Seminal plasma selenium concentrations, sperm mtDNAcn, and semen quality: association and mediation analyses among healthy Chinese men

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Abstract

Objective: To investigate the associations of seminal plasma selenium (Se) concentrations with semen quality and to explore the mediating role of sperm mitochondrial DNA copy number (mtDNAcn). Design: Cross-sectional study with repeated measurements. Setting: Hubei Province Human Sperm Bank of China. Population: A total of 1159 healthy men who repeatedly provided 5617 semen samples were included. Main Outcome Measures: Seminal plasma Se concentration and sperm mtDNAcn of healthy men screened as potential sperm donors were determined using inductively coupled plasma mass spectrometry (ICP-MS) and real-time fluorescent quantitative PCR (RT-qPCR), respectively. Methods: Linear mixed-effects models and mediation analyses were performed. Result(s): After adjusting for potential confounders, we observed positive associations of seminal plasma Se concentrations with sperm concentration and total count (both p-Values for trend < 0.001). Volunteers in the highest vs. lowest quartiles of seminal plasma Se concentrations had 70.1% (95% CI: 53.3%, 88.9%) and 59.1% (95% CI: 40.5%, 80.2%) higher sperm concentration and total count, respectively. We also found inverse associations between within-subject pooled seminal plasma Se concentrations and sperm mtDNAcn, and between sperm mtDNAcn and sperm concentration, total count, total motility, and progressive motility (all p-Values for trend < 0.05). Mediation analyses showed that sperm mtDNAcn mediated 19.7% (95% CI: 15.9%, 25.3%) and 23.1% (95% CI: 17.4%, 33.4%) of the associations between within-subject average seminal plasma Se concentrations and sperm concentration and total count, respectively. Conclusions: Seminal plasma Se concentrations were positively associated with sperm concentration and total count, which was partly mediated by sperm mtDNAcn.

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ABSTRACT

Objective: To investigate the associations of seminal plasma selenium (Se) concentrations with semen quality and to explore the mediating role of sperm mitochondrial DNA copy number (mtDNAcn).

Design: Cross-sectional study with repeated measurements.

Setting: Hubei Province Human Sperm Bank of China.

Population: A total of 1159 healthy men who repeatedly provided 5617 semen samples were included.

Main Outcome Measures: Seminal plasma Se concentration and sperm mtDNAcn of healthy men screened as potential sperm donors were determined using inductively coupled plasma mass spectrometry (ICP-MS) and real-time fluorescent quantitative PCR (RT-qPCR), respectively.

Methods: Linear mixed-effects models and mediation analyses were performed.

Results: After adjusting for potential confounders, we observed positive associations of seminal plasma Se concentrations with sperm concentration and total count (both p -Values for trend < 0.001). Volunteers in the highest vs. lowest quartiles of seminal plasma Se concentrations had 70.1% (95% CI: 53.3%, 88.9%) and 59.1% (95% CI: 40.5%, 80.2%) higher sperm concentration and total count, respectively. We also found inverse associations between within-subject pooled seminal plasma Se concentrations and sperm mtDNAcn, and between sperm mtDNAcn and sperm concentration, total count, total motility, and progressive motility (all p -Values for trend < 0.05). Mediation analyses showed that sperm mtDNAcn mediated 19.7% (95% CI: 15.9%, 25.3%) and 23.1% (95% CI: 17.4%, 33.4%) of the associations between within-subject average seminal plasma Se concentrations and sperm concentration and total count, respectively.

Conclusions : Seminal plasma Se concentrations were positively associated with sperm concentration and total count, which was partly mediated by sperm mtDNAcn.

Keywords: semen quality; selenium; mtDNAcn; repeated measurements; mediation

Tweetable abstract: Sperm mtDNAcn partly mediated the positive dose-response relationships between seminal plasma Se concentrations and sperm concentration and total count.

1 | Introduction

Selenium (Se) is an essential micronutrient required for many physiological functions in humans, including immune function, thyroid hormone metabolism, and antioxidant defense system.[1, 2] Low Se status has been associated with an increased risk of poor immune function, cognitive decline, and all-cause and cancer mortality.[2] Meanwhile, growing evidence shows that Se is essential for male and female reproductive health.[3, 4]

Semen quality is commonly used as a proxy to estimate male fertility. Several previous studies have explored the associations between Se concentrations in different biological samples (e.g. urine, blood, and seminal plasma) and semen quality, but the results remain controversial. For instance, Zeng et al. [5] reported that urinary Se concentrations were positively associated with total sperm count among 394 males from an infertility clinic. In a similar population consisting of 746 men, Wang et al. [6] revealed a positive association between seminal plasma Se concentrations and sperm concentration. In contrast, Akinloye et al. [7] found an inverse association between serum Se concentrations and total sperm count among 60 idiopathic infertile Nigerian men. Meanwhile, a lack of association between Se status and human semen quality was also reported.[8, 9] These previous studies mostly relied on a single measurement of Se concentrations and semen quality, which is prone to measurement error, given the considerable high within-individual variabilities in urinary Se concentrations[10, 11] and sperm quality parameters.[12-15] More importantly, little is known about the mechanisms underlying the associations between Se status and semen quality, which are critical to improve insights for disease development and prevention.

