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Research Article
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Research Article

Hypoxia-induced alteration of RNA modifications in the mouse testis and sperm‡

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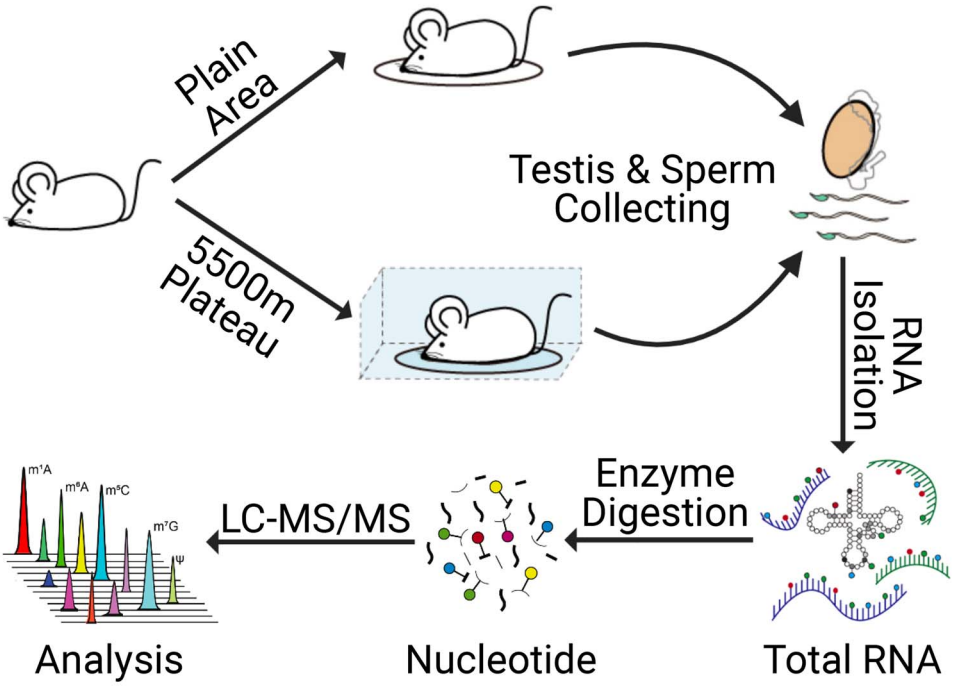
Abstract

Hypobaric hypoxia as an extreme environment in a plateau may have deleterious effects on human health. Studies have indicated that rush entry into a plateau may reduce male fertility and manifest in decreased sperm counts and weakened sperm motility. RNA modifications are sensitive to environmental changes and have recently emerged as novel post-transcriptional regulators in male spermatogenesis and intergenerational epigenetic inheritance. In the present study, we generated a mouse hypoxia model simulating the environment of 5500 m in altitude for 35 days, which led to compromised spermatogenesis, decreased sperm counts, and an increased sperm deformation rate. Using this hypoxia model, we further applied our recently developed high-throughput RNA modification quantification platform based on liquid chromatography with tandem mass spectrometry, which exhibited the capacity to simultaneously examine 25 types of RNA modifications. Our results revealed an altered sperm RNA modifications signature in the testis (6 types) and mature sperm (11 types) under the hypoxia model, with 4 types showing overlap (Am, Gm, m^7G, and m^2_2G). Our data first drew the signature of RNA modification profiles and comprehensively analyzed the alteration of RNA modification levels in mouse testis and sperm under a mouse hypoxia model. These data may be highly related to human conditions under a similar hypoxia environment.

Summary sentence

Using high-throughput LC–MS/MS, our study revealed an altered signature of RNA modifications in the mouse testis and sperm under a hypoxia model. These data may be strongly related to human conditions in a similar hypoxia environment.

Graphical Abstract



Key words: RNA modification, hypoxia, testis, sperm.

Introduction

The plateau region has extreme environmental conditions such as hypobaric hypoxia, high-altitude, and intensive ultraviolet radiation, which pose great challenges to human survival. Rush entry into the plateau region and short-term exposure to a high-altitude environment will induce the human altitude sickness reaction and reproductive defects. Previous studies have investigated the effect of hypoxia at a high-altitude on semen quality in humans [1] and spermatogenesis in mice and rats [2–5], and found that hypoxia exposure has a deleterious effect on the human male reproductive system [6], including decreased serum reproductive hormone levels and declined sperm count, sperm density, motility, and survival rate with an infertility syndrome. Molecular mechanism studies have indicated that high-altitude exposure and hypoxia may induce sperm nuclear DNA damage and alter sperm mtDNA copy numbers, thus leading to the spermatogenesis defect and impaired sperm motility. However, whether another type of molecular signaling is involved in hypoxia-induced male reproductive injury remains to be clarified.

