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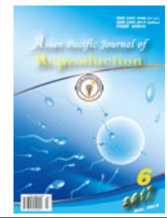

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Effect of low level, short wavelength ultraviolet radiation on sperm chromatin

Rishika Sharma[1], Nidhi Rajput[1], Kanthi Bansal[2], Hyacinth Highland[1]✉

[1]*Department of Zoology, BMTC and Human Genetics, School of Sciences, Gujarat University, Ahmedabad*

[2]*Safal Fertility Foundation and Bansal Hospital, Navrangpura, Ahmedabad*

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ABSTRACT

Objective: To evaluate the effects of low level ultraviolet (UV) radiation on sperm chromatin structure. **Methods:** The target study was divided into three groups: (i) males with proven fertility (*n*=40) was taken as Group Ⅰ (control); (ii) Oligoasthenozoospermic (OAT) cases as Group Ⅱ (*n*=36); (iii) males with unexplained infertility (MUI) cases (*n*=42) as Group Ⅲ. Specific techniques were used to study the impact of UV radiation (Pre and Post UV exposure) on the sperm nuclear DNA viz. Aniline blue staining was for detection of immature chromatin. Chromomycin A3 fluorescence staining was used to determine protamine-DNA dissociation by intense fluorescence of protamine deficient sperm cells and neutral comet assay was for evaluation of DNA fragmentation. Statistical analysis was carried out using Student's *t*-test (GraphPad Prism Version-6). Level of significance was considered at $P<0.05$. **Results:** The data revealed that spermatozoa of OAT and MUI cases when exposed to UV-radiation showed higher percentage of immature sperm compared with normal fertile individuals. Higher percentage of Chromomycin A3 fluorescence in OAT and MUI cases revealed impaired DNA-protamine binding with subsequent DNA fragmentation after exposure to UV. **Conclusions:** In the present study, sperm chromatin is at higher risk of DNA fragmentation in the infertile (OAT and MUI) cases due to UV irradiation (254 nm). Hence, short duration UV exposure is detrimental to sperm DNA which could affect ensuing generations.

1. Introduction

The sperm cell has proven to be the most versatile and perhaps the most enigmatic of all the varied cells in the living world[1]. The human sperm is replete with distinctive organelles and is a highly specialized cell that does not grow, divide, replicate their DNA nor synthesize protein. Yet evidence of mRNA indicates that it does undertake a certain level of gene expression. The prime function of this cell is to ensure that its haploid genome reaches the site of fertilization intact. In spite of the high degree of specialization, spermatozoa prove to be extremely vulnerable to environmental stressors and are the major contributors to poor fertility[2]. The defects underlying the defective spermatozoa are due to both genetic and environmental factors[3]. According to Aitken and Sawyer[4], poor DNA integrity and active production of reactive oxygen species (ROS) are responsible for faulty spermatozoon and this could be due to their exposure to environmental toxicants. Environmental factors have the potency not only to alter the sperm parameters (count, motility, cellular morphology) but also to impact the genetic integrity[5] by chromatin remodelling, inducing apoptotic processes of DNA degradation and increasing spermatic DNA fragmentation[6]. Furthermore, Kumar *et al*[7] have elucidated the

✉Corresponding author: Dr. Hyacinth Highland, Department of Zoology, BMTC and Human Genetics, School of Sciences, Gujarat University, Ahmedabad-380009.
E-mail: hnhighland@gujaratuniversity.ac.in
First author: Rishika Sharma, Department of zoology, Biomedical Technology, and Human Genetics, Gujarat University, Ahmedabad 380009, Gujarat India.
E-mail: rishikasharma1503@gmail.com

