

## **Association between sperm chromatin status and macroscopic sperm parameters in human**

### **العلاقة بين حالة كروماتين النطف ومعايير النطف العيانية في الإنسان**

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#### **Abstract**

Most macroscopic parameters indicate accessory gland dysfunctions or congenital defects , which may also effect sperm chromatin status by increasing oxidative stress and apoptosis .

The current study aimed to find out the possible relationship between sperm chromatin status and the parameters of macroscopic semen examination .

This study was carried out between June 1<sup>st</sup>. 2014 and November 1<sup>st</sup>. 2014 including one hundred (100) selected infertile men who was treated at the Fertility Center in Al-Sadr Medical City in Al-Najaf Province . The study also included twenty (20) healthy fertile volunteers as a control .

Aniline blue (AB) staining was used to differentiate between normal condensed chromatin (unstained) and abnormal decondensed chromatin (blue), while toluidine blue (TB) was used to differentiate between normal intact sperm DNA (blue) and abnormal damaged sperm DNA (purple) .

Abnormal macroscopic semen parameters (except semen appearance and pH) and abnormal sperm chromatin status were seen to be increased in significant levels in the infertile group as compared to fertile men (control) . The results also revealed that there was a high significant negative correlation ( $p<0.01$ ) between both sperm chromatin decondensation and sperm DNA damage and each of ejaculate volume, liquefaction time, and a positive association with semen viscosity , while no relationship was observed with semen appearance and pH .

The study concluded that abnormal semen macroscopic parameters may indicate sperm DNA fragmentation or chromatin decondensation . It is recommended that aniline blue and toluidine blue staining are used to assess sperm DNA fragmentation or chromatin decondensation when bad macroscopic parameters are noticed in the first part of seminal fluid analysis .

#### **المستخلص**

إن أغلب الفحوصات العيانية للسائل المنوي تؤثر وجود اعتلال في عمل الغدد التنكاثرية المساعدة أو عيوباً خلقية ، وهذه الأمور ربما تؤثر أيضاً على حالة كروماتين النطف من خلال زيادة الاجهاد التأكسدي والموت الخلوي .

هدفت الدراسة الحالية للبحث عن العلاقة المحتملة بين حالة كروماتين النطف والفحوصات العيانية للسائل المنوي . تم إجراء هذه الدراسة للمدة من الأول من حزيران 2014 إلى الأول من تشرين الأول 2014 ، إذ تضمنت الدراسة إجراء فحص السائل المنوي والفحوصات الوراثية لعينة مكونة من (100) من الرجال العقيمين الذين يعالجون في مركز الخصوبة في مدينة الصدر الطبية في محافظة النجف الأشرف ، كما شملت الدراسة (20) شخصاً من الرجال الخصيين (مجموعة السيطرة) .

تم استعمال صبغة أزرق أنيلين (AB) للتمييز بين الكروماتين المكثف (السوي غير المصطبغ) للخلايا النطفية وبين الكروماتين غير المكثف (غير السوي المصطبغ باللون الأزرق) ، كما تم استعمال صبغة أزرق تولويدين (TB) للتعرف على الحامض النووي الرايبوزي منقوص الأوكسجين DNA والتمييز بين حالته السوية (اللون الأزرق) وحالته المحطمة (اللون البنفسجي) .

بينت النتائج أن المعايير العيانية غير السوية (باستثناء المظهر ودرجة الحموضة) وكذلك حالة كروماتين النطف غير السوية في الرجال العقيمين قد أظهرت زيادة بمستويات معنوية مقارنة مع الرجال الخصيين ، كما بينت الدراسة وجود ارتفاع معنوي ( $P<0.05$ ) في النسبة المئوية للنطف ذات الـ DNA المحطم في مجموعة (أسوياء النطف) من الرجال العقيمين مقارنة مع الرجال الخصيين . كما بينت النتائج وجود علاقة عكسية ذات دلالة إحصائية عالية المعنوية ( $P<0.01$ ) بين حالتي تكثف الكروماتين وتضرر الـ DNA وبين كل من حجم السائل المنوي ، وزمن الإماعة ، بينما كانت العلاقة طردية مع لزوجة السائل المنوي ، ولم تكن هناك علاقة مع المظهر ودرجة الحموضة .

