Advancing preservation: plastination and deplastination of human osteochondral units for enhanced histological analyses in Education and Forensic Sciences

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SUMMARY

In the realm of medical education and research, the preservation of anatomical specimens has traditionally relied on formaldehyde-based fixatives, posing challenges such as loss of 3D orientation, unpleasant odor, and tissue alterations during extended storage. A more contemporary approach introduced in the late 20th century is plastination, which replaces water and fat with durable polymers, providing odorless and stable specimens for ultra-long-term storage. Despite the success of plastination, its impact on subsequent histopathological studies, particularly regarding special stains and immunohistochemistry (IHC), remains largely unexplored. This study delves into assessing the feasibility of staining deplastinated osteochondral units (OCU). Employing a comprehensive multi-step methodology involving fixation, decalcification, plastination, deplastination, paraffin embedding, and various staining protocols, the study meticulously evaluates tissue integrity and staining outcomes using human OCU.

Significantly, the plastinated and deplastinated OCUs exhibit well-preserved tissue morphology in Hematoxylin and Eosin staining, successful glycosaminoglycan staining with Safranin O, and effective visualization of collagen types II and X through IHC analysis. A noteworthy observation is the potential to stain plastinated bone-containing specimens after deplastination, addressing the drawbacks of long-term tissue preservation using formalin. This innovation allows subsequent staining as needed, and the direct handling of plastinated specimens enhances three-dimensional visualization, rendering them valuable tools in forensic examinations. These findings open promising avenues for refining methodologies in medical education and forensic settings. The establishment of reliable staining and deplastination protocols holds the potential to significantly improve result accuracy and reproducibility, ultimately expanding applications in these critical fields.

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INTRODUCTION

Preservation of anatomical specimens plays an important role in advancing medical education and research. Traditionally, anatomical specimens were preserved by immersing them in fixatives containing formaldehyde, which is a widely utilized preservative in both educational and research settings (Brenner, 2014). Formalin based fixatives act by cross linking proteins thereby preventing decomposition. Despite being a common practice, formalin-fixed specimens pose challenges such as difficulties in maintaining 3D orientation, irritating odour and tanning of tissues, leading to color and texture loss (Elshaer and Mahmoud, 2017; Fischer, 1905). Additionally, the potential for issues related to the upper respiratory tract and the reported carcinogenicity of formalin have led to the exploration of alternative methods (Swenberg et al., 2013). One such alternative is plastination, a technique developed by Dr. Gunther von Hagens in the late-20th century (Pashaei, 2010). Plastination involves replacing water and fat in biological specimens with curable polymer chemicals such as silicon, epoxy, or polyester, resulting in durable, odorless, hardened specimens that can be stored for ultra-long periods at room temperature (Jean and Fasel, 1988; Nel, 1997; Gubbins, 1990). Plastinates, due to their realistic tissue appearance, are now increasingly used by medical professionals and students for interactive learning (Magiros et al., 1997; Riederer, 2014). The process of plastination involves a multi-step approach, including fixation, dehydration, forced impregnation, positioning, and curing of the specimen. Numerous studies have successfully applied this process to various types of soft tissues (Marchese et al., 2013; O'Sullivan and Mitchell, 1995). Additionally, plastinates could be particularly useful in forensic contexts, offering advantages such as long-term stability, absence of hazardous chemicals, and resistance to decay, thereby providing compelling courtroom presentations and ensuring accurate representation of evidence. It could also be used to store specimens exhibiting extremely rare disorders, increasing their numbers over time, and enabling studies with better statistical strength due to a larger sample size.

Plastination, unfortunately, protects the tissue from any histopathological studies on a later date. Deplastination, representing the reversal of plastination, is hypothesised to remedy this deficit based on the belief that samples can be restored to their natural form. Studies using deplastinated soft tissue samples have reported successful staining while maintaining morphological characteristics (Frierson et al., 1988; Baygeldi et al., 2022; Rahul et al., 2020a). However, data on the staining of deplastinated dry body tissues such as bones and cartilage are notably limited. There is also a lack of data on the reactivity of deplastinated tissues to immunostaining procedures like immunohistochemistry (IHC).

