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Immunohistochemical expression of P-selectin, SP-A, HSP70, Aquaporin 5 and Fibronectin in Saltwater drowning and Freshwater drowning

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Abstract:	The diagnosis of drowning is one of the most difficult in forensic medicine. The aim of this study was to analyse pulmonary tissue reactions in death by drowning. In particular, we focused on the immunohistochemical expression of P-selectin, SP-A, HSP70, AQP-5 and fibronectin to investigate our expression in drowning and to understand whether there are differences between Saltwater drowning (SWD) and Freshwater drowning (FWD), which may indicate a different pathophysiology. We retrospectively investigated 10 cases of SWD (Mediterranean Sea) from the Institute of Legal Medicine of Genoa (Italy), and 10 cases of FWD (Lake of Geneva) from the University Center of Legal Medicine of Geneva (Switzerland). As control group, we examined 10 cases of death by acute external bleeding, characterized by minimal respiratory distress. As compared to controls, in SWD cases the results showed a decrease of SP-A expression with membrane patterns. Furthermore, we observed a greater SP-A expression with granular pattern in drowning cases without statistically significant difference between SWD and FWD. For the markers AQP-5, HSP70, fibronectin and P-Selectin, no statistically significant differences were found between SWD, FWD and controls.
Author Comments:	
Response to Reviewers:	Reviewer #1 Thank you very much for the constructive comments and suggestions. We followed you precious guidance to improve our paper. Below we provide a list of changes, point by point 1.Indeed the number of investigated cases is low and this because we have applied severe exclusion criteria to avoid confounding factors. This is stated in the text.

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	2.This was a deliberate choice as in drowning cases it is often difficult to determine the manner of death (accident vs suicide, for exemple). Such an information would be inaccurate by definition Moreover we think that the manner of death has not a relevant influence on the findings of our study. For these reasons we do not provide this information in the text. 3.Diagnostic criteria of salt- and fresh-water drowning have been added, according to your suggestion 4.Number and location of sampling have been added, following your suggestion. 5.A table (n. 1ESM) with macroscopic and histological findings for each case has been added. Immunohistochemical information is provided in table 2ESM. 6.More histological data (information on the extent and the frequency of the findings) have been added in the text and in table 1ESM (for each case). 7.An exemplary figure of SPA reaction in FWD has been added, according to your suggestion. 8.The suggested reference has been added. 9.The diagnosis of drowning is a diagnosis by exclusion. For this reason, SPA is useful in that it brings useful information that has to be integrated in the general context: immunohistochemical data alone (without circumstantial, macroscopic and histological data) is not sufficient to make diagnosis as it is not specific. 10.The introduction was shortened, according to your suggestion. 11.Spelling errors have been corrected. Reviewer #2 Thank you very much for the constructive comments and and suggestions. We followed you precious guidance to improve our paper. Below we provide a list of corrections point by point. 1.Heat shock proteins (HSPs) play a pivotal role in cellular repair and adaptation to stress. As reported in the article this antibody has been studied in the lung. This protein is activated in various stress conditions (such as asphyxia) and not only by heat. Our aim was to investigate whether the different pathophysiology stress between SWD and FWD could induce a different expression of this protein.

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Ref.: Ms. No. IJLM-D-19-00118
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Immunohistochemical expression of P-selectin, SP-A, HSP70, Aquaporin 5 and Fibronectin in Saltwater drowning and Freshwater drowning

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Abstract

The diagnosis of drowning is one of the most difficult in forensic medicine. The aim of this study was to analyse pulmonary tissue reactions in death by drowning. In particular, we focused on the immunohistochemical expression of P-selectin, SP-A, HSP70, AQP-5 and fibronectin to investigate our expression in drowning and to understand whether there are differences between Saltwater drowning (SWD) and Freshwater drowning (FWD), which may indicate a different pathophysiology.

We retrospectively investigated 10 cases of SWD (Mediterranean Sea) from the Institute of Legal Medicine of Genoa (Italy), and 10 cases of FWD (Lake of Geneva) from the University Center of Legal Medicine of Geneva (Switzerland). As control group, we examined 10 cases of death by acute external bleeding, characterized by minimal respiratory distress.

As compared to controls, in SWD cases the results showed a decrease of SP-A expression with membrane patterns. Furthermore, we observed a greater SP-A expression with granular pattern in drowning cases without statistically significant difference between SWD and FWD. For the markers AQP-5, HSP70, fibronectin and P-Selectin, no statistically significant differences were found between SWD, FWD and controls.

Keywords: drowning; aquaporin 5; surfactant protein A; fibronectin; P-selectin; heat shock protein 70; forensic histopathology.

