

## CONCISE REPORT

# Effect of bisphosphonates on viability, proliferation, and dexamethasone-induced apoptosis of articular chondrocytes

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**Background:** Bisphosphonates (BP) increase bone mass in patients with rheumatoid arthritis and are effective in the prevention and treatment of steroid-induced osteoporosis. However, little is known about their direct effects on chondrocytes.

**Objectives:** To study the influence of BP on articular chondrocytes in vitro and to investigate whether BP can prevent steroid-induced apoptosis of articular chondrocytes.

**Methods:** Bovine articular chondrocytes were cultured and incubated with different concentrations of clodronate, pamidronate, risedronate, or dexamethasone. In the second part of the study, BP were added to the chondrocyte cultures one hour before co-incubation with dexamethasone. Viability and proliferation were evaluated using propidium iodide staining and tritium labelled thymidine incorporation. Apoptosis was measured with annexin V staining or the TUNEL method.

**Results:** Only high concentrations ( $>10^{-6}$  mol/l) of clodronate, pamidronate, and risedronate induced a decrease in the viability and proliferation of chondrocytes. None of the BP at concentrations ranging from  $10^{-12}$  to  $10^{-3}$  mol/l induced apoptosis. Growth retardation and apoptosis induced by dexamethasone ( $10^{-7}$  mol/l) was prevented by addition of pamidronate ( $10^{-6}$  mol/l) or risedronate ( $10^{-8}$  or  $10^{-6}$  mol/l).

**Conclusion:** Bisphosphonates in therapeutic concentrations are safe for articular chondrocytes in vitro. Moreover, pamidronate and risedronate prevent dexamethasone-induced growth retardation and apoptosis of chondrocytes. These findings add evidence for a chondroprotective effect of nitrogen-containing BP, especially in patients treated with corticosteroids.

Bisphosphonates (BP) are strong inhibitors of osteoclast mediated bone resorption. These compounds increase bone mass in patients with rheumatoid arthritis (RA)<sup>1</sup> and are effective in the prevention and treatment of steroid-induced osteoporosis.<sup>2</sup> Furthermore, several studies have shown that BP might have an additional anti-inflammatory effect in experimental arthritis and RA owing to an increased apoptosis of macrophages.<sup>3</sup> In contrast with their effects on osteoclasts and macrophages, the influence of BP on cartilage has been much less studied. A few reports point to some chondroprotective effect of BP,<sup>4,5</sup> but little is known about their direct consequences on chondrocytes.

This study aimed at investigating whether BP have an influence on viability, proliferation ability, and apoptosis of articular chondrocytes in vitro. In a second part of the study we investigated whether BP can prevent corticosteroid-

induced growth retardation and apoptosis of articular chondrocytes.

## METHODS

### Chondrocyte culture

Bovine chondrocytes were obtained from the tarsometatarsal joint immediately after slaughter. Cells were isolated using hyaluronidase (Boehringer Mannheim), protease (Sigma), and collagenase type 1A (Sigma) as described previously.<sup>6</sup> Primary chondrocytes were cultured during 24 hours in Dulbecco's modification of Eagle's medium (DMEM) 10% fetal bovine serum (FBS) and identified as chondrocytes by staining for S-100 and collagen II production.

### Incubation with bisphosphonates or dexamethasone

In one experiment chondrocytes were washed with DMEM 10% FBS and incubated for 48 hours with the BP clodronate, pamidronate, or risedronate at different concentrations ( $10^{-12}$ ,  $10^{-10}$ ,  $10^{-8}$ ,  $10^{-6}$ ,  $10^{-4}$ , or  $10^{-3}$  mol/l) or dexamethasone in a therapeutic and pharmacological concentration (respectively  $10^{-7}$  and  $10^{-5}$  mol/l) in DMEM 2% FBS containing 100 U/ml bovine interferon  $\gamma$  (Novartis Animal Health Inc) and the proinflammatory cytokines human interleukin  $1\alpha$  (5 ng/ml) and human tumour necrosis factor  $\alpha$  (5 ng/ml; Peprotech House).<sup>7</sup> The culture medium (DMEM 2% FBS) was enriched with  $\text{CaCl}_2$  (final concentration 4.5 mmol/l) when cells were incubated with BP.<sup>8</sup> As positive control apoptosis was induced with 0.01 mM  $\text{H}_2\text{O}_2$  during the last 12 hours of incubation.

