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ORIGINAL ARTICLE

Molecular pathology of pulmonary surfactants and cytokines in drowning compared with other asphyxiation and fatal hypothermia

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Abstract Drowning involves complex fatal factors, including asphyxiation and electrolyte/osmotic disturbances, as well as hypothermia in cold water. The present study investigated the molecular pathology of pulmonary injury due to drowning, using lung specimens from forensic autopsy cases of drowning (n=21), acute mechanical asphyxia due to neck compression and smothering (n=24), and hypothermia (cold exposure, n=11), as well as those of injury (n=23), intoxication (n=13), fire fatality (n=18), and acute cardiac death (n=9) for comparison. TaqMan real-time reverse transcription polymerase chain reaction was used to quantify messenger RNA (mRNA) expressions of pulmonary surfactant-associated proteins A and D (SP-A and SP-D), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10. SP-A and SP-D mRNA levels were lower for drowning, mechanical asphyxiation, fire fatality, and acute cardiac deaths than for hypothermia and injury. TNF-α, IL-1β, and IL-10 mRNA levels were higher for drowning or for drowning and injury than for other groups; there was no significant difference between fire fatality, involving airway injury due to inhalation of hot/irritant gases, and other control groups. These observations suggest characteristic molecular biological patterns of pulmonary injury involving suppression of pulmonary surfactants and activation of early-phase mediators of inflammation in drowning, with high mRNA expression levels of pulmonary surfactants in fatal hypothermia; however, there was no significant difference among these markers in immunohistochemical detection, except for SP-A. These mRNA expressions can be used as markers of pulmonary injury to assist in investigations of the pathophysiology of drowning and fatal hypothermia in combination with other biochemical and biological markers.

Keywords Molecular pathology · Drowning · Hypothermia · Immunohistochemistry · Real-time RT-PCR · Cytokines · Pulmonary surfactant-associated protein

Introduction

Drowning is defined as suffocation by submersion, especially in water; it continues to be a common cause of accidental death in the general population [1]. However, death from drowning is not only caused by fatal submersion [2] but involves complex factors including asphyxiation and electrolyte/osmotic disturbances, as well as hypothermia in cold water. Therefore, diagnosis of death due to drowning for a body retrieved from water is one of the most difficult tasks in forensic casework.

The pathophysiology of drowning depends on various factors including asphyxia and pulmonary damage from aspiration of an immersion medium, a subsequent alteration in blood components, and systemic electrolytic and metabolic deterioration [3–5]. Previous studies have shown that pulmonary surfactant-associated protein A (SP-A) is useful for investigating pulmonary alveolar injury and acute respiratory distress due to drowning [3–10]. Inflammation-related biomarkers may also be useful to investigate these pulmonary pathologies. With respect to this, tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-10 (IL-10) are major proinflammatory cytokines

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that play important roles in excessive self-injuring inflammation, ultimately leading to multiple organ dysfunctions [11, 12].

This study analyzed the expressions of inflammatory cytokines, including TNF-α, IL-1β, and IL-10, along with pulmonary surfactant-related proteins A and D (SP-A and SP-D) in autopsied human lungs, using a real-time reverse transcription polymerase chain reaction (RT-PCR) system to examine their validity for determining drowning and related fatalities, including fatal hypothermia.

Material and methods

Subjects and samples

Serial medicolegal autopsy cases within 3 days postmortem (median, 22.9 h) at our institute were examined: total, n=119; 83 males and 36 females; 24–89 (median, 58.0) years of age (Table 1). Based on routine macromorphological, micropathological, biochemical, and toxicological findings, the causes of death were classified: drowning (n=21: salt water, n=7 and fresh water, n=14), compared to controls, including acute mechanical asphyxia (n=24: hanging, n=12; strangulation, n=11; smothering, n=1) and acute cardiac death (ACD; n=9), as well as injury (n=23), intoxication (n=13), fire fatality (n=18), and hypothermia (cold exposure, n=11). In addition, a case of fatal hypothermia in a cold bath (an elderly woman in her 80s with dementia, about 2 days postmortem) was investigated as an example to differentiate between drowning and hypothermia; this case had typical pathological and biochemical signs of fatal hypothermia, including multiple dark-brownish gastric erosions, reddish left heart blood with high oxyhemoglobin saturation, bilateral iliopsoas bleeding with edema, and acetonemia, accompanied by scattered intraalveolar edema with hemorrhage in the lung as a possible sign of mild water aspiration.

