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Morphological changes in striated muscle fibres caused by components of the Thiel embalming method

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Background: *Thiel-fixed body donors are highly valued for surgical training courses. The pronounced flexibility of Thiel-fixed tissue has been postulated to be caused by histologically visible fragmentation of striated muscle. The aim of this study was to analyse whether a specific ingredient, pH, decay, or autolysis could cause this fragmentation in order to modulate the Thiel solution to adapt specimen flexibility specifically to the needs of different courses.*

Materials and methods: *Striated muscle of the mouse was fixed for different time periods in formalin, Thiel solution, and its individual ingredients, and analysed by light microscopy. Further, pH-values of Thiel solution and its ingredients were measured. In addition, unfixed muscle tissue was histologically analysed including Gram staining to investigate a relationship between autolysis, decomposition, and fragmentation.*

Results: *Muscle fixed with Thiel solution for 3 months was slightly more fragmentated than muscle fixed for 1 day. Fragmentation was more pronounced after 1 year of immersion. Three individual salt ingredients showed slight fragmentation. Decay and autolysis had no effect on fragmentation, which occurred regardless of the pH of all solutions.*

Conclusions: *Fragmentation of Thiel-fixed muscle is dependent on fixation time and most likely occurs due to salts present in the Thiel solution. Adjustment of the salt composition in the Thiel solution with verification of the influence on the fixation effect, fragmentation and flexibility of the cadavers could be performed in further studies.* (Folia Morphol 2024; 83, 1: 83–91)

Keywords: tissue fixation, skeletal muscle, light microscopy, fragmentation, saturated salt solution, formalin, boric acid, pH

INTRODUCTION

Walter Thiel's stated intention was to develop a fixation method where cadavers tissues should have the smallest possible alteration with respect to colour, consistency, shape and volume and therefore facilitate anatomy teaching [37]. Since then, many studies investigated the advantages of soft embalmed Thiel-fixed cadavers, such as joint pliability, tissue suppleness and appearance with realistic colour [2, 9, 13, 16, 23, 31, 46]. These characteristics are favourable for surgical training [5, 9, 14, 18, 20, 30, 32, 33, 40, 43, 44]. However, some less favourable aspects regarding