RNA modifications have been known to play pivotal roles in RNA structure formation and RNA function performance during holistic biological processes such as mRNA localization, stability, splicing, and mRNA translation [7], and are critical in post-transcriptional regulation during gene expression and embryo development. Recently, researchers found that mRNA m^6A (methylation at the N6 position of adenosine) modification mediated by methyltransferase like 3/methyltransferase like 14 (Mettl3/Mettl14) was involved in mammalian spermatogenesis by regulating spermatogonial differentiation and meiosis initiation via impacting RNA metabolism [8, 9]. m^6A demethylase alkB homolog 5 (ALKBH5) defection also can impair mRNA export and cause the mRNA processing factor to assemble in nuclear speckles, leading to spermatocyte apoptosis and male infertility [10, 11]. There are other modifications such as m^5C (methylation at the C5 position of cytidine), mediated by NOP2/Sun RNA methyltransferase 2 (NSun2) are also related to the fertility and deletion of NSun2 specifically blocked meiotic progression of germ cells into the pachytene stage [12]. However, the function of other types of RNA modification in spermatogenesis and the male reproductive system remains unclear. Our previous studies showed that mouse sperm RNA contained various types of RNA modifications, and m^5C and m^2G (methylation at the N2 position of guanosine) modifications were elevated in sperm 30–40 nt RNA fractions under paternal high-fat diet (HFD) conditions [13, 14], which can serve as an epigenetic information carrier to transmit paternally acquired metabolic disorder to the next generation, along with sperm sncRNA alteration under HFD [14–16]. These results suggested that RNA modification in the male reproductive system may be sensitive to environmental stress. Hence, by using our previously established high-throughput RNA modification detection platform, we comprehensively revealed the alteration of RNA modification profiles in mouse testis and sperm under hypoxia exposure, indicating that RNA modification may be crucial for cellular response to hypoxia environmental exposure in the male reproductive system.

Materials and methods

Animals

Male C57BL/6J mice at 7 weeks old were purchased from the Laboratory Animal Center of Chongqing Medical University. Mice were maintained under optimal conditions for a temperature of 23 ± 3°C,

relative humidity of 40–70% with a 12 h light/12 h dark cycle, and fed with food and water *ad libitum*. After 1 week to acclimatize to the environment, mice were randomly divided into normoxic control group and hypoxia-exposed group. Mice in the hypoxic group were raised in a hypobaric chamber, which was specifically produced by GuizhouFenglei Air Ordnace Co. Ltd (Guangdong, China). After mice were transferred into the chamber, the pressure adjusting device was started to decrease the air pressure by steps of 180 m/min simulated altitude changes. About 30 min later, the oxygen level inside the hypobaric chamber was about $PO_2 = 10.68$ KPa, to simulate a high-altitude of 5500 m above sea level. The oxygen level in the hypobaric chamber was decreased to simulate a high-altitude of 3000 m when clean bedding, food, and water were replaced within 20 min twice a week during 5 weeks of treatment. The mouse caretakers got into the hypobaric chamber through a connected buffer chamber whose air pressure was able to be adjusted up to the same level as the hypobaric chamber. Testis and sperm samples were isolated separately for morphological observation, sperm quality evaluation, and RNA extraction at the end of treatment. All animal experimental manipulations were approved by the Ethics Committee of Chongqing Medical University.

Testis morphological observation

The testis was collected and fixed in 10% neutral buffered formalin for paraffin embedding. Sections were stained with hematoxylin and eosin for light microscopic observations (BX63, Olympus Corporation, Japan).

Sperm concentration and morphological observation

The sperm were released from the cauda epididymis and vas deferens into 2-ml normal saline (NS) and incubated at 37°C for 10 min. After blending gently, 10 µl of the sample were placed in a counting chamber. Sperm concentration and deformation rate were assessed using a computer-assisted sperm assay (SCA CASA System; Microptic S.L., Barcelona, Spain).

The rest of the sperm were centrifuged at 600 g for 5 min, suspended, and adjusted to 2×10^5 cells/ml with NS. One milliliter of cell suspension was centrifuged at 500 g for 5 min by Cell Concentrator (CytoPrep, YingTai, Changsha, China) to place the sperm onto slides. After being fixed in 4% paraformaldehyde and stained with hematoxylin and eosin, sperm observation and image collection were performed under a microscope (BX63, Olympus Corporation, Japan). Sperm deformation was classified into the following abnormalities: blunt hooks, two heads, round heads, two tails, folded tails, and short tails, according to previous studies [17].