Tissue specimens were taken from consistent sites without injury or other pathology in the upper lobes of bilateral lungs at autopsy and were immediately submerged in 1 ml RNA stabilization solution (RNAlaterTM, Ambion, Austin). Total RNA was isolated with ISOGEN (Nippon Gene, Toyama) according to the manufacturer's instructions, and stored at −80 °C until use. 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.

TaqMan RT-PCR and relative quantification of mRNA

RT-PCR was performed using the TaqMan Gene Expression Master Mix kit on an ABI 7500 Fast Real-Time PCR System (PerkinElmer Applied Biosystems, Foster City). The contents of the amplification mixture and the thermal cycling conditions were set according to previous protocols [13]. Amplification of TNF-α, IL-1β, IL-10, SP-A1b, SP-A2b, and SP-D messenger RNA (mRNA), together with endogenous references described below, was performed [14]. Primers and probes spanning the junction of bordering exons for these mRNAs were synthesized using the GenBank nucleotide database [11, 15–17]. According to the manufacturer's instructions, the relative quantification of mRNA transcripts was carried out using the comparative threshold method. The expression level of each mRNA was expressed as the ratio of the target normalized against endogenous references, for which potential quantitative

Table 1 Case profiles

Cause of death	Male/female	Age (years), range (median)	Survival time (h), range (median)	Postmortem time (h), range (median)	Heart weight (g), range (median)	Total lung weight (g), range (median)	Amount of pleural effusion (ml), range (median)
Drowning (n=21)	13/8	34–85 (61.0)	<0.5	13–63 (28.1)	225–605 (355)	595–1870 (1145)	0–750 (27.0)
Salt water (n=7)	4/3	36–70 (63.0)	<0.5	13–61 (23.0)	225–605 (370.0)	595–1870 (1215)	0–550 (80.0)
Fresh water (n=14)	9/5	34–85 (59.5)	<0.5	20–63 (28.3)	225–500 (350.0)	775–1500 (1125)	0–750 (26.0)
Asphyxia (n=24)	14/10	26–87 (62.5)	<0.5	9–58 (22.3)	200–560 (337.5)	415–1800 (1005)	0–35 (0.0)
ACD (n=9)	9/0	54–67 (62.0)	<0.5	16–31 (23.6)	275–650 (485.0)	1085–1805 (1330)	0–235 (0.0)
Injury (n=23)	21/2	29–69 (54.0)	<0.5	10–38 (17.5)	225–430 (335.0)	435–1515 (625)	0–815*[a] (35.0)
Intoxication (n=13)	9/4	24–74 (42.0)	<0.5–36 (6.0)	19–62 (29.0)	190–545 (365.0)	650–1610 (1290)	0–300 (25.0)
Fire fatality (n=18)	9/9	31–89 (64.0)	<0.5	10–37 (21.1)	230–500 (317.5)	430–1465 (850.0)	0–40 (0.0)
Hypothermia (n=11)	8/3	51–82 (65.0)	6–24 (6.0)	15–37 (26.6)	260–440 (340.0)	370–1860 (620)	0–35 (0.0)
Total (n=119)	83/36	24–89 (58.0)	<0.5–36 (0.5)	9–63 (23.1)	190–650 (340.0)	370–1870 (985)	0–815[a] (2.0)

Survival time and postmortem time were estimated from pathological and circumstantial evidence

ACD acute cardiac death

[a] Amount of pleural effusion including blood and/or blood clots

references for normalizing real-time PCR data were generated for each sample using three common housekeeping genes, β2-microglobulin (β2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin [18, 19].

Immunohistochemistry of SP-A, SP-D, IL-1β, IL-10, and TNF-α in the lung

Serial sections 4-μm thick were prepared from formalin-fixed paraffin-embedded lung tissue specimens. Monoclonal mouse anti-SP-A (1:100; Dako, Kyoto) and anti-SP-D (1:40; Abcam, Tokyo), and polyclonal rabbit anti-TNF-α (1:100; Abcam, Tokyo), anti-IL-1β (1:100; Abcam, Tokyo), and anti-IL-10 (1:400; Abcam, Tokyo) were used in an Envision System (Dako, Kyoto), followed by color development with diaminobenzidine, according to the manufacturer's instructions with counterstaining with hematoxylin [20].

