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High doses of alcohol increase urinary testosterone-to-epitestosterone ratio in females

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Abstract

The effect of alcohol (1.2 and 2.0 g/kg) on the urinary testosterone-to-epitestosterone (T/E) ratio was studied by two experiments each conducted with four healthy females and males. The intake of 2.0 g/kg of ethanol within 5 h in the evening significantly increased plasma testosterone concentration and ratio of T/E in urine collected next morning in females. The results suggest that alcohol increases the T/E ratio more in females than in males. The effect of high doses of alcohol on urinary T/E ratio must be kept in mind when doping tests are performed during training periods.

Keywords: Steroids; Testosterone; Epitestosterone; Alcohol

1. Introduction

The use of testosterone to enhance athletic performance is prohibited in sports. In order to reveal such an abuse, urine tests have been endorsed. Administration of exogenous testosterone increases the ratio of testosterone to its enantiomer epitestosterone (T/E) in urine both in males and females from the normal range around 1 [1]. Although plasma testosterone levels experience some diurnal variation, the ratio of T/E remains invariable [2]. Neither has a vigorous exercise a significant effect on this ratio [3], nor the phase of menstrual cycle in females [4]. There is a vast variety of factors which affect the T/E ratio. An exceptionally high ratio could be due to physiological or pathological conditions, e.g. low epitestosterone excretion, enzyme deficiencies, tumour androgen production or dietary factors [3,5–8]. According to the rules of the International Olympic Committee, banned use of exogenous testosterone is estimated by a urinary glucuronide ratio of T/E higher than 6 provided that there is no evidence of a physiological or pathological reason [9,10].

Ethanol has several effects on androgen production and metabolism, e.g. through the effects on the redox state in liver [11] and through an enzyme inhibition or induction [12,13]. Acute alcohol consumption impairs sex steroid production in males when it is ingested in amounts which produce hangover [14]. Prolonged use of alcohol induces gonadal impairment by a direct effect on the testis and interferes with the function of the hypothalamic–pituitary–gonadal axis [15]. Alcohol does not affect plasma levels of female sex hormones during the follicular phase of the menstrual cycle [16]. However, Välimäki et al. [17] reported that acute alcohol intake at doses of 1.2 g/kg may increase plasma

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testosterone concentration in females. Falk et al. [2] demonstrated in males that alcohol intake (≥1 g/kg of body weight) increases urinary testosterone concentration, while epitestosterone level remains unaltered. In their study the consequent mean increase in the T/E ratio was 41%.

We have recently presented a case report on a female powerlifter who was observed to have an elevated ratio of T/E due to the intake of a high amount of alcohol in the previous night before the out of competition doping test [18]. We performed this study to compare the effect of ethanol on T/E ratio in males and females.

2. Experimental

2.1. Conditions

Two experiments both with four men and four women were conducted. The volunteers aged from 19 to 24 years, and they were of normal weight (Table 1). The subjects used no medication excluding contraceptives. All subjects were moderate drinkers and had no history of alcohol abuse. Their serum γ-glutamyl transferase and aminotransferase levels were within normal range. Alcohol consumption was prohibited during the study.

Written informed consent was obtained from all volunteers. The Ethical Committee of National Public Health Institute approved the study protocol.

In the first experiment, 1.2 g/kg (w/w) of ethanol was given to the subjects. Absolute ethanol was mixed with orange juice to yield a 20% (v/v) solution. Urine was collected daily from 8 a.m. to 12 a.m. on four successive days beginning on the day preceding the alcohol intake. Serum samples for hormone assays were collected at noon on the day preceding the alcohol intake (day −1) and on days 1 and 2 post alcohol. Ethanol was drunk between 7 p.m. and 10 p.m. On the day of drinking a whole blood sample was drawn at 10.30 p.m. for the assay of blood alcohol concentration [19]. The subjects were not requested to follow any special diet during the study. However, during the alcohol session a light meal was served.

