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Effects of Chlamydia trachomatis infection on sperm chromatin condensation and DNA integrity

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SUMMARY:

The study was performed to investigate the relation of *Chlamydia trachomatis*infection to sperm chromatin/DNA integrityin a population of infertile men(male partner of infertile couples) from Iran. Blood,semen and first-voidurine samples were obtained from 250 infertile men.Data were analysed with regard to the results of (i) serological analysis forspecific antibodies to *C. trachomatis* in serum; (ii) the presence of *C. trachomatis* and DNA in first-voidurine; and (iii) in the semen sample of the male partner,in addition to sperm analysis, four different tests (Aniline Blue, Chromomycin A3, Acridine Orange and TUNEL) were used to detect sperm chromatin and DNA abnormalities.The main conclusions of the results were: 1) no evidence of *C. trachomatis* infection in semen samples was found; 2) sperm DNA fragmentation and chromatin studies were not correlated with *C. trachomatis* diagnosis; 3) the percentage of DNA fragmentation is positively correlated with the percentage of immotile sperm but negatively with semen volume, normal morphology; and 4) in sperm chromatin evaluations, only the percentage of chromatin protamination was related to male age.

Keywords:Infertility, C. trachomatis, Sperm DNA.

1- Introduction

Chlamydia trachomatis is one of the most prevalent sexually transmitted diseases worldwide. Previous work has shown that *C.trachomatis* IgG antibodies are correlated with tubal factor infertility and reduced pregnancy rates. However, the relevance of testing for *C.trachomatis* of the male partner is controversial. There has been little research on the organism in Iran and the incidence in infertile couples remains unknown. Our previous work (Dehghan Marvast, Aflatoonian, Talebi, Eley, & Pacey, 2016) showed thatthe prevalence of *C. trachomatis* was comparable to study populations elsewhere in the world and there was also a low level of concordance within couples. Men with such an infection in FVU samples (PCR positive) had only lower semen volume compared with men without infection(Dehghan Marvast, Aflatoonian, Talebi, Ghasemzadeh, & Pacey, 2015). Elevation of IL6 and 8 were observed in *C. trachomatis* positive men and this varied with diagnostic method. IL-6 &IL-8 levels were correlated with each other and the concentration of leucocytes, also the IL-8 was correlated negatively with semen volume and positively with age (Dehghan Marvast et al., 2015).

This study investigates the role of *Chlamydia trachomatis* (*C. trachomatis)* infection on sperm chromatin/DNA in male partners of infertile couples. A direct effect of *C. trachomatis* EBs on spermatozoa that can lead to infertility is premature sperm death (Al-Mously & Eley, 2015) through an apoptosis mechanism which is induced by *C. trachomatis* lipopolysaccharide (LPS) (Gallegos et al., 2008; Ploskonos & Nikolaev, 2013; Sellami et al., 2014). The mechanism as defined in different studies involves excessive production of reactive oxygen species (ROS) in leucocytes which has been induced by *C. trachomatis* lipopolysaccharide(Fraczek & Kurpisz, 2015) and ROS is mediator of apoptosis(Sellami et al., 2014; Tafuri, Ciani, Iorio, Esposito, & Cocchia, 2015).Patients with genitourinary infection by *C. trachomatis* and *M. Genitalium*showed increased sperm DNA fragmentation in comparison with fertile controls(Gallegos et al., 2008,(Ahmadi, Mirsalehian, & Bahador, 2016). This increase is proportionally greater than the influence on classical semen parameters and could result in a

decreased fertility potential (Gallegos *et al.,* 2008, (Robinson et al., 2012).Elevated levels of sperm DNA fragmentation are related to morphological abnormalities(Maettner et al., 2014) and it is believed that normal sperm morphology can be a valuable predictor of the fertilization rate if it is evaluated by strict criteria (Kruger *et al*., 1999).Assessment of DNA integrity may be a valuable marker of fertility for both animals and men. High level of chromatin damage has negative impact on both natural and ART conception (Bach & Schlegel, 2016; Oleszczuk, Giwercman, & Bungum, 2016).Although experimentally induced *in vivo*DNA damage of sperm is not possible in humans, strong associations have been shown between paternal genome damage by chemotherapeutic agents and embryo development in animals (Fernandez-Gonzales *et al*., 2008). The importance of studying sperm DNA integrity has been further intensified by the growing concern about transmission of genetic disease through ICSI (Palermo, Neri, & Rosenwaks, 2014).

Therefore in this studyfor first time in our knowledge the effects of *C. trachomatis* on spermDNA integrity was evaluated by different tests to examine thepercentage of sperm DNA abnormality in same time in infected males of infertile couples. In other word, the hypothesis that high levels of sperm DNA abnormality would be related to *C. trachomatis* infection was tested.

