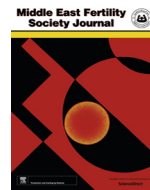




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Original Article

A simple, rapid and economic manual method for human sperm DNA extraction in genetic and epigenetic studies

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ABSTRACT

Over the recent years, isolation of high quantities of pure, intact, double-stranded, highly concentrated and not contaminated genomic DNA is prerequisite for successful and reliable large scale genetic and epigenetic analysis and also high quantities of pure DNA are required for these types of studies. The DNA extraction methods developed for human somatic cells are not effective for sperm because of the high rating of nuclear compaction and DNA integrity in sperm cells. In the present study, an economic, reliable and simple method of DNA extraction procedures, were examined to ascertain their relative effectiveness for extracting DNA from human sperm. The quality and quantity of the extracted DNA were assessed by spectrophotometric measurements, methylation-specific PCR (MSP) amplification, and gel electrophoresis. This method was applied successfully from sperm samples (N = 50, age = 33.9 ± 7.8 years) with DNAs absorbency ratios of A260/A280 (1.82 ± 0.03) and A260/230 (1.95 ± 0.16). Gel electrophoresis revealed non-degraded and suitable quality genomic DNA for epigenetic and genetic analysis. The purity and quality of this protocol was closer to the optimum value and no contamination during manual extraction was observed. It concluded that this described method might have a sufficient quality for subsequent molecular analysis and general genetic and epigenetic methods.

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1. Introduction

Over the recent years, epigenetic and genetic studies are in progress so isolation of high quantities of pure, intact, double-stranded, highly concentrated and not contaminated genomic DNA is prerequisite for successful and reliable large scale analysis and also high quantities of pure DNA are required. The objective of this work was to evaluate the method for extraction of genomic DNA from sperm samples in terms of DNA quantity, concentration, purity, and integrity as well as utility and applicability for subsequent genetic and epigenetic studies. The organization and compaction of human sperm chromatin are differing from somatic cells [1]. In the somatic cell to decrease the volume of chromatin,

nuclear DNA is enveloped around the histones octamer packaged into a solenoid structure. Sperm chromatin configuration is structured by protamines in a particular style to keep the chromatin condensed in the nucleus during transport of the paternal genome in the male and female reproductive tracts [1–3]. Through the spermatogenesis, the most of the sperm cytoplasm loss, the motile tail growth, and somatic-type histones change with protamine to developing disulfide bridges bond [1,3]. Therefore sperm chromatin is denser than (at least six times) the other cells, so the densely packaged genetic information transferred to the embryo [1–3]. Epigenetic has an important part in the regulation of gene expression and altered by the DNA sequence (DNA methylation) or chromatin-associated proteins (i.e., histones) [4]. Distinct the genome, the epigenome is greatly variable between cells reply to cellular and environmental tension [1,5]. These stress can disrupt normal chromatin organization which is vital for fertility and health of the offspring [4]. Thus, for this studies achieving the methods that could extract DNA easily and inexpensively were required. A wide variety of methods have been advanced to extract DNA from several types of human somatic cells [6] but DNA isolation techniques used routinely for human somatic cells have

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proven ineffective for sperm [1,7]. The purpose of this article was to provide an economical, simple and reliable method to extract human sperm DNA which could be used in genetic and epigenetic studies.

2. Material and methods

2.1. Study design and sample collection

Semen samples (N = 50, age = 33.9 ± 7.8 years) were collected from men who attended andrology center at Avicenna Infertility Clinic (Tehran, Iran), during April to May 2016. Semen analysis was performed according to the World Health Organization criteria. The patients with azoospermia were excluded from the study. All of the experimental procedures of the study were approved by the Ethics Committee of Avicenna Research Institute and Human Rights Committee. The written consent form was obtained from all of the participants and kept in their medical records.

2.2. Methods

Semen samples were allowed to liquefy for 30 min at 37°C before analysis with computer-assisted semen analysis (CASA) according to the WHO outline [8]. Samples were analyzed for concentration, motility, and vitality. The total semen was centrifuged at 3400 rpm for 5 min to remove supernatant. The pellets were resuspended and washed twice with 1000 μL sterile phosphate buffered saline (PBS, 0.5 M) at 3400 rpm for 5 min to eliminate cellular and non-cellular components earlier to the addition of lysis buffer. The final pellets were stored at -20°C .

