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ORIGINAL ARTICLE

Increase in clusterin-containing follicles in the adenohypophysis of drug abusers

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Abstract The hypothalamic–pituitary–adrenocortical (HPA) system in drug abusers may be affected due to disorders of the hypothalamic dopaminergic system. The present study investigated alterations in the adenohypophysis of middle-aged drug abusers (40–60 years of age), using clusterin-containing mixed cell-follicles as the indicator, in which clusterin (apolipoprotein J) is a multifunctional glycoprotein related to neurodegeneration. The paraffin-embedded adenohypophyses of methamphetamine and psychotropic drug abusers (n=76) were compared with those of non-abusers (n=82). The number of follicles was larger in drug abusers independent of the immediate cause of death, although the size was not significantly different. When cell types forming the follicles were immunohistochemically examined, drug abusers showed an increase of prolactin (PRL) cells and gonadotroph cells and a reciprocal decrease of growth hormone cells, suggesting hypofunction of dopaminergic neurons in the hypothalamus, while there was no change in the adrenocorticotropic hormone and thyroid-stimulating hormone cells. These increases of the clusterin-containing follicles and PRL cells in the follicles may be related to the dysfunction of dopaminergic neurons in the hypothalamus of chronic drug abusers and may be useful for investigating drug abuse in forensic casework.

Keywords Mixed cell-follicle · Human pituitary · Methamphetamine · Psychotropic drug · Clusterin

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Introduction

Investigation of cases of drug abuse-linked fatal intoxication, other traumas or diseases are an important part of forensic pathology. However, postmortem interpretation of chronic abuse and related physical disorders is often very difficult due to a lack of specific findings. Previous studies showed various pathological findings in drug abusers, which included cardiopulmonary abnormalities [1], myocardial hypertrophy [2] and cerebral hemorrhage [3] in amphetamine abusers and gastric ulcers [4], atrophy of the hippocampus [5] and brainstem ischemia [6] in psychotropic drug abusers. However, there appears to be few investigations of the human endocrine system in drug abusers from a medicolegal aspect [7].

The hypothalamic–pituitary–adrenal (HPA) system may be related to susceptibility to drug abuse, since most abused drugs have an affinity to dopaminergic transmission [8, 9]. Furthermore, activation of the HPA axis and ascending catecholaminergic neurons plays a critical role in metabolic and behavioral adaptation to various forms of stress. Persistent alterations in these systems may be involved in the pathophysiology of some psychiatric disorders [10–13]. Thus, pathology of the adenohypophysis may be useful for postmortem investigation of neurological disorders in drug abusers.

Mixed cell-follicles in the adenohypophysis are composed of various hormone-producing cells and contain clusterin (apolipoprotein J), which is a multifunctional glycoprotein related to neurodegeneration. Clusterin was recently found in senile plaques in the brains of patients with Alzheimer's disease [14], and it was further shown that clusterin binds abnormal proteins [15, 16], prion disease [17], and ischemia [18]. Clusterin may be related to a compensation mechanism involving aggregation of soluble proteins, inhibition of complement-mediated cell damage, adaptive regeneration, or apoptosis [19, 20]. These findings suggest that clusterin in the pituitary mixed cell-follicles may increase in response to cell damage due to various forms of stress. A previous study showed an age-dependent increase in clusterin-containing follicles in the human adenohypophysis [21]: the number was lower in younger subjects than in the elderly. However, individual differences were evident in middle-aged subjects, showing no age-dependency. This suggests the contribution of factors other than aging, which may include drug abuse [8, 9]. For investigating drug abuse-related changes of the adenohypophysis, the use of clusterin as a marker of apoptosis [20] may be useful, considering previous studies regarding age determination and postmortem changes using apoptotic markers [22, 23].

The present study investigated the number and size of clusterin-containing mixed cell-follicles and component cells in the adenohypophysis of middle-aged abusers of methamphetamine (AMP) and psychotropic drugs from a forensic pathological perspective.

