



# Effects of Ketorolac on Fibroblasts and Osteoblasts an *In-Vitro* Study

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## Abstract

**Objective:** Ketorolac and other nonsteroidal anti-inflammatory drugs are routinely used in the postoperative period. However, their effect on fracture and tissue healing remains unclear and controversial. The primary outcome of this investigation was to assess and compare the potential cytotoxicity of clinically relevant concentrations of ketorolac on human osteo- and fibroblasts.

**Methods:** Two cells lines, human osteosarcoma cells with the morphology of human fibroblasts and human osteoblasts were both cultured in a modified Eagle's medium and exposed to different concentrations of ketorolac from 0.9375 to 7.5 ug/ml for either 2 days (short time exposure) or 9 days (long time exposure).

Live cell count was assessed using trypan blue staining. Viability was measured by the tetrazolium bromide assay. Proliferation tests were performed with the help of the colorimetric bromodeoxyuridine assay. Caspase-3 activity was performed by fluorimetric assay.

**Results:** Treatment of cells showed a dose- and time dependent decrease of cell count, viability and proliferation. Osteoblasts were more sensitive to the effects of ketorolac. Apoptosis was noted, but not to the extent to explain the results.

**Conclusion:** This study showed a dose- and time dependent cytotoxic effect of ketorolac on both osteoblasts and fibroblasts. Osteoblasts were more sensitive than fibroblasts. Apoptosis was observed but not to the extent to explain the results, suggesting other mechanisms being involved in the toxicity induced by ketorolac on these cells.

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**Keywords:** Ketorolac; Fibroblasts; Apoptosis; Cytotoxicity.



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## Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are cornerstone means for the management of postoperative pain and a large number of medical inflammatory conditions [1]. NSAIDs and particular ketorolac have been introduced as part of periarthicular local infiltration technique [2,3]. In the operative setting fibroblasts play a crucial role in granulation tissue remodeling and wound contraction[4]. Osteoblasts are the biological machinery involved in endochondral bone formation during fracture repair [5]. Several studies have shown negative effects of NSAIDs on wound bone healing and especially on chondrocytes after topical infiltration or continuous infusion [6,7].

However, there is still controversy regarding at the effect of NSAIDs on bone healing in humans and the results of in-vitro, animals and human studies do not permit to draw conclusive information [8]. Ketorolac was shown an ex-vivo skin growth model to have cytotoxic effect on the growth of epithelial cells[9], but a systematic review could not draw any definitive conclusions on soft tissue healing[10]. Therefore, the behavior of osteoblasts and fibroblasts is of major importance of tissue healing after orthopedic surgery. The aims of this work were to compare in-vitro the effects of different concentrations of ketorolac on osteoblasts and fibroblasts.

## Methods

### Fibroblasts and Osteoblasts

Human osteosarcoma cells (LGC Standard GmbH, Wesel, Germany), which are osteoblast-like cell types with the morphology of human fibroblasts were used. These cells have the characteristics of proliferative wound fibroblasts according to Jukkola et al. [11] Additionally human osteoblasts (hfOB 1.19, LGC, Promochem; catalogue number: CRL-11372) were involved and both cultured in  $\alpha$ -modified Eagle's medium (MEM; LGC Standard GmbH) with 10% fetal bovine serum (FBS; LGC Standard GmbH) and 10'000U/l penicillin/streptomycin (LGC Standard GmbH) at 37°C and 5% CO<sub>2</sub>.

### Drugs

Ketorolac (Toradol®; Roche) was purchased from Hoffmann-La Roche, Basel, Switzerland.

### Experimental groups

To cover the different dosages used in clinical practice, we administered ketorolac at different concentrations from 0.9375 µg/ml to 7.5 µg/ml [12-15].

Cells were exposed to ketorolac for 2 days (short time exposure) followed by another incubation time of 1, 4 or 7 days with normal medium without NSAIDs or cells were exposed permanently to ketorolac for 3, 6 or 9 days (long time exposure). To provide stable and constant drug concentrations, the NSAID-containing medium was changed every second day with the solutions mentioned above.

Analogical, control cells were incubated with medium only and changes of medium performed as in the treated group. All experiments were triplicated and the mean value was considered.

