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Serum Concentrations of Estradiol and Free T_4 Are Inversely Correlated With Sperm DNA Damage in Men From an Infertility Clinic

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ABSTRACT: Sperm DNA damage adversely affects male fertility and contributes to poorer embryo development and lower pregnancy rates. Endogenous hormones are critical to spermatogenesis and maintenance of male reproductive function and likely play an important role in human sperm DNA integrity, but this relationship is not fully understood. The present study measured serum hormone levels and sperm DNA damage with the neutral comet assay in 362 male partners of infertile couples. When sperm concentration and other potential confounding variables were included in multiple linear regression, serum estradiol and free T_4 levels were inversely associated with sperm DNA damage. Among other statistically significant associations that were observed, an interquartile range (IQR) increase in estradiol was associated with a 6.3% decline (95% confidence intervals: −9.7%, −2.9%) in comet extent and a 16.2% (−22.4%, −9.2%) decline in the percentage of DNA in the comet tail (Tail%), whereas an IQR increase in free T_4 was associated with a 24.4% (−31.5%, −17.4%) decline in Tail%. Likewise, in multiple logistic regression, men in the highest estradiol quartile had an 81% reduced risk of having a comet extent value in the highest quartile compared with men in the lowest estradiol quartile. Men in the highest free T_4 quartile had 92% decreased odds of being categorized in the highest Tail% quartile compared with men in the lowest free T_4 quartile. These results suggest that estradiol and free T_4 may have a protective effect against sperm DNA damage, but future mechanistic and epidemiologic studies are needed to confirm these findings.

Key words: Chromatin, comet assay, epidemiology, hormone, human.

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Recent advances in preclinical and clinical biologic markers of male reproductive health have confirmed the importance of sperm chromatin/DNA integrity to fertility and pregnancy outcomes. Growing and consistent evidence shows that sperm DNA damage adversely affects male fertility, contributing to poorer embryo development and lower pregnancy rates among partners of men undergoing assisted reproductive treatments (Duran et al, 2002; Morris et al, 2002; Agarwal and Allamaneni, 2004; Lewis and Aitken, 2005; Borini et al, 2006). Because of their ability to predict infertility and/or adverse pregnancy outcomes, assays to assess sperm chromatin/DNA integrity have been proposed for regular use in the clinic to improve infertility diagnosis and treatment (Agarwal and Said, 2003; Erenpreiss et al, 2006). To date, 4 potential mechanisms of increased sperm DNA damage or abnormal sperm chromatin/DNA structure have been proposed (Sakkas et al, 1999; Agarwal and Said, 2003; Erenpreiss et al, 2006): 1) deficiencies in recombination during spermatogenesis, leading to cell apoptosis; 2) abnormal spermatid maturation; 3) abortive apoptosis; and 4) oxidative stress. Because markers of sperm DNA damage or chromatin integrity are still relatively new, factors contributing to these sources of damage remain largely unclear. However, their discovery is vital for the development of preventative or treatment measures (Aitken and De Luliis, 2007).

Endogenous hormones are critical to spermatogenesis and maintenance of male reproductive function, although many of the basic mechanisms involved are still not completely understood (Lo and Lamb, 2004). Follicle-stimulating hormone (FSH), luteinizing hormone (LH), inhibin B, and testosterone all serve important and well-known functions in the male hypothalamopituitary-gonadal axis and male reproduction (Lo and Lamb 2004), but in recent years important

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roles for estradiol (O'Donnell et al, 2001; Hess, 2003; Akingbemi, 2005) and thyroid hormones (Jannini et al, 1995; Krassas and Pontikides, 2004; Trokoudes et al, 2006) in spermatogenesis, germ cell survival, and apoptosis have been described. However, much remains unknown about the relationships and potential roles of hormones in human sperm DNA integrity and damage. Two previous studies assessed the relationship between hormone levels and DNA damage, as measured by the sperm chromatin structure assay (SCSA), with conflicting results. Richthoff et al (2002) reported that sperm DNA damage was inversely correlated with both testosterone and estradiol levels among 267 healthy young Swedish men, whereas Appasamy et al (2007) reported no associations between sperm DNA damage and FSH, inhibin B, or testosterone in 129 men from a United Kingdom infertility clinic.

To our knowledge, no human studies have assessed the relationship between hormone levels and sperm DNA damage measured using assays other than SCSA (eg, terminal deoxyribonucleotide transferase–mediated nick-end labeling or comet assay). In addition, no previous studies have been found that explored the association between thyroid hormone levels and sperm DNA damage. The present study was conducted to assess the relationships between serum hormone levels and sperm DNA damage, as assessed by the neutral comet assay, in men recruited from an infertility clinic. The neutral comet assay has proven useful in epidemiologic studies because it is a simple and sensitive assay that measures DNA double-strand breaks, a critical DNA lesion for normal cell survival (Singh and Stephens, 1998).

Methods

Subject Recruitment

Between April 2000 and July 2004, men between 18 and 54 years were recruited from the Vincent Memorial Andrology Laboratory at Massachusetts General Hospital (MGH) and invited to participate in a study to assess the effects of environmental exposures on male reproductive health. Approximately 65% of eligible men agreed to participate. The primary reason cited by nonparticipants was lack of time. Exclusionary criteria included prior vasectomy or current use of exogenous hormones. A retrospective review of anonymized clinic records of nonparticipants, who met the same eligibility criteria as the study subjects, found that there were no differences between participants and nonparticipants in regards to age or semen parameters (Hauser et al, 2005). Heights and weights were measured, and all men completed a brief nurse-administered questionnaire at the time of recruitment and provided health information. The Harvard School of Public Health, MGH, and University of Michigan Human Subjects Committees approved the study, and all subjects signed an informed consent.

