Comparative assessment of the morphology and antigenicity of human osteochondral units using formalin and coagulant fixatives

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SUMMARY

Fixation is crucial for preserving tissue integrity during processing, and the most commonly used cross-linking fixative in immunohistochemistry is neutral buffered formalin, which requires antigen retrieval as a crucial step. The successful use of newer coagulant fixatives like methacarn and EMA to preserve isotopes and eliminate the need for antigen retrieval has been reported recently, but their role in decalcified osteochondral unit samples remains unknown. Limited information on the use of coagulant fixatives as formalin substitutes makes it important to comparatively evaluate their effects on osteochondral samples and the impact of antigen retrieval on different days. Osteochondral units from a patient with osteoarthritis who underwent total knee-replacement surgery were fixed with three studied fixatives (Formalin, Methacarn, EMA) and divided into four portions, for different time periods (Day 1, 3, 7, 10). Sections were decalcified, stained with Safranin O, Alcian Blue, Toluidine Blue, PicroSirius Red, Hematoxylin and Eosin, and immunohistochemical analysis of Collagen type II and type X with and without antigen retrieval was conducted.

Formalin showed better hematoxylin uptake than coagulant-based fixatives, while all fixatives preserved tissue morphology without necrosis or cellular degeneration with comparable staining quality. Methacarn and EMA-fixed tissues showed higher uptake of collagen type II compared to formalin-fixed tissues, with collagen type II uptake occurring only in the cartilage region and collagen type X uptake occurring only in the bone region. The study highlights the effectiveness of methacarn and EMA as efficient alternatives to formalin, preserving tissue morphology and antigen specificity.

Keywords: Formalin – Methacarn – EMA fixative – Osteochondral unit – Antigen retrieval – Collagen type II

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Submitted: April 28, 2023. **Accepted:** June 7, 2023

https://doi.org/10.52083/CVZF3774

INTRODUCTION

Fixation is a process that occurs through a combination of physical and chemical mechanisms, and it involves reactions where the fixative acts by slowly diffusing into the tissues (Dey, 2018). Fixation is crucial to maintain the cellular architecture and composition of biopsy tissue specimens during processing, thereby preserving tissue integrity for further study. Following fixation, the tissues can be embedded in paraffin wax and used for further analysis, including immunohistochemistry and molecular studies. Furthermore, fixation is vital in preserving the spatial relationships between carbohydrates, proteins and other intracellular bioactive molecules, which enables their examination and analysis. Neutral Buffered Formalin (NBF), Methacarn, and EMA (mixture of ethanol, methanol and acetic acid) are all fixatives commonly used in histology to preserve tissue samples (Howat and Wilson, 2014). Methacarn and EMA are both coagulative and alcohol fixatives.

NBF fixation is a widely used and optimized fixative that preserves tissue morphology, aids in examining cell architecture and identifying abnormalities (Thavarajah et al., 2012). However, it is known to cause tissue artifacts, tissue shrinkage and distortion, particularly in delicate tissues such as brain or lung tissue. Another disadvantage of NBF fixation is that it can affect the antigenicity of specific proteins, leading to false negative results in the immunohistochemical analysis, thereby leading to loss of time and money due to the standardization of any IHC test (Buesa, 2008). Despite these disadvantages, NBF remains the most commonly used fixative in histology due to its effectiveness, reliability, and ease of use.

Methacarn is an effective coagulant fixative for preserving specific tissues, like nervous and muscular tissues, with minimal shrinkage and distortion (Shibutani et al, 2000). It also preserves protein antigenicity for accurate immunohistochemical analysis. However, it requires longer fixation times and can be more expensive. EMA fixative is a non-formalin coagulant fixative commonly used for preserving tissue morphology and antigenicity. Unlike formalin, which crosslinks proteins, EMA fixative penetrates the tissue and denatures the proteins, thus preserving their native conformation and antigenicity. This makes EMA fixative particularly useful for immunohistochemical analysis, as it allows for more accurate detection of specific antigens. EMA fixative is also compatible with molecular analysis techniques, such as RNA and DNA extraction (Rahman et al., 2022). However, like other non-formalin fixatives, EMA fixative is more expensive and may require longer fixation times (Nietner et al., 2012). Despite these limitations, EMA fixative is a valuable tool in histology for preserving tissue morphology and antigenicity for further analysis.

Decalcification is an essential prerequisite for the immunohistochemical analysis of bone or tissue that contains bone (Alers et al., 1999). Bone's hydroxyapatite crystals, which provide strength, make it challenging to section and analyze (Dermience et al., 2015). Decalcification removes mineral salts, making it easier to section, but it can affect the organic components needed for histological analysis. Fixation before decalcification is necessary to preserve the tissue's cellular and fibrous components (Shields and Heinbockel, 2018). Decalcification and tissue fixation may result in poor antibody binding due to epitope masking or crosslink formation (D'Amico et al., 2009). Therefore, using an appropriate antigen retrieval technique is crucial for examining bone-containing tissue through immuno-histochemical analysis.

Since information about the use of coagulant fixatives as a substitute for formalin fixatives is significantly limited, and each fixative has its own set of benefits and drawbacks, the primary objective of this study was to comparatively evaluate the fixatives for their effect on morphology and antigenicity in osteochondral samples. Since coagulant fixatives have been suggested to be time-dependent and preserve antigenicity more effectively, the study also compared the impact of performing antigen retrieval versus not performing it on different days.

MATERIALS AND METHODS

Tissue sample procurement and processing

All methods used in the study were per the regulations set by the Institution's Review Board and Ethics Committee, as stated in the declaration of

Helsinki. After obtaining written informed consent from the patient, human osteochondral units (OCUs) were harvested from a patient undergoing total knee replacement as a part of the treatment for osteoarthritis (Radiological Kellgren Lawrence score of Grade 4). The osteochondral units were harvested using a Colibri hand drill equipped with a 12 mm Impact Bi-Metal Hole saw (Sutton Tools). The osteochondral units were harvested from a non-weight-bearing area of the joint that contained preserved cartilage.