2- Materials and Methods

2-1- Participants

Sequential couples (n=324) attending the Research and Clinical Centre for Infertility (Yazd, Iran)presenting with primary and secondary infertility were screened for inclusion. All couples were approached with information and were asked to participate unless one or both of them had: (i) abnormal karyotype; (ii) history of chemotherapy or radiotherapy treatment; (iii) previous sterilisation; (iv) low semen volume (<1.0 ml) or retrograde ejaculation in the male partner; (v) hypogonadotropichypogonadism; (vi) a genital tract anomaly.Using these criteria, seventy-four couples were excluded and the remainder (n=250) were enrolled, with each partner giving informed consent.The study was approved by the Ministry of Health Research Ethics Committee,

Iran (Declaration no=11P/88k-A), as well as The University of Sheffield, School of Medicine Research Ethics Committee (SMBRER147).

2-2- Sampling

Of the 250 infertile couples enrolled each individual provided a 2-ml blood sample (1.5-ml serum) and 20-40 ml urine and also provided a semen sample.Blood was collected into a tube without any anticoagulant and within 6 hours was centrifuged (blood was clotted) at 1500 g for 10 minutes then the serum removed and stored at -20ºC.First void urine samples for both partners of infertile couples as well as the fertile controls were stored in arefrigerator immediately after collection and DNA extraction (see below)was performed within 2 days.

2-3- Semen analysis

Men produced their ejaculates after at least 48 hours sexual abstinence and semen analysis was performed according to World Health Organisation (1999) guidelines. Briefly, after 60 min liquefaction of semen at 37ºC the microscopic and macroscopic examinations wereperformed. Macroscopic examination included liquefaction, appearance, volume, viscosity, PHof semen sample. Semen PH was measured by PH paper and semen volume was measured by a graduated test tube(BD Biosciences, Bedford, USA)with manufacture scale reading from the base of meniscus.

Microscopic investigation included concentration, motility, agglutination of spermatozoa and the presence of cellular elements. For the assessment of sperm concentration the haemocytometer (Hawksley, London, UK) method was used. Motility was assessed by classifying 200 spermatozoa, and grading them to progressive motility; non-progressive motility and immotility. Motility was assessed at x400 magnification on a light microscope (Carl Ziessaxiostor plus, Kirchdorf, Germany).

IgA and IgG antibodies were assessed by the mixed antiglobulin reaction (MAR). The MAR tests were performed by mixing semen with latex particles coated with human IgG or IgA. Then,

a mono specific anti–human-IgG antiserum was added to the mixture using spermMar kit (Fertipro N.V., Aalter, Belgium). The presence of IgG or IgA is shown with the formation of a mixed agglutination. The number of sperm with beads attached was counted and expressed as a percentage.

For each patient two semen smears were prepared to assess sperm morphology and the dried slide was fixed in equal volume of Ethanol-Ether for 10 minutes, before being stored in the fridge and then stained by Papanicolaou staining method (WHO, 1999). Stained morphology slides were stored at room temperature (RT)for transportation to Sheffield and assessment by CASMA . Seminal plasma was removed after centrifuging fresh semen at 1000g 5 minutes then was stored at -80ºC before transportation back to Sheffield to measure inflammatory markers.

2-4- Sperm chromatin/DNA assessments

Four different tests were used to detect sperm chromatin and DNA abnormalities. To detect sperm single or double DNA strand breaks the TUNEL assay was performed for each specimen. The Calbiochem (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay) kit (Merck Chemicals Ltd, Nottingham, UK) was used for detection of sperm DNA fragmentation. Briefly, each sample was first centrifuged at 1000 g for 5 minutes and the pellet resuspended in tris-buffered saline (TBS) to give an appropriate sperm concentration of $1x10^6$. A 100µl aliquot was used for preparing the smear and slides were dried overnight. Methanol 100% was used for fixation for 1 minute; fixed slides were stored at -80ºC before transportation back to Sheffield for TUNEL assay. Fixed sample was covered with 1ml TBS for 15 minutes at RT then the excess liquid was tapped off. The rehydrated fixed sample was covered with 100 µl of 20µg/ml Proteinase K, and incubated at RT for exactly 5 minutes. The slide was then washed 2-3 times with 1 ml of TBS. The rehydrated permeablised sample was covered with 100 µl 1x TdT eqilibrium buffer and was incubated at RT for 30 minutes. The permeablised equilibrated sample was then covered with 60 µl of TdT labeling reaction mix, covered with a cover slip, and place in humidified chamber at 37˚C for 60-90 minutes. After removing the cover slip, slide was twice incubated in TBS for 1 minute at RT, and the labelled sample covered with 15 µl mounting medium with propidiumiodied. The slide was then covered with a cover slip and observed under

FITC fluorescence at final magnification of ×1000 using oil immersion (Olympus, Tokyo, Japan).

