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1 **Ibuprofen-loaded calcium phosphate granules: a new bone substitute for local**  
2 **relieving symptoms of osteoarthritis**

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33 **ABSTRACT**

34 Musculoskeletal diseases often demand a drug treatment at the specific site of injury or  
35 defect site. In this context, the use of calcium phosphates is attractive as it allows both the  
36 bone substitution and the local delivery of a drug substance. In this work, we present a  
37 drug delivery device that combines calcium phosphate bioceramic granules and  
38 ibuprofen, a widely used anti-inflammatory drug.

39 After verifying *in vitro* biocompatibility of the ibuprofen-loaded calcium phosphate granules  
40 on murine preosteoblastic cells (MC3T3), we evaluated *in vitro* efficiency of the drug  
41 substance released from the bioceramic using rheumatoid arthritis synoviocytes. Our data  
42 document that ibuprofen-loaded calcium phosphate granules reduced inflammatory  
43 response and increased apoptosis of synoviocytes. *In vivo* study showed that both  
44 unloaded, and ibuprofen-loaded calcium phosphate granules induced a progressive  
45 osteogenesis, but in the case of ibuprofen-loaded implants, bone ingrowth was more  
46 limited in first weeks. However, as far as concerns inflammation, while unloaded granules  
47 showed inflammation up to 4 weeks, ibuprofen loaded granules did not show any  
48 significant inflammation. Ibuprofen concentration determination in blood samples showed  
49 that a very small amount of the drug reached the general circulation which render this  
50 drug delivery system suitable for both bone substitution and reduction of inflammation at  
51 the implantation site.

52 Thus, this new drug carrier could be used to locally relieve inflammatory bone diseases  
53 symptoms including rheumatoid arthritis but, beyond this study, this kind of granules could  
54 be considered for the delivery of therapeutic agents such as antibiotic, analgesic or  
55 anticancer drugs.

56 **Keywords:** Drug delivery system; Ibuprofen; Calcium Phosphate bone substitute; *in vitro*  
57 and *in vivo* anti-inflammatory effect; Rheumatoid Arthritis.

58

## 59 **1. Introduction**

60 Inflammatory diseases represent one of the major health concerns worldwide. In  
61 particular, rheumatoid arthritis (RA) is an inflammatory joint disease related to bone and  
62 cartilage degradation and progressive disability [1, 2]. In the past two decades, several  
63 studies focused on RA pathogenesis and risk factors to the development of the disease.  
64 Thus, genetics, sex and environmental factors are now well described as risk factors [1].  
65 More recently, epigenetics, particularly of genes involved in immunity, have been linked to  
66 RA pathogenesis [3]. Synovial membrane inflammation due to invasion of immune cells  
67 and activation of both resident immune cells and fibroblast-like synoviocytes (FLS) lead to  
68 osteoclastogenesis and chondrocyte catabolism which are responsible for articular  
69 destruction [4]. It is well established that cytokines and chemokines in the inflammatory  
70 milieu of synovial compartment are essential in RA pathogenesis [5]. Among these  
71 mediators, tumor necrosis factor (TNF), interleukin-1 (IL-1) and IL-6 play key roles in this  
72 process. They are described to be responsible for the production of high levels of matrix  
73 metalloproteinases by synovial cells and chondrocytes [6]. However, other cytokines such  
74 as IL-8 which have been described to promote the formation of new blood vessels in  
75 synovial membrane may also play a role in RA pathogenesis [7] [7-9]. As far as concerns  
76 therapeutic strategies, reversing inflammation remains the main approach in this disease.  
77 Thus, disease-modifying antirheumatic drugs (DMARDs) such as glucocorticoids target  
78 inflammation and offer symptomatic and disease-modifying effects. Non-steroidal anti-  
79 inflammatory drugs are also used to relieve disease symptoms but are not disease-  
80 modifying [10]. Given key roles played by TNF and IL-6 in RA development and  
81 progression, biological DMARDs neutralizing both cytokines have been developed to treat  
82 the disease [11-13]. Despite real advances in therapeutic strategies, current treatments

83 are hindered by numerous side effects, due to the chronic nature of the disease, often  
84 requiring treatment for extended duration.

85 A major improvement would consist in providing adapted drug concentrations at the site  
86 of action with limited systemic toxicity. This highlights the need for a drug delivery system  
87 (DDS) that would function as a reservoir for drug gradual release in the vicinity of the  
88 damaged tissue [14, 15]. As previously described, RA is associated with both  
89 inflammation and bone degradation. In the present study, we propose to use a bone  
90 substitute to deliver an anti-inflammatory drug. Among the synthetic materials used for  
91 bone tissue replacement and regeneration, calcium phosphates have attracted extended  
92 attention because of their biocompatibility, osteoconductivity and similarity to the natural  
93 bone mineral phase [16-19]. In addition, several works have focused on their use as drug  
94 carriers [14, 19-29].

95 In this work, porous calcium phosphate granules loaded with ibuprofen, a widely used  
96 non-steroidal anti-inflammatory drug (NSAID), are considered for their potential to locally  
97 treat inflammatory symptoms and replace damaged bone tissue. *In vitro*, loaded granules  
98 reduced significantly human RA fibroblast-like synoviocytes (FLS) proliferation and both  
99 IL-6 and IL-8 pro-inflammatory cytokines production. *In vivo*, implantation of loaded  
100 granules in osseous site also demonstrated anti-inflammatory effect, without preventing  
101 osteogenesis around the implants.

102

## 103 **2. Materials and methods**

### 104 **2.1 Preparation of ibuprofen-loaded calcium phosphate granules**

105 Porous calcium phosphate granules (710-1000 $\mu$ m) were fabricated by high shear wet  
106 granulation (Mi-Pro, Pro-C-epT, Zelzate, Belgium) as described in previous works [30,  
107 31]. Briefly, 90% calcium phosphate (Cooper, France) was granulated with 10%

108 pregelatinized starch (Sepistab ST 200, Seppic, France) also acting as a pore former  
109 after removal by heat treatment at 900°C (Kanthal Super, Rapid High Temperature  
110 Furnace, Bulten-Kanthal, Sweden). The resulting porous granules showed a regular and  
111 spherical shape (Supplementary data, eFigure 1) and were composed mainly of  $\beta$ -  
112 tricalcium phosphate as shown by X-ray diffraction patterns (Supplementary data, eFigure  
113 2). Mercury porosimetry measurements (Autopore IV 9500, Micromeritics Instruments  
114 inc., Norcross, GA) indicated a total porosity equal to 58% and composed of 2 pore size  
115 diameter ranges: 100-400 nm and 400-1600 nm (Supplementary data, eFigure 3).  
116 Previous work has shown that this intragranular porosity allows the loading of the  
117 therapeutic substance and the control of its release according to a diffusion mechanism  
118 [31].

