Histomorphometric analysis of biological responses following the use of pure hydroxyapatite and hydroxyapatite with collagen: a study in dorsal subcutaneous tissue of rats

Análise histomorfométrica das respostas biológicas após o uso de hidroxiapatita pura e hidroxiapatita com colágeno: um estudo no tecido subcutâneo dorsal de ratos

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ABSTRACT
Purpose: The aim of this study was to evaluate the biological responses resulting from the implantation of two types of experimental hydroxyapatite – Pure Ha (HaP) and Ha with collagen (HaCol) and compare them with a third type HaAlobone (commercial) – on dorsal subcutaneous tissue of female rats. Methods: Forty-five animals were used (15 in each group), which were sacrificed 7, 15, and 30 days after operation. The specimens were fixed, stained with hematoxylin and eosin, and then evaluated for inflammatory reactions with a light microscope. Results: The three experimental groups showed a high inflammatory response after 7 days. The inflammatory response was seen to decrease sharply after 15 days. After 30 days, the foreign body reactions were seen to reduce significantly, and an organized collagen tissue was observed. The results showed that the types of hydroxyapatite tested – HaP and HaCol – are biocompatible. When compared with the commercially available hydroxyapatite, these new biomaterials showed similar biocompatibility performance. CLINICAL SIGNIFICANCE: The new hydroxyapatite tested are considered biocompatible.

Keywords: Hydroxyapatites, Materials Testing, In Vitro Techniques.

RESUMO
Objetivo: O objetivo deste estudo foi avaliar as respostas biológicas resultantes da implantação de dois tipos de hidroxiapatita experimental - Ha Puro (HaP) e Ha com colágeno (HaCol) e compará-las com um terceiro tipo de HaAlobone (comercial) - no tecido subcutâneo dorsal de ratos fêmeas. Métodos: Foram utilizados 45 animais (15 em cada grupo), que foram sacrificados 7, 15, e 30 dias após a operação. As amostras foram fixadas, coradas com hematoxilina e eosina, e depois avaliadas para reações inflamatórias com um microscópio de luz. Resultados: Os três grupos experimentais mostraram uma alta resposta inflamatória após 7 dias. A resposta inflamatória diminuiu acentuadamente após 15 dias. Após 30 dias, as reações de corpos estranhos diminuíram.
significativamente, e um tecido colágeno organizado foi observado. Os resultados
mostraram que os tipos de hidroxiapatita testados - HaP e HaCol - são biocompatíveis.
Quando comparados com a hidroxiapatita comercialmente disponível, estes novos
biomateriais mostraram um desempenho de biocompatibilidade semelhante.
SINALIZAÇÃO CLÍNICA: Os novos hidroxiapatitas testados são considerados
biocompatíveis.

**Palavras-chave:** Hidroxiapatitas, Teste de Materiais, Técnicas In Vitro.

**1 INTRODUCTION**

Over 2.2 million bone transplantation procedures are performed annually
worldwide in a variety of fields, including orthopedics, neurosurgery, and dentistry.\(^1\)
Although autologous bone grafts remain the gold standard for bone grafting procedures
due to their superior osteogenic potential, their use is associated with various
complications such as hematoma, soft tissue breakdown, pain, and prolonged recovery
times.\(^2,3\) The concerns of limited supply and donor site complications are still maintained.
Thus, the development of a fully synthetic, readily available, and osteogenic or
osteoc conducive bone substitute as an adjunct to autologous tissue grafts is strongly
encouraged and considered as a great milestone in the clinical field.

Bioresorbable materials as pure hydroxyapatite (HaP) - \(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2\) - have
been clinically applied as bioactive materials and to substitute hard tissues in several
medical specialty fields because of their excellent biocompatibility and
osteoc conducive.\(^4,6\) Porous hydroxyapatite should present the following requirements for
the formation of a suitable osteoc conducive scaffold in bone regeneration:
biocompatibility, osteoconductivity, interconnected porous structures, mechanical
strength, and biodegradability. These factors are required for an appropriate strategy to
deal with a type of bone tissue engineering called "porous osteoconductive scaffold",
which operates in combination with osteoinductive and osteogenic molecules or cells.\(^7,9\)

A material is considered biocompatible when it does not produce harmful
reactions or toxic elements in the tissues around it or adverse systemic responses as the
result of elements, ions, and/or compounds that it releases.\(^10\) In addition, it should be free
of potentially sensitizing agents, which might result in an allergenic response and should
not present any carcinogenic potential.\(^11,12\) Biocompatibility reflects the nature and degree
of interaction between biomaterials and host tissues and is one of the critical concerns in
biomaterials research. Subcutaneous implantation of scaffold is the initial step in order to
determine its biocompatibility. Subcutaneous implantation of hydroxyapatite and other synthetic materials has been widely used in previous studies to examine its biocompatibility.

