Zinc Transporter ZIP12 Maintains Zinc Homeostasis and Protects Spermatogonia From Oxidative Stress During Spermatogenesis

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Research Article

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Abstract

Background: Overwhelming evidences now suggest oxidative stress is a major cause of sperm dysfunction and male infertility. Zinc is an important non-enzyme antioxidant with a wide range of biological functions and plays a significant role in preserving male fertility. Notably, zinc trafficking through the cellular and intracellular membrane is endorsed by precise families of zinc transporters, i.e. SLC39s/ZIPs and SLC30s/ZnTs. However, the expression and function of zinc transporters in the male germ cells were rarely reported. The aim of this study is to determine the crucial zinc transporter responsible for the maintenance of spermatogenesis.

Methods: In the present study, we investigated the expression of all fourteen ZIP members in mouse testis and further analyzed the characteristic of ZIP12 expression in testis and spermatozoa by qRT-PCR, immunoblot and immunohistochemistry analyses. To explore the antioxidant role of ZIP12 in spermatogenesis, an obese mouse model fed with high-fat-diet was employed to confirm the correlation between ZIP12 expression level and sperm quality. Furthermore, ZIP12 expression in response to oxidative stress in a spermatogonia cell line, C18-4 cells, was determined and its function involved in regulating cell viability and apoptosis was investigated by RNAi experiment.

Results: We initially found that ZIP12 expression in mouse testis was significantly high compared to other members of ZIPs and its mRNA and protein were intensively expressed in testis rather than the other tissues. Importantly, ZIP12 was intensively abundant in spermatogonia and spermatozoa, both in mice and humans. Moreover, ZIP12 expression in testis significantly decreased in obese mice, which associated with reduced sperm zinc content, excessive sperm ROS, poor sperm quality and male subfertility. Similarly, its expression in C18-4 cells significantly declined in response to oxidative stress. Additionally, reduced ZIP12 expression by RNAi associated with a decline in zinc level subsequently caused low cell viability and high cell apoptosis in C18-4 cells.

Conclusions: The zinc transporter ZIP12 is intensively expressed in testis, especially in spermatogonia and spermatozoa. ZIP12 may play a key role in maintaining intracellular zinc level in spermatogonia and spermatozoa, by which it resists oxidative stress during spermatogenesis and therefore preserves male fertility.

Background

Infertility is defined as a failure in pregnancy after 12 months of unprotected sexual intercourse, affecting approximately 15% of couples according to a recent epidemiological study[1, 2]. In these cases, up to 50% of all infertility is associated with male factors, mainly caused by defects in sperm concentration, morphology, and motility. As an etiological factor, excessive reactive oxygen species (ROS) resulted in oxidative stress contributes to 30–80% of cases of male infertility[3, 4]. ROS is the intracellular production of free radicals, including superoxide anions ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), proxyl ($\text{OOR}\cdot$), and hydroxyl ($\text{OH}\cdot$)[5]. Excessive ROS that overwhelms endogenous antioxidant defenses can lead to

nuclear DNA damage and mitochondria dysfunction, lipid peroxidation, shorten telomere length, and epigenetic changes in target cells[6, 7].

Spermatogenesis is a continuous and sophisticated cell differentiation process that involves a wide repertoire of spermatogenic cells, including spermatogonia, spermatocytes, spermatids, and spermatozoa[8]. Overwhelming evidence now suggests that ROS is a major cause of impaired spermatogenesis and sperm dysfunction, and acts as a detrimental factor in the etiology of male infertility owing to impairment of both the structural and functional integrity of spermatozoa[9, 10]. Since sperm cells have most of their cytoplasm been extruded and their transcription are generally repressed, they neither have the cytoplasmic storage capacity nor the transcriptional ability to defense against ROS induced by environmental challenges. Therefore, the excess ROS must be continuously inactivated by antioxidants in the niche of the genital tract, such as zinc[11].

Zinc is a member of non-enzyme antioxidants and has significant roles in the antioxidant network through multiple mechanisms, including regulation of oxidant production, maintaining the activity of Cu/Zn-superoxide dismutase (SOD), modulation of zinc-regulated transcription factors, increasing metallothionein expression, regulation of GSH metabolism, modulation of protein kinases and phosphatases and regulation of redox signaling[4, 12, 13]. Notably, zinc has a relatively high concentration in the male reproductive system and exerts essential roles in maintaining spermatogenesis and protecting the testis against oxidative stress. For instance, zinc is a co-factor of metalloenzymes for most enzymatic reactions[9, 12], which are involved in DNA replication and packaging, DNA transcription, steroid receptor expression, and protein synthesis[13–16]. Meanwhile, zinc is assembled in the testis during spermatogenesis and plays the main role in the adjustment of the spermatogonia proliferation, spermatocytes meiosis, and the epithelial integrity of the male reproductive tract[10–12, 17–19]. Moreover, zinc can mitigate testis injury induced by stressors such as heavy metals, fluoride, and heat[12]. On the other hand, zinc deficiency is associated with abnormal endocrine in testes[14, 20], a decrease in sperm concentration of the ejaculate[14], and less sperm motility[21]. In the clinics, the therapy of male infertility was considered to be benefit from a dietary zinc supplement[4, 22–25], although the research data were sometimes contradictory.

