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Research Article

Sperm chromatin and ICSI Outcome

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Abstract

Background: All male partners of couples who achieved a pregnancy during the first 3 months attempting to conceive had < 30% sperm with DNA fragmentation index (DFI). (DNA fragmentation index was calculated as the ratio of spermatozoa with abnormal chromatin to all spermatozoa counted per sample) and 20% of couples who never achieved a pregnancy had > 30% sperm with DFI. Moreover 84% of the men who initiated pregnancy in the first 3 months of pregnancy had sperm DNA damage levels of < 15% ***(Evenson et al., 1999)*** **. Objectives**: The aim of this study was to find out the relationship between sperm chromatin status and ICSI results (fertilization rate and embryo quality). **Patients and methods:** The study is a prospective observation study **Patient(s**): *90 infertile couples.* Intervention(s): A total of 90 semen samples were collected from couples undergoing ICSI and were analyzed according to WHO criteria. Each sample was evaluated for sperm chromatin integrity using three cytochemical assays and semen processing by swim up method. The ICSI was carried out according to a long-term pituitary down regulation protocol**. Measure(s***):* The relation between sperm chromatin integrity and ICSI outcomes (fertilization rate and embryo quality) was examined. **Results** There was no significant correlation between the results of chromatin assays Acridin orange(AO) ,Aniline blue(AB) and Tolludine blue stain(TB,) and fertilization outcomes following ICSI. There were significant negative relations between embryo quality grades with percentage of abnormal chromatin using Acridin orange ,Aniline blue and Tolludine blue stain. There were significant negative correlations between embryo quality grades and percentage of abnormal chromatin using mean DFI for the three stains. **Conclusion:** The impact of sperm DNA damage on fertilization rates remains controversial, but there is a negative effect on embryo development and pregnancy rates

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Keywords: Sperm Chromatin, Intracytoplasmic Sperm Injection

Introduction

The field of Assisted Reproductive Technology (ART) including Intracytoplasmic Sperm Injection (ICSI), invitro fertilization (IVF) and intrauterine insemination (IUI) has been significantly developed in the last two decades and consequently demand for such treatments have dramatically increased. Among various assisted reproductive techniques, ICSI has become a preferred and widely applied method for helping infertile couples to achieve conception (***Tarlatzis et al., 2007).*** There is also an increased tendency for ICSI than IVF, according to Human Fertilization and Embryology Association (HFEA) statistics ***(Patient's Guide National Data Statistic., 2008).*** However, despite the incredible success and broad use of ICSI, these techniques carry some risks. It is argued that through bypassing the natural selection of sperm-zona penetration, the risk of oocyte fertilization by defective sperm will be increased which then can lead to malformations in the offspring ***(Karpman et al., 2005).*** During ICSI, we attempt to select the best spermatozoa according to its morphology and motility as markers

of normality and viability. However, according to the recent studies the routine sperm analyses such as sperm count, motility and morphology, are not reliable indicators of sperm function and chromatin integrity ***(Sills et al., 2004).*** Therefore sperm with normal motility and morphology, but containing chromatin abnormality will be given the chance to penetrate and fertilize the eggs. Sperm functional assay is considered as the valuable diagnostic tool in evaluation and prediction of male infertility ***(Boe-Hansen et al., 2006).***

Different studies have assessed the diagnostic potential of this assay in the outcome of assisted reproductive technologies (ART). Sperm chromatin is tightly packaged to protect DNA during the transit that occurs before fertilization. Any abnormality in chromatin structure and integrity could lead to failure in fertilization. A variety of tests have been described to assess sperm chromatin status that are considered as valuable predictive tools for in vivo and in vitro fertilization outcomes. However, there were widespread controversies over the relationships between sperm chromatin status and ICSI outcomes. Based on various studies of different cases subjected to ICSI program, abnormal sperm factors lead to low fertilization rate and poor embryo quality ***(Velez et al .,2008).*** In contrast there are studies indicating that fertilization rate, embryo cleavage and pregnancy rates are independent of sperm parameter in ICSI ***(Lin et al.,2008).***Aim of the study to assess the importance of sperm chromatin integrity on ICSI outcomes, and to determine the relationship between sperm chromatin status using three cytochemical staining assays (AB) for abnormal persistence of histones, (TB) and (AO) for DNA abnormality and ART results (fertilization rate and embryo quality) during the ICSI treatment.

