Advantages of a Combined Method of Decalcification Compared to EDTA

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ABSTRACT Decalcification of mineralized tissues is an essential step during tissue processing in the routine histopathology. The time required for complete decalcification, and the effect of decalcifier on cellular and tissue morphology are important parameters which influence the selection of decalcifying agents. In this study, we compared a decalcifying solution (ETDA) composed of both acid and chelating agents to a classical and well-known decalcifying agent (EDTA). To this purpose, the optic density of bone radiographs, residual calcium analysis, bone sample weight, and histological and immunohistochemical analysis were performed. Our data suggest that, similarly to EDTA, the ETDA solution completely removes the calcium ions from the samples enabling easy sectioning. However, unlike the EDTA, this agent takes much less time. Furthermore, both agents showed comparable decalcification efficacy, and similarly, they did not produce cellular, tissue or antigenicity impairments. Therefore, ETDA may be a suitable option when it is necessary an association between a rapid and complete removal of calcium minerals, and a suitable preservation of structure and antigenicity of tissues. *Microsc. Res. Tech. 78:111–118, 2015.*

INTRODUCTION

The decalcification of mineralized tissues can be understood as the dissolution of the hydroxyapatite complex (An and Martin, 2003), and represents one of the most important and indispensable steps in the histotechnology (Emans et al., 2005), since it is often necessary to allow hard tissue sectioning and processing (Tinling et al., 2004).

The velocity of the decalcification process and the effects of decalcifying agents on tissue staining characteristics are important parameters that influence the selection of decalcifying solutions (Sanjai et al., 2012). The rate of decalcification is a determinant factor for the preservation of cell structures and components (Pitol et al., 2007). Basically, demineralizing solutions can be categorized into (i) those originating from organic acids (i.e., acetic, formic or citric acids), (ii) inorganic acids (i.e., hydrochloric, nitric or sulfuric acids), or (iii) calcium chelating solutions such as diaminoethylene tetraacetic acid (EDTA).

In the past, different types of decalcifying solutions have been used for different purposes. The Morse solution, for example, is a strong acid decalcifying solution that has been widely used in the technique of RNA detection by in situ hybridization. Another solution of an acidic nature is 5% trichloroacetic acid used in the TUNEL technique for the determination of double-strand DNA (Emans et al., 2005). In contrast, EDTA is widely used in immunolocalization because of its excellent ability to preserve the tissue antigenicity (Cho et al., 2010).

Usually, strong acid solutions such as hydrochloric acid are implicated in negative effects concerning the preservation of tissue morphology and antigenicity, in addition to the DNA integrity. In fact, although decalcifying agents of an acidic nature provide rapid decalcification, when used for long periods of time they usually induce deterioration of cell content, chromatin in particular, in addition to the DNA hydrolysis (Callis and Sterchi, 1998). These effects might be limiting since complete DNA degradation in the nucleus cells can inhibit fluorescence in immunofluorescence techniques and prevent the execution of in situ hybridization with the use of DNA probes.

On the other hand, the use of chelating agents avoids the above problems but has the disadvantage of requiring a longer decalcification time, with complete decalcification at times taking several weeks depending on the size and degree of mineralization of the

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sample (Callis and Sterchi, 1998). In agreement with this, it has been reported that DNA fragments ranging in length from 5 to 21 kb were detected in biopsies of bone decalcified with EDTA solution, indicating full preservation of genetic material (Wickham et al., 2000).

Currently, there is an intensive search for decalcifying solutions with a profile close to ideal, i.e., a decalcifier that does not damage cell structures or their components or genetic material and that preserves tissue antigenicity. In view of that, we present here a combination method of decalcification that uses both acid and chelating agent, which has been used successfully in our laboratory. It is composed of an association of hydrochloric acid (12%), EDTA (0.07%), sodium tartrate (0.014%), sodium and potassium tartrate (0.8%) and water (bring to 1,000 mL), that we, arbitrarily, named ETDA, to differentiate it from the classic EDTA. Variations in the relative proportions of the components of this decalcifying solution are found in literature (DeVilliers et al., 2011, Kashireddy and Chakravarthy, 2010).

The aim of this study was to compare the known chelating agent EDTA with ETDA according to the time and efficacy of decalcification, and the ability to preserve tissue morphology and antigenicity. For this purpose, the following tools were used in this work: (i) the optical density of bone radiographs, (ii) the residual calcium (Ca^{2+}) concentration during different decalcification times, (iii) the variation in sample weight as a function of decalcification time, (iv) hematoxylin and eosin (H&E) histological staining, and (v) osteocalcin immunoreactivity.

