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From Cell Biology to Tissue Engineering

The comparative analyses of decalcification procedures and methyl benzoate pre-treatment on tissue preservation and antigenicity in human acetabular labra

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Summary. The histological processing of musculoskeletal tissue might be challenging. The alteration of tissue composition e.g. by calcification of soft tissue in the elderly, after trauma or surgical interventions makes the histological processing of fixed tissue difficult. Additional steps of decalcification are then needed that probably affect the staining quality. In the present work, the effects of different decalcification agents and the intermedium methyl benzoate on histological staining methods and immunohistochemistry have been compared.

Acetabular labra were fixed with 4% paraformaldehyde, left untreated or decalcified using 30% ethylenediaminetetraacetic acid (EDTA; Chelaplex®) or 6% trichloroacetic acid (TCA) for 1-4 days to investigate the effects of decalcification duration. Moreover, samples were pretreated with methyl benzoate or conventionally paraffin embedded independent of decalcification procedure and duration. The specimens were evaluated using hemalaun-eosin, Azur II- methylene blue staining or immunohistochemistry against ankyrin B to visualize nerve fibers.

Decalcification with Chelaplex® or TCA reduced cutting artifacts without affecting the tissue morphology and proteoglycan staining but decreased antigenicity in immunohistochemistry. Interestingly, methyl benzoate further reduced cutting artifacts without altering tissue morphology and elevated antigenicity for Chelaplex® decalcified tissue samples in immunohistochemistry.

The decalcification with Chelaplex® or 6% TCA preserves tissue morphology and proteoglycan staining similar to non- decalcified tissue but facilitates section processing. In immunohistochemistry both decalcification agents decreased antigenicity. Chelaplex® decalcified, methyl benzoate treated samples yielded an improved antigenicity.

Key words: Decalcification, EDTA, Trichloroacetic acid, Methyl benzoate, Ankyrin B

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Introduction

In histological examinations of musculoskeletal tissue, the anorganic matrix impedes access to the organic components and decreases antigenicity (Ehrlich et al., 2009). This problem is not solely restricted to bony tissue. Similar problems are present at the attachment of ligaments or cartilage to the bone or in pathologies promoting ossification (Ebraheim et al., 1991; Dequeker et al., 1997; Seldes et al., 2001; Jimenez et al., 2014). In those cases decalcification is the procedure of choice. There are several decalcification agents and protocols available (Bourque et al., 1993; Ehrlich et al., 2009; Begum et al., 2010). Fast decalcification agents, like mineral acids, shrink the tissue and impair the immunoreactivity of matrix proteins or the staining by cationic dyes, like methylene

blue (Melrose et al., 2004; Ehrlich et al., 2009). Other rapid decalcification agents, e.g. RDO- Gold or Krajian solution, preserve the tissue structure but affect vulnerable structures like nerves or nerve endings (Begum et al., 2010). After RDO-Gold and Krajian decalcification a disorganized and disrupted staining for myelin was reported (Begum et al., 2010). Thus, organic chelators with an appropriate calcium release, such as ethylenediaminetetraacetic acid (EDTA), are preferred and widely used facilitating the process of tissue sectioning (Ehrlich et al., 2009; Schmitz et al., 2010; González-Chávez et al., 2013). EDTA decalcification is a time-consuming procedure but preserves tissue morphology, proteoglycans and antigenicity with only slight impairments (Mori et al., 1988; Buma et al., 1992; Bourque et al., 1993; Melrose et al., 2004; Begum et al., 2010; Schmitz et al., 2010; González-Chávez et al., 2013;). Therefore, EDTA decalcification is referenced investigating the grade of osteoarthritis or neuronal degeneration (Iwamoto et al., 1995; Glasson et al., 2010). An alternative is presented by low concentrated acids like 6% trichloroacetic acid (TCA) with a shorter time for decalcification compared to EDTA and a preserved immunoreactivity (Alers et al., 1999; Begum et al., 2010). Nonetheless, the acidity of TCA might alter antigen epitopes and staining behavior (Pollard et al., 1987; Bourque et al., 1993). An additional attempt to ameliorate histological processing of musculoskeletal tissue is the application of intermedia like methyl benzoate known for improved tissue and antigenicity preservation (Sato et al., 1986; Le Minor, 1987). In contrast to other clearing agents, methyl benzoate seems to soften the tissue (Schäperclaus, 1992; Tasci et al., 2010; Malentacca and Lajolo, 2015). Comparable clearing agents such as methyl salicylate in combination with EDTA were used for histological investigations previously (Bland and Ashhurst, 2001). However, the effects of this combined application on tissue preservation, proteoglycan staining and immunohistochemistry in comparison to other decalcification procedures were not completely elucidated.

Therefore, the present study was carried out to compare the decalcification methods with 6% TCA or Chelaplex® (EDTA) and to figure out a possible benefit by methyl benzoate treatment.