استنتجت الدراسة أن المعايير العيانية غير السوية للسائل المنوي قد تؤثر حصول حالة تكثف الكروماتين وتضرر الـDNA للنطف . أوصت الدراسة أن استعمال تصبيغ كروماتين النطف بواسطة صبغة أزرق أنيلين (AB) أزرق تولويدين (TB) للتحري عن حالتي تكثف الكروماتين وتضرر الـDNA على التوالي ، وذلك في حالة ملاحظة معايير غير سوية للفحص العياني لسائل المنوي .

## **INTRODUCTION**

The assessment of male infertility has been routinely relied on semen analysis , which is the key predictor in the evaluation of male reproductability . Although have a little clinical importance, macroscopic semen parameters are still used in the infertility clinic laboratories as one part of the routine seminal analysis (1-2) .

The macroscopic portion in a standard sperm analysis evaluates coagulum formation, liquefaction, volume, viscosity, appearance, and pH (3) . It was observed that these parameters have conserved their normal values which remained quite unchanged since the first use of the semen analysis . However, the latest versions of the WHO laboratory manuals have exhibited some differences in liquefaction time (4) .

Most macroscopic parameters indicate accessory gland dysfunctions or congenital defects , for example : low pH may indicate obstructional problems, long liquefaction time may refer to congenital absence of the seminal vesicles, low volume may point to Retrograde ejaculation, and hyperviscosity may be associated to prostate disorders resulting from chronic inflammation (5-7) .

Today, it is well known that the quality and integrity of sperm chromatin is very important in the reproductive potential of men. Sperm DNA is known to contribute to half of the genomic material of the embryo (8). Sperm chromatin is much more compact in somatic cells, with the aim of protecting the paternal genetic materials against damaging factors. Abnormalities in the male genome characterized by damaged sperm DNA may be indicative for male infertility, the biological effect of abnormal sperm chromatin structure depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it. (9)

To evaluate sperm chromatin status, sperm chromatin decondensation and DNA fragmentation are the most two common methods . The colorimetric methods are one of the simple and inexpensive methods for the assessment of chromatin integrity , among of which are aniline blue (AB) and toluidine blue (TB) staining . AB is used to detect sperm immaturity (i.e. sperms with immature or decondensed chromatin) , while TB is used to estimate sperm DNA damage or fragmentation (10) .

There are little previous studies that focused on investigating the relationship between macroscopic semen parameters and the level of sperm chromatin decondensation and DNA damage; perhaps because these parameters are less clinically used in the diagnosis of infertility . However, no laboratory practitioners can make routine seminal analysis without macroscopic seminal parameters (11) .

This study aims to find out the relationship between sperm chromatin status and the parameters of macroscopic semen examination .

## **Materials and METHODS**

### **1. Study subjects :**

The study included one hundred (100) selected infertile men who attended to fertility Center in Al-Sadr Medical City in Al-Najaf Province between June 1<sup>st</sup>. 2014 and November 1<sup>st</sup>. 2014. The study also included twenty (20) healthy volunteer fertile men who have one or more than one child.

### **2. Semen and Serum Collection**

Semen samples were collected by masturbation , after 3–5 days of abstinence, in wide mouth disposable plastic container (12) .

The semen was centrifuged at 3000 (rpm) for 10 minutes to obtain the plasma . Also a total of 5 ml of blood was obtained from the patients control men as well and centrifuged to separate .

### 3. Seminal Analysis :

Routine seminal analysis was achieved according to the criteria and procedures submitted by WHO (2010) (1) .

### 4. AB staining

Slides are prepared by smearing 5 µl of semen sample. The slides are air-dried and fixed for 30 minutes in 3% glutaraldehyde in phosphate-buffered saline (PBS) . The smear is dried and stained for 5 minutes in 5% aqueous aniline blue solution (pH 3.5). Sperm heads containing immature nuclear chromatin stain blue, and those with mature nuclei do not take up the stain (Figure 1) . The percentage of spermatozoa stained with aniline blue is determined by counting 200 spermatozoa per slide under bright field light microscopy (13) .

