IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)
e-ISSN: 2279-0853, p-ISSN: 2279-0861.Volume 17, Issue 10 Ver. 3 (October. 2018), PP 25-32
www.iosrjournals.org

A Fluorescence Microscopy Study of Seminal Fluid In Infertile Males Using Acridineorange Dye To Assess DNA Integrity Along With Semen Analysis

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Abstract: *According to World Health Organization(2000), infertility is the inability of the sexually unprotected, active couple to achieve pregnancy in one year of marriage . Present study is aimed to assess DNA damage in sperms using Acridine Orange fluorescence technique in infertile malesto correlate the results of DNA damage with other semen parameters and to select the healthy semen donors in sperm banks as part of treatment of infertility. Collaborating the findings from our study and the previous studies in the literature, it is concluded that fluorescence microscopy of seminal fluid using acridine orange is an easy test to perform and a relatively cheap method as compared to other methods which are expensive and require more machinery, equipment's and skilled technical staff. Although easy to perform and relatively cheap, it does not loses its relevance in semen analysis in comparison to other tests.*

Key words*: Semen Analysis, Infertility, Asthenozoospermia, Acridine Orange, Fluorescence Microscopy, DNA, Teratozoospermia, Oligoasthenozoospermia*

Date of Submission: 01-10-2018 Date of acceptance: 13-10-2018

I. Introduction

According to World Health Organization(2000), infertility is the inability of the sexually unprotected, active couple to achieve pregnancy in one year of marriage . About 25% of couples do not achieve pregnancy within 1 year, 15% seek medical treatment for infertility and less than 5% remain unwillingly childless. Approximately in 50% of the cases, the underlying etiology lies in men alone.[1]

Determination of quality of seminal fluid is important before common procedures like artificial insemination or IVF in unexplained infertility[2]. The apparent increased incidence of male infertility, in parallel with the widespread use of InVitro Fertilization (IVF), raises concern as to the impact of advanced assisted conception techniques in transmitting genetic anomalies to the offspring. Genetic testing plays an important role not only to identify the etiology of male infertility but also helps in counselling as well as in the prevention of the transmission of genetic defects to the offsprings via assisted reproduction.[3]

Investigation of the male partner in an infertile couple is primarily based on the routine semen analysis. Poor sperm quality is represented by abnormal semen parameters, including low sperm concentration, poor sperm motility and abnormal sperm morphology[4]. It normally takes around 10 to 30 minutes (average 20 minutes) at room temperature for the seminal fluid to change from a thick gel into a liquid. Liquefaction occurs because of presence of fibrinolysin. In case, liquefaction does not take place within 30 minutes, it needs further investigation as it indicates infection.[5]

A more specified measure is motility grade.A man can have total number of sperms far above the limit of 15 million sperm cells per millilitre, but still have bad quality because too few of them are motile. However, if the sperm count is very high, then a low motility (for example, less than 40%) might not matter. The other way round, a man can have a sperm count far less than 15 million sperm cells per millilitre and still have good motility, if more than 32% of those observed sperm cells show good forward movement. The motility of sperms is divided into four different grades:

Grade(a)**:**Sperms with progressive motility. These are the strongest and swim fast in a straight line. Sometimes it is also denoted motility IV.

Grade (b): (non-linear motility): These also move forward but tend to travel in a curved or crooked motion. (Motility III).

Grade(c):These have non-progressive motility because they do not move forward despite the fact that they move their tails (motility II).

