

Histopathological Differentiation of Drowning in Freshwater and Saltwater in Rats: Forensic Point of View

Diferenciación Histopatológica de Ahogamiento en Agua Dulce y Agua Salada en Ratas: Punto de Vista Forense

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SUMMARY: The postmortem diagnosis of death by drowning is one of the most difficult issues in forensic pathology. We investigated possible evidence differentiating saltwater drowning from freshwater drowning by histopathological changes in brain, heart, lungs, liver, and kidneys tissues. A cross section descriptive study was carried out on eighteen 12-week-old male Wistar rats; they were divided equally into 3 groups. Group 1: control group; Group 2: death by drowning in freshwater; Group 3: death by drowning in saltwater. Immediately after death, all tested organs were removed and fixed for histopathological examination. The brain of freshwater group depicted degenerated neurocytes with dystrophic changes in the form of shrunken cell, pyknotic nuclei and deeply eosinophilic cytoplasm. The heart showed clear evidence of myocyte injuries in saltwater drowning compared to the control and freshwater groups. The kidneys of rats drown in saltwater revealed more glomerular destruction with no differences in tubulo-interstitial changes in comparison with those drown in freshwater. In the lungs, the changes in freshwater were restricted to the alveoli, and the bronchial changes were more distinctive in saltwater. No disturbed liver architecture was seen in both test groups, however hydropic degeneration, congested vessels, and sinusoids were more distinct in saltwater group. In conclusion, diagnostic differentiation between fresh and saltwater drowning was reliable in rats' lungs and heart with minimal differentiation in liver, kidneys, and brain. Further studies of drowning with different staining techniques will help to clarify the potential role of histopathological changes in body organs as indicator of drowning.

KEY WORDS: Drowning; Saltwater; Freshwater; Histopathology; Medico-legal.

INTRODUCTION

Deaths by drowning are a serious public health problem worldwide. Egypt is considered as one of the richest countries with water bodies including rivers, ponds, wells in addition to widespread seacoast. Under such circumstances, there is no wonder about the high frequency of medicolegal examiners calls to investigate deaths due to drowning. Nile rivers, lakes and seas are huge places of large incidence of drowning (Kim *et al.* 2000).

It is noteworthy that death from drowning can be defined as a death caused by submersion in a fluid. In acute

drowning, hypoxemia and irreversible cerebral anoxia are the main mechanisms of death (DiMaio & DiMaio, 1989). The World Health Organization defines drowning as "the process of experiencing respiratory impairment from submersion in liquid," with possible outcomes of death, morbidity, or no morbidity (van Beeck *et al.*, 2005).

Drowning is the third leading cause of unintentional injury death worldwide, accounting for 7 % of all injury-related deaths and 360,000 estimated worldwide annual drowning deaths with increased risk among children, males

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and individuals who have access to water bodies. Global estimates may significantly underestimate the actual public health problem related to drowning (Layon *et al.*, 2009).

The confirmation of diagnosis and determination of the cause of death in cases of drowning is considered one of the most difficult practices in forensic medicine field (Piette & De Letter, 2006). In most drowning cases, the findings retrieved after the postmortem external examination and autopsy lack any specific features and the scientific community scarcely appreciates the laboratory investigations. Differentiation between submersion death and body immersion death is considered as one of the highly targeted fields for forensic investigation (Saukko & Knight, 2015). This could be attributed to different factors including the development and advancement of body decomposition that may be caused by delay in body recovery and/or transport to mortuary.

The autopsy diagnosis of death by drowning can be one of the most difficult problems in forensic pathology because in the developing countries set up the time required to complete the inquest formalities and transport of the body to the mortuary is enough to cause the decomposition, which masks most of the *postmortem* evidences of cause of death. Most of the bodies recovered from water are in various stages of decomposition. Some murderers dispose of the dead bodies of their victims in the rivers, seas and wells to simulate death due to drowning (Sugimura *et al.*, 2010).

The exploration of new methods and signs for the precise postmortem diagnosis of drowning continues to be a priority in forensic sciences (Pérez-Cárceles *et al.*, 2012). This includes the evaluation of victims' in the different drowning environment; among them, the two most common liquids for immersion are seawater and freshwater (Piette & De Letter, 2006). Although a well-recognized cause of death, confirming drowning in bodies recovered from water poses a great challenge to the forensic pathologist because of the nonspecific postmortem findings and unreliable ancillary tests (Sugimura *et al.*, 2010).

Until this point in time, indeed, forensic scientists exert great effort to develop a genuine test for the confirmation of diagnosis of drowning, but the medical-legal diagnosis is still depending on the combination of numerous signs and exclusion of other apparent causes of death (Piette & De Letter, 2006). As well, they are looking for methods of differentiation between the different immersion fluids in cases of drowning, in such manner, the water biochemical analysis played non-conclusive role in the differentiation between seawater and freshwater in relation to their components and contaminants (Pérez-Cárceles *et al.*, 2012).

Here, the current study presents the comprehensive findings of a double-blind research. We seek to investigate structural abnormalities in various organs that might be associated with death by drowning in fresh and saltwater.

MATERIAL AND METHOD

A cross-sectional descriptive study was carried out on eighteen male Wistar rats aged 12-week-old and weighted 220–240 g at the beginning of the experiment. They were housed individually in well-aerated metal cages under standard conditions and were fed on a balanced powdered laboratory chow and tap water ad libitum. After one week of adaptation in a room with good ventilation, controlled temperature (24 ± 2 °C) and lighting (12-h light: 12-h dark), rats were divided equally and randomly into 3 groups (n= six each group). The used rats were fasted overnight.

- Group 1(G1): Control group; not exposed to drowning
- Group 2(G2): Freshwater drowning group
- Group 3(G3): Saltwater drowning group

40 mg/kg body weight of sodium thiopental was injected intraperitoneally (ip) as an anesthetic agent in the rats of the two test groups (G2, G3) in order to block the pain of the experimental animal and then placed in a cage that was then immersed into freshwater (G2) and saltwater (G3) at room temperature. The survival period, time between the beginning of fluid immersion and death was estimated. Immediately after death, the brain, heart, lungs, liver, and kidneys were harvested, weighed, and fixed in 10 % formaldehyde. Histopathological examinations were performed from the prepared Specimens. The control group of experimental animals were anaesthetized with sodium thiopental 40 mg/kg body weight intraperitoneally (ip) and then scarified by cervical dislocation at the same time of death in both test groups. The lungs of the experimental animals in each group were excised to determine the weight of the left and right lungs; the previous procedure was repeated for kidneys.

The water used in the experiment was collected in two points:

- 1) Freshwater: was collected from the Nile River of Egypt.
- 2) Saltwater: was collected from the red sea in Suez governorate, Egypt.

For collection of both types of water, 20 meters were counted, inside of the water, beginning from the edge; at 10o'clock (a.m). The collected samples were analyzed

by a salinity meter to calculate the salinity. The measured values were 31.06 ‰ in seawater and 0.03 ‰ in freshwater.