The OCUs were divided using a microtome blade into three parts for different fixatives, with each piece further divided into four portions for the specified time points (Fig. S1). The three fixatives used were a) 10% neutral buffered formalin, b) Methacarn and c) EMA, ensuring that the volume of the fixative was 15 times the volume of the tissue. The fixation time points included the following days: 1, 3, 7, and 10. The OCUs were decalcified by immersing the joints in a solution of 10% EDTA (Qualigens, Cat.No. Q12635, pH 7.4). The joints were kept on a biological magnetic stirrer at 650 rpm, 25˚C, for a period of 35 days. The decalcified OCUs were processed by an automated tissue processor (Leica TO 10120), dehydrated by gradually increasing the ethanol concentration, and then infiltrated with wax and paraffin-embedded. 5 µm sections were placed on poly-L-lysine (SIGMA, P8920) coated slides using the semi-automated Leica microtome (RM-2245). To verify the accumulation of glycosaminoglycans (GAG), the sections were stained with Safranin O, Alcian blue and Toluidine blue. To assess for collagen and for total collagen, the OCUs were subjected to routine staining using PicroSirius red, immunohistochemical analysis for Collagen type II and type X, and Hematoxylin and Eosin staining.

Staining protocol

a) Hematoxylin and Eosin (HE) staining

HE staining was carried out following standard protocols. In summary, transverse sections were stained with hematoxylin solution for 8 minutes, then dipped twice in 1% acid alcohol, rinsed with tap water, and dipped in lithium carbonate. Subsequently, the sections were stained with eosin solution for 30 seconds. The sections were dehydrated using graded alcohol and cleared using xylene.

b) GAG stains: Safranin O, Alcian Blue and Toluidine blue

To perform Safranin O fast green (Fischer Scientific, Cat No:39962), staining, the sections were first treated with Weigert's Iron Hematoxylin, followed by 1% acid alcohol, 0.05% fast green solution, 1% acetic acid, and 0.1% Safranin O solution. Alcian blue staining (pH: 2.5, Cat No: J60122, Alfa Aesar, US) was done by incubating the slides with the dye for 5 minutes and counterstaining with neutral red. For Toluidine blue (Qualigens, C.I.52040) staining, the sections were incubated with 0.1% dye solution for 5 minutes.

c) Collagen stain: PicroSirius red, Collagen type II and type X

0.1% PicroSirius red (Qualigens, C.I.35782) dye was applied to assess the total collagen, followed by Hematoxylin counterstain. For the immunohistochemical analysis of Collagen type II, the tissue sections underwent sequential antigen retrieval using pronase (1 mg/ml, Roche CAS 9036-06-0) and hyaluronidase (2.5 mg/ml, Sigma, Cat No:H3506). While for Collagen Type X, the sections were retrieved using chondroitinase ABC (0.1 units/ml: 1 h, C3667, Sigma Aldrich) and pepsin (pH 2.2, 1 mg/ml:15 min, R2283, Sigma Aldrich). Subsequently, the slides were incubated overnight with mouse monoclonal anti-collagen type II antibodies (5 g/mL, DSHB, II-II6B3) or collagen type X (1 in 200 dilutions, ab49945, Abcam). Subsequently, staining with HRP-labelled goat anti-mouse immunoglobulin secondary antibody (31430, Pierce) at a concentration of 1:100, and 3,3-diaminobenzidine (DAB, Sigma Cat No: D5637) solution and counterstaining with Hematoxylin was performed. For immunohistochemical staining namely collagen type II and X, the sections were stained with and without antigen retrieval. Negative controls included parallel staining, albeit the primary antibody.

All slides were dehydrated, cleared with xylene, mounted with DPX (Merck, DD9DF69319) and scanned using an EVOS FL AUTO slide scanner.

RESULTS

Hematoxylin and Eosin (HE) staining

The comparative assessment showed that the NBF fixative resulted in better haematoxylin uptake compared to the alcohol-based fixatives, Methacarn and EMA (Fig. 1, Fig. S2, Fig. 9). None of the tested fixatives affected the visualization of tissue features on the slide, and no signs of necrosis or cellular degeneration were observed, indicating that all fixatives were effective in preserving tissue morphology. The cartilage lacuna contained chondrocytes with a bright eosinophilic cytoplasm, and the surrounding territorial matrix appeared more basophilic (Fig. 2). Conversely, the lacuna in bone samples was typically empty, and, if cellular, did not exhibit the staining property of the territorial matrix. Notably, lamella could be appreciated in the region of bone (Fig. 2). Finally, the staining of the cartilage gradually changed basophilic towards the bony side and abruptly stopped to give rise to an eosinophilic bony layer, giving rise to a wavefront-like appearance (Fig. 2). Overall, these findings suggest that NBF is a reliable fixative for preserving both the tissue morphology and staining properties in cartilage and bone samples.

GAG stains: Safranin O, Alcian Blue and Toluidine blue

Hyaline cartilage's extracellular matrix includes ground substance and collagen fibers, with the former composed of water, proteoglycans (PGs) and glycosaminoglycans. Safranin O stains PGs and GAGs, forming a red-to-orange complex that indicates high content. Fixative exposure duration did not affect staining quality, as all tested fixatives produced comparable results (Fig. 3). However, the cartilage in slides preserved with EMA and Methacarn fixatives showed less safranin O staining, possibly due to the tissue retaining the cytoplasmic stain (Fast green) better, requiring a more extended differentiation period or might be due to the poorer uptake of Safranin O, requiring a longer duration of exposure to the stain (Fig. 3, Fig. 9). The cellular features were preserved with no signs of necrosis or cellular degeneration. Notably, robust Safranin O uptake was observed in the cartilage, particularly in the territorial matrix.

