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Assessment of Spermatic Chromatin Decondensation by the Toluidine Blue Assay in Infertile Patients in Cotonou

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Abstract

Background: A high level of sperm chromatin decondensation results in loss of DNA quality and it is associated with poor embryonic prognosis in Assisted Reproductive Technology (ART). This had lead to introduction of sperm DNA compaction testing in assessment of clinical male infertility. Toluidine blue assay is one of most commonly used tests to measure chromatin decondensation in research studies and also in clinical routine use. So the objective of our study was to assess the level of sperm chromatin decondensation by toluidine blue assay in men dealing with couple infertility. **Methods:** The sperm samples of 96 patients referred for infertility, over a 4-month period from September 2020 to January 2021, were stained with toluidine blue and observed under an optical microscope. These were evaluated according to the World Health Organization. **Results:** The 25 - 34 age group was the most represented among infertile men. There was a non-significant correlation between age and the level of spermatic chromatin condensation (r = 0.0348). Spermatic parameters had averages above standards norms, apart from that of low vitality. Spermatic chromatin decondenization affected 96% of infertile patients. Asthenozoospermia was the most common abnormality. Among standard parameters of spermogram, only the concentration of spermatozoa in the semen was weakly correlated (r = 0.18) with sperm chromatin decondensation. **Conclusion:** The level of sperm chromatin condensation is, on the one hand, a parameter independent of the age of the subject; on the other hand, allows to evaluate male infertility, like the classic parameters of the spermogram and spermocytogram. However, in view of the high probability of changes in the nuclear quality of sperm intrinsic to the analysis process

and sperm concentration, an improvement of the toluidine blue test using the swim-up technique and associated with image processing software is possible.

Keywords

Male Infertility, Decondensation, Spermatic Chromatin, Toluidine Blue

1. Introduction

Infertility according to the WHO is defined as the inability to obtain a clinical pregnancy after at least 12 months of regular, frequent, unprotected sexual intercourse [1]. It is a public health problem in the world and affects 10% to 15% of couples [2] [3] [4]. In sub-Saharan Africa, the prevalence of couple infertility, which ranges from 12.7% to 16.9%, does not reflect that of the world population due to the lack of data on this condition [5]. The spermogram and spermocytogram explore male fertility by studying the quantitative and cytological parameters of the sperm, but do not assess the quality of the genetic material of the sperm [6]. In recent decades it has been well known that good quality genetic material is very important for conception [7]. Hence the importance of its exploration can be done by studying the fragmentation of sperm DNA and/or the decondensation of sperm chromatin. Sperm chromatin decondensation studies the quality of the tertiary structure of sperm DNA, which must be compacted in the nucleus of the spermatozoon by replacing histones with protamines [8]. Thus, regions that do not contain protamines or do not have a good quality of fixation of protamines to DNA, will lead to an increased risk of alteration of the DNA such as denaturation, break and fragmentation when exposed to aggressions. These attacks are represented by oxidative stress, an infectious or inflammatory process, environmental factors (toxic, hydrocarbons, insecticides, heat) or iatrogenic factors (radiotherapy, chemotherapy) [9] [10].

The sperm chromatin decondensation can be evaluated by flow cytometry tests, which are not feasible in routine and staining tests that are: the toluidine blue test, the aniline blue test and the A3 chromomycin test. The Toluidine Blue (TB) test is a simple and inexpensive test. Smears spread out to be stained by TB can also be used for morphological evaluation of cells.

The objective of this study was to assess correlation between age, standard spermogram and spermocytogram parameters, and sperm chromatin decondensation *in vivo* by toluidine blue assay in infertile men. The findings of our works could be useful for the prognosis of pregnancy occurrence for infertile couples undergoing ART.

2. Material and Method

2.1. Type of Study

This was a prospective descriptive and analytical study carried out over a period

of 05 months (from September 1, 2020 to January 31, 2021).

2.2. Population

96 men admitted to the laboratory for a spermogram/spermocytogram examination were included in our study. Consent was obtained from our patients and the study was approved by ethical comitte of our institution.

➢ **Inclusion criteria**

- Men followed up for the exploration of a couple infertility;
- Men having met necessary pre-analytical conditions according to the WHO latest guidelines (2010).

➢ **Exclusion criteria**

- Azoospermia;
- Severe to moderate oligozoospermia.

