CASE REPORT
https://doi.org/10.5653/cerm.2019.46.1.36
pISSN 2233-8233 · eISSN 2233-8241
Clin Exp Reprod Med 2019;46(1):36-40

A point of confusion for embryologists in the identification of viable spermatozoa by the eosin-nigrosin test

Huanhua Chen, Hong Zhou, Jinhui Shu, Xianyou Gan, Caizhu Wang, Ruoyun Lin

Reproductive Medicine Center, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning, China

A viable spermatozoon is a prerequisite for fertilization in intracytoplasmic sperm injection (ICSI). Thus, it is crucial to select viable but immotile spermatozoa on the day of ICSI. We report conflicting results in the identification of viable but immotile spermatozoa between the eosin-nigrosin staining and the laser test, which resulted in confusion for embryologists during assisted reproductive technology (ART). Three patients' semen samples that showed no motile spermatozoa are described in this report. To identify viable spermatozoa, we used both the eosin-nigrosin test and the laser test for each sample, and repeated the semen analysis twice in each patient. Viable but immotile spermatozoa selected by the laser test were used for ICSI. Viable spermatozoa were detected by both the eosin-nigrosin and laser tests in two patients (case 1, 95.00% vs. 24.21% and 92.68% vs. 22.22%; case 2, 41.18% vs. 23.48% and 39.81% vs. 22.52%), indicating consistent results between the two methods. In the third patient, the eosin-nigrosin test yielded viability rates of 20.75% and 19.14%, while the result of the laser test was 0%. Thus, testicular aspiration was performed to collect viable sperm from this patient. Normal fertilization was achieved after the injection of viable but immotile spermatozoa selected from these patients by the laser test, resulting in the birth of two healthy babies. Our study documents a case where the eosin-nigrosin test showed a limitation in identifying viable but immotile spermatozoa for ART, while the laser test may overcome this limitation. Larger samples may be required to corroborate the clinical value of the laser test.

Keywords: Eosin-nigrosin test; Immotile spermatozoa; Intracytoplasmic sperm injection; Laser

Introduction

Evaluation of spermatozoa vitality using the eosin-nigrosin staining test is recommended by the fifth edition of the World Health Organization (WHO) laboratory manual for the examination and processing of human semen. As such, eosin-nigrosin staining has been an important and routine tool for clinicians to determine the possibility of assisted reproductive technology (ART) treatment, especially for patients with completely immotile sperm [1,2]. In theory, if viable spermatozoa are identified using the eosin-nigrosin staining test, it is reasonable for clinicians to decide that this sperm has a potential for fertilization and can be used for intracytoplasmic sperm injection (ICSI). However, we recently encountered a case in which the results of eosin-nigrosin staining conflicted with those obtained using laser-assisted detection. Many studies have shown that the laser test is an effective and rapid method of identifying viable sperm for ICSI [3-5], but conflicting results between these two tests may create confusion among embryologists regarding whether these patients are suitable for ART treatment. We share our experience with such a discrepancy herein.

Received: Sep 26, 2018 · Revised: Nov 15, 2018 · Accepted: Nov 16, 2018
Corresponding author: **Hong Zhou**
Reproductive Medicine Center, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, No. 225, Xinyang Road, Nanning City 530003, China
Tel: +86-771-5802003 Fax: +86-771-5802003 E-mail: yuhongting@hotmail.com

Case

1. Case 1

A 32-year-old man and his 27-year-old wife presented to our reproductive center with primary infertility of 1 year and 6 months' dura-

tion. The husband's follicle-stimulating hormone (FSH) level was 1.82 mIU/mL, and a karyotype of 46-XY with Y-chromosome microdeletions within the normal limits. Repeated semen analysis performed at another reproductive institute showed severe asthenozoospermia with a complete absence of motile spermatozoa. Repeated semen analysis at our center showed severe asthenoteratospermia (sperm motility rate, 0%; normal sperm morphology rate, 0.5% and 0.8% according to the WHO manual) [6].

2. Case 2

A 41-year-old man and his 40-year-old wife presented with a 22-month history of inability to conceive a planned second child. His wife had delivered a healthy girl 16 years previously. The husband stated that their sex life was normal, with anterograde ejaculation, no impotence, and no premature ejaculation. The karyotype of the man was 46-XY with Y-chromosome microdeletions within the normal limits. Repeated semen analysis at our center showed asthenozoospermia (sperm vitality, $<10\%$ according to the WHO manual) [6]. No motile sperm was observed in the multiple semen samples obtained though ejaculation on the day of ovum pick-up.

