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Ultra-performance liquid chromatography/tandem mass spectrometry for accurate quantification of global DNA methylation in human sperms

Xiaoli Wang[a], Yongshan Suo[c], Ruichuan Yin[a], Heqing Shen[b,**], Hailin Wang[a,*]

[a] State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China
[b] Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
[c] Reproduction Center of Zaozhuang Maternity and Child Care Hospital, Zaozhuang 277102, China

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ABSTRACT

Aberrant DNA methylation in human sperms has been proposed to be a possible mechanism associated with male infertility. We developed an ultra-performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method for rapid, sensitive, and specific detection of global DNA methylation level in human sperms. Multiple-reaction monitoring (MRM) mode was used in MS/MS detection for accurate quantification of DNA methylation. The intra-day and inter-day precision values of this method were within 1.50–5.70%. By using 2-deoxyguanosine as an internal standard, UPLC–MS/MS method was applied for the detection of global DNA methylation levels in three cultured cell lines. DNA methyltransferases inhibitor 5-aza-2′-deoxycytidine can significantly reduce global DNA methylation levels in treated cell lines, showing the reliability of our method. We further examined global DNA methylation levels in human sperms, and found that global methylation values varied from 3.79% to 4.65%. The average global DNA methylation level of sperm samples washed only by PBS (4.03%) was relatively lower than that of sperm samples in which abnormal and dead sperm cells were removed by density gradient centrifugation (4.25%), indicating the possible aberrant DNA methylation level in abnormal sperm cells. Clinical application of UPLC–MS/MS method in global DNA methylation detection of human sperms will be useful in human sperm quality evaluation and the study of epigenetic mechanisms responsible for male infertility.

1. Introduction

DNA methylation, as one of the most important epigenetic modifications in mammalian tissues, plays crucial roles in many biological processes such as gene transcription, genome stability, and embryogenesis [1–3]. Aberrant DNA methylation, manifested as genomic DNA hypomethylation and gene-specific hypermethylation, has been found to be associated with the pathogenesis of various human diseases [4,5]. Hypermethylation of tumor suppressor genes leads to transcriptional silencing and results in malignant transformation eventually [6,7]. Genomic DNA hypomethylation, which can affect chromosome structure and activate oncogenes, has been discovered in many human cancers, such as breast cancer, ovarian epithelial carcinoma and colorectal cancer [8–10]. DNA methylation will be a useful biomarker for disease diagnosis and prognostics.

Male infertility is a common problem in today's man health care research [11]. Protamine deficiency and oxidative DNA damage may be associated with infertility of some male patients, but for most patients the etiology remains unknown [12–14]. Several studies implicated the possible effects of aberrant DNA methylation on male infertility. Aberrant methylation of sperm DNA, such as abnormal genomic imprinting, was found in some infertile men [15,16]. In mammalian sperm line, global DNA methylation level was lower than that of somatic cells, reflecting the hypomethylation of satellite sequence [17,18]. DNA methylation erasure and *de novo* DNA methylation occurs during spermatogenesis [19,20]. Abnormal spermatogenesis will occur if the DNA methylation related epigenetic process is disrupted because of genetic or environmental factors [21,22]. In this sense, it is expected that DNA methylation of sperm line will be a good biomarker to evaluate the fertilizing ability of spermatozoa and to study other human diseases, such as Prader–Willi syndromes [23].

Methods based on polymerase chain reaction (PCR) amplification or methylation-sensitive restriction reaction, such as methylation-specific PCR (MSP) [16,24], methylight [22,25] and end-labeling assay [26], were used for the analysis of locus-specific DNA methylation in sperms. Immunohistochemical 5-methylcytosine staining technique was applied for the measurement of global DNA methylation in genomic sperm DNA [14,23]. However, this method is relatively non-specific and non-sensitive,

* Corresponding author. Tel.: +86 10 62849600; fax: +86 10 62849600.
** Corresponding author. Tel.: +86 592 6190771; fax: +86 592 6190771.
E-mail addresses: hlwang@rcees.ac.cn (H. Wang), hqshen@iue.ac.cn (H. Shen).

