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A Comparison of Three Different Agents of Decalcification for a Histological Examination of Bone Tissues

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Abstract

Objectives: Bone-decalsification is still a time consuming and laboring process in histopathology laboratories. In this study, we have aimed a comparision of decalsification degrees and staining properties of compact bone tissue decalcificated by formic acid, Biodec-R, and Decalcifier II as decalcification agents.

Materials and Methodology: A total of 6 healthy male rats (200-220 g) were used in this study. Rats were decapitated by cervical dislocation. Femurs were removed and 0.5 cm long pieces from these femurs were fixed in 10% formaldehyde for 36 hours. Subsequently, the bone-tissues were stored in decalcification fluids at room temperature for six days. The bone-tissue samples were processed by routine tissue procedures. They were further processed for light microscopic examination and stained with hematoxylin-eosin, Gomori's trichrome, and Periodic acid-Schiff. We have examined the bone sections under a Leica DFC280 light microscope and Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K).

Results: When all three decalcification agents were applied for equal time periods and at the same experimental design, it was observed that formic acid is more effectible for the preservation of natural structure of the bone tissue and on the quality of the staining properties. It was observed that Biodec R and Decalcifier II are similar to each other in terms of staining properties and preservation of structural details of cells and tissue.

Conclusion: Formic acid decalcification is adviced for histologic staining and for a higher qualitiy of microscopic view during histological examination of compact bone tissues.

Key Words: Bone; Decalcification; Formic Acid; Biodec-R; Decalcifier II.

Kemik Dokunun Histolojik İncelemesi İçin Üç Farklı Dekalsifikasyon Ajanının Karşılaştırılması

Özet

Amaç: Kemik dekalsifikasyonu histopatoloji laboratuvarlarında hala zahmetli ve zaman alıcı bir süreçtir. Bu çalışmada, kemik dekalsifikasyonu için kullanılan formik asit, Biodec-R ve Decalcifier II dekalsifikasyon ajanları ile kompakt kemik dokularının dekalsifikasyon derecesi ve boyanma özelliklerinin karşılaştırılması amaçlandı.

Gereç ve Yöntem: Çalışmada toplam altı adet sağlıklı erkek sıçan (200-220 g) kullanıldı. Sıçanlar servikal dislokasyonla dekapite edildi ve her iki femurları çıkarıldı. Femurlardan alınan 0.5 cm uzunluğundaki parçalar %10'luk formaldehitte 36 saat süreyle tespit edildi. Daha sonra kemik dokuları dekalsifikasyon sıvılarında altı gün oda ısısında bekletildi. Dekalsifikasyonun ardından kemik doku örnekleri rutin doku takip işlemlerinden geçirilerek ışık mikroskobik inceleme için hazırlandı. Lamlar üzerine alınan 6 µm kalınlığındaki kesitlere hematoxylin eosin, Gomori's trichrome ve Periodic acid-Schiff boyamaları yapıldı. Kesitler Leica DFC 280 ışık mikroskobu ve Leica Q Win görüntü analiz sisteminde (Leica Micros Imaging Solutions Ltd.; Cambridge, U.K) incelendi.

Bulgular: Her üç dekalsifikasyon ajanı aynı süre, aynı deney dizaynıyla uygulandığında; formik asitin kemik dokunun doğal histolojik yapısını en iyi koruduğunu ve boyanma özelliklerinin belirgin şekilde daha kaliteli olduğu gözlendi. Biodec-R ve Decalcifier II'nin hücre ve doku detaylarının korunması ve boyanma özellikleri açısından birbirine benzer olduğu saptandı.

Sonuç: Kompakt kemik dokunun histolojik incelenmesinde formik asit dekalsifikasyonu, histolojik boyanma ve mikroskopik görüntü kalitesi açısından tercih edilebilir.

Anahtar Kelimeler: Kemik; Dekalsifikasyon; Formik Asit; Biodec-R; Decalcifier II.

