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Original Article

Quick recovery and characterization of cell-free DNA in seminal plasma of normozoospermia and azoospermia: implications for non-invasive genetic utilities

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Abstract

We established a quick and reliable method for recovering cell-free seminal DNA (cfsDNA), by using the binding-washing-elution procedure on the DNA purification column. Low variations (below 15%) among the triplicate values of cfsDNA quantity verified the reproducibility of our cfsDNA recovery method. Similar cfsDNA yield and size distribution between seminal plasma acquired by filtration and centrifugation confirmed the presence of cfsDNA. To investigate the general characterization of cfsDNA, the quantitation and size distribution of cfsDNA from normozoospermic and azoospermic semen were analyzed by real-time PCR and electrophoresis, respectively. CfsDNA concentration in semen with normozoospermia (n = 11) was 1.34 ± 0.65 μg mL^{-1}, whereas a higher cfsDNA concentration was observed in azoospermia (2.56 ± 1.43 μg mL^{-1}, n = 9). The continuous distribution of DNA fragments ranging from ~1 kb to 15 kb and a spectrum of multiples of 180-bp fragments were observed in each normozoospermic and azoospermic sample. Distinct characteristic DNA ladder fragmentations in some azoospermic samples implicated that cfsDNA originate partly from apoptotic cells. CfsDNAs of 36 selected azoospermic patients with known information of Y chromosome microdeletion were subjected to the same microdeletion analysis by multiplex PCR and PCR amplification of sY114 (1 450 bp). All multiplex PCR reactions with cfsDNA amplified successfully and provided the same result as leukocyte DNA. PCR amplification of sY114 gave a 1 450-bp amplicon as expected. Our data suggested the potential use of cfsDNA in search of biomarker or diagnostic procedures.

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1 Introduction

The ejaculated semen is a mixture of secretions from the testes, epididymis, seminal vesicles, prostate and bulbourethral glands discharged from the male urethra. Therefore, human semen contains many chemicals, of which some have been diagnostic biomarkers for disease diagnosis or function evaluation of male reproductive organs. For instance, the levels of seminal fructose, MHS-5 antigen and protein-C inhibitor have been used as markers for the seminal vesicular function [1], acid phosphatase, citric acid and zinc for prostate [2] and choline glycerophosphatide

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for epididymis. On the other hand, because male reproductive organs are small and many of them are composed of tiny canals, and some patients fear the impairment caused by biopsy or puncture, invasive methods are not easy to be applied and accepted. As a consequence, seeking non-invasive diagnostic biomarkers are important for the male reproductive system.

Cell-free nucleic acids, including DNA (cfDNA) and RNA, exist ubiquitously on or out the cell surface of living organisms [3]. They were found to be released by dying cells. There is also evidence that they can be actively secreted by living cells and may have biological roles in the organism [3, 4]. In recent years, their potential clinical application, such as detection and prognosis of cancers, prenatal diagnosis and medicolegal investigation, has become a focus of greater interest [3, 5–13]. Cell-free nucleic acids isolated from blood plasma [6–8], amniotic fluid [9], saliva [10], bronchial lavage [11], cerebrospinal fluid [12] and urine [13] are considered as valuable materials for diagnostics.

The presence of cell-free seminal DNA (cfsDNA) and its association with sperm parameters have been reported recently by Chou *et al*. [14]. In their study, the cfsDNA was not extracted but was analyzed directly by loading little volume (50 μL) of semen on the modified isocratic capillary electrophoresis. However, the concentration and characterization of cfsDNA remain largely unknown. Considering the prominent apoptosis during spermatogenesis, we presumed that cfDNA is abundant in semen and may be a new biomarker for the disease diagnosis or functional evaluation of semen-secreting organs. Herein, quick isolation of cfsDNA was established and characterizations of concentration and size distribution of cfsDNA from normozoospermia and azoospermia semen were described. The possible origin of cfsDNA and its advantages in genetic utilities were also discussed.

2 Materials and methods

2.1 Semen donors

All the semen samples used in this study were from specimens remaining after routine andrological analyses. The study was approved by the Institutional Review Board, and informed patient consent was obtained for the use of semen samples. The criteria for normozoospermia and azoospermia were according to guidelines of WHO (World Health Organization) [15]. Azoospermic semen with any abnormal parameter other than sperm amounts was excluded. Semen specimens were obtained by masturbation after 3–5 days of sexual abstinence and were allowed to liquefy for 30–60 min at room temperature.