### Co-incubation experiments

In three experiments at a time, cells were preincubated with clodronate, pamidronate, or risedronate in different concentrations. After one hour, dexamethasone at a therapeutic ( $10^{-7}$  mol/l) or pharmacological concentration ( $10^{-5}$  mol/l) was added for a further co-incubation during 48 hours.

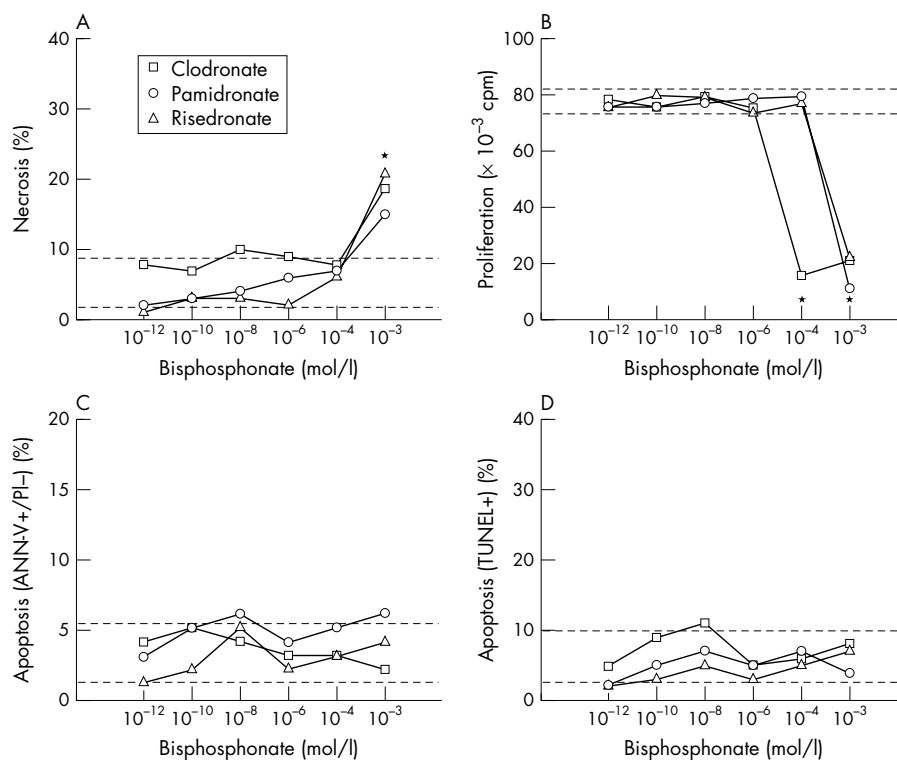
### Viability of chondrocytes

Chondrocytes were incubated for five minutes with 50  $\mu\text{g/ml}$  propidium iodide.<sup>9</sup> Ten thousand cells were measured on the flow cytometer. Propidium iodide (PI) positive cells were considered to be non-viable cells. Results were expressed as a percentage of non-viable PI+ cells.

### Chondrocyte proliferation

Chondrocyte proliferation after 48 hours was determined by a tritium labelled thymidine incorporation method.<sup>9</sup> All experiments were done in quadruplicate. Results were expressed as counts per minute (cpm).

**Abbreviations:** BP, bisphosphonates; cpm, counts per minute; DMEM, Dulbecco's modification of Eagle's medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; PI, propidium iodide; RA, rheumatoid arthritis



**Figure 1** Influence of clodronate, pamidronate, and risedronate on chondrocyte necrosis (A), proliferation (B), and apoptosis (C/D). (A) Percentage of PI+ necrotic cells (10 000 cells were measured). (B) Proliferation of cells (tests were done in quadruplicate). (C) Percentage of annexin V positive, PI- apoptotic cells (10 000 chondrocytes were measured). (D) Percentage of TUNEL positive apoptotic cells (10 000 cells were measured). Dashed lines represent ranges of control cultures. \* $p < 0.05$ : chondrocytes incubated with bisphosphonates v control cultures.

#### Apoptosis measured by annexin V staining

The chondrocytes were resuspended in 100  $\mu$ l HEPES buffer containing 140 mM NaCl, 2.5 mM CaCl<sub>2</sub> and stained with 5  $\mu$ l annexin V-FITC (Pharmingen) and 10  $\mu$ l PI (50  $\mu$ g/ml) for 15 minutes at room temperature in the dark. Five thousand cells were measured on a FACScan flow cytometer and analysed with WinMDI software. Results were expressed as the percentage (PI negative and annexin V positive) apoptotic cells.

