Comparison of the Absolute Level of Epigenetic Marks 5-Methylcytosine, 5-Hydroxymethylcytosine, and 5-Hydroxymethyluracil Between Human Leukocytes and Sperm

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ABSTRACT

5-Methylcytosine is one of the most important epigenetic modifications and has a profound impact on embryonic development. After gamete fusion, there is a widespread and rapid active demethylation process of sperm DNA, which suggests that the paternal epigenome has an important role during embryonic development. To better understand the epigenome of sperm DNA and its possible involvement in a developing embryo, we determined epigenetic marks in human sperm DNA and in surrogate somatic tissue leukocytes; the analyzed epigenetic modifications included 5-methyl-2'-deoxycytidine, 5-hydroxymethyl-2'-deoxycytidine, and 5-hydroxymethyl-2'-deoxyuridine. For absolute determination of the modification, we used liquid chromatography with UV detection and tandem mass spectrometry techniques with isotopically labeled internal standards. Our analyses demonstrated, for the first time to date, that absolute global values of 5-methyl-2'-deoxycytidine, 5-hydroxymethyl-2'-deoxycytidine, and 5-hydroxymethyl-2'-deoxyuridine in sperm are highly statistically different from those observed for leukocyte DNA, with respective mean values of 3.815% versus 4.307%, 0.797 versus 2.945 per 10⁴ deoxynucleosides, and 5.209 versus 0.492 per 10⁶ deoxynucleosides. We hypothesize that an exceptionally high value of 5-hydroxymethyluracil in sperm (>10-fold higher than in leukocytes) may play a not yet recognized regulatory role in the paternal genome.

5-hydroxymethylcytosine, 5-hydroxymethyluracil, 5-methylcytosine, epigenetics, leukocytes, sperm

INTRODUCTION

Sperm DNA is tightly packed as a result of the process of histone replacement with sperm-specific protamine proteins. Sperm passes its paternal genetic and epigenetic information to the embryo via the male pronucleus. After gamete fusion, the protamines must be removed, which is linked with a widespread and rapid DNA demethylation process, which suggests that the paternal epigenome has an important role during embryonic development [1, 2]. This DNA demethylation takes place before the first round of replication, which indicates an active process [3].

Although accumulated evidence has suggested that an active demethylation process is possible in mammalian cells, its molecular mechanism has remained enigmatic [4, 5]. Discoveries described in two independent studies in 2009 [6, 7] demonstrated that 5-methylcytosine (5-mCyt) is oxidized to 5-hydroxymethylcytosine (5-hmCyt) in mammalian DNA and that genomic DNA may contain approximately 0.003% to 0.6% 5-hmCyt [8]. After the rediscovery of 5-hmCyt in 2009, the results of a plethora of studies have confirmed the pivotal role of this modification in active DNA demethylation. The most plausible mechanisms of active demethylation include the involvement of ten-eleven translocation proteins (TETs) in the oxidation of 5-mCyt to form 5-hmCyt [4].

A second plausible scenario for active DNA demethylation involves 5-hmCyt deamination by AID/APOBEC cytosine deaminases to yield 5-hydroxymethyluracil (5-hmUra), which in turn may be replaced by unmethylated cytosine through the BER pathway [4, 9]. In mouse embryonic stem cells, 5-hmUra:Gua mismatches are repaired very quickly. Therefore, 5-hmUra, which is formed during the deamination process of 5-hmCyt (see above), could trigger a rapid demethylation process [10]. Importantly, it was shown that DNA demethylation of paternal DNA is completed very quickly, usually within 4 to 6 h after fertilization, before the occurrence of the first round of DNA replication [11, 12].

To better understand the epigenome of sperm DNA and its possible involvement in the developing embryo, we determined the unique DNA epigenetic code in human sperm DNA and in surrogate somatic tissue leukocytes; the analyzed epigenetic modifications included 5-methyl-2'-deoxycytidine (5-mdC), 5-hydroxymethyl-2'-deoxycytidine (5-hmdC), and 5-hydroxymethyl-2'-deoxyuridine (5-hmdU). For absolute determination of 5-hmdC and 5-hmdU, we used ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) techniques with isotopically labeled internal standards and HPLC-UV for 5-mdC quantification.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the bioethics committee at Nicolaus Copernicus University in Toruń, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Bydgoszcz, Poland (No. KB/396/2009). We obtained written informed consent from all participants involved in this study.

Subjects

The subjects consisted of 108 men (mean age, 32 yr). Information regarding body weight, height, smoking, eating habits, and age was obtained from a questionnaire. Peripheral blood and semen samples were collected from all the men. Semen samples were obtained by masturbation after at least 3 days of...
ejaculatory abstinence. Isolation of leukocytes from venous blood, as well as sperm separation, was described previously [13].