3. Case 3

A 35-year-old man and his 27-year-old wife had been married for 2 years with primary infertility. The medical records of the husband showed that their routine private sex life was normal, with anterograde ejaculation, no impotence, and no premature ejaculation. The right testis volume was 20 mL, while the left testis was not found during body examination. The husband's FSH level was 9.24 mIU/mL, and his karyotype was 46-XY with Y-chromosome microdeletions within the normal limits. Two rounds of semen analysis showed a complete absence of motile spermatozoa according to the WHO criteria. Both the eosin-nigrosin staining and the laser test were used to analyze the viability rate of each semen sample. No motile sperm was detected by laser, so this couple was advised to undergo collection of testicular spermatozoa in the following ART treatment cycle.

4. Detection of the viability rate of immotile sperm

Both eosin-nigrosin staining and the laser test were used to analyze the viability rate of each semen sample. Semen analysis was repeated twice in each patient. The semen collection interval was 3–7 days in each patient. One-step eosin-nigrosin staining was performed, following the WHO guidelines [6]. Laser-assisted selection of viable but immotile sperm was performed as described by Aktan et al. [7]. Briefly, laser assessment of sperm viability was performed by a direct laser shot to the tip of the sperm flagellum, using laser energy of approximately 200 μJ with about 2 ms of irradiation. Spermatozoa that responded to the laser shot by curling their tail were regarded as presumably viable and selected for ICSI.

5. Testicular sperm aspiration

The patient was maintained in the supine position and disinfected using routine protocols. Anesthesia was performed by 2% lidocaine for spermatic cord block. A 50-mL syringe containing 0.5 mL of Quinn's 1020 medium (Sage, Trumbull, CT, USA) and a 16-gauge needle were used for each sample. These samples were then independently minced using two sterile needles in a dish containing 2 mL of fertilization medium (Quinn's 1020).

6. Ovarian stimulation protocols and oocyte collection

Controlled ovarian stimulation was carried out after downregulation with Diphereline. FSH was administered at a dose of 200 IU per day. When the dominant follicles reached a diameter of 18 mm, ovulation was induced by an intramuscular injection of 10,000 IU of human chorionic gonadotropin (hCG). Oocytes were recovered by transvaginal aspiration of follicles under ultrasound guidance 36 hours after the hCG injection.

7. Embryo culture and pregnancy determination

Fertilization was checked by the detection of two pronuclei and two polar bodies at 16–18 hours after ICSI. Only zygotes displaying two pronuclei were transferred into Quinn's 1026 medium (Sage) supplemented with 10% serum protein substitute (SPS, Sage) for days 0–3, and then were moved to Quinn's 1029 medium (Sage) supplemented with 10% SPS for 2 more days. A biochemical pregnancy test was carried out 14 days after embryo transfer by measuring serum β-hCG. Clinical pregnancy was confirmed if a gestational sac was assessed on transvaginal ultrasonography at 6–8 weeks after transfer.

8. Spermatozoa viability detection results and clinical outcomes

Viable spermatozoa were detected by the eosin-nigrosin test and the laser test in the first two patients (case 1, 95.00% vs. 24.21% and 92.68% vs. 22.22%; case 2, 41.18% vs. 23.48% and 39.81% vs. 22.52%), and the results of the two methods were consistent. In the third patient, the viability rates obtained using the eosin-nigrosin test were 20.75% and 19.14%, while the viability rate obtained using the laser test was 0% (Table 1).

As shown in Table 2, for all three patients, normal embryo fertilization with normal cleavage was achieved. After the transfer of fresh embryos, the wife of one patient achieved a singleton pregnancy and delivered a full-term healthy boy, while the wife of the other patient did not become pregnant and no frozen embryo was available. In case 3, because of the risk of ovarian hyperstimulation, fresh embryo transfer was cancelled, and four blastocysts were vitrified. The

Table 1. Comparison of spermatozoa viability between the eosin-nigrosin and laser tests

Case	Semen sample	Abstinence day	Volume (mL)	Density ($\times 10^6$/mL)	Eosin-nigrosin test			Laser test			Did the two results conflict?
					Total sperm (n)	Survival sperm (n)	Survival rate (%)	Total sperm (n)	Survival sperm (n)	Survival rate (%)	
1	1	4	4.9	40.0	280	266	95.00	252	60	24.21	No conflict
	2	4	3.7	23.0	246	228	92.68	216	48	22.22	
2	1	7	2.6	35.0	204	84	41.18	230	54	23.48	No conflict
	2	4	2.1	43.0	216	86	39.81	222	50	22.52	
3	1	2	3.5	11.2	212	44	20.75	230	0	0	Conflict
	2	4	2.8	18.0	209	40	19.14	206	0	0	