Materials and methods

Specimens

Considering the age-dependency of the number of mixed cell follicles, middle-aged subjects (40–60 years of age) were examined since the follicle number was found to be independent of age in this age group [21, 24]. The adenohypophyses of AMP and psychotropic drug abusers (n=76) were compared with those of non-abusers (n=82). The cases included abusers of AMP (n=25), tricyclic antidepressants (TCA, n=12), benzodiazepine (BZO, n=8), barbiturate (BAR, n=16), and combined abusers involving AMP and psychotropic drugs (n=15: TCA, n=3; BZO, n=4; BAR, n=8). These cases did not include those with complications of evident metabolic or hormonal disorders. Causes of death of abusers consisted of AMP and/or psychotropic drug intoxication (n=7), natural diseases (n=14), fire fatalities (n=18), drowning (n=6), blunt injury (n=16), sharp instrument injury (n=7), asphyxia (n=5), and hypothermia (n=3). Control groups comprised natural death (n=28), fire fatalities (n=12), drowning (n=10), blunt injury (n=10), sharp injury (n=10), asphyxia (n=7), alcoholic poisoning (n=2), and hypothermia (n=3). All the drug abusers showed pathological and circumstantial evidence of long-term drug abuse over the years. Cases in which medications were administered in the hospital antemortem were excluded.

Immunohistochemistry

The hypophysis was divided into two portions cutting along the frontal plane. Half of the gland was fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.2) for 12 h, embedded in paraffin, then serial sections, 4 μm thick, were cut.

Since the follicle contents are known to be periodic acid-Schiff (PAS)-positive [21, 25], the first section was stained with PAS. The subsequent sections were used in immunohistochemical studies to identify cells that form follicles. The following primary antibodies were used: rabbit anti-human growth hormone (GH; 22 K) serum (1:8,000; NIDDK, USA), rabbit anti-adrenocorticotropic hormone (ACTH; 1–24 N-terminal) serum (1:8,000; supplied by the Department of Anatomy, Jikei University School of Medicine, Japan) [26], rabbit anti-human follicle-stimulating hormone serum (1:7,000; Scantibodies Laboratory, USA), rabbit anti-human PRL serum (1:8,000; Biogenesis, UK), and rabbit anti-human thyroid-stimulating hormone (TSH) serum (1:5,000, NIDDK). Immunostaining was conducted by the avidin–biotin complex method, and color was developed with 3,3′-diaminobenzidine (DAB). Additional serial sections were used in a double-staining study using anti-human ACTH antibodies and bovine anti-human S-100 protein antibodies (1:5,000, supplied by the Department of Anatomy, Jikei University School of Medicine) [27]. Color was developed with naphthol (4-chloro-1-naphthol) for alkaline phosphatase and DAB for peroxidase. Hematoxylin and eosin (HE) staining was performed to observe the morphology of follicles and follicle-forming cells.

Identification of mixed cell-follicles

As previously described [21], for the pars intermedia follicles, the flattened cells that joined together in a ring around the follicle as indicated by HE staining were found to contain S-100 protein or ACTH by immunohistochemical study [28]. For mixed cell-follicles, however, the eosinophilic cells were mainly GH-secreting cells and the remaining cells were positive for various adenohypophysial hormones and S-100 protein. Since the present study measured the mixed cell-follicles present in the adenohypophysis, follicles of the pars intermedia formed exclusive-

ly by ACTH-positive and S-100-positive cells [28], respectively, were excluded. Follicles larger than 5 μm in diameter could be clearly detected and were measured in the present study.

Number of mixed cell follicles

The number of follicles was determined by a color image analyzer, CIA-102 (Olympus, Tokyo Japan) using PAS-stained sections. Since the sizes of the follicles depended on age, the number of mixed cell follicles was corrected using the formula reported by Yoshimura and Ishikawa [29].

Size of mixed cell-follicles

The diameters of follicles (distance between the inner wall of the follicle-forming cells) were measured using a confocal laser microscope (Zeiss, LSM-510, Jena, Germany), equipped with a 40× objective lens at a discrimination limit of 5 μm. A 543-nm Hene laser was used to visualize PAS staining. Subsequent image restoration and analysis including the length of mixed cell-follicles was performed using commercial LSM 510 VisArt. When the PAS-positive portion was not in contact with the inner wall of the follicle, the distance between the inner walls of the follicle-forming cell was measured to determine follicle size.

