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The Influence of Calcitriol and Tacalcitol on Proliferation of Fibroblasts Cultured from Nasal Polyps*

Badanie wpływu kalcitriolu i tacalcitolu na proliferację fibroblastów otrzymanych z polipów nosowych

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Abstract

Background. Recurrent polyposis resulting in the necessity of repeated polypectomy encouraged the search for new pharmacological therapeutic methods. At present, locally acting glycocorticosteroids have the greatest value in the treatment of nasal polyposis. Polyp growth is connected with inflammation and the proliferation of fibroblasts.

Objectives. An evaluation of the influence of calcitriol and tacalcitol on the proliferation of fibroblasts extracted from nasal polyps.

Material and Methods. The study involved 14 tissue samples of nasal polyps sampled during polypectomies. Testing was performed on the polyps cells after the sixth passage after the primary culture was established. Three days after the culture was started, nutrient medium without additional serum was added to the cells and after a further 24 hours the medium was replaced by nutrient medium with tacalcitol and calcitriol in defined concentrations.

Results. Tacalcitol and calcitriol *in vitro* decreased the proliferation of fibroblasts extracted from nasal polyps. Inhibition was most effective at concentrations of 10^{-4} M and 10^{-3} M.

Conclusions. Experimental data suggest tacalcitol to be more effective at the same concentration. This study may indicate the direction of further investigations in the potential pharmacological treatment of nasal polyps (*Adv Clin Exp Med* 2007, 16, 2, 213–219).

Key words: nasal polyps, tacalcitol, calcitriol, fibroblasts, proliferation.

Streszczenie

Wprowadzenie. Nawracająca polipowatość nosa wymagająca powtarzania polipektomii u tego samego pacjenta zmusza do poszukiwania nowych farmakologicznych metod leczenia. Obecnie największe znaczenie w leczeniu zachowawczym polipów nosa mają miejscowo działające glikokortykosteroidy. Wzrost polipów jest związany z procesem zapalnym oraz proliferacją fibroblastów.

Cel pracy. Ocena wpływu kalcitriolu i tacalcitolu na proliferację fibroblastów otrzymanych z polipów nosa.

Materiał i metody. Do badań włączono 14 próbek tkanki pochodzącej z polipów nosa pobranych podczas zabiegu ich usunięcia. Badania zostały przeprowadzone po VI pasażu od założenia hodowli pierwotnej. Trzy dni po założeniu hodowli kultury były zanurzane w roztworze z dodatkiem takalcitolu i kalcitriolu w zdefiniowanych stężeniach.

Wyniki. Tacalcitol i kalcitriol *in vitro* hamują wzrost fibroblastów pochodzących z polipów nosowych. Najbardziej skuteczne stężenia to 10^{-4} M oraz 10^{-3} M.

Wnioski. Uzyskane wyniki sugerują, że tacalcitol jest bardziej skuteczny w tych samych stężeniach. Obecne doświadczenia mogą wyznaczać dalszy kierunek badań dotyczących farmakologicznego leczenia polipów nosa (*Adv Clin Exp Med* 2007, 16, 2, 213–219).

Słowa kluczowe: polipy nosowe, tacalcitol, kalcitriol, fibroblasty, proliferacja.

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Recurrent polyposis resulting in the necessity of repeated polypectomies encouraged to search for new pharmacological therapeutic methods. At present, locally acting glucocorticosteroids have the greatest value in the treatment of nasal polyposis [1, 2], but considering their well-known side effects, further investigations on other anti-inflammatory and anti-proliferation drugs are being conducted [3, 4]. Some of the research concerns cyclopentanoperhydrofenantren generics, with vitamin D among them. The active form of vitamin D₃, 1,25-dihydrocholecalciferol, except for its known influence on calcium-phosphorus metabolism, has immunoregulative properties. Vitamin D and its synthetic analogues are used to treat rickets and osteoporosis and have also been applied as antiproliferative factors in recent years [5].

According to the studies published so far, polyp growth is connected with the inflammatory process and the proliferation of fibroblasts. Various mediators of inflammation produced by fibroblasts may play a role in the pathogenesis of nasal polyposis. Considering that fibroblasts are basic cells in the structure of polyps, the influence of the vitamin D analogues tacalcitol and calcitriol on fibroblasts extracted from nasal polyps in *in vitro* culture seems interesting. The aim of this study was to evaluate the influence of calcitriol and tacalcitol on the proliferation of fibroblasts extracted from nasal polyps.