Table 2. Embryo development and clinical outcomes of the three patients

Variable	Case 1	Case 2	Case 3
Age of the female partner (yr)	27	40	27
Spermatozoa source	Ejaculation	Ejaculation	Testicular biopsied
Did the results of the eosin-nigrosin and laser tests conflict?	No conflict	No conflict	Conflict
No. of oocytes retrieved	9	9	27
No. of meiosis II oocytes	6	5	23
Fertilization rate	66.67 (6/9)	60.00 (3/5)	86.96 (20/23)
Cleavage rate	100.00 (6/6)	100.00 (3/3)	100.00 (20/20)
Good embryo rate	16.67 (1/6)	33.33 (1/3)	50.00 (10/20)
No. of embryos transferred	2	2	Cancel transfer
Clinical pregnancy	Yes	No	-
Delivery of a baby	Healthy	-	-[a]

Values are presented as percent (number) unless otherwise indicated.
[a]The wife of the third patient successfully achieved intrauterine pregnancy and delivered a healthy full-term baby in the subsequent frozen-thawed cycle.

wife of the third patient achieved a successful intrauterine pregnancy in the subsequent frozen-thawed cycle, and delivered a full-term healthy baby.

Discussion

In our report, in the first two cases, a clinically significant percentage of spermatozoa was classified as viable using the eosin-nigrosin test and by the laser test. Based on these findings, the clinicians speculated that sufficient spermatozoa were available for ICSI. However, no viable spermatozoa were observed using the laser test in the third case. Therefore, the patient was advised to undergo testicular collection of spermatozoa in the following ART cycle. These conflicting results between the eosin-nigrosin test and the laser test caused confusion in the embryologists.

Several techniques have been developed to identify viable but immotile spermatozoa [8-14]. The eosin-nigrosin test and the hypo-osmotic swelling test are recommended for the diagnostic evaluation of spermatozoa vitality in the latest edition of the WHO laboratory manual [6] for the examination and processing of human semen. The eosin-nigrosin test is based upon the sperm head membrane integrity, as nonviable spermatozoa exhibit deficient cell membrane structures, permitting eosin to enter the cells and stain them, whereas viable spermatozoa resist the dyes and remain unstained. However, once exposed to toxic dyes, spermatozoa are no longer suitable for fertilization, which limits the application of this technique to the diagnostic evaluation of the spermatozoa survival rate before ART. The hypo-osmotic swelling test is time-consuming, operationally complex, and operator-dependent [14]. Furthermore, it is not 100% accurate and requires a high level of experience and skill to perform [9]. Furthermore, spontaneously developed tail swellings may influence the accuracy of the hypo-osmotic swelling test, and it is not suitable for cryopreserved ejaculated spermatozoa [15]. Lasers show a thermal effect; when the tail of a spermatozoon is irradiated by a low-energy laser, the local temperature increases, causing the change of cell membrane proteins [5]. Spermatozoa showing a curling reaction are considered to be presumably viable, whereas dead spermatozoa show no reaction. The laser test has several advantages. First, it can be performed with normal culture media, and it does not require any chemical reagents. Second, the laser test is easy to perform and the spermatozoa can be directly used for ICSI. Third, the laser energy level can be adjusted as needed. A further study confirmed that the percentage of immotile spermatozoa classified as viable by the laser test was similar to that detected by the hypo-osmotic swelling test [7].

https://doi.org/10.5653/cerm.2019.46.1.36

The use of viable but immotile spermatozoa, either from the ejaculate or extracted from a testicular biopsy, has resulted in successful pregnancies [8,16], indicating that immotile spermatozoa can successfully fertilize an ovum.

The conflicting results presented in our report may be attributed to the different principles of these techniques. The eosin-nigrosin test is based on the integrity of the sperm head membrane, whereas laser identification is based on protein activity and the integrity of the tail membrane. Studies have shown that immotile spermatozoa from the ejaculate may undergo pathological epididymis degradation, and the degradation of the sperm head and tail membrane occur independently [17]. It has been proven that protein activity is the basis of a series of molecular events during the process of fertilization [18]. Application of a laser to select viable spermatozoa with protein activity has yielded highly satisfactory pregnancy outcomes [8,16]. However, no reports have described the direct use of spermatozoa stained by the eosin-nigrosin test for ICSI. Therefore, testicular sperm extraction is advised for such patients to obtain spermatozoa for fertilization, which can yield a very satisfactory fertilization rate.

In conclusion, this report identified a limitation of the eosin-nigrosin test in ART. The viable immotile sperm identified by the eosin-nigrosin test do not necessarily show protein activity. As is known, the series of molecular events during the process of fertilization require protein activity. Therefore, we suggest that laser-assisted detection may be preferable to the eosin-nigrosin test for identifying viable but immotile spermatozoa for ICSI.

Conflict of interest

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

Acknowledgments

This work was supported by the Guangxi National Science Foundation (Project No. 2013GXNSFAA019258), a project grant from the Research Foundation of Guangxi Medical and Health (Project No. S201612) and a project grant from the Self-raised Foundation of the Guangxi Health Commission (Project No. Z20170777).