Three semen smears were prepared to evaluate sperm chromatin assessment.Three semen smears were prepared to evaluate sperm chromatin assessments.The cytochemical tests includingAniline Blue (AB) for the detection of residual histones;Chromomycin A3 (CMA3) for the evaluation of sperm protamination and Acridine Orange (AO) for detection of sperm chromatin condensation anomalies (AO) were performed according to previous studies (Talebi, Khalili, Vahidi, Ghasemzadeh, & Tabibnejad, 2013; Talebi, Sarcheshmeh, Khalili, & Tabibnejad, 2011). The sperm concentration was adjusted again to 1×10^6sperm per ml, however 10µl was used to prepare the smear and air dried slides were then fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 4ºC overnight for Acridine Orange (AO) and Chromomycin A3 (CMA3) staining. Air dried slides for Aniline Blue (AB) staining were fixed in 3% (v/v) buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 minutes at RT. The prepared fixed slides were stored at 4ºC in Yazd to stain and evaluate later in the study. The rest of semen sample was stored at -80ºC to extract DNA.

DNA was extracted from all urine and semen samples using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. DNA was stored at -20ºC prior to transfer of specimens to the UK.Frozen sera and extracted DNA from urine and semen were transferred on dry ice to Sheffield at the end of the recruitment phase. Upon arrival in Sheffield the samples were stored at -20ºC prior to further analysis as outlined below.

*2-5- C. trachomatis*serology

To detect specific IgA, IgMandIgGantibodies to *C. trachomatis* an immunofluorescence assay (SeroFIA™*C. trachomatis*) kit was used (Savyon, Ashdod, Israel). Two people examined each slide and a positive resultdeclared when both were in agreement. Positive and negative controls on each slidewere included from the kit.

2-6- C. trachomatis PCR

Nested plasmid PCR for *C. trachomatis* was conducted according to a previously published methodon all extracted DNA from urine and semen and two pairs of primers (directed against the cryptic plasmid) used to detect *C. trachomatis*aspreviouslydescribed (Claas*et al*., 1990).Products were analyzed by gel electrophoresis in 1.0% (w/v) agarose with ethidium bromide staining.Positive results were compared with *C. trachomatis* plasmid ($pCTT_1$) sequence, accession: M19487 (J03304).

3- Results:

3-1- Semen parameters and sperm DNA fragmentation

There was a statistically significant negative correlation between high percentage of sperm DNA fragmentation and semen volume (P= 0.046) and the percentage of sperm with normal morphology (P= 0.003). In addition, there was a positive correlation with percentage of immotile sperm (P=0.044)(Table 1,2).

3-2- Semen parameters and sperm chromatin condensation

Briefly, the only significant correlation was seen between the percentage of CMA3 (evaluation of protamination) and male age (P=0.025). There was no correlation between age, duration of infertility and semen parameters with AB staining or AO results(Table 1,2).

3-3- Sperm DNA measurements

Comparison were made between C. trachomatis-infected and uninfected men with respect to thepercentage of sperm DNA fragmentation (TUNEL assay) and the percentage of sperm with chromatin abnormality using three different chromatin tests. Table 1and figure 1 show the result obtained using the TUNEL assay for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG. Briefly, the results show no significant differences in terms of DNA fragmentation between *C. trchomatis* positive and negative men, regardless of whether this was

diagnosed by IgM, PCR or IgG. The results obtained using AB staining (Figure 2) for the detection of residual histones for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG. Briefly, the results show no significant differences in terms of chromatin abnormality between *C. trchomatis* positive and negative men, regardless of whether this was diagnosed by PCR or IgG. Unfortunately no results were available for diagnoses made using IgM (see below). Also, the results obtained using AO staining (Figure 3) for detection of sperm chromatin condensation anomalies for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG. Briefly, the results show no significant differences in terms of chromatin abnormality between *C. trachomatis* positive and negative men, regardless of whether this was diagnosed by PCR or IgG.Again no results were available for diagnoses made using IgM (see below). The CMA3 staining used for evaluation of protamination for *C. trachomatis* diagnoses made using (i) IgM, (ii) PCR of urine DNA, and (iii) IgG, and the results show no significant differences in terms of chromatin abnormality between *C. trchomatis* positive and negative men, regardless of whether this was diagnosed by PCR or IgG. The data of IgM were not available.

Although, we measured the IgM for all serum samples, but, sperm chromatin studies were not performed on all 250 men. The fixed slides have been prepared for a sub-sample of 180 men. The matched samples for the three different staining (AB, AO and CMA3) were only achieved for a sub-sample of 80 men and therefore did not include any of the IgM positives.

