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Original Article

Expression of PARP1 in primary infertility patients and correlation with DNA fragmentation index a pilot study

Swetasmita Mishra [a], *Rajeev Kumar* [b], *Neena Malhotra* [c], *Rima Dada* [a,*]

[a] *Laboratory for Molecular Reproduction and Genetics, Department of Anatomy, All India Institute of Medical Sciences, New Delhi 110029, India*

[b] *Department of Urology, All India Institute of Medical Sciences, New Delhi 110029, India*

[c] *Department of Obstetrics & Gynaecology, All India Institute of Medical Sciences, New Delhi 110029, India*

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ABSTRACT

Purpose: Sperm DNA damage is associated with poor assisted reproductive technique (ART) outcome, birth of offspring with major congenital malformation, recurrent spontaneous abortions after assisted and spontaneous conception. The presence of sperm in the ejaculate with high levels of DNA damage indicates either a defect or deficiency in sperm DNA repair mechanisms. ADP ribosylation brought about by poly ADP ribosylases (PARP) of proteins helps in recognition of DNA breaks and recruitment of DNA base excision repair (BER) enzymes. This study was planned to analyse the expression levels of PARP1 (PARP family member) in the sperm samples from both infertile (with normal semen parameters) and control (fertile) men and correlate with sperm DNA damage.

Methods: The study included both infertile men ($n = 29$) and controls ($n = 17$). The expression level of PARP1 was quantified by qPCR. For all samples Comet assay and sperm chromatin structure assay (SCSA) was performed and DNA fragmentation index (DFI) calculated.

Results: Relative quantification showed that the level of expression of PARP1 was significantly ($P < 0.0001$) lower and percentage DFI significantly ($P = 0.0015$) higher in sperm samples from infertile men compared to controls. There was a negative correlation between PARP1 levels and sperm DNA damage.

Conclusion: Abnormally low expression of DNA repair enzymes explains for the persistence of DNA damage in sperm. Low levels of PARP can explain for persistence of sperm DNA damage. This may be aetiology of infertility or recurrent pre implantation losses following assisted reproduction in cases with idiopathic infertility.

* *Corresponding author*. Tel.: +91 (0) 11 26593517; fax: +91 (0) 11 26588663.
E-mail address: mk_official@rediffmail.com (R. Dada).

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1. Introduction

A large number of infertility cases are idiopathic. Several infertile men are normozoospermic but recent studies have shown alteration at molecular levels in sperm (increased sperm DNA damage, severe oxidative stress) in such cases. Infertility affects 1 in 5 couples in the reproductive age group. In the era of assisted reproduction, majority of infertile couples are opting for these techniques. However recent studies have reported that men with normal and abnormal sperm parameters may have high levels of seminal oxidative stress and DNA damage.[1,2] High DNA damage may explain for poor outcome of assisted reproductive technique (ART), impaired fertilization, recurrent spontaneous abortion, congenital malformation and even cancer (ongoing studies in our laboratory). Persistence of DNA damage in germ cells is critical which shows that there may be defects/deficiency in DNA repair mechanisms.[3–5] This may also aid in understanding the aetiology of increased incidence of gonadal and extragonadal tumours in infertile men.[6] Infertile men have 4–5 fold increase in incidence of gonadal tumours. DNA damage in sperm occurs by 3 main causes. Firstly, environmental exposure to various agents like pollutants (insecticides, pesticides, phthalates, bisphenol A and electromagnetic radiations), xenobiotics and high temperature, secondly as a result of persistence of nicks following meiosis and germ cells and third cause being defective chromatin packaging and abortive apoptosis.[7,8] Due to these causes DNA molecules undergo alterations in the form of base modifications, single strand breaks (SSBs), double strand breaks (DSBs), and intrastrand or interstrand cross-links. In order to maintain genomic integrity, nature has armed the cells with repair mechanisms to withstand or repair these lesions caused by various agents. These are base excision repair (BER), nucleotide-excision repair (NER), mismatch repair (MMR), and recombinational repair which includes homologous recombination (HR) and non-homologous end-joining (NHEJ). The post meiotic male germ cell undergoes extensive remodelling with replacement of majority of histones with transition proteins and the protamines. Thus results in sperm genome being one-sixth to one-twentieth size of genome of somatic cell and being transcriptionally inert. In a recent study from our laboratory we have established defective protamination, high seminal free radical levels and high sperm DFI by Comet assay and sperm chromatin structure assay.[9] All pathways of DNA repair are active before sperm undergoes compact packaging and become transcriptionally inert. Following this only BER pathway is active however recruitment of enzymes of this pathway is dependent on detection of DNA damage by ADP ribosylation of proteins by poly ADP ribosylases (PARP). Sperm accumulate maximum oxidative DNA damage in epididymis where they are stored and thus are exposed to high free radical levels. The male gamete is prone to several types of genetic changes in the form of mutations and breaks in sperm DNA that affect fertility.[10,11] In our previous studies we documented increased mitochondrial sequence variations and low antioxidant levels in infertile men.[12,13] Accumulation of mitochondrial sequence variations results in dysfunctional mitochondria and abortive apoptosis. This may result in persistence of sperm with DNA damage. Sperm in these men are more susceptible to oxidative injury. Oxidative damage severely affects the histone bound nucleosomal DNA and the guanine rich telomeric DNA. This damage leads to chromosomal instability and loss of genomic integrity. Maintenance of sperm chromatin structure and integrity is essential for embryo formation and birth of healthy offspring.[14] Higher degree of DNA damage results in decreased fertilization rate, improper embryonic development, decreased implantation and live birth rate. Sperm DNA damage is proposed to be a major cause of spontaneous abortions and may also result in increased incidence of children with retinoblastoma.[15,16]

