Title

Cranioplasty with autologous bone flaps cryopreserved with dimethylsulphoxide: Does tissue processing matter?

Authors

Vicente Mirabet, PhD[1] Email: mirabet_vic@gva.es

Daniel García, MD[2] Email: daniel1990gs@gmail.com

Amparo Roca, MD[2] Email: amparocabarber@gmail.com

Arnold R Quiroz, MD[2] Email: arnold81492@hotmail.com

Joan Antón, MD[2] Email: joan_93_4@hotmail.com

Miguel Rodríguez-Cadarso, MD[2] Email: migcad94@gmail.com

Dolores Ocete, MD[3] Email: ocete_mar@gva.es

Lucas Aranda, MD[3] Email: aranda_luc@gva.es

Ana Melero, PhD[4] Email: ana.melero@uv.es

Antonio J Guillot, RF[4] Email: antonio.guillot@uv.es

Nuria Yagüe, MD[1] Email: yague_nur@gva.es

Isabel Guillén, PhD[5] Email: iguillen@uch.ceu.es

Carlos Botella, MD PhD[2] Email: botella_car@gva.es

Affiliations

[1]Cell and Tissue Bank, Centro de Transfusión de la Comunidad Valenciana. Valencia. Spain.

[2]Service of Neurosurgery, Hospital Universitario y Politécnico La Fe. Valencia. Spain

[3]Service of Microbiology, Consorcio Hospital General Universitario. Valencia. Spain

[4]Department of Pharmacy, Pharmaceutical Technology and Parasitology, Universitat de València. Valencia. Spain.

[5]Department of Pharmacy, Faculty of Health Sciences, Universidad Cardenal Herrera-CEU. Valencia. Spain

Corresponding Author

Vicente Mirabet, Cell and Tissue Bank, Centro de Transfusión de la Comunidad Valenciana. Avenida del Cid, 65-A. 46014. Valencia. Spain

Corresponding Author's Email: mirabet_vic@gva.es

Key Words

Decompressive craniectomy, autologous bone flap, bone processing, bone storage, cryopreservation, cranioplasty.

Short title

Bone processing for cranioplasty

Abstract

Objective: The aim of this paper was to study the outcome of patients who underwent cranioplasty with cryopreserved autologous bone after decompressive craniectomy.

Methods: Data from 74 patients were retrospectively analyzed. They were divided into groups according to the storage time and the age at cranioplasty. To assess its predictive potential for complication, factors were related to successive stages (preoperative, craniectomy, tissue processing, cranioplasty, and postoperative). Cooling and warming rates applied on bone flap were calculated. The ability to inhibit microbial growth was determined exposing bone fragments to a panel of microorganisms. The concentration of antibiotics eluted from the bone was also determined. A bone explant culture method was used to detect living cells in the thawed cranial bone.

Results: Hydrocephalus was significantly more frequent in pediatric patients (26.7%) than in adults (5.1%). The overall rate of bone flap resorption was 21.6% (43.7% of them requiring reoperation). Surgical site infection after cranioplasty was detected in 6.8% of patients. There was no correlation between infection as postoperative complication and previous microbiological positive culture during processing. The etiology of craniectomy did not influence the risk of bone flap contamination. Vancomycin was the only antibiotic detected in the supernatant where the bone was incubated. Outgrowth from bone explants was observed in 36.8% of thawed skulls. An early start of bone flap processing at the tissue bank had a positive effect on cell viability.

Conclusion: The outcome after autologous cranioplasty is a multifactorial process, which is modulated by patient-, surgery-, and bone-related factors.

Abbreviations:

ATCC: American type culture collection

BFR: bone flap resorption

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethylsulphoxide

GM: growth medium

HPLC: high performance liquid chromatography

PRP: platelet-rich plasma

PL: platelet lysate

SD: standard deviation

UV: ultraviolet light

Introduction

Craniectomy is widely used for the surgical treatment of elevated intracranial pressure refractory to pharmacotherapy. Once the patient recovered from the primary insult, the function of the explanted tissue must be restored, providing protection, improvement in neurologic status, and cosmesis.[1]

Two main choices are available to reconstruct calvarial defects:[2,3]

- The use of the previously explanted autologous bone, which offers several advantages: low cost, precise matching (perfect fit and curvature) and immunological tolerance.
- If the original tissue is not available or its clinical use is not indicated, a synthetic plate (methyl methacrylate, titanium, hydroxyapatite, etc.) can be used.

The first strategy, autografting, requires the storage of the tissue between craniectomy and cranioplasty, which can be performed by two methods:[4]

- Intracorporeal, by subcutaneous implantation within the patient's body (abdomen, thigh, or scalp).
- Extracorporeal, using tissue banking practices to keep the bone biologically safe and clinically efficient.

The clinical experience in this field has shown two main risks conditioning the outcome of the re-implanted bone: surgical site infection and bone flap resorption (BFR). Several factors have been studied as predictors for clinical complications: etiology of craniectomy, young age, comminuted fracture, size of the bone flap, location of cranioplasty, presence of an intracranial device, time between craniectomy and cranioplasty.[5-7] But some aspects of these issues do remain controversial.

This study was designed with a double aim: to determine the complication rates (infection, bone flap resorption, reoperation, hematoma and hydrocephalus) of patients undergoing cranioplasty with autologous bone in our center, and to evaluate both the presence of viable cells within the thawed cranial bone and its antimicrobial potential. Additionally, we want to highlight the importance of close collaboration between neurosurgeons (as responsible for tissue extraction and reimplantation, and patient management), and bankers (as responsible for tissue processing and storage).

Methods

Patients

In this study we retrospectively reviewed 74 cases of patients who underwent decompressive craniectomy and later cranioplasty at La Fe Polytechnic University Hospital (Valencia, Spain), from January 2011 to November 2019. The study complied with the policies of the institutional review board.

Demographic and lifestyle parameters were recorded. Clinical data were grouped as: preoperative (etiology for craniectomy, medical comorbidities), craniectomy (bone size, fragmentation), tissue processing (microbiological cultures), cranioplasty (length of bone storage), postoperative (follow-up period, complications).

We considered: infection, when the bone flap was removed due to this cause and the use of antibiotics was indicated; BFR, two types were established according to Dünisch et al.[8] Type I was a thinning of the bone flap and/or a beginning resorption along the rims of the flap. Type II was characterized by a circumscribed, complete lysis of the bone within the flap, including tabula interna and externa with loss of the bony protection of the brain.