Introduction

Worldwide, drowning ranks third among the causes of accidental fatalities [1], claiming over 370,000 lives annually. Generally the post-mortem diagnosis of drowning is one of exclusion based on several autopsy signs such as mushroom-like foam around the mouth and the nose, subpleural petechiae (Paltauf's hemorrhages), water in the stomach, acute pulmonary emphysema, pleural effusion and eventually the presence of diatoms in bone marrow, spleen and brain [2].

Although there are traditionally described pathophysiological differences between Freshwater drowning (FWD) and Saltwater drowning (SWD), relative to the inverse osmolality gradients created by the two drowning mediums, scarce and blurry macroscopic/histological differences between SWD and FWD have been reported in the literature [2-6]. From a histological point of view, aqueous emphysema in FWD and pulmonary edema in SWD are described as typical features [7].

Also a difference in the volume of pleural fluid has been reported, that is generally more abundant in SWD than in FWD [8]. However, Yorulmaz et al. were not able to show any significant difference in pleural fluid between FWD and SWD [9]. Several studies [10-12] have described the ultrastructural differences between FWD and SWD via experimental drowning models. According to the latter, changes in FWD appeared to be predominantly osmotic in nature, as evidenced by

severe cell disruption, mitochondrial alteration and endothelial destruction in the pulmonary tissue. Conversely, vacuole formation and discontinuities in the alveolar cell lining were found in SWD, but not in FWD.

Other lines of research have focused on immunohistochemical evaluations of the lung tissue in death by drowning [13-14] but nevertheless no reliable and accurate technique has been validated in the routine forensic practice. One of the most studied immunohistochemical markers of drowning is the surfactant protein A (SP-A) [14-18]. This protein is produced by type II alveolar cells and physiologically lines the alveoli and smallest bronchioles. As it is the major surfactant protein, its deficiency causes respiratory distress [19-20]. Hypoxia increases its production, causing its precipitation and formation of aggregates of SP-A in the alveolar spaces, likely due to the exertions of forced breathing [20].

SP-A distribution patterns remain evident with advanced autolysis inasmuch as they are relatively resistant to decomposition [21]. Zhu et al. showed that the intra-alveolar aggregates of SP-A were significantly more present in FWD than SWD [14]. Some authors described a greater granular expression of SP-A in drowning victims [15,18]. According to Pérez-Cárceles et al. the immunohistochemical staining pattern of SP-A is useful for differentiating drowning, but is not helpful to differentiate FWD from SWD [17].

Aquaporins (AQPs) are a family of channel proteins that facilitate rapid influx or outflux of water molecules from cells [22]. The Aquaporin 5 (AQP-5) is the largest water channel in the lung tissue [23-24]. One study showed that hypertonic stress induces the expression of AQP-5 in murine lungs [25]. In an experimental study (on murine specimens), Hayashi et al. reported that AQP-5 mRNA is suppressed in FWD [26]. In the scientific literature we found inconclusive results as to its immunohistochemical expression patterns and only marginal debate as to their value [26].

The P-selectin is a transmembrane glycoprotein that interacts with selectin ligands on leukocytes and represents an endothelial adhesion molecule involved in leukocyte margination [27]. Ortmann and Brinkmann showed an intense and homogeneous staining pattern of P-selectin in acute non-

inflammatory lung injuries, such as in hanging and drowning. In the study, however, normal expression of Selectin-P in healthy lung tissue or eventual differences in expression between FWD and SWD were not evaluated [28].

In a recent study, Cecchi et al. concluded that P-selectin pulmonary expression is not specific because moderately expressed in natural deaths as well as in mechanical asphyxia cases, including cases of FWD and SWD [19].

As a marker, Fibronectin has been extensively studied for forensic research, especially in the context of myocardial ischemia [29-30]. The fibronectin has a major role in biological processes such as cell adhesion, invasion, differentiation and proliferation [31,32] and is early activated within inflammatory and tissue repair processes following trauma [33,34]. The fibronectin receptors are presents on the surface of alveolar macrophages and play a role in inflammatory repair processes [35]. Heat shock proteins (HSPs) play a pivotal role in cellular repair and adaptation to stress [36-38]. The heat shock protein 70 (HSP70) protects cells from a host of stresses, including heat, hypoxia, oxidative stress, and fluctuations in pH [39].

Based on a dedicated literature search conflating the postmortem immunohistochemistry findings in lung tissue, our aim was to assess the usefulness of these markers in drowning and to detect any differences in their expression between FWD and SWD.

Materials and Methods

We retrospectively selected 10 cases of SWD (Mediterranean Sea) from the Institute of Legal Medicine of Genoa (Italy) and 10 cases of FWD (Lake Geneva) from the University Center of Legal Medicine of Geneva (Switzerland).