Statistical analyses

Comparisons between groups were performed using the Bonferroni corrected p value of nonparametric Fisher's F test for multiple comparisons, and Mann–Whitney U test for two-group comparisons. These analyses were performed using Microsoft Excel and Statview (version 5.0; SAS Institute Inc.), and $p<0.05$ was considered significant. The results of the analysis are shown as box-plots, with 50 % of the data shown as box-plots and 50 % of the data summarized in the box. The line in each box represents the median, and the lines outside of each box represent the 90 % confidence interval [21, 22].

Results

Stability of relative mRNA quantification with regards to gender, age, survival period, postmortem interval, and endogenous reference genes

Simultaneous RT-PCR of mRNA of three housekeeping genes (β2M, GAPDH, and β-actin) showed high correlations of C_T values of β2M to those of GAPDH ($r=0.978$, $p<0.0001$) and β-actin ($r=0.970$, $p<0.0001$), as well as between those of GAPDH and β-actin ($r=0.975$, $p<0.0001$). When normalized against β2M mRNA, there was no gender-related difference in each target mRNA level, or age, survival time, or postmortem time dependency for all cases and individual groups, excluding injury cases and fire fatality ($R=0.019$–0.691; $p=0.056$–0.991). Age dependency was significant for TNF-α in injury cases ($R=0.547$, $p<0.01$), and a postmortem time-dependent decrease was detected for SP-D and TNF-α in fire fatality ($R=0.538$ and $R=0.508$, respectively; $p<0.05$); however, there was no postmortem time dependence within 48 h ($p>0.05$). These results were similar when normalized against GAPDH or β-actin mRNA. The stability mRNA assays were partly confirmed by re-examination of the same RNA samples.

Relationship of each mRNA level between bilateral lungs

When normalized against β2M mRNA, the correlation between bilateral lungs was different for target mRNA and was also dependent on the cause of death (Table 2): it was moderate to high for SP-A2b, SP-D, TNF-α, and IL-1β in drowning cases, for SP-A1b and IL-1β in acute injury death, for SP-A1b and SP-D in acute mechanical asphyxiation and intoxication cases, for SP-A1b, SP-A2b, SP-D, and TNF-α in fire fatality, and for SP-A1b, SP-A2b, and TNF-α in hypothermia cases, but was insignificant for all factors in ACD and for IL-10 in all causes of death. The results were similar when normalized against GAPDH or β-actin mRNA.

mRNA levels with regard to the cause of death

When normalized against β2M mRNA, pulmonary SP-A1b, SP-A2b, and SP-D mRNA expressions were highest for hypothermia and tended to be lower for injury cases. Other groups, including drowning, showed lower levels than those of hypothermia and/or injury (Figs. 1 and 2). These findings were similar when normalized against GAPDH or β-actin mRNA.

TNF-α and IL-1β expressions tended to be higher for drowning and injury, significantly so for IL-1β compared to other groups, excluding injury and intoxication (Figs. 1 and 2). IL-10 mRNA expression was higher for drowning than for other groups, excluding intoxication (Figs. 1 and 2). There was no significant difference between fresh- and saltwater drowning.

Total lung weight (the sum of the combined lung weight and the amount of pleural effusion) was greater for drowning than for asphyxia, injury, fire fatality, hypothermia, and ACD, and right lung SP-D and IL-10 mRNA levels in drowning cases correlated with lung weight ($r=0.449$, $p<0.05$ and $r=0.627$, $p<0.05$, respectively).

In the elderly case of fatal hypothermia in a cold bath, accompanied by possible pathological signs of mild water aspiration, these mRNA expression patterns in the lungs were consistent with those for fatal hypothermia but different from those for drowning, showing high mRNA levels of SP-A1b (left, 547.22; right, 1,857.38), SP-A2b (left, 3,878.95; right, 20,811.38), and SP-D (left, 153.44; right, 688.19), but low mRNA levels of TNF-α (left, 1.57; right, 10.57), IL-1β (left, 0.40; right, 5.28), and IL-10 (left, 0.83; right, 5.36).

