Comparison of the Effects of Two Different Fixation Methods on Lung Morphology: An Experimental Study

İki Farklı Tespit Yönteminin Akciğer Morfolojisine Etkilerinin Karşılaştırılması: Deneysel Bir Çalışma

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ABSTRACT Objective: Enlargement of the alveoli is considered to be the most important parameter to assess the degree of emphysema and expansion. Alveolar enlargement is primarily defined by morphological criteria. Lung morphology can be of considerable forensic interest and it may contribute to the differentiation of drowning from other types of asphyxiation. Level of alveolar expansion is critical to differentiate between stillborn infants and those born alive but died shortly after birth. A detailed morphological analysis on the state of lung expansion may be interfered by the collapse of the lung, which usually occurs after opening the thoracic cage at autopsy resulting in loss of negative pleural pressure. **Material and Methods:** The degree of alveolar expansion and distortion of alveolar shape were analyzed morphometrically on samples of rat lungs which were fixed and dissected by different methods. This study comprised three groups including the control group, with 10 rats in each group. Some shape descriptors for the alveoli were measured using an image processing and analysis algorithm. **Results:** The median alveolar area was 0.062 mm² in the control group, 0.10 mm² in the drowning group, and 0.088 mm² in the *in situ* fixed group, respectively. In this experimental study, when fixation was completed before dissection, the morphometric measurements regarding the alveolar shape descriptors, especially in the alveolar area, major-minor alveolar axis and circularity variables were significantly different between groups ($p<0.05$). **Conclusion:** We conclude that *in situ* fixation of the lungs may provide important data in selected cases in terms of diagnosis.

Key Words: Drowning; lung; image processing, computer-assisted; tissue fixation; stillbirth; live birth

ÖZET Amaç: Alveollerin genişlemesi, amfizem ve ekspansiyonun derecesini değerlendirmede en önemli değişken olarak kabul edilir. Alveoler genişleme esas olarak morfolojik kriterlere göre tanımlanmıştır. Akciğer morfolojisi adli tıbbın ilgi alanına girmekte ve suda boğulmalar ile asfiksinin diğer tipleri arasındaki farkın ortaya konulmasına katkı sağlamaktadır. Alveoler genişlemenin derecesi, ölü doğumlar ile canlı doğup kısa bir süre sonra ölen bebekleri ayırt etmede kritik bir rol oynar. Otopside, göğüs kafesi açılırken negatif plevral basıncın kaybı sonucu meydana gelen akciğer kollapsı, akciğer genişlemesinin detaylı morfolojik analizini kısıtlayabilir. **Gereç ve Yöntemler:** Değişik biçimlerde fiksasyona ve diseksiyona tabi tutulan sıçan akciğeri örneklerinde, alveoler genişlemenin derecesi ve alveoler şekil değişikliği morfometrik olarak analiz edilmiştir. Bu çalışmada, biri kontrol olmak üzere her biri 10 sıçan içeren üç grup bulunmaktadır. Bir görüntü işleme ve analizi algoritması kullanılarak, alveollerin şekillerine ilişkin bazı tanımlayıcı değişkenler ölçülmüştür. **Bulgular:** Ortanca alveol alanı sırasıyla kontrol grubunda 0,062 mm², suda boğulma grubunda 0,10 mm², *in situ* tespit edilen grupta 0,088 mm² saptandı. Bu deneysel çalışmada, diseksiyon öncesi fiksasyon uygulandığında, alveol şekilleri ile ilgili morfometrik ölçüm sonuçları, özellikle alveoler alan, alveoler major-minör aks ve dairesellik değişkenleri açısından gruplar arasında istatistiksel olarak belirgin derecede farklılık olduğu saptandı ($p<0,05$). **Sonuç:** Seçilmiş vakalarda, akciğerlerin *in situ* fiksasyonunun tanı açısından önemli veriler sağlayabileceği değerlendirilmiştir.

