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Histological findings and immunohistochemical surfactant protein A (SP-A) expression in asphyxia: Its application in the diagnosis of drowning

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Summary. The histopathological alterations that permit the diagnosis of death by asphyxia are very unspecific, although pulmonary alterations are of great importance in this respect. The postmortem diagnosis of drowning, particularly, continues to be one of the most difficult in forensic pathology. The aim of this study is to jointly evaluate microscopic findings and immunohistochemical surfactant protein A (SP-A) expression in the upper and lower lobes of lungs in different causes of death, and their possible application to the diagnosis of drowning. We studied 120 cadavers from subjects with a mean age of 48.73 years (SD 19.45; range 2-86 years), and with a mean post-mortem interval of 30 hours (SD 39.59; range 3-216 hours). According to the scene, cause and circumstances of death, and autopsy findings, cases were classified into groups as follows: (a) drowning (n=47); (b) other asphyxia (n=44) and (c) other causes (n=29). In the upper and lower lobes of lungs, histological studies of H&E staining and immunohistochemical surfactant protein A expression were made. The presence and severity of congestion, haemorrhage and oedema, together with immunohistochemical SP-A expression, may have a diagnostic value in differentiating asphyxia and drowning from other causes of death, and drowning from other types of asphyxia. Our findings suggest that both lobes should be investigated to establish the diagnosis, although the findings in the upper lobe might be the most important for differentiating the exact cause of death.

Key words: Forensic Pathology, Asphyxia, Surfactant protein A, Lungs

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Introduction

Asphyxia represents a varied group of causes of death, among them those whereby death is caused by breathing an atmosphere poor in oxygen or where there has been a physical interruption to the flow of air in the air passage. Drowning also forms part of this group, even when electrolytic alterations due to the entrance of water seem more important than the lack of oxygen. The histopathological alterations that permit the diagnosis of asphyxia are very unspecific and may not even be present in this type of death, but may be observable in other types of traumatic or natural death (Knight, 1996), where pulmonary alterations are of great importance. Histological, immunohistochemical, immunological and biochemical studies have been described as useful tools for the forensic evaluation of pulmonary tissue (Du Chesne et al., 1996; Betz et al., 1997; Ortmann and Brikmann, 1997; Fineschi et al., 1998; Osuna et al., 1998).

Drowning is a leading cause of injury-related deaths in the world, surpassed only by motor vehicle-related injuries and falls (Hudson et al., 2007). The postmortem diagnosis of drowning continues to be one of the most difficult in forensic pathology. The main positive findings for drowning are related to the penetration of the drowning medium into the airways. The ideal diagnostic test as definite proof for drowning still needs to be established, and more research is necessary (Piette and De Letter, 2006). Histological investigations are of primary importance to demonstrate drowning-related pulmonary changes, such as oedema, congestion, haemorrhage and aquosum emphysema. These drowning-related pulmonary changes are not specific, and previous studies suggest that their heterogeneous distribution in the lungs makes it difficult to find strong evidence of drowning (Fornes et al., 1998). Studies to investigate and improve the reliability of complementary

methods for the diagnosis of drowning are warranted (Lunetta et al., 2002).

Surfactant is a complex mixture of phospholipids and proteins, and the importance of the surfactant-associated proteins (SP-A, SP-B, SP-C, and SP-D) extends beyond their contributions to the surfactant function (Greene et al., 1999). The best characterized function of surfactant is the ability to reduce surface tension at the alveolar air-liquid interface, stabilizing alveoli and terminal airways at low lung volumes. Surfactant protein A (SP-A), the most abundant surfactant protein, has been used as a clinical diagnostic marker in different situations of acute lung injury (Eisner et al., 2003).

In experimental studies, Morita et al. (1985) pointed to an increase in pulmonary surfactant protein in cases of asphyxia, and proposed that this might be used in forensic medicine. More recently, Funayama et al. (1994) and Zhu et al. (1996) proposed the use of SP-A immunohistochemistry in forensic pathology, since it had the added advantage that the immunohistochemical distribution of SP-A is fairly stable against decomposition.

The aim of this study is to jointly evaluate microscopic findings and immunohistochemical surfactant protein A expression in the upper and lower lobes of the lung in different causes of death, and their possible application to the diagnosis of drowning.