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so it cannot be used for precisely quantitative assessment of global DNA methylation in sperm. Other methods, such as methyl-acceptance assay (MAA) [27] and cytosine extension assay (CEA) [28], can only afford indirect information about global DNA methylation level, and their sensitivity and reproducibility are also not satisfactory. Therefore, it is highly desirable to develop a sensitive and reliable method for accurate quantification of global DNA methylation in sperm. Nowadays, global methylation is usually analyzed by high-performance liquid chromatography (HPLC) [29,30]. However, the relatively low sensitivity, long running time and large quantities of genomic DNA required, will limit their application in large-scale clinical analysis. With the development of electrospray ionization (ESI) technique for mass spectrometry analysis (MS), liquid chromatography (LC)/MS has been used for characterization and quantification of biological samples, such as nucleic acids and proteins. Friso used on-line LC/ESI-MS for the quantitative determination of global DNA methylation by measuring methylated cytidine residues in hydrolyzed genomic DNA using isotopically labeled internal standard [31]. However, complete digestion of RNA by enzyme is required to avoid RNA contamination. To avoid the use of expensive isotopic label, Song and Liu developed a LC–MS/MS to analyze 5-methyl-2′-deoxycytidine in enzyme-digested genomic DNA using 2′-deoxyguanosine as an internal standard [32,33]. Due to its high sensitivity and specificity, LC–MS/MS will be a good tool for genomic DNA methylation analysis [34,35].

In this work, we developed UPLC–MS/MS method for detection of global DNA methylation in human sperms by taking advantages of ultra resolution, ultra speed and sensitivity of UPLC technology and high selectivity and specificity of tandem MS detection. The ratio of 5-methyl-2′-deoxycytidine to 2′-deoxyguanosine was used for evaluation of global DNA methylation level. Commercial unmethylated and methylated DNA with a specific sequence and genomic DNA extracted from human cells treated by DNA methyltransferases inhibitor 5-aza-2′-deoxycytidine were used for the confirmation of our developed method. UPLC–MS/MS technology will offer a good opportunity for the accurate quantification of DNA methylation in human sperms, in which small variation in DNA methylation level may have important biological implications. This method will be helpful to study the epigenetic mechanisms of male infertility and other human genomic imprinting disorders.

2. Experimental

2.1. Reagents

5-Methyl-2′-deoxycytidine (5mdC), 2′-deoxycytidine (dC), 2′-deoxyguanosine (dG), thymidine (T), 2′-deoxyadenosine (dA), 5-aza-2′-deoxycytidine (5-Aza-dC) and snake venom phosphodiesterase I were purchased form Sigma (St. Louis, MO, USA). Deoxyribonuclease I (DNase I), calf intestinal alkaline phosphatase (CIP) were obtained from New England BioLabs (Ipswich, MA, USA). Microcon centrifugal filter devices were obtained from Millipore (Bedford, MA, USA). Methanol was of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was prepared by a Milli-Q water system (Millipore, Bedford, MA, USA). Cell-culturing reagents were obtained from Hyclone (Logan, UT, USA).

2.2. Apparatus

Nucleosides separation was achieved by Agilent 1200 Series Rapid Resolution LC system equipped with a vacuum degasser, binary pump SL, high performance autosampler SL with thermostat and thermostatic column compartment. A reversed-phase Zorbax SB-C18 2.1 × 100 mm column (1.8-μm particle size) was used in this experiment. Agilent 6410B Triple Quadrupole mass spectrometer (Santa Clara, CA, USA) with an electrospray ionization source was applied for mass spectrometric detection. MassHunter workstation software version B.01.03 was used for data acquisition and qualitative analysis, and its version B.01.04 was used for the quantitative analysis.

2.3. Cell culture and DNA isolation

Human hepatocellular carcinoma cell line HepG2 and lung adenocarcinoma cell line A549 were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in 5% CO_2 at 37 °C. Renal proximate tubular epithelial cell HK2 were cultured in DMEM-F12 medium, and other conditions were the same as for HepG2. HepG2 cells (5×10^5 cells) were seeded in the culture medium for 24 h and then treated with 0.01, 0.1, 1 and 5 μM of 5-Aza-dC for 72 h. Control cells were cultured in the same way, without treatment of 5-Aza-dC. Cells were harvested after 72 h treatment and genomic DNA was extracted using a Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacture's instruction. DNA concentration and quality were estimated by measuring the absorbance at 260 nm and 280 nm.