INTRODUCTION

Its dynamic structure with a cycle of constant construction and demolition, the difficulty of macroscopic dissection techniques and the differences in tissue processing make histopathological examination of bone tissue an arduous task. It is difficult to obtain a section even with a good processing since cells and matrix proteins make up only 1/3 of the tissue while inorganic matrix (calcium hydroxyapatite) make up the remaining 2/3. For this reason, calcium should be removed in routine examinations of bone tissue. Decalcification is the technique for removing mineral from bone or other calcified tissues while preserving all the essential microscopic elements. Bone decalcification is performed for various reasons, such as routine staining of paraffin sections, enzyme histochemistry, immunohistochemistry (IHC), immunocytochemistry (ISC) and electron microscopy. It is important to minimize the loss of enzymes, antigens, proteoglycan, cellular infrastructure and proteoglycan infrastructure of bone tissue during fixation and decalcification (1).

Decalcification solution should be selected according to the type of bone tissue and staining and the desired decalcification rate. There are ready-to-use agents and a wide range of formulas to be prepared in the laboratory. There is no single formula that can be recommended for decalcification procedure. It is essential for researchers to establish their own standards for each study by checking section and staining. Nevertheless, there are certain formulations that can be used for routine decalcification.

There are two main types of decalcification agents: chelating agents and organic or mineral acids. Chelation agents take up calcium ions from the bone. Ethylenediaminetetraacetic acid (EDTA) is the most frequently used chelating agent. It is a safe but a slow decalcification solution. The organic or mineral acids make up a solution of calcium ions. The type of acid (weak or strong) used in the solution may accelerate the rate of decalcifying process, but it also entails the risk of deterioration of the tissue components and staining properties. Weak acids are formic acid, picric acid, acetic acid and so on and strong acids are nitric acid and hydrochloric acid (HCl). There are also solution with a mixture of these two types of acids, such as acetic acid-formalin, formic acid-formalin solution, hydrochloric acid-formic acid and nitric acid-formalin (2). The choice of decalcifying agent depends on the degree of tissue mineralization, the purpose of the study and the staining techniques (3).

Factors like temperature, agitation, vacuum microwave and electric current are also effective in the process of decalcification. Heat may accelerate a chemical reaction three times more at every 100 C°. Increased temperatures shorten the decalcification time (4). Agitation increases the interaction between object and decalcifying solution, and provides a uniform staining by removing dissolved minerals from the tissue. With vacuum pumping, undissolved calcium mixes with acid and turns into dissolved calcium salts and carbondioxide (CO_2). CO_2 should be immediately removed from the environment in order to maintain chemical equilibrium and reaction rate (5,6). Microwave irradiation affects the decalcification time in acidic solutions by increasing the penetration of the decalcifying solution and accelerating the reaction between the solution and tissue molecules (7,8). Ca^{+2} ions are released faster in the decalcifying solution in the presence of an electric field (9). These Ca^{+2} ions should be removed from the environment as well.

Various methods are used to determine the end-point of decalcification. This can be done mechanically by touching the sample with a needle tip or by weighing the sample. Decalcification can also be checked, using chemical and radiologic methods. In radiological method, photomicrographs of the softening tissue are taken at different stages of the decalcification process using X-ray, and the results are compared with a standard curve. This method is not preferred because this equipment is not available in histology laboratories. In chemical method, Ca salt is searched in the decalcified solution. 0.5 cc saturated ammonium oxalate is added to 5 cc decalcification solution until it becomes alkali. If Ca is present, a white precipitate forms and new solution is added until the fluid becomes clear again. If the fluid remains clear after 30 minutes, decalcification is complete (10,11,12).

The aim of the present study is to compare the effects of %5 formic acid and two commercial products, namely Decalcifier II (Surgipath Europe Ltd. Peterborough, UK) and Boidec-R (Bio-Optica Milano, Italy) on decalcification and bone tissue staining characteristics in compact bone decalcification.

