ABSTRACT

Purpose: Previous experiments in rats have indicated that there are histological changes in skeletal muscle in drowning deaths; these changes include muscle fibers that contain ragged red fibers (RRF). The purpose of this study was to examine whether these changes also occur in humans.

Methods: Histologic and histochemical examinations of three muscles (diaphragm, pectoralis, and psoas) were performed on 24 cadavers with three different causes of death: 8 drowning, 8 hanging, and 8 sudden cardiac disease. Muscle samples were stained with hematoxylin-eosin, MGT, nicotinamide adenine dinucleotide-tetrazolium reductase, succinate dehydrogenase, ATPase, and acid phosphatase via standard staining procedures.

Results: There were statistically significant differences in the detection of RRFs in these cohorts. Additionally, several other cytoarchitectural changes (whorled and core-like fibers) were observed in the diaphragm in the drowning cohort and to a lesser extent in the hangings. These structural abnormalities were not observed in the sudden cardiac disease deaths, thus suggesting a common mechanism for the production of these muscular changes that is not shared in the cardiac death group. The mechanism is most likely intense hypoxia and mechanical trauma of the respiratory muscles in the setting of active blood circulation with intense muscle contraction.

Conclusion: Our results confirmed that there are histological changes in the diaphragm in drownings and, to a lesser extent, in hangings.

Key Words: drowning, hanging, sudden cardiac death, skeletal muscle, ragged red fibers, cytoarchitectural changes.

INTRODUCTION

The forensic diagnosis of drowning as a cause of death remains a challenge, and it is mainly a diagnosis of exclusion [1]. Moreover, it is difficult to distinguish drowning from other asphyxial deaths [2].

An autopsy of a drowning death may find a white or hemorrhagic foamy fluid (froth) in the nostrils, mouth, and airways and the lungs may be edematous and voluminous with the apposition or overlapping of the medial edges [3, 4]. There are other commonly observed findings in drowning deaths, such as brain swelling and hemorrhages of the pleural surface and petrous or mastoid bones; however, there are no pathognomonic findings of drowning [3, 5]. Most of the signs that are observed in bodies retrieved from the water are merely indicative of immersion; alternatively, these signs also may also occur in other causes of death. It must be emphasized that there is no single finding specific for drowning and that all findings commonly associated with drowning must be interpreted within the context of both the history and the circumstances of the death [4, 6].

Numerous ancillary tests to diagnose drowning have been proposed over the years, including chloride, strontium, magnesium, and iron concentrations to demonstrate hemodilution and/or hemoconcentration [7-9]. Postmortem left ventricular and vitreous humor sodium concentrations in saltwater drowning have been shown to be significantly higher than in freshwater drowning most likely due to differential fluid distribution [6]. Nevertheless, none of the aforementioned tests have a high diagnostic predictive value. Even the more exotic diatom test, which is considered by many to be the "gold standard" test for drowning [1, 10-12], has been challenged by others [13-16]. There is a high rate of false positives in non-drowning deaths due to the ubiquity of diatoms in nature, as well as the ability of diatoms to enter the systemic circulation from atmospheric air, food and drink, and not solely from the body of water.

The value of histological analyses into deaths by drowning also should be viewed with caution. The histological investigation of drowning-related findings primarily focuses on the lungs and the skin, with the latter used mainly to attempt to determine the post-mortem interval (PMI) [17]. The lungs are the organs that are most affected by the active inhalation of the drowning medium (e.g., water). Except for 10-15% of drowning victims, who are considered to be victims of "dry drowning" [3], the reported microscopic findings of drowning include intracellular swelling, intra-alveolar hemorrhagic edema, emphysema aquosum, rupture of the terminal air spaces, alveolar dilatation, thinning of the inter-alveolar septa, inflammatory infiltrates around the pulmonary vessels, a marked increase in alveolar macrophages, and increased phagocytic activity [4, 17, 18].