Mitochondrial DNA copy number (mtDNAcn), a measure of mitochondrial genome abundance,[16] has been proposed to be a sensitive biomarker for semen quality and mitochondrial dysfunction.[17-19] Previous studies have shown that sperm mtDNAcn is strongly associated with sperm concentration, total count, and motility.[20-24] Se has a protective effect against oxidative stress due to its unique antioxidant properties.[25] Sperm Se content has been positively correlated with sperm mitochondrial volume both in humans and some animals (e.g., boars, horses, bulls, and rams).[26] Meanwhile, several animal studies have demonstrated that Se treatment can significantly improve sperm mitochondrial function.[27-29] Therefore, we suspecte that sperm mtDNAcn might play an important mediating role in the association between low Se status and impaired semen quality.[19]Because Se concentrations in seminal plasma are direct markers for Se status in the male reproductive tract,[30] we investigated the associations between seminal plasma Se concentrations, sperm mtDNAcn, and semen quality and the mediating role of sperm mtDNAcn among healthy men screened as potential sperm donors who repeatedly provided semen samples.

2 | Methods

2.1 | Study design and subjects

From April 2017 to July 2018, a total of 1487 healthy men screened as potential sperm donors were recruited from the Hubei Province Human Sperm Bank, as described in our previous studies.[31, 32] Briefly, volunteers were eligible if they completed at least a high school degree, aged between 22 and 45 years, and had no sexually transmitted or genetic diseases. At recruitment (day 0), all volunteers completed a questionnaire that collected data on demographic characteristics, lifestyle habits, and reproductive history. Physical examinations were also performed to measure weight, height, and waist circumstance, etc. All volunteers provided a semen sample for the initial assessment of semen quality; additional semen samples for further screening or formal sperm donation were also collected during the following 5 study intervals (e.g., 1-15, 16-31, 32-63, and [?] 64 days since baseline). Finally, 1159 volunteers with 5617 semen examinations were included in our subsequent analyses (**Figure 1**).

2.2 | Semen collection

Semen samples were collected at baseline and each follow-up visit into a trace-element-free sterile polypropy-

lene container by masturbation in a private room at the Hubei Province Human Sperm Bank. We measured sperm quality parameters for each semen sample. Due to the preciousness of semen samples and the considerable within-person variability in seminal plasma Se concentrations, the first semen specimens collected at each pre-specified study interval [i.e., days 0 (baseline), 1-15, 16-31, 32-63, and [?] 64 from initial recruitment] were pooled in equal volumes to reflect the average Se status over an approximately 3-month period,[33, 34] which corresponds to the duration of spermatogenesis (**Figure 1**).

2.3 | Measurement of sperm quality parameters

Sperm quality parameters including semen volume (mL), sperm concentration (million/mL), and motility (%) were evaluated by qualified laboratory technicians according to the World Health Organization (WHO) guidelines,[35]as described previously.[31, 32] A weighing method was used to measure semen volume. Sperm concentration and motility were assessed by placing 10 μL of each well-mixed semen sample into a clean Makler chamber utilizing an optical microscope and a cytometer. Total sperm count (million per ejaculation) was calculated as semen volume × sperm concentration.[36] Daily internal quality controls were performed to guarantee that the interday and intraday variations were less than 10%.

2.4 | DNA extraction and mtDNAcn measurement

Detailed information about DNA extraction and mtDNAcn measurement has been described elsewhere.[24, 37] Concisely, sperm DNA was extracted from the last semen specimen taken within 90 days from baseline recruitment using the TIANamp Genomic DNA Kit (TIANGEN Biochemical Technology Co., Ltd, Beijing, China). The extracted DNA samples were quantified using the Nanodrop spectrophotometer (ND-1000; Thermo Scientific Inc. DE, USA) and then immediately stored at -80 refrigerators until mtDNAcn measurement. Sperm mtDNAcn in the DNA samples was measured by real-time fluorescent quantitative PCR (RT-qPCR). To examine the amplification specificity, each run was completed by a melting curve analysis. All DNA samples were analyzed in triplicates. The mean cycle threshold (Ct) value of three measurements was utilized to calculate the mtDNAcn based on $2^{-\Delta\Delta^{c}\tau}$method.[38] The intra- and inter-assay coefficients of variation were less than 5%.

2.5 | Determination of seminal plasma Se concentration s

Seminal plasma Se concentrations were quantified using inductively coupled plasma mass spectrometry (Agilent 7700x ICP-MS; Agilent Technologies, USA), using the method described previously.[6, 39] Concisely, a 150-μL within-subject pooled seminal plasma was transferred to a 5-mL trace element-free polyethylene tube and then acidized by 2.85 mL 1.0% HNO_3. Spiked within-subject pooled seminal plasma and standard reference materials (SRMs) 1640a and 2670a were used as reference controls. The limits of quantification (LOQ) for Se in seminal plasma was 1.10 μg/L. All specimens had values higher than the lowest detectable level, and the measured concentrations were substituted with the mean concentration + 3 × standard deviation (SD) for those with measured concentrations higher than this value.[40]

2.6 | Within-person variability of seminal plasma Se concentrations

To assess the reproducibility of seminal plasma Se concentrations, we measured Se concentrations in 232 repeated seminal plasma samples collected from 93 randomly selected volunteers across the pre-specified 5 study intervals [i.e., days 0 (baseline), 1-15, 16-31, 32-3, and [?] 64 from initial recruitment] (**Figure 1**).