Material and methods

Forensic autopsy cases

In a study approved by the Ethics Committee of the Institute of Forensic Medicine, we studied 120 cadavers (103 males and 17 females) selected from medico-legal autopsies performed in the Institute of Forensic Medicine, Cádiz (South of Spain). The mean age of the subjects was 48.73 years (SD 19.45; range 2-86 years), and the mean post-mortem interval was 30 hours (SD 39.59; range 3-216 hours). To minimise post-mortem artifacts, the bodies were refrigerated.

Cases were classified into different groups according to the autopsy findings and scene, cause and circumstances of death, as follows: (a) drowning (n=47); (b) other asphyxia (n=44) and (c) other causes (control group) (n=29).

The age of the victims and post-mortem interval (mean hours ± SD) for the diagnostic groups and subgroups of “drowning”, “other types of asphyxia” and “other causes of death” are shown in Table 1.

Methods

Serial 4 µm sections were prepared from the tissue specimens of the upper and lower lobes of both lungs, fixed in 10% formalin and routinely processed for paraffin embedding. To avoid the possible interference of blood distribution due to post-mortem hypostasis in the pulmonary histological and immunohistochemical findings, lung samples were taken from zones free of hypostasis phenomena. Hematoxylin&Eosin (H&E) staining was carried out and pulmonary surfactant detection by immunohistochemistry. The anti-human SP-A mouse monoclonal antibody PE-10 (Dako, Kyoto Japan) (Kuroki et al., 1985) diluted 100-fold dilution was used with a 30 min incubation at room temperature, using a universal streptavidin/biotin immunoperoxidase detection system (OmniTags kit) and DAB (Shandon/Lipshaw/Immunon, Pittsburgh, Penn.) according to the manufacturer's instructions. The tests were performed by two different observers.

A total of 960 sections were evaluated. Histological studies with H&E staining were used to evaluate oedema, congestion and haemorrhage in each of the sections, classifying the severity of the findings into four groups: - negative, + mild, ++ mild/moderate, +++ severe.

SP-A was immunohistochemically demonstrated in two patterns in drowning, and other asphyxia (1) membranous or linear staining of the alveolar interior surface and the interface of the intra-alveolar effusion and (2) staining the intra-alveolar aggregated granular deposit (Zhu et al., 2000a,b).

The SP-A immunostaining reactivity in the alveolar type II cells and alveolar surface was assessed as follows: - negative, + weakly positive and discontinuous line of staining, ++ positive, +++ strongly positive. The findings in the intra-alveolar space were classified into four categories: - negative, + weakly positive, ++ positive with a few massive aggregates of stained granules, +++ intensely and diffusely positive with massive aggregates of stained granules (Zhu et al., 2000a).

Statistical analysis

For statistical analysis of the data the SPSS 14.0

Table 1. Age, and post-mortem interval (in hours). Mean and standard deviation (SD) values for the diagnostic groups.

Groups	n	%	Age (years old)		Postmortem interval (h)	
			Mean	SD	Mean	SD
DROWNING	47	39.2	48.9	18.9	36.0	44.0
Freshwater drowning	15	12.5	47.2	22.2	37.0	54.3
Seawater drowning	32	26.7	49.7	17.4	35.5	39.2
OTHER ASPHYXIAS	44	36.7	50.4	20.2	31.1	46.0
Hanging	33	27.5	50.0	20.9	24.3	35.7
Suffocation	9	7.5	54.7	19.7	41.5	59.5
Aspiration	2	1.7	39.5	3.5	93.5	105.3
OTHER CAUSES	29	24.2	45.8	19.4	18.5	6.0
Multiple trauma	17	14.2	53.1	16.6	20.2	4.6
Gunshot	8	6.7	34.5	13.7	15.5	4.6
Cardiovascular disease	4	3.3	37.5	29.5	17	11.1
TOTAL	120	100	48.7	19.4	30.0	39.5

software was used. Descriptive analysis of the data (mean, median, standard deviation and range) and bivariate correlation (Spearman correlation) were carried out. Association between variables was ascertained with Pearson's $\chi 2$ test. The Kruskal-Wallis test, a non-parametric test for more than two independent samples, was used to compare groups. Also, specific contrasts for each variable (grouped according to diagnostic category) were carried out using the non-parametric Mann-Whitney test for two independent samples. The Kruskal-Wallis test was used to compare each variable among the three diagnostic groups, and the Mann-Whitney test to compare pairs of diagnostic groups. A probability level of $P \leq 0.05$ was considered significant.

