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ABSTRACT

Glucocorticoid (GC) treatment is the main cause of secondary osteoporosis. There are some controversies about the relationships between alveolar bone loss and bone loss at the appendicular and axial skeleton.
Objective: To assess, in parallel, the effects of GCs on alveolar bone and on the tibia in a mice model.
Methods: 5-month-old male Swiss-Webster mice were randomized into two groups. Pellets releasing 5mg/kg/day of prednisolone or control pellets were subcutaneously implanted for 28 days. After euthanasia, the right tibia and the right hemimandible of each mouse were analyzed by histomorphometry and microcomputed tomography. Alveolar bone consists of a thin slab between the incisor and the molar roots connected with the alveolar processes. A 2D-frontal section was done through the pulp chamber of the first molar and was used to measure the thickness of the alveolar bone slab. A 2D-sagittal section was done through the pulp chamber of the 3 molars and was used to measure bone volume in the alveolar processes.
Results: At day 28, thickness and bone volume of alveolar bone were significantly decreased in the GC group ($P < 0.05$). At the tibia, GCs decreased bone formation with a reduced mineral apposition rate and bone formation rate and a significant decrease in BV/TV and Tb.Th ($P < 0.05$).
Conclusion: Although the amount of alveolar bone is very low in the mouse, this study shows that GCs can induce an alveolar bone loss in long term treated animals.

1. Introduction

The mandible forms the lower jaw and is composed of two types of bone tissue: the basal bone which forms the body of the mandible, and the alveolar bone. Alveolar bone is the bony structure that supports and anchors the teeth roots in association with the periodontal ligament. It has a high plasticity and is remodeled at a high rate. There are considerable variations in alveolar bone across individuals and bone mass at the jaw highly depends of local factors (see review in [1]). Mechanical stimulation of alveolar bone during mastication is crucial in keeping the teeth and underlying bone healthy. Loss of teeth is followed by an irreversible alveolar bone resorption; similar findings are encountered in untreated dental diseases leading to periodontitis. A number of metabolic bone diseases can also induce alveolar bone loss, such as vitamin D-resistant rickets [2] or hyperparathyroidism [3]. The fact that osteoporosis affects the severity of periodontal disease is controversial as well as the relationship between alveolar bone loss and generalized osteoporosis [4].
Glucocorticoid-induced osteoporosis (GIOP) is the main cause of secondary osteoporosis. Long-term oral treatment with glucocorticoids (GCs) is associated with an increased risk of osteoporosis or fracture [5-7]. GCs have direct and indirect effects on bone cells with a

suppression of bone formation and an increased bone resorption (see review in [8]). The common findings in histomorphometric studies of axial or appendicular skeleton are a reduction in bone volume and trabecular thickness. In human, the effects of GCs at the mandible have been seldom analyzed. Animal models of mandibular bone loss have been principally described in rodents [9, 10]. Results of these studies are difficult to interpret because of the various techniques used. The purpose of this study was to analyze, in parallel, alveolar and tibia bone loss using histomorphometric and microCT in the mouse receiving GCs.

2. Materials and Methods

2.1. Animals and experimental procedures

Five-month-old male Swiss-Webster mice were obtained from Janvier (Le Genest-Saint-Isle, France). Mice were maintained on commercial rodent chow (UAR, France) and water was available ad libidum. They were housed in a room maintained at 24°C with a 12-h light/dark cycle. The animal ethical committee of University of Angers approved all procedures used. Mice were randomized into 2 groups with 10 animals in each. Slow release pellets, composed of a biodegradable matrix, were used (Innovative Research of America, Sarasota, Fl, USA). One group received pellets releasing 5 mg/kg/day of prednisolone (GC group), the other group received pellets composed of the carrier binder excipients without GC (PBO group). Pellets were implanted subcutaneously under the neck for 28 days [11]. Calcein was given intraperitoneally (10 mg/kg), 7 and 2 days before euthanasia to determine bone mineralizing surfaces and bone formation rates. Euthanasia was done at day 28 after isoflurane inhalation, exsanguination by cardiac puncture followed by cervical dislocation. The right tibia and the right hemimandible were carefully dissected and placed in formalin for 24 hours, then transferred to absolute acetone until use.

