[1]INVESTIGATING THE ANTI-INFLAMMATORY PROPERTIES OF CHOLECALCIFEROL IN RATS WITH CARRAGEENAN-INDUCED INFLAMMATION

Nina Doncheva[1], Lilia Grozlekova[3], Anita Mihaylova[1], Hristina Zlatanova[2], Delian Delev[2], Ilia Kostadinov[2]

[1]Department of Pharmacology, Toxicology and Pharmacotherapy, Faculty of Pharmacy, Medical University of Plovdiv, Bulgaria

[2]Department of Pharmacology and Clinical Pharmacology, Faculty of Medicine, Medical University of Plovdiv, Bulgaria

[3]Department of Anatomy, Histology and Embryology, Faculty of Medicine, Medical University of Plovdiv, Bulgaria

Abstract

Introduction: Beyond its effect in regulating calcium-phosphate homeostasis vitamin D has pleiotropic effects in the body. Recent data suggest that it is considered to possess anti-inflammatory properties. Objective: to investigate the effect of cholecalciferol (vitamin D_3) in rats with carrageenan-induced inflammation. **Material and method:** Male Wistar rats were divided in 6 groups (n=8): control; diclofenac (25 mg/kg bw), and four experimental groups - vitamin D_3 500 and 1000 IU/kg bw, and vitamin D_3 500 and 1000 IU/kg bw with diclofenac 12,5 mg/kg bw. Cholecalciferol was given by oral lavage for 2 weeks. Inflammation was induced by 1% carrageenan injection into the right hind-paw. The volume of the inflamed paw was measured at 2nd, 3rd, 4th and 24th hour. The hind paws were removed for morphological analysis. **Results:** in diclofenac-treated rats the paw volume was significantly lower compared to control at 3rd, 4th and 24th hour ($p<0.05$, $p<0.001$ and $p<0.01$, respectively). The animals with both doses of cholecalciferol significantly reduced the inflammation compared to control at 4th hour ($p<0.05$). The co-administration of cholecalciferol and diclofenac statistically reduced the inflammatory oedema in all measurements compared to control. The effect of the combination at the 4th and 24th hour was greater than that of vitamin D3 alone at both studied doses ($p<0.01$ and $p<0.05$, resp.). Histological examination revealed that cholecalciferol reduced the inflammatory response whereas co-administration with diclofenac led to almost complete lack of inflammatory changes as did diclofenac alone. **Conclusion:** cholecalciferol exerts anti-inflammatory properties and potentiates the effect of non-steroidal anti-inflammatory drugs.

Key words: *cholecalciferol, inflammation, carrageenan, diclofenac, cyclooxygenase*

Introduction

Vitamin D is a steroidal hormone with a variety of physiological effects in our body. The active form of vitamin D ($1,25(OH)_2$ vitamin D_3, calcitriol) is synthetized by two sequential steps in the liver and kidneys. It is often recognized for its role in calcium-phosphate metabolism and bone maturation [1]. However, beyond its essential role in calcium homeostasis vitamin D has been found to express a number of pleiotropic effects and non-skeletal health benefits, such as antimicrobial and anti-inflammatory activity, oxidative stress reduction, immunomodulation, etc. [2]. It is suggested that vitamin D regulates the immune/inflammatory response via modulating the production of inflammatory cytokines which are essential in the pathogenesis of inflammatory diseases [3]. Several studies have linked lower levels of vitamin D to a higher risk of some chronic inflammatory and autoimmune diseases such as asthma, inflammatory bowel disease, rheumatoid arthritis and neuroinflammation [4]. The role of cholecalciferol in affecting inflammatory and immune responses is supported by the presence of the nuclear vitamin D receptor (VDR) in most immune cells [5]. A link between the lack of VDR and increased nuclear factor κB (NFκB)

[1] Corresponding author: e-mail: Nina.Doncheva@mu-plovdiv.bg; tel. +359888729728

activity has also been established. NFκB is a transcription factor which is essential for immunomodulation and takes part in the pathophysiology of different chronic inflammatory diseases [6].