**DNA Extraction and DNA Hydrolysis to Deoxynucleosides**

The pellet of the cells (leukocytes or spermatozoa) was dispersed by vortexing in ice-cold buffer (10 mM Tris-HCl, 5 mM edetic acid disodium salt, 0.15 mM deferoxamine mesylate, pH 8.0). A solution of SDS (to a final concentration of 0.5%) and RNase A and T1 (final concentrations, 5 μg/ml and 5.5 Kunitz units/ml, respectively) was added, and the mixture was gently vortexed. After incubation for 30 min at 37°C, proteinase K was added (to a final concentration of 1 mg/ml). In the case of sperm cells, dithiothreitol was added to a final concentration of 10 mM. Then, the mixture was gently vortexed and incubated at 37°C for 1 h. The mixture was cooled to 4°C and transferred to a centrifuge tube with phenol/chloroform/isoamyl alcohol (25:24:1) and was vortexed vigorously. After extraction, the aqueous phase was treated with a chloroform/isoamyl alcohol mixture (24:1). The DNA supernatant was treated with 2 volumes of cold absolute ethanol in order to precipitate high-molecular-weight DNA. The precipitate was removed with a plastic spatula, washed with 70% ethanol, dried, and dissolved in 0.1% (v/v) CH₃COOH containing 0.2 mM ZnCl₂.

Dissolved DNA samples (100 μl) were mixed with 1 U of nuclease P1. Samples were incubated at 37°C for 1 h. Thereafter, 20 μl of 1% (v/v) NH₄OH and 1.3 U of alkaline phosphatase were added to each sample following incubation at 37°C for 1 h. All DNA hydrolysates were ultrafiltered using a cutoff of 5000-Da filter units.

**Determination of 5-mdC**

The presence of 5-mdC and 2'-deoxyctydine (dC) in DNA hydrolysates was determined using HPLC with UV detector. Separation of the 2'-deoxyribonucleosides was performed using a Synergi 4-μm Hydro-RP 80A column (150 × 2 mm) equipped with a 5-μm precolumn (Phenomenex). The nucleosides were eluted using a mobile phase of 25 mM KH₂PO₄, 1% methanol, and 0.1% acetic acid. The flow rate was 0.4 ml/min, and the injection volume of samples was 10 μl. The eluate was monitored with UV detector at 286 nm (5-mdC detection) and 278 nm (dC detection). Linear calibration curves were obtained in the concentration ranges of 62.5 to 1000 μM for dC and 3.125 to 100 μM for 5-mdC. The 5-mdC contents in genomic DNA have been expressed as relative values of 5-mdC to the sum of dC and 5-mdC (percentage).

**UPLC-MS/MS Analysis of 5-hmdC and 5-hmdU**

The [^{15}N₂]^{13}C₀,^{15}N₀][^{15}N₂]^{15}C₀-5-hmdU was synthesized by the oxidation of [^{15}N₂]^{13}C₀-2'-deoxythymidine (Cambridge Isotope Laboratories) with Na₂S₂O₈ (20 min of reaction at 60°C) using the optimized method by Rahman et al. [14]. Synthetic internal standards were purified with preparative HPLC (5-μm Luna C18 column, 250 × 10 mm; Phenomenex) in 0.5% acetic-acetonitrile gradient (0.5%–15%) and characterized by UPLC-MS/MS and UV analyses. Genuine standards of 5-hmdC and 5-hmdU were obtained from Berry & Associates, Inc., 2'-deoxythymidine and dC were obtained from Sigma, and [D₃]-5-hmdC was obtained from Toronto Research Chemicals.