Drowning is a diagnosis by exclusion: elements that supported diagnosis were circumstantial evidences (e.g. witnessed drownings, suicide notes), presence of foam in the airways. In every case in which diagnosis of drowning was made, we excluded another cause of death by complete

postmortem investigation including systematic histological examination and toxicological investigations.

As control group (CC), we included 10 cases of death by acute external bleeding, which is characterized by minimal respiratory distress.

The exclusion criteria for all groups were the following: age >65 y/o, inhalation/aspiration of blood and/or gastric contents, chronic respiratory or cardiac disease (documented by medical records, available in every included case, or by post-mortem investigations), cardio-pulmonary resuscitation, macroscopic or microscopic signs of putrefaction; postmortem interval (PMI) >72 h, ethanol or drug intoxication.

Histological and immunohistochemical techniques

Pulmonary tissue samples were collected from right and left lung in non-hypostatic areas. Two samples (central and periphery lobe regions) were collected from each lobe. Only one sample from each lung was selected for this study (random selection for the lobes). According to our internal protocols, the pulmonary samples were collected by means of a scalpel blade to avoid any artefacts, then fixed in 4% formaldehyde solution in PBS for 24h, dehydrated in alcohol and then embedded in paraffin. 5µm-thick paraffin-embedded sections were cut and processed for a standard histological examination (hematoxylin–eosin staining) and for immunohistochemistry (IHC) reactions.

IHC reactions was performed in all cases by using the following antibodies:

- Aquaporin 5 (AQP-5) Rabbit/Abcam ab9230 (diluted 1:200)
- P-Selectin (CD62P) Mouse/Abcam ab6632 (diluted 1:200)
- Surfactant Protein A (SP-A) Rabbit/Millipore AB3420-I (diluted 1:200)
- Heat Shock Protein (HSP70) Mouse/Millipore 386032 (diluted 1:200)
- Fibronectin (Fb) Rabbit/Dako A0245 (diluted 1:2000)

For IHC of AQP-5, HSP70 and SP-A the samples were de-waxed, hydrated, and subjected to epitope retrieval with Dako Target-1X at 95°C. For CD62P and Fb, samples were de-waxed, hydrated and subjected to epitope retrieval with K proteinase (to increase membrane permeability to antibodies). The staining procedure was performed as follows: incubation with blocking solution (1% H_2O_2 in 10% methanol with aluminum); wash buffer; incubation with bovine serum albumin; incubation at room temperature with primary antibodies in Dako REAL^{TM}; wash buffer; incubation with biotinylated immunoglobulins; wash buffer; incubation with antibodies Streptavidin-horseradish peroxidase (only for HSP70 and Fb) or solution ABC-Vectastain^{TM} (for AQP-5, CD62P, SP-A); wash buffer; DAB (3,3'-diaminobenzidine) for AQP-5, CD62P, SP-A, HSP70 or aminoethyl carbazole (AEC) for Fbas a chromogenic substrates; wash in distilled water and nuclear staining with hemalaum.

Analysis of immunostaining

A general and overall evaluation of each slide was performed by using an optical microscope at several magnifications (x2, x4, x10, x20, x40). For each immunohistochemical slide, 7 microscopic fields (x20 magnification) were randomly selected and assessed in order to the intensity and the distribution of the positive immunostaining with a semiquantitative method. The reaction intensity of AQP-5, HSP70, CD62P and Fb was graduated semiquantitatively as absent (score 0), mild (score 1), moderate (score 2) and intense (score 3).

According to Zhu et al. [40], the reaction for SP-A was assessed in order to the distribution of the immunostaining and scored in order to the reaction intensity as follows:

- Membranous or linear staining: negative (score 0), sparsely and weakly positive (score 1), diffusely and clearly positive (score 2), strongly positive (score 3);
- Granular staining: negative (score 0), weakly positive (score 1), positive with a few massive aggregates of stained granules (score 2), intensely and diffusely positive with many massive aggregates of stained granules (score 3).

The slides were investigated under blinded conditions by a single investigator trained in forensic histopathology and experienced with IHC investigations. Areas of atelectasis were not taken into account so as to avoid undue interference with the results. To lessen the possibility of false positives from artefacts at the margins of lesions, these areas were also excluded from the count.

Statistical analysis

A student's t-test was used to evaluate the differences in grading and number of lung parenchyma between FWD versus SWD, FWD versus control group and SWD versus control group. Significance was assumed for $p<0.05$.

Results

Macroscopic pulmonary findings

The macroscopic examination of the lungs showed different degrees of acute emphysema (in FWD only) congestion and edema of the parenchyma in both FWD and SWD. The mean pulmonary weight of the right lung was 684 g in SDW group, 650 g in FWD group. The mean pulmonary weight of the left lung was 633 g in SDW group, 606 g in FWD. The macroscopic findings of each case are shown in Table 1 (ESM).