Carrageenan-induced paw edema is an experimental model of inflammation which is widely used for evaluating anti-inflammatory properties of biologically active substances. The aim of our study was to investigate the effect of cholecalciferol (vitamin D_3) in rats with carrageenan-induced paw inflammation.

Material and Methods

Adult male Wistar rats were used. They were housed under controlled laboratory conditions (12-hour light-dark cycles, room temperature 22 ± 2ºC, air humidity 55 ± 5%). Tap water and food were supplied *ad libitum*.

Ethical statement

Permission was obtained from the Ethics Committee at Medical University of Plovdiv, protocol № 1/13.02.2020 and the Animal Health and Welfare Directorate of the Bulgarian Food Safety Agency, permit № 249/22.11.2019.

Experimental design

To evaluate the anti-inflammatory activity of cholecalciferol in rats with carrageenan-induced inflammation the animals were randomly divided into 6 groups (n=8): control group: olive oil 0,1 ml/100g bw, diclofenac (reference substance) 25 mg/kg bw and 4 experimental groups - cholecalciferol 500 and 1000 IU/kg bw; cholecalciferol 500 and 1000 IU/kg bw + diclofenac 12,5 mg/kg bw. The control group was treated with olive oil because it was used to dissolve cholecalciferol.

Cholecalciferol was given orally for 2 weeks prior to the experiments. Diclofenac was applied only at the day of testing; 0,1 ml 1% carrageenan was injected into the right hind-paw of the rats on day 15.

The anti-inflammatory effect was evaluated by digital water plethysmometer (Ugo Basile). The volume of the hind paw was measured before carrageenan administration and on the 2nd, 3rd, 4th and 24th hour thereafter. The displacement value was recorded. The percentage of edema inhibition was calculated with the equation:

$$\% \text{ change in paw volume} = \frac{PVt - PV_0}{PV_0} \times 100,$$

PV_0 is the initial paw volume, PVt is the paw volume on 2nd, 3rd, 4th and 24th hour following the carrageenan injection.

Histological assay

The hind paws with carrageenan-induced inflammation were removed and fixed in buffered formalin. This was followed by tissue dehydration with graded series of alcohol washing. Samples were then blocked in paraffin, sliced and stained with hematoxylin-eosin (H-E).

Statistical analysis

Statistical analysis was performed by IBM SPSS Statistics 19.0. All data are shown as mean±SEM. Data were analyzed by one-way ANOVA, followed by Tukey post hoc test. A value of $p<0.05$ was accepted for statistically significant.

Results

1. Anti-inflammatory effect of cholecalciferol in rats with carrageenan-induced paw inflammation

In diclofenac treated rats the paw volume was significantly lower compared to the control at 3rd, 4th and 24th hour ($p<0.05$, $p<0.001$ and $p<0.01$, resp.). The animals treated with both doses of cholecalciferol markedly reduced paw inflammation in comparison to the control at the 4th hour ($p<0.05$). The rats treated with both doses of vitamin D3 and diclofenac significantly decreased the inflammation compared to the control during all testing hours (2nd hour - $p<0.05$; 3rd hour – $p<0.05$ and $p<0.01$; 4th hour – $p<0.001$; 24th hour – $p<0.01$ and $p<0.05$). In comparison to the rats treated only with cholecalciferol the animals treated with the lower dose of cholecalciferol and diclofenac notably reduced the paw volume during all tests ($p<0.05$), whereas the rats with diclofenac and the higher dose of cholecalciferol reached significance only on the 4th and 24th hour ($p<0.01$) (Fig. 1).