Notably, to sustain homeostasis of zinc, two families of proteins are responsible for the transport of Zn^{2+} across the cellular and intracellular membranes in opposite directions [26]. The zinc transporter proteins (ZnT; encoded by SLC30a gene family) transport Zn^{2+} out of the cytosol, whereas the Zrt-, Irt-like proteins (ZIP; encoded by SLC39a gene family) transport Zn^{2+} into the cytosol from the intracellular compartments or the extracellular fluid [27]. These two types of zinc transporters are conserved in mammals. Human genome sequencing has identified 10 members of ZnT family (referred to as ZnT1–10) and 14 members of ZIP family (referred to as ZIP1–14)[15]. It was reported that decreased zinc intake is associated with spermatogenesis deficiency, but independent of circulating and testis zinc levels[15]. Therefore, it indicates that the impaired spermatogenesis may be attributed to the dysfunction of zinc transporters.

Herein, we firstly clarified the expression of zinc transporters in testis and identified a key zinc transporter ZIP12 in spermatogonia. In addition, we elucidated its role in maintaining spermatogenesis in defense of oxidative stress and demonstrated that ZIP12 expression in spermatogonia was indispensable for male reproduction.

Methods

Animals and obese model

The male 3-week-old C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center and acclimated in the animal facility for at least 1 week before experimentation. Male mice were divided into two groups randomly: the control diet (CD) group and the high-fat diet (HFD) group. Control diet contained 50% carbohydrate, 22% crude protein, 4% crude fat, 5% cellulose, 8% minerals, 1% vitamins, 10% water, whereas high-fat diet contained 20% lard, 25% starch, 15% dextrose, 5% sucrose, 8% casein, 2% cholesterol, 1% cholate, 5% cellulose, 5% soybean oil, 5% minerals, 1% vitamins, 8% water. All mice were weighed every week. Both groups had ad libitum food and water access and were maintained on a 12 h light and 12 h darkness cycle. The mice fed with CD or HFD for 10 weeks were employed for animal experiments as previously described[28]. Animal experiments were conducted according to the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction. This research program was approved by the ethics committee of Shanghai Jiao Tong University School of Medicine (No. A2019-029).

Gene expression analysis

Mice hearts, livers, spleens, lungs, kidneys, brains, small intestines, testes, C18-4 cells, and GC-1 cells were homogenized in the TRIzol reagent (Invitrogen, US). cDNA was reverse transcribed from 1 µg RNA using PrimeScript RT Master Mix (TaKaRa, Japan). SYBR green-based quantitative reverse transcription-polymerase chain reaction (RT-qPCR) was used to measure the expression of ZIPs in testes and the ZIP12 expression in different organs from mice. Primers used for PCR were listed in Supplementary Table 1. PCR conditions were set as follows, 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 43 s. Each PCR was run at least in triplicate. Finally, the data were analyzed by using the $2^{-\Delta\Delta CT}$ method to measure the relative gene expression levels.

Western blot analysis

Protein from C18-4 cell lysates or mouse tissues was resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Then polyvinylidene difluoride (PVDF) membranes (Millipore, Germany) were used to transfer the protein. The membranes were blocked by using 5% bovine serum albumin (BSA) for 1 h, then incubated at 4°C overnight with the primary antibodies against either ZIP12 (Abcam, 1:5000) or β -actin (Abcam, 1:5000), followed by incubation with secondary antibody conjugated to HRP (Jackson, West Grove, PA, USA, 1:10000 dilution). Then enhanced chemiluminescence (Millipore, Germany) was used to generate the signals detected by a Luminescent Image Analyzer (Image Quant LAS 4000, GE

imagination at work, USA) according to the manufacturer's protocol. Western blot has been repeated at least three times to confirm the reproducibility of the result [28, 29].

Immunohistochemistry (IHC) analysis

IHC was performed as described previously [30]. Tissues fixed in Bouin's solution were embedded in paraffin. Then, specimens were sliced into 5 μ m thick sections and mounted on glass slides, followed by deparaffinization and rehydration, and subsequently followed by antigen retrieval through boiling the tissue for 15 min in 10 mM citrate buffer, pH 6.0. The Histostain LAB-SA Detection kits (Invitrogen, MD, USA) were applied according to the manufacturer's instructions. Primary antibody exposure against ZIP12 (1:100 dilution) and the normal rabbit IgG (control) was performed overnight at 4°C. The sections were stained using DAB and nuclei were counterstained with hematoxylin. Digital images were captured under a microscope (Olympus BX53).

