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Exposure to per- and polyfluoroalkyl substances (PFASs) in serum versus semen and their association with male reproductive hormones☆

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ABSTRACT

Given that per- and polyfluoroalkyl substances (PFASs) exhibit different distribution in the serum and semen of adult men, improving our understanding of the predictors of PFAS concentrations in paired serum and semen samples from an individual is essential. Here, we investigated and compared the effects of emerging and legacy PFAS concentrations in serum and semen on reproductive hormone levels in serum within a Chinese adult male population. We explored the relationships among perfluorooctanoate (PFOA), perfluorononanoate (PFNA), perfluorooctane sulfonate (PFOS), and chlorinated polyfluorinated ether sulfonate (6:2 Cl-PFESA) in serum and semen with reproductive hormones in serum among 651 adult men from Nanjing, China. Significant relationships among all analyzed serum and semen PFASs and decreased total testosterone (total T) were found. Serum and semen PFOA levels were associated with significant decreases in free T. Furthermore, the levels of sex hormone-binding globulin (SHBG) were significantly decreased in association with PFNA, PFOS, and 6:2 Cl-PFESA exposure. Negative relationships between the total T/luteinizing hormone (LH) ratio and semen concentrations of selected PFASs were also observed. After adjustment of PFAS concentrations (in both semen and serum), stronger associations of PFASs with total T, free T, estradiol (E2), SHBG, and total T/LH were observed in semen than in serum. We found that 84.8% of the associations between serum PFOA with total T were mediated by semen PFOA. Thus, elevated PFAS exposure may have negative effects on male reproductive health, and semen PFAS may be a better exposure indicator for the male reproductive system than serum PFAS.

1. Introduction

Per- and polyfluoroalkyl substances (PFASs) are anthropogenic chemicals used extensively as surfactants in many consumer products and industrial manufacturing processes, such as carpets, textiles, leather, ski wax, photographic emulsifiers, and fire-fighting foams (Renner, 2001; Wang et al., 2014). Due to their wide use and persistent properties, PFASs are often emitted into the environment, with global distribution now evident (Yamashita et al., 2008; Young and Mabury, 2010). Human exposure to PFASs is ubiquitous and primarily through environmental sources, daily food intake, and drinking water (Shan et al., 2016; Zhang et al., 2011). Due to the slow elimination rate and biodegradability of long-chained PFASs, they can easily accumulate in the human body (Olsen et al., 2007; Yeung et al., 2009). These compounds have been detected in many different matrices, including cerebrospinal fluid, serum, milk, urine, and semen (Beser et al., 2019; Song et al., 2018; Wang et al., 2018a; Worley et al., 2017).

In vitro and male mouse studies have shown that perfluorooctanoate (PFOA) exposure can directly lead to lower testosterone and higher estradiol levels (Biegel et al., 1995; Zhang et al., 2014). Li et al. (2018) revealed the perfluorooctane sulfonate (PFOS) exposure can result in decreased testosterone levels, but no significant effects on in luteinizing or follicle-stimulating hormone levels. In animal models, adverse reproductive effects following PFAS exposure have also been demonstrated, with considerable impact on sex hormones (Kato et al., 2015; Steves

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et al., 2018; Zhang et al., 2016). Due to these findings in laboratory animals, there is growing public concern that ubiquitous exposure to PFASs may affect human male reproductive health.

Several recent epidemiological studies have focused on the relationship between PFAS exposure and male reproductive health (Joensen et al., 2013; Lopez-Espinosa et al., 2016; Raymer et al., 2012). However, among these human studies, there is considerable variation in the results of PFAS exposure and male reproductive health. Some studies have found that PFAS exposure is not significantly associated with either semen quality or reproductive hormones (Lewis et al., 2015; Olsen et al., 1998; Petersen et al., 2018). In contrast, other studies have found a correlation between PFAS exposure and altered semen parameters and sex hormones (Louis et al., 2015; Toft et al., 2012; Tsai et al., 2015). Thus, the relationship between PFAS exposure and serum male reproductive hormones remains somewhat inconsistent and unclear.

Most previous epidemiological studies on male reproductive health have explored associations based on PFAS concentrations in serum (Joensen et al., 2013; Lewis et al., 2015; Specht et al., 2012). However, PFOS and PFOA concentrations may be distributed differently in serum and semen samples (Raymer et al., 2012). Although serum PFASs can represent multi-route and integrative exposure levels within an individual, they may not directly reflect authentic exposure levels of target reproductive organs. Compared to that in other matrixes, pollutants (e.g., phthalate metabolites and vanadium) in semen may be more sensitive biomarkers for assessing reproductive system health (Wang et al., 2016b, 2018b).

We recently reported clearer relationships between semen PFAS concentrations and semen quality parameters than serum PFAS concentrations (Pan et al., 2019). Thus, we conducted a large-scale study in Nanjing, China, to examine whether exposure to PFASs (based on paired serum and semen concentrations) is associated with changes in serum reproductive hormones in healthy men. We then compared the associations between serum and semen PFAS levels and serum reproductive hormones.

