

Using Sperm chromatin Staining Techniques as a Predictive Diagnostic Tool for Male Infertility

استعمال تقنيات تصبغ كروماتين النطف كأحدى الوسائل التشخيصية التنبؤية لحالة العقم عند الرجال

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الخلاصة

خلفية البحث : إن من الحقائق العلمية الثابتة اليوم أن الفحص الروتيني للسائل المنوي يعد عاملاً تنبؤياً ضعيفاً للقدرة الإخصابية للرجال ، ولذلك فإن حاجة متزايدة بدأت تبرز لاكتشاف فحوصات وظيفية جديدة في عملية تقييم العقم عند الرجال .

الهدف : هدفت هذه الدراسة إلى التحري عن القدرة التنبؤية لاختبارات سلامة الكروماتين (الصبغين) للخلايا النطفية باعتبارها من الوسائل المساعدة في تشخيص بعض حالات العقم .

المنهجية : تم إجراء هذه الدراسة للمدة من كانون الثاني 2013 إلى تشرين الأول 2013 ، إذ تضمنت الدراسة إجراء فحص السائل المنوي والفحوصات المناعية لعينة مكونة من (100) من الرجال العقيمين الذين يراجعون 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) .

النتائج : أظهرت النتائج وجود ارتفاع عالي المعنوية ($P<0.01$) في النسبة المئوية للنطف ذات الكروماتين غير المكثف والـ DNA المحطم في الرجال العقيمين 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 طردية ذات دلالة إحصائية عالية المعنوية ($P<0.01$) بين حالتي تكثف الكروماتين وتضرر الـ DNA وبين كل من تركيز النطف وحركة النطف والحركة التقدمية للنطف .

الاستنتاجات : استنتجت الدراسة أن لفحوصات تجزئة الـ DNA للخلايا النطفية (باستعمال صبغة TB) قدرة تنبؤية يمكن أن تساهم في التمييز بين الرجال العقيمين والخصيين زيادة على الفحص الروتيني للسائل المنوي .

التوصيات : أوصت الدراسة بإمكانية استعمال تصبغ كروماتين النطف بواسطة صبغة TB في عملية تشخيص العقم عند الرجال ، كما أوصت بإجراء المزيد من البحوث لتأكيد القدرة التشخيصية لفحص حالة تكثف كروماتين النطف (بواسطة AB) قبل أن تكون جاهزة للاستعمال في العمل الروتيني في مختبرات الخصوبة .

Abstract

Background : It is a scientific fact today that routine seminal analysis is only a poor predictor of male reproductive potentiality , so that there is an increased need for finding out new functional tests in the assessment of male infertility .

Objective : The aim of the present study was to investigate the predictive value of sperm chromatin integrity assays as a complementary tool to assist in diagnosis of infertility .

Method : This study was carried out between January 2013 and November 2013 including one hundred (100) selected infertile men who attended to fertility Center in Al-Sadr Medical City . 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), this is the first study in Iraq that employed (TB) to assess sperm DNA fragmentation .

Results : The results showed that there was a high significant difference ($p<0.01$) in sperm chromatin decondensation and sperm DNA damage between infertile and fertile men . The study also revealed a significant difference ($p<0.05$) in sperm DNA damage between normozoospermic and fertile men, but no significant difference ($p>0.05$) was found in the sperm chromatin condensation . Both sperm chromatin condensation and sperm DNA damage showed a significant positive correlation with sperm morphology, and a significant negative correlation with sperm concentration, motility and progressive motility.

Conclusions : The study concluded that sperm DNA fragmentation (by TB staining) has good predictive value in discriminating infertile and fertile men over seminal fluid analysis .

Recommendations : TB and AB staining of sperm chromatin can be used in the course of infertility diagnosis . However, more investigations may be needed to confirm the discriminating power of sperm chromatin condensation assays (by AB staining) to be ready to use in the routine workup in the fertility clinics .