This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The research protocol with animal experimentation was approved by the Research Ethical Committee (REC), Faculty of Medicine, Suez Canal University (Research # 4184). All surgery was performed under anesthesia and every effort was made to minimize suffering.

Histopathological examination. For microscopic evaluation, brain, heart, lungs, liver, and kidneys were fixed in 10 % formalin for 24 h, and standard dehydration in ascending series of ethanol (70, 80, 95, and 100 %). Tissue samples were then cleared in xylene and embedding in paraffin wax. Sections (5 mm) were cut in a microtome and stained with hematoxylin and eosin (H-E). The sections were then viewed and photographed.

The brain, heart, lungs, liver, and kidneys histological slides were examined and scored under a light microscope by a blinded pathologist to the experimental groups for quantifying the extent of their damage.

Brain sections. Light microscopic examination assessed the integrity of the following structures: **The gray matter** with its well-organized regularly arranged six layers. The molecular layer appears rather fibrous with few neurons and neuroglia cells. **The granular layers** (inner and outer) are formed of stellate-shaped nerve cells with large round nuclei. The inner and outer pyramidal cell layers are formed of medium and large sized cells, respectively. The innermost layer constitutes the pleomorphic layer and is formed of a variety of nerve cells. **The ground** substance between the nerve cells is normally occupied with homogenous neuropil. **The white matter** is formed of homogeneously stained nerve tracts running down the cortex. **Hippocampal region:** shows normal cellular composition in all the three layers (molecular, Purkinje, and granular) (Khodeary *et al.*, 2010).

Heart sections. The examination evaluated the following parameters: cytoplasmic granular vacuolization, absence of nuclei in myocytes, interstitial edema, congested capillaries and lymphocytic infiltrate and according to the extent of cardiac tissue involved (Jokinen *et al.*, 2011).

Lung sections. The examination evaluated the following parameters: edema, hemorrhage and /or congested alveolar capillaries, cell infiltration, and alveolar septal thickening and according to the extent of lung tissue involved (Ji *et al.*, 2016). The right lower lung lobe was excised and immediately

immersed into 10 % formalin. The samples were sectioned and stained with hematoxylin and eosin for light microscopy. The severity of microscopic injury was graded from 0 (normal) to 4 (severe) based on the following categories: neutrophil infiltration, interstitial edema, hemorrhage, and hyaline membrane. The sum of all scores was combined to calculate a composite score as previously described (Ji *et al.*, 2016).

Liver sections. The examination evaluated the following parameters: liver architecture, hepatocytes cell injury, hydropic degeneration, congested vessels, portal inflammation, congested portal vessels and according to the extent of hepatic tissue involved (Ishak *et al.*, 1995). The hepatic scoring was according to modified staging (score 1): architectural changes, fibrosis and cirrhosis and Modified histological activity index (HAL) grading (score 2): necro inflammatory scores (Ishak *et al.*, 1995).

Kidney sections. The examination evaluated the following parameters: Tubules, interstitium and Glomerular damage and according to the extent of renal tissue involved (Cao *et al.*, 2000).

Statistical analysis. was done using SPSS software, version 23 for windows (SPSS, Inc., Chicago, IL, USA). Number of rats per each group was stated in the table or figure legends. After ascertaining the homogeneity of variance between treatment groups by Bartlett's test and to analyze mean differences between experimental groups for each parameter separately, one-way analysis of variance (ANOVA) followed by Tukey-Kramer test were performed. Values of $P < 0.05$ were considered statistically significant.

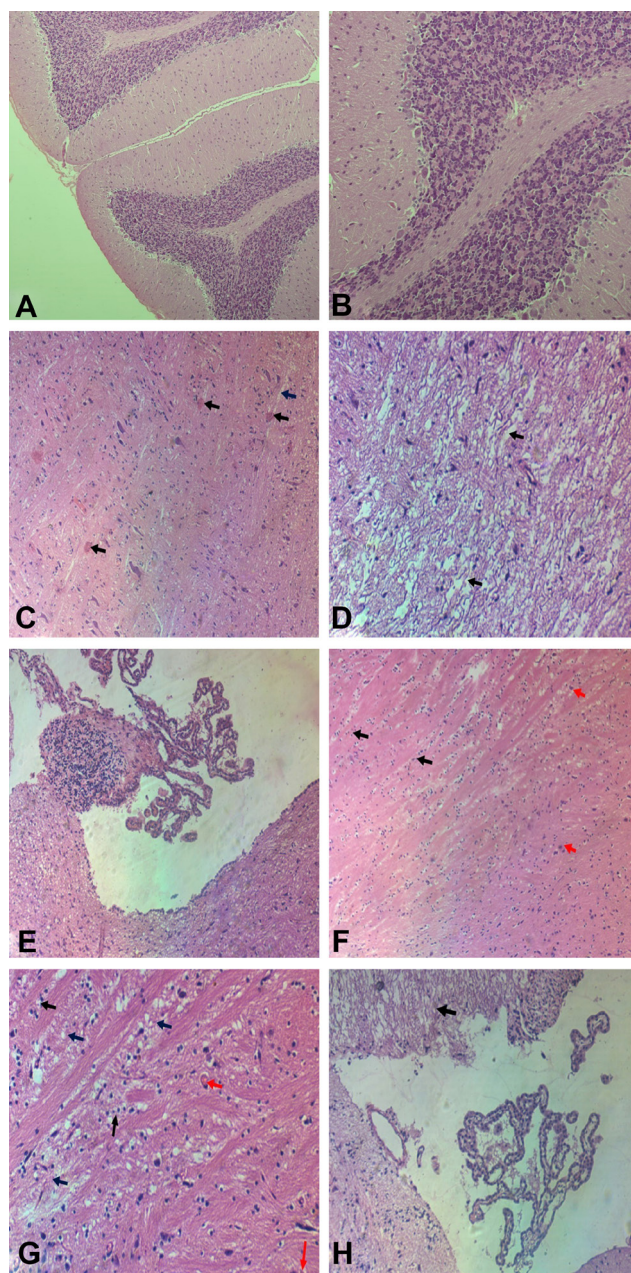
RESULTS

The survival period, time between the beginning of fluid immersion and cardiac arrest, was shorter than 3 minutes in both test groups. The lung weights were higher in both groups of drowning rats compared to normal weights in control group.

Histopathological changes in the brain. Microscopic examination of brain tissue in control group showed multiple large pyramidal cells with distinct apical dendrites, large open face vesicular nuclei and visible nucleoli. Different cells and blood capillaries are scattered within the neuropil (Fig. 1A, B). freshwater drowning group showed interstitial edema, degenerated neurocytes with dystrophic changes in the form of shrunken cell, pyknotic nuclei and deeply eosinophilic cytoplasm "Red neurons" and infiltration of choroid plexus with chronic inflammatory

Table I. The microscopic findings in the brain of control and freshwater drowning and saltwater drowning rats. (n=6).