Table 1
Characteristics of the subjects

Subject	Sex	Age (years)	Height (cm)	Weight (kg)	Use of contraception pills	Day of menstrual cycle	BAC (g/g)
Experiment 1							
HR	Female	23	170	52	Yes	21	0.74
PP	Female	23	174	64	Yes	26	0.60
HE	Female	20	163	52	No	23	0.61
LH	Female	21	160	54	No	17	0.64
RR	Male	20	186	84			0.53
LR	Male	22	179	78			0.76
MP	Male	20	189	96			0.75
KK	Male	20	180	79			0.73
Experiment 2							
HR	Female	24	170	53	Yes	9	1.04
ER	Female	20	173	60	Yes	17	1.77
MT	Female	22	170	71	Yes	25	2.00
TS	Female	22	168	55	Yes	13	1.65
RR	Male	20	186	85			1.40
RR	Male	19	181	77			1.27
MP	Male	21	189	98			1.16
LR	Male	22	179	77			1.47

BAC=blood alcohol concentration 30 min after discontinuing of alcohol intake (1.2 g/kg in Experiment 1; 2.0 g/kg in Experiment 2).

Experiment 2 followed the same protocol as the first one, but the volunteers received 2.0 g/kg (w/w) of alcohol. Four of the volunteers were the same as in the first experiment. Due to the higher dose of alcohol the time for drinking was increased to 5 h (up to 10 p.m.) in order to maintain the constant rate of drinking.

All samples were stored at −20°C until analysis. The samples were analysed for urinary testosterone, epitestosterone, androsterone, etiocholanolone, 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone and creatinine. Serum samples were assayed for testosterone, luteinizing hormone, sex hormone binding globulin, estradiol, androstenedione, dehydroepiandrosterone and dehydroepiandrosteronesulphate.

2.2. Analytical procedures

The quantitation of glucuronide conjugates of testosterone (Makor Chemicals, Jerusalem, Israel), epitestosterone (Steraloids, Wilton, MA, USA), androsterone (Merck, Darmstadt, Germany), etiocholanolone (Makor Chemicals), 11β-hydroxyandrosterone (Sigma, St. Louis, MO, USA) and 11β-hydroxyetiocholanolone (Makor Chemicals) in urine were carried out by gas chromatography–mass spectrometry using selected-ion monitoring (GC–MS-SIM). The method was modified from the standard screening procedure used for anabolic steroids [20]. All assays were carried out in duplicate. Five levels of standards covering the ranges of 1–100 ng/ml for testosterone and epitestosterone, 250–4000 ng/ml for androsterone and etiocholanolone, 125–2000 ng/ml for 11β-hydroxyandrosterone and 62.5–1000 ng/ml for 11β-hydroxyetiocholanolone were prepared in water and treated in the same way as other samples. The following compounds were used as internal standards: 16,16,17-d_3-testosterone (Sigma) (30 ng/ml) for testosterone, 2,2,4,6,6-d_5-epitestosterone (synthesized by Wähälä et al. [21]) (30 ng/ml) for epitestosterone, and methyltestosterone (Diosynth, Oss, Netherlands) (500 ng/ml) for all other steroids. A 2-ml aliquot of urine was introduced into a reversed-phase C_{18} cartridge (Sep-Pak C_{18}, Waters, Millford, MA, USA) which was previously activated with 2.5 ml of methanol and 5.0 ml of water. After washing with 5 ml of water, the sample was eluted with 3 ml of methanol and evaporated to dryness. The residue was dissolved in 1 ml of 0.1 *M* phosphate buffer (pH 7) and hydrolyzed enzymatically by adding 50 μl of β-glucuronidase from *Escherichia coli* K12 (Boehringer Mannheim, Mannheim, Germany) and incubated for 1.5 h at 50°C. After adding 250 μl of 7% potassium carbonate solution, the sample was extracted with 5 ml of *n*-pentane for 5 min. The organic phase was separated and evaporated to dryness and derivatized with 50 μl of N-methyl-N-trimethylsilyltrifluoroacetamide, trimethyliodosilane and dithioerythritol (1000:2:4, v/v) for 15 min at 60°C. Samples were analysed on HP 5890E/HP 5972A GC/MS (Hewlett-Packard, Palo Alto, CA, USA) using a fused-silica capillary column (HP 1, 16 m×0.2 mm I.D., film thickness 0.11 μm). Helium was used as a carrier gas (constant flow 0.5 ml/min). The temperatures of the injection port and transfer line were 300°C and 280°C, respectively. The oven was first programmed from 180 to 230°C at 3°C/min and finally to 310°C at 30°C/min. A 3-μl volume of sample was injected (split ratio 1:28). The bis-trimethylsilylated steroids were detected (dwell time 20 ms) using the following ions: *m/z* 301.1 (methyltestosterone); *m/z* 432.2 (testosterone and epitestosterone); *m/z* 434.4 (androsterone and etiocholanolone); *m/z* 522.5 (11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone); *m/z* 435.3 (d_3-testosterone) and *m/z* 436.3 (d_5-epitestosterone). The inter-assay coefficients of variation (C.V.s) were <9% for testosterone and epitestosterone ($n=9$) and <14% all other compounds ($n=9$).