MATERIALS AND METHODS

Animals

Seven male *Wistar* rats weighing 300–350 g from the Animal House of the University of São Paulo, Ribeirão Preto Campus, were used. The animals were housed in plastic cages with a maximum of four per cage and kept under conditions of controlled temperature of 23 ± 1°C, with free access to balanced ration and water. The study was conducted according to the norms of the Ethics Committee for Animal Experimentation of the University of São Paulo (CETEA, protocol n° 016/2012).

Bone Samples Collection and Decalcification Processes

To compare the two decalcification methods (EDTA vs. ETDA) we employed femur samples of *Wistar* rats. The animals were sacrificed by CO_2 inhalation, the right and left femurs were removed and cleaned of soft tissue, weighed on a precision scale and then radiographed together with an aluminum scale using the same radiographic pattern. After these procedures, the femurs were fixed in buffered 4% paraformaldehyde (PFA) for 24 h and then subjected to decalcification process with EDTA (left femurs) or ETDA (right femurs). The femur samples were maintained on polypropylene tubes containing 9 mL of EDTA or ETDA and changes of decalcification solutions were made as presented next.

Decalcification Endpoint

The endpoint of decalcification was evaluated by manipulation of the tissue specimens (An and Martin, 2003). In this procedure, the femur samples were maintained in EDTA or ETDA solution until they acquired the appropriate softness for histological processing which was assessed by folding and probing (puncture with a stylet) of the specimens. This condition was considered to be the decalcification endpoint and was accomplished in 24 days for EDTA and 24 h for ETDA solution.

The Efficacy of Decalcification Agents

The analysis of decalcification efficacy was performed at three aleatory intervals obtained by dividing the total decalcification time (endpoint time) into 0–6, 6–12, and 12–24 h for ETDA solution, or days for EDTA solution. Each sample was individually immersed in each solution for the scheduled time and then transferred to another tube containing and equal volume of solution (9 mL). After each solution change, decalcification efficacy was assessed on the basis of the variation in sample mass, optical density of radiographic image, and the calcium concentration remaining in the solutions (residual Ca^{2+} concentration). Each method is described in detail below.

Variation in Sample Mass

The samples were weighed on a precision scale (Gehaka® model BG 200, São Paulo Brazil) immediately before being subjected to the decalcification process and at the time points scheduled for each decalcifying agent.

Optical Density of the Radiographic Image

Each femur sample was radiographed four times. The femurs were radiographed immediately before being subjected to the decalcification process, again, immediately after each of the two solution changes, and finally, at the decalcification endpoint. Radiography is considered an efficient method for the determination of whether or not calcium was fully removed in a given sample (Carson and Christa, 2009).

All radiographic images were obtained using a single radiography pattern, with anteroposterior incidence using the same Rx apparatus (Super M-80 Philips®) with digital development. An aluminum scale with 12 steps of known thickness was used as the densitometry reference and radiographed together with each sample (Fig. 1).

The radiographic images were analyzed using a tone intensity histogram in the luminosity channel of the Adobe Photoshop® software (version CS5). In this histogram, the horizontal axis represents the intensity values which range from the darkest (0 value located on the left in the histogram) to the lightest (255 value, located on the right). The number of pixels is represented on the vertical axis (Fig. 2). Thus, an image with low values on the *x* axis of the histogram has details concentrated in shadows, whereas when these values are high the image will have highlighted details (denser bone tissue); similarly, intermediate values indicate that the details are concentrated in medium tones (Oliveira et al., 2006; Watanabe et al., 2006).

After the determination of the mean value of luminous intensity of each step on the aluminum scale (Fig. 1), the total area of each femur was selected with

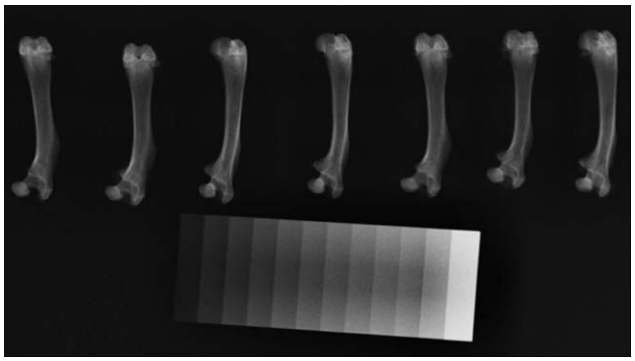


Fig. 1. Radiographs of femurs and the reference of densitometry showing the lower (darker), and the higher (lighter) thickness.