2.2. Experimental design

To confirm the presence of cfsDNA and the effect of seminal DNase on its stability, seminal plasma of three normozoospermia was obtained by either low-speed centrifugation to avoid cell lyses (400 × *g* for 10 min and the seminal plasma was then centrifuged again at 2 000 × *g* for 20 min) or by high-speed centrifugation (16 000 × *g* for 5 min). Seminal plasma obtained by high-speed centrifugation was subjected to cfsDNA isolation directly, whereas the supernatant after low-speed centrifugation was processed by two means before cfsDNA recovery: filtration through filters with 0.22 μm pore size or high-speed centrifugation (16 000 × *g* for 5 min) after incubation at 37ºC for 0 h, 6 h and 24 h. DNA quantification and size distribution was then assessed. Triplicate DNA extraction and quantification of five normozoospermia samples was performed to test the reproducibility of our DNA recovery method.

A total of 11 normozoospermic and 9 azoospermic semen samples were involved in the cfsDNA quantification and size distribution. To verify the use of cfsDNA for genetic diagnosis and research, 36 selected azoospermia with known information of Y chromosome microdeletion (9 with microdeletion) were subjected to PCR for the same microdeletion analyses.

2.3 CfsDNA recovery

CfsDNA recovery was carried out with a binding-washing-elution procedure at room temperature. A total of 800 μL of seminal plasma was mixed with equal volume of DNA-binding buffer (6 mol L^{-1} guanidine-HCl, 10 mmol L^{-1} urea, 10 mmol L^{-1} Tris-HCl, 20% Triton X-100, pH 4.4) and 8 μL of protease K (20 mg mL^{-1}, Amersco). The mixture was incubated at 70ºC for 10 min and then 400 μL of isopropanol was added. The mixture was homogenized by vortex mixing and transferred into a polypropylene membrane spin column for DNA purification (Generay, Shanghai, China) and was centrifuged at 8 000 × *g* for 15 s. The flow-through was discarded, and 500 μL of washing buffer (20 mmol L^{-1} NaCl, 2 mmol L^{-1} Tris-HCl, pH 7.5,

80% ethanol) was added into the spin column and was centrifuged at 8 000 × *g* for 15 s. The flow-through was discarded and the spin column was centrifuged at 12 000 × *g* for 1 min. A total of 40 μL of elution buffer (10 mmol L^{-1} Tris-HCl, PH 8.0, 20 μg mL^{-1} RNase A) was added to the membrane of spin column. The spin column was placed in a new 1.5 mL collection tube and centrifuged for an additional 1 min at 12 000 × *g* to elute DNA.

2.4 Measurement of the quantity and size distribution of cfsDNA

To measure the quantity of cfsDNA, real-time PCR was performed as described previously [16] with an Mx3000P thermocycler (Stratagene) using SYBR GREEN I fluorescence detection of amplified products. Primers for the real-time PCR targeting hGDF gene were as follows: forward 5'-CGCAGAGGTCAGGAAACTGTC-3', reverse 5'-GGCAGGTACACATGACGGTCT-3'. Triplicate measurements were done per sample, and a four-step experimental run protocol was applied as follows: 95ºC for 10 min (initial denaturation); 35 cycles of 25 s at 95ºC (denaturation); 30 s at 60ºC(annealing), 30 s at 72ºC (elongation) and 8 s at 84ºC (fluorescence measurement). A melting curve was generated at the end of every run to ensure product uniformity. Calibration curves were constructed by the use of a concentrated cfsDNA in serial dilutions. The concentration of cfsDNA for calibration curves was high and can be easily analyzed by an ultraviolet photometer (Biometra, Göttingen, Germany).

The cfsDNA (0.5–2 μg) was loaded on a 1% agarose gel for the determination of its size distribution. To improve the resolution of electrophoresis, step-wise increases in voltage were applied. The gels were run from 4 V cm^{-1} for 60 min, followed by 6 V cm^{-1} for 60 min, up to 8 V cm^{-1} for 45 min. After electrophoresis, the gels were stained in SYBR Gold staining solution (Invitrogen, CA, US) for 30 min and then were visualized under an ultraviolet transillumination (Biometra).