### 5. TB staining

Slides are prepared by smearing 5 µl of semen sample. The smears are air-dried, fixed in freshly made 96% ethanol–acetone (1 : 1) at 4°C for 30 minutes, hydrolyzed in 0.1 N Hcl at 4°C for 5 minutes, and rinsed three times in distilled water for 2 minutes each. Smears are stained with 0.05% TB (Merck, Poole, Dorset, UK) for 10 minutes. The staining buffer consists of 50% citrate phosphate (McIlvain buffer, pH 3.5). Under light microscope, sperm heads with good chromatin integrity stain light blue, and those of diminished integrity stain purple (Figure 2) (14) .

### 6. Statistical Analysis:

The analysis of data was performed by using a Megastat (Version 9.4 2005) computer program . Results were expressed as mean ± standard deviation S.D . Independent unpaired student t-test was used to analyze the differences between infertile and control groups .

Chi-Square test was used to find out the significant difference in ASA incidence ,the results of correlation were expressed as a P-value (for significance) , r-value (correlation coefficient) .

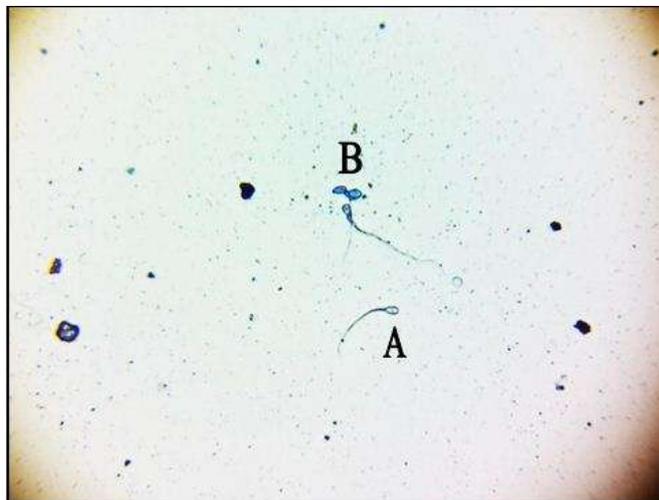


Figure (1) : Sperm Cells stained by Aniline Blue Staining (AB) .

A : Mature Sperm with unstained head (Condensed Chromatin) .

B : Immature Sperm with blue head (Decondensed Chromatin) .

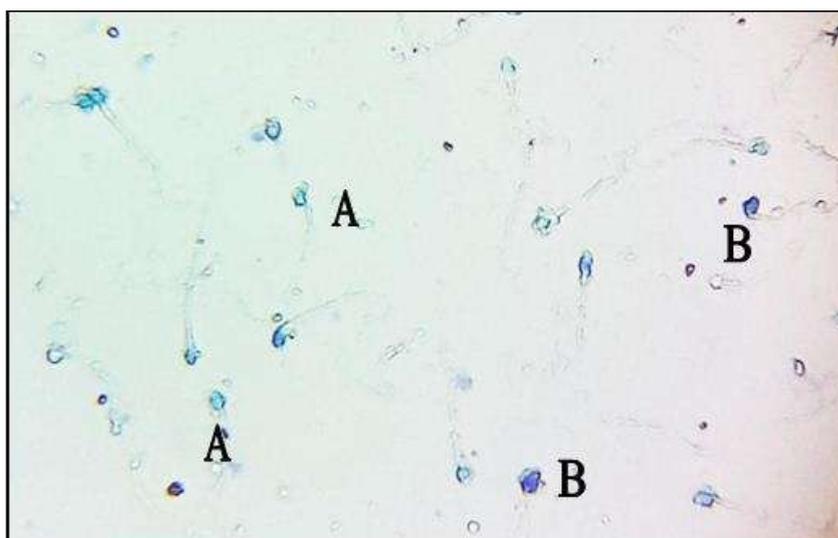


Figure (2) : Sperm Cells stained by Toluidine Blue Staining (TB) .  
 A : Normal Sperm Cells with Blue Heads (Intact DNA) .  
 B : Abnormal Sperm Cells with purple heads (Damaged DNA) .

**RESULTS & DISCUSSION :**

**1. Macroscopic Seminal Parameters of Infertile and Fertile Men**

Tables (1) and (2) showed a high significant difference ( $p < 0.01$ ) in macroscopic seminal fluid investigation between the infertile and fertile groups .

