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# Histological Analysis of an Alkaline Based Tissue Clearing Technique for Fetal Musculoskeletal Tissue

## Análisis Histológico de una Técnica de Limpieza de Tejido de Base Alcalina para Tejido Musculoesquelético Fetal

**Andres Fernando Gomez-Samper[1]; Mario Alberto Latiff[1]; Juan Sebastian Lopez-McCormick[2] & Roberto Javier Rueda-Esteban[3]**

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**SUMMARY:** Tissue clearing techniques are frequently used in the observation and description of anatomical structures and pathways without altering the three-dimensional layout of the anatomical specimen. Tissue optical clearing promotes preservation of three-dimensional structures, which allows the study of the internal anatomy in its original position and original spatial interaction. Among these techniques, Potassium Hydroxide (KOH) maceration clearing is one of the most widely used. However, the histological changes of tissue after KOH maceration have yet to be fully understood. Our aim is to describe the microscopical differences between macerated and normal tissue. To better understand said changes, two human fetuses with a gestation period of 16 to 28 weeks were cleared and processed for histological analysis. Microtome slides of the fetuses' lower limbs were obtained and stained with Hematoxylin & Eosin, Periodic Acid Schiff (PAS), and Masson's trichrome with the purpose of observing the histological and macromolecule composition changes in cleared tissue. Remarkable differences at a histological level regarding the composition of the cellular structures, since diaphanized tissues showed a predominance of extracellular matrix composed of collagen fibers with the absence of most of the nucleated cellular tissue. Phospholipid's saponification, nucleic acids degradation and a change on proteins structural properties are the main factors inducing clearing. At the same time, molecular stability of collagen in alkaline conditions allows the specimen to maintain its shape after the process.

**KEY WORDS: Anatomy & histology; Dawson; Diaphanization; Extracellular matrix; Tissue clearing.**

## INTRODUCTION

The optical clearing of soft tissue is an important tool for examining whole specimens without incurring the need of sectioning to visualize deeper layers. Tissue optical clearing promotes preservation of three-dimensional structures, which allows the observation of the internal anatomy in its original position and original spatial interaction. The different techniques used for clearing are based on various physicochemical phenomena, all based on the idea of modifying the chemical interaction of macromolecules and decreasing light scattering through solid superficial tissues. Spalteholz (1914) proposed the first clearing technique, by applying alcohols to soft tissue in order to dilute optically active metabolites. After Spalteholz (1914), various techniques were invented for clearing soft tissue in anatomical specimens. Within those, Dawson´s clearing of soft tissue technique stands for its practicality and rapid results. This process consists on tissue maceration with aqueous potassium hydroxide (KOH) solutions for observing internal stained bone structures and diverse vascularity of anatomical specimens (Dawson, 1926).

To understand histological changes that could take place in the Dawson´s clearing technique, the chemical and physical phenomena involved in the clearing process must be considered. The basis of the technique lies in two fundamental principles: 1) the medium's refractive index and 2) the chemical effects of alkaline hydrolysis on tissues (Seo *et al*., 2016). The medium's refractive index states that the

[1] Undergraduate Medical Student, Cra 1 Nº 18A - 12, Universidad de los Andes, School of Medicine, Bogotá, Colombia.
[2] Research Trainee, Cra 1 Nº 18A - 12, Universidad de los Andes, School of Medicine, Bogotá, Colombia.
[3] Assistant Professor, Anatomy Laboratory and Class Coordinator, Cra 1 Nº 18A - 12, Universidad de los Andes, School of Medicine, Bogotá, Colombia.

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transparency of a cleared organ increases when the tissue and preservation solution are at the same refractive index values (Richardson & Lichtman, 2015). The chemical effects of alkaline hydrolysis on tissues are based in the fact that mild alkaline corrosion has multiple physicochemical reactions with the various biomolecules contained in the tissue. Thus, we hypothesize that both soft and hard tissue are significantly affected by alkaline hydrolysis, leading to the equalization of the tissue´s refractive index in respect to the medium´s refractive index.

While other techniques such as Spalteholz´s have been biochemically and histologically described (Steinke & Wolff, 2001), histological changes in tissue treated with Dawson´s technique remain unclear. Some studies have reported changes in weight and volume after clearing but none have reviewed the biological and cellular modifications occurring in soft tissue (Bernardes *et al*., 1992). Studies done with this technique may be affected by molecular and cellular changes occurring throughout the entire process, which could alter any results. Our aim is to describe the microscopical differences between macerated and normal tissue.