Results

No statistically significant correlations were observed between degree of severity of the histological findings or the immunohistochemical expression of SP-A and age or postmortem interval.

Classification of the cases into two large groups (asphyxia and non-asphyxia) showed that oedema in the upper right lobe is positive in 80.2% cases of asphyxia and in 19.8% of cases of non-asphyxia (P=0.022), haemorrhage in the upper right lobe is positive in 84.6% cases of asphyxia and 15.4% of cases of non-asphyxia (P=0.032); congestion in the upper left lobe is positive in 75.5% of asphyxia vs 24.5% of non-asphyxia (P=0.003); haemorrhage in the upper left lobe is positive in 86.8% of asphyxia vs 13.2% of non-asphyxia (P=0.005). Significant statistical differences were also found in the pattern of staining in the intra-alveolar space SP-A: positive in 85.9% of asphyxia vs 14.1% of non-asphyxia in the upper right lobe (P<0.0001), and positive in 84.4% asphyxia vs 15.6% in non-asphyxia in the upper left lobe (P<0.0001).

When cases were classified into drowning/non-drowning we found only statistically significant differences in the pattern of staining in the intra-alveolar space SP-A, where it was positive in the upper right lobe in 76.9% of cases of drowning vs 23.1% of non-drowning (P=0.038) and in the upper left lobe 75% of cases of drowning vs 25% in non-drowning (P=0.035).

The distribution of positive cases according to the three diagnostic groups, drowning, other types of asphyxia and other causes, are as follows: oedema in upper right lobe, 40.7%, 39.6% and 19.8%, respectively (P=0.040); haemorrhage in upper right lobe, 38.5%, 46.2% and 15.4%, respectively (P=0.042); haemorrhage in upper left lobe, 37.7%, 49.1% and 13.2% (P=0.013); in the pattern of staining in the intra-alveolar space SP-A, 44.7%, 41.2% and 14.1% in upper right lobe (P<0.0001); 45.5%, 39% and 15.6% in upper left lobe (P<0.0001).

With the diagnostic groups, drowning, other types of asphyxia and other causes, the severest congestion, oedema and haemorrhage appeared in the deaths by drowning and other types of asphyxia, although significant differences in oedema and congestion only existed in the upper lobes of both lungs (Table 2).

When the SP-A immunostaining reactivity in the alveolar type II cells, alveolar surface and intra-alveolar space was analysed, we found the greatest degree of severity in drowned and other types of asphyxia compared with other causes of death, although statistically significant differences in SP-A immunostaining reactivity were only evident in the upper lobes of both lungs (Table 3).

When the cases were grouped into the cases of

Table 2. Severity and distribution of histological findings (H&E) in four lung lobes according to diagnostic group.

	Drowning				Other asphyxia				Other causes				P
	-	+	++	+++	-	+	++	+++	-	+	++	+++	
Right lung-Upper lobe													
Congestion	4.5	11.4	36.4	47.7	0	2.5	32.5	65	3.7	37	33.3	25.9	0.002
Oedema	15.9	27.3	36.4	20.5	10	22.5	40	27.5	33.3	40.7	22.2	3.7	0.036
Haemorrhage	54.5	22.7	6.8	15.9	40	27.5	7.5	25	70.4	18.5	0	11.1	ns
Right lung-Lower lobe													
Congestion	0	41.7	8.3	50	0	16.7	16.7	66.7	20	20	0	60	ns
Oedema	66.7	8.3	0	25	33.3	16.7	25	25	60	20	20	0	ns
Haemorrhage	41.7	16.7	16.7	25	33.3	16.7	8.3	41.7	40	40	0	20	ns
Left lung-Upper lobe													
Congestion		2.9	37.1	60		5.1	20.5	74.4		33.3	45.8	20.8	<0.0001
Oedema	8.6	28.6	42.9	20	7.7	17.9	46.2	28.2	20.8	41.7	33.3	4.2	0.031
Haemorrhage	42.9	34.3	8.6	14.3	33.3	23.1	12.8	30.8	70.8	16.7	0	12.5	ns
Left lung-Lower lobe													
Congestion		22.2	11.1	66.7		12.5	0	87.5		0	50	50	ns
Oedema	22.2	44.4	11.1	22.2	12.5	50	0	37.5	50	50	0	0	ns
Haemorrhage	33.3	22.2	11.1	33.3	12.5	37.5	12.5	37.5	50	0	50	0	ns

