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ORIGINAL ARTICLE

Expression profile and synthesis of different collagen types I, II, III, and V of human gingival fibroblasts, osteoblasts, and SaOS-2 cells after bisphosphonate treatment

Maciej J. K. Simon · Peter Niehoff · Bernhard Kimmig · Jörg Wiltfang · Yahya Açil

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Abstract Bisphosphonates (BP) are used in the treatment of malignant osteolytic processes and postmenopausal osteoporosis. There have been a number of incidents in patients treated with BP. The incidents are described as an osteonecrosis of the jaw (ONJ). The main medications associated with these reports are zoledronic acid (ZOL) and pamidronate (PAM). The clinical presentations describe a deterioration of the jaw bones and surrounding tissues. The purpose of this study was to investigate expression of collagen types I, II, III, and V in human gingival fibroblasts, osteoblasts, and osteosarcoma cells (SaOS-2 cells) by ELISA and reverse transcription PCR (RT-PCR) with constant exposure (28 days) to ZOL and PAM. The real-time PCR indicates that ZOL inhibited gene expression below 16% at any concentration used. Hence, an amplification of extracellular matrix was only possible for PAM at concentrations of 1 μM. The following expression levels were for fibroblasts at a maximum of 31%, exceptionally high for the osteoblasts at 56%, and for SaOS-2 cells the peak was 14%. Principally, a decreased production of collagen was measured. With this in vitro study, we demonstrated how negatively influencing a long exposure to ZOL and PAM can be. Therefore, a reduction in extracellular matrix production of these cell lines under BP exposure could be a possible clinical indication as to why patients experience ONJ and have wound healing problems. However, it remains uncertain as to why an osteonecrosis is mainly found in the jaws and not other bones. As there are many influencing factors, further investigation needs to be pursued.

Keywords Bisphosphonates · Osteosarcoma cells · Osteoblasts · Gingival fibroblasts · Proliferation · Collagen production

M. J. K. Simon · J. Wiltfang · Y. Açil (✉)
Department of Oral and Maxillofacial Surgery,
Universitätsklinikum Schleswig-Holstein,
Campus Kiel, Arnold-Heller-Strasse 3, Haus 26,
24105 Kiel, Germany
e-mail: acil@mkg.uni-kiel.de

M. J. K. Simon
e-mail: maciej.simon@gmail.com

J. Wiltfang
e-mail: wiltfang@mkg.uni-kiel.de

P. Niehoff · B. Kimmig
Department of Radiotherapy (Radiooncology),
Universitätsklinikum Schleswig-Holstein,
Campus Kiel, Arnold-Heller-Strasse 9,
24105 Kiel, Germany

P. Niehoff
e-mail: niehoff@onco.uni-kiel.de

B. Kimmig
e-mail: kimmig@onco.uni-kiel.de

Introduction

Bisphosphonates (BP) are chemotherapeutical compounds used in treatment of patients with osteoporosis and malignant osteolytic processes, such as Paget's disease, hypercalcemia of malignancy, multiple myeloma, and tumor-associated osteolysis [1–6]. The aim of BP is to prevent bone loss. Its method is by blocking the mechanism of osteoclastic bone resorption [7]. The inhibiting mechanism of the BP depends on what generation it is. The newer compounds contain nitrogen atoms and are more potent [8]. Two of the nitrogen-containing BP are zoledronic acid (ZOL) and pamidronate (PAM). These chemotherapeutics are the ones mainly associated with the osteonecrosis of the jaw (ONJ) [9, 10]. This clinical complication of the BP has been reported numerous

times since 2003 [9, 11–15]. ONJ is also often correlated with previous oral surgical procedures, such as tooth extractions, during BP therapy [11, 13, 16]. However, there have been additional risk factors associated with ONJ, such as corticosteroid use, chemotherapy, radiotherapy, trauma, or infection, but the real pathophysiology has not yet been fully clarified. The potency of BP shows that ZOL has a greater potency than PAM and additionally a better patient compliance, which makes it easier to understand why most ONJ cases appear with ZOL. The mechanism of BP on osteoclasts is mostly understood, but the effects on osteoblasts have not been elucidated to its core. There seems to be a paracrine influence from the osteoblasts affecting the net amount of osteoclasts [17].

