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DETERMINATION OF 5-METHYLCYTOSINE, 5-HYDROXYMETHYLCYTOSINE, AND 5-FORMYLCYTOSINE EXPRESSION IN MOBILE AND IMMOBILE SPERM FROM ONE EJACULATION: ANALYSIS OF RELATIONSHIPS WITH SPERM PROPERTIES USING PARTIAL LEAST SQUARE REGRESSION

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ABSTRACT

AIM: To investigate prospective selection markers of donor sperm used in the assisted reproductive technology (ART) of intracytoplasmic sperm injection (ICSI), we measured the concentration of three epigenetic alteration markers; 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), and 5-formylcytosine (5fC), in mobile (i.e., normal shape, motility, morphology) and immobile (i.e., no motility, abnormal shape) sperm from one ejaculation.

METHODS: Mobile and immobile sperm were separated from the seminal plasma of one ejaculation using the original monopolistic centrifugal sedimentation separation method for sperm isolation. Differences in the three prospective epigenetic alteration markers between mobile and immobile sperm were assessed using the Student's t-test. Principal component analysis (PCA) and partial least squares regression (PLS) were performed to determine the relationships between sperm properties and markers, enabling the extraction of most prospective selective markers for ICSI.

RESULTS: Examined markers in mobile and immobile sperm (n = 13) were 5mC_Mobile (0.32 6.47%), 5hmC_Mobile (0.3 2.9%), 5fC_Mobile (0.011 0.159%), and 5mC_Immob (0.37 11.7%), 5hmC_Immob (0.6 20.1%), and 5fC_Immob (0.001 0.315%), respectively. The expression difference between mobile and immobile sperm was significant for 5mC (P = 0.0443, 95% confidence interval) and 5fC (P = 0.0204), while that of 5hmC (P = 0.0773, 95% confidence interval) was significant by 90% confidence interval. Regression analysis showed a correlation of 5mC_Mobile with sperm morphology (r = 0.3514). PCA identified 5mC_Mobile and 5hmC_Mobile as the most suitable factors to identify sperm properties. PLS analysis determined that 5mC_Mobile, 5fC_Mobile, and 5fC_Immob were related to sperm motility, 5mC_Immob to sperm morphology, and 5mC_Mobile and 5fC_Mobile to sperm concentration. 5hmC_Mobile (PLS 2, 3, 4 5, 6) was related to sperm morphology with a high variable importance in projection (VIP) value of 1.4. Overall, concentration of 5hmC_Mobile in donor sperm appears to be the best sperm selection marker for ICSI.

INTRODUCTION

5-methylcytosine (5mC) results from the DNA methylation of a cytosine ring by DNA methyltransferase through epigenetic modification. In somatic cells, the ratio of 5mC in the context of CpG areas is reportedly 2 8% [1, 2]. In human sperm, the reported DNA methylation ratio ranges from 0.2 20% [3-7]. To date, four modified cytosines have been identified in mammalian genomes: 5mC, 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) [4–9]. The ratio of 5hmC ranges from 6.9–34.0% in human sperm [5–9]; however, there are no reports of 5caC and 5fC in human sperm [9–11]. In these epigenetic marker studies, the difference in the ratio of 5mC and 5hmC between normal and abnormal sperm was investigated. Because the authors manually collected the samples using an optical view from over 400 different donors [4], these mixed data may not reflect the characteristics of sperm from one ejaculation. In contrast, Kaneko et al. described a high purification method using density-gradient centrifugation that enabled the collection of normal (mobile and normal shape) and abnormal (immobile and abnormal shape) sperm from one ejaculation [12]. This method could improve the analysis of sperm characteristics and epigenetic alteration markers, which will greatly benefit sperm selection for assisted reproduction technologies (ART).

