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Increased N^6 -methyladenosine in Human Sperm RNA as a Risk Factor for Asthenozoospermia

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Male infertility is a worldwide medical problem. Asthenozoospermia is a common cause of infertility. Epigenetic modifications of DNA and histones have been shown to influence human infertility, but no research has explored whether N^6 -methyladenosine (m^6A) level in RNA is associated with asthenozoospermia. Here, we collected a total of 52 semen samples, including 20 asthenozoospermia patients and 32 healthy controls. An LC-ESI-MS/MS method was used to detect m^6A contents in sperm RNA, and real-time PCR was performed to determine the mRNA expression of demethylase (FTO, ALKBH5), methyltransferase (METTL3, METTL14, WTAP) and an m^6A -selective-binding protein (YTHDF2). We found that m^6A content ($p = 0.033$) and the mRNA expression of *METTL3* ($p = 0.016$) and *METTL14* ($p = 0.025$) in asthenozoospermia patients were significantly higher than those of controls. Increased m^6A content was a risk factor for asthenozoospermia (odds ratio (OR) 3.229, 95% confidence interval (CI) 1.178 – 8.853, $p = 0.023$). Moreover, m^6A content was correlated with the expression of *METTL3* ($r = 0.303$, $p = 0.032$) and with sperm motility (progressive motility: $r = -0.288$, $p = 0.038$; non-progressive motility: $r = -0.293$, $p = 0.037$; immotility: $r = 0.387$, $p = 0.005$). Our data suggest that increased m^6A content is a risk factor for asthenozoospermia and affects sperm motility. Methyltransferases, particularly METTL3, play key roles in increasing m^6A contents in sperm RNA.

Human infertility is a prevalent medical problem, which affects approximately 15% of couples worldwide^{1,2}. Tragically, the infertility rates in some developing countries can reach 30%³. Male infertility contributes to more than 50% of all cases of infertility⁴. Optimal male fertility depends on an adequate number of normal, motile, mature and physiologically functional sperm⁵, which may be influenced by many factors, such as genetic disorders, infections of the genital tract, medical intervention and environmental degradation¹. Decreased semen quality and defective sperm function appear to be common causes of male infertility¹. Many current studies have probed the pathogenesis of infertility, but the interference of different epigenetic alterations in infertility is not fully understood.

Epigenetics is defined as a heritable change affecting gene expression that is not caused by DNA sequence alterations⁶. Epigenetic modifications of DNA and histones have been confirmed as having significant roles throughout spermatogenesis². There are two characteristic features of sperm epigenetic programming. The first feature is a major removal of epigenetic markers occurring in the primordial germ cells (PGCs). The second feature is the reorganization and condensation of the germ cell genome during postmeiotic maturation, including DNA methylation and histone modifications during spermiogenesis². Abnormal sperm DNA methylation levels are related to altered semen parameters⁷. Moreover, many specific RNAs, mRNAs, miRNAs, and Piwi-interacting RNAs (piRNAs) in sperm are vital for male fertilization^{8–11}. In addition to DNA and histone modifications, epigenetic modifications of RNA have been proposed to be another layer of epigenetic regulation. Up to date, more than 100 types of RNA modifications, occurring in mRNA, tRNA, rRNA and small nuclear RNA (snRNA), have been identified¹². Among these modifications, N^6 -methyladenosine (m^6A) modification is the most prevalent in

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	Patients (n = 20)	Controls (n = 32)	p values
pH value	7.1 ± 0.2	7.1 ± 0.1	0.906
Concentration (10 ⁶ /mL)	39.2 ± 23.9	96.6 ± 74.0	0.000
Progressive motility (PR, %)	8.6 ± 6.0	24.7 ± 10.2	0.000
Non-progressive motility (NP, %)	19.8 ± 7.5	44.5 ± 14.2	0.000
Immotility (IM, %)	72.4 ± 10.5	30.8 ± 13.2	0.000
Normal morphology (%)	5.1 ± 3.2	8.2 ± 4.7	0.017
Teratozoospermia index (TZI, %)	1.1 ± 0.1	1.1 ± 0.1	0.291
Sperm deformity index (SDI, %)	1.1 ± 0.1	1.0 ± 0.1	0.029

Table 1. Clinical parameters of semen in controls and patients.

mammalian mRNA¹³. Despite m⁶A modification being first reported in early 1970s¹⁴, its biological function and significance in sperm RNA are largely unknown.