4- Discussion:

Our previous studies investigated the prevalence of *C. trachomatis* and/or *M. genitalium* among infertile couples in Yazd and is the first large scale study undertaken using NAAT and serology in Iran(Dehghan Marvast et al., 2016).The main results of the study showedthat the incidence of

C. trachomatis is comparable with other similar studies, there was no evidence of *M. genitalium* and there is a low level of concordance within couples(Dehghan Marvast et al., 2016). To assess the prevalence of both micro-organisms, a non-invasive technique was carried out using FVU samples. The prevalence of *C. trachomatis* following NPPCR testing on these samplespartners of infertile couples were infected. Also serum samples were obtained from infertile couples to detect IgA, IgM and IgG antibodies to *C. trachomatis* and this showed that the IgM prevalence was 1.2% and 4.0% in the male and female partners of infertile couples respectively. The IgG prevalence was 18.0% and 15.6% in the male and female partners of infertile couples respectively(Dehghan Marvast et al., 2016). The IgA was not observed in the serum samples and this might be due to the half-life of IgA antibodies to *C. trachomatis* which is less than one week and normally used as an indicator of acute infection (Fresse, Sueur, & Hamdad, 2010). The semen samples (semen DNA) were tested for *C. trachomatis* by NPPCR, and no positive samples were detected(Dehghan Marvast et al., 2016).

In current study, sperm chromatin/DNA integrity was assessed by different tests to examine whether it was related to *C. trachomatis* infection in the male partner of infertile couplesfor first time in our population. The main conclusions of the results are: (i) sperm DNA fragmentation (TUNEL) and chromatin studies (AB, AO & CMA3) were not correlated with *C. trachomatis* diagnosis; (ii) the percentage of DNA fragmentation is positively correlated with the percentage of immotile sperm but negatively with semen volume, normal morphology; (iii) in sperm chromatin measurements only the percentage of protamination was related to male age.

Sperm DNA fragmentation assessed by TUNEL assay was not related to *C. trachomatis* infection in the male partners of infertile couples as defined by serology (IgM&IgG) or PCR of urine samples. In addition to the results for DNA fragmentation, the results of this present study also showed *C. trachomatis* infection was not associated with various measures of sperm chromatin condensation such as AB, CMA3 and AO staining. This is in contrast with previous studies that showed sperm DNA fragmentation is higher in *C. trachomatis* infected infertile men (Al-Mously & Eley, 2015) Gallegos *et al*., 2008). This is not a surprise because in this study semen samples were not infected by *C. trachomatis* using PCR which suggests there was no infection in the upper genital tract to affect spermatogenesis. The positive results for infection (urine DNA) indicate lower genital tract infection such as urethritis.

The results of TUNEL assay showed semen volume and percentage of normal morphology were significantly lower in infertile men with a "high" level of DNA fragmentation (based on 75thpercentiles) and the percentage of immotile sperm was significantly higher in "high" level of DNA fragmentation. These findings cannot be compared to other studies because as they used different methods including flow cytometry instead of assessing fixed slides and sperm chromatin investigation rather than double strand DNA break (TUNEL assay).

In this study sperm chromatin integrity was investigated by three methods and showed that the percentage of stained sperm was not directly associated with any semen variable. The results only suggested that the level of protamination as men get older is reduced compared to younger men. CMA3 competes with protamine in the same site of DNA(Agarwal et al., 2016); therefore our results can be explained by age-related alteration of semen parameters that is similar to the work of Sharma et al., (Belloc, Benkhalifa, et al., 2014; Belloc, Hazout, et al., 2014; Johnson, Dunleavy, Gemmell, & Nakagawa, 2015; Sharma et al., 2015) . Although these studies indicated that increasing male age was associated with a decreasing in semen volume, sperm motility and morphology but ,Nijs*et al*., (2009) did not find any male age-related influences on routine semen parameters or sperm DNA measurements. There is an extensive literature about the relationship between semen parameters and sperm DNA damage (Zidi-Jrah et al., 2016).For example, researchers found DNA damage was negatively correlated with semen parameters and the strongest correlation was with sperm morphologyand motility(Belloc, Benkhalifa, et al., 2014; Cassuto et al., 2012; Evgeni, Charalabopoulos, & Asimakopoulos, 2014; Simon et al., 2014; Utsuno, Oka, Yamamoto, & Shiozawa, 2013).Saleh have not found relationship between semen parameters and DNA fragmentation(Saleh et al., 2002);howeverMagdi*et al*., (2015) found higher level of DNA fragmentation in infertile men compared to a fertile group(Magdi, Darwish, Elbashir, & Elawady, 2015). However, these investigations are between infertile menand DNA damage without taking into account *C. trachomatis* infection.