Material and Methods

The material consisted of 14 tissue samples of nasal polyps taken during polypectomies in 12 men and 2 women aged from 29 to 73 years. Polyps with eosinophil infiltration were diagnosed in 8 cases and those with neutrophil infiltration in 4 cases, while in 2 cases there was no predominant inflammatory cell type detected. No treatment was applied in any of the cases in the 3 months before surgery. All the polyp tissues were disinfected with betadine, rinsed with PBS, and then transported in special ice boxes to the Department of Molecular Biology and Medical Genetics in Katowice, where the cell cultures were started. The vitamin D analogues used in the experiment were tacalcitol *in subs* and calcitriol *in subs*, both from the Institute of Pharmacy in Warsaw.

Testing was performed on the polyp fibroblasts after the sixth passage after the primary culture was established. All the cells were cultured at 37°C in an atmosphere of 5% CO₂ and constant humidity. RPMI 1640 with addition of 10% FBS and antibiotics (penicillin, streptomycin, gentamicin) was used as the culture medium. The culture

was started on 96-well plates beginning with a concentration of 5×10^3 cells per well. Three days after the culture was started, nutrient medium without serum was added to the cells and after further 24 hours the medium was replaced by nutrient medium with tacalcitol and calcitriol at concentrations of 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M, and 10^{-8} M. The initial solution of 10^{-3} M was obtained after tacalcitol and calcitriol were dissolved in 70% ethanol. Fibroblast proliferation was evaluated 24, 48, and 72 hours after the cultures were started. Phosphate-buffered saline (PBS) was added to the cultures and they were frozen for 24 hours at -78°C . A CyQUANT Cell Proliferation Assay Kit (Molecular Probe) was used to detect cell survival. The method relies on fluorescent dye bound by DNA in the cells. A WALLAC 1420 VICTOR² TM (Perkin Elmer) with filters for 480 nm (excitation) and 535 nm (emission) was used to measure fluorescence. Because individual cells were dying, the concentration of cells in culture never exceeded 10^{-3} M.

Statistical Analysis

The extent of inhibition of fibroblast proliferation after the test substances were added was calculated in the cultures and compared with the control group. Statistical analysis was performed using the Statistica program. Student's *t*-test and one-factor variance analysis were applied to evaluate the differences between the cultures grouped according to the substances added and the control group. Differences were considered as statistically significant with $p < 0.05$.

Results

Average arithmetical values of fibroblast proliferation after the tested substances were added are presented in Tables 1 and 2 and Figures 1 and 2. Both tacalcitol and calcitriol inhibited fibroblast proliferation at certain concentrations (Figures 3 and 4). With both substances the proliferation was most inhibited at the concentrations of 10^{-4} M and 10^{-3} M. Statistically significant influence in comparison with the control group was observed only in the cultures where tacalcitol was added in concentrations from 10^{-4} M to 10^{-3} M after 48 hours. No inhibition of fibroblast proliferation was observed at lower concentrations, i.e. at 10^{-8} M to 10^{-6} M. Proliferation was stimulated by the analogues at low concentrations (Table 1).

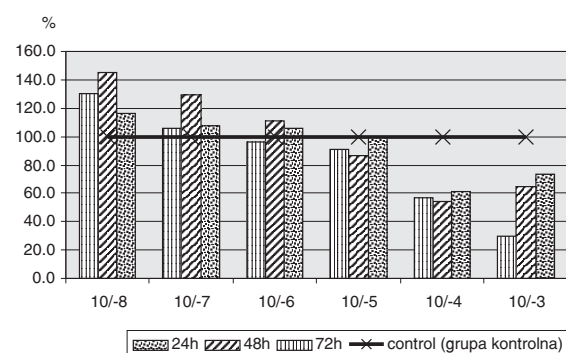
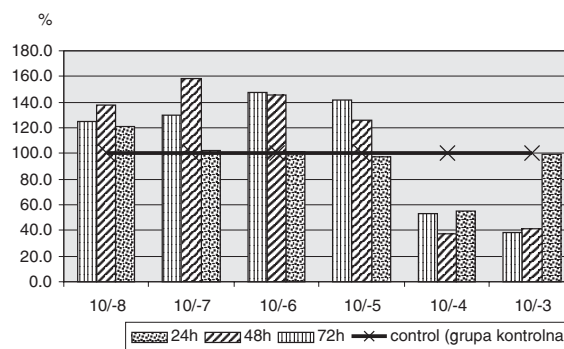
The influence of both substances on histologically different polyps (eosinophilic and neutrophilic) was compared. Both pharmaceuticals