Base lesions and single stranded breaks are mainly repaired by BER pathway. DNA damage triggers post translational modification of nuclear proteins especially histones. Modifications include mono or poly(ADP-ribosyl)ation of these proteins. Histone H1 and H2B are the mainly modified histones that further remodel chromatin structure. These changes act as information bank for proteins involved in DNA repair and transcription.[17] The post translational modification is brought about by PARP. The modification of proteins in the form of ADP ribosylation in response to DNA damage helps in recognition of DNA breaks and recruitment of the DNA base excision repair enzymes by the cells.[18] BER pathway involves a family of enzymes called PARP. PARP utilizes nicotinamide adenine dinucleotide (NAD+) for its activity. There are 17 members in the PARP super family. PARP1 is the most widely studied member of the PARP super family. PARP1 finds its importance in the BER pathway. It detects and binds to SSBs. It gets poly–ADP ribosylated itself using NAD+ and further ribosylates other BER enzymes like XRCC1 and polymerase beta and consequently assembles these repair enzymes to the SSB sites. PARP1 is also involved in NER pathway.[19,20]

To determine the diagnostic and prognostic role of sperm nuclear DNA fragmentation, it is required to understand the impact of DNA damage in ejaculated sperm on pre and post implantation embryonic development. The presence of PARP1 is documented in ejaculated spermatozoa. Sperm of infertile patients expressed lower levels of PARP1 as compared to the sperm of fertile donors which is suggestive of the involvement of PARP1 and its homologues in the maintenance of DNA integrity. Maintenance of DNA integrity is vital for normal fertilisation, pre & post implantation growth, embryogenesis and birth of healthy offspring. Ongoing study from our laboratory has shown that loss of paternal sperm DNA integrity is associated with recurrent pregnancy loss, congenital malformation & retinoblastoma (in non familial childhood cancer). Thus it is important to discuss the cause of DNA damage & why it is persisting in sperm. Immature sperm fractions express lower levels of PARP-1, -2, and -9 compared with the mature sperm fractions of both normal and infertile subjects which is suggestive of the role of PARP homologues in sperm maturation.[21] These reports suggest that PARP has an important role in maintaining genomic integrity in germ cells. Thus analysis of PARP levels is important as it is the key molecule for recruitment of downstream factors of BER pathway.

So, in order to study the underlying interplay between the DNA repair enzyme (PARP1) and sperm DNA damage, we

aimed to evaluate the differential expression of PARP1 in germ cells of males with primary idiopathic infertility with normal semen parameters compared to proven fertile controls in order to understand the aetiology of persistence of DNA damage in sperm.