Keywords : Predictive value, Aniline Blue , Toluidine Blue , sperm Chromatin, Male infertility

INTRODUCTION

It has been documented that about 50% of the infertility problems are , at present , attributed to male factors . In fertility clinics, potential infertile men are screened for the sperm quality by semen analysis, in particular investigating concentration, motility and morphology according to WHO guidelines ⁽¹⁾ .

The semen analysis has traditionally been the cornerstone in the evaluation of the infertile male and remains the initial text of choice. However, at present, these parameters appear to be poor predictors of the reproductive potentiality since infertile men may often have normal semen analysis . Therefore , additional specific investigations are required to assess the functional status of sperm ⁽²⁾ .

Recently, increasing attention has been paid to sperm chromatin structure as one of the parameters determining male fertility ⁽³⁾ . According to Zini *et al.*, sperm DNA integrity assays may prove to be better markers of male fertility potential than the conventional semen parameters ⁽⁴⁾ .

DNA abnormalities include disrupted (damaged) DNA integrity and chromatin prematurity (decondensation) which may occur during spermatogenesis, or as a result of DNA packing at spermiogenesis. Environmental stress, gene mutations, and chromosomal abnormalities can disturb the highly refined biochemical events that occur during spermatogenesis. This can ultimately lead to an abnormal chromatin structure that is incompatible with fertility ⁽⁵⁾ .

The integrity of sperm DNA is a keystone of reproductive success ; because normal sperm genomic material is required for a normal fertilization, pregnancy, live birth, and postnatal child well being. Therefore, abnormal sperm chromatin may result in male infertility, recurrent abortion, increased congenital anomalies , testicular cancer in offsprings as well as assisted reproductive failure ^(6, 7) . It was found that the compact structure of sperm nuclear chromatin is important for the protection of genetic integrity during transport of the paternal genome through the male and female reproductive tracts ⁽⁸⁾ .

Thus, more knowledge on sperm chromatin damage including : etiology, mechanism, detection methods, clinical outcomes and treatment may provide better diagnostic and

prognostic capabilities than standard sperm parameters for both in vivo and in vitro fertilizing ability of human spermatozoa ⁽⁹⁾ .

In this context, sperm chromatin decondensation and DNA fragmentation are two kinds of tests for sperm chromatin integrity . Among the most reliable and easy methods to assess chromatin integrity 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 evaluate sperm DNA damage or fragmentation ⁽¹⁰⁾ .

AIM OF THE STUDY

This study aims to use AB and/or TB staining methods as complementary tests in the evaluation of male infertility .

METHODOLOGY

1. Study subjects :

The study included one hundred (100) selected infertile men who attended to fertility Center in Al-Sadir Medical City . 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 ⁽¹¹⁾ .

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 ⁽¹²⁾ .

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) ⁽¹³⁾ .

RESULTS

1. Seminal Parameters of Infertile and Fertile Men

The values and statistical difference of seminal parameters between infertile and fertile men is illustrated in table (1) .

Table (1) : Major Seminal Fluid Parameters of Infertile and Fertile Men

Parameter	Infertile Men (No. = 100)	Fertile Men (No. = 100)	P-Value
Sperm Concentration (million/ml)	40.2 ± 31.8	76.8 ± 34.6	0.000
Sperm Motility (%)	45.3 ± 28.8	68.2 ± 28.4	0.000
Progressive motility (%)	33.4 ± 26.2	62.1 ± 23.71	0.000
Abnormal Morphology (%)	75.65 ± 18.72	51.9 ± 12.13	0.000
Agglutination (%)	9.87 ± 8.41	2.31 ± 1.56	0.000
WBC Count (million/ml)	2.39 ± 2.75	0.64 ± 0.49	0.000