2.3. Semen Samples

We collected semen samples after 3 - 5 days of sexual abstinence. The analysis was performed 30 min after collection, according to the WHO latest guidelines (2010). It also included sperm vitality, motility, concentration and leukocyte concentration. Sperm morphology was assessed using Kruger's Criteria.

2.4. Data Collection Technique and Tools

- Socio-demographic, clinical data;
- Spermogram and spermocytogram data;
- Sperm chromatin decondensation test data.

These data collected from the bench sheets and patient results are reported on survey sheets.

- **Sperm chromatin decondensation parameters**

Test used: Toluidine blue

a) Principle

Toluidine blue or trimethylthionine hydrochloride is a basic nuclear dye that has a strong affinity for basophilic molecules including nucleic acids, which will then be colored in different shades of blue (orthochromatic) in animal cells. Thus, in the spermatozoon, toluidine blue colors the nucleus more or less intensely, depending on the degree of chromatin opening: strongly condensed DNA will only allow little dye to penetrate, giving the nucleus a very pale blue, while a weakly condensed DNA will accept more dye. Toluidine blue becomes strongly incorporated, with shades of purple or dark blue in damaged chromatin.

b) Technique

This technique was performed using fresh semen samples with at least 5 millions spermatozoa:

- Spread 10 µl of semen on the slide
- Allow to dry in the open air

- Fix the smear slides for 30 minutes at 4˚C in an equal volume mixture of 95˚ ethanol and acetone
- Allow to dry in the open air
- Incubate at 4˚C in 0.1 N HCl for 5 minutes.
- Perform a quick wash in distilled water
- Dilute the toluidine blue powder to 1% with distilled water
- Prepare toluidine blue at 1% in McIlvain buffer (50% citrated phosphate buffer, pH 3.5).
- Incubate the slides for 15 minutes at room temperature in toluidine blue solution buffered at a concentration of 0.035%.
- Dehydrate the slides by successive baths of 05 seconds each, using increasing degree ethanol (70˚, 80˚ then 95˚)
- Lighten with Xylene at room temperature for 3 min
- Make an assembly of slats with Eukitt's balm.

A total of 200 spermatozoa are observed and counted with an optical microscope. Then we calculated the frequency of spermatozoa with decondensed chromatin on 200 spermatozoa to obtain the rate of spermatozoon chromatin decondensation.

c) Sperm chromatin decondensation reference values

- Rate < 20%: good quality
- Rate between 20% and 25%: average quality
- Rate ≥ 25%: poor quality and poor embryonic prognosis [11] [12]

N.B.: toluidine blue, previously diluted to 1% in distilled water, is stored at a temperature of 4˚C.

2.5. Statistical Analysis

The results were expressed in percentages and frequencies for descriptive statistics. For analytic stastistical, Fischer's exact test was used for qualitative data comparisons because of some proportions less than 5. Pearson's correlation test was used to compare quantitative data.

The analyses were performed using Epi Info 7 and STATA 14.0 softwares.

3. Results

Our study examined the level of sperm chromatin decondensation tested with toluidine blue. It was performed in 96 infertile patients recruited over a 20-week period. A rate of spermatic chromatin decondensation < 25% was low, corresponding to a good embryonic prognosis and a rate of sperm chromatin decondensation ≥ 25%, corresponded to a bad embryonic prognosis.

3.1. Age of Patients

The average age of the patients was 37.60 ± 7.25 years with extremes of 25 years and 63 years. Our patients were divided into three age groups (25 - 34; 35 - 39; ≥40 ans). The majority of patients (36.46%) were between 25 and 34 years old.

3.2. Standard Parameters of Spermogram and Spermocytogram

The averages of all the parameters are higher than the standard norms except that of vitality (Table 1).

3.3. Spermogramm and Spermocytogram Abnormalities

Asthenozoospermia was the most common abnormality (Figure 1).

AT: Asthenoteratozoospermia;

A: Asthenozoospermia;

OAT: Oligoasthenoteratozoospermia;

OA: Oligoasthenozoospermia;

O: Oligozoospermia;

T: Teratozoospermia.

3.4. Sperm Chromatin Decondensation

The mean sperm chromatin decondensation rate was 80.07% ± 19.31% with extremes of 18% and 99% (Figure 2).

Figure 3 illustrates the morphological appearance of some spermatozoa after staining with toluidine blue.