This research analyzed the biological behavior – biocompatibility - of two types of hydroxyapatite developed by the Chemistry Department of the State University of Ponta Grossa (UEPG), i.e., pure hydroxyapatite (HaP) and hydroxyapatite with collagen. It was compared with a third biomaterial that has been routinely used in Brazil (HaAlobone-commercial). The synthetic biomaterial used in our research (HaP and HaCol) was a nanocrystalline hydroxyapatite (nHaP) with particle size smaller than 50 nm. The results indicated that the materials had been found to be biocompatible.

2 MATERIALS AND METHODS

The research project was approved by the Ethics Committee on Animal Use at UEPG in accordance with Process CEUA 22/2012, Protocol 08258/2012. A pilot study was performed prior to the experiment to determine the ideal anesthetic for the animals, to practice the surgical procedures, and to determine the surgical protocol, including biopsies, to minimize animal suffering.

2.1 OBTAINING AND CHARACTERIZING THE BIOMATERIAL

The hydroxyapatite powder was obtained by the precipitation method, combining $\text{H}_3\text{PO}_4$ (Nuclear P.A. 85%) with $\text{Ca(OH)}_2$ (Vetec, P.A. 95%) at a molar Ca/P ratio of 1.67 in an aqueous medium. After the acid solution was added, the pH was adjusted to 10 with $\text{NH}_4\text{OH}$ (Reatech, P.A. 28%). The precipitate was aged for 76 h, filtered under vacuum, and the resultant precipitate of each sample was dried in an oven at 100 °C for 24 hours. The hydroxyapatite obtained by this method had a nanocomposite structure (particles) and an average below 50 nm, whereas the particle agglomerates had an average that ranged from 6 to 9 micrometers (Figure 1). Other authors used mechanical methods to obtain a similar nanocrystalline hydroxyapatite with crystallite size ranging from 22 nm to 39 nm. In the present research, bovine collagen was added and solubilized in water by the ultrasound method for one hour and then lyophilized (HaCol, Figure 2). The characterization of the third material (HaAlobone®) by scanning electron microscopy with field emission (FEG-SEM) and XRD is shown in figure 3.
2.2 ANALYSIS OF BIOCOMPATIBILITY

This study used 45 female rats, in which the following three different biomaterials were implanted: pure hydroxyapatite (HaP) - UEPG; hydroxyapatite-collagen (HaCol) – UEPG; and commercial hydroxyapatite (Alobone®, Osseocon Biomaterials, Rio de Janeiro, Brazil). The animals were obtained from controlled reproduction at the UEPG vivarium. The rats were of the Rattus norvegicus albinus species and the Wistar variety. The animals were aged around three months, and their body weight ranged from 250 to 300 grams. They were randomly distributed in different groups. The animals were sacrificed (each group, n = 15 - and each of the three materials were implanted in five animals) at 7, 15, and 30 days to verify the biological behaviour of the tissues and cells around the implanted biomaterial.

The surgical procedures were performed at the Oral Pathology Laboratory of the Department of Dentistry at State University of Ponta Grossa (UEPG), and the biopsied specimens were processed and stained at the Municipal Medical Pathology Laboratory of Ponta Grossa.

2.3 SURGICAL PROCEDURE AND POST-OPERATIVE CARE

The animals underwent general intraperitoneal anesthesia (IP) using 100 mg/ml ketamine, (injectable general anesthetic based on 1.16 g hydrochloride ketamine, 10 ml, Ceva Brasil®) by dilution of 3.75 ml of this ketamine solution together with the muscle relaxant xylazine, 100 mg/ml (xylazine, 200mg; 10 ml bottle, veterinary use, imported, Hertape Calier®), at a ratio of 0.5 ml xylazine diluted in 5.75 ml distilled water. Each animal was administered with 0.2 ml per 100 g weight. The use of ketamine, together with xylazine produced anesthesia and sedation of the animals within 30-40 minutes.19

Forty-five rats were used in the experiments, and each one received a submucosal incision for the placement of the biomaterial (on the right side of the animal) and another incision, starting from the first (left side of the animal). The control consisted only of a subcutaneous dorsal avulsion (left side of the animal). The animals were divided into the following groups: (n = 15 for each group): Group 1: pure hydroxyapatite - HaP; Group 2: hydroxyapatite with collagen – HaCol; and Group 3, commercial hydroxyapatite HaAlobone®. The surgical wound on the dorsal area was used to prevent irritation by contact and auto-cannibalism.