Component cells of mixed cell-follicles

The cell types forming mixed-cell follicles were investigated using serial sections immunostained with adenohypophysial hormone antibodies.

Characterization and identification of follicle components

The other half of the pituitary gland was used for characterization and identification of follicle components. Following the report by Ogawa et al. [30], extracts were electrophoresed using a 10% agarose gel, then blots with chemiluminescence enhanced by the ECLplus Western Blotting Detection System (Amersham Pharmacia) were exposed to X-ray film. Immunostaining with rabbit anti-clusterin-α/β antibodies (1:1,000, Santa Cruz, USA) was also performed on tissue sections of the pituitary gland and hypothalamus in representative cases of fatal AMP and/or psychotropic drug intoxication (n=5), as well as natural death (n=5).

Toxicological analyses

AMP and antipsychotropic drugs were detected by gas chromatography/mass spectrometry.

Statistical analyses

Comparisons between groups were performed using the Scheffe test. These analyses were performed using Microsoft Excel and Statview (version 5.0, SAS Institute) and a P value less than 0.05 was considered significant. The results of the data analysis are shown as box plots, for which 50% of the data are summarized in the box. The line in each box represents the median and the lines outside of each box represent the 90% confidence interval. The sensitivity and specificity levels, to distinguish the two groups using a cutoff for each marker, were estimated by means of receiver-operating characteristics (ROC) analysis; ROC curves were constructed by plotting sensitivity against (1−specificity). The area under the curves were calculated and analyzed by one-tailed test. The optimal compromise between sensitivity and specificity was determined graphically [31].

Results

The number of mixed cell-follicles in the adenohypophysis

The drug abusers showed a larger number of mixed cell-follicles than those in the control groups (p<0.0001), independent of the cause of death and subject age (40–60 years; Figs. 1a and 2). Drug abusers who died due to causes other than drug(s) showed a moderately larger number of mixed cell-follicles compared to those with acute drug intoxication fatalities (p<0.05; Fig. 1b). The number (mean) of follicles in the control groups was 14–25 (21), while that for drug abusers was 18–30 (25); BZO, 18–24 (21); TCA, 20–28 (24); BAR, 22–27 (24); AMP, 20–30 (25); and combined abuse involving AMP and psychotropic drug(s), 24–30 (27). The combined-abuse groups showed the most marked increase (r<0.0001). Fatal hypothermia cases did not include AMP abusers and psychotropic drug abusers showed a slightly lower mean value compared with that for AMP abusers. However, there were no significant differences between abusers of AMP and psychotropic drug (BZO, TCA, or BAR; Fig. 3). Although male subjects showed a slightly larger number of follicles than female subjects, there was no significant difference.

When analyzed by ROC, the sensitivity and specificity in distinguishing drug abusers and non-drug abusers using the number of follicles at a cutoff value of 23 were 0.73 and 0.85, respectively.

The size of follicles

Although a tendency toward increased follicle size was observed in drug abusers, there was no significant difference in the present series. The follicle size (mean) was 42–73

