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INCREASED SERUM AND CEREBROSPINAL FLUID SEX STEROID HORMONE LEVELS IN HYPOTHERMIA

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Abstract: *Background.* Sex steroid hormones are affected by the process of death. The objective of the present study was to compare the concentrations of sex steroid hormones in blood and cerebrospinal fluid (CSF) of people who died from various causes, especially hypothermia, and to examine correlations between sex steroid hormone levels and the pathophysiology of various causes of death.

Methods. Using chemiluminescent enzyme immunoassay, the concentrations of testosterone (TE) and estradiol (E2) were analyzed in blood samples obtained from the right heart chambers and CSF of 233 bodies (150 males and 83 females, age range: 21 to 96 years, median age 64.0 years), for which an autopsy was performed within 72 hours of death. TE and E2 levels were assessed separately in males and females after grouping the causes of death as blunt injury, sharp instrument injury, fire fatality, asphyxia, drowning, intoxication, hyperthermia, hypothermia and sudden cardiac death.

Results. TE and E2 concentrations in serum and CSF in both males and females were high in subjects who died from intoxication. E2 levels were also elevated in males in whom the cause of death was hypothermia, while serum TE concentrations were elevated in females who died from hypothermia.

Conclusion. The present study showed that sex steroid hormone concentrations are affected by the cause of death, especially hypothermia, as seen in autopsy cases.

Keywords: sex steroid hormone, testosterone, estradiol, intoxication, hypothermia.

INTRODUCTION

In the field of forensics, various biochemical markers are measured to clarify the pathophysiological findings indicative of the cause of death [1]. Among them, hormones, which are physiologically active substances released from various secretory glands for the purpose of maintaining homeostasis, are affected by the pathological condition before death and in the agonal phase [2-4]. So far, various hormones have been analyzed and suggested as being usable as markers indicative of a specific cause of death or pathological condition. Previous studies have shown that prolactin and thyroid hormone levels are elevated in conditions that cause acute systemic hypoxia [2]. High levels of prolactin are also seen in cases of drug intoxication [3]. In addition, it was reported that cortisol, which is classified as a steroid hormone, is highly secreted into the blood during hypothermia (cold exposure) [4]. However, no previous reports have examined pathophysiological conditions in the forensic cases of the sex steroid hormones TE and E2. Sex steroid hormones, which, like cortisol, are classified as steroid hormones, might also be highly secreted into the blood during hypothermia.

Many reports have described the synthetic pathways of TE and E2 [5-10]. In males, TE is produced when luteinizing hormone binds to Leydig cells [6], and approximately 95% of circulating TE is produced in the testes [7,8]. On the other hand, most of the E2 in

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males is formed by irreversible conversion of TE to E2 by aromatase found in adipose tissue [9]. In females, follicle-stimulating hormone promotes E2 production by its effects on ovarian granulosa cells [10,11], while 50% to 60% of TE is derived from androstenedione conversion in peripheral tissues, 30% is directly produced in the adrenal gland, and 20% is produced in the ovaries [12,13]. E2 has the strongest physiological activity among estrogens, twice as high as that of estrone and ten times higher than that of estriol.

The objective of the present study was to compare the concentrations of sex steroid hormones in the blood and cerebrospinal fluid (CSF) of people who died from various causes, especially hypothermia, and to examine the correlations between sex steroid hormones and the pathophysiology of various causes of death.

MATERIALS AND METHODS

Autopsy samples

Serial forensic autopsy samples were obtained within 72 hours postmortem at our institute between 2010 and 2017, excluding putrefactive and highly destructive cases. A total of 233 autopsy cases in which serum and CSF samples could be collected (150 males, 83 females, age range: 21 to 96 years, median age 64.0 years), including 11 hypothermia cases (4 males, 7 females) were analyzed (Table 1). For the present examination, the survival period was established based on witness and circumstantial evidence, and the postmortem period was estimated based on pathological findings. Specimens were collected aseptically using syringes to obtain blood from the right heart chambers and CSF. Samples were subsequently stored at −80 °C until use.