On top, the nitrogen-containing BP affecting tumor cells described a reduction of the survival, proliferation, adhesion, migration, and invasion of tumor cells in vitro [18]. The consequences of treatment with BP for other cell types, like osteoblasts and fibroblasts, are not yet fully investigated nor understood.

The purpose of this study was to investigate the effects of BP on these three cell types, gingival fibroblasts, osteoblasts, representing the oral cavity, and SaOS-2 cells. Therefore, we exposed these cells in vitro with constant exposure to BP (28 days) and performed an ELISA and a quantitative analysis of the expression of collagen types I, II, III, and V with the real-time PCR.

Materials and methods

Human cell culture

Human osteoblasts and fibroblasts were isolated from the samples of the iliac crest and of the gingiva, respectively. The osteoblast samples derived from healthy patients, without BP therapy, undergoing reconstruction surgery for cleft lip and palate from our department of oral and maxillofacial surgery. All patients had been informed about this study and had signed a letter of informed consent (Ethic number D 402/07). Preparation of the tissue samples and further processing was performed as previously described [19, 20]. Human osteogenic sarcoma cells (SaOS-2 cells) were purchased from DSZM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Spongiosa cells of the sample and SaOS-2 cells were defined as osteoblast-like cells by the determination of osteoblast markers (biosynthesis of osteocalcin and activity of alkaline phosphatase). Fibroblasts from gingival samples were obtained during routine surgical procedures. Small tissue fragments were incubated in Dulbecco's MEM (Seromed T 043-01) with fetal calf serum (FCS) under physiological conditions at 37°C and 95% air and 5% CO_2. Further additions to the fibroblasts were 10^5IU penicillin, 100 mg/l streptomycin, 2 mM L-glutamine and 1 mM L-ascorbic acid 2-phosphate. The gingival fibroblasts were tested and exhibited the typical morphologic fibroblast patterns. The tests for osteocalcin and alkaline phosphatase were negative.

The cells were subcultured in DMEM with FCS at 37°C and 95% air and 5% CO_2, and with 10^5IU penicillin, 100 mg/l streptomycin, 2 mM L-glutamine and 1 mM L-ascorbic acid 2-phosphate for fibroblasts; and for osteoblast-like cells, 100 nM dexamethasone (Biochrom, Berlin, Germany) was added. Cells were subcultured in second and third transfers at a density of $2.9\times10^6/cm^2$. For the transfers, a PBS–EDTA/Trypsin (10:1) solution 0.05% / 0.02% (*w*/*v*) (Biochrom) was used. Each cell line was seeded on multiple six-well plates at a density of 5×10^5 cells.

Table 1 Primers used for PCR and their sequences (sense and antisense)

Proteins		Primer sequences
GAPDH	Sense:	5′ GAG TCA ACG GAT TTG GTC GT 3′
	Antisense:	5′ GAC AAG CTT CCC GTT CTC AG 3′
Osteocalcin	Sense:	5′ TTC TGT GGG TGG AAG GAG AC 3′
	Antisense:	5′ GGG GAG AGC CCC TAT TTA AG 3′
Collagen type I	Sense:	5′ CCC CAG CCA CAA AGA GTC TA 3′
	Antisense:	5′ CTG TAC GCA GGT GAT TGG TG 3′
Collagen type II	Sense:	5′ ATG AGG GCG CGG TAG AGA C 3′
	Antisense:	5′ CGG CTT CCA CAC ATC CTT AT 3′
Collagen type III	Sense:	5′ AGG GGA GCT GGC TAC TTC TC 3′
	Antisense:	5′ CGG ATC CTG AGT CAC AGA CA 3′
Collagen type V	Sense:	5′ CGA TCC TGT GGA TGT CCT G 3′
	Antisense:	5′ TGG CCT TCT GGA AAG AGT TC 3′
ALP	Sense:	5′ GAG TCA ACG GAT TTG GTC GT 3′
	Antisense:	5′ GAC AAG CTT CCC GTT CTC AG 3′