2.7 | Statistical analyses

Descriptive statistics were conducted for the distribution of volunteers' demographics, seminal plasma Se concentrations, sperm mtDNAcn, and sperm quality parameters. To satisfy normality assumption, seminal plasma Se concentrations, sperm mtDNAcn, and sperm quality parameters were $\log_{10}$-transformed. The reproducibility of seminal plasma Se concentrations was assessed based on the intraclass correlation coefficients (ICCs, 0-1), which was calculated as the between-subject variance divided by the total variance.[41]

We categorized study volunteers into quartiles according to the distribution of within-subject pooled seminal plasma Se concentrations. Linear mixed-effects models were used to examine the associations be-

tween the quartiles of within-subject pooled seminal plasma Se concentrations and repeated sperm quality parameters.[42] Linear regression models were used to investigate the associations between within-subject pooled seminal plasma Se concentrations and sperm mtDNAcn and between sperm mtDNAcn and within-subject average sperm quality parameters. The linear trend was evaluated by modeling the quartiles of seminal plasma Se concentrations as continuous variables using integer values (i.e., 1-4). Covariates were selected a *priori* relied on our prior findings,[6, 11] including demographic characteristics (i.e., age and education level), reproductive history (i.e., history of having ever fathered a child), lifestyle factors (i.e., smoking, alcohol consumption, and abstinence time), physical examination (i.e., BMI, and waist-hip ratio), liquefaction time, and sampling season (i.e., only adjusted in the linear mixed model). To explore potential non-linear associations of seminal plasma Se concentrations with sperm mtDNAcn and sperm quality parameters, restricted cubic splines were conducted by modeling Se concentrations as continuous variables.

Mediation analyses were implemented to explore whether sperm mtDNAcn mediated the association between within-subject average seminal plasma Se concentrations and sperm quality parameters (*medeff* command in Stata).[43] We used linear regression models to examine the associations of exposure-outcome, exposure-mediator, and exposure-mediator-outcome, respectively. The medicated proportion by sperm mtDNAcn was defined as the ratio of indirect effect to the total effect.

Several sensitivity analyses were conducted to assess the robustness of associations between seminal plasma Se concentrations and semen quality. First, we used within-subject average sperm quality parameters across all visits to evaluate the influence of different sampling numbers between participants. Second, we additionally adjusted for seminal plasma concentrations of cobalt, copper, iron, manganese, molybdenum, and zinc to account for the joint associations of these redox metals.[44] Third, we excluded semen samples that were not used for within-subject pooling to avoid potential bias. Finally, we assessed the potential influence of changes in lifestyle and diet factors by excluding participants who did not meet donation criteria at recruitment. All data analyses were performed utilizing Stata software (version 15.0; Stata Corporation, College Station, TX, USA) and R software (version 4.0.2; R Development Core Team).

3 | Results

3.1 | Characteristics of volunteers

The 1159 volunteers had a mean (SD) age, BMI, waist-hip ratio, and abstinence duration of 28.1 (5.3) years, 22.8 (3.3) kg/m^2, 0.8 (0.1), and 6.2 (3.3) days. The majority of volunteers had less than a college degree (65.3%), never fathered a child (72.9%), never smoked (54.0%), and drank alcohol occasionally (60.2%). Due to the Chinese Spring Festival, only 14.6 semen samples were collected during winter (**Table 1**).

3.2 | Distribution of seminal plasma Se concentrations, sperm mtDNAcn, and semen quality

Se concentrations were detected in all pooled within-subject samples (n= 1159). The median (interquartile range, IQR) seminal plasma concentrations of Se were 29.8 (23.7-36.6) μg/L. Among 1159 volunteers recruited in the present study, 989 were measured for mtDNAcn. The median (IQR) sperm mtDNAcn was 0.8 (0.5-1.2). We collected 5617 semen samples from 1159 men. The median (IQR) semen volume, sperm concentration, total sperm count, total motility, and progressive motility were 2.8 (2.0-4.0) mL, 61.0 (42.0-68.0) million/mL, 158.4 (113.1-224.0) million per ejaculate, 64.0 (54.0-67.0) %, and 60.0 (50.0-65.0) %, respectively (**Table 2**).