Table 2 Correlation of relative mRNA expressions between left and right lung specimens

	Drowning	Asphyxia	ACD	Injury	Fire Fatality	Intoxication	Hypothermia
SP-A1	i.s.	R=0.786 P<0.0001	i.s.	R=0.525 P=0.021	R=0.943 P<0.0001	R=0.764 P=0.017	R=0.981 P<0.0001
SP-A2	R=0.867 P<0.0001	i.s.	i.s.	i.s.	R=0.651 P=0.0034	i.s.	R=0.978 P<0.0001
SP-D	R=0.440 P=0.046	R=0.512 P=0.011	i.s.	i.s.	R=0.927 P<0.0001	R=0.671 P=0.012	i.s.
TNF-α	R=0.795 P<0.0001	i.s.	i.s.	i.s.	R=0.881 P<0.0001	i.s.	R=0.805 P=0.0089
IL-1β	R=0.975 P<0.0001	i.s.	i.s.	R=0.863 P<0.0001	i.s.	i.s.	i.s.
IL-10	i.s.	i.s.	i.s.	i.s.	i.s.	i.s.	i.s.

SP-A pulmonary surfactant-associated protein A, *SP-D* pulmonary surfactant-associated protein D, *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β, *IL-10* interleukin-10, *ACD* acute cardiac death, *i.s.* insignificant

Immunohistochemistry

SP-A immunopositivity was detected linearly or membranously on the internal surface of the alveoli, and granularly in the alveolar spaces and macrophages. There was no difference between fresh- and saltwater drowning. Granular and linear/membranous stainings were dominant in cases of mechanical asphyxiation and intoxication, respectively (Fig. 3a, b), and the combined staining pattern was seen in drowning, fire fatality, and ACD (Fig. 3c). SP-A staining was not evident in hypothermia cases (Fig. 3d).

SP-D immunopositivity was detected in macrophages. Immunoreactivity of IL-1β was clearly detected in macrophages in the alveolar spaces, endothelial cells, and type I and II epithelial cells in the lung (Fig. 4a, c). IL-10 showed immunoreactivity in the ciliated epithelia in the tracheal branches and macrophages in the alveolar spaces. TNF-α was hardly detected in the lung (Fig. 4b, d). There was no significant difference in these immunopositivities among the causes of death.

Discussion

In this study, the stability of relative mRNA quantification using RT-PCR for autopsied lung specimens was established for target and reference genes; endogenous reference markers (β2M, GAPDH, and β-actin) showed high correlations in simultaneous assays, and relative mRNA expression levels of individual target genes, normalized against each endogenous reference, showed no significant deviation in re-examination. Detailed investigations detected mild age-dependences and/or postmortem decreases for relative mRNA expression levels of TNF-α and SP-D in