2.4. Sperm collection and DNA preparation

The study was approved by local ethic committee. Semen samples were collected by masturbation from 24 men who were undergoing evaluation for infertility at reproduction department of the hospital. The patients had been informed our research purpose and we are permitted to use the samples. After liquefaction, all the semen samples were divided into two groups. Samples from group 1 were only washed by Phosphate Buffered Saline (PBS) three times at 1500 rpm for 10 min. While samples from group 2 were first centrifuged at 1500 rpm for 15 min using Suprasperm gradients (1 ml semen, 2 ml 40%, 2 ml 80%) and then washed by PBS three times to remove abnormal and dead sperms. Sperm cells were collected separately for global DNA methylation analysis.

Unlike somatic cells, sperm cells have relatively compacted chromatin structures, which is tightly packaged by protamine molecules [12]. Genomic DNA from sperm cells was extracted using Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacture's instruction with some modifications. Sperm cells and nuclei lysis solution were first mixed thoroughly. For complete lysis, dithiothreitol (1 mol/L) and proteinase K (20 mg/ml) were added at 55 °C for 2 h. The cell lysate was then incubated with RNase A at 37 °C for 30 min, and protein was removed by protein precipitation solution. Genomic DNA was precipitated using isopropanol and washed by 70% ethanol twice. DNA concentration and purity were determined by measuring the optical density (OD) at 260 nm and 280 nm.

2.5. DNA enzymatic digestion

DNA digestion was performed as described previously [36]. Genomic DNA (1 μg) extracted from human sperms or cultured cells was digested with 1 U DNase I, 2 U CIP and 0.005 U snake venom phosphodiesterase I at 37 °C for 24 h. Microcon centrifugal filter device with a 3000 D cutoff membrane was used to remove protein from the digested DNA samples by centrifuging at 12,000 rpm for 60 min.

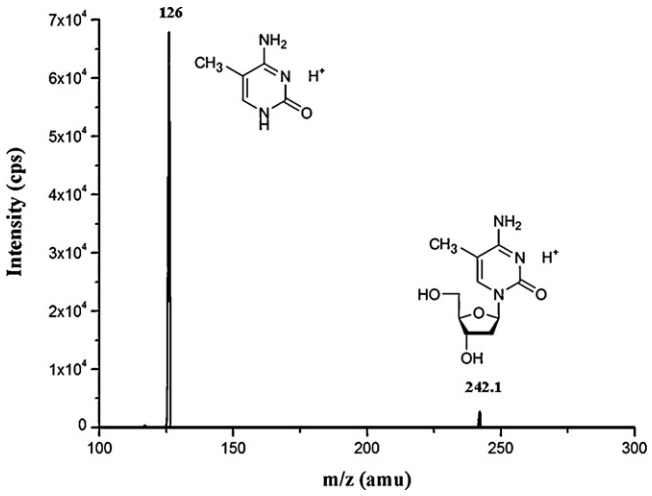

Fig. 1. The product ion mass spectra of 5mdC. Fragment ions at *m*/*z* 242.1 and *m*/*z* 126 were corresponding to the protonated 5mdC and 5-methylcytosine generated by glycosidic cleavage.

2.6. UPLC–MS/MS analysis

The mobile phase consisted of 5.0% methanol and 95% water (plus 0.1% Formic Acid) was used for UPLC separation of the nucleosides at a flow-rate of 0.3 ml/min. Enzymatically digested DNA sample (5 μL each) was injected into for UPLC–MS/MS analysis and each run took 10 min. Mass spectrometry conditions were as follows: ionization mode, ESI-positive; capillary voltage, 3500 V; nitrogen drying gas temperature, 300 °C; drying gas flow, 9 L/min; nebulizer, 40 psi. For MS/MS analysis of nucleotides, the fragmentor voltage was 90 V, collision energy was performed at 5 eV and scan time was 100 ms. Multiple-Reaction Monitoring (MRM) mode was used for the UPLC–MS/MS analysis by monitoring transition pairs of *m*/*z* 242.1/126.0, *m*/*z* 228.1/111.9, *m*/*z* 268.1/152.0, *m*/*z* 252.1/136.0, *m*/*z* 243.1/127.0, corresponding to 5mdC, dC, dG, dA and dT.