119 For the drug loading, 3.5 g of porous granules were immersed in 20 mL of an ethanolic  
120 ibuprofen solution (100 mg.mL<sup>-1</sup>) (Ibuprofen 50, BASF, Germany). The solvent was then  
121 evaporated using a Rotavapor (Büchi, Switzerland) and the granules were vacuum-dried  
122 at 20°C in an oven during 14h. The drug content in the loaded granules was equal to 36%  
123 [31]. For the granules used for *in vitro* and *in vivo* experiments, sterilization at 200°C for  
124 3h (UL 30, Memmert, Germany) was performed before drug loading.

## 125 **2.2. *In vitro* study**

126 Before investigation of the *in vitro* effect of ibuprofen released from the porous granules, a  
127 preliminary study (Supplementary data, eTable 1 and eFigure 4) was realized to verify the  
128 stability of the drug substance in the culture medium. Results indicated that ibuprofen was  
129 degraded in the presence of foetal calf serum (FCS), so release of the drug was  
130 performed in culture medium without FCS (called medium 3 in the following). Complete  
131 medium was used only after 6 hours contact time with the cells (the time estimated to be  
132 necessary for ibuprofen to enter into the cells).

133 Release of ibuprofen was established using 20 mg of ibuprofen-loaded granules put into a  
134 cell culture insert (Greiner) and immersed in 2 mL of medium 3. Results indicated that,  
135 after a lag time of about 20 minutes, ibuprofen concentration increased in the medium to  
136 reach a concentration of 3.6 mg.mL<sup>-1</sup> at 540 minutes, corresponding to a 100% release.  
137 The delay observed at the beginning can be related to the time necessary for the diffusion  
138 of ibuprofen through the porosity of the insert. The 100% release of ibuprofen confirmed  
139 results obtained previously in other experimental conditions [30] which demonstrated the  
140 availability of the total loaded ibuprofen quantity.

### 141 **2.2.1. Preparation of rheumatoid arthritis synoviocytes**

142 Rheumatoid arthritis (RA) synoviocytes were isolated from fresh synovial biopsies  
143 obtained from six RA patients undergoing hip arthroplasty. All patients fulfilled the 1987  
144 American Rheumatism Association criteria for RA. The mean age of the patients was 61.2  
145 ± 3.6 years (range 58–67 years). The mean disease duration was 8.4 ± 2.1 years. At the  
146 time of surgery, the disease activity score (DAS 28) was greater than 3.2. These activities  
147 were approved by local institutional review boards, and all subjects gave written informed  
148 consent. Synovia were rinsed and digested with 1.5 mg.mL<sup>-1</sup> collagenase-dispase,  
149 1 mg.mL<sup>-1</sup> hyaluronidase, and 0.15 mg.mL<sup>-1</sup> DNase I for 3–4 hours at 37°C as previously  
150 described [32]. After centrifugation, cells were resuspended in DMEM supplemented with  
151 10% fetal calf serum (FCS), 2mM L-Glutamine, 100 U.mL<sup>-1</sup> penicillin, and 100 µg.mL<sup>-1</sup>  
152 streptomycin (Gibco BRL) in a humidified atmosphere containing 5% (v/v) CO<sub>2</sub> at 37°C.  
153 After 48 hours, non adherent cells were removed. Adherent cells (macrophage-like and  
154 fibroblast-like synoviocytes) were cultured in complete medium, and, at confluence, cells  
155 were trypsinized and only the fibroblast-like synoviocytes (FLS) were passed.

### 156 **2.2.2. Cell culture**

157 MC3T3 cells were purchased from the American Type Culture Collection (LGC  
158 Standards, Middlesex, UK). Cells were grown in Iscove's Modified Dulbecco's Medium  
159 (IMDM) supplemented with 10 % fetal calf serum (ID Bio, Limoges, France) and  
160  $100 \text{ U.mL}^{-1}$  penicillin and  $100 \mu\text{g.mL}^{-1}$  streptomycin (Gibco BRL).  
161 RA FLS were cultured 45–60 days before experimentation. This delay allowed the  
162 elimination of all possible interactions resulting from any preoperative treatment (with  
163 NSAIDs, analgesics, DMARDs, or steroids). Between passages 4 and 8, RA FLS were  
164 trypsinized. Cell count and viability were determined and cells were seeded in culture  
165 plates or flasks (Sarstedt). For all experiments, RA FLS were allowed to adhere and grow  
166 for 48 hours in culture medium before exposure to treatment.

### 167 **2.2.3. Cell treatment**

168 Cells were seeded at  $1.10^4.\text{cm}^{-2}$  for MC3T3 or  $3.10^3.\text{cm}^{-2}$  for human RA FLS in 24-well  
169 culture plates and were allowed to adhere and grow for 48h. Then 20 mg of ibuprofen-  
170 loaded granules or unloaded granules were put into cell culture insert (Greiner) and  
171 placed in the wells of the 24-well culture plate for 45, 75 and 135 minutes. Of note that  
172 2 mL of culture medium 3 were used (Supplementary data, eTable 1 and eFigure 4). After  
173 45, 75 and 135 minutes, inserts were removed. Then, after 6 hours (the time estimated to  
174 be necessary for ibuprofen to enter into the cells) the medium was removed and replaced  
175 by a complete medium (medium 4 with 2mM L-Glutamine) for 24 and 48h (Supplementary  
176 data, eTable 1 and eFigure 4). Of note, that cells were also treated with an ibuprofen  
177 solution at  $0.5 \text{ mg. mL}^{-1}$ , following the same protocol treatment as for ibuprofen-loaded  
178 granules. This concentration was retained on the basis of ibuprofen release trial  
179 (Supplementary data, eFigure 5) that showed a concentration variation between 0.1 and  
180  $0.7 \text{ mg.mL}^{-1}$  for a 45 to 135 minutes immersion time. The ibuprofen solution was freshly



181 prepared in medium 3 before each experiment. In all experiments, the unloaded granules  
182 and ibuprofen solution treatment represented respectively negative and positive control.