3.3 | Reproducibility of seminal plasma Se concentrations

The between-subject variance (0.016; 64%) was much higher than within-subject variance (0.009; 36%) for repeated seminal plasma Se concentrations among 93 men with 232 measurements. The estimated ICC for repeated Se measurements was 0.64, indicating fair to good reproducibility (Table S1).[41]

3.4 | Associations between seminal plasma Se concentrations, sperm mtDNAcn, and sperm quality parameters

In the fully adjusted models, volunteers in the highest vs. lowest quartiles of seminal plasma Se concentrations had 70.1% (95% CI: 53.3%, 88.9%) and 59.1% (95% CI: 40.5%, 80.2%) higher sperm concentration and total

count, respectively (**Table 3**). These positive dose-response relationships were further demonstrated in the cubic spline models when seminal plasma Se concentrations were modeled as continuous variables (**Figure 2**). We found inverse associations between within-subject pooled seminal plasma Se concentrations and sperm mtDNAcn both in crude and adjusted linear regression models (both P for trend < 0.05), which, again, was further confirmed in the cubic spline models when we modeled seminal plasma Se concentrations as continuous variables (**Figure 2**).

Afer adjusting for potential confounders, volunteers in the highest vs. lowest quartiles of within-subject pooled seminal plasma Se concentrations had a 13.0% (95% CI: -23.6%, -1.1%) lower sperm mtDNAcn (**Table 3**); and sperm mtDNAcn was inversely associated with sperm concentration [-40.8% (95% CI: -46.4%, -34.8%) for the highest vs. lowest sperm mtDNAcn quartile; P for trend < 0.001], total count [-41.2% (95% CI: -47.8%, -33.8%); P for trend < 0.001], total motility [-8.1% (95% CI: -12.6%, -3.4%); P for trend $= 0.010$]), and progressive motility [8.6% (95% CI: -13.4%, -3.7%); $P$for trend $= 0.008$] (Table S2).

3.5 | Mediation and sensitivity analyses

Mediation analyses showed that sperm mtDNAcn mediated 19.7% (95% CI: 15.9%, 25.3%) and 23.1% (95% CI: 17.4%, 33.4%) of the association between within-subject average seminal plasma Se concentrations and sperm concentration and total count, respectively (**Figure 3)** . Sensitivity analyses showed that the associations between seminal plasma Se concentrations and semen quality were materially unchanged when we used the within-subject average sperm quality parameters (Table S3), when we further adjusted for seminal plasma concentrations of cobalt, copper, iron, manganese, molybdenum, and zinc (Table S4), when we excluded semen samples that were not used for within-subject pooling (Table S5), and when we excluded participants who did not meet donation criteria at baseline recruitment (Table S6).

4 | Discussion

4.1 | Main findings

Among 1159 healthy young men who provided 5617 semen samples, we found positive dose-response relationships between within-subject pooled seminal plasma Se concentrations with sperm concentration and total count. Besides, we observed inverse associations between seminal plasma Se concentrations and sperm mtDNAcn and between sperm mtDNAcn and sperm concentration, total count, total motility, and progressive motility. Mediation analyses demonstrated that sperm mtDNAcn mediated 19.7% and 23.1% of the associations between seminal plasma Se concentrations and sperm concentration, as well as total count, respectively.

4.2 | Strengths and limitations

This study has several strengths. First, we collected repeated semen samples from each volunteer to measure seminal plasma Se concentrations and sperm quality parameters, which reduced measurement error.[15, 45] Second, we measured Se concentrations in seminal plasma, which directly reflect Se status in the male reproductive tract. Third, our study participants are healthy men screened as potential sperm donors, which is more representative than previous study populations recruited from infertility clinics. Nevertheless, there are also several limitations. First, we measured sperm mtDNAcn at a single time point, which may have resulted in measurement error. Second, we measured seminal plasma Se concentrations in within-subject pooled samples to reflect the average Se status across the duration of spermatogenesis, which, however, made it impossible to determine the potential window of susceptibility to Se status for semen quality.[11] Third, although we have adjusted for many covariates, residual confounding from unmeasured factors cannot be fully ruled out. Finally, as with any observational study, we cannot establish causal relationships.

4.3 | Interpretation in light of other evidence

Se is essential for testosterone biosynthesis and the normal development of spermatozoa.[4] Consistent with this notion, our results indicated that seminal plasma Se concentrations were positively associated with sperm

concentration and total sperm count in a dose-dependent manner. In support of our findings, Liu, et al. [46] reported positive associations between seminal plasma Se concentrations with sperm concentration and total count among 1136 men being investigated for infertility. Zeng et al. [5] reported a positive association between urinary Se concentrations and total sperm count among 394 males from an infertility clinic. Calogero et al.[47] reported higher Se concentrations in blood and seminal plasma among 48 normozoospermic men compared to 131 men with asthenozoospermia or oligozoospermia. Eroglu et al. [48]reported that serum and seminal plasma Se concentrations were positively associated with sperm concentration, motility, and morphology among 59 men attending an infertility clinic. In a double-blind randomized study, Safarinejad et al. [49] reported that Se supplementation improved semen quality among 68 infertile men. However, controversial findings were also reported among healthy men and male partners of couples from infertility clinics.[7, 50] The inconsistency between studies is not unexpected given the differences in Se status, biological matrices (e.g., blood, urine, and seminal plasma), the composition of the study populations (e.g., healthy vs. infertile men), and sample size.