Immunofluorescence (IF) analysis

For IF staining, sperm smears or testicular sections were prepared on slides and C18-4 cells were cultured on glass-bottom dishes (Cellvis, Mountain View, CA, USA). Then, slides or dishes were fixed with 4% paraformaldehyde for 20 min at 4°C. Nonspecific binding sites were blocked with 10% BSA/PBS for 60 min at room temperature, followed by 0.1% TritonX-100 permeable treatment for 10 min. Sections were incubated with the ZIP12 antibodies (Abcam, 1:200 dilution), and/or PLZF antibodies (Santa Cruz Biotechnology, 1:200 dilution, USA) overnight at 4°C, respectively. Then, fluorescence-labeled secondary antibodies (donkey anti-rabbit Alexa Fluor 488 or donkey anti-mouse Alexa Fluor 555, 1:200 dilution; Jackson ImmunoResearch) were used. Nuclei were counterstained with DAPI (Sigma-Aldrich). The fluorescence signals were detected under a laser scanning confocal microscope (Carl Zeiss LSM-510, Germany) equipped with an argon laser (488 nm), a He/Ne laser (543 nm), an EC Plan-NEOFLUAR 63 $\times$ /1.25 objective, and an LD LCI Plan-APOCHROMAT 25 $\times$ /0.8 objective (Zeiss). Digital images were taken and processed using Aim software (Zeiss Systems)[29].

Assessment of sperm parameters

Preparation and analysis of mouse sperm were performed as described previously [31]. The cauda epididymis was dissected and then placed in pre-warmed (37°C) Tyrodé's Buffer (Sigma-Aldrich, USA) to allow dispersion of sperm. After incubation for 15 minutes, supernatant up-streamed sperm were segregated and sperm motility was analyzed by computer-assisted sperm analysis (CASA) (Hamilton Thorne, USA).

For teratozoospermia analysis, the sperm suspension was initially smeared on a glass slide. After dryness, the slide was fixed and stained by the method of Diff-Quick (BRED Life Science Technology Inc., Shanghai, China) according to the manufacturer's protocol. Finally, the slide was viewed under a microscope (Nikon, ECLIPSE E600, Japan). Sperm samples obtained from ten HFD-fed mice and ten normal diet-fed mice respectively were detected and at least 200 spermatozoa in every sample were included.

Intracellular zinc analysis

For detection of zinc in sperm, up-streamed sperm suspension (1×10^5 per mL) was incubated with the zinc probe, ZnAF-1 (final concentration at 1 μ g/mL, Abcam) or Zinquin ethyl ester (final concentration at 25 μ M, Dojindo) for 30 min at 37°C. After washing with PBS, the fluorescence signals were detected using flow cytometry (Becton Dickinson, Beckman Coulter). Fluorescence of ZnAF-1 was excited at 485 nm and detected at 525 nm, and fluorescence of Zinquin ethyl ester was excited at 368 nm and detected at 490 nm. The CellQuest software analyzed the emission originated from at least 30000 events (Beckman Coulter), and three experiments were repeated for each sperm sample. Meanwhile, the distribution of zinc staining in sperm was viewed under fluorescence microscopy (Olympus BX53, Tokyo, Japan).

Cell culture and RNAi experiment

The immortalized mouse spermatogonial stem cells, C18-4 cells were cultured with DMEM/F12 medium containing 10% FBS in six-well plates. The cells were divided into three groups, i.e., a blank group without any treatment, ZIP12-shRNA group transfected with mouse SLC39A12 shRNA plasmids containing an shRNA expression cassette and a green fluorescent protein (GFP) marker (obtained from Sangon Biotech, Shanghai, China), and control group transfected with control plasmids. The effective ZIP12-specific and control shRNA sequences are as follows: *ZIP12*, 5'-ctcaggttcttggttacataagcaggaa-3'; control, 5'-gcactaccagagctaactcagatagtact-3'[32]. Efficacy of zinc transporter ZIP12 expression knockdown was confirmed by Western blot analysis.

Determination of intracellular ROS

The levels of intracellular ROS were evaluated using the peroxide-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Dojindo, Kumamoto, Japan). The sperm were exposed to a serum-free medium containing 10 μ M DCFH-DA in the dark for 30 min and then washed thrice with cold PBS. The fluorescence intensity was measured by flow cytometry (FACS Calibur, Becton-Dickinson, Sunnyvale, CA).