B

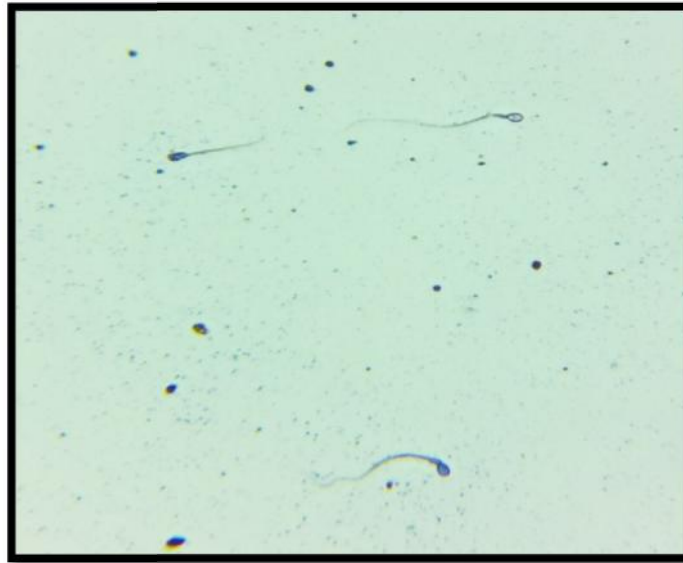
A

B

**Sperm Cells
Blue Staining**

**Sperm with
(Condensed**

**Sperm with
(Decondensed**



**Figure (1) :
stained by Aniline
(AB) .**

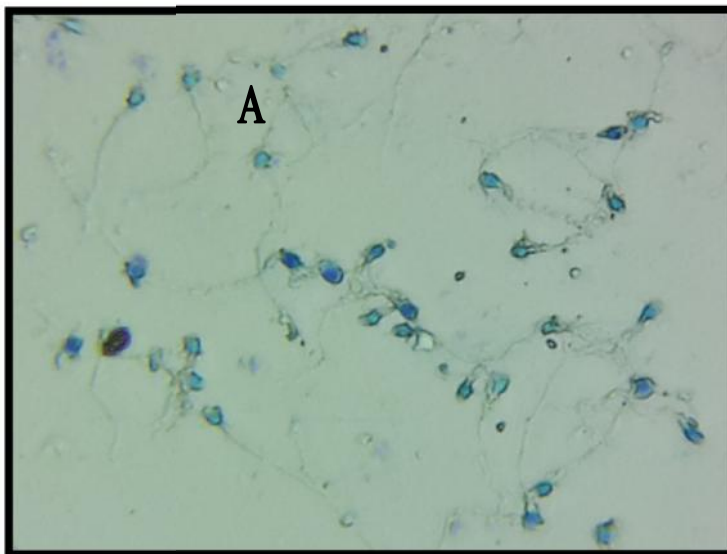
**A : Mature
unstained head
Chromatin) .**

**B : Immature
blue head
Chromatin) .**

B

**Sperm Cells
Toluidine
(TB) .**

**Sperm Cells
(Intact DNA)**



**Figure (2) :
stained by
Blue Staining
A : Normal
with Blue Heads
.**

B : Abnormal Sperm Cells with purple heads (Damaged DNA) .

2. Sperm Chromatin Integrity of Infertile and Fertile Men

As shown in tables (2) , there is a high significant increase in the percentage of sperm with abnormal AB and TB staining in infertile men compared to fertile donors .

3.Sperm Chromatin Integrity of Normozoospermic and Fertile Men

As shown in figure (3) , there is a high significant increase in the percentage of sperm with abnormal AB and TB staining in normozoospermic men compared to fertile donors .

Table (2) : Abnormal AB & TB staining of Infertile and Fertile Men

Parameter	Infertile Men (No. = 100) M \pm SD	Control (Fertile) (No. = 20) M \pm SD	P-Value
Blue AB (%)	35.9 \pm 19.4	25.15 \pm 11.32	< 0.01
Purple TB (%)	43.6 \pm 23	24.45 \pm 12.63	< 0.001

