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In vitro Evaluation of the Calcification Inhibitory Properties of Policosanol, Genistein, and Vitamin D (Reduplaxin®) either Alone or in Combination

Carla Iacobini[a] Valeria Fassino[b] Sandro Mazzaferro[c, d] Lida Tartaglione[d]

[a]Department of Clinical and Molecular Medicine, Sapienza University, Rome, Italy; [b]Department of Experimental Medicine, Sapienza University, Rome, Italy; [c]Department of Translation and Precision Medicine, Sapienza University, Rome, Italy; [d]Department of Internal Medicine and Medical Specialities, Policlinico Umberto I, Rome, Italy

Keywords
Vascular calcification · Vitamin D · Policosanol · Genistein · Vascular smooth muscle cells trans-differentiation

Abstract
Introduction: The process of vascular calcification has severe clinical consequences in a number of diseases, including diabetes, atherosclerosis, and end-stage renal disease. In the present study, we investigated the effect of policosanol (Poli), genistein (Gen), and vitamin D (VitD) separately and in association to evaluate the possible synergistic action on inorganic phosphate (Pi)-induced calcification of vascular smooth muscle cells (VSMCs). ***Methods:*** Primary human VSMCs were cultured with either growth medium or growth medium supplemented with calcium and phosphorus (calcification medium) in combination with Poli, Gen, and VitD. Alizarin Red staining, mineralization, and the protein expression of RUNX2 and superoxide dismutase-2 (SOD2) were investigated. ***Results:*** All three substances tested were effective at reducing osteogenic differentiation of VSMCs in a dose-dependent manner. Poli+Gen, Poli+VitD, Gen+VitD treatment induced a greater inhibition of calcification and RUNX2 expression compared to single compounds treatments. Moreover, the association of Poli+Gen+VitD (Reduplaxin®) was more effective at inhibiting VSMCs mineralization and preventing the increase in RUNX2 expression induced by calcification medium but not modified SOD2 expression. ***Conclusions:*** The association of Pol, Gen, and VitD (Reduplaxin®) has an additive inhibitory effect on the calcification process of VSMCs induced in vitro by a pro-calcifying medium.

Introduction

Vascular calcification is an active and multifactorial process regulated by osteo/chondrogenic reprogramming of vascular smooth muscle cells (VSMCs) [1]. This process shares many similarities with bone mineralization [2]. *Trans*-differentiation of VSMCs stimulated by various pathological factors, particularly phosphate, is important for vascular calcification [3, 4]. During calcification of VSMCs, various stimuli induce the expression of osteogenic markers such as core-binding factor α-1

Carla Iacobini and Valeria Fassino contributed equally to this work.

karger@karger.com
www.karger.com/kbr

Correspondence to:
Lida Tartaglione, l.tartaglione@policlinicoumberto1.it

(CBFA1, also known as Runx2) [5, 6]. Policosanol (Poli) is a very long saturated fatty alcohol widely found in many plants including sugarcane, rice bran, and corn.

The main function of Poli is to increase high-density lipoprotein cholesterol levels and, by acting as a hypocholesterolemic agent, lower low-density lipoprotein cholesterol levels [7, 8]. In addition, Poli is reported to alleviate cardiovascular disease, reduce blood pressure, and inhibit fat production [9–11].

The isoflavones genistein (Gen) is a soy-derived phytoestrogen. Some experimental and clinical studies show that in postmenopausal women isoflavones exhibit potential beneficial features against chronic diseases such as cardiovascular disease and osteoporosis [12].

Vitamin D (VitD) is one of the most important steroid hormones in the body. Its predominant function is the regulation of skeletal health and mineral homeostasis through modulating calcium and phosphate metabolism. VitD has also been reported to serve nonskeletal functions, the majority of which are highly implicated in cardiovascular outcomes. Low serum levels of VitD, which are prevalent in older adults, have been associated with an increase both in cardiovascular risk and in postural instability (and thus of bone fractures). Several pieces of evidence point to a significant and protective role of VitD on the vasculature [13]. In the present study, we investigated the effect of Poli, Gen, and VitD (Reduplaxin®) separately and in association to evaluate the possible synergistic action on inorganic phosphate (Pi)-induced calcification of VSMCs.