2. Materials and methods

2.1. Study population

The subjects recruited in this study were male partners from couples who visited the Reproductive Medical Center at Nanjing Jinling Hospital, Nanjing, China. Details are provided in our previous study (Pan et al., 2019). Briefly, from June 2015 to July 2016, 738 men agreed to participate in the study after being informed of our intention (45% invited). All participants were invited to complete a questionnaire, which included basic information (age, body weight, height, occupation), living habits (smoking and drinking), and medical history. Body weight and height were recorded to calculate body mass index (BMI). Occupations were identified to determine whether potential PFAS exposure hazards existed. Average number of cigarettes during the last three months was recorded. Regular drinking was defined as 'yes' if the subjects drank an alcoholic beverage at least once a week during the last three months, and 'no' if they had not. Self-reported reproductive system disease (e.g., testicular cancer, azoospermia, cryptorchidism, varicocele, urogenital infections, testicular hydrocele, hypospadias, or sexually transmitted diseases) and self-reported abstinence time were required in the questionnaire. On the day of their clinic visit, participants who were celibate for at least two days were asked to provide semen and blood samples. The Human Subject Committee of Nanjing Jinling Hospital approved the study and all men provided informed consent at the time of registration.

In our previous study, we excluded 74 participants: i.e., 65 with self-reported or later discovered reproductive system disease, six with insufficient semen volume to detect semen PFAS concentrations, two that self-reported medicine usage aimed at improving reproductive function, and one with occupational exposure to fluorochemicals (Pan et al., 2019). In the current study, we excluded a further 13 participants due to incomplete reproductive hormone measurements. In total, 651 subjects were used for further statistical analysis.

2.2. Serum sampling

Each participant provided a venous blood sample. Due to the effects of diurnal variation on serum sex hormone levels, the hour of blood sampling and fasting status were recorded (Brambilla et al., 2009). Blood sampling time in hours was categorized into morning (07:30 a.m.–09:30 a.m.), noon (09:31 a.m.–12:00 p.m.), and afternoon (14:00 p.m.–18:00 p.m.). Venous blood samples were centrifuged to obtain serum samples. Serum samples were immediately analyzed to determine reproductive hormones, with the remaining aliquot stored at −80 °C and shipped on dry ice to the Institute of Zoology for PFAS analysis.

Serum reproductive hormone levels, including total testosterone (total T), estradiol (E2), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and sex hormone-binding globulin (SHBG), were measured by chemiluminescence assay using an automated Unicel Dxi 800 Access Immunoassay System (Beckman Coulter, Inc., USA). The limits of detection (LODs) were 0.35 nmol/L for total T, 73 pmol/L for E2, 0.2 IU/L for FSH and LH, and 0.33 nmol/L for SHBG. The intra-assay and inter-assay coefficients of variation (CVs) for these reproductive hormones were all below 5% and 8%, respectively. For E2 levels below the LOD, we used LOD/2 for statistical analysis. The free androgen index (FAI) was calculated by dividing total T by SHBG, and free T was calculated from concentrations of total T and SHBG using a constant albumin level of 43.8 g/L (Vermeulen et al., 1999). We also calculated the total T/LH ratio, which is a good indicator of Leydig cell function.

2.3. Semen sampling

Participant semen samples were collected at the hospital concurrently with venous blood samples and were provided via masturbation into a sterile jar. The semen samples were immediately transferred to −80 °C for later detection of PFAS exposure levels.

2.4. PFAS analysis

Unprocessed seminal fluids and serum samples were directly analyzed to determine the levels of 16 target PFASs, including two emerging PFASs (6:2 and 8:2 Cl-PFESAs) and l4 legacy PFASs. Detailed information is provided in our previous study (Pan et al., 2019). Analyses of semen and serum samples were conducted using ion-pair extraction, as previously described (Pan et al., 2019). Target compounds were separated by an Acquity UPLC™, and then detected using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA, USA). One blank and one SRM1957 sample (NIST, USA) were also extracted in accompaniment with the analyzed samples in each batch (20 samples). The limits of quantitation (LOQs) of the PFASs ranged from 0.01 to 0.20 ng/mL in serum and 0.002–0.10 ng/mL in semen. The average recoveries for PFASs in the two matrixes ranged from 84% to 112%. Measurements below the LOQ were replaced with the LOQ$\sqrt{2}$ value for analysis. Details on the UPLC-MS procedures, LOQs, and matrix recoveries are described in our previous study and thus are not shown here (Pan et al., 2019).

2.5. Statistical analysis

Basic descriptive statistics were provided for population characteristics, semen and serum PFAS concentrations, and serum reproductive hormone levels for the entire sample. Due to the non-normal distributions of PFAS levels in semen and serum, Spearman's Rank Correlation Test were used to examine the associations of PFAS concentrations between serum and semen samples. Separate multivariate linear regression models were used to examine the relationships between serum or semen PFAS levels and reproductive hormones. In all analyses, age and BMI were treated as continuous variables, and abstinence time (<7 d vs. ≥ 7 d), alcohol use (yes vs. no), fathering a pregnancy (yes vs. no), and fasting status (yes vs. no) were treated as dichotomous variables (Jiang et al., 2003). Smoking status (none, < 1, 1–9, 10–19, and ≥20 cigarettes/d) and blood sampling time (morning, noon, and afternoon) were considered as dummy variables. Based on previous research, age (years), BMI (kg/m^2), and smoking status were included first in the final models as covariates of associations with reproductive hormones (Joensen et al., 2013; Vested et al., 2013; Wang et al., 2016a, 2016c). Other potential covariates (abstinence time, alcohol use, fathering a pregnancy, blood sampling time, and fasting status) were individually added to the final models if they resulted in 10% or more change in effect estimates for semen or serum PFAS levels and outcomes. As blood sampling time and fasting status both met this criterion, they were added to the final models. We used age, BMI, smoking status, blood sampling time, and fasting status to adjust all models of associations of reproductive hormones with semen or serum PFASs.

