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ASSESSMENT OF SPERM DNA DAMAGE, APOPTOSIS AND SEMINAL PLASMA OXIDATIVE STRESS IN INFERTILE MEN

Gynaecology

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ABSTRACT

Objectives: To compare the levels of sperm DNA damage, apoptosis and oxidative stress parameters between infertile men and fertile controls.
Main Outcome Measure(s): Assessment of sperm DNA damage in infertile men and fertile controls by Ladder assay, apoptosis by Caspase-3 and seminal plasma antioxidant Glutathione (GSH) levels and lipid & protein oxidative stress parameters by MDA (Malondialdehyde), protein carbonyl (PC) respectively in both the groups.
Result(s): significant difference in the count, motility, abnormal morphology and WBC count was observed between cases and controls. Characteristic smear depicting DNA damage was observed 26samples (n=40) in the study group and 15 samples (n=40) in the control group and this difference was found to be statistically significant (p-0.021).Caspase-3 activity assessing apoptosis was 1.3 folds higher in the study group as compared to controls and this difference of OD in both cases and controls were statistically ($p = 0.001$) highly significant. Similarly, the level of oxidative stress parameters MDA & PC were significantly higher in the study group as compared to controls whereas antioxidant capacity as denoted by GSH was significantly ($p = 0.001$) lower in study group.
Conclusion(s): This study indicate that DNA damage as assessed by both quantitative (Caspase-3) and qualitative (ladder) test were significantly higher in infertile men as compare to fertile control. The levels of oxidants (MDA & PC) were also significantly higher and antioxidants capacity (GSH) was significantly lower in the infertile group. Therefore, the assessment of sperm DNA damage, Apoptosis and OS may be helpful to evaluate the cause of idiopathic male infertility and for the treatment.

KEYWORDS

Oxidative stress (OS), Malondialdehyde (MDA), Caspase -3, Ladder assay, protein carbonyl

INTRODUCTION

Infertility affects 15% of the married couples and in about 50% of cases male factor is the predominant causative factor where both qualitative and quantitative defects are seen in sperm production[1,2].

Though standard semen analysis is routinely used in the evaluation of male infertility, the exact mechanism behind the poor semen parameters is rarely understood. Varicocele, cryptorchidism, hypogonadism and infection are found to be the leading causes of infertility but approximately 30–40% is found to be idiopathic. Genetic (nuclear and mitochondrial) alteration has also been focused as an important aetiology in male infertility.

Reactive Oxygen Species (ROS) are highly reactive oxidising agents belonging to the class of free radicals. ROS at low levels facilitate hyperactivation, capacitation, acrosome reaction, motility, fertilisation and oocyte adhesion of spermatozoa, but higher concentrations of ROS create an oxidative stress (OS) and damage a variety of biomolecules such as lipids, amino acids, carbohydrates, protein and DNA and adversely affect the sperm function[1,2].

Hence any excess ROS must be constantly inactivated in order to maintain normal functions. This function is taken up by the anti-oxidants present in cellular plasma. When there is an excessive production of ROS or impaired anti-oxidant defense mechanism, oxidative stress occurs which is harmful to spermatozoa[1].

Excessive generation of ROS in semen may be associated with reduced sperm fertilizing potentials. Spermatozoa are rendered dysfunctional by lipid peroxidation and altered membrane function, together with impaired metabolism, morphology, and motility. Lipid peroxidation triggers the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, and structural damage to DNA, and cell death. The level of lipid peroxidation can be quantified by Malondialdehyde (MDA) levels, which is the end product of lipid peroxidation (LPO). Human spermatozoa possess the defense enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) or the reduced sulphhydral form (GSH). Estimation of these might be useful in the prediction of sperm fertilizing potentials[3].

Infertile men possess substantially more sperm DNA damage than do fertile men and that this DNA damage may adversely affect reproductive outcomes. Therefore, it is important to identify extent of DNA damage before initiation of therapy so that strategies that may reduce sperm DNA damage may be instituted.

The present study is thus planned, to assess the existing levels sperm DNA damage, apoptosis and oxidative stress in infertile men and normal fertile men.

MATERIALS AND METHOD

Study Population

Couples attending the infertility clinic of Department of Obstetrics and Gynaecology were evaluated for the factor of infertility. Male partners of women with normal fertility work-up viz. confirmed ovulation, patent tubes and absence of any endometrial or adnexal pathology formed the study group. Two abnormal semen analyses reports at 2-3 weeks interval, of oligozoospermia and/or asthenozoospermia, as per WHO guide line 1999, were considered essential for inclusion in study group (sperm density <20 million/ml, motility <50%). Men with normal semen parameters and having fathered a child in the preceding one year were taken as controls.