Materials and Methods

Cell Culture and Osteogenic Trans-Differentiation

Primary human aortic smooth muscle cells (HAoSMCs) commercially obtained from Promocell (Heidelberg, Germany) were cultured in smooth muscle cell growth medium according to manufacturer's instructions at 37°C in a humidified atmosphere containing 5% CO_2. In order to induce VSMC osteogenic *trans*-differentiation, VSMC were seeded into 24-well plates and cultured in growing medium (GM). After confluence, the cells were incubated for 7 or 14 days in DMEM added with 2 mM $CaCl_2$ and 3 mM βglycerophosphate (calcifying medium, CM). The calcifying medium was replaced by a fresh medium every 3 days. All the experiments were performed in triplicate using cells between the 3rd and 6th passages.

Cell Treatments

To evaluate the inhibition of vascular calcification, the cells were exposed to Poli (25–50 μg/mL); Gen (1–10 nM); VitD (25–50 ng/mL) or vehicle (control) during the whole experiment duration. Also, synergistic effects were tested by exposing cells to Poli, Gen, and VitD associations.

Table 1. List of primary antibodies

Target	Host	CAT N
RUNX2	Rabbit mAb	#12556 (Cell Signaling)
SOD2	Rabbit mAb	#135141 (Cell Signaling)
B-ACTIN	Mouse mAb	#5441 (SIGMA Aldrich)

Analysis of VSMC Calcification

To compare the differences in VSMC calcification among different treatments, we analyzed the presence of calcified nodules using the Osteogenesis Assay Kit (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. In brief, cell monolayers were fixed in 4% paraformaldehyde and stained with 2% Alizarin red solution to visually detect the presence of mineralization. The quantitative analysis of calcified mineral was then performed by the extraction at low pH (10% acetic acid) and absorbance value was measured at 405 nm in a microplate reader (Varioskan Lux, Thermofisher).

Western Blot

Total cell extracts were harvested in RIPA buffer supplemented with protease inhibitors (Roche, Mannheim, Germany) and centrifuged at 14,000 × *g* for 10 min at 4°C. Total protein was quantified using the Bradford protein assay reagent (Sigma), after which protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membranes were then blocked with 5% milk prepared in Tris-buffered saline containing Tween 20 and incubated with the specific primary antibodies mentioned below. Signals were detected using enhanced chemiluminescence reagents (Clarity Biorad) according to the manufacturer's protocol. Densitometric analysis of the blotted membrane was performed using a ChemiDoc Imaging System (Bio-Rad laboratories S.r.l. Italy). Runx2 and superoxide dismutase-2 (SOD2) antibodies were obtained from Cell Signaling Technology (Dallas, TX, USA) and β-Actin antibody from Abcam (Cambridge, MA, USA). The primary antibodies used for Western blot assay are listed in Table 1.

Statistical Analysis

All experiments were repeated at least three times. Results are expressed as the mean ± standard deviation of triplicate independent experiments. Statistical analysis was performed using the Student's *t* test. Differences between groups were considered significant when $p < 0.05$.