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dissection, appearance or recognition of tissue, as compared to formalin-fixed cadavers, are reported [24, 42], particularly muscular disorganization and noticeable histological alterations [19, 32]. Although Thiel fixation has been in use for 30 years, and several studies have investigated the histological effects of Thiel fixation, there is still no sufficient explanation why cadavers retain such a high pliability. Benkhadra et al. [5] and McDougall et al. [27] specifically addressed this question in a histological investigation: Benkhadra et al. [5] studied Thiel-fixed muscle tissue from human body donors and demonstrated a "cut-up and minced" appearance of the striated muscle fibres (MF), while nuclei were not visible. They hypothesized boric acid to be the cause of this observed fragmentation, due to corrosive properties of acids on muscle tissue and its proteins [5]. Similarly, McDougall et al. [27] described "loss of nuclear staining, loss of definitive striation, cell structure integrity and considerable muscle fibre fragmentation". Interestingly, these findings were also detected in muscle tissue fixed in a modified Thiel solution without boric acid so that they proposed that, in addition to boric acid, salts in the Thiel solution might be responsible for fragmentation [27]. Other studies also described histological alterations of Thiel-fixed muscle tissue, but without focusing on potential reasons. In a study using human skeletal muscle fixed with a modified Thiel solution (i.e. no intrathecal, rectal, tracheal and gastral injection and minimum immersion time shortened from 3 to 2 months), washed-out appearance of the muscle, absence of cell membranes and nuclei were observed [16]. However, the MF were mainly cut transversely, and fragmentation was neither described nor visible. In another study in rats perfused immediately postmortem with Thiel solution, muscle tissue showed a blurred image, impaired integrity and poorly demarcated nuclei after immersion in Thiel solution for 2 months [4]. Images from this study can be interpreted to reveal muscle tissue that had been pulled apart, some showing fragmentation (described as an impairment of integrity) and missing nuclei. Notably, on images of saturated salt solution (SSS) preparations, individual fragmentations of striated MF were visible as well. A study on formalin-fixed bovine striated muscle [38] revealed impaired tissue after subsequent 7-month fixation in boric acid, but fragmentation in tissue stored in 0.9% NaCl. More recently, only few fragmentations were seen in Thiel-fixed rabbit muscle tissue, but more pronounced fragmentation in SSS-fixed tissue after 2 days [34]. So far, the effects of the individual ingredients of the Thiel solution have not been investigated but could contribute to the understanding of the detailed morphological effect of Thiel solution on muscle tissues. This could open the possibility of adjusting the composition of the Thiel solution according to properties of individual ingredients to retain the advantages of Thiel fixation while reducing the disadvantages (consistency, dissection, hyperlaxity). Anatomical institutes might adapt the tissue pliability according to its use, either in dissection courses or for clinical courses depending on the surgical discipline, approach, or a specific technique. By identifying an ingredient responsible for fragmentation and pliability, cadavers could be used more purposefully. The suitability of Thiel muscle tissue for biomechanical studies is controversial [4, 10–12, 15, 17, 21, 25, 26, 28, 35, 39, 41, 43, 45, 47]. If the biomechanical properties could be normalized by adaptation of the ingredients, it would open new possibilities for scientists working biomechanically with Thiel muscle tissue. The aim of this study was to investigate the effect of the whole Thiel solution, as well as its individual ingredients, on skeletal muscle and to find a possible explanation for the histologically visible fragmentation. Since rodents are widely used for research on muscle morphology, striated muscle of the mouse was selected for this purpose [27]. We analysed the effect of Thiel solution and its individual components, i.e., formaldehyde, boric acid, ethylene glycol, p-chlorocresol, and the salts ammonium nitrate, potassium nitrate and sodium sulphite and the influence of pH as well as the time of autolysis and decay.

MATERIALS AND METHODS

Tissue sampling and muscle preparation

Muscles from 4 mice that were originally part of another experiment approved by the Canton of Bern (number: BE 61/18) were collected. In the original experiment, the mice were euthanized by intraperitoneal injection of pentobarbital (100 mg/kg). Once death was confirmed and tissues were collected for the original experiment, we immediately harvested muscles from the limbs and back for subsequent experiments (Fig. 1).

Fixation with formalin, Thiel solution and Thiel ingredients

Muscle tissue was immersed in the respective fixative, either Thiel's immersion solution (Supplementary material 1 — see journal website), 4% buffered for-

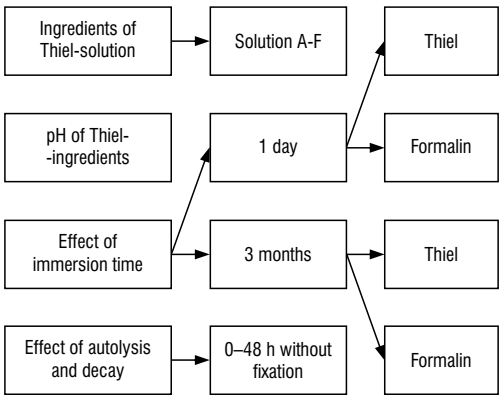

Figure 1. Flowchart of investigations using formalin solution, Thiel solution and its ingredients A–F (compare Table 1).

Table 1. Aqueous solutions of the Thiel-fixation ingredients

Solution A = Ammonium nitrate (10%)
Solution B = Boric acid (3%)
Solution C = Chlorocresol (0.18%)
Solution D = Ethylene glycol (10%)
Solution E = Potassium nitrate (5%)
Solution F = Sodium sulfite (7%)

Note: The concentrations are in line with the final concentration in the immersion solution.