Alcian blue stains the sulfated and carboxylated glycosaminoglycans in cartilage, giving rise to a blue or turquoise coloration, depending on the pH of the staining solution. Neutral red, conversely, selectively stains chondrocyte nuclei, producing

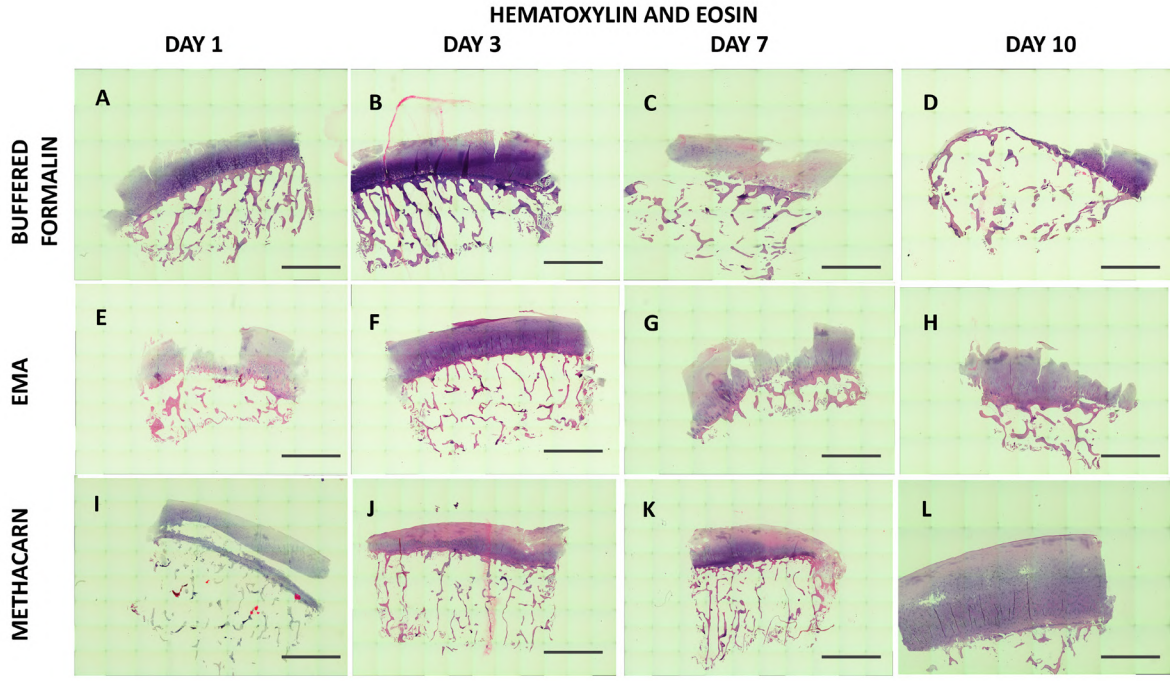


Fig. 1.- Hematoxylin and Eosin staining of the osteochondral unit sections following fixation by either NBF, Methacarn or EMA, and decalcification by 10% EDTA. Magnification 10X. NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio. Scale bars = 100 µm.

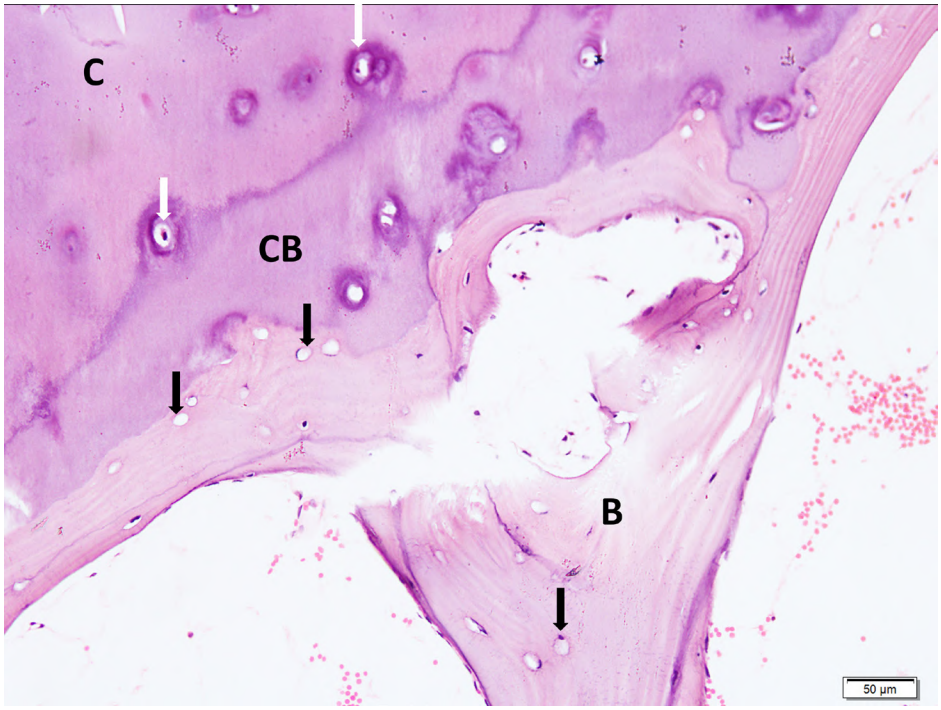


Fig. 2.- Cartilage bone junction fixed with NBF for 3 days taken with the objective set at 20X magnification. Chondrocytes seen as eosinophilic cells in the lacuna of the cartilage(C) with the territorial matrix surrounding them staining basophilic (white arrows). Bone (B) lacuna predominantly appear empty with no surrounding basophilia (black arrows). Lamella can be seen. There is a well-defined area in the junction (CB) which stains basophilic and resembles a wavefront. Scale bar = 50 µm.