Grade(d): These are immotile and fail to move at all (motility I).[6]

It has also been reported that sperm concentration, motility and morphology might be affected by sperm DNA fragmentation[7]. There is now clinical evidence to show that damage to human sperm DNA may adversely affect reproductive outcomes and that spermatozoa of infertile men possess substantially more DNA damage than do spermatozoa of a fertile men.[8] The routine parameters of sperm analysis, as evaluated by microscopical examination are usually insufficient to predict sperm fertilizing capacity. Acridine orange (AO) staining of spermatozoa may be used to upgrade the information obtained by a semen analysis. Acridine orange test (AOT) is a simple microscopic procedure based on acid conditions to denaturant DNA followed by staining with acridine orange dye. It is based on the principle that DNA possesses a different susceptibility to partial denaturation induced by heat shock or by contact with an acidic solution. The AOT measures the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA). It was observed that ≥50% green fluorescence in sample is a normal cut-off value for AOT in sample from fertile donors. AOT using fluorescence microscopy provides a general picture of the status of DNA denaturation. As a test for DNA integrity, this cytochemical method allows the differentiation between double strand/ (green fluorescence) and single-strand (red fluorescence) DNA because of the metachromatic properties of AO. Results of the AO fluorescence staining of semen smears have been reported to be an important indicator of human sperm fertilizing potential, and the AO testing of semen samples has been suggested as a practical and clinically significant procedure to determine sperm quality during infertility investigations.[9]

II. Materials And Methods

In the present study, 100 male cases comprising of samples of 80 infertile and 20 fertile persons with prior approval of the institution's Thesis and Ethical Committee, Government Medical College, Amritsar were selected. From each patient presenting with complaint of infertility in department of Pathology, Government Medical College, Amritsar, seminal fluid was examined. Patients were instructed for semen collection, including a defined pre-test abstinence of sexual intercourse for a period of 2 to 5 days.

In all cases, patients wereasked to collect Semen by means of masturbation into a specimen cup or by intercourse with the use of special semen collection condoms that do not contain substances toxic to sperm. All samples for evaluation, were allowed to liquefy for at least 30 minutes at 37°C in an incubator and then evaluated for sperm count, concentration, motility, and morphology.

Acridine Orange Stainingprocedure:1ml of semen was taken in test tube and spermatozoa were washed with distilled water thrice using a centrifuge machine. The supernatant was discarded and sediment was used to prepare thick smears on clean glass slides. The thick smear was air dried for 2 hours. The smeared slides werefixed in Carnoy's Solution for 2 hours. The slides were than rinsed with distilled water twice. Then the slide was dipped in Mcllvaine Phosphate Citrate Buffer [PH=4] for 5 minutes. Smear was then stained with freshly prepared Acridine Orange solution (0.19 mg/ml) of Mcllvaine Phosphate Citrate Buffer i.e. for 8 minutes in darkness.Smear was covered by glass cover slip and assessed on same day under Nikon Fluorescence Microscope with 460 nm filter. The percentage of illumination was limited to 40 seconds per field.

Scoring of AO "Green" and AO "Orange Red" Sperm: The percentage of sperm with normal DNA have been determined on each slide as follows: We have selected 10 or more individual fields and evaluated a total of 200 sperms in each field under a Nikon fluorescence microscope with 6400 magnification and excitation of 450–490 nm. Observation of the fields was not to exceed 40 seconds so as to prevent fading. The normal spermatozoa were recorded as ''green'' whereas abnormal as ''orange red.'' Each stained slide was evaluated immediately after staining.

Statistical Analysis: The results were compiled and statistically analyzed, using chi square test with level of significance <0.05 (p value).The study included the patients who presented with primary infertility in the age group of 21-50 years and were willing to participate in it.None of the patient gave any history of stress, anxiety, depression and chronic illness in infertile group.

Cut off reference values for semen characteristics as published in consecutive WHO manuals which were being followed in the study are: (1) Volume (ml) ≥1.5, (2) Sperm count (10^6/ml) ≥15, (3) Total sperm count (10^6) ≥39, (4) Total motility (%)≥40, (5) Progressive motility≥32%(a+b), (6) Morphology (%)≥4.