Isolation of total RNA

The sperm were collected as previously described. Sperm suspension was filtered with a 38-µm cell strainer to remove tissue debris. The sperm were treated with a somatic cell lysis buffer (0.1% SDS, 0.5% Triton 100× in nuclease-free H_2O) for 40 min on ice to eliminate somatic cell contamination, pelleted by centrifugation at 600 g for 5 min, and then the sperm pellet was resuspended and washed in a 10 ml PBS and centrifuged at 600 g for 5 min twice for RNA isolation procedure.

Total RNA was extracted with a Trizol reagent (No. 15596026; Invitrogen; Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol. One-milliliter Trizol was added to a microtube with pulverized testis tissue or sperm and vortexed vigorously. Then, a sample was incubated at room temperature for 5 min. Next, 200-µl chloroform was added to the samples, vortexed 15 s, and then incubated for 10 min at room temperature, and centrifuged at 12 000 g for 15 min at 4°C. The aqueous phase was collected into a microtube and combined with an equal volume of isopropanol. After gently mixing and incubating it at −80°C for 30 min, the mixture was centrifuged at 12 000 g for 15 min at 4°C. The final RNA pellets were resuspended with RNase-free water for RNA concentration measurement by UV spectrometer.

Detection of modified nucleosides in RNA molecules by LC–MS/MS

Purified RNAs (50–100 ng) were incubated with 5-U/ul alkaline phosphatase (no. P5521, Sigma Chemica; Merck KGaA, Darmstadt, Germany), 0.01-U/ul phosphodiesterase I (no. 20240Y; Biological; US), and 5-U/ul benzonase (No. E8263; Sigma; Merck KGaA, Darmstadt, Germany) in a microtube at 37°C for 3 h. Then, the enzymes in the mixture were removed by centrifugation using 3K devices with Omega Membrane (Pall Nanosep). Mass spectrometry analysis was performed on a Xevo-TQ-S mass spectrometer connected to an Acquity-UPLC I-class system (Waters Corporation, America) and equipped with an electrospray ionization source. The MS system was operated in positive ion mode using a multiple reaction monitoring (MRM) scan model, except for pseudouridine, which was operated in negative ion mode. Liquid chromatography with tandem mass spectrometry (LC–MS/MS) data were acquired and processed by MassLynx (version 4.1) software. The percentage of each modified ribonucleoside was normalized to the total amount of quantified ribonucleosides with the same nucleobase. For example, the percentage of $m^2{}_2{}^7G$ = mole concentration ($m^2{}_2{}^7G$)/mole concentration ($m^2{}_2{}^7G + Gm + G + m^1G + m^2G + m^2{}_2G + m^7G$).

Statistical analysis

Data are presented as the mean ± SEM, and each experiment was repeated at least three times. Statistical analyses were conducted using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The data were analyzed by an independent-sample student *t*-test. Differences were considered statistically significant if $P < 0.05$.

Results

Hypoxia exposure induced histological alteration in mice testis

We first established the hypoxic model of mice. As shown in Figure 1A, mice were raised in a hypobaric chamber, and air pressure and oxygen concentration were simulated to a plateau with a 5500-m altitude for 35 days. Then, the mouse testis and sperm were collected promptly. The testis of hypoxia group appeared slight hyperemia with an increased weight (Figure 1B), but there is no significant difference compared to the control (Figure 1C). In a seminiferous tube, the arrangement of spermatogenic cells was disordered, spermatogonia appeared to slough into the lumen, and the number of mature sperm was decreased. Moreover, the luminal boundary was degraded and lacked the organized clusters of maturing germ cells that normally characterize the luminal border of the seminiferous epithelium. The subepithelial basal membrane partly became unclear or discontinuous (Figure 1D).