2. Materials and methods

2.1. Spermatozoa purification

Institutional ethical clearance was obtained and informed consent was taken from the patients and the controls. The female partners of all these cases were normal after complete clinical, gynaecological, hormonal and radiological examination. Human ejaculates were obtained from 17 healthy volunteers of proven fertility, and 29 male partners of couple experiencing primary infertility within age group of 18–45 years. Semen analysis was assessed by World Health Organization (1999) criteria. These ejaculates were obtained from the Department of Gynaecology and Obstetrics and Department of Urology, AIIMS, New Delhi. Sperm cells were separated using 80 & 40% density gradient media – (PureCeption, USA). The cells were examined under microscope for any somatic cell contamination. These separated spermatozoa were then stored at −80 °C till RNA was isolated, which were then reverse-transcribed and investigated by qPCR analysis.

2.2. RNA extraction, cDNA synthesis and qPCR

Total RNA was isolated from spermatozoa using RNeasy Mini Kit (Qiagen, USA). RNA was quantified by spectrophotometry by determining the ratio of optical density at 260/280 nm using a NanoDrop spectrophotometer (Thermo Scientific, USA). RNA was treated with DNase I (Promega, USA) and reverse-transcribed into complementary DNA (cDNA) using random hexamer priming and Revert Aid™ M-MuLV reverse transcriptase (Fermentas, USA). From each sample, 1000 ng of RNA was used for cDNA synthesis. Quantitative analysis of each gene was performed by CFX96 Real Time System (Bio-Rad, USA) using SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, CA). Amplification reactions were performed in a 20 μl final volume containing 10 μl SsoFast™ EvaGreen Supermix, 1 μl primer and 4 μl cDNA. Amplification was done for 35 cycles each gene. To normalize the amount of expressed mRNA the internal housekeeping gene β-actin was used and each cDNA product was tested in triplicate. The relative quantification of target genes normalized to β-actin was calculated by 2-ΔCt method. The relative gene expression levels between controls ($n = 17$) and patients ($n = 29$) were compared by two sample *t*-tests using GraphPad software. If the *P* value was <0.05, the difference was considered to be statistically significant.

2.3. Comet assay

Comet assay on sperm was done according to a previously published protocol.[22] Briefly, the sperm embedded in microgel agarose slides were lysed and then electrophoresed to allow the migration of DNA fragments. The agarose slides were neutralised and further processed before staining with YOYO-1 Iodide.

2.4. Image analysis

The sperm comet images were observed on an Olympus microscope (BX 51) at 400× magnification. The analysis was done using Komet 5.5 software and percentage DNA in sperm comet head was assessed for quantifying the sperm DNA damage. Sperm with more DNA damage (higher DNA fragmentation) have smaller DNA fragments. On electrophoresis, the smaller DNA fragments will migrate faster owing to low molecular weight of the fragment. Under such conditions the percentage DNA in comet head will be lesser for a sperm with greater DNA damage as compared to sperm with intact or less DNA damage. For each sample 200 sperm comets were analysed.

2.5. Sperm chromatin structure assay (SCSA)

The frozen aliquot from each ejaculate was thawed in a water bath at 37 °C for 30 s and diluted to a concentration of 2×10^6 sperm/ml in tris sodium chloride-EDTA (TNE) buffer to a total of 200 μl in a falcon tube. Immediately, 0.4 ml of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% v/v Triton X-100, pH 1.2) was added to the Falcon tube. After exactly 30 s, 1.2 ml of acridine orange (AO)-staining solution [6 μg AO (chromatographically purified) (Polysciences, Inc. – USA) per ml citrate buffer (0.037 M citric acid, 0.126 M Na2HPO4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0)] was added. For every six test samples, one standard reference sample was analysed to ensure instrument stability. The samples were analysed using a FAC Scan flow cytometer (BD Biosciences, USA), with an air-cooled argon laser operated at 488 nm and a power of 15 mW. The green fluorescence (FL1) was collected through a 515–545 nm bandpass filter, and the red fluorescence (FL3) was collected through a 650 nm longpass filter (DNA in spermatozoa having abnormal chromatin structure has increased red fluorescence). The sheath/sample was set on 'low', adjusted to a flow rate of 200 events/s when analysing a sample containing 2×10^6 sperm/ml. Immediately after the addition of the AO staining solution, the sample was placed in the flow cytometer and run through the flow system. All the samples were assessed in duplicate at one month interval and the average was taken. After complete analysis of sample, the X-mean (red fluorescence) and Y-mean (green fluorescence) values were recorded manually after selecting gate for sperm cells using FlowJo software (Oregon, USA). Strict quality control was maintained throughout the experiment. Post-acquisition, DFI calculation was performed offline using Flowjo software. The sperm cells are gated after excluding debris and high DNA stainability (HDS) cells and mean values of red and green fluorescence were recorded manually. The DNA fragmentation index was then calculated by the formula, DFI = mean red fluorescence/(mean red fluorescence + mean green fluorescence). Statistical analysis of data was performed between infertile and fertile groups using Student's *t*-test and Mann–Whitney test.