2.5. PCR for Y chromosome microdeletions

For each patient with azoospermia, cfsDNA and leukocyte DNA were isolated and used for the screening of Y chromosome microdeletion. Leukocyte DNA was extracted from peripheral blood lymphocytes as described previously [17]. Leukocyte DNA isolated from peripheral blood lymphocytes of healthy women served as negative control.

A total of 15 previously published sequence tagged sites (STSs) were amplified in four multiplex PCR reactions to screen Y chromosome microdeletions, as described in our previous study [17]. Accession numbers of GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) for these STSs and their primers are as follows: Three AZFa-specific STSs including sY81 (accession number G42821), sY84 (G12019) and sY86 (G49207); five AZFb-specific STSs including sY117 (G11996), sY127 (G11998), sY130 (G12000), sY134 (G12001) and sY136 (G40974); and seven AZFc-specific STSs including sY145 (G66538), sY152 (G12039), sY239 (G65822), sY254 (G38349), sY255 (G65827), sY277 (G42070) and sY283 (G42071) were used to detect AZFa, AZFb and AZFc deletions, respectively. The *SRY* gene was included as an internal control. The PCR amplification was performed on a Gradient thermal cycler (Eppendorf, Hamburg, Germany) in a final volume of 25 μL. The PCR mixture consisted of 10 mmol L^{-1} Tris-HCl (pH 8.3), 50 mmol L^{-1} KCl, 2 mmol L^{-1} $MgCl_2$, 200 μmol L^{-1} dNTPs, 1.5 IU TaKaRa Taq DNA polymerase (TaKaRa Biotechnology, Dalian, China) and 0.4 μmol L^{-1} of each primer. The thermal profile used for multiplex PCR was as follows: After a 5-min denaturation at 94ºC, PCR was carried out for 35 cycles using a denaturation step at 94ºC for 30 s, an annealing step at 57ºC for 30 s and an extension step at 72ºC for 30 s, followed by a final extension at 72ºC for 5 min. The multiplex PCR products were analyzed on a 2% agarose gel.

Conventional PCR targeting sY114 (accession number G12044; 1450 bp amplicon) was carried out to test whether cfsDNA should be the suitable template for large-size DNA amplification, taking leukocyte DNA as positive control. The conventional PCR was carried out as the multiplex PCR described above, except the time of extension was 80 s. The conventional PCR products were analyzed on a 1% agarose gel.

2.6. Statistical analysis

The data are presented as mean ± SD. CfsDNA concentration comparison between normozoospermic and azoospermic semen was performed using the SPSS software (version 11.5) by unpaired *t*-test after checking for normal distribution by means of the Kolmogorov–Smirnov test. All statistical tests were two-tailed and $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Presence of cfsDNA and its stability

Quantity and size distribution of DNA recovered from filtrated seminal plasma was similar to DNA of seminal plasma acquired by centrifugation (16 000 × *g* for 5 min) with or without initial cell removal by low-speed centrifugation (Figure 1). In addition, no significant degradation of cfsDNA was observed after seminal plasma incubation at 37℃ for 6 h. After 24-h incubation, DNA quantity was slightly reduced, whereas the size distribution remained largely unchanged. The variations amongst the triplicate values of cfsDNA quantity were generally < 15% (data not shown).

3.2 Quantity of cfsDNA in normozoospermia and azoospermia

The concentration of cfDNA in seminal plasma of 11 normozoospermia was 1.34 ± 0.65 μg mL^{-1}, with ranges from 0.51 to 2.73 μg mL^{-1}. Whereas in the 9 azoospermic semen, the cfsDNA concentration was 2.56 ± 1.43 μg mL^{-1}, with ranges from 0.82 to 5.45 μg mL^{-1}. Concentrations of cfDNA in seminal plasma were statistically different between the normozoospermic and azoospermic samples ($P < 0.05$).

3.3 Size distribution of cfsDNA

It was shown by electrophoresis that cfsDNA represent a population of heterogenic size molecules. Smears ranging from 180 bp to 15 kb, were detected in cfsDNA of every normozoospermia and azoospermia samples. Interestingly, in all of the 11 normozoospermia and 9 azoospermia, 180-bp smear-like DNA fragments, and two or three times this size were observed (Figure 2). Especially, distinct characteristic DNA ladder fragmentation was observed in some patients with azoospermia.

