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ORIGINAL ARTICLE

Postmortem serum erythropoietin levels in establishing the cause of death and survival time at medicolegal autopsy

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Abstract Circulating erythropoietin (EPO) is mainly produced in the kidneys, depending on blood oxygen level. The present study investigated the postmortem serum EPO levels with regard to the cause of death and survival time. Serial medicolegal autopsy cases of postmortem time within 48 h (n=536) were examined. Serum EPO levels were within the clinical reference range in most cases. Uremic patients with medical administration of an EPO agent (n=11) showed a markedly high level (140–4,850 mU/ml; median, 1,798 mU/ml). Otherwise, an elevation in serum EPO level (>30 mU/ml) was mainly seen in protracted deaths due to blunt injury and fire fatality, depending on the survival time (r=0.69, p<0.0001, and r=0.45, p<0.0001, respectively), and in subacute deaths from gastrointestinal bleeding and infectious diseases. However, mildly to moderately elevated serum EPO levels were sporadically found in acute deaths due to mechanical asphyxiation, fire fatality, and acute ischemic heart disease, and in fatal hypothermia cases, especially for elderly subjects. Protracted deaths due to mechanical asphyxiation and ischemic heart disease did not show any survival time-dependent increase in serum EPO level (p> 0.05). EPO was immunohistochemically detected in the tubular epithelia and interstitial cells, showing no evident difference among the causes of death, independent of survival time or serum level. These findings suggest that serum EPO can be used as a marker for investigating anemia and/or hypoxia as a consequence of fatal insult in subacute or prolonged deaths, or a predisposition to traumatic deaths or fatal heart attacks in acute deaths.

Keywords Forensic pathophysiology · Postmortem blood biochemistry · Erythropoietin · Anemia · Hypoxia

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Introduction

In determining the cause of death, not only the traumas or diseases that are immediately responsible for death but also predispositions and/or complications as contributory factors are of medicolegal importance [1–7]. For contributory factors, anemic and/or hypoxic conditions due to preexisting disorders may be involved in the death process as a susceptibility to diseases or traumas, while these conditions may also be involved in the death process as a consequence of fatal diseases or traumas [8–10]. However, pathological investigation of hypoxia/anemia due to a preexisting disorder or that due to a fatal insult is very difficult unless hematological disorders are detected.

Erythropoietin (EPO) is produced in a spectrum of tissue cells depending on the oxygen level [11–13]. However, circulating EPO is mainly produced by cells close to the proximal tubules in the kidneys and increases as early as 1–2 h after the initiation of anemia and/or systemic hypoxia, showing a relatively short half-life of about 5–9 h [14–19]. Thus, serum EPO may be used as a marker for evaluating preexisting hypoxia/anemia before the onset of acute fatal insult or the severity and duration of hypoxia/anemia after a fatal insult in postmortem investigation of death. The present study investigated serum EPO levels in cadaveric blood in serial medicolegal autopsy cases to examine the relationship to the cause of death and survival time.

Materials and methods

Autopsy material

Serial medicolegal autopsy cases with postmortem times within 48 h (n=536) during a period of 8 years (2000–2007) were examined. Case profiles are shown in Table 1. For all groups, clearly accountable cases were collected. Blood samples were collected from the left and right cardiac chambers, and peripheral external iliac vein using sterile syringes. Serum was immediately separated by centrifugation and stored at −20°C until use. Routine formalin-fixed kidney specimens were used for immunostaining. Case history, and pathological and toxicological data were collected from autopsy documents. The postmortem interval was defined as the time from estimated time of death to autopsy. Survival time was the period from the onset of fatal insult to death. For this purpose, used were cases where witness and/or circumstantial evidence has been well established to confirm survival and postmortem times estimated based on their pathological findings [4, 9].