The DNA hydrolysates were spiked with internal standards, labeled with stable isotopes, and injected into the column in amounts corresponding to 500 fmol [^{15}N₂]^{13}C₀,^{15}N₀][^{15}N₂]^{15}C₀-5-hmdU and 2 pmol [D₃]-5-hmdC. Chromatographic separation was performed with an ACQUITY UPLC instrument (Waters), consisting of a binary gradient pump built-in vacuum degasser sample manager, column heater, and photodiode array detector. The HPLC was operated using MassLynx 4.1 software (Waters). A 1.7-μm Kinetex C18 column was used (150 × 2.1 mm) with a Krude Katcher Ultra 0.5-μm in-line filter (both from Phenomenex). The flow rate was 300 μl/min, and the injection volume was 2 μl. Separation was accomplished by gradient elution for 10 min using a mobile phase of 0.2% acetic acid and acetonitrile (1%–10% for 5 min, then re-equilibration with 1% acetonitrile for 5 min). The amount of 2'-deoxythymidine and dC was determined by UV detection at 280 nm with external calibration. Subsequent mass spectrometric detection was performed using a Quattro Premier XE tandem quadrupole mass spectrometer equipped with an electrospray ionization source (Waters). Ion source parameters were as follows: capillary voltage of 3.8 kV, extractor voltage of 3.5 V, source temperature of 120°C, nitrogen desolvation gas flow of 800 L/h, nitrogen cone gas flow of 50 L/h, and desolvation temperature of 350°C. Collision-induced dissociation was performed with argon 6.0 at 3 × 10⁻⁶ bar pressure as the collision gas. Electrospray ionization was performed in the positive ion mode. For all analytes, protonated molecular ion [M+H]⁺ was selected by the first mass filter. The instrument response was optimized by infusion of the 10 μM genuine compounds (5-hmdU and 5-hmdC) dissolved in water in the mobile-phase stream through a T-connector (10 μl/min) using a 4.1 AutoTune feature (MassLynx). To obtain highest sensitivity, the most abundant fragmentation pathway/transition patterns for each compound were selected and acquired with a dwell time of 50 milliseconds. The transition patterns, cone voltage, and collision energy for each compound are summarized in Table 1.

**Statistical Analysis**

For the statistical analysis, a data analysis software system (STATISTICA, version 10.0; StatSoft, Inc.) was used. For normal distribution, variables were analyzed by the Kolmogorov-Smirnov test with Lilliefors correction. A two-sided Student t-test for independent samples was performed. Statistical significance was set at P < 0.05. All results are expressed as means ± SDs.

**RESULTS**

The level of 5-mdC in DNA isolated from leukocytes reached a mean value of 4.307%. This level was significantly higher than in DNA isolated from spermatozoa, where the level reached 3.815% (Fig. 1A). This difference was highly statistically significant (P < 1 × 10⁻¹¹). Determination of 5-hmdC revealed a significantly (P < 1 × 10⁻¹¹) higher level of the modification in DNA isolated from leukocytes (mean value, 2.945 per 10⁴ deoxynucleosides [dNs]) compared with DNA isolated from spermatozoa (mean value, 0.797 per 10⁴ dNs) (Fig. 1B). The 5-hmCyt differences between leukocytes and sperm were similar to those determined with ELISA in recent work by Jenkins et al. [15]. Conversely, the mean level of 5-hmdU found in DNA isolated from spermatozoa (5.209 per 10⁴ deoxynucleosides) compared with DNA isolated from leukocytes (0.492 per 10⁶ dNs) (Fig. 1C). Determination of 5-hmdU was performed in approximately half of the sperm probes because of insufficient DNA concentrations in samples. In the case of leukocyte probes, in most samples the 5-hmdU level was below the limit of quantification (0.2 per 10⁶ dNs) despite a sufficient DNA concentration. Comparative levels of the above-mentioned modifications are summarized in Table 2. No significant differences were observed for all analyzed DNA modifications among subjects with different semen parameters such as sperm concentration, total sperm number, and sperm motility. We demonstrated previously that the 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) level in sperm DNA is approximately 25% higher than in leukocyte DNA [13]. However, in the present study we did not find any correlation between previously determined 8-oxodG and 5-

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**Table 1. Characteristics of analyzed compounds and internal standards used for MS quantification.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Internal standard</th>
<th>Transition pattern</th>
<th>Cone voltage (V)/collision energy (eV)</th>
<th>LOD (fmol)*</th>
<th>LOQ (fmol)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-hmdU</td>
<td>[^{13}C₀,^{15}N₀][^{15}N₂]^{15}C₀-5-hmdU</td>
<td>259→143</td>
<td>20/12</td>
<td>1.5</td>
<td>5</td>
</tr>
<tr>
<td>5-hmdC</td>
<td>[D₃]-5-hmdC</td>
<td>258→142</td>
<td>20/12</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

*LOD indicates limit of detection on column (signal/noise ratio >3, measured in matrix). †LOQ indicates limit of quantitation on column (signal/noise ratio >10, measured in matrix).
hmdU or 5-hmdC levels in sperm DNA and leukocyte DNA of the studied subjects.

**DISCUSSION**

Recent data provide evidence that the sperm epigenome may have a crucial role in development [1, 2]. Thus, the sperm epigenome implies a poising of the paternal genes during embryonic development. Epigenetic marks may have a significant role in the establishment of totipotency of the embryo and may help in understanding genomic imprinting and some causes of reduced fertility, as well as possible disease risk [1, 2, 16].

