Beneficial role of the phytoestrogen Genistein on vascular calcification

Sabrina B. Cepeda, Marisa J. Sandoval, María B. Rauschemberger, Virginia L. Massheimer

PII: S0955-2863(17)30007-4
DOI: doi: 10.1016/j.jnutbio.2017.08.009
Reference: JNB 7827

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 4 January 2017
Revised date: 6 July 2017
Accepted date: 19 August 2017

Please cite this article as: Cepeda Sabrina B., Sandoval Marisa J., Rauschemberger María B., Massheimer Virginia L., Beneficial role of the phytoestrogen Genistein on vascular calcification, *The Journal of Nutritional Biochemistry* (2017), doi: 10.1016/j.jnutbio.2017.08.009

Beneficial role of the phytoestrogen Genistein on vascular calcification

Sabrina B. Cepeda, Marisa J. Sandoval, María B. Rauschemberger and Virginia L. Massheimer*

* Instituto de Ciencias Biológicas y Biomédicas del Sur (INBIOSUR), Universidad Nacional del Sur (UNS), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Departamento de Biología, Bioquímica y Farmacia, San Juan 670, B8000ICN, Bahía Blanca, Argentina.

Runing tittle: bone vascular action of genistein

E-mail addresses: sabrina.cepeda@uns.edu.ar (Sabrina B. Cepeda), mbrausch@criba.edu.ar (María B. Rauschemberger), msandova@criba.edu.ar (Marisa J. Sandoval)

*Corresponding author:
Virginia L Massheimer, PhD
Cátedra de Bioquímica Clínica II
Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur
San Juan 670. B8000ICN. Bahía Blanca. Argentina
Tel: 54-291-4595100. FAX: 54-291-4595130
E-mail: massheim@uns.edu.ar

Funding sources: grants from the SGCyT, Universidad Nacional del Sur, Bahía Blanca, Argentina (PGI 24/B203); Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina, PIP D-4061).

Key words: genistein; osteoblasts; monocytes; vascular cells; vascular calcification

ABSTRACT

Although soy phytoestrogen are proposed to prevent or improve postmenopausal vascular and bone diseases, the currently available data are controversial and unclear. In this study we evaluated the molecular and biochemical action of Genistein on the cellular events involved in vascular calcification. Rat monocytes, aortic vascular cell and osteoblasts cultures *in vitro* exposed to Gen were employed. Gen down regulated the expression of cell adhesion molecules involved in stable leukocyte attachment. Using flow cytometry we found that the PE significantly diminished monocyte integrins CD11b, CD11c and CD18 expression either under basal and pro-inflammatory environment. At endothelial level, Gen also reduced Intercellular Adhesion Molecule 1 mRNA expression. On vascular muscle cells, the PE markedly reduced cell proliferation and migration. When vascular calcification was studied, muscle cells transdifferentiation into osteoblasts like cells was evaluated. Cells were cultured in osteogenic medium for 21 days. The expression of alkaline phosphatase and the presence of calcified nodules in the extracellular matrix were selected as features of muscle transdifferentiation. Calcified muscle cells exhibited higher levels of alkaline phosphatase activity and enhanced deposition of calcium nodules respect to native cells. Both osteoblastic markers were significantly reduced after Gen treatment. In contrast to this anti-osteogenic action, on bone cells Gen promoted osteoblasts growth, enhanced alkaline phosphatase activity and increased matrix mineralization. Its mitogenic action on osteoblasts directly depends on nitric oxide endothelial production stimulated by the PE. The data presented suppose a beneficial role of Gen on bone and vascular cells, with a cross link between both systems.

1. Introduction

Soy is the major source of plant derived phytoestrogens (PE). This term is generally used to define non-steroidal compounds that are either of plant origin or metabolically derived from plant precursors. There are mainly three subtypes of PE: isoflavones, lignans and coumestans. Due to their structural similarity with 17 beta estradiol (E_2), these compounds have estrogen like functions and may exhibit estrogen agonist or antagonist activity [1]. They act through interaction with estrogen receptors (ER). Although the overall evidence reported in the literature suggests that PE are less potent estrogens than the natural E_2, these plant derived compounds exhibit higher affinity for ERα and ERβ [2]. Especially, the isoflavones genistein (Gen) and daidzein have higher binding preference for ERβ. It has been also reported that PE modulate signalling pathways associated with cell growth and proliferation through activation of membrane G-protein coupled estrogen receptors (GPERs) [3]. Indeed epigenetic alterations of target genes expression has been proposed as a possible mechanism of the potential beneficial role of Gen in cancer [4].

Soy PE intake may reduce the risk of cardiovascular disease (CVD) upon the sudden loss of ovarian function during menopause. This hypothesis is supported by the low rates of CVD in Asian populations where the diet is particularly rich in soy [5]. Although some experimental and clinical studies show that in postmenopausal women isoflavones exhibit potential beneficial features against chronic diseases such as CVD and osteoporosis, the currently available data are controversial and insufficient [2]. Additional work is required for a complete comprehension of the risk/benefit of FE administration on menopause women health.

Bone and vascular homeostasis depend on cellular, endocrine and metabolic signals that flow bidirectionally between both systems. Bone never forms without vascular interactions. Indeed, skeletal trauma and impaired skeletal healing are commonly associated with altered vascular function [6]. Arterial calcification is also associated with osteoporosis, especially in postmenopausal women [7]. Both bone mineralization and vascular calcification are cell mediated processes sharing common mechanisms, based on the presence of bone-related proteins and bone-related cells at the site of calcification. Atherosclerotic calcification of the intima is one of the most prevalent form of vascular mineralization. Atherosclerosis is a chronic inflammatory disease characterized by vascular injury, loss of vascular architecture, and finally, occlusion of the damaged blood vessels [8]. The process starts with endothelial dysfunction, event characterized by imbalance in the production of vasodilator and vasoconstrictor factors, followed by a marked decrease in the bioavailability of nitric oxide (NO) which leads to a pro-oxidant, proinflammatory and prothrombotic features [9]. The initial response to vascular injury is mediated by endothelial permeability impairment and inflammatory cytokines secretion that consequently cause platelet adhesion to the activated endothelium. In turn,

activated platelets promote the recruitment of monocytes, enhancing their transendothelial migration, and macrophage activation. Besides the important role of blood soluble factors, oxidized lipids accumulation, inflammatory cytokines and oxidative stress in atherosclerotic plaque development, intimal macrophages and foam cells have a crucial role in lesion formation and progression. The scavenger receptor CD36 placed on macrophage surface mediates cellular uptake of oxidized LDL, and thus facilitate cholesterol loading and macrophage trapping in the arterial intima. CD36 also triggers cellular immobilization through the reactive oxygen species (ROS) generation and actin polymerization thus trapping foam cells within the intima [10]. The later stage of atherosclerotic lesion involves vascular tissue replacement by osteogenic like cells, extracellular matrix mineralization and intima calcification. This occurs as result of osteogenic transformation of vascular cells induced by the inflammatory microenvironment. Therefore, vascular smooth muscle cells (VSMC) transdifferentiation represents an adapting response in an attempt to survive in that harmful environment [11]. Approximately 15% of human atherosclerotic plaques exhibit full calcification, with histological structure almost indistinguishable from bone trabecular architecture [12].

