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ORIGINAL ARTICLE

Forensic application of intrarenal aquaporin-2 expression for differential diagnosis between freshwater and saltwater drowning

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Abstract Aquaporins (AQPs) are a family of homologous water channel proteins. In this study, the expressions of AQP1, 2, and 4 were immunohistochemically examined in kidney samples to evaluate their forensic applicability to differentiate between freshwater drowning (FWD) and saltwater drowning (SWD). Kidney samples were obtained from 51 drowning cases (23 FWD and 28 SWD) and 19 non-drowning cases. AQP1 was expressed in the proximal tubules and glomeruli, and AQP4 was localized in the collecting ducts. However, there were no significant differences in AQP1 and AQP4 expressions among FWD, SWD, and control groups. Immunohistochemically, AQP2 was predominantly expressed in the apical plasma membrane of the collecting duct principal cells in all kidney samples of FWD and SWD. Morphometrically, AQP2 expression at the apical plasma membrane of collecting ducts was significantly enhanced in SWD group, compared with FWD and control groups. On the other hand, AQP-2 expression was significantly lower in FWD group than in control group. Moreover, in drowning cases, there was no correlation between post-submersion intervals and AQP expression. From a forensic aspect, immunohistochemical detection of AQP2 in the kidney can be considered a valuable marker to differentiate between FWD and SWD.

Keywords Drowning · Immunohistochemistry · Aquaporin-2

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Introduction

A body recovered from water does not always imply that death was due to drowning. At present, the diagnosis of drowning is routinely based on the combination of a complete autopsy, histopathological findings, toxicological analyses, and the diatom test. However, froth around the nostrils and mouth and lung distension, pleural effusion, emphysema aquosum, as well as the detection of "diatom" test from multiple organs, are supportive but not conclusive evidence of drowning [1–3]. Moreover, when drowning is diagnosed, it is also indispensable to further distinguish freshwater drowning (FWD) from saltwater drowning (SWD). Macroscopically, the amount of pleural fluid and lung weights show significant differences between FWD and SWD [3–5]. Histopathologically and ultrastructurally, changes in FWD are predominantly osmotic, as evidenced by edema in alveolar-interstitial spaces and vascular wall, along with severe cellular disruption, organelle swelling, and endothelial destruction. In contrast, abnormally shaped erythrocytes and alveolar epithelium, as well as villous transformation, vacuole formation, and discontinuity of alveolar lining cells are found in SWD but not in FWD [6–9]. In addition, several studies have reported chemical analyses of serum electrolyte levels [10–14], atrial natriuretic peptide, iron, strontium, magnesium, or immunohistochemical detection of intrapulmonary macrophages, surfactant-associated protein A (SP-A) distribution, aquaporin-5 expression [15–17], or the microbiological test of bacterial markers, fecal coliforms, and fecal streptococci [18].

Aquaporins (AQPs) are a family of homologous water channels expressed in many epithelial and endothelial cell types involved in fluid transport [19–22], and 13 members (AQP 0–12) have so far been identified in mammals [22].

Table 1 Cases profiles

	No	M/F	Age (years)		PMI (h)		PSI (h)	
			Range	Mean	Range	Mean	Range	Mean
Control	19	11/8	0.5–84	57.13	5–48.5	21.92	–	–
FWD	23	14/9	0.5–90	65.64	5–72	26.35	0.5–28	8.93
SWD	28	16/12	23–80	58.62	11–72	31.59	0.5–30	10.95

M male, *F* female, *PMI* postmortem interval, *PSI* post-submersion interval, *h* hour

Our previous study showed that immunohistochemical analysis of intrapulmonary AQP5 expression in an experimental drowning model and forensic autopsy samples could differentiate between FWD and SWD, or between FWD and postmortem immersion [17]. AQPs 1, 2, and 4 are the main water channel proteins in the kidney. Thus, in the present study, we immunohistochemically examined the intrarenal expression of AQPs 1, 2, and 4 in order to explore possible markers to differentiate between FWD and SWD.