### Cell count

On days 3, 6 and 9, living cells were counted using the fluorescence DNA quantitation assay (Sigma, Buchs, Switzerland) [16]. The amount of DNA is directly related to the number of

cells. Fluorescent methods for quantitation of nucleic acids depend on the change in fluorescence characteristics of a small molecule or dye upon binding to a nucleic acid. Hoechst 33258 (bisbenzimidazole, H33258) binds specifically to adjacent AT base pairs of DNA from the outside of the helix in the minor groove. Upon binding, both the efficiency and the maximum wavelength of the fluorescence output of H33258 shifts. These fluorescence changes can be measured efficiently by the DyNA Quant 200 fluorometer, which has an excitation wavelength of 365 nm and an emission wavelength of 460. The preferential binding of H 33258 to DNA allows DNA to be quantitated in the presence of RNA, protein, nucleotides, dilute detergents and protein denaturants with minimal interference.

### Cell viability

To measure cell viability *in vitro*, we used the well-established tetrazolium bromide (MTT) (Sigma, Buchs, Switzerland) assay [17]. Mitochondrial dehydrogenases, which are active only in living cells, reduce yellow tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide into purple formazan crystals. Accordingly, conversion of MTT is directly related to cell viability.

### Cell proliferation

The colorimetric bromodeoxyuridine (BrdU) assay (Roche, Basel, Switzerland), which uses BrdU as an analogue of the DNA nucleotide thymidine was chosen to analyze the proliferation of cells. In the cell-DNA incorporated BrdU affects the intensity of absorbance of the final assay-reaction, measured with an enzyme-linked immunosorbent assay (ELISA) reader at 450nm (reference wavelength 620nm) [18,19].

### Fluorometric assays for determination of caspase-3 activity

Measuring caspase-3 activity as a parameter for apoptosis, was performed by fluorometric assay cleaving the fluorogenic caspase-3 substrate Ac-Asp-Glu-Val-Asp-AMC (Calbiochem, Laeufelfingen, Switzerland) by proteolysis [20]. Cells were incubated for 1h at 37°C with 2.5µM substrate. Fluorescence was measured at an excitation wavelength of 360nm and an emission wavelength of 465nm **Statistical analysis**

Values are expressed as mean  $\pm$  standard deviation (SD). Results are presented as a percentage of control. Cell count and ELISA data regarding viability, proliferation rate, caspase-3 activity and results after LPS exposition were analyzed using three-way analysis of variance (ANOVA). Spearman's rank correlation coefficient was computed to assess the relationship between the different variables. OriginPro 8G (OriginLab, Northampton, MA, USA) and SPSS (SPSS, Inc., Chicago, IL, USA) were used for statistical analyses. A probability of  $p < 0.05$  was considered statistically significant.

## Results

### DNA Quantification

The amount of DNA in fibroblast and osteoblast cultures decreased after exposure to ketorolac (**Figure 1a-d**).

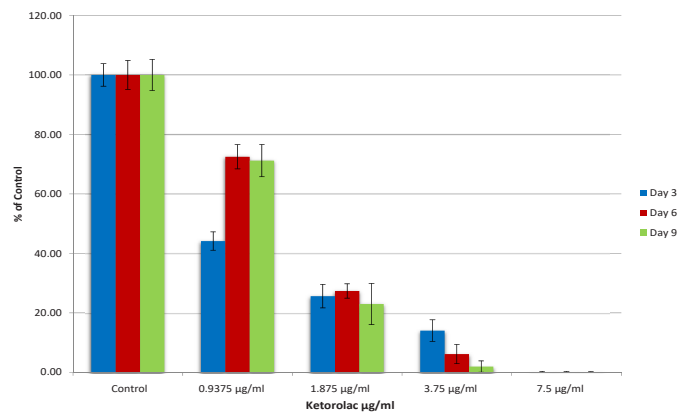
In Fibroblasts, after short time exposure (48hrs), DNA quantification at day three was 44.14 $\pm$ 3.06, 25.62 $\pm$ 3.96, 14.03 $\pm$ 3.61 and 0.08 $\pm$ 0.27% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (**Figure 1a, blue bars**). At day six it was 72.48 $\pm$ 4.09, 27.36 $\pm$ 2.39, 6.14 $\pm$ 3.24 and 0.07 $\pm$ 0.25% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, re-

spectively (Figure 1a, red bars). At day nine it was 71.18±5.42, 22.98±2.39, 1.94±1.88 and 0.08±0.15% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1a, green bars).