ORCID

Huanhua Chen https://orcid.org/0000-0001-8463-7452
Hong Zhou https://orcid.org/0000-0002-9877-5300
Jinhui Shu https://orcid.org/0000-0002-0052-4424
Xianyou Gan https://orcid.org/0000-0002-9742-2626
Caizhu Wang https://orcid.org/0000-0002-7649-6394
Ruoyun Lin https://orcid.org/0000-0002-9779-1552

Author contributions

Conceptualization: HC, HZ. Data curation: HC. Formal analysis: HC. Funding acquisition: XG. Methodology: RL. Project administration: HZ. Visualization: CW. Writing - original draft: HC. Writing - review & editing: JS.

References

1. Bjorndahl L, Soderlund I, Johansson S, Mohammadieh M, Pourian MR, Kvist U. Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitality assessment must change. J Androl 2004;25:671-8.
2. Bjorndahl L, Soderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Hum Reprod 2003;18:813-6.
3. Nordhoff V. How to select immotile but viable spermatozoa on the day of intracytoplasmic sperm injection? An embryologist's view. Andrology 2015;3:156-62.
4. Rubino P, Vigano P, Luddi A, Piomboni P. The ICSI procedure from past to future: a systematic review of the more controversial aspects. Hum Reprod Update 2016;22:194-227.
5. Salman Yazdi R, Bakhshi S, Jannat Alipoor F, Akhoond MR, Borhani S, Farrahi F, et al. Effect of 830-nm diode laser irradiation on human sperm motility. Lasers Med Sci 2014;29:97-104.
6. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: World Health Organization; 2010.
7. Aktan TM, Montag M, Duman S, Gorkemli H, Rink K, Yurdakul T. Use of a laser to detect viable but immotile spermatozoa. Andrologia 2004;36:366-9.
8. Gerber PA, Kruse R, Hirchenhain J, Krussel JS, Neumann NJ. Pregnancy after laser-assisted selection of viable spermatozoa before intracytoplasmatic sperm injection in a couple with male primary cilia dyskinesia. Fertil Steril 2008;89:1826.
9. Barros A, Sousa M, Angelopoulos T, Tesarik J. Efficient modification of intracytoplasmic sperm injection technique for cases with total lack of sperm movement. Hum Reprod 1997;12:1227-9.
10. Ebner T, Tews G, Mayer RB, Ziehr S, Arzt W, Costamoling W, et al. Pharmacological stimulation of sperm motility in frozen and thawed testicular sperm using the dimethylxanthine theophylline. Fertil Steril 2011;96:1331-6.
11. Geber S, Lemgruber M, Taitson PF, Valle M, Sampaio M. Birth of healthy twins after intracytoplasmic sperm injection using ejaculated immotile spermatozoa from a patient with Kartagener's syndrome. Andrologia 2012;44 Suppl 1:842-4.

12. Mangoli V, Mangoli R, Dandekar S, Suri K, Desai S. Selection of viable spermatozoa from testicular biopsies: a comparative study between pentoxifylline and hypoosmotic swelling test. Fertil Steril 2011;95:631-4.
13. Soares JB, Glina S, Antunes N Jr, Wonchockier R, Galuppo AG, Mizrahi FE. Sperm tail flexibility test: a simple test for selecting viable spermatozoa for intracytoplasmic sperm injection from semen samples without motile spermatozoa. Rev Hosp Clin Fac Med Sao Paulo 2003;58:250-3.
14. Zubair M, Ahmad M, Jamil H. Review on the screening of semen by hypo-osmotic swelling test. Andrologia 2015;47:744-50.
15. Hossain A, Osuamkpe C, Hossain S, Phelps JY. Spontaneously developed tail swellings (SDTS) influence the accuracy of the hypo-osmotic swelling test (HOS-test) in determining membrane integrity and viability of human spermatozoa. J Assist Reprod Genet 2010;27:83-6.
16. Nordhoff V, Schuring AN, Krallmann C, Zitzmann M, Schlatt S, Kiesel L, et al. Optimizing TESE-ICSI by laser-assisted selection of immotile spermatozoa and polarization microscopy for selection of oocytes. Andrology 2013;1:67-74.
17. Schaller M, Panhans-Gross A, Bezold G, Korting HC, Wolff H. Images in reproductive medicine: ultrastructural defects in acquired immotile sperm flagellae. Fertil Steril 2000;73:351-2.
18. Dozortsev D, Rybouchkin A, De Sutter P, Dhont M. Sperm plasma membrane damage prior to intracytoplasmic sperm injection: a necessary condition for sperm nucleus decondensation. Hum Reprod 1995;10:2960-4.

https://doi.org/10.5653/cerm.2019.46.1.36