Materials and methods

Antibodies

The following polyclonal antibodies (pAbs) were used for immunohistochemical analysis in the present study: rabbit anti-human AQP1 pAbs (Santa Cruz Biotechnology Inc., CA, USA), goat anti-human AQP2 pAbs (Santa Cruz Biotechnology Inc., CA, USA), and rabbit anti-human AQP4 pAbs (Santa Cruz Biotechnology Inc., CA, USA).

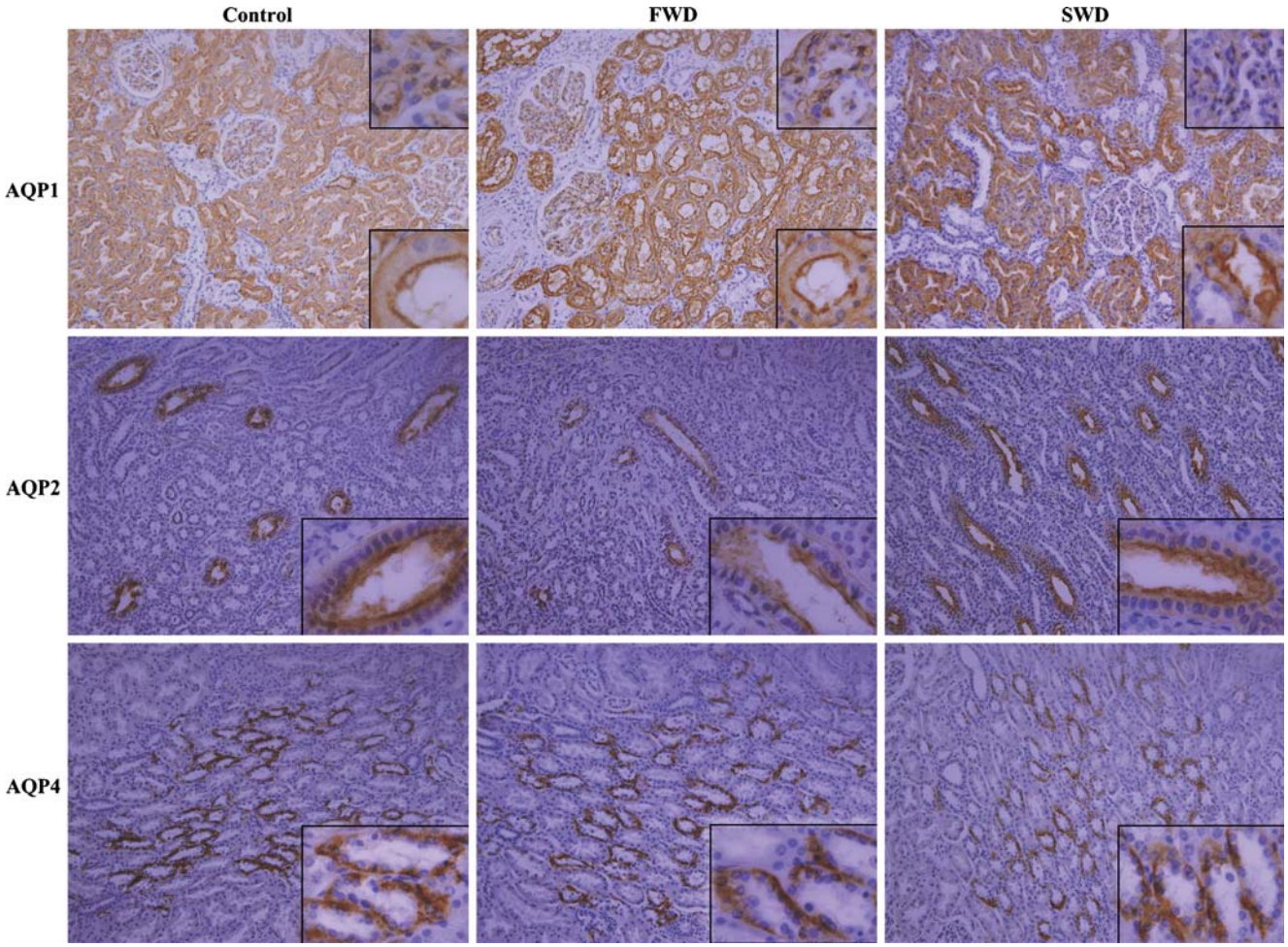

Fig. 1 Immunohistochemical analyses of AQP1, AQP2, and AQP4 expressions in kidney samples of FWD, SWD, and control cases (original magnification, ×200; *inset*, oil immersion, ×1,000). *FWD* freshwater drowning, *SWD* saltwater drowning