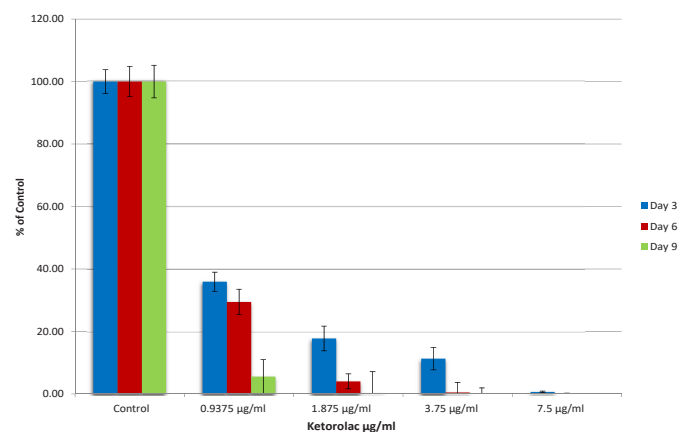
After long term exposure, fibroblasts DNA quantification at day three was 35.93±1.77, 17.76±3.88, 11.31±2.08 and 0.66±1.93% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1b, blue bars). At day six it was 29.45±6.81, 4.02±3.05, 0.48±0.82 and 0.04±0.13% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1b, red bars). At day nine it was 5.55±2.42, 0.18±0.33, 0.03±0.11 and 0.00±0.00% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1b, green bars).

In Osteoblasts, after short time exposure (48hrs), DNA quantification at day three was 45.08±2.95, 1.83±1.58, 0.24±0.61 and 0.57±1.60% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1c, blue bars). At day six it was 28.59±10.28, 0.40±0.65, 0.00±0.00 and 0.00±0.00% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1c, red bars). At day nine it was 5.81±5.39, 0.37±0.89, 0.09±0.18 and 0.07±0.16% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1c, green bars).

After long term exposure, fibroblasts DNA quantification at day three was 27.38±1.97, 0.17±0.44, 0.05±0.16 and 0.84±1.77%

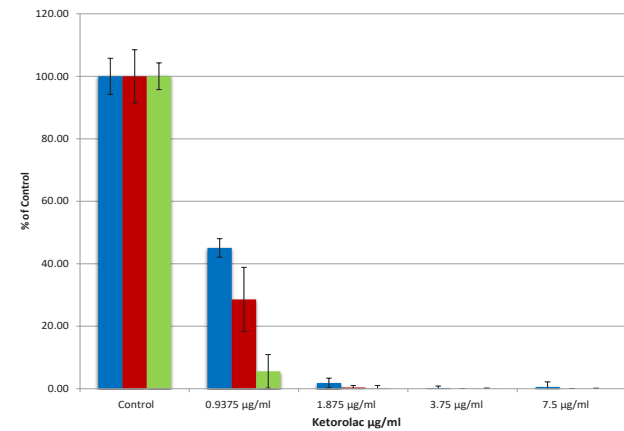


**Figure 1a:** DNA Quantification (cell count) dose /effect (short time exposure). Exposing FIBROBLASTS for 48 hrs to different concentrations of Ketorolac.

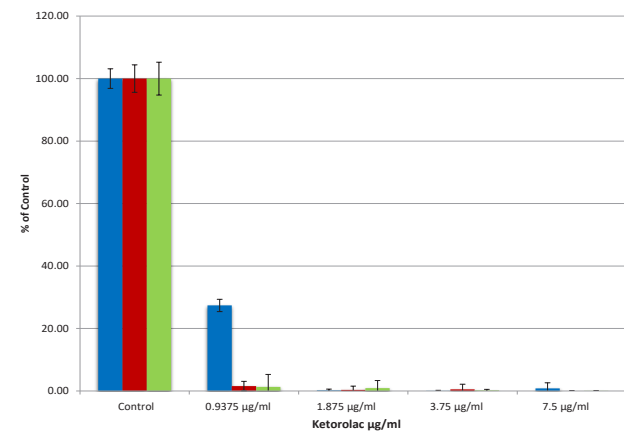


**Figure 1b:** DNA Quantification (cell count) dose /effect (long time exposure). Exposing FIBROBLASTS throughout 9 days to different concentrations of Ketorolac.