2. Histological changes in rats with carrageenan-induced inflammation

The histological examination revealed evidence of pronounced inflammatory response in the controls treated with carrageenan only – severe vasodilation, hyperemia, presence of inflammatory infiltrates with predominant lymphocyte-monocyte cells in the connective tissue (Fig. 2A). In the animals treated only with vitamin D less pronounced inflammatory changes were observed – mild vasodilation and edema, mild to moderate perivascular cell infiltration with monocytes and lymphocytes (Fig. 2C and 2D). The histological assessment of preparations from rats treated with co-administration of vitamin D and diclofenac showed almost complete absence of inflammatory changes similar to the group treated with diclofenac only – preserved integrity of blood vessels, mild or absent vasodilation, almost complete absence of inflammatory infiltrates in the surrounding connective tissue (Fig 2B, 2D and 2F).

Discussion

The present study demonstrates the anti-inflammatory effect of vitamin D_3 in rats with carrageenan-induced model of inflammation. Our results are in agreement with previous experimental studies which found that this vitamin suppresses inflammatory response to carrageenan in mice [7, 8]. The major finding of our study is that cholecalciferol enhances the effect of the non-steroidal anti-inflammatory drug diclofenac. The administration of vitamin D_3 with diclofenac, the dose of which is reduced by half, leads to an anti-inflammatory effect that is comparable with that of the full diclofenac dose and is greater than the effect of cholecalciferol alone. This could be useful for clinical practice because the use of non-steroidal anti-inflammatory drugs is associated with numerous significant adverse drug reactions. Vitamin D supplementation will allow these drugs to be administered at a lower dose which will potentially reduce the risk of adverse effects.

Three phases have been identified in the inflammatory response to carrageenan. The first phase, which occurs within 1 hour of injection is mediated by histamine and serotonin; bradykinin is produced during the second phase and finally prostaglandin synthesis takes place. Their production starts about 3 hours after carrageenan administration [9, 10]. The results from our study showed that cholecalciferol inhibits paw edema at the 4th hour after induction of inflammation. We can speculate that this vitamin inhibits prostaglandin production which contributes to its anti-inflammatory effect. Experimental studies found that in lipopolysaccharide stimulated macrophages $1,25(OH)_2$ vitamin D_3 inhibits cyclooxygenase-2 (COX-2) expression and prostaglandin production. Binding of vitamin D to VDR inhibits the Akt/NF-κB signaling pathway which reduces the expression of COX-2 gene [7]. Nantel F et al. found that carrageenan edema is associated with induction of COX-2 expression [11].

Thus, the observed anti-inflammatory effect of vitamin D in our study might be due to inhibition of COX-2 expression.

The anti-inflammatory effect of vitamin D may also be associated with its immunomodulatory action. This vitamin reduces expression of pro-inflammatory cytokines such as TNF-alfa, IL-1beta and IL-17 [12]. Since reactive oxygen species also play a role in the development of carrageenan edema [10] the antioxidant action of vitamin D may also be involved in its anti-inflammatory effect. Almeida Moreira Leal LK et al found that vitamin D suppresses neutrophils degranulation and reactive oxygen species production by these cells [8]. The involvement of additional mechanisms in the anti-inflammatory effect of cholecalciferol could explain the enhancement of diclofenac's effect when the two drugs are used in combination.

Conclusion

Cholecalciferol exerts anti-inflammatory properties and enhances the effect of non-steroidal anti-inflammatory drugs.

Conflict of Interests: The authors declare that there is no conflict of interests.

Acknowledgement: This study was funded by Medical University of Plovdiv, Bulgaria, grant № DPDP-20/2019.