Osteochondral units (OCU) are known to exhibit resilience to decomposition, enduring for extended periods, and typically being the retrievable specimens at exhumation sites. However, for the processing of bone or bone-containing tissue, in addition to fixation, decalcification is an essential prerequisite for routine, special, or immunohistopathological analysis (Callis and Sterchi, 1998). Decalcification entails extracting mineral salts from the organic matrix, rendering the bone softer, and facilitating the acquisition of micron-sized sections (Dermience et al., 2015; Skinner, 2003). Following paraffin embedding to obtain tissue sections, an additional step preceding immunohistochemical staining for OCU is antigen retrieval (Amirtham et al., 2019; D'Amico et al., 2009). The staining of OCUs following deplastination has not been reported before. Thus, the present study aimed to subject human OCU to plastination followed by deplastination, paraffin embedding, and routine staining using Hematoxylin and Eosin, special staining such as Safranin O and Alcian Blue to assess glycosaminoglycan uptake in the cartilage, Masson trichrome to visualize connective tissues and preservation of antigen epitopes using immunohistochemical staining for Collagen type II specific to cartilage and Collagen type X specific to bone following their specific antigen retrieval.

MATERIALS AND METHODS

Tissue sample procurement and processing

All methodologies employed in this study adhered to the regulations established by the Institutional Review Board and Ethics Committee, following the guidelines outlined in the Declaration of Helsinki. Written informed consent was obtained from the patient before harvesting human OCUs from an individual undergoing amputation due to a traffic accident (Age: 63, male). The collection of osteochondral units was performed using a Colibri hand drill equipped with a 12 mm Impact Bi-Metal hole saw (Sutton Tools). Subsequently, the OCUs were fixed in 10% neutral buffered formalin for a duration of 10 days, ensuring that the volume of the fixative was 15 times the size of the OCU used. Following fixation, the specimens were divided into four parts using a microtome blade. Each of these parts underwent distinct protocols, including processes such as decalcification, plastination, and deplastination of plastinated specimens. Additionally, uncertainty about the necessity of decalcification was considered, leading to the inclusion of four different protocols, as detailed below (Fig. 1).

a) Control: The OCU underwent decalcification for 20 days, followed by paraffin embedding and staining. This arm served as the control.

b) P-DP-DC: Plastination was carried out for a period of 1 week, followed by curing for 28 days, and deplastination for 1 week. This was followed by decalcification for 20 days.

c) DC-P-DP: For this arm, decalcification for 20 days was followed by plastination for 1 week, curing for 28 days, and deplastination for 1 week.

d) P-DP: Plastination was conducted for a period of 1 week, followed by curing for 28 days, and deplastination for 1 week.

Processing of the units

Plastination

Plastination of the sections, which were cylindrical sections of around 1 cm was carried out using the S10 Plastination technique, as described in literature (Hagens, 1986; Shanthi et al., 2015; Suganthy and Francis, 2012). The fixed specimens underwent dehydration at -25°C, initially in 85% acetone for a day, followed by two changes in 100% acetone for 2 days each. Forced impregnation took place in a vacuum chamber at room temperature, with the dehydrated tissue immersed in a 1:100 mixture of S3 and S10 silicone polymers (BIODUR). The reduction of pressure to the desired low levels within the vacuum chamber over 24 hours was performed as previously published by Suganthy and Francis (2012). Curing occurred in a custom-made curing chamber, where the impregnated specimen was exposed to S6 catalyst (BIODUR) as an aerosol for a 24-hour period, resulting in a dry plastinate. The plastinates were set aside in closed ziplock packets for 28 days to ensure complete curing of the tissue. The duration for each step of plastination was reduced taking into consideration the small size of the plastinate and based on the author's past experience in the same field (Francis and Rabi, 2018; Rahul et al., 2020a).

