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Efficacy of testicular sperm chromatin condensation assay using aniline blue-eosin staining in the IVF-ET cycle

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Objective: This study was performed to evaluate testicular sperm chromatin condensation using aniline blue-eosin (AB-E) staining and its effects on IVF-ET.
Methods: Chromatin condensation was analyzed using AB-E staining in 27 cases of testicular sperm extraction. There were 19 cases of obstructive azoospermia (OA) and 8 cases of non-obstructive azoospermia (NOA) in IVF-ET. Mature sperm heads were stained red-pink whereas immature sperm heads were stained dark blue. The percentage of sperm chromatin condensation was calculated from the ratio of the number of red-pink sperm to the total number of sperm analyzed.
Results: The overall percentages of chromatin condensation in OA and NOA were 31.1 ± 11.2% and 26.3 ± 14.4%, respectively. The fertilization rate was significant higher in OA than NOA ($p < 0.05$); however, the rates of good embryos and clinical pregnancy did not show statistical differences. In OA and NOA, statistical differences were not observed in the rate of chromatin condensation, fertilization, good embryos, and clinical pregnancy between the pregnant group and non-pregnant group.
Conclusion: Chromatin condensation is less stable than OA and showed a low fertilization rate in NOA. While there were no significant differences in chromatin condensation results between NOA and OA, we propose that a pattern of decreased chromatin condensation in NOA is one of the factors of low fertilization results requiring further study.

Keywords: Aniline Blue; Azoospermia; Chromatin; Eosin; Testicular Sperm Extraction; Human

Introduction

It has recently been reported that there is a high frequency of sperm DNA damage in male infertility and that this abnormality caused miscarriage and abnormal fertilization [1].

Sperm DNA damage appears in high frequency in abnormal semen and has a negative correlation with the fertilization rate [2,3]. Sperm DNA with more than 30% damage makes natural fertilization difficult [4,5]. Sperm DNA integrity is an important indicator for identify normal sperm, thus, it is necessary to confirm the relationship of sperm DNA damage to its impact on fertilization, embryo development, and pregnancy. There are various ways to measure chromatin/DNA damage in humans, and one of these is a chromatin integrity evaluation method which uses aniline blue and eosin staining [6].

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Unlike the previous studies using ejaculated sperm, the authors of the previous study reported on the effectiveness of the assay method for the identification of chromatin maturity using aniline blue counterstained with eosin in azoospermia and testicular sperm [7].

This study was performed in order to examine the relationship between chromatin characteristics and the results of IVF after aniline blue stain and eosin counterstain in testicular sperm obtained from obstructive- and non-obstructive azoospermia patients.

Methods

1. Subjects

The present study was carried out using the testicular tissue sperm of 27 cases obtained from 25 azoospermic patients participating in an IVF-ET program. After biopsy of testicular tissues from male infertility patients diagnosed with azoospermia, patients were classified as having obstructive or non-obstructive azoospermia, and the testicular sperms obtained by testicular sperm extraction (TESE) or by testicular biopsy were used. Patients who underwent the procedure multiple times were considered independent cases. This study was approved by the Institutional Review Board. The average ages of the male and female patients were 37.3±5.5 years and 33.9±4.8 years, respectively. Nineteen and 8 cycles were undergone in 18 patients with obstructive azoospermia (OA) and 7 patients with non-obstructive azoospemia (NOA), respectively. Of these, the results from 15 and 6 cycles of OA and NOA were compared and analyzed after embryo transfer.

2. TESE and freezing and thawing of the seminiferous tubule

TESE and the freezing and thawing of the seminiferous tubules were conducted according to previous reports [8,9]. TESE was conducted using fine forceps in Ham's F-10 medium after the testicular tissue extraction. The seminiferous tubules with sperms were mixed with CryoSperm (MediCult, Jyllinge, Denmark) and frozen using an automatic cell freezer (CryoMagic-1, Miraebiotech, Seoul, Korea). The ampoules were thawed in tap water for 5 minutes and TESE was performed after 4-5 times washes with the medium.

