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ORIGINAL ARTICLE ***Reproductive biology***

Taurine and its transporter TAUT positively affect male reproduction and early embryo development

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STUDY QUESTION: Are taurine and its transporter TAUT associated with spermiogenesis and early embryo development?

SUMMARY ANSWER: Morphologically abnormal spermatozoa increased after local functional interference by intratesticular injection, and taurine depletion significantly reduced the normal embryo numbers *in vivo* and blastocyst formation rate *in vitro*.

WHAT IS KNOWN ALREADY: Taurine is one of the most abundant amino acids in the male reproductive system and it has been demonstrated that taurine can efficiently improve spermatogenic function in rat models of testicular injury. However, limited information is known about the role of taurine and its transporter TAUT in spermatogenesis and early embryo development.

STUDY DESIGN, SIZE, DURATION: Clinical characteristics from 110 couples who have experienced recurrent pregnancy loss (RPL) were collected from December 2014 to March 2018. According to whether a fetal heartbeat was seen in the previous pregnancy under ultrasonic monitoring, patients with RPL were divided into two groups: an RPL without heartbeat (pregnancy with no fetal heartbeat, ROH) group, and an RPL with heartbeat (one or more pregnancies with fetal heartbeat, RWH) group. Semen samples (21 ROH and 20 RWH) were finally used for metabolomic analysis. Furthermore, semen samples were obtained from 30 patients with teratozoospermia (normal sperm morphology <4%) seeking evaluation for infertility and 25 age-matched control subjects with normal semen quality for western blotting. Animal experiments were performed in CD-1/ICR mice.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Metabolomics was performed to determine the metabolic changes between the ROH and RWH groups. Sperm proteins from patients with teratozoospermia and healthy controls were extracted for detecting TAUT expression using western blot analysis. Immunofluorescence was used to characterize the localization of TAUT in the testis and ejaculated spermatozoa. Functional analysis in mice was performed by intratesticular injection of siRNAs or antagonist (β-alanine) and 5% β-alanine was provided in drinking water to 3-week-old male mice for 5 weeks with the aim of depleting taurine. Murine epididymal spermatozoa were stained with hematoxylin and eosin for morphological assessment. IVF and mating tests were performed in mice for assessing fertility.

MAIN RESULTS AND THE ROLE OF CHANCE: Metabolomic analysis demonstrated that the taurine content was lower in spermatozoa but higher in seminal plasma from the ROH than the RWH group. TAUT expression was lower in spermatozoa from patients with teratozoospermia than controls. Immunofluorescence showed that TAUT was localized to the manchette in mouse elongated spermatids functional analysis showed that morphologically abnormal spermatozoa increased after interference, and this defect increased after supplementation with 5% β-alanine but was improved by 5% taurine supplementation. Supplementation with 5% β-alanine significantly reduced the normal embryo number in the mouse uterus as well as blastocyst formation rate *in vitro*.

LARGE SCALE DATA: N/A

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LIMITATIONS, REASONS FOR CAUTION: The sample size was low and larger cohorts are needed to confirm the positive effect of taurine on human sperm quality. A comprehensive safety examination should be performed to evaluate whether taurine is a possible treatment for teratozoospermia. Furthermore, the specific molecular mechanism of TAUT involvement in spermiogenesis remains to be clarified.

WIDER IMPLICATIONS OF THE FINDINGS: The study provides new insights into the role of taurine and its transporter TAUT in male reproduction and embryo development. The results also indicate that TAUT is a promising molecular candidate for the assessment of sperm quality, which may contribute to the diagnosis and treatment for teratozoospermia.

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Key words: recurrent pregnancy loss / teratozoospermia / taurine / taurine transporter / spermiogenesis / embryo development

Introduction

Recurrent pregnancy loss (RPL) is defined by the American Society for Reproductive Medicine as two or more pregnancy losses that are influenced by many factors and that seriously threatens pregnant women's physical and mental health (Practice Committee of the American Society for Reproductive Medicine, 2020), however, its etiology has not been clarified. Maternal factors, chromosomal abnormalities, immune diseases and endocrine problems are important factors affecting RPL (Khalife *et al.*, 2019), but little attention has been paid to male factors. Recent evidence suggests there is an association between male factors and RPL (McQueen *et al.*, 2019; Haddock *et al.*, 2021; Khambata *et al.*, 2021; Klimczak *et al.*, 2021). Our previous study found that decreased semen quality may increase the risk of unexplained recurrent miscarriage, and numbers of abnormal spermatozoa in RPL male partners were higher than that of healthy fertile men (Zhang *et al.*, 2020).

Metabolomics offers new opportunities for a better understanding of the pathological mechanisms of male infertility. An increasing number of metabolites have been found to be significantly correlated with sperm parameter values and male infertility. Metabolomic analysis of seminal plasma shows that L-carnitine and acetyl-L-carnitine levels are positively correlated with sperm concentration and sperm deformity (Xu *et al.*, 2020), which is consistent with another study on severe oligoasthenozoospermia (Boguenet *et al.*, 2020). Several studies have confirmed that an increase in the carnitine concentration in the epididymal lumen is closely related to sperm motility and L-carnitine administration may effectively improve reproductive competence (Cabral *et al.*, 2018; Jeulin and Lewin, 1996). More recently, metabolomic studies have confirmed there is a strong correlation between energy metabolism and sperm function, multiple metabolites related to the citrate cycle are disrupted in infertility and play an important role in the normal process of spermatogenesis (Zhang *et al.*, 2017; Murgia *et al.*, 2020; Xu *et al.*, 2020).

Taurine, one of the most abundant amino acids in the male reproductive system, has various functions including antioxidant, anti-apoptosis, osmotic adjustment and calcium regulation (Lambert *et al.*, 2015). Accumulating studies have demonstrated that taurine can efficiently improve spermatogenic function in testicular injury models (Liu *et al.*, 2017; Adedara *et al.*, 2018; Ghosh *et al.*, 2019; Abd-Elhakim *et al.*, 2020). Taurine is ubiquitously distributed, and its concentrations are much higher in tissues (such as retina, neurons and muscles) than in body fluids (such as plasma and cerebrospinal fluid), which may closely relies on the high-affinity taurine transporter TAUT (Seidel *et al.*, 2019). Previous studies have shown that taurine is abundant in the testes, epididymis, spermatozoa and seminal plasma (Velazquez *et al.*, 1986; Holmes *et al.*, 1992; Lobo *et al.*, 2000), and the concentration of taurine in the epididymis gradually increases from epididymal caput to cauda (Xu *et al.*, 2003). No key enzymes for taurine synthesis have been found in spermatozoa, hence transporting taurine through TAUT is necessary for spermatozoa to take up sufficient taurine to maintain its biological function (Huxtable and Lippincott, 1982; Li *et al.*, 2006). In Japanese eel testes, taurine and its transporter TAUT are involved in the initiation of germ cell meiosis (Higuchi *et al.*, 2013). Furthermore, adding taurine to culture medium can effectively improve sperm quality and promote early embryo development in many species, including mouse and human embryos (Devreker *et al.*, 1999; Guerin *et al.*, 2001; Brugnon *et al.*, 2013). Recent studies have found that the knockout of taurine-upregulated gene 1 (*Tug1*) leads to infertility in male mice, characterized by reduced epididymal sperm count and increased sperm deformity (Lewandowski *et al.*, 2020).

The aim of this study was to clarify the functional role and potential mechanisms of action of taurine and TAUT in spermatogenesis and early embryo development. The beating of the original human heart tube is generally observed under ultrasound at 6–8 weeks of pregnancy, while recurrent miscarriages with poor embryo quality and no fetal heartbeat occur in earlier stages of pregnancy. In order to explore the potential male etiology of early pregnancy loss without fetal heartbeat, we categorized patients according to whether or not a fetal heartbeat appeared in previous pregnancies under ultrasound monitoring. We found that abnormal sperm numbers in the RPL without heartbeat (ROH) group were significantly higher than that in the RPL with heartbeat (RWH) group. This suggests that male factors could play an important role in early embryo development. Metabolomics was performed to clarify the effect of sperm abnormalities on earlier miscarriages without fetal heartbeat. Subsequent metabolomic analysis demonstrated that taurine levels were decreased in the spermatozoa but increased in seminal plasma of the ROH group. We therefore hypothesize that the imbalance in the taurine concentration between spermatozoa and seminal plasma is associated with the sperm taurine transporter TAUT.