Results

Elevated Calcium and Phosphate Levels Promote Osteogenic Differentiation and Calcification of Human VSMCs

To examine the effect of elevated calcium and phosphate levels on osteogenic differentiation and calcification of human VSMCs, cells were plated in GM or calcifying medium (CM, GM supplemented with 3 mM Pi and 2 mM Ca) for 7 or

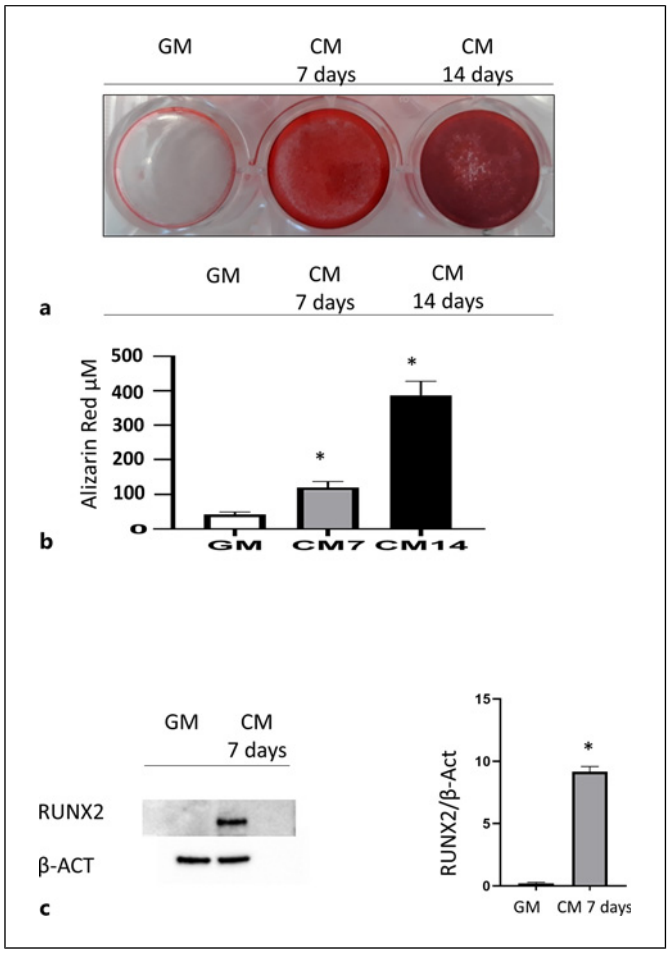

Fig. 1. Osteogenic differentiation of VSMCs. **a** Alizarin Red staining of VSMCs cultured in growth medium (GM) or calcifying medium (CM). **b** Quantitative analysis of mineralization. **c** Western blot analysis of protein expression.