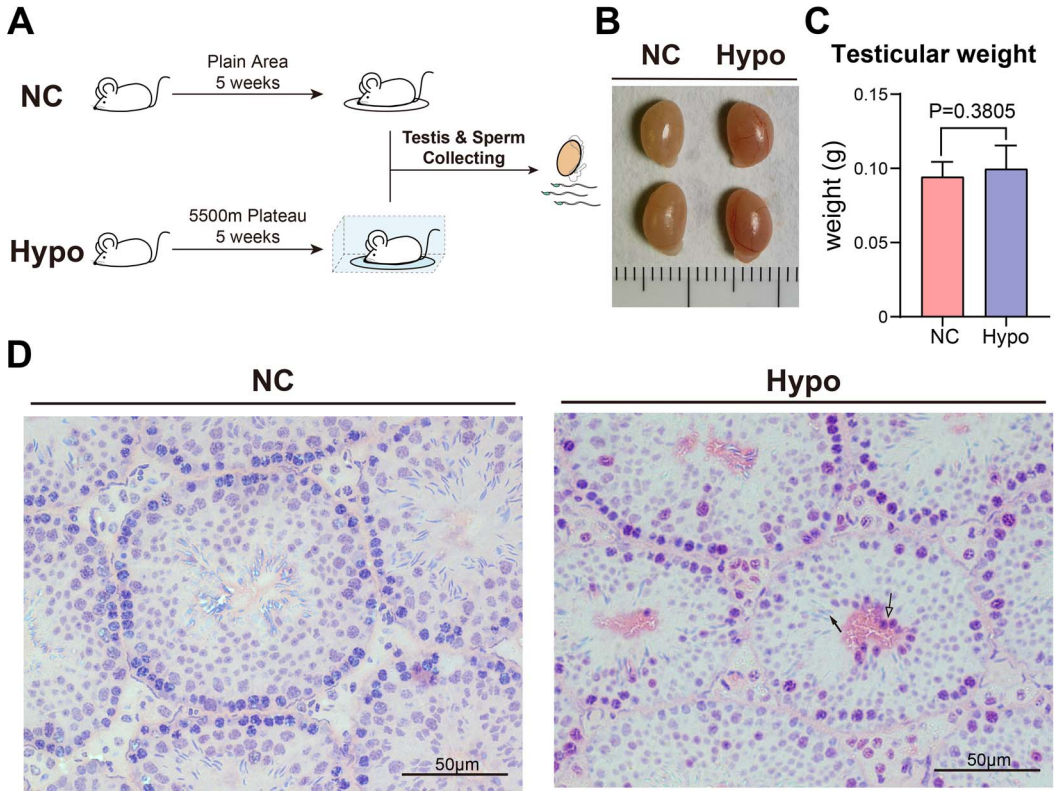


Figure 1. Hypoxia-induced testis injury in the hypoxia mouse model. (A) Schematic illustration of hypoxia exposure to the simulated high-altitude plateau (5500 m) environment and sample collection in mice. (B) Morphological observation of testis. Hyperemia and swelling in the hypoxia group. (C) Testicular weight in hypoxia and normoxia groups, there is no significant difference between the two groups. The *P*-value was determined by two-tailed student *t*-test, two tails. $n = 10$. Data are presented as mean ± SEM. (D) In H&E-stained sections from the hypoxic testis, degenerated germ cells sloughed out into the lumen were visible (hollow arrow), and the number of mature sperm was decreased (solid arrow).

Hypoxia exposure resulted in decreased sperm counts and increased sperm deformation

Given the compromised spermatogenesis as evidenced by histological findings, we further performed morphological observation and sperm count evaluation. The concentration of sperm released from the hypoxia cauda epididymis and the vas deferens was dramatically decreased in the hypoxia group compared with the normoxia group (28.9% vs. 38.6%, $P = 0.0368$, Figure 2A and B). Head and tail malformations were found in both the hypoxia and normoxia condition; however, head malformation was predominant in the hypoxia group (Figure 2C). The deformation rates of sperm were 26.3% in the Hypo group vs. 17.5% in the NC group, and the difference was significant ($P < 0.0001$, Figure 2D).

Alteration of RNA modification levels in mouse testis under hypoxia exposure

To investigate whether RNA modification was involved in the cellular response to hypoxic exposure, the total RNA modification profiles of the mouse testis were determined by using our previously developed high-throughput RNA modification detection platform based on LC–MS/MS, which can simultaneously examine 25 types of RNA modifications (Figure 3A). Then, 19 types of RNA modifications were detected and quantified, and the levels of 6 of them have altered. Am, ac4C, Gm, m^7G, and $m^2{}_2G$ had steadily increased (Figure 3B–F), whereas m^6A was significantly decreased (Figure 3G), and other types were unchanged by hypoxia exposure (Figure 3H–T).