In our laboratory we have previously shown that Gen exhibits a protective effect in female rat aortic tissue due to its regulatory action on endothelial cells (EC) proliferation and apoptosis [13]. The molecular mechanism displayed by the PE involves the participation of ER and the non-genomically activation of NO and cyclo-oxygenase pathway. Moreover, these effects were also detected in rats deprived of ovarian function [14]. Indeed, the isoflavone inhibits platelets aggregation and leucocyte adhesion to endothelial cells either under physiological or inflammatory conditions [13].

Following this line of evidence, the aim of the present study was to evaluate the molecular and biochemical action of Gen on the cellular events involved vascular calcification. Since vascular mineralization implies osteogenic transdifferentiation of vessels cells, the impact of the soy isoflavone on native bone cells was also studied.

2. Materials and Methods

2.1. Materials

Griess reagents were purchased from Britania Laboratories (Buenos Aires, Argentina). Trypsin/EDTA (10X), L-glutamine (100X), amphotericin B (0.25 mg/mL), penicillin/streptomycin (100X), and fetal calf serum were obtained from PAA Laboratories (Pasching, Austria). Dulbecco's modified Eagle's media (DMEM), Gen, Lipopolysaccharides (LPS) from *Escherichia coli* 0127 and all other reagents were purchased from Sigma Chemical Company (St Louis, MO, USA). Matching isotype controls were purchased from BD Biosciences. RT-

PCR RNA kit and Superscript III CellsDirect cDNA synthesis system were purchased from Invitrogen (CarlsBad, CA, USA).

2.2. Animals

Female Wistar rats (3-5 weeks old) were employed. All procedures involving animals and their care were performed at the Unit of Animal Care belonging to the Biology, Biochemistry and Pharmacy Department of the University. The Animal Care Use Committee approved the protocol employed. All the procedures involving animal were carried out in accordance with the guidelines published in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals.*

2.3. Cell cultures and *in vitro* calcification

EC and VSMC cultures were obtained from aortic rings explants isolated from young Wistar rats (3–5 weeks old) as previously described [15]. Briefly, animals were killed by cervical dislocation and the full length thoracic aorta was aseptically removed. Immediately after, the aorta was cleaned of adherent connective tissue, and cut into small ring-shaped segments. Ring explants were seeded on 60-mm matrix-coated petri-dishes containing phenol red-free DMEM supplemented with 20% (v/v) fetal calf serum (FCS), 60 µg/mL penicillin, 10 µg/mL streptomycin, 2.5 µg/mL amphotericin-B, 2 mM L-glutamine, and 1.7 g/L sodium bicarbonate. Explants were incubated at 37°C in 5% CO_2 atmosphere. After 5 days of culture a pure EC culture was obtained, and ring explants were removed and transferred into new culture dishes with fresh DMEM supplemented with 10% (v/v) FCS. Additional transfer of the ring explants resulted in pure culture of VSMC, and the rings were discarded. EC and VSMC cultures were allowed to reach confluence. Cells from passages 2–5 were used for all experiments. Fresh medium containing 10% (v/v) FCS was replaced every 72 h. EC and VSMC identity was performed as previously reported [16].

In order to induce VSMC osteogenic transdifferentiation, VSMC were seeded into 24-well plates and cultured for 21 days in DMEM containing 4 mM $CaCl_2$ and 5 mM β-glycerophosphate (osteogenic medium), as described [19]. The osteogenic medium was replaced by fresh medium every 3 days. The cells were exposed to Gen (0.1-10 nM) or vehicle alone (control) during the last 24 h of culture.

To perform *in vitro* cellular treatments, Gen solutions were prepared in dimethyl sulfoxide (DMSO) and subsequently diluted in isopropanol and PBS. Control groups receive vehicle alone.

2.4. Osteoblast isolation and culture

Calvaria osteoblasts (OB) were obtained from 5-day-old neonatal as previously described [17]. Briefly, calvarias were incubated at 37ºC in PBS containing 4

mM EDTA for 10 min (two periods). Culture medium was discarded, and subsequently calvarias were rinsed in PBS and digested in PBS containing 200 IU/mL collagenase for 15 min (four periods). Cells released during the first digestion were discarded, and those released during the subsequent digestions were spun down and collected after centrifugation (10 min at 300 x g). Then, cells were cultured at 37ºC in 5% CO_2 atmosphere in DMEM supplemented with 15% FCS, 60 µg/mL penicillin,10 µg/mL streptomycin, 0.05 g/L ascorbic acid and 10 mM β-glycerolphosphate. After 24 h, the medium was replaced by fresh DMEM containing 10% FCS, and cells were cultured until 80% of confluence.

2.5. Flow cytometry

Fresh peripheral blood samples were incubated at 37ºC in 5% CO_2 atmosphere and treated with vehicle or Gen during 24 h, LPS for 2 h and Gen plus LPS (added during the last 2 h of isoflavone treatment). The fluorochrome-conjugated monoclonal antibodies used were: CD11b (PE), CD11c (PE), CD18 (FITC), CD14 (APC) and CD45 (PerCP). Aliquots (100 µL) of blood were labeled with the corresponding antibody for 30 min in dark at room temperature and wash twice with PBS solution. Red blood cell lysis solution (BD Biosciences, San Jose, CA, USA) was added and incubated for 10 min at room temperature. Cells were processed by flow cytometry (FACS Canto II, Becton Dickinson, USA) and data were analyzed using Infinicyt 1.6 Software.

To determine whether samples were positive for each marker tested, the mean fluorescence intensity (MFI) obtained as assessed by the mean fluorescence channel was used, and compared with MFI values displayed by isotype-matched controls. Lymphocytes, monocytes and granulocytes were identified and gated according to forward scatter/sideways scatter (FSC/SSC) characteristics and levels of CD45 expression. Monocytes were discriminated with their specific marker, CD14 (gate) and the CD11b, CD11c and CD18 monocytes expression was studied on this gate [18].