Additionally, histological investigations of injuries and hemorrhages, particularly on the neck and chest wall, have been performed in drownings. Intramuscular bleeding was reported as the only significant finding in the human neck, trunk, and arm muscles in drownings. These hemorrhages have been attributed to agonal convulsions, hypercontraction, and overexertion of the affected muscle groups [19]. Moreover, hemorrhagic changes have been reported in the human sternohyoid muscle during drowning [20]. These changes are regarded as vital signs of drowning, as they are likely caused by the extreme strain of the

accessory respiratory musculature from the experience of strong forced breathing in the phase of dyspnea, as well as by the clonic-tonic seizures of the subsequent phase of cerebral hypoxia [21]. Nevertheless, the interpretation of hemorrhages in the neck tissues is controversial. Some authors have stated that in the absence of trauma in that region, these hemorrhages are likely due to hypostasis/lividity [22] or the so-called Prinsloo and Gordon artefact [5].

Experimental findings in rats [23] have demonstrated that there are histological changes in skeletal muscles after death by drowning. By using light and transmission electron microscopy, muscle fibers were found to contain abnormal deposits of red material by modified Gomori trichrome (MGT) staining (ragged red fibers) and other cytoarchitectural changes that are consistent with hypoxia and rhabdomyolysis. In the present paper, we examine if these changes also occur in humans via the histological examination of several skeletal muscles (diaphragm, pectoralis, and psoas) in three different types of death: drowning, hanging, and sudden cardiac disease death.

MATERIALS AND METHODS

Based on the cause of death, 24 cadavers were assigned to one of three groups: 8 drownings recovered from different aquatic environments (drowning death-DD group), 8 hangings (HD group) and 8 sudden cardiac diseases (SCD group). Approximately 5 g of each of the three muscles (diaphragm, pectoralis, and psoas) were sampled during autopsies at the Institute of Legal and Forensic Medicine of Córdoba (Spain).

The selection of the type of muscle was based on their degree of involvement in the respiratory process (the diaphragm as a primary respiratory muscle, the pectoralis as an accessory respiratory muscle, and the psoas as a non-respiratory, postural muscle). All of the autopsy samples were placed in 50 ml plastic capped tubes, stored at -21 °C until analysis, and labelled with a unique number. The histopathologist was blinded to the history.

Light Microscopy

All of the muscles were grossly examined immediately after extraction. For the histological and histochemical analyses, specimens were embedded in OCT (Tissue-Tek®, Sakura), flash-frozen in isopentane cooled with liquid nitrogen (−160 °C), and transversely sectioned at 8 μm by using a cryostat at −20 °C. For each muscle, two sections were processed and stained with hematoxylin-eosin, MGT, nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-tr), succinate dehydrogenase (SDH), ATPase at pH 9.4, and acid phosphatase (AP) via the use of standard staining procedures [24]. The MGT technique can reveal subsarcolemmal mitochondrial clumps and red granular fine patterns inside of the muscle fiber, which correspond to intermyofibrillar mitochondrial concentrations. NADH-tr technique can reveal cytoarchitectural changes (CC) in the muscle fibers (whorled fibers [WF] and core-like fibers [CLF])

Quantitative and Statistical Analyses

Whole transverse histologic sections were examined at 200x magnification. Five randomly selected fields per sample were examined (10 fields per muscle/30 fields per case) that covered an area of 157.5 mm^2 by using a Sony DXC-990P digital camera mounted on a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan). Ragged red fibers (RRF) and fibers with red subsarcolemmal masses (SM) were recorded in each field using the MGT technique by a histologist who was blinded to the samples. Cytoarchitectural changes in the muscle fibers (WF and CLF) were counted based on the NADH-tr technique [25].

The statistical analysis was performed with the PASW Statistics 20 software (IBM-SPSS®) for Windows. In addition to the descriptive analyses, comparisons of the average values were conducted via non-parametric tests, such as the Kruskal-Wallis test and the Mann-Whitney U test, by applying the Finner correction for multiple comparisons. To determine the variation between the histological changes that were obtained (RRF, SM, WF and CLF) and the rest of the variables, the linear regression test and a multiple linear regression analysis were applied. The Spearman correlation test was also performed on the continuous variables. A logistic regression test was performed by comparing groups of death as binary variables. Statistically significant values were indicated by those values with a confidence level over 95% ($p<0.05$).