Biochemical analysis

Amounts of TE and E2 in serum and CSF were measured with a chemiluminescent enzyme immunoassay using the point-of-care testing system PATHFAST (LSI Medience, Tokyo, Japan) according to the manufacturer's protocol [14-17]. For comparison, the clinical serum reference ranges of these hormones are 1.87–8.35 ng/mL and <0.758 ng/mL for TE, and <59.9 pg/mL and <375 pg/mL for E2 in males and females, respectively.

Histopathological and immunohistochemical analyses

Serial 4-μm-thick horizontal sections of formalin-fixed, paraffin-embedded testicular and

Table 1. Case profiles (n = 233)

Sex	Cause of death	n	Age (y)	Postmortem period (h)	Survival period (n)		
			Range (median)	Range (median)	Acute	Subacute	Prolonged
Male	Sharp instrument injury	13	45 – 74 (66)	12 – 60 (27)	6	6	1
	Blunt injury	29	25 – 81 (52)	12 – 60 (32)	9	11	9
	Asphyxia	22	21 – 81 (65)	12 – 60 (37)	15	7	-
	Drowning	12	33 – 85 (64.5)	12 – 72 (33)	10	2	-
	Intoxication	12	21 – 50 (40)	24 – 60 (35)	3	7	2
	- Psychotropic drug poisoning	8	21 – 50 (43)	24 – 48 (33)	2	5	1
	- Methamphetamine poisoning	4	38 – 46 (38.5)	36 – 60 (43)	1	2	1
	Fire fatality	43	28 – 95 (71)	12 – 60 (23)	43	-	-
	Hyperthermia	3	72 – 76 (74)	24 – 36 (26)	-	3	-
	Hypothermia	4	52 – 81 (66.5)	24 – 60 (49)	1	3	-
	Acute cardiac death	12	44 – 76 (65.5)	24 – 60 (35)	10	2	-
	Total	150	21 – 95 (61)	12 – 72 (30)	97	41	12
Female	Sharp instrument injury	3	56 – 86 (61)	12 – 36 (28)	2	1	-
	Blunt injury	16	52 – 93 (75)	12 – 60 (39)	2	2	12
	Asphyxia	11	23 – 87 (57)	12 – 60 (32)	8	3	
	Drowning	6	58 – 96 (78)	42 – 48 (24)	4	1	1
	Intoxication	9	25 – 48 (40)	12 – 60 (37)	1	7	1
	- Psychotropic drug poisoning	5	38 – 45 (40)	12 – 60 (25)	-	4	1
	- Methamphetamine poisoning	4	25 – 48 (41.5)	36 – 48 (41)	1	3	-
	Fire fatality	21	33 – 92 (76)	12 – 60 (20)	21	-	-
	Hyperthermia	8	28 – 92 (76.5)	24 – 60 (38)	-	8	-
	Hypothermia	7	34 – 91 (84)	24 – 60 (57)	-	5	2
	Acute cardiac death	2	25 – 62 (43.5)	24 – 60 (35)	2	-	-
	Total	83	23 – 96 (73)	12 – 60 (34)	40	26	17
Total		233	21 – 96 (64)	12 –72 (31)	137	67	29

ovarian tissue autopsy specimens were prepared for hematoxylin-eosin staining. Rabbit polyclonal antibody to TE (GTX72779; GeneTex Inc., Irvine, CA) for the testis and rabbit polyclonal antibody to E2 (PAA461Ge01; Cloud-Clone Corp., Houston, TX) for the ovary were used at empirically determined dilutions. Immunoreactivity was achieved by the polymer method using Histofine Simple Stain MAX-PO (MULTI) (424154; Nichirei Bioscience Inc., Tokyo, Japan) and Histofine DAB substrate kits (425011; Nichirei Bioscience Inc.) according to the manufacturer's instructions. Immunoreactivity of TE in the testis was analyzed by open-source software Image J (https://imagej.nih.gov/ij/), and the areas stained brown by DAB were quantified. Immunoreactivity of E2 in the ovary was graded by scoring based on the following algorithm: score 0, negative; score 1, weak positivity (slight reaction); and score 2, irregular or diffuse positivity (strong reaction).