2.2. Microcomputed tomography (microCT)

MicroCT analyses were performed using a Skyscan 1172 X-ray computerized microtomograph (Skyscan, Kontich, Belgium) equipped with an X-ray tube working at 80 kV/100 µA. Bones were placed in Eppendorf's tubes and filled with water to prevent desiccation. The tubes were fixed on a brass stub with plasticine and analyzed with a pixel size corresponding to 3.88 µm, the rotation step was fixed at 0.40°, and exposure was done with a 0.5 mm aluminum filter. For each tibia, a stack of 2D-sections was obtained. The CTAn Software (Skyscan, release 1.10.1.0) was used for measuring the bone mass and architecture at the secondary spongiosa of the tibia. The upper limit of the volume of interest was located just after the disappearance of the growth plate and primary spongiosa; the lower limit was located 200 sections below. A threshold was determined interactively to eliminate background noise and to select bone. The volume of interest (VOI) was designed by drawing interactively polygons on the 2D sections. Only a few number of polygons need to be drawn (e.g. on the first section, several at the middle, and on the final section) since a routine facility calculated all the intermediary masks by interpolation. The VOI comprised only trabecular bone and the marrow cavity. The following parameters were measured according to the recommendations of the American Society for Bone and Mineral Research [13]

- Trabecular bone volume (BV/TV_{3D}, in %) represents the percentage of the cancellous space occupied by trabecular bone in the VOI.
- Trabecular thickness ($Tb.Th_{3D}$, in µm), trabecular separation ($Tb.Sp_{3D}$, in µm), and trabecular number ($Tb.N_{3D}$, in mm^{-1}) provide a full description of bone microarchitecture.
- Structure model index (SMI) indicates the composition of trabecular bone in the form of rods or plates. SMI values are comprised between zero (ideal plate structure model) and three (ideal rod structure).

- Trabecular pattern factor ($Tb.P_f$) is an index of trabecular connectivity. $Tb.P_{f\ is}$ low in a well-connected structure and high in a disconnected trabecular network.

For each mandible, the ANT software (Skyscan, release 2.4) allowed the reconstruction of 3D models from the stack of 2D sections. Alveolar bone was measured on two types of 2D sections re-sliced from the 3Dmodels: 5 frontal sections through the middle of the pulp chamber of the first molar was used to evaluate the thickness of alveolar bone; 10 sagittal sections through the pulp chamber of the 3 molars were used to measure bone volume in the alveolar processes (Fig. 1).