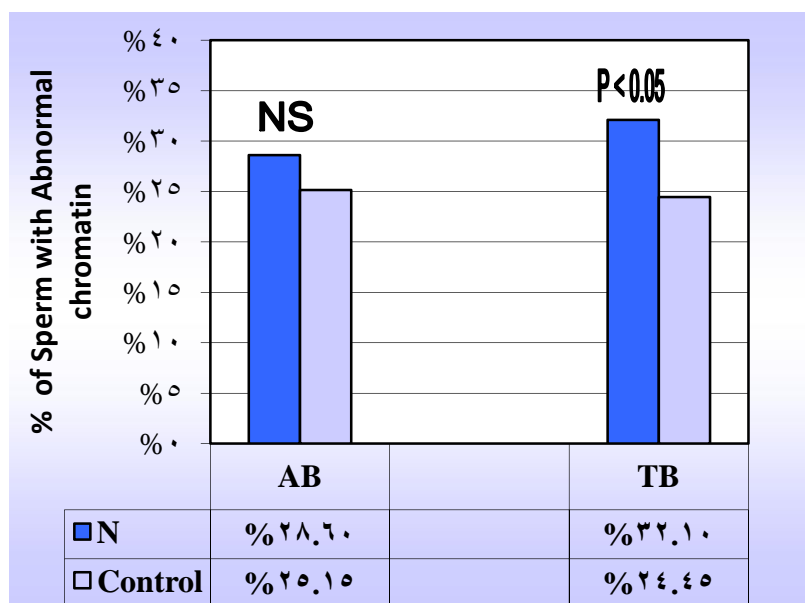


Figure (3) : Abnormal AB & TB Chromatin Staining of Normozoospermic and Fertile (Control) Men

4. Correlation between Seminal Fluid Parameters and Percentage of Abnormal AB & TB Chromatin Staining of Infertile Men

As listed in table (6) , there is a high significant negative correlation ($p < 0.01$) between AB and TB abnormal staining in infertile men and each of : sperm concentration, sperm motility, progressive motility, sperm morphology . the same table shows that no significant correlation was observed between AB and TB abnormal staining in infertile men and each of : sperm agglutination and seminal WBC count .

Table (3) : Correlation between Seminal Fluid Parameters and Percentage of Abnormal AB & TB Chromatin Staining of Infertile Men

Chromatin Stain Parameter	Blue AB (%)	Purple TB (%)
Sperm Concentration (Million/ml)	$r = - 0.232$	$r = - 0.315$
Sperm Motility (%)	$r = - 0.395$	$r = - 0.441$
Progressive Sperms (%)	$r = - 0.338$	$r = - 0.398$
Abnormal Morphology (%)	$r = 0.355$	$r = 0.426$
Agglutination (%)	$r = 0.189$	$r = 0.106$
WBC Count (Million/ml)	$r = 0.161$	$r = 0.121$

Critical (r) value at 0.05 = ± 0.197

Critical (r) value at 0.01 = ± 0.256

DISCUSSION

A conventional seminal fluid analysis (SFA) was done for both infertile and fertile groups , the results , as expected , show a high significant difference ($p < 0.01$) between the two groups (table 1) .

The current study has adopted the new reference limits submitted by WHO manual (2010) ; because it is characterized by simplification and high standardization levels ⁽¹⁴⁾ .

Although the classical SFA is considered to be the main pillar in male infertility investigation , it allows only a rough estimation of the fertility status because it does not provide fair assessment for the functional status of the sperm ⁽²⁾ . The clinical significance of SFA is further diminished by the heterogeneity of human semen which is large enough to establish subtle variations in semen parameters between men in different geographic areas and between different times or conditions within the same subject ⁽¹⁵⁾ .

It was estimated that the predictive value of normal semen testing in anticipation of natural pregnancy is only 60% ⁽¹⁶⁾ , so that recent researches seek to find new predictor tools

by developing and testing specific investigations that can be used to further discriminate between infertile and fertile subjects .

The results of the current study agree with many previous studies recorded a high significant decondensation rate in infertile patients indicating a role for sperm chromatin condensation in causing infertility ^(17, 13) .

During normal spermatogenesis, sperm chromatin become tightly compacted as basic proteins called protamines replace somatic histones ⁽¹⁸⁾ . It was found that DNA compaction enables safe transport of paternal DNA throughout the genital tract, and play an important role in early pregnancy and allows the developing embryo to easily access the genetic information and the “switch on” of genes that are needed during embryo development ⁽¹⁹⁾ .

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 ⁽²⁰⁾ .