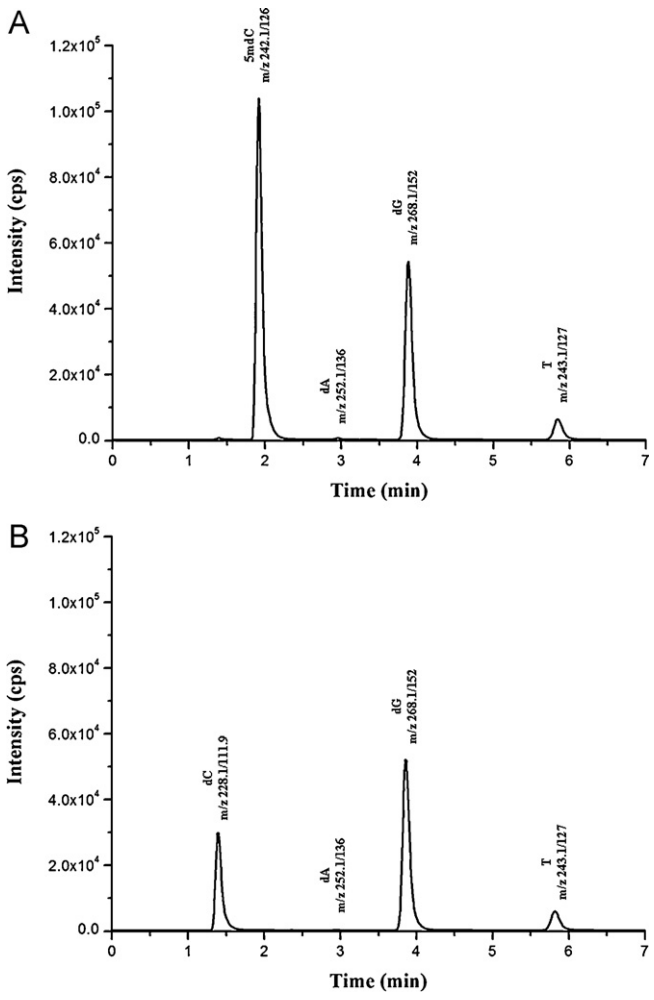

Fig. 2. UPLC–MS/MS chromatograms of DNA hydrolysate from 5-methylcytosine DNA standard (A) and cytosine DNA standard (B). 5mdC (1. 9 min) and dC (1.4 min) were detected by monitoring *m*/*z* 242.1/126 and 228.2/111.9, respectively.

3. Results and discussion

3.1. UPLC–MS/MS detection of 5-methyl-2′-deoxycytidine

In our experiment, MRM mode was selected for highly sensitive quantification of 5-methyl-2′-deoxycytidine (5mdC). In product ion spectra of 5mdC (Fig. 1), *m*/*z* 242.1 and *m*/*z* 126.0 were the precursor and predominant product ions of 5mdC, respectively. They correspond to the protonated 5mdC and 5-methylcytosine produced from glycosidic cleavage of 5mdC. The transition pair of *m*/*z* 242.1/126.0 was then used for detection of 5mdC in MRM mode.

DNA sequences from 5-methylcytosine DNA Standard Set (Zymo Research, Orange, CA, USA) were used for UPLC–MS/MS detection of 5mdC. Cytosine DNA Standard and 5-methylcytosine DNA Standard are linear dsDNA which have the same 897 bp sequence. The only difference is that Cytosine DNA Standard contains unmodified cytosines, while cytosines are fully replaced by 5-methylcytosines in 5-methylcytosine DNA Standard. Cytosine DNA Standard and 5-methylcytosine DNA Standard were digested by DNase I, CIP, and snake venom phosphodiesterase I and the products of digestion were analyzed by UPLC–MS/MS. Chromatographic peaks of 5mdC (1.9 min, Fig. 2A) and dC (1.4 min, Fig. 2B) could be clearly detected in the digested 5-methylcytosine DNA Standard and Cytosine DNA Standard, respectively. Transition pairs of *m*/*z* 268.1/152.0, 252.1/136.0, 243.1/127.0 corresponding to dG, dA and dT were monitored at the same time. Surprisingly, the area under the dT peak is much larger than the area under the dA peak although they have same concentration. This difference may be attributed to the different protonation efficiency of these nucleosides in MS/MS detection. We speculated that it was difficult for thymidine to be protonated, so T peak was relative small. For adenosine, it is supposed to be converted to inosine by a small amount of adenosine deaminase during the process of enzymatic digestion. Complete separation of 5mdC from other nucleosides by UPLC could be achieved within 10 min. Potential interference from other nucleotides was not observed due to the complete separation of these nucleotides, showing high specificity of our method.