A detailed history with specific emphasis on occupational exposure to high temperature working environment, personal habits, including cigarette smoking alcohol consumption, substance abuse etc. were elicited from both the groups. Men with any form of substance abuse were taken as high risk category for the purpose of study and never users were taken as low risk.

In addition, men in the study group were subjected to detailed general physical examination and local examination to document secondary sexual characters, presence of cryptorchidism, varicocele or any other abnormality.

Sample collection

Semen samples from infertile male and controls were obtained by masturbation, without the use of lubricants after an abstinence of 3-5 days. The sample was collected in the semen collection room of Hospital Laboratory Services block of the hospital, in a sterile wide mouthed plastic container.

Semen samples from both the study (the second confirmatory sample of oligoasthenozoospermia) and control groups were taken and after liquefaction semen sample was utilized for various analyses in the following manner:

a) 0.5 ml of sample for standard semen analysis.
b) 5 aliquots of 200 µl each for assessment of DNA damage & apoptosis.
c) 1-1.2 ml sample for separation of seminal plasma to evaluate oxidative stress parameters.

Genomic DNA was extracted as per manufacturer protocol (XNAPS body fluid DNA Minispin Kit, Renogen Biolab, Canada) and extracted DNA was run on 1% agarose gel to check the presence and absence of isolated DNA in the samples and stored at -20˚C until for the ladder assay analysis.

Semen Analysis

After liquefaction, 0.5 ml sample was taken for semen analysis. The following conventional parameters were documented: volume, sperm density, motility, morphology and presence of pus cells with the help of microscope, macular chamber and slides.

Assessment of DNA damage

It was performed by using Ladder assay. The characteristic 'smear' on agarose gel was taken as a positive evidence of DNA damage.

Assessment of apoptosis

Apoptosis measured commercially available ELISA kit caspase-3 as per the manufacturer's protocol (caspase-3/CPP32 Colorimetric Assay Kit, Bio Vision)

Measurement of oxidative stress parameters

MDA and protein carbonyl was estimated as a measure of ROS and total reduced glutathione level was used to depict the antioxidant levels. Standard graph of MDA and GSH was used for calculation.

Statistical Analysis

Quantitative parameters by unpaired-t test and Qualitative parameters by Chi-square test. Coefficient of correlation was analyzed for comparison of semen parameters with DNA damage and oxidative stress parameters.

RESULTS

40 men satisfying the inclusion and exclusion criteria were recruited as the study group. For the control group, 45 men (husbands of women admitted in the postnatal ward) were screened and 5 excluded due to abnormal semen parameters.

Age distribution:

The mean age of cases and controls was 28.4 years and 29.6 years respectively. Both the mean age and distribution of cases amongst different age groups were comparable between the study and control groups.

Semen parameters:

Men recruited as control group were normozoospermic with a sperm count ranging from 50 to 190 million/ml (mean 103.75 million /ml) and rapid linear progressive motility ranging 50 to 90% (mean 79.25%). Of the 40 men in the study group, mild, moderate and severe degree of oligospermia (11-20mill/ml, 6-10 mill/ml and <5 mill/ml) were observed in 19, 14 and 7 patients respectively. A significant difference in the count, motility, abnormal morphology and WBC count was observed between cases and controls.

Assessment of DNA damage:

Characteristic smear depicting DNA damage was observed 26 samples (n=40) in the study group and 15 samples (n=40) in the control group and this difference was found to be statistically significant (p-0.021) (Table 1, Fig. 2). Ladder assay being a qualitative test, was analyzed by chi-square test. Since Ladder assay is a subjective assay of DNA damage, control and study samples were laid side by side on agarose gel plate for easy comparison of the smearing of damaged DNA.

Table 1: Comparison of DNA damage

Ladder assay smear	Study Cases (%)	Control	p-value
Present	26 (65%)	15 (36.5%)	0.021
Absent	14(35%)	25 (63.5%)	

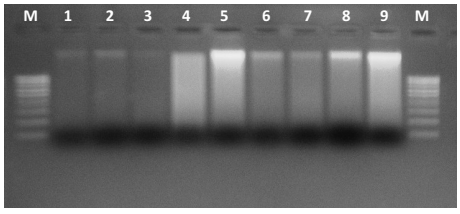

Fig. 2: Ladder Assay Agarose gel, Gel document system, ELISA plate shows smear of damaged DNA in lane 4, 5, 7, 8, 9 in cases and intact band of DNA in 1, 2, 3 in controls. The gel picture was captured by using gel document system.

When the DNA damage results were analyzed according to the degree of oligospermia, 58% cases in the mild oligospermia group exhibited DNA damage as against 71% in the severe oligospermia group. This difference however was not statistically significant (p-0.064)

Of the 25 cases with h/o substance abuse, DNA damage was positive in 17 cases as against 9 cases of DNA damage in the group without substance abuse. This difference was not statistically significant (p=1.000).