Deplastination

After the curing period, the plastinates were immersed in a deplastinating fluid, composed of a 5% solution of sodium methoxide in 95% methanol. The specimens were placed on an agitator, and the deplastinating fluid was changed once every two days for a period of one week. The tissue was then embedded in paraffin wax after dehydration in 95% methanol followed by 100% methanol, cleared with two changes of Xylol, and impregnation in two changes of paraffin wax. Sectioning was performed to obtain tissue sections with a thickness of 5 microns. Methodology followed for deplastination was as initially published by Grondin et.al. (1994) and further refined in the institutional laboratory (Francis and Rabi, 2018; Rahul et al., 2020b).

Decalcification

The OCUs underwent decalcification through immersion in a 10% EDTA solution (Qualigens, Cat.No. Q12635, pH 7.4). The joints were placed on a biological magnetic stirrer at 650 rpm, 25°C, for a duration of 20 days. After decalcification, au-

tomated processing was carried out using a tissue processor (Leica TO 10120). The decalcified OCUs underwent dehydration with a gradual increase in ethanol concentration, followed by infiltration with wax and paraffin embedding. Sections of 5 µm thickness were mounted on poly-L-lysine-coated slides using the semi-automated Leica microtome (RM-2245).

Staining Protocol

Routine staining, including Hematoxylin and Eosin, and special staining using Safranin O, was conducted to evaluate glycosaminoglycan uptake in the cartilage. Masson trichrome staining was employed for visualizing connective tissues. Additionally, immunohistochemical staining for Collagen type II (specific to cartilage) and Collagen type X (specific to bone) was performed to preserve antigen epitopes, following their specific antigen retrieval.

a) Hematoxylin and Eosin (HE) Staining

HE staining was conducted following standard protocols. In brief, transverse sections were stained with Harris hematoxylin (Qualigens Cat No: Q39411) solution for 8 minutes, dipped twice in 1% acid alcohol, rinsed with tap water, and then immersed in lithium carbonate. Subsequently, the sections were stained with eosin solution for 30 seconds.