Toxicological analyses

Drug analyses were routinely performed with gas chromatography/mass spectrometry (GC/MS) using an Agilent model 5975c MSD (Agilent Technologies, Santa Clara, CA). For GC/MS, a 0.5 mL aliquot of the sample was used. For instrumental conditioning, automated GC/MS following solid-liquid phase extraction was performed (column, DB-5MS, 30 m × 0.25 mm i.d.; film, 0.25 μm; column temperature, 100–325 °C; injector temperature, 280 °C; turbocharged carrier gas, helium at a flow rate of 48 mL/s; interface temperature, 300 °C) [18-20].

Statistical analysis

Spearman's rank correlation coefficient was used to compare two values, including TE levels, E2 levels, age of subjects, postmortem period and survival period. For comparisons between groups, the nonparametric Mann-Whitney U test was used. The Kruskal-Wallis test was used for analyses involving multiple comparisons. In the data for this test, the line in each box represents the median, and lines outside each box indicate the 90% confidence interval. Maximum TE and E2 levels in serum and CSF were log-transformed for graphical presentation only. Diagnostic relevance was estimated according to the values obtained for sensitivity, specificity and accuracy (proportion of subjects correctly predicted). Youden's index (sensitivity + specificity − 1) was used to determine the optimal cut-off value. The results are presented as medians. All analyses were performed using SPSS version 9.0 statistical package (SPSS, Chicago, IL). Values of $p < 0.05$ were considered significant.

RESULTS

Correlations between sex steroid hormone levels and sex

Serum TE levels were higher in males than in females ($p < 0.01$). However, no correlation was observed between CSF TE levels and sex. Additionally, no correlation was found between both serum and CSF E2 levels and sex.

TE and E2 levels in males

Correlations between sex steroid hormones and age and postmortem period

Serum TE and E2 levels showed no correlation with age, although a slightly negative correlation was observed for CSF TE and E2 levels with age in males (TE: $r = -0.188$, $p < 0.05$; E2: $r = -0.345$, $p < 0.01$).

No correlations were identified between serum TE levels and the postmortem period. In contrast, a slightly positive correlation was found between CSF TE levels and the postmortem period ($r = 0.176$, $p < 0.05$). Further, a slightly positive correlation was found between serum E2 levels and the postmortem period ($r = 0.343$, $p < 0.01$), although no correlations were identified between CSF E2 levels and the postmortem period.

Correlations between TE and E2 levels in the two types of samples

No correlation was observed between serum and CSF TE levels, while serum E2 levels moderately positively correlated with CSF E2 levels ($r = 0.354$, $p < 0.01$) in males.

Correlations between sex steroid hormone levels and the cause of death

In males, there were no differences in serum and CSF TE levels by cause of death (Fig. 1). On the other hand, both serum and CSF E2 levels were significantly higher in subjects that died from hypothermia and intoxication than in subjects with other causes of death ($p < 0.01–0.05$) (Fig. 2). In the intoxication subgroup, there were no differences in serum and CSF E2 levels between stimulant poisoning and psychotropic drug poisoning. The estimated cutoff values calculated to distinguish higher and lower E2 levels in serum and CSF based on death from hypothermia versus other causes, except for intoxication, were 579.0 pg/mL and 150.5 pg/mL, respectively.

TE and E2 levels in females

Correlations between sex steroid hormones, age and postmortem period

No correlation was found between TE levels and age or the postmortem period in either serum or CSF in females.