Material and methods

30% Chelaplex® (EDTA), toluol, methyl benzoate, Mayer's hemalaun, eosin (Dr. Hollborn und Söhne, Leipzig, Germany), paraformaldehyde (PFA), Tris (Roth, Karlsruhe, Germany), TCA, Triton X-100, methanol, ethanol, xylene, normal goat serum, bovine serum albumin, biotinylated goat anti-mouse IgG (cat.-No B-7264), 3,3'- diaminobenzidine (Sigma-Aldrich, Steinheim, Germany), 15 ml and 50 ml tubes (Falcon, BD Biosciences Discovery Labware, Bedford, MA, USA), low melting paraffin (melting range: 52-54°C), Roti® Plast (melting range: 54-56°C), hydrogen peroxide (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), ExtrAvidin-horseradish peroxidase (cat.-No E2886) from Sigma, St. Louis, MO, USA, superfrost microscope slides (Thermo Fisher Scientific, Rockford, IL, USA), disodium hydrogen phosphate, potassium dihydrogen phosphate, entellan (Merck, Darmstadt, Germany), mouse monoclonal antibody anti human ankyrin B (cat.-No 75-145, clone N105/17, NeuroMab, San Diego, CA, USA), Zeiss Axioskop 2 plus equipped with a Zeiss AxioCam HR camera (Zeiss, Göttingen, Germany), AxioVision 4.6 (Zeiss, Göttingen, Germany)

Ethical statement

Acetabular labra were collected from specimens of the body donor program of the University of Leipzig. Individuals gave their signed consent before death for the use of their bodies for educational purposes in medical school and for research in accordance to the Saxonian Death and Funeral Act of 1994.

Assignment to decalcification and embedding

The acetabular labra were resected using an anterolateral approach and removing the surrounding soft tissue exposing the complete acetabulum. After capsulotomy and hip dislocation, labra were dissected periosteal and fixed using 4% (w/v) PFA in 0.2 mol/l phosphate buffered saline solution (PBS) for at least 48 hours (h). Afterwards, labra were arbitrarily allocated to the different treatment protocols (Fig. 1). Firstly, labra were divided in three equal parts and left untreated (PFA group) or subjected to decalcification procedures: 6% (w/v) TCA or 30% EDTA (30% Chelaplex®). Independently of being decalcified or left untreated, samples were stored in 50 ml tubes containing at least 25 ml of fluid and protected from light. Each treatment had a maximum duration of four days (d), the duration was set on the basis of our own pilot experiments. In the untreated group, the PFA was renewed every second day. At each day, a small part (approximately 1x1x0.5 cm) of the labral parts was dissected and subsequently stored in a 15 ml tube with at least 5 ml PFA at 4°C until further use. PFA was renewed every second day. After decalcification, for each procedure (PFA, 6% TCA or 30% Chelaplex®) and for each time point (1 to 4d) part of samples were subjected to methyl benzoate pre-treatment. Thereafter, samples were paraffin embedded and if not indicated stirring, heating, vacuum agitation was avoided.

Decalcification with 6% (w/v) trichloroacetic acid (TCA)

From the commercially available trichloroacetic acid 6 g were dissolved in 100 ml distilled water without adjusting the pH (Begum et al., 2010). Tissue samples were washed for 5 minutes (min) in 0.2 mol/l PBS before they were treated with 6% TCA at 4°C.

All specimens were completely covered by the solution. The solution was not renewed within the experimental procedure. Before transferring the samples back to PFA at their assigned time point, samples were washed again for 5 min in 0.2 mol/l PBS.

Decalcification with 30% Chelaplex®

After washing, specimens were treated with 30% Chelaplex® (30% EDTA). The complete coverage of the specimens by the fluid was ensured and the samples were stored at 38°C. Chelaplex® was changed every second day.

Methyl benzoate pre-embedding treatment and conventional paraffin embedding

Tissue samples allocated for methyl benzoate pre-treatment were subjected to 50%, 70% and 96% ethanol for 8-12 h for each step. Afterwards, the specimens were rinsed in absolute ethanol for 4 h twice followed by the incubation with methyl benzoate for 8-12 h three times. Subsequently, specimens were treated with toluol for 2 h twice. For each step the solutions were renewed. Thereafter, tissue samples were transferred to a liquid mixture (1:1) of toluol and melted low melting paraffin and incubated twice for 2 h at 60°C in 10 ml glass vials. The temperature was maintained for the next steps. Specimens were then transferred and incubated in low melting paraffin for at least 12 h followed by incubation in Roti® Plast for at least 12 h.

Specimens assigned to conventional paraffin embedding were treated with 70% and 80% ethanol each for 90 min followed by 2.5 h 96% ethanol, absolute ethanol for 2.5 h twice, xylol for 60 min twice and Roti® Plast twice (for 3 h and for 4 h).

Afterwards all tissue samples were embedded using an embedding station. From each block 12 μm thick sections were cut and used for staining.