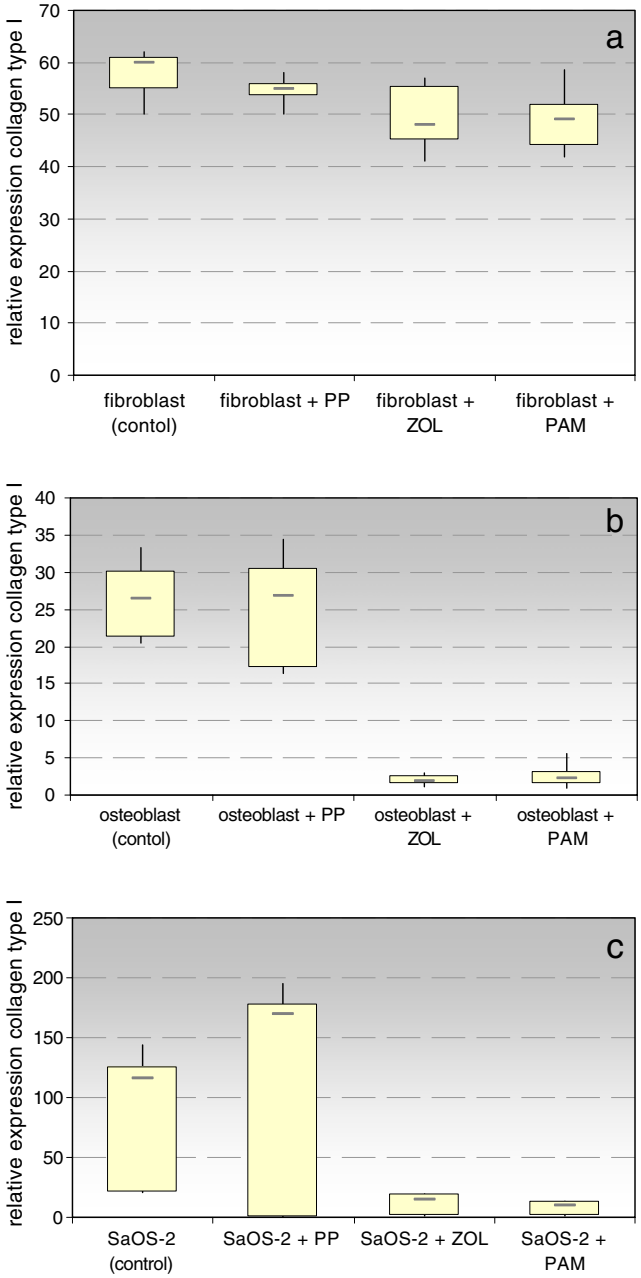

Fig. 1 **a**–**c** Box plots, relative mean expression levels of collagen types I at 1 μM in fibroblasts (**a**, control), osteoblasts (**b**, PP), and SaOS-2 cells (**c**, PP) were significantly higher than with ZOL and PAM. The number of samples (n=6 for each cells), minimum, maximum, and median are shown

Alkaline phosphatase (ALP) activity

ALP activity was determined by cytochemistry with Sigma Diagnostic Kit (86-R, Deisenhofen, Germany), as described previously [19–21].

Osteocalcin synthesis

The expression of osteocalcin was studied using monoclonal antibodies provided by Takara (Takara Shuzo, Co., Ltd., Japan), as described previously [19, 20].

Bisphosphonates (BP)

In order to relate the scientific research to clinical processes, the decision was made to use the most common drugs associated with ONJ [9, 13, 16, 22, 23]: zoledronic acid (ZOL; Zoledronic acid, Zometa, Novartis®, 200 mg, i.v. Nürnberg, Germany) and pamidronate (PAM; Pamidronate, Aredia, Novartis®, 506 mg, i.v.); plus inorganic pyrophosphate (PP; Pyrophosphorsäure techn., Aldrich, Taufkirchen, Germany) as a positive control component. Four different concentrations of each molecular compound were elected and used in this study (1, 5, 10 and 20 μM).

