surrounding molecules with special affinity to lipid, proteins, nucleic acid etc. This modifies the cellular redox balance ultimately resulting in apoptosis. Superoxide anion, hydroxyl radical and hydrogen peroxide are some of the major ROS present in seminal plasma. Various environmental factors (high temperature, pesticides, pollution etc.), lifestyle factors of advanced ages (smoking, stress, obesity etc.) and other factors (infections, autoimmunity, chronic disease etc.) are thought to increase the ROS levels.^{2,3} Small amount of ROS generated by normal spermatozoa is needed for physiological processes such as sperm capacitation, acrosome reaction and sperm–oocyte fusion.

Antioxidants present in seminal plasma act as free radical scavengers to protect spermatozoa against ROS. Role of oral supplementation of antioxidants is still a debatable issue all over the world. Antioxidants are known to decrease the ROS level and protect the cell from oxidative damage. Currently, infertility clinics all over the country recommend a course of oral antioxidants such

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as lycopene, coenzyme Q10 (CoQ10), L-carnitine, zinc, selenium, vitamin E, vitamin C, or/and multi-vitamins without assessing the OS status of male partners. Going beyond the WHO guidelines, by using newer methods, identification of the latent factors responsible for male infertility is an absolute necessity.

Oral supplementation of combined antioxidants (L-Carnitine, Acetyl-L-Carnitine, CoQ10, Lycopene, Zinc, Folic acid, Vitamin B12, Selenium, Fructose, and citric acid) is expected to considerably improve semen parameters of infertile men. Here, the efficacy of these drugs has been tested in a group of patients. Details of the study design and corresponding results are presented in the following section.

OBJECTIVES

The main objectives of the study are as follows:

- Semen parameters such as sperm count, motility, morphology, leukocytes, etc., were analyzed within 1 hour of ejaculation as per WHO protocol before and after oral supplementation of antioxidants.
- To evaluate the ROS and mitochondrial membrane potential (MMP) of semen before and after oral supplementation of antioxidant.
- To estimate the TAC in seminal plasma before and after oral supplementation of antioxidant.
- To estimate the lipid peroxidation (LPO) level in seminal plasma before and after oral supplementation of antioxidant.
- To estimate coenzyme Q10, zinc, and selenium in seminal plasma before and after 6 months of oral supplementation of antioxidant.
- To correlate the data for a better understanding of the role of antioxidant in male infertility and ascertain whether semen parameters of infertile men are significantly improved.

METHODOLOGY

Sample Collection and Processing

Approval for this study was obtained from the research ethics committee of the Institute of Reproductive Medicine (IRM), Kolkata. All types of earlier medication and detailed history were recorded. A 3 to 5-day period of sexual abstinence was ensured. The age group of the men in the study was 32 to 51 years. Duration of infertility ranged from 2 to 10 years. The case history of patients revealed that 36.5% of them were smokers and 24.5% were alcohol consumers, while 10.25% of them consumed tobacco in some form.

One hundred forty patients, suffering from male factor infertility, who had reported to this institution have been included in this study. These patients were advised to

take the above-mentioned combined antioxidant therapy for a period of 3 months. Among them, 115 patients reported after 3 months of oral supplementation. Those patients, who showed less improvement in parameters after 3 months of treatment, were advised to continue the medication for another 3 months. However, 70 patients, receiving these antioxidants, reported after 6 months of oral supplementation.

Semen samples were collected by masturbation in sterile containers from infertile male patients. The sample was divided into three parts. Liquefied semen samples were taken from the first part, which were analyzed as per WHO guidelines (2010). In the second part of the semen sample, reactive oxygen species, MMP were measured using FACS, while the third part was centrifuged at 1000 rpm for 8 minutes to remove white blood cells, red blood cells, and other cellular components. The clear supernatant was preserved at -10°C . Frozen supernatant was used for the assessment of TAC and LPO.

A placebo trial study was conducted on 45 patients of the same profile. Semen parameters were analyzed before and after 3 months of placebo supplementation. Results were compared with the experimental data.