Caspase-3 activity assay

Caspase-3 activity was measured using the Caspase-3 Activity Assay Kit (Dojindo) according to the manufacturer's protocol. In brief, cell suspension (blank group, control group, and ZIP12-shRNA group) was inoculated in 6-well plates. After being cultured for 24 h, cells were treated with 100 μ M H_2O_2 for 24 h and then collected for Caspase-3 activity assay. Collected cells were lysed in the lysis buffer on ice and the cell lysate supernatant was diluted to 1 mg/ml by lysis buffer. Then, substrate and assay buffer was added to the cell lysate supernatant and the mixture was incubated at 37°C for 2h. Finally, Caspase-3 activity was assumed with the relevant optical absorbance detected at 405 nm with a Multiskan GO Spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland).

Cell viability analysis

Cell suspension (100 μ L per well) was inoculated in 96-well plates and cultured at 37°C, 5% CO₂ for 24h. The cells from the blank group, control group, or ZIP12-shRNA group were directly added 10 μ L of CCK-8 solution and incubated for 2 h. The relevant optical absorbance was detected at 450 nm with a Multiskan GO Spectrophotometer.

Statistical Analysis

All data were analyzed using SPSS software (SPSS Statistic 24, Chicago, IL, USA), and the data are reported as mean $\pm$ SEM. Group comparisons were made using Student's t-test where appropriate. One-way analysis of variance (ANOVA) test was used assuming a two-tail hypothesis with $P < 0.05$. The correlations of mean fluorescent intensity and sperm motility or progressive motility were assessed by Spearman correlation analysis. P values of < 0.05 were considered statistically significant[33].

Results

ZIP12 is highly expressed in mouse testis

Since there are 14 members of the ZIP family responsible for the transport of zinc into the cytoplasm, the expression levels of these ZIPs in mice testes were investigated by RT-qPCR. The results showed that the mRNA levels of ZIP6 and ZIP12 were a relatively high expression in the testis (Fig. 1A). Moreover, immunoblotting analysis and immunohistochemistry (IHC) staining showed the abundant expression of ZIP12 in the testis, whereas the expression of ZIP6 in the testis was difficult to be detected (Fig. 1C, E). It should be noted that the protein bands of ZIP12 detected by Western blotting were at both 73 kDa and 46 kDa according to the prediction, while the 46 kDa band was much more intensive than the 73 kDa band.

To better understand the possibly important role of ZIP12 in testis, the expression of ZIP12 in varied mouse tissues was investigated by RT-qPCR and Western blotting. The results showed that ZIP12 mRNA was profusely expressed in testis and brain among 12 mouse tissues including heart, liver, spleen, lung, brain, ovary, uterus, testis, epididymis, and prostate (Fig. 1B), which was concordant with that in Mouse ENCODE transcriptome data published in the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/gene/277468>). Although the protein expression of ZIP12 detected by Western blot had some differences with that in RT-qPCR analysis, its expression level was still high in mouse testis (Fig. 1D).

Notably, IHC analysis showed that ZIP12 was especially localized in the spermatogonia and spermatids within the seminiferous tubules (Fig. 1E). Furthermore, IF analysis confirmed the co-localization of ZIP12 with the spermatogonia marker PLZF in spermatogonia throughout the seminiferous epithelium (Fig. 1F). Because spermatogonia enable the productivity of all kinds of spermatogenic cells throughout the seminiferous epithelium, the special expression of ZIP12 in spermatogonia indicated its potential role in preventing spermatogonia from oxidative damage during spermatogenesis by the maintenance of the intracellular zinc concentration depended on its zinc transport activity.

ZIP12 localization is consistent with zinc distribution in mouse spermatozoon

Since sufficient zinc in spermatozoa is crucial for maintaining sperm function, we investigated the correlation between ZIP12 localization and zinc distribution in spermatozoa. As shown in Fig. 2A, the distribution of zinc in fresh mouse spermatozoa, which was stained by a zinc fluorescence probe ZnAF-1, was primarily clustered in the midpiece of the sperm tail. Meanwhile, immunofluorescent staining of ZIP12 in mouse spermatozoa showed that its localization was prominent in the postacrosomal region of the sperm head and the midpiece of the sperm tail (Fig. 2B). The compatible distributions of ZIP12 with zinc in spermatozoa suggested that ZIP12 may participate in the transmembrane transport of zinc and keeping necessary zinc levels in mouse spermatozoa.

ZIP12 is expressed in spermatogonia and spermatozoa in human

Slc39a12/ZIP12 is highly conserved across mammalian species [34]. To confirm the relevance of our results in mice to humans, we examined ZIP12 expression in human testis and spermatozoa. IHC analysis was performed using testis sections from patients who received castration therapy for prostate cancer, and the result showed that ZIP12 was intensively localized in spermatogonia within the seminiferous tubules (Fig. 3A). Meanwhile, the immunofluorescent staining showed ZIP12 localization in the sperm head and the midpiece of the sperm tail (Fig. 3B). Therefore, these results were coincided with ZIP12 expression pattern in mouse testis and spermatozoa, indicating the similar role of ZIP12 in both mouse and human testes and spermatozoa during the spermatogenesis.