Score severity grade: Negative (-), mild (+), mild/moderate (++), severe (+++). Pearson's $\chi 2$ test; P: probability; ns: non-significant.

greatest severity, the staining patterns in the alveolar surface and alveolar type cells were as follows: in the upper right lobe the percentage of cases scoring ++ and +++ in drowning was 86.4%, while in other types of asphyxia it was 30% and other causes 18.5% (P<0.0001). The figures for the left upper lobe were 85.7%, 28.9% and 8.3% respectively (P<0.0001). Staining in the intra-alveolar space was positive (++ and +++) in the right upper lobe in 63.6% of drownings, 47.5% of other types of asphyxia and 7.4% of other causes (P<0.0001). For the left lung upper lobe, the figures were 68.5%, 52.6% and 8.3%, respectively (P<0.0001).

A non-parametric test (Kruskal-Wallis) was used to compare the severity of the histological and immunohistochemical findings in the diagnostic groups. We found statistically significant differences in oedema, congestion and haemorrhage, and SP-A immunoreactivity in alveolar type II cells, alveolar surface and intra-alveolar space in the upper lobes in both lungs

Table 3. Severity and distribution of immunohistochemical findings (SP-A) in four lung lobes according to diagnostic group.

	Drowning				Other asphyxia				Other cause				P
Score	-	+	++	+++	-	+	++	+++	-	+	++	+++	
Right lung-Upper lobe SP-A membrane	4.5	9.1	61.4	25	5	65	17.5	12.5	0	81.5	14.8	3.7	<0.0001
Right lung-Upper lobe SP-A space	13.6	22.7	31.8	31.8	12.5	40	42.5	5	55.6	37	7.4	0	<0.0001
Right lung-Lower lobe SP-A membrane	16.7	0	33.3	50	12.5	12.5	12.5	62.5	33.3	33.3	33.3	0	ns
Right lung-Lower lobe SP-A space	28.6	28.6	28.6	14.3	37.5	62.5	0	0	100	0	0	0	ns
Left lung-Upper lobe SP-A membrane	2.9	11.4	80	5.7	5.3	65.8	18.4	10.5	0	91.7	8.3	0	<0.0001
Left lung-Upper lobe SP-A space	14.3	17.1	31.4	37.1	7.9	39.5	50	2.6	50	41.7	8.3	0	<0.0001
Left lung-Lower lobe SP-A membrane	0	0	100	0	0	0	25	75	51.7	48.3	0	0	ns
Left lung-Lower lobe SP-A space	0	100	0	0	50	50	0	0	55	45	0	0	ns

SP-A immunostaining reactivity in the alveolar type II cells and alveolar surface: -, negative, +, weakly positive and discontinuous line of staining, ++, positive, +++, strongly positive. SP-A intra-alveolar space: -, negative, +, weakly positive, ++, positive with a few massive aggregates of stained granules, +++, intensely and diffusely positive with massive aggregates of stained granules. Pearson's $\chi 2$ test; P: probability; ns: non-significant.