Materials and methods

Study approval

Clinical characteristics from 110 patients whose partners suffered with RPL were collected at the Reproductive Medicine Center of the Second Affiliated Hospital of Nanjing Medical University from December 2014 to March 2018. In total 41 semen samples were collected for metabolomic analysis. Furthermore, semen samples from 30 teratozoospermic men (normal sperm morphology <4%) seeking evaluation for infertility and 25 age-matched control subjects with normal semen quality were collected for western blotting. The ethics committee of the Second Affiliated Hospital of Nanjing Medical University approved the study. Each participant signed an informed consent related to the study.

Male CD-1/ICR mice (aged 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks) were obtained from the animal center of Nanjing Medical University (Nanjing, Jiangsu, China) and were housed in separated cages with free access to food and water. The room temperature was kept at 25°C ± 1°C under a natural light/dark cycle. All animal experimental procedures were approved by the ethical board of Nanjing Medical University.

Clinical sample collection and metabolomic analysis

Exclusion criteria of maternal factors were abnormal chromosomes, endocrine dysfunction, uterine malformations, reproductive tract infection, immunological disorders and other systemic diseases including hypertension, diabetes mellitus and malignancies. Male partners who exhibited varicocele, cryptorchidism, abnormal chromosomes or a history of surgery on male genital organs were excluded from the study. According to whether a fetal heartbeat was seen in previous pregnancies under ultrasonic monitoring, patients with the loss of two or more consecutive pregnancies were divided into two groups: the RPL without heartbeat (pregnancy with no fetal heartbeat, ROH) group and RPL with heartbeat (one or more pregnancies with fetal heartbeat, RWH) group. Semen was collected by masturbation after 3–7 days of abstinence. The sperm parameters were assessed according to the World Health Organization guidelines (WHO, 2010) using a computer-assisted semen analyzer (Weili Company, Beijing, China). In order to decrease the potential contamination as much as possible, samples with $>1.0 \times 10^6$/ml round cell with a positive peroxidase test were excluded from the study. Finally, 21 ROH and 20 RWH cases and age-matched controls were enrolled and used in the metabolomics study. No reagents other than PBS were added during sample collection thus avoiding potential influence on metabolomic results.

Human spermatozoa and seminal plasma were analyzed by UPLC-Q-Exactive Orbitrap-MS (ultra-high-performance liquid chromatography coupled with hybrid quadrupole-orbitrap mass spectrometry) using Ultimate 3000 Ultra-performance liquid chromatography (Dionex, Germering, Germany) and triple quadrupole tandem mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described in our previous reports (Zhang *et al.*, 2020). In brief, 10 µl samples were disrupted with ultrasound for 1 min, then mixed with methanol to precipitate the protein. The mixture was centrifuged at 16 000*g* for 15 min at 4°C. The supernatant was evaporated after adding 10 µl of each of the three internal standards, 20 µl of ultrapure water was added to redissolve the solid, which was vortexed and centrifuged at 16 800*g* for 10 min, and the supernatant was pipetted for detection. TraceFinder (version 3.1, Thermo Fisher Scientific) was used to process data, including qualitative and quantitative analysis. Identification was carried out by searching HMDB (http://www.hmdb.ca/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) databases, and pathway analysis was performed with the MetaboAnalyst tool (http://www.metaboanalyst.ca/) and Interactive Pathway Explorer (iPath3.0).

Intratesticular injection of Taut siRNA and antagonist

The siRNA sequences were purchased from Shanghai GenePharma Co., Ltd (Shanghai, China): siRNA#1 (sense: 5′-GACGCUGGAACUCAGAUAUTT-3′; antisense: 5′-AUAUCUGAGUUCCAGCGUCTT-3′), siRNA#2 (sense: 5′-GUGGCAUGUAUGUGUUUCATT-3′; antisense: 5′-UGAAACACAUACAUGCCACTT-3′), siRNA#3 (sense: 5′-CUCCGCGUGAGAAUCAAAUTT-3′; antisense: 5′-AUUUGAUUCUCACGCGGAGTT-3′), and Negative siRNA (sense: 5′-UUCUCCGAACGUGUCACGUTT-3′; antisense: 5′-ACGUGACACGUUCGGAGAATT-3′) were used as controls. The siRNAs were diluted to a final concentration of 20 µM and stored at −20°C. The efficacies of the three siRNAs were verified using the mouse spermatocyte-derived GC-2 cell line, as per the manufacturer's instructions, and the one with the highest efficacy (siRNA#1) was used for studies *in vivo*. Approximately 5 µl of individual siRNA or antagonist was injected into the seminiferous tubules of each testis of a 3-week-old ICR mouse using the injection procedure described previously (Shen *et al.*, 2014); 0.2% Fast green FCF (Fast Green FCF is a triaryl-methane food dye, used to stain for the misexpression of transgenes in tissues of interest) (A610452-0005, Sangon Biotech, Shanghai, China) was used as an indicator to ensure that the microinjection was successful. Negative control siRNA or saline was injected in control mice testes.

Study design and treatments

We adopted two similar schemes to interfere with the functions of taurine and TAUT in mouse testes. For the siRNA scheme, all 3-week-old male mice weighing 12–14 g were randomly divided into four groups (n = 35 per group): negative siRNA, *Taut* siRNA, *Taut* siRNA + 5% β-alanine and *Taut* siRNA + 5% Taurine. In the negative siRNA and *Taut* siRNA group, negative siRNA and *Taut* siRNA were injected into the seminiferous tubules, respectively. In the *Taut* siRNA + 5% β-alanine group, β-alanine (5% w/v) (146064, Sigma-Aldrich, Shanghai, China) was added to the drinking water after *Taut* siRNA intratesticular injection to cause systemic taurine depletion, and taurine (T8691, Sigma-Aldrich) was added to drinking water in the *Taut* siRNA + 5% Taurine group after the *Taut* siRNA intratesticular injection. For the antagonist scheme, 3-week-old male mice were divided into four groups (n = 35 per group): control, antagonist (β-alanine, competitive of taurine transporter antagonist) alone, antagonist + 5% β-alanine and antagonist + 5% Taurine. The control group received intratesticular injection of a normal saline solution, and the antagonist groups received intratesticular injection of 50 mM β-alanine. The antagonist + 5% β-alanine and antagonist + 5% Taurine groups

were both given drinking water treated with 5% β-alanine or taurine, respectively, after intratesticular injection of the antagonist. After 3 weeks of treatment, some of the mice were killed for morphological observation of epididymal spermatozoa, and the residual mice were used for mating and IVF when they reached adulthood.

Protein extraction and western blotting

Mouse and human tissues or cells were lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate) in the presence of 1% (w/v) protease inhibitor cocktail (HY-K0010, MedChemExpress, Shanghai, China). The samples were sonicated and homogenized on ice for 1 h, the mixture was centrifuged at 15 000*g* for 45 min at 4°C and the supernatant was collected. The protein concentration of each sample was determined via the BCA method (P0012, Beyotime, Shanghai, China). Then the lysate was mixed with the sample buffer (P0015A, Beyotime) and incubated at 100°C for 5 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to separate proteins and transfer them to polyvinylidene difluoride membranes (162-0177, Bio-Rad, Beijing, China), which were blocked in TBS containing 5% (w/v) non-fat milk powder for 2 h and incubated overnight with antibodies against TAUT (diluted at 1:1000; ab196821, Abcam, Shanghai, China); they were then washed and incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:2000; SA00001, Proteintech, Wuhan, China) at room temperature. Specific proteins were detected with an ECL kit (FD8000, Hangzhou Fude Biological Technology Co., Ltd, Hangzhou, China). The intensities of the bands on film were quantified by using the software Image J (https://imagej.net/Fiji/Downloads).