In this report, we determined three epigenetic markers among mobile and immobile sperm from one ejaculation. Epigenetic alteration markers were also analyzed to create a choice assistant index for the selection of prospective sperm for the ART of intracytoplasmic sperm injection (ICSI). Publication of the International Committee Monitoring Assisted Reproductive Technologies (ICMART) report has led to worldwide increased use of the ICSI approach for male infertility over in-vitro fertilization (IVF) [13, 14]. The selection of prospective sperm from the donor is a key step in the process. Previously, morphological resemblance (e.g., to mobile and non-deformed sperm) was used as a selection criterion for ICSI. Here, we analyzed the relationships among WHO sperm parameters (sperm concentration, motility, morphology) and 5mC, 5hmC, and 5fC levels in sperm using multivariate analyses (regression, principal component analysis (PCA) and partial least squares regression (PLS)), and extracted related epigenetic markers for prospective sperm selection for ICSI.

MATERIALS AND METHODS

Semen collection and sperm analysis

Human ejaculate was obtained from patients who visited the Reproduction Center in the Ichikawa General Hospital, Tokyo Dental College. Consent was obtained from all study participants, who were briefed about the aims of the study and the parameters to be measured. The study was approved by the ethics committees of Meiji Pharmaceutical University and Ichikawa General Hospital. Sperm concentration and motility were measured using a computer assisted image analyzer (C-Men; Compix Inc., PA, USA). The semen sample was diluted twice with saline and centrifuged at 400×g for 10 min, then the sediment was diluted with saline for a total volume of 1.0 mL.

The WHO guidelines (WHO Manual, 5th ed. 2010) [15] were used to classify the sperm into three categories based on concentration: normozoospermia (20×106 sperm/mL), oligozoospermia (< 20×106 sperm/mL), and azoospermia. Sperm head morphology was also evaluated according to these guidelines.

Centrifugation of human sperm in Optiprep and Percoll density gradients

Optiprep (Axis-Shield, Oslo, Norway) was made isotonic by the addition of 20 mM HEPES-buffered Hanks solution with 2.0 mg/ml human serum albumin, pH 7.4, to yield an apparent density of 1.17 g/mL. The condensed sperm was then overlaid onto 0.5 mL Optiprep and centrifuged at 20,000×g for 10 min. The interface layer and sediment were recovered, and the former was further separated using a Percoll (GE Healthcare, NJ, USA) density gradient. For this, 5 mL isotonic 20 mM HEPES-buffered 90% Percoll was made isotonic using powder-type Hanks mixture and 2.0 mg/mL human serum albumin, pH 7.4 (apparent density: 1.12 g/mL), and placed in a conical tip test tube. Next, 1.0 mL Hanks solution was layered on top while undergoing 10 revolutions at an angle of 30° to produce the linear density gradient. The resulting sperm suspension was then loaded onto the gradient and centrifuged in a swing-out rotor at 400×g for 30 min, after which the sediment was recovered [12].

Mobile (mobile and normal shape) and immobile (immobile and abnormal shape) sperm collection

After sperm from one ejaculation classified as normozoospermia (n=13) underwent Optiprep and Percoll density-gradient centrifugation as described above, mobile (mobile and normal shape) and immobile (immobile and abnormal shape) sperm were collected. Next, the DNA from each ejaculate was separated using the Wizard Genomic DNA Purification Kit (Promega, WI, USA) adjusted to an OD of 1.0 (260/280 nm). Each epigenetic alteration marker was quantified using the MethylFlash Global DNA Methylation (5mC/5hmC) ELISA Easy Kit (EpiGentek, NY, USA) and the MethylFlash 5-Formylcytosine (5fC) DNA Quantification Kit (EpiGentek).

Statistical analyses

Student's t-test, regression analysis, PCA, and PLS were performed using JMP Pro 13.2.0 (SAS Institute, Japan).

RESULTS AND DISCUSSION

Separation of mobile and immobile sperm from one ejaculation

The semen profiles of 13 ejaculate specimens, with concentrations ranging from 4800 26,400 × 104 sperm/mL, are shown in Table 1(Tables 1, last of manuscript). The motility and morphology values were 7.7 64.7% and 1.0 13.6%, respectively. Using Kaneko et al.'s [12] centrifugal sedimentation sperm isolation method for sperm in one ejaculation, mobile and immobile sperm were successfully separated. While previous reports [3 8] compared differences in the ratio of 5mC and 5hmC between normal and abnormal (immobile and/or abnormal shape) sperm from mixed specimens, we collected mobile (mobile and normal shape sperm) and immobile (immobile and/or abnormal shape) sperm from one ejaculation. This method enabled more accurate data collection of the sperm properties from one donor, greatly improving sperm selection during the ICSI process. The following data analyses were performed.