Previous studies mostly relied on a single measurement of Se concentrations and semen quality, which may have resulted in measurement error. Among a subgroup of 93 volunteers who provided 232 semen samples, we explored, for the first time, the reproducibility of Se concentrations in seminal plasma. We found that Se concentrations in seminal plasma exhibited fair to good reproducibility probably due to the changes in dietary intake and physiological metabolism,[51] indicating potential exposure misclassification in previous studies that measured Se in a single semen sample. Substantial studies have also reported considerable high within-individual variabilities in sperm quality parameters[12-15] and Se concentrations in urine samples.[10, 11] All these findings emphasize the importance of collecting repeated samples in the future studies.

Se is an important ingredient of glutathione peroxidase enzymes[52] and is considered to shield germline cells from oxidative damage,[53] which, thus, has a protective effect against oxidative stress. Sperm mtDNAcn has been proposed to be a sensitive biomarker for the decline in semen quality and mitochondrial dysfunction induced by oxidative stress.[54] In support of these notions, we found strongly inverse associations between seminal plasma Se concentrations and sperm mtDNAcn and between sperm mtDNAcn and sperm concentration, total count, total motility, and progressive motility. Our mediation analyses further revealed for the first time that sperm mtDNAcn mediated nearly 20% of the association between seminal plasma Se concentrations and sperm concentration and total count. We suspect that higher Se status could suppress oxidative stress, as evidenced by the activity of the antioxidant selenoenzyme glutathione peroxidase,[55-57] which decreased sperm mtDNAcn and eventually reduced the harmful effect induced by oxidative stress on spermatogenesis.[58]

5 | *Conclusions*

Based on repeated measurements study design, we found positive dose-response relationships between seminal plasma Se concentrations and sperm concentration and total count, which was partly mediated by sperm mtDNAcn. Our findings highlight the importance of Se in improving semen quality and provide an original clue of the underlying mechanism related to sperm mtDNAcn.

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Disclosure of interests

The authors of this manuscript have no conflicts of interests to disclose. Completed disclosure of interest forms is available to view online as supporting information.

Contribution to authorship

Heng-Gui Chen analyzed the data. Heng-Gui Chen and Yi-Xin Wang drafted the original manuscript. Yi-

Xin Wang and Weimin Ye lead the study conception, study design, analysis plan, and interpretation of findings. Bin Sun, Ying-Jun Chen, Cheng-Liang Xiong, Tian-Qing Meng, and Peng Duan contributed to the acquisition of data. Bin Sun and Fuxin Lin validated the accuracy of data analysis with a technical review. An Pan, Zhijian Hu, and Carmen Messerlian reviewed and edited the manuscript. All authors read and approved the final manuscript.

Details of ethics approval

The study protocol was approved by the Ethics Committee of the Reproductive Medicine Center of Tongji Medical College, and all volunteers provided informed consent.

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Table 1. Baseline characteristics of the study population [n (%) or mean ± SD]. [a]

Characteristics	Subjects included in the current analysis (n = 1159)	Stratified by seminal plasma Se co
		Q1 (n = 290)
Age, years	28.1 ± 5.3	27.1 ± 4.5
Education level		
Less than college	757 (65.3)	211 (72.8)
College and above	402 (34.7)	79 (27.2)
Ever fathered a child		
No	845 (72.9)	233 (80.3)
Yes	314 (27.1)	57 (19.7)
Smoking status		
Never	626 (54.0)	125 (43.1)
Former	84 (7.3)	21 (7.2)
Current	449 (38.7)	144 (49.7)
Alcohol consumption		
Never	287 (24.8)	69 (23.8)
Occasional	698 (60.2)	174 (60.0)
Former	12 (1.0)	4 (1.4)
Current	162 (14.0)	43 (14.8)
BMI, kg/m^2	22.8 ± 3.3	22.5 ± 3.4
Waist-hip ratio	0.8 ± 0.1	0.8 ± 0.1
Abstinence time, days [b]	6.2 ± 3.3	6.4 ± 3.9
Liquefaction time, minutes [b]	24.8 ± 10.6	23.6 ± 10.1
Season at semen examination [b]	Season at semen examination [b]	
Spring (Mar-May	1086 (19.3)	190 (19.3)
Summer (Jun-Aug)	2049 (36.5)	330 (33.4)
Autumn (Sept-Nov)	1665 (29.6)	293 (29.7)
Winter (Dec-Feb)	817 (14.6)	174 (17.6)

[a]A total of 1 man had missing information on BMI, 1 on waist circumference, and 4 on history of having ever fathered a child, and the median imputation method was used to handle missing data. Demographic characteristics across quartiles of total MET scores were compared using Kruskal-Wallis analyses or χ^2 tests where appropriate.

[b]The numbers shown here are total numbers of semen samples provided by the participant throughout the study period.

Abbreviations: BMI, body mass index; Q1, the first quartile; Q2, the second quartile; Q3, the third quartile; Q4, the fourth quartile; Se, selenium.