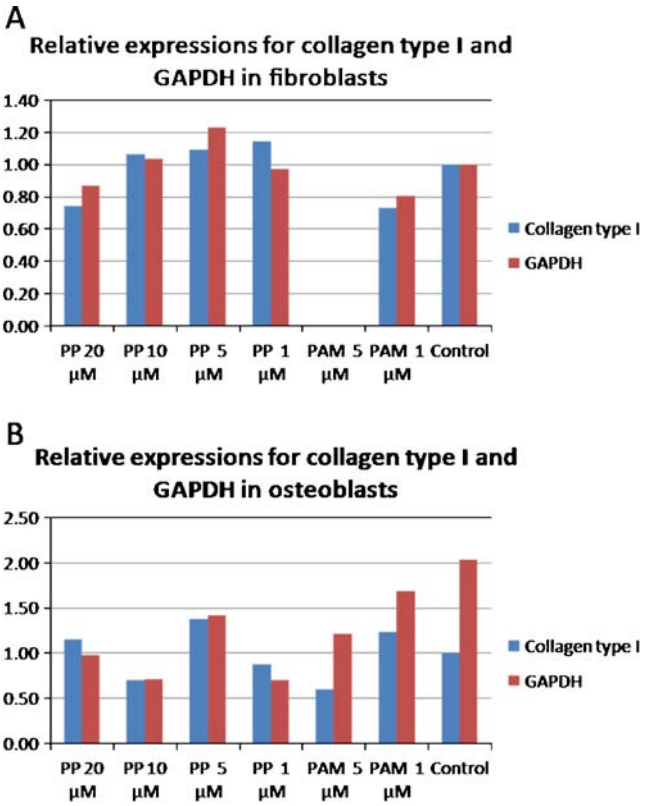

Fig. 2 **a**, **b** Relative expression of GAPDH and collagen type I show great accordance in fibroblasts (**a**) and osteoblasts (**b**) supplemented with PP. PAM induces an expression increase for GAPDH. Osteoblasts have a higher expression of GAPDH than collagen type I as seen in the control (**b**). Therefore, the higher GAPDH expression in PAM-supplemented cell culture medium in osteoblasts is of norm (**b**). PAM in 5 μM concentration did not allow sufficient expressions in fibroblasts (**a**)

Four-week cell culture

After seeding of the cells (at 5×10^4 cells per well) of each cell line, they were cultured in ten groups of six-well plates, each with different concentrations of supplemented growth medium, for 4 weeks. Every third day, the medium was renewed with the three different substrates (ZOL, PAM, PP) and with the four different concentrations. The used growth medium from the cell cultures was saved and pooled from every transfer for further testing. The experiment was terminated on day 28, and analysis was performed.

Enzyme-linked immunosorbent assay (ELISA)

After each week, a sample of 100 µl cell culture medium with their specific supplemented concentrations of the 4-week cell culture was collected and further used in the standardized ELISA. The following assay was performed for collagen type I and finalized with a flowthrough spectrometer.

RNA extraction and cDNA synthesis

RNA was extracted from the final cell-medium solution according to the standard protocols using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from 1 µg of RNA using the QuantiTect Reverse Transcription Kit (Qiagen).

Detection of the transcripts by real-time PCR

Expression of the transcripts was determined by means of PCR using the QuantiTect SYBR Green PCR Kit (Qiagen). One undiluted microliter of the exprimed cDNA was added to 19 µl of SYBR Green PCR Mix (Qiagen), and a real-time PCR was performed using the LightCycler® (Roche, Mannheim, Germany). GAPDH was used for normalizing the threshold cycle (C_t), while H_2O was used as negative control. All measurements were performed six-fold. Every set of experiments comparing the expression of GAPDH, osteocalcin, collagen type I, II, III, and V, and alkaline