Immunofluorescence of isolated testicular cells and spermatozoa

The fresh testes of mice were decapsulated and fixed at room temperature for 2 h with 4% (w/v) paraformaldehyde (PFA) after capsule removal. Approximately 50 mg testis tissues were cut into small pieces with scissors and repeatedly drawn through syringes of different volumes (50, 20, 10 and 2 ml sequentially) to provide a homogeneous suspension. The supernatant was collected after standing for 5 min on ice and centrifuged 1000*g* for 5 min. The precipitation was resuspended in PBS, and the cells were spread onto slides. For immunofluorescent analysis, spermatozoa were collected from the cauda epididymidis, spread onto slides, fixed in 4% (w/v) PFA and blocked with 1% (w/v) bovine serum albumin at room temperature for 2 h. Following incubation with the primary antibody (diluted at 1:1000; ab236898, Abcam) at 4°C overnight, the cells were incubated with FITC-labeled secondary antibody (SA00003, Proteintech) for 1 h. Nuclei were stained using DAPI. Alexa488-conjugated peanut agglutinin (L7381, Sigma-Aldrich) was added at 10 μg/ml to visualize the acrosome. Finally, the images were acquired using a ZEISS LSM 800 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Cell culture and transfection

The mouse GC-2 cell line (ATCC # CRL-2196) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 1% (w/v) penicillin–streptomycin–amphotericin B solution (15140-122, Gibco, Shanghai, China). The GC2 cell line was transiently transfected with either *Taut* siRNA or a negative control siRNA (Shanghai GenePharma Co., Ltd) using Lipofectamine 3000 transfection reagent (Invitrogen, L3000008, Shanghai, China) according to the manufacturer's instructions. The cells were prepared for western blotting 72 h after transfection.

Hematoxylin and eosin staining of mouse epididymal spermatozoa

Spermatozoa were collected from the cauda epididymidis 3 weeks after intratesticular injection, spread onto slides, fixed with 4% PFA and stained with hematoxylin and eosin (H&E) for morphological observation. At least 200 cells were evaluated on each slide in a double-blind manner.

Assessment of fertility

Approximately 5 weeks after intratesticular injection, male mice were mated with two 8-week-old wildtype female mice. The female mice were checked every day for a vaginal plug and separated if pregnant, and embryonic stage E0.5 was defined on the morning of vaginal plug confirmation. The female mice were killed on Day 11.5 of pregnancy, and normal fetuses and fetal resorptions in the uterus were recorded. For IVF, the cauda epididymidis from adult males was punctured and the luminal contents were squeezed into medium, allowing spermatozoa to capacitate in human tubal fluid media for 60 min. *In vivo*-matured oocytes were obtained from female mice. Gonadotrophin was injected i.p. at doses of 7.5 IU pregnant mare serum gonadotropin (Ningbo Sansheng Biological Technology Co., Ltd, Ningbo, China), followed by 7.5 IU hCG (Ningbo Sansheng Biological Technology Co., Ltd) 48 h later. Oocyte–cumulus complexes were collected from the oviducts 14-16 h after hCG administration for fertilization. Moderately motile spermatozoa were added to groups of oocytes within their cumulus masses and the oocytes were transferred to KSOM media 4 h later. Fertilized oocytes were identified by the presence of two pronuclei (2PN). The numbers of 2PN, 2-cell, 4-cell and blastocyst stage embryos were determined 10, 24, 48 and 96 h after fertilization, respectively.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 8.0 Software (https://www.graphpad.com/). All data are expressed as the mean ± SD. Statistical differences between two groups were measured by Student's *t*-test. One-way ANOVA followed by Bonferroni's multiple comparison test was used for multiple comparisons when appropriate. Brown-Forsythe and Welch ANOVA with Tamhane's T2 *post hoc* were used when the SDs were different. Non-parametric data were analyzed with the Kruskal–Wallis test. $P < 0.05$ was considered statistically significant.

Results

Comparison of sperm abnormalities in the ROH versus RWH groups

The clinical data showed that the percentage of abnormal spermatozoa in the ROH group was significantly higher than that of males in the

RWH group ($P < 0.01$). No significant difference was found in the number of abortions, age and BMI of the couples, semen volume, sperm concentration, total sperm count, sperm motility or progressive motility between the two groups (Supplementary Table SI). We investigated the potential of semen parameters to distinguish ROH patients from RWH patients in the study, using receiver operating characteristic (ROC) curve analyses (Fig. 1). ROC analysis suggested that sperm morphology discriminated significantly between ROH patients and RWH patients, which had an ROC curve AUC value of 67.8% ($P < 0.01$).

Metabolomic analyses of spermatozoa and seminal plasma

A total of 41 semen samples from 20 RWH and 21 ROH patients were collected for metabolomic analyses. Taurine, which is the only differential metabolite detected both in the spermatozoa and the seminal plasma, was lower in the spermatozoa ($P < 0.01$) but higher in the seminal plasma ($P < 0.05$) of ROH than RWH patients (Supplementary Tables SII and SIII). ROC analysis suggested that the taurine content of spermatozoa (Fig. 2A) but not seminal plasma (Fig. 2B) discriminated significantly between ROH and RWH patients. The KEGG pathways involving the significantly different metabolites in the spermatozoa and seminal plasma between the ROH and RWH groups are shown, including taurine and hypotaurine metabolism in both spermatozoa (Fig. 2C) and seminal plasma (Fig. 2D). The metabolic connection of the metabolites and metabolic pathways in relation to ROH and RWH patients in the KEGG general metabolic pathway map is shown in Supplementary Fig. S1.

Decrease in sperm TAUT expression in teratozoospermia

TAUT was detected in human and mouse spermatozoa as a single band with a molecular weight of 50 kDa by western blotting (Fig. 3A). A significant decrease in the expression level of TAUT in teratozoospermic patients ($P < 0.001$) was demonstrated when compared with that of normal donors (Fig. 3B and C), and there was no significant difference in the men's age or other semen parameters between the two groups (Supplementary Table SIV). Western blots of many tissues and cells from humans and mice showed that TAUT was widely expressed and that there were different subtypes in different tissues (Fig. 3D).

Detection and localization of TAUT in mouse testis and spermatozoa

The presence of testicular TAUT at different developmental stages in mice was confirmed by western blotting. The results showed that there were two TAUT subtypes of 50 and 65 kDa in the testis and TAUT was expressed in the mouse testis from birth to adulthood (Fig. 4A and B). A significant increase in TAUT protein was found at Week 3 when mouse spermiogenesis begins, suggesting that TAUT may play an important role in spermiogenesis. In the immunofluorescent analysis of spermatozoa from the mouse cauda epididymidis and ejaculate of healthy male donors, TAUT was located in both the sperm axoneme and the acrosome, and it colocalized with PNA (peanut agglutinin, an acrosomal marker) (Fig. 4C). Indirect immunofluorescence of testicular cell suspensions demonstrated that TAUT was distributed in the whole cell bodies of spermatogonia, spermatocytes and round spermatids (Fig. 4D). However, the TAUT signal was found in the post-nuclear region in elongated spermatids, which is where the manchette arises during spermiogenesis (Fig. 4E).

Co-localization of TAUT and α-tubulin in the manchette of elongated spermatids

From the above results, we predicted that TAUT would be located in the manchette. Triple immunofluorescence staining of α-tubulin (a marker for the manchette), TAUT and DAPI to test our hypothesis showed that the TAUT signals were localized in the post-nuclear region of the elongated spermatids, coincident with α-tubulin, suggesting that TAUT is located in the manchette in spermatids from step 8 to step 16 during spermiogenesis (Fig. 5). Moreover, TAUT signals were detected in the acrosomal region during spermiogenesis, which is consistent with its location in the acrosome of mature spermatozoa (Fig. 4C).

Sperm abnormalities after interference with taurine transport

Mouse seminiferous tubules were injected with siRNA against *Taut* or the taurine transport antagonist β-alanine, which introduced siRNA or antagonist into ~70% of the seminiferous tubules (Fig. 6A). Three-week-old mice, the time when TAUT expression significantly increases and mouse spermiogenesis begins, were used for intratesticular injection (Huang *et al.*, 2008). The *Taut* siRNA that induced the highest level of TAUT protein inhibition in the mouse spermatogenic cell line (GC-2) and testis after 72 h was used for subsequent experiment. The TAUT protein was significantly suppressed in the testis 72 h after injection with the *Taut* siRNA 1# (Fig. 6B–E). The proportion of morphologically abnormal spermatozoa increased markedly in the *Taut* siRNA-treated group compared with the negative control siRNA group, principally shown as tail deformity (Fig. 6F). In addition, abnormal spermatozoa increased in the testes of mice given drinking water with 5% β-alanine, but sperm abnormalities were effectively rescued in those given drinking water with 5% taurine after TAUT knockdown. Similar results were found in mice used in the antagonist intratesticular injection scheme (Fig. 6G). H&E staining showed that sperm abnormalities were mainly manifested as tail defects, including curling, folding and cornering tails (Fig. 6H).