The results of the present study also showed that there is a high significant difference in sperm DNA fragmentation between infertile and fertile men (table 2) . Although, many techniques are widely used to evaluate sperm DNA fragmentation, sperm chromatin staining by TB is simple, easy , safe and inexpensive method that can be used to detect sperm DNA damage with scientifically reliable results ⁽¹⁰⁾ .

There is an agreement among many researchers that sperm DNA damage is one of the initial causal factors that reduce the fertilizing capabilities of men ^(14, 21-23) .

The results of the current study revealed that there is a significant difference in sperm DNA damage between normozoospermic and fertile men (figure 3) , but no significant difference was found in the sperm chromatin condensation . this results agrees with many other studies that recorded a high percentage of sperm DNA damage in patients with idiopathic male fertility ⁽²⁴⁻²⁶⁾ .

Accordingly, this result meets the aim of this study to use simple and inexpensive sperm DNA techniques as a complementary tool in the fertility clinics to evaluate male infertility and discriminate between infertile and fertile subjects beside routine seminal analysis .

As shown in the correlation matrix (table 3), there is a significant negative correlation between sperm chromatin decondensation and each of sperm concentration and sperm motility, while significant positive correlation was observed with sperm agglutination .

There are conflicting data to describe the relationship between semen parameters and sperm chromatin decondensation, this may be imputed to the wide variations of semen

parameters recorded among-individuals as a result of biological variability, intra-individual, seasonal and geographical variations that complicate the performance of seminal analysis ⁽²⁷⁾ .

The correlation matrix (table 3) also showed a significant negative correlation between sperm DNA damage and each of sperm concentration and sperm motility, while significant positive correlation was recorded between sperm DNA damage and sperm agglutination .

The debate about the relationship between sperm DNA damage and semen parameters is less controversial ; some researchers demonstrated weak to moderate correlations with classic seminal parameters ⁽²⁸⁻²⁹⁾ , while many other indicated a strong association between sperm DNA damage and standard seminal parameters ^(30 , 21) .

In respect of sperm concentration, the effect of DNA damage on sperm count has been proved as a result of retarded spermatogenesis and apoptosis : Al-Hashimi *et al.* observed a negative correlation between late apoptosis and DNA integrity ⁽³¹⁾ .

Regarding sperm morphology, previous studies agree with the present study in proving strong association between abnormal sperm morphology and sperm DNA damage assessed by different methods ^(30 , 32 , 21 , 8) .

Concerning sperm motility, sperm DNA damage seems to be in close association with sperm motility and grades of progressive motility : Lukanov *et al.*, used cytometry significant negative correlations between DFI and sperm motility in normozoospermic infertile men ⁽³²⁾ , Erenpreiss *et al.* used SCSA to detect DNA damage and found a correlation with progressive motility ⁽³⁰⁾ , Nava-Trujillo *et al.* utilized TB to assess DNA and found a negative correlation with sperm motility ⁽³³⁾ .

It is possible to hypothesize that any single-stranded or double-stranded breaks in sperm genome will greatly hamper the functional status of the damaged DNA resulting in down-regulation of the transcription process . Some researchers reported that when DNA damage results in sperm apoptosis, it is understandable to find many immotile sperms ⁽²²⁾ .

CONCLUSIONS

The study concluded that sperm DNA fragmentation (by TB staining) has good predictive value in discriminating infertile and fertile men over seminal fluid analysis .

RECOMMENDATIONS

TB staining of sperm chromatin can be used in the course of infertility diagnosis . However, more investigations may be needed to confirm the discriminating power of sperm chromatin condensation assays (by AB staining) to be ready to use in the routine workup in the fertility clinics .