References

1. Jeon SM, Shin EA. Exploring vitamin D metabolism and function in cancer. Exp Mol Med 2018; 5: 1-14.
2. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, molecular mechanism of action, and pleiotropic effects. Physiol Rev 2016; 96: 365-408.
3. Yin K, Agrawal DK. Vitamin D and inflammatory diseases. J Inflamm Res. 2014; 7:69-87.
4. Pludowski P, Grant WB, Konstantynowicz J, Holick MF. Editorial: Classic and pleiotropic actions of vitamin D. Front Endocrinol (Lausanne). 2019; 10:341.
5. Mousa A, Naderpoor N, Johnson J, Sourris K, de Courten MPJ, Wilson K, Scragg R, Plebanski M, de Courten B. Effect of vitamin D supplementation on inflammation and nuclear factor kappa-B activity in overweight/obese adults: a randomized placebo-controlled trial. Sci Rep. 2017;7(1):15154.
6. Mousa A, Misso M, Teede H, Scragg R, de Courten B. Effect of vitamin D supplementation on inflammation: protocol for a systematic review. BMJ Open 2016; 6(4): e010804.
7. Wang Q, He Y, Shen Y, Zhang Q, Chen D, Zuo C, Qin J, Wang H, Wang J, Yu Y. Vitamin D inhibits COX-2 expression and inflammatory response by targeting thioesterase superfamily member 4. J Biol Chem. 2014; 289(17):11681-11694.
8. Almeida Moreira Leal LK, Lima LA, Alexandre de Aquino PE, Costa de Sousa JA, Jataí Gadelha CV, Felício Calou IB, Pereira Lopes MJ, Viana Lima FA, Tavares Neves KR, Matos de Andrade G, Socorro de Barros Viana G. Vitamin D (VD3) antioxidative and anti-inflammatory activities: Peripheral and central effects. Eur J Pharmacol. 15; 879:173099.
9. Pérez Gutiérrez RM. Anti-inflammatory effect of birsonimadiol from seeds of *Byrsonima crassifolia*. Food Sci Biotechnol. 2016; 25(2):561-566.
10. Morris CJ. Carrageenan-induced paw edema in the rat and mouse. Methods Mol Biol. 2003; 225: 115-21.

11. Nantel F, Denis D, Gordon R, Northey A, Cirino M, Metters KM, Chan CC. Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation. Br J Pharmacol. 1999; 128(4): 853-9.
12. Wöbke TK, Sorg BL, Steinhilber D. Vitamin D in inflammatory diseases. Front Physiol. 2014; 5: 244.

Figure 1. Effects of choleclaciferol on paw volume in rats with carrageenan-induced inflammation. Data are expressed as mean±SEM (n=8). *p<0.05 compared to control; ** p<0.01 compared to control; *** p<0.001 compared to control; ^ p<0.01 compared to vitamin D3 500 IU/kg; ^^ p<0.01 compared to vitamin D3 1000 IU/kg.