In the adjusted models, serum total T, E2, SHBG, LH, FSH, free T, FAI, and total T/LH levels were ln-transformed before multivariate analysis to obtain normal distributions. All PFAS measurements in semen and serum showed skewed distribution and were ln-transformed to improve distribution. To assess potential non-linear relationships, PFASs were also divided into quartiles and entered into the model as categorical variables with the lowest quartile used as the reference category. The *p*-trends were then conducted by fitting the median levels of PFASs (continuous variables) in each quartile. To explain the linear regression coefficients (beta, β), results were expressed as percentage changes in reproductive hormones with interquartile range (IQR) increment in PFAS concentrations (equation: % change = [2^(β × IQR) − 1] × 100). Adjustment for multiple testes were not performed (Rothman, 1990).

We performed sensitivity analysis to assess the likelihood of effect change of these relationships. Our data also were stratified by age (18 to < 30, 30 to < 50) to examine the potential changes. We also reran analyses by excluding participants that did not fast prior to blood collection. To compare the relationships of serum and semen PFAS concentrations with reproductive hormones, we further performed two other sensitivity analyses. First, PFAS levels in semen and serum of each paired sample were entered in the same multivariate linear regression model to assess the relationships of with male reproductive hormones. Second, we performed mediation analysis to estimate the role of semen PFAS levels on the effects of serum PFAS levels on male reproductive hormones. The indirect effect (IE) was the estimated effect of serum PFASs on serum reproductive hormones that respond by semen PFASs using a linear mediation model, with the *p*-value achieved based on bootstrap analysis. The same confounders were adjusted for mediation analysis. Statistical analysis was conducted using SPSS v18.0 (SPSS Inc., Chicago, IL, USA). Mediation analysis was performed using the SPSS macro written by Valeri and Vanderweele (2013). A *p*-value of <0.05 was regarded as statistically significant.

3. Results

3.1. Population characteristics

Table 1 summarizes the demographic characteristics of the 651 study subjects. Blood samples of 562 (86.5%) participants were drawn between 07:30 and 11:40 a.m. Table 2 shows the distribution and percentage of reproductive hormones (above LOQs) for all remaining subjects. The median values for E2, FSH, LH, SHBG, and total T were 101.0 pmol/L, 3.9 IU/L, 3.8 IU/L, 25.6 nmol/L, and 13.2 nmol/L, respectively.

3.2. PFAS concentrations in serum and semen

The distributions of PFAS levels in serum and semen are similar to those reported in our previous study (Pan et al., 2019). More than 50% of perfluoroheptanoate (PFHpA) and perfluorotetradecanoate (PFTeDA) measurements in both serum and semen were below the LODs and were therefore excluded from our analysis. In addition, the detection rates of perfluorododecanoate (PFDoDA), perfluorotridecanoate (PFTriDA), perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), and 8:2 Cl-PFESA that were lower than 80% in semen were also excluded from analysis. The highest PFAS level was PFOS (mean: 13.5 ng/mL) in serum and PFOA (mean: 0.3 ng/mL) in semen, with 6:2 Cl-PFESA being the third highest in both (i.e., serum, mean: 8.9 ng/mL; semen, mean 0.1 ng/mL). The most abundant PFASs in the two matrices were PFOA, PFNA, PFOS, and 6:2 Cl-PFESA, accounting for 70.1% and 73.1% of total PFAS concentrations in serum and semen, respectively. These four dominant PFASs were a major determinant of serum and semen PFAS concentrations. Thus, we focused our analyses on PFOA, PFNA, PFOS, and 6:2 Cl-PFESA. The distributions of these four dominant PFASs are displayed in Table 3, and other PFASs were shown in the Supplementary Materials (Table S1). The correlations between PFOA, PFNA, PFOS, and 6:2 Cl-PFESA concentrations in the serum and semen samples were significantly positive, with Spearman's Rank Correlation coefficients ranging from 0.646 to 0.826 ($p < 0.001$) (Table S2).

Table 1
Basic characteristics and reproductive hormones of participants ($n = 651$).

Characteristic[a]	Mean ± SD	Number of subjects *n* (%)
Age (year)	29.4 ± 5.4	
Body Mass Index (BMI, kg/m^2)	23.9 ± 3.1	
Abstinence		
<7 days		485 (74.5)
≥7 days		166 (25.5)
Smoking status(cigarettes/day)		
None		352 (54.1)
<1		46 (7.1)
1–9		78 (12.0)
10–19		116 (17.8)
≥20		59 (9.0)
Alcohol use		
No		294 (45.2)
Yes		357 (54.8)
Ever fathered a pregnancy		
No		452 (69.4)
Yes		199 (30.6)
Fasted		
No		282 (43.3)
Yes		367 (56.4)
Blood sampling time		
Morning		161 (24.7)
Noon		401 (61.6)
Afternoon		88 (13.5)

Table 2
Distribution of reproductive hormones among study population (n = 651).