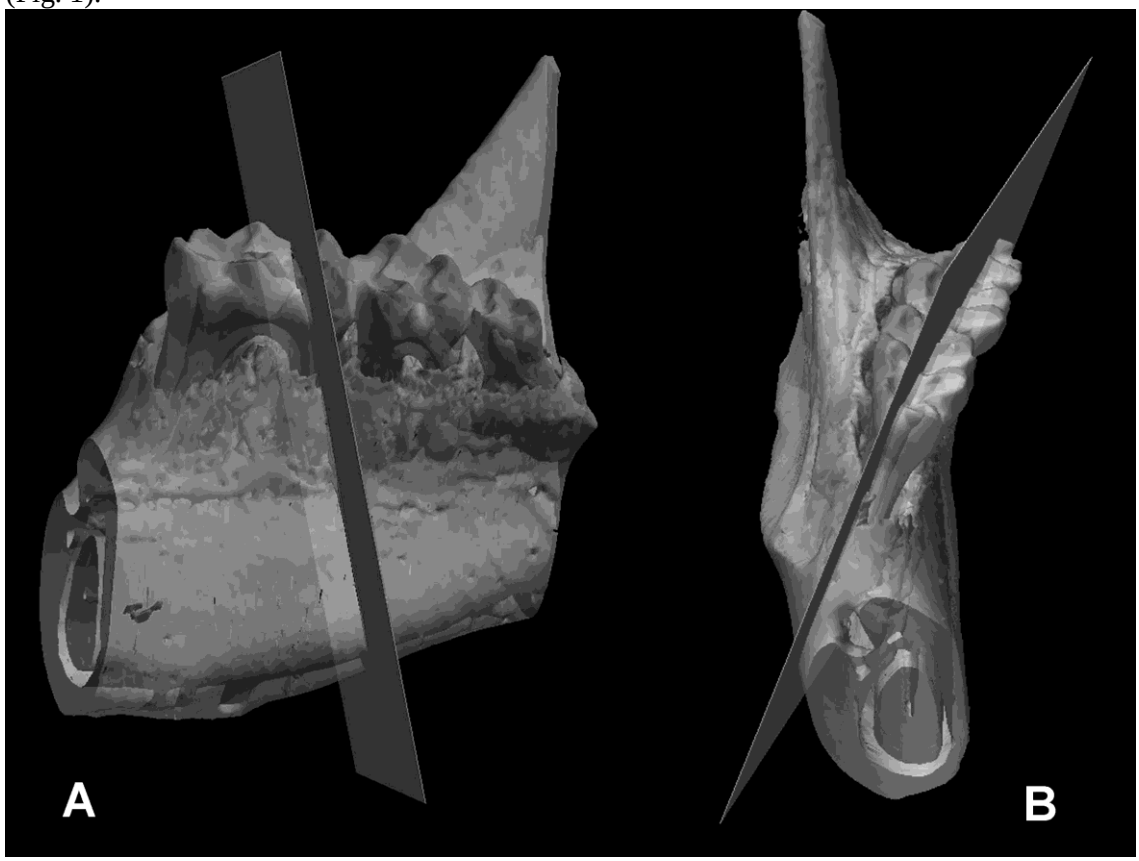

Fig. 1. 3D model of a mouse mandible (bone has been rendered semi-transparent) showing a cutting plane passing through the middle of the pulpar chamber of the first molar to obtain the frontal (A) and the sagittal (B) 2D sections of figures 3 and 4.

2.3. Bone histomorphometry

Tibias and hemimandibles from GC and PBO mice were embedded undecalcified in poly (methylmethacrylate). Tibias were cut-dry (7 µm in thickness) on a heavy-duty microtome equipped with tungsten carbide knives (Leica Polycut S-Rueil Malmaison, France) and stained with a modified Goldner's trichrome. Osteoclasts were identified by a histochemical detection of tartrate resistant acid phosphatase. Histomorphometric analysis was done on an automatic image processor (Leica Quantimet Q550) and a semiautomatic system (Summasketch III, Summagraphics, USA, digitalizing tablet coupled with a PC). The following parameters were measured: BV/TV, Tb.Th, Tb.N, Tb.Sp, osteoid volumes (OV/BV, in %), osteoid surfaces (OS/BS, in %), N.Oc/BAr (number of osteoclasts per mm^2 of bone area). Histodynamic measurements at the tibia included single and double labeled surfaces (resp. sLS and dLS, in %), trabecular mineral apposition rate (Cn.MAR, in µm/d), cortical mineral apposition rate (Ct.MAR, in µm/d), mineralized surface per bone surface (MS/BS, in %), mineralized surface per osteoid surface (MS/OS, in %), adjusted apposition rate (Aj.Ar, in µm/d), bone surface per bone volume (BS/BV, in mm^2/mm^3), bone formation rate per bone surface (BFR/BS, in $\mu m^3/\mu m^2/y$) and bone formation rate per bone volume (BFR/BV, in %/d). Because undecalcified enamel cannot be cut on a microtome, sections of the mandibles were prepared

(350 μm in thickness) on a precision cut-off machine equipped with a diamond saw (Accutom 50, Struers, Denmark). Sections were then stuck onto a plastic plate and observed under fluorescence microscopy.

2.4. Biochemical markers of bone turnover

Blood samples were centrifuged and the serums were collected to measure bone formation markers: procollagen I N-terminal propeptide (PINP) (Rat-Mouse PINP EIA, Immunodiagnostic systems, France), and osteocalcin (Mouse Osteocalcin IRMA test, Immunodiagnostic systems) and bone resorption markers: C-terminal cross-linked telopeptide of type I collagen (CTX) (RatLaps^{TM} EIA, Immunodiagnostic systems). Enzyme-linked immunosorbent assay kits and immunoradiometric assay kits were used according to the manufacturer's recommendation. All samples were assayed in duplicate.

2.5. Statistical analysis

Statistical analysis was performed using the Systat statistical software release 11.0 (Systat Software Inc, San Jose, CA). All data were expressed as mean ± standard error of the mean (SEM). Group differences were searched by the Kruskall-Wallis non parametric test. Differences were considered as significant when $P < 0.05$.