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detrimental effects of environmental factors in reproductive health in Indian men. As mentioned by Wang *et al*[8], the human sperm is therefore more vulnerable to environmental and occupational exposure leading to potent loss in sperm quality. According to WHO report[9], 250 million cases of work related to injuries are reported per year. Individuals associated with welding industry experience have various health hazards apart from injuries. Some of which include infrared radiation and ultraviolet (UV) exposure, fumes, particulate generation and occupational heat stress[10]. In the past few decades, man is increasingly exposed to low wavelength UV. It was also shown by Krutmann[11] that UV light specifically affects cell membrane, alters its membrane permeability and induces DNA damage. UV radiation has detrimental effects on human spermatozoa. It may disrupt the DNA structure through DNA fragmentation and induce ROS formation[12]. In addition, the sperm chromatin structure is known to be the most unique among the varied eukaryotic cells. This exceptionally organized chromatin structure is correlated with the firm packing of the DNA. Any distortion in the chromatin packaging of the DNA due to UV exposure would lead to a direct attack on the highly susceptible sperm DNA. Therefore, the present study aims at evaluating the effect of a specific wavelength of UV radiation which forms a large fraction of the radiation exposure to people working at blast furnaces, welders and industrial heating units. The main aim of this study was to determine the impact of UV radiation on the sperm nuclear integrity in semen of both normal and infertile man. Specifically, the study was designed to ascertain whether spermatozoa of normal volunteers and infertile individuals manifest a differential susceptibility to environmental stressors such as UV radiation.

2. Materials and methods

2.1. Study design

Semen samples were freshly collected from a recognized *in–vitro* fertilization clinic. Preliminary analysis such as count and motility was evaluated in each case using the standard of WHO methods[13], and based on this preliminary analysis the selected cases were grouped as follows:

Group Ⅰ: Normal, control males of proven fertility, aged 25-40 years (n=40)

Group Ⅱ: Males with Oligoasthenozoospermia (OAT), aged 25-40 years (n=36)

Group Ⅲ: Males with Unexplained infertility (MUI), aged 25-40 years (n=42)

The present study has the approval from Human Ethics Committee, Department of Zoology (November-12/2015). Consent from the patients was duly taken.

Patients with history of infection, immune factors, on drug/ medication, with habits of smoking/alcohol were excluded from the study. Samples from each group were placed at a 1 meter distance from a UV source of wavelength 254 nm and evaluated before and after the *in–vitro* exposure to UV light for 15 min. The duration of 15 min was selected based on pilot studies which indicated onset of visible damage only after 15 min. The selected parameters for assessing the sperm chromatin integrity were taken as follows.

2.2. Acidic aniline blue staining

Acidic aniline blue staining described by Hoffmann and Hilscher[14] was for evaluating sperm chromatin maturity. Chromatin maturity is achieved by protamines which are arginine and cysteine rich proteins that tightly compact the sperm DNA. This technique therefore reveals differences in the basic nuclear protein composition of ejaculated human spermatozoa. The percentage of spermatozoa stained with aniline blue was determined by counting 100 spermatozoa per slide under bright field microscopy using an Olympus binocular microscope under 40 × magnification.

2.3. Chromomycin A3 (CMA3) staining

Staining with the fluorochrome CMA3 was carried out to determine protamine deficient spermatozoa or protamine-DNA dissociation according to the technique given by Manicardi *et al*[15]. CMA3 is a guanine-cytosine-specific fluorochrome, and reveals that chromatin is poorly packaged in human spermatozoa via indirect visualization of protamine-deficient DNA. CMA3 and protamines compete for the same binding sites in the DNA. Therefore, high CMA3 fluorescence is a strong indicator for the spermatozoa in low protamination state. A total of 100 spermatozoa were randomly evaluated on each slide. Evaluation of CMA3 staining is done by distinguishing spermatozoa that stain bright yellow (CMA3 positive) from those that stain a dull yellow (CMA3 negative) by using binocular Lawrence and Mayo (LYNX) fluorescent microscope. Photographs were taken at 40 × magnification using a digital camera.