In vivo, the dynamic regulation of m⁶A modification plays important roles through the functional interplay among m⁶A methyltransferases and demethylases¹⁵. The methyltransferase complex, which is composed of methyltransferase like 3 (METTL3), methyltransferase like 14 (METTL14) and Wilms tumor 1 associated protein (WTAP), has been identified as a crucial factor catalyzing the formation of m⁶A with S-adenosyl-L-methionine (SAM) as the methyl donor¹⁶. In contrast to methyltransferases, two demethylases, the alpha-ketoglutarate- and Fe²⁺-dependent dioxygenase fat mass and obesity associated protein (FTO) and AlkB family member 5 protein (ALKBH5), have similar functions as the TET enzyme in DNA demethylation¹⁷; i.e., both FTO and ALKBH5 can remove the methyl group from m⁶A in RNA^{18,19}. In this dynamic modification, methyltransferases act as 'writers', demethylases serve as 'erasers', and m⁶A-selective-binding proteins (YTHDF) represent 'readers' of m⁶A in mRNA. More recently, the YTHDF domain family 2 protein (YTHDF2) has been shown to regulate RNA stability, translation, splicing, transport and localization through selective recognition of methylated RNA^{20,21}.

Unlike DNA methylation, very few studies directly focused on the role of RNA methylation in human disease, particularly m⁶A. Recent advances have demonstrated that FTO expression and m⁶A levels are inversely correlated during adipogenesis²² and the FTO gene encoding an m⁶A demethylase is also associated with human obesity²³. We have reported that the increased mRNA expression of FTO contributed to the reduction of m⁶A in type 2 diabetes mellitus²⁴. However, the association of m⁶A with human fertility can be traced back to 1997, when Bokar and colleagues first revealed using Northern blot analysis that the mRNA of METTL3 (*MT-A70*) was expressed in a wide variety of human tissues, with the highest levels being in testis²⁵. In addition, a recent animal study documented that *Alkbh5*-deficient male mice showed increased m⁶A in mRNA and impaired fertility due to compromised spermatogenesis¹⁹. Based on these experimental results, we hypothesize that m⁶A modification is associated with human asthenozoospermia and plays a role in male infertility.

To verify our hypothesis, in the current study, we first detected the m⁶A contents in sperm RNA from asthenozoospermia patients and healthy controls. Compared with the controls, m⁶A content in asthenozoospermia patients was significantly increased, which is a risk factor for asthenozoospermia. To better understand this phenomenon, we performed real-time PCR to examine the mRNA expression of key genes involved in m⁶A regulation. Our results indicated that the mRNA expression of METTL3 and METTL14 was higher than that of controls. In addition, m⁶A content was closely related to the mRNA expression of METTL3 and clinical parameters of sperm.

Results

Clinical parameters of semen. The clinical parameters of semen from 20 asthenozoospermia patients and 32 healthy controls are given in Table 1. Student's t test indicated that there was no significant difference in semen pH and teratozoospermia index (TZI) between the two groups; nevertheless, asthenozoospermia patients showed significantly lower sperm concentration ($39.2 \times 10^6/\text{mL}$ vs. $96.6 \times 10^6/\text{mL}$), progressive motility (8.6% vs. 24.7%), non-progressive motility (19.8% vs. 44.5%) and normal morphology (5.1% vs. 8.2%) and higher sperm immotility (72.4% vs. 30.8%) and sperm deformity index (1.1% vs. 1.0%) compared with the controls.

LC-ESI-MS/MS method development. The linearity of this method was investigated using 40 pmol rA standard supplemented with m⁶A at different amounts ranging from 8 fmol to 800 fmol (Table S1). The calibration curves were constructed by plotting the mean peak area ratio of m⁶A/rA versus the mean molar ratio of m⁶A/rA based on data obtained from triplicate measurements. The results showed linearity within the range of 0.02–2% (molar ratio of m⁶A/rA) with the coefficient of determination (R^2) higher than 0.9977 (Table S1). Limits of detection (LOD) and limits of quantification (LOQ) for m⁶A were calculated as the amounts of the analytes at signal/noise ratios of 3 and 10, respectively. The LOD and LOQ were 1.2 fmol and 4.0 fmol for m⁶A, respectively (Table S1). Validation of the method was accomplished using the synthesized m⁶A-containing oligonucleotide by comparing the measured m⁶A content to the theoretical m⁶A content (Table S2). m⁶A was determined from RNA hydrolysis products with RSDs being 1.8–10.6% and REs being 2.7–12.1% (Table S3), indicating high accuracy and precision for the determination of m⁶A by LC-ESI-MS/MS.

