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Original Article

Assessment of different decalcifying protocols on Osteopontin and Osteocalcin immunostaining in whole bone specimens of arthritis rat model by confocal immunofluorescence

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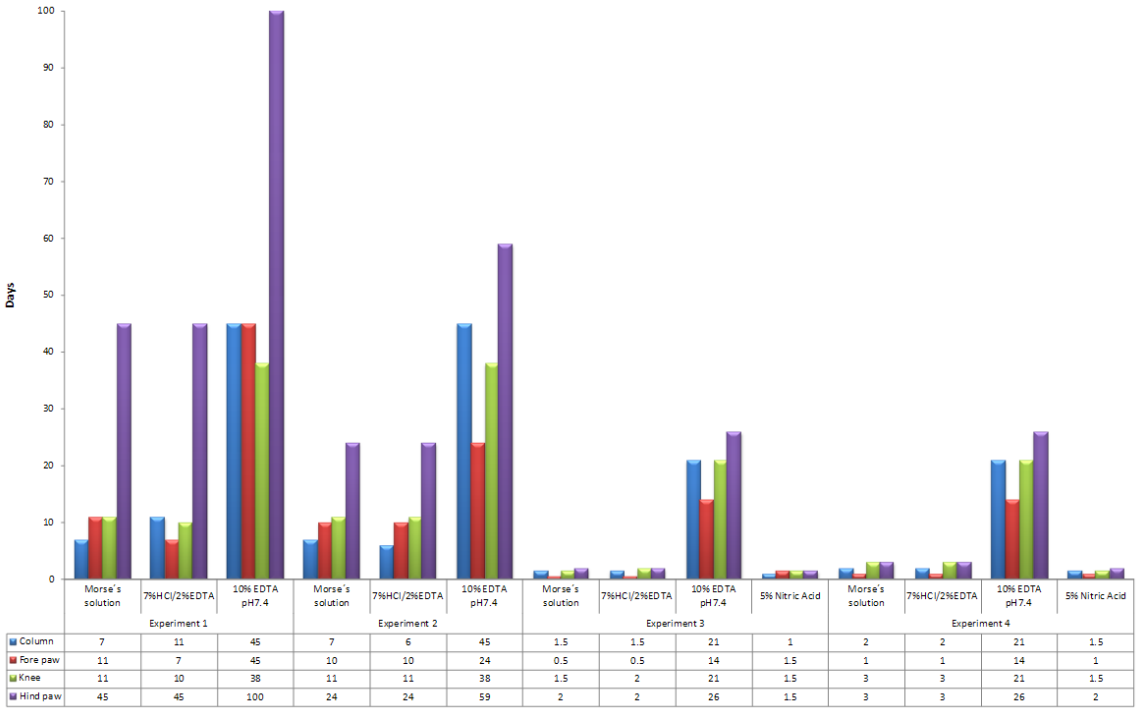
Abstract: Confocal immunofluorescence is a valuable technique for the detection of relevant molecules in the pathogenesis of arthritis in rat models; however, it requires efficient processing of tissues including bone decalcification. The decalcification process must ensure the complete removal of calcium and also a proper preservation of cellular structures and, specially, the antigenicity of the tissue to allow the immunodetection of the molecules of interest. In the present study, we evaluated the effect of four different decalcifying solutions: the Morse´s solution, 10% EDTA (pH 7.4), 7% HCl/2% EDTA and 5% Nitric acid, as well as four different treatments of the tissues (including microwave irradiation) in the processes of decalcification for large pieces of adult rat bones (hind paw, fore paw, knee and column). We assessed the time of decalcification, the easiness of slicing, the morphological preservation and finally, the antigenicity of two different bone proteins (Osteopontin (OPN) and Osteocalcin (OC)) measured by its immunofluorescence intensity under controlled confocal microscopy conditions. Our results showed that the specimen size and the presence of skin are critical factors for the rate of decalcification, and no significant benefit was found if microwave irradiation is applied to the tissue. The comprehensive statistical analysis showed that the optimal solution for the detection of OPN and OC by confocal immunofluorescence is the 5% Nitric Acid, and followed by 10% EDTA (pH 7.4), Ana Morse solution and 7% HCl/2% EDTA.

Keywords: Bone decalcification, arthritis rat model, confocal immunostainig

Introduction

In the study of arthritis, the use of animal models has represented a valid alternative to explore aspects that, for ethical reasons, cannot be studied in humans. Until now, a diversity of rodent models has been described [1, 2]. Currently explored aspects include both, the analysis of Spondyloarthropathies (SpA) inflammatory processes as well as the abnormal bone formation in the entheses in the human SpA [3]. Frequently, the analysis of the pathogenesis relies on histology, where several analytic approaches are suitable. In every case, decalcification represents a potential hurdle to the permanence of the actors involved in the complex picture of the pathogenic process; furthermore the analysis is enriched if the visualization of the entire joint within the slide is possible because it allows locating the specific cellular subtype, protein or nucleic acid within the articular geography.