Table 2. Distribution of within-subject pooled seminal plasma Se concentrations (μg/L), sperm mtDNAcn, and sperm quality parameters.

Parameters	N (%) < LOQ	Mean
Se (n = 1159)	0 (0.0)	30.9
MtDNAcn (n =989)	0 (0.0)	1.0
Sperm quality parameters (n = 5617)	**Sperm quality parameters (n = 5617)**	**Sperm quality parameters (**
Semen volume	-	3.1
Sperm concentration	-	56.6
Total sperm count	-	171.2
Total motility	-	60.3
Progressive motility	-	57.2

Abbreviations: LOQ, limits of quantification; mtDNAcn, mitochondrial DNA copy number; Se, selenium.

Table 3. Percentage change [%Δ (95% CI)] of within-subject pooled seminal plasma Se concentration in relation to repeated sperm quality parameters based on linear mixed-effects models (n = 5617) and sperm mtDNAcn based on linear regression models (n = 989).

Sperm parameters	[?]25th	>25th-50th	>50th-75th	>75th
Semen volume	**Semen volume**	**Semen volume**	**Semen volume**	**Semen volume**
Crude model	0.0	4.8 (-2.0, 12.0)	3.1 (-3.6, 10.2)	-3.6 (-10.0, 3.3)
Adjusted model[a]	0.0	4.0 (-2.6, 11.1)	1.0 (-5.4, 7.8)	-6.5 (-12.7, 0.2)
Sperm concentration	**Sperm concentration**	**Sperm concentration**	**Sperm concentration**	**Sperm concent**
Crude model	0.0	38.0 (25.1, 52.3)	50.4 (36.4, 65.9)	78.7 (61.4, 97.9)
Adjusted model[a]	0.0	36.5 (23.7, 50.7)	45.9 (32.3, 61.0)	70.1 (53.3, 88.9)
Total sperm count	**Total sperm count**	**Total sperm count**	**Total sperm count**	**Total sperm co**
Crude model	0.0	44.0 (27.7, 62.3)	54.3 (36.9, 73.8)	71.6 (51.6, 94.2)
Adjusted model[a]	0.0	41.5 (25.8, 59.2)	46.9 (30.6, 65.3)	59.1 (40.5, 80.2)
Total motility	**Total motility**	**Total motility**	**Total motility**	**Total motility**
Crude model	0.0	-0.8 (-5.1, 3.7)	-1.5 (-5.7, 2.9)	-2.7 (-6.9, 1.8)
Adjusted model[a]	0.0	-1.1 (-5.2, 3.3)	-1.0 (-5.1, 3.4)	-1.7 (-6.0, 2.8)
Progressive motility	**Progressive motility**	**Progressive motility**	**Progressive motility**	**Progressive mo**
Crude model	0.0	-0.9 (-5.4, 3.9)	-1.2 (-5.6, 3.5)	-2.1 (-6.7, 2.7)
Adjusted model[a]	0.0	-1.2 (-5.6, 3.4)	-0.6 (-5.0, 4.0)	-1.1 (-5.7, 3.7)
Sperm mtDNAcn				
Crude model	0.0	-2.9 (-14.4, 10.2)	-6.4 (-17.5, 6.2)	-17.5 (-27.3, -6.4)
Adjusted model[b]	0.0	-0.4 (-12.2, 13.0)	-3.3 (-14.8, 9.7)	-13.0 (-23.6, -1.1)

[a]Models were adjusted for age (continuous), BMI (continuous), waist-hip ratio (continuous), abstinence time (continuous), history of having ever fathered a child (yes or no), education levels (less than undergraduate, or undergraduate and above), smoking status (never, former, or current), alcohol consumption (never, former, occasional, or current), sampling season (spring, summer, fall, or winter), and liquefaction time (continuous).

[b]Models were adjusted for age (continuous), BMI (continuous), waist-hip ratio (continuous), average abstinence time (continuous), history of having ever fathered a child (yes or no), education levels (less than undergraduate, or undergraduate and above), smoking status (never, former, or current), alcohol consumption (never, former, occasional, or current), and average liquefaction time (continuous).

Abbreviations: BMI, body mass index; CI, confidence interval; mtDNAcn, mitochondrial DNA copy number;

Se, selenium.