MATERIAL AND METHODS

In this study three different decalcifying solutions, %5 formic acid, Decalcifier II (Surgipath Europe Ltd. Peterborough, UK) and Boidec-R (Bio-Optica Milano, Italy) were used for the femur bone decalcification of a total of six healthy male rats (200-220 g). The rats were decapitated by cervical dislocation and both femurs were removed. Sections (0.5 cm) taken from the chamber for the femur were fixed in 10% formaldehyde for 36 hours at room temperature. Then, bone tissues were subjected to decalcification in 100 ml Decalcifier II (Hydraulic acid - Ethylenediaminetetraacetic acid) and Boidec-R (10% hydraulic acid – 5% formic acid) and %5 formic acid solutions for six days at room temperature, and the solutions were renewed on alternate days. At the end of decalcification period, the bone tissue samples were washed in running tap water and embedded in paraffin blocks after being exposed routine histological tissue processing. Hematoxylin-Eosin (H&E), Gomori's Trichrome and Periodic acid-Schiff (PAS) stains were applied to 6 µm-thick sections cut from the paraffin blocks for light microscopic examination. The sections were examined using Leica DFC 280 light microscope and Leica in QWin Plus image analysis system (Leica Micros Imaging Solutions Ltd. Cambridge, UK).

RESULTS

Formic acid: In the bone tissue sections decalcified by formic acid and stained with H&E, the periosteal and endosteal layers and vascular structures were clearly distinguishable. The cell nucleus on the periosteum and the osteocytes nucleus in the lacunae were blue-purple with high contrast. The bone matrix on compact and trabecular bone tissue was stained in eosin with red-pink colors and there were weak, irregular basophilic spots stained in light blue. There was high-quality staining in the bone marrow tissue in the medullary cavity that allows for identification of cells (Figure 1a, b). The cell nucleuses in the sections stained with Gomori's Trichrome were purple-black, while bone matrix was light green. The cytoplasmic domains of osteocytes in the lacunae were in the form of transparent spaces. Vascular structures were clearly distinguishable. Cellular staining of the bone marrow was of the quality that allowed cells to be distinguished (Figure 1c). In the sections stained with periodic acid-Schiff, the cells

nucleus in the structure of bone tissue and bone marrow were stained in hematoxylin with blue-purple colors while the bone matrix was stained in pink-violet. Megakaryocytes in the bone marrow were recognizable and the cellular nucleuses in all tissues areas were with high contrast (Figure 1d).

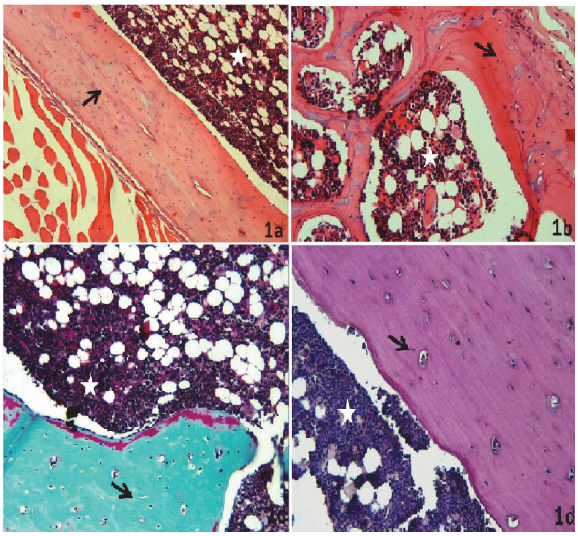

Figure 1. Decalcification with formic acid a) H-E x 10, b) H-E x 20, c) TRC x 20, d) PAS x 20 (arrows: compact bone tissue; asterisk: bone marrow)

