A Study of Role of Reactive Oxygen Species in Idiopathic Male Infertility & Its Management by Antioxidant Therapy in Uttar Pradesh (U.P.)

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ABSTRACT

BACKGROUND
Infertility is defined as the inability to conceive after at 1 year of regular unprotected intercourse. Male contributes to almost half of infertility cases and in almost 30 % of cases, no definite aetiology is identified, and hence, male infertility is labelled idiopathic in these cases. Oxidative energy production mechanisms are almost always accompanied by reactive oxygen species (ROS), generation whose too much concentrations can lead to extensive protein damage and cytoskeletal modifications and inhibit cellular mechanisms. A number of laboratory techniques have been developed to evaluate oxidative stress by measuring ROS level in the semen. In recent times antioxidant supplements have been proposed as useful agents to increase the scavenging capacity of seminal plasma, controversy still surrounds their actual clinical utility.

METHODS
34 male patients were included in this study. Reactive oxygen species detection was done by Flowcytometry using dichlorofluroseciindiacetate (DCFH-DA).

RESULTS
The ROS in the patient group was found to be significantly higher 29.821 (5.6300 than the control group 22.162 (1.6331 having p value < 0.001). The ROS (29.821 ± 5.6300) was found to be significantly reduced after 3 months of antioxidant therapy which got reduced to 19.893 ± 4.2299 respectively.

CONCLUSIONS
Our study demonstrates that infertile men have significantly higher level of ROS (as measured by flowcytometry) & lower sperm count (oligospermia), decreased progressive & total motility and increased immotile sperms as compared to healthy fertile men. This study further proves that antioxidant therapy based on a combination of carnitine, zinc, coq10, lycopene and vitamin C & E for 3 months is associated with a decrease of ROS as measured by flowcytometry & a variable degree of improvement in above mentioned semen parameters.

KEYWORDS
Reactive Oxygen Species, Male Infertility

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BACKGROUND

Infertility is defined as nonachievement of conception after a minimum of 1 year of regular unprotected intercourse. Infertility affects about 15 %[1] of couples worldwide. It's called "male factor infertility" when semen parameters derangement or sexual or ejaculation dysfunction is found during evaluation. Male contributes to almost half of infertility cases and occasionally an aetiology directed treatment strategy can be applied. However, in almost 30 %[2] of cases, no definite aetiology is identified, and hence, male infertility is called idiopathic in such cases. Oxidative energy production is arbitrarily associated with the reactive oxygen species (ROS) production, excessive concentrations of which often leads to cellular damage.[3] A free radical[4] is any molecule that has one or more unpaired electrons. The superoxide ion, the hydroxyl (OH), and the hypochlorite radical are some of the highest reactive radicals of oxygen. Because of their high reaction potential and ability to unleash an uncontrolled chain reaction cascade, ROS can cause extensive cytoskeletal and protein damage along with cellular function inhibition.

Oxidative stress (OS) is a significant contributor to male infertility. A number of laboratory techniques are now available for oxidative stress evaluation in the semen. 20 % – 40 % of infertile men have significantly higher levels of ROS in their semen when compared with normal fertile men. All the more recent studies strongly indicate OS to negatively influence semen parameters, fertilization rate, embryonic development, and pregnancy rate. Therefore, correction of the state of OS could be considered a logical and potential step in infertility management. While antioxidant supplementation[5] has been proposed as a suitable approach to increase the scavenging capacity of seminal plasma, controversy still surrounds its actual therapeutic potential.

Aim

To find the level of ROS in cases of idiopathic male infertility, to ascertain their effect on fertility and to analyse the beneficial effect of antioxidant therapy on the ROS.

METHODS

This study is done in the Department of Urology, Institute of Medical Sciences and Department of Genetics, BHU, Varanasi, from November2016 to September 2018.

Inclusion Criteria

Married Male patients of age between 21 years - 40 yrs presenting with complaint of infertility since more than 1 year and diagnosed as having idiopathic male infertility after excluding other causes presenting to infertility Urology OPD, IMS, BHU, Varanasi.