#### 183 **2.2.4. Cell proliferation and viability**

184 Cell proliferation and viability of MC3T3 or RA FLS were measured using the MTT assay.  
185 Cells were seeded in 24-well culture plates and grown for 48 hours before exposure to  
186 treatment for 24 and 48 hours. MTT was carried out daily as previously [33] described  
187 and experiments were performed in sextuple assays.

#### 188 **2.2.5. Apoptosis quantification: DNA fragmentation**

189 Cells were cultured and treated as described previously, then apoptosis was quantified at  
190 24 and 48h by analyzing DNA fragmentation using the Cell Death Detection ELISA plus  
191 (Roche Diagnostics). Cytosol extracts were obtained in accordance with the  
192 manufacturer's protocol and apoptosis was measured as previously described [34].

#### 193 **2.2.7. IL-6 and IL-8 production**

194 IL-6 and IL-8 proinflammatory cytokines concentration in the medium was evaluated at 24  
195 and 48h using a Quantikine human IL-6 or IL-8 immunoassay kit in accordance with the  
196 instructions of the manufacturer (R&D Systems) and as previously described [32].

#### 197 **2.2.8. Statistical analysis**

198 Data are expressed as the arithmetic means  $\pm$  SD of separate experiments. The  
199 statistical significance of results obtained from *in vitro* studies was evaluated by the  
200 Student's t-test, with probability values of less than 0.05 being considered as significant.

### 201 **2.3 *In vivo* study**

#### 202 **2.3.1. Animal surgery**

203 All implantations were performed on healthy rabbits. Fifteen white New-Zealand rabbits  
204 weighing between 2.5 and 4 kg were randomized into five groups of three animals,  
205 corresponding to the five implantation times under study: 1, 2, 4, 6 and 12 weeks. For

206 each group, two animals were implanted with ibuprofen-loaded granules and the third one  
207 received unloaded granules. Before surgery the animals were acclimatized to their new  
208 environment and fed a standard rabbit diet.

209 The surgical procedure was the following. After intramuscularly anesthesia with  
210 metedomidine ( $0.3 \text{ mg.kg}^{-1}$ ) and ketamine ( $20 \text{ mg.kg}^{-1}$ ), both back legs were shaved and  
211 disinfected. Then incision of skin and muscle was made to access to the femoral  
212 epiphysis and a hole of 4 mm in diameter and 6 mm in depth was created. After rinsing  
213 with physiological saline to remove bone debris, the defects were filled with 76 mg of  
214 implants corresponding to 27 mg of ibuprofen in the case of loaded granules. Following  
215 insertion of the implants, surrounding soft tissues were carefully closed and the surgical  
216 wound was disinfected. After recovering of anesthesia, functional activity was not limited  
217 and the animals received standard post-operative medication (buprenorphine  $0.03\text{-}$   
218  $0.16 \text{ mg.kg}^{-1}$  and marbofloxacin  $0.2\text{-}0.5 \text{ mL.kg}^{-1}$ ) for 3 days. General state of the rabbits  
219 (weight, pain, healing) was followed until euthanasia.

220 For the following of ibuprofen concentration in the general circulation, blood samples  
221 (puncture in the animal's ear central vein) were extracted at J1, J2, J3 and J7. After  
222 centrifugation (2 min, 3000 rpm) samples were frozen until analysis.

223 For euthanasia, an intracardiac lethal injection of T61® (embutramide  $200 \text{ mg.mL}^{-1}$ ,  
224 mebezonium iodide  $50 \text{ mg.mL}^{-1}$  and tetracaine chloride  $5 \text{ mg.mL}^{-1}$ ) was performed. After  
225 sacrifice, soft tissues were removed from the femurs and implants with surrounding bone  
226 were fixed for histology.

### 227 **2.3.2. Histology**

228 Samples were fixed during 24 hours with fresh 10% formaldehyde solution and then  
229 decalcified with RDO®. Some samples had a second decalcification with DCI® in order to  
230 soften the bone tissue and facilitate cutting. They were then dehydrated, embedded in

231 paraffin wax, cut into histological sections and stained with hematoxylin eosin safranin  
232 (HES) for light microscopy observation.

### 233 **2.3.3 Ibuprofen *in vivo* release**

234 Ibuprofen levels in serum were determined by liquid chromatography (Alliance, Waters  
235 2695) coupled to mass spectroscopy (Micromass® MS Technology). Quantification was  
236 performed using methanol/ammonium formiate/acetic acid (69.9/29.9/0.2 V/V/V) as the  
237 mobile phase and the elution speed was fixed to 0.25 mL.min<sup>-1</sup>. Ketoprofen was used as  
238 internal standard. Linearity of standard curve relating ibuprofen concentration was  
239 established from 0.2 to 50 mg.mL<sup>-1</sup>. Detection and quantification limits were equal to  
240 0.04 mg.mL<sup>-1</sup> and 0.2 mg.mL<sup>-1</sup> respectively.

### 241 **2.3.4. Ethics**

242 All experimental protocols with animals were approved by the regional ethic committee for  
243 animal experimentation.

244

## 245 **3. Results**

### 246 **3.1 Effect of ibuprofen-loaded granules on MC3T3 cells**

247 In order to determine the biocompatibility of ibuprofen-loaded granules, we first tested the  
248 effect of these granules on cell proliferation, apoptosis and proinflammatory cytokine (IL-6  
249 and IL-8) production on MC3T3.

250 As shown in figure 1A, ibuprofen at 0.5 mg.mL<sup>-1</sup> or 135 minutes of contact with the  
251 unloaded granules did not significantly modulate MC3T3 cell proliferation. However,  
252 contact with ibuprofen-loaded granules induced significant changes in cell proliferation. A  
253 short contact period (45 or 75 minutes) and therefore a reduced release of ibuprofen  
254 stimulated (140-170%) cell proliferation at 24 and 48h. Conversely, a long contact period  
255 (135 minutes) induced a reduction of proliferation of approximately 70% at 24 and 48h.

