Where we should analyze bone healing after placement of particulate grafts in surgical bone cavities

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Abstract

The evaluation of particulate biomaterial properties used in surgical bone cavities should take into consideration two different environments: first, the events that occur at the interface between particles, the blood clot and the granulation tissue, including osteogenesis; and second, those that occur in the spaces between particles and away from their surface, that is, induced tissue reactions, including osteogenesis. In these spaces, evaluations should include progressive changes in blood clot, granulation tissue and new bone formation. Responses to particulate biomaterials should be evaluated in face of events directly on the surface of the particles, as well as whether these particles will be reabsorbed or not and be replaced with bone to reestablish normal conditions in the site.

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The beginning

Bone repair begins as the surgical cavity is filled with a blood clot, which provides a fibrin meshwork as anchorage for neighboring cells that will adhere to the region.

Neighboring cells that adhere to the clot to repair bone come simultaneously from:

1. The endosteum that covers the trabeculae with osteoblasts and pre-osteoblasts organized as a delicate "wall paper" of very thin connective tissue. On the walls of bone surgical cavities, the trabeculae are sectioned, and the marrow spaces are exposed in windows open to the blood clot formed there.
2. The bone marrow, in the form of tissue stem-cells that differentiate into osteoblasts. The marrow spaces between trabeculae are filled with the bone marrow exposed on the surgical walls of the cavity. In the past, these cells were also called undifferentiated mesenchymal cells. The term "osteoprogenitor cells" has been often used to describe these reserve cells found in the bone marrow.
3. The periosteum, when preserved and re-oriented on the bone surface that underwent operation, because it is rich in osteoblasts and pre-osteoblasts, particularly on the internal surface that is in contact with cortical bone.

The formation of a fibrin meshwork: angiogenesis occurs immediately after that

Before the cells permeate the whole fibrin network, a mesh of newly formed vessels has to be constructed to nourish the cells that will reach and invade the whole clot, from the periphery to the center. After some minutes, angiogenesis begins from the neighboring blood vessels, immediately after clot formation: this phenomenon is characterized by new vessel formation. Angiogenic mediators, particularly growth factors, are released by endothelial cells, platelets, macrophages and cells from the injured region.

Granulation tissue: in minutes and hours

Angiogenic mediators, released at the site by platelets and macrophages that originated in clotted blood in the surgical cavity, promote on the vessels of neighboring bone the proliferation of vascular walls cells, such as angioblasts and endothelial cells. Forming sprouts, they all align unidirectionally towards the center of the cavity. This vascular meshwork of newly formed vascular branches and inflammatory cells, particularly macrophages, characterize the first phases of granulation tissue: a fragile, gel-like tissue still poor in fibers and tissue cells. Its name derives from the large amount of small reddish dots that give it a granulated appearance. Doctors in the past, who had no microscopes, said that the wound was granulating, which meant that it was progressing well into full repair.

The term ***granulation tissue*** indicates repair and connective tissue reconstruction, as the last phase of a successful inflammatory process. Granulation tissues should not be confused with chronic inflammation, a phase of inflammation in which the aggressive agent is resistant and difficult to destroy.

Chronic inflammation is a term that indicates that the aggressive agent is persistent, whereas granulation tissue means that the aggressive agent has been controlled and eliminated. Chronic inflammation is characterized by the encapsulation of the aggressive agent in the center of the agglomerate of mono- and multinucleate macrophages, associated or not with other leukocytes, such as lymphocytes. These macrophage agglomerates are known as ***granulomas*** and are typical of chronic inflammation, that is, when the aggressive agent is persistent.

Primary bone: In a few hours and days

At practically the same time, at a small time difference from the cells of the migrating vessels, neighboring tissue cells are also stimulated to proliferate by cytokines and growth factors primarily released by macrophages and platelets.

These young cells, many still not differentiated, migrate into the granulation tissue components to become part of it. Between vessels and anchored to the fibrin meshwork, migrating cells differentiate into osteoblasts and rapidly lay down a collagen matrix ready to be mineralized. Therefore, rudimentary and randomly distributed trabeculae appear in some hours and reconstruct bone a few days later by filling up the cavity, although this bone primarily has almost no function in terms of load bearing.

This newly formed bone that fills up the cavity at first has the same organization and structure as embryonic bone and, therefore, is called primary, embryonic or immature bone. Its main characteristics are: rich cellularity, little mineralization and disorganized random distribution in granulation tissue.

Secondary or mature bone: in a few days

Primary bone trabeculae are found around any surgical cavity after some hours or days. Granulation tissue maturation is a process that occurs from the periphery to the center of the cavity. While primary bone in the periphery has already replaced granulation tissue, areas of the blood clot surrounded by immature granulation tissue remain in the center of the bone cavity. Maturation and surgical cavity filling are centripetal processes.