Bone, and bone marrow tissue areas in the sections decalcified by Biodec-R were stained in strong eosinophilic red by hematoxylin-eosin staining. The details of the cells were indistinguishable. It was notable that the nucleuses were stained in hematoxylin (Figure 2a, b). In the sections stained with trichrome, the bone tissue matrix was diffuse green and the nucleuses of osteocytes were dark purple. The cells of bone marrow tissue were stained basophilic in diffuse dark purple and the details of the cellular structure were indistinguishable (Figure 2c). In the sections stained with Periodic acid-Schiff, the bone tissue matrix was of diffuse pink color. The osteocytes nucleuses were indistinguishable and lacunars were in the form of small oval spaces. The bone marrow areas were stained in purple weak basophilic manner. The boundaries and nucleus of the cells were indistinguishable (Figure 2d).

In the sections of the bone tissues subjected to decalcification with Decalcifier II, H&E and all tissue areas were stained in dark red-eosinophilic color. It was observed that the nucleuses were not stained in hematoxylin in these sections. The osteocytes nucleuses were indistinguishable in the bone tissue areas. The cells nucleuses of the bone marrow tissue were stained in dark red color in a strong eosinophilic character. The cellular borders and structures were indistinguishable in all tissue areas (Figure 3a, b). In the sections section stained with trichrome, the bone tissue matrix was stained in diffuse green color. The nucleuses of osteocytes within the bone matrix were stained in dark purple. The bone marrow cells were stained basophilic diffuse texture of a dark purple color and the cell details were indistinguishable (Figure 3c). In the sections stained with Periodic Acid-Schiff, the bone tissue matrix was stained in diffuse light pink and the cell nucleuses were indistinguishable. In the bone marrow areas, there were weak basophilic stains with a low contrast. It was not possible to observe the details to distinguish the bone marrow cells (Figure 3d).

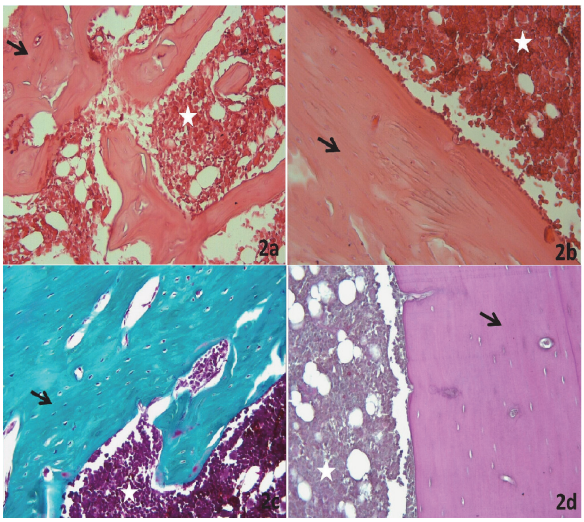

Figure 2. Decalcification with Biodec-R a) H-E x 10, b) H-E x 20, c) TRC x 20, d) PAS x 20 (arrows: compact bone tissue; asterisk: bone marrow)

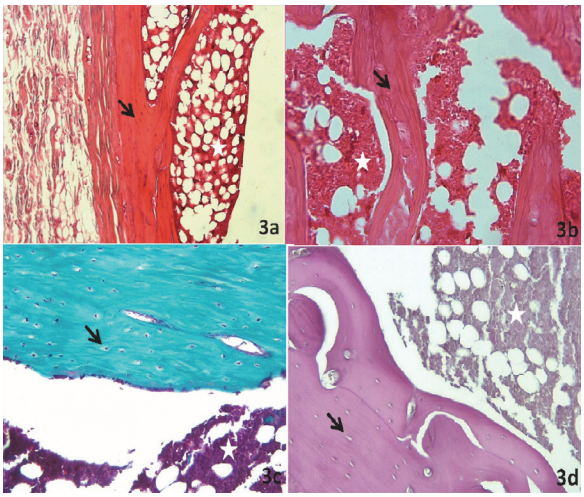

Figure 3. Decalcification with Decalcifier II a) H-E x 10, b) H-E x 20, c) TRC x 20, d) PAS x 20 (arrows: compact bone tissue; asterisk: bone marrow)

DISCUSSION

The decalcification process of the bone tissue that is needed for histological examination is a hard-to-formulate, laborious, and time consuming process. The protection of bone tissue components and their interrelationship along with the staining properties of tissues depend on the decalcification quality and speed.