Harvesting the bone flap

Skin incision: it´s like a question mark, started at widow's peak hairline, but with increased exposure by taking it posteriorly close to the inion, then turning sharply anteriorly and hugging the ear to preserve blood supply. A burr hole was made just above the posterior root of the zygomatic arch; a second one may be made just behind the frontal insertion of the zygomatic arch, inferior to the superior temporal line.

The bone flap proceeded posteriorly from the posterior zygomatic arch using the footplated craniotome. Posteriorly, remained ≈1cm superior to asterion to avoid the transverse sinus. The flap was taken 1cm beyond the lambdoid suture, and then up towards the sagittal suture, crossing the lambdoid suture again (this leaves a small amount of bone posteriorly on which the head can rest post-op). An anterior turn was made 1cm short of the sagittal suture to avoid the superior sagittal sinus, and the sagittal suture was paralleled. The coronal suture was crossed, and the drill was taken as low as possible in the frontal fossa near the midline. Keeping as low as possible, the orbital roof was posteriorly followed towards the second burr hole. The burr holes were then connected.

The dural opening based inferiorly, taken to 1cm short of the craniotomy edge. Dural releasing incisions may be made at intervals up to the bone margin to avoid strangulation of the brain on the dural edge. Then, the duraplasty onlay was carried out with heterologous material as Durarepair® or Duragen®. The dural flap was then replaced on top of the brain and dural substitute, which was not sutured, later the end the skin flap was closed by stiches and staples.

Finally, the tissue was placed in an ethyl-vinyl-acetate bag and sent to the tissue bank. The delivery took <30min.

Bone flap processing at the tissue bank

Tissue processing started as soon as possible (in other case the bone was stored at 4°C until use). All tissue manipulation was performed in a laminar flow cabinet (grade A air quality) in a grade D environment. The bag was heat-sealed and the bone flap was rinsed twice in saline by gently shaking. The supernatant was removed and a sample (≈10%) was taken for microbiological culture. Following, a previously validated antibiotic solution (vancomycin, 50µg/mL; tobramicyn, 50µg/mL; cotri-moxazole, 50µg/mL; and fungizone, 5µg/mL; in Hank's balanced salt solution) was added and incubated for 1h at room temperature.[7] The disinfectant solution was then removed and storage solution (culture medium 199 supplemented with 5% human albumin and 10% dimethylsulphoxide, DMSO) was added. After 5-10min, a sample of storage solution (≈10%) was taken for microbiological culture (measures to avoid false negative results due to residual antibiotics were observed during culture). Uncontrolled freezing was achieved by introduction in a freezer at -80°C and finally stored at this temperature.

When the tissue was required, fast thawing was performed by immersion in a water bath at 37°C. Then, it was rinsed twice with saline and a sample of the washing solution (≈10%) was taken for microbiological culture. The bone flap embedded in saline within the bag was sent to the operating room.

Surgical protocol for cranioplasty

Surgical wound was re-incised, being careful to avoid intracranial penetration of the scalpel. A plane of dissection was then developed between the galea and the dura or the dural substitute, beginning at the most superior aspect of the defect. The temporal muscle was carefully dissected away from the epidural scarring tissue, avoiding cerebrospinal fluid leaks. The deep edge of the inner table was then exposed, without dissecting the epidural space in order to prevent any epidural bleeding.

The bone flap was perforated and dural tack-up sutures placed. The flap was then secured in the defect with titanium plates and screws. A subgaleal drain is left in place, and meticulous haemostasis was achieved prior the standard skin flap closure.

In vitro assays

Bone flaps non-intended for autologous use (because of patient death) and authorized for researching purpose were selected for this study. Thawing was performed as before.

Bone chips (4-8mm^2) of skull were seeded in 3.5cm petri dishes (5-7 fragments per plate, figure 1) as primary explants and cultured with growth medium (GM): nutrient mixture DMEM-Ham F-12 plus penicillin/streptomycin (100IU/100µg/mL, respectively) and supplemented with 10% of human platelet lysate (PL). The PL was generated from whole blood from volunteer donors which was centrifuged at 400g for 7min.

The platelet-rich plasma (PRP, 2/3 of the plasma volume on the packed red cells) underwent two cycles of freezing/thawing. Before preparing complete medium, PL was coagulated by adding calcium chloride. Then, it was squeezed and centrifuged at 900g for 10min and the supernatant used as medium supplement. Duplicate plates were seeded with explants from the edge and from the center of the bone flap. The cultures were incubated at 37°C, 95% humidity and 5% CO_2. Medium was refreshed twice a week. Cultures were assessed for cell growth under inverted microscopy every five days. This methodology is addressed to cells with the ability to migrate from the explants and attach on the plastic surface.

When growth of colonies was observed, GM was switched by osteogenic differentiation medium (GM plus 10mM β-glycerol phosphate, 0.1µM dexamethasone, and 0.2mM ascorbate-2-phosphate). Staining with alizarin red solution was used to detect the presence of mineralization.

Additionally, 10 bone flaps were used for antibiotic testing. Six bone fragments (1 cm^2) were obtained from each bone flap and individually placed in tubes with 10mL of saline:

- One fragment was incubated for 3h at room temperature. Then, the tissue was discarded (after weighing and measuring it) and the tube centrifuged at 3800g for 5min. The supernatant was stored at -80°C until use. The antibiotic concentrations in the supernatants were measured by high performance liquid chromatography (HPLC) using a PerkinElmer® Series 200 equipment, coupled with a UV detector. The stationary phase was a kromasil C18 (5µm) (150×4.6µm) column. The analytical methods were validated in our previous work.[9] Briefly, analyses were performed at room temperature using different mobile phases and wavelengths at 1mL/min flows. Tobramycin was analyzed with ammonium acetate 0.02M pH9:methanol (55:45) and a λ=210nm. For vancomycin we used a phosphate buffer-acetonitrile (90:10) pH3.5 mobile phase and a λ=280nm. Co-trimoxazole was analyzed with an acetonitrile:HPLC-water (20:80) plus triethylamine (0.1% v/v) mobile phase and a λ=240nm. And fungizone, with an acetonitrile:phosphate buffer pH3.5 0.01M (65:35) phase and a λ=345nm. The methods were validated through analysis of standard calibration curves (3x) to estimate inter-day precision. Three different aliquots of each sample were analyzed for each HPLC method, and three replicates of each one were injected. Results of the samples containing quantifiable concentrations were calculated as mean±SD.
- The other five fragments were exposed to a panel of microorganisms (prepared as in our previous work[9]), including the following species: *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 29213), and

Candida albicans (ATCC 90028). Initially, bones were seeded on Muller-Hinton agar medium, and then it was decided to use the broth macrodilution method (soaking the tissue into the culture medium). When at least a 99.9% reduction in the starting inoculum was achieved the effect was considered positive.