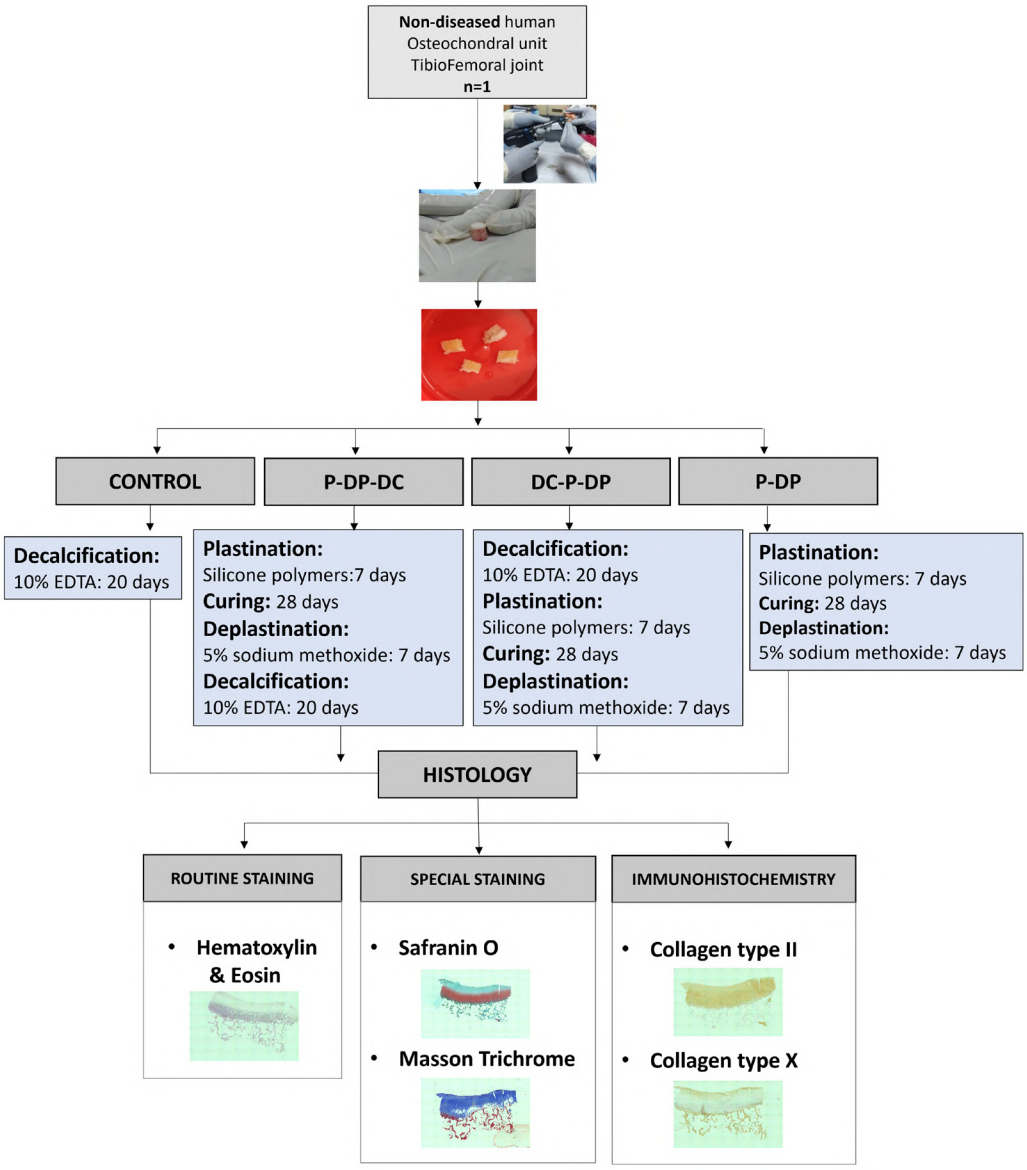

Fig. 1.- Study algorithm depicting the process involved in the processing of human osteochondral units against the four different study arms, following which routine, special staining and immunohistochemical analysis was performed.

b) Glycosaminoglycan (GAG) Stains: Safranin O

For Safranin O fast green staining (Fischer Scientific, Cat No: 39962), sections underwent treatment with Weigert's Iron Hematoxylin (Qualigens Cat No: Q39411) for 5 minutes, followed by 1% acid alcohol dips, 0.05% fast green solution (Fischer Scientific, Cat No: 115381) for 5 minutes, 1% acetic acid (Qualigens Cat No: 21055) for 30 seconds, and 1% Safranin O solution (Qualigens Cat No: Q39962) for 1 hour.

c) Connective tissue Stain: Masson Trichrome

For Masson Trichrome staining, sections underwent treatment with Harris Hematoxylin (Qualigens Cat.No: Q39411) for 5 minutes, differentiation in 1% acid alcohol, alcoholic picric solution (Qualigens Cat No: 35973) for 5 minutes, 1% ponceau red (HiMedia Cat No: 27195) for 10 minutes, 1% Phosphomolybidic acid solution (Qualigens Cat No: 13102) for 10 minutes and 2.5% aniline blue solution (LEO CHEM Cas No: 42755) for 3 minutes, distilled water wash, 1% Phosphomolybidic acid solution for 2 minutes and 1% acidic acid (Qualigens Cat No: 21055) for 1 minutes.

d) Collagen Stain: Collagen type II and type X

For immunohistochemical analysis of Collagen type II, tissue sections underwent sequential antigen retrieval using Pronase (1mg/ml, Roche CAS 9036-06-0) for 30 minutes and hyaluronidase (2.5mg/ml, Sigma, Cat No: H3506) for 30 minutes. For Collagen Type X, sections were retrieved using chondroitinase ABC (0.1 units/ml: 1 h, C3667, Sigma Aldrich) and pepsin (1mg/ml:15 min, R2283, Sigma Aldrich) for 15 minutes. Subsequently, slides were incubated overnight with mouse monoclonal anti-collagen type II antibodies (5µg/mL, DSHB, II-II6B3) or collagen type X (1 in 200 dilutions, ab49945, Abcam). 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 was performed, followed by counterstaining with Hematoxylin (Qualigens Cat.No: Q39411).