3. Results

3.1. PARP1 expression

Mean ΔΔ Ct value of PARP1 in patient samples was lower (0.0302 ± 0.0274) compared to controls (0.2380 ± 0.1160) (Fig. 1). Therefore expression of PARP1 was significantly lower ($P < 0.0001$) in idiopathic infertile men as compared to fertile controls.

3.2. Sperm DNA damage

DNA damage was assessed by two techniques, SCSA and Comet assay. By SCSA, DNA Fragmentation Index (DFI) was found to be significantly higher ($P = 0.0015$) in sperm samples from infertile men (29.02 ± 5.6) as compared to controls (23.37 ± 3.6) (Fig. 2). By Comet assay the DNA fragmentation index (DFI) in infertile men was also found to be higher (37.23 ± 15.18) as compared to controls (12.13 ± 7.18) (Fig. 3). The difference in DFI analysed by SCSA and Comet assay was statistically non significant ($P > 0.05$). There was a negative correlation between PARP1 levels and DFI (Table 1).

4. Discussion

PARPs consist of a family of enzymes, which mediate poly-ADP-ribosylation of proteins a post translational modification.[20–23] This enzyme plays a critical role in spermatogenesis and oogenesis and maintenance of testicular germline.[24] This family consists of about 17 members. But two enzymes, PARP-1 and PARP-2 have been extensively studied. These enzymes are activated by DNA strand breaks which are detected by DNA-binding domains of these enzymes.[25] PARP1 is most abundant and is found in nucleus and highly conserved during evolution and catalyses the cleavage of nicotinamide adenine dinucleotide (NAD+) to form a protein bound branched poly (ADP-ribose) (PAR) units on aspartic and glutamic residues of the enzyme itself and on its substrates.

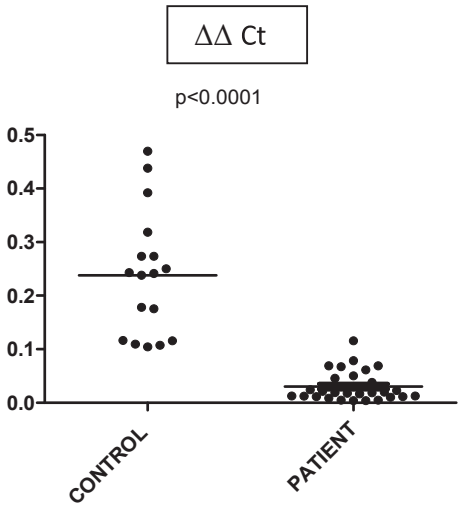

Fig. 1 – Expression of PARP1 in controls and patients.