The detection of relevant molecules within the arthritis pathogenesis can be achieved by several techniques including: the detection of this markers by immunohistochemistry in cells isolated from synovial fluid [4-6] and the detection of RNA expression by Microarray analysis [7] or in situ RNA hybridization [8]. In the specific case of SpA, the detailed study of osteoproliferation process require the identification of specific bone differentiation markers *in situ*, where it can be demonstrated the coexistence and/or



	Experiment 1			Experiment 2			Experiment 3				Experiment 4			
	Morse's solution	7%HCl/2%EDTA	10% EDTA pH7.4	Morse's solution	7%HCl/2%EDTA	10% EDTA pH7.4	Morse's solution	7%HCl/2%EDTA	10% EDTA pH7.4	5% Nitric Acid	Morse's solution	7%HCl/2%EDTA	10% EDTA pH7.4	5% Nitric Acid
Column	7	11	45	7	6	45	1.5	1.5	21	1	2	2	21	1.5
Fore paw	11	7	45	10	10	24	0.5	0.5	14	1.5	1	1	14	1
Knee	11	10	38	11	11	38	1.5	2	21	1.5	3	3	21	1.5
Hind paw	45	45	100	24	24	59	2	2	26	1.5	3	3	26	2

Figure 1. Time in days taken for decalcification.

the overlapping of inflammation with new bone formation. These assessments require detailed study of bony structures and therefore its decalcification [9].

OPN is expressed by various cell types and is present in several tissues [10]. It is now thought that OPN is involved in normal tissue remodeling processes, such as bone resorption, angiogenesis, tissue injury and wound healing as well as in certain diseases, such as atherosclerosis, restenosis, renal diseases, and tumorigenesis. Also, it has been suggested that that OPN plays a critical role in the pathogenesis of rheumatic disease, particularly in bone resorption [11]. OC is the most abundant non-collagenous bone matrix protein [12], is preferentially expressed by osteoblasts and able to bind calcium ions. The OC has a dual role in bone: regulates bone remodelling by modulating osteoblasts and osteoclast activity and acts as a regulator of bone mineralization. The involvement of OC in bone resorption has also been demonstrated, particularly in the regulation of osteoclast formation and activity [13].

The purpose of decalcification is to destroy the inorganic phase of bone, with the removal of the essential element calcium, in such a way that preserves the organic component sufficiently intact to be handled and sectioned by ordinary methods [14]. However some agents that remove the calcium ions completely and rapidly, inversely affect the properties and may also damage the organic counterpart [15]. Moreover, the bone specimen size is an important factor in the decalcification rates. Histological analysis of bone is usually performed with small specimens [16-19], while decalcifications of whole pieces of rat bone, which also contain other tissues such as muscle and skin, have been little studied.

Performing undecalcified bone histology is technically challenging, particularly with large size specimens. It requires variations in technique from those used in standard paraffin embedded histology [20]. There are several strategies to decalcify tissues to allow its slicing into traditional microtomes, in every case a potent chemical reaction is induced that progressively remove the hydroxyapatite crystals softening the tissue, this process -decalcification- is defined in its duration by a series of chemical factors and for most cases is measured in days to weeks depending among other factors in the size of the specimen.

Table 1. Decalcifying solutions scores as measurement of ease of sectioning, morphological preservation and tissue quality after antigen retrieval (AR)

Experiment		Ease of sectioning	H&E Morphological Evaluation								Tissue quality after AR	Total score
			HTS	STS	STA	STCS	STNS	BM	C	Total		
1	Morse´s solution	2*	3	2	1	2	3	2	3	16	NT	18
	7% HCl/2% EDTA	2*	3	2	2	3	3	3	3	19	NT	21
	10% EDTA (pH 7.4)	1*,†	2	2	2	2	2	2	2	14	NT	15
2	Morse´s solution	3*	3	2	2	3	3	2	3	18	NT	21
	7% HCl/2% EDTA	2*	3	2	2	2	2	2	3	16	NT	18
	10% EDTA (pH 7.4)	1*,†	2	2	2	2	3	2	2	15	NT	16
3	Morse´s solution	3	3	3	3	3	3	3	3	21	2	26
	7% HCl/2% EDTA	3	4	3	3	3	3	3	4	23	2	28
	10% EDTA (pH 7.4)	4	4	3	4	4	4	4	4	27	4	35
	5% Nitric Acid	4	3	2	3	3	3	3	3	20	3	27
4	Morse´s solution	3	4	2	2	3	3	3	3	20	2	25
	7% HCl/2% EDTA	3	3	3	3	3	3	3	4	22	2	27
	10% EDTA (pH 7.4)	4	3	3	3	4	4	4	4	25	4	33
	5% Nitric Acid	3	4	3	2	3	3	3	3	21	3	27

HTS: hard tissue staining, STS: soft tissue shrinkage, STA: soft tissue attachment, STCS: soft tissue cytoplasm staining, STNS: soft tissue nuclear staining, BM: bone marrow, C: cortical. NT: Non tested. *Specimens only possible to cut immediately after the paraffin embedding and blocking, not possible to cut days after. †The hind paws were impossible to cut in the microtome.

Several methods to decalcification have been described in the literature. One of the most commonly used decalcifying agent is the ethylenediaminetetraacetic acid (EDTA), whose chelating action promotes tissue decalcification, while preserving its structure; this method, however is time-consuming and it depends upon the specimen size [21]. Also, both strong and weak acids have been reported as decalcification agents; 5% Nitric acid is commonly used [8, 9, 21] and Anna Morse solution (50% formic acid and 20% sodium citrate) apparently scores good results for a short time decalcification [8, 16, 22]. Some variations in EDTA decalcification protocols [8] as well as a combination of strong acids and chelator [23] have been proposed in order to accelerate and improve the process. Moreover, physics methods have been added to chemical solution to increase the rate of decalcification, one of the most reported method is the microwave irradiation [24-26] who has been used in diagnosis laboratories with notable time reduction.