2.4. Neutral comet assay

Neutral comet assay as described by Haines *et al*[16], the single-cell gel electrophoresis was performed to assess the fragmentation in the double stranded DNA on irradiation with short-wavelength UV light. Neutral electrophoresis buffer conditions were used to show that the migration of double-stranded DNA loops from a damaged cell in the form of a tail unwinding from the relaxed super-coiled nucleus was proportional to the extent of damage inflicted on the cell. This finding took the appearance of a comet with a tail when viewed under the fluorescent microscope. The damage is quantified by measuring the displacement between the genetic material of the nucleus ‘comet head’ and the resulting tail. The tail lengths are used as an index for

the damage. Slide was stained with Ethidium bromide and observed under fluorescent microscope (LYNX) at 40 × magnification. Images of 60-70 cells with camera attached to fluorescent microscope were taken. Image analysis software Comet Assay Software Project was used to measure the comet tail. The software measured the DNA in terms of percentage of Head DNA, and percent DNA in tail and tail moment.

2.5. Statistical analysis

Each parameter was expressed as mean±SE. The Student's *t*-test was used and level of significance was calculated at $P<0.05$.

3. Results

3.1. Sperm chromatin maturity

As shown in Table 1, the sperms stained with acidic aniline blue showed a lower percentage of chromatin maturity in the cases of OATs and MUI when compared with normal samples. There was a significant decrease in chromatin maturity after exposure of spermatozoa to UV light ($P<0.001$) (Figure 1).

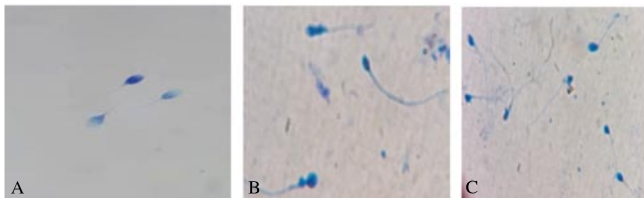

Figure 1. UV irradiated spermatozoa by aniline blue staining in selected cases.
A: Normal; B: OAT; C: MUI.

3.2. Protamine–DNA dissociation

Table 1 shows a highly significant ($P<0.001$) increase in sperm Protamine-DNA dissociation in the cases of OATs and MUI compared with control group. This suggests an increase in protamine-DNA dissociation and disrupted chromatin structure in the cases evaluated (Figure 2).

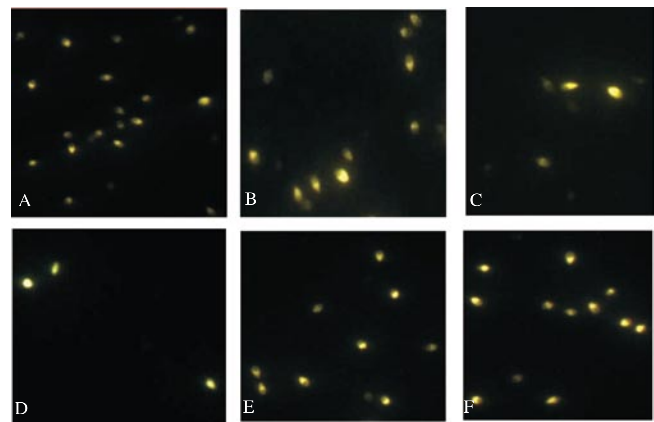

Figure 2. CMA3 staining in the selected cases.
A: Pre-treated normal; B: UV exposed normal; C: Pre-treated OAT case; D: UV exposed OAT; E: Pre-treated MUI; F: UV exposed MUI.

3.3. Comet assay analysis

As shown in Table 1, the results from the comet assay measured the percentage of dispersed chromatin, observed as halo and comet tails formed in each group before and after UV exposure. DNA leaching observed as Halo formation was observed to be significantly higher ($P<0.001$) in the OATs and MUI cases when irradiated with UV light. The percentage of comet tail formation was also seen more in cases with OATs when compared with normal and MUI cases (Figure 3).