This difference indicates that macroscopic seminal parameters have a discriminate power that can be used in the routine infertility test, even though the clinical significance is less than that of the microscopic parameters (2) . Abnormal semen is often low in volume and non-coagulating with acidic pH . Volume variation is of value when it is consistently low and may indicate partial duct obstruction or retrograde ejaculation, while high-volume semen ( $> 8\text{ml}$ ) is often associated with poor-quality semen (15) .

Prolonged liquefaction and high viscosity indicate poor prostatic secretion as in the case of inflammation. If these cases are associated with low sperm motility, the sperm transportation will be compromised. (16) .

Table (1) : Ejaculate volume and liquefaction time of infertile and fertile men

| Parameter               | Infertile Men<br>(No. = 100) M $\pm$ SD | Fertile Men<br>(No. = 20) M $\pm$ SD | P-Value |
|-------------------------|---|--------------------------------------|---------|
| volume (ml)             | 3.2 $\pm$ 2.2                           | 5.82 $\pm$ 1.4                       | <0.001  |
| Liquefaction time (min) | 37.4 $\pm$ 4.2                          | 25.1 $\pm$ 3.71                      | <0.001  |

Table (2) : Semen appearance, viscosity and pH of infertile and fertile men

| Parameter  |                          | Infertile Men (No. = 100) | Fertile Men (No. = 20) | Chi-Square<br>(p-value)    |
|------------|--------------------------|---------------------------|------------------------|----------------------------|
| appearance | normal                   | 15 (15%)                  | 1 (5%)                 | $\chi^2 = 1.44$<br>(0.223) |
|            | abnormal                 | 85 (85%)                  | 19 (95%)               |                            |
| viscosity  | normal                   | 28 (28%)                  | 0 (0%)                 | $\chi^2 = 4.57$<br>(0.03)  |
|            | abnormal                 | 72 (72%)                  | 20 (100%)              |                            |
| pH         | normal<br>(7.2-7.8)      | 22 (22%)                  | 2 (10%)                | $\chi^2 = 1.5$<br>(0.22)   |
|            | Abnormal<br>(<7.2, >7.8) | 78 (78%)                  | 18 (90%)               |                            |

## **2. Sperm Chromatin Integrity of Infertile and Fertile Men**

As illustrated in table (3) , there is a high significant increase in the percentage of sperm with abnormal AB and TB staining in infertile men comparing to fertile donors . The results of the current study revealed a high significant increase in sperm chromatin decondensation (assessed by AB) in the infertile group as compared to fertile men (table3) . These results come in consistence with many previous studies which recorded a high significant decondensation rate in infertile patients indicating that sperm chromatin decondensation is one of the factors associated with infertility (14 , 17) .

There was also a high significant increase in sperm DNA fragmentation (assessed by TB) in the infertile comparing to fertile men (table 3) . This proves the accordance among many investigators that sperm DNA fragmentation is one of the initial factors that minimize the reproductive capabilities of men (18-20) .

The effect of DNA damage and chromatin decondensation on male fertility has been proved to result from retarded spermatogenesis and apoptosis (24) ; some researchers observed that DNA integrity is negatively correlated with late apoptosis(21) ,While others found that the performance of spermatogenesis according to WHO classification is correlated with sperm DNA damage (22), also some other researchers reported that patients with abnormal semen parameters have a higher count of sperms that contain damaged DNA and abnormal sperms that express apoptotic markers (23).

Sperm DNA damage is associated with underprotamination in infertile men, the exact effect of protamine downregulation on male infertility is still unknown, it has been postulated that protamine deficiency makes sperm DNA more sensitive to damage by exogenous and endogenous factors specially during late spermatogenesis, these factors include nucleases, free radicals that can cause oxidative stress or environmental mutagens (25) .