In order to find the most suitable decalcifying agent to use in our SpA studies, we present the comparative evaluation of four different decalcifying agents and the influence of microwave irradiation in regard to the rate of decalcification for large pieces of adult rat bones (hind paw, fore paw, knee and column) by evaluating the time from fixation to decalcification, ease of slicing, morphological preservation under the hematoxylin and eosin (H&E) stain and finally antigenicity measured as immunofluorescence intensity under controlled confocal microscopy for 2 different bone proteins (OPN and OC).

Materials and methods

Animals and tissue preparation

Ten adult male Wistar rats (average body weight of 250 g) were sacrificed by isoflurane inhalation. The hind paws, fore paws, knees and column were removed and placed in 10% buffered formalin for 24 hours. Specimens were rinsed in phosphate-buffered saline (PBS) and separated in four experiments. Experiment 1: whole specimens with skin (hind and fore paws) were decalcified with Morse´s solution, 10% EDTA (pH 7.4) and 7% HCl/2% EDTA; Experiment 2: whole specimens without skin were decalcified with Morse´s solution, 10% EDTA (pH 7.4) and 7% HCl/2% EDTA; Experiment 3: whole specimens without skin were decalcified with Morse´s solution, 10% EDTA (pH 7.4), 7% HCl/2% EDTA and 5% Nitric acid, were irradiated 40 seconds on microwave and were dissected with a sagittal cut in two pieces according the decalcifying process allow it; Experiment 4:

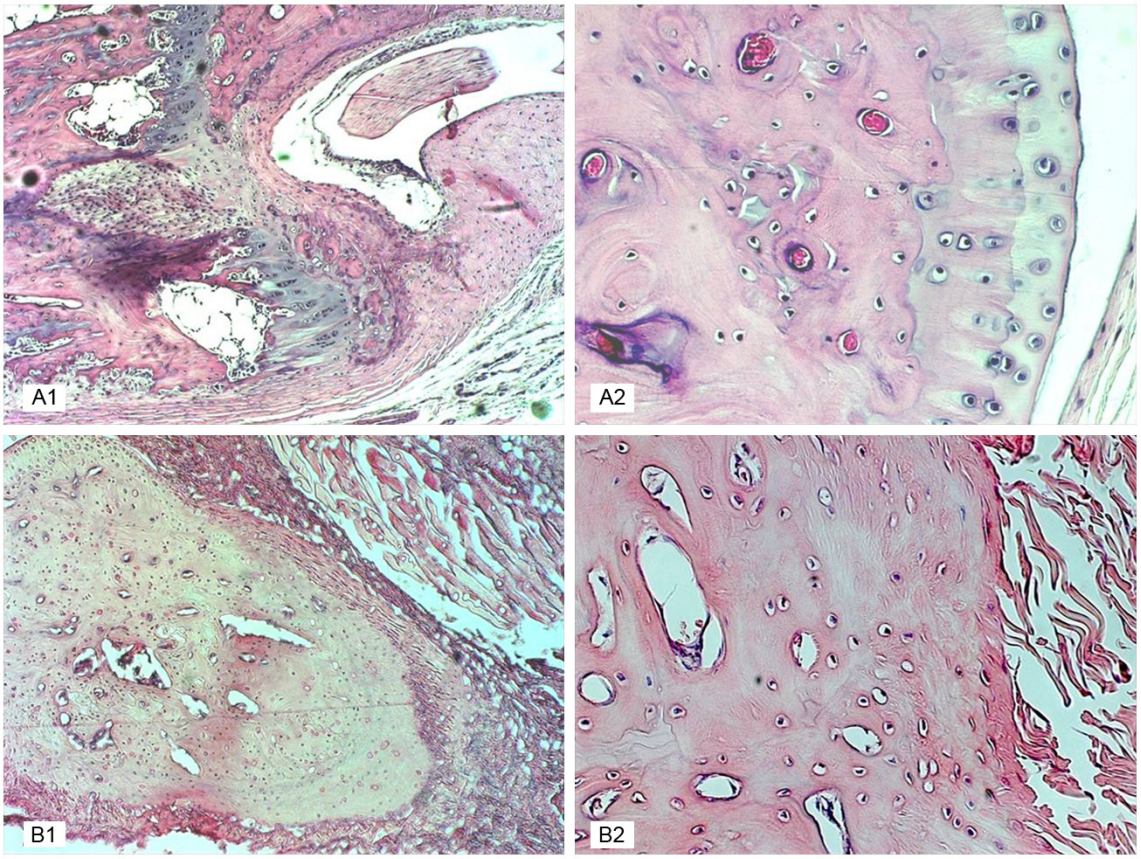


Figure 2. H&E staining of fore paw A) decalcified with 10% EDTA in experiment 3 (highest score) and B) decalcified with Morse's Solution in experiment 1 (lowest score). Magnification: x 100 (1), x 400 (2).

the same condition of experiment 3 without the application of microwave irradiation.

Decalcification was performed at 4°C. 5% Nitric Acid, Morse´s and 7% HCl/2% EDTA solutions were replaced daily and 10% EDTA (pH 7.4) was replaced weekly. The decalcification process and the end point were assessed whit a surgical blade and radiographically wherein the opacity suggested incomplete decalcification.