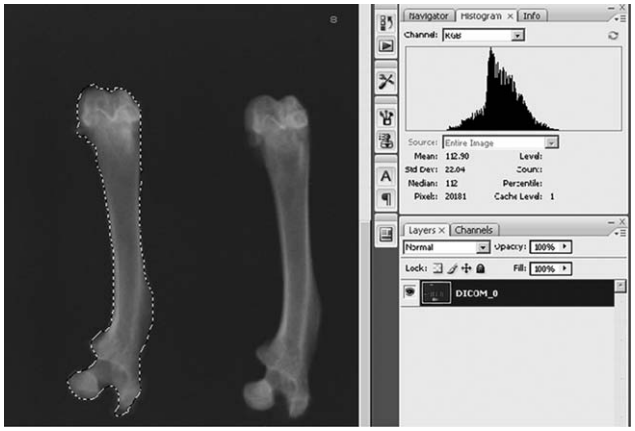


Fig. 2. Image of a femur selected for the determination of optical density (**A**). By selecting the area, the optical density of the sample is determined, and transformed into a histogram. The left side of the histogram shows lower luminous intensity values (darker); greater luminous intensity values are on the right side. The mean value of luminosity obtained in the sample selection is used to calculate the optical density (**B**).

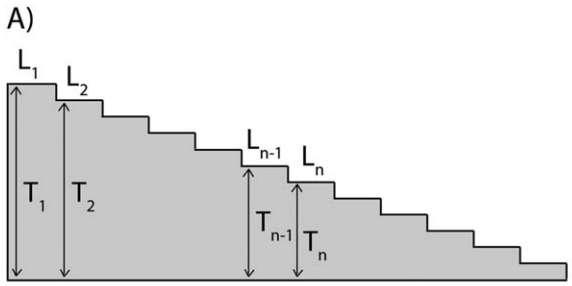
the aid of the selection tool of the Adobe Photoshop® software and the mean value of luminous intensity of the selected area was obtained (Fig. 2).

Each step of the aluminum scale is of known thickness (T) and corresponds to a mean luminosity value (L). The selected bone region will have a mean luminosity value (LO) always between two L values of the reference. Thus it was possible to determine bone density (BD) in millimeters of aluminum by applying the direct proportion of the terms described earlier (Cecim, 2007; Fig. 3).

For the comparison of the different decalcification times, the optical density values in millimeters of aluminum was reported as the percentage of the mean of the values obtained before decalcification (predecalcification, PD). The mean PD value was taken as 100%.

Determination of Residual Ca^{2+} Concentration

The solutions remaining after femur sample decalcification at the different time points were analyzed for the determination of residual Ca^{2+} using a Shimadzu® AA680 (Japan) atomic absorption and emission spectrometer. This chemical test for the determination of



B)

Reference Step	T (mm)
L1	8.0
L2	6.0
L3	5.0
L4	4.5
L5	4.0
L6	3.5
L7	3.0
L8	2.5
L9	2.0
L10	1.5
L11	1.0
L12	0.5

C)

$$BD = T_n - \frac{(T_n - T_{n-1})(L_n - BL)}{L_n - L_{n-1}}$$

Fig. 3. **A**: Optical density from densitometric reference aluminum. **B**: In thicknesses, in millimeters (mm), of each reference step of the aluminum scale. **C**: The equation for determination of optical density is shown in. t, thickness of each reference step; L, mean luminosity of each reference step; BL, mean luminosity of the bone region chosen; BD, bone density in mm.

the presence of calcium in the decalcifying solution is considered to be a precise method for the evaluation of decalcification (An and Martin, 2003).

Role of Decalcification Process on Tissue Morphology and Antigenicity

Histological analysis. The femur samples decalcified with EDTA or ETDA were subjected to a dehydration process starting with a 70% ethanol solution for 24 h, followed by immersion for 1 h in each one of the following solutions: 80, 90, and 100% ethanol. Next, samples were subjected to the diaphanization with 3 Xylene (Synth®) baths (for 1 h), followed by three passages in paraffin wax of 2 h each (Paraffin I, Paraffin II, and Paraffin III). Finally, the paraffin inclusion was performed and 5 μm sections were obtained with a microtome (Leica® RM2145 - Germany). The sections were then stained with H&E for the assessment of the degree of tissue preservation.

Immunohistochemistry Method for Tissue Antigenicity Analysis

Immunohistochemistry was applied to 2 μm sections of the femur samples in order to assess the preservation of tissue antigenicity. The sections were deparaffinized in a decreasing alcohol series (100%, 95%, 70%, 50%, 30%). Next, antigen recovery was performed, with the samples being immersed in sodium citrate