Anahtar Kelimeler: Boğulma; akciğer; görüntü işleme, bilgisayar yardımlı; doku fiksasyonu; ölü doğum; canlı doğum

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Drowning is a common incident in forensic practice and the manner of death can be accidental, suicidal, homicidal, or natural. Autopsy can play a fundamental role in solving such cases while the scene investigation and proper collection of samples are also important.[1-3]

The mechanism of death in acute drowning is irreversible cerebral anoxia. The original concept of drowning deaths was that they were asphyxial in nature, with water occluding the airways.[4]

Enlargement of the alveoli is considered to be the most important parameter in assessing the degree of emphysema and expansion. Alveolar enlargement is primarily defined on morphological criteria; therefore, characterization of these differences using morphological variables is a prerequisite to study the pathogenesis.[5,6]

In these cases, the degree of aeration of the lung parenchyma can be of considerable forensic interest and it may contribute to the differentiation of drowning from other types of asphyxiation. On the other hand, the level of alveolar expansion is of considerable help in differentiating between stillborn infants and those born alive but died shortly after birth.[5-9]

The air content of the lungs may be reduced in various pathologic conditions such as surfactant deficiency or hyaline membrane disease of premature newborns, in cases of bronchial obstruction, or compression of the lung due to pneumothorax, a neoplasm or localised emphysema.[6,10]

A detailed morphological investigation on the state of lung expansion may be hampered by the collapse of the lungs, which usually occurs after opening the thoracic cage at autopsy resulting in loss of negative pleural pressure.[5,11] This phenomenon has already been described in the early 1960s by some pathologists, but has not yet been critically analysed in forensic applications.[5,6,12]

This experimental study aims to analyze the comparative morphology of lungs in drowning cases with emphasis on the effects of dissection and fixation methods.

MATERIAL AND METHODS

The experiments were carried out with the permission of the University Animal Research Ethical Committee. Thirty Adult Wistar rats from the Medical and Surgical Experimental Animal Research Centre of Osmangazi University were used in the experiments.

All rats were sacrificed using Xylasine HCl (10 mg/kg) and Ketamine (50 mg/kg) before autopsy. The groups formed were as follows:

Group I (Control): Dissection was performed after lethal anesthesia. The right lung was removed after opening the right hemithorax and was fixed in 10% neutral buffered formalin solution (n=10).

Group II (Experimental drowning): Tracheal intubations at the upper third were performed using a plastic cannula connected to a 10 ml syringe filled with fresh water. A 10 cm high tubing system connected the syringe directly to the trachea of the tracheostimized, anesthetized rats, and fresh water was infused under gravity until the heart stopped. The right lung was removed after opening the right hemithorax and was routinely fixed in 10% neutral buffered formalin solution (n=10).

Group III (*In situ* fixation group): After the rats were sacrificed with an overdose of anesthetics, the right lung was fixed *in situ* by infusing 10% neutral buffered formalin via the main bronchus, before opening the thoracic cage and pleural cavity. To preserve the lung paranchyme as close to as possible to that in the *in situ* state, the right hemithorax with the thoracic and upper lumbar part of the spinal column was removed en-block and placed in 10% neutral buffered formalin solution (n=10) for further tissue fixation. In this group, the pleural cavity was left intact in order to keep the negative pressure during fixation.

HISTOPATHOLOGICAL AND MORPHOMETRIC EVALUATION

After removal, all pneumectomy samples were submerged into 10% neutral buffered formalin solution and kept in it for at least 72 hours to ensure proper fixation. Then, each lung was sampled by slicing coronally from the apex to the base passing through the hilus. After routine tissue processing, all lung sections were embedded into paraffin blocks. Whole mount sections in standard thickness (4 micrometer) were obtained, stained with Hematoxylin & Eosin, and coverslipped for histopathological examination.

All samples were evaluated by the same pathologist who was blinded to the experimental design and the characteristics of the test subjects.

Of each lung section, three digital images from distinctive areas representing the overall condition of the parenchyma were captured under x25 magnification using an image analysis workstation composed of the following components: A light microscope with a motorized stage (Zeiss Axioscope, Zeiss GmBH, Germany), a digital camera attached to the microscope (Sony AVT Horn 3CCD RGB camera, Sony Inc., Japan), and a frame grabber card (Matrox Meteor, Matrox Inc., Canada) installed on a PC running Microsoft Windows NT 4.0 Service Pack 6a (Microsoft Inc., U.S.A). All images were captured by a digital imaging software (Autocyte Link, ThreePath Imaging Inc., -formerly Autocyte Inc.- U.S.A) in 768x576 pixel in resolution and were saved as a tagged image file format (tiff) file, which is widely used for lossless digital image storage.