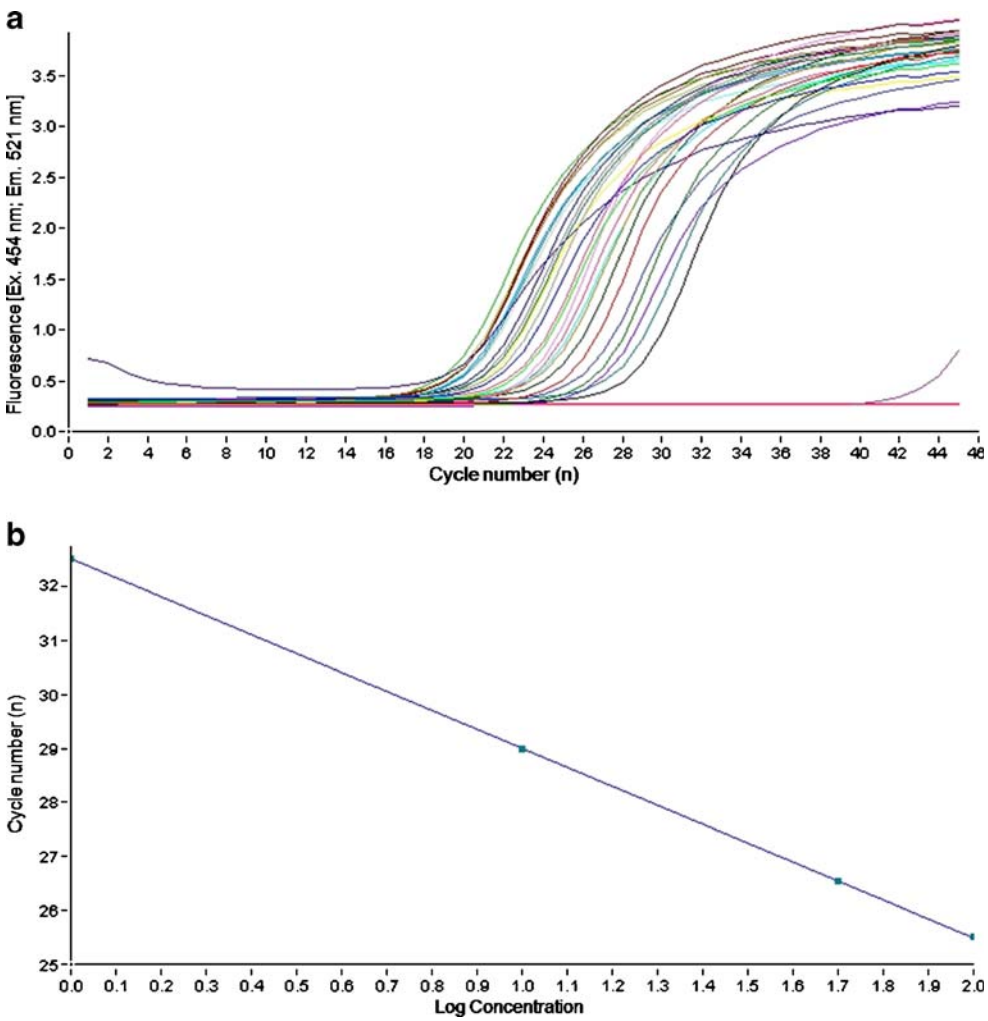

Fig. 3 **a**, **b** Graph of real-time PCR for collagen type I from human osteoblasts. **a** The *curves* represent dilution of template concentrations (1:1; 1:2; 1:10; 1:100). **b** Logarithmic graph for standard curve

phosphatase (ALP) (fibroblasts, osteoblasts) was carried out with cDNA from the same sample. Primer sequences for the transcripts investigated are summarized in Table 1.

Only samples of the PCR producing a single DNA fragment, analyzed by gel electrophoresis, were used for the statistical analysis.

Statistical analysis

For comparison of the frequencies of extracellular matrix protein (collagen type I) and RNA expression in gingival fibroblasts, osteoblasts, and SaOS-2 cells with and without bisphosphonates, non-parametric median tests were employed. Statistical analysis was performed by Wilcoxon signed rank test and a Wilcoxon sum rank test (values considered significant at $p<0.05$) (Fig. 1).