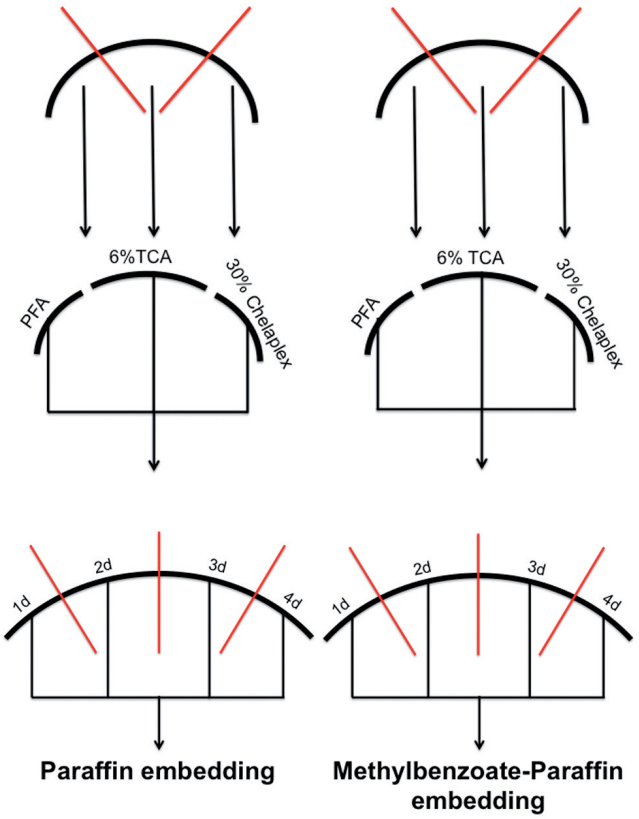

Fig. 1. Assignment to decalcification and embedding or methyl benzoate pre-treatment. Following 4% PFA fixation labra were divided into three equal parts. From each labrum one part was not decalcified (PFA) or decalcified either by 6% TCA or 30% Chelaplex®. Over four days from each part an additional part was resected and stored in 4% PFA until embedding. Independent of decalcification technique or duration labra were paraffin embedded with or without the use of the intermedium methyl benzoate.

Hemalaun-eosin (HE) staining

Assigned slides for hemalaun-eosin (HE) staining were deparaffinized and incubated with Mayer's hemalaun for 5 min followed by 30 min rinsing with tap water. Subsequently, eosin was added for 5 min followed by the dehydration using rising alcohols and mounting on microscope slides with entellan.

Azur II- methylene blue staining

For Azur II- methylene blue staining (AIIMB) sections were deparaffinized and stained with AIIMB (0.125% methylene blue, 0.125% borax solution, 0.125% Azur II solution in distilled water) for 20 seconds. After washing with distilled water sections were drained in rising alcohols and mounted with entellan.

Ankyrin B (AnkB) staining- ExtrAvidin peroxidase staining

To investigate the effects of the different procedures on vulnerable neuronal structures, immunohistochemistry with an anti-ankyrin B (AnkB) antibody was performed as previously described (Pieroh et al., 2014). Briefly, all sections were treated with methanol containing 5% (v/v) of 30% (v/v) hydrogen peroxide for 10 min and washed afterwards 3 times for 10 min with PBS/Triton X-100. Prior to antibody treatment, samples were incubated with normal goat serum (1:20 in PBS/Triton X-100) for 30 min at room temperature followed by incubation with the anti-ankyrin B antibody (1:1000) in PBS/Triton X-100 containing 0.5% (w/v) bovine albumin serum for at least 16 h at 4°C. Thereafter, the slides were washed three times for 10 min with PBS/Triton X-100, incubated with biotinylated goat anti-mouse IgG (1:100) for 60 min and washed

again 3 times for each 10 min with PBS/Triton X-100. Afterwards, the slides were incubated with ExtrAvidin-Horseradish Peroxidase (1:100) in PBS/Triton X-100 for 60 min. The visualization of immunoreactivity was enabled using 3,3-diamino-benzidine followed by coverslipping with entellan.

Microscopic analysis

Stained sections were examined regarding structural or immunoreactive changes related to different decalcification techniques and methyl benzoate pre-treatment using bright field microscopy. Image acquisition was performed with a Zeiss Axioskop 2 plus microscope equipped with a Zeiss AxioCam HR camera (Zeiss, Göttingen, Germany) driven by AxioVision 4.6 software.