Cooling and warming rates

In order to study heat interchange during freezing and thawing processes we placed a thermocouple probe into the cancellous bone between the cortical tables. It penetrated 13mm from the bone rim.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics v22.0. The chi-square test or Fisher's exact test (when expected numbers were less than 5) was carried out to study the associations between the categorical variables. Student's t-test or Mann-Whitney U-test (without normal distribution) were used to compare the means of the continuous variables. A p-value ≤0.05 was considered as statistically significant.

Results

Clinical data

The characteristics of the studied population are shown in table 1. Two cohorts were established as function of the time of bone storage: early, <3 months; and late, ≥3 months. The main difference between these cohorts was the age of patients, because younger patients were included in the early cranioplasty and the older in the late cranioplasty. It conditioned other parameter showing differences (but not significant) as the bone flap size, or some comorbidities associated to adult population.

When the incidence of complications after cranioplasty was studied, hydrocephalus was significantly more frequent in pediatric patients, and BFR after early cranioplasty. No other differences were statistically relevant in the age groups or in those corresponding to late or early cranioplasty (table 2). The overall rate of BFR was 21.6% (16/74 patients), 56.3% (9/16 patients) of them corresponding to type I and 43.7% (7/16 patients) classified as type II. Five out of 7 type II (71.4%) occurred in patients aged 42.8±16.1 years old, with cranioplasty performed after 32±16.1 days of storage. Hematoma appeared as a complication in 11.9% of adult patients but was absent in the pediatric group.

No significant differences in the incidence of positive microbiological cultures associated to the etiology indicating craniectomy were found (data not shown).

Cutibacterium acnes was the most frequently isolated microorganism from samples taken during bone processing at the tissue bank (table 3). The use of a disinfection protocol was effective in reducing

microbiological load. The incidence of positive microbiological cultures dropped from 18.9% in pre-disinfection samples to less than 6% in both post-disinfection and post-thawing samples.

Surgical site infection after cranioplasty was observed in 6.8% of patients. None of the cranial fragments of these patients provided a positive result in any of the microbiological controls performed during bone flap processing.

In vitro assays

All methods used to determine antibiotic concentrations showed linearity (R^2>0.999), specificity and reproducibility, with variation coefficients <10% between standards. The quantification limits were 29ng/mL for tobramycin, 44ng/mL for vancomycin, 69ng/ml for trimethoprim, 290ng/mL for sulfamethoxazole, and 60ng/mL for fungizone. Only vancomycin was detected in 9 out of 10 samples from the supernatant after incubation of the cranial fragments in saline. The mean value was 4.27±3.97µg/mL. Two groups (5 samples each) were established based on the calculated density of the bone fragment: >1500mg/cm^3, releasing 20.9±24.2ng of antibiotic per gram of tissue; and <1500mg/cm^3, releasing 48.7±23.6ng/g. Nevertheless, this difference was not significant. The bone flap with the longer storage (404 days) yielded the highest vancomycin concentration. No detectable amounts of trimethoprim, sulfamethoxazole, tobramycin, or fungizone were found in any of the analyzed samples.

The fragments of the first 7 bones for the bacterial growth inhibition assay, which were seeded on Muller-Hinton agar medium, yielded negative results. Since the bone contact with the strains of microorganisms occurred mainly through the cortical with this culture system, it was decided to immerse the remaining fragments (3 bones) in a liquid medium. With this system, allowing contact with the cancellous bone, bacterial growth inhibition was observed in some cases: 1/3, *Enterococcus faecalis*; 3/3, *Escherichia coli*; 0/3, *Pseudomonas aeruginosa*; 1/3, *Staphylococcus aureus*; and 0/3 *Candida albicans*.

Seven out of 19 cultures seeded with bone explants showed cell growth with spindle-shaped morphology and, in 5 of those primary cultures, the colonies reached enough growth to perform the assay for osteogenic differentiation. The medium with osteogenesis-inducing factors led to calcium deposition on the colonies (figure 2). The presence of living cells was significantly correlated with an early processing at the tissue bank. Nevertheless, BFR after cranioplasty and a positive microbiological culture were not affected by this circumstance (table 4). Similarly, the duration of storage (which lasted from 7 days to 1,345 days in the cell culture assay) did not influence the growth of cell culture (positive, 11.3±7.2months; negative, 12.4±9.7months).

Cooling and warming rates are showed in figure 3.

Discussion

The clinical efficiency of autologous cranioplasty after decompressive craniectomy has been validated by different studies. Nevertheless, the incidence of complications has prompted the research to identify predictive factors to reduce these risks.[5,7,8,10-15] Parameters associated with tissue processing are not usually included among the factors analyzed.

BFR is a complication which can lead to the loss of the autograft. In some cases, depending on its grade and extension, it can require revision surgery for bone replacement. Therefore, adding a new risk to the patient and a significant increase of the cost by using expensive synthetic grafts. Its incidence varies from 2.5% to 50%.[5,8,14-18] The variations in these rates may be attributed to the criterion used to define this complication, as well as the duration of the follow-up (the longer the period the higher the rate). Kim et al.[15] highlighted the need of a standard definition that contained radiologic evidence and the functional status of bone remodeling. Korhonen et al.[19] proposed a score and grading (I, II, III) system aimed to standardize the definition of BFR. The system was based on these variables: extent, estimated remaining bone volume; severity, possible perforations and their measured diameter; and focus, the number of BFR foci within the bone flap. In a recent study, using 3D image analysis system, different grades of BFR have been observed in 77% of patients.[20]

Our overall rate of BFR (26.1%) can be considered as an intermediate level, compared to the limits of the range provided by literature on this subject (2.5% to 50%). In our study, the only significant factor associated with this complication was early cranioplasty, which represented 13/16 cases (81.2%) (table 2).

Anatomic locations for bone flaps, fragmentation, and the use of ventriculoperitoneal shunt implantation for management of hydrocephalus, have been associated with a higher risk of BFR,[8,16,17,22] but not as independent predictive factors.[15]

Indeed, the concept itself has been discussed, proposing two possible primary mechanisms: direct bone resorption by osteoclastic activity or enzymatic degradation of the collagenous matrix, highlighting a disarrangement of the normal physiologic process of bone resorption;[21] or due to avascular necrosis.[19]

In an effort to control the incidence of BFR for patients at high risk of developing this complication, the use of preventive measures, such as the use of angiotensin-converting enzyme inhibitors (as an attenuant of osteoporosis) has been suggested.[23]

BFR is most frequently observed in pediatric patients[24-26], but this was not the case in our study population, which showed a rate of 13.3% (2/15) in the pediatric patients, and 23.7% (14/59) in adults. Rocque et al.[26]

estimated a resorption rate of 1% lower per month older. Several reasons have been argued to explain the increased risk for that population:

- Trauma due to accidents (car, bicycle, and scooter) is the most common indication for craniectomy.[11]
- The thinner and rapidly growing cranium, and comminuted fractures hindering to fit the bone flap.5[,8,27]
- The compressive force of the tightened scalp.[21]

Then, in these patients, shortening the time to cranioplasty could be a strategy to avoid difficulties for close approximation of the fragments.[28] In fact, younger age and early cranioplasty have been related to improved outcome.[29] In our center, BFR in pediatric patients from the early cohort was present in 16.7% (2/12 patients) while it was absent in the late cohort (only including 3 patients, so the comparison was not representative).