Alteration of RNA modification levels in mouse sperm under hypoxia exposure

Previous studies confirmed that sperm RNA harbored abundant modifications in mice. In the present study, 15 of the 25 types of sperm RNA modifications signatures were detected and quantified, and a majority of sperm RNA modifications were markedly altered (Figure 4A–K), with only 4 types of RNA modification (m^6A, ac4C, m^5C, and Ψ) being unregulated in the hypoxia mouse model (Figure 4L–O). Furthermore, there were 4 types of RNA modification, Am, Gm, m^7G, and $m^2{}_2G$, showing an overlap in the testis and mature sperm of mice exposed to the hypoxia environment.

Discussion

Previous studies have revealed that hypobaric hypoxia-induced evident changes in testicular morphology and impaired spermatogenesis in adult and adolescent rats using a hypobaric chamber simulating a high-altitude of ∼5000 m. Moreover, exposing rats to hypoxic conditions at the pre-puberty phase when sperm formation had not started caused damage to spermatogenesis, which could affect sperm production after sex maturity [18]. Increased testicular temperature and vascularization in hypoxic rat testis has been found to be a local

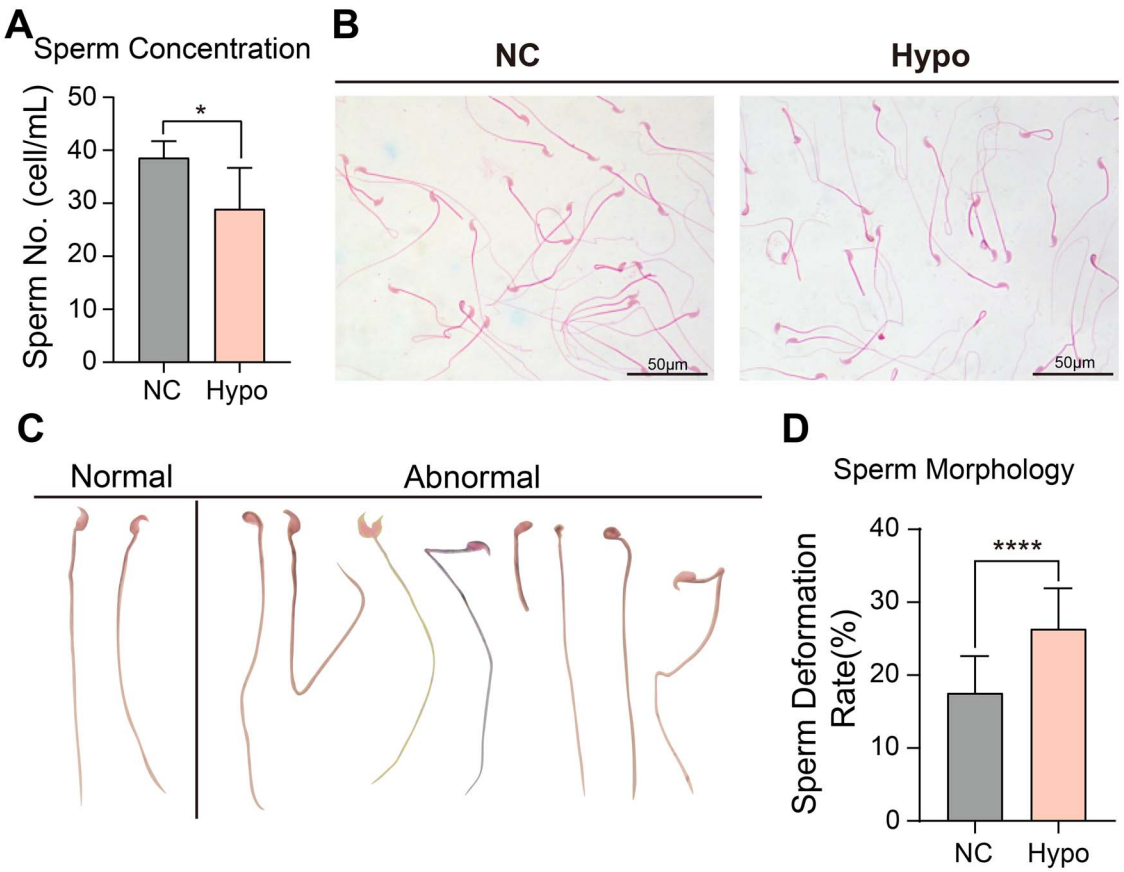


Figure 2. Hypoxia-induced sperm injury in the hypoxia mouse model. (A) Sperm concentration from the cauda epididymis and vas deferens in hypoxia and normoxia group. (B) H&E staining of mouse sperm. Scale bar, 50 μm. (C) Representative images of deformed sperm. (D) Sperm deformation rate. The *P*-value was determined by two-tailed student *t*-test, two tails, $^{*}P < 0.05$, $^{****}P < 0.0001$, $n = 3$. Data are presented as mean ± SEM.

mechanism responsible for a decrease in spermatogenic cell production [19]. In addition, hypoxia inhibited spermatogenesis in rats via decreasing tetraploid spermatogenic cells, which might contribute to the apoptosis of primary spermatocytes and spermatogonia [2].