RESULTS

For the decedents, 70.8% (n=17) were men and 29.2% were women (n=7). The average age was 58.9 years ±17.4 (standard deviation [SD]), and the average post-mortem interval was 23.4 hours ±7.5. Descriptive features of the sample, according to the cause of death are shown in Table 1. The histological and histochemical analyses revealed statistically significant changes in the diaphragm samples for the DD and HD groups compared to the SCD group. No histological lesions were observed in the psoas and pectoralis muscles in any group.

In the DD group, several changes in the diaphragm muscle were noted with H-E staining but were particularly evident with the MGT staining, SDH technique, and NADH-tr technique. There were no changes with either the ATPase at pH 9.4 or acid phosphatase methods. With H-E, most muscle fibers showed apparent normal histology, although scattered granular appearing muscle fibers corresponded with RRF (Fig. 1a). Staining also revealed other fibers that contained peripheral red masses (Fig. 1b), although the latter structures lacked the distinctive ragged red or internal granular red fibers and were classified as SM. Both RRF and SM fibers were also marked with the oxidative techniques SDH and NADH-tr (in these cases the fibers are called *ragged blue fibers*) (Fig. 1c). It is noteworthy that some samples had cytoarchitectural changes such as giant fibers or whorled fibers (WFs) and other fibers with pale central or peripheral areas similar to core lesions (CLFs) (Fig. 1c). In one case, whorled fibers with RRF features were seen (Fig. 1d).

In the HD group, although to a lesser extent, the changes observed in the diaphragm muscle also included RRF, SM, WFs, and CLFs (Fig. 2a-d). In the SCD group, changes were nearly absent (Fig. 3a-d). The distribution of these changes in all groups and statistically significant differences are shown in Fig. 4. Statistical comparison revealed a higher number of RRF in females with respect to males (p=0.004) and a positive correlation of RRF with age (r= 0.6, p=0.002) with the linear regression test. Multiple linear regression analysis, however, found no statistical significance for RRF by sex, but did demonstrate significance for age and cause of death (R^2= 70%). Logistic regression showed significance for RRF (ORc= 1.34; RC 1.004-1.780; p= 0.047) in the DD group in relation to the other groups.

DISCUSSION

In this study, we confirmed the presence of RRFs and SMs in the diaphragm and established them as statistically significant findings in drowning and hanging deaths. The increase in RRF in drowning deaths was statistically, significantly different from both hanging deaths (p=0.019) and sudden cardiac deaths (p=0.003). Additionally, we found a statistically significant difference between hanging deaths and sudden cardiac deaths (p=0.019), being higher in hangings. In relation to the SMs, the results demonstrated the opposite effect. SMs were primarily observed in hanging deaths and to a less extent in drowning deaths but were nearly absent in sudden cardiac deaths. In fact, though both types of changes were separately quantified, they are likely different degrees of the same finding because in areas where muscle fibers were longitudinally sectioned, the appearances of the RRFs affected one segment of the fiber, whereas the other segment showed an SM appearance with peripheral or crescent red masses. The likely explanation for is that the appearance of SMs occurs before the more severe change, such as RRFs. If considered altogether (RRF+SM), these structural abnormalities affected the muscle fibers in the DD group to a greater extent (4.59%) than in the HD (3.36%) and SCD (0.87%) groups. Nevertheless, when grouped together, there were statistically significant differences among the DD and HD groups versus the SCD group (p=0.003), but there were no differences between the DD and HD groups. Therefore, it can be assumed that, in drowning deaths, the mechanism responsible for these changes is more pronounced than in hanging deaths.