In some days, primary bone will be gradually reabsorbed in the peripheral area and in areas contacting the margins of the bone cavity, where osteoblasts lay down a collagen matrix that is much more organized and mineralized and with fewer osteocytes inside. These new trabeculae are laid down in an organized way to respond to local functional demands according to the loads that they bear. This more organized and mineralized, but less cellularized bone is named secondary or mature bone.

The size of the bone cavity and the particulate material in the clot

If the surgical cavity is small, the fibrin meshwork is the ideal site for the phenomena aforementioned. In larger cavities, however, the fibrin meshwork collapses, retracts or undergoes dehiscence, which reduces its volume due to loss of surface area. The fibrin meshwork is the matrix for new bone formation: if its volume is reduced, the area of the bone cavity that will be reconstructed is also reduced.

The surgical bone defect may be said to have reached a critical size when bone reconstruction does not occur adequately because of size, as retraction led to dehiscence of the fibrin meshwork, the origin of granulation tissue.

In these cases, one of the options is to increase anchorage of the fibrin meshwork by adding solid particles to it (Fig 1), so that its fixation is increased and extends beyond the surgical margins. Biomaterial particles act as true pillars, or dowels, for provisional and intermediate anchorage for the fibrin meshwork. Examples of particles to be grafted into blood clots in bone cavities are: autogenous bone fragments, bovine bone, polymers and other several options available in the market.

A particulate graft implant in the blood clot preserves the surface of the fibrin meshwork and also reduces the bone spaces to be reconstructed, initially, by granulation tissue originated in the migration of neighboring cells (Fig 1).

Ideal biomaterial properties: neither antigenic, nor foreign body!

Particles should not be toxic or contaminated to ensure that the fibrin meshwork, platelets and macrophages are the first to find anchorage and interact with their surface. Ideally, macrophages, which also recognize foreign substances and are antigen-presenting cells, should not recognize any foreign proteins. Also ideally, macrophages should not recognize particle surfaces or composition as being foreign, so that particles are not treated as foreign bodies or antigenic substances.