Semen Sample Collection

Semen was collected on site at MGH in sterile plastic specimen cups after a recommended period of abstinence of 48 hours. After liquefaction at 37°C for 30 minutes, semen parameters and characteristics were measured. Measurement of the semen parameters (sperm concentration, motility, and morphology) has been described previously (Hauser et al, 2003). Briefly, we measured sperm count and motility by computer-aided semen analysis using the Hamilton Thorne IVOS 10 Analyzer (Hamilton-Thorne Research, Beverly, Massachusetts). To assess sperm morphology, we evaluated 200 sperm using the Tygerberg strict criteria (Kruger et al, 1988). The remaining unprocessed semen was frozen in 0.25-mL cryogenic straws (CryoBiosystem, San Diego, California) by immersion of the straws directly into liquid nitrogen (−196°C). Previous work in our laboratory showed that this freezing method produced comet assay results that were highly correlated with results from fresh, unfrozen samples (Duty et al, 2002). Semen samples were later analyzed in batches. Straws were thawed by gently shaking in a 37°C water bath for 10 seconds, and the semen was immediately processed for the comet assay.

Neutral Comet Assay

The comet assay procedure used in the present study has been previously described (Singh and Stephens, 1998; Duty et al, 2002). Briefly, 50 μL of a semen/agarose mixture (0.7% 3:1 high-resolution agarose; Amresco, Solon, Ohio) was embedded between 2 additional layers of agarose on microgel electrophoresis glass slides (Erie Scientific, Portsmouth, New Hampshire). Slides were then immersed in a cold lysing solution to dissolve the cell membrane and make chromatin accessible for the enzyme digestion steps. After 1 hour of cold lysis, slides were transferred to a solution with 10 μg/mL of RNase (Amresco) for enzyme treatment and incubated at 37°C for 4 hours. Slides were then transferred to a second enzyme treatment with 1 mg/mL proteinase K (Amresco) and incubated at 37°C for 18 hours. The slides were placed on a horizontal slab in an electrophoretic unit, were equilibrated for 20 minutes, and underwent electrophoresis for 1 hour. DNA in the gel was then precipitated, fixed in ethanol, and dried. Slides were stained and observed with fluorescence microscopy. Comet extent, tail-distributed moment (TDM), and percent DNA located in the tail (Tail%) were measured on 100 sperm in each semen sample using VisComet software (Impuls Computergestutzte Bildanalyse GmbH, Gilching, Germany). Comet extent is a measure of total comet length from the beginning of the head to the last visible pixel in the tail. Tail% is a measurement of the proportion of total DNA that is present in the tail. *TDM* is an integrated value that takes into account both the distance and intensity of comet fragments:

$$TDM = \frac{\sum (I \times X)}{\sum I},$$

where ΣI is the sum of all intensity values that belong to the head, body, or tail and X is the x-position of the intensity value. Comets with high DNA damage (CHD), which are cells too long to measure with VisComet (>300 μm), were counted for each subject and used as an additional measure of DNA damage.

Serum Hormones

One nonfasting blood sample was drawn between the hours of 9:00 AM and 4:00 PM on the same day that the semen sample was collected. Blood samples were centrifuged, and serum was stored at −80°C until analysis. Testosterone was measured directly using the Coat-A-Count radioimmunoassay kit (Diagnostic Products Corp, Los Angeles, California), which has an interassay and intraassay coefficient of variation (CV) of 12% and 10%, respectively, with a sensitivity of 4 ng/dL (0.139 nmol/L). The free androgen index (FAI) was calculated as the molar ratio of total testosterone to sex hormone–binding globulin (SHBG). SHBG was measured using a fully automated system (Immulite; Diagnostic Products Corp) that uses a solid-phase 2-site chemiluminescent enzyme immunometric assay and has an interassay CV of less than 8%. Inhibin B was measured using a commercially available, double-antibody, enzyme-linked immunosorbent assay (Oxford Bioinnovation, Oxford, United Kingdom) with interassay and intraassay CVs of 20% and 8%, respectively; limit of detection of 15.6 pg/mL, and functional sensitivity (20% CV) of 50 pg/mL. Serum LH, FSH, estradiol, and prolactin concentrations were determined by microparticle enzyme immunoassay using an automated Abbott AxSYM System (Abbott Laboratories, Chicago, Illinois). The Second International Reference Preparation (World Health Organization 71/223) was used as the reference standard. The assay sensitivity for LH and FSH were 1.2 international units (IU)/L and 1.1 IU/L, respectively. The intraassay CVs for LH and FSH were less than 5% and less than 3%, respectively, with interassay CVs for both hormones of less than 9%. The testosterone:LH ratio, a measure of Leydig cell function, was calculated by dividing testosterone (nmol/L) by LH (IU/L). The assay sensitivity for estradiol and prolactin were 20 pg/mL and 0.6 ng/mL, respectively. For estradiol, the within-run CV was between 3% and 11%, and the total CV was between 5% and 15%. For prolactin, the within-run CV was less than or equal to 3%, and the total CV was less than or equal to 6%.