## MATERIAL AND METHOD

Samples were extracted from two human fetuses with gestational ages between 16 to 28 weeks. Specimens were obtained for research and education at our Anatomy Laboratory and had been preserved for more than a year in a 10 % formaldehyde solution. The project received approval from the Institutional Review Board of the University of los Andes, under the use of human cadaveric specimens for research for Education in Anatomy, (Minute No. 518 of 2015). Each of the fetus´s lower limbs were dissected and used for histological analysis: two lower limbs were cleared with a 1 % KOH solution, stained with alizarin red using Dawson´s technique and preserved in glycerol, and the two remaining lower limbs were maintained in 10 % formaldehyde solution as controls. Before histological processing, cleared specimens were immersed again in a 10 % formaldehyde solution to eliminate remaining glycerol in which cleared tissue is finally stored to obtain adequate material for histological processing.

After formaldehyde immersion, all cleared specimens were treated with gradual increasing solutions of ethanol from 70 % to 90 % with 5 % changes to extract the remaining quantities of glycerol. Finally, lower limbs were dissected from the fetus and were embedded in paraffin wax for histological processing. From each limb we extracted the following samples: 1) a transverse cut of the tibia and fibula, with complete muscular and epithelial layout of the section, and 2) a transverse cut of the foot at the mid-level of the calcaneus bone showing tarsus, metatarsus, phalanges, nerves, muscles, and associated vasculature. Eight paraffin wax blocks were obtained meaning a total of 2 blocks per specimen that included the 2 macroscopic sections mentioned previously. Three microscopic sections of 7 micrometers wide were cut from each paraffin block and were stained with Hematoxylin and Eosin (HE), Masson´s trichrome and Periodic Acid Schiff (PAS), obtaining a total of 6 slides per fetal specimen. Slides were observed under a light microscope.

## RESULTS

The clearing process exhibited important histological changes in contrast to the formaldehyde preserved tissue. First, the HE stain (Fig. 1) showed that most of the cleared tissue was stained with a lower intensity compared to controls. Moreover, while the histological layout of the muscle did not change, most of the skeletal muscle nuclei were not observable, leaving several empty spots in the cellular layout.

In bone tissue most of peripheral osteocytes were absent and peripheral matrix appeared to be irregular compared to formaldehyde preserved bones. Keratinized stratified squamous epithelia of the skin could not be observed and there was no evidence of preserved vascular or nervous structures in the cleared tissue, which suggest that most of the squamous epithelia was affected by alkaline hydrolysis. PAS staining did not show any additional information when compared to the HE stain.

Masson´s trichrome stain showed a significant difference on soft tissue composition (Fig. 2). While formaldehyde preserved tissue did show red and blue stain depicting the presence of both muscle fibers and collagen respectively, cleared tissue presented exclusively blue stain predominance in soft tissue.

## DISCUSSION

As expected, the biochemical reactions between mild alkaline solution and biological molecules caused a significant maceration of the tissue. Regarding the HE stain samples of cleared muscular tissue, the most relevant finding was the lack of observable nuclei. These results imply that the observed layout was mostly extracellular matrix according to the main biochemical components of the said