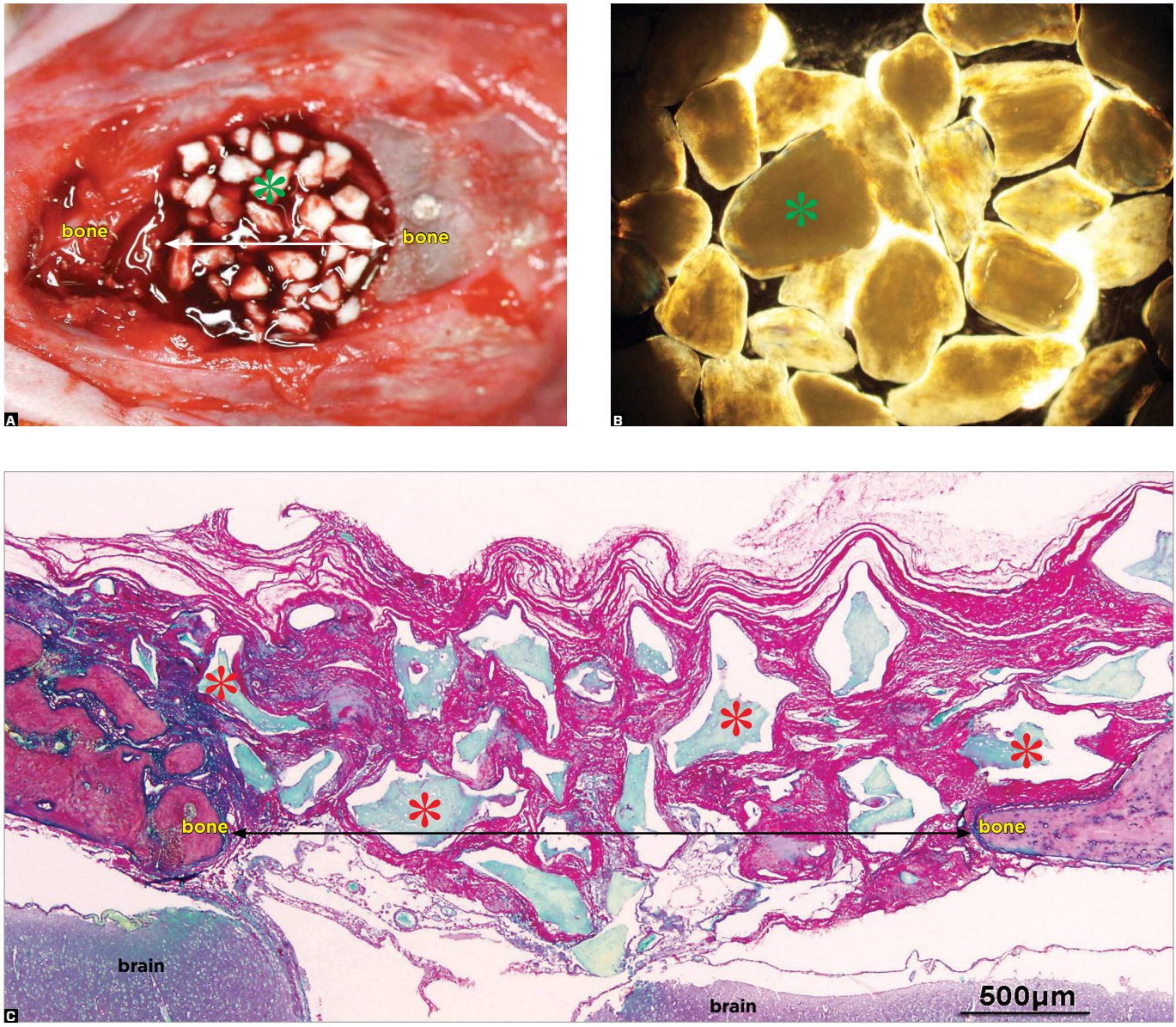


Figure 1 - Spaces between biomaterial particles (asterisks) are initially filled with blood clot (**A**) that turns into granulation tissue in some hours and, in some days, becomes fibrous connective tissue (**C**) or newly formed bone. Bone cavity (arrow) in murine calvarium where particulate biomaterial was placed for experiment (Picrosirius staining, images acquired 60 days after operation).

If no antigens or unusual structure are identified, biomaterial particles act as an inert body over which cells, such as fibroblasts, osteoblasts, cementoblasts and other synthesizing cells, will anchor and lay down an organic matrix to be mineralized. The result would be a true structural and functional integration of newly formed bone and biomaterial particles (Figs 3 and 5).

However, products or biomaterials whose particles are all identified as inert bodies are still rare. Most have particles that are not toxic or contaminated, but macrophages in the blood clot still identifying them as foreign bodies, surround them and remain around them for an indefinite amount of time (Figs 2 and 5). Macrophages surround, adhere to and encapsulate particles in the attempt to isolate them from the rest of the body, forming clusters that are called foreign body granulomas (Figs 2 and 5). In addition to mononucleate macrophages, some younger macrophages join older ones and form multinucleate giant cells of inflammation, or multinucleate macrophages.

In most biomaterial particles used in surgical bone cavities, foreign body granulomas are formed and outlined by a discrete organization of collagen fibers and fibroblasts that surround them. After 30, 60 and 120 days, there will be numerous particles and their corresponding foreign body granulomas in the cavity (Figs 2 and 5).

While in the middle of particles of most biomaterials and their resulting granulomas, the blood clot changes into granulation tissue invaded by osteoblasts, producing primary and, later on, secondary bone (Figs 3 and 4). New bone formation between the particles and granulomas does not occur only when the area has been contaminated or when biomaterials release toxic products that prevent new bone to develop in the neighboring granulation tissue.

When biomaterial particles are inert bodies

The proteins in the structure of biomaterial particles are not identified as an antigen when the particle acts as an inert body, and its surface and structures are recognized, even by macrophages, as the same or as part of the same organism, and adhere to their surface and lay down an organic matrix to be mineralized (Figs 3 and 4). In other words, osteoblasts adhere to their surface, and bone forms over that surface, in continuity to the newly formed