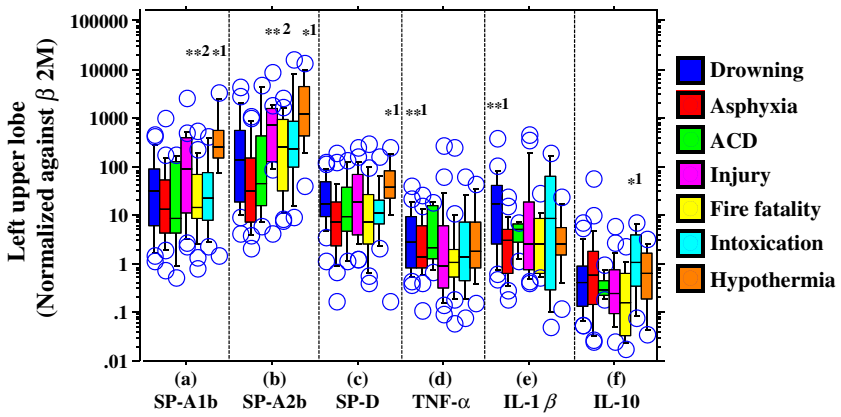

Fig. 1 Transcript quantification in the left upper lobe and the cause of death. **a** SP-A1b, **b** SP-A2b, **c** SP-D, **d** TNF-α, **e** IL-1β, and **f** IL-10. **a** *1* Significantly higher in hypothermia than in other causes of death (Fisher's F test; *p<0.05–p<0.01). *2* Significantly higher in injury than in drowning (Mann–Whitney U test; **p<0.05). **b** *1* Significantly higher in hypothermia (Fisher's F test; *p<0.05–p<0.01). *2* Significantly higher in injury than in asphyxia (Mann–Whitney U test; **p<0.05). **c** *1* Significantly higher in hypothermia than in asphyxia (Fisher's F test; *p<0.05); significantly higher in hypothermia than in intoxication (Mann–Whitney U test; *p<0.05). **d** *1* No significant difference by the Fisher's F test; significantly higher in drowning than in fire fatality (Mann–Whitney U test; **p<0.05). **e** No significant difference by the Fisher's F test; *1* significantly higher in drowning than in other causes, except injury and intoxication (Mann–Whitney U test; **p<0.05– p<0.01). **f** *1* Significantly higher for intoxication than for others (Fisher's F test; *p<0.05–p<0.01)

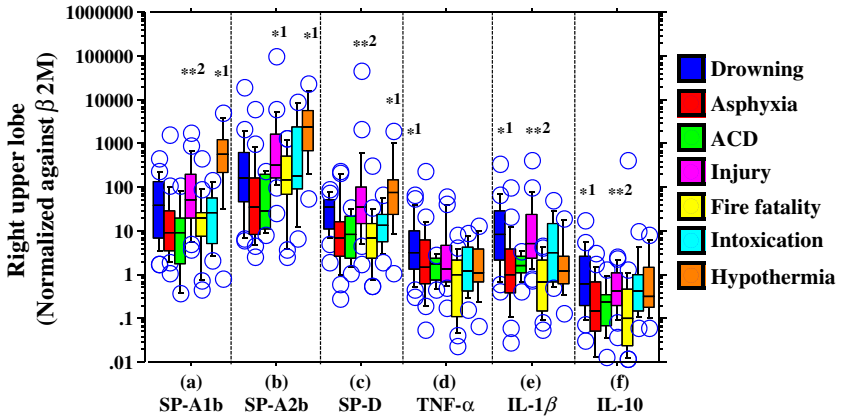

Fig. 2 Transcript quantification in the right upper lobe and cause of death. **a** SP-A1b, **b** SP-A2b, **c** SP-D, **d** TNF-α, **e** IL-1β, and **f** IL-10. **a** *1* Significantly higher in hypothermia than in other causes (Fisher's *F* test; **p*<0.01–*p*<0.001). *2* Significantly higher in injury than in other causes, except drowning and hypothermia (Mann–Whitney *U* test; ***p*<0.05–*p*<0.01). **b** *1* Significantly higher in hypothermia than in other causes, except intoxication and drowning (Mann–Whitney *U* test; **p*<0.01–*p*<0.05). Significantly higher in injury than in asphyxia and acute cardiac death (Mann–Whitney *U* test; **p*<0.05 and **p*<0.01, respectively). **c** *1* Significantly higher in hypothermia than in other causes (Fisher's *F* test; **p*<0.05). *2* Significantly higher for injury than in other causes, except drowning and hypothermia (Mann–Whitney *U* test; ***p*<0.01–*p*<0.05). **d** *1* Significantly higher in drowning than in other causes except injury (Fisher's *F* test; **p*<0.05); significantly higher in drowning than in injury (Mann–Whitney *U* test; *p*<0.05). **e** *1* Significantly higher in drowning than in fire fatality (Fisher's *F* test; **p*<0.05); significantly higher in drowning than in other causes, except injury (Mann–Whitney *U* test; *p*<0.0001–*p*<0.05). *2* Significantly higher in injury than in other causes, except intoxication and drowning (Mann–Whitney *U* test; ***p*<0.0001–*p*<0.01). **f** *1* Significantly higher in drowning than in other causes, except hypothermia (Fisher's *F* test; **p*<0.05); significantly higher in drowning than in injury, fire fatality, and acute cardiac death (Mann–Whitney *U* test; **p*<0.01–*p*<0.05). *2* Significantly higher in injury than in fire fatality and asphyxia (Mann–Whitney *U* test; ***p*<0.05)

some causes of death (injury and/or fire fatality), for which further investigation is needed; however, such findings were not evident in drowning, other asphyxiation deaths, and fatal hypothermia, in which target mRNA expression levels could be successfully evaluated within 2 days of death, as was previously reported [8, 9], and is shown in an example of fatal hypothermia in a cold bath described below.