Immunostaining

Among cases shown in Table 1, the following subjects (n=56; survival time, < 0.5–24 h; postmortem interval, 10–36 h) were randomly selected from each group for immunostaining: blunt head injury (n=6), chest injury (n=5), sharp instrument injury (n=5), asphyxiation (n=5; strangulation, n=3; hanging, n=2), drowning (n=4), fire fatalities (n=8, carboxyhemoglobin [COHb]<60%, n=4; COHb>60%, n=4), hypothermia (n=3), methamphetamine intoxication (n=4), acute ischemic heart disease (n=4), spontaneous cerebral hemorrhage (n=4), gastrointestinal bleeding (n=4), and pneumonia (n=4). The kidney specimens were fixed in

Table 1 Case profile

Cause of death	Case number	Male/female	Age, years (median)	Survival time, h (median)	Postmortem interval, h (median)
Blunt injury[a]					
Head	78	63/15	18–96 (58.0)	<0.5–260 (2.7)	5–40 (14.6)
Others	48	32/16	19–90 (55.5)	<0.5–168 (2.0)	6–40 (20.5)
Sharp instrument injury	40	28/12	19–75 (52.0)	<0.5–10.5 (0.5)	6–47 (15.0)
Mechanical asphyxiation[b]	61	32/29	19–94 (52.5)	<0.5–27 (0.5)	6–48 (23.0)
Drowning[c]	26	15/11	33–88 (61.0)	<0.5–0.7 (0.5)	13–47 (27.5)
Fire fatality					
COHb<60%	63	43/20	22–97 (67.0)	<0.5–320 (0.5)	7–46 (15.9)
COHb>60%	33	23/10	25–90 (64.5)	<0.5–4.0 (0.5)	6–47 (12.6)
Hypothermia	27	22/5	55–90 (68.5)	1–78 (4.0)	8–48 (24.7)
Poisoning[d]	17	11/6	28–74 (43.0)	0.5–36 (3.0)	9–38 (24.6)
Ischemic heart disease	54	44/10	17–87 (60.0)	<0.5–34 (0.5)	7–48 (20.8)
Spontaneous cerebral hemorrhage	27	18/9	39–72 (56.0)	<0.5–29 (2.8)	9–30 (24.6)
Hemorrhagic diseases[e]	22	19/3	50–92 (71.0)	3–72 (3.0)	8–40 (21.0)
Infectious diseases[f]	29	18/11	41–86 (62.0)	3–120 (5.0)	8–40 (21.0)
Uremia with EPO treatment[g]	11	7/4	30–87 (60.0)	1–480 (5.0)	8–31 (23.1)
Total cases	536	375/161	18–97 (61.5)	<0.5–720 (0.5)	5–48 (18.8)

COHb blood carboxyhemoglobin, *EPO* erythropoietin
[a] Traffic accident (n=40), falls (n=63), blows (n=18), and others (n=5)
[b] Hanging (n=13), strangulation (n=26), aspiration (n=18), choking (n=2), and traumatic asphyxia (n=2)
[c] Saltwater (n=5) and freshwater (n=21)
[d] Sedative-hypnotic drugs (n=7), methamphetamine (n=6), alcohol (n=1), cocaine (n=1), and others (n=2)
[e] Gastrointestinal bleeding (n=15) and ruptured aortic aneurysm (n=7)
[f] Pneumonia (n=21) and peritonitis (n=8)
[g] Ischemic heart disease (n=4), leukemia (n=1), hypothermia (n=1), and blunt injury (n=5)

10% formalin neutral buffer solution (pH 7.0–7.5; Wako, Japan) and embedded in paraffin, followed by sectioning at a thickness of 5 μm. The sections were used for hematoxylin–eosin and immunostaining. Polyclonal rabbit anti-human erythropoietin antibody (R&D Systems, Minneapolis, MN, USA, diluted 100-fold) was used. Following incubation with the primary antibody at −4°C for 18 h, the immunoreaction was visualized by the ABC method using the Vectastain Universal Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) and color development with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Dako, Japan) according to the manufacturer's instructions with hematoxylin counterstaining [10]. For the control study to confirm the specificity of immunostaining, phosphate-buffered saline and normal rabbit serum were substituted for the primary antibody.