Abnormal embryo development after taurine depletion

IVF was performed ~5 weeks after siRNA injection, when the male mice of each group reached adulthood with enough mature spermatozoa in cauda epididymidis. The results showed that rates of fertilization and blastocyst formation were reduced in the 5% β-alanine supplementation group compared with the *Taut* siRNA group (Fig. 7A). After male mice were mated with two 8-week-old WT female mice 5 weeks after siRNA injection and pregnant females were killed at E11.5, the number of normal embryos decreased and resorbed embryos increased in mice treated with 5% β-alanine in drinking water, compared with mice given normal drinking water after intratesticular injection (Fig. 7B). There was no significant difference between the

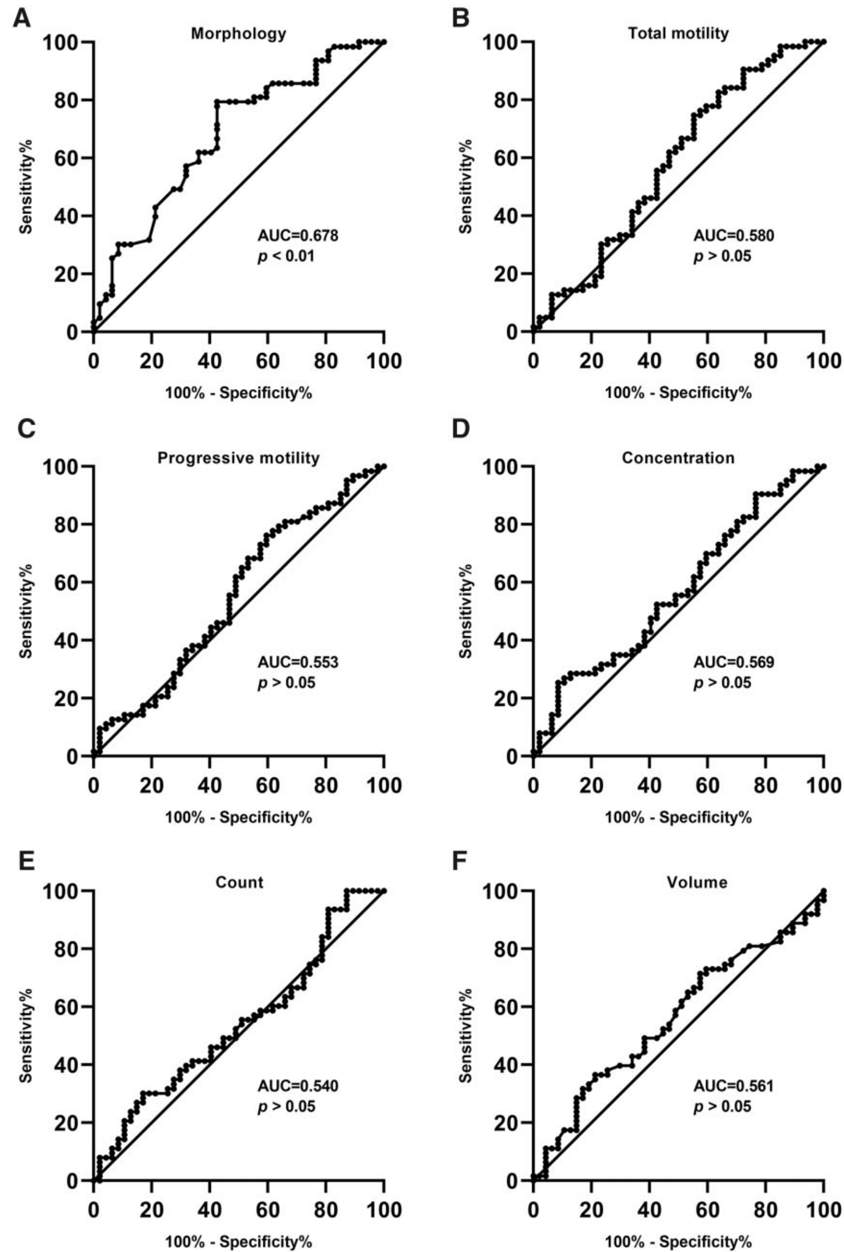

Figure 1. Analysis of semen parameters in male partners of women experiencing recurrent pregnancy loss without a fetal heartbeat. Receiver operating characteristic (ROC) curve analyses for (**A**) abnormal sperm morphology, (**B**) total sperm motility, (**C**) progressive sperm motility, (**D**) sperm concentration, (**E**) sperm count and (**F**) sperm volume. AUC values are presented for each parameter.

other groups. The resorbed embryos were abnormally small and hemorrhagic/necrotic in appearance (Fig. 7C and D). Similar results were found in the antagonist intratesticular injection scheme (Fig. 7E and F).

Discussion

RPL is a devastating experience for most couples and male factors were confirmed to be involved in its pathogenesis (McQueen *et al.*, 2019; Haddock *et al.*, 2021; Khambata *et al.*, 2021; Klimczak *et al.*, 2021). The heart is the first organ formed during mammalian development (Cetnar *et al.*, 2021), representing the earliest stage of embryo development, so abortion before fetal heartbeat formation may represent the earliest abnormal embryo development. To our knowledge, we classified for the first time the patients with RPL according to whether (RWH group) or not (ROH group) a fetal heartbeat appeared in previous pregnancies, which supplements innovatively the existing miscarriage classification. Our study showed that the