3.5. Sperm Chromatin Decondensation and Age

There is no statistically significant association between age and sperm chromatin decondensation (Table 2).

Table 1. Average standard spermogram parameters of patients.

Parameters	Normal values	Means and standard deviations
Volume (ml)	1.5 à 6 ml	2.61 ± 1.41
pH	7.2 à 7.8	7.33 ± 0.18
First hour sperm motility (%)	>32%	35.86 ± 15.18
Vitality (%)	>38%	50.89 ± 17.03
Concentration (millions/ml)	≥15 M/ml	61.36 ± 58.86
Normal shapes (%)	>4%	9.48 ± 9.47

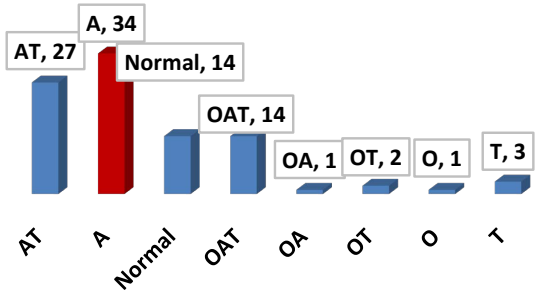

Figure 1. Frequency of spermogram and spermocytogram abnormalities.

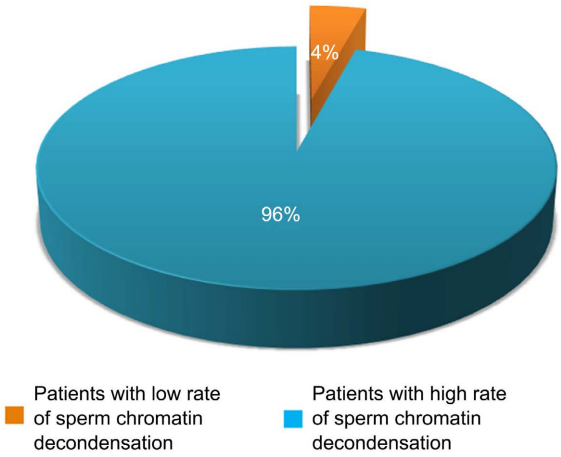

Figure 2. Distribution of infertile patients according to the level of sperm chromatin decondensation.

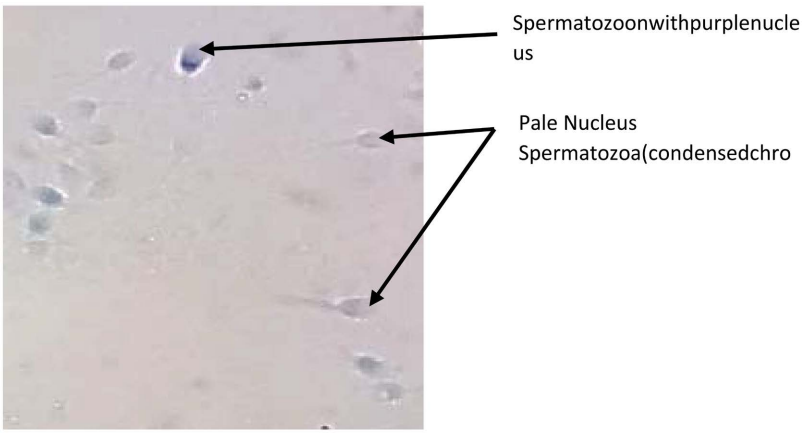

Figure 3. Observation in optical microscopy (X 1000) of toluidine blue coloured spermatozoa (Reproductive Biology Section of the LHRBCMG of the FHS/UAC of Cotonou, Benin).

Table 2. Relationship between sperm chromatin decondensation and age.

Sperm chromatin decondensation	Age range			
	25 - 34	35 - 39	40 - 63	p = 0.55
Low (%)	50	0	50	
High (%)	35.87	30.43	33.70	

3.6. Sperm Chromatin Decondensation and Sperm Concentration

There is no statistically significant association between early spermatozoa motility and sperm chromatin decondensation (Table 3).

3.7. Sperm Chromatin Decondensation and Spermatozoa Morphology

There is no statistically significant relationship between spermatozoa morphology and sperm chromatin decondensation (Table 4).

3.8. Correlation between Age, Spermogram and Spermocytogram Parameters and Sperm Chromatin Decondensation (Table 5)

There was no correlation ($r < 0.1$) between patient age, vitality, spermatozoa morphology and sperm chromatin decondensation.

