Assessment of Sperm Deoxyribose Nucleic Acid Fragmentation Using Sperm Chromatin Dispersion Assay

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Abstract

The integrity of sperm deoxyribose nucleic acid (DNA) is one of the determinants that ensure normal fertilization, implantation, pregnancy, and the generation of a healthy progeny. Assessment of sperm DNA fragmentation has gained importance as a tool to provide significant information regarding sperm quality, and it can independently predict sperm fertilizing potential. The sperm chromatin dispersion (SCD) assay is one of the available techniques to detect sperm DNA damage with results comparable to the gold standard – Sperm Chromatin Structure Assay. We present here a detailed methodology of an alkaline modification of SCD that can be carried out with minimal laboratory equipment. The SCD assay is a modified halo assay that utilizes chemical methods to demonstrate sperm DNA fragmentation. It involves the embedding of sperms in an agarose medium followed by exposure to alkaline denaturation and deproteinization. The results are interpreted under a light microscope. A visual scoring system is utilized to differentiate the sperms with possible DNA fragmentation from those without fragmented DNA and to determine the sperm DNA fragmentation index (SDFI) % for each semen sample. The SDFI % is directly proportional to the quality of sperm. The SCD assay is a simple, cost-effective, and reliable technique that can detect sperm DNA fragmentation, thus providing information regarding sperm functional quality and reproductive capacity. It is of significance in clinical and research areas of andrology and reproductive medicine, toxicology, and pharmacotherapeutics.

Keywords: Andrology, assisted reproduction, male infertility, sperm deoxyribose nucleic acid damage

Introduction

The integrity of sperm deoxyribose nucleic acid (DNA) is one of the determinants that ensures normal fertilization, implantation, pregnancy, and the generation of a healthy progeny. Damage to sperm DNA can interfere with any of the above processes. The assessment of sperm DNA damage has gained importance as a tool to provide significant information regarding sperm quality since it can independently predict sperm fertilizing potential.[1] The presence of increased sperm DNA damage has proven associations with male infertility and repeated pregnancy loss.[2] In assisted reproductive techniques (ARTs), detecting sperm DNA damage aids the selection of sperms and prediction of pregnancy outcome.[3] The detection of sperm DNA damage is of profound interest in the evaluation of drug safety and toxicity, exposure to environmental toxins and pollutants, genotoxicity testing, and pharmacotherapeutics.[4] Although conventional semen analysis is used preferably, sometimes solely, to assess sperm quality in the above-mentioned clinical and research settings, it does not provide sufficient information regarding the sperm genome.[5]

The sperm chromatin dispersion (SCD) Assay was introduced by Fernández *et al*. in 2003 to detect the presence of sperm DNA fragmentation.[6] Its advantages over its predecessor techniques (single cell gel electrophoresis technique, terminal deoxynucleotidyl transferase dUTP Nick End Labeling Assay [TUNEL], sperm chromatin structure assay [SCSA], and acridine orange staining) include its cost-effectiveness and simplicity. Its results are comparable to the gold standard - SCSA.[6]

Access this article online

Quick Response Code:

Website: www.jpharmacol.com

DOI: 10.4103/jpp.JPP_187_16

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How to cite this article: Pratap H, Hottigoudar SY, Nichanahalli KS, Chand P. Assessment of sperm deoxyribose nucleic acid fragmentation using sperm chromatin dispersion assay. J Pharmacol Pharmacother 2017;8:45-9.

Received: 08-12-2016 **Revised:** 14-03-2017 **Accepted:** 14-03-2017

The principle of sperm chromatin dispersion assay

The procedure of SCD involves an initial chemical denaturation of sperm cells, which causes the formation of single-stranded (ss) DNA motifs from the ends of existing DNA breakage sites, followed by the deproteinization of sperm cell nuclear and cell membranes. The resultant "sperm nucleoid," the sperm nucleus surrounded by a halo of relaxed DNA loops, is assessed for DNA damage based on its appearance under the microscope. The ratio of the nucleoid halo:nucleus determines the presence of DNA damage. Sperm nucleoids with minimal/absent DNA damage form large haloes, whereas those with significant damage do not produce prominent haloes.[7,8] The reason for suppression of halo formation by sperm nuclei with DNA damage is not known. An interaction between the ss DNA motifs generated and the sperm head has been suggested.[6]

We present here a detailed protocol of an alkaline modification of SCD standardized in the Department of Anatomy, Jawaharlal Institute of Postgraduate Medical Education and Research, Puducherry, India.