2.6. Reverse transcription-polymerase chain reaction (RT-PCR) assay

ECs were cultured in DMEM (10% FCS) and allowed to grow up to 90% of confluence. Monolayers were starved for 24 h with serum free medium, and then treated with 10 nM Gen or 1 µg/mL LPS. Total cellular RNA extraction and reverse transcription were performed using Superscript III Cells Direct cDNA synthesis system according to the instructions of manufacturer and as previously described [13]. Complementary DNA was then amplified by PCR using a programmed thermocycler (Biometra Uno II; Biometra, Göttingen, Germany). PCR cycles were as follows: Intercellular Adhesion Molecule 1 (ICAM-1) (95ºC, 3 min, 94ºC, 60 s, 64ºC, 60 s, 72ºC, 60 s, 72ºC, 7 min, 32 cycles). Primers sequences were as follows: forward: 5'-CTG CAG AGC ACA AAC AGC AGA G-3', reverse: 5'-AAG GCC GCA GAG CAA AAG AAG C-3'. The expression of housekeeping gene glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) was checked for each set of RT-PCR experiments (forward primer: 5'-TCC CTC AAG ATT GTC AGC AA-3', reverse primer: 5'-AGA TCC ACA ACG GAT ACA TT-3'; amplification steps: 95ºC, 3 min, 94ºC, 30 s, 53ºC, 30 s, 72ºC, 45 s, 72ºC, 7 min, 35 cycles). Negative controls (PCR reaction without RT product) were also processed. PCR amplification products were detected by electrophoresis in agarose gels stained with ethidium bromide. The density of each band on RT-PCR gel was quantified using Image J software (1.43 c version, NIH, Rasband). The mRNA in each sample was normalized against GAPDH mRNA.

2.7. Cell proliferation assay

VSMC and OB proliferation were evaluated by the MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) conversion assay [19]. Cells were seeded into 96-multi-well plates in DMEM supplemented with 10% (v/v) FCS and allowed to grow to 50-60% confluence. Cells were synchronized by placing in serum-free DMEM for 24 h, and further exposed to Gen or vehicle alone (control) in fresh DMEM containing 1% (v/v) FCS. After treatment, the media was removed, and MTT solution (5 mg/mL) was added to each well followed by incubation for 4 h at 37ºC. Immediately after, the medium was carefully removed and the resulting intracellular formazan crystals were dissolved in dimethylsulfoxide, and the absorbance value was measured at 550 nm in a microplate reader (Biotek Synergy-HT). Optical density (OD) is directly proportional to number of proliferating cells.

2.8. Cell migration assay

VSMC were evaluated by scratch wound assay as previously described [22]. VSMC ($3x10^5$ cell/mL) were seeded into 6-multi-well plates (NUNC) dishes with DMEM containing 10% (v/v) FCS. After confluence, the cells were starved from serum in a free serum medium for 24 h, and then wounded by pressing a razor blade down on the dish to cut the cell layer. The blade was then gently moved to one side to remove the half of the monolayer. Immediately, the cells were washed twice with PBS and cultured in fresh DMEM containing 1% (v/v) FCS plus Gen (100 - 0.1 nM) or vehicle. After 72 h the cells were fixed in 0.1% (v/v) glutaraldehyde and stained with Giemsa. Migration was quantified by counting the number of cell nuclei that crossed the line demarcated in at least seven different microscopic fields (40X) representative of each culture plate. Results were expressed as means ± S.D. of number of cells/field [21].

2.9. Alkaline phosphatase (ALP) activity assay

ALP activity of cell lysates was measured using a commercially available kit [24]. After treatment, the culture medium was collected and the assay was performed using p-nitro-phenyl phosphate as a substrate, according to

manufacturer's instruction. Protein content was measured by Lowry [23] method. Results are expressed as IU ALP/mg protein.

2.10. Measurement of extracellular calcium deposition

Extracellular calcium quantification was determined in VSMC-OB and OB cultures. To that end, cells monolayers were decalcified with 0.6 mol/L HCl for 24 h, and calcium content in the supernatant was determined by spectrophotometry using the o-cresolphthalein complexone method (Wiener lab, Argentina). Following decalcification, cells were washed with PBS and solubilized with 1 mol/L NaOH and protein content was measured by Lowry Method. Cellular calcium levels was normalized to total protein content and expressed as µg/mg protein [17].

2.11. Alizarin red staining

The presence of calcified nodules was analyzed by Alizarin red staining [24]. Once finished treatment, cells monolayers (VSMC-OB or OB) were fixed in 4% paraformaldehyde for 10 min and subsequently washed three times with PBS. Cells were stained with 2% Alizarin red solution for 30 min at room temperature and washed three times with distilled water to remove residual stain. The stained culture plates were photographed using a digital camera (Olympus C7070WZ) coupled to an optical microscope (Olympus BX51). The relative intensities of calcified nodules were quantified by extracting with 1 mol/L NaOH, and the absorbance value was measured at 550 nm in a microplate reader (Biotek Synergy-HT).

2.12. Inmunofluorescence staining

VSMC were grown in 96-well optical bottom plates (NUNC). Confluent VSMC were washed twice with PBS and fixed with paraformaldehyde 2% (v/v) in PBS for 15 min. Then, the cells were permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min and incubated with 1% albumin for 1 h to prevent non-specific antibody binding. Cells were immunostained with Texas Red-phalloidin (Sigma-Aldrich) and the nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Immunofluorescence was visualized using an Olympus BX41 microscope and recorded with an Olympus Q-Color 3 digital camera (Olympus America, Center Valley, PA, USA).

2.13. EC-conditioned medium assays

EC cultures at 60–70% confluence were rinsed twice with PBS, and exposed to Gen (10 nM or 1 µM) or vehicle (control) for 30 min at 37ºC in DMEM containing 1% (v/v) FCS. Each EC medium (ECM) was collected for further use in OB proliferation assays. To that end, calvaria cells were seeded on 96-multi-well plates and allowed to grow to 50-60% confluence. The monolayers were deprived for serum for 24 h, and immediately after incubated with freshly ECM

during 24 h. Cell proliferation was evaluated by the MTT as described above [15].

2.14. Statistical analysis

The results presented were obtained from three independent experiments where each individual experimental condition was performed by quadruplicate (n=4). All data are presented as mean ± SD. Different cell cultures were used for each independent experiment. Comparisons between two means were made using Student's t-test, and multiple comparisons with one or two ways ANOVA, followed by Fisher's Least Significant Difference Test, using SPSS Statistics 23.0 software for Windows. P-values lower than 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of Gen on cell adhesion molecules expression

Stable monocyte adhesion to ECs is mediated by leukocyte integrins and a variety of endothelial adhesion molecules such as ICAM-1. We tested the effect of the PE on the regulation of those proteins expression.