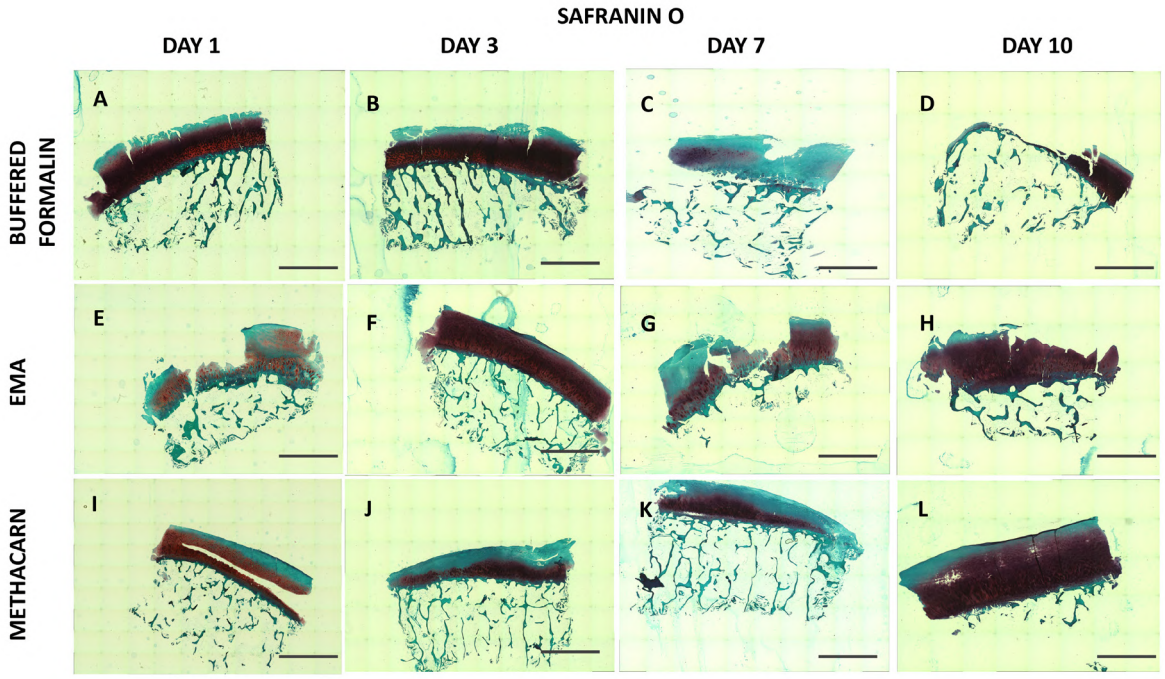


Fig. 3.- Safranin O staining of the osteochondral unit sections following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA. Magnification 10X. NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio. Scale bars = 100 µm.

a red or pink coloration, which contrasts well with the blue coloration of the Alcian blue-stained matrix (Fig. 4, Fig. 9). Our examination revealed that both Methcarn and EMA exhibited poor uptake of the counterstain on the first day, although they produced similar results to the formalin fixative on the other days.

Toluidine blue is a basic metachromatic dye that can selectively stain cartilage proteoglycans, providing insight into cartilage tissue's extracellular

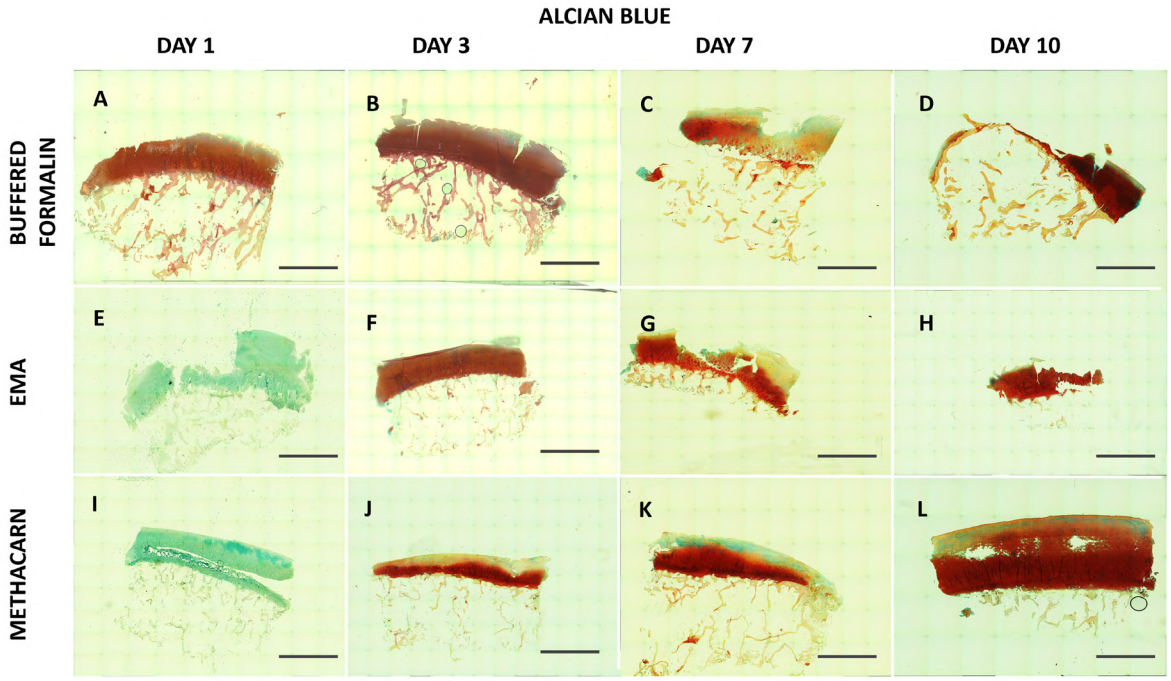


Fig. 4.- Alcian Blue staining of the osteochondral unit sections following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA. Magnification 10X. NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio. Scale bars = 100 µm.