Measurement of ROS

Reactive oxygen species levels were measured in washed sperm cells by chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1-phthalazinedione) as a probe. Two hundred microliters of sperm suspension was placed in the luminometer cuvette (Berthold, Model no. 0727). Next, 2 ml of 100 mM luminol (Sigma, St. Louis, MO) in dimethyl sulphoxide (DMSO) and 4 ml of 2 mg/ml horseradish peroxidase (HRP) in Biggers, Whitten, Whittingham (BWW) media were added and each sample was scanned for 10 minutes. Biggers, Whitten, Whittingham media was used as blank and BWW media plus luminol were used as control. Reactive oxygen species values were expressed as 10^6 counted photons per minute (cpm)/10 million cells.

Measurement of Tac in Seminal Plasma

Nonenzymatic TAC was measured in the seminal plasma using the enhanced chemiluminescence assay. Seminal plasma was diluted 1:20 with deionized water (dH_2O) and filtered through a $0.2\ \mu\text{m}$ millipore filter. Signal reagent was prepared according to the chemiluminescence kit. Aliquots of 20 μl horseradish peroxidase linked immunoglobulin (HRP) were added to 4.98 ml dH_2O . This was further diluted 1:1 to give a working solution with the desired luminescence output (3×10^7 c.p.m.). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble α -tocopherol analogue, was used

as a standard (25–75 $\mu\text{mol/l}$). With the luminometer in the kinetic mode, 100 μl of signal reagent and 100 μl of HRP was added to 700 μl of dH_2O and mixed. The solution was equilibrated to the desired level of chemiluminescence output ($2\text{--}3 \times 10^7$ cpm) for 100 seconds. Aliquots of 50 μl of the prepared seminal plasma were added immediately to the signal reagent and HRP and the chemiluminescence was measured. Suppression of chemiluminescence and the time, from the addition of seminal plasma to 10% recovery of the initial chemiluminescence, were recorded. Total antioxidant capacity was expressed as molar Trolox equivalents.

Measurement of LPO in Seminal Plasma

Five hundred microliters seminal plasma was mixed with 1 ml of solution containing 0.37% TBA and 15% TCA in 0.25 N HCl. Subsequently, the samples were heated at 100°C for 15 minutes in the dark, as the solution of TBA and TCA is photosensitive. Then, it was cooled in ice and centrifuged at 1000 rpm for 10 minutes at 4°C to remove the precipitate. Phosphate-buffered saline (PBS) or the solution containing PBS and TBA: TCA (1:1) were used as blank and control respectively. The supernatant fractions containing MDA of all samples were determined spectrophotometrically at 535 nm.

Measurement of CoQ10 in Seminal Plasma

Coenzyme Q10 extraction from spermatozoa was performed by high-performance liquid chromatography (HPLC) system as described by Surai et al.⁸ Seminal plasma was mixed in 0.7 ml of 1:1 (v/v) mixture of 5% NaCl solution in ethanol following the addition of 3 ml hexane and homogenization for 3 minutes. After centrifugation, the hexane layer was collected and the extraction was repeated twice. Hexane extracts were combined and dried under nitrogen. Then, the residue was redissolved in 150 ml of methanol and injected into HPLC system fitted with an ultraviolet detector. Chromatography was performed using a mobile phase of methanol/water (97:3 v:v) at a flow rate of 1 ml/min. Fluorescence detection and quantification of α -tocopherol were required for excitation and emission wavelengths of 295 and 330 nm respectively. Chromatographic absorbance data were analyzed. Calibration was performed using standard solutions of CoQ10 (Sigma Chemical Co., St. Louis, MO) in methanol. Seminal plasma level of CoQ10 was expressed in nanogram per milliliter (ng/ml).

Determination of Selenium and Zinc

Seminal zinc and selenium concentrations were determined by atomic absorption spectrophotometry (Analyst 700;

PerkinElmer, Waltham, MA). An aliquot of 100 μl of seminal plasma was diluted 100-fold with a solution containing 5 g/l of 25% ammonia, 0.5 g/l ethylenediaminetetraacetic acid (EDTA), and 0.5% Triton X-100 in Millipore water. Standard solutions of zinc and selenium were used to develop standard curves. All samples were prepared in duplicate. Calibration was carried out using blank and standards. All samples were analyzed in duplicate. The results were expressed as $\mu\text{g/ml}$.