Urinary creatinine was determined with a commercially supplied colour reagent kit (Bayer Diagnostics Manufacturing, Orcq-Tournai, Belgium). The inter-assay C.V. was <4% over the whole concentration range.

Serum total testosterone was assayed by a direct competitive radioimmunoassay (RIA) using polyclonal testosterone specific antibody: the bound and unbound tracer (^{125}I-Testo) were separated with solid-phase assay tubes, to which the antibody was conjugated. The manufacturer of the kit was Diagnostic Products Corporation (DPC) (Los Angeles, CA, USA). Sex hormone binding globulin concentrations from 0 to 180 nmol/l had minimal effects to the results (92–104%). The sensitivity of the test

was 0.5 nmol/l and the inter-assay variation ranges from 4 to 7% (C.V.) over the concentration range 3.5–29.5 nmol/l (n=25).

The principles of serum dehydroepiandrosteronesulphate, dehydroepiandrosterone and androstenedione RIA methods were similar to the testosterone assay (kits were from DPC). Dehydroepiandrosterone and androstenedione were extracted prior to RIA. The sensitivity of the dehydroepiandrosteronesulphate assay was 0.1 μmol/l and the inter-assay precision was from 5 to 9% (C.V.) over the concentration range from 0.5 to 13.5 μmol/l (n= 20). The sensitivity of the dehydroepiandrosterone assay was 0.1 nmol/l and the inter-assay variation was from 5 to 10% (C.V.) over the concentration range 4–90 nmol/l (n=20). The sensitivity of the androstenedione assay was 0.4 nmol/l and inter-assay variation was from 5 to 10% (C.V.) over the concentration range 3.2–30 nmol/l (n=20).

Serum estradiol was measured by a direct competitive RIA using a polyclonal estradiol specific antibody and ^{125}I-labelled tracer. Free and bound tracer were separated with a second antibody conjugated to assay solid-phase polypropylene tubes. The RIA kits were from Orion Diagnostica (Turku, Finland). The sensitivity of the assay was 0.02 nmol/l and the inter-assay precision from 4 to 8% (C.V.) over the concentration range 0.18–1.30 nmol/l (n= 25).

Serum luteinizing hormone was measured by an immunofluorometric assay (TR-IFMA) using two monoclonal β-subunit specific antibodies, one of which was conjugated to the assay solid-phase, the other one was labelled with Eu-chelate. The manufacturer of the assay was Wallac (Turku, Finland). The sensitivity of the assay was 0.1 IU/l and the inter-assay variation was from 6 to 8% over the concentration range 4.0–53.7 U/l (n=20). The crossreactivity with human chorionic gonadotrophic hormone was 0.5%.

Serum sex hormone binding globulin was assayed by an immunofluorometric assay (TR-IFMA) using two monoclonal antibodies, one of which was conjugated to the assay solid-phase, the other one was labelled with europium chelate (Wallac). The inter-assay precision (C.V.) was less than 6% over the concentration range 20–90 nmol/l (n=20)

2.3. Statistical analysis

Nonparametric Friedman's test was used for evaluation of the significancies for the data obtained from day −1 to day 1. Probabilities equal to or smaller than 0.05 were regarded as statistically significant. Statistical analyses were carried out using Systat software [22] and the results are expressed as mean ± standard error of mean (S.E.M.). Statistical parameters were calculated both together and differently for either sex and experiment.

3. Results

The results are given in Table 2. In the experiment 1, alcohol intake (1.2 g/kg) did not cause any significant hormonal changes either in males or females. However, in females there was a tendency to increased serum testosterone concentration and urinary ratio of testosterone to creatinine, 125% and 129%, respectively.