Analytical procedure

Serum EPO levels were measured by a specific radio-immunoassay using the Recombigen EPO RIA kit (Nippon DPC, Chiba, Japan) and a standard EPO of recombinant human EPO (epoetin alfa; Kirin Brewery Co., Tokyo, Japan) [20, 21]. The minimum detectable quantity of EPO was 5.0 mU/ml. Inter-assay and intra-assay coefficients of variation were 3.5% and 8.9%, respectively. Diluted human serum showed that the displacement curve was parallel to the standard EPO curve. The mean recovery of EPO added was 93.3%. The clinical reference range for EPO was <36 mU/ml. In addition, serum total protein concentrations were measured by dextran with biuret-type assay (total protein HR-II kit, Wako Pure Chemical Industries, Osaka, Japan) [22], in which clinical reference range was 6.7–8.3 g/dl.

Blood %carboxyhemoglobin (COHb, %) saturation was analyzed on a CO–oximeter system (Ciba Corning 270, New York or Radiometer, Copenhagen, Denmark) [23, 24]. Blood cyanide and alcohol levels were determined by head-space gas chromatography/mass spectrometry [25, 26]. Drug analyses were performed by gas chromatography/mass spectrometry.

Statistical analysis

The Scheffe test was used for multiple comparisons among groups, and comparisons between groups were performed by non-parametric Mann–Whitney *U* test. Analyses were performed using Microsoft Excel and Statview (version 5.0, SAS Institute Inc., SAS Campus Drive Cary, NC, USA). A *P* value of less than 0.05 was considered significant. In Fig. 2, the results of 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. Variables that were not normally distributed were log-transformed for statistical analyses. Diagnostic relevance was estimated based on sensitivity, specificity, and accuracy (proportion of subjects correctly predicted). Youden's index (sensitivity + specificity − 1) was chosen as the best cutoff