Serum E2 levels showed a slightly negative correlation with age in females ($r = -0.233$, $p < 0.05$), while slightly positive correlations were found between serum ($r = 0.313$, $p < 0.01$) E2 levels and the postmortem period. No correlation was found between CSF E2 levels and age in females. In contrast, CSF E2 levels showed a slightly positive correlation between serum ($r = 0.291$, $p < 0.01$) E2 levels and the postmortem period.

Correlations between TE and E2 levels in the two types of samples

Serum TE levels slightly positively correlated with CSF TE levels ($r = 0.264$, $p < 0.05$), while serum E2 levels moderately positively correlated with CSF E2 levels ($r = 0.509$, $p < 0.01$) in females.

Correlations between sex steroid hormone levels and causes of death

In females, serum TE levels were higher in subjects with hypothermia than in subjects with other causes of death ($p < 0.01$). Estimated cutoff values of 2.61 ng/mL were calculated as being able to distinguish higher and lower TE levels (hypothermia vs other groups) in serum. There were no differences in CSF TE levels by cause of death (Fig. 3).

Serum and CSF E2 levels were higher in subjects with intoxication than other causes of death, including hypothermia ($p < 0.05$) (Fig. 4). In the intoxication subgroup, there were no differences in serum and CSF E2 levels between stimulant poisoning and psychotropic drug poisoning. Estimated cutoff values of 508.5 and 108.0 pg/mL were calculated to distinguish higher and lower E2 levels in serum and CSF, respectively.

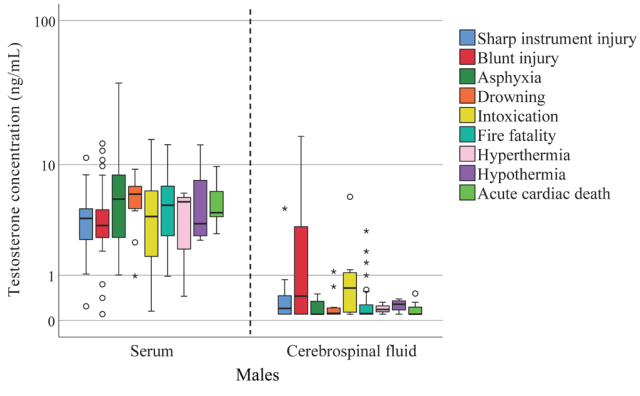

Figure 1. Postmortem serum and CSF testosterone (TE) levels by cause of death in males. No differences in serum and CSF TE levels were seen by cause of death.

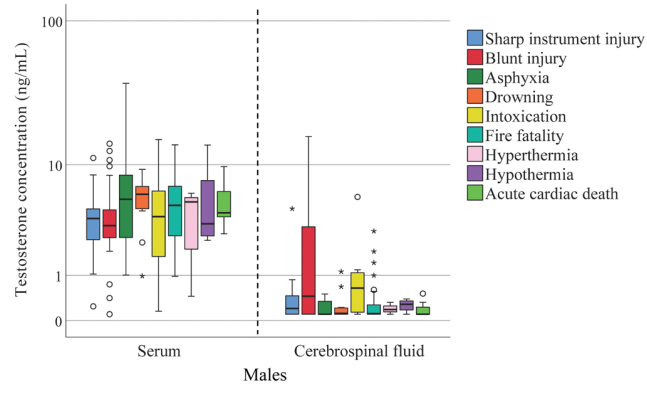

Figure 2. Postmortem serum and CSF estradiol (E2) levels by cause of death in males. Subjects that died from intoxication and hypothermia had significantly higher serum and CSF E2 levels compared with the other causes of death (†$p < 0.05$, ††$p < 0.01$).