There was a weak ($0.1 < r < 0.3$) correlation, but non-significant ($p > 0.05$) between spermatozoa concentration and sperm chromatin decondensation.

There was a non-significant ($p > 0.05$) negative correlation ($r < 0$) between first hour spermatozoa mobility, round cell concentration and spermatic chromatin decondensation.

4. Discussion

4.1. Age and Sperm Chromatin Decondensation

The age groups described as qualitative variables in our study are those indicated by the work of de la Rochebrochard and E, Thonneau P [7].

The average age in our study was 37.60 ± 7.25 years. This average age was superimposed on that found by Hee-Sun Kim and al in Korea, in their study [8].

Table 3. Relationship between decondensation of spermatic chromatin and spermatozoa concentration.

Sperm chromatin Decondensation	Sperm concentration		
	Poor	Normal	p = 0.47
Low (%)	25	75	
High (%)	14.13	85.87	

Table 4. Relationship between the sperm chromatin decondensation and the spermatozoa morphology.

Sperm chromatin decondensation	Spermatozoa Morphology		
	Normal	Abnormal	p = 0.36
Low (%)	25	75	
High (%)	52.17	47.83	

Table 5. Correlation between age, first-hour motility, vitality, concentration, spermatozoa morphology and sperm chromatin decondensation.

Variables	Sperm chromatin decondensation
Age	r = 0.0348 p = 0.74
First-hour motility (%)	r = −0.06 p = 0.59
Vitality (%)	r = 0.00 p = 0.96
Concentration (millions/ml)	r = 0.18 p = 0.07
Normal shapes (%)	r = 0.02 p = 0.84
Round cells concentration (millions/ml)	r = −0.05 p = 0.63

The 25 - 34 age group is the most represented among infertile men, unlike several other studies that show that the risk of infertility and miscarriage increases when the man is over 35 or even 40 years old. This corresponds to the age limit for sperm donors [9] [10]. Only after the age of 35 should reproductive risk factors in the partner be considered, such as: age and low ovarian response, suggesting an interaction between paternal age and maternal risk factors [7] [13] [14] [15] [16].

Furthermore, there is no significant link in our study between the age group and the fertility prognosis related to spermatic chromatin decondensation ($p = 0.55$). The correlation between age and spermatic chromatin decondensation is not significant with a coefficient $r = 0.035$ and $p = 0.74$, as shown by Hee-Sun Kim et al in their work [8].

This could suggest the evaluation of spermatic chromatin decondensation as a microscopic parameter involved in male infertility, regardless of the age of the subject.

4.2. Sperm Parameters and Sperm Chromatin Decondensation

In our study, sperm parameters had averages above standard norms, apart from that of vitality which was low. This could be explained by the fact that the plasmic membrane of non-viable spermatozoa becomes permeable to certain dyes and no longer responds to osmotic pressure variations [17]. In addition, Aoki and al. in the United States, noted that sperm with low levels of protamines have decreased viability and a significant susceptibility to DNA damage [10]. This explains the decrease in vitality in infertile patients.

In terms of mobility, it was impaired in the majority of our patients. Moreover, asthenozoospermia is a disorder very frequently found in infertile patients as reported by Tognifodé in Benin. Often changes in mobility are accompanied by poor quality of spermatic DNA that can be fragmented [18]-[25]. By the way, flagellar pathologies are usually accompanied by changes in the cephalic constituents of sperm [26]. Poorly condensed chromatin, nuclear vacuoles and malformed or absent acrosome are therefore frequently observed.

By cross-referencing the data, we did not find a significant link between sperm mobility, concentration, morphology and fertility prognosis related to sperm chromatin decondenization. In contrast, Hee-Sun Kim *et al.* found a correlation between morphology and the rate of spermatic DNA decondensation. They reported a higher frequency of decondensed chromatin sperm in the abnormal-shaped sperm population. Contrary to these results, Ajina T and al in Tunisia showed a significant correlation between spermatic chromatin abnormalities and those of mobility, concentration, vitality and morphology. This demonstrates the impact of significant abnormalities in the structure of spermatic chromatin on the alteration of standard spermatic parameters [27].