Results

Effects of decalcification agents and durations or methyl-benzoate pretreatment on labral tissue

In HE stained sections tissue preservation and matrix structure were investigated. HE staining yielded the parallel-aligned collagen fiber with the fibrocyte nuclei

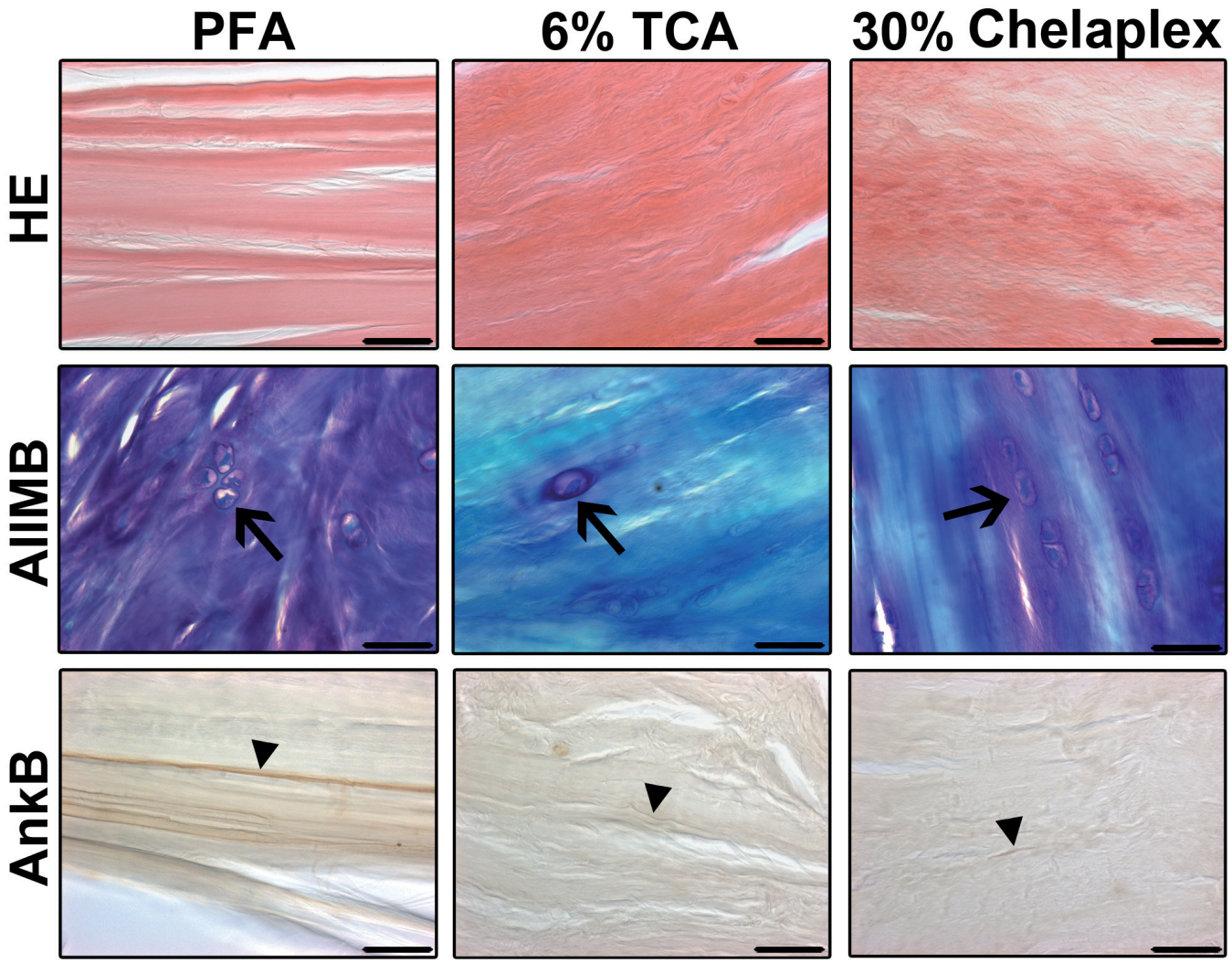

Fig. 2. Comparison of decalcification techniques 1d after application with subsequent methyl benzoate pre-treated tissue samples regarding tissue preservation, cartilage architecture and immunohistochemistry for ankyrin B. Decalcification independent of duration reduced cutting artifacts visible in all stained samples. In HE stained sections, decalcification led to an assumable dissociation of collagen fibers enhancing the opportunity to determine the course and contrast to cellular structures. In AIIMB staining, decalcification altered the metachromatic characteristics of the cartilage and elevated the contrast between cartilage components. Chondrons are marked by arrows. Regarding immunoreactivity (arrow head), decalcification with 6% TCA or 30% Chelaplex® reduced antigenicity. Scale bars: 100 μm.

in between (Fig. 2). In samples treated with methyl benzoate, both decalcification techniques reduced obviously cutting artifacts improving tissue preservation (Figs. 2, 3). In decalcified samples, the collagen fibers seemed dissociated, revealing their course and orientation. Furthermore, the contrast to the cellular structures within matrix was enhanced. Matrix is basically formed by collagen in this region. Obvious differences in terms of staining intensity, loss of nuclear staining or complete dissociation of collagen fiber bundles related to the agent and time of decalcification applied were not observed.

With the AIIMB staining changes of cartilage architecture and proteoglycan staining were examined. The staining represented territories that were in majority small in size and contained only one chondrocyte (Fig. 3). Furthermore, larger territories were found with multiple chondrocytes (Fig. 1) surrounded by force adapted collagen fibers.