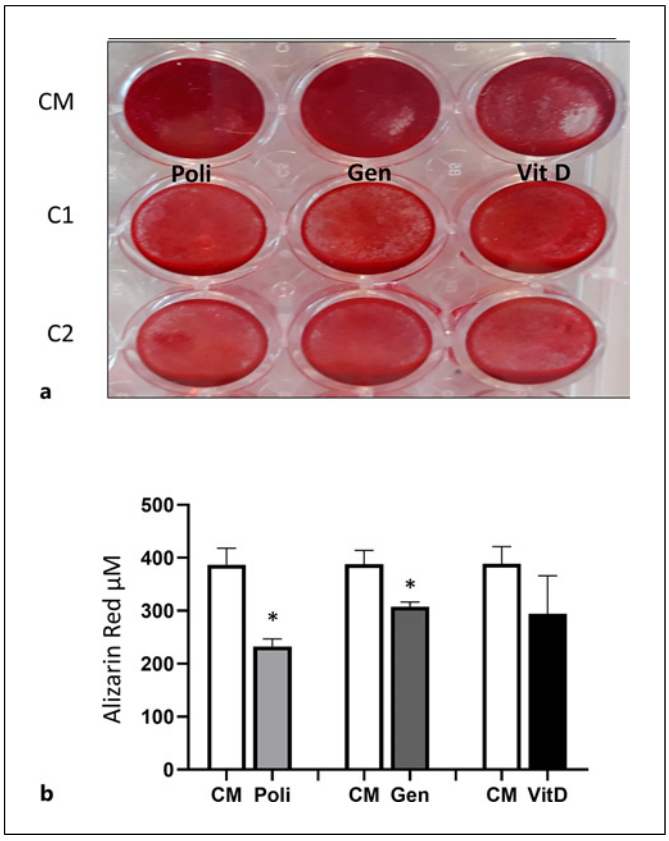

Fig. 2. Dose-dependent inhibition of vascular calcification by Poli, Gen, and VitD. **a** Alizarin Red staining of VSMCs cultured in calcifying medium alone or added with different concentrations (C1, C2) of Poli (C1 = 25 µg/mL or C2 = 50 µg/mL), Gen (C1 = 1 nM or C2 = 10 nM), and VitD (C1 = 25 µg/mL or C2 = 50 µg/mL). **b** Quantitative analysis of mineralization in VSMCs cultured in calcifying medium alone or added with Poli (50 µg/mL), Gen (10 nM), VitD (50 µg/mL).

14 days. Alizarin red staining, used to assess the induction of mineralization, showed that calcium deposition was detected in HAoSMCs cultured in CM, but not in GM (Fig. 1a). Quantification of Alizarin red staining showed 2.5-fold increment in calcium content in CM-treated cells at day 7 and 9.8-fold increment at day 14 compared with GM-treated cells (Fig. 1b). Additionally, we found that Runx2 protein levels were increased by 7.4-fold, in CM-treated VSMCs at day 7 compared with GM-treated cells (Fig. 1c).

Poli, Gen, and VitD Attenuate Osteogenic Differentiation and Calcification of HAoSMC

To identify new agents which could inhibit vascular calcification, HAoSMCs were incubated for 14 days in CM with increasing doses of Poli, Gen, VitD (25–50 µg/mL; 1–10 nM; 25–50 ng/mL, respectively). Alizarin red staining quantification showed that all three substances tested were effective at reducing calcium deposition in a dose-dependent manner (Fig. 2a). In detail, Poli, Gen reduced calcification by about 40 and 20%, respectively. However, inhibition by VitD did not reach significance (Fig. 2b). Further, Western blot analysis showed that Poli and Gen additions down-regulated Runx2 expression in HAoSMCs growth in CM by about 50 and 45%, respectively. However, addition of VitD did not reach significance (Fig. 3a). Poli, Gen, and VitD did not modify SOD2 expression (Fig. 3b). These results suggest that the tested agents may attenuate osteogenic differentiation of VSMCs.