Exclusion Criteria

1. Female patients
2. Unmarried male patients
3. Patients having age less than 21 years and more than 40 years.
4. Patients diagnosed as having obstructive causes like Congenital bilateral absence of vas deferens (CBAVD), Ejaculatory duct obstruction (EJDO). Varicocele, priorvasectomy, Testiculartrauma, urethralinjury, pelvic / perineal trauma.
5. Patients diagnosed with primary / secondary testicular failure, other syndrome, like Klinfelter syndrome, Cartagenner syndrome, VACTERAL Anomalies.
6. Patients with previous bladder, urethral, prostate, retroperitoneal surgery.
7. Patients with previous radio and chemotherapy history.
8. Serious medical co morbidity like chronic liver disease, severe heart disease.
9. Patients having anomalies like unilateral or bilateral cryptorchidism, anorchia.
10. Patients who are chronic drinkers and smokers.

Data Collection and Sample Testing

34 male patients diagnosed as idiopathic male infertility during the study period mentioned above presenting to urology infertility OPD were selected for this study. Patients were discussed about their disease and purpose of this study and informed written consent was obtained before inclusion. Patients were subjected to proper medical history with special attention to above inclusion and exclusion criteria. Thorough physical examination was done including examination of bilateral testis with orchidometer, bilateral vas, penis, urethra, meatus, and DRE.

Semen sample was collected under aseptic conditions in a sterile plastic vial by masturbation by patient himself after a minimum abstinence of 5 days. Semen sample was then analysed at genetics department. All patients were given antioxidant therapy consisting of carnitine-1gm,zinc-10 mg, coq10 – 100 mg, lycopene - 2.5 mg and vitamin C1 gm daily for 3 months &semen parameters were repeated thereafter & compared. 20 healthy male volunteers having no apparent comorbidities in the same age group as patients with completed families were randomly taken as controls. Four patients were lost to follow up and flowcytometry for ROS was repeated in 30 patients after 3 months of antioxidant therapy and results were analysed.

Reactive oxygen species detection was done by Flowcytometry[6] using Dichloroflurosecindiacetate (DCFH - DA).[7] 2′ - 7′ - Dichlorodihydrofluoresceindiacetate (DCFH - DA) is one of the most widely used techniques for directly measuring the redox state of a cell. DCFH - DA is a non-fluorescent & cell permeable DCF precursor and used as an intracellular probe for oxidative stress. DCF collection in cells is measured as an increase in fluorescence at 530 nm when the sample is excited at 485 nm. Fluorescence at 530 nm is measured using a flow cytometer and is assumed to be proportional to the concentration of hydrogen peroxide in the cells.

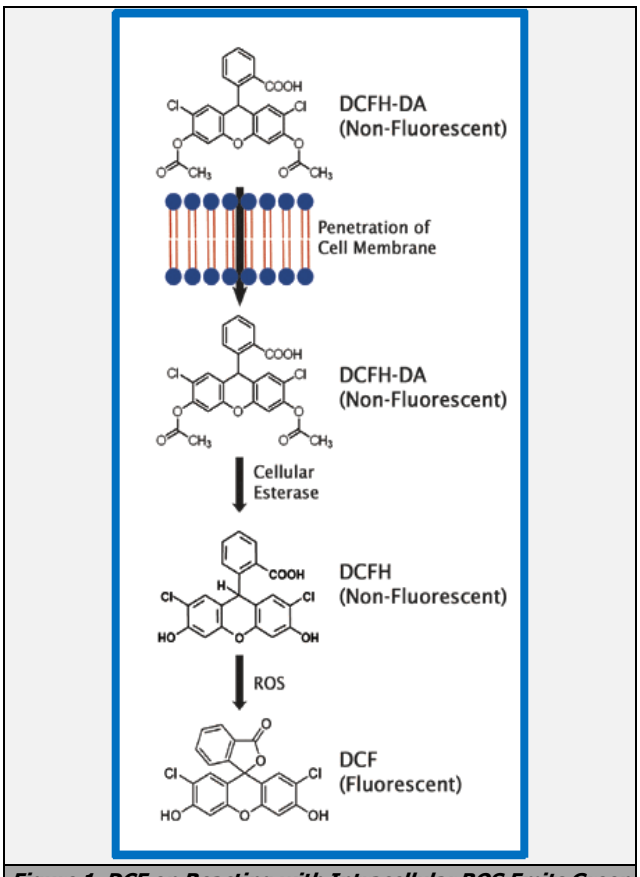

Figure 1. DCF on Reacting with Intracellular ROS Emits Green Fluorescence Data were Expressed as Percentage of Fluorescent Spermatozoa

Statistical Analysis

The results obtained were analyzed statistically using the unpaired t-test, as appropriate. Data were presented as mean ± SD. SPSS was used for statistical analyses. P-values less than 0.05 were considered statistically significant (P< 0.05 = significant).