During detecting and decalcification steps, the proteoglycan content sample should be small as possible to prevent the loss of cellular infrastructure, antigens, and enzymes, but this is not always possible (1). For the identification process of the bone tissue prior to the decalcification on electron microscopy with EDTA, best agent protecting the fine cellular structure and enzymes, 1-2 mm or smaller pieces of bone tissue should be sampled (13). Because it decalcifies faster than EDTA while also preventing proteoglycan loss, ascorbic acid may be used during the process; yet, it does not protect cells and intracellular structures well (14). All tissue decalcification agents, especially strong acids, affect the staining process badly no matter how small the particles are.

Decalcification solutions, either produced for commercial reasons or prepared in the laboratory, are diverse and there is no single formula that can be recommended in this regard. The studies to this day have either focused on finding a brand new decalcification agent or modifying those that are already known (4,15-17). A good decalcification agent should guarantee the complete removal of calcium from the tissues, minimise the possible damage on the cells and tissues, prevent deterioration in the sequential staining, and carry out the decalcification at a reasonable speed (18).

Poorly identified bones are adversely affected by the decalcification process in cases when the detecting fluid does not achieve proper penetration into the tissue due to the thick cortex in compact bones. Therefore bone fragments should not be put into the solution before making sure that they are suitably fragmented. If bones and soft tissues are intact, the detection disrupts the natural ties in the soft tissues and cause separation during cutting (19). Generally accepted process is as follows: cutting, identification, cutting and decalcification. Since the acids in the decalcification fluid disrupt the cellular morphology of unidentified tissues, an identification process prior to decalcification is critical (4). If the decalcification is made using an acid-containing solution, it is reported that washing the tissue under flowing water for 10-15 minutes has a positive effect on the osteoid matrix (4).

In our study, we have evaluated the effects of three different decalcification agents on organic and inorganic components of bone tissues along with their degree of decalcification and staining properties throughout the methods used in routine histology laboratories. All three agents were tested in terms of standard procedures such as temperature, pressure, and motion at the same time and under the same laboratory conditions. Due to the fact that protecting normal structure of bone tissue and staining quality is more important than decalcification time in histologic evaluations, we conducted the tests during the same time period for all three agents.

The first of our agents was the formic acid, a soft acid which is widely used in 2-10% concentrations in routine histological studies for the decalcification of compact bones and bone marrow. This decalcifying agent can also be used in immunohistochemical staining. Small spongy bone fragments are decalcified in two days while larger, dense compact bones become decalcified within 20 days in formic acid solutions. Decalcification fluid must have sufficient volume and the fluid should be renewed every 48 hours. The solution can also contain formalin both for detection and decalcification of the tissues. These solutions do not harm nuclear staining and are safer than HCl, however, their disadvantage is that they decalcify slower than HCl (20). Throughout our studies, we have observed that formic acid groups provided clear and high contrast nuclear staining in all cell types of bone and bone marrow during the H&E staining. Compact and trabecular bone matrix in addition to the bone marrow tissue inside were well-stained so as to allow the identification of cells. The cell nuclei were purple and black, the bone matrix was green, and lacunar areas were marked as spaces in Gomori's trichrome staining. Vascular structures and cells in the bone marrow could be clearly distinguished. In PAS staining, too, all cell nuclei in all the tissue areas were observed in high contrast. In their study on the effect of decalcification agents on cartilage and the effect of decalcification and/or identification solutions on proteoglycans loss, Callis et al. have decided to use 5% formic acid due to the minimal loss of proteoglycan loss during the process and faster decalcification it provided (1).