3. Results

3.1. General findings

Implantation of the pellets was well tolerated without local infection or general complications. Mice implanted with GC pellets lost weight during the first two weeks of the study (maximum 15 % below baseline) but regained weight thereafter; there were no differences in weight at day 28 between the GC and placebo groups. Two mice in each group died before day 28 of undetermined cause.

3.2. Effects of GC excess on the tibia

Histomorphometric and microarchitectural results are expressed in table 1. MicroCT evaluation of GC treated mice showed a significantly reduction in BV/TV_{3D} (-22%; $P<0.01$) and $Tb.Th_{3D}$ (-10%; $P<0.01$) in the proximal tibia at day 28. A marked deterioration of the trabecular connectivity occurred in the GC group with a significant increase in $Tb.P_f$ ($P<0.01$). There were no significant differences in $Tb.N_{3D}$, $Tb.Sp_{3D}$ and SMI between the 2 groups at day 28 (Fig. 2).

		Placebo	GC	*P* value
Histomorphometry 3D				
BV/TV_{3D}	(%)	19.12 ± 0.98	14.85 ± 0.96	<0.01
$Tb.Th_{3D}$	(µm)	51 ± 0.1	46 ± 0.1	<0.01
$Tb.N_{3D}$	(mm-1)	1.65 ± 0.21	3.24 ± 0.22	NS
$Tb.Sp_{3D}$	(µm)	191 ± 10	194 ± 7	NS
$Tb.P_f$		13.62 ± 0.80	18.52 ± 1.18	<0.01
SMI		1.36 ± 0.06	1.52 ± 0.07	NS
Histomorphometry 2D				
BV/TV	(%)	7.55 ± 0.73	5.13 ± 0.78	NS
Tb.Th	(µm)	23.6 ± 1.2	19.3 ± 1.1	<0.05
Tb.N	(mm^{-1})	3.19 ± 0.27	2.67 ± 0.40	NS
Tb.Sp	(µm)	309 ± 30	447 ± 112	NS
N.Oc/B.Ar	(¢/mm^{-2})	8.62 ± 0.95	11.03 ± 1.22	NS
OV/BV	(%)	1.10 ± 1.24	1.24 ± 1.23	NS
OS/BS	(%)	0.57 ± 0.47	0.27 ± 0.24	NS
CnMAR	(µm/d)	0.87 ± 0.07	0.54 ± 0.18	NS
CtMAR	(µm/d)	1.11 ± 0.07	0	NS
sLS	(%)	40 ± 12.86	42.70 ± 11.92	NS
dLS	(%)	54.42 ± 6.80	5.09 ± 1.32	<0.01
MS/BS	(%)	62.33 ± 7.57	24.3 ± 5.35	<0.01
MS/OS	(%)	226.9 ± 64.95	59.48 ± 69.28	<0.05
Aj.AR	(µm/d)	1.82 ± 0.5	0.48 ± 0.80	<0.05
BFR/BS	($\mu m^3/\mu m^2/y$)	200.94 ± 27.61	42.26 ± 19.94	<0.01
BS/BV	(mm^2/mm^3)	86.21 ± 4.33	107.49 ± 6.67	<0.05
BFR/BV	(%/d)	17536 ± 2707	4453 ± 2072	<0.01

Table 1. Trabecular bone microarchitecture parameters (mean ± SEM) and histomorphometric parameters (mean ± SEM) obtained at the proximal tibia in the placebo (PBO) and GCs treated (GC) groups.