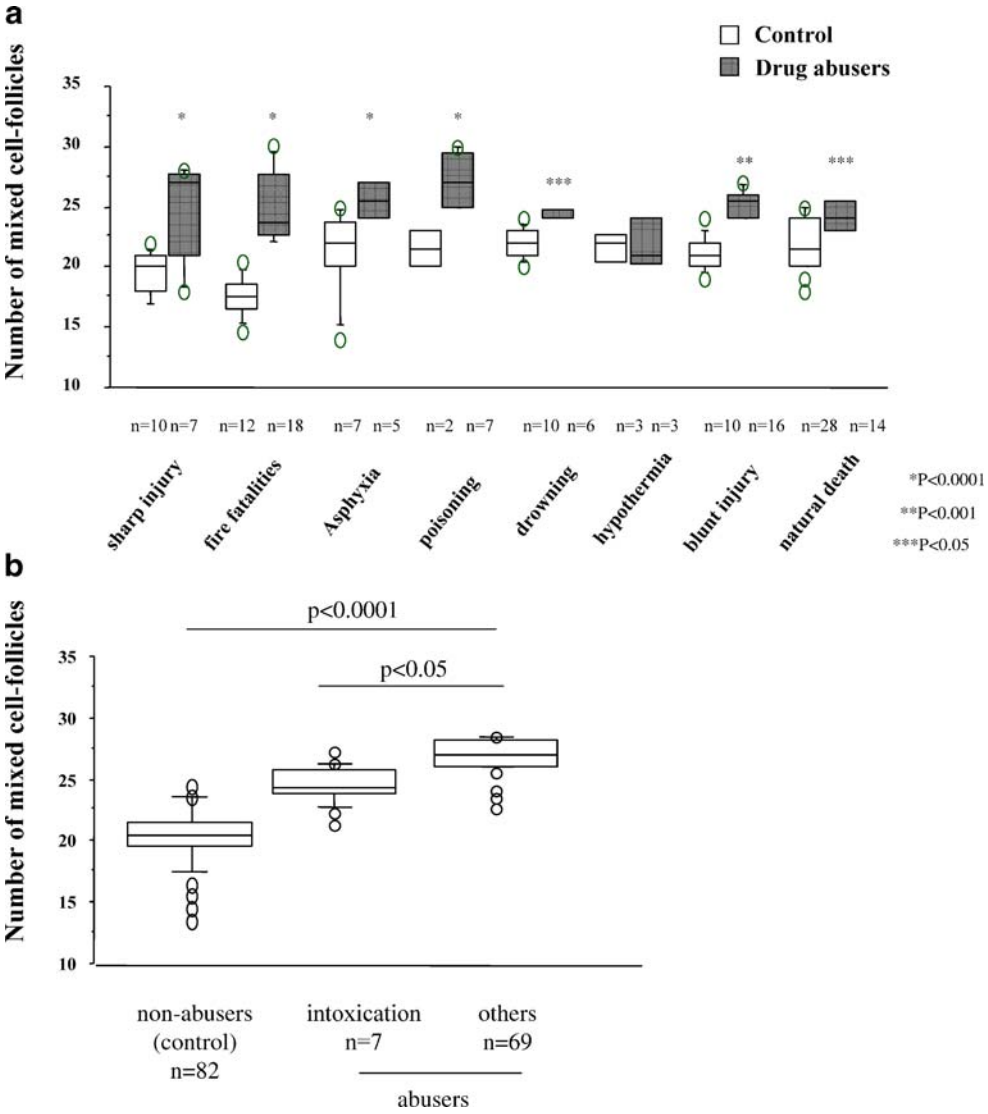

Fig. 1 **a** The number of mixed cell-follicles with regard to the cause of death. Drug abusers showed a larger number of mixed cell-follicles than control groups, independently of the cause of death. **b** Comparison between drug abusers and non-drug abusers. Drug abusers who died of causes other than drug(s) showed a moderately larger number of mixed cell-follicles compared to that of those with acute drug intoxication fatalities

(56) μm in control groups, 49–62 (53) μm in the BZO group, TCA 43–68 (57) μm, BAR 60–69 (65) μm, AMP 41–71 (63) μm, and 42–72 (62) μm in the combined abuse group.

Structure of follicle-forming cells

Mixed cell-follicles consisted of one to three layers of eosinophilic cells, basophilic cells, and chromophobic cells that encircled the follicles in all cases. In control groups, the eosinophilic cells were mainly GH cells (Fig. 2), whereas the PRL and gonadotrophic cells (58.8 and 43.9%, respectively) were predominant in drug abusers, and the number of GH cells decreased to approximately 29%. Increases in the PRL cells was significantly greater for combined abuse (63.9%) and AMP abuse (57.2%) than psychotropic drug abuse (53.5%; $p<0.05$). Hypothermia cases showed a moderate