Ladder assay results in relation to age, socioeconomic status and occupation did not differ significantly between different groups.

In contrast to ladder assay qualitatively depicting early DNA damage, the end stage of irreparable DNA damage resulting in apoptosis could be quantified by Caspase -3 activity.

Caspase -3 activity reported as Optical Density (OD) at 405 nm in the microtiter plate reader varied in the study group from 0.048 – 0.093 with a mean of 0.070 ± 0.010 as compared to a mean of 0.054 ± 0.005 in the control group (p-0.001).

Biochemical Analysis

Apoptosis and Oxidative Stress Assessment

Caspase-3 activity assessing apoptosis was 1.3 folds higher in the study group as compared to controls and this difference was statistically (p = 0.001) highly significant. Similarly, the level of oxidative stress parameters MDA & PC were significantly higher in the study group as compared to controls whereas antioxidant capacity as denoted by GSH was significantly (p = 0.001) lower in study group (Table 2).

Table 2: Comparison of Apoptosis and Seminal Plasma Oxidative Stress Parameters

Biochemical Parameters	Case (mean+ S.D)	Control (mean+ S.D)	p-value
Caspase 3	0.070+0.010	0.054+0.005	**0.001***
MDA (nmol/ml)	4.833 + 1.36	3.525 + 1.514	**0.001***
PC (nmol/mg of protein)	0.914 + .214	0.643 +.140	**0.001***
GSH (mg/dl)	19.08+2.269	20.26+2.080	**0.010***

Independent t test was used to analyze the oxidative stress parameters and apoptosis marker p-value* <0.05 was considered as significant.

With increasing sperm density there was declining trend in the MDA content in both the study and control groups and increasing trend of GSH content with increasing sperm counts (Fig. 3a, 3b, 3c).

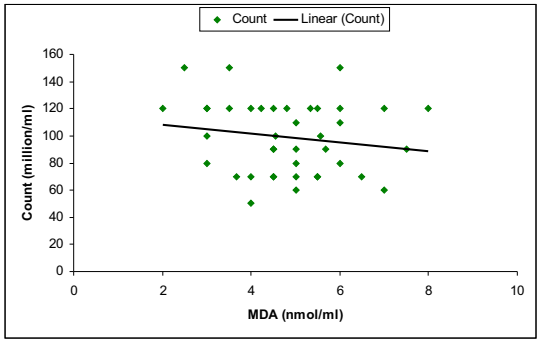

Fig. 3a: Scatter Diagram Depicting Relationship of MDA levels with Sperm Density in controls

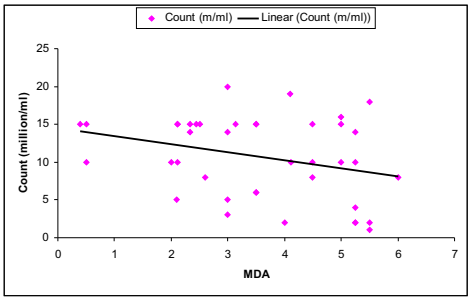

Fig. 3b: Scatter Diagram Depicting Relationship of MDA levels with Sperm Density in cases

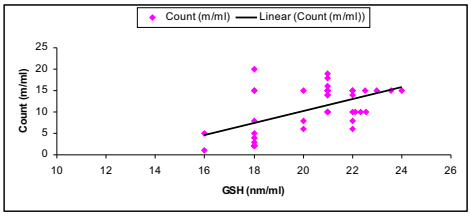

Fig. 3c: Scatter Diagram Depicting increasing trend of GSH content with increasing sperm counts

In present study Pearsons coefficient of correlation was measured between OS parameters (MDA & PC), antioxidant and apoptosis. A negative correlation of MDA & PC with GSH was observed, which, however was not significant No significant correlation was observed with caspase-3 activity (Table 3).

Table 3: Correlation of OS markers with anti oxidant and apoptosis

Variables	GSH	
	R*	p –value
MDA	-0.031	0.849
PC	-0.047	0.777
CASPASE-3	-0.433	.006

*Pearson's correlation coefficient

Categorizing the study group according to WHO Criteria 2010, the mild oligospermic cohort could be further divided into 2 groups with 11-14 million/ ml count (6 cases) and 15-20 million/ml counts (13 cases). Sub group analysis in these cases of mild oligospermia failed to detect any statistical significance in any of the parameters assessed.

DISCUSSION

Though semen analysis is still considered as fundamental diagnostic step in the evaluation of male infertility, it fails to detect the fertilizing capacity of the sperm. Whether oxidative insult is the main factor resulting in DNA damage and subsequent apoptosis thereby causing oligoasthenospermia is the question being addressed in the present study.

Not only were the sperm count and motility significantly different between the groups (inherent to recruitment criteria), even abnormal morphology and WBC count were significantly higher in the study group.