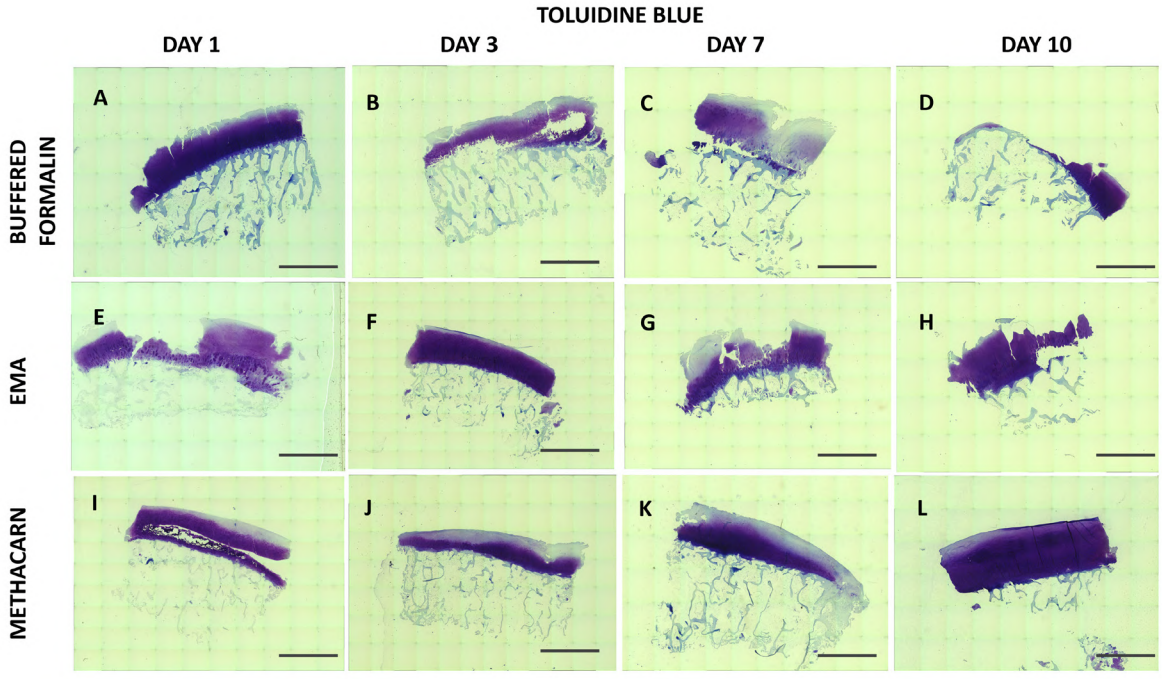


Fig. 5.- Toluidine Blue staining of the osteochondral unit sections following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA. Magnification 10X. NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio. Scale bars = 100 µm.

matrix composition and structure. Comparative staining was observed between the three groups not affected by the fixation period (Fig. 5, Fig. 9).

Collagen stain: PicroSirius red, Collagen type II and X

PicroSirius red staining is a commonly used technique in histology that selectively stains col-

lagen fibers, allowing for visualization and analysis of collagen distribution and density in various tissues. Analysis of the three groups showed that PicroSirius red stained the collagen network uniformly across the different fixative groups and periods (Fig. 6, Fig. 9).

Analysis of collagen type II uptake showed that negative control (Fig. S3) without primary antibody exposure and test slides without antigen retrieval demonstrated only mild DAB uptake, thus considered negative background staining. Conversely, all test slides with antigen retrieval exhibited strong uptake, with methacarn and EMA-fixed tissues showing higher uptake than formalin-fixed slides (Fig. 7, Table 1A). The observation was that uptake occurred solely in the cartilage region with a clear boundary between the cartilage area displaying strong uptake and the bone region with no uptake. The territorial matrix of the cartilage revealed robust uptake, confirming the specificity of the staining. Notably, the bone demonstrated negative uptake of DAB, which could thus serve as an internal negative control for future studies.

Concerning Collagen Type X, examining the negative control without the primary antibody showed a mild DAB uptake in the area just below the surface and the transition zone (Fig. S3). Similar results in test slides without antigen retrieval were also observed, thus considered negative background staining. However, all test slides with antigen retrieval demonstrated strong DAB uptake, with methacarn and EMA-fixed tissues exhibiting comparable uptake to NBF-fixed slides (Fig. 7, Table 1B). Importantly, we observed DAB uptake only in the bone region, with a clear boundary between the cartilage region, which had no uptake, and the bone area with robust uptake (Fig. 8). Intriguingly, the interlamellar planes of the bone exhibited particularly strong DAB uptake (Fig. 8). In contrast, the cartilage region displayed no DAB uptake, suggesting that it can be used as an internal negative control for future studies.

DISCUSSION

Standardizing staining of osteochondral units is crucial for ensuring accurate and reproducible assessment of tissue structure and composition, which can inform diagnoses and treatment decisions for joint-related diseases (Lepage et al., 2019). Fixation of osteochondral samples before staining is vital to preserve tissue structure and prevent artifacts that can distort the tissue's mor-

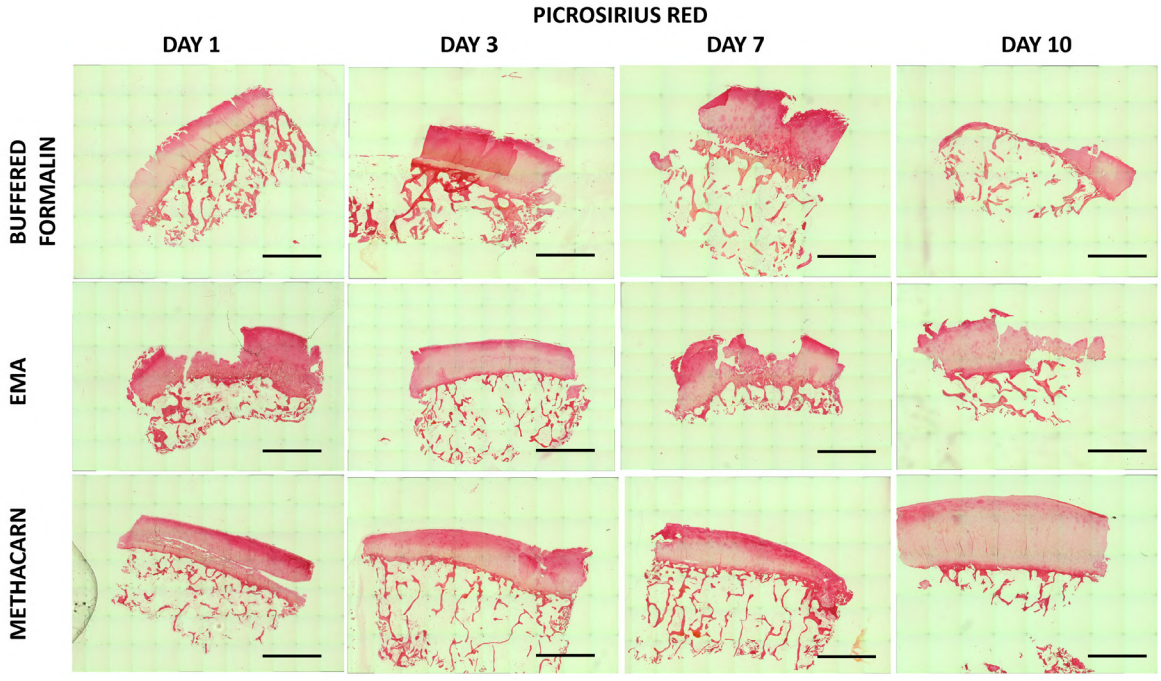


Fig. 6.- PicroSirius Red staining of the osteochondral sections following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA. Magnification 10X. NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio. Scale bars = 100 µm.