Groups	Findings
Control group	<ul style="list-style-type: none"> ▪ Multiple large pyramidal cells with distinct apical dendrites. ▪ Large open face vesicular nuclei and visible nucleoli.
Freshwater group	<ul style="list-style-type: none"> ▪ Different cells and blood capillaries are scattered within the neuropil. ▪ Interstitial edema. ▪ Degenerated neurocytes with dystrophic changes in the form of shrunken cell. ▪ pyknotic nuclei and deeply eosinophilic cytoplasm “Red neurons”.
Saltwater group	<ul style="list-style-type: none"> ▪ Infiltration of choroid plexus with chronic inflammatory cells. ▪ Interstitial edema. ▪ Few degenerated neurocytes “Red neurons”. ▪ Infiltration of brain parenchyma with lymphocytes with loss of neurons. ▪ No chronic inflammation in choroid plexus.



cells (Fig. 1C, D, E). However, saltwater drowning group showed interstitial edema, few degenerated neurocytes “Red neurons”, infiltration of brain parenchyma with lymphocytes with loss of neurons and no chronic inflammation in choroid plexus (Fig. 1F, G, H; Table I).

Histopathological changes in the heart sections. Microscopic examination of cardiac tissue of control group showed intact muscle fibers arranged as bundles with normal connective tissue spaces (Fig. 2A, B). On the other hand, freshwater drowning group showed evidence of myocyte injury; cytoplasmic granular vacuolization, absence of nuclei in some myocytes, interstitial edema and mild lymphocytic infiltrate and congested capillaries (Fig. 2C, D). However, the rats in saltwater drowning group showed evidence of myocyte injury; cytoplasmic granular vacuolization, absence of nuclei in many myocytes, interstitial edema and congested capillaries and mild lymphocytic infiltrate with relatively higher scoring in this group (Fig. 2E, F; Table II).

Fig. 1. A photomicrograph of transverse section in the rat brain stained with H&E stains shows (A) and (B) Micrograph from normal control group showing normal pyramidal cells with basophilic cytoplasm, rounded nucleus and prominent nucleolus. Clear blood capillaries and normal fibrous appearance (C) Freshwater drowning group shows red neuronal degeneration (Black arrows) and interstitial edema (Blue arrow) (H&E, 10x). (D) Freshwater drowning group shows interstitial edema (Black arrows) (H&E, 10x). (E) Freshwater drowning group shows infiltration by mono-nuclear inflammatory cells in the choroid plexus (Black arrow) (H&E, 10x). (F) Saltwater drowning group shows infiltration of brain parenchyma with lymphocytes (Black arrows) with significant loss of neurons. Right side of figure shows few scattered red degenerated neurons (Red arrows) (H&E, 10x). (G) Saltwater drowning group shows infiltration of brain parenchyma with lymphocytes (Black arrows) with significant loss of neurons. Few scattered red degenerated neurons are seen (Red arrows). Interstitial edema is seen (Blue arrows) (H&E, 40x). (H) Saltwater drowning group shows no infiltration by mono-nuclear inflammatory cells in the choroid plexus. Upper part of figure shows interstitial edema (Black arrow) (H&E, 10x).

Table II. Comparison between changes found in the heart sections of control, freshwater drowning and saltwater drowning rats regarding the histopathological grading (n=6).

Pathological changes in the heart	Control group	Freshwater group	Saltwater group
	Histopathological Grading		
Myocyte vacuolization.	0	1	3
Myocyte necrosis.	0	1	3
Mono-nuclear infiltration	0	1	1
Fibrosis	0	0	0
Interstitial edema	0	1	2
Congested capillaries	0	3	3

Absent (grade 0) no lesions detected.

Minimal (grade 1) lesions involved less than 10 % of the heart.

Mild (grade 2) lesions involved 11 %–40 % of the heart.

Moderate (grade 3) lesions involved 41 %–80 % of the heart.

Marked (grade 4) lesions involved greater than 81 % of the heart.