Obesity causes downregulation of ZIP12 expression

In general, systemic and tissue-local oxidative stress statuses are common characteristics of obesity. To probe into the role of ZIP12 and zinc influx in protecting sperm against oxidative stress, we investigated the correlation between obesity related oxidative stress and ZIP12 expression *in vivo*. Then, the male mice were fed with a high-fat diet (HFD) for 10 weeks to ensure that they gained significantly more body weight compared to their age-matched littermates fed with a normal control diet (CD) (Fig. 4A). These obese males (HFD group) exhibited significantly decreased sperm motility and increased sperm abnormality in comparison to that in the CD group (percentage of motility: 38.39 ± 3.46 vs. 77.96 ± 2.38 , $n = 10$, $P < 0.05$; sperm abnormality rate: 66.65 ± 4.40 vs. 44.09 ± 5.78 , $n = 10$, $P < 0.05$) (Fig. 4B, C). Such poor sperm quality in obese mice (HFD group) was associated with a significantly high level of ROS (Fig. 4D) and low level of zinc concentration (Fig. 4E) detected by flow cytometry analysis, indicating that insufficient antioxidative effects such as zinc deficiency may contribute to high ROS or oxidative damage in sperm.

On the other hand, the expression of ZIP12 in testes was detected by Western blot. The result showed that there was a significant decrease in ZIP12 expression in HFD mice testes compared to that in CD mice testes (Fig. 4F). Thus, the inverse correlation of ZIP12 expression with ROS level in obese mice indicated

that the dynamic transport of zinc into the spermatogenic cells or spermatozoa undertaken by ZIP12 may be crucial for preventing sperm cells from oxidative damage during spermatogenesis.

ZIP12 expression in C18-4 is down-regulated in response to H₂O₂ / sodium palmitate-induced oxidative stress

Since the expression of ZIP12 was prominent in the spermatogonia in the seminiferous epithelium, a mouse spermatogonia cell line, C18-4 cell was employed to investigate the function of ZIP12 in response to oxidative stress. Both immunofluorescence staining and Western blot analysis confirmed the expression of ZIP12 in C18-4 cells (Fig. 5A-C). After treated with a ROS inducer, H₂O₂, for 24h, 48h, and 72h, ZIP12 expression level in C18-4 cells significantly decreased (Fig. 5B). Similarly, ZIP12 expression was also declined in C18-4 cells when exposed to a lipotoxic condition induced by sodium palmitate (PA) treatment, which also promotes ROS generation (Fig. 5C). The decreased expression of ZIP12 in C18-4 cells under oxidative stress was consistent with that in testes from obese mice, which suggested that oxidative stress can downregulate ZIP12 expression in testes or spermatogonia, while inadequate amounts of ZIP12 in spermatogonia potentially contributes to insufficient antioxidant defense against oxidative damage.

ZIP12 knockdown reduces intracellular zinc and antioxidant capacity in C18-4 cells

In this research, RNA interference (RNAi) was employed in C18-4 cells to evaluate the antioxidant capacity of ZIP12 in spermatogonia. The expression of ZIP12 protein in C18-4 cells transfected with *ZIP12*-specific shRNA significantly decreased compared to that in control group transfected with control shRNA (Fig. 6A). Then, the intracellular zinc stained by Zinquin, a fluorescent probe, was analyzed by flow cytometry. Consistent with the previous report in neuro-2a cells [32], the mean fluorescent intensity of intracellular zinc significantly decreased when knockdown of ZIP12 in C18-4 cells (positive for GFP signal) (Fig. 6B, C), suggesting that ZIP12 was a key player responsible for maintaining intracellular zinc concentration.

Furthermore, H₂O₂ treatment was applied to assess the antioxidant capacity of ZIP12. C18-4 cells in both the control group and the *ZIP12*-shRNA group were treated with H₂O₂ respectively and then CCK8 assay and Caspase-3 activity were assessed. *ZIP12*-shRNA group exhibited significant low CCK8 level and high Caspase-3 activity in comparison to those in the control group (Fig. 6D, E), which indicated low cell viability and high apoptosis in *ZIP12* knockdown cells. Taken together, insufficient ZIP12 accompanied with decreased intracellular zinc in C18-4 cells can impair the cell antioxidant capacity indeed and finally result in high sensitivity to oxidative stress.