Measurement of ROS by Flow Cytometry

Reactive oxygen species levels were measured in washed sperm cells by fluorescence-activated cell sorting (FACS) using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence marker. Sperm pellet was washed with PBS twice. Pellet was then dissolved in 500 μl of PBS. One microliter of DCFDA was added to the solution and kept at 37°C in dark for 25 to 30 minutes. After incubation, the sample was washed with PBS. Reactive oxygen species generation was measured by FACS.

Determination of Mitochondrial Membrane Potential by Flow Cytometry

Flow cytometry has emerged as the technique of choice for analysis of the MMP ($\Delta\psi$) in whole cells. Membrane-permeable lipophilic cationic fluorochromes are used as probes of $\Delta\psi$; they penetrate cells and their fluorescence is a reflection of $\Delta\psi$. JC-1 (5,5',6,6' tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) is a lipophilic fluorochrome that is used to evaluate the status of the $\Delta\psi$. Stock solution was prepared at 1 mg/ml in DMSO. Stock solution was divided into small aliquots and stored at -20°C until use. Working solution, prepared from the stock solution, must be used immediately after preparation and cannot be stored. Sperm suspension was diluted to a concentration of 5 million sperms/ml in PBS. The sperm suspension was incubated with 0.5 μl of JC-1 for 30 minutes at 37°C in dark. The sample was washed in PBS. The staining properties of JC-1 in each sample were visually confirmed by flow cytometry.

RESULTS AND FINDINGS

Comparison of semen parameters, ROS level, TAC, LPO, and DNA damage in patients, with male factor infertility, before and after 3 and 6 months of oral supplementation have been done. Semen parameters of 45 patients, who were treated with placebo for a 3-month time period, almost remained unchanged showing no improvement in parameters. Seminal parameters and

the other factors of the placebo-treated subjects are summarized below.

Statistical Analysis

All the values were presented as mean \pm SEM. The clinical outcomes between the control and experimental groups were compared using the t-test. STATA 10.0 software (StataCorp, College Station, TX) was used for statistical analysis. Statistical significance is considered to be positive if the p-value is less than 0.05 ($S=p \leq 0.05$, $NS=p > 0.05$)

Pregnancy Outcome

Out of 70 patients, four (6%) got spontaneous pregnancy. Intrauterine insemination (IVI) was performed in 25 couples, where four (16.0%) couples achieved pregnancy. Fifteen couples were waiting for IUI within next few months. *In vitro* fertilization was performed in 13 couples, out of whom five (38.0%) achieved pregnancy. Intracytoplasmic sperm injection was performed in six couples among whom three (50.0%) achieved pregnancy.

DISCUSSION

In the present study, our aim was to evaluate the efficacy of a combination of antioxidant supplementation for 6 months on idiopathic male infertility. The combination included L carnitine, acetyl L carnitine, CoQ10, lycopene, zinc, folic acid, vitamin B12, selenium, fructose, and citric acid. Sperm parameters and the OS biomarker values were estimated both before starting and after completion of therapy.

In the above combination, CoQ10 acts as an electron carrier and produces energy in mitochondria for the movement of spermatozoa. For this energy production, β oxidation of long chain fatty acid is essential. Before entering into mitochondria, fatty acid must be activated. L-carnitine and acetyl carnitine help in the activation and transportation of fatty acid to the mitochondria.⁴ Therefore, combination of L-carnitine and CoQ10 provides a better improvement in sperm motility than CoQ10 alone.

Lycopene, the most abundant carotenoid in nature, plays the most important role in the scavenging system of ROS. A study by Gupta and Kumar⁵ reported significant improvement in semen parameters, especially sperm morphology and sperm concentration, after giving 2000 μ g of lycopene for 3 months. In our study, oral supplementation of lycopene 5000 μ g with CoQ10, L-carnitine, zinc, and folic acid, twice daily for 6 months, showed significant improvement in sperm parameters and reduction of OS.