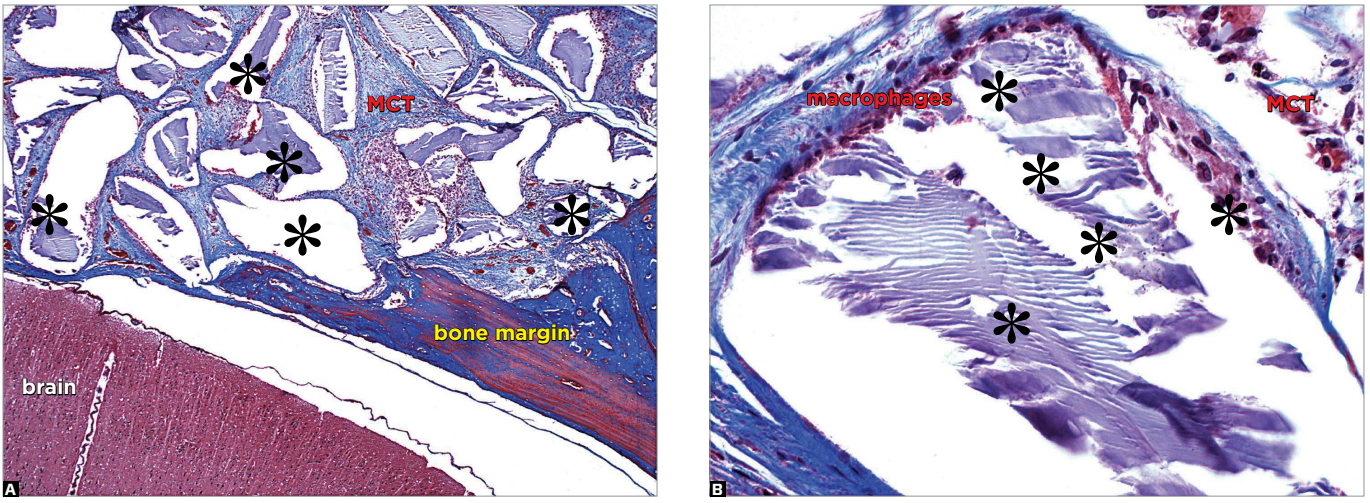


Figure 2 - After some weeks, macrophages and multinucleate giant cells of inflammation adhere to the surface of biomaterial particles (asterisks) and organize as foreign body granulomas to encapsulate particles. Granulation tissue become mature fibrous connective tissue (MCT) in their periphery and in the space between particles and granulation tissue 60 days after operation (Masson's trichrome stain; **A** = 10X; **B** = 25X).

bone in the space between the particles previously occupied by the blood clot and granulation tissue.

Bone formation over biomaterial particles and in continuity to them is a sign of full integration with tissues in the area. After 180 days, bone and remaining biomaterial particles are found in the region. Depending on their composition, particles may be absorbed by osteoclasts and gradually replaced with constantly remodeling bone (Figs 3 and 5).