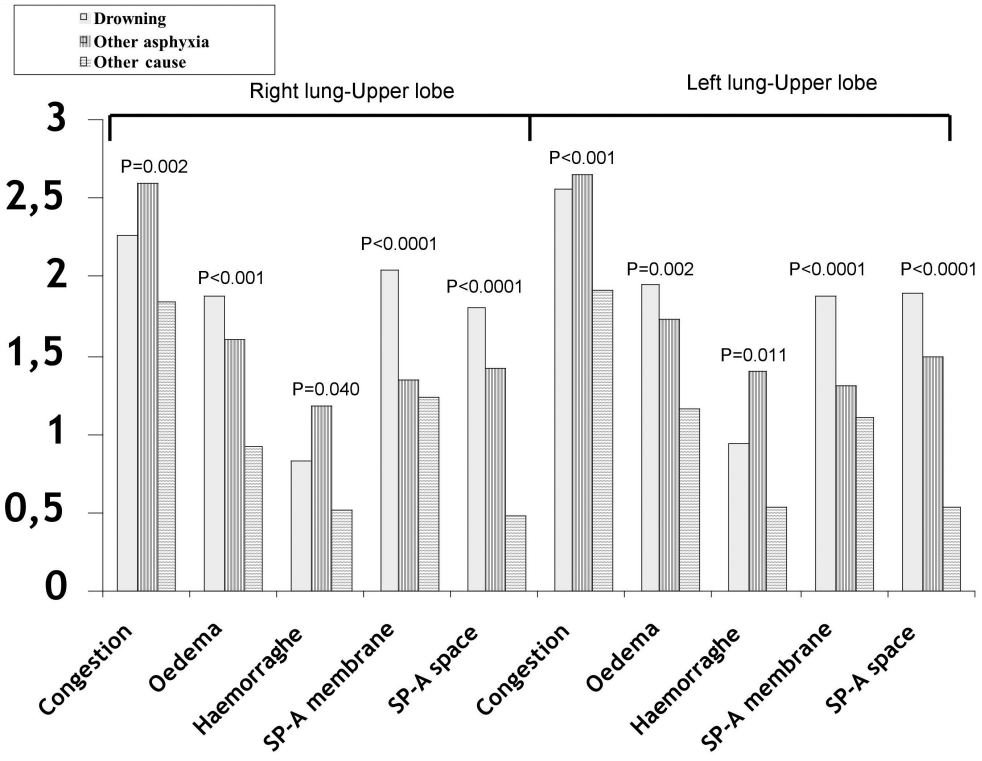

Fig. 1. Kruskal-Wallis test illustrating the differences in the degree of severity of the histological and immunohistochemical findings in the diagnostic groups.

(Fig. 1).

In the Mann-Whitney test (Table 4) differences for the SP-A immunostaining reactivity (alveolar type II cells, alveolar surface and intra-alveolar space) were observed in the upper lobes of both lungs between drowned and other types of asphyxia. Significant differences were also found in the severity of oedema and congestion, and SP-A immunostaining reactivity (alveolar type II cells, alveolar surface and intra-alveolar space) in the upper lobes of both lungs between drowning and other causes of death. Statistically significant differences in congestion, oedema and haemorrhage in the upper lobes of both lungs and SP-A immunostaining reactivity in the intra-alveolar space were found in asphyxia (excluding drowning) and other causes of death (Figs. 2, 3, 4).

There were significant correlations between positivity for SPA aggregates in the alveolar space and

Table 4. Mann-Withney test used to compare the degree of severity of the histological and immunohistochemical findings in the diagnostic groups.

Variable	Groups	Probability
SP-A membrane Right lung-Upper lobe	Drowning - Other asphyxia	<0.0001
SP-A space Right lung-Upper lobe	Drowning - Other asphyxia	0.035
SP-A membrane Left lung-Upper lobe	Drowning - Other asphyxia	<0.0001
SP-A space Left lung-Upper lobe	Drowning - Other asphyxia	0.029
Congestion Right lung-Upper lobe	Drowning - Other cause	0.035
Oedema Right lung-Upper lobe	Drowning - Other cause	0.004
Congestion Left lung-Upper lobe	Drowning - Other cause	0.001
Oedema Left lung-Upper lobe	Drowning - Other cause	0.015
SP-A membrane Right lung-Upper lobe	Drowning - Other cause	<0.0001
SP-A space Right lung-Upper lobe	Drowning - Other cause	<0.0001
SP-A membrane Left lung-Upper lobe	Drowning - Other cause	<0.0001
SP-A space Left lung-Upper lobe	Drowning - Other cause	<0.0001
Congestion Right lung-Upper lobe	Other asphyxia - Other cause	<0.0001
Oedema Right lung-Upper lobe	Other asphyxia - Other cause	<0.0001
Haemorrhage Right lung-Upper lobe	Other asphyxia - Other cause	0.014
Congestion Left lung-Upper lobe	Other asphyxia - Other cause	<0.0001
Oedema Left lung-Upper lobe	Other asphyxia- Other cause	0.001
Haemorrhage Left lung-Upper lobe	Other asphyxia - Other cause	0.004
SP-A space Right lung-Upper lobe	Other asphyxia - Other cause	<0.0001
SP-A space Left lung-Upper lobe	Other asphyxia - Other cause	<0.0001

Diagnostic groups: (1) Drowning (n= 47); (2) Other asphyxia (n=44); (3) Other cause (n=29).