The histological changes observed in the muscles in drowning deaths confirmed the previous results of our research group [23], the most noteworthy of them being RRFs and fibers containing abnormal deposits of the red material observed with the MGT technique. RRFs can be seen in rare muscular disorders, particularly mitochondrial myopathies, and can also be observed in experimental ischemic myopathy [24, 25]. Because these types of changes were only observed in the diaphragm muscle, but not in the psoas and pectoralis muscles, we propose that these fiber abnormalities may be a consequence of the vital role of the diaphragm in respiration. The pectoralis and psoas muscles are not primary respiratory muscles while the diaphragm is the main muscle involved in inspiration and contraction during each breathing cycle [26]. During drowning, the initial reaction is to hold one's breath until a breaking point is reached, after which the individual involuntarily inhales, thus taking in large volumes of water [3]. The hypoxia, in combination with the extreme strain of the accessory respiratory musculature from the experience of the strong forced breathing in the phase of dyspnea, may be responsible for the aforementioned changes. Intense muscular

activity may cause a metabolic stress similar to ischemia which is amplified when the oxygen supply is reduced from a hypoxic condition (such as respiratory insufficiency) [27].

Nevertheless, the fact that these structural abnormalities (RRF and SM) also have been observed in hanging deaths, but not in sudden cardiac deaths, suggests that a common mechanism exists for the production of these muscular changes, which is not shared by the latter group. The microscopic features of these changes suggest two possible mechanisms: intense hypoxia, which is a mechanism common to deaths by drowning and by hanging, and mechanical trauma of the respiratory muscles, which is mainly observed in deaths by drowning and involves intense muscle contractions involved in forced breathing and in trying to expel the water. A similar explanation is offered by Schulz et al. [21] for the hemorrhagic changes reported in neck muscles. They suggested that these hemorrhages are likely caused by the extreme strain of the accessory respiratory musculature from the experience of the strong forced breathing in the phase of dyspnea, as well as by the clonic-tonic seizures in the subsequent phase of asphyxia from cerebral hypoxia. Intense hypoxia also is a feature in SCD. The difference between HD/DD and SCD is the blood circulation. With hanging and drowning, the mechanism of death is a cerebral hypoxia. This allows for the heart to continue beating for many minutes [3]. With SCD, the circulation stops suddenly even though there still is hypoxia and potential hyper-respiratory efforts. Therefore, in order for these microscopic changes to develop, there likely needs to be circulation in the setting of marked hypoxia with hyper-respiratory effort.

Furthermore, provided that RRFs and SMs are likely different degrees of the same finding, and with RRFs being a more evolved stage, the statistically higher number of RRFs observed in the DD group, compared to the HD group, suggests that the mechanism responsible for the production of these changes is more sudden and intense in drowning deaths than in hanging deaths.

Another aspect that deserves consideration is that RRFs were positively correlated with age (r=0.6). This finding has been previously described, however, a percentage of the RRFs above 0.4% is considered to be pathological and not attributable to the ageing process [28]. The mean age of the DD group was higher than the other groups; consequently, this phenomenon may have some influence on the higher numbers of RRFs observed in drowning deaths. Nevertheless, RRFs that are observed in the ageing process affect not only the diaphragm but also other muscles, while in our sample, RRFs were not observed in the pectoralis or psoas muscles.

Statistical analyses demonstrated a significant difference by sex, with RRFs being found more frequently in females than males. Multivariate analysis, however, found no statistical significance for RRF by sex, but did demonstrate significance for age and cause of death (R^2= 70%). There is no obvious pathophysiological reason for this finding, and it may be explained by sampling. Our cohort contained fewer women, and most were from the DD group with a higher average age than the men; both of these factors are involved in the production of RRFs. On the other hand, when considering drowning as a binary variable, logistic regression showed significant results in the number of RRFs (ORc 1.34) in the DD group in relation to the other groups, even taking into account the limited sample size of our study.

The cytoarchitectural changes were indicative of myofibrillar changes due to either fiber disorientation (whorled fibers) or fiber degeneration (core-like fibers). Although these changes are observed in several specific conditions such as muscular dystrophies, they also can be non-specific and incidental [24]. Since the normal alignment of the myofibrils depends on the preservation of the cytoskeleton, the presence of both types of changes in the DD and HD groups may be explained by focal damage in the myofibrils and cytoskeleton, as a consequence of an intense contractile muscular activity. In our view, this phenomenon was greater in drownings than in hangings. We also note that in the SCD group, the amount of core-like fibers that was observed was not negligible. Indeed, the average CLF number was higher in the SCD group than in the HD group, but this was mainly attributed to only two cases. These two cases are the only ones in our sample with known antecedents of chronic alcoholism, which is a condition that may explain the myofibrillar degeneration. It is well known in muscle biopsies of individuals with chronic alcoholism or drug consumption, that fibers with alterations in the intermyofibrillar staining pattern via oxidative techniques, such as core or moth-eaten fibers, are common [29]. None of the remaining subjects of the sample had evidence or a history of a prior neuromuscular disease, which also may influence myopathological findings.