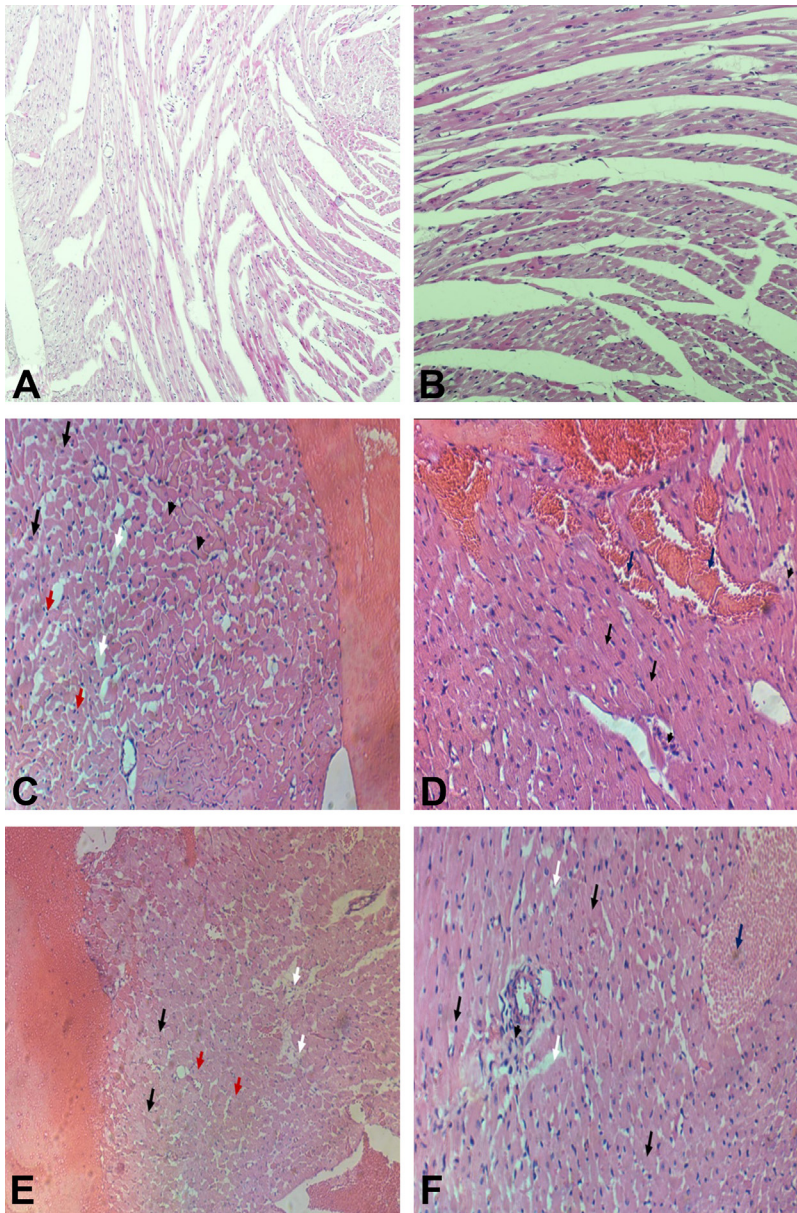
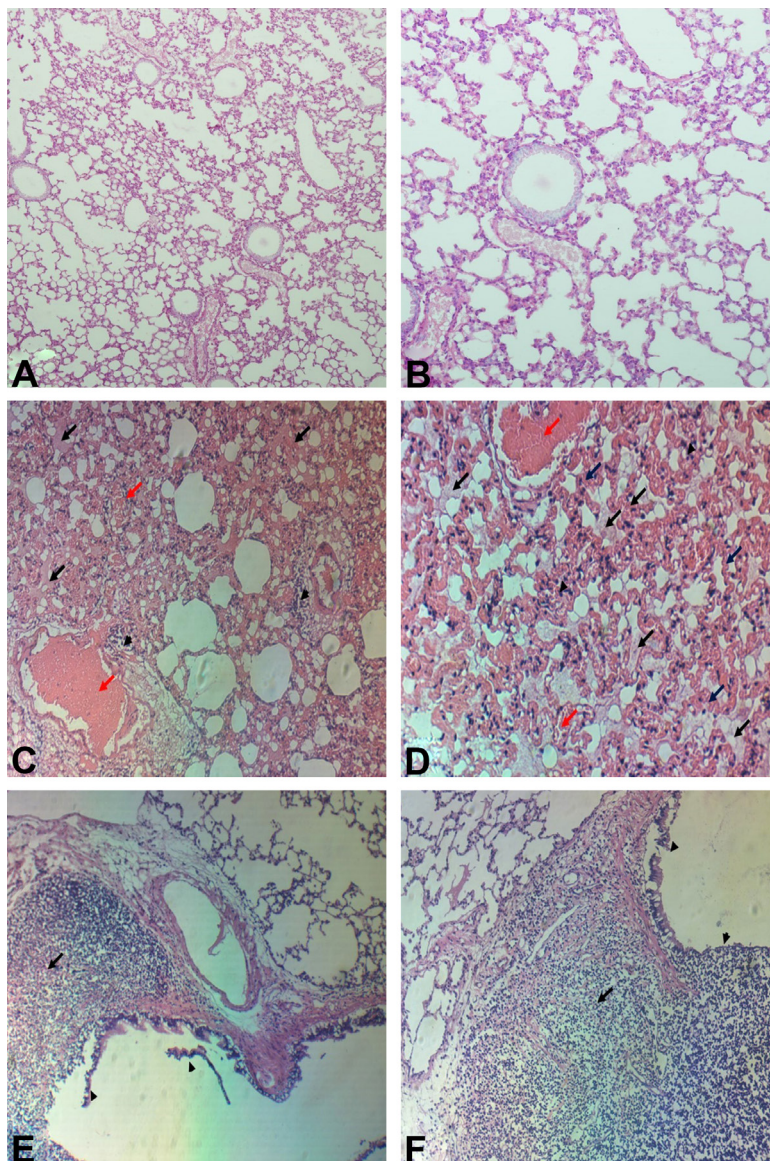


Fig. 2. A photomicrograph of transverse section in the rat heart stained with H&E stains shows (A) and (B) Photomicrograph of heart section in the normal control group showing normal branched cardiomyocytes with central oval vesicular nucleus. The cells are separated with little amount of connective tissue. (C) Freshwater drowning group shows evidence of myocyte injury: cytoplasmic granular vacuolization (Black arrow), absence of nuclei in some myocytes (Red arrows), interstitial edema (White arrows) and mild lymphocytic infiltrate (Arrow heads) (H&E, 10x). (D) Freshwater drowning group shows evidence of myocyte injury: Absence of nuclei in some myocytes (Black arrows), congested capillaries (Blue arrows) and mild lymphocytic infiltrate (Arrow heads) (H&E, 20x). (E) saltwater drowning group shows evidence of myocyte injury: cytoplasmic granular vacuolization (Black arrow), absence of nuclei in many myocytes (Red arrows) and interstitial edema (white arrows) (H&E, 10x). (F) Evidence of myocyte injury: Absence of nuclei in many myocytes (Black arrows), congested capillaries (Blue arrows), interstitial edema (White arrows) and mild lymphocytic infiltrate (Arrow heads) (H&E, 20x).

Table III. Comparison between changes found in the lung sections of control, freshwater drowning and saltwater drowning rats regarding the histopathological grading (n=6).

Pathological changes in the Lung	Control group	Freshwater group "Changes in alveoli"	Saltwater group "Changes in Bronchioles"
Histopathological Grading			
Intra-alveolar edema	0	2	0
Hemorrhage/congested blood vessels	0	2	0
Cell infiltration	0	1	3
Alveolar septal thickening with edema	0	2	0
Total score	0	7	3

(Score 0) Absent: no lesions detected.
 (Score 1) Minimal: lesions involved less than 10 % of the lung section.
 (Score 2) Mild: lesions involved 11 %–40 % of the lung section.
 (Score 3) Moderate: lesions involved 41 %–80 % of the lung section.
 (Score 4) Marked: lesions involved greater than 81 % of the lung.



Histopathological changes in the lung sections. The observed weight of the lungs in G2 and G3 represented higher values in comparison to the control G1 ($p < 0.05$). However, there was no statistically significant difference between the lungs' weights of animals in G2 and G3 ($p > 0.05$).

Microscopic examination of lung tissue of control group showed no lung injury, with samples demonstrating simple columnar epithelium or cuboidal epithelium respiratory bronchioles, and regular wall structures in the

Fig. 3. A photomicrograph of transverse section in the rat lungs stained with H&E stains shows: (A) and (B) Photomicrograph of a section from the normal control group revealing normal lung structure with uniform alveoli and no abnormality detected in both alveolar architecture and epithelium. (C) Freshwater drowning group shows intra-alveolar edema (Black arrows), congested blood vessels (Red arrows) and lymphocytic infiltrate in alveolar walls (Arrow heads) (H&E, 10x). (D) Freshwater drowning group shows intra-alveolar edema (Black arrows), edematous thickening of alveolar walls (Blue arrows), congested blood vessels (Red arrows) and lymphocytic infiltrate in alveolar walls (Arrow heads) (H&E, 40x). (E) Saltwater drowning group shows infiltration by mono-nuclear inflammatory cells in walls of bronchioles (Black arrow), with sloughing of epithelial lining (Arrow head). Alveoli (upper right of figure) showed no remarkable changes (H&E, 4x). (F) Saltwater drowning group shows infiltration by mono-nuclear inflammatory cells in walls of bronchioles (Black arrow), with sloughing of epithelial lining (Arrow head). Alveoli (upper left of figure) showed no remarkable changes (H&E, 10x).

pulmonary alveoli (Fig. 3A, B). So far, freshwater drowning group showed the presence of intra-alveolar edema, congested blood vessels and lymphocytic infiltrate in alveolar walls, edematous thickening of alveolar walls and congested blood vessels (Fig. 3C, D). However, saltwater drowning group showed the presence of infiltration by mono-nuclear inflammatory cells in walls of bronchioles, with sloughing of epithelial lining. Alveoli showed no remarkable change (Fig. 3E, F; Table III).