But what is the best timing to perform cranioplasty? The strategy of performing cranioplasty as soon as clinically safe and feasible seems to be useful to reduce the incidence of complications. A period of three months has been established to consider as early the time between craniectomy and cranioplasty.[7,8,12,30,31] It has been observed that very early cranioplasty (within 45 days after craniectomy) might minimize the risk of some complications whereas late cranioplasty (>90 days) may minimize hydrocephalus but may increase the risk of seizure.[13,32] Conversely, Borger et al.[33] found a significantly higher complication rate in adult patients with early cranioplasty. These authors suggested the consideration of a bias related to the inclusion of patients tending to have prolonged duration of brain swelling and a generally more complicated clinical course. We observed that most of the type II necrosis occurred in adult patients who underwent cranioplasty in the first month after craniectomy. Additionally, in case of early cranioplasty, which can disrupt the progression of the wound healing process, the risk of infection must be controlled.[12,34,35] In any case, there is a growing trend to consider that the issue of the timing might not be a significant factor, as previously thought.[8]

Infection is another complication which must be controlled. Discarding the bone flaps with positive microbiological cultures is a common practice in some neurosurgical teams and tissue banks.[3] It is performed regardless of the nature or degree of contamination. However, no correlation between microbiological culture results and infection status has been demonstrated.[35-37] Operative factors has been highlighted as more important than low numbers of skin flora contaminating the bone flap. In general, allografts must not be considered as sterile unless a validated method to achieve this condition was used.

Our rate of infection (7.5%) can be considered within the expected range showed by other similar studies (ranged from 0% to 25.9%). The microorganisms isolated in the present study are the same as those mentioned in other articles.[3,35-37]

The convenience of antibiotic-impregnated cranioplasties has been suggested in patients at high risk of infection.[38] Our results supported the presence of residual antibiotics in disinfected bone flaps. However, there was no inhibition of the bacterial growth when only the cortical bone was in contact with the culture media. Winkler et al.[398] observed that cortical bone was less accessible to antibiotics than cancellous bone. Moreover, they determined different release kinetics for vancomycin and tobramycin from cancellous bone. The reason why only vancomycin was detected in our work is still unclear but might be related to a higher affinity of some antibiotics for the tissue than for the aqueous solvent. This hypothesis would correlate with the results of the microbial inhibition assay, which suggested the presence of other antibiotics in bone. So, further research will be required to study the affinity of antibiotics for bone and their release rates.

Regarding the relevance of cell viability in the autologous bone flaps, previous works have shown *in vitro* cell growth from explants of fresh samples, but no cell growth has been detected after storage by freezing.[40-42] We had previously observed cell outgrowth from frozen bones.[43] In the present study, the presence of osteogenic cells has been observed in some cultures. Considering that cultured bone explants represented approximately 2% of the entire bone flap, these results support a change regarding the existence of living cells in skull autografts. Additionally, since cell viability in cortical bone is significantly reduced in absence of blood supply, the source for cells growing in culture must be the cancellous bone, which represents a low proportion in the skull. This cancellous bone contains cells with osteogenic potential which can be nourished by diffusion. But the heat generated on bone edges by the high-speed-drill during craniectomy can reduce its porosity (figure 4). Consequently, it could hamper the contact between bones after grafting, hindering revascularization and the migration of osteoprogenitor cells from the recipient into the graft (osteoconduction). For this same reason, the penetration of disinfectant or cryoprotectant solutions into the bone through its edges could also be affected.

Our results also support the use of cryoprotectant agents, in accordance with Fan et al.[44] who found similar microscopic features in DMSO-cryopreserved bone flaps compared to normal fresh skull bone. However, cell viability may be more a consequence of the cooling and warming rates used for freezing and thawing, respectively. The diffusion of cryoprotectant would only reach cancellous stroma at a few millimeters from the rim (being the cortical tables impermeable). Direct freezing at -80ºC yielded a cooling rate around 1.5ºC/min, which is well known to preserve cell integrity.[45] Conversely, thawing at room temperature would not seem an advisable strategy with that aim.

Finally, the presence of bioactive substances to stimulate bone repair in the calvaria should be considered. Unlike other bones in which bone healing is subjected to stimuli from cross-talking between biochemical and

mechanical signals, in the human calvaria the cranial environment does not receive enough physiologic stress and therefore bone healing is promoted mainly by biochemical signaling pathways. The union between bone flap and host bone depends on several elements, which comprise *the regenerative pentagon*: cellular environment, growth factors, bone matrix, mechanical stability and vascularisation.[46] The absence of one or more of these factors predisposes the development of a non-union, which in cranial bone flaps leads to BFR. Frozen bone is well-known to provide osteoconduction and to retain bone morphogenetic proteins from bone matrix, so providing osteoinductive property (differentiation of osteoprogenitor cells in osteoblasts). Considering our findings, osteogenic potential (associated to living cells into the bone flap) could also be deemed in cryopreserved bone flaps.

To reinforce the signaling pathway for bone healing, Anto et al.[47] developed a strategy based on incubating the bone flap (overnight, the day prior to cranioplasty) in PRP harvested from the patient's blood.

The limitations of this study included its retrospective design and single-center nature, the absence of intraoperative factors (as duration of surgery) and the lack of data on the neurological status of patients (such as Glasgow outcome score) and on ventricular width prior surgery. Another limitation was the sample size, which conditioned the strength of the analysis.

Conclusions

Our clinical results are consistent with other studies. The outcome after autologous cranioplasty is a multifactorial process, which is modulated by patient- (age, etiology for craniectomy, comorbidities, smoking, alcohol abuse, elevated body mass index, neurological status), and surgery-related (previous reoperation, operating time, surgeon's skill, rigid fixation, ventriculoperitoneal shunt) factors.