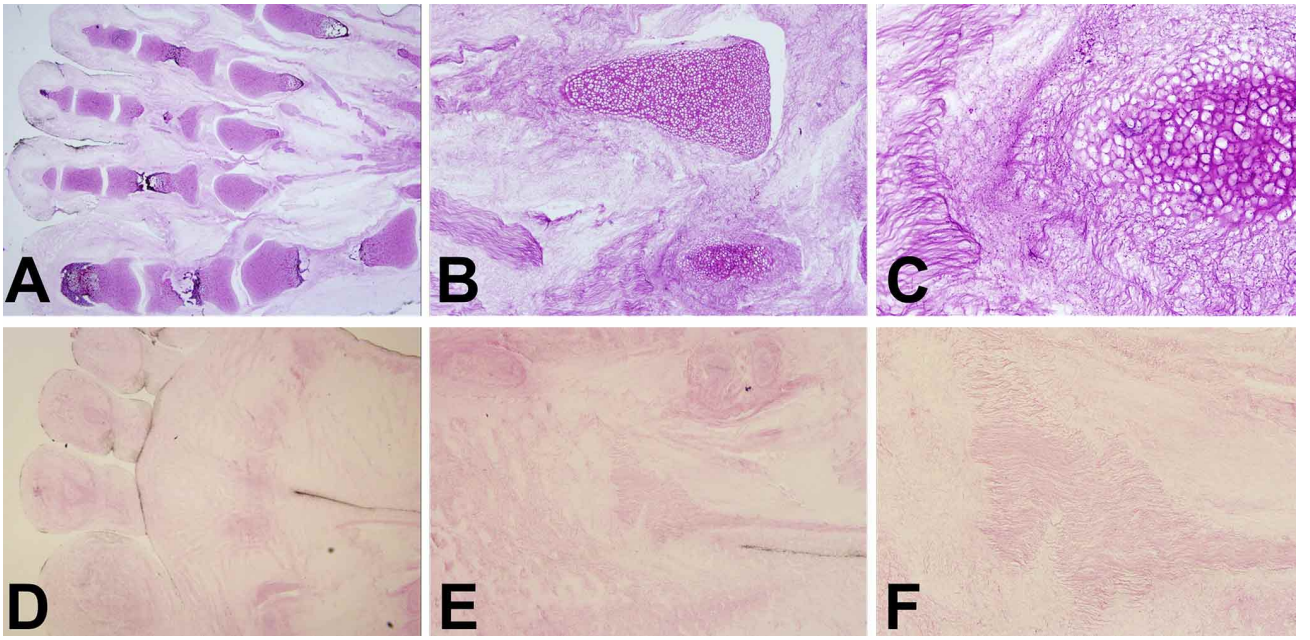


Fig. 1. HE stain of non-cleared (A-C) and cleared (D-F) feet. At low magnification(A, D) shows most macrostructures absence and less staining in cleared specimen. High magnification view of the feet (B, C, E, F) shows acellular fibers on cleared tissue and dominance of connective tissue over other structures contrasting various observable structures in non-cleared tissue.

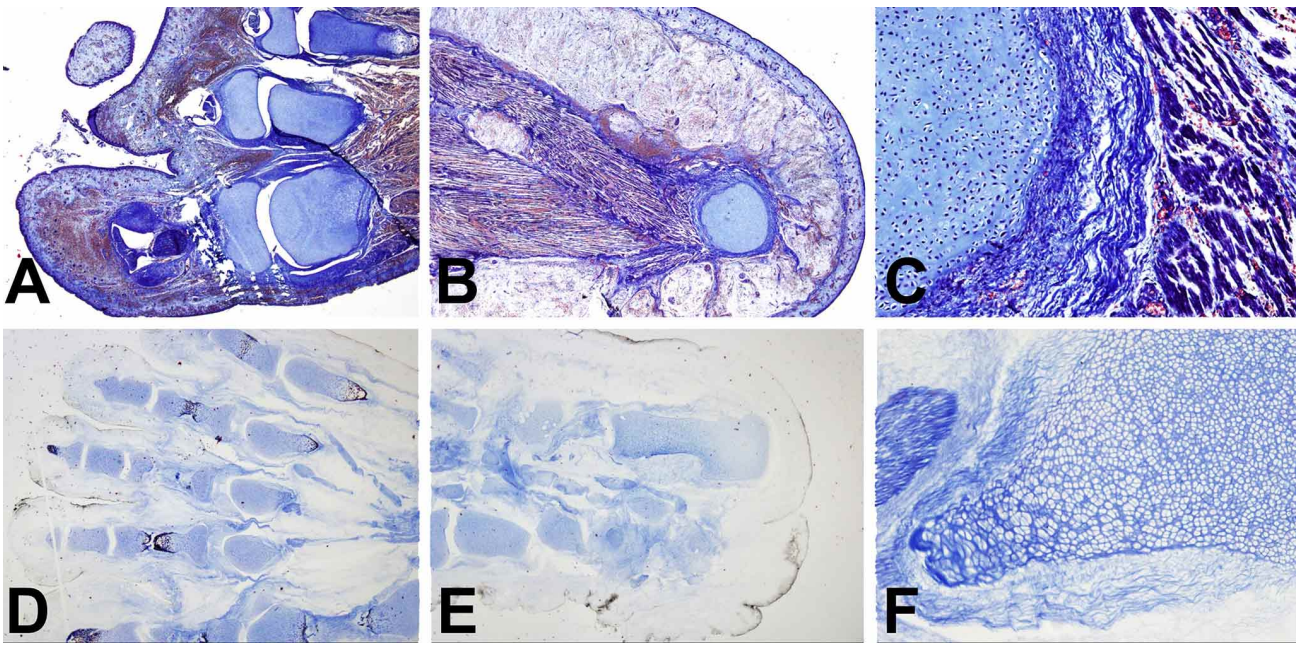


Fig. 2. Masson´s trichrome staining of non-cleared (A-C) and cleared (D-F) feet. While confirming H&E stain results, high magnification view (C, F) shows absence of muscular tissue and dominance of collagen composed tissue in both bony and soft tissue.

structure (Fig. 1). The effect of an alkaline solution on nucleic acids results in their hydrolysis, meaning that in the clearing process the nuclear envelope and DNA were affected by an alkaline hydrolysis reaction (Takeda *et al*., 2000), explaining why there were no observable nuclei in the muscle fibers.