Results

The present study investigated the expression of extracellular proteins in gingival fibroblasts, osteoblasts, and SaOS-2 cells under a constant BP exposure of 28 days. Gene expression was monitored by real-time PCR after reverse transcription of the corresponding mRNA. Six groups of samples were treated and analyzed. The relative mean expression levels of collagen types I, III, and V, osteocalcin, ALP, and GAPDH in untreated cells (controls) were significantly higher than those in treated cells (data not shown). The relative expression levels are demonstrated for collagen type I of fibroblasts, osteoblasts, and SaOS-2 cells treated with the specific growth media (Fig. 1). No expression of collagen type II was observed in any of the samples studied. Otherwise, no significant differences regarding the amounts produced of the different collagen types I, III, and V within one cell line were observed under the influence of PP. However, PP influenced collagen types I, III, and V expression more positively in osteoblast-like cells than in fibroblasts. (Fig. 1a–c; only collagen type I). There is a 5 μg threshold for the amplification of RNA, which was not reached in cell cultures with high concentrations of ZOL and PAM, as supplements. Only concentrations of 1 μM of ZOL and PAM reached the threshold. Due to that, the study was able to show a low gene amplification (<16%) for cells with a 1 μM concentration of ZOL as a supplement. At 1 mM, PAM allowed a greater amplification in fibroblasts, osteoblasts, and SaOS-2 cells (31%, 56%, and 14%, respectively). Consequently, a proper assessment was only possible with data related to BP concentrations at 1 μM, because higher concentrations were toxic to the cells and no sufficient expression of genes was detectable. Comparing our statistical analysis and the box plots (Fig. 1a–c) with the RT-PCR and RNA expression results shows a great resemblance of statistical significance ($p<0.05$). Relative expression of collagen type I and the housekeeping gene GAPDH demonstrates accordance or minor increase of GAPDH versus collagen type I (Fig. 2a, b). The quality of real-time PCR experiments was determined by monitoring reactions with different template concentrations of control collagen type I cDNA (Fig. 3b). There was a linear logarithmic dependency on template concentration. In addition, the expression of mRNA for collagen type I was visualized on a gel electrophoresis scan and supported the set threshold for no sufficient amplification of samples just below the threshold (Fig. 4).

The data of ELISA detection of collagen type I support the findings of reduced mRNA found in the present study after 4 weeks (Fig. 5a–d). Fibroblasts showed a weekly reduction in collagen type I production (Fig. 5a, b). In addition, PAM and ZOL decreased the osteoblastic collagen type I synthesis, whereas the control production of collagen type I was constantly increased (Fig. 5c, d).

Discussion

In the present study, the expression of human collagen types I, II, III, and V, osteocalcin, ALP, and GAPDH from human gingival fibroblasts, osteoblasts, and SaOS-2 cells

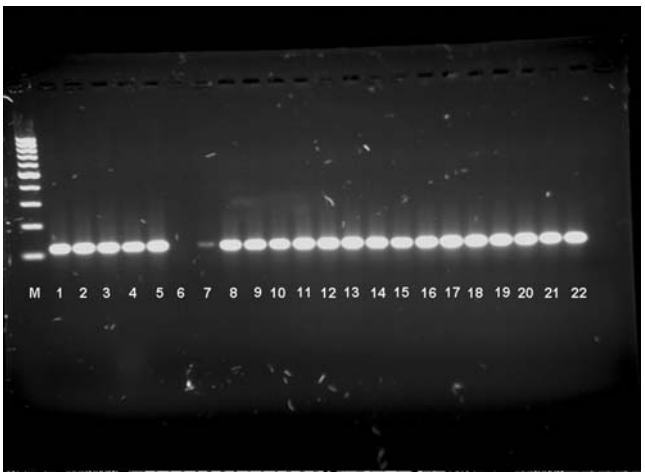

Fig. 4 Expression of mRNA from collagen type I in 22 samples from human cells detected by RT-PCR. No expression was assessed for ZOL at 20 μM (*lane 6*) and only a minor mRNA expression could be visualized under 1 μM ZOL supplementation (*lane 7*) compared to others. *M* marker. *Lanes 1–9*: SaOS-2 cells. *1* Control, *2* 20 μM pyrophosphate, *3* 10 μM pyrophosphate, *4* 5 μM pyrophosphate, *5* 1 μM pyrophosphate, *6* 20 μM zoledronic acid, *7* 1 μM zoledronic acid, *8* 5 μM pamidronate, *9* 1 μM pamidronate. *Lanes 10–15*: fibroblasts. *10* Control, *11* 20 μM pyrophosphate, *12* 10 μM pyrophosphate, *13* 5 μM pyrophosphate, *14* 1 μM pyrophosphate, *15* 1 μM Pamidronate®. *Lanes 16–22*: osteoblasts. *16* Control, *17* 20 μM pyrophosphate, *18* 10 μM pyrophosphate, *19* 5 μM pyrophosphate, *20* 1 μM pyrophosphate, *21* 5 μM pamidronate, *22* 1 μM pamidronate