In this study, we found that hypoxia caused certain damage to the mouse testis and sperm. In general, the histological changes of mouse testis under hypoxia were not as obvious as that of rats [2, 19]. Slight hyperemia and swelling of the testis were observed, but there is no statistical difference of testis weight between two groups. An array of spermatogenic cells was disordered, and some spermatogonia sloughed into the lumen of the seminiferous tube. Moreover, the sperm count significantly decreased and the deformation rates of mature sperm dramatically increased, which is consistent with the results of previous studies [17, 20], indicating the impaired male productive system of mice under hypoxia condition. Vacuolation in the seminiferous epithelium and increase of blood vessels in interstitial tissue steadily shown in the hypoxic rat testis was not obvious in mice [2, 19], which may be related to the differences in the adaptation and responsiveness of species to hypoxia. In summary, our histological observation in the hypoxia-exposed mice seems to be milder than previous reports on rats [2, 19]. It is speculated that hypoxia may induce function impairment rather than apparent tissue damage in mice after exposing to hypoxia.

Spermatogenesis is a highly complex process involved in mitotic and meiosis cell division, in which unique and extensive chromatin and epigenetic modifications occur to bring about specific epigenetic profiles in spermatozoa [21]. Previous studies showed that RNA modifications in sperm were sensitive to environmental changes and could serve as novel post-transcriptional regulators in male spermatogenesis and epigenetic information carriers that transmit paternally acquired phenotypes to the next generation [14, 15, 22–26]. However, the profiles of RNA modification in impaired spermatogenesis caused by hypoxia are unknown to date.

Using the hypoxia mouse model, we further applied our developed high-throughput RNA modification quantification platform based on LC–MS/MS and revealed alterations of the RNA modification signature in the testis and mature sperm. Based on published paper [14], 25 types of RNA modifications examined in this study are more relevant to mammalian species especially on fertility. Six types of RNA modifications (Am, ac4C, Gm, m^7G, $m^2{}_2G$, and m^6A) were significantly regulated in the hypoxic testis; furthermore, more types of RNA modifications (m^1A, m^3C, m^1G, m^2G, $m^2{}_2G$, $m^2{}_2{}^7G$, m^7G, Am, Um, Cm, and Gm) were dramatically altered in hypoxic mature sperm. Interestingly, 4 types of RNA modifications (Am, Gm, m^7G, and $m^2{}_2G$) showed the overlap in the hypoxia testis and mature sperm. The direct roles of all these altered RNA modifications in hypoxia-treated male productive systems remain unclear. It was recently reported that testicular injury in rats, which is induced by DEHP, the environmental endocrine-disrupting chemical, was related to increased m^6A RNA modification of antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) [27]. The altered levels of RNA modification provided another clue