Six planimetric features (Table 1) of the alveoli were measured on these images using ImageJ, an open source image analysis software written in Java language and supported by The National Institute of Health (NIH). For this purpose, a custom-made image analysis routine was developed by one of the authors (YK). Before carrying out measurements, a geometric calibration was done using a special slide provided by Zeiss GmBH, which had a milimetric scale. This calibration allowed the lenght and area of the objects to be expressed in micrometers and micrometer-squares, respectively. The basic steps in the measurement process were as follows: 1) Image processing including shading correction, bacground subtraction, and contrast enhancement prior to segmentation of the alveoli; 2) Converting the image to the binary form and segmentation of the alveoli; 3) Eliminating unwanted objects using adjustable size and shape filters; 4) Eliminating alveoli touching any borders of the image; 5) Measurements of the predefined geometric variables of alveoli; 6) Iteration of the previous steps on all three of the images from the same sample; 7) Recording and displaying the results.

The resulting data set contained 5000 records representing the measured alveoli, and each record was composed of values from 6 planimetric variables (Table 1).

STATISTICAL METHODS

The statistical data analysis was performed using SPSS for Windows release 15.0. Descriptive statistics were expressed as medians (minimum-maximum). The data obtained in the groups were compared with the Kruskal Wallis test. Bonferroni

TABLE 1: Measured geometric variables of alveoli.

Variable	Description *
Area#	Area of selection in square pixels.
Perimeter¥	The length of the outside boundary of the selection.
Major Axis¥	Primary axis of the best fitting ellipse.
Minor Axis¥	Seconday axis of the best fitting ellipse.
Circularity‡	4pi(area/perimeter^2). A value of 1.0 indicates a perfect circle. As the value approaches 0.0, it indicates an increasingly elongated polygon.
Feret's Diameter¥	The longest distance between any two points along the boundary of the object. Also known as the caliper length.

* Descriptions are excerpted from the documentation of ImageJ (http://imagejdocu.tudor.lu/imagej-documentation-wiki/gui-commands/set-measurements)

Micrometer square

¥ Micrometer

‡ Unitless

corrected Mann–Whitney U-test was used as the PostHoc test.

RESULTS

Histological findings in tissue samples obtained from the *in situ* fixed lung showed marked differences compared to those obtained from the routinely fixed lung. The *in situ* fixed lung (group III) and fresh water-drowning group showed expanded alveolar lumina in all sections (Figure 1).

The morphometrically evaluated median values of alveolar area were 62430.87 μm² (≈0,062 mm²) in Group I, 104190.68 μm² (≈0,10 mm²) in Group II, and 88000.6 μm² (≈0,088 mm²) in Group III (Table 2).

Alveolar area, major-minor axis and circularity showed statistically significant differences at a significance level of 0.05. On the other hand, perimeter, and Feret's diameter were not significantly different between the groups.