malin solution, or a solution of the individual ingredients A-F of the Thiel immersion solution (Table 1). Tissue:liquid ratio was at least 1:10 (weight/volume). The Thiel solution and the solutions containing the ingredients (all purchased from Sigma-Aldrich, Buchs, Switzerland) were prepared in-house. Muscle tissue was fixed for 1 day or, according to the original protocol by Thiel [37], 3 months in Thiel immersion solution, or as a control in 4% formalin, at 8°C. To study long-term effects, we also used muscle tissue from Thiel-fixed body donors from our own Institute stored at least 1 year in Thiel immersion solution or Thiel soaked cloths. Since immersion of muscle in the solutions of the single Thiel ingredients A–F (Table 1) must be considered as insufficient fixation, these samples were stored for 20 hours at 4°C to prevent bacterial growth but ensure sufficient diffusion into the muscle to avoid manifestations of autolysis and cytoplasmic vacuolization [7, 36]. Thereafter, the muscles and solutions were stored at room temperature for another 4 hours to warm up and allow the ingredients to chemically interact with the tissue as recommended [3].

pH measurement

To study the effects of pH on the muscle pieces, we measured the pH of the Thiel solution, and of its ingredients, with an IKA KMO 2 basic (IKA Werke GmbH, Staufen, Germany).

Testing of autolysis and natural decay effects

In order to investigate decay and autolysis as a possible cause of fragmentation, some muscles were left unfixed in phosphate-buffered saline (PBS)-soaked cloths at room temperature. After 8, 16, 24 and 48 hours, respectively, the muscle was immersed in Thiel solution for 14 days and prepared for light microscopy following the same protocol. To objectify the presence of decay with bacterial growth, an additional Gram staining was performed with these sections.

Tissue embedding, haematoxylin-eosin and Gram staining and light microscopy analysis

All muscles were dissected, and pieces of 5 × 2 mm cut in longitudinal MF direction, rinsed, and dehydrated using a Tissue Processor Histokinette (Shandon Citadel 1000, Thermo Fisher Scientific, Basel, Switzerland). After 17 hours, the pieces were manually embedded in paraffin (Dr. Grogg Chemie AG, Stettlen-Deisswil, Switzerland). The paraffin blocks were cut with a microtome (Microm HM 355 S, Thermo Fisher Scientific) at a section thickness of 5 μm. Subsequently, sections were stained with haematoxylin-eosin. Gram staining was performed on sections of all autolysis time points. A positive control was used to verify the obtained results. Slides were analysed with a Zeiss Axio Imager M2 microscope (Carl Zeiss AG, Feldbach, Switzerland) equipped with an Olympus UC50 camera (Olympus, Wallisellen, Switzerland) by the following criteria: Cell integrity and appearance, presence and appearance of nuclei, skeletal muscle striation, recognition of surrounding tissue, alignment and fragmentation of MF, whereby fragmentation was defined as transverse interruption of a longitudinally cut MF bundle, with sharp edges, which did not correspond to a cutting artifact. For the Gram-stained slices, the presence and quantity of bacteria was assessed and documented.

RESULTS

Fixation solutions and individual Thiel ingredients

The muscle fixed in formalin for 1 day represented a control tissue with intact MF integrity and align-