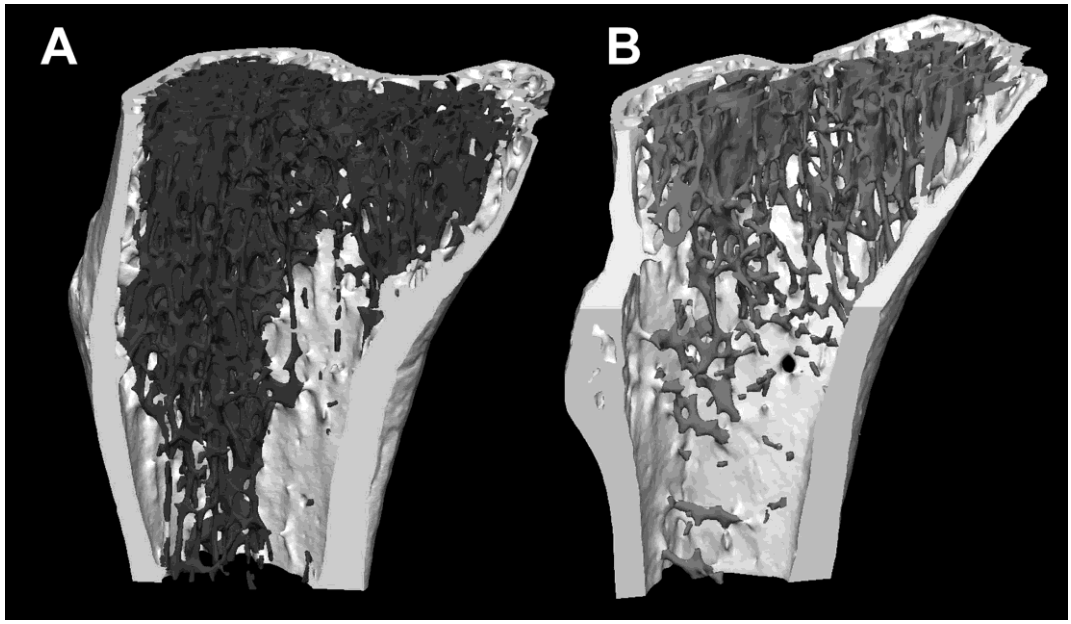

Fig. 2. 3D models of the tibia in (A) PBO and (B) GCs treated groups. Bone volume, trabecular thickness, and trabecular pattern factor are significantly decreased in the GCs group.

Histomorphometric evaluation of GC treated mice showed a significantly reduced Tb.Th (P<0.05) in the proximal tibia compared with PBO group at day 28. BV/TV was found reduced in GC treated mice (-32%), but the difference did not reach significance. Osteoclast number in the GC group was maintained at the same level as in mice in PBO group. Osteoid parameters were not significantly different between the 2 groups. Mineral apposition rate and bone formation rate in the tibia were significantly decreased in GC treated mice with a significant decrease in double-labeled surfaces.

3.3. Effects of GC excess on alveolar bone

In mice, the amount of alveolar bone was found very low. Alveolar bone consisted in a thin bony slab between the incisor and the molar roots, connected with the alveolar process where the teeth are anchored. The thin bony slab between the incisor and the molar roots was measured on 5 consecutive frontal 2D sections (Fig. 3). At day 28, it was found to be 56.7 ± 2.4 µm in the PBO group. In the GC treated group, the thickness was found markedly reduced (46.6 ± 3.3 µm; P<0.05). This thin slab was sometimes fenestrated due to the occurrence of perforations. No significant correlation was observed between the thickness of the bone alveolar slab and $Tb.Th_{3D}$ at the tibia. The bone volume (BV/TV) of the alveolar process was measured between the roots of the first molar on 10 consecutive sagittal sections (Fig. 3). At day 28, it was found to be 66.4 % ± 1.7 in the PBO group. In the GC treated group, BV/TV was found significantly reduced (55.9 % ± 3.4, P<0.05). On the histological sections, the labeled surfaces were observed in a very limited number of mice (even in the PBO group), so histodynamic parameters could not be determined in the alveolar bone (Fig. 4).