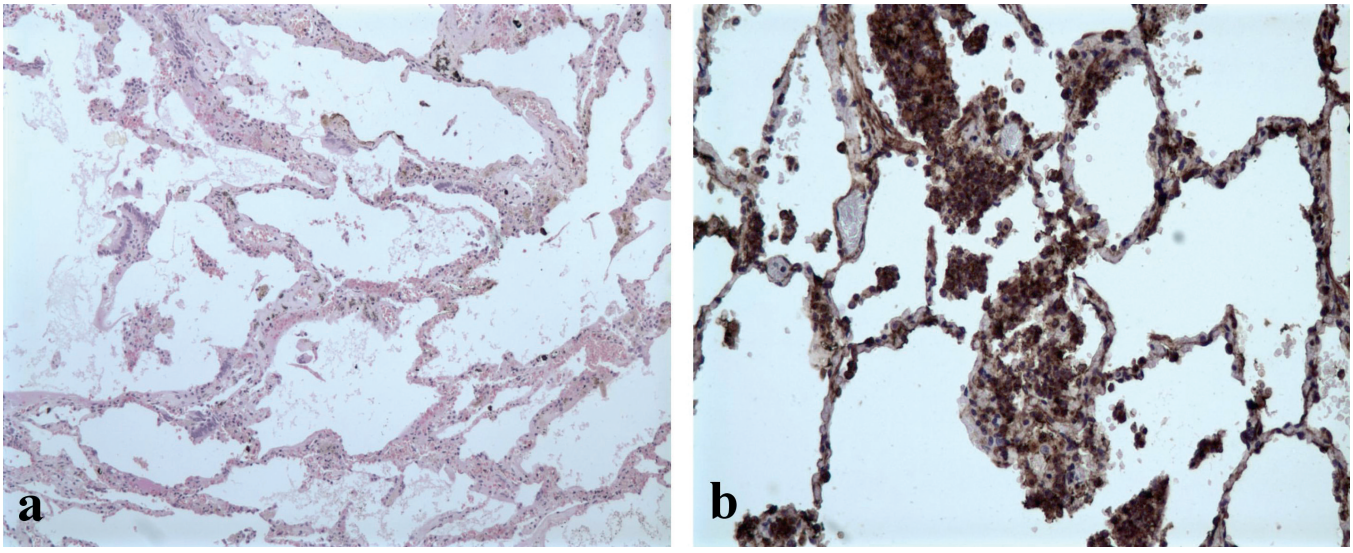

Fig. 2. H&E **(a)** and immunohistochemical expression of SP-A **(b)** in drowning. x 150

congestion, oedema and haemorrhage in the upper lobes. Analysis of the percentage of positive cases and severity of the histological findings obtained by H&E and surfactant-associated protein A (SP-A) immunohistochemistry pointed to no significant differences between the drowning subgroups (salt/fresh) or between