m⁶A contents increased in asthenozoospermia. To investigate whether m⁶A modification differs in asthenozoospermia patients and controls, the m⁶A contents of 52 sperm RNA samples, including 20

Figure 1. The MRM chromatograms of nucleosides and difference of m⁶A contents between the two groups. (A) Standard nucleosides. **(B)** 50 ng sperm RNA from an asthenozoospermia patient. Shown in inset is the enlargement chromatogram of m⁶A. **(C)** Comparative analysis of m⁶A contents in sperm RNA from asthenozoospermia patients (n = 20) and controls (n = 32).

asthenozoospermia patients and 32 controls, were analyzed by LC-ESI-MS/MS in multiple reaction monitoring (MRM) mode (Table S4). The MRM chromatograms of 9 standard nucleosides and the hydrolysis products of 50 ng sperm RNA from an asthenozoospermia patient are shown in Fig. 1A,B. The average contents of m⁶A in asthenozoospermia patients and healthy controls were $0.18 \pm 0.02\%$ and $0.13 \pm 0.01\%$, respectively. Student's t test indicated asthenozoospermia patients had higher m⁶A contents ($p = 0.033$), as shown in Fig. 1C. Importantly, logistic regression analysis revealed that increased m⁶A content in sperm RNA was a risk factor for asthenozoospermia (odds ratio (OR) 3.229, 95% confidence interval (CI) 1.178 – 8.853, $p = 0.023$).

mRNA expression of the regulatory genes of dynamic RNA methylation and its correlation with m⁶A contents. To address why m⁶A contents increased in asthenozoospermia patients, we utilized real-time PCR to determine the mRNA expression levels of the core regulatory genes that would be engaged in dynamic m⁶A modification of RNA (Fig. S1). We used Student's t test to compare the differences in *ALKBH5* and *METTL14* between the two groups, and the Mann-Whitney U test for *FTO*, *METTL3*, *WTAP* and *YTHDF2* comparisons. Asthenozoospermia patients showed significantly higher mRNA expression levels of the methyltransferases, including *METTL3* ($p = 0.016$) and *METTL14* ($p = 0.025$) compared to the healthy controls (Fig. 2B). The average mRNA expression levels of *METTL3* and *METTL14* were 2.4-fold ($p = 0.016$) and 2.5-fold ($p = 0.025$) higher, respectively, than those of controls (Fig. 2B). However, there was no significant difference in *FTO*, *ALKBH5*, *WTAP*

Figure 2. mRNA expression of the regulatory genes of dynamic RNA methylation and the correlation of *METTL3* expression with m⁶A contents. (A,B) Gene expression levels of the regulatory genes of dynamic RNA methylation, normalized to GAPDH, were examined by real-time PCR. Data are expressed as means $\pm$ SEM. (C) Spearman correlation analysis between m⁶A contents and mRNA expression of *METTL3* in all subjects.

or *YTHDF2* mRNA levels between asthenozoospermia patients and controls (Fig. 2A,B). Interestingly, Spearman correlation analysis indicated that the mRNA expression of *METTL3* ($r = 0.303$, $p = 0.032$) (Fig. 2C), *WTAP* ($r = 0.324$, $p = 0.023$) and *YTHDF2* ($r = 0.338$, $p = 0.014$) (Fig. S2) associated with the contents of m⁶A in sperm RNA; while no correlation was found between *FTO* expression and m⁶A contents ($r = 0.268$, $p = 0.057$); Pearson correlation analysis revealed neither *ALKBH5* ($r = 0.055$, $p = 0.700$) nor *METTL14* ($r = 0.194$, $p = 0.172$) was correlated with the contents of m⁶A (Fig. S2). Our data suggested that the increased m⁶A contents in asthenozoospermia patients might be caused by the upregulation of methyltransferase encoding genes, particularly *METTL3*.

m⁶A content associated with clinical parameters of sperm. To test whether m⁶A content is related to the clinical parameters of sperm, we performed Pearson correlation analysis to evaluate the correlation between m⁶A content and several clinical parameters. m⁶A content was negatively correlated with sperm progressive motility ($r = -0.288$, $p = 0.038$) and non-progressive motility ($r = -0.293$, $p = 0.037$), and positively correlated with sperm immotility ($r = 0.387$, $p = 0.005$) (Table 2). No correlation was found between m⁶A contents and other clinical parameters.