3.2. Quantification of global DNA methylation level

To quantify genomic DNA methylation level, the ratio of 5-methyl-2′-deoxycytidine to 2′-deoxyguanosine was estimated, in which dG was chosen as an internal standard. The use of expensive isotope labeled standards could be avoided and the influence of insufficient DNA hydrolysis could be corrected in this way. Serial dilutions of 5mdC to dG were prepared and the ratios of 5mdC to dG were chosen at 0.1%, 0.5%, 1%, 2.5%, 5%, and 10% because global DNA methylation levels were typically varied from 2% to 7% in mammalians. Calibration curve was obtained by plotting the ratio of MRM/MS signal of 5mdC to dG against [5mdC]/[dG] (R^2 = 0.999)

Table 1
Precision of UPLC–MS/MS method for the determination of 5mdC ($n = 3$).

DNA sample	Intra-day		Inter-day	
	Ave. + S.D.	C.V.%	Ave. + S.D.	C.V.%
[5mdC]/[dG] (%)				
1	1.03 ± 0.015	1.50	1.02 ± 0.042	4.08
5	5.17 ± 0.097	1.87	5.14 ± 0.160	3.12
10	9.90 ± 0.167	1.69	9.74 ± 0.272	2.80
Genomic DNA				
A549	4.13 ± 0.134	3.24	4.28 ± 0.244	5.70
HepG2	3.68 ± 0.141	3.83	3.81 ± 0.161	4.24
HK2	5.13 ± 0.099	1.94	5.39 ± 0.307	5.70

and used for the following estimation of global DNA methylation level.

Global DNA methylation levels in three cultured cell lines (HepG2, A549 and HK2) were evaluated by UPLC–MS/MS method. Using the ratio of 5mdC to dG as the indicator of global DNA methylation level, the corresponding average values for HepG2, A549, and HK2 were 3.81%, 4.28%, and 5.39% as indicated in Table 1. It was evident that the global DNA methylation levels of HepG2 and A549 were obviously lower than that of HK2. It was well known that HepG2 and A549 were cancer cell lines, while HK2 was an immortalized proximal tubule epithelial cell line from normal adult human kidney. This was consistent with the fact that DNA methylation levels of tumor-derived genomic DNA were significantly lower than that in normal cell lines [37]. The applicability of the developed UPLC–MS/MS method for global DNA methylation detection was confirmed.

To further test the applicability of UPLC–MS/MS method to DNA methylation detection, genomic DNA hypomethylation caused by 5-Aza-dC, a representative inhibitor of DNA methyltransferase, was assessed. HepG2 cells were treated by 5-Aza-dC (0, 0.01, 0.1, 1 and 5 μM) for 72 h. Genomic DNA was extracted from harvested cells and then enzymatically digested for UPLC–MS/MS detection. Global DNA methylation level decreased with the increasing concentration of 5-Aza-dC. Even treated with 5-AzadC as low as 0.01 μM, the inhibition of global DNA methylation (3.6%) could be observed obviously (Fig. 3). This result demonstrated that UPLC–MS/MS method was applicable to the accurate evaluation of small changes in global DNA methylation level.

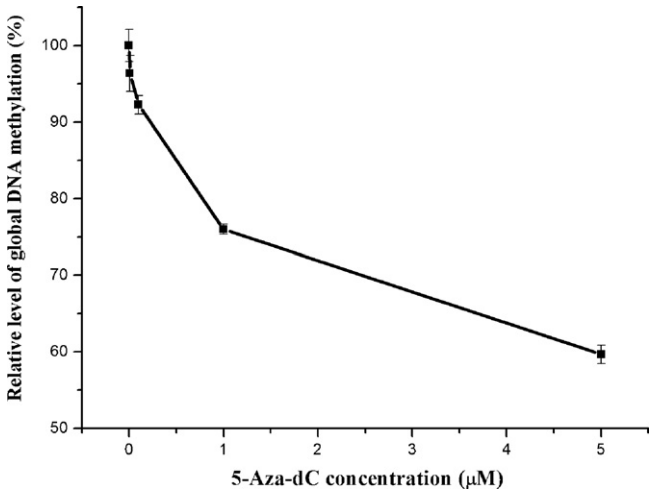

Fig. 3. Influence of 5-Aza-dC on the global DNA methylation level of HepG2 cells. Reduction of global DNA methylation level could be detected after the cells being treated with 0.01 μM of 5-Aza-dC. Treated with 5 μM of 5-Aza-dC for 72 h, the global DNA methylation level of HepG2 decreased about 40%.