All slides underwent dehydration, clearing with xylene, mounting with DPX (Merck, DD9DF69319), and scanning using an EVOS FL AUTO slide scanner.

Assessment and Analysis

All stained slides were assessed by a single investigator with 10 years' experience in histopathology. The investigator was initially blinded to the mode of preparation of the slide except for the control and was asked to give a qualitative report following which he was unblinded to reason out the differences noted.

RESULTS

General findings

It was noted during the sectioning phase that the controls and the deplastinated tissues behaved similarly during sectioning. The non deplastinated sections did not section well and had a higher rate of section separation from the slides and tissue folding mainly near the cartilage bone junction.

a) HE staining

The decalcified slides (Control, DC-P-DP & P-DP-DC) both stained similarly with the cartilage, bone and tidemark clearly distinguishable (Fig. 2A-D). The non-decalcified section (P-DP) showed stronger retention of both the cytoplasmic and nuclear stain with the tidemark not being clearly distinguishable (Fig. 2D). A brownish hue was noted on the cartilaginous side, which could represent the calcified bone.

Cellular arrangement was similar in all sections, with the chondrocytes seen in the lacuna on the cartilage and the osteocytes seen in between the lamellae and on the bone surface. Tissues which underwent deplastination (DC-P-DP, P-DP-DC and P-DP) showed the presence of glassy remnants of silicone polymer as transparent artifacts in the lacuna and marrow spaces.

b) GAG stains: Safranin O

The extracellular matrix of hyaline cartilage consists of ground substance and collagen fibers. The ground substance is composed of water, proteoglycans (PGs), and glycosaminoglycans (GAGs). Safranin O staining specifically highlights PGs and GAGs, creating a red-to-orange complex, indicating their high content. In the control units, we ob-

served a good uptake of Safranin O in the cartilage, particularly in the territorial matrix, with negative uptake of the cytoplasmic stain. Conversely, in the bone, we observed a negative uptake of Safranin O with a strong uptake of the cytoplasmic stain alone. Both stains were taken up in the transition zone (Fig. 2E). Both the decalcified test groups (P DP DC & DC P DP) showed both an increased uptake of Safranin O in the territorial matrix and comparatively decreased uptake in the interterritorial matrix with absent uptake in bone. There was a mild uptake of the cytoplasmic stain in the cartilage and transition zone, with strong uptake in the bone area (Fig. 2F, G). The group without decalcification (P-DP) showed better retention of Safranin O and cytoplasmic stain in the cartilaginous and bony regions respectively. However, the tidemark was not clearly visible (Fig. 2H). Deplastination artifacts were seen in all the deplastinated groups (P-DP-DC, DC-P-DP, P-DP).

c) Connective Tissue staining: Masson Trichrome

Masson's trichrome is a histological staining technique that employs three colours. Typically, this method results in the cartilage matrix appearing blue or green depending on stain used, the nuclei exhibiting a blue-black hue, and the region of calcifying cartilage displaying a red tint, thus enabling the deciphering of the cartilage from the transition zone to the bone.

In the control OCU, the periarticular regions of the cartilage exhibited a blue coloration due to the uptake of the fiber stain, while the bone displayed a red tint resulting from the cytoplasmic stain (Fig. 2I). As anticipated, the transition zone displayed a mixed uptake of both fiber and cytoplasmic stains. However, in all three test arms (P-DP-DC, DC-P-DP, P-DP), negative results were observed in tissues along with deplastination artifacts (Fig. 2J-L). Despite increased fiber stain uptake in the cartilage, bone, and transition zone of the units, the cytoplasmic stain was not evident in the bone or transition zone.

d) Immunohistochemical Collagen Analysis: Collagen Type II and type X

Type II collagen is a fibrous collagen found exclusively in cartilages, constituting more than 90–95% of the collagen present in mature articular cartilage. In the control group, robust expression of collagen II was observed in the cartilage territorial matrix, while tissues containing bone did not exhibit the expected stain uptake (Fig. 3A). Plastinated sections followed by deplastination, regardless of the decalcification order (P DP DC, DC P DP), displayed intense staining for Collagen type II in the cartilage region, with no staining observed in the bone, comparable to the control group. Deplastination artifacts were observed which did not affect the IHC processing, by not taking up C2 immunostaining (Fig. 3B, C). The non-decalcified plastinate-deplastinate sections were incomplete and challenging to mount on slides; however, the retained cartilage exhibited a greater collagen Type II uptake with a more pronounced hematoxylin counterstain (Fig. 3D). In contrast to the control, the test groups exhibited very mild and non-uniform retention of DAB stain in few areas containing bone.