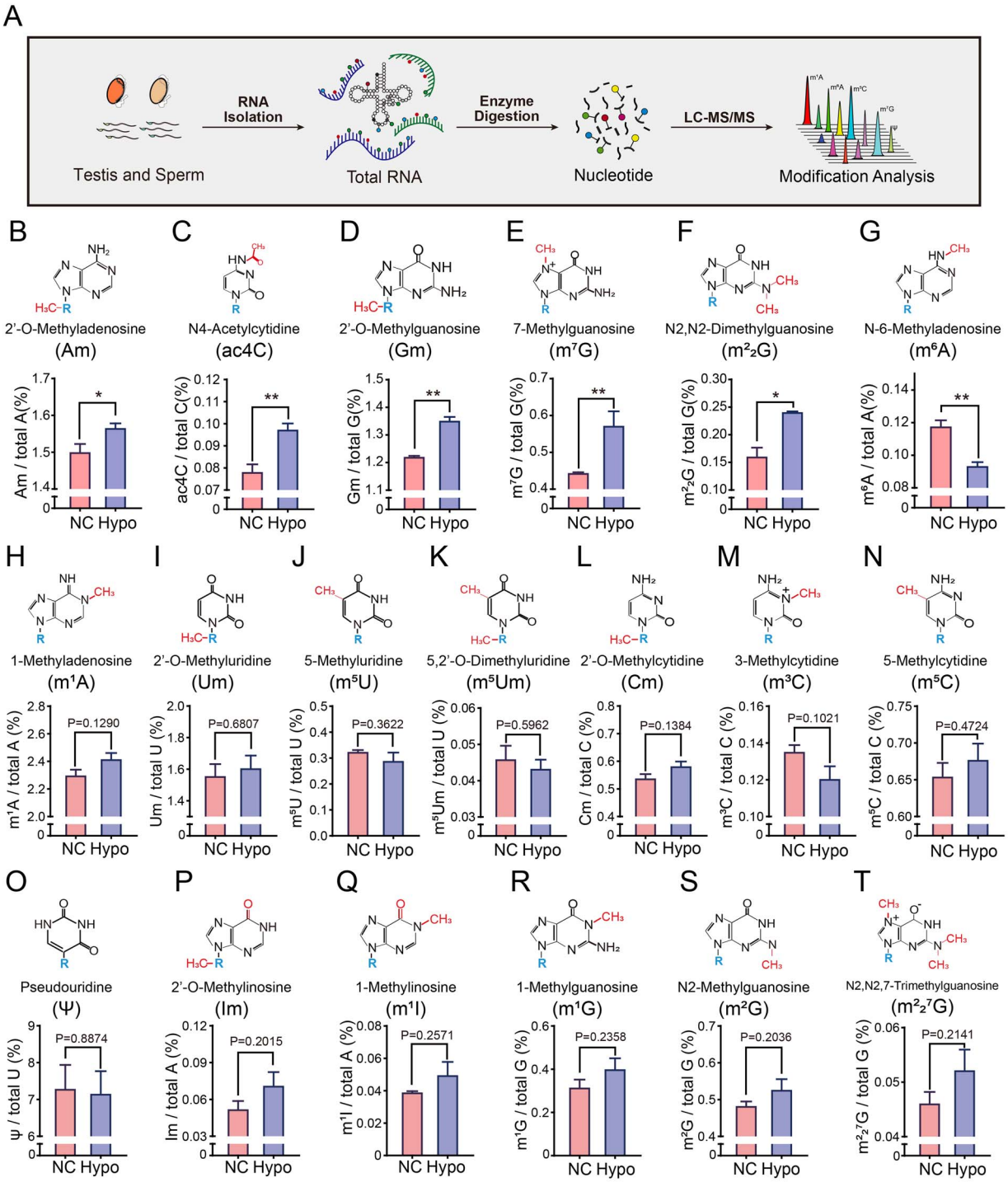


Figure 3. Hypoxia-induced alteration of RNA modification levels in the testis total RNAs in the hypoxia mouse model. (A) Illustration of total RNA modification detection by LC–MS/MS. (B–T) Nineteen types of RNA modifications were detected and quantified in the mouse testis under hypoxia exposure. The levels of RNA modification for Am, ac4C, Gm, m^7G, $m^2{}_2G$, and m^6A were significantly altered (B–G) with other 13 types of RNA modification unregulated (H–T). The *P*-value was determined by the two-tailed student *t*-test, two tails, $^*P < 0.05$, $^{**}P < 0.01$, $n = 3$. Data are presented as mean ± SEM.

to explain impaired spermatogenesis caused by hypoxia, indicating a new direction for future investigation of its mechanism. Native residents at high-altitudes had a similar fertility rate to populations in the plains [28]. However, newcomers into the plateau displayed a weaker ability to reproduce [1]. Whether RNA modifications are involved in adaption to the hypoxic environment or a stress reaction needs to be further explored.

In summary, our data demonstrated that hypoxia-induced impaired spermatogenesis in mice, which is associated with altered RNA modification profiles in testis and sperm. Data obtained here