Decalcification reduced cutting artifacts (Figs. 2, 3), both decalcification procedures seemed to enhance the contrast between extracellular cartilage matrix and cellular components but maintained proteoglycan staining. Decalcification duration or type of decalcification did not lead to differences in staining of the cartilage structure.

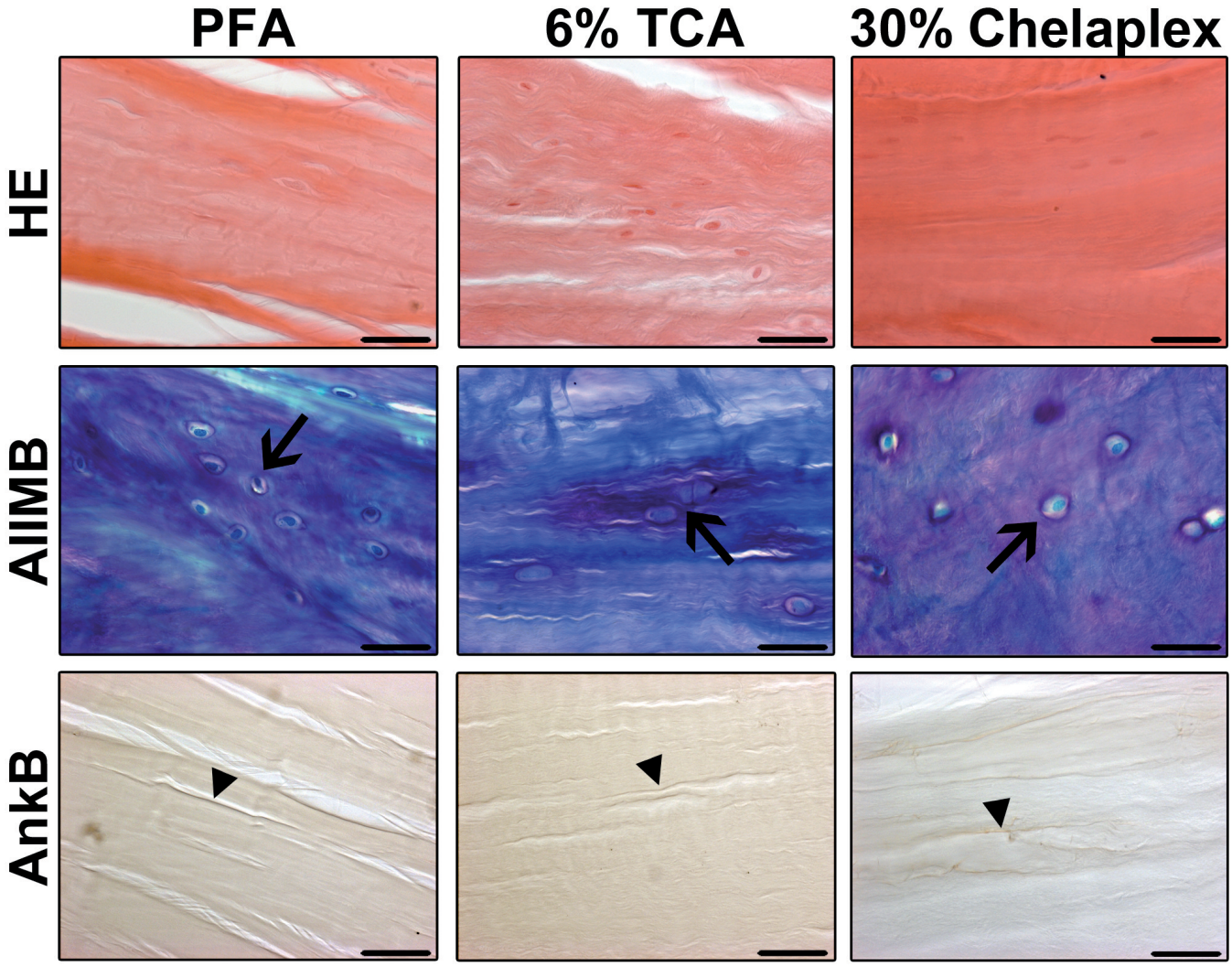

Fig. 3. Comparison of decalcification techniques 3d after application with subsequent methyl benzoate treatment regarding tissue preservation, cartilage architecture and immunohistochemistry for ankyrin B. Besides further reduced cutting artifacts no obvious differences over time were determined between 1 (Fig.2) and 3d in HE and AIIMB staining. Interestingly, decalcification with 6% TCA further impaired antigenicity of AnkB whereas samples decalcified with 30% Chelaplex® presented an appropriate preserved antigenicity. Chondrons are marked by arrows, positive immunoreactivity by arrow head. Scale bars: 100 μm.

For immunohistochemistry AnkB, expressed in mechanoreceptors, nociceptors and free nerve endings, was chosen (Engelhardt et al., 2013). The nerve endings displayed most likely unmyelinated axons in the fibrous part of the labrum (Figs. 2-4). Decalcification with 6% TCA seemed to decrease antigenicity especially with increasing duration of decalcification (Figs. 2, 3). In contrast, decalcification with 30% Chelaplex® and methyl benzoate treatment improved antigenicity, especially with increasing time of decalcification (Figs. 2, 3).