Hormonal measure	Mean ± SD	Minimum	Maximum	Median (25th, 75th)
Estradiol (pmol/L)	100.4 ± 51.4	< LOQ	462.0	101.0 (73.0, 128.0)
FSH (IU/L)	4.5 ± 2.7	0.7	26.4	3.9 (2.9, 5.3)
LH (IU/L)	4.3 ± 2.4	1.1	36.1	3.8 (2.8, 5.1)
SHBG (nmol/L)	26.8 ± 11.6	4.8	87.6	25.6 (18.1, 33.6)
Total T (nmol/L)	13.6 ± 4.3	3.4	40.8	13.2 (10.7, 16.3)
Free T	0.3 ± 0.1	0.1	1.2	0.3 (0.3, 0.4)
FAI	0.6 ± 0.3	0.1	2.6	0.5 (0.4, 0.7)
Total T/LH ratio	3.8 ± 1.9	0.4	17.7	3.4 (2.5, 4.7)

Note: Limits of detection: estradiol (E2) (73 pmol/L), follicle-stimulating hormone (FSH) (0.05 IU/L), luteinizing hormone (LH) (0.05 IU/L), sex hormone-binding globulin (SHBG) (0.23 nmol/L), testosterone (total T) (0.23 nmol/L).

Table 3
Detection rate and concentrations of PFAS in serum and semen from studied subjects (n = 651).

Compound	Percent detected (%)	Arithmetic Mean	SD	minimum	maximum	Median (25th, 75th)	Proportion (%)
PFAS concentration in serum (ng/mL)							
PFOA	100	10.48	8.17	1.66	95.69	8.57 (6.83, 11.11)	24.13
PFNA	100	1.82	1.38	0.27	17.30	1.47 (1.03, 2.23)	3.89
PFOS	100	13.52	19.37	1.47	392.01	9.94 (6.65, 15.65)	25.73
6:2 Cl-PFESA	100	8.91	9.87	0.44	96.06	6.10 (3.36, 9.90)	16.34
PFAS concentration in semen (ng/mL)							
PFOA	100	0.32	0.31	0.04	2.97	0.23 (0.15, 0.37)	36.26
PFNA	95.85	0.03	0.04	< LOQ	0.36	0.02 (0.01, 0.04)	3.64
PFOS	99.85	0.23	0.45	< LOQ	9.90	0.15 (0.09, 0.27)	22.66
6:2 Cl-PFESA	100	0.11	0.13	0.01	1.37	0.07 (0.04, 0.12)	10.50

3.3. Serum PFAS concentrations and reproductive hormones

The associations between serum PFAS concentrations and reproductive hormones based on linear regression models are given in Table 4. All PFASs showed a significantly decreasing trend with increasing total T levels. In addition, all PFASs were negatively correlated with free T and E2 levels and total T/LH. Only PFOA showed a significantly inverse association with free T levels ($p = 0.015$). All PFASs were negatively associated with SHBG levels. Furthermore, elevated PFNA and PFOS concentrations were significantly related to a decrease in SHBG levels ($p = 0.033$ and 0.014, respectively). Though all studied PFASs were associated with increased FSH levels, the results were not statistically significant. No consistent relationships among studied PFAS levels and LH or FAI were identified in the serum samples. Due to the significant associations between certain PFASs and total T, free T, total T/LH, and SHBG, the associations of these sex hormones with the quartiles of PFOA, PFNA, PFOS, and 6:2 Cl-PFESA concentrations in serum were determined, as shown in Fig. S1. All significant associations were generally consistent with PFAS quartile models. We also found a significant negative association between the PFOS quartile and total T/LH and the 6:2 Cl-PFESA quartile and SHBG (Fig. S1).

3.4. Semen PFAS concentrations and reproductive hormones

The associations between PFAS concentrations in semen and reproductive hormones in serum are shown in Table 5. We identified significant negative relationships for all selected PFASs and total T. Significant and inverse associations between PFOA concentrations and free T were also observed ($p = 0.003$). A significant negative correlation was observed between PFOA and 6:2 Cl-PFESA concentrations and E2 levels ($p = 0.044$ and $p = 0.028$, respectively). Moreover, all studied PFASs showed significantly negative correlations with the total T/LH ratio. Significant relationships were found between PFNA, PFOS, and 6:2 Cl-PFESA and decreased SHBG levels. However, we did not find any other significant relationship between PFAS levels and FSH, LH, and FAI levels. To corroborate the significant correlations, we used multivariate linear regression models to analyze the relationship between selected semen PFAS quartiles and total T, free T, E2, SHBG, and total T/LH ratio (Fig. S2). Other reproductive hormones demonstrated non-significant

Table 4
Percentage change (95% CI) in serum reproductive hormones in relation to ln-transformed PFAS levels in serum (n = 651).