III. Observations

The present study was conducted on 100 patientswhich were divided into two groups. Group 1 was control group of 20 Fertile males and group 2 was case group of 80 Infertile males who presented to the Department of Pathology,Government Medical College, Amritsar. Maximum number of cases of infertility were

found in the age group of 31-40years (62.5%) followed by 31.25% cases in the age group of 21-30 years. The mean age of the Group 1 (Fertile) was 33.15yrs and that of Group 2 (Infertile) was 33.63yrs with non-significant p value (p> 0.05). Maximum infertile patients 33.75% presented within 3-4 years of marriage.Out of total 80 infertile cases, maximum number of cases83.75% were urbanites and 16.25% were from rural areas.

In Group 1 which is control group of fertile male, the maximum i.e. 80% patients who presented had no habitual addiction of any kind.In Group 2,maximum number of patients i.e. 30% were drug addicts, followed by 23.75% cases who had habit of alcohol admixed with smoking. 12.5% among them were smokers only, 18.75% were alcoholic and 15% were drug addicts in combination with smoking with significant p value (p<0.05).

In Group 1(Control Group) of fertile male, volume of semen in all cases (100%) came out to be more than 1.5ml per ejaculate and in Group 2 of infertile males, 85% patients had semen volume more than 1.5ml and 15% patients had semen volume less than 1.5ml per ejaculate .So both groups were comparable to each other with significant p value (p< 0.05)

In Group 1 (Control group), the liquefaction time of semen in all the cases was more than 30 minutes. In Group 2, the liquefaction time of semen was within 30 minutes in maximum 72.5% cases and in 27.5% cases, liquefaction time was more than 30 minutes with insignificant p value (p> 0.05)

In Group I (ControlGroup), all cases (100%) had normal sperm count. In Group 2, only 53.75% cases had normal sperm count whereas 46.25% were oligozoospermic with significant p value (p<0.05).

In Group I(Control Group),only 5% case werefound to be Asthenozoospermic, whereas in Group 2, 72.5% caseswere found to be asthenozoospermic followed by 18.75% cases with total motility >40% and 8.75% with immotile spermswith significant p value (p<0.05).

In Group I(Control Group), all cases had progressive sperm motility > 32%In Group 2, 72.5%cases had progressive sperm motility<32%,followed by 18.75% cases with progressive sperm motility >32%.And only8.7 % cases had immotile sperms, with significant p value (p<0.05).

In Group I, in all cases the sperms had normal morphology whereas in Group 2, sperms in 75% cases had normal morphology and 25% were teratozoospermic with significant p value (p<0.05).

In Group 2, most common age group with maximum cases of oligozoospermia (52%), Asthenozoospermia (78%), immotile sperms (12%) and teratozoospermia (30%) was 31-40 years. All these patients were exposed to lifestyle related bad habits and most common semen variable in them was asthenozoospermia. Maximum cases with teratozoospermia were found in drug addicts, so reduced sperm motility and abnormal sperm morphology was significantly related to life style related bad habits with p value (p= 0.027 & p = 0.001) respectively and 46.2% cases were Oligoasthenozoospermic and 20% cases were Oligoasthenoteratozoospermic.In Group 2, maximum (45%) cases had green sperm heads in range of 31-40%, 27.5 in the range of 41-50%, 15% in the range of 21-30%. With increase in the range of green sperm heads the semen characters improved showing significant correlation of DNA damage of sperms with the fertility of men. DNA Damage is significantly related to all the semen parameters with p value (p<0.05).

In 18.75**%** cases of Group 2, the semen parameters were normal but even then DNA damage was evident.The correlation of DNA damage of sperms with the fertility of men.