Discussion

Zinc is an essential element with a wide range of biological functions and plays a significant role in the male reproductive system. However, the zinc transporters responsible for transmembrane zinc traffic during spermatogenesis are still not well understood. In the present study, we demonstrated the abundant

expression of ZIP12 in testes, especially in spermatogonia and spermatozoa of both mice and humans, and confirmed its crucial role in maintaining intracellular zinc concentration and antioxidant ability. Importantly, the expression of ZIP12 in testes or spermatogonia under stress conditions significantly decreased and thereby was associated with a decline in zinc concentration. Therefore, our results strongly suggested a potential mechanism involved zinc traffic in the impairment of spermatogenesis and sperm quality caused by environmental stress.

Zinc is a major non-enzyme antioxidant in sperms and the male genital tract. Numerous studies have proved the importance of zinc on male fertility. Zinc plays a role in chromatin stabilization, the sperm morphological maturation, sperm motility acquirement, the epithelial integrity of male reproductive organs, and the preservation of testosterone level [14, 20]. Zinc deficiency is generally associated with male infertility or subfertility [9, 17], and zinc supplementation in diet is considered as a recommendation for the improvement of sperm quality and male fertility [14, 24, 25]. Moreover, several studies demonstrated the beneficial effects of zinc addition *in vitro* on sperm quality and the fertilization rate in assisted reproductive techniques [35–37].

However, a recent clinical study including 2370 couples investigated the effect of dietary supplements containing zinc and showed that zinc supplementation does not appear to improve pregnancy rates, sperm counts or sperm function [38]. Therefore, it is worth noting that zinc concentration differs between diet intake, blood, and testicular tissues [39], and plentiful zinc in the testicular microenvironment and spermatogenic cells was mainly dependent on zinc traffic afforded by zinc transporters including ZnTs and ZIPs. These two classes of zinc transporters were well-documented to mediate the transfer of zinc across physiological membranes and maintain the proper intracellular zinc concentration.

Previous researches have preliminarily revealed physiological functions of ZnTs and ZIPs mainly according to their distribution. For instance, ZnT1 can regulate zinc absorption and secretion depending on its specific location at the apical or basolateral membrane. ZnT4, ZnT5 and ZnT6 were distributed in the inner membrane of organelles and were responsible for zinc transport into organelles [40]. ZIP4 was mainly expressed in human epidermal keratinocytes, and its deficiency will lead to acrodermatitis enteropathica [41]. On the other hand, ZIP5, ZIP6, and ZIP10 are reported to express in testis [33]. Specifically, ZIP5 is expressed in the plasma membrane of Sertoli cells, while ZIP6 and ZIP10 are mainly distributed in the plasma membrane of spermatozoa [33], which suggested their roles in controlling the zinc influx in these cells. However, the expression and function of zinc transporters in the male germ cells were rarely reported and it remains unclear about their roles in zinc homeostasis when suffering oxidative stress induced by pathological conditions or environmental challenges.

Since the increasing trend of zinc concentration in different periods of developing spermatogenic cells during spermatogenesis and sufficient intracellular zinc is crucial for maintaining cell function [9, 12], we focused on the expression of ZIPs in testis owing to their functions in mediating zinc influx into cells. In the present study, we analyzed the expressions of all fourteen ZIP members in mouse testis and found significantly high expression of ZIP12 in testis in comparison to other ZIPs. Moreover, although ZIP12 is

ubiquitously distributed in tissues, its mRNA and protein levels were abundant in testis. Considering the high concentration of zinc in testis, such intensive expression of ZIP12 in testis indicated its potentially indispensable role in zinc homeostasis and spermatogenic cell development.

Previous studies on ZIP12 mainly aimed at its function in the central nervous system [32, 42–44] and showed its important role in neuronal differentiation. Meanwhile, ZIP12 was also reported to participate in hypoxia-induced remodeling of pulmonary arterioles [45]. It is worthy to note that ZIP12 has the highest affinity to zinc among all the ZIPs proteins [46] and the loss of ZIP12 cannot be compensated by other zinc transporters in the central nervous system [32]. However, the expression and function of ZIP12 in the testis or spermatozoa have never been reported before.

In the current study, we firstly found that ZIP12 was intensively abundant in spermatogonia and spermatozoa, both in mice and humans. Spermatogonia are the primordial cells of spermatogenesis, and any damage to spermatogonia may result in spermatogenic disorder and male infertility. Thus, intensive expression of ZIP12 in spermatogonia suggested its important role in zinc homeostasis during spermatogenesis. Meanwhile, the accordant distribution of ZIP12 with intracellular zinc in spermatozoa indisputable confirmed its role in mediating zinc influx which was potentially involved in resisting oxidative damage and maintaining sperm quality.