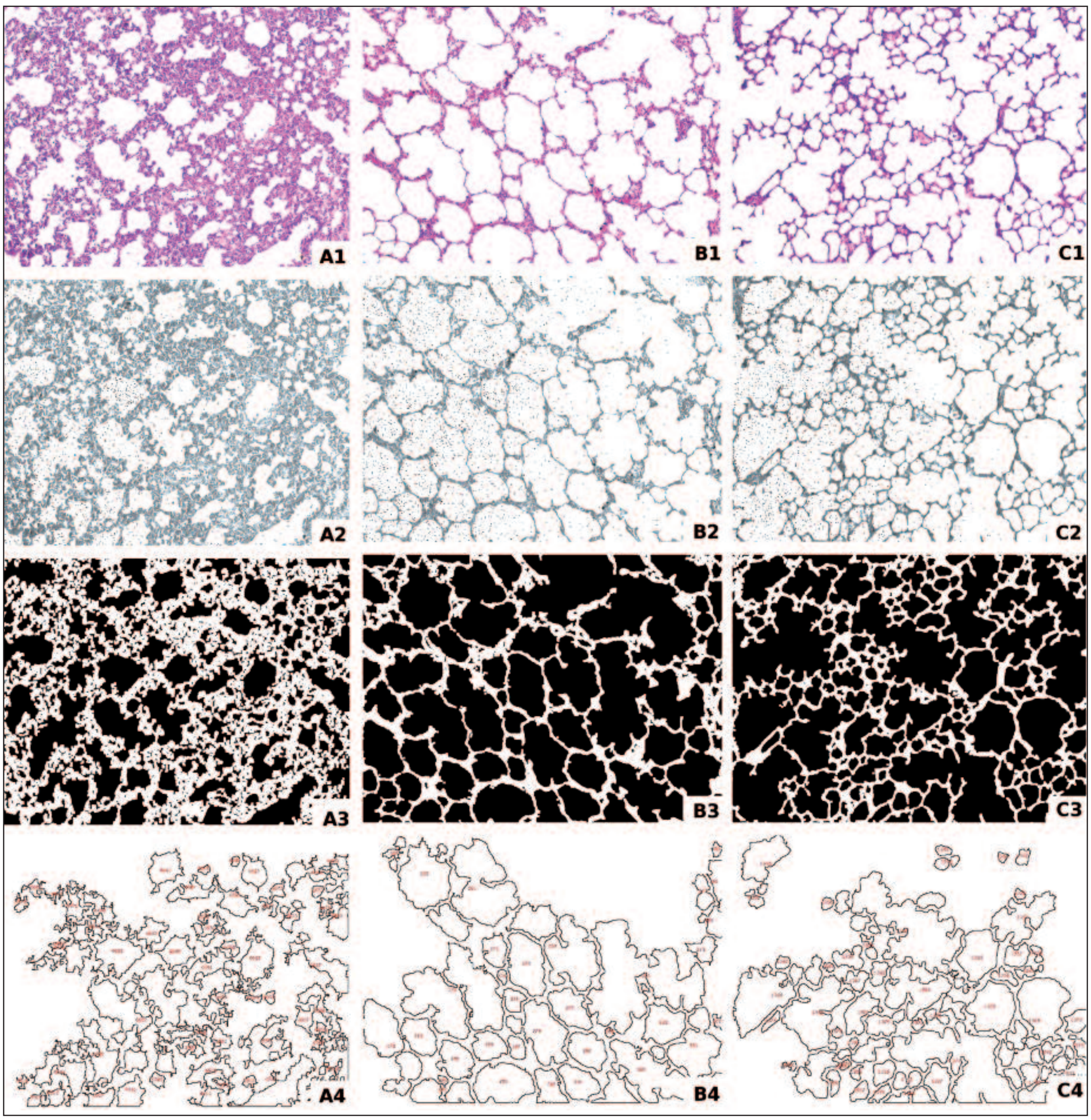

FIGURE 1: Histological sections from Group I (A1,2,3,4), Group II (B1,2,3,4) and Group III (C1,2,3,4). Original images (top row), enhanced greyscale images (second row), binary images (third row), and images containing segmented alveoli on which the measurements were done (bottom row).
(See for colored form http://tipbilimleri.turkiyeklinikleri.com/)

TABLE 2: Alveolar shape descriptors: Medians (minimum-maximum) calculated from the morphometric variables were compared between groups.

Groups		Area (μm^2)	Perimeter (μm)	Major Axis (μm)	Minor Axis (μm)	Circularity (unitless)	Feret's Diameter (μm)
Group I	Median	62430.87	5470.90	1010.27	570.29	0.35	1280.63
	Minimum	56380.19	4730.45	910.93	490.17	0.19	1200.45
	Maximum	99180.48	8010.84	1250.73	680.54	0.45	1680.62
Group II	Median	104190.68	6230.39	1240.10	730.47	0.37	1520.08
	Minimum	65950.22	4930.35	980.63	540.78	0.30	1220.57
	Maximum	155150.83	8110.31	1440.39	870.10	0.43	1700.45
Group III	Median	88000.60	5460.72	1210.94	680.39	0.43	1420.24
	Minimum	54600.87	4870.11	990.64	530.75	0.31	1260.81
	Maximum	113530.32	6240.48	1320.04	730.99	0.47	1550.94
P*		0.003	0.073	0.010	0.005	0.019	0.051
Pairwise comparison**	Group I vs Group II	<0.001		0.009	0.005	0.739	
	Group I vs Group III	0.015		0.007	0.005	0.019	
	Group II vs Group III	0.529		0.739	0.353	0.011	

* Kruskal Wallis test (p<0.05 was statistically significant).

**Bonferroni corrected Mann Whitney U test (p<0.017 was statistically significant).

A significant difference was found between the groups with respect to the alveolar area and the most prominent difference was detected between Group I and Group II (Figure 2).