Decalcifier II was our second agent. It is an effective and fast decalcification agent specially prepared for histology and pathology laboratories. Its long term application, as in most acids, may result in ribonuclease damage caused by intracellular nucleic acids and this, in turn, ends up in basophil loss in histological staining. Although this agent is used more in the decalcification of the bone marrow biopsy materials, it can also be used in compact bone decalcification. In both cases, samples should be checked every 1/2-1 hours. When the application temperature is lowered to 20°C, any histochemical staining can be applied to samples. If an IHC staining is planned, sample tissue should be taken with bone biopsy. In this case, Decalcifier I solution, which is more sensitive for staining, should be preferred. Among HCl containing agents, Decalcifier II agent is three to four times slower (1). Even though it is produced for decalcification in shorter periods of time, we wanted to use this agent to see its staining quality and ability to protect cellular details using the same method, within the same period of time. Studying the bone tissue sections decalcified with Decalcifier II, we have noted that the nuclei were not properly stained in H&E staining, osteocytes nuclei in the bone tissue were not distinguishable, and that the cellular nuclei in the bone marrow had strong eosinophilic characteristics. Neither the borders of the cellular structures in the tissues, nor their details were identifiable. The nuclei of osteocytes within the bone matrix stained with Gomori's Trichrome were dark purple while the cells of the bone marrow tissue were stained diffused dark purple colour; the details of the cells were hard to recognise. Again,

the matrix of bone tissue in the sections stained with PAS method were stained diffused light pink and the cell nuclei were not identifiable. The bone marrow had lost basophils and the staining was in low contrast, which made it hard to distinguish the cells.

The third agent we used was Biodec-R, which is a fast decalcification agent that is capable of operating in all mineralised tissues with salt as its regulatory components. Regulators are added to minimise tissue swelling caused by the acid (21). Another study argues that using formic acid and HCl (modified for transmission and scanning electron microscope) forms of mineralised materials is more effective compared to other agents since they shorten the decalcification process while protecting the cellular structures (19). While Chagas et al. show that Biodec-R is not effective in maintaining cell nucleus (21), Silva et al. claim that it actually protects tissue and cell morphology (19). Using 5% nitric acid, another strong acid-based decalcifying agent, in modifying EDTA and reducing the 135-day-long decalcification period down to 24 days, these researchers have not observed proper cell morphology despite saving a significant amount of time (22). Despite the disruption of tissue integrity, acids can be used in urgent cases. In cases where time factor is not a priority, the satining quality can be achieved by using neutral EDTA, which protects the integrity of soft tissues (23). In our decalcification experiment with Biodec-R, we have similarly observed that the bone and bone marrow tissues with eosinophilic stain were strongly distinguishable whereas the details of the cells were not observable and the nuclei were not stained. In the Gomori's Trichrome staining, the matrix of the bone tissues were green and the nuclei of osteocytes were stained dark purple. The cells of bone marrow tissues were stained with diffused dark basophilic and the details of the cellular structure details could not be seen. The bone tissue matrix stained with periodic acid-Schiff appeared in diffused pink colour but the osteocyte nuclei were not visible while lacunas were observed simply as small oval shapes. The bone marrow appeared poorly stained in basophilic character; the nuclei and boundaries of the cells could not be detected.

After applying these three agents at the same time and the same experimental design, we can conclude that formic acid is best in preserving the bone intrastructure. The staining degree and quality were the same for all three agents. Although Biodec-R and Decalcifier II were way behind formic acid in terms of preserving the details and the quality of staining, it is difficult to state that one agent is superior to the other.

Considering the arguments presented above, we recommend the establishment of a decalcification protocol and to set some standards in order to make it capable of preserving bone and cell structures with a shorter histological decalcification process. To this end, there is need for more researches conducted in different experimental conditions.

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