Otherwise, intrinsic factors to bone flap (bone fragmentation, bone size, presence/absence of living cells, close contact with the bone host, storage duration, osteoinductive and osteoconductive potential) must also be considered to improve its incorporation.

Antibiotic-disinfected and DMSO-cryopreserved cranial bone flap is not merely a passive scaffold since it seems to carry on living cells, bioactive molecules, and antibiotic. Exact contiguity between the edges (bone graft/host bone) appears as a key factor which can positively impact outcome.

Further evaluation from prospective and large-scale studies will be needed (with close collaboration between neurosurgeons and tissue bankers) to clarify remaining controversies about the predictive value of some factors and to elucidate the role of bone processing factors.

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CRediT author statement

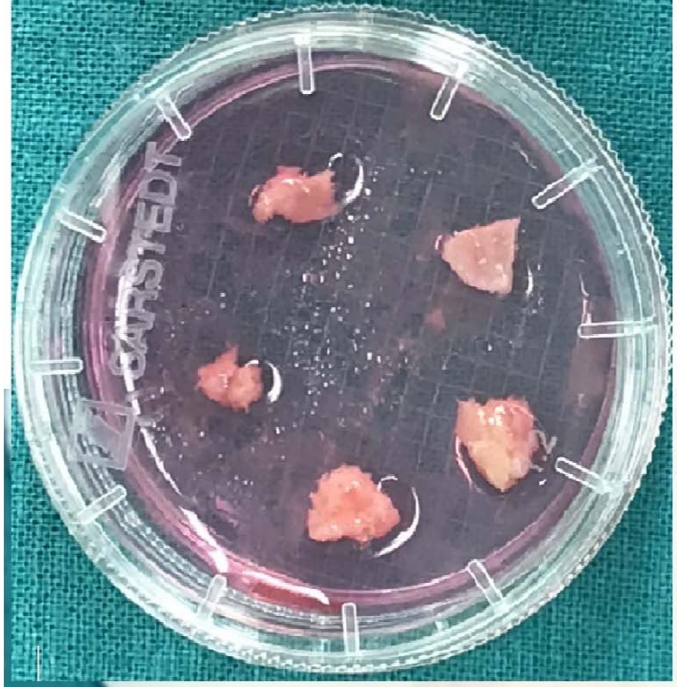
Vicente Mirabet: Conceptualization, Methodology, Supervision, Project Administration, Writing-Reviewing and Editing. Daniel García: Writing-Reviewing, Supervision, Data Curation, Investigation. Amparo Roca: Writing-Reviewing, Data Curation, Investigation. Arnold R Quiroz: Writing-Reviewing, Data Curation, Investigation. Joan Antón: Writing-Reviewing, Data Curation, Investigation. Miguel Rodríguez-Cadarso: Writing-Reviewing, Data Curation, Investigation. Dolores Ocete: Methodology, Investigation, Writing-Reviewing. Lucas Aranda: Investigation, Writing-Reviewing. Ana Melero: Methodology, Investigation, Writing-Reviewing. Antonio J Guillot: Investigation, Writing-Reviewing. Nuria Yagüe: Methodology, Investigation, Writing-Reviewing. Isabel Guillén: Investigation, Writing-Reviewing. Carlos Botella: Conceptualization, Writing-Reviewing, Supervision, Project Administration.

Figure 1. Cancellous bone chips were collected using fine rongeurs and seeded as explants on the culture dish.

Figure 2. Outgrowth of cells from bone explants (left). Cell colony showing calcium deposits stained with alizarin red (right). 200x

Figure 3. Cooling and warming curves. Data recorded by a thermocouple probe placed into the cancellous bone, between the two cortical tables. IB: insulating box (volume: 7,7L; wall thickness: 2,4cm). WB: water bath.

Figure 4. Bone flap edge showing: A, unaffected cancellous bone matrix; B, heat-denatured cancellous bone matrix; C, the bone in A after washing thoroughly; D, the bone in B after washing thoroughly.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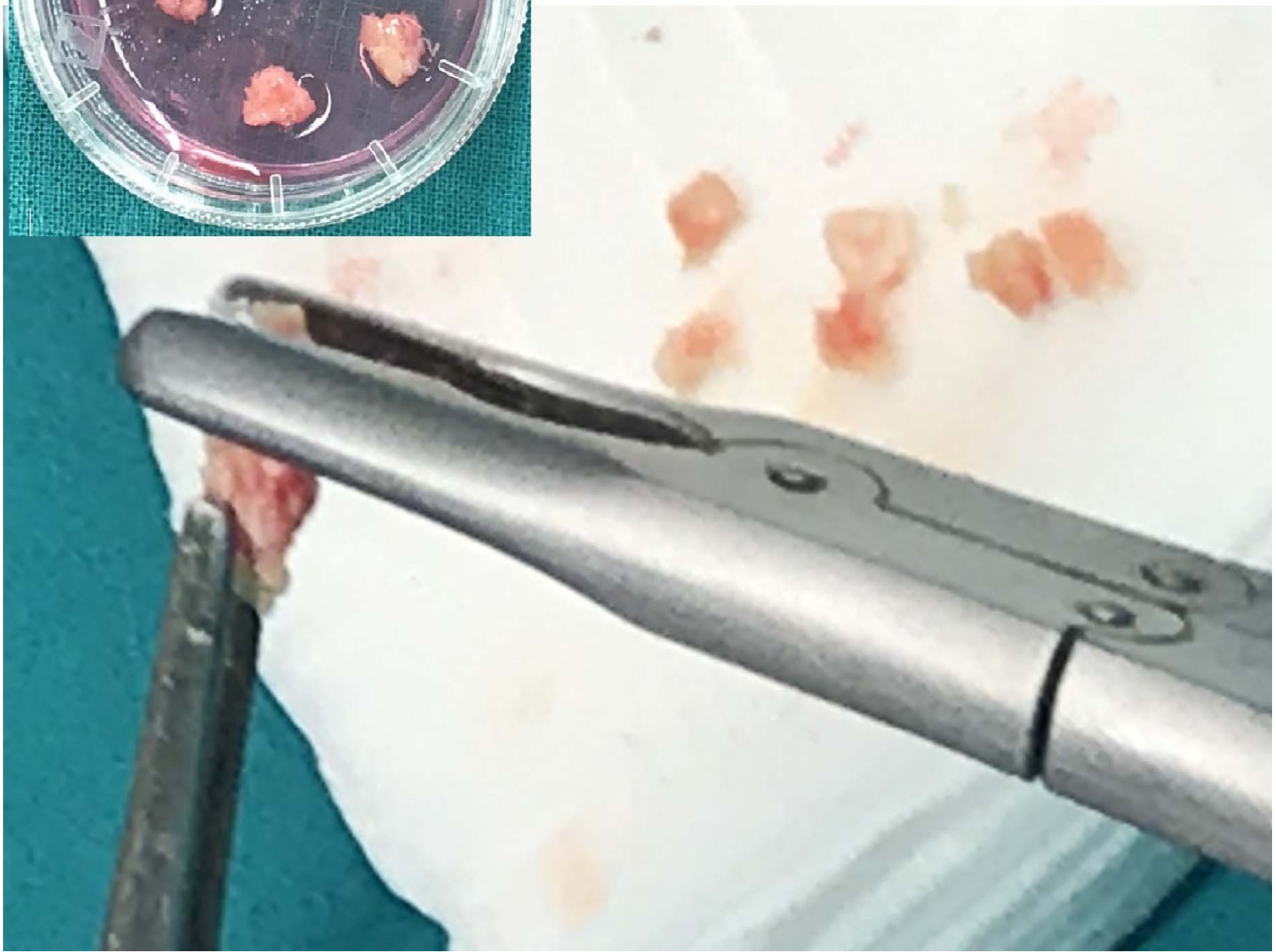