Materials and Methods

Requirements

Reagents

Agarose - low electroendosmosis (EEO) (Molecular Biology grade, MB), agarose - low melting (MB), Triton X-100 (MB), sodium hydroxide (Analytical Reagent grade, AR), sodium chloride (AR), disodium ethylenediaminetetraacetic acid (EDTA) (AR), Tris (AR), dithiothreitol (DTT), absolute ethanol, and Wright stain solution (Sigma).

Equipment

Light microscope, digital pH meter (Deep Vision, model 111, 1205015), electronic weighing balance (Shimadzu AUY 220), microwave oven, magnetic stirrer, refrigerator, water distillation unit (Mono Quartz, model: Borosil 3363), and micropipette 10–100 µl.

Preparation of working solutions

Normal melting point agarose

To prepare normal melting point agarose (NMA) solution, dissolve 0.041 g of low EEO agarose powder in 6.5 mL of phosphate-buffered saline (PBS) in a clean glass beaker and cover it with aluminum foil. Heat the mixture in a microwave for 5–10 s at low power.

The prepared working solution may be stored in the refrigerator at 20°C and reused. A pressure cooker can be used as an alternative to a microwave.

Low melting point agarose

To prepare low melting point agarose (LMA) solution, dissolve 0.03 g of LMA powder in 6.25 mL of PBS in a clean glass beaker. Cover the beaker with aluminum foil and place in a microwave for 7–10 s. The working solution of LMA can be stored in the same way as NMA for further use.

Alkaline denaturation solution

To prepare the denaturation solution, dissolve 58.44 g (1 M) of sodium chloride and 1.2 g (0.03 M) of sodium hydroxide in 1000 ml of distilled water in a beaker and adjust the pH of this solution to 7.5.

Lysis solution

The lysis solution is prepared by dissolving 116 g (2.5 M) sodium chloride, 48.45 g (0.4 M) Tris, 18.6 g (50 mM) EDTA, and 1 g (0.006 M) DTT in 700 mL of distilled water in a clean glass measuring cylinder. Mix 1.5 mL of Triton X-100 in 100 mL of distilled water and add it to the above solution. Adjust the pH of the solution to 7.5. Add distilled water to the final solution until it measures 1000 mL.

Alcohol solutions

To prepare 50% ethanol solution, add 50 mL of distilled water to 50 mL of absolute ethanol. Similarly, a 70% ethanol solution is made by adding 30 mL of distilled water to 70 mL absolute ethanol.

Protocol for slide preparation

Agarose coating of slides

Pipette 100 µL of the prepared NMA on one end of a clean, labeled glass slide and create a uniform layer by gently smearing it with another glass slide angled at 45°. The slide is allowed to dry at room temperature.

Note: The first layer of NMA serves as a base to embed the spermatozoa and requires careful preparation. The NMA is layered only when it reaches room temperature.

Embedding of spermatozoa

The concentration of spermatozoa is detected in the sample with the help of a wet preparation as described in the WHO manual.[9] Based on the sperm concentration in the semen sample, dilute the same with the phosphate buffer to obtain a resultant concentration of 5–10 million spermatozoa/mL. Mix 20 µL of the diluted semen and 80 µL of LMA in a microcentrifuge tube. Gently stir the mixture. Pipette 100 µL semen-LMA mixture onto the NMA coated slide. Place a clean glass cover slip over this and cool it on a prerefrigerated metallic tray at 4°C for 10–20 min.

Note: The semen sample was collected by masturbation into a sterile graduated wide-mouthed plastic container following an ejaculatory abstinence period of 2-7 days. The LMA serves as an inert medium to stabilize the loops of DNA. LMA is used after bringing to room temperature as heat from it can introduce sperm DNA damage.

Denaturation

Once the layers have solidified, take the slide out from the refrigerator, and remove the coverslip without disturbing the underlying layers. Place the slide horizontally in a trough containing 10 mL (per slide) of denaturation solution. The trough is placed in the refrigerator at 4°C for 8–10 min.