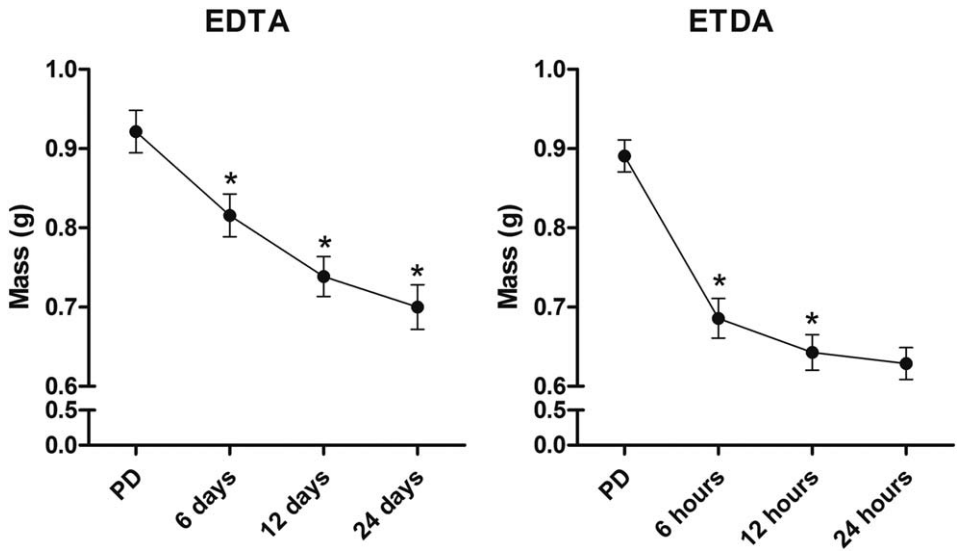


Fig. 4. Mass variation profile of femurs subjected to different times of decalcification with the EDTA or ETDA solution. Abscissa: times of decalcification when the femur mass was measured (PD=pre-decalcification); Ordinate: mass values in grams (g). Data are reported as mean ± SEM ($n = 7$). All points differed from PD values; $^*P < 0.05$, paired t test.

buffer, pH 6.0, and incubated at 90°C for 30 min. The samples were allowed to cool outside the bath and then washed three times for 5 min each in 0.1 M PBS, pH 7.4. Endogenous peroxidase activity was blocked with hydrogen peroxide (Merke®), 0.3% in 0.1 M PBS for 10 min. After three washes (5 min each) in 0.1 M PBS, the sections were subjected to blockade of non-specific sites with 1% bovine serum albumin (BSA, Sigma-Aldrich®), and 1% chicken serum (Vector Laboratories®) in 0.1 M PBS. The sections were then washed once in 0.1 M PBS for 5 min and incubated with 1 : 100 anti-rabbit osteocalcin primary antibody (Santa Cruz®) in 0.1 M PBS for 24 h in a humidified chamber. Three washes of 5 min each in 0.1 M PBS were carried out before the sections were incubated with a biotinylated secondary antibody for 90 min (Anti-sheep IgG, Vectastain, Vector Laboratories®) at 1 : 400 concentration, diluted in 0.1 M PBS. Then, three washes (5 min each) in 0.1 M in 0.1 m PBS were performed, followed by incubation with the Avidin-Biotin-Peroxidase complex (Vectastain solutions A and B, Vector Laboratories®) for 2 h, and two additional washes (5 min each in 0.1 m PBS). Then, osteocalcin immunoreactivity was revealed by the colorimetric method by the addition of 3'3'-diaminobezidine tetrachloride (DAB, Sigma-Aldrich®), and 0.3% H_2O_2 in 0.1 M TBS for 10 min. Finally, the sections were cleared in increasing alcohol concentrations (75%, 95%, 100%), mounted with Entellan (Merck®) and a coverslip.

Statistical Analysis

Data regarding the analysis of variation of femur mass, radiographs optical density, and residual Ca^{2+} concentration were analyzed by paired t test at all-time points compared to the PD condition, in each decalcifier solution group. The unpaired t test was used to analyze the percentage of change from total variation in femur mass. Data are reported as mean ± SEM. In all cases, the level of significance was set at $P < 0.05$.

RESULTS

Femur Samples Mass

Both decalcifying solutions significantly reduced the mass of the femurs compared to PD condition at all-time points (paired t-test; EDTA 6 days × PD $t = 9.65$, $P < 0.001$; 12 days × PD $t = 16.43$, $P < 0.001$; 24 days × PD $t = 33.96$, $P < 0.001$; ETDA 6 h × PD $t = 12.89$, $P < 0.001$; 12 h × PD $t = 15.64$, $P < 0.001$; 24 h × PD $t = 16.51$, $P < 0.001$; Fig. 4).