Hematoxylin–Eosin staining

The standard histological examination of the lung tissue showed different degrees of alveolar vascular congestion, some intra-alveolar hemorrhages and large fields of alveolar edema in all samples from both FWD and SWD groups. Acute pulmonary emphysema was marked and widespread in samples from FWD group. Minimal and focal alveolar distention associated to areas with atelectasia were detected in samples from SWD group. The standard histological examination of the control group showed mild alveolar edema in only 1 cases and a focal vascular congestion in only 1 case. The findings of the standard histological examination are shown in Table 1 (ESM).

Immunohistochemistry

Surfactant Protein-A

Table 2 (ESM) shows the semi-quantitative results for SP-A obtained in the cases analysed. In order to the membranous or linear pattern, we observed a significant expression of SP-A in control group (score of 3 in 5 cases) and a reduced expression in SWD group (score of 1 in 6 cases). We found no statistically significant differences in the intensity of expression between control cases and FWD nor between SWD and FWD. The only statistically significant difference ($p < 0.05$) was found between control group and SWD with a notable reduction of membrane SP-A in SWD as compared to controls was highlighted.

As regards the granular expression of SP-A, this was observed both in the form of free aggregates within alveoli and inside alveolar macrophages. A high intra-alveolar granule score of SP-A was significantly ($p < 0.05$) more frequently observed in cases of drowning (both FWD and SWD) than in control cases (Fig. 1). Finally, no significant difference was observed between FWD and SWD.

Fibronectin

In all groups, a diffuse expression of fibronectin in the alveolar walls was observed. Generally, there was very mild and/or absent expression in the peripheral bronchi (only 3 cases in SWD group, 2 cases in FWD group and 1 case in the control group) (Fig. 2A). An intense expression (score 3) in the alveolar wall was seen in 3 cases of SWD and 3 cases of FWD. Only one case from the control group showed a completely negative reaction. No statistically significant differences were found between groups (Table 2 ESM). In fact, a marked positivity was also observed in the control cases.

P-selectin

With the exception of alveolar capillaries that were uniformly negative, a widespread pattern of expression of P-selectin was found in the endothelia of all types of vessels, especially veins (Fig. 2B). Endothelial cells showed strong intensity (score 3) only in one case in the SWD group, in 3 cases of FWD and in 1 case in CC. Completely negative expression was found only in SWD and CC. No statistically significant differences were observed between the different comparison groups (Table 2 ESM).

Aquaporin 5

AQP-5 was immunohistochemically expressed in the bronchiolar epithelium in all the groups analyzed, albeit non-uniformly (4 cases in SWD, 4 cases in FWD and 5 in controls) in the alveolar wall (type I alveolocytes) (Fig. 2C). As shown in Table 2, the same intensity of expression was recorded in both FWD and SWD. As regards intensity, the bronchial epithelium showed expression (score 3) of AQP-5 in a single case of SWD, 2 cases of FWD and 5 control cases, whereas only in a single case of FWD the antibody was not expressed at all.

No statistically significant differences were found between the different groups analyzed and the control cases.

HSP70

In all the investigated groups, although to different degrees of intensity, expression was observed in the bronchiolar structures (muscular and epithelial), vessels (muscular and endothelial), alveolar walls and endoalveolar macrophages (Fig. 2D). Strong expression of HSP70 was observed in 1 case of SWD and 1 case of FWD. Overall, no statistically significant differences were observed between the different comparison groups (Table 2 ESM). Positivity (to varying degrees of intensity) of the marker was observed in all control cases.

Discussion

The aim of this study was to investigate the expression of selected immunohistochemical markers in death by drowning and to verify whether there are differences in their expression between SWD and FWD, which could indicate a different pathophysiology. We used strict exclusion criteria, that may limit the application of our results to the forensic practice but that are necessary in order to minimise possible variables and interferences that can alter the results and lead to evaluation errors. That is why we managed to include only a limited number of selected cases.

Several studies have described hemodynamic and biochemical differences between fatal drownings in fresh and salt water [41-43]. FWD entails diffusion of hypotonic fluid into the intravascular compartment causing hypervolemia, hemodilution and hemolysis. On the contrary, in SWD the electrolytes present in the drowning medium may, to a limited extent, permeate the bloodstream. Of greater impact, however, plasma and fluid are drawn into the alveoli causing hypovolemia and hemoconcentration due to the concentration gradient [44]. This pathophysiological mechanism would explain the attenuation of AQP-5 mRNA expression in FWD compared to SWD described by Hayashi et al [26]. It is important here to state that the clinical impact of such molecular pathways have been recently criticised [45]. According to some Authors the distinction often made between drowning in fresh water or salt water is not clinically relevant because the volumes of liquid ingested in the event of drowning are too weak to be able to cause volume or electrolyte imbalance [45].