Note: Make sure the slides are immersed thoroughly. Denaturation causes the formation of DNA motifs from the

ends of DNA breakage sites. The alkaline solution, as used in this protocol, is a stronger denaturant than acid solutions. Hence, the former produces haloes with better visibility than the latter when viewed under the light microscope.

Lysis

To initiate lysis, immerse the slide horizontally in 10 ml (per slide) of the prepared lysis solution at room temperature for 30 min.

Dehydration

The slides are placed horizontally and dehydrated sequentially with 50%, 70%, and absolute ethanol for 2 min each. Leave the slides to air dry.

Staining

The Wright's stain solution is mixed with its phosphate buffer in the ratio of 1:1. Approximately 2 mL of the mixture is required for each slide. Cover the slide with the mixture for 15 min and then wash gently with running tap water. Allow it to air dry.

Note: Forceful washing of slide may cause the layers to peel off. The various problems encountered during the procedure, and their troubleshooting is listed in Table 1.

Squam DNA Fragmentation Index

Following the SCD procedure, sperm nucleoids were visualized on the slide under light microscopy. The sperm nucleoid consists of two parts: the central core and the outer halo. Figure 1 shows a sperm nucleoid under × 400 magnification with sperm halo radius marked "*r*" and core diameter "*d*." On comparing these two parameters, four patterns of sperm nucleoids may be recognized as shown in Figure 2:

A. Nucleoids with large-sized haloes ($r > d$)
B. Nucleoids with medium-sized haloes ($r = d$)
C. Nucleoids with very small-sized haloes ($r < d$)
D. Nucleoids with no halo (only core of nucleoid present).

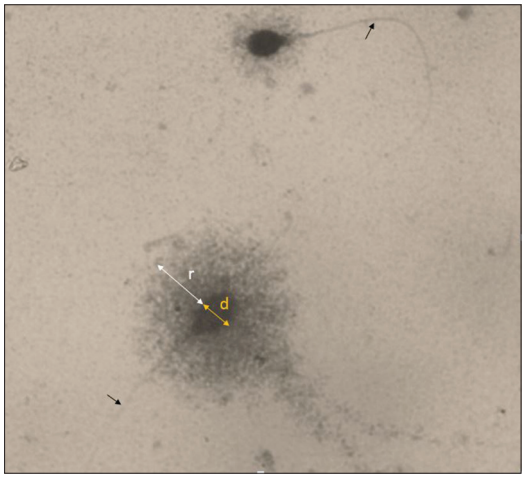

Figure 1: Visual assessment of deoxyribose nucleic acid fragmentation in a sperm nucleoid at ×400 magnification. "*r*" Radius of the halo; "*d*" Diameter of the core; Arrows: Sperm tail

At least 200 spermatozoa were assessed in each slide. The percentage of nucleoids belonging to each of the four patterns was noted. Those with absent or small-sized haloes ($r < d$) were considered to exhibit DNA damage and those with medium-sized ($r = d$) or large-sized haloes ($r > d$) were the spermatozoa without DNA damage. From the above data, sperm DNA fragmentation index (SDFI) was calculated using the formula:

SDFI = 100 × Number of sperm with DNA damage/Number of sperms counted.

Advantages

The protocol described in this paper differs from the initial SCD assay protocol introduced by Fernández *et al*. in 2003. The solutions, namely the lysis and the neutralization buffers used separately[6] have been combined into a single solution. This reduced the time and effort spent on the sample processing. A similar concept has been used in an "improved" SCD protocol, introduced by the same author, and is currently being employed in the commercially marketed SCD kits.[7] The technical advantages gained by the above modification are similar to those mentioned in the "improved" SCD protocol. They are (a) preservation of sperm tail – making differentiation from other cells easier, (b) distinct peripheries of nucleoid halo – for better recognition of nucleoid patterns, and (c) improved precision under bright field microscopy. Apart from using an alkali as the denaturing agent, the other areas of modifications in our protocol include the technique of layering of agarose, reduced the use of DTT in the lysis buffer, duration of the procedure, and staining.