Autopsy samples

A total of 51 cases of drowning (30 males and 21 females) were selected from autopsy documents at our institute. In each case, the cause of death was carefully diagnosed based on autopsy and histopathological findings, toxicological data, and the diatom test. The cases were composed of 23 FWD and 28 SWD. Moreover, as control, 19 non-drowning cases with similar age and sex distribution (each five of blunt injury and fire fatality, each two of acute drug intoxication and brain injury, and each one case of sharp instrument injury, asphyxia, myocardial infarction, diabetes, and sepsis) were also analyzed. The detail profiles of all cases (gender, age, postmortem intervals, and post-submersion intervals) are shown in Table 1.

Immunohistochemical analyses

Kidney samples were fixed in 4% paraformaldehyde solution buffered with neutral phosphate-buffered solution (PBS; pH 7.2), and embedded in paraffin for sectioning (4–6 μm). Hematoxylin and eosin staining was carried out for routine histological analysis. For immunohistochemical analyses [23–25], deparaffinized sections were immersed in 0.3% H_2O_2 in methanol for 30 min to eliminate endogenous peroxidase activity, followed by incubation with PBS containing 1% normal serum corresponding to the secondary IgG and 1% bovine serum albumin to reduce nonspecific reactions. The sections were incubated with rabbit anti-human AQP1 pAbs (1:2,000), goat anti-human AQP2 pAbs (1:1,000), or rabbit anti-human AQP4 pAbs (1:1,000) at 4°C overnight. The immune complex for AQP1 and AQP4 was detected with Envision$^+$ goat anti-rabbit labeled polymer horseradish peroxidase (Dako Cytomation, Kyoto, Japan) at room temperature for 1 h. AQP2-positive signals were detected with the labeled streptavidin biotin horseradish peroxidase system (Dako Cytomation).

Morphometrical analysis

For the evaluation of intrarenal AQP expression, the ratios of the number of AQP1-positive proximal tubules, AQP2, and AQP4-positive collecting ducts to the total number of corresponding renal tubules were calculated in ten randomly selected fields (×400). The average ratio was evaluated as the AQP expression. Moreover, the number