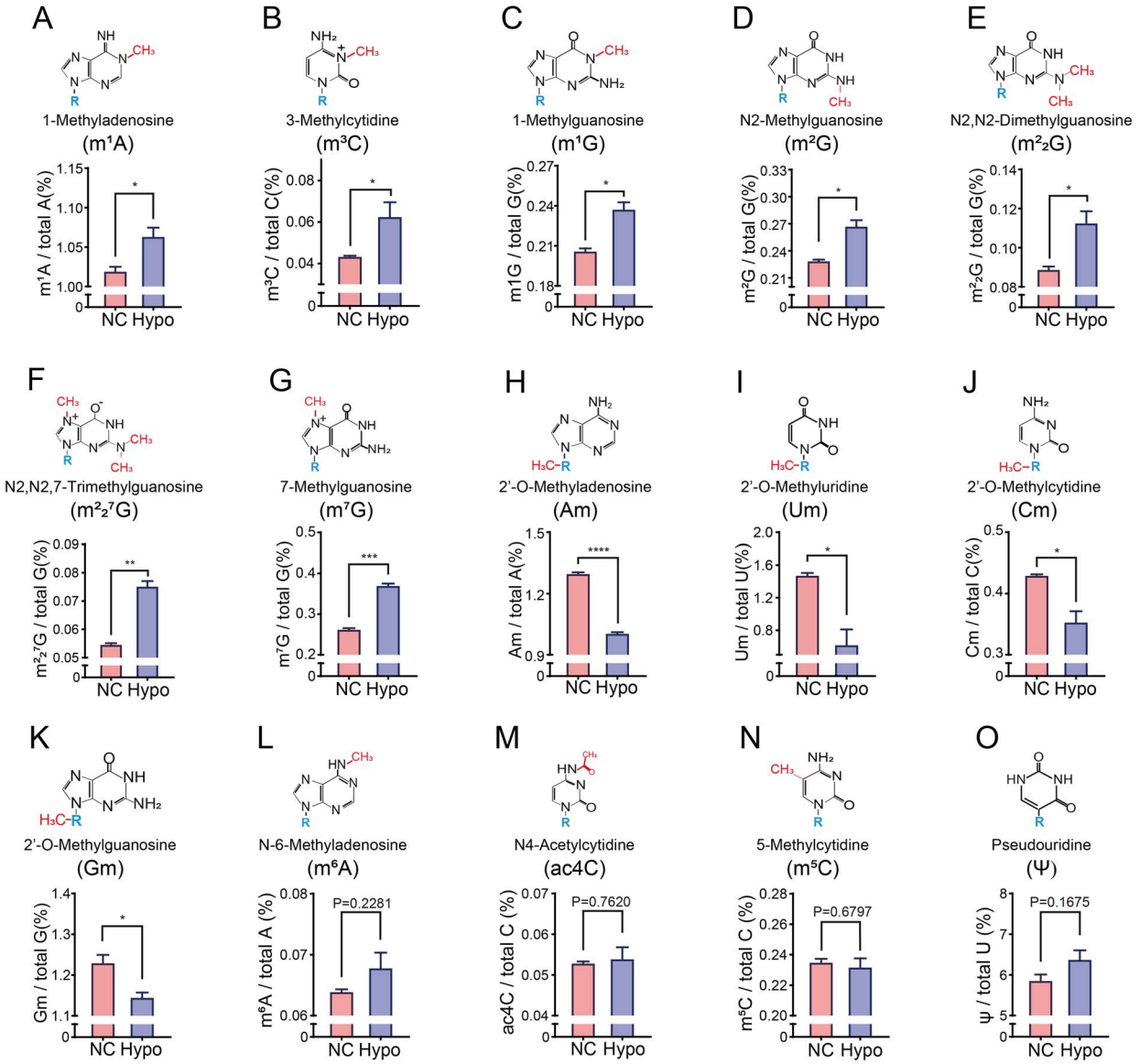


Figure 4. Hypoxia-induced alteration of RNA modification levels in sperm total RNAs in the hypoxia mouse model. (A–O) 15 types of RNA modifications were detected and quantified in mature mice sperm under hypoxia stress. The levels of RNA modification for m^1A, m^3C, m^1G, m^2G, $m^2{}_2G$, $m^2{}_2{}^7G$, m^7G, Am, Um, Cm, and Gm were dramatically altered (A–K), and relative levels of m^6A, ac4C, m^5C, and Ψ were unregulated (L–O). The *P*-value was determined by the two-tailed student *t*-test, two tails, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, $n = 3$. Data are presented as mean ± SEM.

may lay a foundation for future exploration on the role of individual RNA modification in the male productive system under hypoxia and provide a new layer of information in understanding the molecular impact of hypoxia in male reproductive health. In addition, the detected alterations of certain RNA modifications under hypoxia may also lead to future studies relating to their regulatory enzymes.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author's contributions

Y.T., Y.Z., and D.T. conceived of the idea and designed the experiments. T.H. and H.G. performed the mice experiment with the help of Y.X, D.C., and T.H. H.G. performed RNA isolation experiment with the help of X.S, X.J., and L.X. X.S. performed LC–MS/MS and data analysis with the help of H.G. and X.W. Y.Z, H.G., and T.H. wrote the manuscript with the supervision of Y.T., and D.T. and Y.T. revised the manuscript. All authors agreed to the final manuscript.

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Conflict of interest: The authors have no conflicts of interest to disclose.

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