256 Associated with this reduction of cell proliferation for 135 minutes of contact, we showed  
257 (Fig. 1B) that there was induction of the DNA fragmentation indicating an apoptotic  
258 process. This induction of DNA fragmentation was not observed for shorter contact times  
259 and for ibuprofen  $0.5 \text{ mg.mL}^{-1}$  or unloaded granules.

260 Regarding proinflammatory cytokine production, we showed that ibuprofen-loaded  
261 granules reduced significantly production of IL-6 and IL-8 starting from 45 min release  
262 time at both 24 and 48h (Fig. 2A and 2B).

### 263 **3.2 Effect of ibuprofen-loaded granules on human RA FLS**

264 As shown in Fig. 3A and 3B, ibuprofen at  $0.5 \text{ mg.mL}^{-1}$  induced a significant decrease in  
265 cell proliferation and an increase in DNA fragmentation at 24 and 48h. Furthermore, it  
266 was shown that ibuprofen reduced significantly the production of the proinflammatory  
267 cytokines IL-6 (Fig. 4A) and IL-8 (Fig. 4B).

268 Concerning ibuprofen-loaded granules, Fig. 3A shows that cell proliferation was not  
269 inhibited at 45 min release time at both 24 and 48h. However, at 75 and 135 min release  
270 time RA FLS proliferation was significantly reduced at 24 and 48h. As far as concerns  
271 apoptosis induction, we demonstrated that ibuprofen-loaded granules increased DNA  
272 fragmentation at 75 and 135 min time release at both 24 and 48h, but no effect at 45 min  
273 time release was shown (Fig. 3B). Regarding IL-6 and IL-8 production, ibuprofen-loaded  
274 granules reduced significantly production of both proinflammatory cytokines starting from  
275 45 min release time at both 24 and 48h (Fig. 4A and 4B).

276 Of note that unloaded granules (negative control) did not affect cell proliferation, DNA  
277 fragmentation and IL-6 and IL-8 production even at the longer time release (135 min).

278

### 279 **3.3 *In vivo* study**

280 The purpose of this *in vivo* study was first to evaluate the local tissue reaction following  
281 the implantation of granules containing the drug substance or not. Healthy rabbits were  
282 used and qualitative histology of the bone implantation site was realized from one to  
283 twelve weeks after surgery. The second objective was to investigate drug substance  
284 diffusion in the general circulation. Ibuprofen concentration was determined in blood  
285 samples taken during the first 2 weeks of experimentation.

286 Clinical monitoring was good for all the animals whatever the type of implants they  
287 received.

### 288 3.3.1 Histology

289 The main histological observations based on the implantation time are presented in  
290 Table 1. One week after implantation, local granulomatous tissue and invasion of the site  
291 with multinucleated giant cells were observed around unloaded granules, as normal  
292 consequence of surgical trauma. At 2 and 4 weeks, inflammation reaction remained  
293 intense: dense fibrosis and only a slight decrease of the number of multinucleated cells  
294 were noticed. From 6 weeks, inflammation clearly reduced with disappearance of giant  
295 cells. Concerning ibuprofen-loaded granules, very low granulomatous reaction was  
296 identified in the vicinity of the implants and, for all implantation times, it was less than that  
297 around unloaded granules. Implant sites at 1 and 6 weeks are presented in Fig. 3 for both  
298 unloaded and ibuprofen-loaded granules. These illustrations demonstrate that  
299 inflammation reaction at 1 week was intense around unloaded implants then, it started to  
300 reduce at 6 weeks. However, it was very limited around ibuprofen-loaded granules all  
301 along the 6 weeks of implantation.

302 Concerning osteogenesis, histological findings demonstrated gradual new bone formation  
303 in the vicinity of both types of implants as shown on Fig. 5, but unloaded implants showed  
304 higher bone cells infiltration. A slight delay was noticed in the case of ibuprofen-loaded

305 granules; however, this difference reduced after six weeks of implantation and bone  
306 formation was observed around the two types of implants.

### 307 3.3.2 Drug diffusion

308 Ibuprofen concentration in the blood samples are given in Table 2. It can be observed that  
309 levels were low and they were detectable only over the 3 first days after implantation.  
310 Considering a total volume of blood in rabbits of  $56 \text{ mL.kg}^{-1}$ , the maximal concentration of  
311 ibuprofen, if the total quantity was released, was estimated to  $200 \text{ mg.L}^{-1}$ .

312

## 313 4. Discussion

314 In this study, porous calcium phosphate granules were investigated as local ibuprofen  
315 delivery system in order to relieve musculoskeletal disease symptoms. The spherical  
316 shape of these granules presents several advantages. First, it allows for the filling of  
317 complex-shaped bone defects, contrary to blocks, while keeping a regular intergranular  
318 macroporosity above  $100\mu\text{m}$  necessary for bone colonization [31]. Secondly, spherical  
319 granules are also interesting for drug delivery applications because their controlled  
320 surface allows for the deposition of regular and reproducible amounts of therapeutic  
321 agents. The elaboration process and drug functionalization of these Drug Delivery  
322 Systems have been described previously [30, 31, 35], however the therapeutic efficiency  
323 of the drug released (ibuprofen in this study) from these spherical granules has not been  
324 evaluated. Ibuprofen is a nonsteroidal anti-inflammatory drug widely used in in the  
325 treatment of inflammatory diseases and rheumatoid disorders [36]. Ibuprofen is a good  
326 model drug often used for its short biological half-life (2 h), appropriate molecule size  
327 ( $\sim 0.6\text{-}1.0 \text{ nm}$ ) and good pharmaceutical activity [19].

328 Both *in vitro* and *in vivo* biocompatibility of ibuprofen-loaded granules were demonstrated.

329 *In vitro*, MC3T3 cell line and rheumatoid arthritis (RA) fibroblast-like synoviocytes (FLS)

330 were chosen as study models. MC3T3 is a widely recognized good model for testing bone  
331 biocompatibility [37-39] and FLS are critical actors in the pathogenesis of rheumatoid  
332 arthritis [40]. *In vivo*, femoral implantation was performed in healthy rabbits in order to  
333 assess the impact of ibuprofen on local bone tissue reaction.