Table 1. Proliferation of fibroblasts sampled from nasal polyps in *in vitro* cultures after 24, 48, and 72 hours after tacalcitol or calcitriol was added compared with the control group**Tabela 1.** Proliferacja fibroblastów pobranych z polipów nosowych w hodowlach *in vitro* po 24, 48, i 72 godz. od dodania tacalcitolu, kalcitriolu w stosunku do grupy kontrolnej

	Control (Grupa kontrolna)	Time – hours (Czas – godz.)	10 ⁻⁸ M	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M
Tacalcitol	100.0	24	121.4	102.2	102.1	97.5	54.7	84.0
		48	137.5	158.8	145.4	97.3	37.5*	41.5*
		72	124.6	140.3	137.4	93.2	53.0	38.5
Calcitriol	100.0	24	116.7	107.5	105.4	99.6	61.3	73.5
		48	145.3	129.1	111.0	86.6	53.8	64.6
		72	130.3	105.6	96.2	90.5	56.8	45.0

* Statistically significant difference $p < 0.05$.* Różnica istotna statystycznie dla $p < 0,05$.**Table 2.** Proliferation of fibroblasts sampled from nasal polyps with eosinophilic or neutrophilic infiltration in *in vitro* cultures after 24, 48, and 72 hours after tacalcitol or calcitriol was added compared with the control group**Tabela 2.** Proliferacja fibroblastów pobranych z polipów nosowych eozynofilowych i neutrofilowych w hodowlach *in vitro* po 24, 48, i 72 godz. od dodania tacalcitolu i kalcitriolu w stosunku do badań kontrolnych

	Control (Grupa kontrolna)	Time – hours (Czas – godz.)	10 ⁻⁸ M	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M
Tacalcitol Eos/neutro	100.0	24	119.8/ 121.4	101.9/ 102.2	100.7/ 102.1	84.6/ 99.5	34.9/ 74.7	87.0/ 84.0
		48	95.2/ 165.5	106.9/ 158.8	102.8/ 145.4	79.7/ 97.3	25.7*/ 51.5	40.9*/ 41.5*
		72	136.9/ 114.6	149.8/ 99.5	121.7/ 87.4	62.2/ 105.5	30.5/ 74.8	20.1/ 33.5
Calcitriol	100.0	24	104.4 116.7	100.3 107.5	99.7 105.4	94.9 108.6	54.0 76.3	101.0 92.3
		48	139.0 145.3	125.9 129.1	104.7 111.0	83.6 89.6	52.1 69.8	62.8/ 63.6
		72	124.8 130.3	101.8 105.6	96.9 96.2	80.8 87.5	50.0 57.8	50.8 60.0

* Statistically significant difference $p < 0.05$.* Różnica istotna statystycznie dla $p < 0,05$.**Fig. 1.** Proliferation of fibroblasts sampled from nasal polyps in *in vitro* cultures after 24, 48, and 72 hours after calcitriol was added compared with the control group**Ryc. 1.** Proliferacja fibroblastów wyhodowanych z polipów nosowych w kulturach *in vitro* w porównaniu z grupą kontrolną po 24, 48 i 72 godz. po dodaniu kalcitriolu**Fig. 2.** Proliferation of fibroblasts sampled from nasal polyps in *in vitro* cultures after 24, 48, and 72 hours after tacalcitol was added compared with the control group**Ryc. 2.** Proliferacja fibroblastów wyhodowanych z polipów nosowych w kulturach *in vitro* w porównaniu z grupą kontrolną po 24, 48 i 72 godz po dodaniu tacalcitolu