3.4 Y chromosome microdeletions

All PCR reactions, in which cfsDNA was added as the template, were carried out and amplified successfully. Y chromosome microdeletions in the 36 azoospermia cfsDNA were the same as their corresponding leukocyte DNA; nine cases of microdeletions were found: six AZFc microdeletions (all applied AZFc-specific STSs except sY145 were deleted), two AZFb + c microdeletions (all applied AZFb-specific and AZFc-specific STSs except sY117 were deleted) and 1 AZFa + b + c microdeletion (all applied STSs except sY81 and SRY were deleted). PCR amplification of sY114 from cfsDNA gave a 1450-bp amplicon (Figure 2C) as expected, excluding the three patients with AZFb + c or AZFa + b + c microdeletions.

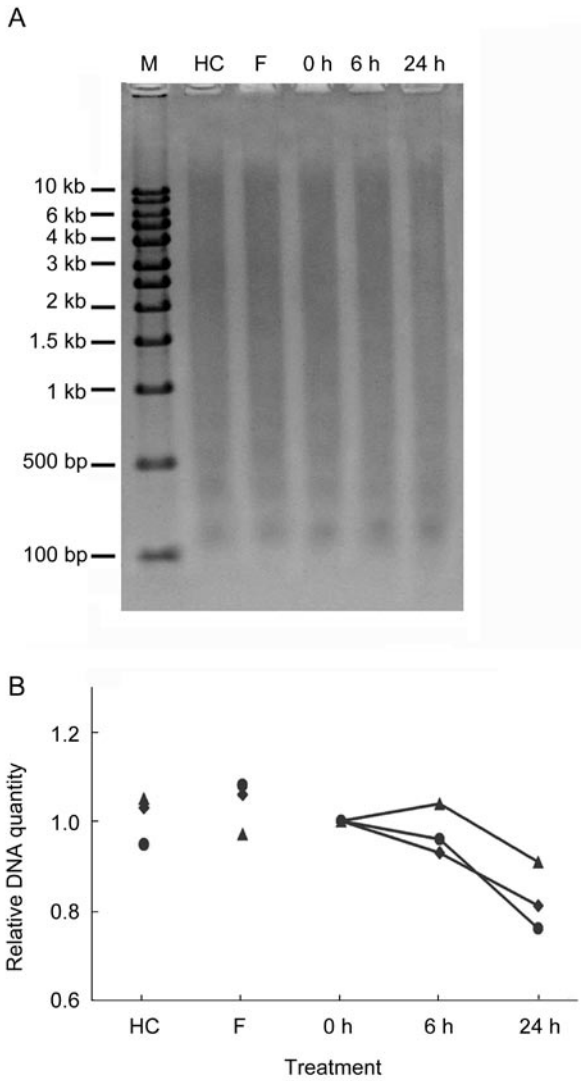

Figure 1. Size distribution (A) and DNA yield (B) of seminal plasma acquired by high-speed centrifugation (HC, 16 000 × *g* for 5 min), filtration after low-speed centrifugation (F) and high-speed centrifugation after low-speed centrifugation and incubation (0 h, 6 h and 24 h, at 37℃). Similar DNA quantity and size distribution amongst these treatments were observed, except the slight decrease of DNA quantity after 24-h incubation. Different icons (diamond, triangle and circle) represent three normozoospermic semen samples, DNA quantity was normalized to the amount measured at 0 h; M, marker.