In addition, first-hour mobility and vitality in our study were negatively correlated with spermatic chromatin condensation; this correlation was not signifi-

cant with respectively (p = 0.59; p = 0.96). The results of our work are superimposable on those of Pourmasumi S. *et al.* in Iran whose study used the tests with toluidine blue and aniline blue, and Hammadeh and al who performed the test with only blue d. aniline [28] [29].

This suggests that chromatin condensation is a reliable parameter that can be analyzed in the exploration of male infertility, regardless of the standard parameters of mobility, vitality, concentration and morphology.

The round cells, which are divided into spermatogenic cells and leukocytes, represent other parameters which, like the presence of reactive oxygen species, involved in sperm chromatin decondensation as revealed by the works of Henkel R *et al.* [30]. The concentration of round cells in the semen is also not correlated with sperm chromatin decondensation. Henkel R and al. reported that the oxidative stress caused by either leukocytes or sperm cells themselves can also lead to sperm chromatin decondensation.

On the one hand, packaging the male genome with protamines offers protection against aggression, including oxidative stress caused by leukocytes. This means that, if there is an under-protamination of the sperm chromatin, the DNA would be exposed and therefore easily accessible to attacks, which can damage it. On the other hand, DNA size requires restructuring and rearrangement of the male genome during chromatin condensation, in order to attenuate the torsional stress of the molecule by negative supercoiling [31].

4.3. Study Limits

Our sample could have included fertile controls and simultaneously been tested with other cytochemical techniques, allowing a better appreciation of the sensitivity and specificity of the toluidine blue test, for the evaluation of sperm chromatin condensation.

Moreover, the search for reactive oxygen species for each sperm sample would have made possible to highlight their impact on the decondensation of sperm chromatin.

In general, the TB method is simple and inexpensive and has the advantage of providing preparations for use on ordinary microscope. Smears coloured with the TB method can also be used for morphological evaluation of cells. However, this method has an inherent limit of repeatability dictated by variations in dye balance and a limited number of cells that can be reasonably noted [32].

An improvement of the toluidine blue test using the swim-up technique and associated with image processing software could have facilitated sperm analysis by reducing the operator's subjectivity, particurarly in the case of medical assistance to procreation.

Erenpreisa J. and al have in their work, associated image cytometry to obtain an algorithm of cytometric images and a finer determination of the nuclear status of the sperm [33]. The sperm heads, coloured with toluidine blue and observed under an optical microscope, fell into one of four categories (dark purple,

dark blue, light blue and light purple). Their study found that the average percentage of dark blue and dark purple headed sperm was higher in infertile men than fertile men.

5. Conclusion

Abnormalities in the nuclear quality of the sperm are likely to compromise the onset of pregnancy at a certain threshold. In our context in developing countries, studying the level of sperm chromatin condensation by toluidine blue assay, could be a routine examination. This study found that infertile patients had a high rate of sperm chromatin decondensation. The level of sperm chromatin decondensation is a parameter that, on the one hand, varies independently of the age of the subject. On the other hand, it may allow to evaluate male infertility, like the classic parameters of the spermogram and spermocytogram, especially in patients undergoing ART.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Zegers-Hochschild, F., Adamson, G.D., De Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., *et al.* (2009) The International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) Revised Glossary on ART Terminology, 2009. *Human Reproduction*, **24**, 2683-2687. https://doi.org/10.1093/humrep/dep343

[2] Kliesch, S. (2014) Diagnosis of Male Infertility: Diagnostic Work-Up of the Infertile Man. *European Urology Supplements*, **13**, 73-82. https://doi.org/10.1016/j.eursup.2014.08.002

[3] Meacham, R.B., Joyce, G.F., Wise, M., Kparker, A. and Niederberger, C. (2007) Male Infertility. *Journal of Urology* **177**, 2058-2066. https://doi.org/10.1016/j.juro.2007.01.131

[4] Skakkebæk, N.E., Giwercman, A. and de Kretser, D. (1994) Pathogenesis and Management of Male Infertility. *Lancet*, **343**, 1473-1479. https://doi.org/10.1016/S0140-6736(94)92586-0

[5] Agarwal, A., Mulgund, A., Hamada, A. and Chyatte, M.R. (2015) A Unique View on Male Infertility around the Globe. *Reproductive Biology and Endocrinology*, **13**, Article No. 37. https://doi.org/10.1186/s12958-015-0032-1