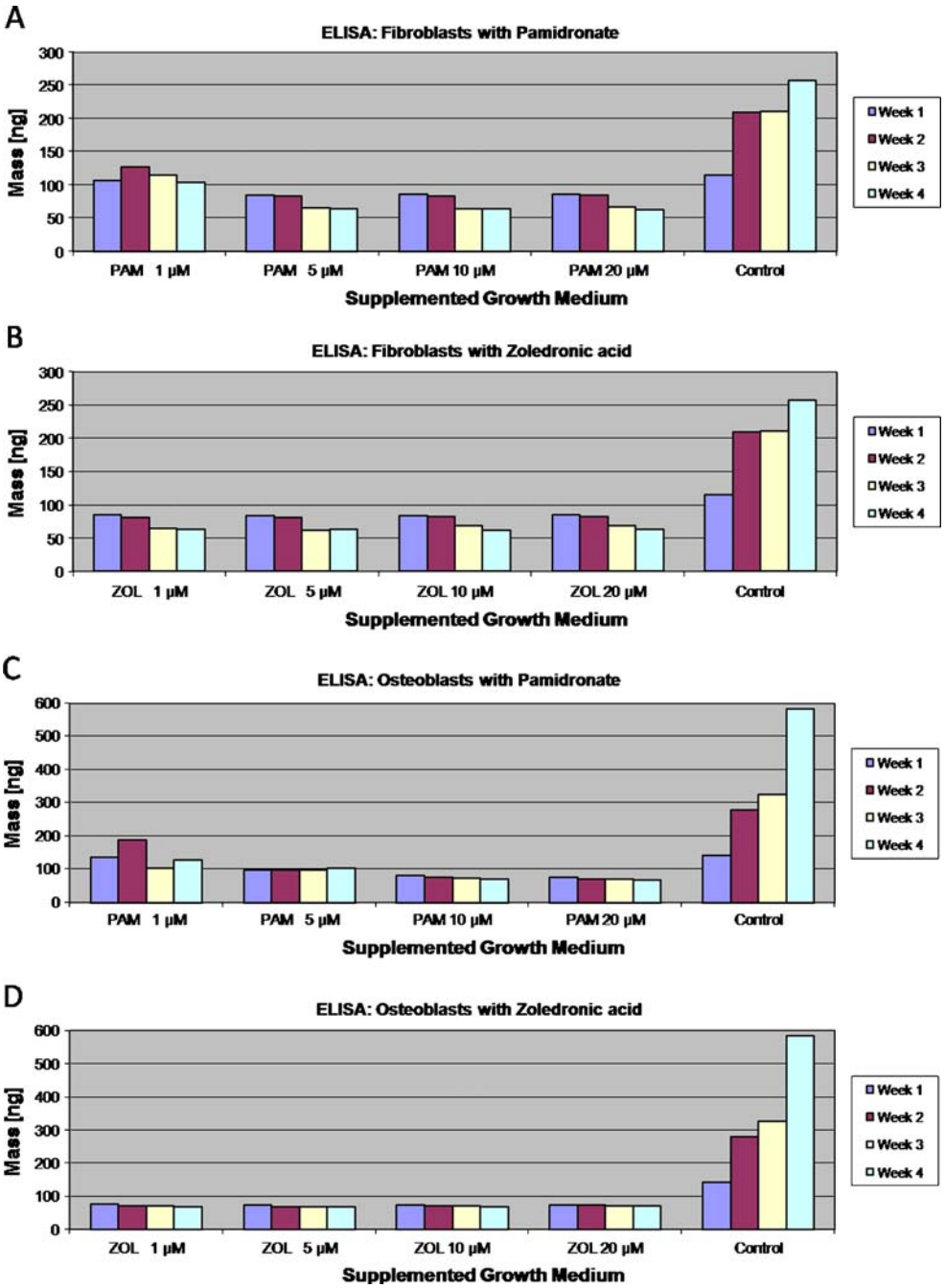

Fig. 5 **a**–**d** Weekly ELISA collagen type I production evaluation of the fibroblasts (**a**, **b**) and osteoblasts (**c**, **d**) treated with ZOL or PAM in the supplemented culture medium. Fibroblasts show a constant decrease in the collagen type I synthesis in each concentration of PAM (**a**) and ZOL (**b**) supplemented. Osteoblasts demonstrated a stronger decrease over the 4-week period when supplemented with PAM (**c**). ZOL significantly inhibited the collagen type I production from the first week on (**d**). Control growth proves a weekly augmentation in all cell lines (**a**–**d**)

treated for 4 weeks with ZOL, PAM, and PP was determined by real-time PCR.