According to the results of our study, a statistically significant difference in the expression of AQP-5 between FWD and SWD would not be detectable by immunohistochemistry alone. As such it should not be relied on to distinguish the two different forms of drowning. Consequently, one may hypothesize that although the hypotonic stimulus due to fresh water aspiration induces a reduction of AQP-5 mRNA in an attempt to prevent hemodilution, the short-lived agonic period is too brief to produce any immunohistochemically appreciable differences in protein levels.

On the contrary, our study results do confirm that there is a rationale to considering SP-A a potentially useful marker in the diagnosis of drowning. Specifically, an intense and diffuse granular

pattern of expression of SP-A was statistically significant ($p < 0.05$) for drowning cases as compared to control cases. Nevertheless, it still remains a relatively non-specific marker, inasmuch as immunohistochemical positivity to it was also described in other types of violent (i.e. mechanical) asphyxia [20].

However, contrary to the findings reported by Zhu et al [14], we failed to observe a statistically significant difference between cases of FWD and SWD. Therefore, our results offer no proof to substantiate the use of this marker in the differential diagnosis between the two forms of drowning.

In contrast to our expectations, we observed a low and statistically significant ($p < 0.05$) linear pattern expression of SP-A in SWD cases as compared to controls. Instead, no significant difference was found between FWD and control cases nor between SWD and FWD.

This result could be explained by the fact that the damage to lung tissue appears greater in cases of drowning in salt water than in fresh water [46]. Underlying the alveolar distress there seems to be more direct (mechanical-asphyxial) damage in SWD, but also a greater activation of the acute inflammatory response. Therefore, it may be hypothesized that the greater the damage to the pulmonary parenchyma in SWD, the greater the degradation of SP-A along the alveolar walls. Meanwhile, in FWD cases, membrane SP-A would present an intermediate expression between those of controls and SWD cases, due to the proportionally lesser lung damage and corresponding inflammatory reaction.

Weis et al [47] reported a substantial increase in its immunohistochemical expression in cases of burn shock as compared to hemorrhagic shock (in which an endothelial expression was detectable in 26%). This result is in contrast to our findings in which a baseline P-selectin expression pattern was observed in almost every case (i.e. 9 out of 10).

Our findings, therefore, support the non-specificity of this marker in the lung, in line with a report by Cecchi et al [20]. Specifically, this marker seems to be physiologically expressed in the endothelia of pulmonary vessels, as our data confirmed. In short, based on our findings, immunohistochemical evaluation of this marker has no role in the diagnosis of drowning.

Likewise, also for the immunohistochemical analysis of fibronectin, there are no reported supporting findings related to its significance. Indeed, diffuse immunohistochemistry staining was observed in pulmonary parenchyma, albeit with nuanced differences in intensity, in all groups. All the more, no statistically significant differences were found between SWD, FWD and CC.

The results we obtained do, however, appear to contrast with those reported by Bohnert et al [33]. In this study, the authors reported that in vital burns pulmonary expression of fibronectin (especially on alveolar macrophages, alveolar epithelial cells and, unassociated with cells, in the matrix of interalveolar septa) was significantly higher than in controls (unselected autopsy cases included fatalities due to both natural and non-natural causes) or in cases of post-mortem burn [33]. The latter maintain that the physiological expression of fibronectin in lung tissue is extremely limited. One must take into account that, unlike the latter study, which considered a heterogeneous control group, in our study the control cases included only cases of hemorrhagic shock with extremely short-lived agonic periods (several minutes). Under such circumstances the pulmonary stress is presumed negligible. In fact, fibronectin is generally present in the pulmonary interstitium and is produced by type II alveolar and bronchial epithelial cells [48].

Lastly, our results indicate that lung HSP70 immunohistochemistry evaluation is of no value in the diagnosis of drowning nor in differentiating between FWD and SWD. Indeed, there was a baseline expression in the bronchiolar epithelium and in the wall of the pulmonary vessels in many control cases, moreover no statistically significant difference in intensity between the different groups was observed. This result may be related to the fact that in drownings (whether SWD or FWD) the agonic period is generally brief and insufficient to result in a marked expression of HSP70. Neither is it significantly higher than control cases, as reported by Marschall et al who investigated burn shock [49]. In this study, the authors reported a significantly higher expression of HSP70 in the tracheal, bronchial and pulmonary epithelia and parenchyma as well as in the intraluminal erythrocytes and leukocytes of victims of fire fatalities compared to a control group of unselected

cases including natural and non-natural causes of death [49]. The authors suggested an increased pulmonary expression of this protein due to thermal damage as compared to control cases.