We found a similar degree of total decalcification with both EDTA and ETDA solutions at the decalcification endpoint. After the total decalcification time (24 days for EDTA or 24 h for ETDA) the EDTA solution reduced femur mass by 24.15 ± 0.99% and the ETDA solution reduced it by 29.40 ± 1.70%. However, of the total femur mass lost throughout the decalcification process, 47.16 ± 3.6% was lost during the first time interval when EDTA was used and 77.92 ± 2.27% when ETDA was used. For the other time intervals of decalcification, the EDTA and ETDA solutions reduced the femur mass by 35.08 ± 2.72% and 16.78 ± 2.16 % in the second interval, and by 17.77 ± 3.28% and 5.30 ± 1.63, respectively, in the last interval. At any of these time points analyzed, there was a statistically significant difference between the EDTA and ETDA groups (unpaired t-test: 0–6 days EDTA × 0–6 hours ETDA, t12 = 2.840, $P = 0.0149$; 6–12 days EDTA × 6–12 h ETDA, t12 = 5.839, $P < 0.0001$; 12–24 days EDTA × 12–24 h ETDA, t12 = 3.405, $P = 0.0052$; Fig. 5).

Optical Density of the Radiographic Image

Both EDTA and ETDA significantly decreased the optical density of the images of the femurs in all time points evaluated (paired t-test; EDTA 6 days × PD $t = 18.00$, $P < 0.001$; 12 days × PD $t = 32.21$, $P < 0.001$; 24 days × PD $t = 40.45$, $P < 0.001$; ETDA 6 h × PD

$t = 15.54$, $P < 0.001$; 12 h × PD $t = 45.39$, $P < 0.001$; 24 h × PD $t = 40.14$, $P < 0.001$; Fig. 6). At the final decalcification times (EDTA 24 days and ETDA 24 h) the EDTA solution had reduced the optical density by 86.22 ± 0.69% and the ETDA solution had reduced it by 76.43 ± 0.43%.

The Residual Ca^{2+} Concentration

Both the EDTA and ETDA solutions removed expressive quantities of Ca^{2+} from the femur samples as measured in the decalcifying solution, as early as during the first time interval of decalcification (EDTA: 9.16 ± 0.43 mg/L; ETDA: 7.74 ± 0.29 mg/L). Additionally, there was significant reduction of Ca^{2+} concentration in the decalcifying solution in the other times analyzed (paired t-test; EDTA 6 × 12 days $t = 10.71$, $P < 0.001$; 6 × 24 days $t = 22.14$, $P < 0.001$; 12 × 24 days $t = 30.39$, $P < 0.001$; ETDA 6 × 12 days $t = 24.77$, $P < 0.001$; 6 × 24 days $t = 26.66$, $P < 0.001$; 12 × 24 days $t = 12.18$; Fig. 7).

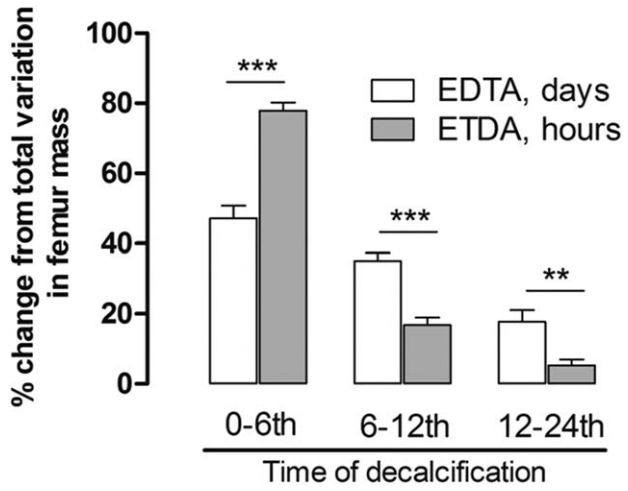


Fig. 5. The analyses of mass variation profile of femurs subjected to three times of decalcification with EDTA or ETDA solution. Abscissa: time of decalcification divided into three intervals: 0–6, 6–12, and 12–24 days for EDTA and 0–6, 6–12, and 12–24 h for ETDA. Ordinate: percentages of the total variation of femur mass. Data are reported as mean ± SEM ($n = 7$). $^{**}P < 0.01$, $^{***}P < 0.001$, unpaired t-test.

Role of Decalcification Process on Tissue Morphology and Antigenicity

Histological analysis with H&E staining showed that both decalcifying solutions maintained good tissue integrity at the end of the decalcification process. The sections showed good preservation of the tissue structure and of the cellular elements (Fig. 8). In addition, tissues decalcified with ETDA presented greater color contrast, especially in the region of the growth plate, probably as a function of the acid characteristic of the ETDA solution.

Immunohistochemistry revealed preservation of tissue antigenicity at least for osteocalcin with both decalcifying solutions. Antigenic labeling for osteocalcin was present in the chondrocytes, in compact bone tissue and in bone marrow and was a little more intense in tissues decalcified with ETDA. When only the primary antibody was absent there was no labeling in these tissues (Fig. 9).