3. Aniline blue-eosin stain

Sperms were stained with aniline blue-eosin as described in a previous report [7]. Briefly, testicular sperms were fixed with 4% formalin for 5 minutes, followed by staining with 5% aniline blue solution adjusted with 4% acetic acid for 5 minutes. After washing, the samples were stained with 0.5% eosin solution for 1 minute and dried. The sperm was then mounted with Permount (SP 15-500, Fisher Sci., Fair Lawn, NJ, USA) and observed under a microscope. The sperm was classified as immature if the head stained dark blue and as mature if the head stained red-pink (Figure 1). On each slide, the number of sperm stained pink was counted and the ratio of pink-stained sperm per 100 sperms was calculated.

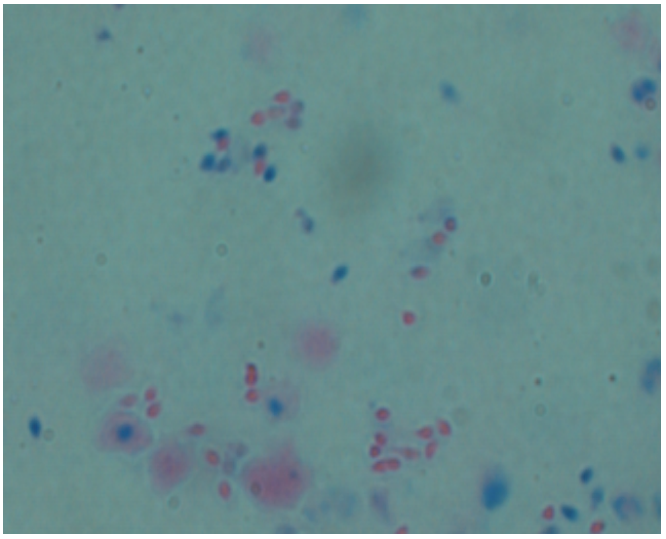

Figure 1. Sperm chromatin integrity assessed by aniline blue-eosin staining. Mature sperm heads were stained red-pink whereas immature sperm heads were stained dark blue (×400).

4. Oocyte aspiration and ICSI

Ovulation induction for oocyte aspiration followed the FSH/hMG and GnRH agonist/antagonist combination method. Ovulation was induced by 5,000-10,000 IU of hCG injection when the size of the dominant follicles reached 18-20 mm in diameter, followed by aspiration of the oocytes guided by vaginal ultrasound after 34-36 hours. The aspirated oocytes were incubated in G-1 medium (Vitrolife, Goteborg, Sweden) supplemented with 10% human serum albumin (HSA) solution (Vitrolife) for 3-5 hours and the cumulus cells were removed by the treatment of 0.1% hyaluronidase enzyme. Then, oocytes were washed and incubated in a gamete incubation medium (Vitrolife). ICSI was performed to the second meiotic metaphase oocytes with the first polar body. Testicular sperms were transferred to the gamete medium (Vitrolife) to assess motility, and when there were no motile sperms, 5 mM pentoxifylline was added to induce motility. A nearly non-motile single sperm was injected into the oocyte after physically immobilizing the sperm's tail and retrieving the sperm using an injection pipette.

5. Assessment of fertilization and embyo

Fertilization was assessed by the presence of two pronuclei 16-18 hours post ICSI. The fertilized egg with 2 pronuclei was transferred to G-1 medium and incubated for 48 hours and followed by further incubation in G-2 medium (Vitrolife) supplemented with 10% HSA solution. The determination of embryonic status was based on the uni-

formity, size, and fragmentation of blastomeres, and these qualities were classified into 5 groups denominated grades I-III. The embryos with equal-sized blastomeres and having no fragmentation were classified as grade I. The embryos with equal-sized blastomeres and having less than 25% fragmentation in the whole volume of the embryo were classified as grade I-1. The embryos with uneven blastomeres and having less than 25% of fragmentation were classified as grade II. If there were uneven blastomeres and 25-50% fragmentation, the embryos were classified as grade II-1. The embryos with more than 50% fragmentation were classified as grade III. Of these, grade I and II embryos were considered good.

6. Embryo transfer and pregnancy confirmation

Grade I and II embryos normally developed into 4-8 cell stages and were then transferred into the uterus. Pregnancy was confirmed by the presence of 5 mIU/mL of β-hCG 12 days after egg aspiration, and defined as clinical pregnancy when a pregnancy sac was identified by ultrasound examination.

7. Statistical analysis

Statistical analyses were carried out by Student's *t*-test and chi-square test, and were considered significant when the *p*-value was less than 0.05.

