Abstract
To evaluate the significance of immunohistochemical staining of ubiquitin (heat shock protein) in the midbrain for the medico-legal diagnosis of fatal asphyxiation and drowning, we investigated forensic autopsy cases of fatal mechanical asphyxia (n = 18), manual/ligature strangulation (n = 9), hanging (n = 4), aspiration/choking (n = 5) and drowning (n = 16). These were compared to control groups (n = 30) consisting of fatalities from brainstem injury (n = 12) and acute myocardial infarction (n = 18). Ubiquitin was clearly demonstrated in the nuclei of pigmented substantia nigra neurons, showing two intranuclear staining patterns: a type of inclusion (possibly Marinesco bodies) and a diffuse staining. The diffuse staining was significantly more frequently observed in cases of drowning. The percentage of total ubiquitin positive neurons was frequently higher in strangulation (5.1–28.4%, mean 17.0%), aspiration/choking (5.3–32.0%, mean 17.6%) and drowning (7.0–34.1%, mean 19.8%), but relatively low in hanging (5.1–12.7%, mean 8.6%), brainstem injury (0–10.4%, mean 5.0%) and acute myocardial infarction (1.5–16.9%, mean 8.3%). These observations suggest that intranuclear ubiquitin immunoreactivity of the pigmented substantia nigra neurons in the midbrain was induced by a fatal severe stress on the central nervous system in asphyxiation and drowning.

Keywords
Ubiquitin · Immunohistochemistry · Pigmented substantia nigra neuron · Asphyxiation · Drowning

Introduction
Asphyxia in a forensic context is usually relevant to mechanical asphyxia due to various mechanisms including strangulation, hanging, smothering and choking or asphyxia. The immediate cause of death involves not only systemic hypoxia due to airway obstruction but also cerebral ischemia and possible nerve effects in cases of pressure on the neck. Although conventional pathology usually greatly contributes to the diagnosis of asphyxial death in a typical case, more complicated cases are not rare and therefore, ancillary evidence may be required. In relation to fatal asphyxia, previous studies have mostly been undertaken to investigate pulmonary pathophysiology [1, 2, 3, 4, 5, 6, 7, 8, 9]. Recently, an increased immunoreactivity of an immediately early gene product, c-fos in the inferior olive of the human medulla oblongata in asphyxia was suggested [10].

Heat shock proteins have been investigated for post-mortem markers of local and systemic responses to various traumas and stress [11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24]. Ubiquitin is a well-known heat shock protein that responds very rapidly to various types of stress [18, 19, 20, 21, 22, 23, 24, 25, 26, 27]. In our previous immunohistochemical investigation, a markedly clear ubiquitin staining was observed in the pigmented substantia nigra neurons in the midbrain and a significant increase in the intranuclear ubiquitin positivity was observed in fire fatalities [28].

In the present study, we examined forensic autopsy materials to evaluate the medico-legal significance of the immunohistochemical staining of ubiquitin in the midbrain for the diagnosis of fatal acute mechanical asphyxiation and drowning.

Materials and methods
Materials
Formalin-fixed paraffin-embedded midbrain tissue specimens (horizontal sections, sampled at the given post-mortem times) of forensic autopsy cases of fatal mechanical asphyxia and drowning (n = 34) at our institute were examined. The cases included manual or ligature strangulation (n = 9, 3 males and 6 females, 43–85 years of age, mean age 65.2 years, survival time < 30 min–1.5 h, 15–50 h post-mortem), hanging (n = 4, 3 males and 1 female,
40–68 years of age, mean age 56.8 years, survival time < 30 min, 23.5–34 h post-mortem) and aspiration or choking (n = 5, all males, 27–75 years of age, mean age 52.4 years, survival time < 30 min, 7–20 h post-mortem) and drowning (n = 16, including 6 cases of possible suicide, 7 males and 9 females, 13–85 years of age, mean age 55.8 years, survival time < 30 min, 5–74 h post-mortem) in fresh water (n = 11) and salt water (n = 5). Control groups (n = 30) consisted of fatalities from brainstem injury (n = 12, 11 males and 1 female, 30–85 years of age, mean age 55.8 years, survival time < 5 min, 8–38 h post-mortem) and acute myocardial infarction (n = 18, 12 males and 6 females, 39–89 years of age, mean age 62.5 years, survival time < 0.5–15 h, 6–34 h post-mortem).

Methods

Tissue sections

Serial sections (4 µm thick) were prepared from the tissue specimens of the midbrain. The tissue sections were used for hematoxylin-eosin (H&E) and immunostaining.

Immunostaining

Polyclonal rabbit anti-ubiquitin serum (Dako A/S, Denmark) was used at a 100-fold dilution and a 3 h incubation at 37 °C with the Vectastain Universal Elite ABC kit (DAB) (Vector Laboratories, Burlingame, Calif.) according to the manufacturer’s instructions (counterstaining with hematoxylin). Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide for 5 min. For the control study to confirm the specificity of immunostaining, phosphate-buffered saline or normal rabbit serum was substituted for the primary antibody.

Quantitative analysis of Marinesco bodies and ubiquitin staining in the nuclei of pigmented neurons of the substantia nigra

Marinesco bodies were identified as eosinophilic nuclear inclusions in addition to the nucleolus in the pigmented neurons in the H&E sections. The neurons with nuclei in which Marinesco bodies were detected were quantitatively analysed, the number of total pigmented neurons and Marinesco body-containing neurons were counted in 10 fields under a 200 × magnification and the percentage was estimated.

Ubiquitin-positive pigmented neurons were quantitatively analysed in a similar manner: the number of neurons with nuclei in which ubiquitin immunoreactivity was detected were counted and the percentage of nuclear ubiquitin positivity (Ub-positive %) was estimated as described above.

Chemical analysis

Blood alcohol levels were determined by head-space gas chromatography/mass spectrometry [29]. Drug analyses were performed by gas chromatography/mass spectrometry.