The specimens were washed in PBS and then embedded in paraffin. The embedding process was carried out in the Microm STP 120 Spin Tissue Processor (Thermo Scientific) which comprised one immersion in 70%, 80%, 96% ethanol (90 minutes each), three immersion in absolute ethanol (60 minutes each), two immersion in xylol (90 minutes each) and two immersion in liquid paraffin at 60°C (120 minutes each). Sections of 5 μm thickness were obtained with a microtome (HM 325 Microm) and placed on adhesive-coated glass slides (3-aminopropyltiethoxysilan, Sigma-Aldrich) to avoid their detachment during immunofluorescence processing.

Immunofluorescence

Sections were deparaffinized in two changes of xylene and dehydrated in descending concentrations of ethanol (100%, 96%, 80%, 70%, and 50%) until water. Antigen retrieval was performed with 0.001 M EDTA at 80°C, then the slides were permeabilized with 0.2% Trition-X100 (Sigma-Aldrich). After blocking with 10% BFS/5% milk for 1 h at RT in humidified chamber, they were incubated with each primary antibody diluted in 1% BFS/PBS at 4°C overnight: rabbit polyclonal anti-Osteocalcin (FL-95) (Santa Cruz Biotechnology, Santa Cruz, CA, USA. sc-30045) and mouse monoclonal anti-OPN (Akm2A1) (Santa Cruz Biotechnology, Inc. sc-21742). After washing in PBS, the sections were incubated with FITC-conjugated secondary antibodies (Santacruz Biotechnology, Inc. sc-23650, sc-2366) at room temperature

Table 2. Semi-quantitative evaluation of OPN and OC detection in bone by immunofluorescence

Experiment		OSTEOPONTIN							OSTEOCALCIN						
		OST	BM	OB	OC	CL	CC	CB	OST	BM	OB	OC	CL	CC	CB
3	5% Nitric Acid	++	++	+++	++	++	++	++	+	+	++	+	-	-	+
	Morse´s solution	+	+	++	+	+	+	+	+	+	++	++	-	-	+
	7% HCl/2% EDTA	+	+	+	+	+	+	+	+	+	+	-	-	-	-
	10% EDTA (pH 7.4)	++	++	++	++	+	++	++	+	+	++	+	-	+	++
4	5% Nitric Acid	++	++	+++	++	++	++	++	+	++	++	+	-	+	+
	Morse´s solution	++	+	++	+	+	+	+	+	++	++	++	-	+	+
	7% HCl/2% EDTA	++	+	++	+	+	+	+	-	+	+	+	-	+	+
	10% EDTA (pH 7.4)	++	++	++	++	+	+	+	+	+	++	++	-	++	++

OST: osteoid, BM: bone marrow, OB: osteoblast, OC: osteoclast, CL: cement line, CC: chondrocyte, CB: chondroblast.

in the dark for 1 h. The absence of primary antibody was used as a negative control. The tissues were visualized and evaluated by epifluorescence microscopy (Axio Imager A1, Carl Zeiss) and photographed with a scanning confocal microscope (LSM 700 Axio Observer, Carl Zeiss). Also a slide of each tissue was stained with H&E and was observed under the optical microscope (Axiostar plus, Carl Zeiss).

Evaluation of decalcifying agent

Decalcification was assessed at four levels during the investigation. First, speed of decalcification was evaluated radiographically and physically (surgical blade); second, ease of sectioning during the microtome cut process; third, the morphological preservation by H&E staining, and fourth, the antigenic preservation by OPN and OC immunofluorescence staining. The ease of sectioning and morphological preservation parameters were graded from 1 to 4 (1-poor, 2-fair, 3-good, and 4-excellent) while the evaluation of antigenic preservation was performed by a semi-quantitative analysis in epifluorescence microscopy, subjectively using +/mild, ++/moderate and +++/strong immunoreactivity. Moreover, a quantitative analysis of fluorescence intensity from the confocal microscopy images was conducted using the ImageJ 1.46r program.

Statistics analysis

One-way ANOVA was used to prove the effect of the four decalcifying solutions in the quantitative analysis of OPN and OC antigenic preservation. Kruskal-Wallis test was performed to determine if there was significant difference between the solutions tested for each of the parameters evaluated along the four experiments. Differences with P <0.05 were interpreted significant.

Results

Time of decalcification

Decalcifying times are shown in **Figure 1**. In all four experiments, the decalcification of the hind paws was the slowest while the fore paws were the fastest. Among the four solutions tested, the decalcification with 10% EDTA pH 7.4 takes the longest time (up to 100 days for hind paws in the experiment 1) and the use of Morse´s solution takes the shorter time (12 hours for fore paws in the experiment 4).

Ease of sectioning

After paraffin embedding, tissues treated under conditions of experiments 1 and 2 showed disadvantage in the microtome cutting (**Table 1**), it was impossible to cut the hind paws with tested solutions in experiment 1 and with 10% EDTA in experiment 2. In experiments 3 and 4, the blocks were cut adequately. After a couple of days, all paraffin embedding specimens from experiments 1 and 2 showed severe signs of dehydration and it was impossible to make additional cuts. The specimens easier to cut were those decalcified with 10% EDTA in experiments 3 and 4, followed by those treated with 5%-Nitric acid.