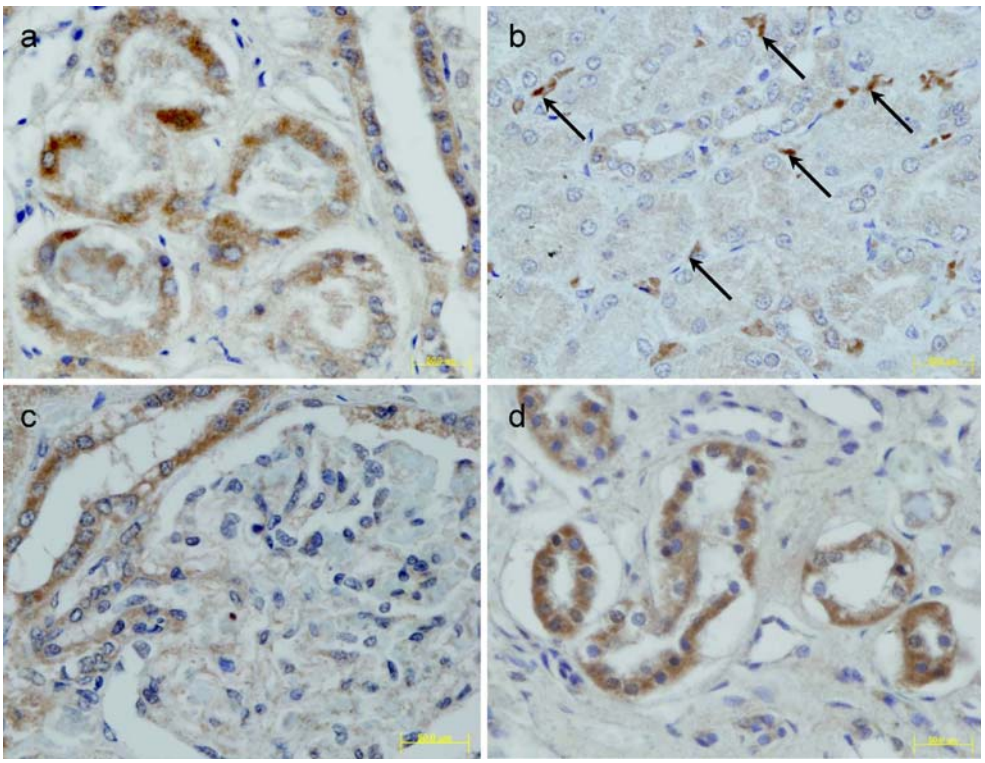

Fig. 1 Immunostaining of erythropoietin (*EPO*) in the kidney. EPO immunopositivity was detected in tubular epithelia (**a**) and interstitial cells (*arrows*) in the vicinity of the proximal tubules (**b**) but was negative in the glomeruli (**c**) in a case of gastrointestinal bleeding (68-year-old male; survival time, about 3 h; 27 h postmortem; serum EPO, 124.7 mU/ml). **d** The distal tubules and collecting ducts were strongly positive in a case of acute myocardial infarction (67-year-old male; survival time, about 1 h; 18 h postmortem; serum EPO, 19.8 mU/ml). EPO immunostaining in the kidney showed no evident relation to the survival time or serum EPO level

value. The usefulness of EPO for differentiating between acute death (survival time ≦ 1 h) and non-acute death (survival time > 1 h) cases was evaluated using receiver operating characteristic (ROC) curves and respective areas under the curve (AUS). All statistical procedures including ROC analyses were performed with the SPSS 9.0 statistical package (SPSS Inc., Chicago, IL, USA) [5, 7, 27].

Results

Immunohistochemical distribution of EPO in the kidneys

Among 56 cases (three to six cases of each cause of death), EPO immunopositivity was detected in the cytoplasm of tubular epithelia (n=56, 100%; Fig. 1 a) and in the nuclei of interstitial cells in the vicinity of the proximal tubules (n=3, 5.4%; Fig. 1b). No glomerular staining was detected (Fig. 1c). In most cases, the distal tubules and collecting ducts (Fig. 1d) were strongly positive, and the proximal tubules were less intensely positive. Positive signals were also detected in the neutrophils, monocytes, and erythrocytes in the vessels. However, these findings showed no evident relationship to the cause of death, survival time, or serum EPO level.

Postmortem interval, age, and gender of subjects

Serum EPO level showed an almost equivalent correlation between left (x_1), right (x_2) cardiac and peripheral (y) blood: $y=0.986x_1-0.072$, $r=0.985$, $n=132$, $p<0.001$; $y=1.057x_2-0.560$, $r=0.939$, $n=132$, $p<0.001$, without any relationship to postmortem interval (<48 h) at each site, age dependency, or gender-related difference for all cases. Considering the topographical stability of serum EPO levels, further analyses were performed primarily using the data from right cardiac blood, which were partly supplemented by those from peripheral or left cardiac blood (n=45).

When cases with hemolysis were excluded, serum total protein levels were within the clinical reference range (6.7–8.3 g/dl) in most cases, showing medians of 7.2 g/dl (mean±SD, 7.0±1.8 g/dl; n=286) and 7.7 g/dl (mean±SD, 7.6±1.9 g/dl; n=293) in left (x) and right (y) heart blood, respectively: $y=0.74x+2.37$, n=286, r=0.84, and $p<0.001$. There was no correlation between the total protein levels and postmortem interval (<48 h; $p>0.05$).