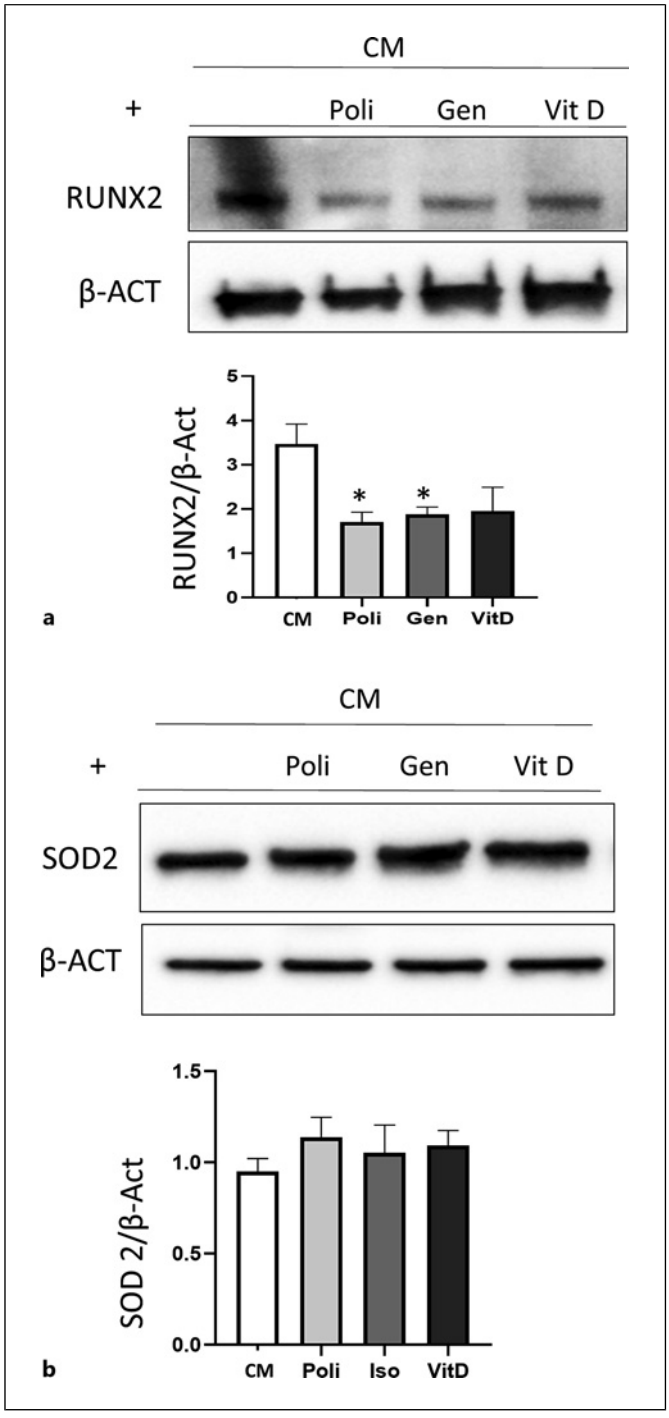

Fig. 3. Effect of Poli, Gen, or VitD treatment. WB analysis of RUNX2 (**a**) and SOD2 protein expression (**b**) in VSMCs.

Synergistic Effect of Poli, Gen, and VitD

To evaluate the effect of Poli, Gen, and VitD association in inhibiting HAoSMC trans-differentiation, the cells were cultured in CM added with the following combination: Poli+Gen; Poli+VitD; Gen+VitD; Poli+Gen+VitD. As shown in Figure 4a, b Poli+Gen, Poli+VitD, Gen+VitD treatment induced a greater inhibition of calcification (respectively, 55, 47, and 42%) and RUNX2 expression (respectively, 55, 42, and 50%) compared to single compounds treatments. Moreover, the association of Poli+Gen+VitD (Reduplaxin®) resulted in a further reduction of HAoSMC calcification and expression of the osteogenic marker RUNX2 even in the presence of a reduced concentration of the single compounds. In detail, the addition of Poli+Gen+VitD combination decreased calcification by 58% as assessed by Alizarin red staining quantification (from 380 μm in the CM to 158 μm) and reduced RUNX2 expression by 57% (Fig. 4c). Conversely, in these conditions, SOD2 protein expression was not significantly affected (data not shown).

Discussion

In the present study, we found that there may be some potential synergistic effect of the association of Poli+Gen+VitD (Reduplaxin®) on the inhibitory effect of the calcium-phosphate induced vascular calcification. Reduplaxin is already present in the Italian registry of food supplements and is in the market since 2018 as oral supplement for cardiovascular health.

Vascular calcification may be considered an osteoblastogenesis-like process that occurs within the artery wall. A great body of evidence shows that it is not a passive event but an active process in which VSMCs play a crucial role.

In essence, vascular calcification is the trans-differentiation of blood vessels into an osteoblastic phenotype and a transformation of vascular tissues into bone-like tissues. This phenomenon typically occurs in aging people, in diabetics, and in renal failure patients [14]. The current knowledge of the pathogenesis of vascular calcification is still incomplete but highlights the role of deranged calcium and phosphorus metabolism and of the balance between calcification inhibiting and promoting factors.

In particular, it is widely acknowledged that renal failure patients develop a severe type of vascular calcification which is pathogenically linked to the derangements in mineral metabolism and of calcium, phosphate, and VitD. Available therapies are based on restriction of dietary phosphate load and/or administration of drugs chelating phosphate in the foods [15]. Despite this, more effective strategies are needed. Available data in the literature indicate that there are several naturally occurring substances that could possibly represent novel adjuvant therapies.