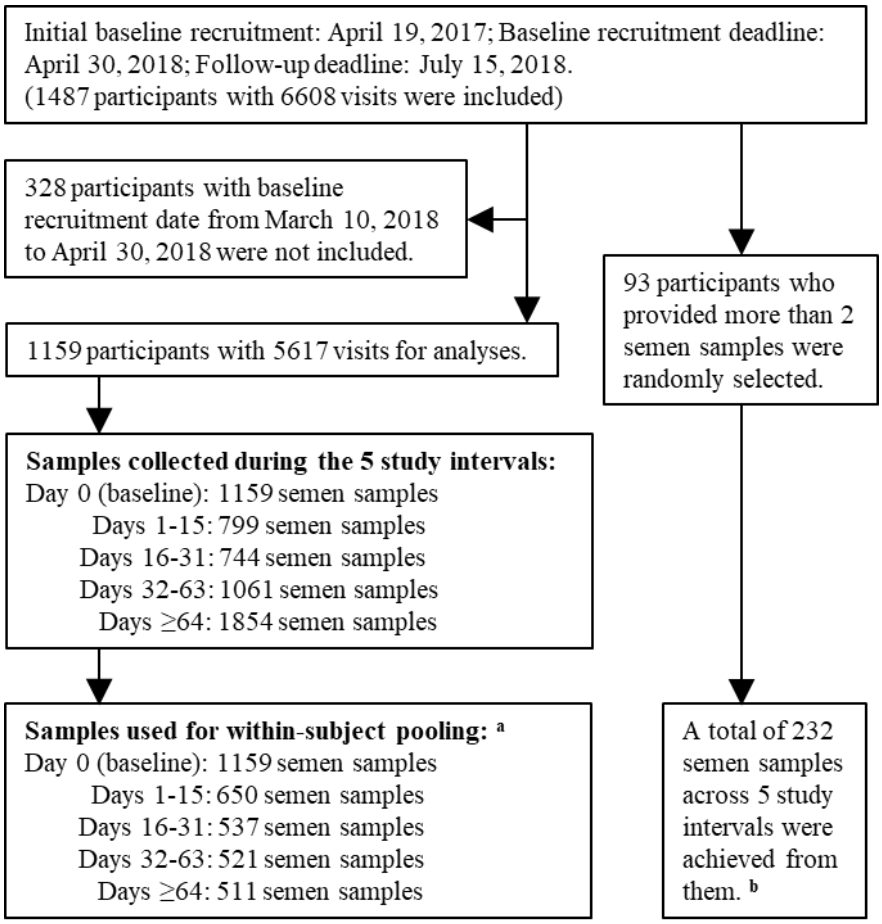
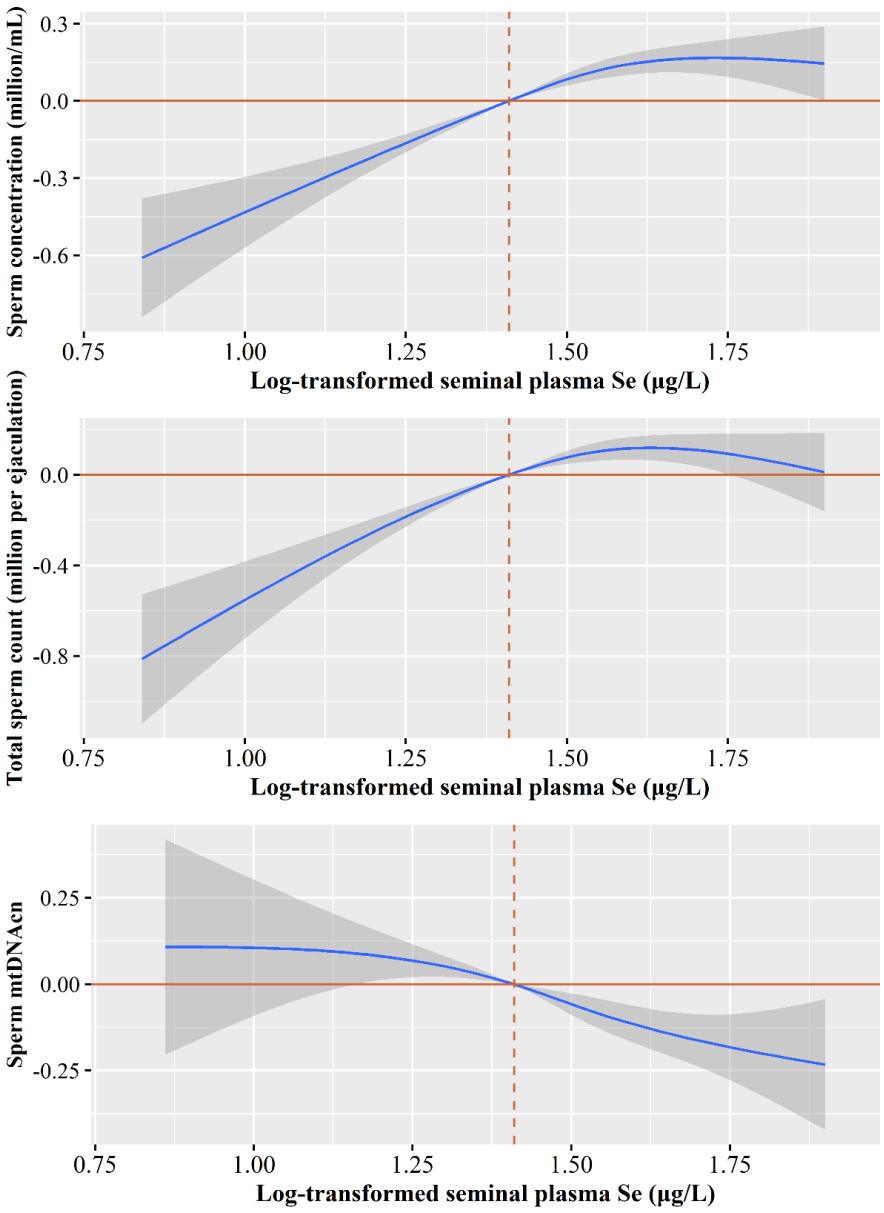


Figure 1. Study flow.