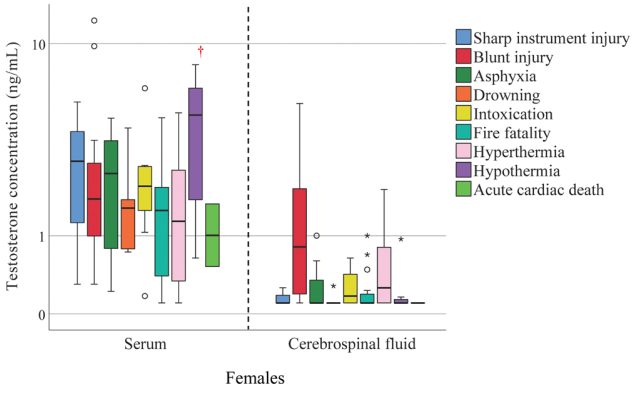

Figure 3. Postmortem serum and CSF testosterone (TE) levels by cause of death in females. Subjects that died from hypothermia had significantly higher serum TE levels compared with the other causes of death (†$p < 0.05$). No differences in CSF TE levels were seen by cause of death.

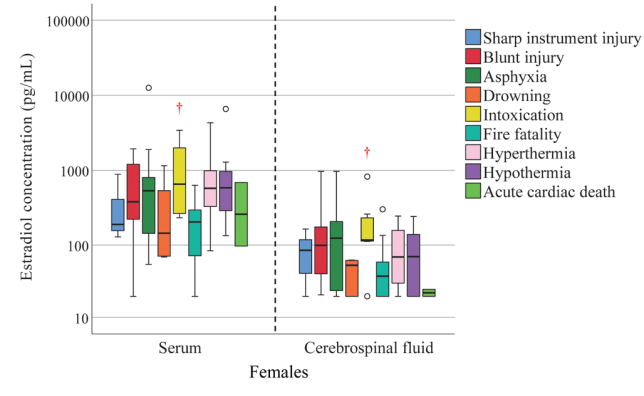

Figure 4. Postmortem serum and CSF estradiol (E2) levels by cause of death in females. Subjects that died from intoxication had significantly higher serum and CSF E2 levels compared with the other causes of death (†$p < 0.05$).

Immunohistochemical analyses

Staining for TE was seen in interstitial tissue (Leydig cells) of the testes (Fig. 5a), although there was no significant difference in TE-immunoreactivity by cause of death (Fig. 6). However, there was a slight correlation between TE-immunoreactivity and serum ($r = 0.192$, $p < 0.05$) and CSF ($r = 0.183$, $p < 0.05$) TE levels. In females, E2 staining was present in theca cells and the corpus albicans of the ovary (Fig. 5b), although no changes in E2-immunoreactivity by cause of death were seen. In addition, no correlations were found between E2-immunoreactivity and E2 levels in serum and CSF (Fig. 7).

DISCUSSION

The forensic pathological diagnosis of hypothermia is often difficult, especially in cases lacking the classic findings of gastric erosions (Wischnewski-Flecke spots) [21] and color differences between the left and right heart blood [22]. Several previous studies have evaluated the influence of hypothermia on endocrine hormone systems. Postmortem serum levels of ACTH were reportedly significantly higher in cases of hypothermia (cold exposure) than other causes of death [23]. In addition, cortisol and corticosterone are increased by cold exposure [4]. However, no reports have examined the association between sex steroid