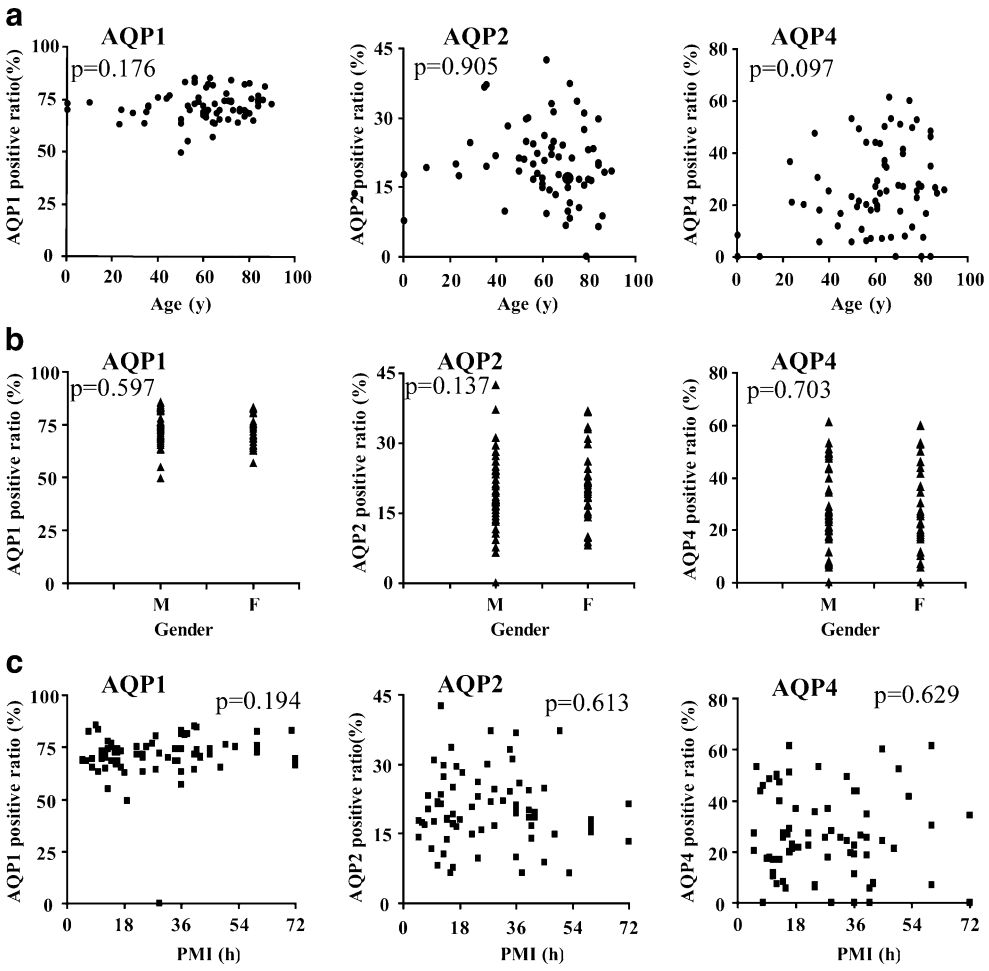

Fig. 2 **a** The relation between age and AQP expression in all cases. **b** The relation between gender and AQP expression in all cases. **c** The relation between postmortem intervals and AQP expression in all cases. These results were obtained with Spearman's correlation coefficient by rank test

of AQP1-positive glomeruli was also calculated in 100 randomly counted glomeruli under microscopy in each section. The morphometrical evaluation was blindly performed by two investigators who had no prior knowledge of the samples.

Statistical analysis

The means and standard error of the mean (SEM) were calculated for all parameters determined in this study. Statistical significance was evaluated using one-way ANOVA with post hoc testing with the Scheffé's *F* multiple comparisons test or Spearman's correlation coefficient by rank test. $p<0.05$ was considered significant.

Results

Immunohistochemical analysis of AQPs in autopsy samples

First, we examined the distribution of AQP1, AQP2, and AQP4 in kidney samples. Consistent with previous observations [26–28], AQP1 was expressed in the apical and basolateral membranes of proximal tubules, and positive staining was also observed in glomeruli in control, FWD, and SWD kidney samples (Fig. 1). In FWD cases, AQP2 was expressed in the apical plasma membrane and cytoplasm of collecting duct principal cells. In contrast, in SWD cases, AQP2 was predominantly expressed at the apical plasma membrane of collecting duct principal cells and weakly in the cytoplasm of these cells. Moreover, the staining intensity of AQP2 in the apical membrane was stronger in the SWD than FWD group (Fig. 1). AQP4 was expressed in the basolateral plasma membrane of collecting ducts in both FWD and SWD cases (Fig. 1).

Intrarenal AQP2 expression was higher in SWD cases

There were no significant differences on age, gender, or postmortem intervals for intrarenal AQP protein expression (Fig. 2). As shown in Fig. 3, the ratio of AQP2 expression at the apical plasma membrane of collecting ducts was significantly enhanced in the SWD group, compared with the FWD and control groups. On the other hand, AQP-2 expression was significantly lower in FWD group than in control group. In contrast, there was no significant difference in the AQP1 or AQP4-positive ratio among FWD, SWD, and control groups (Fig. 3). Additionally, the number of AQP1-positive glomeruli showed no significant differences between these three groups (Fig. 3). Moreover, in drowning cases, there was no correlation between post-submersion intervals and AQP expression (Fig. 3e).