3.1 Age group wise distribution of cases

Age group	**No. of Group 1 (Fertile) cases and %age**		**No. of Group 2 (infertile) cases and %age**	
21-30 years	10	50	25	31.25
31-40 years	7	35	50	62.5
41-50 years	3	15	5	6.25
Total	20	100	80	100

Table 1. Age group wise distribution of cases P value is insignificant (p= 0.072)

3.2.Distribution of cases on basis of years since marriage

Years since marriage	**No. of Group 1 (Fertile) cases and %age**		**No. of Group 2 (Infertile) cases and %age**	
0-2yrs	7	35	17	21.25
3-4yrs	6	30	27	33.75
5-6yrs	3	15	17	21.25
7-8yrs	1	5	12	15
9-10yrs	-	-	3	3.75
11-15yrs	1	5	2	2.5
16-20yrs	2	10	2	2.5
Total	20	100	80	100

Table 2.Distribution of cases on basis of years since marriage P value is insignificant. (p= 0.318)

3.3. Area wise distribution of cases

Area	**No. of Group 1 (Fertile) cases and %age**		**No. of Group 2 (infertile) cases and %age**	
Urban	5	25	67	83.75
Rural	15	75	13	16.25
Total	20	100	80	100

Table 3. Area wise distribution of cases P value is significant (p<0.05)

3.4. Distribution of cases on basis of Exposure to lifestyle related bad habits

Habits	**No. of Group 1 (Fertile) cases and %age**		**No. of Group 2 (infertile) cases and %age**	
Smoking	1	5	10	12.5
Alcohol	3	15	15	18.75
Alcohol + Smoking	-	-	19	23.75
Drug abuse	-	-	24	30
Drug abuse+ Smoking	-	-	12	15
No lifestyle related bad habits	16	80	-	-
Total	20	100	80	100

Table 4. Distribution of cases on basis of Exposure to lifestyle related bad habits P value is significant. (p= 0.000)

3.5. Distribution of cases on basis of volume of semen per ejaculation in both groups

Volume	**Group 1 (Fertile)**		**Group 2(infertile)**	
	No.	**%age**	**No.**	**%age**
<1.5ml	-	-	12	15
>1.5ml	20	100	68	85
Total	20	100	80	100

Table 5. Distribution of cases on basis of volume of semen per ejaculation in both groups P value is significant. (p= 0.039)

3.6. Distribution of cases on basis of Liquefaction time of semen in both groups

Liquefaction Time of semen	**Group 1 (Fertile)**		**Group 2(infertile)**	
	Number	**%age**	**Number**	**%age**
0-30mins	20	100	58	72.5
>30mins	-	-	22	27.5
Total	20	100	80	100

Table 6. Distribution of cases on basis of Liquefaction time of semen in both groups
P value is insignificant. (p= 0.456)

3.7. Distribution of cases on basis of sperm count

Sperm Count	No. of Group 1 (Fertile) cases and %age		No. of Group 2(infertile) cases and %age	
Normal Count	20	100	43	53.75
Oligozoospermic	-	-	37	46.25
Total	20	100	80	100

Table 7. Distribution of cases on basis of sperm count P value is significant. (p= 0.000)

3.8. Distribution of cases on basis of Total sperm motility

Total sperm motility	No. of Group 1 (Fertile) cases and %age		No. of Group 2(infertile) cases and %age	
TM>40%	19	95	15	18.75
TM<40%	1	5	58	72.5
Immotile	-	-	7	8.75
Total	20	100	80	100

TM= Total motility

Table 8. Distribution of cases on basis of Total sperm motility P value is significant. (p= 0.000)

3.9. Distribution of cases on basis of Progressive sperm motility

Progressive sperm motility	No. of Group 1 (Fertile) cases and %age		No. of Group 2(infertile) cases and %age	
PM>32%	20	100	15	18.75
PM<32%	-	-	58	72.5
IM	-	-	7	8.75
Total	20	100	80	100

PM= Progressive Motility, IM=Immotile
Table 9. Distribution of cases on basis of Progressive sperm motility p value is significant. (p=0.000)

3.10. Distribution of cases on basis of sperm morphology of group 1 and group 2

Sperm morphology	**No. of Group 1 (Fertile) cases and %age**		**No. of Group 2(infertile) cases and %age**	
Normal morphology	20	100	60	75
Teratozoospermia	-	-	20	25
Total	20		80	100

Table 10. Distribution of cases on basis of sperm morphology of group 1 and group 2
P value is significant. (p= 0.012)