In males, no significant differences were found in any parameters measured even after the higher dose of alcohol (experiment 2). In females serum testosterone and dehydroepiandrosteronesulphate significantly increased by 154% and 130%, respectively, on day 1 in comparison to the concentrations obtained prior ethanol intake. In females, 2 g/kg of alcohol tented to increase serum luteinizing hormone and androstenedione concentrations, but these changes did not reach the level of statistical significance.

After the intake of 2.0 g/kg of ethanol (experiment 2) the ratio of T/E was elevated. Fourteen hours after the discontinuation of alcohol intake, urinary T/E ratio was significantly (P<0.05) increased by 277% in the whole population (Fig. 1a). This increase was mainly due to the rise of the ratio in females (range 1.9–8.7). Both creatinine related testosterone and epitestosterone in urine were significantly increased by 420% and 142%, respectively, in females (Fig. 1b).

In females urine etiocholanolone/creatinine ratio was significantly (P<0.05) decreased by 33% from that obtained before the intake of alcohol (2 g/kg) (Fig. 1c). Two g/kg of alcohol tended also to decrease the ratio of urinary androsterone to

Table 2
Hormones in serum (S) and urine (U) before and after alcohol intake

		Females			Males		
		before	after		before	after	
Experiment 1							
S-Testosterone	(nmol/l)	1.00± 0.27	1.25± 0.27	(125%)	18.45± 1.51	19.80± 2.52	(107%)
S-Free testosterone	(pmol/l)	9.25± 3.03	11.34± 3.66	(123%)	301.89± 18.29	333.77± 46.13	(111%)
S-Luteinizing hormone	(IU/l)	4.68± 3.11	7.40± 5.97	(158%)	2.93± 0.25	2.95± 0.67	(101%)
S-Estradiol	(nmol/l)	0.48± 0.23	0.51± 0.23	(106%)	0.06± 0.01	0.08± 0.02	(133%)
S-Androstenedione	(nmol/l)	7.70± 2.02	8.28± 1.55	(108%)	7.00± 0.70	9.33± 1.53	(133%)
S-Dehydroepiandrosterone	(nmol/l)	34.75± 10.08	44.75± 11.03	(129%)	46.00± 3.98	57.75± 5.71	(126%)
S-Dehydroepiandrosteronesulphate	(μmol/l)	5.08± 0.99	4.96± 1.12	(98%)	13.23± 1.84	13.70± 1.74	(104%)
U-Androsterone/creatinine	(ng/mg)	1225.69±192.99	1559.31±352.95	(127%)	3153.13±863.96	3339.07±984.98	(106%)
U-Etiocholanolone/creatinine	(ng/mg)	1607.39±135.98	1665.52±180.23	(104%)	3171.18±304.57	3008.66±343.45	(95%)
U-11-Hydroxyandrosterone/creatinine	(ng/mg)	597.25±137.18	671.99± 88.51	(113%)	772.20±276.87	763.10±260.37	(99%)
U-11-Hydroxyetiocholanolone/creatinine	(ng/mg)	212.33± 38.68	185.76± 36.81	(87%)	238.42±117.40	251.80±142.66	(106%)
U-Epitestosterone/creatinine	(ng/mg)	7.80± 3.46	9.62± 3.46	(123%)	34.05± 6.60	29.17± 4.85	(86%)
U-Testosterone/creatinine	(ng/mg)	3.02± 0.43	3.89± 0.74	(129%)	27.50± 14.14	25.00± 12.72	(91%)
Experiment 2							
S-Testosterone	(nmol/l)	0.70± 0.27	1.08± 0.39	(154%)*	18.03± 2.86	16.05± 1.34	(89%)
S-Free testosterone	(pmol/l)	3.65± 1.25	5.78± 2.07	(158%)*	298.04± 42.47	259.72± 21.58	(87%)
S-Luteinizing hormone	(IU/l)	0.50± 0.26	0.83± 0.33	(166%)	3.03± 0.87	5.98± 2.77	(197%)
S-Estradiol	(nmol/l)	0.02± 0.00	0.02± 0.00	(100%)	0.05± 0.01	0.04± 0.01	(80%)
S-Androstenedione	(nmol/l)	3.88± 0.82	5.80± 0.66	(149%)	5.53± 0.80	6.95± 0.55	(126%)
S-Dehydroepiandrosterone	(nmol/l)	29.50± 8.39	29.25± 8.93	(99%)	35.50± 4.63	41.25± 4.64	(116%)
S-Dehydroepiandrosteronesulphate	(μmol/l)	4.10± 1.24	5.35± 1.27	(130%)*	12.03± 1.14	13.60± 1.27	(113%)
U-Androsterone/creatinine	(ng/mg)	1794.31±304.96	1325.50±270.29	(74%)	2418.93±448.08	1970.62±255.48	(81%)
U-Etiocholanolone/creatinine	(ng/mg)	1988.17±182.91	1333.91±256.78	(67%)*	2332.13±311.09	1905.98±424.10	(82%)
U-11-Hydroxyandrosterone/creatinine	(ng/mg)	558.52±117.70	569.39±141.25	(102%)	780.68±159.63	689.96± 89.52	(88%)
U-11-Hydroxyetiocholanolone/creatinine	(ng/mg)	163.03± 64.85	175.47± 53.42	(108%)	190.02± 85.46	183.61± 87.31	(97%)
U-Epitestosterone/creatinine	(ng/mg)	2.19± 0.17	3.11± 0.42	(142%)*	34.01± 10.29	31.63± 7.28	(93%)
U-Testosterone/creatinine	(ng/mg)	3.24± 0.41	13.61± 3.32	(420%)*	20.18± 10.40	25.22± 13.05	(125%)