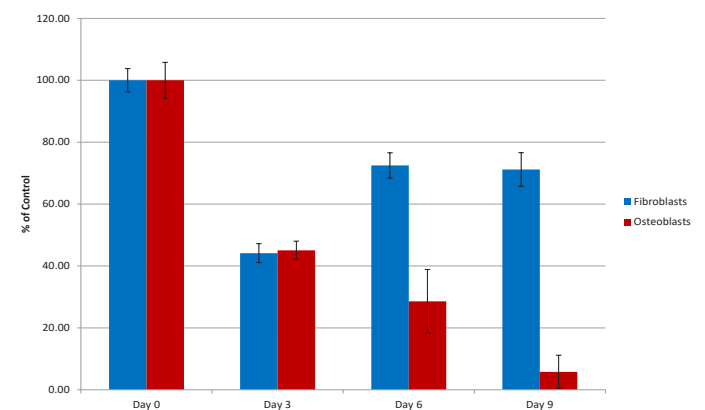
at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1d, blue bars). At day six it was 1.58±1.50, 0.37±1.21, 0.50±1.66 and 0.00 ± 0.00% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1d, red bars). At day nine it was 1.31±3.98, 0.93±2.4, 0.17 ± 0.33 and 0.01±0.04% at ketorolac concentrations of 0.9375, 1.875, 3.75, 7.5 µg/ml, respectively (Figure 1d, green bars).



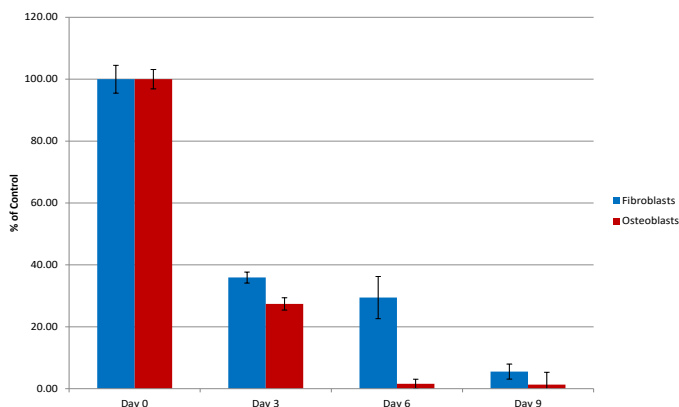
**Figure 1c:** DNA Quantification (cell count) dose /effect (short time exposure). Exposing OSTEOLASTS for 48 hrs to different concentrations of Ketorolac.



**Figure 1d:** DNA Quantification (cell count) dose /effect (long-time exposure). Exposing OSTEOLASTS throughout 9 days to different concentrations of Ketorolac.



**Figure 2a:** DNA Quantification (cell count) time course (short time exposure). After short time exposure to Ketorolac 0.9375 µg/ml.



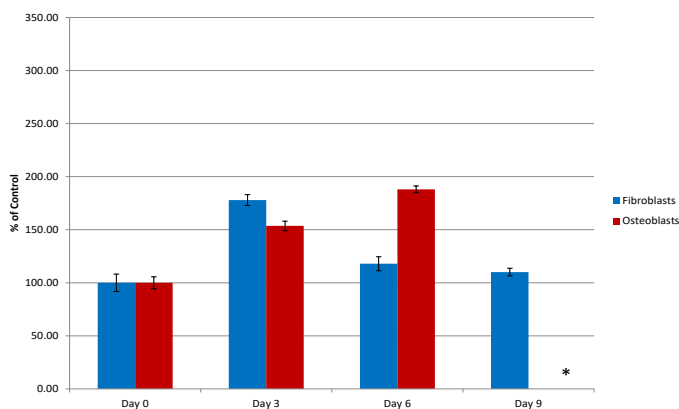
**Figure 2b:** DNA Quantification (cell count) time course (long-time exposure). After longtime exposure to Ketorolac 0.9375 µg/ml.