Morphological preservation

The H&E staining (**Figure 2**) showed that after decalcification process, experiments 3 and 4 allowed the best preservation of cellular structures (**Table 1**) having the highest scores using

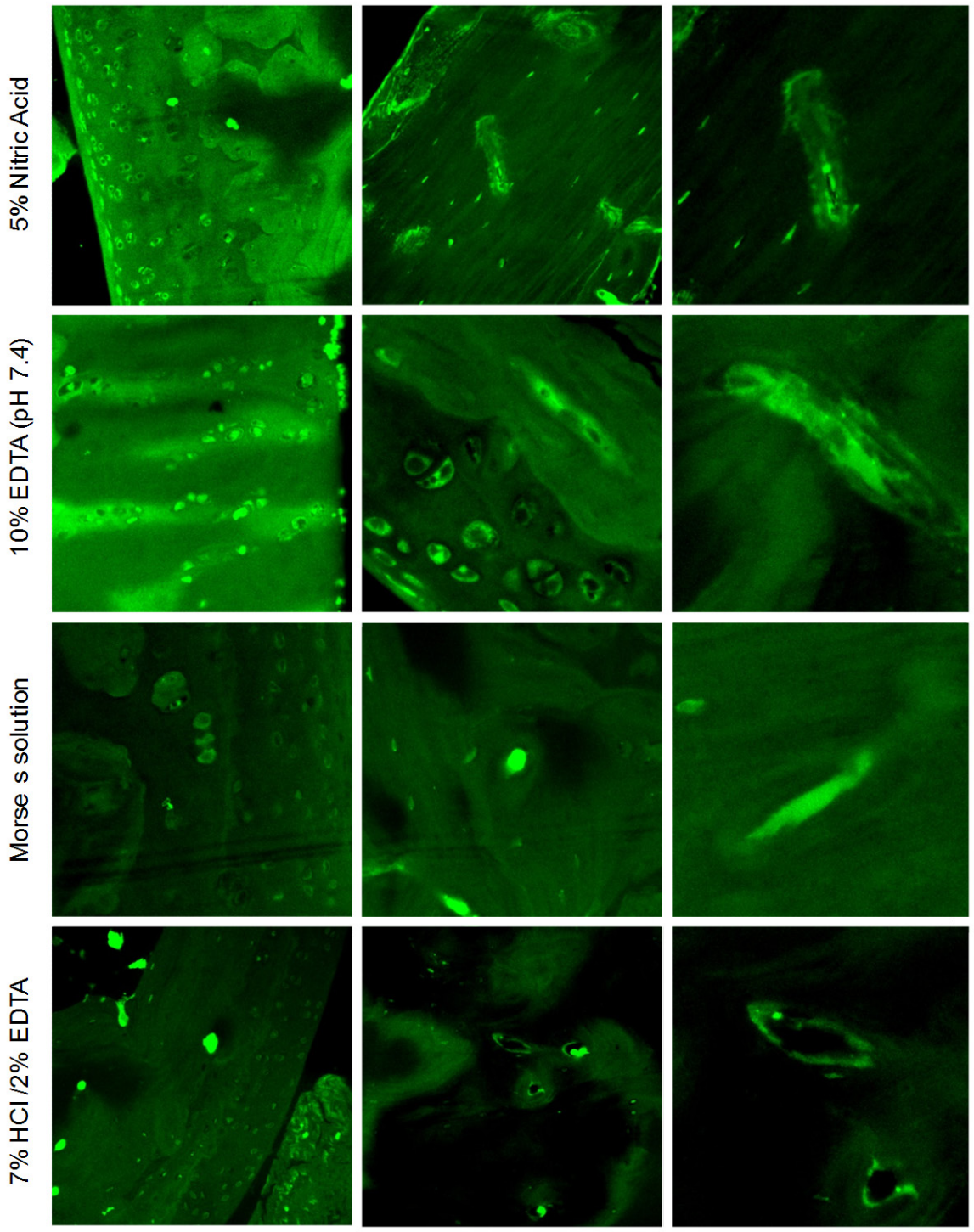


Figure 3. Confocal immunofluorescence detection of OPN in rat bone using 5% Nitric Acid, 10% EDTA (pH 7.4), Morse´s solution or 7% HCl/2% EDTA decalcifying solution.

10% EDTA in such experiments. Morphological observations on the soft tissue shrinkage and attachment as well as bone marrow were the most affected with the conditions of experiments 1 and 2.

Antigenic preservation

The semi-quantitative evaluation results are shown in **Table 2**. The stronger fluorescence intensity signals were obtained decalcifying