Free T_4, total T_3, and thyroid-stimulating hormone (TSH) concentrations were also determined in serum by microparticle enzyme immunoassay (AxSYM Automated System; Abbott Diagnostics). The assay sensitivity for free T_4 and total T_3 were 0.01 ng/dL and 0.15 ng/mL, respectively. The interassay CVs for both hormones were less than 9%. For TSH, the ultrasensitive hTSH II assay (Abbott Diagnostics) was used and has a functional sensitivity of 0.03 μIU/L and interassay CVs of less than 8%.

Statistical Analysis

Data analysis was performed using SAS version 9.1 (SAS Institute Inc, Cary, North Carolina). Descriptive statistics on subject demographics were calculated, along with the distributions of hormone levels and comet assay measures. Hormone levels and comet measures were stratified by demographic categories, and a student's t test or 1-way analysis of variance was conducted to investigate differences between categories and the potential for confounding. Spearman correlation coefficients were used to determine correlations among hormones, among comet measures, and between comet parameters and hormone levels.

Multivariate linear regression was used to explore continuous relationships between hormone levels and measures of sperm DNA damage. Serum concentrations of testosterone, estradiol, inhibin B, free T_4, and total T_3 closely approximated normality and were used in statistical models untransformed, whereas the distributions of FSH, LH, SHBG, FAI, prolactin, and TSH concentrations were skewed left and transformed to the natural log (ln) for statistical analyses. Comet extent, TDM, and Tail% were modeled untransformed. The number of CHD in each subject's semen sample was not normally distributed, and an arcsine transformation was used (Zar, 1984). To improve interpretability, the regression coefficients were back-transformed and expressed as a change in the dependent variable (ie, comet measures) for an interquartile range (IQR) increase in hormone levels. To explore the shape of hormone-DNA damage relationships, comet measures were also regressed on hormone quartiles. Finally, hormones associated with high levels of each of the DNA damage measures were also explored. For this analysis, each comet measure was divided into quartiles, and multivariate logistic regression was used to calculate odds ratios (ORs) for having a comet measure in the highest quartile among increasing hormone quartiles.

Inclusion of covariates was based on statistical and biologic considerations (Hosmer and Lemeshow, 1989). Age and body mass index (BMI) were modeled as a continuous variable, smoking status was dichotomized by current smoker vs never-smoker or former smoker, and race/ethnicity was categorized into 4 groups: white, African American, Hispanic, and other. The period of abstinence prior to semen sample collection was modeled as a 5-category ordinal variable, and timing of blood sample by season (winter vs spring, summer, or fall) and time of day (9:00 AM–12:59 PM vs 1:00 PM–4:00 PM) were considered for inclusion in the models as dichotomous variables. Semen quality parameters (sperm concentration, motility, or morphology) were additionally considered for inclusion as continuous variables because of relationships with comet measures (Trisini, et al. 2004) and hormone levels (Meeker et al, 2007). Sperm motility and morphology were normally distributed, whereas sperm concentration followed a log-normal distribution and was transformed using ln prior to inclusion in the models.

Results

Of the 477 men recruited, hormone data and comet assay data were both available for 371 men. The primary reason for this difference was because the comet assay was not introduced until several months into the study.

Nine additional subjects were excluded for taking hormone medications, and the remaining 362 men comprised the final study population in the present report. Age, BMI, sperm concentration, and sperm motility were similar between the final 362 men and the 106 men for which hormone and comet data were not available. Demographic characteristics of the men are presented in Table 1. Most of the men were white (85%) and had never smoked (74%). The mean (standard deviation) age and BMI were 36 (5.2) years and 28 (4.9), respectively. Distributions of comet assay DNA damage measures and hormone levels are presented in Table 2. Most men in the study had hormone levels within MGH reference ranges, when applicable.

In preliminary bivariate analyses (Spearman correlations) for the continuous variables, age was positively associated ($P < .05$ for all associations listed in the text) with FSH and LH levels but inversely associated with estradiol, FAI, free T_4, and total T_3 levels. BMI was associated with decreased levels of LH, inhibin B, testosterone, and SHBG but increased levels of estradiol, FAI, and TSH. Among comet measures, there was a positive association between age and CHD and an inverse association between BMI and Tail%. Among hormones, FSH was positively correlated with LH, and both FSH and LH were positively correlated with prolactin but inversely correlated with inhibin B. Testosterone was positively associated with LH, SHBG, and estradiol. There was an inverse association between estradiol and inhibin B and positive associations between estradiol and SHBG, free T_4, and total T_3. In addition, inhibin B was positively associated with SHBG but negatively associated with both prolactin and total T_3.

Among the categoric variables, men whose clinic visits occurred in the winter had higher comet extent and lower serum inhibin B and free T_4 levels compared with those in the spring, summer, or fall. There were positive associations between the ordinal abstinence period categories and LH and FSH levels—men with abstinence time of longer than 6 days had higher LH and FSH levels than men who abstained 3 or less days. Current smokers had higher total T_3 concentrations and lower TSH and prolactin concentrations than former and nonsmokers. Men with blood samples collected in the morning (between 9:00 AM and 12:59 PM) had higher testosterone and TSH levels and lower prolactin levels compared with those with blood samples collected in the afternoon.