The advantages of the SCD assay are the minimal requirement of laboratory equipment, the simplicity of the process, and cost efficiency. The SCD test results can be interpreted with

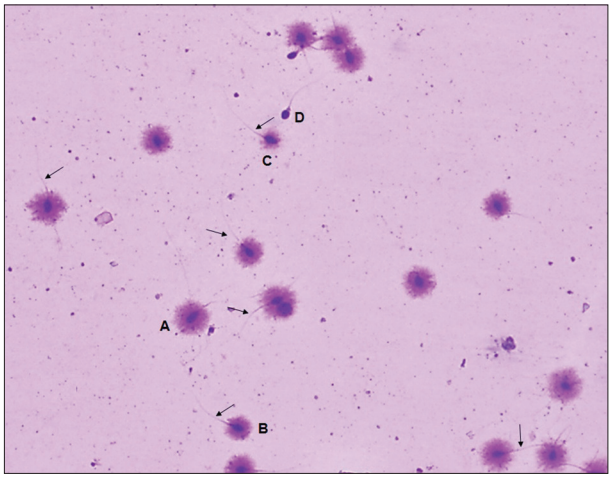

Figure 2: The four patterns of sperm nucleoids observed at ×100 magnification on a study sample of sperms following sperm chromatin dispersion test. (A) Nucleoids with large-sized haloes, (B) Nucleoids with medium-sized haloes, (C) Nucleoids with very small-sized haloes, and (D) Nucleoids without a halo. Arrows: Tail of sperm

Table 1: Troubleshooting in the procedure of sperm chromatin dispersion assay

Problem	Step	Cause	Suggestion
Peeling of agarose layer	Layering of agarose	Incomplete drying/increased thickness of the agarose layer	Allow the agarose layer to air dry until the shiny glaze disappears
Early solidification of agarose while layering	Layering of agarose	Evaporation of water content during heating of stored agar	Prepare new agarose solution. Keep solution loosely covered with silver foil during heating
Overcrowding of spermatozoa making it difficult to assess	Embedding of spermatozoa	Improper dilution	Assess sperm concentration using the WHO guidelines[9] before diluting
Absence of haloes	Denaturation, lysis	Inadequate exposure to denaturation or lysis solutions	Ensure the presence of 10 mL of denaturation and lysis solutions for each slide. Prolong exposure to 12-14 min for denaturation and 30-35 min for lysis

accuracy and clarity using a bright field microscopy, and unlike the SCSA, it does not require a flow cytometer.[10] When compared to the TUNEL assay, the SCD has significantly more sensitivity under the bright field microscope.[11] The commercially available kits based on SCD protocol, namely "Halosperm" and "Halomax" are relatively expensive. In contrast, the manual technique employed by us is simple, cost-effective, especially when a large sample size is considered.[10] The SCSA is, however, the gold standard for detection of sperm DNA fragmentation with maximum statistical robustness.[10]

An SDFI of <30% is considered as an essential prerequisite for the initiation and maintenance of pregnancy, irrespective of the status of semen analysis parameters such as sperm count, morphology, or motility.[6,12] A study conducted to decipher the link between SDFI and intrauterine insemination outcome reported that only 1 out of 23 women artificially inseminated with semen with SDFI >27% had successful pregnancy.[12] While the cut-off for SDFI varies between 27% and 30%, its applicability has been studied only in SCSA and is yet to be standardized for use in the SCD assay. Another area of possible future research is the possibility of designing a test that can detect sperm DNA fragmentation without affecting the viability of the sperms which is not feasible with SCD or SCSA. This test will allow the direct use of the selected sperms for clinical purposes as in assisted conception or research.