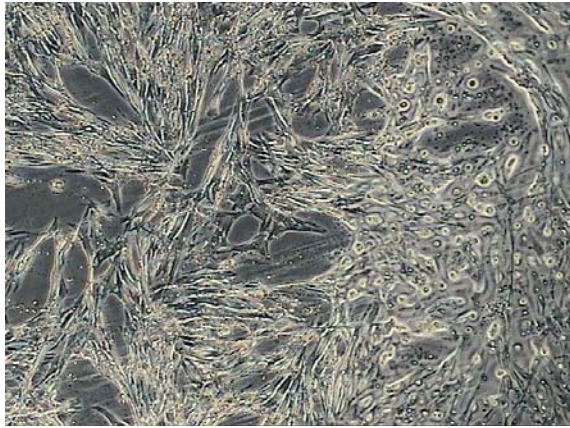


Fig. 3. Fibroblast proliferation in the control group

Ryc. 3. Hodowla fibroblastów z polipów nosa – badanie kontrolne

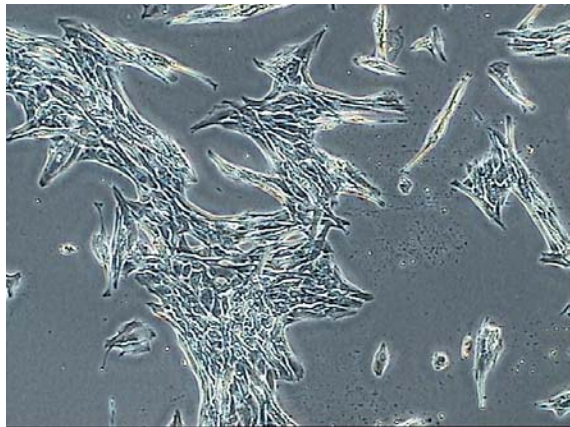


Fig. 4. Inhibition of fibroblast proliferation after tacalcitol and calcitriol was added

Ryc. 4. Hodowla fibroblastów z polipów nosa po dodaniu tacalcitolu – zahamowanie proliferacji

inhibited fibroblasts sampled from eosinophilic polyps more efficiently (Table 2). But the differences were not statistically significant ($p > 0.05$).

Discussion

The active form of vitamin D and its analogues became a subject of interest of interdisciplinary research in the fields of molecular biology, biochemistry, and immunology because of their therapeutic applications [6–8]. Calcitriol, as used in the treatment of patients with improper growth and impaired cell differentiation, is connected with its increased concentration over the physiological level. This results in improper calcium processes (hypercalcemia, hypercalcuria, organ calcification). Vitamin D analogues preserve the antiproliferative and differentiation properties of the vita-

min, but are deprived of its negative influence. The exact mechanism of calcitriol metabolic activity has not been explained. It is known that the VDR (vitamin D receptor) nuclear receptor, belonging to a family of ligand-dependent receptors, mediates in vitamin D's function [9]. The VDR has a domain structure common to other nuclear receptors with minor modifications. The E domain, located on the carboxyl end of the receptor and differentiated in its amino-acid sequence, is responsible for receptor binding [10]. The RXR nuclear receptor for 9-cis retinoic acid cooperates with the VDR. VDR expression, except for classic bone, intestine, and parathyroid gland cells, also occurs in other cell types, such as keratinocytes, lymphocytes, monocytes, and neoplastic cells [5]. Inflammatory cell infiltration of joints together with decreased vitamin D blood concentration were detected in rheumatoid arthritis, a disease of unknown etiology. In rats with an artificially provoked inflammatory condition in joints, vitamin D and its analogues inhibited the progression of the disease [11, 12]. Patients with arthritis in the course of psoriasis presented significant improvement after treatment with vitamin D [13].

Ligands of the VDR inhibit expression of proto-oncogene c-myc, K16 (keratin 16), EGF-receptor (epithelial growth factor receptor), IL-2, IL-6, IL-8, TNF-alpha (tumor necrosis factor-alpha) and IFN-gamma [14, 15]. These are gene products which promote inflammation and proliferation. This may suggest that VDR ligands may have therapeutic effect in clinical practice. The mechanism of the inhibiting influence on those genes remains unknown. The suppressive impact of vitamin D and its analogues on GM-CSF (granulocyte-macrophage colony-stimulating factor) expression, described by Towers, may play role in this process [16].

Vitamin D, in addition to its classical biological function in calcium-phosphorus homeostasis preservation, has a regulative influence on the proliferation and differentiation of normal and neoplastic cells [17, 18]. *In vitro* research also revealed its influence on apoptosis [19]. The antiproliferative influence of calcitriol and tacalcitol on fibroblasts sampled from nasal polyps shown in the present study seems to be interesting considering the potential future application of vitamin D and its analogues in the treatment of nasal polyposis. Tacalcitol, a synthetic vitamin D₃ analogue, inhibits epidermal cell proliferation in psoriasis and is used as a drug in this disease [20, 21]. According to Gerritsen [22], the mechanism of its action at the cellular level is based on the reduction of inflammatory cell number in patients' skin [22]. Castelijns, using an immunohistochemical

method, evaluated the level of keratin 14 and 16 in patients with psoriasis. The author observed reduction of the markers after tacalcitol was applied. This reflects lower keratinocyte proliferation [20]. Calcitriol, the most active vitamin D₃ metabolite, influences cells through proliferation inhibition and differentiation modification [23]. Calcitriol analogues inhibit tumor cell proliferation *in vitro* and increase the antitumoral effect of cytostatics [6, 8, 24–26].