3.11. Distribution of cases Age group wise in relation to semen characteristics in Infertile group (Group 2).

Age group	**Sperm Count No. of cases and %age**				**Sperm motility No. of cases and %age**						**Sperm morphology No. of cases and %age**			
	NSC	**%**	**OZ**	**%**	**TM>40**	**%**	**TM<40**	**%**	**IM**	**%**	**NM**	**%**	**TZ**	**%**
21-30 yrs (25)	18	22.5	7	8.75	8	10	16	20	1	1.25	23	28.75	2	2.5
31-40 yrs (50)	24	30	26	32.5	5	6.25	39	48.75	6	7.5	16	20	34	18.75
41-50 yrs (5)	1	1.25	4	5	2	2.5	3	3.75	-	-	2	2.5	3	3.75
TOTAL	43	53.75	37	46.25	15	18.75	58	72.5	7	8.75	41	51.25	39	48.75

NSC = Normal Sperm Count, OZ= Oligozoospermia, TM = Total Motility, IM = Immotile, TZ = Teratozoospermia, NM= Normal Morphology
Table 11. Distribution of cases Age group wise in relation to semen characteristics in Infertile group (Group 2).

3.12. Relation of semen characters with lifestyle habits of infertile (study) group 2.

Characteristics	**(Smoking) No. of cases and %age in group (N=10)**		**(Alcohol) No. of cases and %age in group (N=15)**		**(Alcohol +Smoking) No. of cases and %age in group (N=19)**		**(Drug abuse) No. of cases and %age in group (N=24)**		**(Drug abuse +smoking) No. of cases and %age in group (N=12)**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	**N**	**%**
NSC (43)	7	8.75	10	12.5	13	16.25	9	11.25	4	5
OZ(37)	3	3.75	5	6.25	6	7.5	15	18.75	8	10
TM>40%(15)	4	5	4	5	3	3.75	2	2.5	2	2.5
TM<40%(58)	5	6.25	11	13.75	15	18.75	21	26.25	6	7.5
IM (7)	1	1.25	-	-	1	1.25	1	1.25	4	5
NM(60)	9	11.25	14	17.5	17	21.25	16	20	4	5
TZ (20)	1	1.25	1	1.25	2	2.5	8	10	8	10
TOTAL(80)	30	37.5	45	56.25	60	75	72	90	36	45

NSC = Normal Sperm Count, OZ= Oligozoospermia, TM = Total Motility, IM = Immotile, TZ = Teratozoospermia, NM= Normal Morphology
Table 12. Relation of semen characters with lifestyle habits of infertile (study) group 2.

3.13. Distribution of all cases on basis of Acridine Orange staining

Approx. % of Green sperm Heads with normal DNA	**No. of Group 1 (Fertile)**		**No. of Group 2(infertile)**	
	cases	**(%)**	**cases**	**(%)**
0-10%	-	-	5	6.25
11-20%	-	-	5	6.25
21-30%	-	-	12	15
31-40%	-	-	36	45
41-50%	-	-	22	27.5
51-60%	6	30	-	-
61-70%	8	40	-	-
71-80%	2	10	-	-
81-90%	3	15	-	-
91-100%	1	5	-	-
Total	20	100	80	100

Table 13. Distribution of all cases on basis of Acridine Orange staining
P value is significant. (p= 0.000)

3.14.Distribution of cases on basis of acridine orange staining and relation with sperm count for Group 2 (infertile group).