Comparison of methyl benzoate vs. non-methyl benzoate pre-treated samples

Independent of the applied decalcification agent, methyl benzoate treatment reduced cutting artifacts compared to non-methyl benzoate treated specimen possibly due to the softening effects of methyl benzoate (Figs. 3, 4, Table 1). Best-preserved samples were obtained when specimens were long decalcified with 30% Chelaplex® and treated with methyl benzoate (Fig. 3, Table 1). No obvious differences in HE staining and

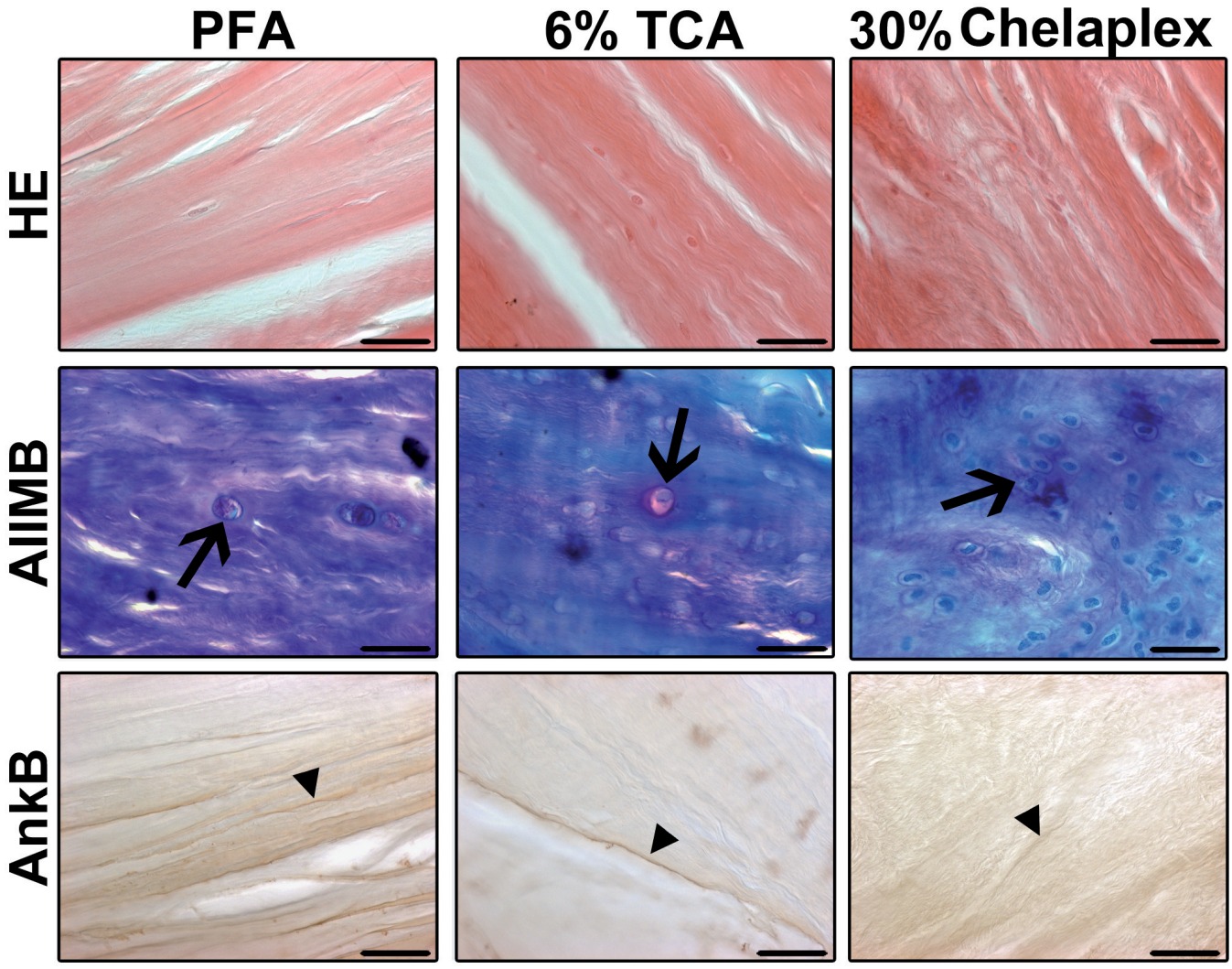

Fig. 4. Comparison of decalcification techniques 3d after application without subsequent methyl benzoate pre-treatment tissue samples regarding tissue preservation, cartilage architecture and immunohistochemistry for ankyrin B. Compared to methyl benzoate pre-treated samples, non- methyl benzoate pre-treated samples yielded a higher amount of cutting artifacts independent of the applied decalcification agent. No differences were detected regarding the tissue integrity (HE) and proteoglycan staining (AIIMB) when methyl benzoate pretreated samples were compared to those without methyl benzoate pretreatment. In non- methyl benzoate pre-treated samples decalcification impaired antigenicity for both decalcification agents. Thus, methyl benzoate treatment did not affect the immunoreactivity of AnkB but enhanced the antigenicity following Chelaplex® decalcification. Chondrons are marked by arrows, positive immunoreactivity by arrow head. Scale bars: 100 μm.