Student's t-test

Each epigenetic alteration marker was quantified in 13 ejaculate specimens (Tables 1). The proportion of 5mC varied significantly between mobile and immobile sperm (P = 0.0443, 95% confidence interval), ranging from 0.32 6.47% in mobile sperm and 0.37 11.7% in immobile sperm; so did 5fC (P = 0.0204, 95% confidence interval), ranging from 0.011 0.159% and 0.001 0.315%, respectively. The proportion of 5hmC ranged from 0.3 2.9% and 0.6 20.1%, respectively; however, the difference was significant (P = 0.0773, 90% confidence interval). Our results confirm the previously reported expression differences in the three epigenetic alteration markers between mobile and immobile sperm in one ejaculation.

Multivariate analysis

Multivariate regression analysis and principal component analysis (PCA)

We next applied multivariate regression analysis to extract relationship factors among sperm properties and epigenetic alternation markers. Only the factor 5mC_Mobile was correlated to sperm morphology (r = 0.3514); the other epigenetic alteration markers showed no significant correlation to sperm parameters. PCA was then applied to the above data. Figure 1

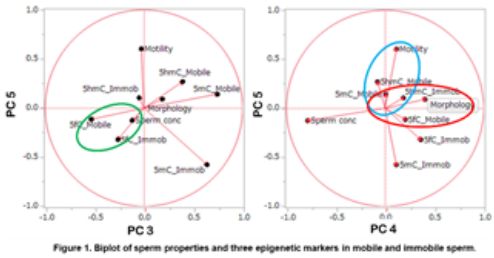

Figure 1. Biplot of sperm properties and three epigenetic markers in mobile and immobile sperm.

shows the selected biplot from PC1 PC6. In PC4 PC5, 5mC_Mobile, 5hmC_Immob, and 5fC_Mobile were related to sperm morphology, and 5mC_Mobile, 5hmC_Mobile, and 5hmC_Immob were related to sperm motility. Sperm concentration was only related to 5fC_Mobile and 5fC_Immob in PC3 PC5. 5mC_Mobile and 5hmC_Mobile were extracted as relation factors to sperm properties by PCA.

Partial Least Squares regression (PLS)

The potential explanatory variables (epigenetic alteration markers: 5mC_Mobile, 5mC_Immob, 5hmC_Mobile, 5hmC_Immob, 5fC_Mobile, and 5fC_Immob) were assessed using PLS to determine possible relationships among them. PLS is better suited to examine relationships among explanatory variables [16,17], as there is a possibility of decay of data analysis precision in full regression and PCA. In the PLS analysis, explanatory variables were extracted by covariance calculation, so that an additional projection of related factors to that found by PCA could be extracted. Figure 2 shows the PLS plot of

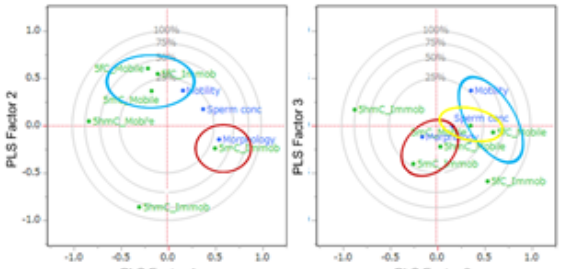

Figure 2. PLS factor plots of sperm properties and three epigenetic markers in mobile and immobile sperm

selected variables. In PLS 1 2, 5mC_Mobile, 5fC_Mobile, and 5fC_Immob were related to sperm motility (blue ring), while 5mC_Immob was related to sperm morphology (red ring). In PLS 2 3, 5mC_Mobile and 5fC_Mobile were related to sperm concentration (yellow ring) and sperm motility (blue ring), and 5hmC_Mobile and 5mC_Immob were related to sperm motility (red ring). 5hmC_Mobile (PLS 2, 3, 4 5, 6 not in Figure) was related to sperm morphology. In addition, the explanatory variables with the greatest relation to the objective variables (sperm concentration, motility, morphology) were calculated as numerical values (variable importance in projection; VIP) with regard to sperm parameters (sperm concentration, motility, morphology) (Fig. 3) [16,17]. In this analysis, 5hmC_Mobile was highly related to the three sperm properties (Fig. 3), and was