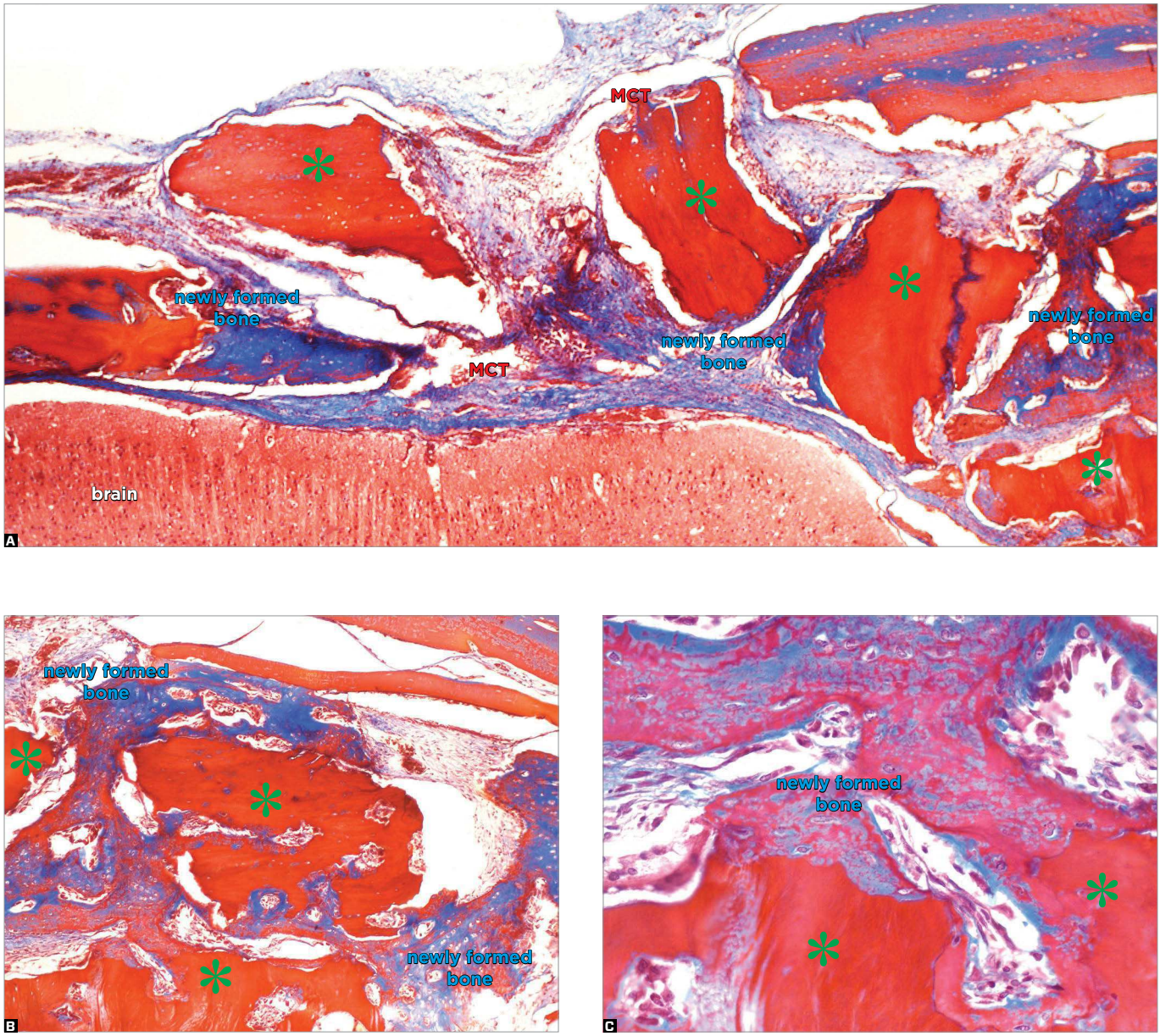


Figure 3 - Tissue sections shows progression of granulation tissue into new bone formation on surface of particles (interface between particle and neighboring tissues undergoing reorganization) and in the space between particles (asterisks). Some biomaterial particles indicate partial resorption and replacement with new bone resulting from bone remodeling 60 days after operation (Masson's trichrome; **A** and **B** = 10X; **C** = 40X.

Biomaterial particles may remain for an indefinite time or disappear

If foreign body granulomas form around biomaterial particles, macrophages are rarely able to phagocyte them, but they never stop trying and remain there indefinitely. No clinical symptoms or discomfort are felt, but there will be obstacles to tooth movement or to place dental osseointegrated implants in the area. Granulomas hardly ever eliminate all biomaterial particles from the site where they were placed originally (Figs 2 and 5).

Some materials accept osteoblast adherence to their surface, and a mineralized bone matrix is laid down, with which the graft becomes integrated (Figs 3 and 5). In this case, as bone is constantly remodeling, the area of bone repair will not be stable indefinitely and will be gradually remodeled.

If osteoclasts can absorb biomaterial particles, the particles will be gradually remodeled and replaced with new bone in the site at the same time as integrated bone. After some months, there will be not even microscopic signs of biomaterial particles at the site. This occurs, for example, with particles of autogenous bone placed in surgical cavities.

Some bovine bone biomaterials have the same properties as autogenous bone, integrate with newly formed bone on their surface and undergo remodeling along months or years. However, other bovine bone biomaterials do not have the same characteristics and behave as foreign bodies, indefinitely forming foreign body granulomas around it and, therefore, remaining in the site indefinitely.

In sum:

1. Some materials increase anchorage of the fibrin meshwork, cooperate with or favor bone formation around their particles and, later on, are absorbed and remodeled together with bone in the region where they were applied.

2. Other materials remain in the implantation site for an indefinite time, even when bone forms over their particles and surfaces. In this case, they do not remodel together with the bone around it because osteoclasts do not absorb them, although they do not form foreign body granulomas around themselves.

3. Most biomaterials leave their particles in the site, which remain encapsulated by foreign body granulomas for an indefinite amount of time, even when bone is formed between the particles. Therefore, there is no clinical discomfort.

When associated with biomaterial particles placed in surgical cavities, there is inflammation with pus, a sign of bacterial contamination. This may be explained by the fact that the material was contaminated or the surgical cavity was invaded by bacteria, usually *staphylococci* and *streptococci*.

What to measure or evaluate in *in vivo* studies about biomaterials

In a surgical cavity where biomaterial particles are used to assist blood clot anchorage, evaluation of effects should take into consideration two "environments," or sites:

1. **The direct particle-tissue interface around it (Fig 5) —** Cells and tissue components in the interface with the surface of biomaterial particles:
   a) may originate from granulation tissue, osteoblasts and mineralized bone matrix that forms directly on its structure; or

b) may form a cluster of macrophages and multinucleate giant cells of inflammation that characterize a foreign body granuloma; or
c) in longitudinal studies, may indicate whether their particles are remodeling together with bone in the region, or whether they are not absorbed by osteoclasts.

2. **The space between particles and their direct tissue reactions (Fig 4)** — Particles and their surface reactions leave a space that was originally filled by the blood clot, later by granulation tissue, and finally by bone. In general, this space is filled with mature bone in a few months.

When foreign body granulomas form around the particles, bone formed in the space between granulomas has a three-dimensional distribution that looks somehow like honeycombs.

In sum, this space should be evaluated to define whether granulation tissue becomes young fibrous connective tissue in some weeks and, right after that, immature and, subsequently, mature bone.

If a biomaterial actively affects tissue reactions in the space between particles, it is probably releasing products during its interaction with cells on its surface, and these products are eventually diffused around it.