The limitations of this study were (i) lack of control group as this was not easy to get fertile men to take part in the study and (ii) sperm DNA chromatin assessment was performed only on a sub-sample of n=80 men and this did not include any of the IgM positive men and (iii) the method used for TUNEL assay was only assessing fixed slides and not flowcytometry which is more accurate. This is because flowcytometry was not available.

Implication of sperm DNA testing is controversial in infertility investigations. It might help couples with a history of long term unexplained infertility and ART failure(Vandekerckhove, De Croo, Gerris, Abbeel, & De Sutter, 2016). The American Society for Reproductive Medicine (ASRM) guideline (2013) suggested that although sperm DNA damaging is more common in infertile men, however, the clinical utility of testing is not recommended in routine infertility work-up, as these tests are not reliable to predict outcome of pregnancy (ASRM, 2013). In addition, due to lack of efficient treatment there is no more help for couples even if sperm DNA abnormalities were detected (ASRM, 2006). Also ASRM Practice Committee (2006) did not find relationship between sperm DNA damage and reproductive outcome either in spontaneous or assisted conception. Barratt *et al*., (2010) reported the results of an ESHRE workshop in 2009 which concluded sperm DNA abnormalities may have most effect on IUI pregnancy rates and pregnancy loss after IVF and ICSI. However, there are clinical and assay uncertainties because of the lack of a robust clinical test. The larger issues including: who to test, when to test and how to treat patients with abnormal results have not yet been resolved. To confirm the results of literatures, further larger, well designed and controlled prospective studies will be required.

5- Conclusion:

The main conclusions of the results are: (i) sperm DNA fragmentation (TUNEL) and chromatin studies (AB, AO & CMA3) were not correlated with *C. trachomatis* diagnosis; (ii) the percentage of DNA fragmentation is positively correlated with the percentage of immotile sperm but negatively with semen volume, normal morphology; (iii) in sperm chromatin measurements only the percentage of protamination was related to male age.

Table 1: Age, duration of infertility, semen parameters (mean ± SD) in low and high levels of DNA fragmentation using TUNEL assay (based on 25th and 75th percentiles).

Variable	DNA fragmentation 25th percentile (n=64)	DNA fragmentation 75th percentile (n=67)	P-value
Age[a](years)	32.53 ± 4.51	32.22 ± 5.29	P=0.717
Duration of infertility[a](years)	5.72 ± 2.85	6.27 ± 3.91	P=0.360
Semen volume[a] (ml)	3.48 ± 1.78	2.92 ± 1.48	P=0.046*
pH[a]	8.26 ± 0.49	8.20 ± 0.42	P=0.843
Sperm concentration[a] million/ml	62.37 ± 66.82	68.13 ± 39.53	P=0.548
Percent progressive Motile[b]	45.63 ± 25.64	54.15 ± 18.96	P=0.094
Percent immotile[b]	33.12 ± 14.61	44.97 ± 28.57	P=0.044*
Percent normal morphology[b]	6.95 ± 3.78	5.05 ± 3.45	P=0.003*
Leucocytes[a], million/ml	1.35 ± 1.57	1.58 ± 1.29	P=0.349

a: parametric independent t test; b: non parametric independent t test

*P-value<0.05

Table 2: Age; duration of infertility; semen parameters (median) and their corresponding correlation with DNA integrity measured by Aniline Blue (AB), Acridine Orange (AO) and Chromomycine A3 (CMA3) for a sub-sample of 80 men.

Variable	Median(range) (n=80)	Correlation with %AB	Correlation with %AO	Correlation with %CMA3
Age(years)	33 (22-49)	P=0.961 r=0.006	P=0.157 r=0.160	P=0.025* r=0.252
Duration of infertility (years)	4.3 (1-18)	P=0.765 r=0.034	P=0. 280 r=0.122	P=0.179 r=0.115
Semen volume(ml)	3.5 (1.0-9.5)	P=0.496 r=0.077	P=0.182 r=0.151	P=0.643 r=0.053
pH	8.5 (6.0-9.0)	P=0.897 r=0.015	P=0.857 r=0.020	P=0.203 r=0.145
Sperm concentration million/ml	74.0 (3.0-310.0)	P=0.124 r=0.173	P=0.982 r=0.003	P=0.466 r=0.083
Percent progressive motile	59.5 (0.0-95.0)	P=0.099 r=0.186	P=0.912 r=0.012	P=0.611 r=0.058
Percent immotile	32.0 (6.0-100.0)	P=0.574 r=0.064	P=0.447 r=0.086	P=0.422 r=0.092
Percent normal morphology	6.3 (0.5-14.5)	P=0.390 r=0.097	P=0.450 r=0.086	P=0.264 r=0.127
Leucocytes, million/ml	1.1(0.15-14.4)	P=0.092 r=0.192	P=0.818 r=0.027	P=0.061 r=0.215

***P-value<0.05**

Table 3: Detection of residual histone (Aniline Blue),evaluation of protamination (Chromomycine A3), sperm DNA fragmentation (TUNEL assay)**,** sperm chromatin condensation anomalies (AO) in*C. trachomatis* positive men for: (i) IgM; (ii) DNA and (iii) IgG.