To corroborate our interpretation, Doberetz et al [50] also reported that the expression of HSP70 in lung tissue was higher in cases of burn shock than in the control cases and the degree of immunohistochemical expression depends considerably on the survival time. In fact, the longer the survival time, the greater the expression of HSP70, with long-term survivors having extremely high levels.

Conclusion

In conclusion, in contrast to the results reported by different authors in the literature, in our study showed none of the investigated markers (i.e. AQP-5, HSP70, fibronectin and P-selectin) showed a statistically significant difference between SWD and FWD. Only the immunohistochemical expression of SP-A may, be of help for forensic practice, including its evaluation with data from circumstantial, macroscopic and histological investigation.

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Figure Legends

Fig. 1: Immunohistochemical expression of SP-A in saltwater drowning (A x10), freshwater drowning (B x10) and control group (C x10). Granular pattern of SP-A was significantly more frequently detected in drowning than in control cases.

Fig. 2: Immunohistochemical expression of fibronectin, P-selectin, AQP-5 and HSP70. A) Fibronectin showed a diffuse expression in the alveolar walls x10. B) P-selectin was found in the endothelia of all types of vessels x10. C) AQP-5 was immunohistochemically expressed in the bronchiolar epithelium in all the groups analysed x10. D) HSP70 was expressed in the bronchiolar structures (muscular and epithelial), vessels and alveolar walls x10. No statistically significant differences were observed for these types of markers.

	Saltwater Drowning		Freshwater Drowning		Control Group	
	Macroscopic findings	**Histological findings**	**Macroscopic findings**	**Histological findings**	**Macroscopic findings**	**Histological findings**
Case n.1	External foam, subpleural petechiae, lung congestion and edema	Mild alveolar edema, severe vascular congestion with intra-alveolar hemorrhages	External foam, subpleural petechiae, overdistension of lung, water in the stomach	Mild alveolar edema, moderate vascular congestion, acute pulmonary emphysema	Pale lungs	Mild alveolar edema
Case n.2	External foam, subpleural petechiae, water in the stomach, lung congestion and edema	Moderate alveolar edema, mild vascular congestion with intra-alveolar hemorrhages	External foam, overdistension of lung, lung congestion and edema	Moderate alveolar edema, moderate vascular congestion, acute pulmonary emphysema	Normal	Normal
Case n.3	External foam, water in the stomach, lung congestion and edema	Mild alveolar edema, mild vascular congestion, alveolar distention and atelectasia	External foam, subpleural petechiae, overdistension of lung, water in the stomach, lung congestion and edema	Mild alveolar edema, moderate vascular congestion, acute pulmonary emphysema	Normal	Normal
Case n.4	External foam, subpleural petechiae, water in the stomach, lung congestion and edema	Moderate alveolar edema, severe vascular congestion with intra-alveolar hemorrhages	External foam, subpleural petechiae, overdistension of lung, lung congestion and edema	Mild alveolar edema, severe vascular congestion, intra-alveolar hemorrhages, acute pulmonary emphysema	Pale lungs	Normal
Case n.5	External foam, subpleural petechiae, water in the stomach, lung congestion and edema	Moderate alveolar edema, moderate vascular congestion with intra-alveolar hemorrhages, alveolar distention and atelectasia	External foam, subpleural petechiae, overdistension of lung, lung congestion and edema, water in the stomach	Severe alveolar edema, moderate vascular congestion, intra-alveolar hemorrhages, acute pulmonary emphysema	Normal	Normal
Case n.6	External foam, subpleural petechiae, water in the stomach, lung congestion	Moderate alveolar edema, severe vascular congestion	External foam, subpleural petechiae, lung congestion and edema, water in the stomach	Severe alveolar edema, moderate vascular congestion, intra-alveolar hemorrhages, acute pulmonary emphysema	Pale lungs	Normal
Case n.7	External foam, subpleural petechiae, lung congestion and edema	Moderate alveolar edema, moderate vascular congestion	External foam, subpleural petechiae, overdistension of lung, lung congestion and edema	Moderate alveolar edema, moderate vascular congestion, intra-alveolar hemorrhages, acute pulmonary emphysema	Pale lungs	Normal
Case n.8	External foam, subpleural petechiae, lung congestion and edema	Severe alveolar edema, severe vascular congestion with intra-alveolar hemorrhages	External foam, subpleural petechiae, overdistension of lung, lung congestion water in the stomach	Mild alveolar edema, moderate vascular congestion, acute pulmonary emphysema	Normal	Focal vascular congestion
Case n.9	External foam, subpleural petechiae, water in the stomach, lung congestion and edema	Mild alveolar edema, severe vascular congestion, alveolar distention and atelectasia	External foam, subpleural petechiae, overdistension of lung, lung congestion and edema, water in the stomach	Severe alveolar edema, severe vascular congestion, intra-alveolar hemorrhages, acute pulmonary emphysema	Normal	Normal
Case n.10	External foam, subpleural petechiae, water in the stomach, lung congestion and edema	Moderate alveolar edema, moderate vascular congestion	External foam, subpleural petechiae, overdistension of lung, lung congestion and edema, water in the stomach	Moderate alveolar edema, moderate vascular congestion, acute pulmonary emphysema	Normal	Normal