In the pairwise comparisons, alveolar area, major axis, and minor axis variables were significantly different in both Group I-Group II, and Group I-Group III matchings (p<0.017) (Figure 3 and 4). Circularity, on the other hand, showed a significant difference between Group II and Group III (p<0.017) (Figure 5).

DISCUSSION

In the forensic practice, the recognition of death through asphyxiation can present problems if there are no indicative external or internal injuries or signs of obstruction of the respiratory tract, and the conditions of death are not known in sufficient detail.[4,13,14] Postmortem diagnosis of death by asphyxia involves not only an assessment of circumstantial, documentary, and anatomo-pathological data, but also histological examination.[6]

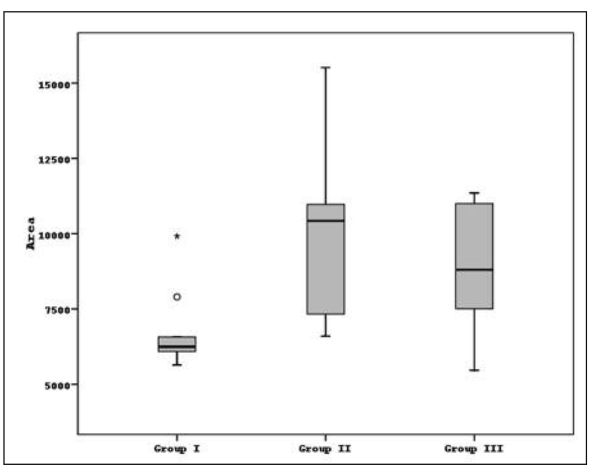

FIGURE 2: Alveolar area values of the groups.

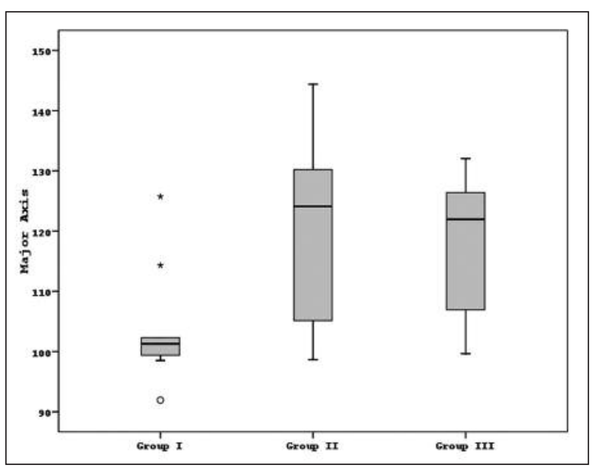

FIGURE 3: Major axis values of the groups.

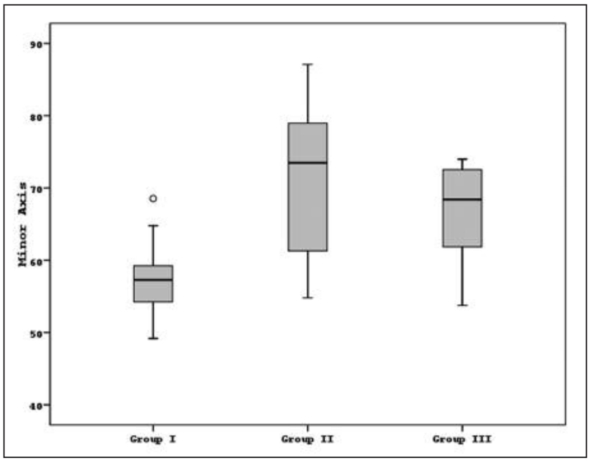

FIGURE 4: Minor axis values of the groups.

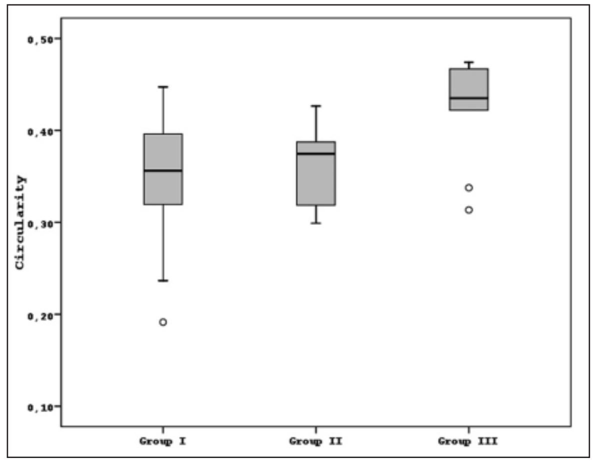

FIGURE 5: Circularity values of the groups.