Concerning IHC with Collagen Type X, a network forming type of collagen, ~~is~~ primarily found in hypertrophic chondrocytes within cartilage and areas containing bone. In the control group, positive and anticipated staining was observed specifically in the bone-containing region below the tidemark, with an increased expression of collagen X evident in the interlamellar plane. Conversely, no staining was observed in the cartilage-containing area (Fig. 3E). In all three test groups (P-DP-DC, DC-P-DP, P-DP), collagen type X uptake was evident in the bone-containing area and the interlamellar zone, with a non-specific uptake observed in the cartilage area, though with noticeable demarcation (Fig. 3F-H). Deplastination artifacts, indicated by a glassy appearance, were observed in the test groups but were unaffected by the stain uptake.

DISCUSSION

Anatomical specimen preservation has long relied on using formaldehyde-based fixatives, with well-documented challenges such as 3D orientation maintenance, unpleasant odor, and color degradation over time as well as having a detrimental effect on the associated personnel and on the environment (Elshaer and Mahmoud, 2017). In re-

sponse to these drawbacks, plastination emerged as a promising ultra-long-term alternative, offering durable, odorless, and stable specimens. Plastinates have thus found an increasing utility in medical education and forensic contexts due to their realistic tissue appearance and absence of hazardous chemicals (Jean and Fasel, 1988; Nel, 1997; Pashaei, 2010). The process of deplastination has further opened the window for utilising these plastinates for histopathological studies in the future. However, the role of deplastination on special stains and immunohistochemical studies is absent. The application of plastination to OCUs, specifically involving bones and cartilage, further poses unique challenges, necessitating innovative approaches to maintain tissue integrity. This study explores the use of plastination and subsequent deplastination on human OCUs, focusing on the staining outcomes using various histological techniques.

The study employed a multi-step methodology, including fixation, decalcification, plastination, deplastination, paraffin embedding, and staining protocols for OCUs. The necessity of decalcification was underscored for obtaining micron-sized sections for plastinated OCUs. Remarkably, the plastinated and deplastinated OCUs exhibited comparable results to the conventionally processed control specimens in terms of Hematoxylin and Eosin (HE) staining, revealing preserved tissue morphology without signs of necrosis or cellular degeneration. Safranin O staining for GAG content exhibited successful outcomes, with decalcified plastinated-deplastinated OCUs displaying better staining than non-decalcified counterparts. Masson's trichrome staining highlighted the challenges associated with deplastination artifacts, emphasizing the need for further evaluation of slide adhesion agents in non-decalcified tissues. Immunohistochemical analysis of Collagen Type II and Type X demonstrated the preservation of the antigen epitopes even after plastination and deplastination procedure. Another notable finding was the timing of decalcification, which did not affect the staining uptake, whether performed before or after the plastination.

The obtained results underscore the unique benefits of the plastination and deplastination process. They showcase the feasibility of staining plastinated bone-containing specimens after