REFERENCES

- [1] WHO (World Health Organization) (2010) . "WHO Laboratory Manual for The Examination of Human Semen and Sperm Cervical Mucus Interaction". 5th ed. World health organization. Cambridge University Press .
- [2] Sharma, R. S. ; Gaur, K. K. ; Manocha P. M. ; Tomar D. ; Khan, A. A. ; Tripathi, V. ; Chattree, V. *et al.* (2005) . Semen Characteristics : Advancement in Andrological Assessment . *Indian Journal of Clinical Biochemistry*, 20 (1) .
- [3] Shamsi, MB. ; Imam, SN. and Dada, R. (2011) . Sperm DNA Integrity Assays : Diagnostic and Prognostic Challenges and Implications in Management of Infertility. *J Assist Reprod Genet*, 28: 1073-1085 .
- [4] Zini, A. ; Bielecki, R. ; Phang, D. and Zenzes, MT. (2001) . Correlations between two markers of sperm DNA integrity, DNA Denaturation and DNA fragmentation, in fertile and infertile men. *Fertil Steril*, 75: 674-677.
- [5] Agarwal, A. and Said, T. (2003) . Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update*, 9 : 331-345.
- [6] Wu, T. F. and Chu, D. S. (2008) . Sperm Chromatin : Fertile Grounds for Proteomic Discovery of Clinical Tools . *Molecular and Cellular Proteomics*, 7(10) : 1876 – 1886 .
- [7] Aitken, RJ. and Sawyer. D. (2003) .The human spermatozoon not waving but drowning. *Adv Exp Med Biol*, 518:85-98.
- [8] Fortunato, A. ; Leo, R. ; Casale, S. ; Nacchia, G. ; Liguori, F. and Tosti, E. . (2013) Sperm DNA Fragmentation Assays Correlate with Sperm Abnormal Morphology and the Pregnancy Outcome . *J IVF Reprod Med Genet*, 1:1 . available at : <http://dx.doi.org/10.4172/jfiv.1000101> .
- [9] Hekmatdoost, A. ; Lakpour, N. and Sadeghi, M.R. (2009) Sperm Chromatin Integrity: Etiologies and Mechanisms of Abnormality, Assays, Clinical Importance, Preventing and Repairing Damage . *Avicenna J Med Biotech*, 1(3) : 147-160 .
- [10] Kim, H-S. ; Kang, M.J. ; Kim, S.A. ; Oh, S.K. ; Kim, H. ; Ku S-Y. *et al.* (2013) . The utility of sperm DNA damage assay using Toluidine blue and aniline blue staining in routine semen analysis . *Clin Exp Reprod Med.*, 40(1) : 23-28 .
- [11] Pal, P. C. ; Rajalakshmi, M. ; Manocha, M. ; Sharma, R. S. ; Mittal, S. and Rao, O. N. (2006) . Semen quality and sperm functional parameters in fertile Indian men . *Andrologia*, 38: 20-25.
- [12] Hammadeh, M. ; Zeginiadov, T. and Rosenbaum, P. (2001) . Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. *Arch Androl*, 46 : 99–104.
- [13] Erenpreiss, J. ; Jepson, K. ; Giwerzman, A. ; Tsarev, I. ; Erenpreisa, J. and Spano, M. (2004) . Toluidine blue cytometry test for sperm DNA conformation: comparison with the flow cytometric sperm chromatin structure and TUNEL assays. *Hum Reprod*, 19:2277–2282.
- [14] Haidl, G. (2011) . New WHO-reference limits-Revolution or Storm in a teapot? . *Asian Journal of Andrology*, 13 : 208–211 .
- [15] Keel, B. A. (2006) . Within- and Between-Subject Variation in Semen Parameters in Infertile Men and Normal Semen Donors. *Fertil Steril.*, 85 : 128–134 .
- [16] Van der Steeg, J. W. ; Steures, P. ; Eijkemans, M. J. ; Habbema, J. D. F. ; Hompes, P. G. A. ; Kremer, J. A. M. *et al* (2011) . Role of semen analysis in subfertile couples. *Fertil Steril*, doi:10.1016/ fertnstert .