Discussion

The majority of male infertility results from asthenozoospermia. Despite tremendous efforts to characterize the genetic basis for this disorder, almost half of male infertility cases are of unknown etiology²⁶. So far, few reports described RNA epigenetic alterations in human asthenozoospermia. Given the importance of m⁶A in RNA stability, splicing regulation, miRNA attenuation, RNA editing prevention, disease acceleration and epigenome control²⁷, we first established a highly sensitive LC-ESI-MS/MS method to measure the m⁶A content in sperm RNA.

	Pearson correlation coefficient	<i>p</i> values
pH value	0.043	0.760
Concentration (10 ⁶ /mL)	−0.238	0.089
Progressive motility (PR, %)	−0.288	0.038
Non−progressive motility (NR, %)	−0.293	0.037
Immotility (IM, %)	0.387	0.005
Normal morphology (%)	−0.039	0.792
Teratozoospermia index (TZI, %)	−0.033	0.823
Sperm deformity index (SDI, %)	0.001	0.996

Table 2. Pearson correlation analysis of m⁶A content with clinical parameters of semen.

Even though there are many methods to detect m⁶A in RNA, such as MeRIP-Seq (m⁶A-specific methylated RNA immunoprecipitation combined with next-generation sequencing)²⁸, the facile enzymatic ligation-based method²⁹, the selective Polymerase method³⁰, SMRT reverse transcription³¹ and the LC-ESI-MS/MS method²⁴. We chose the LC-ESI-MS/MS method to determine m⁶A contents in the semen samples as its high sensitivity, relative low cost and satisfying qualitative and quantitative ability.

One of the most obvious and distinctive features of asthenozoospermia is weak sperm motility, which is defined as the proportion of progressively motile sperm. In our study, correlations existed between m⁶A contents and sperm progressive motility, non-progressive motility and immotility, suggesting that increased m⁶A in sperm RNA from asthenozoospermia patients might be a leading cause of the reduction of sperm motility. Previous studies have demonstrated that the nucleus of mature sperm contains a complex population of RNAs⁸, and differential expression of sperm mRNA is correlated with sperm motility and male reproduction³². We also found that m⁶A was present in human sperm RNA, and asthenozoospermia patients had higher m⁶A contents. Thus, we propose that the abnormal m⁶A modification in sperm RNA may affect the steady mRNA expression of certain genes related to sperm motility, such as *cysteine-rich secretory protein 2 (CRISP2)*^{33,34} and *human β-defensin 1 (DEFB1)*³⁵. However, determining which genes are responsible will require further exploration.

The discovery and characterization of m⁶A demethylases and methyltransferases and an m⁶A-selective-binding protein reveals the functional and reversible regulatory mechanism of m⁶A modification. Theoretically, the altered mRNA expression levels of demethylases, methyltransferases and m⁶A-selective-binding protein encoding genes may lead to abnormal m⁶A modification and further impact on the fundamental biological process and development of disease³⁶. Our results demonstrated that *METTL3* played a major role in altering the contents of m⁶A in asthenozoospermia patients and influenced fertility. *METTL3* (MT-A70) was confirmed as one component of the N⁶-adenosine methyltransferase complex. Bokar and colleagues²⁵ isolated the methyltransferase complex from HeLa cell nuclear extracts and named the 70 kDa protein, which exhibited methyltransferase activity, MT-A70 or *METTL3*. Together with *METTL14*, another methyltransferase, *METTL3* forms a stable heterodimer to mediate mammalian nuclear m⁶A methylation¹⁶. Interestingly, WTAP itself has no methyltransferase activity, but it can affect cellular m⁶A by interacting with the *METTL3*-*METTL14* complex¹⁶. Recent studies have shown that *METTL3* participates in the regulation of a variety of life processes by catalyzing the formation of m⁶A, including stem cell self-renewal and differentiation³⁷, controlling the circadian clock³⁸ and marking primary microRNAs for recognition and processing³⁹.

It is worth noting that *ALKBH5* deficiency can result in the elevation of m⁶A in male mice and further impair fertility¹⁹, whereas our data revealed that *METTL3* but not *ALKBH5* played a key role in human male infertility, especially in asthenozoospermia. The possible reason for this difference between humans and mice might be the diversity of the gene expression model.