Outcome	PFOA		PFNA		PFOS		6:2 Cl-PFESA	
Serum	Percentage change (95% CI)	p	Percentage change (95% CI)	p	Percentage change (95% CI)	p	Percentage change (95% CI)	p
Total T(nmol/L)	−3.10 (−5.32, −0.84)	0.008*	−3.99 (−7.01, −0.87)	0.013*	−3.36 (−6.40, −0.22)	0.036*	−3.62 (−6.52, −0.62)	0.009*
Free T	−2.70 (−4.83, −0.53)	0.015*	−2.77 (−5.69, 0.25)	0.072	−1.64 (−4.60, 1.41)	0.287	−2.68 (−5.49, 0.21)	0.068
FAI	−1.37 (−3.99, 1.32)	0.316	0.35 (−3.31, 4.15)	0.854	1.67 (−2.04, 5.51)	0.383	−0.15 (−3.65, 3.47)	0.932
Estradiol (pmol/L)	−2.32 (−6.31, 1.84)	0.270	−2.08 (−7.56, 3.73)	0.475	−5.09 (−10.39, 0.52)	0.075	−4.86 (−9.96, 0.53)	0.076
SHBG (nmol/L)	−1.76 (−4.61, 1.17)	0.236	−4.32 (−8.12, −0.37)	0.033*	−4.94 (−8.71, −1.02)	0.014*	−3.47 (−7.15, 0.36)	0.075
FSH (IU/L)	0.30 (−3.41, 4.14)	0.878	2.11 (−3.06, 7.54)	0.431	3.28 (−1.93, 8.77)	0.221	4.66 (−0.40, 9.98)	0.072
LH (IU/L)	−1.47 (−4.77, 1.95)	0.395	−0.61 (−5.18, 4.18)	0.799	1.22 (−3.42, 6.10)	0.612	0.031 (−4.11, 4.93)	0.894
Total T/LH ratio	−1.66 (−5.20, 1.82)	0.345	−3.40 (−7.92, 1.34)	0.157	−4.53 (−8.99, 0.15)	0.058	−3.91 (−8.22, 0.60)	0.088

Note: Statistical models adjusted for age, body mass index (BMI), smoking status, blood sampling time, and fasting status. *Significant associations detected at $p < 0.05$.

Table 5
Percentage change (95% CI) in serum reproductive hormones in relation to ln-transformed PFAS levels in semen ($n = 651$).

Outcome	PFOA		PFNA		PFOS		6:2 Cl-PFESA	
Semen	Percentage change (95% CI)	p	Percentage change (95% CI)	p	Percentage change (95% CI)	p	Percentage change (95% CI)	p
Total T (nmol/L)	−5.56 (−8.40, −2.62)	<0.000*	−5.27 (−8.27, −2.18)	0.001*	−4.20 (−7.13, −1.18)	0.007*	−4.25 (−7.30, −1.10)	0.009*
Free T	−4.42 (−7.12, −1.55)	0.003*	−2.76 (−5.71, 0.29)	0.075	−2.36 (−5.22, 0.58)	0.115	−2.91 (−5.87, 0.14)	0.061
FAI	−1.98 (−5.41, 1.66)	0.282	1.25 (−2.48, 5.12)	0.516	1.15 (−2.45, 4.87)	0.536	0.12 (−3.58, 3.97)	0.949
Estradiol (pmol/L)	−5.49 (−10.60, −0.17)	0.044*	−2.97 (−8.45, 2.84)	0.308	−5.13 (−10.29, 0.33)	0.065	−6.31 (−11.61, −0.69)	0.028*
SHBG (nmol/L)	−3.67 (−7.36, 0.17)	0.059	−6.44 (−10.17, −2.55)	0.001*	−5.29 (−8.94, −1.49)	0.007*	−4.37 (−8.22, −0.35)	0.033*
FSH(IU/L)	−1.21 (−5.98, 3.81)	0.633	1.15 (−4.01, 6.59)	0.669	0.53 (−4.42, 5.74)	0.837	4.11 (−1.22, 9.73)	0.133
LH (IU/L)	−0.78 (−5.16, 3.81)	0.743	0.98 (−3.70, 5.89)	0.687	0.84 (−3.67, 5.57)	0.718	1.43 (−3.30, 6.38)	0.560
Total T/LH ratio	−4.83 (−9.12, −0.35)	0.035*	−6.19 (−10.61, −1.56)	0.009*	−5.00 (−9.32, −0.48)	0.031*	−5.59 (−10.06, −0.9)	0.020*

Note: Statistical models adjusted for age, body mass index (BMI), smoking status, blood sampling time, and fasting status. *Significant associations detected at $p < 0.05$.

associations with PFAS quartiles and are not shown. We did not find a significant negative relationship between E2 levels and 6:2 Cl-PFESA modeled as a quartile outcome. However, most of the aforementioned significant associations were unchanged, regardless of whether the individual PFASs were modeled as quartiles or continuous variables. Significant relationships were observed across PFNA quartiles with decreasing free T levels ($p = 0.013$).

3.5. Sensitivity analysis

The association between PFAS and selected reproductive hormones stratified by age and fasting status are shown in Tables S3 and S4. In stratified analysis, these significant relationships largely remained among subjects ≤30 years old (Table S3), while no significant associations between PFASs and reproductive hormone levels were found in subjects above 30 years old (Table S4). We re-analyzed serum or semen PFASs and reproductive hormones by excluding subjects who did not fast prior to blood sampling, and observed that the significant associations remained suggestive or significant (these results are provided in supporting information Table S5). Lack of significant association in the stratified analysis may be due to reduced sample number. When paired serum and semen samples for PFASs and reproductive hormones were modeled together, we observed statistically significant associations changed (Table 6). Association trends between semen PFASs and reproductive hormones are consistent with above results but most statistical significance after adjustment were disappeared. All p-values of serum PFASs were higher than those of corresponding semen PFASs. We did not identify any significant associations for serum PFASs.

Significant associations were found between all semen and serum PFAS concentrations as well as total T levels (Table S2 and Table 5). Mediation analysis was performed to estimate the proportion of the effect of serum PFAS exposure on total T levels mediated by semen PFASs (Fig. 1). Results showed a statistically significant mediation effect by semen PFOA levels, which mediated 84.8% [IE: −0.06(-0.11, −0.01)] of the negative association between serum PFOA levels and total T levels. In contrast, semen PFOS contributed to a large proportion (98.3%) of the negative association between serum PFOS and total T, although the mediation effect did not reach statistical significance ($p = 0.086$). There were no significant mediations by semen PFNA and 6:2 Cl-PFESA.