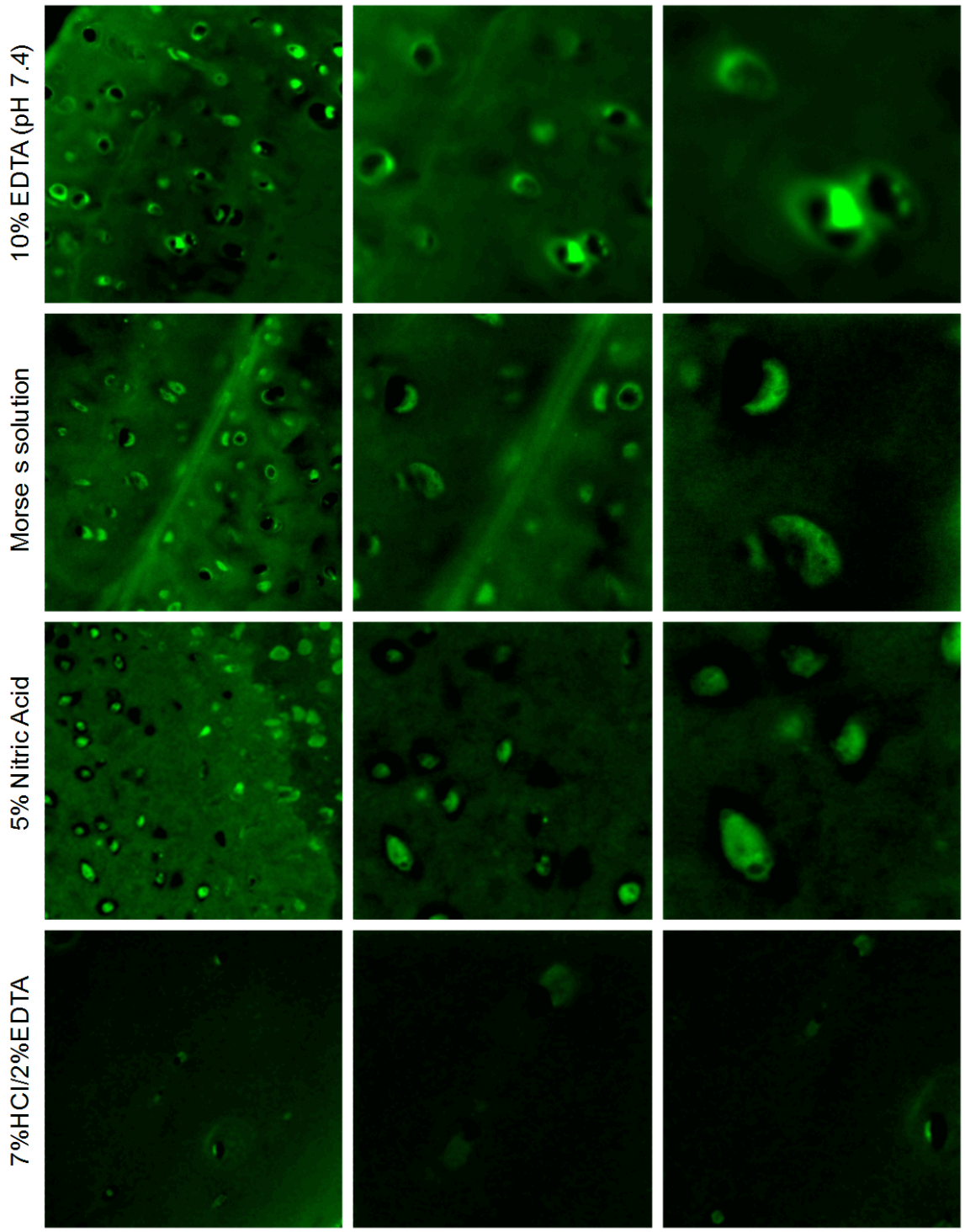
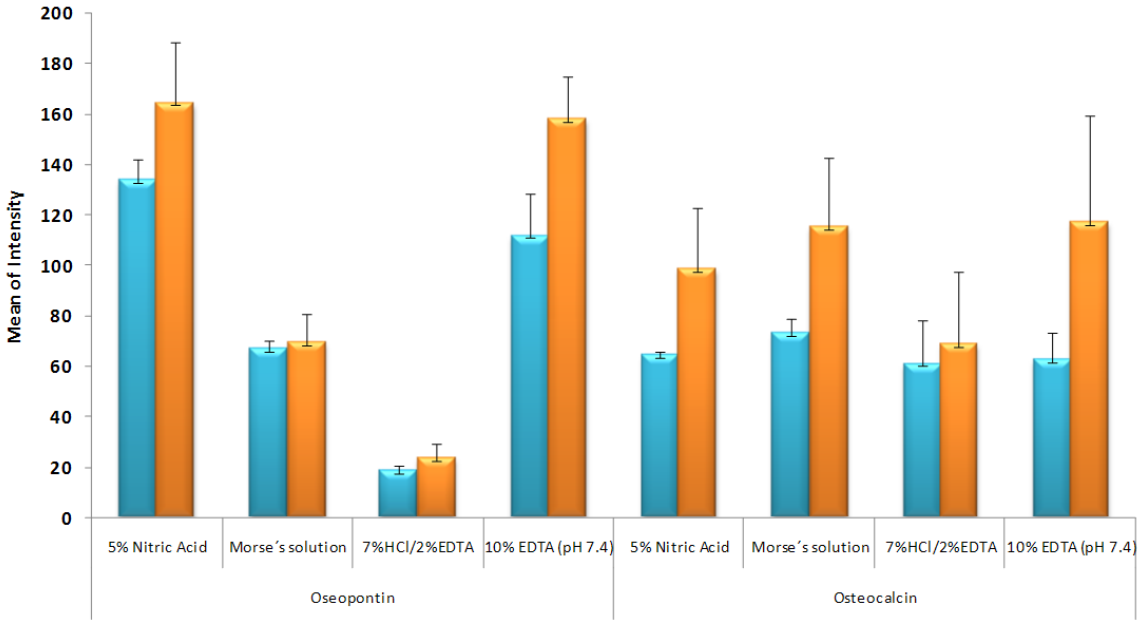


Figure 4. Confocal immunofluorescence detection of OC in rat bone using 5% Nitric Acid, 10% EDTA (pH 7.4), Morse´s solution or 7% HCl/2% EDTA decalcifying solution.

with 5% Nitric Acid and 10% EDTA (pH 7.4) to OPN and OC respectively, while milder signals for both antibodies was observed in tissues decalcified with 7% HCl/2% EDTA. The application of microwave irradiation (experiment 3) decreased the detection intensity of both antibodies compared with unirradiated tissue (experiment 4).