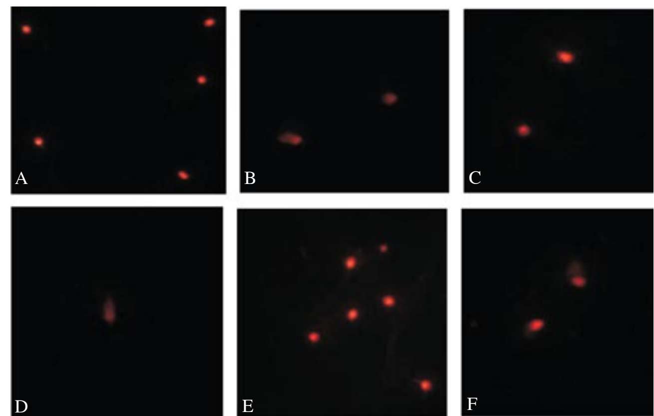

Figure 3. DNA damage in sperm by comet assay in the selected cases.
A: Pre-treated normal; B: UV exposed normal; C: Pre-treated OAT; D: UV exposed OAT; E: Pre-treated MUI; F: UV exposed MUI.

Table 1
Percentage of sperm chromatin maturity (aniline blue staining), protamine binding efficiency (CMA3 staining) and neutral comet assay in normal, OATs and MUI (mean±SE).

Groups	Aniline blue		CMA3 staining		Neutral comet assay			
	Pre-UV treated	UV-exposed	Pre-UV treated	UV-exposed	Pre-UV treated (halos)	UV-exposed (halos)	Pre-UV treated (comet)	UV-exposed (comet)
Ⅰ-Normal (*n*=40)	90.7±0.5	84.8±0.3	45.30±2.90	53.4±7.1	10.63±2.94	11.60±0.90	2.80±0.40	4.3±1.1
Ⅱ-OATs (*n*=36)	30.7±1.4#	23.0±0.8#	65.04±9.60#	84.2±7.5#	24.30±4.10#	37.30±0.95#	7.00±0.70#	14.0±0.9#
Ⅲ-MUI (*n*=42)	45.6±2.3#	31.8±1.2#	24.70±10.20#	64.7±5.3#	22.00±0.72#	35.80±4.70#	6.83±0.60#	10.6±2.3#

#significantly different compared with Group Ⅰ, $P<0.001$.

4. Discussion

Sperm chromatin being a very complex structure, its compaction, integrity and competence is taken as essential criteria for spermatozoon to be fertile. DNA integrity in sperm is essential for the accurate transmission of genetic information and in turn, maintenance of intact DNA for future generations. It is an independent measure of sperm quality that provides better diagnostic and prognostic capabilities than standard sperm parameters for male fertility potential[17]. Aitken and Sawyer[4] had observed that poor DNA integrity and excessive ROS production deteriorated the quality of the sperm, and one such cause attributed for which was the negative impact through environmental stressors. Exposure to UV radiation can therefore lead to DNA damage causing its fragmentation and inducing cell apoptosis[18]. The effect of UV irradiation had previously been reported based on experiments mainly on spermatozoa from marine species, which were naturally exposed to such putative damage with effects being reported on sperm motility and fertilization ability[19–22], membrane integrity[23] and DNA integrity[24]. Torres *et al*[25] reported that UV radiation attributes to decline in motility. The study carried out by Amaral *et al*[26] suggested that UV irradiation caused ROS induced damage in human spermatozoa. Buck Louis *et al*[27] have confirmed that persistent environmental pollutants work as reproductive toxicants. These reports have therefore triggered the objectives of the present study towards analysis of sperm nuclear changes in infertility and changes related to environmental stress impacts, specifically radiation in the UV region.