Monocytes integrins (CD11b, CD11c and CD18) expression was evaluated using flow cytometry. Monocytes (CD45 positive cells) population was selected as CD14+ leukocytes and by size determined by the forward scatter. Side scatter vs CD14+ blood leukocytes showed an homogeneous population. Figure 1A shows dot and histogram plots of basal expression of each molecule. As shown in Fig 1B, the expression of CD11b, CD11c and CD18 was significantly diminished after 24 h of Gen treatment (47-70% below control, $p<0.001$). When monocytes were incubated in a proinflammatory environment (LPS) a markedly enhancement of integrin expression was observed (28-35% over control, $p<0.001$). Meanwhile, when blood cells were exposed to the PE prior LPS addition, the incremental increase induced by LPS was completely blunted.

Using RT-PCR technique the effect of Gen on the regulation of ICAM-1 mRNA expression was studied. The incubation of EC with 10 nM Gen (24 h) did not modify mRNA levels compared to control group, meanwhile, as was expected, LPS treatment clearly increased its expression. The preincubation of EC with Gen before LPS addition, prevented the enhancement in ICAM-1 mRNA induced by the proinflammatory agent (Fig. 1C).

3.2. Effect of Gen on VSMC proliferation and migration

Muscle vascular cells mobilization, and alterations in proliferation patterns initiate intima calcification. Therefore, using MTT assay, the effect of 10 nM Gen on VSMC cell growth was evaluated. Fig 2 shows that, 24 h of Gen treatment (10 nM) significantly reduced cell proliferation, effect that was sustained toward 72 h (22 –14% of inhibition, 24-72 h).). As can be observed in the images provided in Fig. 2B, the cell morphology was not affected by Gen treatment. The inhibitory action of VSMC proliferation was observed in a wide range of Gen concentrations (Table 1). The results were confirmed by cell counting (data not shown).

In order to assess the role of Gen on VSMC migration, wounding assays were performed. The microphotographs presented in Fig. 3A show that, Gen treatment reduced cell migration respect to non-treated cells in all concentrations tested. Fig 3B show that the number of cells/field in the denuded area of the monolayers exposed to the PE was strikingly lower respect to control group (86-79% of reduction, 0.1-100 nM Gen).

3.3. Effect of Gen on VSMC osteogenic transdifferentiation

To explore the effect of Gen treatment on VSMC-OB, cells cultures were incubated for 21 days in osteogenic medium. As features of muscle cell transdifferentiation, we selected the osteoblastic marker ALP, and the presence of calcified nodules in the extracellular matrix. In Fig. 4 it can be observed that, VSMC exhibited a minimal ALP activity. Gen did not modify this basal level. A strikingly enhanced in the enzyme activity was detected in VSMC-OB (20 fold increase respect to VSMC). When mineralized muscle cells were incubated with Gen for 24 h, a reduction in ALP activity was detected, at all PE concentrations tested.

Figure 5 shows the effect of the isoflavone on matrix mineralization. Once again, VSMC had low level of extracellular calcium deposition meanwhile VSMC-OB exhibited a markedly increase, evidenced either by quantification of calcium by HCl leaching (Fig 5A) or by Alizarin red staining (Fig 5B).

After Gen treatment calcium content and calcium nodules formation declined significantly (17- 49% decrease respect to control, 0.1-10 nM Gen).

3.4. Effect of Gen on OB cells

The results described above suggest an anti-osteogenic action of Gen. In order to evaluate whether this activity was also exhibited on osteogenic linage cells, the direct role of Gen on calvaria osteoblasts growth and differentiation was studied. The OB marker of differentiation (ALP) was measured at different time interval of cell culture. As it can be observed in Fig 6, at early time of osteoblast culture (3-7 days), lower level of ALP was detected. The maximal activity of the OB marker arose after 12-15 days of culture. On this phase of OB differentiation, a significant stimulatory action of Gen was evidenced. This pro-

osteogenic action of Gen was maximal at 12 days of culture (35; 60% above control, 10 nM; 1uM Gen).The PE also affected extra cellular matrix mineralization. On differentiated OB, the calcium deposition (Fig.7 A) and calcium nodules formation (Fig.7 B) were higher in OB exposed to Gen respect to control cells.

When the effect of the isoflavone on OB growth was tested, we found that the direct treatment of OB with Gen did not modify cell proliferation (Fig 8A). Having in mind that bone homeostasis depends on vascular signals, we evaluated whether the modulation of OB proliferation could depend on endothelial diffusible factors released by isoflavone stimulation. To that end, experiments using ECM were carried out. When OB were incubated with ECM obtained from EC exposed to different concentrations of Gen, bone cell proliferation was stimulated (Fig. 8B). Due to the fact that it has been reported that NO regulates OB growth [27] and in view of our previous evidence about Gen stimulatory action on endothelial NO production [13], additional experiments were performed using ECM obtained from EC preincubated with L-NAME (nitric oxide synthase inhibitor) prior to Gen treatment. Fig. 8C shows that, when endothelial nitric oxide synthase activity was suppressed, the enhancement in OB proliferation induced by Gen was blunted. Indeed, it can also be observed that OB treatment with 100 mM sodium nitroprusside (NPS), a nitric oxide donor, significantly enhanced cell proliferation (130% above control).

4. Discussion

The results presented in this work postulate a beneficial role of Gen either at vascular and bone levels, with a cross link between both systems. The isoflavone modulates the cellular events implicated in vascular lesions, such as stable monocyte adhesion, VSMC migration and transdifferentiation. The molecular mechanism displayed by Gen involves down regulation of leukocyte integrins and ICAM-1 gene expression, as well as inhibition of osteogenic markers of calcification, mediated by its direct action on monocytes, EC and VSMC respectively. In contrast with this anti-osteogenic effect elicited in vascular wall, on bone cells Gen promotes growth and differentiation into mature OB. The assays performed using ECM revealed that the bone action of the PE depends on its modulatory effect on EC.