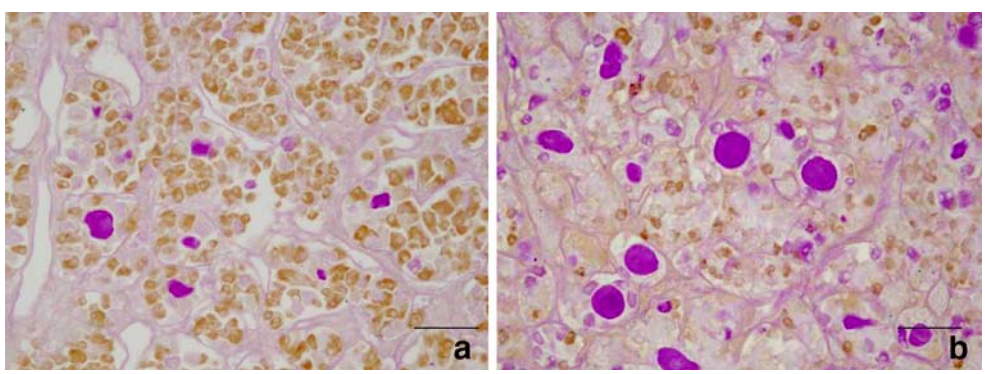

Fig. 2 Mixed cell-follicle in the adenohypophysis in a case of natural death in a 43-year-old man (**a**) and a fatality due to abuse amphetamine and psychotropic drugs (**b**) in a 60-year-old man. Double-staining with PAS and immunostaining using anti-human growth hormone antibodies. *Bars* indicate 60 μm

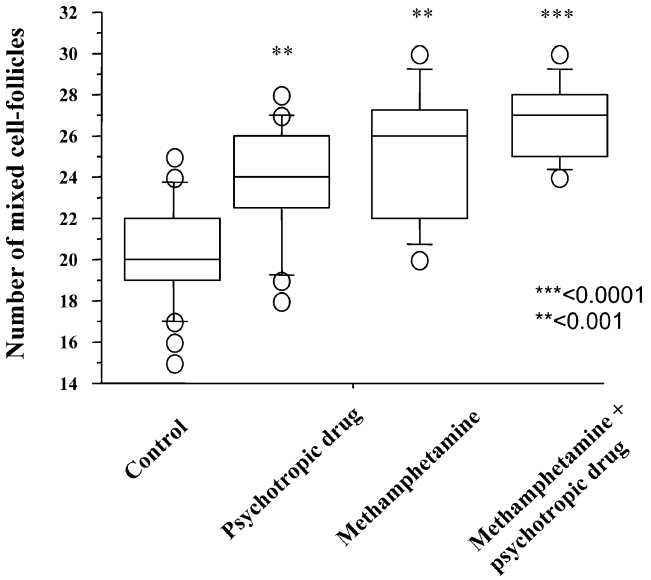

Fig. 3 The number of mixed cell-follicles with regard to the abused drug in abusers including those with fatal intoxication and the other causes of death. Psychotropic drug abusers showed a mildly lower mean value compared with that for AMP abusers. There was no significant difference between abusers of AMP and psychotropic drugs

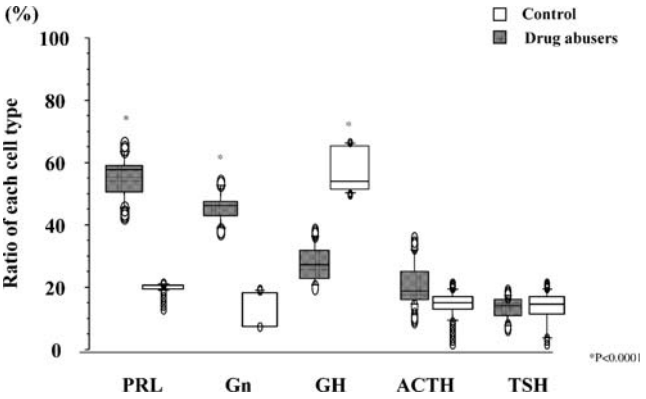

Fig. 4 Graph showing the ratio of each cell type forming mixed cell-follicles in drug abusers and non-abusers (*Gn* gonadotrophs). In the control groups, the cells were mainly GH cells, whereas the PRL and gonadotrophic cells (58.8 and 43.9%, respectively) were predominant in drug abusers and the number of GH cells decreased to approximately 29%

increase in the PRL cells for abusers (52.5%) compared with that for non-abusers (19.3%; $p<0.05$). The PRL/GH cell ratio was significantly higher for drug abusers (2.6) than for control groups (0.3; $p<0.0001$). When analyzed by ROC, the sensitivity and specificity for distinguishing drug abusers and non-drug abusers using the PRL/GH cell ratio at a cutoff value of 0.4 were 0.75 and 0.87, respectively. The numbers of ACTH and TSH cells did not show significant changes in drug abusers (Fig. 4).