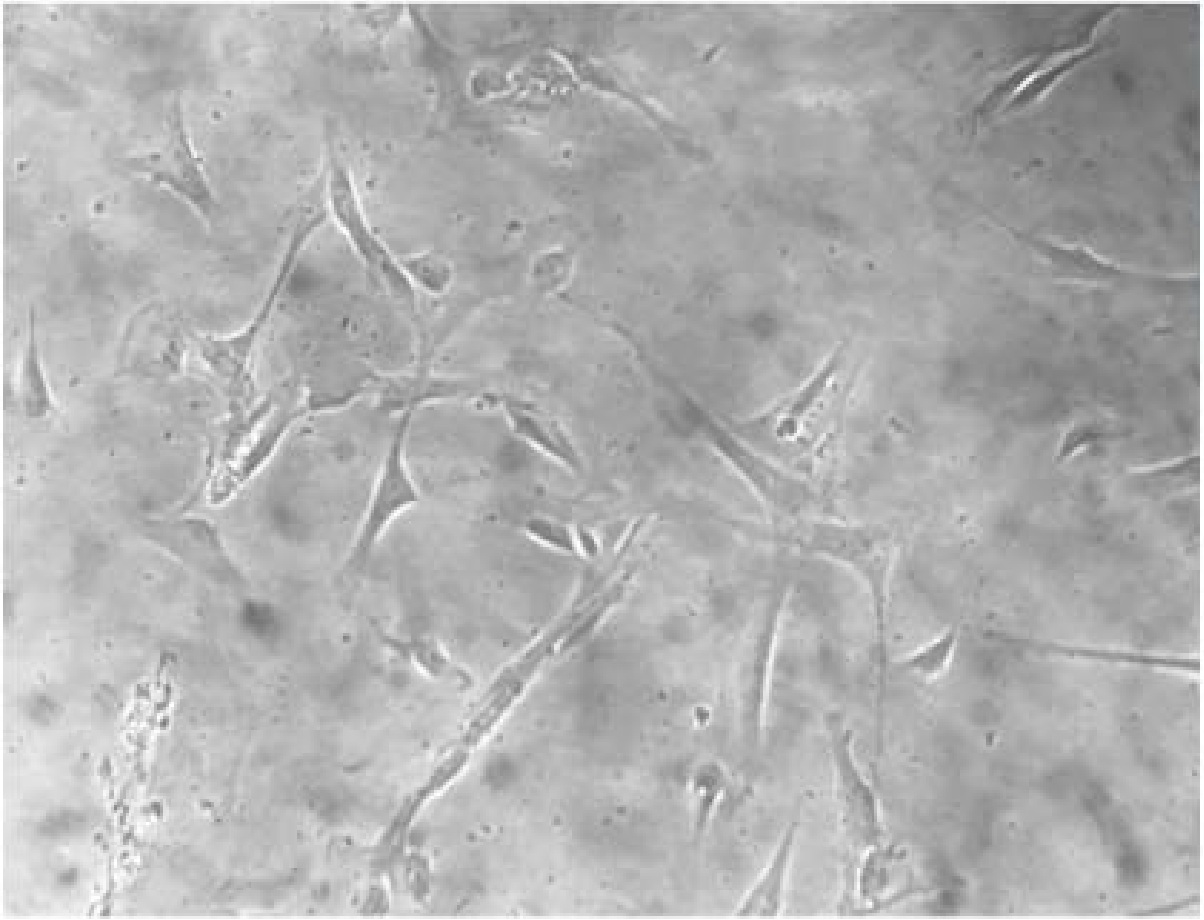

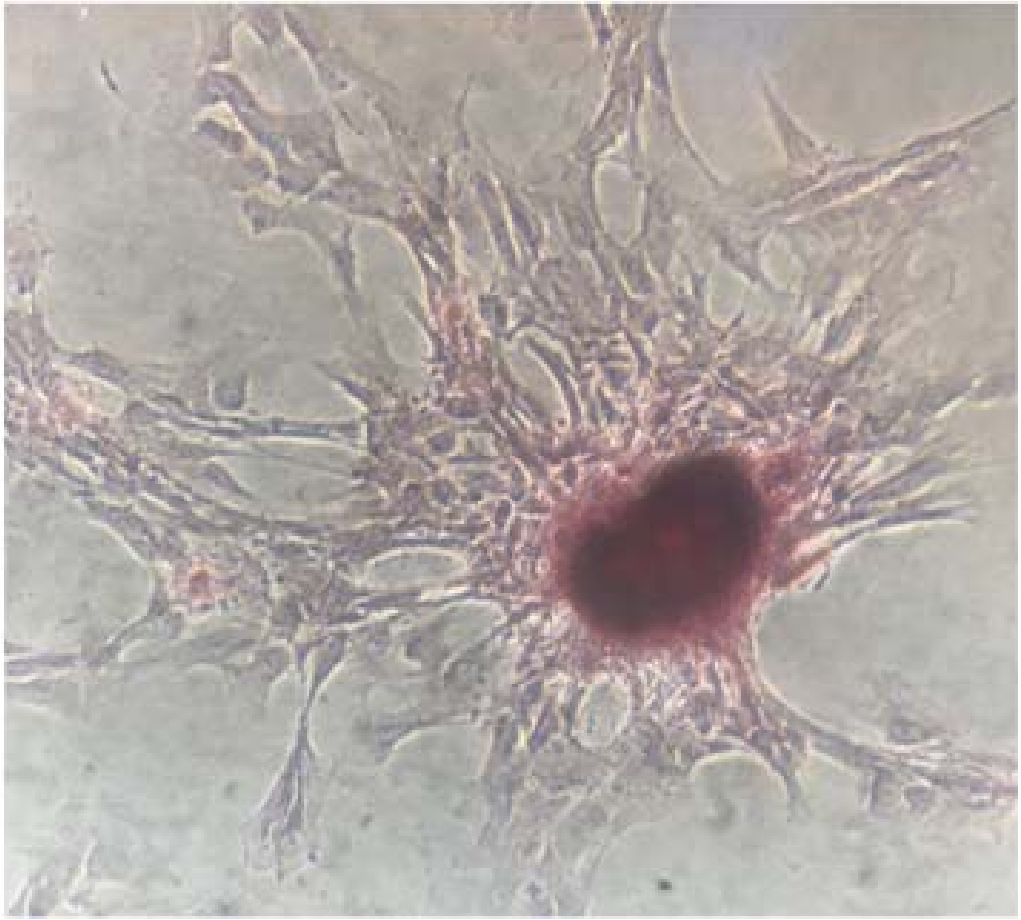

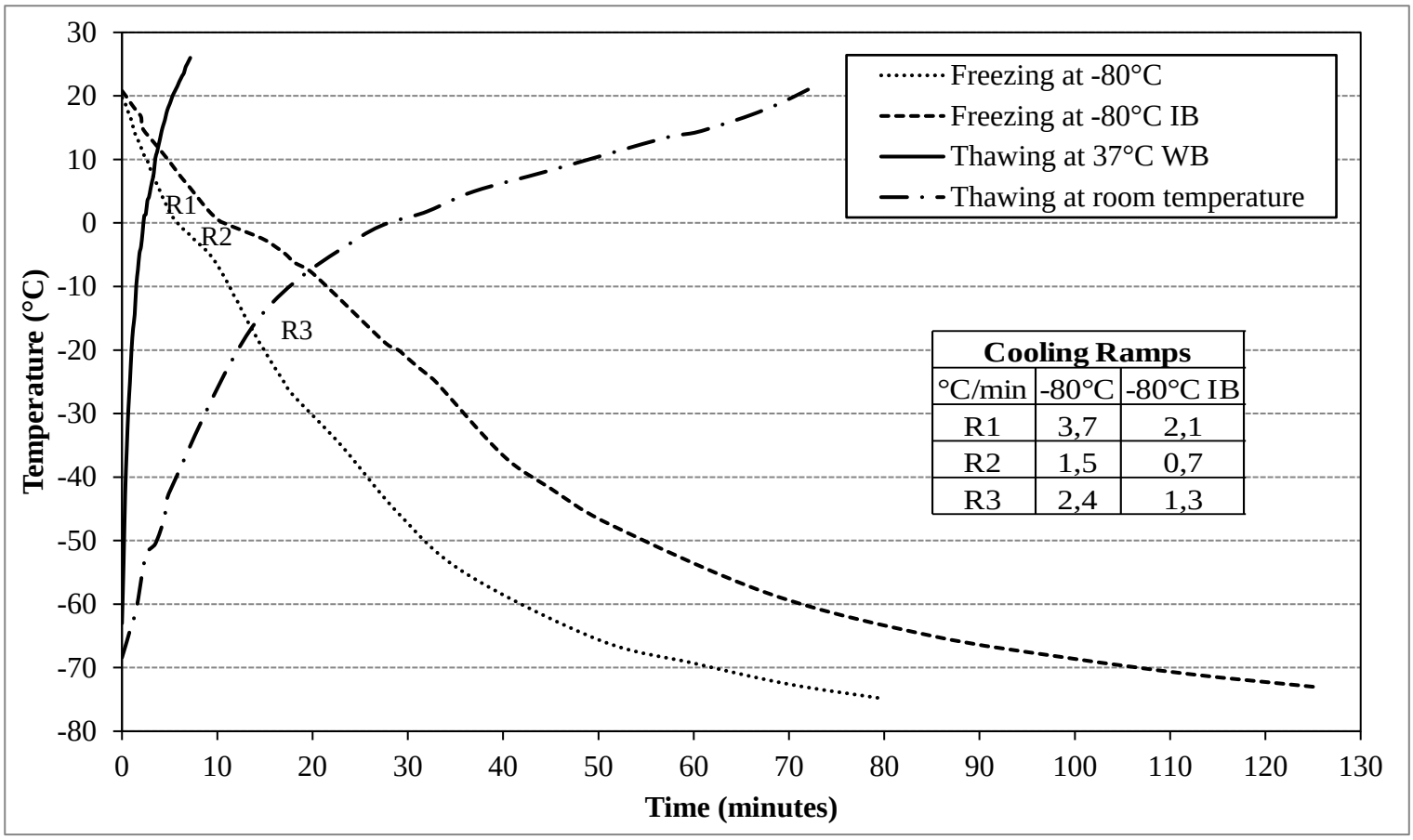

Cooling Ramps		
°C/min	-80°C	-80°C IB
R1	3,7	2,1
R2	1,5	0,7
R3	2,4	1,3

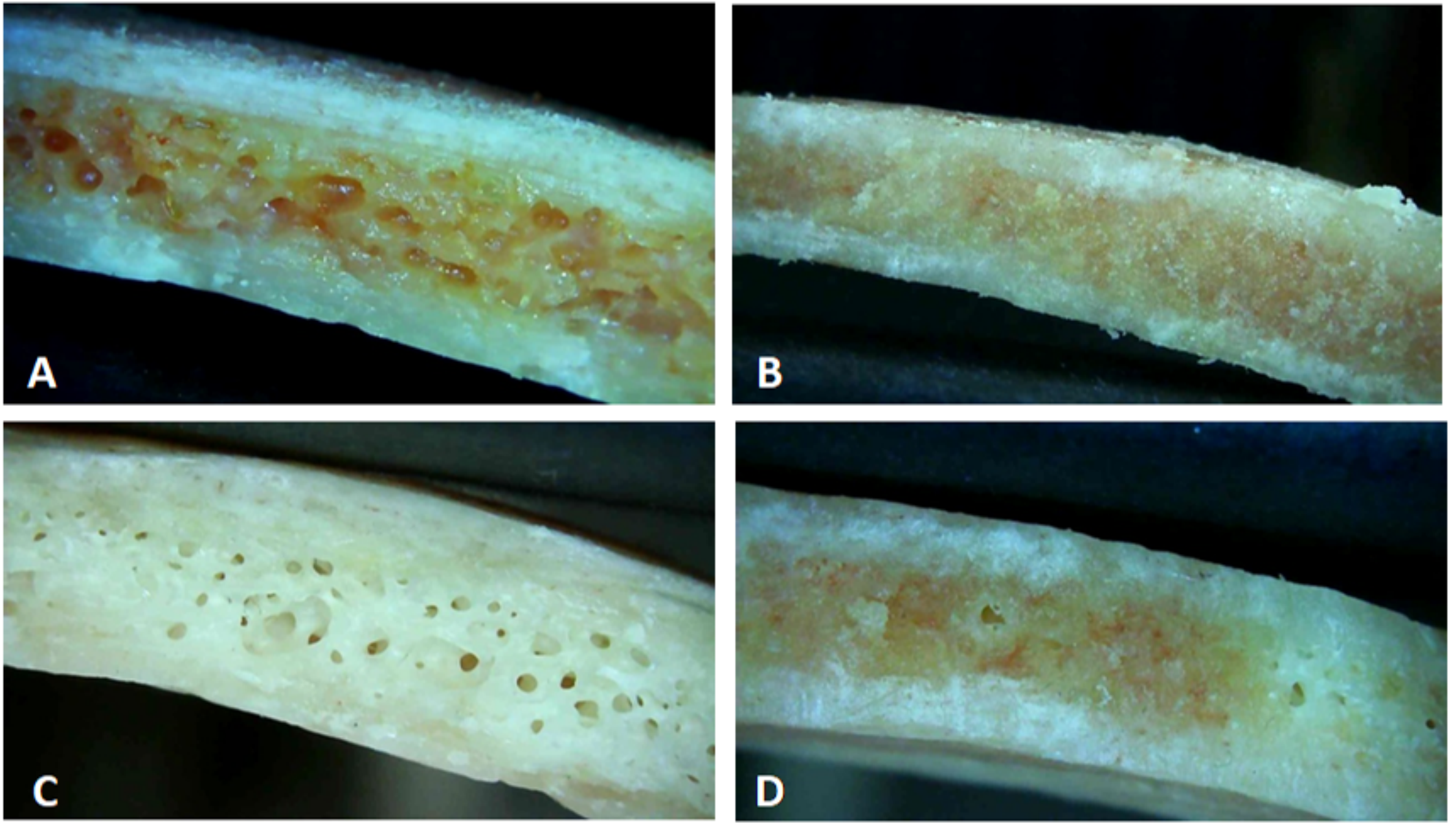
A
B
C
D

Table 1. Comparative baseline characteristics of all the patients and grouped in the early and late cranioplasty cohorts. Values represent percentage of patients (%) or mean±standard deviation.

Patient and bone flap characteristics	All	Time to cranioplasty		p Value
		Early <3 months n= 40	Late ≥3 months n= 34	
Age at craniectomy				
Mean (years)	39.7±19.6	35.1±20.2	45.2±17.7	0.024
<16 years (%)	20.3	30	8.8	
16-59 years (%)	63.5	62.5	64.7	
≥60 years (%)	16.2	7.5	26.5	
Male patients (%)	56.8	52.5	61.8	0.423
Smoking (%)	24.3	17.5	32.4	0.138
Alcohol abuse (%)	6.8	5	8.8	0.656
Diabetes mellitus (%)	5.4	5	5.9	0.867
Obesity (CMI>30) (%)	13.5	12.5	14.7	1.000
Arterial hypertension (%)	32.4	22.5	44.1	0.048
Ventriculoperitoneal shunt (%)	13.5	10	17.6	0.497
Indication for craniectomy (%)				
Traumatic edema	5.4	10	0	0.120
Traumatic intraparenchymal hematoma	9.5	7.5	11.8	0.696