In atheromatosus plaque generation, vascular injury prompts activation, adhesion, and aggregation of platelets to endothelium. Under this condition, several cytokines, chemokines, vasoactive molecules and growth factors are released by the surrounding cells in response to the inflammatory microenviroment [26]. In fact, monocyte chemoattractant protein-1 (MCP-1) represents one of the main chemokines that promotes monocytes/macrophages migration and infiltration. In turn, cytokines released from platelets contribute to monocytes activation to macrophages [27]. The platelet-monocyte aggregates

adhered to endothelium surface promote a secondary recruitment of monocytes [28]. We have previously demonstrated, using cell adhesion assays, that Gen prevents platelets and monocytes adhesion to endothelial cells. In this work we further analyze monocyte-EC providing evidence thas support that Gen acts either at monocyte and EC level. Under inflammatory stress, Gen reduces the expression of the surface integrins CD11b, CD11c, CD18 responsible for leukocyte attachment, and at endothelial side inhibited ICAM-1 expression induced by LPS. ICAM-1 is implicated in the early steps of transendothelial cell migration process through its interaction with activated integrins that ensures stable monocyte adhesion to the endothelium, and thereby enables them to migrate to the underlying tissue [29]. ICAM-1 and integrin genes expression can be promoted by several mediators (thrombin, TNF-α, IL-1β, and LPS) [30,31]. It has been demonstrated that activation of ERs by estradiol inhibits LPS-induced CD11b-expression [32]. Indeed, estradiol blunts monocytes adhesion to EC through a rapid signaling pathway mediated by a subset of ER located at cell membrane [33].Moreover, recent evidence in the literature showed that sex steroids blocks the initiation of atherosclerosis through the sialylation of EC-CAMs and consequently prevents the capture of monocytes [34].

VSMC behaviour inside artery wall depends on the microenvironment created by surrounding cells and factors. In healthy blood vessel, these cells exist in a quiescent differentiated state with low rate of cell proliferation and turnover. After macrophages recruitment, the inflammatory environment conducts to VSMC proliferation and migration to the intima leading to the progression of sclerotic lesion [35]. Thus, VSMC change their contractile phenotype to a synthetic one, with an enhancement of proliferation and often motile features [36]. Here we showed that Gen reduced VSMC growth and mobilization in a wide range of concentrations. At 48-72 h of cell culture a significant decrease in cell growth was detected even in control cells. Nevertheless the cellular integrity was preserved, as seen in fluroscence staining images. Since our data belong from *in vitro* assays using isolated cells, the contribution of surrounding environment cannot be addressed. Similar profile of murine VSMC growth regulated by estradiol has been reported by other authors, whom attributed the decrease in cell growth to an enhancement in cellular apoptosis [37] or alterations in the phenotypic state of the cells [38,39]. Since estradiol and Gen act through estrogen receptor, perhaps this feature may be related to molecular effects elicited down stream ER activation. This fact could be focused in future investigations. In agreement with our present results, *in vivo* and *in vitro* studies demonstrated that isoflavones inhibit VSMC growth, migration and induce apoptosis in human and rabbit aortic cells, as well as in muscle cells isolated from spontaneously hypertensive rats [40]. Emerging research data propose that Wnt proteins are important regulators of VSMC behaviour via activation of β-catenin signal pathways, with potential functional significance in atherosclerosis and restenosis [41]. As known Wnt signal is one of the principal

cascade involved in osteoblastogenesis [42]. This reported evidence could support the notion of a molecular linkage between bone-vascular systems.

Vascular calcification may be considered an osteoblastogenesis like process that occurs within artery wall. Although it was once thought to be a passive physico-chemical event of extracellular mineralization, in the last two decades a great body of evidence shows that it is an active cellular pathobiological process where inflammation, macrophages and VSMC play a crucial role [41]. One of the main cellular events of vessel skeletonized is the osteogenic transdifferentiation of VSMC [11]. Our findings show that Gen attenuate osteogenic transdifferentiation of VSMC evoked by reduction of ALP activity enhancement, extracellular calcium deposition and nodules formation exhibited by VSMC-OB. It has been reported that another isoflavone, puerarin, inhibits osteoblastic differentiation of VSMC through the ER/PI3K/Akt signal pathway [44]. The biomineralization of the VSMC is associated with up-regulation of calcification genes such as Runx2, BMP-2 (bone morphogenetic protein-2), the sodium-dependent phosphate cotransporter Pit-1; and TNAP (tissue non-specific alkaline phosphatase). Under physiological conditions TNAP is not expressed in arterial muscle cells, and its induction means irreversible modification towards mineralization by decreasing inorganic pyrophosphate levels [45]. Data from our laboratory reported that VSMC-OB express calcification markers (Runx2; TNAP1), as well as high ALP activity and great amount of mineralization nodules [17].

PE are proposed to be anti-resortive and bone sparing agents. Although the evidence is controversial, several clinical studies suggest a positive relationship between isoflavones and bone health [46]. Oral administration of soy isoflavone extract to ovarectomized rats promoted preosteoblasts differentiation into OB and enhanced mineralization, as well as improved bone mineral density and trabecular bone volume [47]. Indeed, PE stimulated *in vitro* OB differentiation of mouse bone marrow derived mesenchymal stem cells [48]. The inhibition of VSMC transdifferentiation reported in this work could be considered as an anti-osteogenic effect. However we found that Gen possesses the ability to regulate similar events in opposite ways according to the cellular type. On bone cells Gen promotes OB differentiation. The significant stimulatory action of Gen on OB-ALP activity after 12 days of culture, suggest a pro-osteoblastogenic effect of the PE. This was confirmed with the consequent extracellular matrix mineralization induced by the isoflavone. Surprisingly no direct action of the PE on OB growth was detected. However the ECM assays demonstrated an indirect regulation of OB proliferation mediated by the own action of Gen on EC. Although further studies are required to fully identify the mediators of this effect, the evidence suggests that NO could be involved in this close link between bone and vascular systems. NO appears to play an important regulatory role on bone metabolism, stimulates osteoblast proliferation, osteocalcin synthesis and *in vitro* formation of mineralized matrix, and keeps the osteoclast mediated bone

resorption under control [49]. Indeed NO can also facilitates fracture healing, and promotes mechanical stress bone formation [50].

Overall our data suppose a beneficial role of Gen on bone and vascular cells with a cross link between both systems. Whether the administration of PE could be helpful to prevent or reverse vascular diseases that seriously compromise blood vessels architecture remains to be clarified. Further investigations in animal models will be conducted for a better understanding of the pathophysiological relevance of Gen action.

Disclosures

None

Acknowledgments

This research was supported by grants from the SGCyT, Universidad Nacional del Sur, Bahía Blanca, Argentina (PGI 24/B203); Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina, PIP D-4061).

Reference List

[1] Brzezinski, A, A Debi. Phytoestrogens: the "natural" selective estrogen receptor modulators? Eur J Obstet Gynecol Reprod Biol 1999;85:47-51.

[2] Rietjens, IM, J Louisse, K Beekmann. The potential health effects of dietary phytoestrogens. Br J Pharmacol 2016.

[3] Prossnitz, ER, M Barton. The G-protein-coupled estrogen receptor GPER in health and disease. Nat Rev Endocrinol 2011;7:715-26.