Indeed, the isolation of sperm RNA is a challenging work owing to the intrinsic heterogeneous cells exist in the ejaculate and the low quantity of RNA present in Spermatozoon. Although we cannot absolutely ensure that no RNA isolated from other cellular structures in sperm purified by a typical Percoll gradient centrifugation, the potential influences are likely limited.

In summary, we first reported that methyltransferases (*METTL3* and *METTL14*), particularly *METTL3*, play key roles in the dynamic modification of m⁶A in asthenozoospermia patients and that increased m⁶A can impair sperm motility. According to our results, we deduce a process of dynamic modification of m⁶A in asthenozoospermia patients (Fig. 3). The underlying mechanisms of m⁶A modification in asthenozoospermia still need further investigation.

Materials and Methods

Chemicals and reagents. Adenosine (rA), uridine (rU), cytidine (rC), guanosine (rG), 2'-deoxyadenosine (dA), thymidine (T), 2'-deoxycytidine (dC), 2'-deoxyguanosine (dG) were purchased from Sigma-Aldrich (Beijing, China). N⁶-methyladenosine (m⁶A) was from Hanhong Chemical Co., Ltd. (Shanghai, China). S1 nuclease and alkaline phosphatase (CIAP) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Phosphodiesterase I was from Sigma-Aldrich (St. Louis, MO, USA). Chloroform and formic acid were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Chromatographic grade methanol was purchased from Merck (Darmstadt, Germany). All water was purified by a Milli-Q apparatus (Millipore, Bedford, MA). Stock solutions of the ribonucleosides and 2'-deoxynucleosides were prepared in Milli-Q water at a concentration of 5 mmol/L.

Figure 3. A process of the dynamic m⁶A modification in asthenozoospermia. Increased mRNA expression of *METTL3* and *METTL14* contributes to the elevation of m⁶A content which affects sperm motility and further results in male infertility.

Semen sample collection. The ethics committee of Zhongnan Hospital of Wuhan University has approved this study. The study was carried out in accordance with the approved guidelines of the ethics committee of Zhongnan Hospital of Wuhan University and informed consent was obtained from each participant at the time of semen sample collection. According to the 5th edition of the WHO laboratory manual for the examination and processing of human semen²⁶, 20 asthenozoospermia patients and 32 healthy controls from the Productive Medicine Center of Zhongnan Hospital donated semen samples by masturbation after 2–7 days of abstinence. All participants were without leukocytospermia or reproduction tract infection. To eliminate the influence of age on the results, in subjects selection we ensured no significant difference in age between healthy controls and asthenozoospermia patients (32.2 ± 5.5 years VS. 32.4 ± 4.5 years, $p = 0.901$). We defined asthenozoospermia as forward progression (progressive motility and non-progressive motility) sperm $<40\%$ and healthy control as forward progression sperm $\geq 40\%$ or progressive motility sperm $\geq 32\%$. Fresh semen samples were incubated at 37°C for 30 min to allow for liquefaction before further processing.

Sperm purification and RNA extraction. Human sperm was purified from 2–3 mL of liquefied semen samples using density gradient centrifugation²⁶. Briefly, the sample was transferred to a 15 mL conical tube (RNase-Free), and then 6–10 mL (0.01 mol/L) phosphate-buffered saline (PBS) was added and the tube inverted several times to wash the sperm. After centrifugation at $300 \times g$ for 15 min at 4°C , the supernatant was discarded and the sediment was resuspended in 1 mL of PBS. The sample was carefully overlaid onto a two-layer (40–80%) Percoll gradient to avoid disturbing the interface. Following another centrifugation at $400 \times g$ for 20 min, the supernatant was discarded and the sperm pellet was resuspended in 2 mL of PBS. To confirm the purity of spermatozoa preparation, the sperms were examined by microscope (Olympus, Japan) after purification, and no lymphocytes, epithelial cells or bacteria was found under 5 microscopic vision fields ($400\times$) (Fig. S3). Then the sperm RNA was extracted from the purified sperm suspension in a biosafety cabinet using a commercial RNeasy Pure Cell/Bacteria Kit (Qiagen, Beijing, China) according to the manufacturer's instructions. RNA was quantified by spectrophotometry (ND-2000, NanoDrop Inc., USA).

Oligonucleotides. A 15-mer m⁶A-containing oligonucleotide was synthesized according to a previously described method⁴⁰. The 10-mer RNA (5'-AUCUAUAUGC-3') was purchased from Takara Biotechnology Co., Ltd. (Dalian, China).