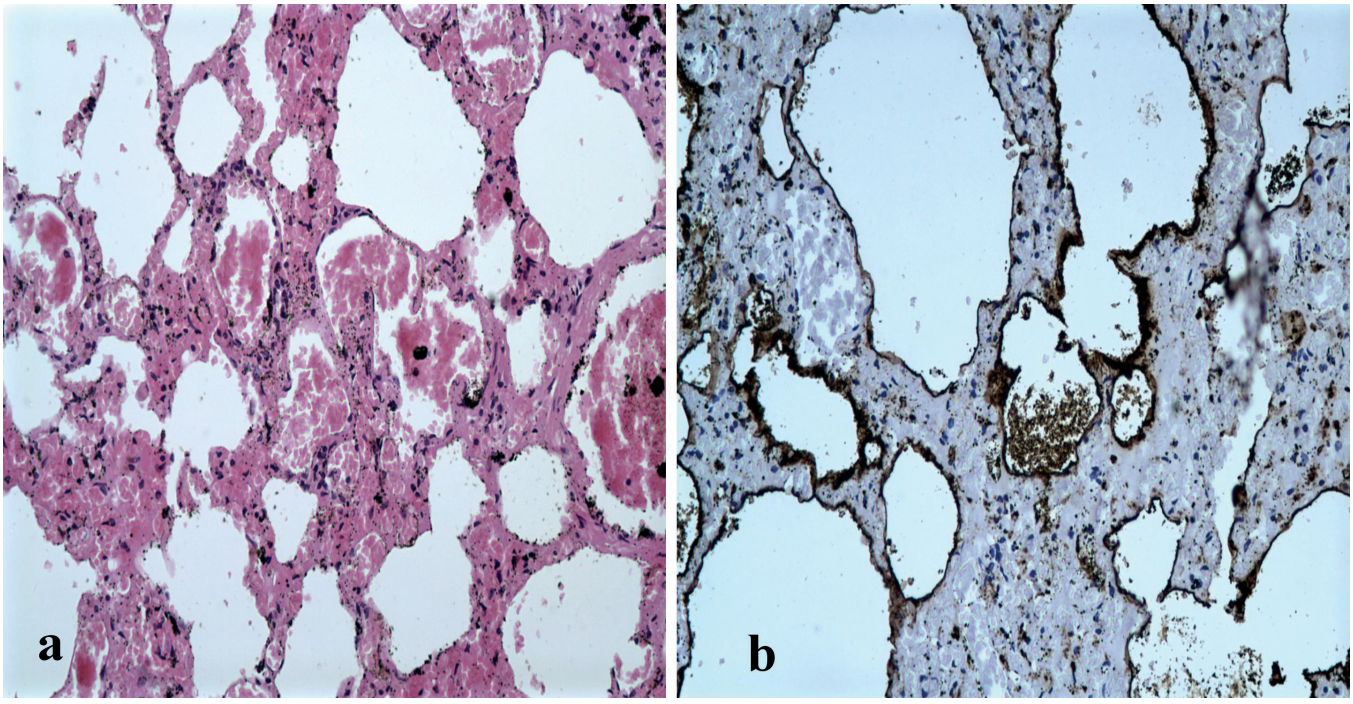

Figure 3. H&E **(a)** and immunohistochemical expression of SP-A **(b)** in asphyxia (hanging). x 150

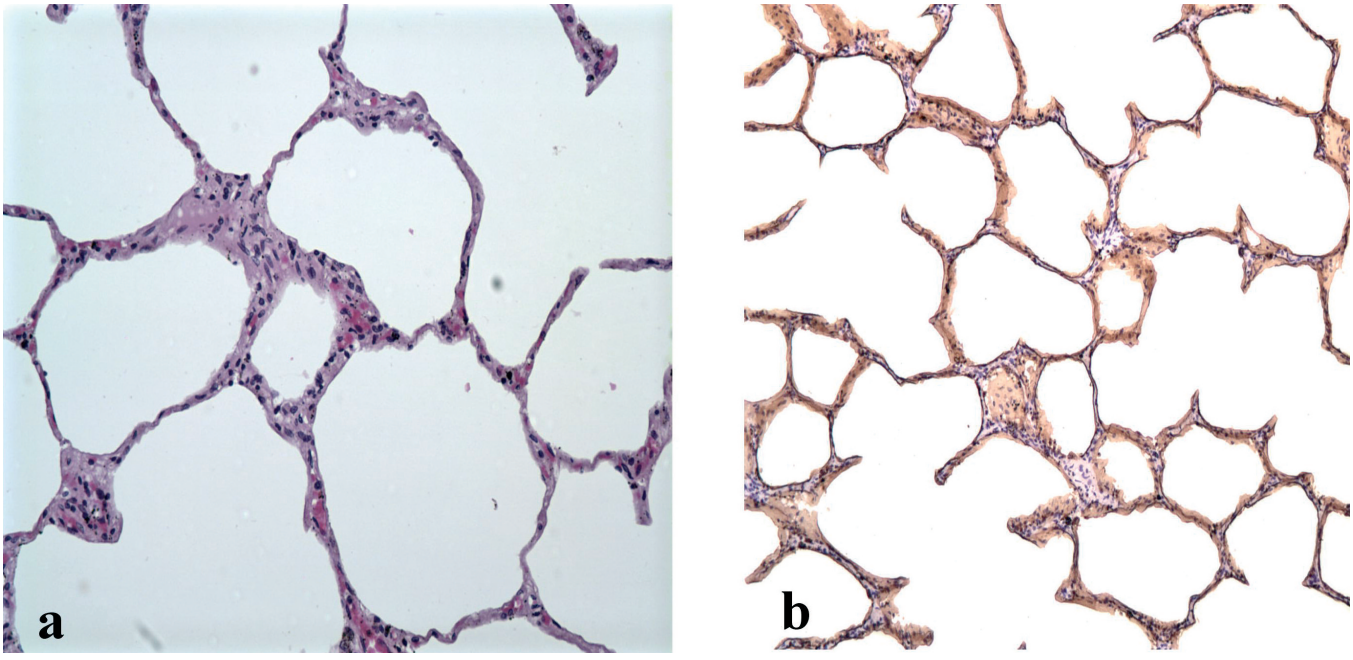

Figure 4. H&E **(a)** and immunohistochemical expression of SP-A **(b)** in control group (gunshot). a, x 150; b, x 125