Histopathological changes in the liver. Microscopic examination of the hepatic tissue in the control group revealed normal characteristic hepatic architecture with radially arranged hepatocytes, regularly sized nuclei, intervening sinusoids, and normal central vein (Fig. 4A). Freshwater group showed no disturbed liver architecture. There is evidence of hepatocytes cell injury: hydropic degeneration and congested vessels, congested vessels, and hepatic sinusoids (Fig. 4B, C, D). However, saltwater group showed no disturbed liver architecture is seen. There is evidence of hepatocytes cell injury: hydropic degeneration and focal spotty inflammation (Score 2). There is mild portal inflammation (Score 1) with mild congested portal vessels. There is mild cloudy swelling of hepatocytes and mild

congested hepatic sinusoids (Fig. 4E, F, G, H; Table IV).

Histopathological changes in the kidneys. Microscopic examination of renal tissue of control group revealed no abnormal changes in the glomeruli and interstetium (zero score: Fig. 5A, B). Freshwater group showed enlargement of glomeruli with mesangial expansion and hypercellularity (2+) with patent capillary lumens and no change in capillary wall thickening. Tubules show evidence of acute tubular injury (2+): irregular dilatation of tubules with irregularity in cell lining and focal loss of brush margin, swelling of epithelial lining of tubules and focal epithelial necrosis. Interstetium shows congested capillaries (Fig. 5C, D).

On the other hand, saltwater group showed enlargement of glomeruli with mesangial expansion, hypercellularity and congested glomerular capillaries (4+) with patent capillary lumens and no change in capillary wall thickening. Tubules show evidence of acute tubular injury (2+): irregular dilatation of tubules with irregularity in the cell lining and focal loss of brush margin, swelling of epithelial lining of tubules and focal epithelial necrosis. Interstetium shows congested capillaries (Fig. 5E, F; Table V).

Table IV. The microscopic findings in liver of control and freshwater drowning and saltwater drowning rats. (n=6)

Groups	Findings
Control group	No disturbed liver architecture. Radially arranged hepatocytes. Regularly sized nuclei, intervening sinusoids, normal central vein
Freshwater group	No disturbed liver architecture. There is evidence of hepatocytes cell injury: hydropic degeneration, congested vessels and sinusoids
Saltwater group	No disturbed liver architecture. There is evidence of hepatocytes cell injury: mild hydropic degeneration, mild congested sinusoids and focal inflammation (Score 2). There is mild portal inflammation (Score 1).

DISCUSSION

Bodies recovered from water (either seawater or freshwater) are commonly subjected to forensic pathology examination to establish a definitive cause of death and release the medico-legal ambiguity in such cases (van Beeck *et al.*, 2005). The accepted mechanism of death caused by drowning is from asphyxiation/ hypoxia from inhaling the immersed liquid, which can occur within minutes. Drowning is a difficult diagnosis because the autopsy findings can be minimal, and the typical features are nonspecific. This can be complicated by other factors such as the absence of witnesses to the event, prolonged immersion time, and postmortem decomposition (Cala *et al.*, 2013).

Anesthetization and intratracheally instilled salt or freshwater in dogs or rabbits are the most common methods used to explore the drowning pathogenesis (Chen *et al.*, 2016). However, many obvious drawbacks have been identified in intratracheally instilled water method. Firstly, seawater instilled intratracheal does not imitate the actual normal drowning scene, as about 10-20 % of death in drowned bodies was from laryngeal spasm (i.e. Dry drowning) without fluid aspiration (Lunetta *et al.*, 2004). Secondly, it was found that animals under anesthesia loss the response of stress induced by drowning. Previous researchers demonstrated that in actual drowning condition, the drowning