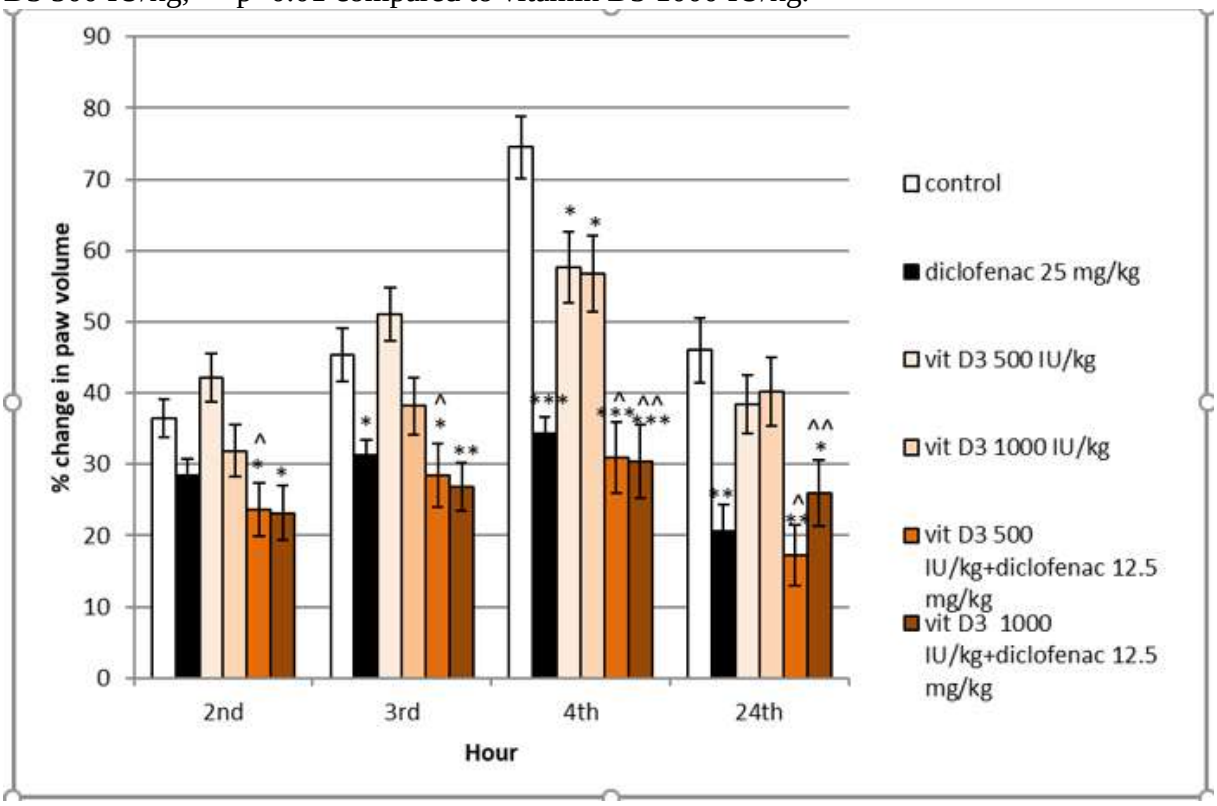
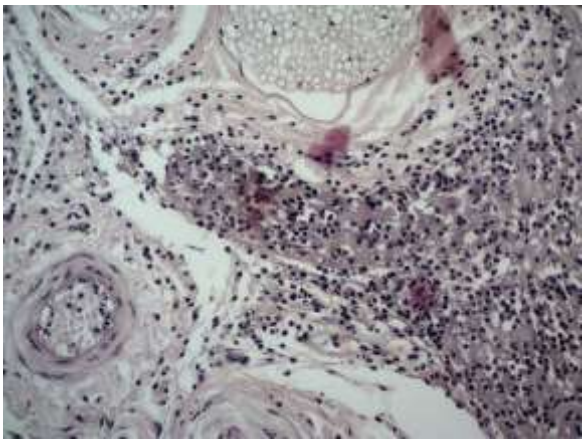
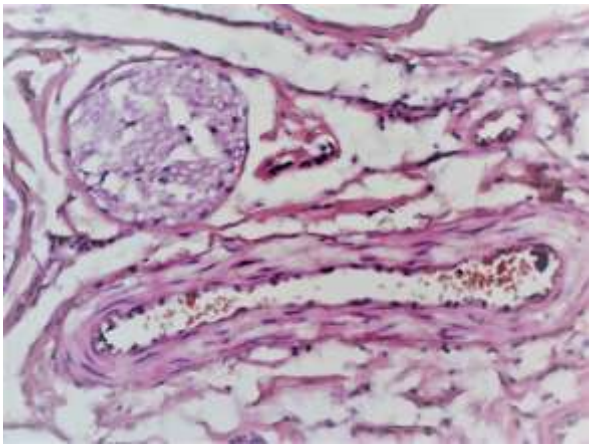