Materials and Methods

Patients and Methods

The study is a prospective observational study that conducted on 90 infertile couples from those attendant to International Islamic Center for Population Studies and Research (IICPSR)- Al Azhar University for ICSI procedure.,Informed written consent was obtained from all couples included in this study according to ethical regulation of raesearchs involving the human subjects of International Islamic Centre for Population Studies and Research (IICPSR)-ART unit, Al Azhar University, Cairo, Egypt,

Inclusion criteria

Couples with primary infertility
Fresh semen samples
Semen samples count with more than 1×10^6 sperm/ml
The female partner age <40 yrs
Female BMI 20-30 kg/m^2
Female baseline FSH (b-FSH) <12 IU/L

Exclusion criteria

Cry-preserved semen samples
Semen samples with less than 1×10^6 sperm/ml
Previous failed ICSI more than three trials
Secondary infertility
Less than two follicles after induction of ovulation

Male partners included in this study were subjected to the following:

1. Complete history taking.
2. General and local examination.
3. semen analysis was assessed according to WHO criteria.
4. Scrotal ultrasonography.
5. Measurement of sperm DNA damage expressed as the DNA fragmentation index using Toluidine Blue, Aniline Blue and acridine orange staining and viewed under microscope.

Statistical analysis

Data were analysed using Statistical Package for Social Science (SPSS) version 20.0.Categorical variables were described using number and percntages, continuous variables were described using mean and statndard diviasion.Comparison of the categorical variables was performed using Chi square test. Continuous variables were compared using Student t- test for normally distributed variables and Mann-whitney U test for not normally distributed variables. P values of less than 0.05 were regarded statistically significant. Power of significance probability; P value > 0.05 insignificant. P value < 0.05 significant. P value < 0.01 highly significant and p value < 0.001 very high significant., persons correlation co efficient was used.

Results

Table (1): Relationship between Embryo quality and DFI by Acridin Orange

	Acridin orange DFI						P-value
	< 15%		15 – 30%		> 30%		
	No.	%	No.	%	No.	%	
Embryo quality A	91	49.5	66	23.6	5	7.6	0.000
Embryo quality B	65	35.3	78	27.9	10	15.2	0.007
Embryo quality C	20	10.9	70	25.0	23	34.8	0.000
Embryo quality D	8	4.3	66	23.6	28	42.4	0.000
Total	184	100.0	280	100.0	66	100.0	

Chi-square test was used; P-value < 0.05 was considered significant. P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant.

There were statistically significant negative correlations between embryo quality grades with parentage of abnormal chromatin using Acridin orange staining .

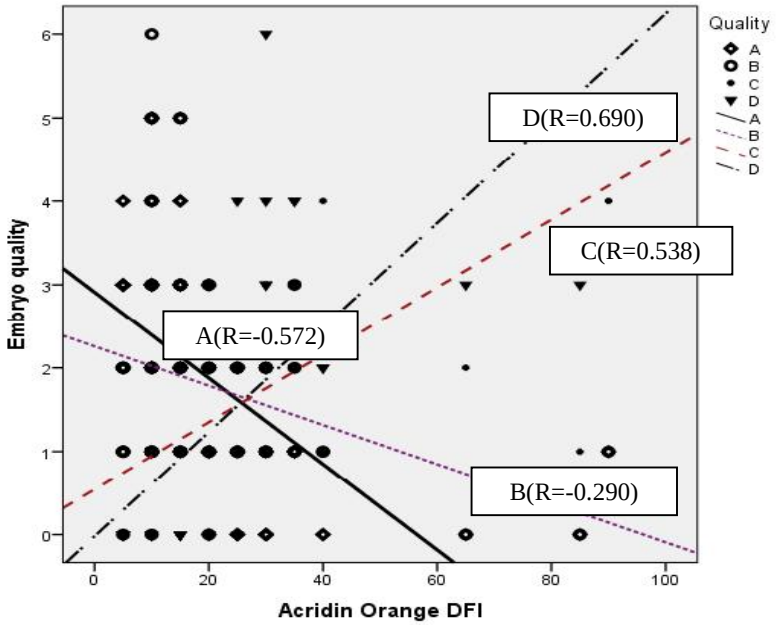
Table (2): Relationship between Embryo quality and DFI by Tolludine blue