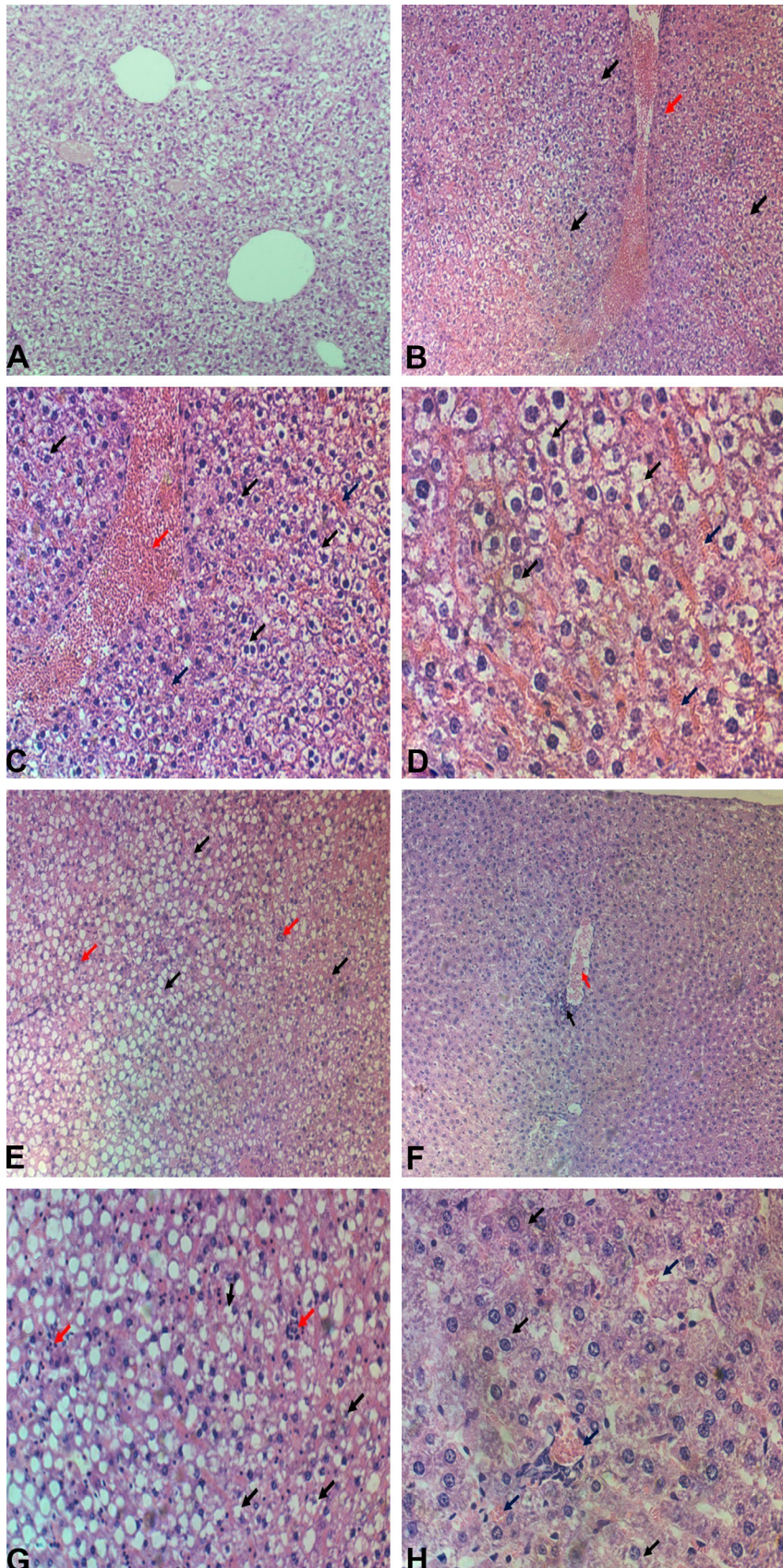


Fig. 4. A photomicrograph of transverse section in the rat liver stained with H&E stains shows (A) normal control group shows normal liver section with its central vein (CV), blood sinusoids arranged radially toward the CV. Hepatocytes are arranged in anastomosing cords with deep acidophilic cytoplasm and vesicular rounded nuclei with obvious nucleoli. (B) Freshwater drowning group shows no disturbed liver architecture. There is evidence of hepatocytes cell injury: hydropic degeneration (Black arrows) and congested vessels (Red arrow) (H&E, 10x). (C) Freshwater drowning group shows hepatocytes hydropic degeneration (Black arrows), congested vessels (Red arrow) and hepatic sinusoids (Blue arrows) (H&E, 20x). (D) Freshwater drowning group shows hepatocytes hydropic degeneration (Black arrows) and congested hepatic sinusoids (Blue arrows) (H&E, 40x). (E) Saltwater drowning group shows no disturbed liver architecture. There is evidence of hepatocytes cell injury: hydropic degeneration (Black arrows) and focal inflammation (Red arrows) (H&E, 10x). (F) Saltwater drowning group shows mild portal inflammation (Black arrow) with mild congested portal vessels (Red arrow) (H&E, 10x). (G) Saltwater drowning group shows mild hydropic degeneration of hepatocytes (Black arrows) and focal spotty inflammation (Red arrows) (H&E, 20x). (H) Saltwater drowning group shows mild cloudy swelling of hepatocytes (Black arrows) and mild congested hepatic sinusoids (Blue arrows) (H&E, 40x).

Table V. Comparison between changes found in the renal sections of control, freshwater drowning and saltwater drowning rats regarding the histopathological grading (n=6).

	Control group	Freshwater group	Saltwater group
Tubules- interstitium	0	2+	2+
Glomerular damage	0	2+	4+
Tubules and interstitium		Glomerular damage	
No lesion (0)		no sclerosis(0)	
Very mild focal dilatation, with evidence of acute tubular injury (10 %tubules) (1+)		1-25 % mesangial expansion and congested glomerular capillaries (1+)	
Large number of dilated tubules with evidence of acute tubular injury (10-50 % of tubules)with congested capillaries in interstitium (2+)		25-50 % mesangial expansion and congested glomerular capillaries (2+)	
Extensive dilatation of tubules with cystic formation and/or protein cast with widening and chronic inflammation of the interstitium (3+)		50-75 % mesangial expansion and congested glomerular capillaries (3+)	
Complete atrophy of the tubules, fibrosis of interstetium (4+)		75-100 % mesangial expansion and congested glomerular capillaries (4+)	
		Sclerosing glomeruli (5+)	