Enzymatic digestion of RNA. RNA (200 ng) was first denatured by heating at 95°C for 5 min and then chilling on ice for 2 min. After adding 1/10 volume of S1 nuclease buffer (30 mM CH_3COONa , pH 4.6; 280 mM NaCl; 1 mM ZnSO_4) and 150 units of S1 nuclease, the mixture (20 μL) was incubated at 37°C for 4 h. The solution was subsequently added to 1/10 volume of alkaline phosphatase buffer (50 mM Tris-HCl, 10 mM MgCl_2 , pH 9.0), 0.002 units of venom phosphodiesterase I and 15 units of alkaline phosphatase. Then, the incubation was continued at 37°C for an additional 2 h followed by extraction with an equal volume of chloroform twice. The resulting aqueous layer was collected and lyophilized to dryness and reconstituted in 100 μL of water. Next, 30 μL of the obtained samples was subjected to liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis.

Measurement of m⁶A content by LC-ESI-MS/MS. Analysis of nucleosides was performed on the LC-ESI-MS/MS system consisting of an AB 3200 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization source (TurboIonSpray) and a Shimadzu LC-20AD HPLC (Tokyo, Japan) with two LC-20AD pumps, a SIL-20A autosampler, a CTO-20AC thermostatic column compartment, and a DGU-20A3 degasser. Data acquisition and processing were performed using AB SCIEX Analyst 1.5 Software (Applied Biosystems, Foster City, CA). The HPLC separation was performed on a Hisep C18-T column (150 mm × 2.1 mm i.d., 5 µm, Weltech Co., Ltd., Wuhan, China) with a flow rate of 0.2 mL/min at 35 °C. Formic acid in water (0.1%, v/v, solvent A) and formic acid in methanol (0.1%, v/v, solvent B) were employed as the mobile phase. A gradient of 5 min 5% B, 10 min 5–30% B, 5 min 30–50% B, 3 min 50% B–5% B and 17 min 5% B was used.

The mass spectrometry detection was performed under positive electrospray ionization mode. The target nucleosides were monitored by multiple reaction monitoring (MRM) mode using the mass transitions (precursor ions → product ions) of m⁶A (282.2 → 150.1), rA (268.4 → 136.2), rU (245.4 → 113.1), rC (244.4 → 112.2), rG (284.5 → 152.2), dA (252.4 → 136.2), T (243.3 → 127.2), dC (228.4 → 112.2), dG (268.4 → 152.4). The MRM parameters of all nucleosides were optimized to achieve maximal detection sensitivity.

Quantification of mRNA expression by real-time PCR. For mRNA quantifications of *FTO*, *ALKBH5*, *METTL3*, *METTL14*, *WTAP* and *YTHDF2*, cDNA was synthesized by DNase treatment and reverse transcription (TOYOBO, Osaka, Japan) and real-time PCR was performed on a CFX96 Touch TM Real-Time PCR Detection System (BioRad) with iTaq TM Universal Supermixes (BioRad) and intron-spanning primers, which are listed in Table S5. The mRNA levels of the target genes were normalized to that of the reference gene *GAPDH*, and the results are expressed as the means ± SEM.

Statistical analyses. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, USA). A normality test was used to explore the data distribution, $p > 0.05$ was considered to be normal distributed. Two-tailed Student's *t* test and Mann-Whitney *U* test were used to compare the differences in normal and non-normal distributed data between the two groups, respectively. The correlations of m⁶A contents with gene expression and semen parameters were assessed by Pearson correlation coefficient for normal distributed data and Spearman correlation coefficient for non-normal distributed data. Logistic regression analysis was used to evaluate whether m⁶A contents associated with asthenozoospermia risk. $p < 0.05$ was considered to be statistically significant.

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Author Contributions

Y.Y., Y.-Q.F., B.-F.Y. and S.-M.L. designed research; Y.Y., W.H., J.-T.H., F.S. and E.-F.Y. conducted research; M.Z., J.-T.H. and S.S.-Q. collected the semen samples and clinical informations; W.H. and J.X. detected m⁶A contents; Y.Y., W.H., B.-F.Y. and S.-M.L. prepared the initial manuscript draft; Y.Q.-F., B.-F.Y. and S.-M.L. edited and revised subsequent drafts; S.-M.L. had primary responsibility for final content.

Additional Information

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