Selenium may protect the sperm from oxidative damage of DNA and is required for normal testicular development, spermatogenesis, and motility.⁶ The mechanism by which selenium fights against OS is precisely not known; selenoenzyme and sperm capsular selenoprotein glutathione peroxidase may mediate its effect.^{7,8}

Zinc and folic acid combination increases normal morphology and sperm concentration. Folic acid and vitamin B12 help in the synthesis of healthy DNA.⁹

In the present study, following therapy with nutraceuticals having a combination of L carnitine, acetyl L carnitine, CoQ10, lycopene, zinc, folic acid, vitamin B12, selenium, fructose and citric acid for 6 months, an improvement in all the sperm parameters, except sperm morphology, was statistically significant.

After completion of the above therapy for 6 months, pregnancy rate with or without the help of antiretroviral therapy (ART) procedure was much higher.

Intracytoplasmic sperm injection in six couples, showing no improvement in sperm parameters but with reduction in ROS level, achieved 50% pregnancy rate; therefore, these antioxidant combinations help in improving fertilization potential of the spermatozoa by reducing OS.

To establish our evaluation, a larger study for a prolonged duration is needed.

CONCLUSION

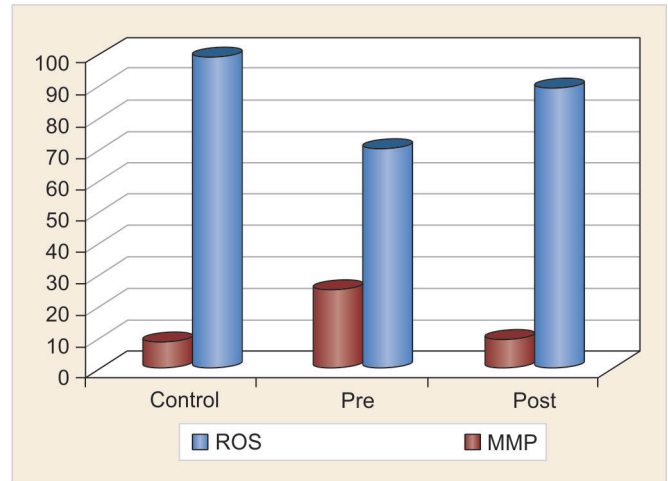
Comparison of semen parameters, ROS level, TAC, LPO, some antioxidant level in seminal plasma (CoQ10, zinc, and selenium) and MMP in patients with male

Table 1: Semen parameters, ROS, TAC and LPO measurement before and after 3 months of the combined antioxidant supplementation

Parameters	Before antioxidant supplementation (n = 115) (a)	Third months after antioxidant supplementation (n = 115) (b)	p-value
Total count (M/ml)	19.30 \pm 4.15	25.20 \pm 6.95	0.4668 (NS)
Motility (%)	16.80 \pm 2.40	27.50 \pm 3.10	0.0068 (S)
Morphology (%)	15.75 \pm 1.30	20.35 \pm 1.95	0.0509 (NS)
Leukocyte concentration (M/ml)	9.80 \pm 0.89	8.25 \pm 0.56	0.1418 (NS)
ROS (cpm/10 million cells)	67245.30 \pm 9870.45	38630.50 \pm 5432.60	0.0118 (S)
TAC Semen (μ M of trolox equivalent)	2124.65 \pm 95.70	2435.80 \pm 108.65	0.0327 (S)
LPO (nM/10 ⁴ cells)	765.30 \pm 31.20	695.70 \pm 23.45	0.0759 (NS)

NS: Not significant; S: Significant

factor infertility, before and after 3 and 6 months of the combined antioxidant supplementation, has been done. One hundred fifteen patients among the total cohort (140) reported after 3 months of antioxidant supplementation (Table 1). Seventy patients reported after 6 months of medication, as they were advised to continue the medicine for another 3 months due to poor improvement. After analysis of the semen parameters of those 70 patients, significant improvement of parameters was noted after 6 months of treatment (Table 2). Sperm count, sperm motility, ROS and TAC level show statistically significant improvement ($p < 0.05$) after 6 months of medication. Sperm motility, one of the important parameters, shows an encouraging result after 6 months of therapy ($p = 0.0001$). Here, it is worth mentioning that ROS levels have a higher value (~25%) prior to the treatment and came down to a compromised level after 6 months of antioxidant supplementation (~9%) (Graph 1). Mitochondrial membrane potential level also improved after the drug supplementation (Graph 1). Coenzyme Q10 and zinc level in seminal plasma also increased significantly ($p = 0.0388$, $p = 0.0492$) after 6 months of medication