These products may be cytokines and growth factors that stimulate osteogenesis or, alternatively, release toxic chemicals that induce inflammation and prevent repair by osteogenesis in this space between particles.

Imaging studies can be performed to evaluate this progression

Imaging studies, such as periapical radiographs, CT scans and micro-CT imaging, may be used to evaluate the following parameters along weeks and months:

1. Persistence, or not, of biomaterial particles when their radiopacity differs from that of the bone where they were placed. Some particles simulate the same radiopacity of normal or newly formed bone, which makes this analysis difficult. Some of the biomaterials are processed bone fragments that retain the same physical properties of bone. Ideally, they should be replaced with bone during remodeling along time.

2. Whether newly formed bone is between particles or in close contact with them on its surface. For those purposes, images should be acquired as very thin micro-CT sections that provide high resolution and clearly show details. However, when doing so, it is not possible to compare microscopic and CT findings, not even when the best scanners are used. When not very mineralized or when still very thin, bone does not generate a detectable image.

3. Bone formation between biomaterial particles placed in a specific site. This test has the same restrictions and limitations described in the item above. Micro-CT sections should be very thin to provide a high level of resolution and show details very clearly. However, it is not possible to compare microscopic and CT findings, not even when the best scanners are used. When not very mineralized or still very thin, bone does not generate a detectable image.

4. Full bone remodeling, as well as particle remodeling, starting at the moment when the same compatible trabecula is seen and in continuity with areas neighboring the surgical cavity.

5. Repair or preservation of bone surface at a desired level, as previously planned.

Shape and size of particles affect results

If the particles adjust to each other in the middle of the blood clot, the space for the clot is reduced (Fig 1). In contrast, if the particles are more distant from each other because of their larger size or irregular shape, the space for the blood clot is larger.

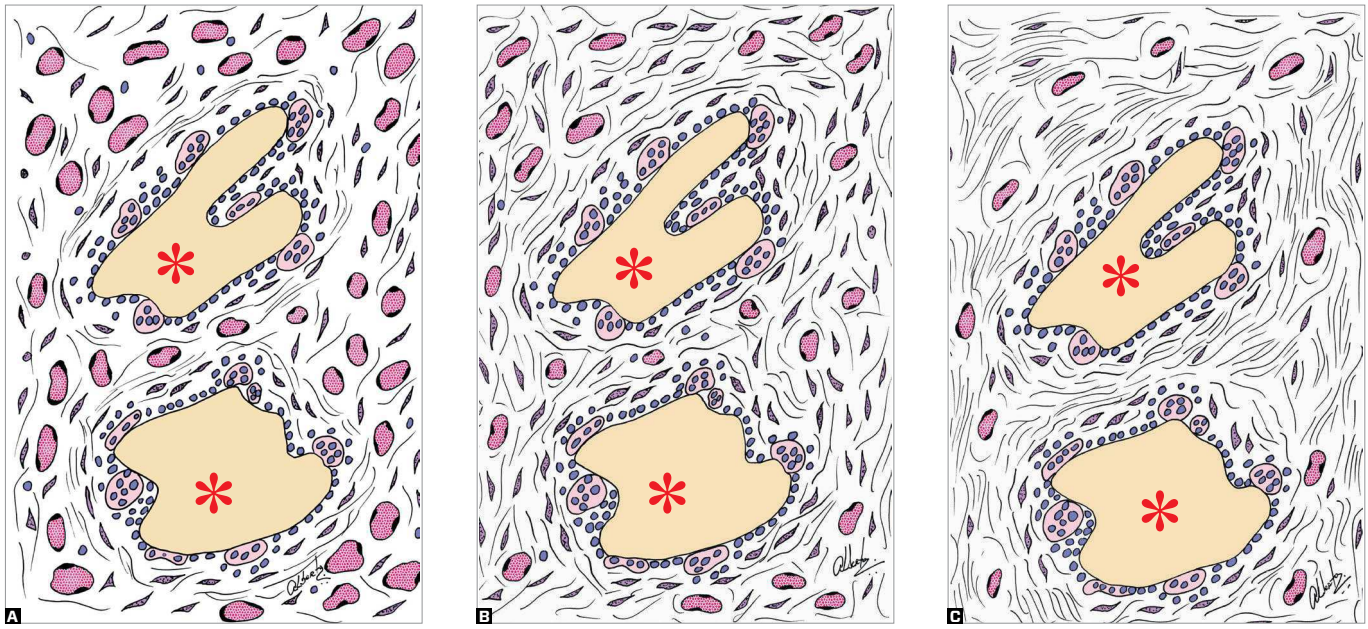
When bone is formed on the surface of particles, osteogenic cells from the granulation tissue originated in the blood clot should adhere to this surface. Particles should not replace all the blood clot and its fibrin meshwork, nor occupy all of its space, and the fibrin meshwork is the true primary matrix for reparative osteogenesis.