After anesthesia, trichotomy was performed using an electrical appliance (a hair/beard trimmer) in the dorsal region as well as antiseptis (topical solution of 10%
PVP, 100 ml flask; Geyer\textsuperscript{\textregistered} manufacturer). The incision and avulsion were performed using Metzenbaum Duflex\textsuperscript{\textregistered} 14 scissors with a blunt tip (SS White, Rio de Janeiro, Brazil). The initial incision was made in the median sagittal line in the middle third of the back, and it measured approximately 15 mm. The avulsion, which was performed on the right side in the same manner as on the left side, was about 18 mm in length and 15 mm width (Figure 4). The animals showed no bleeding or any other systemic condition that might exclude them from the study. A metal-amalgam port (Golgran\textsuperscript{\textregistered}, São Paulo, Brazil) was used for the placement of the biomaterials with a uniform internal depth so that the amount of implanted material in the submucosa was the same for all the animals (0.3 grams). The hydroxyapatite was previously hydrated with saline solution. After the placement of the biomaterials, interrupted sutures were used on the animals’ back with nº 3 black silk thread (Ethicon, Johnson & Johnson, São Paulo, SP, Brazil) so that the healing occurred by first intention.

After the surgical procedures, the animals received analgesic and anti-inflammatory ketoprofen (IP) that was injected in a single dose at a 2 mg/kg ratio, and they were placed in special cages (exposed to low light) to recover from the anesthesia. The temperature in the immediate post-operative environment ranged from 27 to 30 °C to avoid hypothermia. The immediate consumption of water was monitored to prevent dehydration. On the following days, the food was regular, and the administration of water was ad libitum. There was no postoperative death of the animals.

2.4 OBTAINING BIOPSIES

The animals (n = 15 for each period) were sacrificed 7, 15, and 30 days after surgery by an overdose of isoflurane - 240 ml bottle - (inhalation solution indicated in humans for the induction and maintenance of general anesthesia). Under the isoflurane effect, each animal underwent cervical dislocation with a metal bar to confirm euthanasia. Marks of sutures were observed on the back of the animals, which facilitated the removal of the biopsy from an exact location. The biopsies from the animal's back were performed in line with the following routine: trichotomy and incision with Metzenbaum Duflex\textsuperscript{\textregistered}-14 scissors with a blunt tip to remove all the submucosal area with the biomaterial (hydroxyapatite), including the part of the dorsum that only received incision and avulsion (control).
2.5 HISTOTECHNICAL PROCEDURE AND HISTOMORPHOMETRIC ANALYSIS

The biopsies were fixed in 4% formalin that was buffered for 48 hours. The pieces were submitted to the standard method for histotechnical staining - hematoxylin and eosin. The pieces were placed in a paraffin block for the cutting by microtome (approximately 5-7 mm thickness) for both the hydroxyapatite biomaterial (experimental) and the surgical avulsion of the dorsal area (control). The slides were analysed (by a single professional calibrated with the kappa method to assess the level of concordance), and a descriptive and semi-quantitative analysis was performed that considered representative parameters of the repair process such as inflammatory infiltrate, the predominance of certain inflammatory cells and signs of repair to the connective tissue. The numerical correlation references adopted for the presence or absence of those parameters were as follows: connective tissue repair (presence of collagen fibers) and inflammatory infiltrate (predominance of a particular type of inflammatory cell): 0= Absent: no inflammatory cells infiltration. 1= Mild: scattered chronic inflammatory cells without tissue changes. 2= Moderate: focal inflammatory cell infiltration with tissue changes but without necrosis. 3= Severe: severe infiltration of inflammatory cells with or without abscess formation.17