4. Discussion

The current study measured PFAS concentrations in serum and semen and examined the associations between selected PFASs (i.e., PFOA, PFNA, PFOS, and 6:2 Cl-PFESA) and reproductive hormones from an adult male population in China. PFAS exposure is usually analyzed by measuring the levels of PFASs in serum. In the current study, however, PFASs were also measured in semen. The higher concentration of PFASs in serum than in semen demonstrated here is consistent with previous studies (Raymer et al., 2012; Song et al., 2018). In general, the concentrations of PFASs in semen were 1–2 orders of magnitude lower than that in serum, which was likely the result of different binding ability of PFASs to proteins in specific tissues (Kudo et al., 2007). Studies have demonstrated that PFCAs bind to serum proteins, especially serum albumin (Han et al., 2003; Jones et al. 0.2003). However, the high positive correlations between semen and serum PFASs indicated that semen may be used as a PFAS exposure biomarker. Compared with serum, the lower PFAS levels in semen only represent reproductive organs with chronic PFASs exposure. Very few reports have focused on the concentrations of PFASs in semen. In this study, the detected PFAS concentrations in semen were relatively low. Compared with results from other studies, semen PFOA (mean: 0.3 ng/mL) and PFOS concentrations (mean: 0.2 ng/mL) were lower than that found in general populations from the US and from Guangdong Province,

Table 6
Percentage change (95% CI) in selected serum reproductive hormones in relation to paired of ln-transformed PFAS levels in semen and serum samples ($n = 651$).

Outcome	Matrix	PFOA		PFNA		PFOS		6:2 Cl-PFESA	
		Percentage change (95% CI)	p	Percentage change (95% CI)	p	Percentage change (95% CI)	p	Percentage change (95% CI)	p
Total T (nmol/L)	semen	−4.25 (−9.20, −1.12)	0.012**	−4.76 (−8.84, −0.50)	0.029**	−4.17 (−8.74, 0.63)	0.087	−3.40 (−8.66, 2.15)	0.224
	serum	−0.34 (−3.49, 2.86)	0.831	−0.79 (−5.00, 3.62)	0.721	−0.04 (−4.93, 5.10)	0.987	−1.01 (−6.10, 4.35)	0.705
Free T	semen	−3.67 (−7.52, 0.26)	0.068	−1.61 (−5.65, 2.61)	0.449	−2.78 (−7.22, 1.86)	0.236	−1.81 (−6.91,3 .58)	0.503
	serum	−0.78 (−3.77, 2.26)	0.613	−1.70 (−5.70, 2.47)	0.418	0.58 (−4.13, 5.52)	0.813	−1.31 (−6.16, 3.80)	0.609
Estradiol (pmol/L)	semen	−5.98 (−12.22, 0.70)	0.080*	−2.55 (−9.27, 4.65)	0.477	2.11 (−5.68, 10.54)	0.606	−5.82 (−13.99, 3.12)	0.194
	serum	1.62 (−3.49, 7.05)	0.537	2.14 (−4.83, 9.63)	0.556	−2.74 (−10.35, 5.52)	0.504	3.78 (−4.74, 13.06)	0.396
SHBG (nmol/L)	semen	−4.00 (−9.04, 1.31)	0.140	−6.52 (−11.56, −1.18)	0.017**	−3.93 (−9.69, 2.19)	0.203	−4.14 (−10.70, 2.91)	0.243
	serum	0.34 (−3.68, 4.48)	0.872	0.12 (−5.24, 5.78)	0.967	−1.87 (−7.91, 4.56)	0.559	−0.28 (−6.74, 6.63)	0.935
Total T/LH ratio	semen	−6.31 (−12.14, −0.17)	0.044**	−7.19 (−13.09, −0.89)	0.026**	−4.00 (−10.77, 3.29)	0.274	−6.66 (−14.15, 1.49)	0.107
	serum	1.72 (−3.02, 6.74)	0.480	1.57 (−4.83, 8.41)	0.638	−1.39 (−8.53, 6.31)	0.715	1.32 (−6.38, 9.65)	0.745

Note: Statistical models adjusted for age, body mass index (BMI), smoking status, blood sampling time, and fasting status. * $0.05 \leq p < 0.10$. ** $p < 0.05$.