- [17] Foresta, C. ; Zorzi, M. ; Rossato, M. and Varotto, A. (1992) . Sperm Nuclear Instability and Staining with Aniline Blue : abnormal persistence of histones in spermatozoa in infertile men. *Int J Androl*, 15: 330–7.
- [18] Ward, W. S. and Coffey, D. S. (1991) . DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod*, 44 : 569–74.
- [19] Barratt, C. L. ; Aitken, R. J. ; Bjorndahl, L. ; Carrell, D. T. ; de Boer, P. ; Kvist, U. *et al.* (2010) . Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications--a position report. *Hum Reprod*, 25 : 824–38.
- [20] De Iuliis, G.N. ; Thomson, L.K. ; Mitchell, L.A. ; Finnie, J.M. ; Koppers, A.J. ;Hedges, A., et al. (2009) . DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod*, 81:517–524.
- [21] Rahim, A. I. (2010) . Assessment of Human Chromatin Structure in /relation to Sperm Parameters and Assisted Reproductive Technologies . Master Thesis, Al-Nahrain University/ Institute of Embryo Research and Infertility Treatment .
- [22] Alani, G. T. ; Khonda, S. S. and El Yaseen, H. D. (2011) . Analysis of DNA Damage and Oxidative Stress in Human Spermatozoa and Some Biochemical Changes in Seminal Plasma and their Correlation with Semen Quality of Infertile Men . *The Iraqi Postgraduate Medical Journal*, 10(1) : 81-88 .
- [23] Bungum, M. (2012) . Role of Sperm DNA Integrity in Fertility . Embryology - Updates and Highlights on Classic Topics, Pereira, L.V. (Ed.), Available from : <http://www.intechopen.com/books/embryology-updates-and-highlights-on-classic-topics/role-of-sperm-dnaintegrity-in-fertility> .
- [24] Saleh, R. A. ; Agarwal, A. ; Nelson, D. R. ; Nada, E. A. ; El-Tonsy, M. H. and Alvarez, J. G. (2002) . Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study . *Fertility and Sterility*, 78(2) : 313-318 .
- [25] Bungum, M. ; Humaidan, P. ; Axmon, A. ; Span, M. ; Bungum, L. ; Erenpreiss, J. and Giwercman A. (2007) .Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod*, 22:174-179.
- [26] Al-Hashimi, A. F. (2012) . Role of DNA Integrity of Spermatozoa in Male Infertility . *IRAQI J MED SCI*, 10 (1) : 4-11 .
- [27] Natali, A. and Turek, P. J. (2010) . An Assessment of New Sperm Tests for Male Infertility – Review Article . *Urology*, 77 (5) : 1027-1034 .
- [28] Giwercman, A. ; Richthoff, J. ; Hjollund, H. ; Bonde, J. P. ; Jepsen, K. ; Frohm, B. *et al.* (2003) . Correlation between sperm motility and sperm chromatin structure assay parameters. *Fertil Steril*, 80 ;1404-12.
- [29] Rasool, E. A. ; Abdul-Rasheed, O. F. and AL-Hashimi, A. F. (2012) . Comparison Between Different DNA and Conventional sperm parameters in Infertile Men . *Al-kindy Col Med J*, 8(2) : 40-47 .
- [30] Erenpreiss, J. ; Elzanaty, S. and Giwercman, A. (2008) . Sperm DNA damage in men from infertile couples . *Asian J Androl*, 10 (5): 786–790 .
- [31] AL-Hashimi, A. F. (2007) . Analysis of Oxidative Stress, DNA Damage and Apoptosis in Human Spermatozoa and their Correlation with Semen Quality in Infertile Men . PhD Thesis, Al-Nahrain University , College of Medicine .
- [32] Lukanov, T. , Lichev D. ; Konova, E. ; Emin, A. ; Ayvazova, N. ; Kotzev, R. *et al.* (2008) . Investigation of Sperm DNA Fragmentation by Sperm DNA Integrity Assay . *Journal of IMAB - Annual Proceeding (Scientific Papers)*, book 1 .
- [33] Nava-Trujillo, H. ; Quintero-Moreno, A. ; Finol-Parra, G. ; Carruyo, G. ; Vilchez-Siu, V. ; Osorio-Meléndez, C. *et al.* (2011) . Relationship among damaged chromatin, motility and viability in cryopreserved spermatozoa from Brahman bulls . *Rev Colomb Cienc Pecu*, 24:116-122 .