The conventional semen analysis following the WHO guidelines is used universally in clinical and research areas that require screening and analysis of semen.[5,13,14] However, it does not analyze the properties of sperms at a molecular level, especially its genetic integrity.[5] The manual method of the conventional semen analysis is highly subjective with considerable inter- and intraobserver variations in the results.[15] Hence, its use as a sole measurement of semen quality may not identify hidden chromosomal, nuclear, or cell membrane anomalies.[16] For example, in assisted reproduction, the use of sperms with DNA damage may fertilize ova but negatively affect implantation and embryo development, thereby affecting the procedure's outcome.[5]

The SCD assay has applications in diverse fields of medicine and research. In andrology, it is relevant for screening and diagnosis of male infertility.[1] It may be beneficial in the selection of sperms before ART and prediction of pregnancy outcome following ART.[3] In pharmacology, the detection of sperm DNA damage has been used to assess male fertility status following chemotherapy, radiation exposure, and exposure to environmental toxins/pollutants.[4] It has also been employed in the study of the beneficial role and side effects of drugs on male reproductive capacity.[17,18]

Conclusion

The SCD assay can demonstrate the presence of DNA fragmentation in spermatozoa. It involves a simple, yet cost-effective procedure and gives reliable results. The inclusion of SCD test along with the regular semen analysis is likely to augment information regarding sperm fertilizing capacity. The detection of sperm DNA fragmentation using SCD is of importance to clinicians and researchers in the fields of andrology, genetics, reproductive medicine, toxicology, and pharmacotherapeutics.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-45.
2. Absalan F, Ghannadi A, Kazerooni M, Parifar R, Jamalzadeh F, Amiri S. Value of sperm chromatin dispersion test in couples with unexplained recurrent abortion. J Assist Reprod Genet 2012;29:11-4.
3. Schulte RT, Ohl DA, Sigman M, Smith GD. Sperm DNA damage in male infertility: Etiologies, assays, and outcomes. J Assist Reprod Genet 2010;27:3-12.
4. Barratt CL, Aitken RJ, Björndahl L, Carrell DT, de Boer P, Kvist U, *et al.* Sperm DNA: Organization, protection and vulnerability: From basic science to clinical applications – A position report. Hum Reprod 2010;25:824-38.
5. Sergerie M, Laforest G, Bujan L, Bissonnette F, Bleau G. Sperm DNA fragmentation: Threshold value in male fertility. Hum Reprod 2005;20:3446-51.
6. Fernández JL, Muriel L, Rivero MT, Goyanes V, Vazquez R, Alvarez JG. The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. J Androl 2003;24:59-66.
7. Fernández JL, Muriel L, Goyanes V, Segrelles E, Gosálvez J, Enciso M, *et al.* Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. Fertil Steril 2005;84:833-42.

8. Gosálvez J, Gosálbez A, Arroyo F, Fernández JL, López-Fernández C. Assessing sperm DNA fragmentation in the field: An adaptation of sperm chromatin dispersion technology. Biotech Histochem 2008;83:247-52.
9. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed. Geneva: World Health Organization; 2010.
10. Evenson DP. The Sperm Chromatin Structure Assay (SCSA®) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility. Anim Reprod Sci 2016;169:56-75.
11. Zhang LH, Qiu Y, Wang KH, Wang Q, Tao G, Wang LG. Measurement of sperm DNA fragmentation using bright-field microscopy: Comparison between sperm chromatin dispersion test and terminal uridine nick-end labeling assay. Fertil Steril 2010;94:1027-32.
12. Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. Hum Reprod 2004;19:1401-8.
13. Owumi SE, Odunola OA, Gbadegesin MA, Nulah KL. Protective effect of *Juglans nigra* on sodium arsenite-induced toxicity in rats. Pharmacognosy Res 2013;5:183-8.
14. Raga DD, Pocsidio GN, Herrera AA. Effects of the oral administration of nonpolar extract from *Ardisia squamulosa* Presl (*Myrsinaceae*) leaves on spermatogenesis in rats. Pharmacognosy Res 2011;3:260-5.
15. Mahfouz RZ, Sharma RK, Said TM, Erenpreiss J, Agarwal A. Association of sperm apoptosis and DNA ploidy with sperm chromatin quality in human spermatozoa. Fertil Steril 2009;91:1110-8.
16. 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.
17. Gharagozloo P, Gutiérrez-Adán A, Champroux A, Noblanc A, Kocer A, Calle A, *et al.* A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: Promising preclinical evidence from animal models. Hum Reprod 2016;31:252-62.
18. Roodbari F, Abedi N, Talebi AR. Early and late effects of Ibuprofen on mouse sperm parameters, chromatin condensation, and DNA integrity in mice. Iran J Reprod Med 2015;13:703-10.