	AB	CMA3	TUNEL	AO
IgM $^{+}$ (n=0)	n/a	n/a	8.66 ± 10.66	n/a
IgM $^{-}$(n=80)	29.81± 15.6	39.87 ± 10.6	12.69 ± 8.25	35.68 ± 12.20
P value	n/a	n/a	P=0.407	n/a
DNA $^{+}$(n=11)	28.0 ± 15.2	35.0±8.2	11.18 ± 5.94	37.54 ± 7.90
DNA $^{-}$(n=69)	30.10 ± 14.8	39.80 ± 10.6	12.82 ± 8.31	35.42 ± 12.60
P value	P=0.657	P=0.156	P=0.673	P=0.462
IgG+(n=21)	28.0 ± 13.9	38.54 ± 8.22	12.21 ± 8.28	35.52 ± 12.90
IgG−(n=59)	28.85 ± 15.4	40.63 ± 12.3	12.71± 8.01	35.71 ± 12.17
P value	P=0.689	P=0.299	P=0.592	P=0.957

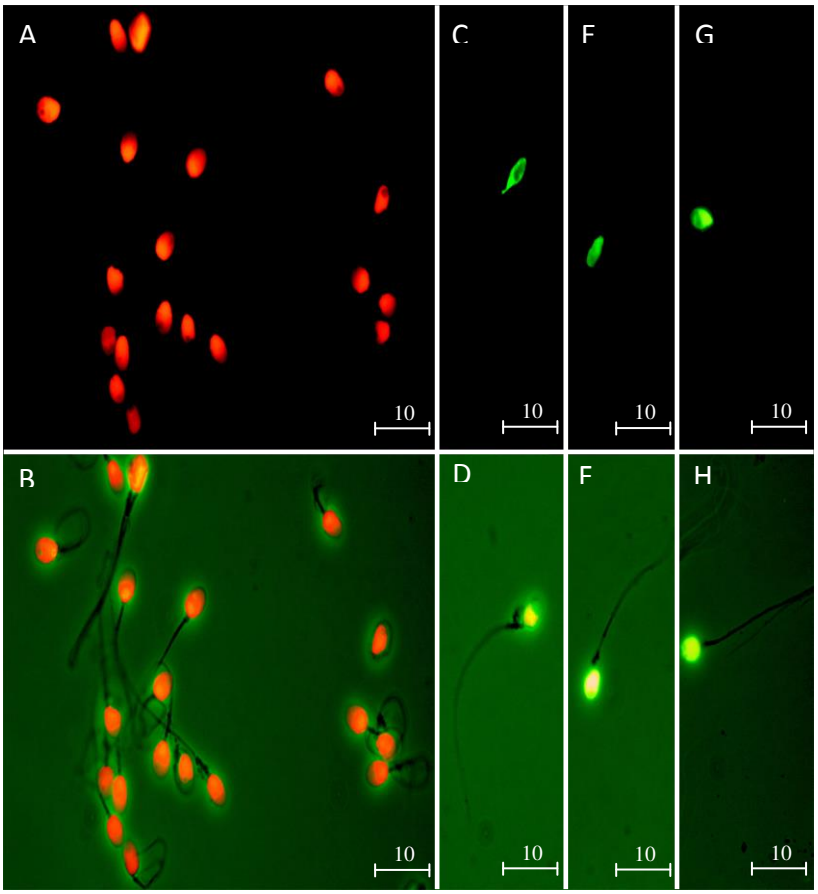


Figure 1: DNA fragmentation assay using TUNEL.Panels A& B show sperm with intact DNA, whereas C-H show sperm with damaged DNA. Upper panels show fluorescent signal and lower panels show corresponding image using visible light