proteoglycan staining were detected between methyl benzoate treated and non-methyl benzoate treated samples (Figs. 3, 4, Table 1). The application of methyl benzoate enhanced the contrast between cellular and matrix components in AIIMB staining (Fig. 3, Table 1). In immunohistochemistry, the presence or absence of methyl benzoate had no effect on visualization of neuronal structures in non- decalcified and with 6 % TCA decalcified tissues (Figs. 3, 4). In contrast, pre-treatment with methyl benzoate increased the antigenicity in 30% Chelaplex® decalcified sections ameliorating the detection of positive immunoreactivity by reducing the background staining (Fig. 3, Table 1). Although decalcification using 30% Chelaplex® enhanced the background staining similar to 6% TCA in non-methyl benzoate treated samples (Fig. 4), methyl benzoate reduced the background staining, but only for decalcification with 30% Chelaplex®. It should be mentioned that except for the combination of 30% Chelaplex® and methyl benzoate treatment the duration of decalcification was related to decreased antigenicity (Fig. 2-4). Notably, the paraffin blocks of PFA group (4d) regularly broke during the cutting process indicating a higher stiffness and/or calcification remnants in tissue.

Though decalcification agents reduced cutting artifacts and preserve tissue morphology, they decreased antigenicity. Interestingly, in specimens pretreated with methyl benzoate cutting artifacts were further reduced possibly due to its softening effects (Schäperclaus, 1992; Tasci et al., 2010; Malentacca and Lajolo, 2015).

Discussion

By the anorganic matrix of musculoskeletal tissue the histological processing and staining is impeded, especially for bone (Ehrlich et al., 2009). Nonetheless, the attachment of ligaments/cartilage to the bone or pathologies, such as labral tears, osteoarthritis, calcific deposition promoting ossification also impair the histological assessment of tendons, ligaments or joint labra (Ebraheim et al., 1991; Dequeker et al., 1997; Seldes et al., 2001; Jimenez et al., 2014). Changes in the anorganic matrix in those tissues might not be so severe as in bone, but the histological processing is still challenging. Furthermore, the composition of soft and hard tissue as well as their transition may aggravate histological processing. Therefore, additional and modified histological methods processing this kind of tissue are required. The application of decalcification agents facilitates tissue processing of musculoskeletal tissue but an impairment of antigenicity and tissue morphology has been discussed (Melrose et al., 2004; Ehrlich et al., 2009; Begum et al., 2010). Organic chelators such as EDTA (Chelaplex®) or low concentrated acids, e.g. 6% TCA, maintain tissue integrity and immunoreactivity and were thus intensively used in histology (Mori et al., 1988; Buma et al., 1992; Bourque et al., 1993; Alers et al., 1999; Melrose et al., 2004; Begum et al., 2010; Schmitz et al., 2010; González-Chávez et al., 2013). Nonetheless, they differ in their mode of action. EDTA is a chelator acting at a neutral pH, binding the calcium to form a precipitating stable complex (Kiviranta et al., 1980). In contrast, acids remove the insoluble calcium to form soluble calcium salts (Eggert and Germain, 1979; Alers et al., 1999). Though decalcification with EDTA (Chelaplex®) is time-consuming, its result with mineral acids such as TCA is a poor staining of nucleic acids and an overstaining of the cytoplasm in HE stained sections (Mori et al., 1988; Graner et al., 1995; Melrose et al., 2004). In addition, EDTA decalcification seemed to be more cautious, resulting in a preserved tissue integrity even in samples with a transition of soft to hard structures (Bourque et al., 1993). Though EDTA and TCA decalcification were intensively discussed in earlier studies, their combination with additional procedures such as methyl benzoate treatment was not evaluated. Intermedia like methyl benzoate are widely used to soften the tissue (Sato et al., 1986; Le Minor, 1987). In contrast to other intermedia or clearing agents, such as xylene or benzene, methyl benzoate softens the tissue without affecting morphology and tissue composition (Schäperclaus, 1992; Tasci et al., 2010; Malentacca and Lajolo, 2015).

As described previously EDTA and TCA decalcification improved tissue-processing regarding decreased cutting artifacts (Ehrlich et al., 2009), methyl benzoate treatment further reduced cutting artifacts.

Table 1. The comparative analyses of decalcification procedures and methyl benzoate pre-treatment on tissue preservation and antigenicity.