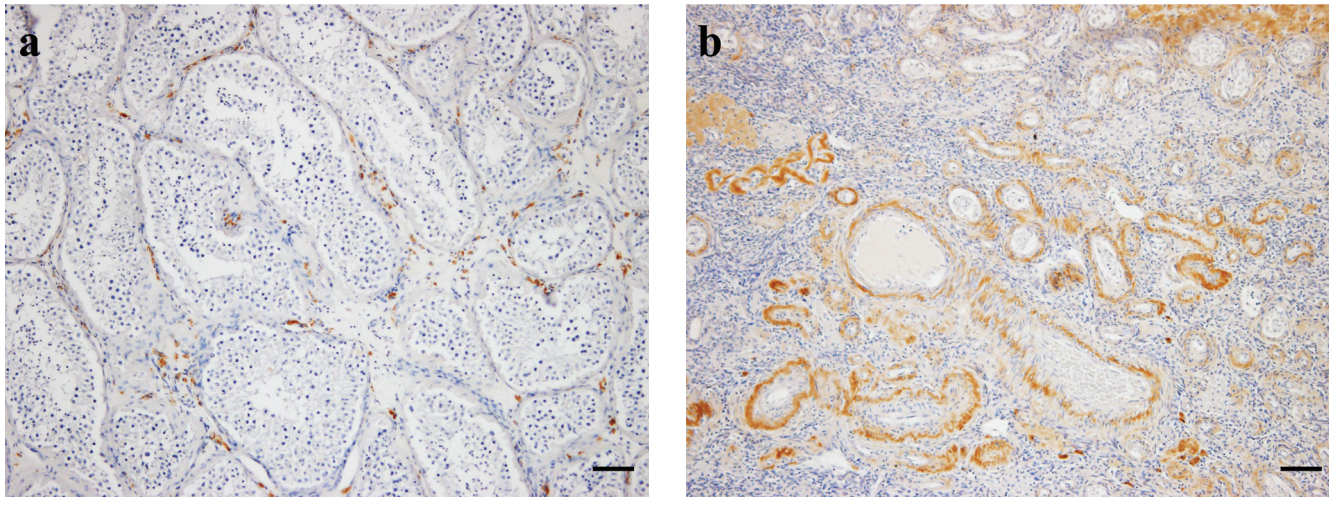

Figure 5. Immunostaining for testosterone (TE) in the testis and estradiol (E2) in the ovary. Micrographs showing immunostaining for (a) TE in the testis and (b) E2 in the ovary in cases of hypothermia (original magnification ×100). The scale bars represent 50 μm.

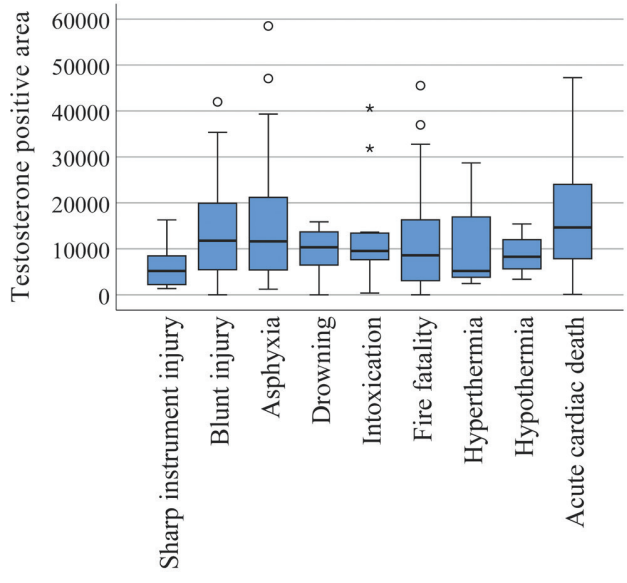

Figure 6. Quantitative evaluation of testicular immunohistochemical staining for testosterone (TE). There were no significant differences in TE-immunoreactivity by cause of death.