Multivariate linear regression results for the association of hormone levels with sperm DNA damage are presented in Table 3, both with and without (ln-transformed) sperm concentration included in the models. The associations of sperm DNA damage with FSH, LH, inhibin B, and total T_3 levels but not testosterone, estradiol, FAI, and free T_4 levels were confounded by sperm concentration. Following adjustment by sperm concentration, there were significant but inconsistent associations between sperm DNA damage measures and the levels of LH, inhibin B, and testosterone. For comet extent, there were statistically significant or suggestive declines of 8.2 μm (95% confidence intervals [CI]: −12.7 to −3.8 μm), 4.7 μm (−9.7 to 0.3), and 3.9 μm (−8.1 to 0.2) associated with IQR increases in the levels of estradiol, free T_4, and total T_3, respectively. An IQR increase in estradiol was also associated with a 2.21-μm (−4.0 to −0.4) decline in TDM and a 4.6% (−6.4% to −2.6%) decline in Tail%. Based on the population median values of comet extent (131 μm), TDM (57 μm), and Tail% (28%), for an IQR increase in estradiol, these coefficients represent declines of 6.3% (95% CI: −9.7% to −2.9%), 3.9% (−7.1% to −0.7%), and 16.2% (−22.4% to −9.2%), respectively. Both free T_4 and total T_3 levels were positively associated with CHD. However, there were suggestive inverse associations between free T_4 and total T_3 and comet extent and significant inverse associations between both thyroid hormones and Tail%. For an IQR increase in free T_4, the coefficients represent declines in the study population median (95% CI) of 3.6% (−7.4%

Table 1. *Subject demographics (N = 362)*

Characteristic	Mean (SD)	N (%)
Age	36.2 (5.16)	
BMI[a]	27.7 (4.87)	
Race		
White		307 (85%)
African American		13 (4%)
Hispanic		16 (4%)
Other		26 (7%)
Abstinence period[a]		
≤2 days		90 (25%)
3 days		111 (31%)
4 days		62 (17%)
5 days		37 (10%)
≥6 days		58 (16%)
Smoking[a]		
Never-smoker		266 (74%)
Ever-smoker		95 (26%)
Current smoker		33 (9%)
Former smoker		61 (17%)
Season of blood sample		
Winter		75 (21%)
Spring, summer, or fall		287 (79%)
Time of blood sample		
9:00 AM–12:59 PM		158 (44%)
1:00 PM–4:00 PM		203 (56%)

Abbreviations: BMI indicates body mass index; SD, standard deviation.

[a] Information on BMI missing for 1 patient, abstinence period missing for 4 patients, and smoking status missing for 2 patients.

Table 2. *Distribution of serum hormone levels and sperm DNA damage measures (N = 362)*

	Mean	Selected Percentiles						
		5th	10th	25th	50th	75th	90th	95th
FSH, IU/L	8.97	3.67	4.51	5.77	7.50	10.2	14.7	18.0
LH, IU/L	11.1	4.90	5.88	7.37	10.1	13.5	16.9	19.9
Inhibin B, pg/mL	174	75.5	97.0	128	166	202	272	301
SHBG, nmol/mL	28.2	12.2	15.3	20.3	26.2	34.0	42.9	49.3
Testosterone, ng/dL	422	223	254	324	407	504	603	653
Testosterone:LH ratio	43.8	17.3	22.5	30.5	40.3	54.0	68.3	77.4
FAI	0.57	0.32	0.35	0.42	0.53	0.67	0.81	0.94
Estradiol	28.5	<20	<20	23	29	36	43	46
Prolactin	12.8	5.67	6.49	8.27	11.4	15.5	10.3	25.4
Free T_4, ng/dL	1.22	0.92	0.97	1.05	1.18	1.35	1.53	1.67
Total T_3, ng/mL	0.97	0.70	0.76	0.83	0.96	1.08	1.22	1.27
TSH, μIU/mL	1.68	0.59	0.78	1.04	1.43	1.98	2.71	3.58
Comet extent, μm	132	77.4	84.3	108	131	154	182	209
Tail distributed moment, μm	58.1	35.9	39.3	47.9	57.0	67.1	75.7	85.5
DNA in comet tail, %	33.2	14.1	16.5	20.4	28.4	44.8	53.9	62.6
Cells with high damage, count	8.78	0	0	1	4	11	22	31

Abbreviations: FAI indicates free androgen index; FSH, follicle-stimulating hormone; IU, international units; LH, luteinizing hormone; SHBG, sex hormone–binding globulin; TSH, thyroid-stimulating hormone.

to 0.2%) for comet extent and 24.4% (−31.5% to −17.4%) for Tail%. Results in Table 3 were similar when sperm motility was added to the models in place of sperm concentration (results not shown).

When sperm DNA damage measures were regressed on hormone quartiles, most of the statistically significant relationships presented in Table 3 remained significant. Regression coefficients followed significant monotonic trends indicative of a dose-dependent relationship. For example, the regression coefficients for a change in comet extent among increasing estradiol quartiles (1 through 4) were 0 (reference group), −8.7 (95% CI: −19.4 to 2.06), −14.4 (−24.9 to −3.98), and −22.4 (−33.7 to −11.2), respectively ($P < .0001$). However, there were a few differences between the regression analysis using hormone quartiles as compared with regression results when hormone levels were modeled as continuous variables (Table 3). In the quartile analysis, there were statistically significant but nonmonotonic trends in the associations for both free T_4 and total T_3 levels with CHD. There was also a nonmonotonic positive trend in the relationship between total T_3 and comet extent, which was inconsistent with the inverse association between total T_3 and comet extent in the original regression analysis (Table 3). In addition, the association between total T_3 and Tail% was not found in the quartile regression analysis.