#### Apoptosis measured by end labelling of fragmented DNA

The chondrocytes were fixed with 1% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes. After washing with PBS, cells were fixed in 70% ethanol at  $-20^{\circ}\text{C}$  for one hour. Subsequently, a two colour staining method for labelling DNA breaks was performed, using an Apo BrdU kit (Pharmingen) to detect apoptotic cells. Five thousand cells were measured on a FACScan flow cytometer and analysed with WinMDI software. Results were expressed as the percentage (TUNEL positive) apoptotic cells.

#### Statistics

An analysis of variance and Student's *t* test were performed to analyse differences between samples.

### RESULTS

#### Incubation with dexamethasone or bisphosphonates

The percentage of PI+ cells varied between 2% and 9% in control cultures (without BP or dexamethasone) and increased to 23–27% if incubated with dexamethasone  $10^{-7}$  mol/l and to 33–37% with dexamethasone  $10^{-5}$  mol/l. The range of proliferation was  $74\text{--}83 \times 10^3$  cpm in control cultures,  $8\text{--}17 \times 10^3$  cpm with dexamethasone  $10^{-7}$  mol/l, and  $5\text{--}12 \times 10^3$  cpm with dexamethasone  $10^{-5}$  mol/l. The percentage of apoptotic chondrocytes in control cultures was 0–4% with the annexin V

method and 1–6% with the TUNEL assay. With dexamethasone  $10^{-7}$  mol/l apoptotic cells increased to 23–31% (annexin V method) or 26–30% (TUNEL-assay). Dexamethasone  $10^{-5}$  mol/l induced apoptosis in 39–45% (annexin V method) or 42–47% (TUNEL assay) of chondrocytes.

The percentage of PI+ cells was only higher when cells were incubated with  $10^{-3}$  mol/l clodronate, pamidronate, and risedronate, compared with control chondrocytes ( $p=0.02$ ) (fig 1A).

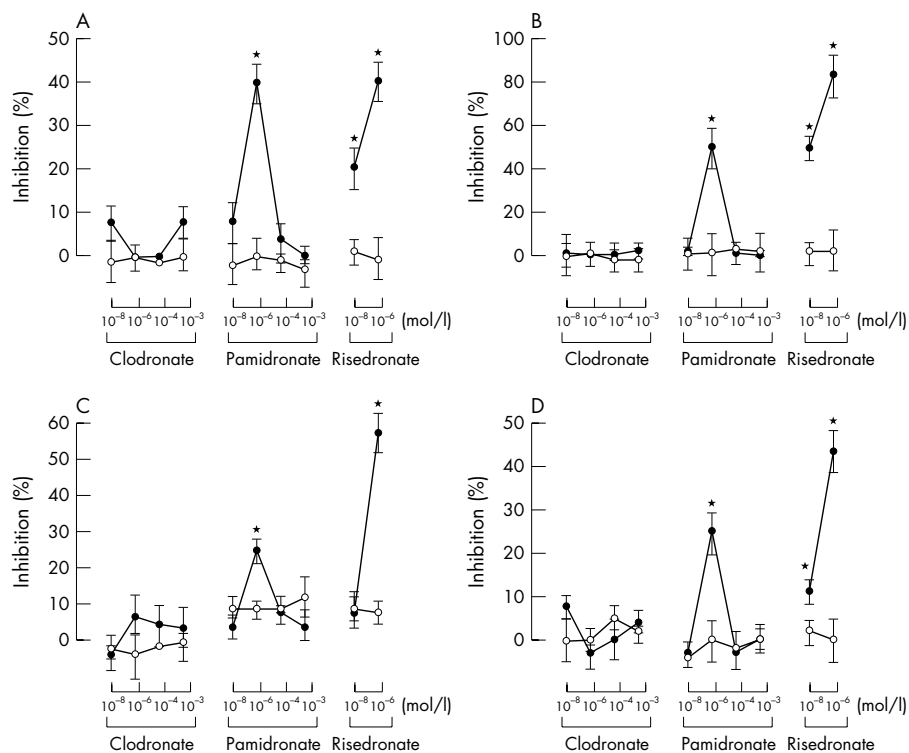
Only at high concentrations of clodronate, pamidronate, and risedronate ( $10^{-4}$  and/or  $10^{-3}$  mol/l) was there a decrease in proliferation, determined by incorporation of tritium labelled thymidine, compared with control chondrocytes ( $p=0.03$ ) (fig 1B).