Additionally, a lack of epithelia and bone tissue was also observed after HE staining. This is mostly due to degradation of cellular membranes, which exposed the cellular content to the alkaline hydrolysis reaction. The biochemistry of the phospholipid cellular membrane is prone to suffer changes in a basic pH environment. This occurs

due to the process of saponification between fatty acid chains of phospholipids and potassium hydroxide (Wrolstad, 2005). It is also significant, that when using HE stain, cleared tissue appeared less stained than non-cleared samples. This may be due to dilution of most macromolecules in the alkaline solution.

Cleared soft tissues stained with Mason's trichrome showed collagen fibers predominance. Collagen fibers are important for cellular and tissular adhesion, since those are one of the components of the extracellular matrix. The fact that these fibers were preserved, essentially maintaining extracellular matrix structure, was previously reported by Wen *et al*. (2010) at an in vivo optical glycerol skin clearing.

It was proven that most of the tissue proteins were degraded except for collagen, which appears to have maintained the macroscopic structure of the specimens. Due to KOH usage in Dawson's technique, alkaline hydrolysis affected proteins and caused degradation into simple amino acids (Griggs, 1921). Collagen proteins are unaffected in cleared tissue because of its particular amino acid sequence, as they do not easily degrade in an alkaline medium (Eastoe, 1955). Besides the degradation of proteins as a contributing factor for tissue transparency, KOH generates racemization of optically active amino acids, stimulating the transparency of cleared tissue (Griggs, 1921).

PAS technique didn´t show polysaccharide staining, which is the target molecule of this particular stain. Results of PAS were mainly negative, which demonstrates that the alkaline hydrolysis process affected polysaccharides. Concerning muscle fibers, this means that glycogen reserves were diluted during the clearing process or previous tissue preservation.

## CONCLUSION

Phospholipids saponification, nucleic acids and polysacharide degradation and a change on the protein´s structural properties are the main factors inducing clearing. At the same time, molecular stability of collagen in alkaline conditions allows the specimen to maintain its shape after the process.

With the understanding of these phenomena, the sum of all described cleared tissue changes during Dawson´s optical clearing method explains the refractive index changes. Yet, clearing will affect any further study focusing on a macromolecule layout perspective as cleared organs or tissues do not preserve their original contents after said process.

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**GOMEZ-SAMPER, A. F.; LATIFF, M. A.; LOPEZ-MCCORMICK, J. S. & RUEDA-ESTEBAN, R. J.** Análisis histológico de una técnica de limpieza de tejido de base alcalina para tejido musculoesquelético fetal. *Int. J. Morphol., 40(3)*:557-561, 2022.

**RESUMEN:** Las técnicas de limpieza de tejido se utilizan con frecuencia en la observación y descripción de estructuras y vías anatómicas sin alterar el diseño tridimensional de la muestra anatómica. El aclaramiento óptico de tejidos promueve la preservación de estructuras tridimensionales, lo que permite el estudio de la anatomía interna en su posición original y la interacción espacial original. Entre estas técnicas, el aclarado por maceración con Hidróxido de Potasio (KOH) es una de las más utilizadas. Sin embargo, los cambios histológicos del tejido después de la maceración con KOH aún no se han entendido por completo. Nuestro objetivo es describir las diferencias microscópicas entre el tejido macerado y el normal. Para entender mejor dichos cambios, dos fetos humanos con un período de gestación de 16 a 28 semanas fueron aclarados y procesados para análisis histológicos. Se obtuvieron microtomos de las extremidades inferiores de los fetos y se tiñeron con hematoxilina y eosina, ácido peryódico de Schiff (PAS) y tricrómico de Masson con el fin de observar los cambios histológicos y de composición de macromoléculas en el tejido aclarado. Diferencias notables a nivel histológico en cuanto a la composición de las estructuras celulares, ya que los tejidos diafanizados mostraban un predominio de matriz extracelular compuesta por fibras de colágeno con ausencia de la mayor parte del tejido celular nucleado. La saponificación de los fosfolípidos, la degradación de los ácidos nucleicos y un cambio en las propiedades estructurales de las proteínas son los principales factores que inducen la depuración. Al mismo tiempo, la estabilidad molecular del colágeno en condiciones alcalinas permite que la muestra mantenga su forma después del proceso.

**PALABRAS CLAVE: Anatomía e histología; Dawson; Diafanización; Matriz extracelular; Limpieza de tejidos.**

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Corresponding author:
Roberto Javier Rueda-Esteban
Cra 1 Nº 18A- 12
Universidad de los Andes
Bogotá
COLOMBIA

E-mail: rj.rueda32@uniandes.edu.co