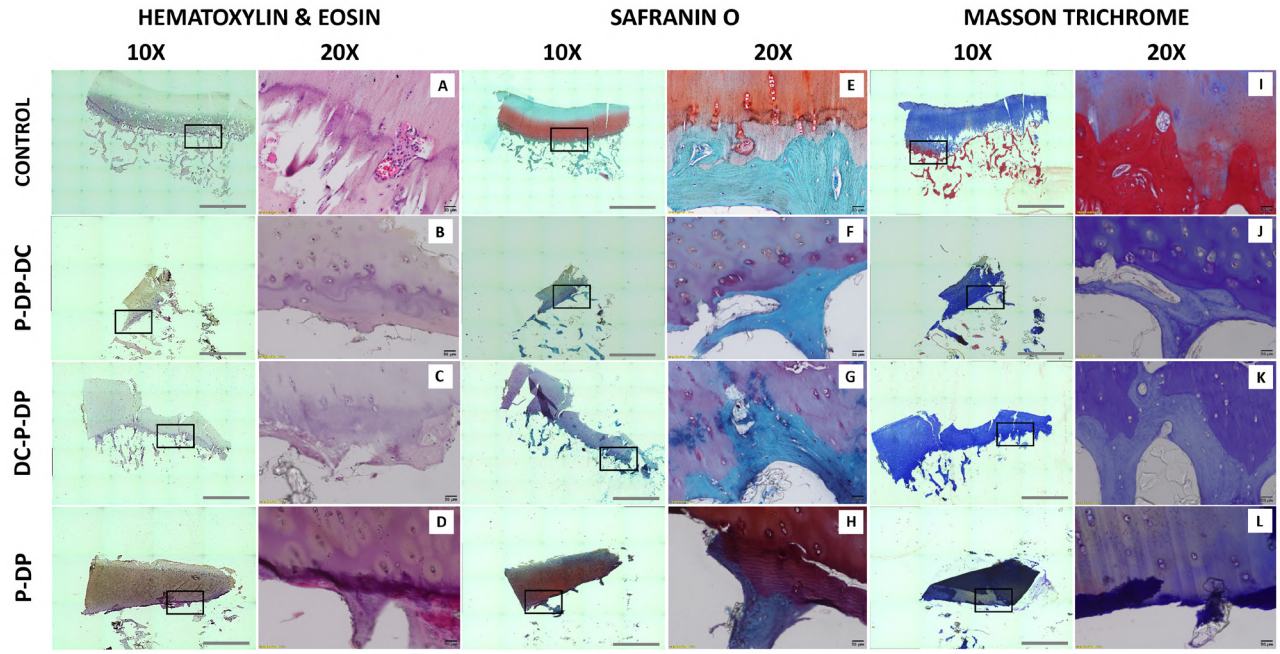

Fig. 2.- Hematoxylin and Eosin, Safranin O and the Masson Trichrome staining of the osteochondral unit sections processed as follows: a) **Control**: The OCU underwent decalcification for 20 days, followed by paraffin embedding and staining. b) **P-DP-DC**: Plastination was carried out for a period of 1 week, followed by curing for 28 days, and deplastination for 1 week. This was followed by decalcification for 20 days. c) **DC-P-DP**: For this arm, decalcification for 20 days was followed by plastination for 1 week, curing for 28 days, and deplastination for 1 week. d) **P-DP**: Plastination was conducted for a period of 1 week, followed by curing for 28 days, and deplastination for 1 week.