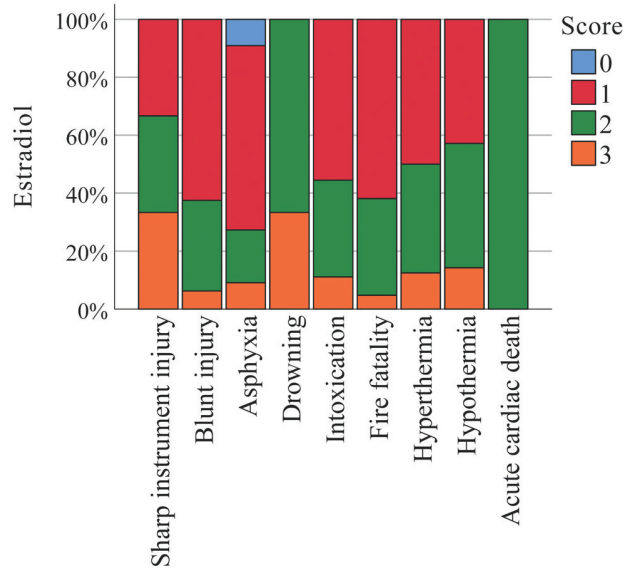

Figure 7. Evaluation of stainability in ovarian estradiol (E2) immunohistochemical staining. No changes in E2-immunoreactivity by cause of death were seen.

hormones and hypothermia. Sex steroid hormones, which, like cortisol, are classified as steroid hormones, might also be highly secreted into the blood during hypothermia. Therefore, we examined sex steroid hormone levels in relation to various causes of death, particularly hypothermia.

Generally, males and females show differences in the amount of hormones secreted [13]. In the present study, the serum TE concentration was significantly higher in males than in females, although there were no sex-based differences in serum E2 concentrations. The absence of a sex-based difference in serum E2 levels in the present study might be because the amount of E2 secretion decreases with age and/or due to pathophysiological differences in various causes of death, although TE concentrations in both serum and CSF in females did not show any correlations with age [24]. This suggests that the age of subjects needs to be considered when investigating the pathophysiological significance of E2 concentrations in females. In males as well, TE and E2 concentrations did not correlate with age, except for CSF E2 concentration.

TE concentrations in serum and CSF in both males and females were not impacted by the postmortem time. This might suggest that the TE concentration is relatively stable in the postmortem period in both males and females. In contrast, E2 concentrations in serum and CSF were impacted by the postmortem time. This might be because the half-life of E2 is longer than that of TE (2 to 8 hours) [25], and E2 might be synthesized in tissues during the survival period, increasing its concentration. In this study, there were no correlations between TE and E2 concentrations in serum and CSF, respectively, in males, and there was only a slight correlation between TE and E2. On the other hand, in females, there was a correlation between these sex steroid hormones in serum and CSF. Compared to females, males have higher TE secretion relative to E2 synthesis, which might be the reason for the lack of a correlation between TE and E2 levels in males in this study [13].

When comparing the cause of death, TE and E2 concentrations in serum and CSF were high in subjects with intoxication, except for TE levels in males. In addition, E2 levels in males were also elevated in hypothermia. Serum TE levels were elevated in females with hypothermia. The increased TE in hypothermia might have been caused by cold exposure, as with cortisol secreted by the adrenal glands [4]. In addition, we believe that the increase in TE only in females is related to the higher proportion of adrenal-derived TE in females than in males. However, immunohistochemical staining showed no changes relative to the cause of death in the secretion of TE and E2 or localization of these hormones. Although there was a slight correlation between TE-immunoreactivity and TE concentration in males, it was difficult to evaluate this microscopically. In addition, since age has a large effect on the ovaries, it is possible that E2-immunoreactivity was independent of E2 concentration.

There is a limitation to the present study. Increases in E2 and TE levels might be affected by drugs. Hence, the medications taken by a person before death need to be considered when investigating the pathophysiological significance of postmortem sex steroid hormone levels.

In conclusion, E2 levels were high in males who died from hypothermia, while hypothermia was associated with high serum TE levels in females. Although the results of evaluation of postmortem sex steroid hormones are affected by various factors, such as sex, age and antemortem medications, postmortem evaluation of sex steroid hormone levels might be able to assist in the pathological evaluation of the causes of death.

Conflict of interest

The authors declare that they have no conflict of interest.

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Ethical statement

This study was approved by the ethics review board of the Osaka City University Medical School.

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