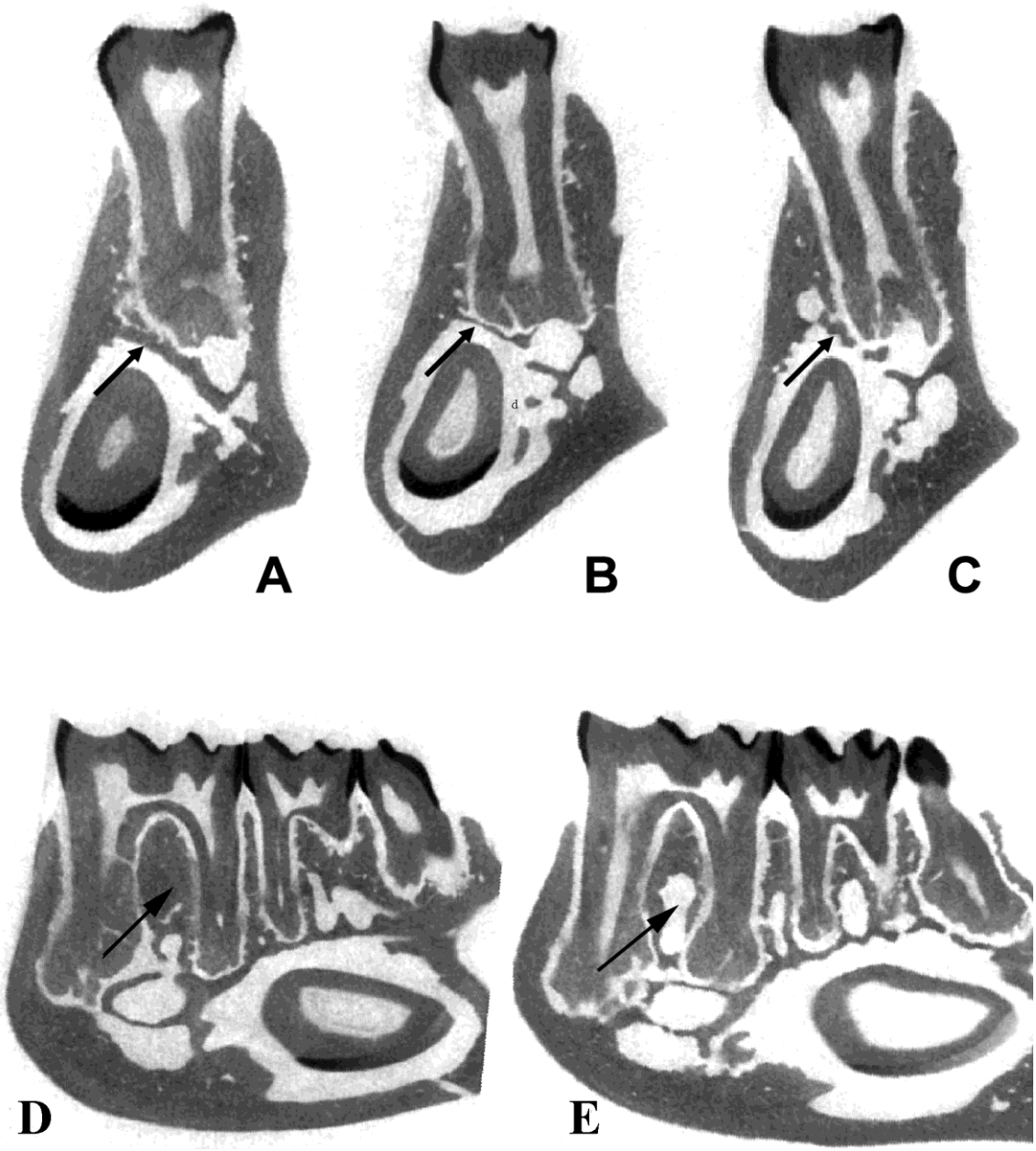

Fig. 3. 1st raw: 2D frontal slices obtained with the cutting plane from reconstructed 3D models of the mandible in (A) PBO and (B, C) the GCs treated mice.
2nd raw: 2D sagittal slices obtained with the cutting plane from reconstructed 3D models of the mandible in (D) PBO and (E) the GCs treated mice.