For the assessment of hormonal predictors of sperm DNA damage, adjusted ORs were calculated to assess the odds of being in the highest quartile for each of the DNA damage measures among increasing hormone quartiles. Increased estradiol levels were associated with significantly reduced odds of being in the highest comet extent, TDM, and Tail% quartiles (Figure 1), potentially suggesting a protective association. The adjusted ORs for being in the highest quartile for DNA damage comparing the highest estradiol quartile to the lowest estradiol quartile were 0.19 (95% CI: 0.08–0.47), 0.28 (0.12–0.69), and 0.25 (0.11–0.56) for comet extent, TDM, and Tail%, respectively. Free T_4 levels were also associated with lower odds of having high comet extent or Tail% measures but was not associated with TDM (Figure 2). The OR for the highest Tail% quartile among men in the highest compared with the lowest free T_4 quartile was highly protective (OR = 0.08; 95% CI: 0.03–0.23). Conversely, total T_3 levels were suggestively associated with increased odds of being in the highest quartile for comet extent, TDM, and CHD, although the relationships did not follow a monotonic trend (Figure 3). Of the 4 DNA damage measures, testosterone was only associated with a suggestive decrease in odds for being in the highest Tail% quartile. Adjusted ORs for being above the 75th percentile for Tail% among testosterone quartiles 1 through 4 were as follows: 1.0 (reference group), 0.96 (95% CI: 0.48–1.92), 0.71 (0.33–1.53), and 0.52 (0.22–1.21); $P = .09$.

Because of the strong associations between both estradiol and total T_4 with Tail%, and the significant correlation coefficient between estradiol and total T_4, both hormones were entered into the linear and logistic Tail% multivariate models simultaneously while adjustments were also made for other potential confounders (age, BMI, sperm concentration, abstinence time, smoking, season, time of day, and SHBG level). Estradiol and total T_4 levels both remained significant predictors of Tail% in these models (data not shown),

Table 3. *Adjusted*[a,b] *regression coefficients for change in sperm DNA damage associated with an interquartile range increase in serum hormone levels (N = 362)*

	Adjusted Coefficients, Excluding Sperm Concentration (95% CI)				Adjusted Coefficients, Including Sperm Concentration (95% CI)			
	Comet extent	TDM	Tail%	CHD[c]	Comet Extent	TDM	Tail%	CHD[c]
FSH	6.61 (1.99, 11.3)	2.06 (0.27, 3.86)[d]	1.90 (−0.02, 3.83)[e]	0.19 (0.02, 0.36)[d]	2.13 (−2.75, 7.01)	0.68 (−1.24, 2.59)	0.97 (−1.11, 3.04)	−0.01 (−0.19, 0.17)
LH	3.49 (−2.24, 9.20)	2.45 (0.25, 4.65)[d]	−2.22 (−4.58, 0.15)[e]	0.24 (0.03, 0.46)[d]	0.27 (−5.38, 5.92)	1.51 (−0.69, 3.72)	−3.07 (−5.46, −0.70)[d]	0.12 (−0.09, 0.33)
Inhibin B	−2.81 (−6.81, 1.18)	−1.41 (−2.96, 0.11)[e]	2.59 (0.96, 4.29)[d]	−0.29 (−0.44, −0.14)[d]	0.03 (−4.00, 4.07)	−0.59 (−2.15, 0.96)	3.48 (1.85, 5.18)[d]	−0.19 (−0.33, −0.04)[d]
SHBG	−0.43 (−5.72, 4.88)	−0.58 (−2.63, 1.47)	0.21 (−1.98, 2.40)	−0.01 (−0.20, 0.20)	−0.99 (−6.09, 4.11)	−0.75 (−2.75, 1.25)	0.09 (−2.08, 2.26)	−0.03 (−0.22, 0.16)
T[a]	2.52 (−4.14, 9.18)	2.34 (−0.22, 4.86)[e]	−4.86 (−7.56, −2.16)[d]	0.12 (−0.13, 0.36)	2.52 (−3.78, 8.82)	2.34 (−0.14, 4.86)[e]	−4.86 (−7.38, −2.16)[d]	0.12 (−0.12, 0.36)
T:LH	−2.53 (−7.88, 2.81)	−1.49 (−3.55, 0.57)	−0.23 (−2.44, 1.98)	−0.18 (−0.38, 0.02)[e]	0.03 (−0.086, 0.15)	−0.009 (−0.06, 0.04)	0.007 (−0.04, 0.06)	−0.001 (−0.01, 0.003)
FAI	1.40 (−4.13, 6.91)	1.69 (−0.44, 3.81)	−3.20 (−5.46, −0.95)[d]	0.06 (−0.14, 0.27)	1.84 (−3.47, 7.15)	1.82 (−0.25, 3.89)[e]	−3.11 (−5.37, −0.87)[d]	0.08 (−0.12, 0.28)
Estradiol[a]	−8.71 (−13.4, −4.03)[d]	−2.34 (−4.16, −0.52)[d]	−4.68 (−6.50, −2.73)[d]	0.07 (−0.10, 0.25)	−8.19 (−12.7, −3.77)[d]	−2.21 (−4.03, −0.39)[d]	−4.55 (−6.37, −2.60)[d]	0.10 (−0.07, 0.26)
Prolactin	−1.90 (−7.60, 3.82)	−0.74 (−2.95, 1.48)	1.00 (−1.36, 3.37)	−0.10 (−0.31, 0.11)	−3.34 (−8.86, 2.18)	−1.18 (−3.34, 0.98)	0.69 (−1.66, 3.05)	−0.16 (−0.36, 0.04)
Free T_4	−5.67 (−10.8, −0.52)[d]	−0.57 (−2.57, 1.44)	−7.14 (−9.15, −5.13)[d]	0.24 (0.05, 0.43)[d]	−4.68 (−9.66, 0.29)[e]	−0.26 (−2.21, 1.70)	−6.93 (−8.94, −4.95)[d]	0.28 (0.10, 0.47)[d]
Total T_3	5.70 (−0.11, 11.5)[e]	2.25 (0.01, 4.50)[d]	−1.07 (−3.48, 1.34)	0.29 (0.07, 0.50)[d]	−3.90 (−8.05, 0.24)[e]	−0.21 (−1.85, 1.42)	−5.78 (−7.45, −4.13)[d]	0.24 (0.08, 0.39)[d]
TSH	0.23 (−4.17, 4.62)	−0.04 (−1.74, 1.65)	0.67 (−1.14, 2.49)	0.05 (−0.11, 0.22)	−0.25 (−4.47, 3.97)	−0.19 (−1.84, 1.47)	0.57 (−1.23, 2.36)	0.03 (−0.12, 0.19)