The serum and urine values refer to samples taken on the days preceeding and following alcohol intake.
* $P<0.05$ (nonparametric Friedman's test), significance of difference for the data through days −1 and 1.

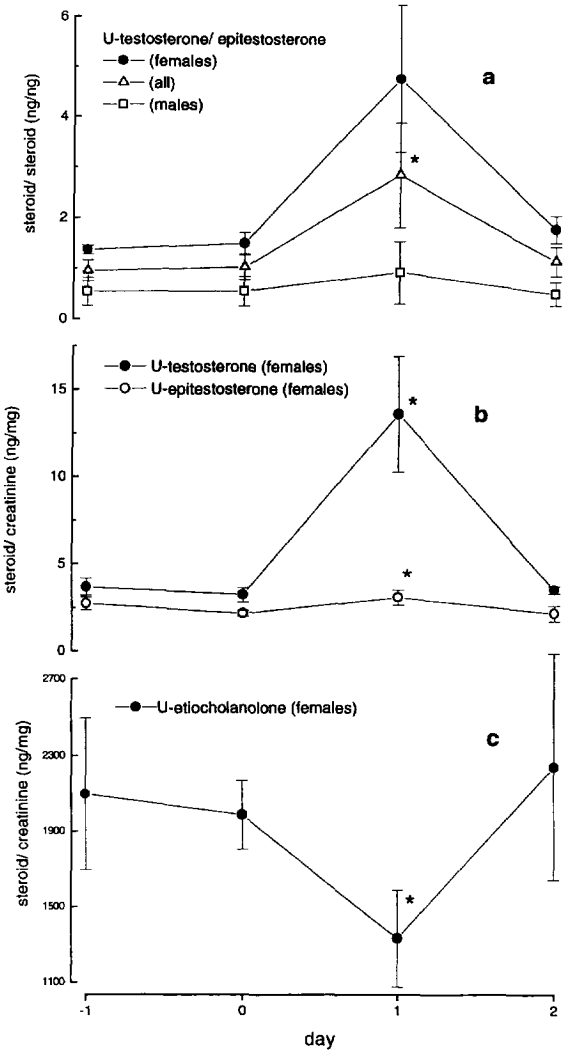

Fig. 1. Mean (±S.E.M.) urinary ratio of testosterone to epitestosterone in males and females (a), testosterone/creatinine and epitestosterone/creatinine in females (b) and etiocholanolone/creatinine in females (c) after 2 g/kg (w/w) of alcohol on day 0. *=$P<0.05$ (nonparametric Friedman's test) for the data through days −1 and 1.

creatinine in females. However, this decrease did not reach the level of statistical significance.