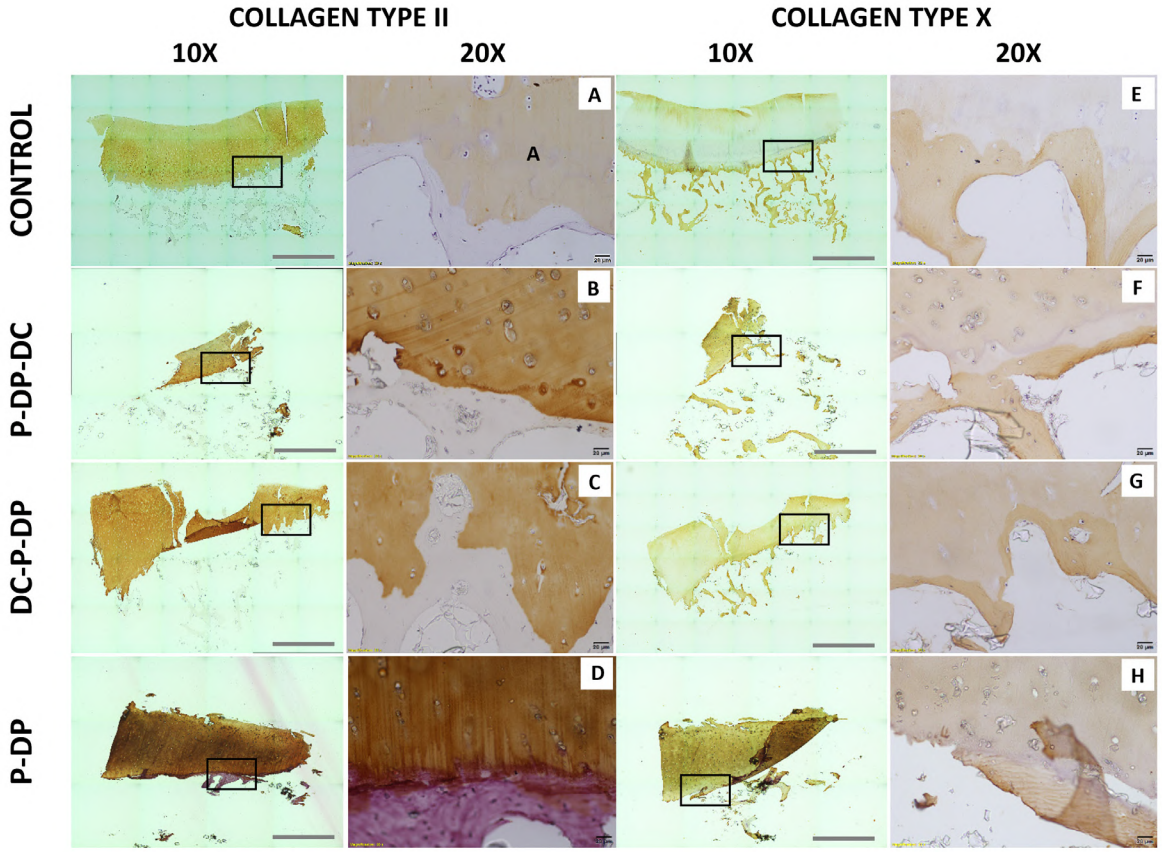

Fig. 3.- Immunohistochemical staining of the osteochondral unit sections for Collagen type II and X processed as follows: a) **Control**: The OCU underwent decalcification for 20 days, followed by paraffin embedding and staining. b) **P-DP-DC**: Plastination was carried out for a period of 1 week, followed by curing for 28 days, and deplastination for 1 week. This was followed by decalcification for 20 days. c) **DC-P-DP**: For this arm, decalcification for 20 days was followed by plastination for 1 week, curing for 28 days, and deplastination for 1 week. d) **P-DP**: Plastination was conducted for a period of 1 week, followed by curing for 28 days, and deplastination for 1 week.

deplastination, presenting a viable alternative for specimens, especially rare samples requiring prolonged preservation. This innovation addresses the drawbacks of long-term tissue preservation using formalin, allowing for subsequent staining as needed. The added advantage of direct handling facilitates three-dimensional visualization of specimens, enhancing their utility in forensic examinations. These unique benefits make the plastination and deplastination process a promising avenue for future research and application.

The findings of this study hold promise for enhancing the standardization of timings associated with staining and deplastination processes, effectively mitigating artifacts and potentially minimizing non-specific staining, emphasizing the need for further standardization. This development is poised to have far-reaching implications, contributing to the refinement of methodologies in both educational and forensic settings. The establishment of reliable protocols for staining and deplastination could significantly improve the accuracy and reproducibility of results, fostering broader applications in these critical fields.

CONCLUSION

In the conclusions of this study, several key findings are established:

1. The study demonstrates the comparability of paraffin-embedded tissue and deplastinated tissue histologically.
2. It elucidates the effect of decalcification on deplastinated tissue, both histologically and through Immunohistochemistry, for the first time.
3. The study confirms the preservation of antigenicity in deplastinated tissue for the first time.
4. It identifies the presence of histological artifacts specific to the process of plastination/deplastination for the first time.

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