Relationship to the cause of death and survival time

Serum EPO level was within the clinical reference range (<36 mU/ml) in most cases (n=342/536, 63.8%), as shown in Fig. 2. A markedly high value (140–4,850 mU/ml; median, 1,798 mU/ml) was seen in uremic patients under artificial hemodialysis with medical administration of an EPO agent (n=11), which included cases of death from blunt injury (n=5), hypothermia (n=1), ischemic heart disease (n=4), and leukemia (n=1), independent of the survival time. Serum EPO level (mU/ml) was usually low for acute deaths (n=196) due to asphyxia (n=55), drowning (n=25), fires (n=81), and ischemic heart disease (n=35), showing a nonparametric distribution with a mean±SD of 28.8±33.9 and a median value (90% confidential range) of 21.2 (11.0–54.3). There was no significant difference between head and non-head blunt injury, between subgroups of mechanical asphyxiation (hanging, strangulation, aspiration, and others), between freshwater and saltwater drowning, or between fire fatalities with blood COHb levels of <60% and >60%. Serum EPO levels were higher for non-acute deaths due to blunt injury (n=64: head, n=40; other, n=24), fire fatality (n=14), and hemorrhagic diseases (gastrointestinal bleeding, n=11) compared with those in acute death cases of each group (Fig. 2).

ROC analysis showed that the sensitivity and specificity in distinguishing acute death (survival time≦1 h) and non-acute death (survival time>1 h) were 0.71 and 0.75 for

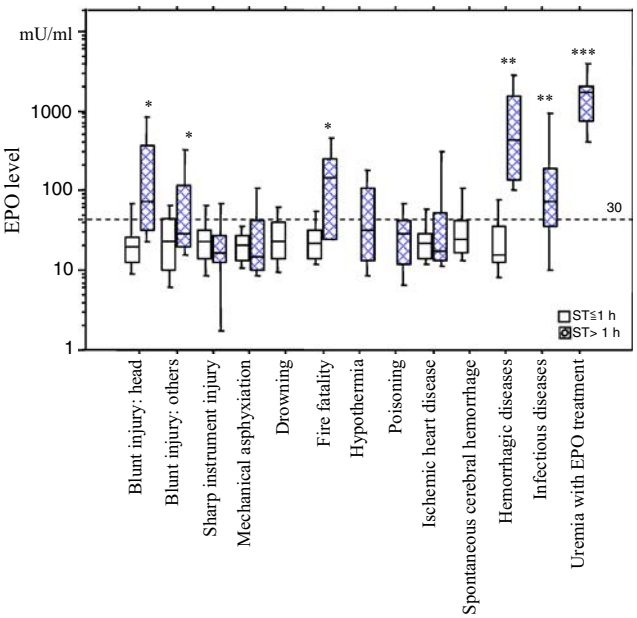

Fig. 2 Erythropoietin (*EPO*) levels of the right heart blood with regard to the cause of death. The results of individual comparisons by Mann–Whitney U test are shown. *$p<0.001$, significantly higher in non-acute death (survival time>1 h) than in acute death (survival time<1 h). **$p<0.001$, significantly higher for gastrointestinal bleeding (n=15, survival time>1 h) and infectious diseases (n=29, survival time>1 h) than other diseases (acute cardiac death, n=54; spontaneous cerebral hemorrhage, n=27; and ruptured aortic aneurysm, n=7). ***$p<0.001$, significantly higher for uremia with EPO treatment than for other causes of death. The *short-dashed line* shows the cutoff value (30 mU/ml) of serum EPO levels. There was no difference between salt- and freshwater drowning between fire fatalities with a higher (>60%) and lower (<60%) carboxyhemoglobin level, or between pneumonia and peritonitis

EPO at a cutoff value of 30 mU/ml, respectively. For blunt injury and fire fatality groups, the serum EPO level depended on the survival time (<1 week; r=0.69, p<0.0001, and r=0.45, p<0.0001, respectively; Fig. 3a and b). There was no significant difference between head and non-head blunt injury. Non-acute deaths from infectious diseases (n=29) also showed elevated serum EPO levels (5.0–8,310 mU/ml; median, 74.1 mU/ml; Fig. 2). However, mildly to moderately elevated serum EPO levels (>30 mU/ml) were sporadically detected in cases of acute death due to mechanical asphyxiation (n=8/56; 31.0–106 mU/ml; median, 36.8 mU/ml), drowning (n=9/26; 35.1–84.9 mU/ml; median, 46.8 mU/ml), fire fatality (n=22/82; 32.0–374.0 mU/ml; median, 52.9 mU/ml), and ischemic heart disease (n=9/35; 30.1–101.0 mU/ml; median, 57.7 mU/ml), and also in fatal hypothermia cases (n=14/27; 32.1–1,380 mU/ml; median, 105.5 mU/ml), which included mostly elderly subjects (age> 60 years; n=39/62, 62.9%). Non-acute deaths due to mechanical asphyxiation and ischemic heart disease did not show any survival time-dependent increase in serum EPO level (p>0.05, Fig. 3c and d).