To explore the role of ZIP12 in defense of oxidative stress, an obese mouse model was employed to investigate the correlation between ZIP12 expression level and sperm quality. Obesity was well demonstrated as a cause of systemic and tissue-local oxidative stress and thereby led to poor sperm quality [47, 48]. Previous studies reported that zinc contents in the skin, muscle, and bone were reduced in obese mice [49], and serum zinc concentration was significantly lower in obese children than that in matched normal-weight children [50–52]. Meanwhile, the expressions of some zinc transports, such as ZNT1 and ZNT6 in brain[53], ZIP14 in adipose[54, 55], and ZnT4, ZnT5, ZnT9, ZIP1, ZIP4 and ZIP6 in leukocyte[56], were inversely correlated with obesity. Herein, our study provided similar evidence that ZIP12 expression in testis significantly decreased in obese mice, which was associated with reduced sperm zinc content, high sperm ROS level, poor sperm quality and male subfertility. Thus, reduced ZIP12 expression in testis may be a cause of insufficient zinc content and elevated oxidative stress in sperm, which may further lead to male infertility.

Considering the role of ZIP12 in zinc homeostasis or antioxidant defense, we were curious about the cause of obesity-associated ZIP12 down-regulation in testis. A previous study showed the altered expressions of zinc transporters in hepatocyte via oxidative stress [57]. Due to the tight association of obesity with oxidative stress, our results also demonstrated the decrease of ZIP12 expression in response to oxidative stress in a spermatogonia cell line C18-4 cells. Consistent with its reduced expression in obese testis, ZIP12 expression in C18-4 cells was significantly declined in response to the H₂O₂ or PA induced oxidative stress. Therefore, our study documented that downregulated ZIP12 expression induced by oxidative stress can subsequently lead to a decrease of zinc-associated antioxidant activity, and thereby aggravate oxidative stress in turn.

Furthermore, the effect of ZIP12 on spermatogonia was also evaluated by ZIP12 knockdown in C18-4 cells. Consistent with the previous report in neuro-2a cells [32], we also found a decrease of intracellular zinc in C18-4 cells by ZIP12-shRNA knockdown. In addition, our results indicated that ZIP12 played a crucial role in the defense of oxidative damage and the maintenance of cell viability, whereas reduced ZIP12 expression in C18-4 cells will result in insufficient antioxidant capacity and be prone to apoptosis. It should be point out that, we are aware of the limitation of RNAi assay in vitro in current study. Thus, additional studies in vivo, such as *ZIP12* knock out mice, are required to understand the precise role of ZIP12 in the spermatogonia involved in spermatogenesis and male fertility.

Conclusions

In summary, a zinc transporter ZIP12 is highly expressed in the testis, especially in spermatogonia and spermatozoa. It participates in mediating zinc influx and is crucial for zinc homeostasis during spermatogenesis. By maintaining intracellular zinc levels, ZIP12 may play a key role in reducing oxidative stress in spermatogonia and spermatozoa (Fig. 7). In contrast, insufficient ZIP12 accompanied with decline in intracellular zinc level can cause an impairment of antioxidative capacity and finally lead to male subfertility or infertility ultimately.

Abbreviations

CASA: computer-assisted sperm analysis; CCK-8: cholecystokinin-8; CD: control diet; DAPI: 4',6-diamidino-2-phenylindole; DIC: Differential interference contrast; GFP: green fluorescent protein; H₂O₂: hydrogen peroxide; HFD: high-fat diet; IF: immunofluorescence; IHC: immunohistochemistry; PA: sodium palmitate; PLZF: promyelocytic leukemia zinc finger; RNAi: RNA interference; RT-qPCR: quantitative reverse transcription polymerase chain reaction; SE: Standard Error; SEM: standard error of mean; shRNA: short hairpin RNA; SOD: superoxide dismutase; SPSS: statistic package for social science; ZIP: Zrt-, Irt-like protein; ZnT: Zinc transporter protein.

Declarations

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author's contributions

XZ, CY, WW, LS and CJ performed experiments. XZ and CY analyzed data and prepared the manuscript. YL and ZD edited the manuscript. LW, YL and ZD supervised the project and finally approved the manuscript. All authors read and approved the final manuscript.

Ethics approval

All animal care and experimental protocols used in this study were approved by the institutional animal care and the ethic committee of Shanghai Jiao Tong University School of Medicine. Use of the human testicular section and semen sample were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine and all experiments were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The expression of ZIP12 in mouse testis. A Relative expression of ZIP mRNAs detected in mouse testis by RT-qPCR. Data were presented as means±SE from three independent experiments. B Relative expression of ZIP12 mRNA detected in various mouse tissues by RT-qPCR. Data were presented as means±SE from three independent experiments. C Western blot detection of ZIP12 protein and ZIP6 protein in mouse testis respectively, indicating the protein bands of ZIP12 at 73 kDa and 46 kDa as predicted. D Western

blot detection of ZIP12 protein in the different tissues. The relative levels of ZIP12 protein were normalized to β -actin. E Immunohistochemical analysis of ZIP12 and ZIP6 expression in the testis. Red arrows indicated spermatids and spermatogonia positive for ZIP12 staining. Control represents normal rabbit IgG staining. Scale bar = 50 μ m. F Immunofluorescent staining of ZIP12 (red) in testis. Spermatogonia (white arrows) were stained with PLZF (green). Cell nuclei were stained with DAPI (blue). Scale bar = 50 μ m.