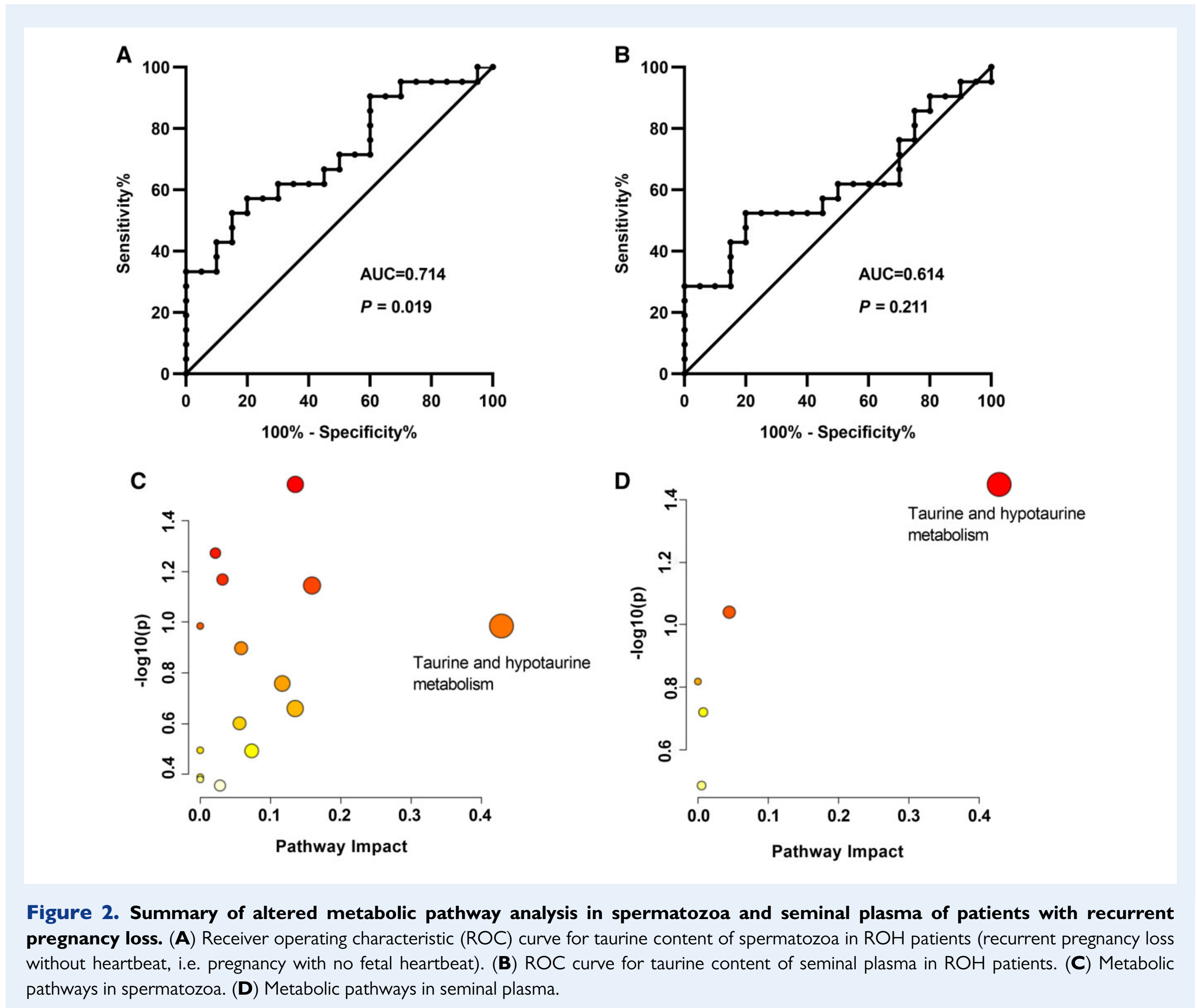

Figure 2. Summary of altered metabolic pathway analysis in spermatozoa and seminal plasma of patients with recurrent pregnancy loss. (**A**) Receiver operating characteristic (ROC) curve for taurine content of spermatozoa in ROH patients (recurrent pregnancy loss without heartbeat, i.e. pregnancy with no fetal heartbeat). (**B**) ROC curve for taurine content of seminal plasma in ROH patients. (**C**) Metabolic pathways in spermatozoa. (**D**) Metabolic pathways in seminal plasma.

percentage of morphologically abnormal spermatozoa in partners of the ROH group was significantly higher than that of the RWH group. Furthermore, our findings demonstrate for the first time that TAUT levels are reduced in ejaculated spermatozoa from teratozoospermic patients compared with normal donors. This study revealed a novel molecular mechanism underlying the pathogenesis of teratozoospermia, in which the downregulation of sperm TAUT and depletion of taurine contribute to the impairment of sperm morphology, which may eventually influence embryo development.

The metabolomic results give a possible explanation for the higher percentage of sperm abnormalities in the ROH than the RWH group. As our metabolomic analysis of semen demonstrated, taurine content was decreased in the spermatozoa but increased in the seminal plasma of ROH compared with RWH patients and previous studies have shown that key enzymes for taurine synthesis are not expressed in spermatozoa, these unbalanced intra- and extracellular taurine concentrations are likely to be associated with its transporter TAUT (Huxtable and Lippincott, 1982; Li *et al.*, 2006). That western blots demonstrated a reduced TAUT expression in ejaculated spermatozoa from teratozoospermic patients is consistent with our hypothesis. We further hypothesize that downregulation of the TAUT protein and deficiency of taurine could result in impaired sperm morphology, which may then affect early embryo development, as was observed in RPL patients without fetal heartbeat.

TAUT (gene name *Slc6a6* in mice and *SLC6A6* in human) is a member of the solute carrier (SLC) family. Research is increasingly showing that ion channels, including the SLC family, are vital during spermatogenesis. *Slc26a8* knockout male mice have been documented to be infertile because of severe defects in sperm structure (Toure *et al.*, 2007; Dirami *et al.*, 2013). Concurrently, *Slc4a2* and *Slc22a14* knockout male mice are also infertile owing to abnormal spermatogenesis (Medina *et al.*, 2003; Maruyama *et al.*, 2016). It has been reported that taurine and its transporter TAUT are essential for the initiation of

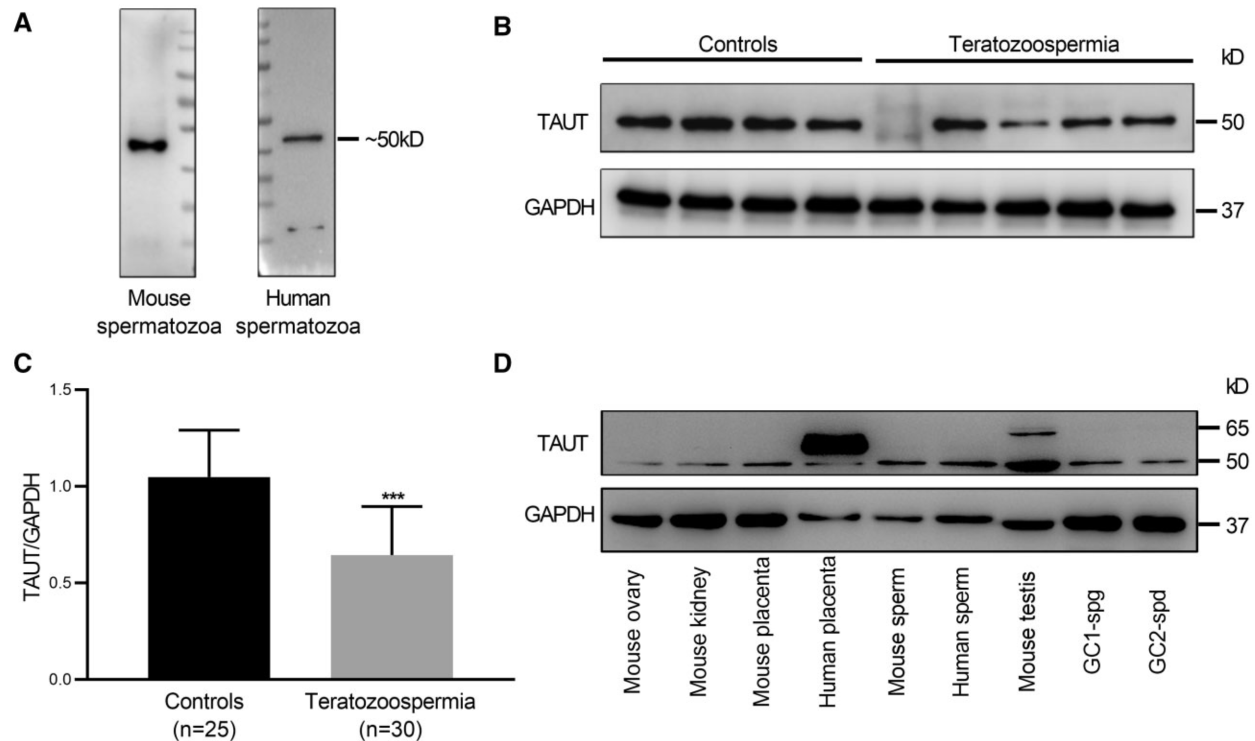

Figure 3. Levels of TAUT as analyzed by western blotting. (**A**) Detection of TAUT (taurine transporter) in mouse and human spermatozoa. (**B**) Representative results of western blotting analysis of TAUT protein in spermatozoa from teratozoospermic men and healthy donors. GAPDH was used as a loading control. (**C**) Levels of TAUT protein in controls and men with teratozoospermia. (**D**) Expression of TAUT in different tissues and cells from human and mouse. No negative control was used because of the wide expression of TAUT protein. All data are expressed as mean ± SD; $^{***}P < 0.001$ versus normal group by Student's *t*-test.

sperm meiosis (Higuchi *et al.*, 2013). Our study also demonstrated that taurine and its transporter TAUT are required for mouse spermatogenesis.