Antibody	Decalcifying solution	Mean	SD	p value	F value	Mean	SD	p value	F value
		Osteoid				Cells			
Oseopontin	5% Nitric Acid	133.57	8.25	0.000	141.05	164.25	24.31	0.00	864.99
	Morse´s solution	66.95	3.34			69.50	11.12		
	7%HCl/2%EDTA	18.73	2.14			23.43	5.70		
	10% EDTA (pH 7.4)	111.75	16.51			157.70	16.90		
Osteocalcin	5% Nitric Acid	64.26	1.41	0.193	1.74	98.54	23.95	0.00	15.76
	Morse´s solution	73.13	5.45			115.23	27.14		
	7%HCl/2%EDTA	61.02	16.91			68.80	28.46		
	10% EDTA (pH 7.4)	62.73	10.34			117.19	41.87		

Figure 5. Quantitative analysis of fluorescence intensity in OPN and OC detection. The table shows the one-way ANOVA results and the graphic bars represent the mean and standard deviation.

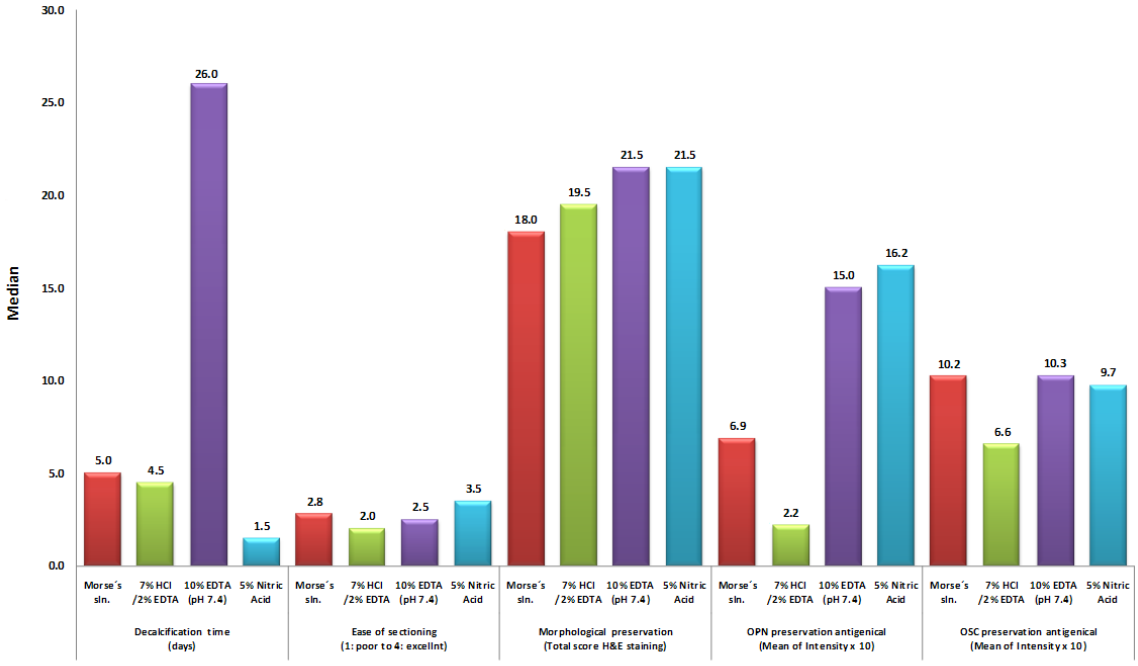
The **Figures 3** and **4** show the detection of OPN and OC in bone by confocal immunofluorescence, and the **Figure 5** the results of statistical analysis of their expression. OPN is better preserved in osteoid and cells using 5% Nitric acid. OC is best detected with 10% EDTA in cells and there was no significant difference between solutions for the detection in osteoid.

The **Figure 6** shows the overall statistical analysis of the four experiments. In decreasing order 5% nitric acid, 10% EDTA, Morse´s solution and 7% HCl/2% EDTA are the solutions that showed better results for the detection of OPN and OC by immunofluorescence.

Discussion

Detection of osteoproliferation markers in spA bone requires standardization of a decalcification protocol that preserves tissue to observe entire joint/entheseal structures and also ensures antigenic preservation, coupled with the anterior, would ideally a should be non-time consuming.

In our work, decalcification times were reduced from experiment 1 to 4 having the longest times in experiment 1 for all specimens. Skin removal resulted in a reduction of about 28% over that time; this can be explained by the increased rate of penetration of the fixative and decalcifying solutions throughout the tissue. Decalcification times reported for experiments 1 and 2 are very high in comparison with other studies in rodent bone, which have been reported times between 7 to 27 days with EDTA, 24 hours to 35 days with Ana Morse solution, and 2 to 7 days with nitric acid [8, 9, 16, 22, 27]; however, in those studies the specimens sizes were smaller than those analyzed here.