4 Discussion

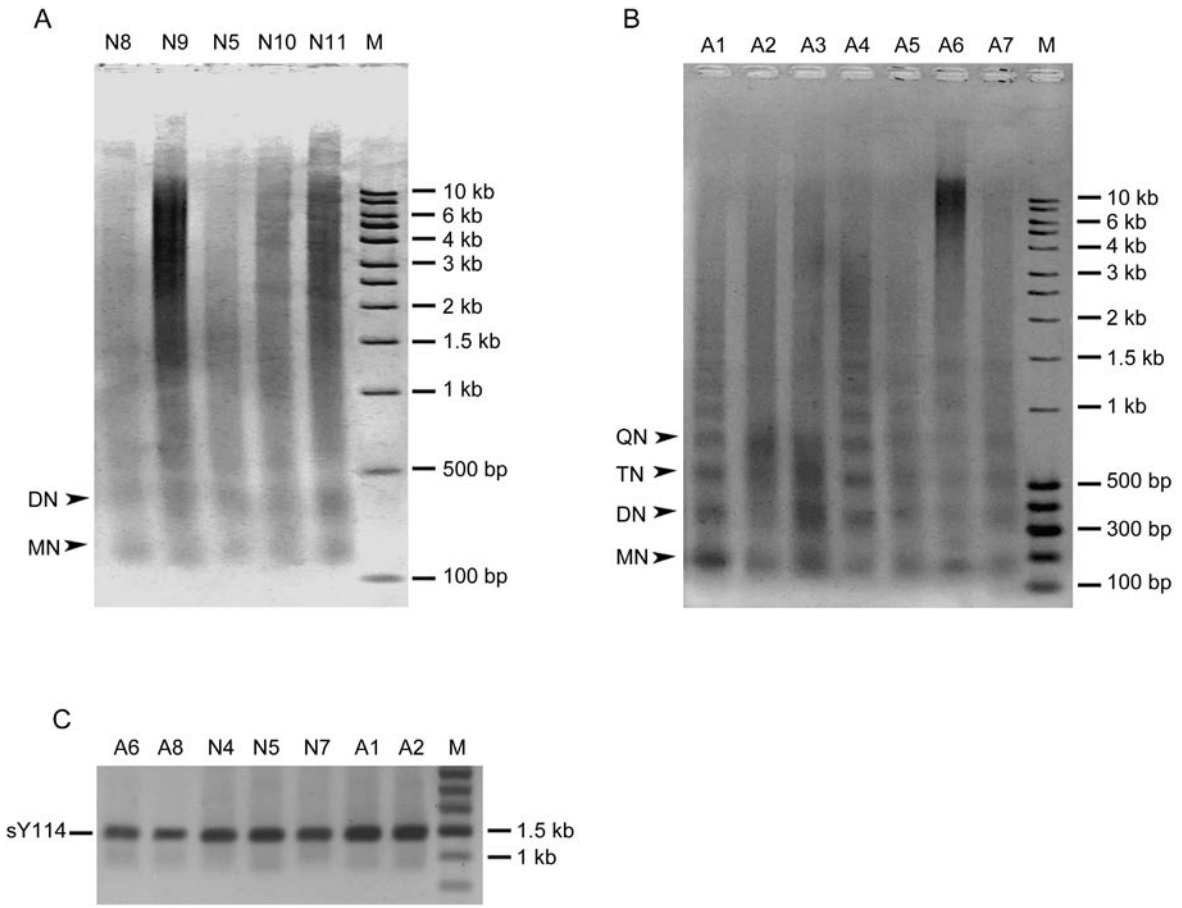

Figure 2. Size distribution of cfDNA in semen of normozoospermia (A) and azoospermia (B), and the amplification of sY114 (C). The continuous distribution of DNA fragments ranging from ~1 kb to 15 kb and a spectrum of multiples of 180-bp fragments, including mononucleosomal (MN), dinucleosomal (DN), trinucleosomal (TN) and quadrinucleosomal (QN) were observed. Distinct characteristic DNA ladder fragmentation was observed in some azoospermia samples (lane A1, A4~A7). PCR amplification of sY114 from cfsDNA gave a 1 450-bp amplicon (C) as expected, which confirmed that cfsDNAs present an alternative for PCR template. N, normozoospermia; A, azoospermia; M, marker.

In the present study, a quick and reliable method for recovering the cfsDNA was established. By using the binding-washing-elution procedure on the DNA purification column, the cfsDNA isolation can be accomplished within 30 min. Moreover, the cfDNA concentration in semen was found to be high, and can be easily analyzed by an ultraviolet photometer and routine electrophoresis. In contrast, cfDNAs in other human biological fluids need to be quantitated using fluorescence-based assays, radioimmunoassay, quantitative PCR *etc.* [5]. Thus, the developed method is easy to manipulate and might be suitable for wide application in andrology laboratory.

This study also confirmed the presence of cell-free DNA in human semen. Semen filtrated through the 0.22-μm filter resulted in similar cfsDNA yield and size distribution with seminal plasma acquired by high-speed centrifugation with or without initial cell removal by low-speed centrifugation. Therefore, recovered DNA from seminal plasma does not seem to be the result of cell lyses during high-speed centrifugation or filtration.