Results

The testicular sperm chromatin maturity of the OA and NOA patient groups were 31.1 ± 11.2% and 26.3 ± 14.4%, respectively. The value was slightly higher in the OA group than in the NOA group. The rates of fertilization, embryonic development, and healthy embryo generation in OA and NOA groups were compared (Table 1). The fertilization rate of the OA group (74.2%) was significantly higher ($p < 0.05$) than that of the NOA group (51.4%). The embryonic development rates were not different between the two groups, whereas the healthy embryo generation rates were 49.1% and 71.9% in the OA and NOA groups, respectively, though the difference was not statistically significant. The fertilization rate of the OA group (60.0%) was higher than that of the NOA (83.3%). The clinical pregnancy rates in OA and NOA groups were 46.7% and 33.3%, respectively, though the difference was not statistically significant.

The rates of fertilization, embryo development, and healthy embryo generation in the OA group were measured and presented in Table 2. The chromatin maturity rates in the pregnant and non-pregnant groups were 34.1 ± 13.8% and 26.7 ± 7.4%, respectively. This rate was higher in the pregnant group, though the difference was not statistically significant. The rates of fertilization, embryo development, and healthy embryo generation were also higher in the pregnant group, though the difference was not statistically significant.

In the NOA group, the rates of fertilization, embryo development, and healthy embryo generation between the pregnant and non-pregnant groups were compared. The chromatin maturity rates in the pregnant group and the non-pregnant group were 30.3 ± 9.5% and 26.3 ± 16.7%, respectively. The rate in the non-pregnant group was slightly higher than pregnant one; however, there was no statistical difference (Table 3). The fertilization rate was higher in the pregnant group, whereas the healthy embryo generation rate was higher in the non-pregnant group. However, there were no statistical differences between groups.

Table 1. Comparison of sperm chromatin condensation, embryo quality and clinical pregnancy outcomes in azoospermia

	Obstructive (n = 19)	Non-obstructive (n = 8)
Sperm chromatin condensation	31.1 ± 11.2	26.3 ± 14.4
Retrieved oocytes	338	98
Good oocytes	267	72
Fertilized oocytes	198/267 (74.2)[a]	37/72 (51.4)[a]
Cleavage embryos	108/112 (96.4)	32/32 (100.0)
Good embryos	53/108 (49.1)	23/32 (71.9)
Transferred cycles	15	6
Pregnancies	9 (60.0)	5 (83.3)
Clinical pregnancies	7 (46.7)	3 (50.0)

Values are presented as mean ± SD or number (%).
[a]$p < 0.05$.

Table 2. Comparison of sperm chromatin condensation, fertilization and embryo quality in obstructive azoospermia

	Pregnant (n = 7)	Non-pregnant (n = 8)
Sperm chromatin condensation	34.1 ± 13.8	26.7 ± 7.4
Retrieved oocytes	133	126
Good oocytes	112	91
Fertilized oocytes	88/112 (78.6)	61/91 (67.0)
Cleavage embryos	48/49 (98.0)	46/48 (95.8)
Good embryos	27/48 (56.3)	21/46 (45.7)

Values are presented as mean ± SD or number (%).
Statistical difference was not observed between two groups.

Table 3. Comparison of sperm chromatin condensation, fertilization and embryo quality in non-obstructive azoospermia

	Pregnant (n = 3)	Non-pregnant (n = 3)
Sperm chromatin condensation	26.3 ± 16.7	30.3 ± 9.5
Retrieved oocytes	46	44
Good oocytes	34	32
Fertilized oocytes	22/34 (64.7)	15/32 (46.9)
Cleavage embryos	17/17 (100.0)	15/15 (100.0)
Good embryos	12/17 (70.6)	11/15 (73.3)

Values are presented as mean ± SD or number (%).
Statistical difference was not observed between two groups.

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Discussion

The DNA packaging of sperm chromatin differs from the chromatin of somatic cells. The human sperm nucleus consists of approximately 85% protamine and 15% histone proteins. During spermatogenesis, after binding to DNA, histone is replaced by transition protein in the spermatocyte and further replaced by protamine in the spermatid [10]. In most somatic cells, nuclear DNA coils to form a supercoil around a histone octamer, but the DNA helix in mammalian sperms are arranged into a linear configuration. For this condensation, chromatin is stabilized by the disulfide bond between DNA and protamine [11].