Discussion

Previous animal studies suggested that the site of EPO production was peritubular fibroblasts in the renal cortex [28–30]. In the present study, EPO immunopositivity was intensely detected in the distal tubules and collecting ducts, and the interstitial cells in the vicinity of the proximal tubules were also positive in some cases. Relationship of these EPO immunopositivities to the cause of death, survival time, or serum EPO level was not interpretable in the present study; thus, further investigations are necessary. However, serum EPO levels were different among the cause of death, suggesting the usefulness of biochemical serum EPO assay.

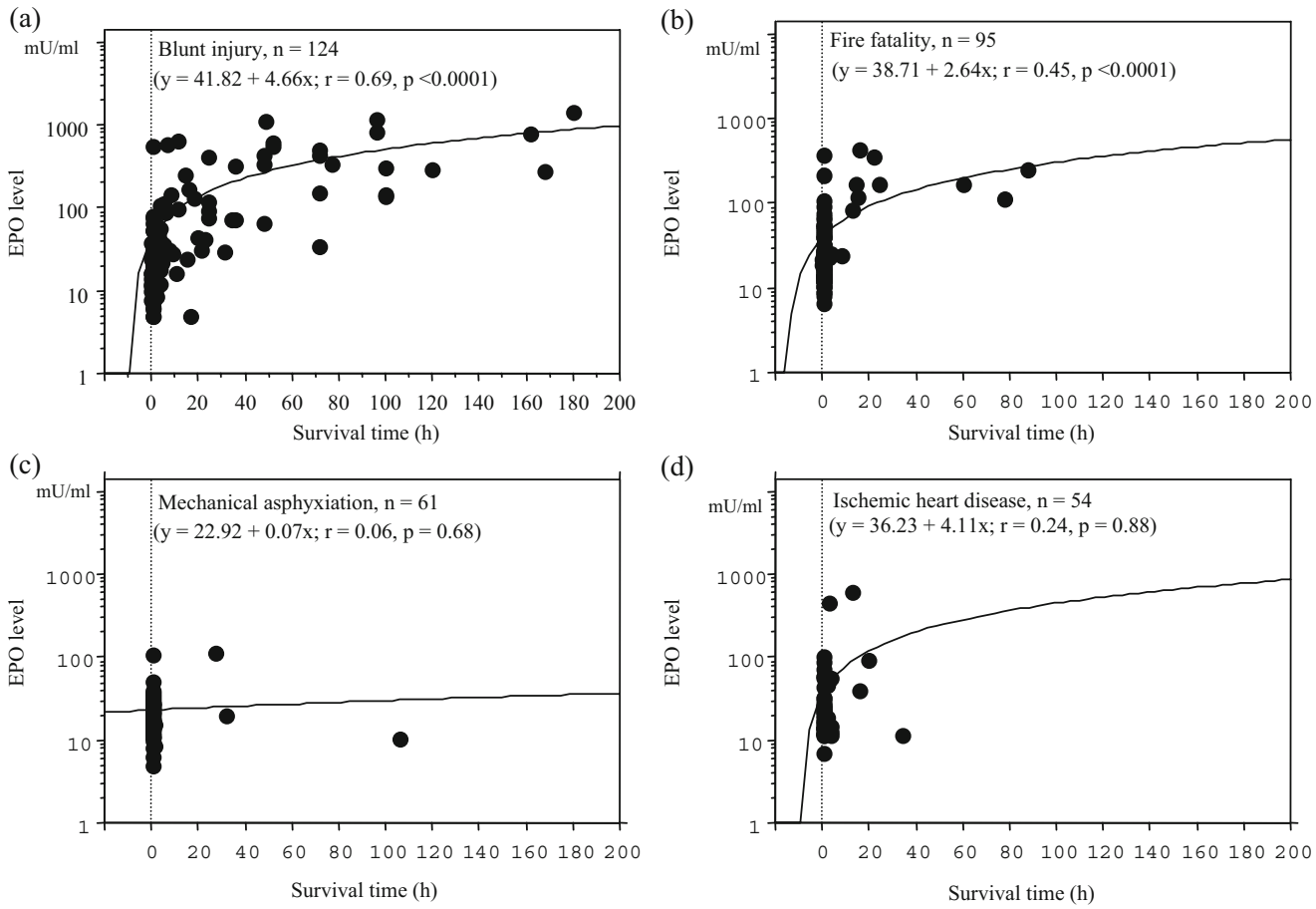

Fig. 3 Correlation between serum erythropoietin (*EPO*) levels and survival time (<1 week). **a** Blunt injury (n=124) including head injury (n=76) and non-head injury (n=48): y=41.82+4.66×(r=0.69, n=124, p<0.0001). **b** Fire fatality including acute death (n=80) and delayed death (n=15): y=38.71+2.64×(r=0.45, n=95, p<0.0001). **c** Mechanical asphyxiation including hanging (n=13), strangulation (n=26), aspiration (n=18), choking (n=2), and traumatic asphyxia (n=2): y=22.92+0.07×(r=0.06, n=61, p=0.680). **d** Ischemic heart disease (n=54): y=36.23+4.11×(r=0.24, n=54, p=0.880)

The present study showed the stability of serum EPO and total protein during an early postmortem period (<48 h). There was no difference due to blood sampling site. For serum EPO level, 90% confidential range for acute death cases was 11.0–54.3 mU/ml, and the cutoff value, as was estimated for distinguishing acute and non-acute deaths, was <30 mU/ml, which was similar to clinical reference range (<36 mU/ml). Under these conditions, uremic patients under artificial hemodialysis with medical administration of an EPO agent showed a markedly high serum EPO level of >1,000 mU/ml, independent of the cause of death and survival time, indicating interference due to medical intervention before death. Such findings may be seen for patients within about 12 h after intravenous injection of an EPO agent when the elimination rate is considered [31, 32].