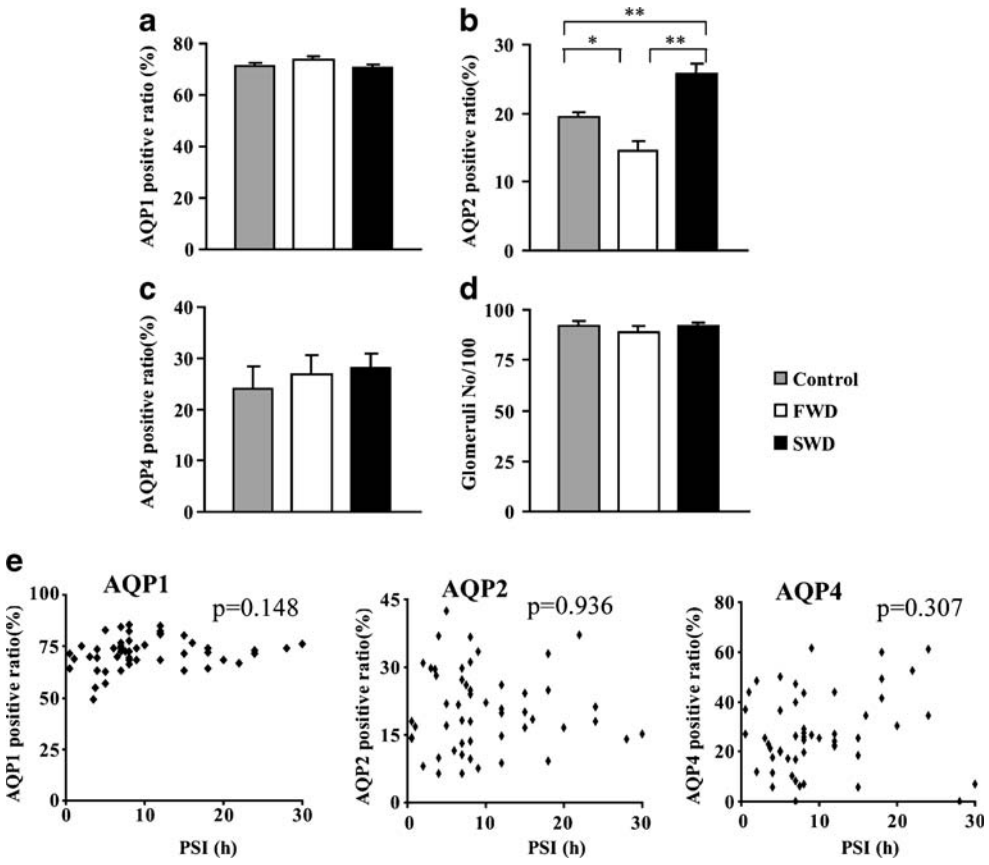

Fig. 3 **a-c** Average ratios of AQP1, AQP2, and AQP4 in the corresponding renal ductules in kidney samples. **d** The number of AQP1-positive glomeruli in kidney samples. All values represent the means ± SEM ($n=23$ in FWD; $n=28$ in SWD; $n=19$ in control). *$p\leq0.05$, **$p\leq0.01$; these results were obtained with one-way ANOVA with post hoc testing with the Scheffé's *F* multiple comparisons test. **e** The relation between post-submersion intervals and AQP expression in drowning cases. These results were obtained with Spearman's correlation coefficient by rank test

Discussion

In forensic practice, the cause of death must be definitely diagnosed. Because drowning is due to the inhalation of either fresh or seawater, resulting in lung damage and ventilation-perfusion mismatching [29], several lines of accumulating evidence have focused on lung lesions, serum electrolyte concentration [2–6, 10–14], or immunohistochemical detection of intrapulmonary SP-A protein distribution, macrophage amount, and the diatom test [1, 2, 15–17]. However, there is still no reliable technique for differentiating between FWD and SWD. Recently, several water channel proteins that can regulate osmolarity throughout the body have been cloned [22, 30, 31]. Our previous study demonstrated that immunohistochemical detection of intrapulmonary AQP5 expression was suitable for differential diagnosis of FWD and SWD [17].