Graph 1: Reactive oxygen species and MMP (analyzed by FACS)

(Table 3). It is clear that LPO level has a higher value in case of infertile men and decreased after 3 and 6 months of the drug supplementation.

Semen parameters of 45 patients, who were treated with placebo for a 3 months time period, almost remained unchanged showing no improvement in parameters.

Table 2: Semen parameters, ROS, TAC, LPO and DNA damage before and after 6 months of the combined antioxidant supplementation

Parameters	Before administration (n = 70) (a)	After 6 months of administration (n = 70) (b)	p-value
Total count (M/ml)	21.75 ± 2.60	34.48 ± 4.49	0.0154 (S)
Motility (%)	18.75 ± 1.40	35.40 ± 2.88	0.0001 (S)
Morphology (%)	19.55 ± 1.45	24.18 ± 2.20	0.0811 (NS)
Leukocyte concentration (M/ml)	9.95 ± 0.83	7.80 ± 0.72	0.0524 (NS)
ROS (cpm/10 million cells)	65860.55 ± 10870.40	28662.80 ± 6789.20	0.0043 (S)
TAC Semen (μM of trolox equivalent)	2346.72 ± 97.15	2820.75 ± 119.65	0.0025 (S)
LPO (nM/10 ⁴ cells)	734.24 ± 36.10	645.60 ± 29.32	0.0587 (NS)

NS: Not significant; S: Significant

Table 3: Coenzyme Q10, zinc and selenium measurement before and after 6 months of the combined antioxidant supplementation

Parameters	Before antioxidant administration (n = 70) (a)	Sixth months after antioxidant administration (n = 70) (c)	p-value
CoQ10 concentration (ng/ml)	54.76 ± 3.26	66.95 ± 4.85	0.0388 (S)
Zinc (μg/ml)	16.15 ± 2.16	22.60 ± 2.42	0.0492 (S)
Selenium (μg/ml)	23.46 ± 4.92	26.75 ± 6.45	0.6857 (NS)

NS: Not significant; S: Significant

Table 4: Semen parameters, ROS, TAC, LPO and DNA damage of placebo-controlled recipients

Parameters	Before placebo administration (n = 45) (a)	After 3 months of placebo administration (n = 45) (b)	p-value
Total count (M/ml)	14.90 ± 4.73	15.40 ± 4.97	NS
Motility (%)	18.10 ± 3.72	20.62 ± 3.09	NS
Morphology (%)	24.10 ± 2.99	25.70 ± 3.96	NS
Leukocyte concentration (M/ml)	7 ± 0.49	6.650 ± 0.73	NS
ROS (cpm/10 million cells)	59257.23 ± 4223.52	55480.76 ± 3644.45	NS
TAC Semen (μM of trolox equivalent)	2212.0 ± 148.9	2278.05 ± 123.3	NS
LPO (nM/10 ⁴ cells)	737.3 ± 27.23	725.4 ± 28.18	NS

NS: Not significant

Seminal parameters and the other factors of the placebo-treated subjects are summarized in the Table 4.

From the above results, it is very clear that continuous 6 months oral supplementation of antioxidant combination with universally recommended dosage (L-Carnitine, acetyl-L-carnitine, CoQ10, lycopene, zinc, folic acid, vitamin B12, selenium, fructose, and citric acid) has an encouraging improvement in semen parameters of idiopathic male infertility.

CONCLUSION

The administration of the above mentioned combined antioxidant therapy may play a positive role in treatment of male factor infertility of unknown origin where deterioration of sperm parameters and functions is mainly due to OS. However, further study is highly needed regarding this experiment for validating the trend.

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