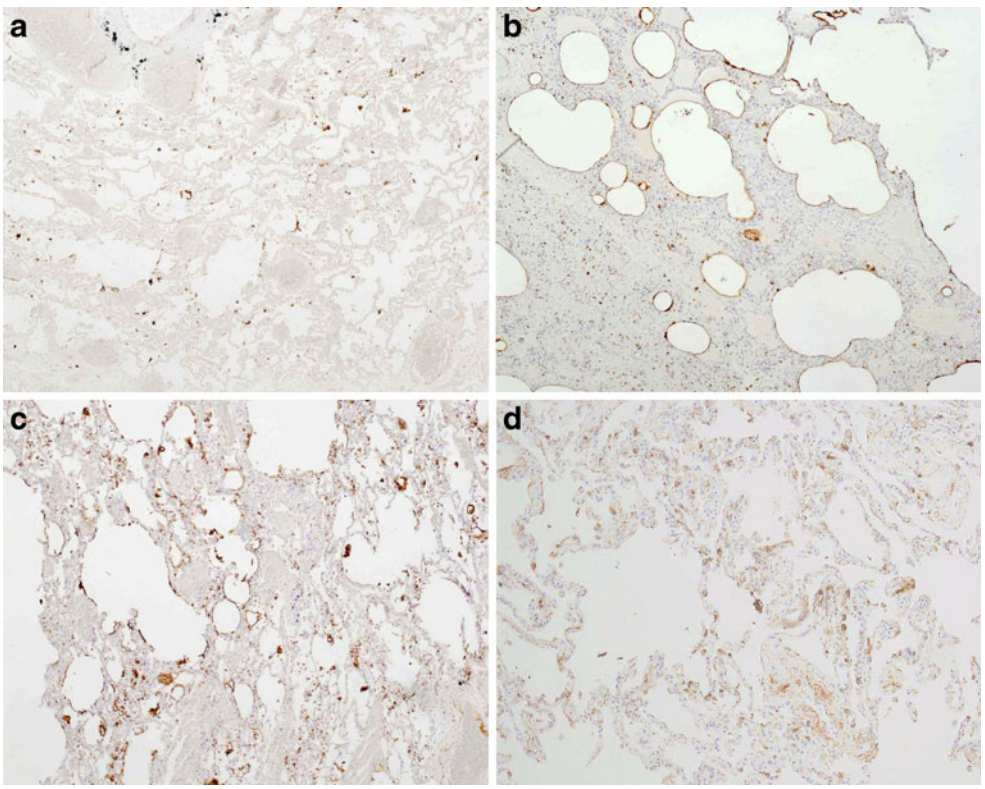

Fig. 3 Immunostaining of pulmonary SP-A. Intra-alveolar granular staining in an acute mechanical asphyxiation case (**a**), membranous or linear staining on the internal surface of the alveoli in an intoxication case (**b**), and combined pattern in cases of drowning (**c**), and not featured in cases of hypothermia (**d**). Original magnification, ×100