Figure 2

ZIP12 localization and zinc distribution in mouse spermatozoa. A Fluorescent staining of zinc by using ZnAF-1 (green) in fresh spermatozoa. White arrow indicated zinc staining in the midpiece of the mouse sperm tail. Differential interference contrast (DIC) images show the sperm shape. Scale bar = 50 μ m. B Immunofluorescent staining of ZIP12 (red) in mouse sperm. Acrosomes were stained with PNA (green). Cell nuclei were stained with DAPI (blue). White arrows indicated ZIP12 staining in the postacrosomal region of the sperm head and the midpiece of the sperm tail. Scale bar = 50 μ m.

Figure 3

ZIP12 localization in testis and spermatozoa from human specimens. A Immunohistochemical analysis of ZIP12 expression in human testis. The red arrow indicated the spermatogonia positive for ZIP12 staining. Control represents normal rabbit IgG staining. Scale bar = 50 μ m. B Immunofluorescent staining of ZIP12 (red) in human spermatozoa, indicating its distribution in the head and the midpiece of tail. Cell nuclei were stained with DAPI (blue). DIC images show the sperm shape. Scale bar = 20 μ m.

Figure 4

Comparison of sperm parameters, ROS level, and ZIP12 expression in obese mice. A Comparison of time-dependent increases in body weight between CD (n = 10) and HFD groups (n = 10). B Sperm motility in mice fed either CD or HFD analyzed by CASA (n = 10). C Sperm morphology analysis of CD-fed mice and HFD-fed mice using Diff-Quick method (n = 10). D Comparison of sperm ROS level in CD and HFD groups (n = 7). E Fluorescent intensity of Zinc in mouse sperm stained by ZnAF-1 and analyzed by flow cytometry (n = 5). F Western blot detection of ZIP12 protein in CD (n = 3) and HFD (n = 8) mice. The relative levels of ZIP12 protein were normalized to β-actin. Data were presented as means ± SEM. *P < 0.05, **P < 0.01, ****P < 0.0001.

Figure 5

ZIP12 expression in response to oxidative stress in spermatogonia cell line C18-4 cells. A Immunofluorescent staining of ZIP12 (red) in C18-4 cells. Cell nuclei were stained with DAPI (blue). Scale bar = 50 μm. B Western blot detection of ZIP12 protein in C18-4 cells after 100 μM H₂O₂ treatment for 24h, 48h, 72h. The relative levels of ZIP12 protein were normalized to β-actin. Data were presented as means ± SEM from three independent experiments. C Western blot detection of ZIP12 protein in C18-4 cells after 50 μM PA treatment for 24h and 48h. The relative levels of ZIP12 protein were normalized to β-actin. Data were presented as means ± SEM from six independent experiments. *P < 0.05, **P < 0.01, ****P < 0.0001.

Figure 6

Knockdown of ZIP12 decreased zinc concentration and the antioxidant capacity in C18-4 cells. A ZIP12 protein was detected in the control shRNA group and ZIP12 shRNA group by Western blot. The relative levels of ZIP12 protein were normalized to β -actin. Data were presented as means $\pm$ SEM from three independent experiments. B-C Flow cytometric analysis of the fluorescent intensity of zinc on the control ($n = 5$) and the ZIP12 shRNA group ($n = 5$). (B) One representative sample showed P2 region, which represented cells transfected with control shRNA or ZIP12-specific shRNA that were positive for GFP signal. (C) Mean fluorescent intensity of zinc of the two groups indicated the decrease of zinc level in ZIP12-knock down C18-4 cells. Data were presented as means $\pm$ SEM from four independent experiments. D CCK-8 proliferation assay in control shRNA ($n = 4$) or ZIP12-specific shRNA ($n = 4$) transfected C18-4 cells with the treatment of 100 μ M H₂O₂. Data were presented as means $\pm$ SEM from

four independent experiments. E Relative Caspase-3 activity in C18-4 cells transfected with control shRNA or ZIP12-specific shRNA and treated with 100 μ M H₂O₂. Data were presented as means $\pm$ SEM from three independent experiments. *P <0.05, ****P <0.0001.

Figure 7

Schematic representation of ZIP12 function in spermatogenesis. The zinc transporter ZIP12 is intensively expressed in the testis, especially in spermatogonia and spermatozoa. It mediates the zinc influx into cytosol and involves in maintaining intracellular zinc level, which plays a key role in the defense of oxidative stress.

Supplementary Files

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- [SupplementaryTable1.xlsx](#)