Subarachnoid hemorrhage	10.8	15	5.9	0.275
Ischemic stroke	20.3	12.5	29.4	0.071
Tumor	10.8	17.5	2.9	0.063
Spontaneous intraparenchymal hematoma	25.7	17.5	35.3	0.081
Subdural hematoma	16.2	20	11.8	0.338
Infection	1.4	0	2.9	0.459
Mean bone flap size (cm^2)	71.6±16	68.3±16.2	72.96±18.2	0.085
Bone flap fragmentation (%)				0.394
Present	18.9	22.5	14.7	
Absent	81.1	77.5	85.3	
Mean time to cranioplasty (weeks)	25.1±31.2	6.3±3.5	47.3±34.7	
Mean follow-up after cranioplasty (months)	20.2±18.6	19.54±18.5	21.07±18.9	0.782

Table 2. Incidence of complications in patients grouped by time between craniectomy and cranioplasty (early and late) or age at craniectomy. Values represent percentage of patients (%).

Type of complication	Time to cranioplasty			Age (years) at craniectomy			
	Early <3 months n= 40	Late ≥3 months n= 34	p Value	<16 n= 15	16-59 n=47	≥60 n= 12	p Value
Infection (%)	10	2.9	0.366	6.7	6.4	8.3	0.971
Bone necrosis (%)			0.008				0.407
Type I	17.5	5.9		6.7	14.9	8.3	
Type II	15	2.9		6.7	12.8	0	
Reoperation (%)	17.5	23.5	0.521	20	23.4	8.3	0.511
Hematoma (%)							
Epidural	2.5	8.8	0.328	0	6.4	8.3	0.564