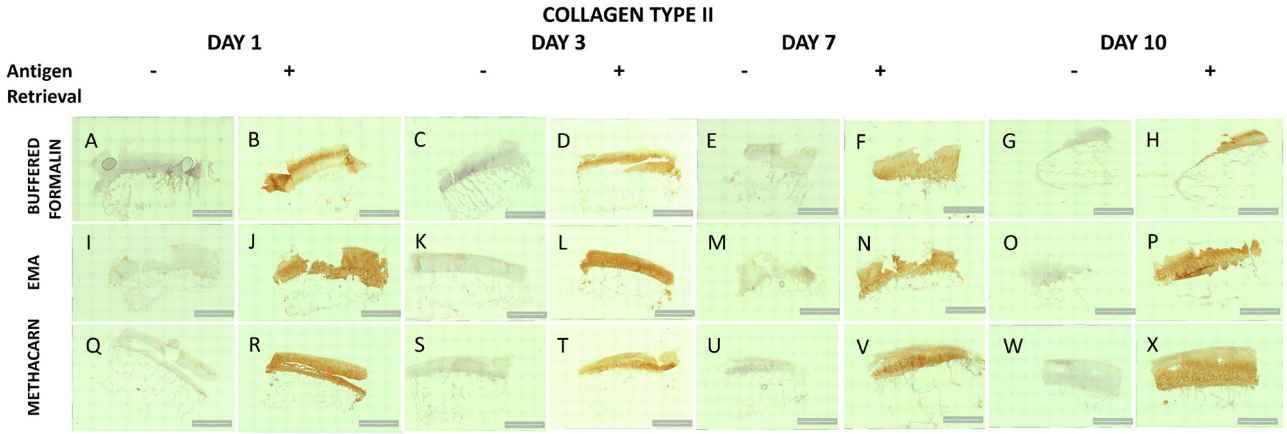


Fig. 7.- Immunohistochemical staining of the osteochondral unit sections for collagen type II following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA with and without antigen retrieval. The retrieval was performed using pronase and hyaluronidase. Magnification 10X. NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio. Scale bars = 100 µm.

Table 1. A) Grading of Collagen type II and B) Collagen type X stain uptake by the osteochondral sections following fixation by either NBF, Methacarn and EMA, and decalcification with and without antigen retrieval.

A: Collagen Type II									
Days of Fixation		**Day 1**		**Day 3**		**Day 7**		**Day 10**	
Antigen Retrieval Pronase + Hyaluronidase		With	Without	With	Without	With	Without	With	Without
Fixative	NBF	+	-	+	-	+	-	+	-
	Methacarn	++	-	++	-	++	-	++	-
	EMA	++	-	++	-	++	-	++	-
B: Collagen Type X									
Days of Fixation		**Day 1**		**Day 3**		**Day 7**		**Day 10**	
Antigen Retrieval Chondroitinase ABC + Pepsin		With	Without	With	Without	With	Without	With	Without
Fixative	NBF	++	-	+	-	++	-	+	-
	Methacarn	+	-	++	-	++	-	+	-
	EMA	++	-	+++	-	++	-	+	-

Grading scale: no stain uptake (-), good stain uptake (+), very good stain uptake (++).

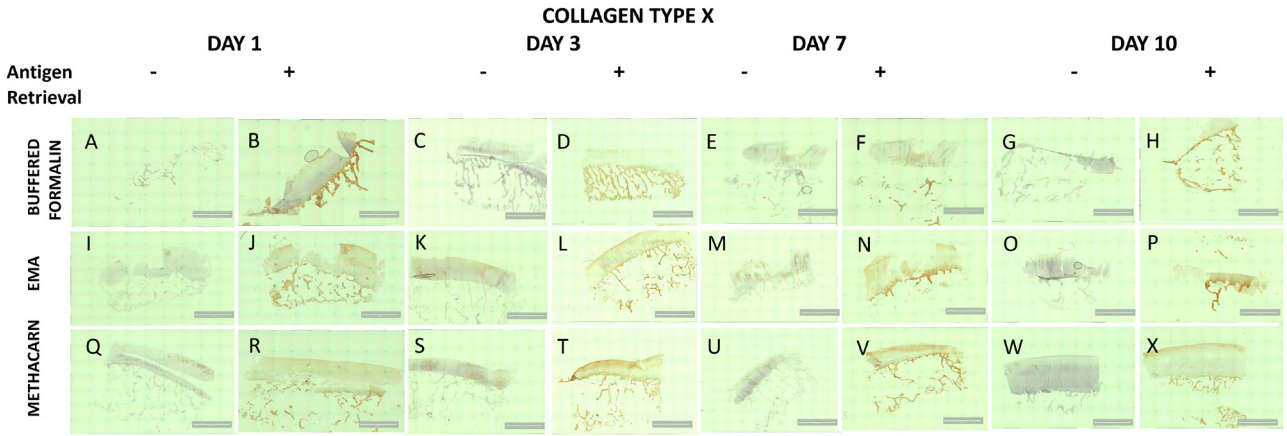


Fig. 8.- Immunohistochemical staining of the osteochondral unit sections for collagen type X following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA with and without antigen retrieval. The retrieval was performed using Chondroitinase ABC and pepsin. Magnification 10X. NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio. Scale bars = 100 µm.