**Cell Viability (ketorolac 0.9375 µg/ml)**

After short time exposure (48hrs), in fibroblasts, viability of surviving cells was 177.95±5.10, 117.91±6.53, 110.08±3.64% at days 3,6 and 9, respectively (Fig. 3a, blue bars). In osteoblasts it was 153.58±4.50, 188.03±3.14, 614.08±34.38%\* at days 3,6 and 9, respectively (Fig. 3a, red bars).

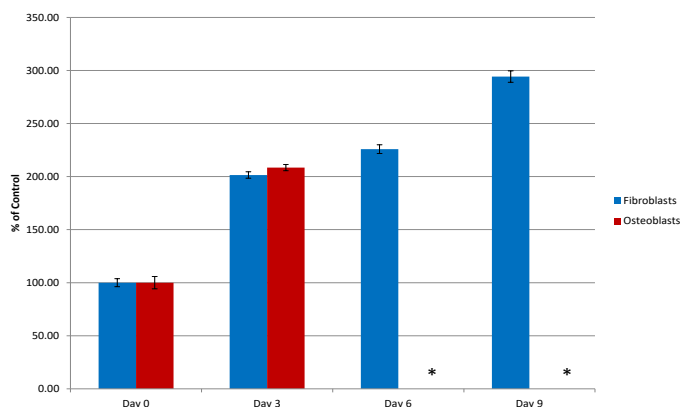
After long term exposure, fibroblasts viability of surviving cells was 201.50±4.30, 225.84±6.80, 294.22±2.37% at days 3,6 and 9, respectively (Fig. 3b, blue bars). In osteoblasts it was 208.43±4.48, 814.69±5.6\*, 243.26 ± 0.97%\* at days 3,6 and 9, respectively (Fig. 3b, blue bars).

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value). For better readability graph bars are omitted in the figures for these values.



**Figure 3a:** VIABILITY of surviving cells Ketorolac 0.9375 µg/ml. After short time exposure to Ketorolac 0.9375 µg/ml.

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value). For better readability graph bars are omitted in the figures for these values.



**Figure 3b:** VIABILITY of surviving cells Ketorolac 0.9375 µg/ml. After longtime exposure to Ketorolac 0.9375 µg/ml.

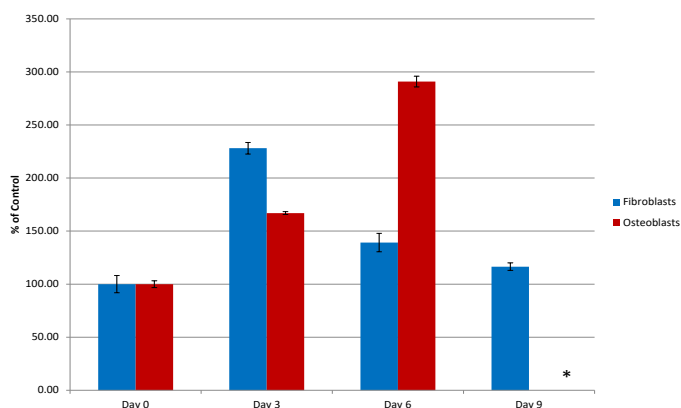
\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value). For better readability graph bars are omitted in the figures for these values.

**Proliferation Rate (ketorolac 0.9375 µg/ml)**

After short term exposure (48hrs), in fibroblasts, proliferation rate of surviving cells was 228.09±5.38, 139.16±8.67, 116.5±3.53% at days 3,6 and 9, respectively (Fig. 4a, blue bars). In osteoblasts it was 166.96±1.36, 290.91±5.17, 1027.08±5.77%\* at days 3,6 and 9, respectively (Fig. 4a, red bars).