Combined analysis of number of mixed cell-follicles and PRL/GH cell ratio

Using cutoff values of 23 for the number of follicles and 0.4 for the PRL/GH cell ratio, about 82.5% (n=33/40) of drug abusers showed positive findings for both indicators: 15 of 15 cases for combined abusers; 7 out 10 cases for AMP abusers, and 11 out 15 cases for psychotropic drug abusers. However, positivity was observed only in 23.2% (n=19/82) of non-abusers.

Immunostaining of clusterin in the hypothalamus

There was no detectable clusterin positivity in drug abusers.

Characterization and identification of follicle components

A band was identified at approximately 50 kDa on Western blotting (Fig. 5), and it was positive for clusterin by immunostaining (Fig. 6).

Discussion

Drug abuse causes deaths due to acute overdose toxicity, as well as other traumas or diseases associated with long-term

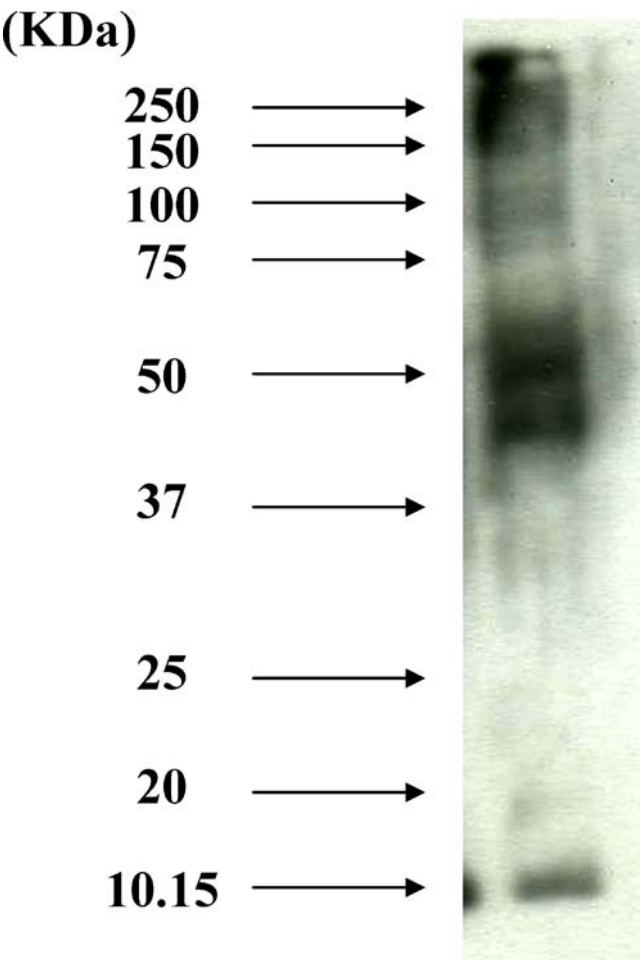

Fig. 5 Western blotting of extracts from mixed cell-follicles. A band was identified at approximately 50 kDa

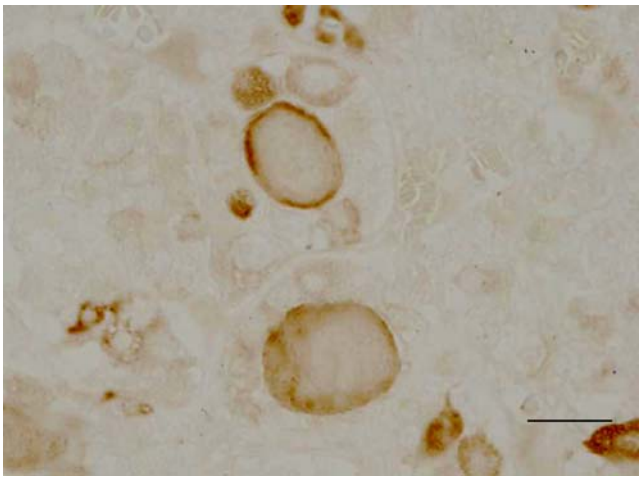

Fig. 6 Micrograph showing immunostaining of clusterin in the adenohypophysis. *Bar* indicates 30 μm