different types of asphyxia (hanging/suffocation/aspiration).

Discussion

The differential diagnosis of death by asphyxia and other causes, especially drowning and other causes, remains a problem in forensic medicine. Congestion, haemorrhage, intra-alveolar and interstitial oedema, emphysema and atelectasia are more frequent in deaths by asphyxia than in deaths from other causes and, although they all provide useful information, none is specific.

According to our results, the presence of oedema, congestion and haemorrhage in all the lung lobes is more frequent in death by asphyxia than death by other causes, although statistically significant differences are only found in the upper lobes of both lungs. Although oedema predominates in drowning and haemorrhage in other types of asphyxia, these histological findings do not permit the two causes of death to be differentiated. In an attempt to identify histological diagnostic parameters, Fornes et al. (1998) analysed several histomorphometric findings and observed that significant differences existed in alveolar wall/cavity parameters between the drowned subjects and those dying from asphyxia, which encouraged them to suggest the possible diagnostic value of these parameters.

To investigate the causes of death and the process of death in forensic pathology, it is especially important to identify morphological characteristics and to analyse markers that indicate physiopathological changes due to lesions in different organic systems (Maeda et al., 2003). Surfactant is synthesised in the type II alveolar epithelial cell and packaged into a secretory granule, the lamellar body (Rooney et al., 1994). One of the most common phenomena in the process of asphyxia is heavy forced breathing, which we can assume to be one of the main differences from other causes of death. Perhaps due to this, and to the direct action of water, in the case of drowning, pulmonary surfactant is released and distributed differently than in cases of death by other causes, since a physical distortion of the type II cell at high lung volumes is probably the major stimulus for surfactant release (Doyle et al., 1999). This explains why pulmonary surfactant might be regarded as a possible indicator for diagnosing deaths from hypoxia, including drowning (Morita et al., 1985; Lorente et al., 1990; Zhu et al., 1996).

Using SP-A immunohistochemistry, Zhu et al. (2000a) found no significant differences between asphyxia and control groups in the staining pattern in the alveolar surface and alveolar-type cells. However, in the staining pattern of the intra-alveolar space, many prominent massive aggregates of granular SP-A positive staining were found exclusively in the asphyxia group, which led the authors to suggest that the immunohistochemistry of SP-A could be useful for distinguishing asphyxia from other causes of death. The same authors subsequently demonstrated a significantly high level of intraalveolar aggregates of SP-A more frequently in drowning than in other causes of death (Zhu et al., 2002; Maeda et al., 2003).

In our study, too, the SP-A immunostaining pattern of the intra-alveolar space permitted asphyxia to be distinguished from other cause of death, since we found a higher proportion of cases with massive aggregates of granular SP-A in asphyxia that in the former cases. However, the differences were only statistically significant in the upper lobes of both lungs, which may be due to the functional differences (ventilation/perfusion ratio) between the different lung regions.

As Table 2 shows, the severity of congestion was similar in the upper and lower lobes for drowning and other types of asphyxia, whereas other cases showed different distribution patterns; severe congestion was more frequent in the lower lobes. However, such a difference was not evident with SP-A immunostaining. In contrast, differences in the distribution of SP-A immunopositivity were seen between drowning and asphyxia (Table 3), underlining the importance of SP-A immunohistochemistry.

Although more research is necessary, we propose that not only the presence of congestion, haemorrhage and oedema (especially in the upper lobes), but also their severity and immunohistochemical SP-A expression, may have a diagnostic value for differentiating asphyxia and drowning from other causes of death, and drowning from other types of asphyxia.

Our findings suggest that both lobes should be investigated to establish diagnosis, although the findings in the upper lobe might be the most important for differentiating the exact cause of death.

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