RESULTS

34 male patients of age between 21 to 40 years with complaint of infertility after trying for baby since more than 1 yr. were included in this study. Table 1 shows the characteristics of study population.

	Mean ± SD
Age	29.794 ± 5.5256
BMI	25.356 ± 2.7523
Duration infertility	4.926 ± 2.4126
Days abstinence	5.059 ± 1.1532
Liquefaction time	36.471 ± 3.5949
Volume	2.476 ± 1.2858
pH	7.385 ± 0.2986
Concentration	24.025 ± 21.1888
Total no ejaculate	55.767 ± 55.4793
PM	29.582 ± 12.0937
NP	30.936 ± 15.8119
IM	43.471 ± 19.9609
Total Motile	45.577 ± 17.7842
Round Cells	13.400 ± 2.0736
ROS pre	29.821 ± 5.6300
ROS 3 month	19.893 ± 4.2299

Table 1. Characteristics of Study Population

	Group	Mean ± SD	T-Value	P-Value
Age	1	29.794 ± 5.5256	0.839	0.405
	2	28.550 ± 4.7625		
	Group	Mean ± SD	t - value	p - value
Liquefaction time	1	36.471 ± 3.5949	0.489	0.627
	2	36.000 ± 3.0779		
	Group	Mean ± SD	t - value	p - value
Volume	1	2.476 ± 1.2858	- 0.588	0.559
	2	2.672 ± 0.9696		
	Group	Mean ± SD	t - value	p - value
pH	1	7.385 ± .2986	0.506	0.615
	2	7.345 ± .2523		

Table 2. Comparison of Age & Semen Characterstics between Case & Control Group

Age-The mean age of the cases was 29.794 ± 5.5256 yrs while that of the control group was 28.550 ± 4.7625. Thus both cases and controls were of same reproductive age group. The liquefaction time of both the case & control group was similar. The semen volume of both the case & control group was similar. The semen ph of both the case & control group was similar

Variable	Mean ± SD 1	Mean ± SD 2	T = Value	P - Value
PM	29.582 ± 12.0937	53.750 ± 8.1232	- 7.931	< 0.001
NP	30.936 ± 15.8119	29.000 ± 12.9371	0.463	0.645
IM	43.471 ± 19.9609	17.250 ± 11.8405	5.336	< 0.001
Total Motile	45.577 ± 17.7842	82.750 ± 11.8405	- 8.311	< 0.001
Round cell	13.400 ± 2.0736	0.0 ± 0.0	-	-
ROS Pre	29.821 ± 5.6300	22.162 ±1.6331	5.918	< 0.001

Table 3. Comparison of Motility & ROS Level between Case & Control Group

The progressive motility of the cases was 29.582 ±12.0937 while that of the controls was 53.750 ± 8.1232. Thus, PM of cases was very significantly less in cases as compared to the control group. Similarly the immotile fraction was significantly higher in the control group while the total motile sperms were significantly less in the case group as compared to the control group. The ROS in the patient group was found to be significantly higher29.821 ± 5.6300 than the control group 22.162 ± 1.6331 having p value < 0.001.

	Median (Group 1)	Median (Group 2)	P - Value
Concentration	19.300 (11.300 - 31.000)	85.000 (70.500 - 119.000)	< 0.001
Total count per ejaculate	50.900 (21.290 - 69.225)	245.450 (176.250 - 333.200)	< 0.001

Table 4. Comparison of Sperm Count between Case & Control Group

The mean conc. of the cases was 19.300 (11.300 - 31.000) & total count were 50.900 (21.290 - 69.225) which were significantly less than that of control group which was 85.000 (70.500 - 119.000) & 245.450 (176.250 - 333.200).