Alignment analysis indicated that the gene of human *SLC6A6* and mice *Slc6a6* is highly homologous. In our study, TAUT was widely expressed in several tissues, with different subtypes, which is consistent with the existing research in placenta and lymphocytes (Roos *et al.*, 2004; Iruloh *et al.*, 2007; Kusinski *et al.*, 2010). The expression level of testicular TAUT significantly increased from Week 3, when mouse spermiogenesis begins. Further immunofluorescence showed that the TAUT signal was seen in the whole cell bodies of spermatogonia, spermatocytes and round spermatids but migrated to the post-nuclear region in elongated spermatids and was localized to the manchette during spermiogenesis. Spermiogenesis happens in the last phase of spermatogenesis, during which the nucleus is remodeled by chromatin condensation, excess cytoplasm is removed, acrosomes are formed and sperm tails are assembled (Lehti and Sironen, 2016). The proteins needed for tail assembly are transported through the manchette by intra-manchette transport (IMT) to the base of the tail and then into the developing sperm tail via intra-flagellar transport (IFT) (Gunes *et al.*, 2020). Our data strongly support an involvement of TAUT in spermiogenesis not only because of the significantly increased content in testes of 3-week-old mice but also owing to the particular localization at the microtubules of the manchette and flagellum. In addition, we have shown that TAUT is localized in the acrosome and tail of mature spermatozoa, indicating that it may affect their motility and fertilizing ability. However, we found no difference in the fertilization and blastocyst formation rate in the *Taut* siRNA group and the antagonist group compared with controls, which could be associated with gradual functional recovery of injected seminiferous tubules and compensation of these residual normal seminiferous tubules. Further studies on conditional knockout mice will be conducted.

Functional analysis of TAUT was performed by knocking down its expression in mice, which is a method widely used in our experimental center (Shen *et al.*, 2014; Wu *et al.*, 2015; Zhou *et al.*, 2021). To our knowledge, transcription and translation almost cease during spermiogenesis (Steger, 2001). The introduction of siRNA into spermatogenic cells degrades the target mRNA and the transcription of new mRNA correspondingly stops. Therefore, the subsequent decline in protein levels of the target gene can be maintained for a long period of time. The results showed that the *Taut* siRNA or antagonist-treated mice had increased numbers of morphologically abnormal spermatozoa, and

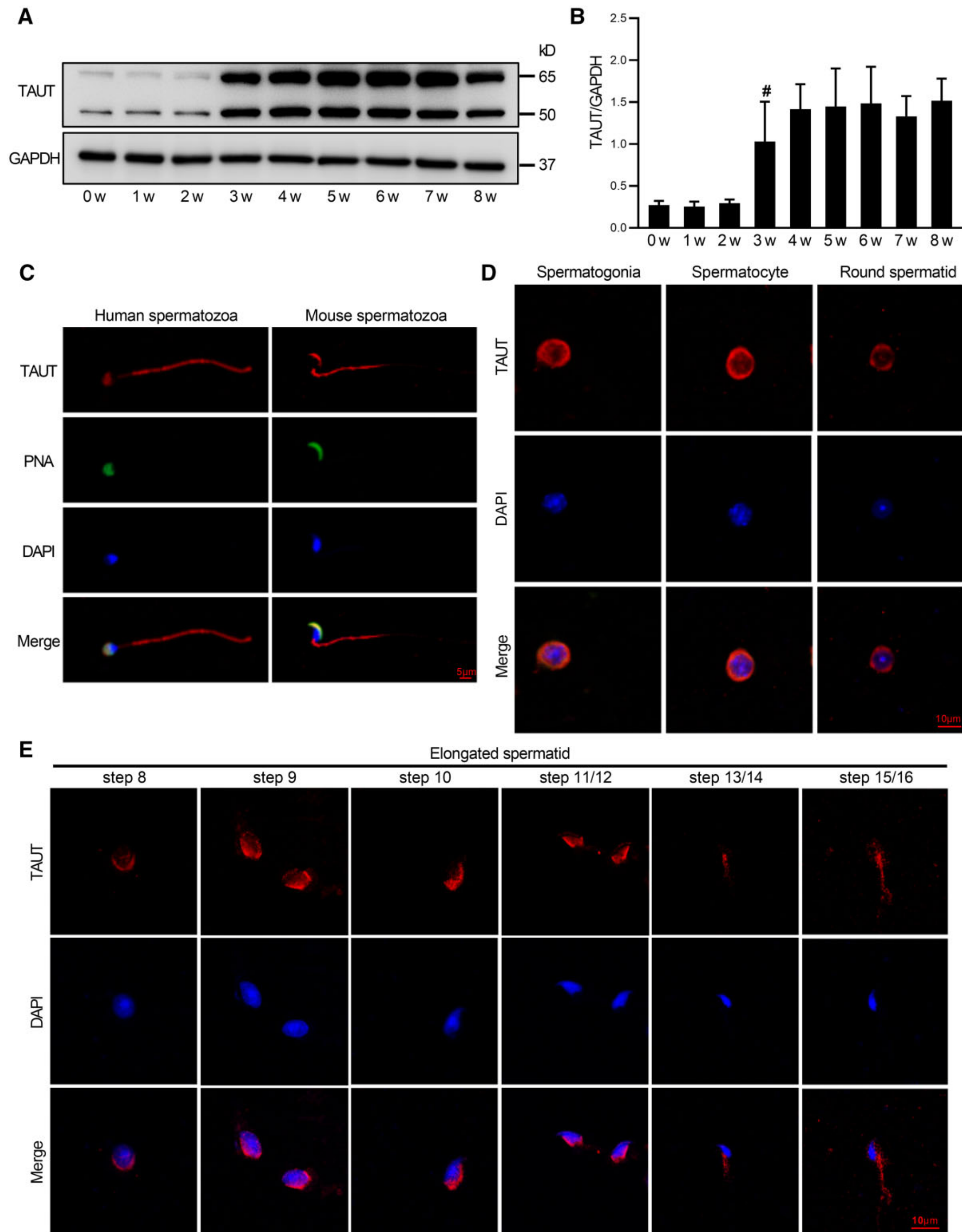

Figure 4. Levels of TAUT in mouse testes at different developmental stages and immunofluorescent staining of TAUT in mouse spermatogenic cells and mature spermatozoa. (**A**) Western blot analysis of TAUT (taurine transporter) protein in mouse testes at different developmental stages (w: weeks). No negative control was used because of the wide expression of TAUT protein. (**B**) Quantification of the level of TAUT protein using GAPDH as a loading control. #The time of siRNA or antagonist intratesticular injection. (**C**) Double immunofluorescent staining of TAUT (red) and PNA (green) in mature spermatozoa. TAUT was localized in the axoneme of the sperm tail and acrosome, colocalized with PNA in the acrosome. Scale bar, 5 μm. (**D**, **E**) Distribution of TAUT (red) in spermatogenic cells at different developmental stages. Scale bar, 10 μm. All data are expressed as mean ± SD of three independent experiments. All nuclei were stained with DAPI (blue).