[6] Owolabi, A.T., Fasubaa, O.B. and Ogunniyi, S.O. (2013) Semen Quality of Male Partners of Infertile Couples in Ile-Ife, Nigeria. *Nigerian Journal of Clinical Practice*, **16**, 37-40. https://doi.org/10.4103/1119-3077.106729

[7] De la Rochebrochard, E. and Thonneau, P. (2002) Paternal Age and Maternal Age Are Risk Factors for Miscarriage; Results of a Multicentre European Study. *Human Reproduction*, **17**, 1649-1656. https://doi.org/10.1093/humrep/17.6.1649

[8] 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. *Clinical and Experimental Reproductive Medicine*, **40**,

23-28. https://doi.org/10.5653/cerm.2013.40.1.23

[9] British Andrology Society (BAS) (1999) British Andrology Society Guidelines for the Screening of Semen Donors for donor Insemination (1999). *Human Reproduction*, **14**, 1823-1826. https://doi.org/10.1093/humrep/14.7.1823

[10] Aoki, V.W., Emery, B.R., Liu, L. and Carrell, D.T. (2006) Protamine Levels Vary between Individual Sperm Cells of Infertile Human Males and Correlate with Viability and DNA Integrity. *Journal of Andrology*, **27**, 890-898. https://doi.org/10.2164/jandrol.106.000703

[11] Sharma, R.K., Sabanegh, E., Mahfouz, R., Gupta, S., Thiyagarajan, A. and Agarwal, A. (2010) TUNEL as a Test for Sperm DNA Damage in the Evaluation of Male Infertility. *Urology*, **76**, 1380-1386. https://doi.org/10.1016/j.urology.2010.04.036

[12] Simon, L. and Lewis, L.E. (2011) Sperm DNA Damage or Progressive Motility: Which on Is Better Predictor of Fertilization *in Vitro*? *Systems Biology in Reproductive Medicine*, **57**, 133-138. https://doi.org/10.3109/19396368.2011.553984

[13] American Society for Reproductive Medicine (ASRM) (1998) Guidelines for Gamete and Embryo Donation. *Fertility and Sterility*, **70**, 1S-13S.

[14] De la Rochebrochard, E. and Thonneau, P. (2003) Paternal Age over 40 Years: An Important Risk Factor for Infertility. *American Journal of Obstetrics & Gynecology*, **189**, 901-905. https://doi.org/10.1067/S0002-9378(03)00753-1

[15] Dunson, D.B., Colombo, B. and Baird, D.D. (2002) Changes with Age in the Level and Duration of Fertility in the Menstrual Cycle. *Human Reproduction*, **17**, 1399-1403. https://doi.org/10.1093/humrep/17.5.1399

[16] Watanabe, Y., Cornet, D., Merviel, P., Mandelbaum, J., Antoine, J.M. and Uzan, S. (2000) Influence of Husband's Age on Outcome of a Shared Oocyte Donation Program. *Fertility and Sterility*, **74**, S78-S79. https://doi.org/10.1016/S0015-0282(00)00933-X

[17] Astolfi, P., De Pasquale, A. and Zonta, L.A. (2004) Late Paternity and Stillbirth Risk. *Human Reproduction*, **19**, 2497-2501. https://doi.org/10.1093/humrep/deh449

[18] Gandini, L., Lombardo, F., Paoli, D., Caponecchia, L., Familiari, G., Verlengia, C., *et al.* (2000) Study of Apoptotic DNA Fragmentation in Human Spermatozoa. *Human Reproduction*, **15**, 830-839. https://doi.org/10.1093/humrep/15.4.830

[19] Zini, A., Bielecki, R., Phang, D. and Zenzes, M.T. (2001) Correlations between two Markers of Sperm DNA Integrity, DNA Denaturation and DNA Fragmentation, in Fertile and Infertile Men. *Fertility and Sterility,* **75**, 674-677. https://doi.org/10.1016/S0015-0282(00)01796-9

[20] Marchetti, C., Obert, G., Defosez, A., Formstecher, P. and Marchetti, P. (2002) Study of Mitochondrial Membrane Potential, Reactive Oxygen Species, DNA Fragmentation and Cell Viability by Flow Cytometry in Human Sperm. *Human Reproduction*, **17**, 1257-1265. https://doi.org/10.1093/humrep/17.5.1257