Abbreviations: CHD indicates comets with high DNA damage; CI, confidence intervals; FAI, free androgen index; FSH, follicle-stimulating hormone; IU, international units; LH, luteinizing hormone; SHBG, sex hormone–binding globulin; T, testosterone; TDM, tail-distributed moment; TSH, thyroid-stimulating hormone.

[a] All testosterone and estradiol models were also adjusted for SHBG.

[b] Adjusted for age, body mass index, abstinence period, current smoking, season (winter vs other), and time (morning vs afternoon) of blood sample.

[c] Variable was arcsine transformed.

[d] $P < 0.05$

[e] $P < .1$.

and there was no evidence of collinearity in the models (ie, standard errors and CI were not inflated).

Discussion

In the present study, which used several statistical approaches to explore consistency of results, serum concentrations of estradiol and free T_4 were inversely associated with sperm DNA damage. Relative to the median values for the 3 measures of DNA damage, an IQR increase in serum estradiol was associated with statistically significant 6.3%, 3.9%, and 16.0% decreases in comet extent, TDM, and Tail%, respectively, after adjustments were made for potential confounding variables. An IQR increase in free T_4 was associated with a statistically significant 24.4% decline in Tail%. In multiple logistic regression using hormone quartiles, adjusted ORs for high DNA damage among men in the highest estradiol quartile compared with the lowest estradiol quartile were 0.19 (95% CI: 0.08–0.47), 0.28 (0.12–0.69), and 0.25 (0.11–0.56) for comet extent, TDM, and Tail%, respectively. Men in the highest free T_4 quartile were at an unexpected 92% reduced risk (OR = 0.08; 95% CI: 0.03–0.23) for being in the highest Tail% quartile compared with men in the lowest free T_4 quartile.

Several other statistically significant or suggestive associations between hormone levels (FSH, LH, inhibin B, testosterone, and total T_3) and sperm DNA damage were observed, although they were not as strong or consistent as those for estradiol and free T_4. Confounding by sperm concentration of the associations involving FSH, LH, and inhibin B was not unexpected because associations between semen quality parameters and sperm DNA damage (Trisini et al, 2004) and between these hormones and semen quality (Meeker et al, 2007) were both previously reported in cohorts that overlapped with the men in the present study. However, after sperm concentration or motility was considered in the multivariate models, the strong relationships involving estradiol and free T_4 remained, suggesting these associations exist independent of semen quality parameters.

To our knowledge, this is the first human study to explore associations between sperm DNA damage and thyroid hormone levels, and our findings for an inverse association between estradiol and sperm DNA damage are consistent with 1 previous study (Richthoff et al, 2002). These researchers used the SCSA, as opposed to the neutral comet assay used in the present study, and reported a statistically significant inverse correlation between estradiol and DNA fragmentation index (DFI; a measure of DNA denaturation following SCSA)