334 Overall, *in vitro*, our data document that Ibuprofen loaded granules inhibited MC3T3 and  
335 RA FLS cell proliferation accompanied by apoptosis induction. Furthermore, ibuprofen  
336 loaded granules, reduced significantly IL-6 and IL-8 pro-inflammatory cytokine production.  
337 However, the effect of ibuprofen-loaded granules was clearly more intense on RA FLS  
338 cells than on MC3T3. Of note that these effects were not observed with unloaded  
339 granules (negative control) on both cell types.

340 *In vivo*, implantation of unloaded or ibuprofen-loaded granules in osseous site induced  
341 bone formation with a slight delay of bone cells infiltration in the case of ibuprofen-loaded  
342 granules in the first 6 weeks. Inflammatory response was clearly dependent on the  
343 presence of ibuprofen on the granules, revealing the activity of the drug released from  
344 calcium phosphate granules. Indeed, compared to unloaded granules, very few signs of  
345 post-operative inflammation (granulomatous reaction, presence of multinucleated giant  
346 cells) were found in the case of ibuprofen-loaded granules. These results demonstrated  
347 the local action and efficacy of ibuprofen-loaded granules to both decrease the  
348 inflammatory response and to induce neo-osteogenesis around the granules.

349 As histological observations were quite similar for the two groups (ibuprofen loaded  
350 granules vs. unloaded granules) beyond 6 weeks, results demonstrated that loaded  
351 granules succeeded in their function of drug delivery system, with an ibuprofen release  
352 period that could be estimated around six weeks. Of note that, no degradation of granules  
353 was observed over the implantation period. This confirmed the results obtained *in vitro* in  
354 a previous study that showed that ibuprofen release was mainly governed by diffusion

355 [31]. As far as concerns drug diffusion, our data document that only a very small amount  
356 of the drug substance reached systemic circulation and it may be expected that the risk of  
357 side effects using loaded granules is extremely low. It can be suggested, then, that the  
358 drug remained inside or around the implantation site, what is sought for a local action.

359

## 360 **5. Conclusion**

361 Our data document that ibuprofen loaded porous calcium phosphate granules can be  
362 used to local relieving symptoms in musculoskeletal diseases such as rheumatoid  
363 arthritis. However, further investigation has to be done to develop this drug delivery  
364 system. In order to further check the efficacy of the ibuprofen loaded granules, animal  
365 models of inflammation might be used. Then, adjustment of drug substance quantity and  
366 release kinetics has also to be performed.

367 Overall, using porous calcium phosphate granules as drug delivery system is of great  
368 interest to counteract several diseases symptoms. Indeed, porous calcium phosphate  
369 granules demonstrated their capability to support osteogenesis while locally releasing a  
370 drug substance including non-steroidal anti-inflammatory drugs –as in this study- but also  
371 antibiotics, analgesics and anticancer drugs in order to repair/regenerate the damaged  
372 tissue.

373

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379



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## Figures and Tables legends

471

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473

474 Figure 1: Effect of ibuprofen-loaded granules on (A) MC3T3 proliferation and (B)  
475 apoptosis (\*p<0.05 for 24h group, #p<0.05 for 48h group)

476

477 Figure 2: Effect of ibuprofen-loaded granules on (A) IL-6 and (B) IL-8 production in  
478 MC3T3 cells (\*p<0.05 for 24h group, #p<0.05 for 48h group)

479

480 Figure 3: Effect of ibuprofen-loaded granules on (A) human rheumatoid arthritis fibroblast-  
481 like synoviocytes proliferation and (B) apoptosis (\*p<0.05 for 24h group,  
482 #p<0.05 for 48h group)

483

484 Figure 4: Effect of ibuprofen-loaded granules on (A) IL-6 and (B) IL-8 production in human  
485 rheumatoid arthritis fibroblast-like synoviocytes (\*p<0.05 for 24h group, #p<0.05  
486 for 48h group)

487

488 Figure 5: Microscopic observations (x100) of histological sections after two weeks of  
489 implantation for unloaded granules (A and C) and ibuprofen-loaded granules (B  
490 and D) (DG: dissolved granule, GT: granulation tissue, CB: cancellous bone,  
491 NB: new bone, GC: giant cells)

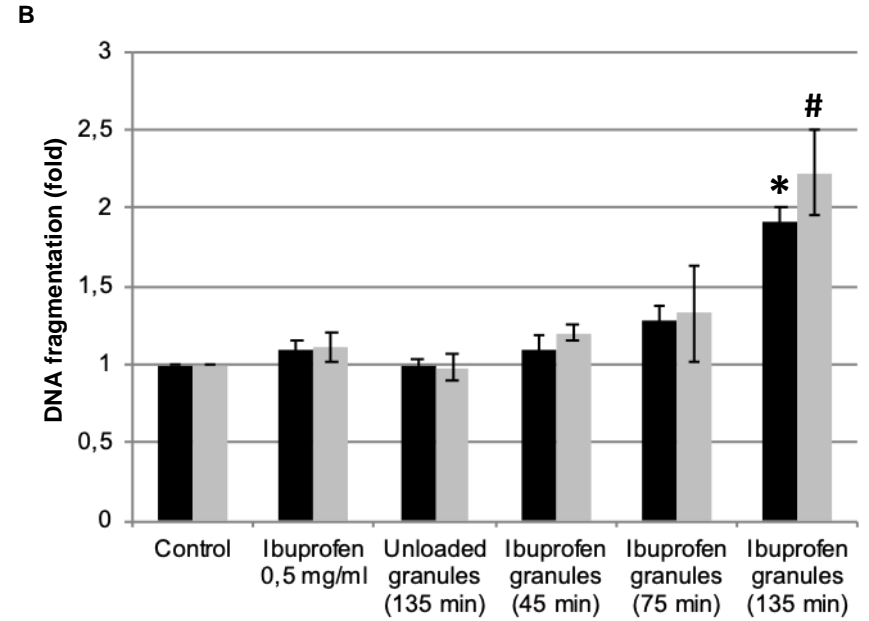
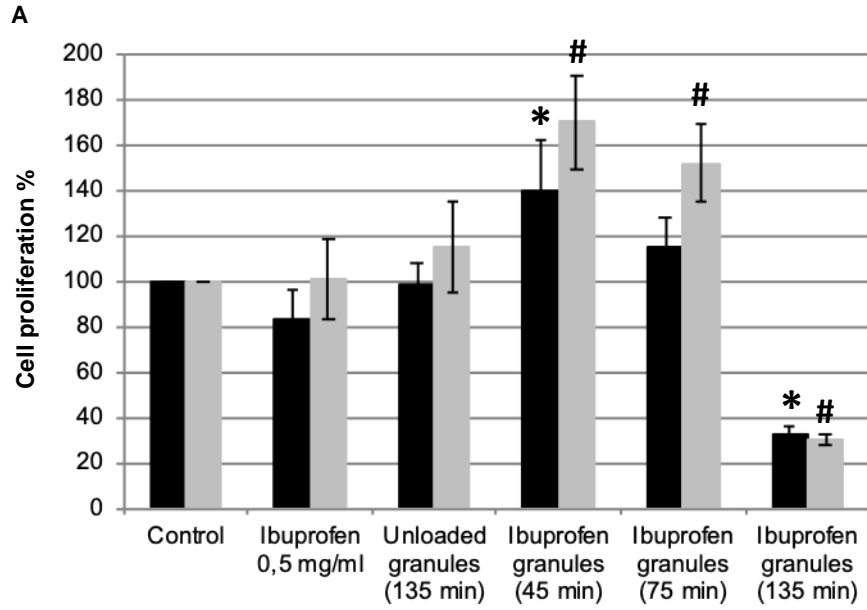
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493 Table 1. Description of histological sections as a function of implantation time, for  
494 unloaded and ibuprofen-loaded granules