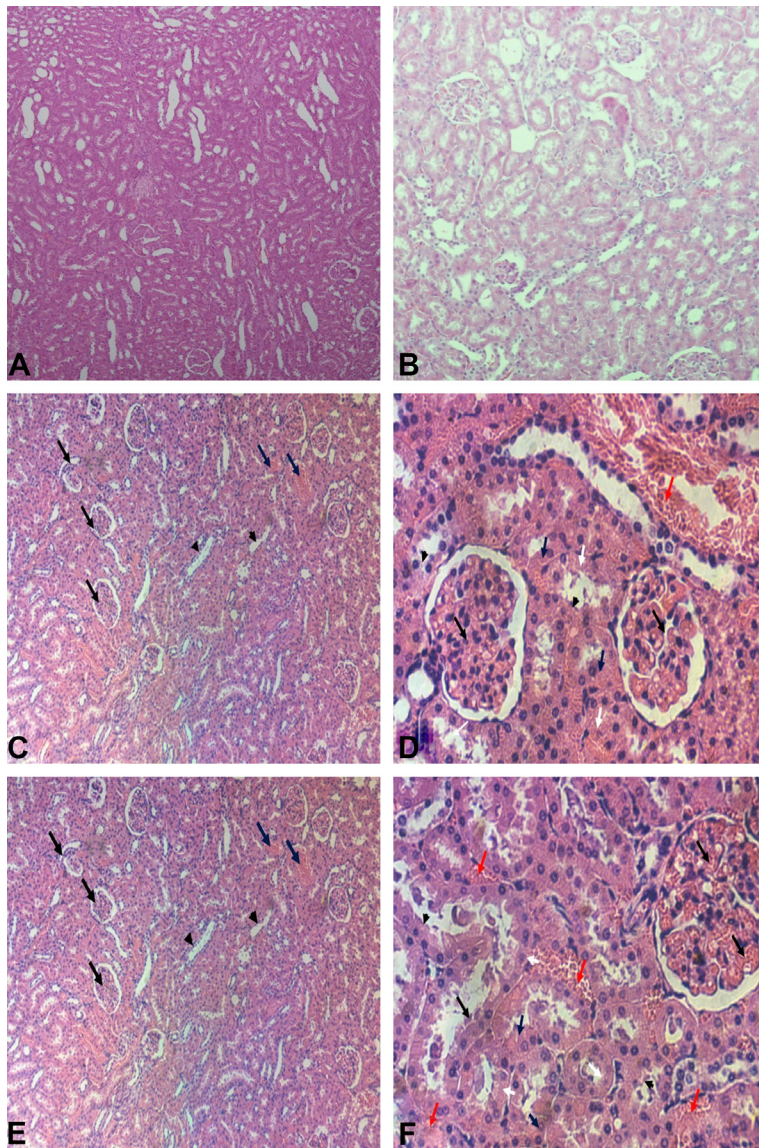


Fig. 5. A photomicrograph of transverse section in the rat kidney stained with H&E stains shows (A) and (B) Photomicrograph of a section from the normal control group showing normal renal cortex and medulla. The cortex shows normal glomeruli surrounded by patent urinary space, proximal convoluted tubules have narrow lumen and lined with large acidophilic cells that have rounded vesicular nuclei, also it shows distal convoluted tubules with wide lumen lined with cubical light acidophilic cells. Normally arranged renal medullary tubules. (C) Freshwater drowning group shows enlargement of glomeruli with mesangial expansion and hypercellularity (Black arrows). Tubules show irregular dilatation (Arrow heads). Interstitium shows congested capillaries (Blue arrows) (H&E, 10x). (D) Freshwater drowning group shows enlargement of glomeruli with mesangial expansion and hyper cellularity, with patent capillary lumens and no change in capillary wall thickening (Black arrows). Tubules show evidence of acute tubular injury: irregular dilatation of tubules with irregularity in cell lining and focal loss of brush margin (Arrow heads), swelling of epithelial lining of tubules (Blue arrows) and focal epithelial necrosis (White arrows). Interstitium shows congested capillaries (Red arrow) (H&E, 40x). (E) Saltwater drowning group shows enlargement of glomeruli with mesangial expansion and hyper cellularity (Black arrows). Tubules show irregular dilatation (Arrow heads). Interstitium shows congested capillaries (Blue arrows) (H&E, 10x). (F) Saltwater drowning group shows enlargement of glomeruli with mesangial expansion and congested glomerular capillaries (Black arrows), with patent capillary lumens and no change in capillary wall thickening. Tubules show evidence of acute tubular injury: irregular dilatation of tubules with irregularity in cell lining and focal loss of brush margin (Arrow heads), swelling of epithelial lining of tubules (Blue arrows) and focal epithelial necrosis (White arrows). Interstitium shows congested capillaries (Red arrow) (H&E, 40x).

cases, particularly non-swimmer, regularly explicit dread and despondency feeling in the form of significant stress responses (Sood *et al.*, 2014). In addition, the time lapse between instillation of copious water intra-tracheally in animals and death may last many hours. Therefore, it is difficult to disclose the key pathophysiological hallmarks of the drowning (Farrugia & Ludes, 2011). In the current study, we created the drowning model using seawater and freshwater by directly immersed the rats into water.

In the investigation of a body recovered from water, a wide range of possibilities other than drowning have to be considered (Saukko & Knight, 2015). The diagnosis of drowning is established through critical evaluation of the victim's individual characteristics, the circumstances, autopsy findings (DiMaio & Dana, 2006), and other investigations such as the diatom test (Levkov *et al.*, 2017). The current study was carried out to investigate the probability and capability of evidence that differentiate between fresh and saltwater using histopathological investigation of different body organs.

The mechanism of death in salt and freshwater drowning had been depicted in previous studies with significant difference between both. Swann & Brucer (1946) in an experimental study exhibited that various hemodynamics and biochemistries changes occur in fresh and saltwater drowning. When the drowning occurs in saltwater, there is diffusion of water electrolytes into the circulation by concentration gradient. Simultaneously, the changes that occur are lung edema, hemoconcentration, hypovolemia and hypoproteinemia. However, drowning in freshwater water, the hypotonic water of the lungs diffused rapidly into the circulation; bring about hypervolemia, hemodilution, hemolysis and serum electrolytes abatement except potassium. Hemolysis leads to increased potassium level in blood (Timperman, 1972).

The survival period after submersion showed no difference in both test groups (fresh and saltwater) as it was nearly 3 minutes. This in agreement with Farrugia & Ludes (2011) who reported that the submersed animals usually lost their consciousness within three minutes. Previous animal study carried out by Yamamoto *et al.* (1983) demonstrated that submersion of rats in freshwater, isotonic and hypertonic saline has no survival time difference.

Gross picture of drowning represents that, large quantities of froth and fluid flow from nasal openings, Clear or pink frothy foam found in the trachea and airways in animals drowned in freshwater, larger quantities of froth found in saltwater drowning than in freshwater drowning animals, areas of hemorrhage or congestion were dispersed

on the surface of the lung. These results are in agreement with Munro & Munro (2013) who reported that, the gross appearance of the lungs in freshwater and saltwater drowning is similar. The heart shows marked enlargement in its right side and the great veins were congested, and the left heart chambers were empty.

Hypoxia and ischemia are considered as the principal modes for tissue damage to the central nervous system in cases of drowning. This could be attributed to the fact that, the nervous tissue in general and brain tissue in specific as their reserves of metabolic substrates are limited and they cannot withstand the deprivation of oxygenation for long time. Therefore, the higher the intensity and the longer the duration of the hypoxia, the greater the tissue damage of the brain and nervous system. This irreversible damage could be observed in the limbic system, basal nuclei and cerebral tissue within 4–10 minutes (Montenij *et al.*, 2011).

In the current study, histopathological changes in the brain tissue among freshwater and saltwater drowning animals showed interstitial edema, degenerated neurocytes with dystrophic changes in the form of shrunken cell, pyknotic nuclei and deeply eosinophilic cytoplasm "Red neurons" and infiltration of choroid plexus with inflammatory cells. These results are in agreement with Neubauer & James (1998) who reported also that, oxygenation is the most critical function of blood flow and a sudden reduction in oxygen resulting in failure of membrane integrity of some cells and necrosis.

In addition, microscopic evidence of injury to the myocytes of rates that were drowned in freshwater were observed along with granular vacuolization of the cardiac cells cytoplasm, disappearance of nuclei in some cardiac cells and mild lymphocytic infiltrate as well as interstitial edema with congested capillaries. On the other hand, the histological examination of myocardial cells of the rats drowned in saltwater, revealed changes similar to freshwater drowned rates, in the form of evidence of injury to the myocytes; cytoplasmic granular vacuolization, vanishing of nuclei in many myocytes and mild lymphocytic infiltrate along with interstitial edema and congested capillaries.

Christe *et al.* (2008) reported that 40 % of drowning cases showed an enlarged heart at multi slice computed tomography (MSCT) at autopsy. The size of the right atrium was increased in 60 % of the cases, the size of the right ventricle in 40 %; values were 40 % and 20 %, respectively, at autopsy. The left heart side was increased in only 10 % of drowning in MSCT (one case).

In the current study, it was found that the lungs weights in rats of both test groups (fresh and saltwater) were increased in comparison to non-drowned control group. However, no significant difference has been identified between lungs weights in both test groups (2 and 3). The finding was quite similar to Nishitani *et al.* (2006).