[4] Remely, M, L Lovrecic, AL de la Garza, L Migliore, B Peterlin, FI Milagro, AJ Martinez, AG Haslberger. Therapeutic perspectives of epigenetically active nutrients. Br J Pharmacol 2015;172:2756-68.

[5] Zhang, X, XO Shu, YT Gao, G Yang, Q Li, H Li, F Jin, W Zheng. Soy food consumption is associated with lower risk of coronary heart disease in Chinese women. J Nutr 2003;133:2874-8.

[6] Thompson, B, DA Towler. Arterial calcification and bone physiology: role of the bone-vascular axis. Nat Rev Endocrinol 2012;8:529-43.

[7] London, GM. Bone-vascular cross-talk. J Nephrol 2012;25:619-25.

[8] Ross, R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999;340:115-26.

[9] Libby, P, PM Ridker, A Maseri. Inflammation and atherosclerosis. Circulation 2002;105:1135-43.

[10] Curtiss, LK. Reversing atherosclerosis? N Engl J Med 2009;360:1144-6.

[11] Pugliese, G, C Iacobini, FC Blasetti, S Menini. The dark and bright side of atherosclerotic calcification. Atherosclerosis 2015;238:220-30.

[12] Serrano, CV, Jr., M Oranges, V Brunaldi, A de Matos Soeiro, TA Torres, JC Nicolau, JA Ramires. Skeletonized coronary arteries: pathophysiological and clinical aspects of vascular calcification. Vasc Health Risk Manag 2011;7:143-51.

[13] Sandoval, MJ, PH Cutini, MB Rauschemberger, VL Massheimer. The soyabean isoflavone genistein modulates endothelial cell behaviour. Br J Nutr 2010;104:171-9.

[14] Polini, N, MB Rauschemberger, J Mendiberri, J Selles, V Massheimer. Effect of genistein and raloxifene on vascular dependent platelet aggregation. Mol Cell Endocrinol 2007;267:55-62.

[15] Cutini, PH, VL Massheimer. Role of progesterone on the regulation of vascular muscle cells proliferation, migration and apoptosis. Steroids 2010;75:355-61.

[16] Campelo, AE, PH Cutini, VL Massheimer. Cellular actions of testosterone in vascular cells: Mechanism independent of aromatization to estradiol. Steroids 2012;77:1033-40.

[17] Cutini, PH, MB Rauschemberger, MJ Sandoval, VL Massheimer. Vascular action of bisphosphonates: In vitro effect of alendronate on the regulation of cellular events involved in vessel pathogenesis. J Mol Cell Cardiol 2016;100:83-92.

[18] Wu, H, RM Gower, H Wang, XY Perrard, R Ma, DC Bullard, AR Burns, A Paul, CW Smith, SI Simon, CM Ballantyne. Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. Circulation 2009;119:2708-17.

[19] Toledo-Piza, AR, E Nakano, RE Rici, DA Maria. Proliferation of fibroblasts and endothelial cells is enhanced by treatment with Phyllocaulis boraceiensis mucus. Cell Prolif 2013;46:97-108.

[20] Cutini, PH, AE Campelo, E Agriello, MJ Sandoval, MB Rauschemberger, VL Massheimer. The role of sex steroids on cellular events involved in vascular disease. J Steroid Biochem Mol Biol 2012;132:322-30.

[21] Pedram, A, M Razandi, M Aitkenhead, CC Hughes, ER Levin. Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J Biol Chem 2002;277:50768-75.

[22] Kanno, Y, T Into, CJ Lowenstein, K Matsushita. Nitric oxide regulates vascular calcification by interfering with TGF- signalling. Cardiovasc Res 2008;77:221-30.

[23] Lowry, OH, NJ Rosebrough, Alfarr, RJ Randall. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.

[24] Viale-Bouroncle, S, M Gosau, C Morsczeck. Collagen I induces the expression of alkaline phosphatase and osteopontin via independent activations of FAK and ERK signalling pathways. Arch Oral Biol 2014;59:1249-55.

[25] Saura, M, C Tarin, C Zaragoza. Recent insights into the implication of nitric oxide in osteoblast differentiation and proliferation during bone development. ScientificWorldJournal 2010;10:624-32.

[26] Deshmane, SL, S Kremlev, S Amini, BE Sawaya. Monocyte chemoattractant protein-1 (MCP-1): an overview. Journal of Interferon & Cytokine Research 2009; 29:313-326.

[27] Steinhubl, SR, DJ Moliterno. The role of the platelet in the pathogenesis of atherothrombosis. Am J Cardiovasc Drugs 2005;5:399-408.

[28] Ed Rainger, G, M Chimen, MJ Harrison, CM Yates, P Harrison, SP Watson, M Lordkipanidzé, GB Nash. The role of the platelets in the recruitment of leukocytes during vascular disease. Platelets 2015;26:507-520.

[29] Lawson, C, S Wolf. ICAM-1 signaling in endothelial cells. Pharmacol Rep 2009;61:22-32.

[30] Rao, RM, L Yang, G Garcia-Cardena, FW Luscinskas. Endothelial-dependent mechanisms of leukocyte recruitment to the vascular wall. Circ Res 2007;101:234-47.

[31] van Gils, JM, JJ Zwaginga, PL Hordijk. Molecular and functional interactions among monocytes, platelets, and endothelial cells and their relevance for cardiovascular diseases. J Leukoc Biol 2009;85:195-204.

[32] Faas, MM, H Moes, G van der Schaaf, MJ Heineman, VP de. Monocyte activation, but not granulocyte activation, is inhibited in the presence of developing ovarian follicles. J Reprod Immunol 2006;70:21-32.

[33] Lu, Q, GR Schnitzler, K Ueda, LK Iyer, OI Diomede, T Andrade, RH Karas. ER Alpha Rapid Signaling Is Required for Estrogen Induced Proliferation and Migration of Vascular Endothelial Cells. PLoS One 2016;11:e0152807.

[34] Naftolin, F, H Mehr, A Fadiel. Sex Steroids Block the Initiation of Atherosclerosis. Reprod Sci 2016;23:1620-5.

[35] Qiu, J, Y Zheng, J Hu, D Liao, H Gregersen, X Deng, Y Fan, G Wang. Biomechanical regulation of vascular smooth muscle cell functions: from in vitro to in vivo understanding. J R Soc Interface 2014;11:20130852.

[36] Louis, S, L Saward, P Zahradka. Both AT(1) and AT(2) receptors mediate proliferation and migration of porcine vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 2011;301:H746-H756.

[37] Song, J, Y Wan, BE Rolfe, JH Campell, GR Campell. Effect of estrogen on vascular smooth muscle cells is dependent upon cellular phenotype. Atherosclerosis 1998;140:97-104.