2.6 STATISTICAL ANALYSIS

The data were statistically analyzed using the non-parametric Kruskal–Wallis test with Dunn’s test (for multiple comparisons) to study the response of the subcutaneous tissue to different biomaterials. The statistical analysis was performed using IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 21 for Windows. The observed significance levels, as indicated by an asterisk in the figures, were considered statistically significant at $P < 0.05$. In our research, we used the kappa statistics. In the periodontal literature, the kappa statistics is frequently used to confirm an adequate degree of examiner agreement.20

3 RESULTS

Figure 5 shows the inflammatory response at 7, 15, and 30 days for all the groups, and Figures 6, 7, and 8 illustrate the histopathology of the experimental groups.
3.1 SEVEN DAYS

The results showed stages of moderate to severe inflammation. At seven days, there were significant differences (*) from the control, which did not contain any biomaterial, compared to HaP, HaCol, and HaAlobone, which were the groups that had the highest initial levels of inflammation (Figures 6A, B and C). In all groups, the initial inflammatory response was acute, with a significant presence of neutrophils (PMNs), and exudate was present in some cases. Granulation tissue was clearly observed. However, in 7 days in the control group, acute inflammation was no longer observed.

3.2 FIFTEEN DAYS

At 15 days, there was a decrease in the inflammatory response in all groups, but there was a difference between the control, which did not present inflammation, and all the other experimental groups (Figures 7A, B, and C). The predominant cells in the experimental groups were macrophages. The connective tissue contained bundles of loose collagen fibers; however, new blood vessel formation was observed.

3.3 THIRTY DAYS

At 30 days, there were no differences between the groups. The inflammatory response had significantly reduced, but the presence of macrophages and few multinucleated giant cells were observed. The connective tissue contained dense bundles of organized collagen fibers and fibroblasts were present. There was a non-inflammatory connective tissue capsule in any of the groups (Figures 8A, B, and C). In contrast, in the control group, the regeneration around the surgical wound was complete. In the control side, there was no difference at 7, 15, and 30 days, and the score 0 for inflammation predominated.

After seven days, in all the groups where biomaterials were included, there was an increase in the scores relating to inflammatory response. At 15 days, the scores were reduced (no significant difference), and they remained at the same level between days 15 and 30. However, there was a significant difference between the scores at seven and 30 days because the biomaterials induced intense inflammatory responses in the early days and were reduced entirely at 30 days, demonstrating the biocompatibility of the biomaterials tested.

In all the samples, the materials under study were surrounded by a non-inflammatory connective tissue capsule. At day 15, the fibrous capsule with regularly
oriented collagen fibers appeared to be very thin and not fully defined (Figure 4). However, the thickness of the capsule increased over time. At day 30, the biomaterial was surrounded by denser connective tissue with a defined and compact cellular organization (Figure 5). At this stage, the material showed signs of reabsorption due to a reduction in its presence in the tissue (Figure 5). The main feature of this phase was the absence of infiltration of inflammatory cells, which meant the absence of chronic inflammation.

4 DISCUSSION

The aim of this study was to evaluate the reaction of tissues to two types of synthetic hydroxyapatites (HaP and HaCol) produced by UEPG, and to compare them to a third commercial type (HaAlobone®). The implantation of biomaterials within the subcutaneous connective tissue of rats has been widely accepted as an essential test to access the biocompatibility of biomaterials in various specialties. In conjunction with previous reports, the results of the present study have shown that the histological response could be used as a preliminary source of information on biocompatibility.

In addition to biocompatibility, the physical architecture of the scaffold is of great importance. One of the main challenges in tissue engineering is designing suitable structures that meet the critical requirements for application in regenerative medicine. Both natural and synthetic polymers have been used for this purpose; however, the ability to adjust the material properties and tailor its performance in terms of tissue response and biodegradation makes synthetic polymers more attractive than natural polymers. In vivo biocompatibility is the most essential criterion to determine the validity and feasibility of a biomaterial.

The results of this study showed that after seven days of biomaterial implantation, there was a high inflammatory reaction (Figures 6A, B, and C). Neutrophils predominated, and the formation of exudate occurred in some cases. There were no areas of tissue necrosis.

The incorporation of pure hydroxyapatite with collagen may be able to improve the body's response to a material that is implanted in vivo. Isikli, Hasirci, and Hasirci found that various naturally derived polymers have been successfully implanted in hydroxyapatite nanofibrous scaffolds. The aim of such an approach would be to increase biomimetics, which is a property of collagen. However, the results of our research found that there was no significant difference in the biological behavior of these two materials.
(HaP and HaCol). It means that the incorporation of collagen does not favor the tissue regeneration processes.