The current study depicted the presence of the presence of intra-alveolar edema, edematous thickening of alveolar walls and congested blood vessels in freshwater drowning. These findings in agreement with Orłowski *et al.* (1987) who reported that experimental animal research explicit that drowning in isotonic and hypertonic fluids causes less alveolar damage, while in freshwater drowning, direct alveolar injuries occur in extensive manner. However, saltwater group in this study showed the presence of infiltration by mono-nuclear inflammatory cells in walls of bronchioles, with sloughing of epithelial lining. Alveoli showed no remarkable change. The data obtained in our study is broadly consistent with the major trends described by Giammona & Modell (1967) who reported that pulmonary edema occurs in hypertonic solution drowning (as occurs in drowning in seawater) due to aspiration and subsequent great increase in osmolar gradients.

When freshwater enters the alveoli, the surfactant is destroyed causing collapse of the alveoli with loss of its opening ability during breathing. A mismatch can happen when some lung parts are deprived from oxygen to be absorbed and the blood is pumped to these parts. Because of this effect, oxygen concentration in the blood decreases and leads to ventilation-perfusion mismatch (Orłowski *et al.*, 1987).

Saltwater does not devastate surfactant; rather it washes it away and causes damage of the membrane between the capillary blood vessel and the alveolus inducing acute lung injury (ALI). Small amount of water is sufficient to compromise surfactant leading to alveolar collapse. Lung edema is a major feature in saltwater aspiration due to high osmotic saltwater which can pull water through vascular endothelial cells and alveolar epithelial cells (Layon *et al.*, 2009). The lack of surfactant in drowning, regardless of the type of water, causes deterioration of function of the lung (Zhang *et al.*, 2014).

On the other hand, aspiration of freshwater in cases of drowning leads to surfactant dilution within the alveoli leading to a decrease in the surface tension of the surfactant lining the alveoli and their subsequent collapse. These results are consistent with those of Armstrong & Erskine (2018) whose results revealed microscopic edema of the alveoli with relative higher frequency in the freshwater drowning group.

The diagnosis is often based on the anatomic-pathological observations during the autopsy. These observations are mainly the following (Timperman, 1972): edema and congestion of the lungs, presence of hemorrhagic intra-parenchymatous foci, or diffuse, patchy sub-pleural areas of hemolysis, presence of liquid in the airways, in the thoracic cavity and the stomach (DiMaio & Dana, 2006). Pulmonary histology can sometimes provide a useful complement to the macroscopy, without being highly specific (Piette & De Letter, 2006).

The *postmortem* diagnosis of drowning depends on microscopic and macroscopic findings (Van Hoyweghen *et al.*, 2015). Externally, one of the substantial findings is the foam existence at the nostrils or mouth, or both. Internally, waterlogged, over-distended and over weighted lungs are found with overlapping of pericardium in severe condition. Pleural effusion, decreased weight of spleen, middle ears hemorrhage and fluid accumulation in the stomach are other macroscopic findings. Concerning the microscopic findings, hematoxylin and eosin staining used in histological investigations depict intra-alveolar edema and dilatation of the alveolar spaces with secondary compression of the septal capillaries (Seo *et al.*, 2013).

Microscopic examination of renal tissue of rats drowns in freshwater showed enlargement of glomeruli with mesangial expansion and hypercellularity (2+) with patent capillary lumens and no change in capillary wall thickening. Tubules show evidence of acute tubular injury (2+): irregular dilatation of tubules focal epithelial necrosis. Interstitium shows congested capillaries.

When it comes to the rats drowned in saltwater, the microscopic examination revealed close changes to that of the freshwater drowned rats, in the form of, enlarged glomeruli along with mesangial expansion in addition to hypercellularity and congested glomerular capillaries (4+) as well as patent capillary lumens that was not associated with changes in capillary wall thickening, moreover the tubules displayed evidence of acute tubular injury (2+).

These results agree with the results of Seong *et al.* (2012), who reported that; acute tubular necrosis may result from drowning in salt and freshwater. The morphological changes occur mainly in the proximal tubules, susceptible to ischemic injury, including loss of polarity, loss of the brush margin and redistribution of integrins and Na⁺/K⁺-ATPase to the apical surface. Calcium and reactive oxygen species also have a role in these morphological changes, in addition to subsequent cell death resulting from necrosis and apoptosis. Both viable and non-viable cells are shed into the tubular lumen.

CONCLUSION

Based on the results presented here, diagnosis of drowning in salt and freshwater was effectively differentiated in rats' lungs and heart with minimal differentiation in liver, kidneys, and brain. Further studies with different stain techniques will permit an overall statistical assessment and hopefully, assist to illustrate the postulated role of histopathological changes in different body organs as indicator of drowning. Further research that reflects the impacts needs to be added.

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IBRAHIM, M. A.; HAGRAS, A. M.; KHALIFA, A. M.; ALI, Z. A.; EID, G. A.; REHMAN, A. & ELSERAFY, O. S. Diferenciación histopatológica de ahogamiento en agua dulce y agua salada en ratas: punto de vista forense. *Int. J. Morphol.*, 40(4):1134-1146, 2022.

RESUMEN: El diagnóstico post mortem de muerte por ahogamiento es uno de los temas más difíciles de la patología forense. Investigamos la posible evidencia que diferencia el ahogamiento en agua salada del ahogamiento en agua dulce por cambios histopatológicos en los tejidos del cerebro, el corazón, los pulmones, el hígado y los riñones. Se realizó un estudio descriptivo de corte transversal en dieciocho ratas Wistar macho de 12 semanas de edad; se dividieron por igual en 3 grupos. Grupo 1: grupo control; Grupo 2: muerte por ahogamiento en agua dulce; Grupo 3: muerte por ahogamiento en agua salada. Inmediatamente después de la muerte, se extirparon todos los órganos analizados y se fijaron para el examen histopatológico. El cerebro del grupo de agua dulce mostró neurocitos degenerados con cambios distróficos en forma de células encogidas, núcleos picnóticos y citoplasma profundamente eosinofílico. El corazón mostró una clara evidencia de lesiones de miocitos en los ahogamientos en agua salada en comparación con los grupos de control y de agua dulce. Los riñones de ratas ahogadas en agua salada revelaron una mayor destrucción glomerular sin diferencias en los cambios túbulo-intersticiales en comparación con las ahogadas en agua dulce. En los pulmones, los cambios en agua dulce se restringieron a los alvéolos y los cambios bronquiales fueron más distintivos en agua salada. No se observó una arquitectura hepática alterada en ambos grupos de prueba, sin embargo, la degeneración hídrica, los vasos congestionados y los sinusoides fueron más distintos en el grupo de agua salada. En conclusión, la diferenciación diagnóstica entre ahogamiento en agua dulce y salada fue confiable en los pulmones y el corazón de las ratas con una diferenciación mínima en el hígado, los riñones y el cerebro. Estudios adicionales de ahogamiento con diferentes técnicas de tinción ayudarán a aclarar el papel potencial de los cambios histopatológicos en los órganos del cuerpo como indicador de ahogamiento.

PALABRAS CLAVE: Ahogamiento; Agua salada; agua dulce; Histopatología; Médico-legal.

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