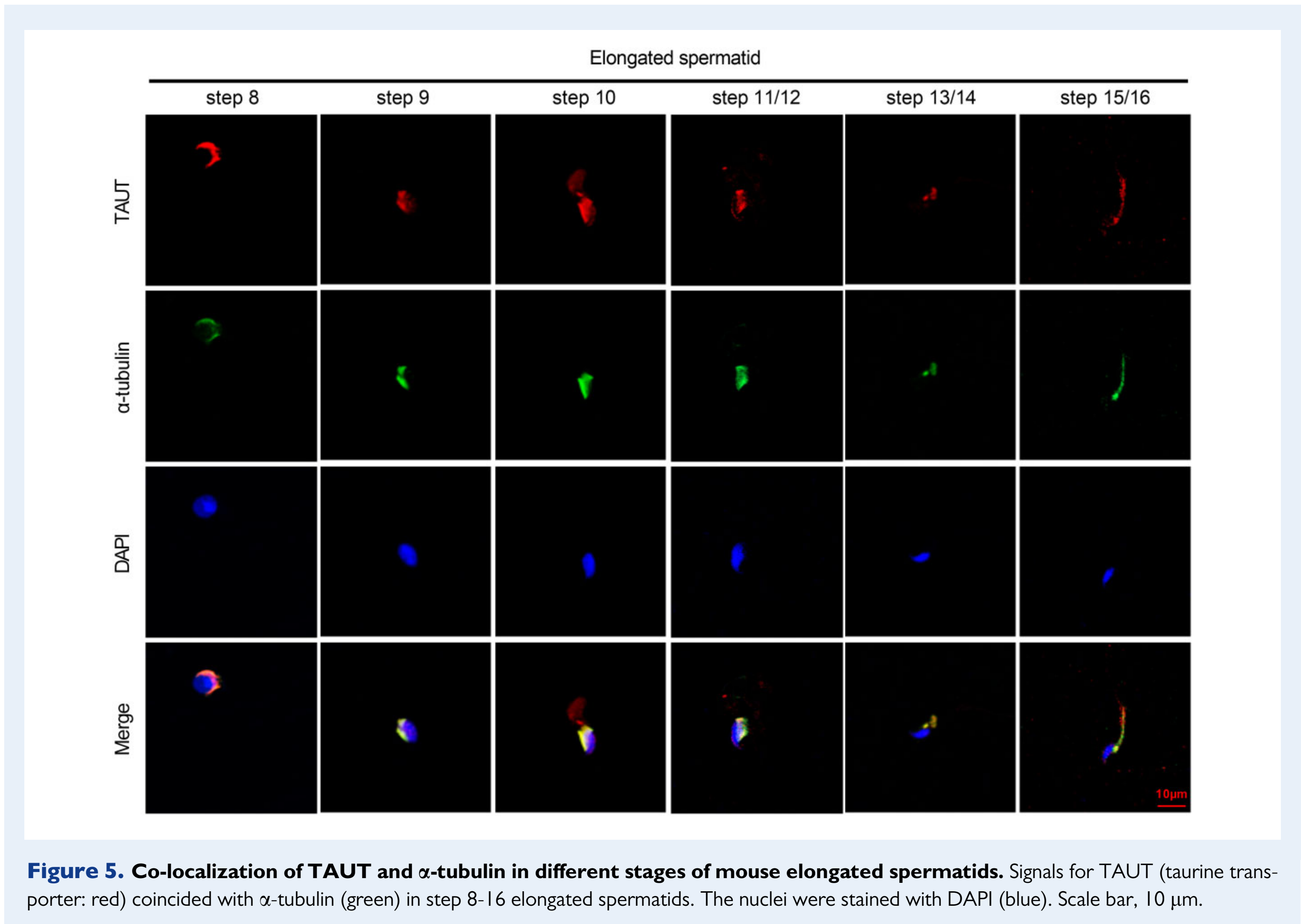

Figure 5. Co-localization of TAUT and α-tubulin in different stages of mouse elongated spermatids. Signals for TAUT (taurine transporter: red) coincided with α-tubulin (green) in step 8-16 elongated spermatids. The nuclei were stained with DAPI (blue). Scale bar, 10 μm.

sperm tail defects were particularly common, possibly owing to the particular localization at microtubules of the manchette and flagellum. Similar abnormalities have been seen in other models of interference in the synthesis of many microtubule-associated proteins (Mendoza-Lujambio et al., 2002; Lehti et al., 2013; Liu et al., 2017). The sperm defects in our experiment deteriorated further after supplementation with 5% β-alanine for 3 weeks but were effectively improved by taurine supplementation. This result is consistent with previous studies in aged rats (Yang et al., 2010). β-Alanine is an efficient taurine transporter antagonist that can competitively bind to TAUT and effectively inhibit the cellular uptake of taurine (Jong et al., 2010; Baek et al., 2012; Rasmussen et al., 2016). Adding 5% β-alanine in the drinking water for 5 weeks can reduce the content of taurine by ∼40% in rats (Ericson et al., 2011). We have provided strong evidence that the decreased expression of TAUT and taurine depletion may be contributory pathogenic factors in patients with teratozoospermia.

Sperm quality is of vital importance to normal embryo development. A recent study showed that paternal diet-induced obesity partially transmits defective epigenetic signatures of developmental importance via the spermatozoa to the embryo, leading to embryo loss (Deshpande et al., 2020). Similar results have also been found after estrogen receptor agonist administration (Dumasia et al., 2017). Our study showed that down-regulation of the TAUT protein and deficiency of taurine could result in impaired sperm morphology and there were fewer normal embryos and more resorbed embryos in mouse uterus after β-alanine administration. This reveals that taurine and its transporter may positively affect early embryonic development through the regulation of sperm morphology. Taurine appears to have a beneficial effect on the early embryo development before formation of the fetal heart, as was clinically observed in ROH patients who showed more sperm abnormalities and a lower taurine content than RWH patients. Subsequent IVF results demonstrated that taurine depletion decreases the fertilization and blastocyst formation rates. Previous investigations have indicated that adding taurine to culture medium can improve early embryo development (Devreker et al., 1999; Guerin et al., 2001) and dietary taurine supplementation during pregnancy provides significant protection against oxidative stress in embryos (Shivananjappa and Muralidhara, 2012). These data are line with our clinical observations that couples with RPL with higher sperm abnormalities show earlier abnormal embryo development, characterized by miscarriage without a detectable fetal heartbeat. However, apart from being a competitive taurine transporter antagonist, the chronic