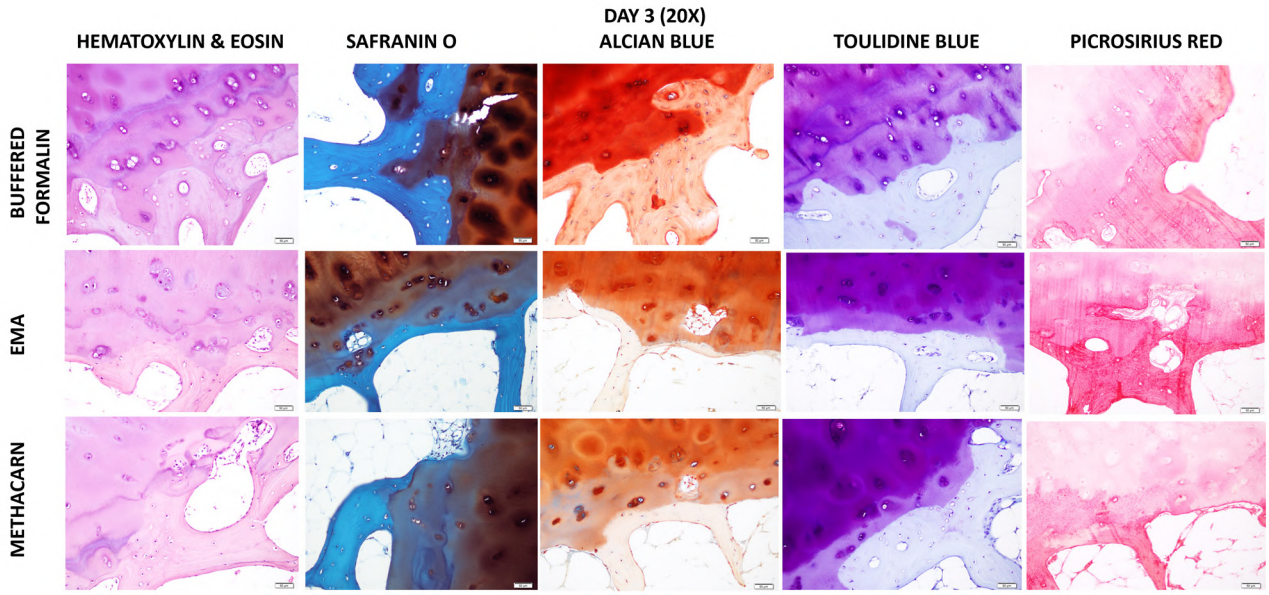
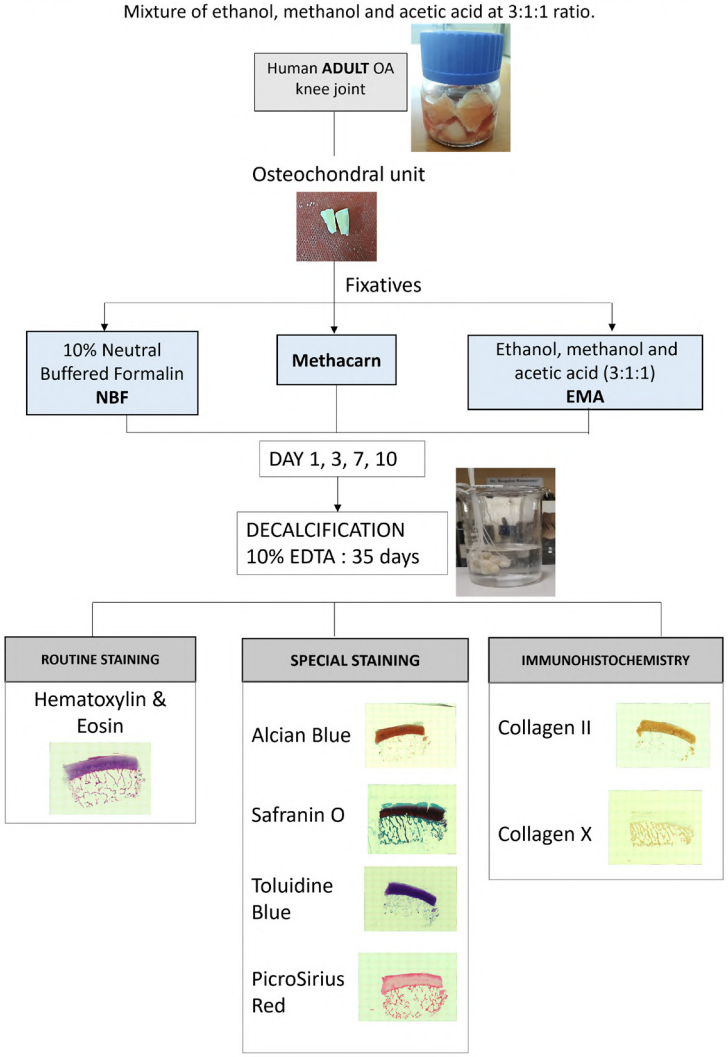


Fig. 9.- Cartilage bone junction fixed with either NBF, EMA and Methacarn for 3 days taken with the objective set at 20X magnification. Scale bars = 50 µm.

Figure S1: Study algorithm depicting the procedure for processing the osteochondral unit, derived from human adult OA knee joints using the three fixatives: NBF, Methacarn and EMA, the time period for fixation and staining used. NBF: 10% Neutral Buffered Formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio.