2.2.1. DNA extraction method

For thawing the samples, the pellet had been allowed to sit at room temperature for 15 min. To make A lysis buffer (10 \times), 5 ml Tris-HCl 1 M, 1 ml NaCl 5 M and 2.5 ml MgCl_2 1 M should be added to 41.5 ml H_2O . Then 1000 μL of A lysis buffer (1 \times) was added, vortexed and placed at room temperature for 15 min, to resuspend the cells. This tube was centrifuged at 3400 rpm for 10 min and the supernatant was discarded to remove somatic cells. This step was repeated once again. Then 500 μL TRIzol and 50 μL proteinase K were added to the micro tube and strongly mixed by inverting the tube for 40 times. The micro tube was placed in a 56°C water bath and incubated for overnight. The tube was removed from the water bath and let cool at room temperature. Then 500 μL chloroform was added and mixed strongly by inverting for 40 times until observed white color. Adding chloroform created three separated phase. The tube was located in 4°C for 15 min and after that was centrifuged at maximum speed for 15 min, 4°C . The higher clear phase (about 400 μL containing DNA) was removed to a new tube and 800 μL pure cold ethanol and 40 μL cold 3 M sodium citrate were added to simplify DNA sedimentation. It was inverted smoothly until DNA strands form then stored at -20°C for 1 h. After 1 h the tube was centrifuged at maximum speed for 20 min, 4°C . The supernatant carefully was empty out. The white DNA pellet was washed twice in 600 μL 70% ethanol then the alcohol solution was removed by reversing the tube to drain. DNA was left at room temperature overnight to more rehydrate in 100 μL Tris-HCl or ddH $_2\text{O}$.

2.2.2. Assessment of DNA quantity and purity by spectrophotometer

Quantity and purity of DNA extracted with this method were assessed using a NanoDrops ND-1000 spectrophotometer (PeqLab, Erlangen, Germany) which calculates the concentration of double-stranded DNA (dsDNA). The DNA concentration and ratio of the 260/280 and 260/230 absorbance were automatically measured

to define DNA purity. The ratio of absorbance at 260 nm and 280 nm was used to assess protein contamination while the ratio of absorbance at 260 nm and 230 nm was calculated to assess guanidine contamination. The spectrophotometric measurements used for DNA quality assessment with higher values associated with better DNA purity and quantity.

2.2.3. Assessment of whole genomic integrity by gel electrophoresis

The integrity of DNA extracted by this method was assessed by gel electrophoresis. Specifically, 2 μL of each DNA extract was electrophoresed on a 1.5% agarose gel containing nucleic acid gel stain (Smobio, Hsinchu City, Taiwan) and was visualized by U.V. illumination.

2.2.4. Assessment of DNA quality for epigenetic research

We assessed the epigenetic quality by assessing the methylation status of Kelch-like ECH-associated protein 1 (Keap1) in extracted DNAs. Bisulfite-modification of extracted DNAs was performed manually by modifying 2 μg of extracted and denatured DNAs with NaOH and sodium bisulfite to convert unmethylated cytosines to uracil [9,10]. The modified DNA was amplified by MSP using specific primers to distinguish unmethylated (changed) from methylated (unchanged) sequences [11,12]. PCR reactions were performed using the master mix PCR buffer (6 μL), primers (50 pmol each per reaction), and bisulfite-modified DNA (50 ng) in a final volume of 12 μL . Reactions were hot-started at 95°C for 5 min and amplified by Mastercycler Gradient (Eppendorf, Hamburg, Germany) for 35 cycles, followed by a final 4-min extension at 72°C . Two sets of primers were used separately to check for methylated or unmethylated islands. The human methylated and non-methylated DNA set (Zymo Research, California, United States) were used respectively as methylated and unmethylated DNA controls. Each PCR reaction products were directly electrophoresed on a 1.5% agarose gel, which was later stained with nucleic acid gel stain (Smobio, Hsinchu City, Taiwan) to allow DNA visualization under UV illumination.

2.2.5. Statistical analysis

The mean \pm SD of parameters were performed with SPSS software (V. 16, Chicago, Illinois, USA). The Pearson's was used for correlation of parametric variables. $P \leq .05$ was considered to be statistically significant.

3. Results

Total sperm samples were used for DNA extraction. After the extraction procedures, DNA was directly quantified by spectrophotometry. Concentration and total yield of extracted DNA were assessed using the NanoDrops ND-1000 spectrophotometer. No failure was detected in the manually prepared samples and 50 samples were included in this procedure. Descriptive statistics of this method for all samples were summarized in Table 1. As

Table 1

The summarized results indicated the mean \pm SD of parameters.

Parameters N = 50	Range	Mean \pm SD
Age	20-56	33.9 \pm 7.89
Sperm concentration (*10 ⁶)	1-150	51.78 \pm 21.95
Volume (mL)	1-7	3.12 \pm 1.40
Vitality (%)	18-95	86.90 \pm 11.79
PH	7.30-7.60	7.47 \pm 0.07
A (%)	0-25	6.90 \pm 7.16
B (%)	2-40	29.34 \pm 7.40
C (%)	5-30	17 \pm 5.24
DNA concentration (ng/ μL)	147.20-4157.80	1126.14 \pm 810.75
DNA 260/280 absorbance ratio	1.70-1.88	1.82 \pm 0.03
DNA 260/230 absorbance ratio	1.70-2.35	1.95 \pm 0.16

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