The concentration of cfDNA in semen is much higher than other human biological fluids, with a mean value of 1.34 μg mL^{-1} in normozoospermia and

2.56 μg mL^{-1} in azoospermia. As for other fluids of healthy people, cfDNA concentrations are 1.8–35 ng mL^{-1} in blood plasma [5], 9.4 ng mL^{-1} in amniotic fluid [9] and 0.3–200 ng mL^{-1} in human urine [18, 19]. The high concentration of cfDNA in the semen may be due to (1) DNA released by dying cells, including by apoptosis and necrosis, and possibly by a new form of cell death termed Netosis [20]. Especially, germ cell apoptosis during spermatogenesis in mammalian testis has been estimated to result in the loss of up to 75% of the potential mature spermatozoa [21]; (2) DNA secreted by cells of glandular organs. Secretions of the seminal vesicles, prostate and bulbourethral glands make up about 90% of the volume of semen. Given the long intervals (abstinence days, 3–5 days in the present study) between two ejaculations, it is reasonable to presume that large amounts of DNA are secreted and stored, then mixed into the semen during ejaculation; (3) Seminal plasma contains many chemicals, some of which may protect cfsDNA from degradation by binding with cfsDNA. In addition, some chemicals, especially some cations, such as Ca^{2+}, Mg^{2+} and Zn^{2+}, may affect the activity of seminal DNases, which have been found in human semen with high activity dependent on Ca^{2+}, Mg^{2+} and other chemicals [22, 23]. (4) It cannot be excluded that some pathological conditions, such as some inflammation, cancer, trauma etc., which cannot detected by the current WHO guidelines, may contribute to the high cfsDNA concentrations.

The characterizations of cfsDNA concentration and size distribution implicated that cfsDNA originate partly from apoptotic cell. First, the continuous distribution of DNA fragments ranging from ~1 kb to 15 kb and a spectrum of multiples of 180-bp fragments were observed in the cfsDNA recovered from each sample included in this study. Especially, distinct characteristic DNA ladder fragmentations were observed in some patients with azoospermia. In cfDNA of blood plasma, it has been suggested that high molecular weight DNA is released from cells dying by necrosis, and the multiples of 180-bp fragments is reminiscent of the oligonucleosomal DNA ladder characteristic for programmed cell death (apoptosis) when genomic DNA is degraded by a caspase-activated DNase [3, 24]. Second, the average cfsDNA concentration of azoospermia was almost twofold higher than normozoospermia. Therefore, the obvious multiples of 180-bp DNA fragments may mainly originate from genomic DNA of apoptotic cells, and the increased cfsDNA concentration may be due to prominent pathological apoptosis of spermatogenetic cells in azoospermia. If this hypothesis is proven to be true, prominent different cfsDNA characterization, such as low DNA quantity and indistinct DNA ladder, may be expected in obstructive azoospermia. It is also reasonable to presume that the cfsDNA concentration and its size distribution are related to spermatogenesis and testis phenotype.

Considering the high concentration and broad size distribution of cfsDNA, it could be suitable for many molecular biological methods, such as DNA methylation and mutation, which have become focuses of great interest [3, 5–7, 12, 13]. PCR is an indispensable step for these and other molecular biological methods. The preliminary study is designed to verify that the cfsDNA could be the template for PCR. The result of multiplex PCR and conventional PCR confirmed that cfsDNA present a quick and non-invasive alternative for PCR template, and can be employed in the analysis of genomic DNA information of semen-secreting organs, such as methylation and mutation detection for tumors and *de novo* germline mutation. Therefore, cfsDNA can possibly be used to develop biomarkers that provide reproductive tract-specific DNA information or for disease diagnosis.

To sum up, a quick and convenient procedure for the cfsDNA recovery was developed, and the cfsDNA concentration and size distribution were assessed. The DNA ladder characteristic and relative higher concentration of cfsDNA in azoospermia implicated that cfsDNA originate partly from apoptotic germ cells. These preliminary results indicate that cfsDNA can possibly be used for further investigation of this DNA in search of biomarker or diagnostic procedures. Other information of the cfsDNA, such as methylation and oxidation, deserves further investigation.

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