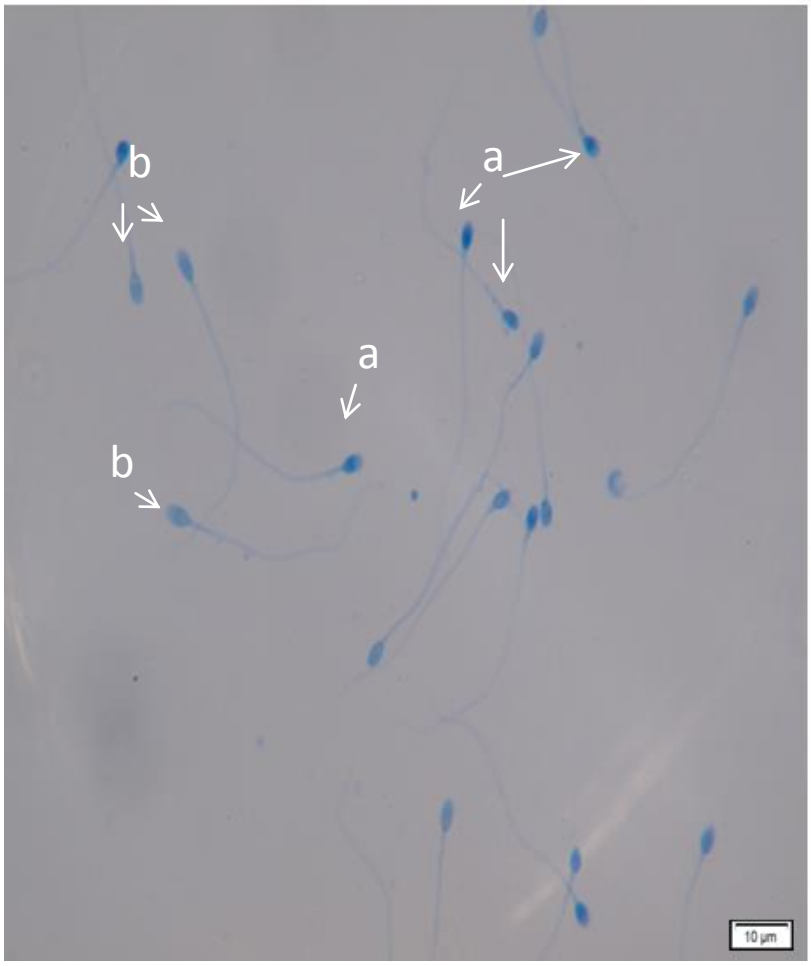
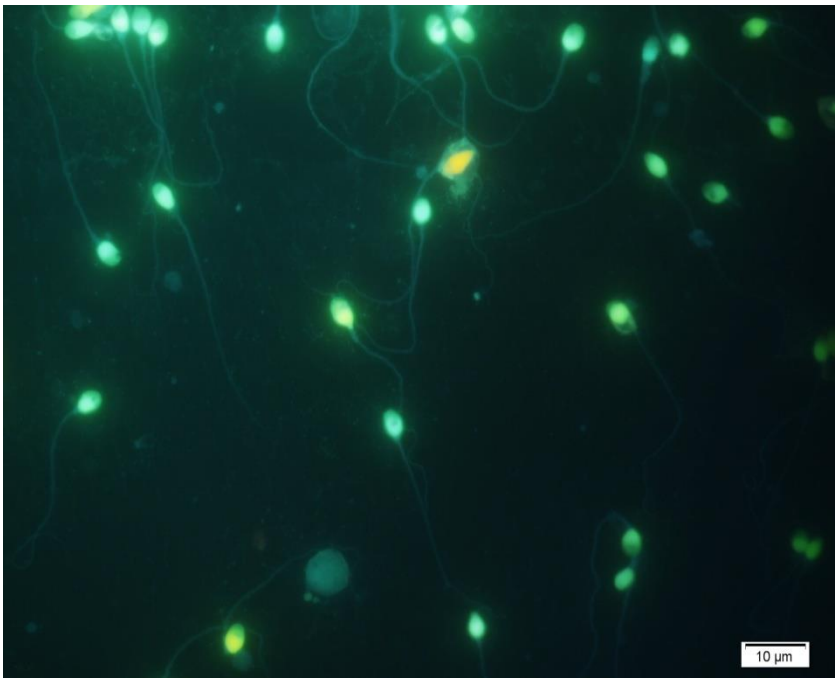