Table (3) : Abnormal Aniline Blue & Toluidine Blue staining of Infertile and Fertile Men

| Parameter     | Infertile Men<br>(No. = 100)<br>M ± SD | Control (Fertile)<br>(No. = 20)<br>M ± SD | p-value |
|---------------|--|---|---------|
| Blue AB (%)   | 55.68 ± 18.9                           | 24.87 ± 5.48                              | < 0.001 |
| Purple TB (%) | 33.5 ± 13.4                            | 18.66 ± 6.22                              | < 0.001 |

## **3. Correlation between seminal fluid macroscopic parameters and percentage of abnormal AB & TB chromatin staining of infertile men**

As listed in table (4) , there is a high significant negative correlation ( $p < 0.01$ ) between AB abnormal staining in infertile men and each of : ejaculate volume and liquefaction time . the same table also shows a significant negative correlation ( $p < 0.05$ ) between TB abnormal staining in infertile men and each of : ejaculate volume and liquefaction time .

Table (5) shows that sperm chromatin damage and decondensation (assessed by TB AB respectively) are associated with semen viscosity , while no association was seen with semen pH as in table (6) .

As shown in (table 4) , there is a high significant negative correlation between ejaculate volume and each of sperm chromatin decondensation and fragmentation, while a significant positive correlation is shown with semen liquefaction time , while tables (5) and (6) shows that both sperm chromatin decondensation and fragmentation are affected by semen viscosity and pH respectively .

Sperm chromatin decondensation and fragmentation seems to have similar causes that leads to low semen volume, some researchers found that ejaculate volume is related to resistance to sperm chromatin decondensation, they demonstrated that patients with reduced prostatic secretions, evidenced by low semen volume, have higher levels decondensation that due to low concentration of zinc that is derived from prostatic secretions (26) . In contrast to the present study , some

researchers used chromatin structure assay (SCSA), and found a significant positive correlation between DNA fragmentation index DFI, this may be attributed to the difference in the method used in the estimation of DNA damage(27) .

Some researchers found that a high significant negative significant correlation ( $r = - 0.242$  ;  $P = 0.01$ ) was assessed between AB test and liquefaction time, this may be explained by deficiency in the prostatic secretions, which results in elongating liquefaction time and increasing free radicals that cause DNA damage (28-29) . Recent studies reported that oxidative stress associated with semen hyperviscosity may cause infertility by damaging sperm membrane and DNA (6) .

Table (4) : Correlation between ejaculate volume and liquefaction time and percentage of abnormal AB & TB chromatin staining of infertile men

| Chromatin Stain Parameter | Blue AB (%)   | Purple TB (%) |
|---------------------------|---------------|---------------|
| volume (ml)               | $r = - 0.278$ | $r = - 0.285$ |
| Liquefaction time (min)   | $r = - 0.203$ | $r = - 0.210$ |

Critical (r) value at 0.05 =  $\pm 0.192$

Critical (r) value at 0.01 =  $\pm 0.248$

Table (5) : Association between semen viscosity and the percentage of sperm with abnormal Aniline Blue and Toluidine Blue staining of infertile Men

| Sub-group Chromatin Stain   | Infertile Men with normal viscosity (No. = 28) | Infertile Men with abnormal viscosity (No. = 72) | (p value) |
|-----------------------------|--|--|-----------|
| Abnormal Aniline Blue (%)   | $18 \pm 5.6$                                   | $78 \pm 16.8$                                    | $< 0.001$ |
| Abnormal Toluidine Blue (%) | $12 \pm 2.3$                                   | $45 \pm 12.4$                                    | $< 0.001$ |

Table (6) : Association between semen pH and the percentage of sperm with abnormal Aniline Blue and Toluidine Blue staining Fertile Men

| Sub-group Chromatin Stain   | Infertile Men with normal pH (No. = 34) | Infertile Men with abnormal pH (No. = 66) | (p value) |
|-----------------------------|---|---|-----------|
| Abnormal Aniline Blue (%)   | $49 \pm 22.9$                           | $61 \pm 29.6$                             | 0.07      |
| Abnormal Toluidine Blue (%) | $28 \pm 17.4$                           | $39 \pm 19.5$                             | 0.08      |

The study concluded that abnormal semen macroscopic parameters may indicate sperm DNA fragmentation or chromatin decondensation .

It is recommended that aniline blue and toluidine blue staining are used to assess sperm DNA fragmentation or chromatin decondensation when bad macroscopic parameters are noticed in the first part of seminal fluid analysis .

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