CONCLUSIONS

In conclusion, our results on human autopsy samples confirmed previous experimental findings in rats, indicating that there are histologically evident changes in the diaphragm in deaths caused by drowning and, to a lesser extent, in hangings. The observed structural abnormalities consisted of muscle fibers that contained abnormal deposits of red material identified via modified Gomori trichrome staining as ragged red fibers, as well as other cytoarchitectural changes (whorled and core-like fibers). These structural abnormalities were observed in DD and HD groups, but not in sudden cardiac deaths, thus suggesting a common mechanism for the production of these muscular changes that is not shared by the latter group. This mechanism most likely involves intense hypoxia and mechanical trauma of the respiratory muscles, which is linked to intense muscle contractions that are due to the overstrain involved in forced breathing in the presence of active circulation. Future research is needed to establish the possible usefulness of these structural abnormalities as ancillary tests that may assist in the diagnosis of these deaths.

KEY POINTS

- Structural abnormalities are observed in the diaphragm after drowning and hanging deaths
- Structural abnormalities are not observed in these muscles after sudden cardiac deaths
- These changes include ragged red fibers, whorled, and core-like fibers
- The mechanism involved is most likely intense hypoxia and mechanical trauma of the respiratory muscles in the setting of active blood circulation with intense muscle contractions.

Compliance with Ethical Standards

Funding: None.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Spanish legislation and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The research was approved by the Córdoba's Institute of Legal and Forensic Medicine teaching and research committee (23092016).

Informed consent: Informed consent was obtained from relatives of all individual participants included in the study.

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Figure captions

Fig. 1 Transverse sections of the diaphragm muscle in DD group. a: Three muscle fibers (arrows) of granular appearance. Insert: detail of a granular fiber in which fragmentation of its sarcoplasm can be seen and with a slightly internalized nucleus. b: Several fibers with features of RRF (arrows) and others with peripheral red subsarcolemmal masses (SM) (arrowheads). c: Whorled muscle fibers (asterisks) beside to core-like fibers, with areas lacking oxidative histochemical activity (arrowheads) and to two ragged blue fibers (equivalent to the ragged red fibers) (arrows). d: The arrow marks a whorled fiber with accumulations of red material that characterize an RRF. a: 20x; insert: 40x, H&E; b, d: 20x, NADH-tr; c: 40x, MGT

Fig. 2 Transverse sections of the diaphragm muscle in HD group. a: Two RRF (arrows) with marked accumulations of red material in the periphery. Insert: Detail of one of these RRF in which the fragmentation and reddish granulation of its sarcoplasm is checked. b: An RRF (arrow) and two SM (arrowheads). c: A ragged blue fiber (arrow) (equivalent to the RRF) with peripheral and internal areas of marked oxidative staining. d: A muscle fiber with a core type change (arrow), and three fibers presenting peripheral accumulations of oxidative activity (arrowheads). a: 20x, insert 40x, MGT; b: 40x, MGT; c, d: 40x NADH-tr

Fig. 3 Transverse sections of the diaphragm muscle in SD group. No abnormalities are observed. a: 20x, H&E stain; b: 40x, MGT stain; c:40x, NADH-tr stain; d: 40x, SDH

Fig. 4 Structural abnormalities in the diaphragm muscles, according to the causes of death
RRF: Ragged Red Fibers * DD>HD (p= 0.019); DD>SCD (p= 0.003); HD>SCD (p= 0.019)
SM: Subsarcolemmal Masses ♣ DD>SCD (p= 0.015); HD>SCD (p= 0.017)
WF: Whorled Fibers ♦ DD>SCD (p< 0.001); HD>SCD (p= 0.006)
CLF: Core-like Fibers ♥ DD>HD (p= 0.012)