A survival time-dependent elevation in serum EPO level was detected for blunt injuries and fire fatality, suggesting the systemic influence of anemia/hypoxia following massive bleeding and/or tissue damage [33–35]. Similar findings for gastrointestinal bleeding and infectious diseases may be related to the severity and duration of bleeding/anemia or advanced hypoxia in the death process [35–38]. Further investigation of injury cases is necessary to clarify individual factors that may contribute to the elevation in serum EPO level, which may include the severity of bleeding or tissue damage and survival time.

However, serum EPO level showed no significant survival time-dependency for protracted deaths due to mechanical asphyxiation and ischemic heart disease. Furthermore, mild to moderate elevation in serum EPO levels was seen in some cases of acute death due to mechanical asphyxia, fire fatality, and acute ischemic heart disease, and also in some cases of fatal hypothermia, especially for elderly subjects. These findings suggest that an elevated serum EPO level can also be an indication of preexisting anemia, which may have been a predisposition to the fatality [39, 40].

In conclusion, the present study showed that serum EPO was stable during an early postmortem period (<48 h), and the elevation (>30 mU/ml) was mainly seen for protracted deaths due to injury and fire fatality, and incidentally in other cases. These findings suggest that serum EPO can be used as a marker for investigating anemia and/or hypoxia as a consequence of fatal insult in subacute or prolonged deaths, or a predisposition to traumatic deaths or fatal heart attacks in acute deaths.

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