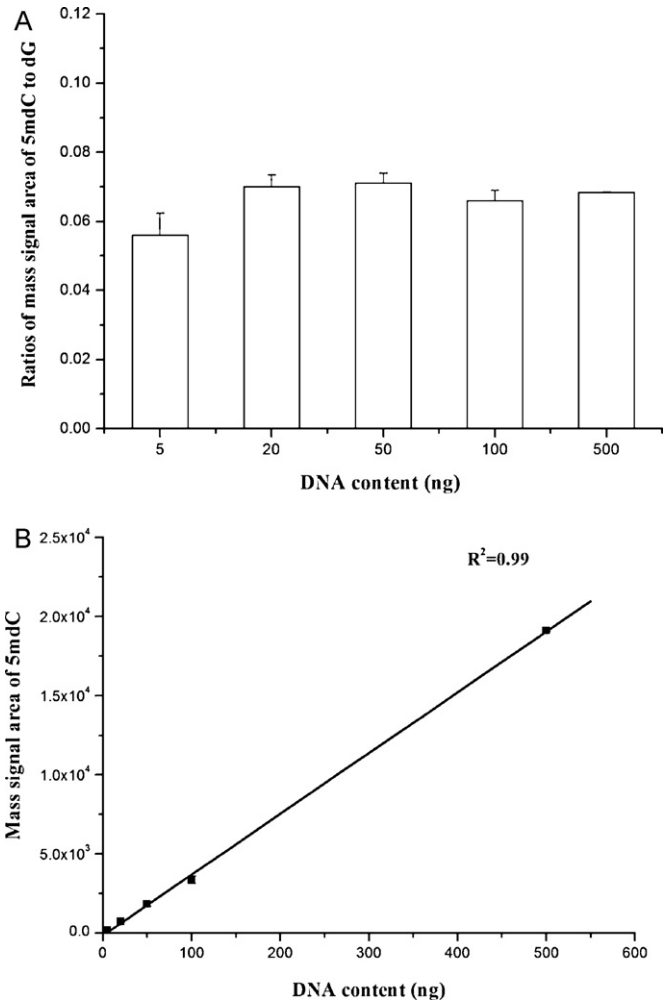

Fig. 4. The change in the MS signal ratio of 5mdC to dG by varied DNA content from 5 to 500 ng (A) and the linear relationship between the mass signal area of 5mdC and DNA contents (B).

3.3. Validation of UPLC–MS/MS method

For UPLC–MS/MS method validation, intra-day and inter-day precision were evaluated for the ratio of 5mdC to dG at 1%, 5% and 10%, and the results were summarized in Table 1. The intra-day precision values showed as covariance (C.V.) varied from 1.50% to 1.87% and the inter-day precision values ranged from 2.80% to 4.08%. For global DNA methylation detection of cultured cell lines, the intra-day precision values ranged from 1.94% to 3.83% and the inter-day precision values varied from 4.24% to 5.70%. All these values were within the accepted guidance for industry (bioanalytical method validation), showing little variability and good reproducibility of the UPLC–MS/MS method.

Detection limit of UPLC–MS/MS method was also estimated using a series of diluted A549 genomic DNA (5 ng, 20 ng, 50 ng, 100 ng, and 500 ng). There was a linear relationship ($R^2 = 0.99$) between the mass signal area of 5mdC and DNA content analyzed (Fig. 4B), while the ratios of mass signal area of 5mdC to dG remained constant (C.V. 8.15%) even when the amount of DNA assayed varied from 20 ng to 500 ng (Fig. 4A). Based on the UPLC–MS/MS results, with 5 ng genomic DNA analyzed, it still could be detected when the global DNA methylation level was as low as 0.16% (S/N = 3). Therefore, by taking advantage of the low DNA consumption and high sensitivity, UPLC–MS/MS method could be used for global DNA methylation analysis.