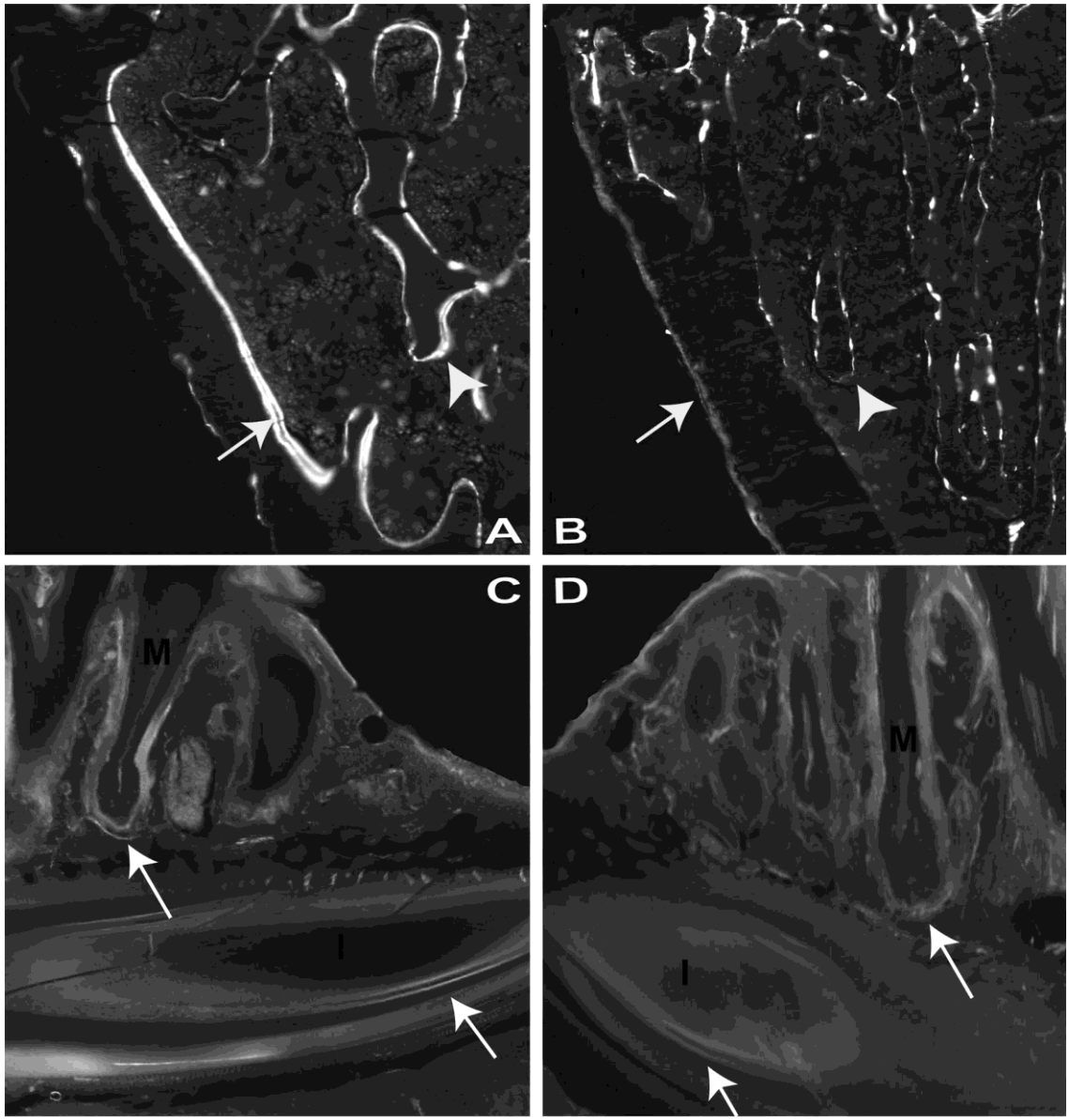

Fig. 4. Labeling with calcein viewed under UV light, original magnification 400. Double regular labels along cortical (arrow) and trabeculae (arrowhead) of the tibia in PBO group (A). Reduced mineralizing surfaces in a GC treated mouse (B). At the mandible, the labeling with calcein is rare, it appears at the periphery of the incisive (I) and the molar roots (M). There was no obvious difference between PBO (C) and GC (D) group.

3.4. Effects of GC excess on bone turnover markers

At day 28, serum CTX and PINP levels were significantly higher in the GC group compared with the PBO group ($P<0.01$). Serum osteocalcin levels did not differ significantly between the two groups.

4. Discussion

In this study, long term GCs administration in mice led to a decrease in alveolar bone (represented in the mouse by alveolar processes connected with a bony slab between the incisor and the molar roots). Even if alveolar bone is low in the mouse, GCs induced a reduction of bone volume in the processes; furthermore thinning and perforations occurred in the thin slab of underlying bone. As expected, this study also showed a decrease in Tb.Th and BV/TV at the tibia, an altered microarchitecture and decreased MAR and BFR.