DISCUSSION

In this study, we compared the profile of the decalcifying solution ETDA to that of EDTA, a decalcifying agent that has been long used in histotechnology.

In a first stage, we determined the decalcification endpoint for each solution determined by the sample folding and probing (An and Martin, 2003). We observed that the ETDA solution required a much shorter time (24 h) for appropriate sample demineralization than the EDTA solution (24 days). This result was expected since the ETDA solution has acid characteristics and its low pH dissolves hydroxyapatite and removes calcium from the mineral/organic matrix in a rapid and efficient manner (An and Martin, 2003). In

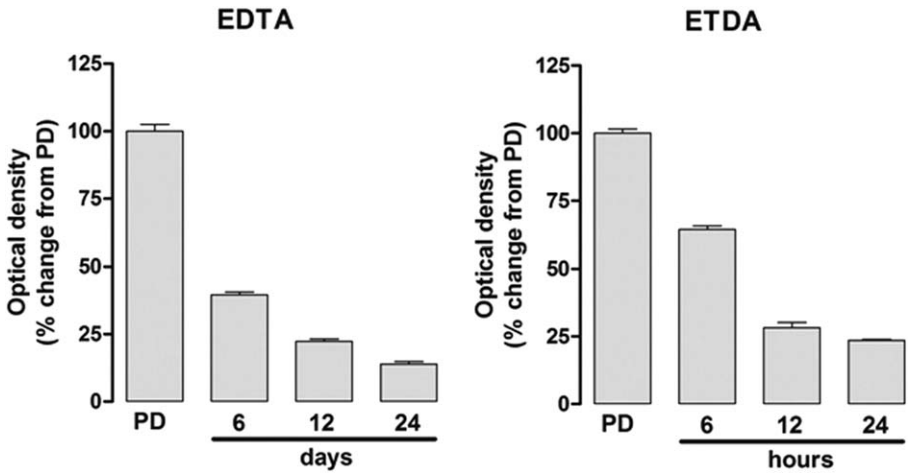


Fig. 6. Optical density variation profile of femurs subjected to different times of decalcification with the EDTA or ETDA solution. Abscissa: time of decalcification when the radiographic images of the femurs were obtained; Ordinate: optical density values in mm aluminum reported as percentage of the mean values obtained before decalcification (PD, predecalcification). The mean PD value was assumed to be 100%. Data are reported as mean ± SEM ($n = 7$). All groups differed from one another ($P < 0.05$, paired t test), with exception of ETDA 12 × 24 h.

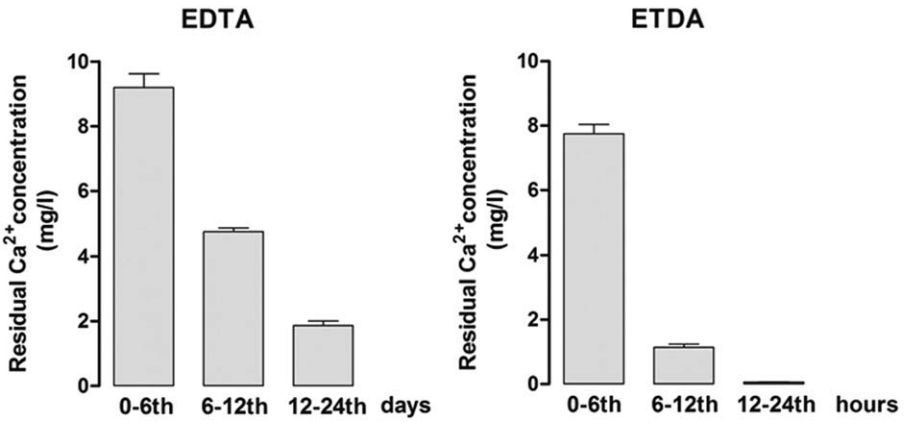


Fig. 7. Variation in the Ca^{2+} concentration of decalcifier solutions. Abscissa: time of decalcification when the Ca^{+2} concentrations in the solution were determined. Ordinate: Ca^{+2} concentration (mg/L) in the decalcifying solution. Data are reported as mean ± SEM ($n = 7$). All groups differed from one another ($P < 0.05$, paired t test).