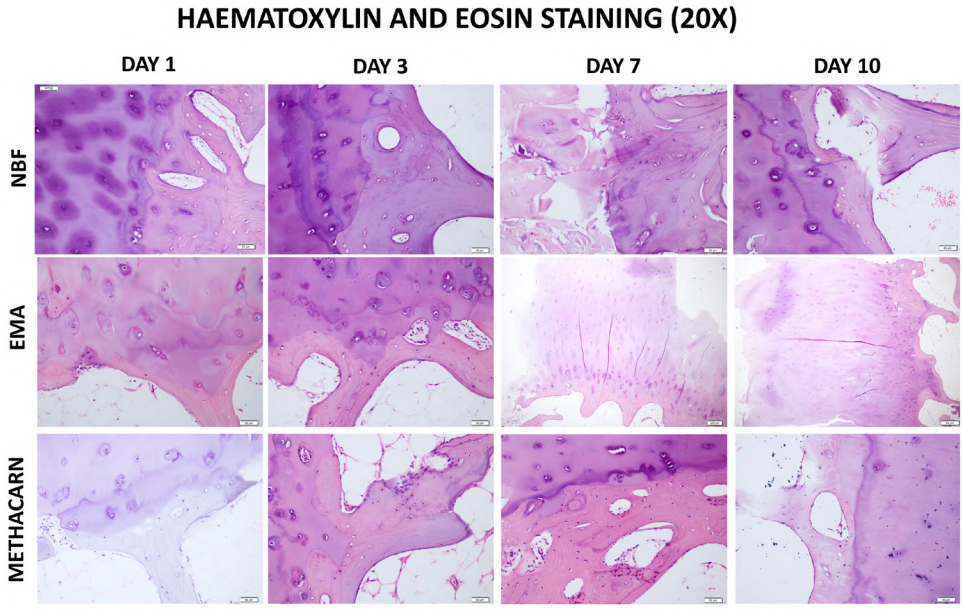


Figure S2: Hematoxylin and Eosin staining of the osteochondral sections for following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA. Magnification 20X.
NBF: 10% Neutral buffered formalin, EMA: Mixture of ethanol, methanol and acetic acid at 3:1:1 ratio.

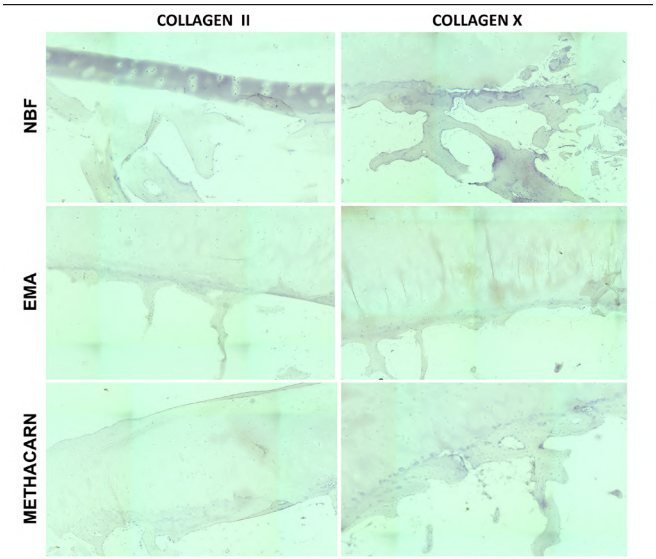


Figure S3: Negative controls: Immunohistochemical staining of the osteochondral sections for collagen type II and collagen following fixation by either NBF, EMA and Methacarn, and decalcification by 10% EDTA with antigen retrieval but without primary antibody incubation.

phology and composition, thereby ensuring reliable and consistent staining results for accurate histological analysis. Being a complex tissue that requires additional processing such as decalcification and antigen retrieval, the need to explore and optimize alternative strategies becomes paramount (Amirtham et al., 2019; Orth et al., 2015).

NBF, methacarn, and EMA are common coagulant fixatives used in histology. NBF provides good preservation of tissue structure but can result in tissue shrinkage (Thavarajah et al., 2012). Methacarn preserves lipids and yields less shrinkage but is time-consuming (Shibutani et al., 2000). EMA is a newer fixative that provides

excellent tissue morphology and antigenicity preservation with minimal protein cross-linking (Rahman et al., 2022). The choice of fixative depends on the study's specific needs and the tissue being analyzed.

The present study evaluated the efficacy of these different fixatives in preserving tissue morphology and staining properties in cartilage and bone samples. Hematoxylin and eosin (HE) staining showed that NBF fixative resulted in better haematoxylin uptake compared to alcohol-based fixatives. All fixatives effectively preserved tissue morphology without necrosis or cellular degeneration. Safranin O staining showed that all tested fixatives produced comparable staining quality, with robust uptake especially observed in the territorial matrix of the stained cartilage. Alcian blue staining revealed poor uptake of the counterstain on the first day with coagulative fixatives like Methacarn and EMA fixatives, while Toluidine blue staining was not affected by the fixative or the fixation period. Picrosirius red staining was uniform across all fixative groups and periods.

The analysis of collagen type II and type X uptake showed that the negative control and test slides without antigen retrieval had mild DAB uptake and were considered negative background staining. However, all test slides processed with antigen retrieval exhibited strong DAB uptake, with methacarn and EMA-fixed tissues showing higher uptake of collagen type II than formalin-fixed tissues. The uptake occurred solely in the cartilage region for collagen type II and only in the bone region for collagen type X. The territorial matrix of the cartilage for collagen type II and the interlamellar planes of the bone for collagen type X exhibited particularly strong DAB uptake.

Our novel findings highlight the utility of different fixatives and their effects on routine, special and immunohistochemical staining in cartilage and bone tissue research. The coagulant fixatives displayed superiority to formalin with stronger collagen type II uptake. However, antigen retrieval was required with all fixatives unlike IHC studies performed without decalcification. A minimum period of 3 days is required for coagulant fixatives to provide staining consistent with formalin fixatives. Overall, methacarn and EMA serve to be good alternatives to formalin for the processing of cartilage or bone-containing tissues, with better preservation of its morphology and antigen specificity. Further studies using different tissue samples and a larger cohort would provide insights into the wider use of alternative fixatives based on the obtained immunohistochemical assays individually for specific antigen targets.

ACKNOWLEDGEMENTS

We would like to acknowledge Dr. Soosai Manickam Amirtham, Ms. Esther Rani and Mr. Ashok Kumar for technical support, and the Centre for Stem Cell Research (A unit of inStem Bengaluru), Christian Medical College, Vellore for infrastructural support. This project was supported by the Department of Biotechnology (BT/PR32777/MED/31/415/2019), Govt. of India and Fluid Research Grant (IRB Min No: 14299), Christian Medical College, Vellore.

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