Parameter evaluated	Kruskal-Wallis Test										Global result	
	Overall ranking in the four experiments											
	1		2		3		4		H value	p value	Rank	Solution
	Solution	Ave Rank	Solution	Ave Rank	Solution	Ave Rank	Solution	Ave Rank				
Decalcification time	5% Nitric Acid	9.9	Morse´s solution	24.3	7%HCl/4%EDTA	24.4	10% EDTA pH7.4	46.1	31.200	0.000	1	5% Nitric Acid
Ease sectioning	5% Nitric Acid	86.4	10% EDTA pH7.4	54.3	Morse´s solution	52.2	7%HCl/2%EDTA	48.1	16.410	0.001	2	10% EDTA pH7.4
Morphological preservation	5% Nitric Acid	61.7	10% EDTA pH7.4	59.2	7%HCl/2%EDTA	51.6	Morse´s solution	42.3	6.290	0.099	3	Morse´s solution
OPN Antigenic preservation	5% Nitric Acid	150.5	10% EDTA pH7.4	144.8	Morse´s solution	79.5	7%HCl/2%EDTA	27.0	163.670	0.000	4	7%HCl/2%EDTA
OC Antigenic preservation	10% EDTA pH7.4	93.1	Morse´s solution	89.9	5% Nitric Acid	76.3	7%HCl/2%EDTA	30.3	40.910	0.000		

Figure 6. Assessment of different decalcifying solutions on OPN and OC detection in whole rat bone specimens by confocal immunofluorescence. The table shows the Kruskal-Wallis test results and the graphic bars represent the medians of each parameter.

It is well known that the decalcification time depends of the specimen size [23, 28], agreeing with the above, the reduction of time between experiment 2 and 3 (in which the main difference was the smaller sizes by the sagittal cut) was the most significant, with a decrease of about 73% over time. This effect is also clearly observed between specimens analyzed, regardless of the solution used, the forepaws are the most rapidly decalcified while the hind paws are the ones that take longer time.

Under the conditions employed in our experiments, no significant difference in times was observed between experiments 3 and 4, however some studies have shown a reduction of time by using microwave irradiation as a decalcification accelerator [29, 30].

Another interesting variable analyzed was the amount of decalcifying solution used for the experiments, finding a reduction around 90% in solutions spending between experiments 1 and 4, which show us that, besides the reduction on time, the conditions of the experiments 3 and 4 significantly reduce the use of reagents in the laboratory cutting processing costs.

After paraffin embedding, using a process reported for bone [23, 31], severe problems with the tissues from experiments 1 and 2 were observed, it was not possible to cut the hind paws, because it showed a gummy consistency, and even though was possible to cut all other specimens, after a few days the tissues were dehydrated inside the paraffin block, and was impossible to cut them again. The penetration of both, the fixing and decalcifying solutions depends, besides size, on the kind of tissue involved [32, 33] and an inadequate protocol at any of these steps can result in an incorrect hydration and insufficient paraffin penetration, resulting in the impossibility of the cuts or the increase in troubles in the microtome process [28, 34]. This may explain the observed in whole and with skin specimens

(experiments 1 and 2), contrary to what was found with smaller pieces (Experiments 3 and 4) in which the paraffin embedded the entire tissue and cuts were made more easily. Of tested solutions, 5% Nitric acid and 10% EDTA showed to be the decalcifying agents who allow obtain better cuts, while in the most of tissues treated with Morse´s solution a certain level of disintegration was detected during microtome cutting.

Studies of the effect of decalcifying solution over the morphological preservation are controversial [8, 15, 16, 27, 35, 36] and depends largely on the type of tissue and the conditions of fixation and decalcification, so it is difficult to label one as the most appropriate or less adequate solution. In our study, this effect was observed when in an independent way were analyzed the 4 experiments, where the 10% EDTA was the solution that best preserved tissue structures in the experiments 3 and 4; however, this was not the better in experiments 1 and 2. Moreover, putting together the data from the four experiments and performing a global analysis, 5% Nitric acid was the solution that persevere the tissue morphology better.

Although one of the goals of this work was be able to analyze whole pieces of bone, the sagittal cut of the specimens did not affect the microscopic evaluation allowing the observation of structures for our interest, and was essential since it produces the greatest reduction in decalcification times, prevents tissues hydration after paraffin embedding, and makes it easier the microtome cut.

The histological detection of OPN and OC in osteoid, bone marrow, cement line and cells in bone has been reported by several authors [31, 37, 38]. Our results are consistent with those already reported for OPN, however for OC in semi-quantitative analysis there was no significant detection in cement line.

The fluorescent intensity quantitative analysis of images obtained by confocal microscopy and the statistical analysis, showed that there are significant differences in the bone tissue antigenicity for the detection of OPN and OC depending on the decalcifying solution used, and coinciding with semi-quantitative analysis, the solutions that best allow the detection of OPN and OC are 5% Nitric Acid and 10% EDTA (pH 7.4) respectively.

The global analysis of the effect of decalcifying solution throughout the detection process of OPN and OC in whole rat bone specimens shown that the most appropriate solution for the process of detection of these antigens by immunofluorescence is 5% nitric acid, followed by 10% EDTA, then the Morse´s solution and finally 7% HCl/2% EDTA. The decalcifying solution was a variable which significantly affects the decalcification time, ease of sectioning and OPN and OC immunogenicity. However, there was no significant effect of the solution on morphological preservation of decalcified tissues.

EDTA is a decalcifying that, despite taking long time to decalcification, has proven to be one of the best in morphological and antigenic preservation [9, 22, 27, 37]. However according to our results, 5% nitric acid was found to be the most favorable solution for the detection of OPN and OC by immunofluorescence, this solution was the highest ranked in most parameters, offering the shorter decalcification time, the improved ease of cut, as well as good morphological and antigenic preservation. The 10% EDTA (pH 7.4) with the exception of decalcification time, proved to be the second best decalcifying solution for detection of these markers.

Disclosure of conflict of interest

The authors do not have any financial interests to disclose, this paper received no funding from commercial sources and there is no conflict of interests.

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