Table 1 (ESM): Macroscopic and histological findings.

Case N°	SP-A Granular Pattern			SP-A Membranuos Pattern			Fibronectin		
	SWD	FWD	Controls	SWD	FWD	Controls	SWD	FWD	Controls
1	**2**	**3**	**1**	**1**	**2**	**3**	**3**	**1**	**1**
2	**1**	**2**	**0**	**1**	**3**	**2**	**1**	**1**	**1**
3	**1**	**3**	**1**	**1**	**2**	**2**	**1**	**3**	**2**
4	**3**	**3**	**2**	**1**	**1**	**3**	**2**	**2**	**1**
5	**2**	**1**	**0**	**2**	**2**	**2**	**2**	**1**	**1**
6	**2**	**1**	**1**	**3**	**2**	**1**	**3**	**3**	**3**
7	**1**	**2**	**0**	**2**	**1**	**3**	**1**	**3**	**1**
8	**1**	**1**	**0**	**1**	**2**	**2**	**2**	**1**	**2**
9	**2**	**2**	**0**	**2**	**2**	**3**	**2**	**1**	**0**
10	**1**	**2**	**1**	**1**	**1**	**3**	**3**	**1**	**1**
Case N°	HSP70			Aquaporin-5			P-Selectin		
	SWD	FWD	Controls	SWD	FWD	Controls	SWD	FWD	Controls
1	**1**	**2**	**0**	**2**	**2**	**1**	**2**	**1**	**1**
2	**2**	**2**	**2**	**1**	**1**	**3**	**2**	**3**	**2**
3	**1**	**1**	**1**	**2**	**1**	**2**	**0**	**1**	**0**
4	**2**	**2**	**1**	**2**	**3**	**3**	**1**	**1**	**2**
5	**2**	**3**	**2**	**2**	**1**	**3**	**2**	**3**	**3**
6	**2**	**2**	**1**	**1**	**1**	**3**	**2**	**1**	**1**
7	**1**	**1**	**1**	**1**	**0**	**3**	**1**	**2**	**1**
8	**1**	**2**	**2**	**1**	**3**	**2**	**3**	**3**	**1**
9	**3**	**1**	**1**	**3**	**2**	**1**	**1**	**2**	**1**
10	**2**	**1**	**1**	**1**	**2**	**2**	**2**	**1**	**1**

Table 2 (ESM): Mean degree of the intensity of SP-A, AQP-5, HSP70, P-selectin and Fb in saltwater drowning, freshwater drowning and control cases.

Fig. 1

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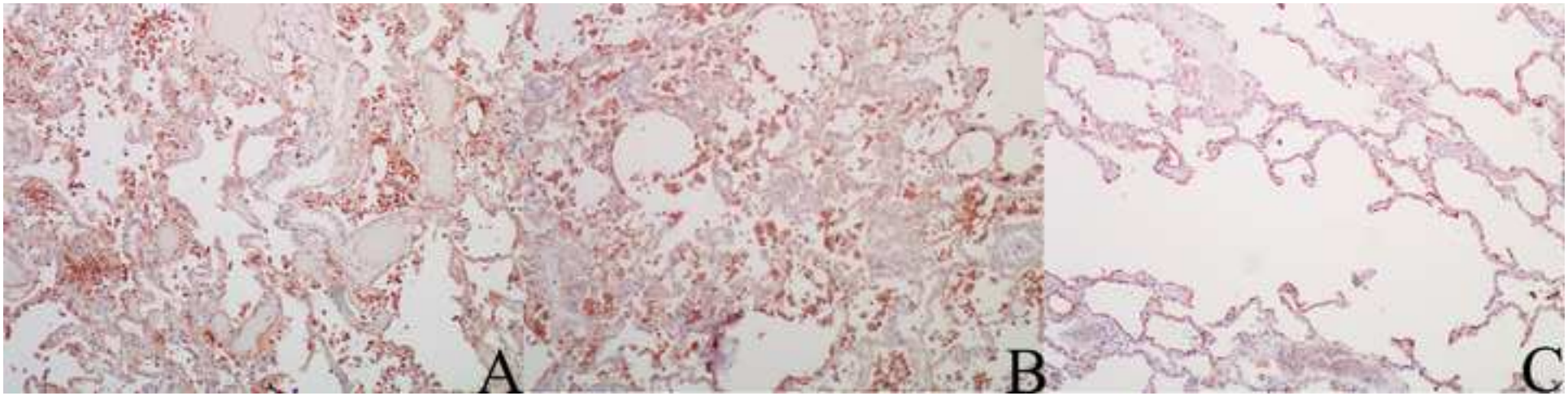