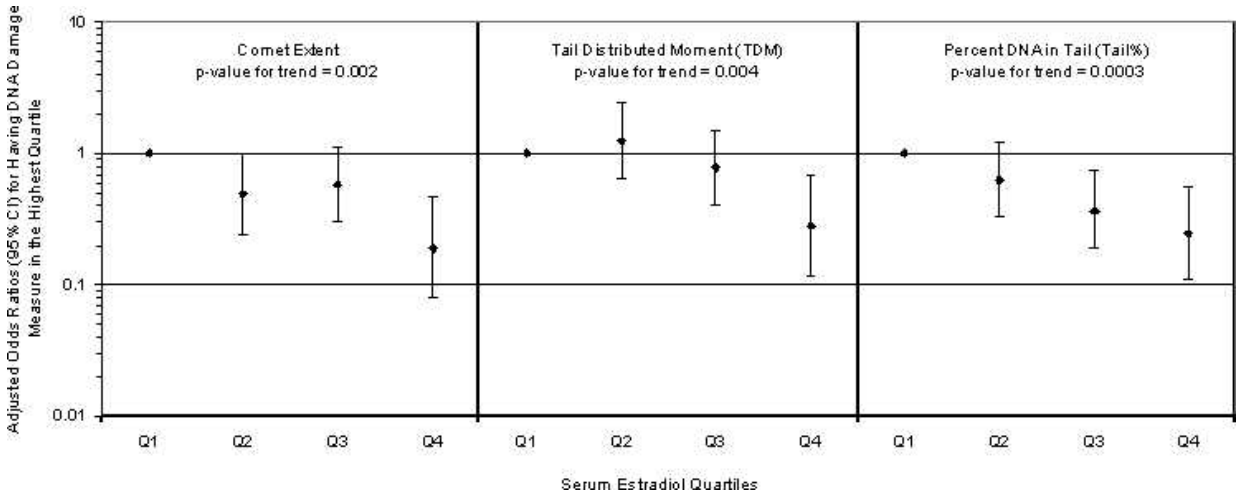

Figure 1. Adjusted (for age, body mass index, abstinence period, smoking, season, time of blood sample, natural log [ln]–transformed sex hormone–binding globulin, and ln-transformed sperm concentration) odds ratios for having DNA damage measures in the highest quartile with increasing serum estradiol quartiles (N = 362).

among 278 Swedish military conscripts. They also found that DFI was inversely correlated with testosterone levels, although the correlation was not as strong as with estradiol. Another recent study reported no associations between DFI and FSH, inhibin B, or testosterone among 129 men undergoing infertility evaluation (Appasamy et al, 2007). However, only bivariate relationships were tested, and the study did not account for confounding variables in multivariate analysis.

Estradiol is produced by testosterone aromatization, but individual differences in aromatase activity results in varying correlations between the 2 hormones across a population. In addition, estradiol in the human male may have important functions independent of those of testosterone. For example, low estradiol levels serve as a better predictor than testosterone levels of bone loss and bone density among elderly men (van den Beld et al, 2000; Amin et al, 2006) and of carotid artery intima-media thickness in middle-aged men (Tivesten et al, 2006). There have been recent advances in our understanding of the presence and role of estradiol in the male. Although traditionally considered the female sex hormone, through a number of experimental models, it has been shown that estradiol plays a vital role in normal sperm cell development and function (O'Donnell et al, 2001; Hess, 2003; Akingbemi, 2005). Studies of estrogen receptor α (ERα) knockout and aromatase knockout mice first suggested an indirect role of estrogens in male fertility (Eddy et al, 1996; Hess et al, 1997; Robertson et al, 1999). Male ERα knockout mice

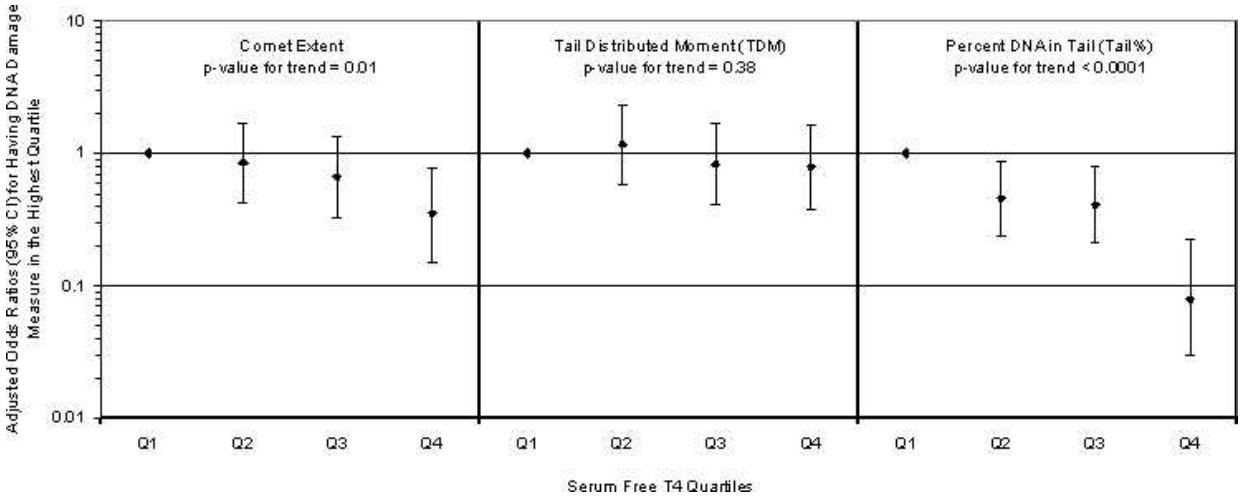

Figure 2. Adjusted (for age, body mass index, abstinence period, smoking, season, time of blood sample, and natural log–transformed sperm concentration) odds ratios for having DNA damage measures in the highest quartile with increasing serum free T_4 quartiles (N = 362).