influence on the central nervous system, which is mainly related to dysfunction of the hypothalamic dopaminergic system [32]. For drug abusers, both short-term and long-term serial and parallel toxic processes may occur, causing neuronal metabolic deterioration involving an increase in oxidative stress, which can be enhanced by hyperthermia and finally resulting in apoptotic and necrotic neuronal death [33–36]. These neuronal changes were mainly detected in acute intoxication [32]. However, long-term abuse may cause more significant changes in the hypophysis than in the neuronal system.

Previous reports have described that stress induces persistent changes in the HPA axis function [37]. These changes involve an elevation in basal corticosterone levels due to sensitization of ACTH.

However, long-term abuse of psychotropic drugs or amphetamines may cause hyperprolactinemia due to modulation of the hypothalamic dopaminergic system through blockage of dopamine 2 receptors [38, 39]. This may be related to hyperfunction of the PRL cells in the hypophysis and hypofunction of the endocrine cells including GH cells, which may be associated with an increase in clusterin-containing follicles [21, 39].

The present study used Western blotting and immunostaining to demonstrate that humans also possess mixed-cell follicles containing clusterin. On Western blotting, detection of clusterin at 50 kDa suggested a complex form of α and β subunits [30, 40].

In the present study, increases in the clusterin-containing follicles in the adenohypophysis, PRL, and gonadotrophic cells in the follicles and a reciprocal decrease in GH cells were observed in long-term drug abusers, independent of the immediate cause of death. However, there were no changes in the size of follicles or in the numbers of ACTH and TSH cells. Moreover, there were no clusterin-positive cells in the hypothalamus of drug abusers. These findings can be explained by the modulation of the hypothalamic dopaminergic system due to the drug effects described. In the Parkinson disease model rat, apolipoprotein was not increased in dopaminergic neurons, although an increase was observed in neuroglias [41, 42], suggesting that apolipoprotein including clusterin does not increase in the dopaminergic neurons in Parkinson disease. The findings in the present study suggest reduced dopamine secretion in hypothalamic neurons.

The increase in gonadotrophic cells suggests the influence of long-term AMP or psychotropic drug abuse on sexual functions [43]. Since the increase in mixed cell-follicles and PRL cells was mildly observed in hypothermia cases of psychotropic drug abusers, psychotropic drugs may also have substantial influence on the hormone balance [44, 45]. Using cutoff values for the number of follicles and PRL/GH cell ratio, drug abusers showed highly positive findings for both indicators (82.5%), while the positivity was observed only in 23.2% of non-abusers. When the effect of drugs on the hypothalamic–pituitary axis is considered, the increase in PRL/GH cell ratio may be a more specific indicator for drug abuse than that in the number of mixed cell-follicles. These changes in the adenohypophysis were the most evident for AMP and combined abusers, suggesting potent effects of AMP on dopaminergic neurons [24]. However, some other factors including disturbed sex hormone metabolism in advanced liver cirrhosis should also be taken into consideration [46].

As described, findings of increased mixed cell-follicles in drug abusers differed from the age-dependent increase, showing an increase in the PRL/GH cell ratio. These findings suggest that the follicles may increase due to various kinds of stress.

Investigation of the follicle components may be useful to determine the responsible stress. In this respect, further investigations are necessary to consider metabolic or hormonal disorders due to intrinsic diseases including liver cirrhosis.

In conclusion, the present study showed that clusterin-containing follicles and PRL cells in the hypophysis were increased in drug abusers. These findings may be related to the dysfunction of dopaminergic neurons in the hypothalamus in chronic drug abusers and may be useful for investigating drug abuse in forensic casework.

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