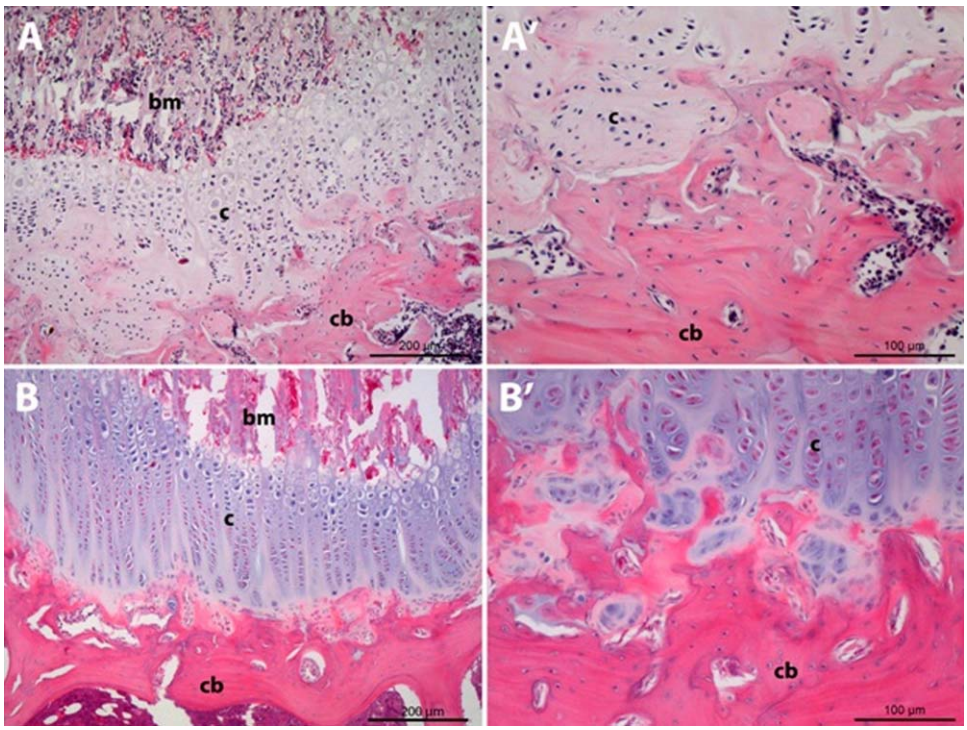


Fig. 8. Histological section (H&E) from femoral epiphysis of rat decalcified with EDTA (A and A') or ETDA (B and B'). (c) chondrocytes, (cb) compact bone, and (bm) bone marrow. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

contrast, EDTA is a chelating agent that binds to Ca^{2+} in the outer layer of the hydroxyapatite crystal, reducing the size of the crystal during the decalcification process. This process can be very slow compared with the acid decalcification profile.

Precise determination of the decalcification endpoint is critical to optimal staining, and higher-level analyses. Ideally, samples should be taken from the decalcifier as soon as all calcium has been removed from the tissue to minimize exposure of the specimens to this agent. Over-decalcification, particularly with the strong acid decalcifiers, spoils the staining and can cause maceration of the softer tissue elements. On the other hand, specimens that are incompletely decalcified may be difficult or impossible to section. Probing or bending is generally the most commonly used method for endpoint determination, and it is adequate for determining when a sample is ready to be sectioned (An and Martin, 2003).

To evaluate the decalcification efficacy of each decalcifying solution we divided the total decalcification time into three aleatory sequential intervals comprising 6 h or days, each, respectively, for ETDA and EDTA. During