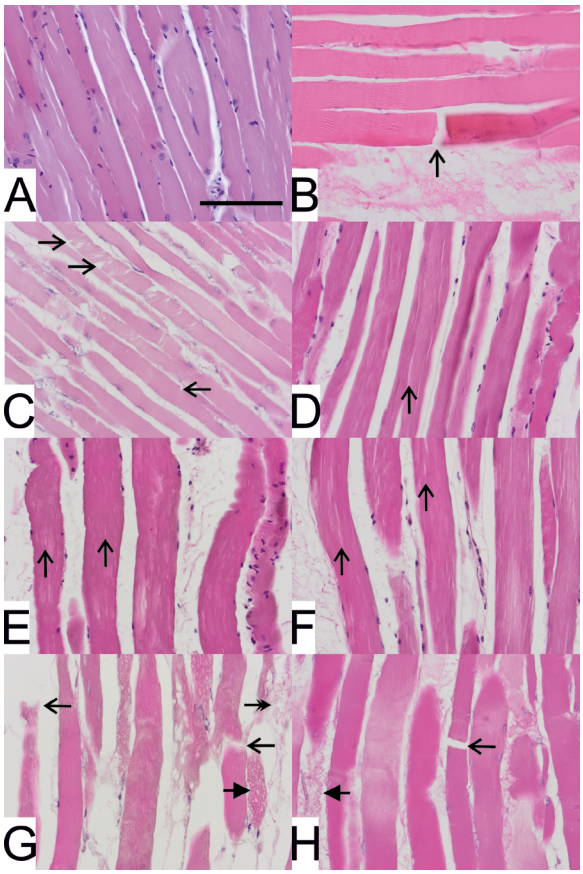

Figure 2. Histological analysis of muscle tissue fixed for 1 day stained with haematoxylin-eosin. **A.** formalin fixation; **B.** Thiel fixation. Arrow shows a fragmentation; **C.** Ammonium nitrate. Arrows show some indentations; **D.** Boric acid. Arrow shows a longitudinal tear; **E.** Chlorocresol solution. Arrows show the longitudinal and vacuole like alterations; **F.** Ethylene glycol. Arrows show longitudinal tears; **G.** Potassium nitrate. Open arrowhead points to shredded tears while the filled arrowhead shows a vacuolated muscle bundle. The double arrowhead points to amorphous surrounding tissue; **H.** Sodium sulfite. Open arrowhead shows a fragmentation, filled arrowhead shows amorphous surrounding tissue. Scale bar: 100 μm.

ment, correct staining, visible striations, intact nuclei, and demarcated and identifiable surrounding tissue (Fig. 2A). The muscle fixed in Thiel solution for 1 day showed few fragmentations, but with a well-preserved striation of MF (Fig. 2B). In general, the MF appeared slightly pale, loose and only moderately pulled apart. The architecture of the tissue appeared slightly disturbed and disintegrated. No indentations, which may be interpreted as beginning fragmentations, were visible. The nuclei appeared ballooned and pale. The surrounding tissue was generally well demarcated and intact, but with a slightly dissolved endomysium. In general, all MF bundles fixed in solutions of the Thiel ingredients A–F were slightly pulled apart compared to the MF bundles of formalin-fixed muscle (Fig. 2C–H). In detail, the muscle fixed in solution A (ammonium nitrate, Fig. 2C) showed some perpendicular tears, the striation was preserved. Mainly at the sides of the MF bundle, there were small indentations at the level of the I zone, which were sporadically continuous throughout the MF bundle. The nuclei were ballooned and pale. The surrounding tissue was recognizable. Muscle from solution B (boric acid, Fig. 2D) showed a preserved striation. Dominant were longitudinal tears along the MF. The nuclei were present and regularly stained. The surrounding tissue was intact. Muscle fixed in solution C (chlorocresol, Fig. 2E) revealed slightly pulled apart MF with curly borders, intense staining, few tears, and a visible striation in some areas. Dominant were short and elongated tears along the MF, which appeared like long vacuoles, but no fragmentation. The MF appeared more pulled apart than in muscle from solution B. There was a normal shape and staining of the nuclei. Muscle from solution D (ethylene glycol, Fig. 2F) also showed a preserved striation. Dominant were elongated tears along the MF, but more pronounced than in muscle of solution B (Fig. 2D). There were fuzzy borders but only few transverse tears. The muscle showed normal nuclei. The surrounding tissue appeared partly amorphous. In muscle of solution E (potassium nitrate, Fig. 2G), striation was almost invisible with some blurred MF. The muscle showed tears, partially looking like shredded and partially like classic fragmentations. In some areas, the MF also possessed a lot of vacuoles so that the muscle structure was unrecognizable. Nuclei were mostly absent or ballooned and pale. The surrounding tissue was partly. In the muscle of solution F (sodium sulfite, Fig. 2H), the MF bundles were arranged more densely together. Striation was visible, and the borders appeared blurry. Some classical fragmentation was seen here (open arrowhead) with otherwise intact muscle. Unlike in the muscle from solution A, incipient indentations were rarely seen. Nuclei were present, but appeared slightly pale and ballooned, the surrounding tissue was mostly amorphous.