In assisted reproductive technology (ART), the methods used to evaluate sperm DNA damage which influence the fertilization and pregnancy rates were practiced by integrity assessments of sperm DNA or sperm chromatin. Aniline blue staining method was used as one of sperm chromatin integrity (chromatin packaging) evaluation methods to identify immature sperms due to an abnormal histone-protamine transition [12-14]. Due to the presence of lysine-rich histone in the immature sperm, the sperm head is stained dark blue by aniline blue. However, the head of mature sperm do not stain due to the presence of cystein- and arginine-rich protamine contained in them. According to these principles of the aniline blue staining method, we know that a large amount of histone is present in sperm with abnormal DNA [15,16]. However, a precise evaluation was difficult because the mature sperm were not stained by the conventional staining method. To address this problem, a staining method using aniline blue counterstained with eosin on ejaculated sperm has been reported [6].

In general, an egg can be fertilized by a sperm with stable DNA. Fertilization by sperm with abnormal DNA is also possible, despite a lower pregnancy rate [17]. In male patients with severe infertility problems, the percentage of sperm DNA damage appears to be high. Therefore, when practicing ICSI, the probability of using sperm with damaged DNA is high, and due to the high apoptotic activity during development into a blastocyst and, in the event of a successful pregnancy, the chance of a successful ongoing pregnancy is significantly low [18,19]. When performing ICSI, though sperm may appear to be normal, sperm chromatin may be abnormal, and the higher the rates of abnormal chromatin the more the initiation or complement of decondensation interfere and cause pregnancy failure [20]. Unrecoverable abnormal paternal genes may also be transmitted in sperm, influencing blastocyst development [21]. Thus, even though the motility and shape of sperm may be normal, the possibility that genetically abnormal sperm is injected cannot be ruled out [22]. The relationship between a minute increase in DNA damage in these sperms and abnormal blastocyst development, failure of implantation, and miscarriage has been reported. By evaluating sperm chromatin integrity, the diagnosis and prognosis of male infertility can be determined [23-25].

However, Tomlinson et al. [26] reported that there was no correlation among apoptosis, fertilization rate, and mean embryonic scores in incubation at 2-3 days *in vitro* for ejaculated sperm. This is due to the fact that a paternal gene is expressed after 4 cell stages of embryonic development regulated by the maternally-derived mRNA in humans [27], and a previous report [28] demonstrated that the paternal gene is activated after embryonic genome activation. On the other hand, studies that have analyzed the roles of abnormal paternal genes in respect to miscarriage have not been performed properly. This has been attributed to the fact that evaluation of fine chromosomal damage (DNA nick, double-strand break) has not actively been performed though analyses of numerical and structural abnormalities [29].

It has been reported that sperm with damaged DNA has a negative influence on pregnancy outcome. However, there is some debate regarding which methods are appropriate to measure the effects of sperm DNA damage in infertile patients. The majority of previous reports have attempted to determine a diagnosis and prognosis of male infertility by means of identifying the relationship among abnormal blastocyst development, implantation failure, miscarriage, and an increase in DNA damage. In this study originated from identify chromatin abnormality, our results are somewhat different from previous reports. Because there are no criteria regarding fertilization rates and embryo development according to chromatin maturity, evaluation criteria need to be established. The benefits of aniline blue-eosin staining for the evaluation of chromatin abnormality are the simplicity of the procedure and the ease of confirming the results. Using this method, it has been reported that it could be possible to predict the pregnancy failure rate for women aged below 35 years old than over 35 years old [6]. However, the study was performed using ejaculated sperm. In the author's previous report [7], the characteristics of aniline blue-eosin staining in testicular sperm were described and the present study has a meaning in the evaluation of relevance to IVF results.

In this study, the degree of chromatin condensation identified by aniline blue-eosin staining in non-obstructive azoospermia patients is slightly lower than that of inobstructive azoospermia patients ($p < 0.1$), likely accounting for the reduced pregnancy rate. The possibility of predicting pregnancy and embryo development is not different between two groups. More analysis is therefore needed to determine whether this result is due to a small number of repetitions or the abnormality in sperm chromatin may be overcome during embryo development.