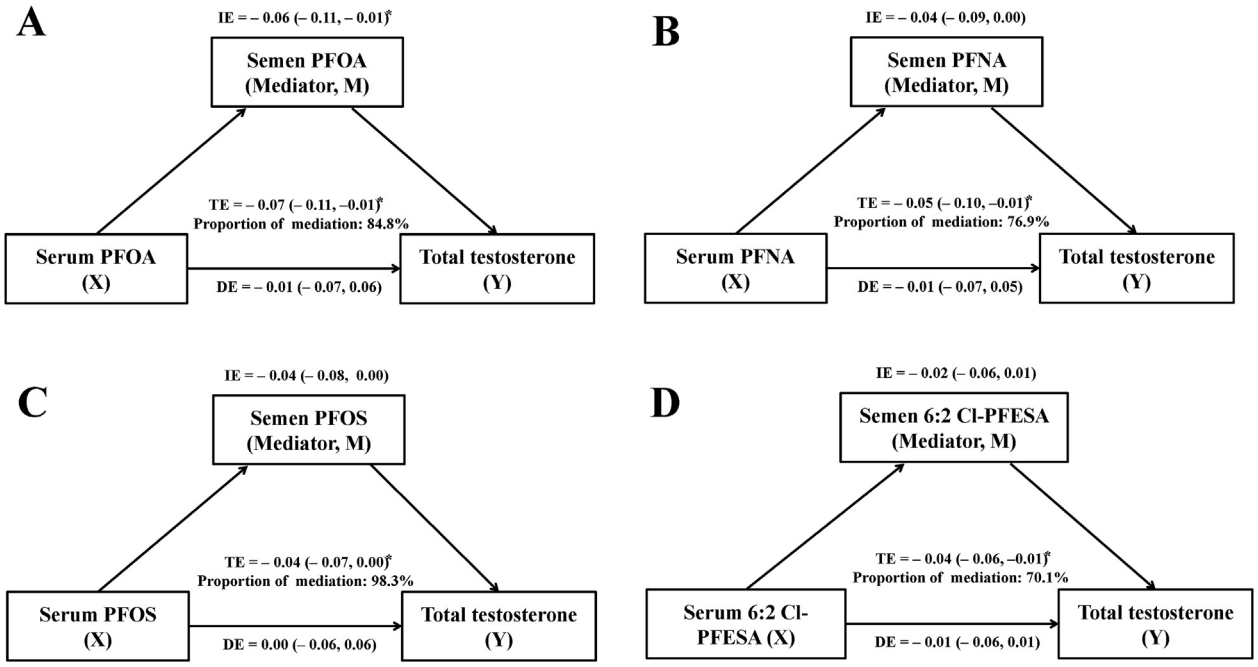

Fig. 1. Mediation analysis of estimated effect (95% CI) of serum PFASs on total testosterone (T) levels with corresponding semen PFAS concentrations. (A) PFOA, (B) PFNA, (C) PFOS, and (D) 6:2 Cl-PFESA. Figure shows semen PFAS concentrations as potential mediators, estimates of indirect effect (IE), direct effect (DE), total effect (TE), and proportion of mediation. IE, DE, and TE are presented as β (95% CI) and proportion of mediation was calculated as ratio of IE to TE. Models were adjusted for age, body mass index (BMI), smoking status, blood sampling time, and fasting status. *Significant associations detected at $p < 0.05$.

China (Raymer et al., 2012; Song et al., 2018), but higher than that from Italy (Di Nisio et al., 2019). We note that PFOA levels in exposed men from Italy (mean: 0.12 ng/mL) were lower than that reported in our study (Di Nisio et al., 2019). Here, PFOA showed the highest mean concentration in semen (mean: 0.3 ng/mL), whereas PFOS (mean: 5.3 ng/mL) was found at the highest level in semen samples collected from Guangdong (Song et al., 2018). In addition, individual PFAS levels detected in the above Guangdong semen samples were higher than those from Nanjing, implying marked regional differences in the extent and source of exposure.

Our data showed a significant trend of increased serum PFOA, PFNA, PFOS and 6:2 Cl-PFESA concentrations with decreased total T levels in serum, in accordance with the results observed in semen (Tables 4 and 5). Moreover, these significant negative relationships did not change for PFAS quartiles associated with total T levels (Figs. S1 and S2). In support of negative association between PFAS concentrations and total T levels, two previous epidemiological studies also found inverse correlations between certain PFAS levels in serum and total T in Taiwan and Denmark (Joensen et al., 2013; Tsai et al., 2015). However, other studies have reported different results, with no significant relationship observed between PFASs and total T (Lewis et al., 2015; Olsen et al., 1998). Di Nisio et al. (2019) reported that seminal fluid PFASs was positively associated with T levels in exposed populations with higher semen PFAS concentrations and supported this result by antagonism of PFOA on the binding of T with androgen receptor. However, majority of toxicological studies in rodents have demonstrated that PFAS exposure, including that of PFOA, PFNA, and PFOS, can reach and interfere with the testes by reducing T secretion (Biegel et al., 1995; Lopez-Doval et al., 2014b; Singh and Singh, 2019; Zhang et al., 2014). Our previous study also found significant associations between serum PFASs with several semen quality parameters in the same population (Pan et al., 2019). Based on our results and earlier animal-study evidence, we speculate that PFAS exposure would have an adverse effect on testes and fertility in males.

In the present study, we found a significant decrease in free T levels (a marker of biologically active T) with increasing PFOA levels in semen and serum. Joensen et al. (2013) only found higher serum PFOS concentrations to be significantly associated with decreased free T levels. Other studies observed that there were no relationships between serum PFAS and free T levels (Tsai et al., 2015; Den Hond et al., 2015). Thus, a clear correlation is uncertain and requires further investigation. Animal studies have demonstrated that PFOS and PFOA can exert some anti-androgenic effects by inhibiting T synthesis from Leydig cells (Biegel et al., 1995; Li et al., 2018). We found a decreased total T/LH ratio with increased PFAS exposure in semen samples, which could result in adverse effects on the testes due to poor Leydig cell function with higher PFAS levels (Li et al., 2018). Furthermore, these results would supply evidence that PFAS exposure affects testis function and further clarify the potential biological mechanism between PFAS exposure and disturbed male reproductive system.