4. Discussion

The results agree with our earlier findings on a female powerlifter, who was observed to have an elevated T/E ratio after drinking high amount of alcohol during the night before the doping test [15]. The results demonstrate further that acute alcohol intake affects urinary T/E ratio proportionally more in females than males. We could not demonstrate statistically significant change in T/E ratio in males as did Falk et al. [2]. This was probably due to the relatively high variation in T/E ratios and small number of subjects.

Sex difference in acute alcohol response may lie, at least in part, in the fact that the origin of testosterone is different in males and females. In females the ovaries and the adrenals contribute equally to testosterone production, each supplying about 25% of the total circulating level [23]. The rest, 50% of the circulating testosterone, is derived from peripheral conversion of androstenedione in the liver, skin, brain and adipose tissue. In men, on the other hand, the majority of testosterone is of gonadal origin [23,24]. Moreover, the basic androgen concentrations in females are essentially lower allowing a more distinct outcome due to small changes.

Alcohol may change both the synthesis and the metabolism of testosterone. Elevated serum and urinary testosterone may be derived from an effect promoting the synthesis of testosterone or an effect preventing its metabolism.

The body's endocrine reaction to alcohol is quite similar as to a general stress, where plasma cortisol concentration is increased [25]. Alcohol stimulates steroid production by a direct mechanism in the adrenal cortex [26]. On the other hand, adrenal androgens are produced mainly as intermediates in the formation of cortisol [23]. Consequently, when corticosteroid production is increased the production of androgen may increase too. Although the exact site of this alcohol effect is not known, it is likely that alcohol increases accordingly the production of testosterone precursors. This is in agreement with increased serum concentrations of dehydroepiandrosteronesulphate, the major adrenal androgen [23], found in females after 2 g/kg alcohol intake. Slightly elevated levels of serum dehydroepiandrosterone and androstenedione in females after the intake of a higher dose of alcohol support further the theory of alcohol stimulated adrenal androgen production. This is in agreement with the finding that serum androstenedione levels accompany those of cortisol [23].

Another mechanism by which alcohol may enhance the conversion of androgen precursors to testosterone is its increasing effect on the ratio of oxido–reduction couple NADH to NAD^+ in liver leading to increased steroid oxidation. This redox effect on the levels of circulating conjugated steroids is seen even with small single dose of alcohol (0.3 g/kg), but the effect may depend on the mode of conjugation [27,28]. Although previous studies have demonstrated the redox effect of alcohol on sulphate conjugates only, it is not excluded on glucuronide conjugates [28]. The metabolic alteration is likely to affect testosterone levels especially in females, in whom proportionally larger amounts of androgens are produced via extragonadal conversion of androgen precursors [20].

Although alcohol increased serum luteinizing hormone concentrations in females of experiment 2, this hormone was probably not responsible for increased testosterone concentrations. This is suggested by the finding that serum estradiol concentrations remained unchanged, and if the increased testosterone concentrations had been due to luteinizing hormone stimulation in the ovaries, then serum estradiol concentration should have been concomitantly increased [29]. Increased luteinizing hormone concentrations found in females after alcohol reflect most likely the physiological changes during advanced menstrual cycles.

Acute intake of ethanol is known to cause enzyme inhibition and slow down the metabolism of several compounds oxidized via cytochrome P450 enzyme [12,13]. Testosterone metabolites are included these compounds and thus increases in testosterone excretion during the study may be explained in part by this reduced catabolism [23]. Decrease in the urinary concentrations of androsterone and etiocholanolone, two main metabolites of testosterone, suggests further that alcohol diminished the metabolism of testosterone [23].

Increased amounts of adrenal androgens may have led to higher serum testosterone levels, which could be a reason for the increased ratio of T/E. Tamm et al. demonstrated that after administration of androstenedione, testosterone and epitestosterone glucuronides were also increased [30], but in our work the urinary concentrations of these glucuronides were different. Consequently, epitestosterone is either metabolized or excreted in the different way as testosterone.

The elevation of the T/E ratio by high doses of alcohol should be taken into account when performing out of competition doping tests. Although females may be more sensitive to this increase due to their lower basic androgen concentrations, the increase in T/E ratio cannot be ruled out in males.

In conclusion, ethanol may stimulate adrenal steroid production and affect the redox balance in liver leading to increased ratio of T/E excreted into the urine. However, the doses of alcohol must be considerably high to increase this ratio. Alcohol intake has more profound effect on the T/E ratio in females than in males.

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