Of comparable significance, another study analyzed the expression of a messenger polypeptide from osteoblasts. This polypeptide affects osteoclasts in their bone resorption characteristics [24]. However, the concentrations of BP and exposition time were different. Additionally, they did not notice an expression of any inhibiting factors of fibroblasts. In contrast, in an in vivo model, slight bone formation was observed under BP influence in the first week [17]. Interestingly, the formed bone became necrotic from the second week and an overall reduction in bone formation was histopathologically registered. More in line with our study in collagen expression, Santini and colleagues investigated the gene expressions of VEGF, PDGF, and βCTX (β-crosslinked type I collagen C telopeptide) [6, 25]. They noted a reduction of gene expression even after one single infusion of ZOL indicating a possible antiangiogenic effect and an impaired bone-forming effect of ZOL.

In the present survey, a significant reduction in the expression of collagens was detected. In cells treated with high concentrations of ZOL, a quantitative threshold of

mRNA was not reached in cell cultures; therefore, a real-time PCR was only possible to execute at 1 μM concentration. We also noted that sufficient threshold levels of cells treated with PAM were only reached in 1 mM concentration.

The housekeeping gene GAPDH was being questioned to be of proper use as a control gene with BP by Valenti et al. [26]. Their results showed that amino-BP reduced in a dose-dependent manner the expression of the GAPDH gene. Nonetheless, their analysis was performed on breast and prostate cancer cell lines and not on benign human osteoblasts and fibroblasts. The atypical characteristics of malign cells are well known and, as various studies [27–29] show that GAPDH can be up and down-regulated, an internal control in each study should be performed and thereafter decided whether it is appropriate to use. The current study expressed a clear concordance of GAPDH and collagen type I under BP treatment (Fig. 2a, b).

In addition to the current study of real-time PCR experiments, minor deviations could be excluded (Fig. 2a). Any deviation of measuring points from the standard curve is minimal (Fig. 2b). Consequently, the method of PCR is not the reason for any of the observed variations. Furthermore, present ELISA results demonstrate a reduction pattern of collagen type I over a 4-week period (Fig. 5a–d) and support the real-time PCR results.

Previously, many clinical observations of ONJ [11–13, 16, 30] have been published, but no analyses of the gene expressions of human gingival fibroblasts and osteoblasts with this long exposition time to BP have been performed. Therefore, it should be of clinical interest that we measured a reduced expression of collagens in those cells treated with BP under constant exposure.

With this collected data, we see a possible link and influence of BP treatment on the gene expression. Furthermore, Sedghizadeh and colleagues [31] recently identified large areas of biofilms, mostly compromised of bacteria, on affected specimens from ONJ patients. These bacterial biofilms additionally compromise the already poor wound healing processes that have been observed under BP [32, 33]. Therefore, new clinical and therapeutic aspects have to be taken into account and they need to be monitored and further analyzed.

In summary, our results indicate a reduction in collagen expression of fibroblasts, osteoblasts, and SaOS–2 cells, with the focus on bone and gingival formation. BP probably acted not only in restricting the collagen expression but also were most probably toxic to cells in this constant exposure study. Therefore, a reduction in the overall gene transcripts is a normal consequence. Comparing the properties of ZOL and PAM, ZOL was shown to be more potent and confirmed our expectations (Fig. 4). Our findings could possibly supplement clinical reports discussing why patients under BP treatment develop necrotic bone, destruction of the surrounding tissue, and have wound healing difficulties. For that reason, future studies will have to determine whether altered collagen expression is the key point, or whether the toxicity of the substrate itself is, or if the general homeostasis is compromised. Furthermore, it would be interesting to investigate the time period when protein production ceases and no active bone formation or gene expression is noticeable. Lastly, the influences of additional environmental factors, such as bacteria or other medications, in addition to the BP exposure, are of significant interest.

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Conflict of interest The authors declare that they have no conflict of interest.

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