In the kidney, seven aquaporin members (AQP1, 2, 3, 4, 6, 7, and 8) have been identified [22, 31–33] of which four members (AQP1, 2, 3, 4) are responsible for renal tubular water transport and body water balance [30, 31]. Consistent with previous observations, AQP1 was expressed in the apical and basolateral membranes of the proximal tubule, descending thin limb epithelium, and glomeruli [22, 26, 27], and AQP2, 3, and 4 in the collecting duct principal cells [31]. Our observations implied significant differences in the intrarenal expression of AQP2, but not AQP1 or AQP4 between FWD and SWD.

In the present study, moderately positive signals for AQP2 were found in the cytoplasm and the apical plasma membrane of collecting duct principal cells in FWD cases. In contrast, in SWD cases, AQP2 was localized predominantly in the apical plasma membrane and faintly labeled at the cytoplasm of those cells. Biochemical studies demonstrated that AQP2 could be recycled between the cytoplasm and apical plasma membrane [22, 32, 34–37]. Once AQP2 is inserted into the apical membrane, water permeability of the collecting ducts increases, enabling water reabsorption [36, 37]. Collectively, it is considered that the apical plasma membrane AQP2-positive reaction is more bioactive. Thus, in the present study, we evaluated collecting ducts with apical membrane AQP2-positive reactions.

Acute hypotonic conditions reduced the plasma membrane expression of AQP2 and increased intracellular expression of AQP2 [38]. In line with this, decreased AQP2 labeling at the apical plasma membrane was observed in FWD cases. Decreased AQP2 expression at the apical plasma membrane was due to relocation of the apical plasma membrane expressing AQP2 into the cytoplasm and not due to increased AQP2 synthesis [38]. In this study, we also observed moderate AQP2 staining in the cytoplasm of collecting duct principal cells in FWD cases. It is well-known that FWD can cause hypervolemia, marked hemodilution, hemolysis, and the decrease of serum electrolytes, except potassium, by the transportation of hypotonic water into microvessels [39, 40]. Our data indicated that AQP2 expression at the apical plasma membrane was downregulated in FWD. Decreased expression of AQP2 at the apical plasma membrane immediately following hypotonic stress may be a protective mechanism limiting apical water entry, thus reducing cell swelling and hemodilution in FWD.

In contrast, increased AQP2 apical plasma membrane expression in the collecting ducts was observed in SWD cases. This observation may be supported by evidence that acute NaCl-derived hypertonic solution incubation (≤30 min) induced rapid plasma membrane accumulation of AQP2 in rat kidney tissue slices and several kidney epithelial cell lines in vivo and in vitro [41]. This finding further supports our observation of significant increased AQP2 expression at the apical plasma membrane in SWD cases compared with FWD.

From the viewpoint of forensic pathology, our results imply AQP2 at least has potential as a marker to differentiate between FWD and SWD. Immunohistochemical detection of AQP2 expression at the apical plasma membrane in autopsy kidney samples might be valuable for the differential diagnosis of drowning.

When examining forensic samples, the influence of the postmortem interval is always taken into consideration. Based on our previous study, decomposition or autolysis did not have great influence on cytokine immunoreactivity in skin wound samples with a 3-day interval [42–45]. In the present study, kidney samples with a postmortem interval from 5 h to 72 h were used; thus, autolysis does not seem to have a significant influence on the immunoreactivity of AQP.

Finally, post-submersion intervals between 0.5 and 30 h had no significant affects on intrarenal AQP expression. Thus, when we encounter drowning cases without sufficient supportive data for FWD or SWD, immunohistochemical analysis of intrarenal AQP2, in conjunction with other markers, will greatly contribute to the differential diagnosis of FWD and SWD.