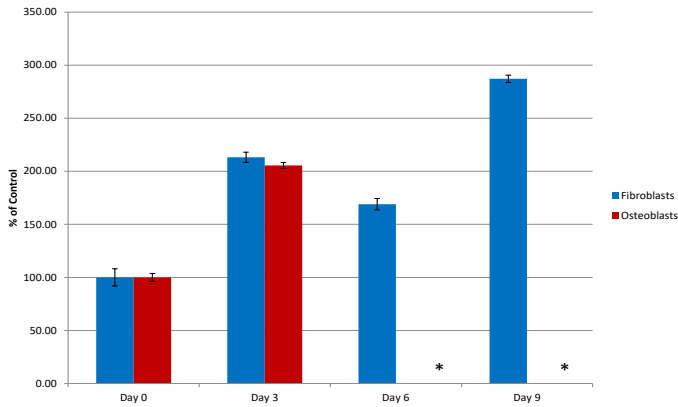
After long term exposure, fibroblasts viability of surviving cells was 213.24±4.79, 168.94±5.39, 287.14±3.49% at days 3,6 and 9, respectively (Fig. 4b, blue bars). In osteoblasts, was 205.50±2.83, 404.59±6.04\*, 25.67±0.55%\* at days 3,6 and 9, respectively (Fig. 4b, red bars).

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value). For better readability graph bars are omitted in the figures for these values.

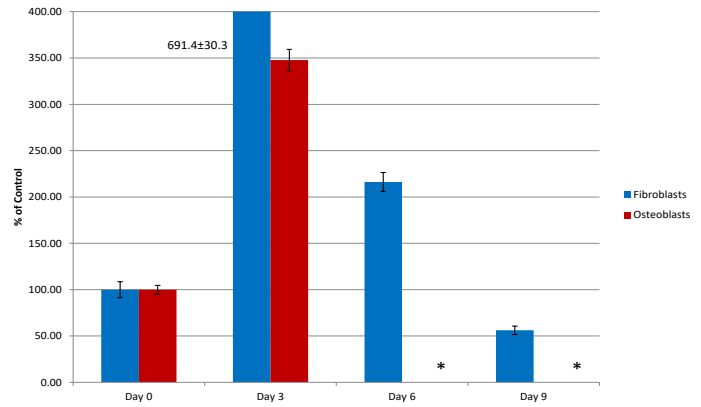


**Figure 4a:** PROLIFERATION of surviving cells Ketorolac 0.9375 µg/ml. After short time exposure to Ketorolac 0.9375 µg/ml.

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value). For better readability graph bars are omitted in the figures for these values.



**Figure 4b:** PROLIFERATION of surviving cells Ketorolac 0.9375 µg/ml. After long time exposure to Ketorolac 0.9375 µg/ml.



**Figure 5b:** Caspase activity of surviving cells ketorolac 0.9375 µg/ml. After long time exposure to ketorolac 0.9375 µg/ml.

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value). For better readability graph bars are omitted in the figures for these values.

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value) For better readability graph bars are omitted in the figure for these values.

**Caspase 3 activity (ketorolac 0.9375 ug/ml)**

After short time exposure (48hrs), in fibroblasts, caspase 3 activity of surviving cells was 306.56±21.87, 57.12±4.13, 81.19±8.83% at days 3,6 and 9, respectively (Fig. 5a, blue bars). In osteoblasts it was 158.45±8.53, 108.73±7.73, 75.15±5.03%\* at days 3,6 and 9, respectively (Fig. 5a, red bars).

**Discussion**

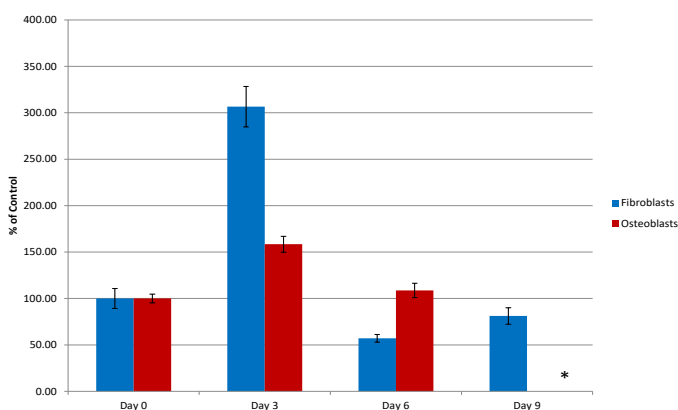
This study showed that ketorolac had a dose- and time dependent exposure toxic effect on both fibroblast and osteoblast cells, which was significantly more pronounced on osteoblasts. Apoptosis was noted in all cells, but not to the extent to explain the results, suggesting other mechanisms to be involved in this occurrence.