By accumulation of PARs, chromatin acquires an open configuration which facilitates transcription. An enzyme called poly (ADP-ribose) glycohydrolase (PARG) regulates PAR protein levels in chromatin. PARP1 is localized to constitutive heterochromatin region like centromere and telomere. During excessive DNA damage this enzyme is cleaved into 24 kDa and 84 kDa domains as a marker of apoptosis. PARP1 plays an important role during germ cell differentiation during spermiogenesis and chromatin remodelling and thus is expressed more in the basal region of seminiferous tubules but not in the luminal region. The expression of PARP1 decreases with the development of the sperm. This decrease in PARP1 levels may be due to changes in chromatin structure during remodelling in spermatogenesis thus PARP1 has a role in meiosis and spermiogenesis. PARP1 modifies histones and remodel chromatin structure which recruits other proteins involved in DNA repair and gene regulation.[17] Therefore this enzyme can interact with coactivators and DNA-binding factors, which further bind to enhancer promoter region.[26] PARP1 has a well defined role in apoptosis and oxidative stress. In oxidative stress induced DNA damage PARP1 recruits DNA repair protein XRCC1 to the site of damage. For breaking down of oxidatively damaged histones it activates 20S proteosome.[27] DNA fragmentation (DFI > 30%) is a major cause of idiopathic male infertility and DFI > 26% was found in idiopathic recurrent spontaneous abortions and is a major cause of low success rate of IVF/ICSI.[1] Sperm DNA damage prior to post meiotic remodelling of sperm DNA occurs in BER pathway. However following sperm genome compaction, BER is the only DNA repair pathway which is active. Also sperm DNA damage is corrected by oocyte repair mechanism following fertilisation, however this is dependent on type of DNA damage and quality of oocyte. Accumulations of etheno nucleosides (oxidative DNA damage by products) inhibit oocyte BER and with ageing oocyte may have defective genetic filter. This may result in persistence of sperm DNA damage. This may not impair fertilisation, but affect early embryogenesis.[28] Recent studies have shown role of sperm DNA/mRNA in early embryonic development and genes of developmental importance like HOX, WNT are found in the histone bound fractions of sperm DNA and thus are prone to oxidative damage and other environmental insult. Thus it is crucial that sperm DNA damage is repaired to prevent damage to histone bound DNA fraction, which translates genes of critical developmental importance. Thus the role of PARP1 as a key gene for DNA repair is crucial. In this pilot study we have observed relatively lower expression of PARP1 in the idiopathic infertile men (with normal semen parameters) as compared to healthy controls. In future we will further validate these findings by immunoblotting using PARP1 antibodies and quantify DNA damage by gamma-H2AX staining. These men have high levels of sperm DNA damage as confirmed by SCSA and Comet assay. Increased DFI and decreased PARP1 expression in the infertile men may explain for the inefficient DNA repair in the sperm and may explain for the persistence of DNA damage in sperm of infertile men with normal and abnormal sperm parameters. This may be the underlying link of male infertility and testicular cancer and recently it has been documented that, male infertility may be an early marker of testicular cancer.[29–31] This may be explained by accumulation of highly