	- methyl benzoate			+ methyl benzoate		
	PFA	6% TCA	30% Chelaplex®	PFA	6% TCA	30% Chelaplex®
Cutting Artifacts	+++	++	++	++	+	o*
Proteoglycan Staining	o	o	o	o	O	o
Contrast Cartilage Matrix Components	o	+	+	o	++	++
Ankyrin B immunoreactivity	+	+	+	+	+	+++
Antigenicity	+	++*	++*	+	++*	o*

o: indicates the optimal status regarding the investigated characteristic or the initial state. The amount of + shows the manifestation of the characteristic. *: indicates an increase of the characteristic with a prolonged time of decalcification.

Interestingly, TCA decalcification neither alters the staining intensity nor leads to an overstaining of the cytoplasm or nuclei as earlier reported (Graner et al., 1995; Emans et al., 2005). As recently reported, TCA treatment seemed to lead to collagen fiber dissociation in the present study (Ehrlich et al., 2009). Though this dissociation was absent in Chelaplex® decalcified and methyl benzoate treated samples, in Chelaplex® decalcified and non- methyl benzoate treated samples the dissociation was also found. However, in previous studies a dissociation of collagen fibers was not reported following EDTA decalcification.

Mineral acids are known to impair proteoglycan staining when using cationic dyes such as methylene blue (Melrose et al., 2004; Schmitz et al., 2010; Hunziker et al., 2014). But so far, it remains unclear whether these alterations are also found following decalcification with EDTA or 6% TCA.

The AIIMB staining in decalcified sections seemed to be more intense and with a higher contrast possibly due to the solubilized amount of proteoglycans by decalcification (Ehrlich et al., 2009). Differences between the used decalcification agents were not observed.

Nerves and the antigenicity of their proteins are highly sensitive to histological procedures (Begum et al., 2010). Therefore, their preservation represents an appropriate indicator for a cautious decalcification. The decalcification with EDTA is known to preserve surface antigens (Mori et al., 1988; Bourque et al., 1993) and neuronal proteins, but recent publications yielded, despite a good immunoreactivity, also a slight disorganization of myelin basic protein immunoreactiviy (Begum et al., 2010). In contrast, decalcification with 6% TCA revealed an optimal immunoreactivity of myelin basic protein (Begum et al., 2010). On the other hand, mineral acids are known to decrease the intensity of immunoreactivity whereas EDTA decalcified samples displayed the most intense immunostaining (González-Chávez et al., 2013).

In the present study, AnkB as a membrane protein expressed in all mechanoreceptors, nociceptors and free nerve endings was chosen to stain almost all nerval structures and to investigate differences in immunohistochemistry upon decalcification and methyl benzoate treatment (Engelhardt et al., 2013). In contrast to other nerval markers AnkB labelling depicts almost all nerval structures. Besides a reduced antigenicity in decalcified samples, no differences from non-decalcified slides were obtained. No effects depending on the used decalcification agent were determined. Independent of the applied decalcification agent or used staining, the duration of decalcification did not lead to differences in morphology or immunohistochemistry as known for EDTA but not for TCA decalcification (Hukkanen et al., 1993; Ichikawa et al., 1997; Miao and Scutt, 2002).

In plastic embedding methyl benzoate was shown to maintain antigenicity and act as plasticizer (Erben, 1997). Interestingly, the combination of EDTA, respectively Chelaplex®, and methyl benzoate treatment partially enhanced antigenicity in immunohistochemistry and improved the detection of positive immunoreactivity. This effect was absent in tissue decalcified with 6% TCA.

It is of note that the present study has some limitations. In contrast to previous studies (Eggert and Germain, 1979; Emans et al., 2005; Begum et al., 2010; Guibas et al., 2014), only two different decalcification agents were tested. The effect of different fixation agents was not examined (Bourque et al., 1993; Miao and Scutt, 2002; Hunziker et al., 2014). Furthermore, the duration of decalcification was set based upon our own pilot experiments. Although the duration of decalcification might be crucial, a general protocol for all tissues and especially for all tissue sizes might be not appropriate as EDTA decalcification was shown to be strongly dependent on slice thickness (Kiviranta et al., 1980). As previously shown the success of decalcification can be monitored by chemical (Begum et al., 2010) or radiographic (Guibas et al., 2014) methods. Both methods reveal an option to objectively assess the grade of decalcification and subsequently determine the suitable starting time point for cutting the specimens. Here, the introduction of a histological score might be an opportunity (Guibas et al., 2014). However, such scores are observer dependent and they need a validation. Currently no validated score is available to compare decalcification methods of musculoskeletal tissue, thus their introduction was avoided in the present study.

In conclusion, the decalcification with EDTA (Chelaplex®) or 6% TCA preserves tissue morphology and proteoglycan staining similar to non- decalcified tissue but facilitates section processing. Both decalcification agents maintain immunoreactivity for AnkB though a decreased antigenicity was detected. The addition of methyl benzoate following EDTA decalcification enhanced antigenicity and improved the detection of positive immunoreactivity. Besides, methyl benzoate treatment further improved histological sectioning without affecting HE and AIIMB staining.

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