Different animal models have been proposed to study the pathophysiology of GIOP with contradictory findings probably due to background factors such as the dose of GC or the age of the animals. We have used a mice model in which bone loss was induced by a continuous release of GC; it has already been described as a suitable model for GIOP [11]. The secondary spongiosa of the tibia is recognized to reflect the bone changes observed in humans. Decreased bone formation is the most significant event leading to GIOP; the depressed osteoblastic activity is evidenced by a reduction in MAR, Aj.AR and BFRs after double-labeling with calcein. The consequence is a significant decrease in BV/TV and Tb.Th at the proximal tibia metaphysis. These two parameters are principally affected by long-term GC treatment in animals [11, 12] and also described on human bone biopsies [13, 14]. Discrepancies between 2D and 3D results are due to the fact that microCT overestimates BV/TV and Tb.Th compared with histomorphometry, as previously shown [13]. Altered trabecular connectivity was also evidenced by a significant increased $Tb.P_f$ in the GC group. GCs have been shown to increase the osteoclast number, most likely by extending osteoclast life-span [15]. In our study, osteoclast number was not significantly different in the GC and PBO groups; a 28 day GCs administration in the mouse corresponds to a long term treatment, equivalent to ~ 3-4 years in human as previously shown by others [12]. There was no significant difference in osteoid parameters between the 2 groups but there was a trend of decreasing osteoid surfaces in GC group, suggesting a lower bone turnover. Previous studies have shown a decrease in osteoid parameters during a GC short term administration [16] or a long term administration in mice [12].

Our study showed a significant reduction in alveolar bone. Measurements of BV/TV at the alveolar processes and thickness of the underlying bony slab were the only reliable parameters at the mandible of mice. Studies dealing with mandibular alveolar bone in the mouse are rare [17, 18] and have never concerned GIOP. Furthermore, this is the first study describing both 2D and 3D parameters in parallel at the tibia and at the mandible. Rat is the most frequently animal model used to study the effects of GCs at the mandible and different measurement tools have been described, (microCT, pQCT, histomorphometry 2D) [19-25]. Reduction in mandibular cortical thickness has been reported [19] and an altered volume of alveolar bone has also been shown [21]. However, data concerning the effects of GC on bone in rats are conflicting and bone volume may be increased [26]. Rabbit [27, 28] and sheep [29, 30] models have also been proposed combining the effects of GCs with calcium diet or hypogonadism. In humans, studies dealing with the impact of GCs on mandible are even rarer [31-35]. A significant correlation between alveolar bone loss and hip BMD was reported in 30 patients with chronic obstructive pulmonary disease (COPD) treated with inhaled GCs for at least one year. BMD at the mandible was decreased especially in male and edentulous patients [33].

In our study, the bone marker CTX was increased. Similar results were reported in a 28 days study in the mouse [36]. The results concerning bone formation markers were more surprising: PINP was significantly higher in GC treated mice group compared with PBO group after 4 weeks of GC treatment ($P<0.01$). On the contrary, osteocalcin remained unchanged. Previous studies have shown a decrease in bone formation markers in GC treated animals, principally osteocalcin [11, 12, 36, 37]. PINP measurement has been less studied; In GC treated BALB/c mice, GC increased CTX and decreased both PINP and osteocalcin after the 1st week [38]. Later, CTX values decreased and PINP tend to return to pretreatment values. PINP and osteocalcin are two bone formation markers involved at different stages of osteoblast differentiation: PINP is believed to be a marker expressed at the early or the proliferation phase; osteocalcin is a marker of more differentiated osteoblasts [39, 40]. This difference of expression could explain the discrepancy between the results but further studies are needed concerning the evaluation of the different bone markers.

Mice is a good model for GIOP, nevertheless the amount of alveolar bone is very low and do not permit a study of all histomorphometric parameters. Furthermore, histological analysis at the molar socket and in the inter-radicular area cannot be done by routine methods due to the presence of the enamel which impairs sectioning on bone microtomes equipped with tungsten carbide knives. MicroCT is a validated tool in the study of bone microarchitecture. It was easy to recognize the region of interest and 2D sections could be obtained in the very same area in all mice. Today, there is a growing body of interest concerning alveolar bone in clinical practice

and it has received little consideration up to now. The interest for osteoporosis, osteonecrosis of the jaw, periodontitis, and implant placement is increasing in medical and dental practice. Our study emphasized the negative impact of GCs on mandible alveolar bone.
In conclusion, our results confirm that long term administration of GCs induces a decreased bone formation with a reduction in mineral apposition rate and a decrease in bone volume and trabecular thickness in long bones in mice. The present study also showed, for the first time, that GCs can induce a decrease in alveolar bone in the mouse.

Acknowledgments

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Conflicts of interest: none

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