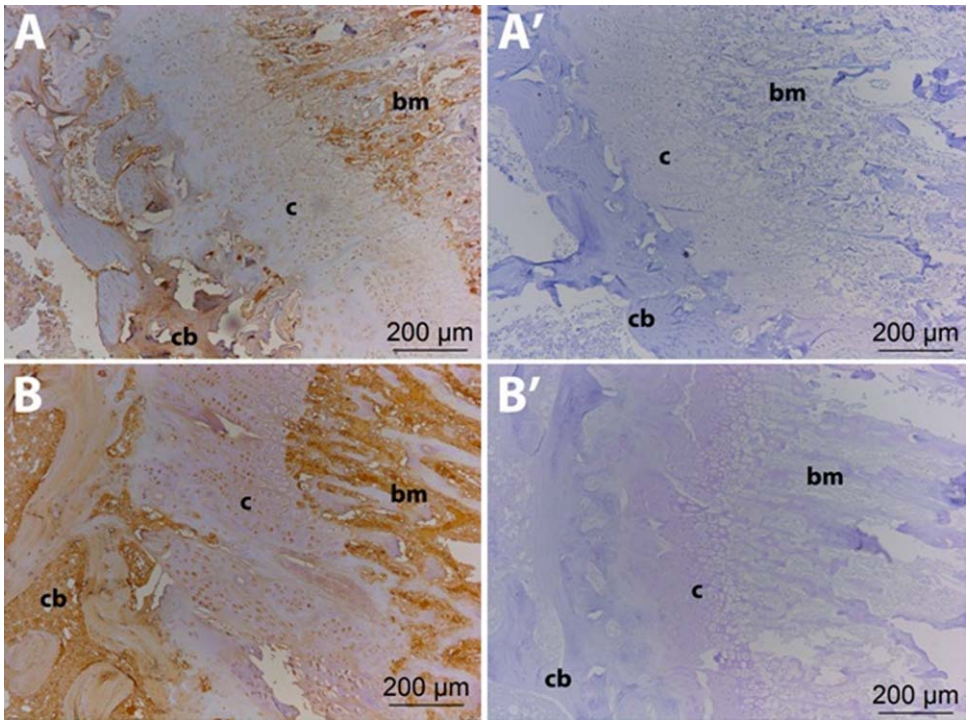


Fig. 9. Immunoreactivity for osteocalcin in femoral epiphysis of *Wistar* rat. Osteocalcin immunoreactivity is shown in (**A**) and (**B**) for samples decalcified with EDTA and ETDA, respectively. In (A') is a negative control for samples decalcified with EDTA and in (B') for ETDA. (c) Chondrocytes, (cb) compact bone, and (bm) bone marrow. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

these intervals, the evaluation of decalcification status included the measuring of sample mass and density, and the analyses of residual Ca^{2+} present in the decalcifying solution.

Analysis of the variation of sample mass revealed that, at the end of the decalcification process both solutions reduced this mass at similar proportions (about 24% for EDTA and 29% for ETDA). The evaluation of each solution profile showed that almost 80% of the total mass lost throughout the decalcification process occurred during the first time interval with the use of the ETDA solution (first 6 h) and a little less than 50% occurred with the use of EDTA (first 6 days).

Evaluation of the amount of residual Ca^{2+} in the decalcifying solution showed that both ETDA and EDTA agents were much more efficient during the first time interval of contact with sample. In the second decalcification interval there was a drastic reduction of the quantity of Ca^{2+} removed from the samples, especially when ETDA was used. Similar results were seen in the last interval, in which the new ETDA solution removed negligible amounts of Ca^{2+} from the sample. This slight difference between the two agents may be easily explained by the longer time intervals scheduled for the EDTA. It is fully expected that greater residual Ca^{2+} deposit be measured in the first intervals when the samples have total Ca^{2+}contents. Regarding the optical density of the radiographic images we found that, at the end of the entire process, both solutions had reduced this density in a similar manner (EDTA by about 86% in 24 days, and ETDA by about 76% in 24 h). This evaluation method of decalcification is efficient, and takes the advantage of its economic and easy reproducible profile. Furthermore, optical densitometry is a technique that is becoming outstanding because of the advances in the area of informatics (Lousada et al., 1998).

Histological analysis of femur tissue samples stained with H&E showed that neither decalcifying solution impaired the preservation of the cellular and tissue characteristics after the entire decalcification process. This result is expected for EDTA. This chelating agent binds to calcium ions from the external layer of the apatite crystals. When all calcium ions from this outer layer are removed, they will be replaced by ions from the deeper layers decreasing the crystal size gradually. In this way, an excellent preservation of tissue components are produced (An and Martin, 2003). Similarly, the samples decalcified with ETDA produced well-defined structures, without loss of tissue morphology. Also, no decrease in the ability of nuclei staining was seen which may result from hydrolytic cleavage of the N-glycosidic and phosphate ester bonds of DNA when the specimens are exposed to acid agent (Bancroft and Stevens, 1990).

Similar results for both decalcifying agents were observed regarding the preservation of tissue antigenicity. Osteocalcin expression was substantially detected in chondrocytes, bone marrow, and compact bone. Osteocalcin is the most bone-specific of the noncollagenous bone matrix proteins (Hughes and Aubin, 1998). It is expressed by osteoblasts late in the differentiation process, and it has been reported that osteocytes produce high levels of osteocalcin, even higher

than osteoblasts (Mikuni-Takagaki et al., 1995). The γ-carboxylated form of osteocalcin binds hydroxyapatite and is abundant in bone extracellular matrix. Osteocalcin has become a biochemical marker routinely used clinically to look at bone remodeling (Gundberg, 2003).

In agreement with our results, several studies have shown that decalcification in EDTA maintained the integrity of tissues and did not interfere significantly in the antigenic reactivity of important proteins (Mukai et al., 1986; Mori et al., 1988; Bourque et al., 1993; Frank et al., 1993). Likewise, when we used the ETDA similar results were obtained.

In conclusion, the ETDA decalcifying solution proved to be efficient in the process of bone tissue demineralization. In comparison to the universally accepted EDTA, ETDA shows similar decalcification efficacy with the great advantage of requiring an expressively shorter total time for decalcification. Further, no perceptible cellular damage was detected, and tissue antigenicity was preserved. Thus, the use of this decalcifying solution may improve the research and clinical routines of mineralized tissue processing by drastically reducing the time needed for the execution of this process.

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