		Mean ± SD	P - Value
Pair 1	ROS_pre	29.887 ± 5.7053	< 0.001
	ROS_3 month	19.893 ± 4.2299	

Table 5. Comparison of ROS Level in Case Group before & after 3 Months of Antioxidant Therapy

The ROS (29.887 ± 5.7053) was found to be significantly reduced after 3 months of antioxidant therapy which got reduced to 19.893 ± 4.2299 respectively.

DISCUSSION

Present concept of 'idiopathic infertility' describes the condition as "even after laboratory testing to identify the pathology the results remain inconclusive and etiology unidentified in approx. 40 – 60 % of case". In these patients, oligozoospermia is frequently observed with more severe forms manifesting as azoospermia and asthenozoospermia. Idiopathic infertility male patients have no clear history of fertility problems and both clinical findings and endocrinal evaluation test results are normal. However, carefully performed semen analysis frequently detects a host of semen parameter abnormalities. The prevalence of idiopathic infertility amongst all causes is reported to range from 40 – 60 %. Most studies from the last ten years show idiopathic male infertility (IMI) to be a disease of varied and obscure aetiology in most circumstances.

Currently only few modalities are available to diagnose patients with IMI and even fewer appropriate intervention options are available. The harmful consequences of reactive oxygen species (ROS) and free radical- arbitrated damages to spermatozoa and seminal plasma are well deliniated; however, their deeper effects on cytoskeletal anatomy and molecular physiology have yet to be understood. Such a study should help to access the impact of ROS on spermatogenesis in a broader perspective.

Our study is one of those which aims to find the level of ROS in cases of idiopathic male infertility, to ascertain their effect on fertility and to analyse the beneficial effect of antioxidant therapy on the ROS. Free radicals were first described more than a century ago & during last 50 years it has been elucidated that almost all oxidative biochemical reactions are mediated by free radicals. After this, free radicals were found in biological systems and are considered to be responsible for many neurodegenerative disorders, various malignancies, vascular disorders and aging. The mechanism of their actions was gradually elucidated in successive years and they were discovered to be regulators of many metabolic pathways but were also found to be themselves regulated by hormones like insulin.

Though free radicals act as damaging agents in many processes they are also mediators of many normal functions in living organisms. Various metabolic and physiological processes release Reactive oxygen species as normal by products, however when released in excessive amount they cause oxidative stress.

Sperms produce ROS in physiological amounts which plays vital role in functioning of sperm during capacitation, acrosome reaction (AR), and fertilization. Damaged sperms will likely be the source of ROS. The important ROS produced by human sperm are hydrogen peroxide, superoxide anion and hydroxyl radicals. Human seminal plasma has sufficient level of antioxidants to scavenge ROS and prevent ROS mediated sperm damage. In usual state of affairs there is an apt equilibrium between oxidants and antioxidants. An increase in the levels of ROS in semen will result in oxidative stress (OS) inside spermatozoa.

Agrawal[4] showed that OS and the excessive production of ROS have been associated with impaired sperm motility, concentration, and morphology. Aforementioned variables are important predictors of a person's capacity to produce viable sperm. Decreased progressive motility may be the result of a cascade of events that occur during Lipid peroxidation, such as decreased ciliary microtubule protein phosphorylation.

It is estimated that significant proportional relation between Ros mediated sperm DNA damage and reduced motility is probably because both sperm chromatin compaction and motility acquisition are parallel events culminating at the occassion of passing of the maturing male gamete in the epidydimis and distal reproductive tract. As a result of increased oxidative stress, motility decreases, preventing defective spermatozoa from participating in the process of fertilization, thus confirming studies signifying sperm motility as an accurate criterion in the evaluation of infertility. In our study it is found that patients having higher level of ROS had decreased progressive &total motility and increased immotile sperms.

Individuals having decreased sperm concentrations (oligozoospermia) have decreased chances of fertilization, which is attributed to increased ROS levels in their seminal plasma. As pointed before, pathological levels of ROS are known to induce spermatozoa apoptosis, resulting in cell death and a reduced sperm count. Numerous studies have indicated a higher percentage of apoptotic spermatozoa in oligoasthenozoospermic subjects than in normozoospermic men. In fact, elevated levels of ROS, known to induce apoptosis, were reported in mature spermatozoa from infertile men as compared to fertile donors.[8] Additionally, the presence of ROS in varicocele patients disclosed an inverse relationship between ROS and sperm concentration.