A very high density of particles in the blood clot may not be desirable because there may be no space left for the blood clot to reorganize for repair. In other words, pushing down, compressing or condensing particles into the bone cavity may be unfavorable. Ideally, the material should accommodate, fit to, distribute along or adapt to the blood clot and the cavity walls.

Biomaterials in the form of single dense blocks are reparative only in their periphery, as described for each of the particles separately. They behave as a single large particle. When there is only one porous block, full of spaces, as a true sponge, the blood clot permeates all of its structure with the fibrin meshwork, filling them up and serving as a scaffold for repair.

When the biomaterial particles are absorbable and remodel together with newly formed bone, the larger they are, the longer they will remain in place (Fig 5). The clasts act on the periphery and surface of particles. Smaller biomaterial particles, whose granulation is finer, remodel in bone at an earlier stage, if they have that property. When the biomaterial particle is not absorbable, remodeling does not take place, regardless of shape or size.

The variable granulation options that commercial biomaterials have should respond to the different clinical needs in terms of time and repair duration that each case requires in our daily routine. This is directly associated with the type of cell and tissue reaction induced by the biomaterial particles on the surface and between particles.



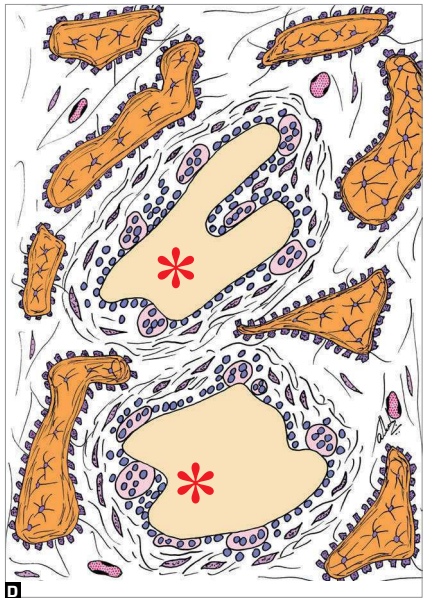


Figure 4 - Morphological tissue and cell changes observed and measured in the evaluation of reactions to particulate biomaterials placed in surgical cavities, particularly in the spaces between particles (asterisks):

In **A**, granulation tissue is immature, has numerous blood vessels (red) and a few fusiform fibroblasts with few collagen fibers (lines). Collagen fibers tend to encapsulate and peripherally isolate particles covered by macrophages and derive multinucleate giant cells of inflammation identified by blue nuclei and pink cytoplasm — in this case organized as a foreign body granuloma.

In **B**, there are fewer blood vessels and more fibroblasts and collagen fibers, and particle encapsulation increased. These are characteristics of mature granulation tissue, which begins production of still immature fibrous connective tissue.

In **C**, there are even fewer blood vessels, more marked collagen fiber bundles organized together with a predominance of fibroblasts, and no inflammatory cells. This condition may remain unchanged for an indefinite amount of time when some biomaterials are used.

In **D**, space between particles may receive neighboring osteoblasts, together with fibroblasts, and start focal new bone formation, which increases gradually as they unite and form large ossification areas, as may occur when some biomaterials are used.