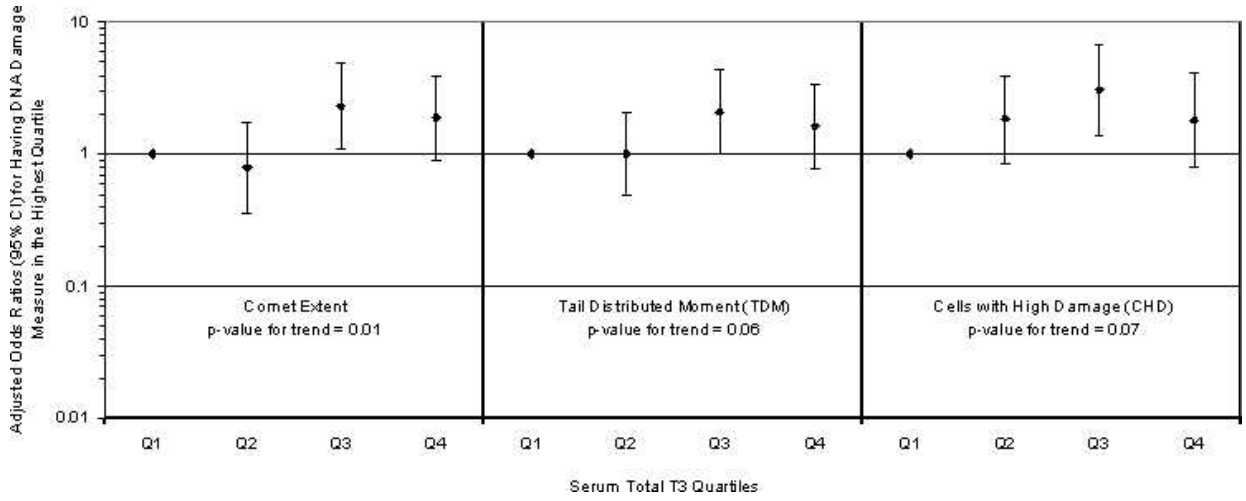

Figure 3. Adjusted (for age, body mass index, abstinence period, smoking, season, time of blood sample, and natural log–transformed sperm concentration) odds ratios for having DNA damage measures in the highest quartile with increasing serum total T_3 quartiles (N = 362).

were infertile, with postpubertal degeneration of the testes and disrupted spermatogenesis (Eddy et al, 1996; Hess et al, 1997). Estradiol is produced in the testes from aromatized testosterone, and progressive disruption of spermatogenesis and infertility was also observed among aromatase knockout mice (Robertson et al, 1999). A direct role of estradiol as a germ cell survival factor was then demonstrated in the human testis in vitro, where estradiol was shown to inhibit testicular apoptosis much more effectively (100- to 1000-fold) than testosterone (Pentikainen et al, 2000). Estradiol has also been shown to induce spermatogenesis in gonadotropin-deficient (*hpg*) mice (Kula, 1988; Singh et al, 1995; Ebling et al, 2000). Results of the present study, in which we found that estradiol levels were inversely associated with sperm DNA damage, support the hypothesis that estradiol is associated with sperm development, maintenance, and function in humans.

Thyroid hormones impact most tissues and systems in the human body, and infertility is a common clinical manifestation of thyroid hormone deficiency in adult males (Nussey and Whitehead, 2001; Krassas and Pontkides, 2004). Although much in this area is still unknown, especially for subclinical alterations in thyroid hormone levels, there is evidence that thyroid hormones have important functions on fetal Sertoli cell maturation (Holsberger and Cooke, 2005) and on Leydig cell differentiation and steroidogenesis in the postnatal testis (Mendis-Handagama and Ariyaratne, 2004) and can stimulate testosterone and estradiol production and secretion by varying the pituitary's responsiveness to LH (Velazquez and Bellabarba Arata, 1997; Maran, 2003). Thyroid hormones may be involved with germ cell survival and mitotic germ cell DNA synthesis through a paracrine signal from the Sertoli cells (Jannini et al, 1993, 1995), which may provide a mechanism for the relationship between thyroid hormone levels and sperm DNA damage in the present study. However, because most experimental studies to date have focused on thyroid hormone action in the developing testes, more work is needed on the role of thyroid hormones in spermatogenesis and reproductive function. The influence of thyroid hormones on steroidogenesis may also partially explain our observation of a correlation between thyroid hormones and circulating estradiol and testosterone. However, despite the correlation between estradiol and thyroid hormones in men from the present study, when both estradiol and free T_4 were entered as independent variables into multiple linear regression models, they both remained significantly associated with declined sperm DNA damage with no indication of collinearity.

Inconsistent results between the various DNA damage measures obtained by the neutral comet assay regressed on the same independent variable have been observed in previous studies, and it has been hypothesized that the different comet assay parameters may reflect different types of DNA strand breaks (Meeker et al, 2004). Specifically, because of the lack of correlation between TDM and Tail%, it was hypothesized that a high TDM may be more likely to be associated with double-strand breaks, whereas a high Tail% may reflect single-strand breaks (Meeker et al, 2004). Thus, in the present study, TDM was more highly inversely associated with estradiol, which may reflect a protective relationship between estradiol and double-strand breaks. Conversely, Tail% was more highly inversely associated with free T_4, which may signify a protective relationship between free T_4 and single-strand breaks. However, because both hormones were significantly and

independently associated with Tail%, if this hypothesis holds, then both free T_4 and estradiol may have a protective association with single-strand breaks.

In summary, we found statistically significant associations between serum levels of several hormones and sperm DNA damage, most notably protective associations involving estradiol and free T_4. Estradiol and thyroid hormone play a role in calcium metabolism (Khosla et al, 1998; Lindblom et al, 2001; Kumar and Prasad, 2003) and may prevent elevated intracellular concentrations of calcium (Hilton et al, 2006; Marino et al, 2006) and subsequent DNA damage (Ray et al, 1993; Liu and Huang, 1996; Bentle et al, 2006). However, at this time it is not known whether hormones are in the causal pathway for sperm DNA damage or if both decreased hormone (estradiol, free T_4) levels and increased sperm DNA damage are related to another unmeasured factor that is a common cause of both. For example, oxidative stress was associated with decreased steroidogenesis and increased DNA damage in mouse testes (Kaur and Bansal, 2004), although a role for hormones in the DNA damage causal pathway was not ruled out in the study. Additional studies are needed to elucidate the nature of the relationship between hormones and DNA damage in human sperm.

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