In experimental studies, Poli effectively reduced the expression of phosphate-stimulated osteogenic genes and restored the expression of a VSMCs marker like smooth muscle alpha-actin [16]. Similarly, in vitro models, Gen showed a beneficial role at vascular levels by inhibiting

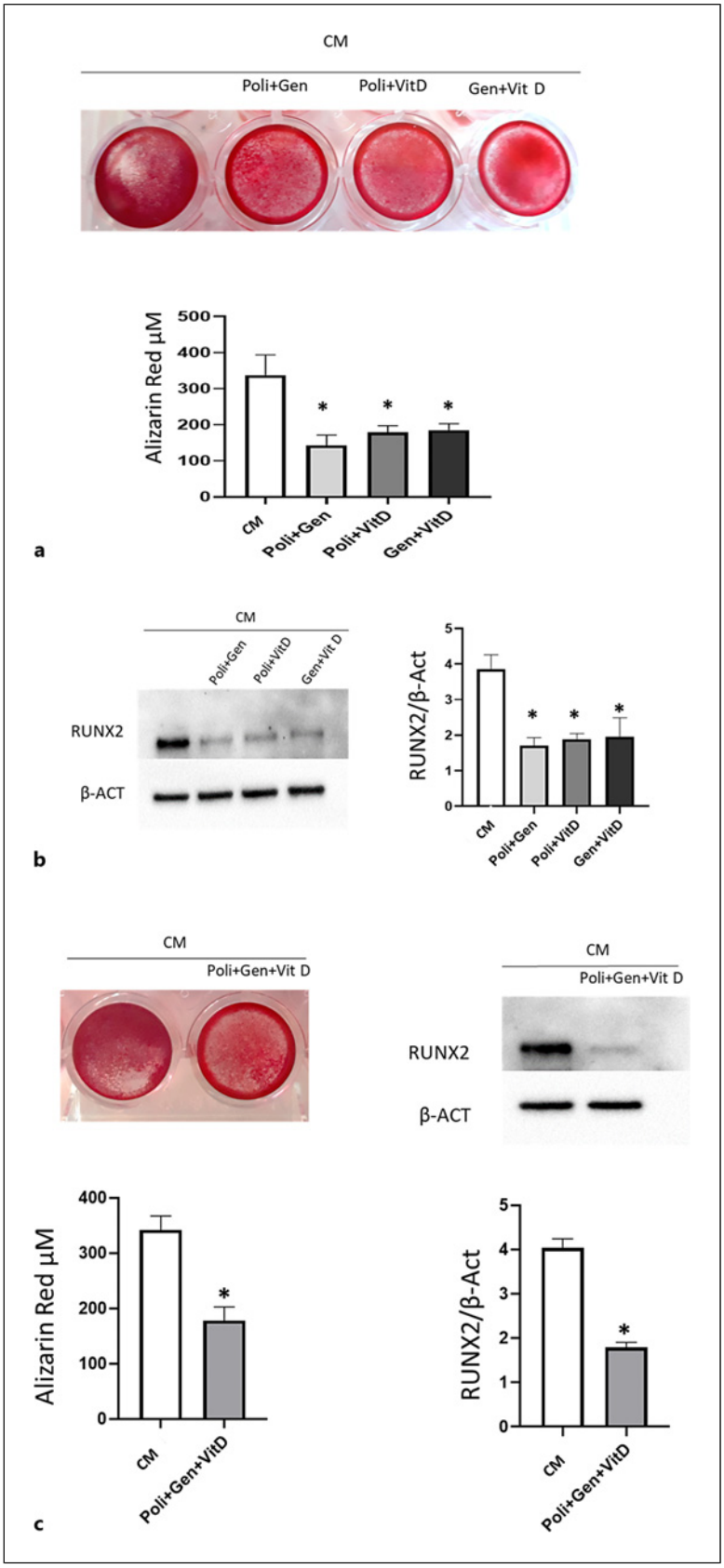

Fig. 4. Synergistic effect of Poli, Gen, VitD in inhibiting vascular calcification: Alizarin red staining, quantitative analysis of mineralization and WB of protein expression in **a**. **b** VSMCs growth in calcifying medium alone or added with the following combinations Poli+Gen; Poli+VitD; Gen+VitD (Poli: 50 µg/mL; Gen: 10 nM; VitD: 50 ng/mL). **c** VSMCs growth in calcifying medium alone or added with the combination of Poli+Gen+VitD (Poli: 30 µg/mL; Gen: 5 nM; VitD: 50 ng/mL.

Table 2. Effects of Poli, Gen, and VitD on calcification and osteogenic markers of *HAoSMC*

	Reduction of calcium deposition	Reduction of Runx2 expression	Alteration of SOD2 expression	Dose-dependence effect
Poli	++	++	Absent	Yes
Gen	++	++	Absent	Yes
VitD	+ (*)	++	Absent	Yes
Poli + Gen	+++	+++	Absent	Not tested
Poli + VitD	+++	+++	Absent	Not tested
Gen + VitD	+++	+++	Absent	Not tested
Pol + Gen + VitD	++++	++++	Absent	Not tested

Poli, policosanol; Gen, genistein; VitD, vitamin D. (*) VitD reduced calcium deposition; however, the reduction did not reach significance.

the expression of osteogenic markers of calcification in VSMCs [17]. As for VitD, its biological role is always under evaluation for potentially relevant clinical effects (besides treatment of osteoporosis) including prevention of vascular calcification through both direct receptor-mediated and indirect biologic effects [13].

To our knowledge, no data describe the potential synergistic effect played by the association of these drugs. Our results clearly indicate that the combination of Poli, Gen, and VitD (Reduplaxin®) may attenuate the osteogenic differentiation of VSMCs.