In the present study, the chromatin maturity of the sperm was visualized with acidic aniline blue stain which indicated that protamines were essential for DNA compaction and also for conferring mechanical and chemical stability to spermatozoa[28]. As discussed by Steger *et al*[29], during the later stages of spermatogenesis, the spermatid nucleus underwent specific modifications (*i.e.* replacement of histones with transition nuclear proteins and then finally with protamines) and condensation in order to have a compact DNA. Protamines play a key role in compaction thereby conferring stability of sperm nucleus[29,30], which in-turn safeguards the sperm genome from external stressors[31]. Normally sperm chromatin has tightly packaged DNA by protamines, however 15% of the DNA remains packaged by histones in a non-random association. These retained histones are kindred with nuclear periphery and telomeres, and thought to be involved with fertilization and early embryo development[32]. Therefore, samples from Group Ⅱ and Ⅲ showed a lower percentage of spermatozoa with mature chromatin when compared with normal individuals, which indicated that infertile individuals would have more percentage of retained histones thus resulting in poor chromatin compaction and rendering the spermatozoa more vulnerable to external as well as environmental stressors[33]. Hence a clear association could be established between impaired sperm chromatin maturation and male infertility. In the present investigation, the protamine-DNA dissociation in the sperm was evaluated by CMA3 assay. CMA3 is a guanine-cytosine-specific fluorochrome that competitively binds to DNA at protamine binding sites. Hence cells darkly stained with CMA3 suggest that chromatin is poorly packaged in human spermatozoa. Moreover, the increased percentage of CMA3 stained cells reflects dissociation of protamine binding to DNA in infertile samples. A higher percentage of sperm showing CMA3 fluorescence in the infertile group is therefore an indicator of low protamination state of spermatozoon and subsequent impaired chromatin packaging. Interestingly, CMA3 staining has been shown to be increased in the sperm cells of infertile patients by Nasr-Esfahani[34] and may also affect spermatogenesis and quality of semen[35]. Our observations revealed that CMA3 staining gave a high positive correlation with the data obtained from acidic aniline blue indicated chromatin maturity. This suggested that in infertile samples with lower chromatin maturity, the sperm nuclear chromatin has higher incidence of protamine dissociation from the DNA.

DNA integrity analysis is a better diagnostic and prognostic marker of sperm reproductive potential, which is the main aim of this study. This analysis laid great emphasis on determining the alteration in the sperm chromatin integrity which could lead to greater accessibility of the sperm DNA to damage. In the infertile cases, the high percentage of immature sperm, higher percentage of DNA-Protamine dissociation (CMA3 assay) were also positively correlated with the results of Comet assay showing elevated DNA fragmentation, with formation of comet tails in an increased number of spermatozoa.

Climate change, global warming, high environmental temperatures are multiple stressors that impact sperm function may compromise reproductive efficiency. Exposure to varied environmental degrading factors has been linked to reproductive defects, transmitted through damaged sperm DNA. Low wavelength UV radiation was selected as stress parameters because individuals were more exposed to this physical agent during industrial smelting, welding, metal fabrication and also from the environment. The findings obtained here reveal that UV irradiation at 254 nm has a deleterious impact on sperm DNA and proteins, causing disorganization in the sperm chromatin and fragmentation of DNA. This finding has a direct bearing on welders and persons occupationally exposed to this wavelength of UV radiation.

The present study has been directed towards the evaluation of deleterious effect of low level, short wavelength UV radiation (254 nm) on human spermatozoa in-vitro which could be directly proportional in causing molecular aberrations to spermatozoa and hence decreasing the fertility. Occupational exposures of UV radiation may prove to be harmful not only to the individual exposed to it but also to the future generations as it brings about disruption in the structure of chromatin thereby increasing DNA vulnerability to

fragmentation. The vitality and integrity of the human spermatozoa are highly compromised in the individuals when they are occupationally exposed to UV radiation. The present study has also revealed that exposure to UV radiation damages spermatozoa of all three groups including male with unexplained infertility, OAT and normal fertile.

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