The morphology of pulmonary paranchyme histology can supply significant findings and can contribute, for example, to the diagnosis of drowning or other types of asphyxiation. A diagnosis can be based, for example, on prominent alveolar expansion (acute emphysema) in cases of mechanical asphyxiation. The evaluation of lung tissue can also contribute to a differentiation between stillborn infants and babies born alive but died shortly after birth.[5,6]

A number of reports described a method of intrathoracal *in situ* fixation of the lungs after removal of the thorax at autopsy, which includes fixation in formaldehyde solution.[5,6,12] This procedure allows pulmonary expansion and the air space volume is comparable to that during life.[5,6]

In order to demonstrate the influence of postmortem lung retraction on the degree of alveolar expansion, a histomorphometrical analysis was performed in lungs from 30 sacrified rats in our study. Tissue specimens of the lungs were obtained at autopsy and routinely fixed after retraction (in group I and II), whereas the lungs were fixed *in situ* before opening the thoracic cage ('*in situ* fixed lung') in group III.

Significant gross and microscopic differences between the groups were present. The *in situ* fixed lungs (group III) were totally expanded and they filled out the thoracic cavity similar to that in *in vivo* state. On the other hand, the lungs in group I were collapsed as usual. Furthermore, there was considerable pulmonary expansion in the drowning group.

The median alveolar area was 62430.87 μm² and 104190.68 μm² in the routinely fixed group I and II and 88000.6 μm² in the *in situ* fixed group in this study. The morphometrical differences in the alveolar area, major-minor axis and circularity were the most evident among the measured variables.

In the pairwise comparisons, alveolar area, major axis, and minor axis variables were significantly different in both Group I-Group II, and Group I-Group III matchings. Circularity showed a significant difference between Group II and Group III. These findings are consistent with the data presented in the study of Hausmann et. al., while we have observed an additional difference for circularity between the routinely fixed and *in situ* fixed lungs.

We believe our results provide some evidence for significantly reduced alveoler area in the routinely fixed lung, which can be of special forensic interest in cases of death by drowning.

On the other hand, the above-cited drowning-related alveolar changes are not specific. In addition, their non-uniform distribution in the lungs can make them difficult to evaluate histologically. Thus, quantification of lesions by histomorphometry might provide more objective and consistent data than qualitative histology.[5,6,11]

Based on our findings and a review of the literature, we conclude that *in situ* fixation of the lungs may provide important data in selected drowning cases.[5,11] This can also be of special importance in cases of suspicious infanticide or stillbirth, where it may provide more reliable information on the degree of aeration of the lung tissue.[5]

Our findings are supported by the results of a morphometric investigation of emphysema aquosum in the elderly reported by Kohlhase and Maxeiner, recently.[11] The authors concluded that without intrathoracic fixation, drowning in old persons (with senile lung emphysema) could not be clearly diagnosed by means of histology alone, because emphysema aquosum is often masked by lung collapse at autopsy.[11]

Moreover, Hausmann et al. examined lung samples from a 9-month-old, otherwise healthy infant who died of sudden infant death syndrome. The results of the morphometrical analysis revealed a significant decrease (about 27%) in the size of alveoli of the fixed lung. In addition, the routinely fixed lung contained several atelectatic areas, which were not detectable in the *in situ* fixed lung. Therefore, it can be suggested that the evaluation of lung tissue can also contribute to the differentiation between stillborn infants and babies either born alive or died shortly after birth.[5]

Even though such diagnostic approaches are well known and thoroughly investigated, it must be emphasized that standardization of the technique is essential for these findings to be of diagnostical use.[5,15] Naturally, the *in situ* fixation technique has a number of limitations. Furthermore, the presented procedure probably involves too much time and effort for routine use. Nevertheless, the *in situ* fixation technique can stil be recommended particularly in the above-mentioned cases, in which the expansion of the lung is of special interest.[5]

The fact that our quantitative data showed significant differences between the drowned and the sacrificed animals regarding alveolar size and shape variables can be of diagnostic value. These results also indicate that the *in situ* fixation technique is useful in preserving the complex anatomical structures in lung. This can offer a better perspective on the evaluation of lung paranchyme expecially in the forensic study of respiratory mechanism, on the alveolar level.

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