In this study it was found that patients having higher level of ROS had lower sperm count (oligospermia), decreased progressive &total motility and increased immotile sperms. This study also demonstrates that infertile men had significantly higher level of ROS (as measured by flowcytometry) in their semen as compared to healthy fertile men (P value < 0.001).

Antioxidants

Antioxidants present in seminal plasma perform the function of scavenging ros and protecting sperms from damage. Common antioxidants present in semen which protect sperm from excessive ROS and OS-induced damage and altogether represent the total antioxidant capacity (TAC) of semen are Superoxide dismutase (SOD), catalase, glutathione peroxidase system selenium and selenoproteins such as the phospholipids hydroperoxide glutathione peroxidase (PHGPx) and the glutathione reductase system vitamins A, C and E, glutathione, spermin, thiols, carnitine, and zinc. Additionally, the possible protective antioxidant role of co q 10 & lycopene[7] have been reported in literature. Above mentioned antioxidants work in conjunction as a protective network and quite a number of them become radicals themselves while scavenging oxidants. Remaining antioxidants in the network ensure that they are regenerated back to their original structures. For example, vitamin C and glutathione regenerate vitamin E.

Treatments with Antioxidants

According to study of Mohamad Hammadeh & Alixides Filippos A (2009),[5] carried out on patients with significant levels of DNA damage, it was shown that increasing the intake of antioxidant could result in an improvement in pregnancy outcomes with 3 months treatment with the immune-modulating and anti-oxidants (beta-glucan, papaya, lactoferrin, and vitamins C and E) in patients with asthenoteratozoospermia associated with leukocytosis.

Piomboni et al. (2008) found a significant reduction in seminal fluid leukocyte concentrations (2.2 ± 0.9 vs. 0.9 ± 0.2) and consequently an increase in the percentage of morphologically normal sperm (17.0 ± 5.2 % vs. 29.8 ± 6.5%), total progressive motility (19.0 ± 7.8 vs. 34.8 ± 6.8) & chromatin integrity with antioxidant treatment.

In a prospective study[9] performed on men with oligospermia (5 – 20 million / ml) a statistically significant ($p = 0.009$) increase in sperm count after antioxidant therapy was seen. Univariate logistic regression analysis showed that men treated with antioxidant therapy presented with the probability of having a normal sperm count that was 20 - fold (OR = 20.1; CI 95 % = 1.05 – 43.2; $p = 0.014$) higher than a untreated men was recorded.

Our study also shows that antioxidant therapy based on a combination of carnitine, zinc, coq 10, lycopene and vitamin C&E for 3 months is associated with a decrease of OS as measured by flowcytometry.

CONCLUSIONS

Several studies suggest that raised ROS levels play an important role in the pathogenesis of idiopathic male infertility. Increasing levels of ROS have been implicated in derangement of various semen parameters and it fairly seems that increasing ROS can cause decrease in number of sperms (oligospermia) and also negatively affect total & progressive motility. Inspite of progress in reproductive sciences it remains to be established what's the exact role of ROS and what are it's physiological and pathological levels. Spermatogenesis being a complex process involves different type of cells at various stages, mutations in DNA at any step, could disturb the formation of morphologically and functionally normal mature spermatozoa thus leading to infertility. Much larger studies are required for elucidation of the correlation of pathological levels of ROS which may lead to DNA damage for in depth understanding of male infertility aetiology. A suitable antioxidant therapy may benefit idiopathic infertility patient identified with increased Ros level. As motility defects and DNA damage are frequently manifested as OS imbalance thus early OS diagnosis and prompt antioxidant treatment may prevent OS induced DNA damage.

It can also be concluded that increasing ROS level is associated with deranged semen parameters like oligospermia, decreased progressive & total motility and increased immotility & consequently idiopathic male infertility. The present findings of this study indicate that idiopathic male infertility may be associated with increased ROS level and can be alleviated to some extent by antioxidant treatment.

Data sharing statement provided by the authors is available with the full text of this article at jebmh.com.
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