After long term exposure, caspase 3 activity of surviving cells was 691.24±30.35, 216.28±10.19\*, 56.17±4.63% at days 3,6 and 9, respectively (Fig. 5b, blue bars). In osteoblasts it was 347.87±11.56, 286.24±6.201\*, 0.00±0.00%\* at days 3,6 and 9, respectively (Fig. 5b, red bars).

Ketorolac was chosen since it is in common use for postoperative analgesia as well for surgical site analgesia local infiltration (LIA) [21,22]. The dosages of ketorolac were chosen in order to be within the range of those found in animal and human studies [23,24].

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value>90% of mean value). For better readability graph bars are omitted in the figures for these values.

This work demonstrated that osteoblasts were significantly more sensitive than fibroblasts after exposure of ketorolac. The negative effects of ketorolac as well as other NSAIDs drugs have already been described on osteoblasts and concurred with our results [25,26]. However, as compared to fibroblasts, the higher sensitivity of osteoblasts can be explained since these cells are the most specialized cells within the connective-tissue family [27]. For both types of cells, the proliferative properties of surviving cells is quite remarkable and may explain in part the controversy regarding the interaction of NSAIDs with bone healing after short term exposure [8]. Fibroblasts are considered to be the least specialized cells in the connective-tissue family. They are responsible throughout the body to secrete a non-rigid extracellular matrix, which play a major role when a tissue is injured [27]. Fibroblasts are also considered to be the most versatile cells of this family since they possess a great capacity to differentiate into other members of the family [27]. These properties can partly explain the lesser sensitivity of ketorolac compared to osteoblasts.



**Figure 5a:** Caspase activity of surviving cells Ketorolac 0.9375 µg/ml. After short time exposure to Ketorolac 0.9375 µg/ml.

\*DNA Quantification value was low (<6% of DNA present) and results scattered (Standard Deviation value >90% of mean value). For better readability graph bars are omitted in the figures for these values.

The interaction between ketorolac and other NSAIDs on different tissues is crucial since the versatility of fibroblasts has a clinical impact. Indeed, studies suggested that a direct conversion of fibroblasts to osteoblasts could be considered a novel strategy for bone regeneration in elderly [28]. Yamamoto et al. established a procedure to directly convert human fibroblasts into osteoblasts by transducing some defined factors which were cultured in osteogenic medium. These converted osteo-

blasts had a similar gene expression profiles as normal osteoblasts and were used to contribute to bone repair after transplantation into mice with bone defects [29].

The increase in caspase 3 suggests, that apoptosis is at least in this model one of the mechanisms for cell death induced by NSAIDs, which is in accordance with published literature. Mohan et al demonstrated in corneal fibroblast cultures that diclofenac and ketorolac increased cell death through apoptosis after treatment with TNF- $\alpha$  [30]. However, the extent of apoptosis in this work is not sufficient to make this mechanism the sole explanation for our findings. Further studies are welcome to have a better understanding regarding the interaction of these cells with ketorolac and probably other NSAIDs.

This work has some limitations. First, we incubated osteoblasts and fibroblasts only with ketorolac, which is known to have a high selectivity to inhibit COX1. Other experiments with a more COX2 selectivity could have shown different results. Second, we did not further explore the possible mechanisms of cell death at the level of mitochondria or cellular permeability.

In conclusion, in this model ketorolac showed a concentration, time- and exposition dependent toxicity on osteoblasts and fibroblasts when exposed to concentrations consistent with those measured in daily clinical practice. The results also demonstrated that osteoblasts are more sensitive than fibroblasts to the toxic effect of ketorolac. Apoptosis is involved for cellular death in both types of cells, but this mechanism alone cannot explain this outcome. Further studies are needed to have a better understanding of the toxic mechanisms of ketorolac on these cells.

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**Conflict of interest:** None.

**Presentation:** None.

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