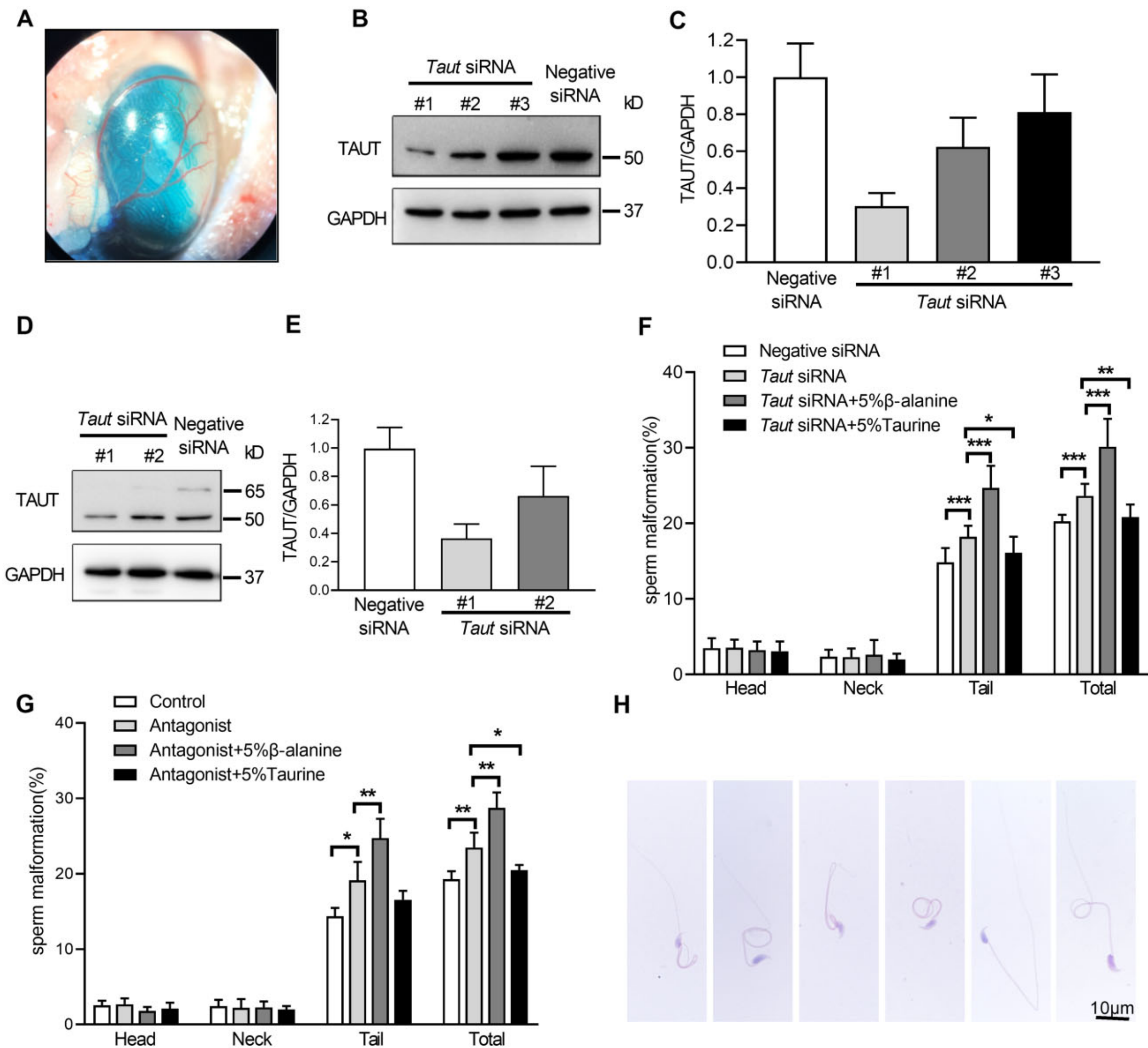

Figure 6. Effect on mouse sperm morphology of interference with taurine transport *in vivo* **by intratesticular injection.** (**A**) Diagram of the intratesticular injection. Approximately 70% of the seminiferous tubules in 3-week-old mouse testes were injected with siRNAs or antagonist (β-alanine) mixed with indicator (blue). (**B, C**) Inhibition efficiency of three *Taut* (taurine transporter) siRNAs on GC-2 cell line. (**D, E**) Inhibition efficiency of two selected *Taut* siRNAs on testicular TAUT protein. (**F**) Effect of *Taut* siRNA injection and taurine depletion on sperm morphology. (**G**) Effect of antagonist injection and taurine depletion on sperm morphology. All experiments were performed in triplicate in individual mice. (**H**) Shapes of spermatozoa from the cauda epididymidis of mice treated with *Taut* siRNA 1#. Scale bars, 10 μm. One-way ANOVA followed by Bonferroni's *post hoc* comparisons tests and Brown-Forsythe and Welch ANOVA with Tamhane's T2 *post hoc* multiple comparison tests were performed for statistical analyses. Non-parametric data were analyzed with the Kruskal–Wallis test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

administration of β-alanine causes cellular oxidative damage and significantly changes energy metabolism, as reported previously (Gemelli *et al.*, 2013, 2018), which may negatively affect spermatogenesis and early embryo development. Moreover, whether epigenetic factors are involved in the pathogenesis of abnormal embryo development caused by taurine depletion needs to be clarified.

In summary, we propose a new classification method for RPL and have revealed that there is a decrease in sperm TAUT in teratozoospermia. Inhibition of cellular taurine uptake by β-alanine and taurine transporter knockdown during spermatogenesis resulted in an increase in sperm abnormalities. Taurine depletion, through adding β-alanine into the drinking water of males, increased sperm abnormalities, which may result in abnormal embryo development as observed in our study.

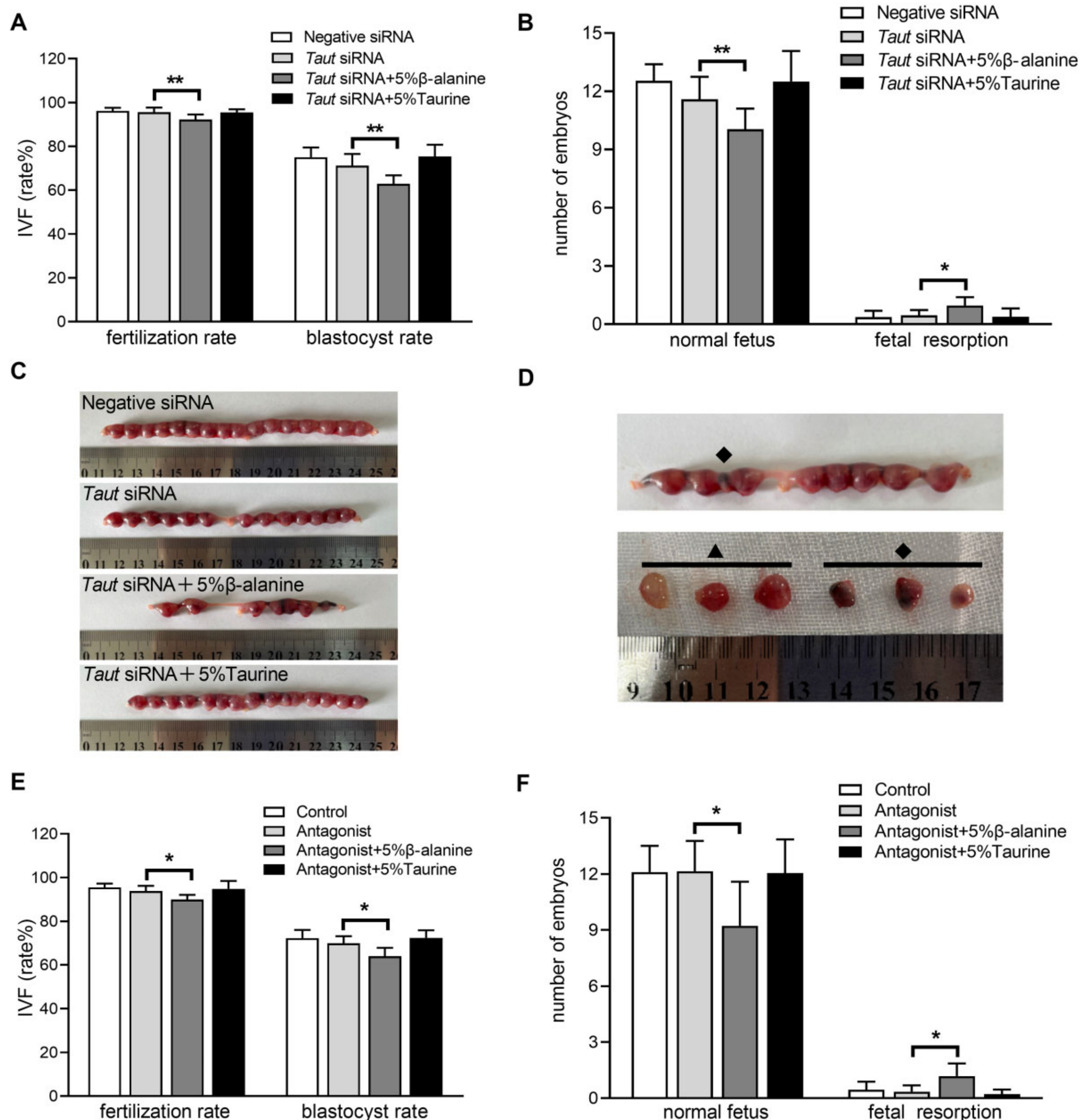

Figure 7. Reproductive abnormalities in mice treated with 5% β-alanine. (**A**) Quantification of fertilization and blastocysts in IVF assays of the siRNA scheme. (**B**) Mating tests of male mice in four groups from the siRNA scheme with normal adult female mice. (**C**) Representative images of embryonic Day 11.5 embryos of the four groups. (**D**) Representative images of appearance of viable and resorbed embryos. ▲, viable embryos in the uterus; ◆, resorbed embryos in the uterus. (**E**) Quantification of fertilization and blastocysts in IVF assays of the antagonist scheme. (**F**) Mating tests of male mice in four groups from the antagonist scheme with normal adult female mice. All experiments were performed in triplicate in individual mice. One-way ANOVA followed by Bonferroni's *post hoc* comparisons tests and Brown-Forsythe and Welch ANOVA with Tamhane's T2 *post hoc* multiple comparison tests were performed for statistical analyses. Non-parametric data were analyzed with the Kruskal–Wallis test. $^{*}P < 0.05$; $^{**}P < 0.01$.

Moreover, our data strongly suggest an influence of TAUT on the development of the sperm tail in spermatids owing to its localization at the manchette via its role in the IMT or IFT mechanisms. Taurine supplementation may effectively improve sperm quality and pregnancy outcomes in patients with teratozoospermia. However, the specific molecular mechanism of TAUT involvement in spermiogenesis remains to be clarified. Conditional knockout of *Taut* will be employed in further investigations.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

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

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Authors' roles

H.W. and X.Z. are co-first authors and contributed equally to this work. Y.Q. and H.W. developed the hypothesis and designed the research. H.W. and X.Z. carried out the data collection, and contributed to the draft and revision of the manuscript. H.W. and J.Y. performed the research. H.W., J.Y. and Y.C. contributed to the sample collection and analyzed the data. H.W. and R.F. provided funding and experimental platform. X.Z., H.W. and R.F. revised and edited the manuscript. T.F. contributed to the sperm analysis. Y.Q. contributed to the design of the study and the patient recruitment, and critically revised the manuscript. All authors provided a critical review and approved the final manuscript. The order of their names was established on the basis of their individual contributions to this study.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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