	Tolludine blue DFI						P-value
	< 15%		15 – 30%		> 30%		
	No.	%	No.	%	No.	%	
Embryo quality A	83	48.0	75	25.6	4	6.3	0.000
Embryo quality B	64	37.0	81	27.6	8	12.5	0.007
Embryo quality C	18	10.4	72	24.6	23	35.9	0.000
Embryo quality D	8	4.6	65	22.2	29	45.3	0.000
Total	173	100.0	293	100.0	64	100.0	

Chi-square test was used

P-value < 0.05 was considered significant., P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant.

This table show the relation between tolludine blue DFI and embryo quality. There were significant negative relations between embryo quality grades and percentage of abnormal chromatin using Tolludine blue stainin



Chi-square test was used

P-value < 0.05 was considered significant., P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant

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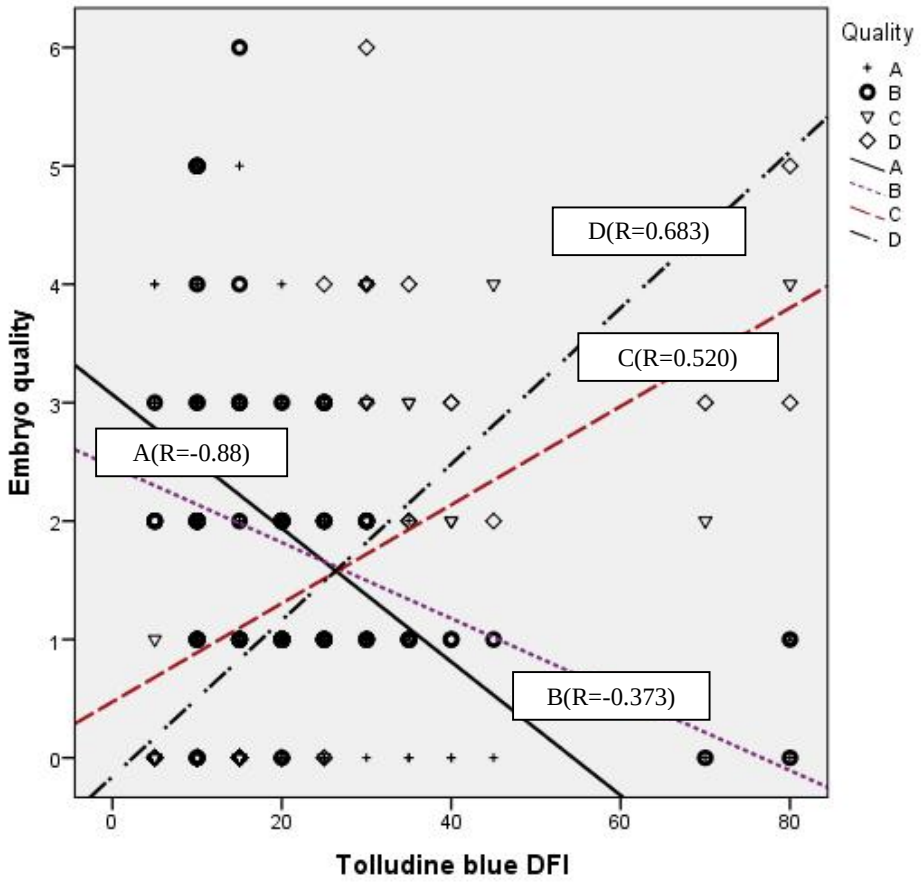

Table (3): Relationship between Embryo quality and DFI by Aniline blue

	Aniline blue DFI						P-value
	< 15%		15 – 30%		> 30%		
	No.	%	No.	%	No.	%	
Embryo quality A	61	55.0	97	27.5	4	6.1	0.000
Embryo quality B	40	36.0	104	29.5	9	13.6	0.007
Embryo quality C	7	6.3	82	23.2	24	36.4	0.000
Embryo quality D	3	2.7	70	19.8	29	43.9	0.000
Total	111	100.0	353	100.0	66	100.0	

Chi-square test was used

P-value < 0.05 was considered significant., P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant

This table show the relation between aniline blue DFI and embryo quality., There were statistically significant negative correlations between embryo quality grades with percentage of abnormal chromatin using Aniline blue staining .