No literature has been found about the influence of vitamin D analogues on fibroblasts sampled from nasal polyps nor the influence of this vitamin on their proliferation. The antiproliferative impact of calcitriol on human pulmonary fibroblasts is well known [27]. Arroyo [7] explored IL-6 and IL-8 expression after application of calcitriol at concentrations lower than or equal 10^{-9} M in *in vitro* cultures of human fibroblasts. Some of the cultures were stimulated with sulfur mustard. Decreased IL-6 and IL-8 expression was the effect of calcitriol application. Similarly, after calcitriol was added, human keratinocytes in the cultures without sulfur mustard stimulation presented increased IL-6 and IL-8 expression and decreased expression of the cytokines in the cultures that were stimulated with sulfur mustard. The author maintained that the cytokine suppression provoked by calcitriol depended on the kind of cells in the culture. The influence of calcitriol on human keratinocyte proliferation was assessed in the same study. The proliferation was most inhibited at the concentration of 10^{-6} M [7]. The influence of vitamin D and its analogues, i.e. tacalcitol, calcipotriol, and maxacalcitol, on human keratinocytes *in vitro* proliferation was also described by Takahashi [28]. Maximum inhibition occurred at a concentration of 10^{-7} M, but no statistically significant differences between vitamin D and its analogues were observed regarding their antiproliferative properties. However, the inactive vitamin D form did not influence keratinocyte proliferation [28]. Fukuoka revealed inhibition of RANTES (regulated on activation, normal T-cell expressed and secreted) and IL-8 synthesis after tacalcitol was added to human keratinocyte cultures [29, 30]. The most intensive suppression of RANTES synthesis occurred at a concentration of 10^{-8} M for tacalcitol, and IL-8 was most suppressed at a concentration of 10^{-7} M. Yaron explored the influence of 1,25-(OH)₂D₃ and 24,25-(OH)₂D₃ on *in vitro* human synovial fibroblast cultures. Fibroblasts were inhibited at a con-

centration of 10^{-7} M 1,25-(OH)₂D₃. The anti-inflammatory effect of 1,25-(OH)₂D₃ was evaluated in the same study through evaluation of IL-1-β metabolites, i.e. prostaglandin E₂ (PGE₂) and collagenase. The concentration of 10^{-7} M revealed the most intensive inhibitory effect [31].

Considering the experimental results of the present study, one may conclude that the concentrations of 10^{-4} M and 10^{-3} M seemed to inhibit proliferation most effectively. In some cases, especially polyps with eosinophilic infiltration, strong antiproliferative properties were also observed at the concentration of 10^{-5} M and 10^{-6} M. More increased tacalcitol and calcitriol antiproliferative activity was observed at higher concentrations than in other studies by other authors [28]. This may result from the absence of calcium in the cultures. Calcium was present in the amount of 0.01 mM in the Takahashi study, where the maximum tacalcitol activity was at 10^{-7} M [28]. Gniadecki observed keratinocyte proliferation inhibition after active vitamin D at a concentration of 10^{-8} M was added in the presence of 0.01 mM calcium [32]. The author suggests that vitamin D activity depends on the amount of calcium in the culture. Stimulation of keratinocyte proliferation was achieved at concentrations from 10^{-11} M to 10^{-9} M in the presence of 1.8 mM calcium [32]. The results confirm stimulation of fibroblast proliferation after low concentrations of vitamin D analogues are added, as observed in the present study. Similarly, Studzinski in *in vitro* keratinocyte culture achieved cell proliferation stimulation at concentrations of 10^{-10} M to 10^{-12} M [33].

The authors conclude that tacalcitol and calcitriol decreased *in vitro* the proliferation of fibroblasts sampled from nasal polyps. Inhibition was most effective at concentrations of 10^{-4} M and 10^{-3} M. Experimental data suggest that tacalcitol is more effective at the same concentration. Statistically significant activity of tacalcitol occurred after 48 hours at concentrations of 10^{-4} M and 10^{-3} M. Both tacalcitol and calcitriol inhibit the proliferation of fibroblasts sampled from polyps with eosinophilic infiltration slightly more than those with neutrophilic infiltration. However, the differences were not statistically significant. Lower concentrations, i.e. from 10^{-8} M to 10^{-6} M, did not inhibit fibroblast proliferation. The present study may indicate a direction for further investigation into the potential pharmacological treatment of nasal polyps.

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