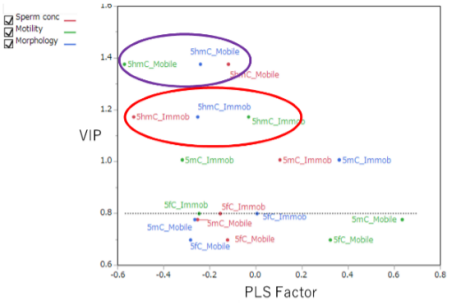

Figure 3. VIP (variable importance for projection) and PLS Factor plot of sperm condition, mobility, and morphology in mobile and immobile sperm.

a key factor for the selection of the "most fit sperm" for ICSI. While 5mC_Mobile and 5fC_Mobile had low VIP scores (< 0.8), these factors were also effective in ICSI. Taking additional information from the WHO guideline into account, it appears that the concentration of 5hmC_Mobile in donor sperm constitutes a suitable criterion for sperm selection in ICSI.

CONCLUSION

Selection of the most suitable sperm from a donor has emerged as a crucial consideration in ART and especially ICSI. Usually, "well mobile and normal shape sperm" are selected as the "most fit sperm". Here, we propose the quantification of epigenetic markers in donor sperm to evaluate sperm quality. For the first time, we report the quantification of 5mC, 5hmC, and 5fC expression in mobile and immobile sperm separated from one ejaculation. Analyzes by Student's t-test of these three markers showed significant differences among mobile and immobile sperm. Regression analysis indicated a correlation of 5mC_Mobile to sperm morphology only (r = 0.3514). Using PCA analysis, 5mC_Mobile, 5hmC_Mobile, and 5hmC_Immob were shown to be related to sperm properties. Covariance values derived from a PLS analysis showed that 5mC_Mobile and 5fC_Mobile were related to sperm concentration; 5mC_Mobile, 5hmC_Mobile, 5fC_Mobile, and 5fC_Immob were related to sperm motility; and 5hmC_Mobile (PLS 2, 3, 4 5, 6 not in Figure) was related to sperm morphology. From the VIP and PLS plot, 5hmC_Mobile was highly related to the three sperm properties, and was a key factor for the selection of "most fit sperm" for ICSI.

Conflict of interest

The authors declare that they have no conflict of interest.

Table 1: The percentage of 5-mC, 5-hmC, and 5-fC in mobile and immobile sperm from one ejaculation

Specimen	Sperm conc. (× 104)	Motility	Morphology	5mC_Mobile	5mC_Immob	5hmC_Mobile	5hmC_Immob	5fC_Mobile	5fC_Immob
529	7800	7.7	1	0.61	0.69	2.9	10.6	0.018	0.001
530	17600	45.5	10.5	0.32	0.37	0.17	1.37	0.014	0.047
534	9200	42.2	5.9	0.6	5.38	0.3	20.1	0.011	0.01
535	15200	35.5	13.6	0.95	11.7	0.17	1.74	0.009	0.039
669	12400	64.5	7.4	4.69	3.52	1.44	0.69	0.01	0.104
670	26400	50	1.5	1.33	1.83	0.78	0.6	0.091	0.138
671	8000	60	5.6	1.73	3.03	1.49	0.91	0.159	0.118
676	14600	32.9	4.6	1.89	3.82	0.83	1.04	0.117	0.315
677	4800	35.4	2.4	6.47	5.05	1.7	1.09	0.023	0.052
678	26200	38.9	1.8	1.95	2.37	0.69	0.89	0.004	0.079
679	5800	19.6	2.7	3	4.21	1.24	2.53	0.03	0.339
680	20400	64.7	10.5	4.82	5.58	2.06	2.34	0.016	0.199
681	23600	27.1	2.5	4.63	10.71	1.81	1.82	0.055	0.047

Conc., condition; 5mC_Mobile, 5mC in mobile sperm; 5mC_Immob, 5mC in immobile sperm. Both sperm types were separated from one ejaculation.

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