Duration of fixation

Muscle pieces immersed in formalin for 3 months (Fig. 3A) showed no notable difference to those stored for 1 day in formalin solution (Fig. 2A). Some areas appeared less dense with MF slightly pulled apart in the longitudinal direction, but for the most

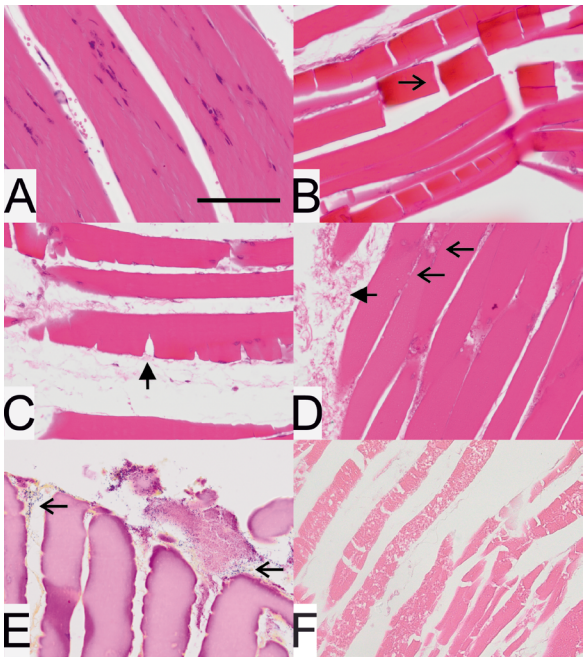

Figure 3. Histological analysis of muscle tissue stained with haematoxylin-eosin (**A–D, F**) and Gram stain (**E**); **A.** Formalin fixed, 3 months; **B.** Thiel fixed, 3 months. Arrow shows a fragmentation; **C.** Thiel fixed, 3 months. Arrow points to indentation (also visible in panel B); **D.** Muscle unfixed for 48 hours with signs of autolysis. The open arrowheads show vacuolization and the filled arrowhead amorphous tissue; **E.** Same muscle treated as in panel D, after Gram staining. The arrows point to gram-positive cocci; **F.** Muscle fixed for more than 1 year in Thiel-solution. In addition to fragmentation, the structure in the muscle fibres is considerably dissolved and the striation almost no longer recognizable. Scale bar: 100 μm.

part revealed intact tissue with single transverse tears, intact nuclei and visible striations, and intact surrounding tissue. On the contrary, the MF preserved in the Thiel solution for 3 months, were pulled apart and not dense with clear and classical fragmentations of the tissue (Fig. 3B). There were also indentations present (Fig. 3B, C). Striation was visible as well as the nuclei, which, however, were ballooned and pale. The surrounding tissue was moderately dissolved. Thiel tissues from body donors of our institute (Fig. 3F) fixed for more than 1 year showed a further increase in fragmentation and disintegration of the MF, looked like perforated, pale, and nuclei where missing.

pH-value

Table 2 summarizes the pH values of all solutions used. The pH value of the Thiel solution was 7.7 and therefore slightly within the basic range. While the pH of potassium nitrate, ethylene glycol and chlorocresol was slightly within the basic range, the pH of ammonium nitrate and boric acid was within the acidic range. The boric acid solution (pH 4.7) was the solution with the lowest pH. The pH value of the formalin solution was 7.2.