[38] Hung, X, Y Jin, D Zhou, G Xu, J Huang and L Shen. IQGAP1 modulates the proliferation and migration of vascular smooth muscle cells in response to estrogen. International journal of molecular medicine 2015;35:1460-1466.

[39] Vazquez-Padron, RI, D Lasko, S Li, L Louis, IA Pestana, M Pang, C Liotta, A Fornoni, A Aitouche, SM Pham. Aging exacerbates neointimal formation, and increases proliferation and reduces susceptibility to apoptosis of vascular smooth muscle cells in mice. J Vasc Surg 2004;40:1199-207.

[40] Gencel, VB, MM Benjamin, SN Bahou, RA Khalil. Vascular effects of phytoestrogens and alternative menopausal hormone therapy in cardiovascular disease. Mini Rev Med Chem 2012;12:149-74.

[41] Mill, C, SJ George. Wnt signalling in smooth muscle cells and its role in cardiovascular disorders. Cardiovasc Res 2012;95:233-40.

[42] Liu, F, S Kohlmeier, CY Wang. Wnt signaling and skeletal development. Cell Signal 2008;20:999-1009.

[43] Böstrom, KI, NM Rajamannan, DA Towler. The regulation of valvular and vascular sclerosis by osteogenic morphogens. Circ Res 2011;109:564-77.

[44] Lu, Q, DX Xiang, HY Yuan, Y Xiao, LQ Yuan, HB Li. Puerarin attenuates calcification of vascular smooth muscle cells. Am J Chin Med 2014;42:337-47.

[45] Chistiakov, DA, AN Orekhov, YV Bobryshev. Vascular smooth muscle cell in atherosclerosis. Acta Physiol (Oxf) 2015;214:33-50.

[46] Sirotkin, AV, AH Harrath. Phytoestrogens and their effects. Eur J Pharmacol 2014;741:230-6.

[47] Bedell, S, M Nachtigall, F Naftolin. The pros and cons of plant estrogens for menopause. J Steroid Biochem Mol Biol 2014;139:225-36.

[48] Chang, KL, YC Hu, BS Hsieh, HL Cheng, HW Hsu, LW Huang, SJ Su. Combined effect of soy isoflavones and vitamin D3 on bone loss in ovariectomized rats. Nutrition 2013;29:250-7.

[49] Wimalawansa, SJ. Nitric oxide and bone. Ann N Y Acad Sci 2010;1192:391-403.

[50] Wimalawansa, SJ. Rationale for using nitric oxide donor therapy for prevention of bone loss and treatment of osteoporosis in humans. Ann N Y Acad Sci 2007;1117:283-97.

FIGURE LEGENDS

Figure 1. Gen regulation of cell molecule adhesion expression.

A-B: monocytes were incubated with 1 μg/mL LPS 2 h or 10 nM Gen 24 h in absence or presence of LPS added during the last 2 h of Gen treatment.
Panel A shows light scatter profiles and CD14 positivity of selected blood monocytes; dot plot and histogram of CD11b, CD11c and CD18 expression in control samples. Values express the MFI. **Panel B** shows the quantification data expressed as % respect to control (MFI: 885,2 ± 33, 955,2 ± 25 and 913,5 ± 25, CD11b, CD11c and CD18 respectively), Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4). *p<0.05; **p< 0.01; ***p<0.001 vs control. **Panel C:** ECs were incubated for 24 h with 10 nM Gen, 1 μg/mL LPS or Gen plus LPS (added during the last 19 h of Gen treatment). A representative gel photograph of PCR amplification products is shown. The expected band size for ICAM-1 and GAPDH product are indicated. Bars show the relative intensity of each band determined by densitometric analysis. Data are presented as ICAM-1 mRNA relative to GAPDH mRNA. Results represent the mean ± SD of three separated experiments. *p<0.0 vs control **p<0.01 vs LPS.

Figure 2. Effect of Gen on VSMC proliferation. Cells were treated with 10 nM Gen at the indicated times. **(A)** Cell proliferation was measured by MTT assay as described under Materials and methods section. **(B)** microphotographs of representatives fields of fluorescence staining of indicated conditions (200X magnification).
Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4) *p< 0.05; ***p<0.001 vs each control.

Figure 3. Effect of Gen on VSMC migration. Hemi half confluent VSMC monolayers were removed by scraping. Detached cells were washed with PBS, and the remaining monolayer was treated during 72 h with the indicated concentrations of Gen or vehicle alone. Dotted lines indicate the boundary between the unscratched and scratched areas. **(A)** Images of representative fields of each condition after Giemsa staining (40X magnification). The scale bar represents 340 μm. **(B)** Bars show the mean ± SD of number of migrated cells/field from three separated experiments in which each experimental condition have been performed by quatriplicate (n=4). *p < 0.05; **p < 0.01.

Figure 4. Effect of Gen on VSMC-OB ALP activity. VSMC were cultured 21 days in DMEM or in osteogenic medium. Gen was added at the indicated concentration during the last 24 h of incubation period and ALP activity was measured as described under Materials and methods section. Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4). *p< 0.05 vs VSMC-OB control group.

Figure 5. Effect of Gen on VSMC-OB: extracellular mineralization. VSMC were cultured 21 days in DMEM or in osteogenic medium. Gen was added at the indicated concentration during the last 24 h of incubation period. (**A)** Extracellular calcium deposition measured by HCl leaching calcium. **(B)** Calcium nodules formation. Images show representative fields of Alizarin red staining (40X magnification). The scale bar represents 340 μm. **(C)** Quantification of Alizarin red staining of calcified nodules. Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4). *p< 0.05 vs control group.

Figure 6. Effect of Gen on OB ALP activity. Cells were allowed to growth for the indicated time interval. Gen was added during the last 72 h of each time of cell culture. ALP activity was measured as described in methods. Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4). *p< 0.05; ** p<0.01 vs each control group.

Figure 7. Effect of Gen on OB extracellular calcium deposition. Cells were allowed to growth for the time interval indicated. Gen was added during the last 72 h of each time of cell culture. **(A)** Measurement of extracellular calcium content. **(B)** Images show representative fields of Alizarin red staining (40X magnification). The scale bar represents 340 μm. **(C)** Quantification of Alizarin Red staining of calcified nodules. Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4). *p< 0.05; **p<0.01 vs control group.

Figure 8. Effect of Gen on OB proliferation. (A) Cell were treated with Gen at the indicated concentrations for 24 h. Cell proliferation was measured by MTT assay as described under methods. **B)** OB were incubated during 24 h, with the ECM obtained from EC cultures exposed for 30 min to the indicated concentrations of Gen. **(C)** Left and middle bars: OB were incubated with ECM obtained from EC cultures exposed for 30 min to 10 nM Gen in the absence or presence of L-NAME. Right bars: OB were incubated for 24 h with 100 uM NPS or vehicle alone. Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4). **p< 0.01; ***p<0.001 vs each control group.