Studies demonstrated that after the decrease of testosterone levels, increased LH levels at the pituitary occurs due to the negative feedback of hypothalamic-pituitary-testicular (HPT) axis (Damassa et al., 1976). In the current study, T levels decreased significantly following PFAS exposure; however, there was no significant change in LH levels with increased semen or serum PFASs. Although a suggestive positive association between serum PFAS and FSH levels was observed, it was weak and did not reach statistical significance, which were in accordance with previous findings reported in Joensen et al. (2013). The relationships contrasts with several previous studies. Raymer et al. (2012) reported that only serum PFOA and PFOS were positively associated with LH. Tsai et al. (2015) found that certain serum PFASs were inversely associated with FSH and testosterone levels. Vested et al. (2013) showed positive associations between utero PFOA exposure and LH and FSH in adult men. Furthermore, Lopez-Doval et al. (2014a) found that PFOS (0.5 mg/kg/day, gavage) exposure in adult male rats reduced LH and T release and stimulated FSH secretion,

eventually hindering HPT axis activity. However, the effects observed at high exposure levels in laboratory animals may differ from those at low environmental exposure levels in humans. The three sex hormones are regulated by physiological activity of the HPT axis (Schlatt and Ehmcke, 2014). If PFAS exposure do exert an indirect effect on pituitary-hypothalamic function, it could explain why the corresponding changes happened in these three sex hormones.

A competitive relationship exists between PFOS and T or E2 for binding to SHBG when the PFOS exposure level is far higher than environmental concentrations (Jones et al., 2003). In present study, a reduction in SHBG levels with increasing PFOS were observed, which is similar to previous reports, which described these associations in females aged 12–17 (Tsai et al., 2015). Specht et al. (2012) reported a slight increase in SHBG levels associated with PFOA exposure in fertile men from Greenland, in stark contrast to our observations. We suggest that disturbance in SHBG levels can occur in humans exposed to PFAS at environmental levels. We further speculate that the decrease in SHBG, which resulted in lower total T levels without variation in FAI or LH, may be indicative of androgenic action.

In present study, we did not observe the significant association of serum PFASs with E2 levels. In support of our findings, five weeks of oral gavage exposure to PFOS in adult male mice did not affect serum E2 levels (Qu et al., 2016). Most epidemiological studies assessing the relationship between serum PFASs and E2 levels report similar results to our study, which failed to observe significant associations (Joensen et al., 2009; Raymer et al., 2012). However, the negative relationship between semen PFOA and E2 in our study is compatible with previous toxicological studies, which demonstrated that PFOA exposure can interfere with estrogen biosynthesis *in vivo* or *in vitro* (Seacat et al., 2002).

After semen and serum PFASs were both included in statistical models, significant associations between male reproductive hormones and semen PFASs were not observed between semen PFASs and serum PFASs. The results suggest that semen PFAS concentrations are more closely related to male reproductive hormones than serum PFAS concentrations. Mediation analysis found that semen PFOA concentrations may be a mediator of the relationship between serum PFOA concentrations and T levels. Thus, these results support our hypothesis that semen PFAS levels are a more direct biomarker of exposure for male testis and would be more appropriate for use in biomonitoring studies of the male reproductive system. Thus, the selection of specific matrices as internal dose biomarkers can allow for accurate assessment of human tissue exposure to compounds such as PFASs.

The main advantage of this study is that we assessed PFAS levels in both serum and semen as biomarkers of exposure and comprehensively evaluated their relationships with serum reproductive hormones, which are indicators of male reproductive health. However, there are several limitations of this study. First, our study subjects were recruited from a reproductive medical center, and thus our conclusions may not extend to the general population. Second, only one blood sampling was conducted without performing other effective strategies to lower the diurnal and temporal variation of reproductive hormone levels caused by changes in eating habits, lifestyle factors, or daily activities. Third, we only detected PFAS levels in current samples without knowledge of exposure levels during the most vulnerable developmental stage. Such exposure misclassification might distort real relationships between PFASs exposure and reproductive hormones. Fourth, we could not exclude the possibility of false positive findings in the present study due to multiple comparisons. Finally, more relevant reproductive hormones (e.g., inhibin B, pituitary prolactin, and gonadotropin-releasing hormone) should be detected and added for further analysis. This would help clarify the causal relationship between PFAS exposure at environmental levels and sex hormones, and the possible mechanisms of the adverse effects of PFAS exposure on male reproductive health.

5. Conclusions

In this study, we investigated and compared the effects of serum and semen PFAS exposure on reproductive hormones in adult subfertile Chinese males. Overall, we found that increasing exposure to serum and semen PFASs was associated with decreased total T, free T, total T/LH, and SHGB levels. These results provide evidence that PFAS exposure impacts male reproductive dysfunction by altering reproductive hormones. Stronger associations between PFAS concentrations and selected reproductive hormones in semen samples than serum samples also suggest that semen PFASs are a more direct biomarker of exposure and male reproductive health than serum PFASs. These results indicate that semen PFAS levels as exposure indicators may more accurately reflect the relationship with T levels compared to serum PFASs.

Author contribution statements

Qianqian Cui: carried out the experiments, handle the data and wrote the manuscript. Yitao Pan: contributed to the interpretation of the results. Jinghua Wang: helped carry out the experiments. Hongxiu Liu: provided supports in software technology. Bing Yao: collected samples and data interpretation. Jiayin Dai: conceived the original idea, supervised the projected and write the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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