Wang et al.\textsuperscript{31} found that the physical-chemical characteristic of the surface of biomaterials is one of the decisive factors for the adequate adsorption of proteins to occur. The surface topography, such as the roughness, porosity, pore size, and particle size, determines the size of the surface area that interacts with the protein molecules. Thus, the adsorption of proteins is critical for a sequence of biological activities to occur. In the early days of implantation, strong adsorption of plasma proteins and other macromolecules present at the site of injury was observed. This interaction and the restoration of tissues, fluids, and organic macromolecules occurs directly on the surface of the biomaterial; it is referred to as the \textit{Vroman effect}, and it is governed by the biomaterial physical and chemical properties.

At the stage of 15 days after surgery, the predominant cells in the experimental groups were macrophages. The connective tissue was still with bundles of loose collagen fibers; however, the formation of new blood vessels was observed. The regenerative and reparative processes were very active. It should be noted that in the bone matrix, the hydroxyapatite implants provided a suitable environment to stimulate bone development in terms of structural composition, which was very similar to the bone. Although heterotopic ossification can occur in ectopic tissues such as subcutaneous tissues, this was not observed. At this 15-day stage, the inflammatory reaction took the chronic form, and it could be understood as a protective response from the body against the presence of different components. When a biomaterial is toxic or incompatible with tissues, then inflammation persists; it can acquire forms of acute relapse, leading to extensive tissue damage and primary necrosis. The magnitude and duration of the inflammatory response are directly linked to the biocompatibility of a material.\textsuperscript{32,33}

At 30 days, there were no differences between the groups. In the biomaterials tested, the inflammatory response decreased to levels similar to the control. The presence of macrophages and multinucleated giant cells was observed. The organization of the collagen fibers of the connective tissue was present, and the predominant cells were fibroblasts. All groups presented a non-inflammatory connective tissue capsule, while in the control side, the regeneration was complete, and there were no signs of inflammation.

After 30 days, the organization of the connective tissue around the biomaterial, which was disordered, scattered, and missing in some areas at 15 days, became an organized fibrous capsule with a denser thickness. Ge et al.\textsuperscript{34} found that after 90 days, the
thickness of the fibrous capsule around the Ha-chitin materials had decreased due to the organization and the maturation of the fibrous scarring process. The scar tissue still comprised fibroblasts, macrophages, and giant cells, which are called resident cells. Legrand et al. [35], argued that the formation of the fibrous capsule by long collagen fibers and resident cells is part of the tissue reaction to a foreign body. This fibrous capsule may be regularly refurbished and disappear when the biomaterial is resorbed or when it is incorporated into the calcification process. A biomaterial is biocompatible when the intensity of the tissue reaction decreases over time. A material cannot be biocompatible when an inflammatory response remains within its environment, and the fibrous capsule remains fairly thick. Small particles of biomaterials (in contrast to larger particles) can evoke a more intense local inflammatory response, which is characterized by the presence of multinucleated giant cells and macrophages by releasing pro-inflammatory kinins such as Platelet-derived growth factor (PDGF) and Fibroblast growth factor (FGF). This influences the behaviour of the fibroblasts and subsequently induces the thickening of the fibrous capsule.

In this study, a decrease was observed in the amount of biomaterial in the connective tissue, and this was a form of in vivo degradation over 30 days. Kashaba et al. [36] indicated that it is possible to speculate that the soluble fragments of calcium phosphate that are released into the tissue suffer removal by local lymphatic drainage. This hypothesis may explain why the inflammatory reaction decreased with time and why the healing of the connective tissue occurred for all the experimental materials 90 days after implantation. When there is a moderate inflammatory reaction with macrophages and giant multinucleated cells, these phagocytes show full activity. This might suggest that the large particles of calcium phosphate that were not removed by the local lymphatic drainage or were digested by macrophages and giant cells may remain in the connective tissue, causing a persistent chronic inflammatory reaction that does not allow complete healing of the connective tissue.