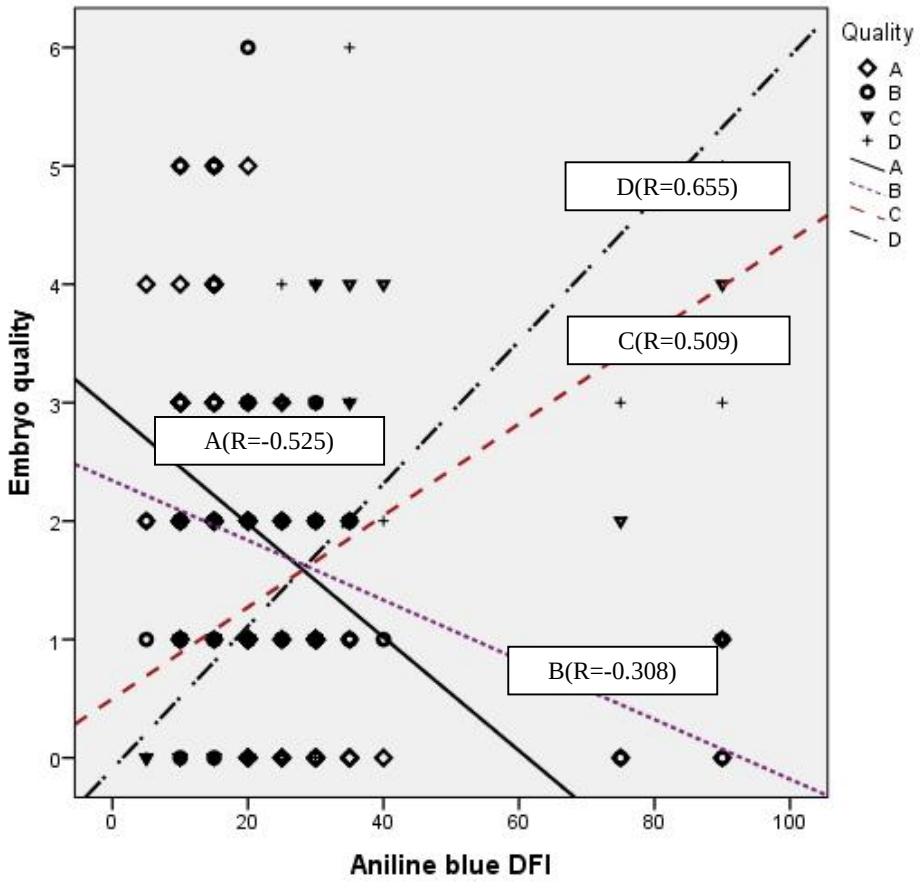

Table (4): Relationship between embryo quality and mean of DFI by 3 methods

	Mean of 3 methods						P-value
	< 15%		15 – 30%		> 30%		
	No.	%	No.	%	No.	%	
Embryo quality A	98	49.5	58	23.5	6	7.1	0.000
Embryo quality B	72	36.4	69	27.9	12	14.1	0.007
Embryo quality C	20	10.1	64	25.9	29	34.1	0.000
Embryo quality D	8	4.0	56	22.7	38	44.7	0.000
Total	198	100.0	247	100.0	85	100.0	

Chi-square test was used

P-value < 0.05 was considered significant, P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant

This table show the relation between mean of DFI by 3 methods and embryo quality., There were statistically significant negative relations between embryo quality grades with percentage of abnormal chromatin using mean of DFI by 3 methods

Table (5): Relationship between Embryo quality and pregnancy outcome

	Outcome		**P-value**
	Negative pregnancy	**Positive pregnancy**	
	Mean ± SD	**Mean ± SD**	
Embryo quality A	1.69 ± 0.20	2.11 ± 0.20	0.116
Embryo quality B	1.67 ± 0.17	1.89 ± 0.19	0.191
Embryo quality C	1.57 ± 0.16	0.97 ± 0.18	0.015
Embryo quality D	1.53 ± 0.21	0.74 ± 0.18	0.010

Mann-Whitney Test
* Statistical significant difference (P < 0.05)
P-value < 0.05 was considered significant., P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant

This table show that bad Embryo quality is associated with negative pregnancy

Table (6): Relationship between semen parameters and DFI by Acridin Orange

	Acridin orange DFI			**P. value**
	< 15%	15 – 30%	> 30%	
	Mean ± SD	Mean ± SD	Mean ± SD	
Sperm count (10^6 per mL)	**24.8+4.9**	**26+5.8**	**27.6+4.3**	**0.304**
Progressive motility	**17.2+15.3**	**23.4+15.3**	**28.2+19.3**	**0.087**
abnormal forms	21.06+13.21	28.59+11.96	60.91+15.94	0.000

ANOVA test was used
P-value < 0.05 was considered significant,
P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant
This table show that asignificant negative relation between only abnormal forms of sperms and DFI by Acridin Orange

Table (7): Relationship between semen parameters and DFI by Tolludine blue

	Tolludine blue DFI			**P. value**
	< 15%	15 – 30%	> 30%	
	Mean ± SD	Mean ± SD	Mean ± SD	
Sperm count (10^6 per mL)	**24.1+5.3**	**26.5+5.5**	**26.5+4.1**	**0.138**
Progressive motility	**19.8+17.2**	**22+14.9**	**25+18.8**	**0.637**
abnormal .forms	17.32+8.22	29.2+12.55	61.25+13.51	0.000

ANOVA test was used
P-value < 0.05 was considered significant,
P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant

This table show that a significant negative relation between only abnormal forms of sperms and DFI by Tolludine blue

Table (8): Relationship between semen parameters and DFI by Aniline blue

	Aniline blue DFI			**P. value**
	< 15%	15 – 30%	> 30%	
	Mean ± SD	Mean ± SD	Mean ± SD	
Sperm count (10^6 per mL)	**24.8+4.6**	**26+5.8**	**26.9+4.2**	**0.513**
Progressive motility	**20.3+18.5**	**21.9+14.4**	**23.6+20**	**0.851**
abnormal .forms	19.57+12.42	28.75+13.89	56.36+18.85	0.000

ANOVA test was used
P-value < 0.05 was considered significant,
P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant

This table show that asignificant negative relation between only abnormal forms of sperms and DFI by Aniline blue

Table (9): Relationship between semen parameters and mean of DFI by 3 methods

	Mean of 3 methods			**P. value**
	< 15%	15 – 30%	> 30%	
	Mean ± SD	Mean ± SD	Mean ± SD	
Sperm count (10^6 per mL)	**24.6+4.9**	**26.5+5.8**	**26.8+4.6**	**0.231**
Progressive motility	**17.5+16.2**	**25+14.7**	**22.5+18.5**	**0.130**
abnormal .forms	19.43+10.56	29.02+12.46	57.86+15.15	0.000

ANOVA test was used
P-value < 0.05 was considered significant,
P-value < 0.01 was considered as highly significant. P-value > 0.05 was considered insignificant

This table show that a significant negative relation between only abnormal forms of sperms and mean of DFI by 3 methods .

Discussion

Sperm DNA damage is multifactorial and may be due to various pathologic conditions including cryptorchidism, cancer, fever, age, infection, leukocytospermia, and varicocele **(Sakkas et al; 1999; Evenson & Wixon, 2005).** Several other studies have evaluated DNA structure and integrity in human ejaculated sperm samples and have reported a negative effect of high percentages of DNA damaged spermatozoa on pregnancy rates **(Larson *et al*., 2000; Larson-Cook *et al*., 2003; Saleh *et al*., 2003b; Tesarik *et al*., 2004b).** In other studies, there seems to be a consensus that no pregnancies resulted after ART if >27% of sperm in the net sample showed DNA denaturation by sperm chromatin structure assay (SCSA) **Gandini *et al*. (2004)** demonstrated ICSI pregnancies even with semen samples characterized by a high SCSA DFI (>30%); . In a larger study on the predictive value of sperm chromatin structure assay(SCSA) in relation to the outcome of IVF and ICSI**. Bungum *et al*. (2004)** reported that a DFI level >27% is not incompatible with pregnancy following IVF and ICSI. Another study by **Saleh *et al*. (2003),** using the SCSA, has demonstrated a significant increase in the extent of DNA damage in infertile patients with varicocele.