Approx. % of Green sperm Heads with normal DNA	**No. of cases**	**Sperm count**			
		Number of Cases with normal count	**%**	**Number of Cases with Oligozoo-spermia**	**%**
0-10%	5	1	1.25	4	5
11-20%	5	-	-	5	6.25
21-30%	12	4	5	8	10
31-40%	36	17	21.25	19	23.75
41-50%	22	21	26.25	1	1.25
TOTAL	80	43	53.75	37	46.25

Table 14.Distribution of cases on basis of acridine orange staining and relation with sperm count for Group 2 (infertile group).
P value is significant. (p= 0.00)

3.15. Distribution of cases on basis of acridine orange staining and relation with sperm morphology for Group 2.

Approx. % of Green sperm Heads with normal DNA	**No. of cases**	**Sperm morphology**			
		No. of cases with normal Morphology	**%**	**No. of cases with Teratozoo-spermia**	**%**
0-10%	5	1	1.25	4	5
11-20%	5	1	1.25	4	5
21-30%	12	7	8.75	5	8.75
31-40%	36	30	37.5	6	7.5
41-50%	22	21	26.2	1	1.25
TOTAL	80	60	75	20	25

Table 15. Distribution of cases on basis of acridine orange staining and relation with sperm morphology for Group 2. P value is significant. (p= 0.00)

3.16. Distribution of cases on basis of acridine orange staining and relation with sperm motility for Group 2.

Approx. % of Green sperm Heads with normal DNA	**No. of cases**	**Sperm Motility**					
		TM>40	**%**	**TM<40**	**%**	**IM**	**%**
0-10%	5	-	-	2	2.5	3	3.75
11-20%	5	-	-	4	5	1	1.25
21-30%	12	-	-	10	12.5	2	2.5
31-40%	36	4	5	31	38.75	1	1.25
41-50%	22	11	13.75	11	13.75	-	-
TOTAL	80	15	18.75	58	72.5	7	8.75

Table 16. Distribution of cases on basis of acridine orange staining and relation with sperm motility for Group 2. P value is significant. (p= 0.00)
TM= Total Motility, IM = ImmotileGreen sperm heads represent normal DNA
Orange sperm heads represent denatured (damaged) DNA

IV. Discussion

In Group 2(Infertile) of the present study, the maximum no of infertility cases 62.5 % were in the age group of 31-40 years with mean age being 33.63yrs for Group 2and 33.15yrs for Group I and hence,both groups were comparable with each other for the study.There are few studiesin the literature and our findings are similar with the studies of O.P Omosigho et al [10], A.C Varghese et al[11,] Kumar et al [12], Mankar S B et al [13.]

In Group 2 of present study, the maximum 33.75% patients of infertility presented within 3 to 4 years of marriage. Whereas, the study by AkhtarS et al[14] in Bangladesh reported the time period as 4.7 years, Elussein EA et al[15] in Sudan as 5.2 yearsandaccording to a study by Mankar SB et al[13] mean duration of infertility was 4.56±3.85 years.The maximum cases in Group 2of present study were urbanites i.e. 83.75% and 16.25% were from rural back ground.Similar findings were seen by Kumar et al[12], who reported that maximum cases i.e. 81.7%of infertility were urbanites and rest were from rural background.

In Group 2 of present study,the majority of 85 % of patients had semen volume more than 1.5ml. The liquefaction time of semen was within 30 minutes in maximum cases i.e. 72.5% patients and in 27.5% patients, liquefaction time was more than 30 minutes. Nandini Bet al[16] in her study analyzed that 7.45% of the samples were of inadequate quantityand liquefaction time of semen was within 30 minutes in maximum cases.In Group I, all cases had normal sperm count, normal morphology and only 5% were asthenozoospermic. In Group 2,

53.75%, had normal sperm count whereas 46.25% were oligozoospermic. Also in this group 72.5% had progressive motility <32%,followed by 18.75% cases with progressive motility >32% and 8.75% cases with immotile sperms had significant p value ($p<0.05$).72.5% cases werefound to be asthenozoospermic followed by 18.75% cases with total motility >40% and 8.7% cases with immotile sperms.Also75% patientshad normal morphology and 25% had abnormal morphology with significant p value ($p<0.05$).