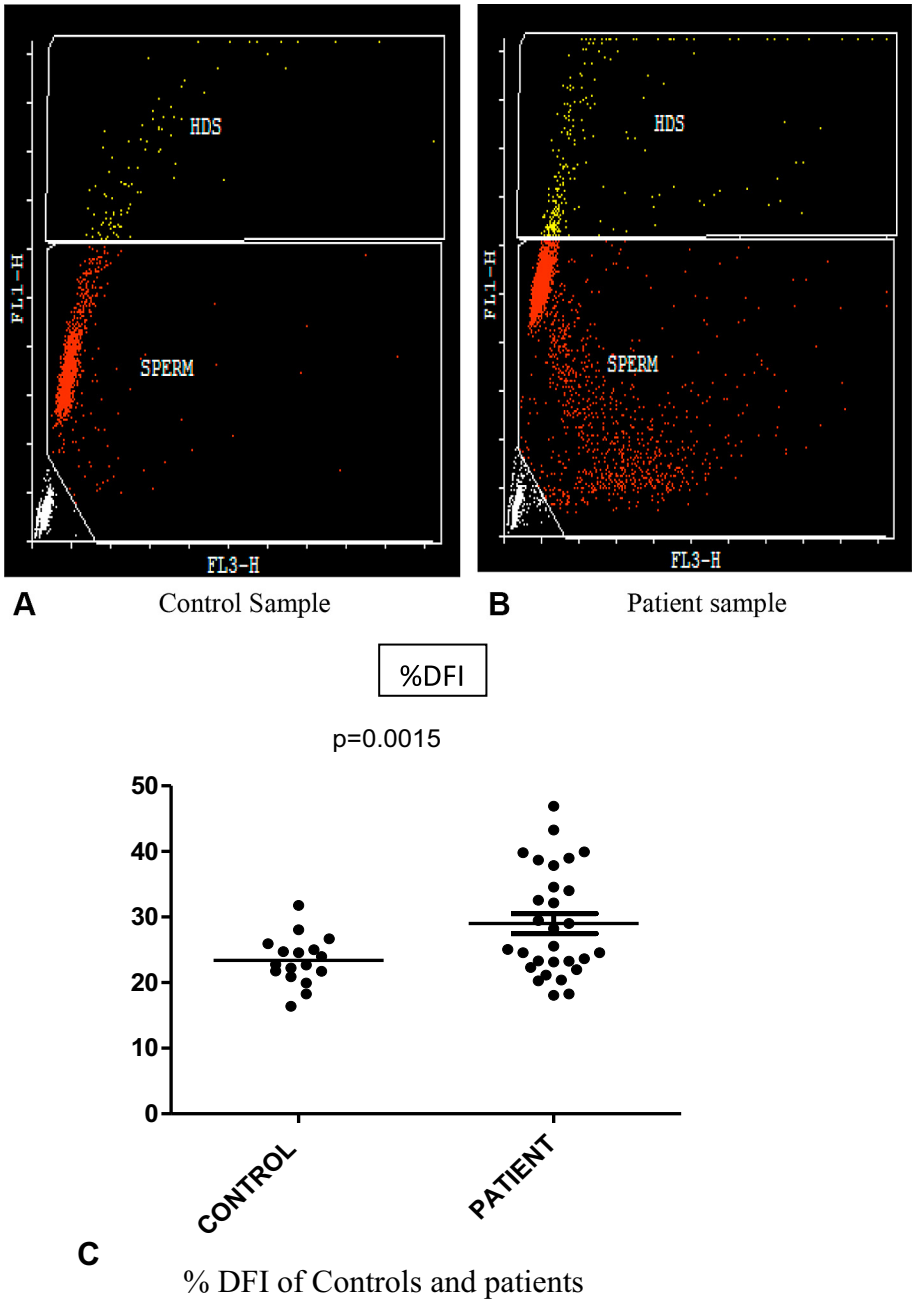

Fig. 2 – Pseudocolour dot plot cytograms of semen samples by SCSA. X-axis represents fragmented DNA and Y-axis represents native DNA.

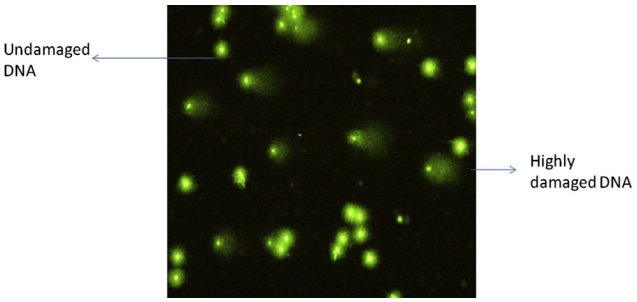

Fig. 3 – Results of Comet assay.

mutagenic base (8-hydroxy-deoxy-guanosine), SSB, DSB, shorter telomeres (which confer genomic integrity), lower levels of DNA repair enzymes and dysfunctional mitochondria all found in sperm of infertile men. In a pilot study of 50 children with retinoblastoma, we found father had high levels of DNA damage and even more in cases who was alcoholic and smoker. Thus life style factors can profoundly impact DNA integrity. Previous studies in our laboratory have documented that, chief cause of DNA damage is oxidative stress and in few cases mutations in protamines & transition protein genes.[9,32] These mutations disrupt packaging of sperm DNA into a compact toroid. However DNA damage is usually

Table 1 – DFI and Mean ΔΔ Ct values of controls and patients.

	Controls ($n = 17$)	Patients ($n = 29$)
Mean % DFI (SCSA)	23.37 ± 3.6	29.02 ± 5.6
Mean ΔΔ Ct Value	0.2380 ± 0.1160	0.0302 ± 0.0274

repaired by various repair mechanisms. Thus it is important to study why this damage persists. As PARP1, which is activated following DNA damage is one of key factors and recruits other factors once activated, it is important to access if its expression levels are optimal or there are defects in downstream genes and pathways. Further studies are required to investigate the underlying causes of lower expression levels of PARP1 in the germ cells. Whether this is due to sequence alterations and promoter methylation in the PARP1 gene itself or there are some other modulating factors that affect the expression of this crucial enzyme in the sperm cells of idiopathic infertile men remains unanswered. Such studies will aid in understanding the aetiology of paternal sperm DNA damage.

Conflicts of interest

All authors have none to declare.

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