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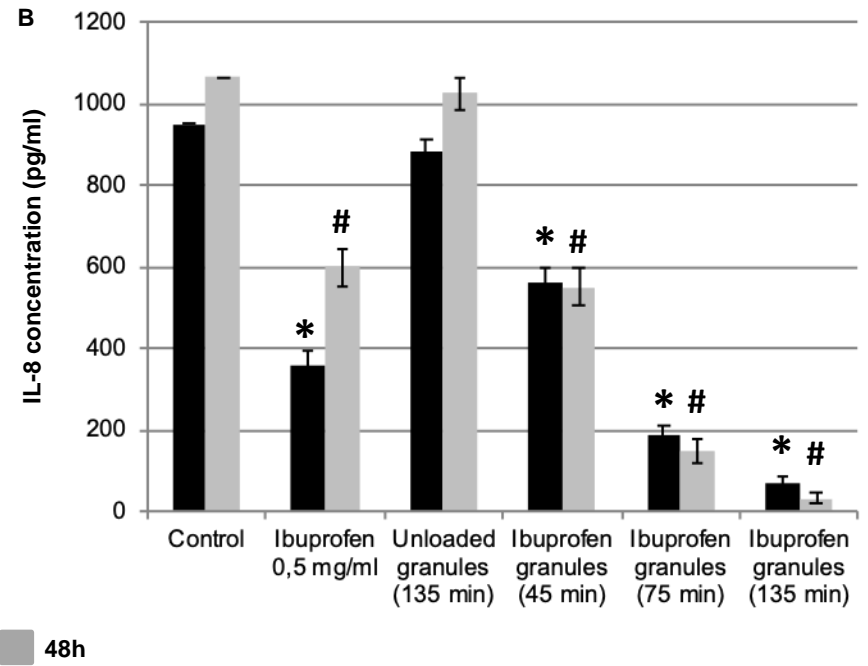
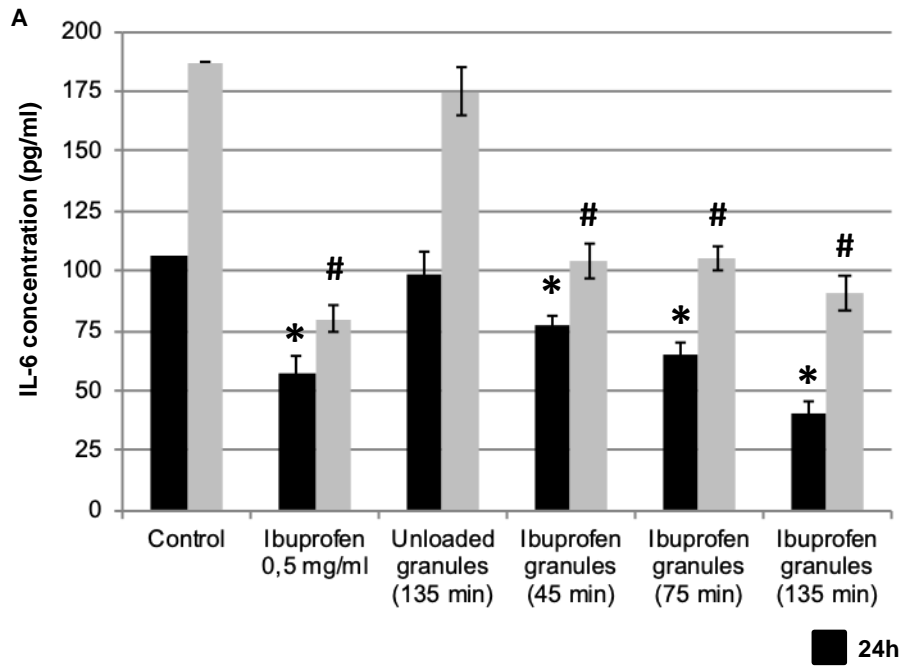
496 Table 2. Ibuprofen concentration in blood samples