The results regarding the biocompatibility of the biomaterials investigated (HaP and HaCol), are significant, since they do not differ from several other studies with positive biocompatibility results already carried out with animals. [13-17,36-38] The subcutaneous tissues were seen to present, at 30 days, a significant reduction in the inflammatory infiltrate and an increase in the thickness of the collagen fibers, which are evident signs of biocompatibility. It means that these biomaterials exhibit promising potential as bone substitutes.
Finally, this study presents some limitations. The concept that animal research, particularly that relating to pharmaceuticals and environmental agents, maybe a poor predictor of human experience is not new.\textsuperscript{39} In our research, the ectopic nature of the biomaterial implantation (in subcutaneous conjunctive tissue on the rats’ back) imposes some limits to this model regarding the observation of bone growth, differently from what occurs in calvaria [40]. Due to this limitation, further studies must be carried out on the calvaria of rats.

5 CONCLUSIONS

Based on the results of this study, the pure hydroxyapatite and hydroxyapatite with collagen tested in the dorsal submucosa of rats showed no toxicity and were biocompatible materials. There were no significant differences between both biomaterials that were tested regarding biocompatibility. Compared with the third type of hydroxyapatite (HaAlobone\textsuperscript{®}), the two types of hydroxyapatite produced did not show significant differences in terms of biocompatibility.
REFERENCES


FIGURES LEGEND

Figure 1. Scanning Electron Microscopy (SEM). Pure Hydroxyapatite - HaP: In (A) chemical composition of hydroxyapatite, mapping the distribution of chemical elements by minerals; in (B) distribution and percentage of constituent chemical elements; in (C), SEM micrograph showing the spheroid and microparticulate form of the biomaterial.

Figure 2. Hydroxyapatite with collagen – HaCol. In (A) and (B), hydroxyapatite with collagen. In (C), SEM micrograph.

Figure 3. Commercial Hydroxyapatite - HaAlobone: In (A) and (B) the hydroxyapatite composition. In (C), SEM micrograph.

Figure 4. Surgical procedures: after trichotomy a uniform and carefully incision and avulsion were performed using Metzenbaum Duflex® 14 scissors with a blunt tip. The avulsion, which was performed on the right side in the same manner as on the left side, was approximately 18 mm in length and 15 mm wide.

Figure 5. Comparison between different groups at each time point (Kruskal-Wallis test with Dunn's post-test). Subcutaneous tissue response after 7, 15 and 30 days. Scores: 0 = absent; 1 = mild; 2 = moderate; 3 = severe. (*) P<0.05, with HaP and HaCol after 7 days. (**) P<0.05, with HaAlobone®, HaP and HaCol after 14 days. Comparison between different times in the same group (Kruskal-Wallis test with Dunn's post-test). Different letters (P <0.01 significance) for biomaterials.
Figure 6. In (A) HaP - 7 days, the biomaterial (red arrows) was observed in the connective tissue with dense inflammatory infiltrate. There was a predominance of neutrophils. In (B) HaCol - 7 days, the biomaterial (red arrows) was distributed in the tissue with inflammatory infiltrate. There was little presence of exudate (yellow arrow). In (C) HaAlobone®- 7 days, the biomaterial (red arrows) was surrounded by connective tissue and inflammatory response was visible (Images HE, 400X).

Figure 7. In (A) HaP, 15 days, the biomaterial (red arrow) was surrounded by the fibroblastic proliferation process. The presence of multinucleated giant cells and macrophages (yellow arrows) near the biomaterial was observed. In (B) HaCol, 15 days, around the biomaterial (red arrows) the fibroblast proliferation process had already started. There was a reduction in the inflammatory infiltrate and macrophages predominated. Multinucleated giant cells were present (yellow arrows). In (C) HaAlobone®, 15 days, it was observed that around the biomaterial (red arrows) the process of fibroblastic proliferation had already begun. There was a reduction in the inflammatory infiltrate and macrophages predominated (yellow arrows) (Images, HE, 400X).

Figure 8. In (A), HaP, 30 days, the formation of a fibrous capsule was observed around the biomaterial (red arrows). Inflammatory infiltrate was not evident; there were few macrophages and giant cells were present (yellow arrow). In (B) HaCol, 30 days, the biomaterial was surrounded by a fibrous capsule (red arrows). There was minimal inflammatory infiltrate, with the presence of macrophages and multinucleated giant cells (yellow arrows). In (C) HaAlobone®, 30 days, the formation of a fibrous capsule around the biomaterial (red arrows) was evident. Inflammatory infiltrate was not visible. Macrophages and multinucleated giant cells were present (yellow arrows) (Images, HE, 400X).