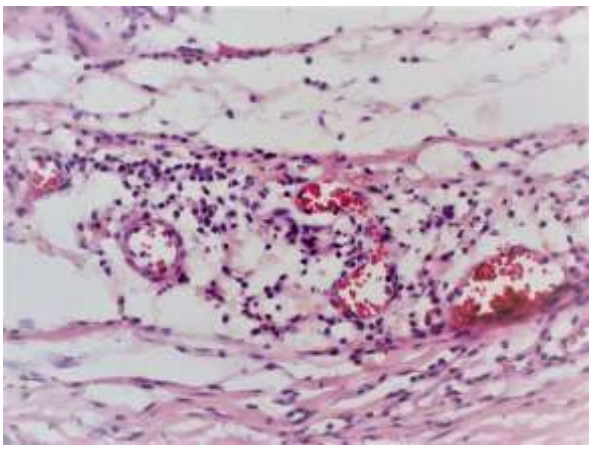
Figure 2. Microphotograph of the inflamed plantar tissue (x200) stained with H-E A) control B) diclofenac 25 mg/kg bw; C) vitamin D_3 500 IU/kg; D) vitamin D_3 1000 IU/kg; E) vitamin D_3 500 IU + diclofenac 12.5 mg/kg bw; F) vitamin D_3 1000 IU + diclofenac 12.5 mg/kg bw;



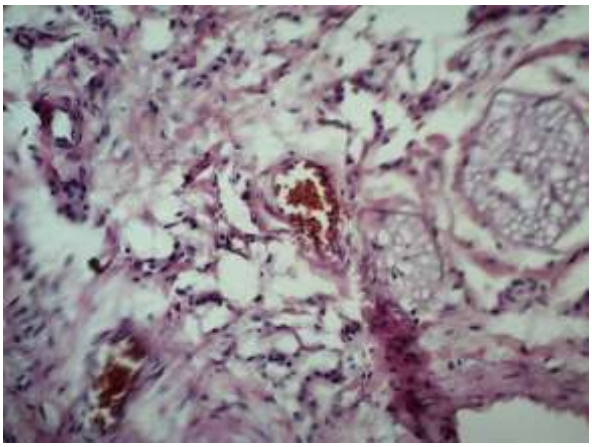
2A



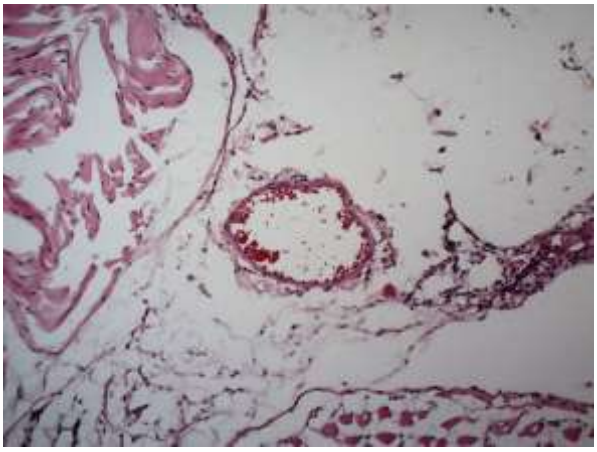
2B



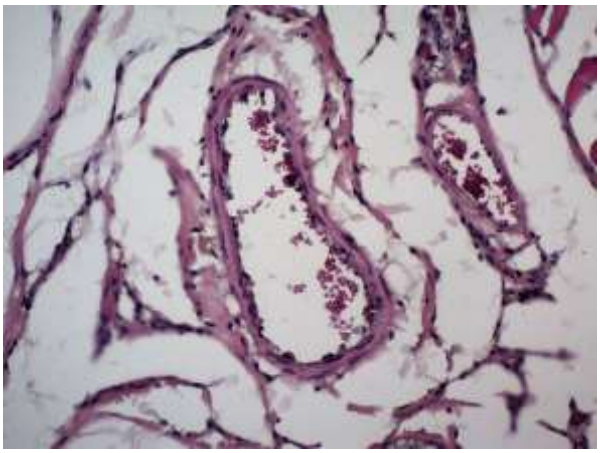
2C



2D



2E



2F