Table 2. pH of Thiel and formalin solution and the solutions A–F

Solution	pH
Thiel solution	7.7
Formalin 4%	7.2
Ammonium nitrate 10% (A)	6.36
Boric acid 3% (B)	4.7
Chlorocresol 0.18% (C)	7.93
Ethylene glycol 10% (D)	7.87
Potassium nitrate 5% (E)	8.58
Sodium sulfite 7% (F)	8.73

Autolysis and natural decay effects

In general, there were no differences regarding fragmentation and nuclei between muscle pieces placed immediately in Thiel solution (Fig. 2B) and those stored up to 48 hours more before fixation (Fig. 3D), but there were differences in autolysis, condition of the surrounding tissue and bacterial growth. At all tested time points, unfixed tissue showed only few and diffusely formed tears, rather than fragmentations with the classic shape. The nuclei were present, but paleness and ballooning increased with the time before fixation. After remaining unfixed for 8 hours, signs of autolysis like vacuolization and disintegration, especially of the connective tissue around the muscle, were already visible (Fig. D). Signs of autolysis and disintegration increased with the duration the tissue remained unfixed. After 48 hours, the MF bundles appeared partly amorphous and with loss of striation, no classic fragmentations occurred. After 8 hours left unfixed, bacterial growth increased as compared to immediately fixed tissue. Bacteria appeared in clusters and were often present within the amorphous surrounding tissue (Fig. 3E). Progression of autolysis varied locally and over time, with the difference between 0–8 hours postmortem being greater than between 8–48 hours. Thus, fragmentation was not dependent on autolysis and decay.

Discussion and Conclusions

In this study, we investigated the influence of the Thiel solution and its individual ingredients on striated muscle fibres in the mouse. We used light microscopy to identify an ingredient responsible for

fragmentation and consequently the pliability of Thiel cadavers. For that purpose, we also ruled out autolysis and natural decay as a possible confounding factor.

While muscle fixed in formalin served as 1-day and 3-months control, and showed regular histology, muscle fixed in Thiel solution for one day already showed disintegrated and less dense MF with few fragmentations and some indentations, which we interpreted as beginning fragmentations. In muscles fixed according to Thiel [37], an increase in fragmentation and disintegration was visible after 3 months. In muscle tissue after Thiel fixation for more than 1 year, there was a further increase in fragmentation and strong disintegration, which looked like perforated muscle. This suggests both potentiation of the ingredients in the complete solution and progression of the effects over time. So far, Thiel-fixed muscle has been demonstrated to show a similar impairment of muscle tissue compared with other studies; however, in our study, the nuclei were still present, but paler than after formalin fixation [4, 16, 34, 38].

This time dependent effect on muscle histology, which presumably influences the cadaver pliability could be considered and adapted depending on the use of the cadavers by the institutes.

To specify the effect of each ingredient of the Thiel solution, we immersed muscle pieces in the single ingredients for 24 hours. Since this procedure corresponds to an incomplete fixation, we proceeded similar to the method of Cocariu et al. [7] and Baur et al. [3]. Striated muscle tissue was stored at 4°C for only 20 hours of immersion, in order to omit signs of decay and autolysis. Then the tissue was thawed until room temperature for another 4 hours to allow the chemicals to react. The histological effects were compared to those of muscle pieces fixed in 4% formalin and Thiel solution for 24 hours, respectively. Several ingredients of the Thiel solution could be identified to cause fragmentations in the muscle, predominantly sodium sulphite and potassium nitrate, and to a lesser extent ammonium nitrate, all belonging to the group of salts. This supports the hypothesis of McDougall et al. [27], that salts may play a role in tissue alteration. They had found fragmentation of tissue fixed in a modified Thiel solution without boric acid, which is supported by our finding that boric acid as a single component did not cause any fragmentation. In another study, boric acid exerted a slightly corrosive effect on striated muscle tissue at high concentration, but no classic fragmentation was visible [38]. While low pH with its corrosive effect has been suggested as a possible cause of fragmentation [5], muscle pieces immersed in boric acid solution did not show any significant fragmentation, so that we do not consider boric acid or low pH to be the cause of fragmentation. Further, while our pH measurements showed that boric acid solution possessed the lowest pH of all ingredients, Liao postulated boric acid to be almost neutralized in Thiel solution [26].