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References

1. Piette MH, De Letter EA (2006) Drowning: still a difficult autopsy diagnosis. Forensic Sci Int 163:1–9
2. Lorin de la Grandmaison G, Paraire F (2003) Place of pathology in the forensic diagnosis of drowning. Ann Pathol 23:400–407

3. Yorulmaz C, Arican N, Afacan I, Dokgoz H, Asirdizer M (2003) Pleural effusion in bodies recovered from water. Forensic Sci Int 136:16–21
4. Morild I (1995) Pleural effusion in drowning. Am J Forensic Med Pathol 16:253–256
5. Zhu BL, Quan L, Li DR et al (2003) Postmortem lung weight in drownings: a comparison with acute asphyxiation and cardiac death. Leg Med (Tokyo) 5:20–26
6. Brinkmann B, Fechner G, Puschel K (1983) Lung histology in experimental drowning. Z Rechtsmed 89:267–277
7. Brinkmann B, Fechner G, Puschel K (1983) Ultrastructural pathology of the alveolar apparatus in experimental drowning. Z Rechtsmed 91:47–60
8. Reidbord HE, Spitz WU (1966) Ultrastructural alterations in rat lungs. Changes after intratracheal perfusion with freshwater and seawater. Arch Pathol 81:103–111
9. Nopanitaya W, Gambill TG, Brinkhous KM (1974) Fresh water drowning. Pulmonary ultrastructure and systemic fibrinolysis. Arch Pathol 98:361–366
10. Lorente JA, Villanueva E, Hernandez-Cueto C, Luna JD (1990) Plasmatic levels of atrial natriuretic peptide (ANP) in drowning. A pilot study. Forensic Sci Int 44:69–75
11. Lorin de la Grandmaison G, Leterreux M, Lasseuguette K, Alvarez JC, de Mazancourt P, Durigon M (2006) Study of the diagnostic value of iron in freshwater drowning. Forensic Sci Int 157:117–120
12. Piette M, Timperman J, Parisis N (1989) Serum strontium estimation as a medico-legal diagnostic indicator of drowning. Med Sci Law 29:162–171
13. Azparren JE, Fernandez-Rodriguez A, Vallejo G (2003) Diagnosing death by drowning in freshwater using blood strontium as an indicator. Forensic Sci Int 137:55–59
14. Zhu BL, Ishida K, Taniguchi M et al (2003) Possible postmortem serum markers for differentiation between fresh-, saltwater drowning and acute cardiac death: a preliminary investigation. Leg Med 5(Suppl 1):S298–S301
15. Locali RF, Almeida M, Oliveira-Junior IS (2006) Use of the histopathology in the differential diagnosis of drowning in fresh and salty water: an experimental model establishment in rats. Acta Cir Bras 21:203–206
16. Zhu BL, Ishida K, Quan L et al (2002) Pulmonary immunohistochemistry and serum levels of a surfactant-associated protein A in fatal drowning. Leg Med (Tokyo) 4:1–6
17. Hayashi T, Ishida Y, Mizunuma S, Kimura A, Kondo T (2008) Differential diagnosis between freshwater drowning and saltwater drowning based on intrapulmonary aquaporin-5 expression. Int J Legal Med 123:7–13
18. Lucci A, Campobasso CP, Cirnelli A, Lorenzini G (2008) A promising microbiological test for the diagnosis of drowning. Forensic Sci Int 182:20–26
19. Jeyaseelan K, Sepramaniam S, Armugam A, Wintour EM (2006) Aquaporins: a promising target for drug development. Expert Opin Ther Targets 10:889–909
20. Verkman AS (2005) More than just water channels: unexpected cellular roles of aquaporins. J Cell Sci 118:3225–3232
21. Verkman AS (2002) Aquaporin water channels and endothelial cell function. J Anat 200:617–627
22. Nielsen S, Frokiaer J, Marples D, Kwon TH, Agre P, Knepper MA (2002) Aquaporins in the kidney: from molecules to medicine. Physiol Rev 82:205–244
23. Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2008) Expression of oxygen-regulated protein 150 (ORP150) in skin wound healing and its application for wound age determination. Int J Legal Med 122:409–414
24. Nosaka M, Ishida Y, Kimura A, Kondo T (2009) Time-dependent appearance of intrathrombus neutrophils and macrophages in a stasis-induced deep vein thrombosis model and its application to thrombus age determination. Int J Legal Med 123:235–240
25. Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2009) Detection of fibrocytes in human skin wounds and its application for wound age determination. Int J Legal Med May 123:299–304
26. Bedford JJ, Leader JP, Walker RJ (2003) Aquaporin expression in normal human kidney and in renal disease. J Am Soc Nephrol 14:2581–2587
27. Maunsbach AB, Marples D, Chin E, Ning G, Bondy C, Agre P, Nielsen S (1997) Aquaporin-1 water channel expression in human kidney. J Am Soc Nephrol 8:1–14
28. Mobasheri A, Marples D, Young IS, Floyd RV, Moskaluk CA, Frigeri A (2007) Distribution of the AQP4 water channel in normal human tissues: protein and tissue microarrays reveal expression in several new anatomical locations, including the prostate gland and seminal vesicles. Channels (Austin) 1:29–38
29. Rumbak MJ (1996) The etiology of pulmonary edema in freshwater near-drowning. Am J Emerg Med 14:176–179
30. Kwon TH, Nielsen J, Moller HB, Fenton RA, Nielsen S, Frokiaer J (2009) Aquaporins in the kidney. Handb Exp Pharmacol 190:95–132
31. Ksiazek K, Witowski J (2002) Aquaporin water channels in water balance regulation in the kidney. Przegl Lek 59:1013–1017
32. Nielsen S, Kwon TH, Christensen BM, Promeneur D, Frokiaer J, Marples D (1999) Physiology and pathophysiology of renal aquaporins. J Am Soc Nephrol 10:647–663
33. Nejsum LN (2005) The renal plumbing system: aquaporin water channels. Cell Mol Life Sci 62:1692–1706
34. Agre P, Nielsen S (1996) The aquaporin family of water channels in kidney. Nephrologie 17:409–415
35. Takata K, Tajika Y, Matsuzaki T, Aoki T, Suzuki T, Abduxukur A, Hagiwara H (2004) Molecular mechanisms and drug development in aquaporin water channel diseases: water channel aquaporin-2 of kidney collecting duct cells. J Pharmacol Sci 96:255–259
36. Nielsen S, Chou CL, Marples D, Christensen EI, Kishore BK, Knepper MA (1995) Vasopressin increases water permeability of kidney collecting duct by inducing translocation of aquaporin-CD water channels to plasma membrane. Proc Natl Acad Sci USA 92:1013–1017
37. Yamamoto T, Sasaki S, Fushimi K et al (1995) Vasopressin increases AQP-CD water channel in apical membrane of collecting duct cells in Brattleboro rats. Am J Physiol 268:C1546–C1551
38. Tamma G, Procino G, Strafino A et al (2007) Hypotonicity induces aquaporin-2 internalization and cytosol-to-membrane translocation of ICln in renal cells. Endocrinology 148:1118–1130
39. Swann HG, Brucer M, Moore C (1947) Fresh water and seawater drowning; a study of the terminal cardiac and biochemical events. Tex Rep Biol Med 5:423–437
40. Swann HG, Brucer M (1949) The cardiorespiratory and biochemical events during rapid anoxic death; freshwater and seawater drowning. Tex Rep Biol Med 7:604–618
41. Hasler U, Nunes P, Bouley R, Lu HA, Matsuzaki T, Brown D (2008) Acute hypertonicity alters aquaporin-2 trafficking and induces a MAPK-dependent accumulation at the plasma membrane of renal epithelial cells. J Biol Chem 283:26643–26661
42. Hayashi T, Ishida Y, Kimura A, Takayasu T, Eisenmenger W, Kondo T (2004) Forensic application of VEGF expression to skin wound age determination. Int J Legal Med 118:320–325
43. Kondo T, Tanaka J, Ishida Y, Mori R, Takayasu T, Ohshima T (2002) Ubiquitin expression in skin wounds and its application to forensic wound age determination. Int J Legal Med 116:267–272
44. Kondo T, Ohshima T, Mori R, Guan DW, Ohshima K, Eisenmenger W (2002) Immunohistochemical detection of chemokines in human skin wounds and its application to wound age determination. Int J Legal Med 116:87–91
45. Kondo T, Ohshima T, Eisenmenger W (1999) Immunohistochemical and morphometrical study on the temporal expression of interleukin-1alpha (IL-1alpha) in human skin wounds for forensic wound age determination. Int J Legal Med 112:249–252