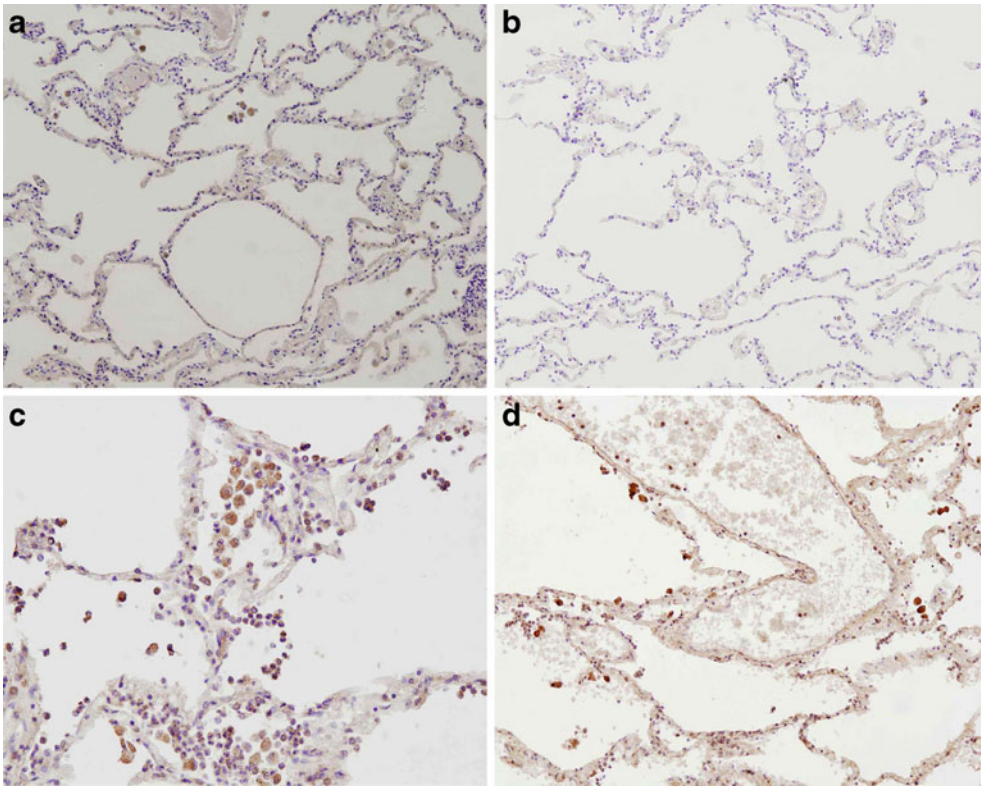

Fig. 4 Immunostaining of SP-D, TNF-α, IL-1β, and IL-10 in the lungs of a drowning case. SP-D immunopositivity is detected in macrophages in the alveoli (**a**). TNF-α is almost negative (**b**), but IL-1β positivity is clearly seen in macrophages in the alveoli, endothelial cells, and type I and II epithelia (**c**). IL-10 shows immunoreactivity in the ciliated epithelia in the tracheal branches and macrophages in the alveolar spaces (**d**). Original magnification, **a** and **b** ×100, and **c** and **d** ×400

Previous studies suggested the down-regulation of SP-A mRNA expression accompanied by the appearance of granular SP-A staining in the alveolar spaces, typically in cases of mechanical asphyxiation, drowning, and fire fatality, to be a molecular biological sign of acute respiratory distress, which can occur rapidly with a short survival time [3–10]. Similar findings of SP-A were detected in the present study. SP-D also had a lower mRNA expression in these fatalities. These observations suggest the dysfunction of pulmonary alveolar surfactants in drowning and other deaths accompanied by acute respiratory distress, which may be associated with delayed pulmonary damage in prolonged deaths under clinical care. In fatal hypothermia, however, mRNA expression levels of SP-A and SP-D were not suppressed; SP-A1b can be a very stable and effective marker for interpreting fatal hypothermia.

In addition, drowning cases had activation of inflammatory responses, involving increased TNF-α, IL-1β, and IL-10 mRNA expressions, significantly so for IL-1β and IL-10. For these markers, however, there was no significant difference between fire fatality, involving airway injury due to inhalation of hot/irritant gases, and other control groups. These findings suggest that water aspiration can cause substantial pulmonary injury involving the activation of early-phase mediators of inflammation, which may induce advanced inflammatory reactions involving respiratory distress in prolonged deaths. Pulmonary IL-1β mRNA presented a clearer increase in drowning cases than the hypoxia-induced factors investigated in the previous study [23, 24], showing significant differences from mechanical asphyxiation and ACD. Topographical differences in the mRNA expression level of each marker can indicate the localization of related pathology, reflecting the severity of lung damage due to respective insults; however, there was no significant difference in immunohistochemical detection of these markers.

As above, the present study demonstrated characteristic molecular biological patterns of pulmonary injury involving suppression of pulmonary surfactants and activation of early-phase mediators of inflammation in drowning, with high expression levels of pulmonary surfactants in fatal hypothermia. Application of this procedure is limited to fresh victims without evident decomposition but may be useful to reinforce pathological and biochemical findings; when this procedure was used in the case of death in a cold bath (about 2 days postmortem), for example, molecular pathological profiles supported the pathological and biochemical findings of fatal hypothermia.

In conclusion, the present study has suggested the dysfunction of pulmonary surfactants due to acute respiratory distress and the activation of early-phase inflammatory mediators caused by water aspiration in drowning, with high mRNA expression levels of pulmonary surfactants in fatal hypothermia. These mRNAs can be used as molecular biological markers of pulmonary injury to assist investigations of the pathophysiology of drowning and fatal hypothermia in combination with other biochemical and biological markers.

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