In our work herein, we compared epigenetic marks in sperm DNA with those characteristic for DNA of somatic cells represented by surrogate tissue leukocytes. To analyze absolute values of 5-mdC, 5-hmdC, and 5-hmdU, we used liquid chromatography MS/MS techniques with isotopically labeled internal standards, the gold standard for this kind of analysis.

We demonstrated that global cytosine methylation is highly statistically reduced in sperm DNA compared with leukocyte DNA (Fig. 1). Our findings agree with those by Ehrlich et al. [17], who demonstrated that human sperm DNA has a significantly lower methylation level than a number of somatic tissues, including lymphocytes. It is noteworthy that the level found in our study for leukocytes (4.307%) is a typical value for somatic tissue. It is a surprising finding because cytosine methylation, usually at deoxycytidine-deoxyguanosine (CpG) dinucleotides, is commonly thought to be a transcription repressive mark, and the sperm genome is transcriptionally silent. Our results may be explained, at least partially, by the finding that the methylation pattern of testicular DNA displays many more hypomethylated loci in non-CpG islands outside gene promoters than somatic tissues [18]. It is possible that sperm hypomethylation may have some regulatory role during early embryonic development.

Notably, 5-hmCyt is the key player in the active demethylation process. Moreover, some data suggest that it is not simply a demethylation intermediate but may itself have a role in gene regulation/activation [19]. Therefore, our finding that the 5-hmdC level is almost 4 times lower in sperm than in leukocytes suggests the potential role of this modification as a transcription activator [20] and that the sperm genome is transcriptionally silent. Of note, we were able to detect 5-hmdC in all analyzed samples despite the fact that previous analyses using a relative quantification test (ELISA) were unable to detect it in multiple sperm probes [15] and that human blood was among the tissues with the lowest level of 5-hmCyt (approximately a 20-fold lower level than in brain) [21]. Moreover, our absolute values of this modification agreed well with that characteristic for mouse spermatocytes [22].

The most striking difference concerns the 5-hmdU level, which was found to be more than 10-fold higher in spermatozoa than in leukocytes. This ratio is probably much higher because despite a sufficient DNA concentration in

![FIG. 1. Comparison of the levels of analyzed DNA modifications in spermatozoa and leukocytes. A) Level of 5-mdC. B) Level of 5-hmdC. C) Level of 5-hmdU.](https://www.biolreprod.org/)

**TABLE 2. Levels of DNA modifications (expressed as mean ± SD).**

<table>
<thead>
<tr>
<th>Type of human cells</th>
<th>5-mdC per (dC + 5-mdC) (%)</th>
<th>5-hmdC per 10^6 dNs</th>
<th>5-hmdU per 10^6 dNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td>4.307 ± 0.065</td>
<td>2.945 ± 0.490</td>
<td>0.492 ± 0.174</td>
</tr>
<tr>
<td>(n = 107)</td>
<td>(n = 108)</td>
<td>(n = 24)</td>
<td></td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>3.815 ± 0.079</td>
<td>0.797 ± 0.165</td>
<td>5.209 ± 1.790</td>
</tr>
<tr>
<td>(n = 92)</td>
<td>(n = 91)</td>
<td>(n = 57)</td>
<td></td>
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</table>
approximately 80% of leukocyte samples the 5-hmdU level was below the limit of quantitation (0.2 per 10^6 dNs). Therefore, an important question concerns the possible role of this modification in sperm DNA and the relevant question refers to the possible origin of 5-hmUra in cellular DNA.

Originally, 5-hmUra was identified as an oxidatively modified DNA base derivative (as the product of thymine oxidation) [23]. However, there is some experimental evidence suggesting that DNA oxidation may not be the main source of 5-hmUra in cellular DNA. The 5-hmUra: Ade base pair generated during the oxidation reaction is not miscoding and does not perturb the DNA structure [24, 25]. Indeed, in some bacteriophage 5-hmUra completely replaces thymine [26]. Moreover, the 5-hmUra: Ade base pair is a poor substrate for glycosylase activity, while the 5-hmUra: Gua mispair, which is formed during active demethylation, is excised with 60 times greater capacity (thymidine DNA glycosylase does not excise 5-hmUra when paired with adenine) [27]. These results suggest that there is no need for the removal of 5-hmUra paired with adenine. Therefore, the existence of 5-hmUra glycosylase activity, which very quickly and efficiently removes this modification from cellular DNA [9, 28, 29], leads to the proposal that this activity was directed to remove 5-hmUra that is formed during enzymatic oxidation/deamination of 5-mCyt (paired with guanine [see below]). Interestingly, the yield of the formation of 5-hmUra exposed to ionizing radiation (an efficient source of reactive oxygen species [ROS]) is very low compared with other DNA bases (approximately one order of magnitude lower than 8-oxodG) [23, 30].