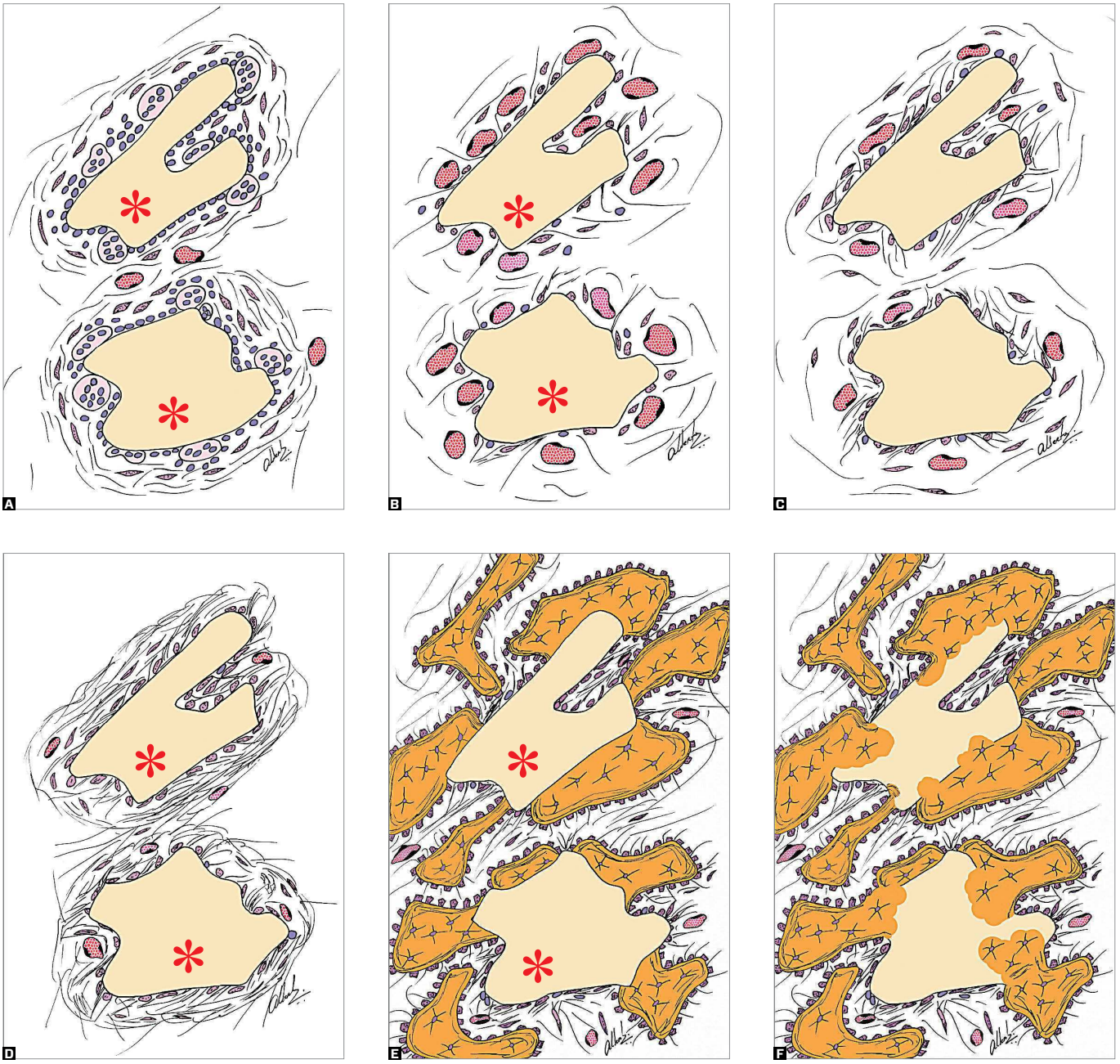


Figure 5 - Morphological tissue and cell changes to be observed and measured in evaluation of reactions to particulate biomaterials placed in surgical cavities, particularly on particles surface (asterisks):

In **A**, foreign body granuloma organizes on surface and immediate periphery of particles of most biomaterials in a few days, being nourished by neighboring blood vessels (red). Macrophages and multinucleate giant cells of inflammation derived from macrophages adhere to particle surface for phagocytosis. This reaction may go on for an indefinite amount of time.

In **B**, on particles surface of some biomaterials, induction to form foreign body granuloma does not occur, and fibroblasts adhere to it to begin production of collagen fibers. In this phase, there are numerous blood vessels and macrophages diffusely distributed between them. At this point, structures organize on particles surface to form immature granulation tissue.

In **C**, there are even fewer blood vessels, more marked collagen fiber bundles organized together with predominant fibroblasts, and no inflammatory cells, which characterizes mature granulation tissue and immature connective tissue.

In **D**, particles integrate to mature fibrous connective tissue that is newly formed, and fibroblasts and collagen fibers are mingled in surface structure. This may remain like that for an indefinite amount of time when some biomaterials are used.

In **E**, as in some biomaterials, neighboring osteoblasts, together with fibroblasts, may adhere to particle surface, which begins new bone formation and gradually increases its area over biomaterial and leads to structural bone-particle integration and formation of large ossification areas, although without biomaterial remodeling.

In **F**, structural bone-particle integration, characterized by large ossification areas, occurs simultaneously with development of focal particle resorption areas and their replacement with bone. Particles are gradually replaced with bone during normal continuous remodeling of human bones.

Final considerations

The evaluation of the properties of particulate biomaterials in surgical bone cavities should take into consideration two different environments.

1) Events, including osteogenesis, that occur directly in the interface between the particles, the blood clot and the granulation tissue.

2) The behavior of the blood clot, granulation tissue and new bone formation in the spaces between the particles distant from their surfaces.

The most preponderant reaction should be associated with what occurs directly on particles surface and whether, along time (weeks and months) the particles will be absorbed to give room to bone that will reestablish normal conditions in the site.

Finally, evaluations should investigate whether biomaterial particles undergo osseointegration or remodeling after some weeks or months. If findings are positive, osseointegrated implants and orthodontic tooth movement may be successfully attempted.

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