Importantly, our results showed that the association of Poli+Gen+VitD (Reduplaxin®) resulted in a further reduction both of HAoSMC calcification and of the osteogenic marker RUNX2 expression in the presence of reduced concentrations of the single compounds (Table 2). It is thus possible to hypothesize that the association treatment could exert additive effects in blunting the process of vascular calcification by positively influencing the intracellular pathways ultimately leading to calcium-phosphate deposition in the vessel walls.

The study has limitations. First, we did not evaluate the expression of ALP, a well-known marker of VSMCs trans-differentiation into osteoblast like cells. However, we evaluated the expression of RUNX2, a transcription factor that is a master regulator of the complex gene regulatory network leading to osteoblastogenesis and to the eventual ALP activity. Second, we did not thoroughly investigate the pathomechanism of the tested substances. In fact, we know from both in vivo and in vitro studies that the inhibition of vascular calcification process can be driven by several mechanisms like: modulation of antioxidant enzymes/inflammatory cytokines [18], direct induction of scavenging ROS [19], or by activation of different pathways involved in insulin-induced genes expression [16].

Conclusions

The association of Pol, Gen, and VitD (Reduplaxin®) allows dose reduction and has an synergistic inhibitory effect of the calcification process of VSMCs induced in vitro by a pro-calcifying medium.

Statement of Ethics

Ethics approval and written informed consent were not required due to national guidelines.

Conflict of Interest Statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Author Contributions

Conceptualization and writing – original draft: C.I., V.F., S.M., and L.T.; formal analysis: C.I., V.F., and L.T.; funding acquisition: S.M.; investigation and validation: C.I. and V.F.; methodology: C.I,. V.F., S.M., and L.T.; project administration and supervision: S.M. and L.T.

Data Availability Statement

All data generated during this study are included in this article. Further inquiries can be directed to the corresponding author.

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