Clodronate, pamidronate, and risedronate at all concentrations did not induce apoptosis of chondrocytes as measured by annexin V or TUNEL assay (figs 1C and D).

#### Co-incubation with bisphosphonates and dexamethasone

With the therapeutic dose of dexamethasone ( $10^{-7}$  mol/l) the percentage of PI+ cells decreased when chondrocytes were co-incubated with pamidronate  $10^{-6}$  mol/l ( $p=0.04$ ) or risedronate  $10^{-6}$  mol/l and  $10^{-8}$  mol/l ( $p=0.04$  and  $p=0.03$ , respectively) (fig 2A). Dexamethasone-induced growth retardation of chondrocytes was inhibited if cells were co-incubated with pamidronate ( $10^{-6}$  mol/l) ( $p=0.02$ ) or risedronate ( $10^{-8}$  mol/l or  $10^{-6}$  mol/l) ( $p=0.03$ ,  $p=0.05$ , respectively) (fig 2B). Addition of risedronate ( $10^{-8}$  or  $10^{-6}$  mol/l) or pamidronate ( $10^{-6}$  mol/l), but not clodronate, did inhibit partly the dexamethasone-induced apoptosis (respectively  $p=0.02$ ,  $p=0.03$ , and  $p=0.04$ ). Similar results were found with annexin V analysis (fig 2C) and the TUNEL assay (fig 2D).

Bisphosphonates could not prevent necrosis, growth retardation, or apoptosis of chondrocytes induced by dexamethasone  $10^{-5}$  mol/l (fig 2).



**Figure 2** Influence of clodronate, pamidronate, and risedronate on chondrocytes co-incubated with dexamethasone 10<sup>-7</sup> mol/l (closed symbols) or 10<sup>-5</sup> mol/l (open symbols). Results expressed as percentage of inhibition of: (A) necrosis; (B) growth retardation; (C) apoptosis measured by annexin V; and (D) apoptosis measured by TUNEL. Bars represent the standard error of the mean of three experiments. \*p<0.05: chondrocytes incubated with both dexamethasone and bisphosphonates v chondrocytes incubated with dexamethasone alone.

## DISCUSSION

Cartilage destruction in RA is closely related to the degree of apoptosis of articular chondrocytes.<sup>10</sup> The results of our in vitro study show that there is no induction of apoptosis of articular chondrocytes by different concentrations of BP, suggesting that these compounds are safe for cartilage metabolism. This is in accordance with in vivo observations of chondrocytes in growth plates: pamidronate in children with severe osteogenesis imperfecta did not alter the growth rate or the appearance of the growth plates.<sup>11</sup>

Therapeutic use of corticosteroids reduces bone formation by increasing osteoblast apoptosis and reduces growth cartilage thickness, probably by increasing apoptosis of chondrocytes.<sup>12</sup> Dexamethasone suppresses type 2 collagen expression and proliferation of rat articular chondrocytes.<sup>13</sup> In our study dexamethasone reduced the viability and proliferation ability of bovine articular chondrocytes in vitro and induced apoptosis. An important finding is that this growth retardation and apoptosis might be inhibited by pamidronate and risedronate at therapeutic concentrations but not by clodronate. BP can be divided into two classes, which may explain some of the pharmacological differences: those that can be metabolised into non-hydrolysable analogues of ATP such as clodronate and those that are not metabolised but that can inhibit protein prenylation. The latter are the more potent nitrogen-containing BP such as pamidronate and risedronate.<sup>3</sup>

The results of our study correspond with the growing evidence that despite their pro-apoptotic consequences on osteoclasts and macrophages, BP may have opposite effects on other cells. It has been shown that BP can enhance the differentiation and bone forming activities of osteoblasts.<sup>14</sup> Furthermore, it has been demonstrated that BP prevent steroid-induced apoptosis of osteoblasts in vitro and in vivo.<sup>15</sup>

In conclusion, our results show that BP are safe for articular chondrocytes in vitro. Moreover, to our knowledge this study is the first to demonstrate a protective effect of BP on

steroid-induced apoptosis and growth retardation of articular chondrocytes. This observation adds more evidence for a chondroprotective effect of nitrogen-containing BP, especially in patients treated with corticosteroids.

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