Usually, fixation of body donors is not performed immediately, but 1 to 2 days postmortem after their arrival at an anatomical institute. As some studies used human muscle tissue from body donors, we also investigated the role of autolysis and decay on muscle histology and observed time-dependent morphological changes, which did not resemble fragmentations. Notably, these alterations were present from the beginning and did not increase after 48 hours, in contrast to bacterial growth and signs of decay and autolysis. This makes the influence of autolysis and decay on fragmentations unlikely. Therefore, we conclude that the fragmentations observed in Thiel-fixed muscle tissue can be attributed to the fixative itself, particularly the group of salts, i.e. sodium sulfite, potassium nitrate and to a lesser extent ammonium nitrate. While fragmentation was defined as disruption of the MF, it must be mentioned that in all individual ingredients, as well as in the Thiel tissue, the MF were also less dense, less contiguous and appeared pulled apart. There exist several histological studies, which have fixed muscle pieces in either sodium chloride or SSS [1, 4, 34, 38] and they showed similar tissue that was neither dense, nor contiguous and fragmented. In agreement with our findings, these studies showed a similar effect when muscle was fixed in solutions containing salts, as a possible reason for fragmentation [27]. We suggest a physiochemical process, which may underlie the principle of solvatization of salts. Anions and cations are enclosed in a hydration shell of water splitting them apart. The dissolved anions and cations, in our case mostly sodium and potassium, move around or into the myofibrils, due to the surrounding osmotic pressure or by directly binding the charged groups of the myofibrils. The bound and charged molecules lead to electrostatic repulsion, thus degrading the myofibrils and disturbing the spatial myofibrillar structure due to entropically driven forces.

Both physiochemical processes enable further water to enter in, as well as around the myofibrillar

structures, as a result of lattice changes [8, 22, 29]. These processes might well be the cause of indentations or, if increasing, fragmentation with less dense MF. Degradation and enhanced water retention pulls the MF apart, both longitudinally and perpendicularly as visible in light microscopy.

Another issue that leads to fragile tissue, and therefore presumably contributes to fragmentation, is the fact that, in Thiel solution, formalin concentration is significantly reduced. Methylene bridges crosslinking adjacent proteins, amines and related nucleophiles are missing [6, 16, 26]. We histologically noticed this fragility when the paraffin blocks were rotated during the cutting process on the microtome. Cutting the MF vertically instead of horizontally resulted in a significant increase of small tissue cracks and deformation in MF (data not shown). The lower formalin content and the effect of the salts might be a cumulative process. Adjusting the amount of salts in the Thiel solution, could be a possibility to decrease the amount of salt and water entering and binding to the MF, therefore reducing the lattice rearrangement. We assume that the reduced amount of formalin and the myofibrillar degradation due to salts could be the origin of the fragmentations in muscle tissue. This, in turn, reduces the muscle resistance while moving joints, described as pliability.

Limitations of the study

However, there are some limitations of the study. We tested the ingredients of the Thiel solution and their effects separately. Due to decay and autolysis, the long-term effect of the individual ingredients could not be investigated. Also, Thiel solution and potential effects due to altered properties of the solution were not analysed after 1 year. In addition, ingredients belonging to the same chemical group may have an amplifying effect.

Although this was not investigated, we consider the chemical effects to be similar to the effects of the complete Thiel solution. The chemical effect on muscle of mice and humans could be different. However, since they have basically the same histological structure, we assume that the effect of the chemicals is the same. In addition, similar images have been published in literature with fragmentations of human striated muscle. Nevertheless, further studies are needed to confirm our findings as well as the suitability of an adjusted solution as a fixative. The anticipated improved properties could then be evaluated for their potential applications to improve biomechanical properties for research and surgical training.

CONCLUSIONS

To conclude, we investigated the effects of Thiel solution on striated muscle to find an explanation for the observed fragmentation visible of Thiel-fixed tissue, which is proposed as reason of the cadaver joint pliability. The pH as well as autolysis and decay could be excluded. Fragmentations occurred after Thiel fixation and were time dependent. Ingredients belonging to the group of salts showed to cause fragmentations, disturbed myofibrillar lattice, likely by repulsion of actin and myosin as well as enhanced water binding. Adjustments of salt concentrations and altered tissue pliability might be advantageous for work with Thiel-cadavers.

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Conflict of interest: None declared

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