Figure 2: Sperm DNA integrity measurement using AB staining for detection of residual histones: (a) = abnormal spermatozoa (dark blue) and (b) = normal spermatozoa (pale blue or unstained).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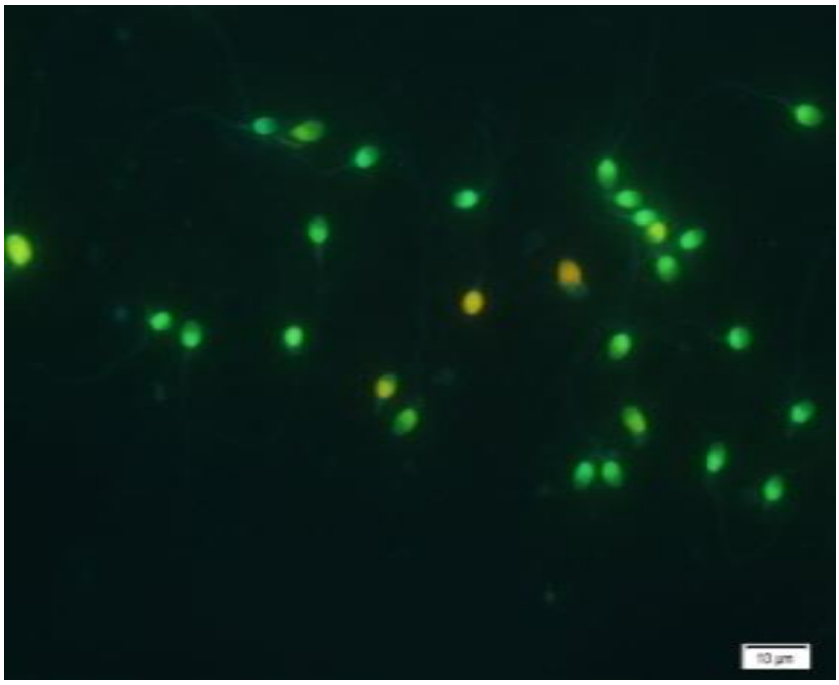

Figure 3: Sperm DNA integrity measurement using AO staining to detect sperm chromatin condensation anomalies: (a) = abnormal spermatozoa (orange-red; denatured DNA) and (b) = normal spermatozoa (green; double stranded DNA).

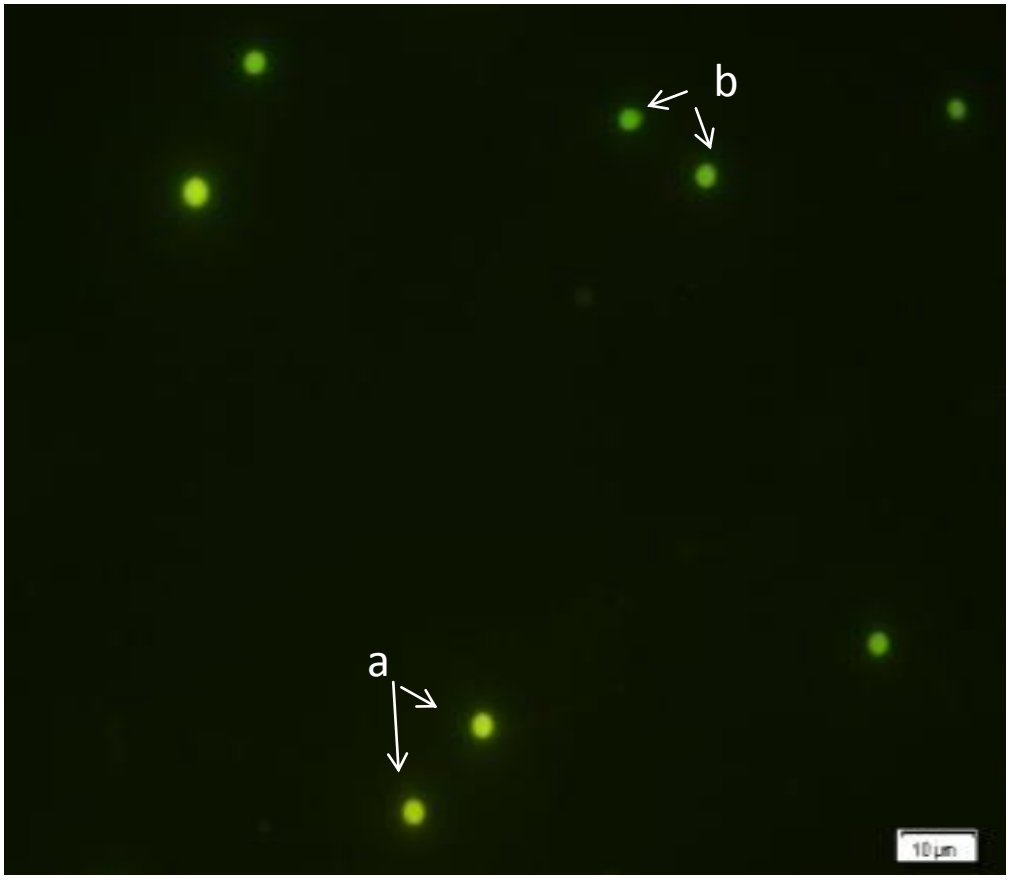

Figure 4: Sperm DNA integrity measurement using CMA3 staining for evaluation of protamination: (a) = abnormal spermatozoa (bright yellow) and (b) = normal spermatozoa (yellowish-green).

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