Therefore, the present results clearly show that ART results are affected by the degree of sperm chromatin damage. Further studies are

needed to develop and standardize a reliable method for the evaluation of chromatin damage, to verify its influence on pregnancy, as well as to develop a method for selecting a proper sperm with undamaged chromatin for ART.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

References

1. Hofmann N, Hilscher B. Use of aniline blue to assess chromatin condensation in morphologically normal spermatozoa in normal and infertile men. Hum Reprod 1991;6:979-82.
2. Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. Biol Reprod 1997;56:602-7.
3. Spanò M, Bonde JP, Hjollund HI, Kolstad HA, Cordelli E, Leter G. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team. Fertil Steril 2000;73:43-50.
4. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, et al. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 1999;14:1039-49.
5. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl 2002;23:25-43.
6. Wong A, Chuan SS, Patton WC, Jacobson JD, Corselli J, Chan PJ. Addition of eosin to the aniline blue assay to enhance detection of immature sperm histones. Fertil Steril 2008;90:1999-2002.
7. Park YS, Shon JH, Lee JS, Seo JT. Efficacy of the aniline blue-eosin staining method for testicular sperm chromatin condensation assay in azoospermia. Korean J Androl 2010;28:107-11.
8. Park YS, Lee SH, Song SJ, Jun JH, Koong MK, Seo JT. Influence of motility on the outcome of in vitro fertilization/intracytoplasmic sperm injection with fresh vs. frozen testicular sperm from men with obstructive azoospermia. Fertil Steril 2003;80:526-30.
9. Park YS, Choi SJ, Lee SH, Park DW, Lim CK, Jun JH, et al. Comparative results of embryo development and clinical pregnancy using sperm retrieved from fresh and frozen-thawed testicular tissue from patients with obstructive and non-obstructive azoospermia. Korean J Reprod Med 2009;36:301-10.
10. Golan R, Shochat L, Weissenberg R, Soffer Y, Marcus Z, Oschry Y, et al. Evaluation of chromatin condensation in human spermatozoa: a flow cytometric assay using acridine orange staining. Mol Hum Reprod 1997;3:47-54.
11. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-45.
12. Auger J, Mesbah M, Huber C, Dadoune JP. Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. Int J Androl 1990;13:452-62.
13. Hammadeh ME, al-Hasani S, Stieber M, Rosenbaum P, Küpker D, Diedrich K, et al. The effect of chromatin condensation (aniline blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic sperm injection programme. Hum Reprod 1996;11:2468-71.
14. Hammadeh ME, Zeginiadov T, Rosenbaum P, Georg T, Schmidt W, Strehler E. Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. Arch Androl 2001;46:99-104.
15. Foresta C, Zorzi M, Rossato M, Varotto A. Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. Int J Androl 1992;15:330-7.
16. Laberge RM, Boissonneault G. Chromatin remodeling in spermatids: a sensitive step for the genetic integrity of the male gamete. Arch Androl 2005;51:125-33.
17. Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum Reprod 2002;17:990-8.
18. Thompson-Cree ME, McClure N, Donnelly ET, Steele KE, Lewis SE. Effects of cryopreservation on testicular sperm nuclear DNA fragmentation and its relationship with assisted conception outcome following ICSI with testicular spermatozoa. Reprod Biomed Online 2003;7:449-55.
19. Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. Fertil Steril 2004;82:378-83.
20. Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 1998;69:528-32.
21. Jones GM, Trounson AO, Lolatgis N, Wood C. Factors affecting the success of human blastocyst development and pregnancy following in vitro fertilization and embryo transfer. Fertil Steril 1998;70:1022-9.
22. Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, Leter G, et al. Full-term pregnancies achieved with ICSI despite high levels of

http://dx.doi.org/10.5653/cerm.2011.38.3.142

sperm chromatin damage. Hum Reprod 2004;19:1409-17.

23. Larson KL, DeJonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. Hum Reprod 2000;15:1717-22.
24. Zini A, Bielecki R, Phang D, Zenzes MT. Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil Steril 2001;75: 674-7.
25. Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 2003;80:895-902.
26. Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M, Sakkas D. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. Hum Reprod 2001;16:2160-5.
27. Tesarík J, Kopecný V, Plachot M, Mandelbaum J. Activation of nucleolar and extranucleolar RNA synthesis and changes in the ribosomal content of human embryos developing in vitro. J Reprod Fertil 1986;78:463-70.
28. Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, Bianchi PG, et al. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod 2000; 15:1112-6.
29. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil Steril 2004;81:1289-95.