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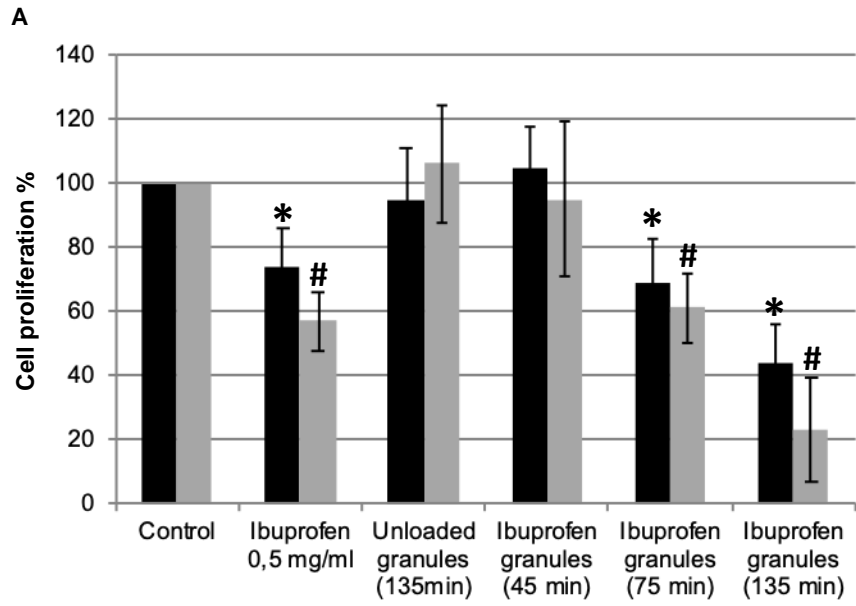


■ 24h    ■ 48h

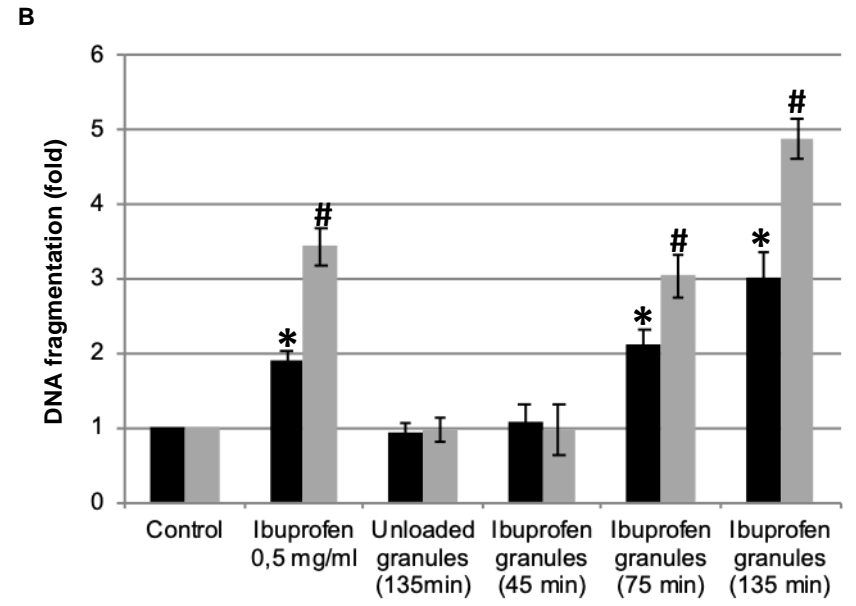
**Figure 1**



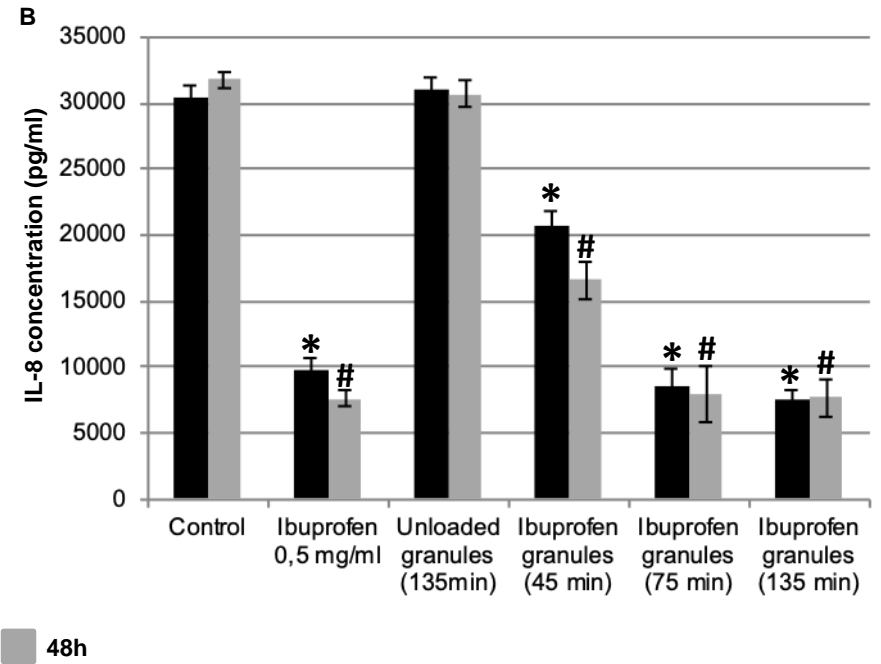
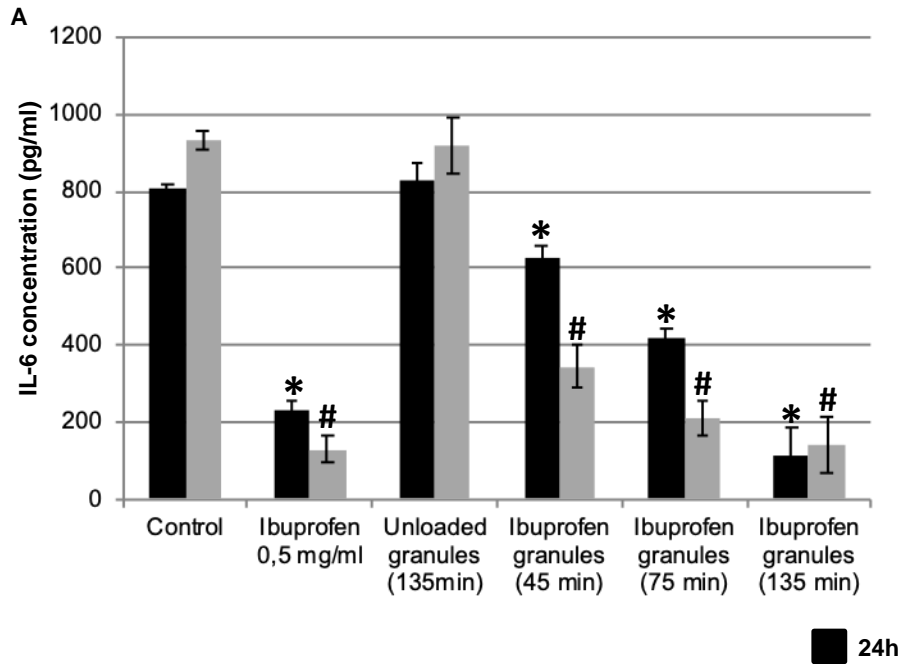
**Figure 2**