Because DNA in Escherichia coli is not fully protected by proteins, the level of oxidatively modified bases should be higher in this organism than in DNA isolated from mammalian cells. In agreement with this, our previous work showed that the 8-oxoG level (a well-recognized marker of oxidatively damaged DNA) is substantially higher in E. coli DNA than in pig liver DNA [31]. However, our results also show that the level of 5-hmdU is undetectable in E. coli cells (limit of detection, 0.2 per 10^6 dNs). Of note, we found no correlation between the levels of 5-hmdU and 8-oxodG (data not shown). All these data suggest that oxidative stress is not the main source of 5-hmUra in DNA, although we cannot exclude the possibility that part of the analyzed modification may be the result of ROS reaction with thymine.

There is some experimental evidence to support the notion that 5-hmUra has an important role in active DNA demethylation: overexpression of AID and TET1 protein leads to global accumulation of 5-hmUra [28, 32]. However, there are also data indicating that AID/APOBEC family members preferentially deaminate unmodified cytosine and discriminate against 5-substituted cytosine substrates with increasing size [33].

An important question concerns the possible role of 5-hmUra in sperm DNA. The simplest answer is that the modification is a transient intermediate derived from 5-hmCyt to 5-hmdU by thymine deaminase [34]. Moreover, APOBEC4, another member of the cytosine deaminase family, was reported to be expressed in testis [35].

However, it cannot be excluded that 5-hmUra itself performs some regulatory function similar to that recently shown for other intermediates of the active demethylation process, namely, 5-formylcytosine (5-fCyt) and 5-carboxycytosine (5-caCyt). It has been shown that these modifications could serve as transcription regulators [20, 36, 37]. Interestingly, 5-fCyt, 5-caCyt, and 5-hmUra are recognized by thymidine DNA glycosylase [38], and a complex of the enzyme with these modifications may regulate transcription independently of its repair activity [39].

The most attractive hypothesis for the role of 5-hmUra in spermatozoa would be similar to that recently shown for 5-fCyt: it was suggested that it may be a regulatory element of ‘‘ready to go’’ (poised) genes [37]. Likely, 5-hmUra may be also formed as the product of enzymatic reaction during hydroxylation of the methyl group of thymine, with analogy to the formation of base J in Leishmania, trypanosomes, and flagellates. In Leishmania, thymine residue is hydroxylated in the specific position of DNA to form 5-hmUra (in the next step, a glucose is attached to the modification) [40]. The hydroxylation reaction is catalyzed by recently described members of the TET-JBP family of dioxygenases [39]. Although there is no evidence for the presence of base J in mammalian DNA, 5-hmUra may have a related function to base J (i.e., its presence may be associated with gene silencing and may help in the formation of repressive chromatin [39, 40]). Indeed, when the present article was in press, it was demonstrated that thymine is a substrate for TET-induced oxidation to produce 5-hmUra; it was also observed that the 5-hmUra: Ade base pair may influence binding of transcription factors and chromatin-remodeling proteins [10].

Intriguingly, the level of 5-hmdU is relatively stable in different somatic tissues and similar to that observed in lymphocytes (approximately 0.5 per 10^6 dNs in human colon, colorectal cancer, and pig liver), while the level of 5-hmC varies greatly among these tissues, ranging from 0.29 in leukocytes up to 2.9 per 10^3 dNs in colon tissue (data not shown). Of note, the 5-hmdU level in sperm DNA detected in our study is similar to that characteristic for mouse embryonic stem cell DNA [10].

In summary, our analyses demonstrated, for the first time to date, that absolute global values of 5-mdC, 5-hmC, and 5-hmdU in sperm are highly statistically different from those characteristic for leukocyte DNA. We hypothesize that the exceptionally high value of 5-hmdU in sperm may have some as yet unrecognized regulatory role in the paternal genome. As mentioned in the Introduction, the paternal epigenome has an important role during embryonic development. Moreover, the DNA epigenetic code in human sperm may have an essential role in preimplantation development, male fertility problems, and pregnancy outcomes [41, 42]. Therefore, our results should be read in the context of the conclusion by Jenkins and Carrell [2] in their review article: ‘‘As we learn more about the true effects of epigenetic alteration in sperm, how they arise and how they affect fecundity, we will be more capable of addressing the growing issue of male factor infertility in prevention, diagnosis, and treatment.’’

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