[a] The first semen samples collected from each study subject at five study intervals were pooled in equal volumes.

[b] The first semen samples collected from each study subject at five study intervals were used to investigate the reproducibility of seminal plasma selenium.

Figure 2. The restricted cubic spline for the associations of within-subject pooled seminal Se concentrations (log_{10}-transformed) with repeated sperm quality parameters (n = 1159 subjects, 5617 semen samples) and sperm mtDNAcn (n = 989 subjects, 989 semen samples). [a]



[a]The blue solid lines represent the effect estimates, and the shadowed parts are 95% confidence intervals. The reference values were set at the 25th percentile (i.e., 1.41 μg/L for selenium).

In the analyses between seminal plasma Se and sperm quality parameters, models were adjusted for age (continuous), BMI (continuous), waist-hip ratio (continuous), abstinence time (continuous), history of having ever fathered a child (yes or no), education levels (less than undergraduate, or undergraduate and above), smoking status (never, former, or current), alcohol consumption (never, former, occasional, or current), sampling season (spring, summer, fall, or winter), liquefaction time (continuous), seminal plasma cobalt (continuous), seminal plasma copper (continuous), seminal plasma iron (continuous), seminal plasma manganese (continuous), seminal plasma molybdenum (continuous), and seminal plasma zinc (continuous).

In the analyses between seminal plasma Se and sperm mtDNAcn, models were adjusted for age (continuous), BMI (continuous), waist-hip ratio (continuous), average abstinence time (continuous), history of having ever

fathered a child (yes or no), education levels (less than undergraduate, or undergraduate and above), smoking status (never, former, or current), alcohol consumption (never, former, occasional, or current), and average liquefaction time (continuous), seminal plasma cobalt (continuous), seminal plasma copper (continuous), seminal plasma iron (continuous), seminal plasma manganese (continuous), seminal plasma molybdenum (continuous), and seminal plasma zinc (continuous).

Abbreviations: BMI, body mass index; Se, selenium.

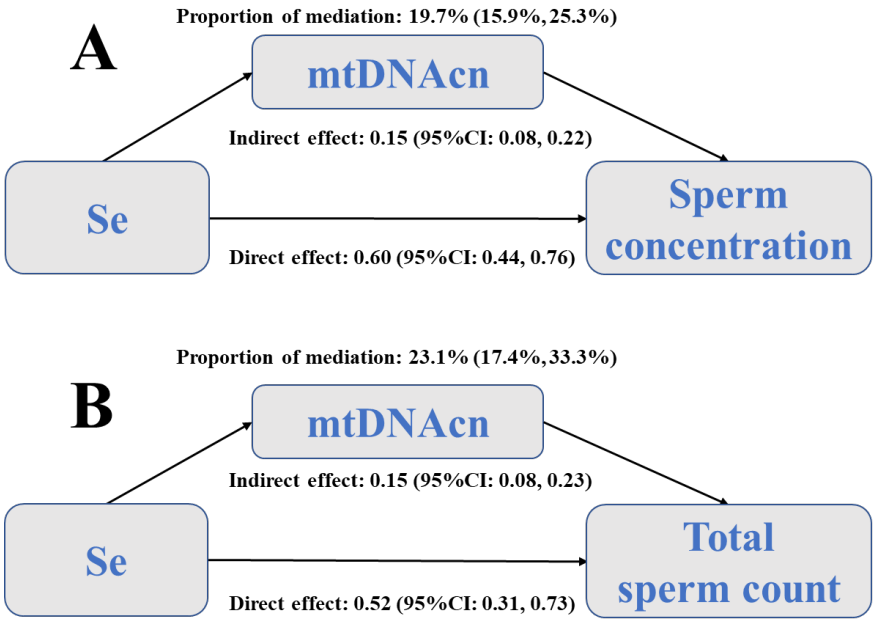


Figure 3. The estimated proportions of association between within-subject average seminal plasma Se concentrations and sperm quality parameters mediated by sperm mtDNAcn (n = 989). [a]

[a] Models were adjusted for age (continuous), BMI (continuous), waist-hip ratio (continuous), average abstinence time (continuous), history of having ever fathered a child (yes or no), education levels (less than undergraduate, or undergraduate and above), smoking status (never, former, or current), alcohol consumption (never, former, occasional, or current), and average liquefaction time (continuous), seminal plasma cobalt (continuous), seminal plasma copper (continuous), seminal plasma iron (continuous), seminal plasma manganese (continuous), seminal plasma molybdenum (continuous), and seminal plasma zinc (continuous).

Abbreviations: BMI, body mass index; CI, confidence interval; mtDNAcn, mitochondrial DNA copy number; Se, selenium.