[21] Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, B., Lejeune, H., *et al.* (2003) Sperm DNA Fragmentation Decreases the Pregnancy Rate in an Assisted Reproductive Technique. *Human Reproduction*, **18**, 1023-1028. https://doi.org/10.1093/humrep/deg228

[22] Liu, C.H., Tsao, H.M., Cheng, T.C., Wu, H.M., Huang, C.C., Chen, C.I., *et al.* (2004) DNA Fragmentation, Mitochondrial Dysfunction and Chromosomal Aneuploidy in the Spermatozoa of Oligoasthenoteratozoospermic Males. *Journal of Assisted Reproduction and Genetics*, **21**, 119-126. https://doi.org/10.1023/B:JARG.0000029495.22787.83

[23] Huang, C.C., Lin, D.P., Tsao, H.M., Cheng, T.C., Liu, C.H. and Lee, M.S. (2005) Sperm DNA Fragmentation Negatively Correlates with Velocity and Fertilization Rates But Might Not Affect Pregnancy Rates. *Fertility and Sterility*, **84**, 130-140. https://doi.org/10.1016/j.fertnstert.2004.08.042

[24] Piasecka, M., Gaczarzewicz, D., Laszczynska, M., Starczewski, A. and Brodowska, A. (2007) Flow Cytometry Application in the Assessment of Sperm DNA Integrity of Men with Asthenozoospermia. *Folia Histochemica et Cytobiologica*, **45**, S127-S136.

[25] Varum, S., Bento, C., Sousa, A.P., Gomes-Santos, C.S., Henriques, P., Almeida-Santos, T., *et al.* (2007) Characterization of Human Sperm Populations Using Conventional Parameters, Surface Ubiquitination, and Apoptotic Markers. *Fertility and Sterility*, **87**, 572-583. https://doi.org/10.1016/j.fertnstert.2006.07.1528

[26] Tognifode, M.V., Lokossou, S., Aboubakar, M., Ogoudjobi, M., Hounkpatin, B., Tonato-Bagnan, A., *et al.* (2018) Paramètres du spermogramme dans l'infertilité du couple: Cas d'une clinique privée du Bénin a propos de 210 cas. *Journal de la Société de Biologie Clinique du Bénin*, No. 28, 41-45.

[27] Ajina, T., Ammar, O., Haouas, Z., Sallem, A., Ezzi, L., Grissa, I., *et al.* (2017) Assessment of Human Sperm DNA Integrity Using Two Cytochemical Tests: Acridine Orange Test and Toluidine Blue Assay. *Andrologia*, **49**, Article ID: e12765. https://doi.org/10.1111/and.12765

[28] Pourmasumi, S., Khoradmehr, A., Rahiminia, T., Sabeti, P., Talebi, A.R., Ghasemzadeh, J. (2019) Evaluation of Sperm Chromatin Integrity Using Aniline Blue and Toluidine Blue Staining in Infertile and Normozoospermic Men. *Journal of Reproduction & Infertility*, **20**, 95-101

[29] Hammadeh, M.E., Zeginiadov, T., Rosenbaum, P., Georg, T., Schmidt, W. and Strehler, E. (2001) Predictive Value of Sperm Chromatin Condensation (Aniline Blue Staining) in the Assessment of Male Fertility. *Archives of Andrology*, **46**, 99-104. https://doi.org/10.1080/01485010117363

[30] Henkel, R., Bastiaan, H.S., Schüller, S., Hoppe, I., Starker, W. and Menkveld, R. (2010) Leucocytes and Intrinsic ROS Production May Be Factors Compromising Sperm Chromatin Condensation Status. *Andrologia*, **42**, 69-75. https://doi.org/10.1111/j.1439-0272.2009.00967.x

[31] Balhorn, R. (1982) A Model for the Structure of Chromatin in Mammalian Sperm. *Journal of Cell Biology*, **93**, 298-305. https://doi.org/10.1083/jcb.93.2.298

[32] Agarwal, A. and Said, T.M. (2018) Sperm Chromatin Assessment. In: *Textbook of Assisted Reproductive Technique*, 2nd Edition, 93-106.

[33] Erenpreisa, J., Erenpreiss, J., Freivalds, T., Slaidina, M., Krampe, R., Butikova, J., *et al.* (2003) Toluidine Blue Test for Sperm DNA Integrity and Elaboration of Image Cytometry Algorithm. *Cytometry Part A*, **52A**, 19-27. https://doi.org/10.1002/cyto.a.10015