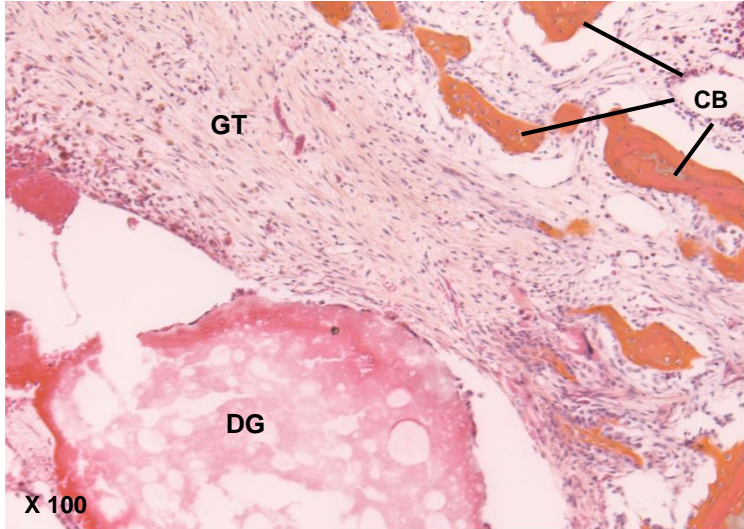
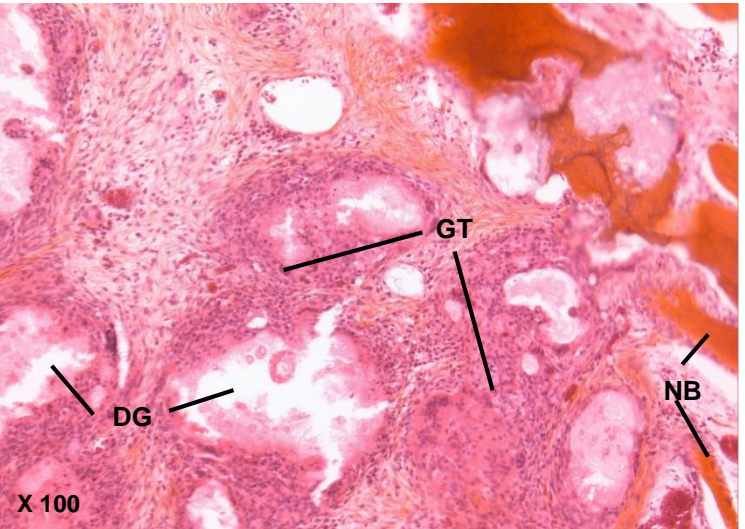

■ 24h    ■ 48h



**Figure 3**



**Figure 4**

	Unloaded granules	Ibuprofen-loaded granules
One week	<p><b>A</b></p>  <p>GT</p> <p>GT</p> <p>X 100</p>	<p><b>B</b></p>  <p>GT</p> <p>DG</p> <p>CB</p> <p>X 100</p>
Six weeks	<p><b>C</b></p>  <p>GT</p> <p>DG</p> <p>NB</p> <p>X 100</p>	<p><b>D</b></p>  <p>OB</p> <p>CB</p> <p>DG</p> <p>GT</p> <p>X 100</p>

**Figure 5**



**Table 1. Description of histological sections as a function of implantation time, for unloaded and ibuprofen-loaded granules**

<b>Time (weeks)</b>	<b>unloaded granules</b>	<b>Ibuprofen-loaded granules</b>
<b>1</b>	<ul style="list-style-type: none"> <li>- Normal articular surface</li> <li>- Replacement of periosteum and bone tissue by granulomatous tissue</li> <li>- Invasion of the site with multinucleate giant cells</li> </ul>	<ul style="list-style-type: none"> <li>-Very low granulomatous reaction with multinucleate giant cells</li> </ul>
<b>2</b>	<ul style="list-style-type: none"> <li>- Inflammatory reaction</li> <li>- Fibrosis</li> <li>- Invasion of the grain porosity by bone cells</li> </ul>	<ul style="list-style-type: none"> <li>- Presence of a few inflammatory cells</li> <li>- No bone cells</li> </ul>
<b>4</b>	<ul style="list-style-type: none"> <li>- Persistence of fibrosis and granulomatous reaction</li> <li>- Decrease of the number of multinucleate giant cells</li> </ul>	<ul style="list-style-type: none"> <li>-Same intensity of granulomatous reaction as observed at one week</li> </ul>
<b>6</b>	<ul style="list-style-type: none"> <li>- Continuation of granulomatous reaction</li> <li>- Decrease of the number of multinucleate giant cells</li> <li>- Beginning of bone formation</li> </ul>	<ul style="list-style-type: none"> <li>- Similar intensity of the inflammation reaction as observed with non-loaded granules at six week</li> </ul>
<b>12</b>	<ul style="list-style-type: none"> <li>- Absence of multinucleate giant cells</li> <li>- Development of a new tissue rich in osteoblasts and osteocytes around calcium phosphate granules</li> </ul>	<ul style="list-style-type: none"> <li>- Similar intensity of the inflammation reaction as observed with non-loaded granules at six week</li> <li>- Development of a new tissue rich in osteoblasts and osteocytes around calcium phosphate granules</li> </ul>

**Table 2. Ibuprofen concentration in blood samples**

<b>Days post implantation</b>	<b>Ibuprofen concentration (mg.L<sup>-1</sup>)</b>
1	0.250 (0.026)
2	0.07
3	0.04
7	ND
14	ND

Mean value ( $\pm$  S.D.)

ND: Not detected