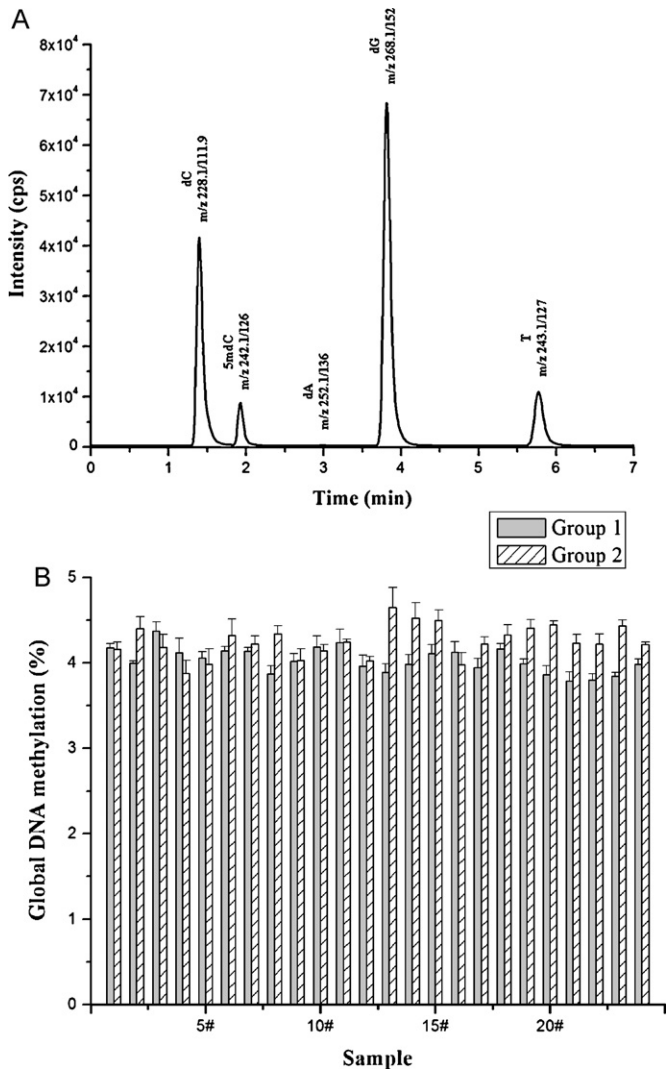

Fig. 5. UPLC–MS/MS chromatogram of DNA hydrolysate from human sperms (A) and the global DNA methylation levels in human sperms (B). Sperm samples of group 1 were only washed by PBS, while abnormal and dead sperm cells were removed by density gradient centrifugation from samples of group 2.

3.4. Analysis of global DNA methylation level in human sperm samples

With the confirmation of UPLC–MS/MS method described above, global DNA methylation level in human sperm samples was detected. Semen samples were divided into two groups according to different pre-treatment protocols. Genomic DNA extracted from sperm cells was digested to nucleosides using DNase I, CIP and snake venom phosphodiesterase I, and the digestion products were assayed using UPLC–MS/MS method. 5mdC (1.9 min) and dC (1.4 min) were detected in the UPLC–MS/MS chromatogram of genomic DNA extracted from human sperm cells, with complete separation of all nucleotides assayed (Fig. 5A).

By calculating the ratio of 5mdC to dG, global DNA methylation level of detected human sperm ranged from 3.79% to 4.65% (Fig. 5B). Comparing the results from sperm of group 1 with that of group 2, average global DNA methylation level of group 1 ($4.03 \pm 0.15\%$) was relatively lower than that of group 2 ($4.25 \pm 0.18\%$). In group 2, abnormal and dead sperm cells were removed by density gradient centrifugation, so we speculated that the lower average global DNA methylation level of group 1 may be associated with the existence of abnormal and dead sperm cells. Aberrant DNA methylation reprogramming may occur during spermatogenesis in abnormal and dead sperm cells. From the preliminary result, we considered that pre-treatment of semen samples might influence the detection of global DNA methylation level and aberrant DNA methylation may partly contribute to abnormal spermatogenesis and compromised sperm function. The present result need to be tested in a larger cohort/population.

4. Conclusion

In summary, we describe a UPLC–MS/MS method for detection of global DNA methylation levels in human sperm cells. Compared with conventional methods used for global DNA methylation detection of human sperms, the ultra resolution, high sensitivity and specificity of UPLC–MS/MS method make it a very useful tool for global DNA methylation assay. With the accurate quantification of sperm DNA methylation level using UPLC–MS/MS, DNA methylation could be used as a good biomarker for clinical evaluation of human sperm quality and elucidation of epigenetic decisions in mammalian germ cells.

Acknowledgments

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