In the present study substance abuse did not show any have significant effect on semen parameters. There are controversial reports about the detrimental effect of cigarette smoking on sperm quality.

In the present study, DNA damage as assessed by ladder assay which gives a qualitative measure was found to be significantly higher (p-value 0.0001) between groups. DNA damage assessment by ladder assay has been mostly applied in blood samples. The same was utilized in the present study due to the simple methodology and easy availability of the test in the institution. Studies reporting on sperm DNA damage have mostly utilized either qualitative methods by COMET or SCSA (Sperm chromatin structure assay) assay or quantitative assessment by 8-OHdG (8 hydroxyguanidine).

In the present study there was no correlation of DNA damage (measured by apoptosis) with the substance abused (smoking, alcohol etc).

In present study there were no relation of age with DNA damage. Singh et al demonstrate significant increase in sperm DNA damage with increase in age, a significant increase in mean comet extent ($p<0.004$). Percentage of sperm with highly damaged DNA exhibit a significant positive regression with age.

In the present study apoptosis was measured by caspase-3 activity and a statistically significant (0.001), difference was observed between Similarly Grunewald et al analysed caspase-3 activation in semen samples from subfertile males with oligoasthenoteratozoospermia and teratozoospermia revealed elevated levels of the active protease when compared to samples from healthy fertile donors[4].

In the present study there was a significant ($p=0.001$) increase of MDA content in cases as compared to controls (4.83 nmol/ml vs 3.52 nmol/ml). Similar reports of high MDA levels in oligoasthenospermic men have been reported by Tavilani et al[5] and Chaudhari et al[6]. Badade et al in addition to high MDA levels found negative co-relation of MDA with sperm count and motility[7].

Estimation of PC being a multistep lengthy procedure which is difficult besides time consuming, has been done only in a few studies.

In the present study, significantly higher content of PC in cases as compared to controls was observed in cases as compared to controls ($p=0.000$).

Saraniya et al observed significantly higher levels of malondialdehyde (MDA), protein carbonyl (PC) and protein-bound sialic acid (SA) in seminal plasma in 26 abnormal semen as compared with 24 normal semen samples[8].

In the present study, anti oxidant capacity was analysed by determining GSH levels and, there was significant difference ($p<0.001$) between groups. Similar results of significantly higher GSH levels have been reported by Chaudhari et al[6].

In the present study there was a negative but non significant correlation of GSH with MDA of seminal plasma. On the contrary Tavilani et al evaluating multiple antioxidants like CAT (catalase), SOD (superoxide dismutase) & GPX (glutathione peroxidase), reported a positive but again non significant correlation with MDA[5].

Chaudhari et al revealed that GSH levels were statistically significant and negatively correlated with MDA level[6].

Similar to MDA, in the present study PC content of seminal plasma in the cases was negatively correlated (-0.096) with GSH but not significant.

CONCLUSION

The present study showed a significant difference between the infertile men and fertile controls the exact cause of this difference is not known. Hence in these idiopathic cases, we attempted to see the difference of DNA damage, Apoptosis and OS parameters between infertile and fertile patients. GSH which denotes antioxidants capacity was significantly lower in the study group.

REFERENCES

1. Ochsendorf FR. Sexually transmitted infections: impact on male fertility. Andrologia 2008;40:72–75.
2. Venkatesh S, Deecaraman M, Kumar R, Shamsi MB, Dada R. Rate of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. Indian J Med Res 2009;129:127-137.
3. Venkatesh S, Riyaz AM, Shamsi MB, Kumar R, Gupta NP, Mittal S, et al. Clinical significance of reactive oxygen species in semen of infertile Indian men. Int J Endro 2009;41;251-256.
4. Grunewald S, Sharma R, Paasch U, Glander HJ, Agarwal A. Impact of caspase activation in human spermatozoa. Microsc Res Tech 2009;72(11):878-88.
5. Tavilani H, Goodarzi MT, Vaisi-raygani A, Salimi S, Hassanzadeh T. Activity of antioxidant enzymes in seminal plasma and their relationship with lipid peroxidation of spermatozoa. Int Braz J Urol 2008;34(4):485-91.
6. Chaudhari AR, Das P, Singh R. Study of oxidative stress and reduced glutathione levels in seminal plasma of human subjects with different fertility potential. Biomed Res 2008;19(3):207-210.
7. Badade ZG, More K, Narshetty J. Oxidative stress adversely affects spermatogenesis in male infertility' Biomed Res 2011;22(3):323-328.
8. Saraniya A, Koner BC, Doureradjou P, Selvaraj N, Shivagourou V. Altered malondialdehyde, protein carbonyl and sialic acid levels in seminal plasma of microscopically abnormal semen. Andrologia 2008;40(1):56-7.