In this study, three different cytochemical staining methods including Toluidine Blue, Aniline Blue, and Acridine Orange assays were applied in order to assess different aspects of sperm DNA integrity., Aniline Blue staining to detect excessive presence of histones; Toluidine Blue and fluorescence Acridine Orange, as a sensitive structural probes, were used for assessing sperm chromatin structure and packaging **(Erenpreisa et al., 1992)** The TB is a classic nuclear dye used for

external metachromatic and orthochromatic staining of chromatin, which overall is negatively charged, It becomes heavily incorporated in sperm chromatin. The AO assay measures the chromatin). This stain is a sensitive structural probe for chromatin structure and packaging, assessed by microscopy. **(Erenpreisa et al.,2003)**.

Our results showed that when the cases were divided in three groups according to chromatin abnormality, no significant differences in fertilization rates were found among the three groups using Acridine Orange, Aniline blue and Toluidine blue.

Our results were in agreement with **(Zini et al.,2005)** that found no significant correlation between Chromomycin A3, Acridine Orange and Toluidine Blue with fertilization rate in infertile couples undergoing IVF/ ICSI. Also, our results were in agreement with the finding of Hammadeh and his colleagues, that reported no significant correlation between sperm with abnormal chromatin and fertilization rate with regard to 30% cut off for DNA fragmentation using Aniline Blue staining **(Hammadeh et al.,1996)**,.

In contrary to our results there were broader studies showing negative associations between frequency of sperm DNA fragmentation and fertilization rate and embryo development **(Avendaño et al., 2009).**

Therefore, there are still controversies over the effect of sperm chromatin status on fertilization rate **(Collins et al., 2010) ,**

In this study There were significant negative correlations between embryo quality grades and percentage of abnormal chromatin using Acridin orange, Tolludine blue and Aniline blue staining as showen in tables.(1,3 and 5).

By using Acridin orange correlations between embryo quality grades and percentage of abnormal chromatin were R=-0.572, R=-0.373, R=0.520 and R=0.683 for embryo quality A,B,C and D respectively.

By using Tolludine blue correlations between embryo quality grades and percentage of abnormal chromatin were R=-0.88, R=-0.290, R=0.538 and R=0.690 for embryo quality A,B,C and D respectively. By using Aniline blue correlations between embryo quality grades and percentage of abnormal chromatin were R=-0.525 susceptibility of sperm nuclear chromatin to acid induced denaturation in situ by quantifying the metachromatic shift of AO fluorescence from green (native chromatin) to red (denatured ,R=-0.308, R=0.509 and R=0.655 for embryo quality A,B,C and D respectively.

There were significant negative correlations between embryo quality grades and percentage of abnormal chromatin using mean DFI by the three stains.

As regard to the former results we can predict that abnormal chromatin may affect embryo quality following ICSI procedure.

There were statistically significant negative relations between pregnancy rate and percentage of abnormal chromatin using Aniline blue staining only but there was no statistically significant relation between pregnancy rate and percentage of abnormal chromatin using Acridin orange and Tolludine blue staining .

an explanation for the non significant correlation between fertilization rates and sperm chromatin abnormalities may be due to the fact that assays for sperm chromatin abnormalities were performed on raw semen samples that contained large numbers of immotile, nonviable or degenerated sperm with abnormal chromatin. However, through pre-ICSI processing techniques (gradient centrifugation, swim up or glass wool, etc.) most abnormal sperms are removed and the resultant processed semen contain motile sperm with normal morphology **(Boomsma et al., 2004),** this is because processed sperm possess greater quality, assessment of the correlation between sperm parameters in raw semen sample and fertilization ability of the processed semen samples add further to the controversies. In other words, detected sperm in raw semen does not reflect the population of normal selected sperm applied in the ICSI treatment.

This is especially true for oligoasthenoteratozoospermic (OAT) samples that contain largely non-viable and immotile sperm. Indeed, spermatozoa of OAT samples recovered after processing techniques are significantly improved sperm parameters **(Zollner et al., 2001).** There is olso no significant Relationship between semen count and progressive motility with DFI by the three stains, but There is asignificant Relationship between semen abnormal forms with DFI by the three stains as showen in tables (6,7.8 and 9)

Conclusion

The impact of sperm DNA damage on fertilization rates remains controversial, but there is negative effects on embryo development and pregnancy rates.

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