Fig. 2

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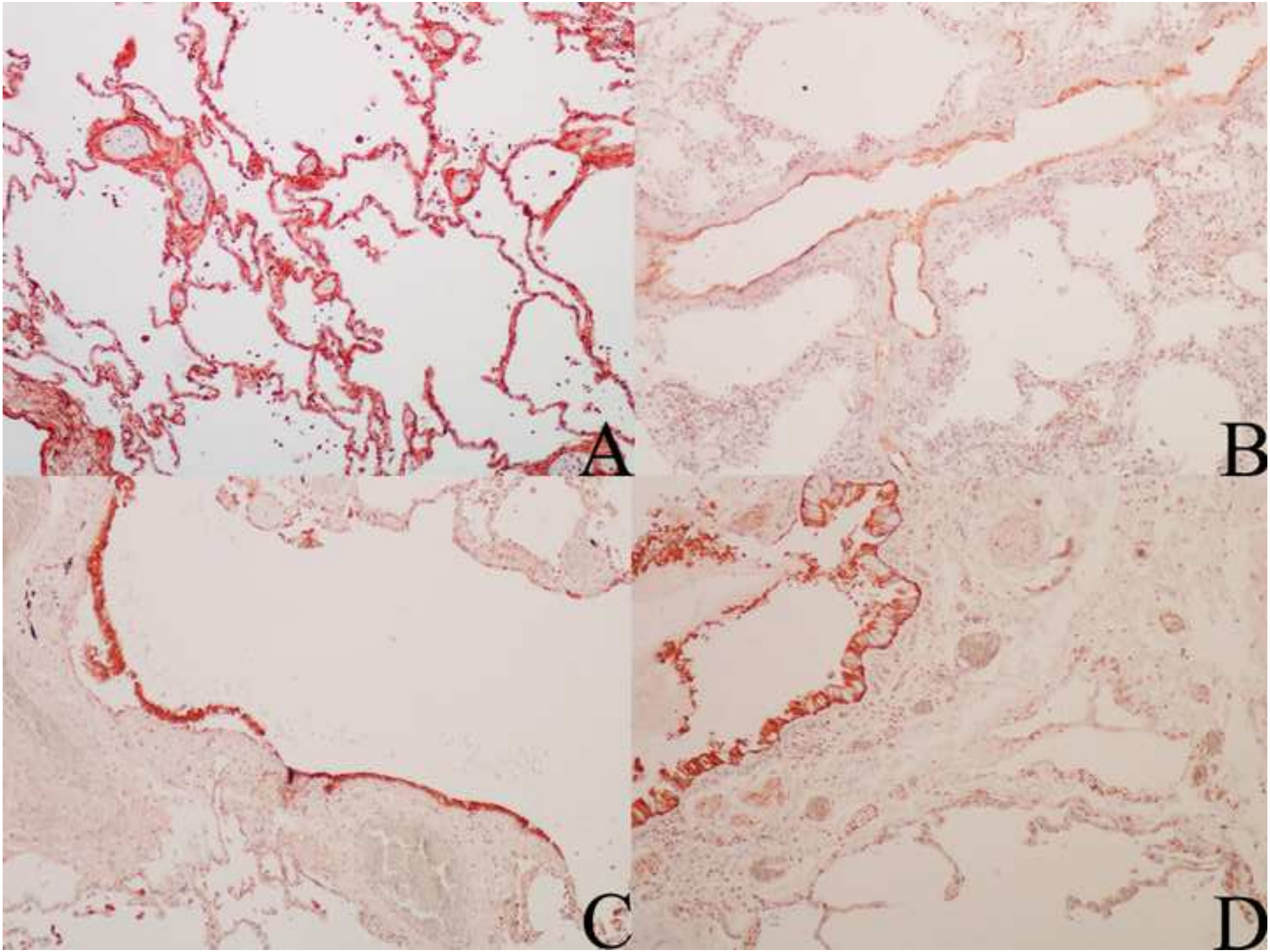

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