In Group Iof present study, 5% had history of smoking whereas in Group 2, 12.5% patients gave history of smoking and among them, 30% were oligozoospermic. Hence smokers (30%) of Group 2 had lower sperm count as compared to non-smokers of Group I. This finding is comparable with the findings of Close et al[17],andChia et al[18].In Group 2 of the present study, 50%smokers had decreased sperm motility (Asthenozoospermia) than non-smokers of Group1. Similar findings were reported by Shaarawy and Mahmoud[19],Rantala and Koskimies[20] and Moskova and Popov[21] with more than 50% cases with decreased motility. According to Kumar et al[12] low sperm motility was significantly associated with smoking which goes in favour of the present study.

Whereas in another study by Gaur DS et al[22] of alcoholics,Teratozoospermia was the most common variable in 72% cases.Another study by Emanuele MA et al[23] found that alcohol consumption affected the reproductive system at all levels. A recent study in Nigeria by Ok Onofua F et al[24] found a significant effect of alcohol consumption on infertility especially moderate to heavy alcohol intake.But previous studies of Jensen et al[25] and Curtis KM et al[26] had found no association between alcohol consumption and male infertility.

Among 30% drug addicts of Group 2, 62.5% were oligozoospermic, 95.5% were asthenozoospermic and 64.3% had abnormal sperm morphology. Low motility and abnormal sperm morphology was significantly related to drug addiction and this finding is consistent with the finding of Kumar et al[12] in drug exposed subjects.

In Group 2 of present study, maximum cases with normal sperm count were seen in the category of green sperm heads (native DNA) in the range of 41-50%. Maximum cases 37.5% with normal morphology had green sperm heads in the range of 31-40% and only 1.25% case with normal morphology was reported in the category of green sperm heads in the range of both 0-10% and 11-20%.

Maximum cases 13.75% with total motility more than 40% were seen in the category with green sperm heads in the range of 41-50% and no case was reported with total motility more than 40% with green sperm heads in the range of 0-30%. All the semen parameters are significantly related to DNA damage with p value ($p<0.05$).Tejada et al[27] and few other studies have concluded that in the analysis of DNA integrity using acridine orange fluorescence microscopy method, less than 50% of green sperm heads is taken as abnormal and might be responsible for infertility. With increase in the range of green sperm heads, the semen characters improved showing the correlation of DNA damage of sperms with the fertility of men.This finding is comparable to other studies of Bungum et al[28,] Kumar et al[12]and A.C Varghese et al[11.]

Similarly, Sergey I Moskovtsev al[29] in his study indicated that DNA damage was significantly correlated to semen parameters with p value ($p<0.05$), which goes in favour of the present study. Whereas, Khalilietal[30] in his study failedto show any significant difference in the DNA integrity of the spermatozoa between infertile and fertile men, which is reverse of the present study.

V. Conclusion

Decline in semen quality is in direct proportion with the DNA damage of the sperms. Patients exposed to the bad lifestyle changes have poor semen quality along with more DNA damage. In urban areas, more patients are affected maybe because of radiation exposure due to mobile towers/high tension wires, excessive use of Laptops, Cell Phones and prolonged sitting at their work place. In 18.75% cases of Group 2, the semen parameters were normal but even then DNA damage was evident which proves that fluorescence microscopy of seminal fluid using acridine orange is an independent parameter for assessing male fertility. Despite having normal semen parameters, patients in our study were infertile because of defects in their DNA.

Collaborating the findings from our study and the previous studies in the literature, it is concluded that fluorescence microscopy of seminal fluid using acridine orange is an easy test to perform and a relatively cheap method as compared to other methods which are expensive and require more machinery, equipment's and skilled technical staff. Although easy to perform and relatively cheap, it does not loses its relevance in semen analysis in comparison to other tests.

Conflict of interest: None declared
Ethical Clearance: Approved from ethical committee of the department.

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Dr. Gaurav Pawar1, A Fluorescence Microscopy Study of Seminal Fluid In Infertile Males Using Acridineorange Dye To Assess DNA Integrity Along With Semen Analysis ."IOSR Journal of Dental and Medical Sciences (IOSR-JDMS), vol. 17, no. 10, 2018, pp 25-32.