Subdural	2.5	2.9	1.000	0	2.1	8.3	0.382
Intraparenchymal	0	2.9	0.459	0	0	8.3	0.073
Hydrocephalus (%)	10	8.8	1.000	26.7	4.3	8.3	0.035

Table 3. Incidence of positive microbiological cultures depending on the source of the sample (washing or cryoprotective solutions). The isolated microorganisms were coagulase negative Staphylococcus (CNS), Cutibacterium acnes (CA), and Corynebaterium spp (CO). Values represent percentage of positive results (%).

Sample	Positive culture	CNS	CA	CO
Washing solution (pre-disinfection)	18.9%	35.7%	50%	14.3%
Cryoprotective solution (post-disinfection)	4%	0%	100%	0%
Washing solution (post-thawing)	5.4%	25%	75%	0%

Table 4. Influence of the time between harvesting and the start of bone processing on explant culture, bone necrosis after re-implantation, and microbiological culture of solutions in contact with the tissue.

	Time between collection to processing	p Value
Cell growth (n=17)		0.003
Positive	4.4±3.6 hours	
Negative	12.5±4.9 hours	
Bone flap resorption (n=74)		0.536
Absent	12.5±7.2 hours	
Present	13.8±6.6 hours	
Microbiological culture (n=74)		0.928
Negative	12.7±6.6 hours	
Positive	12.8±8.7 hours	