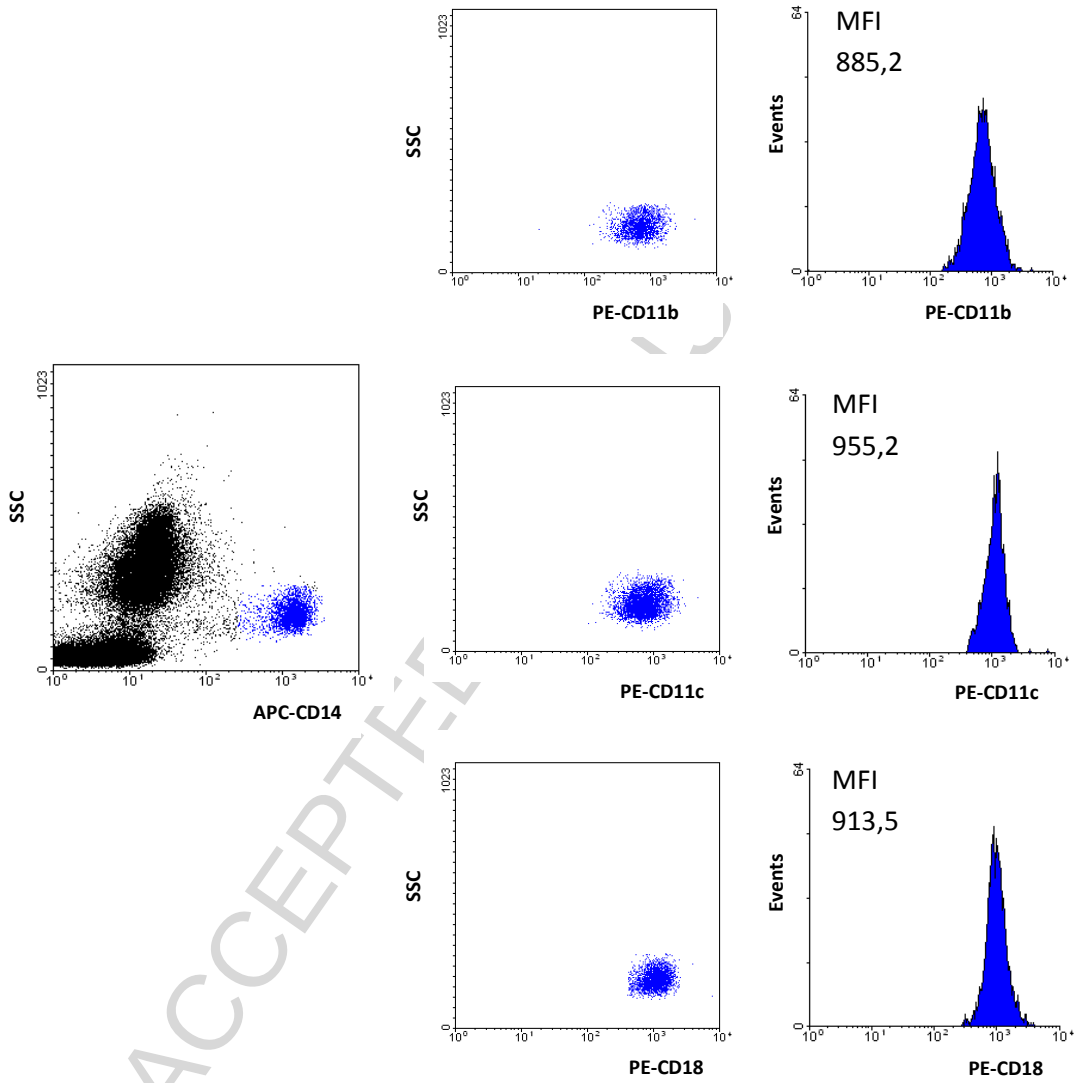
Table 1. Effect of Gen on VSMC cells proliferation

Treatment (24 h)	Proliferation (OD units)		
	Mean (±SD)	P	% inhibition
Control	0.562 (±0,03)		
100 nM	0.472 (±0,05)	0.01	16
10 nM	0.420 (±0,03)	0.001	25
1 nM	0.451 (±0,03)	0.001	20
0 .1 nM	0.455 (±0,04)	0.001	19

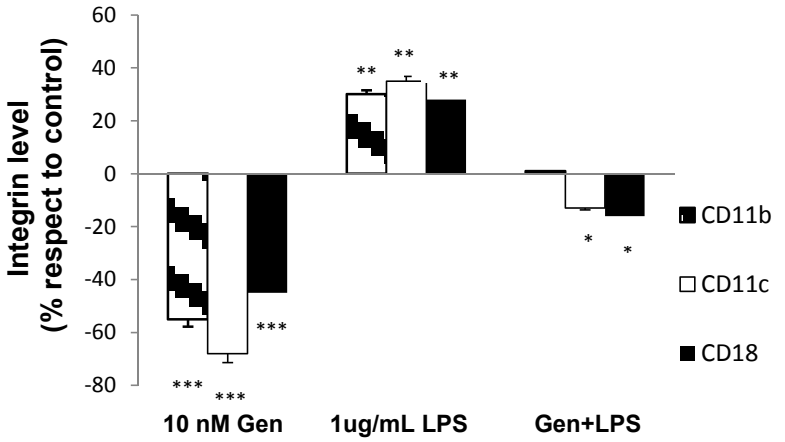
VSMC were treated with Gen or vehicle (control) at the indicated concentrations for 24 h. Cell proliferation was measured by MTT assay as described under methods section. Results represent the mean ± SD of three separated experiments in which each experimental condition have been performed by quatriplicate (n=4).

Figure 1

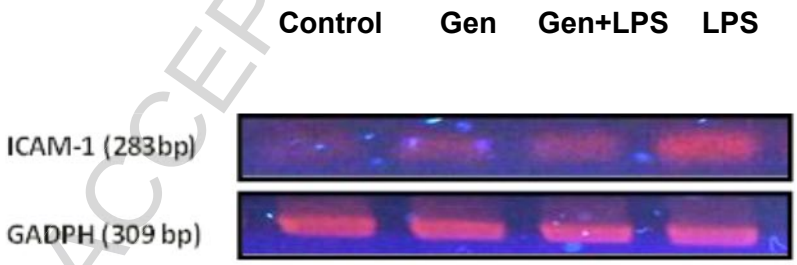
(A)



(B)



(C)



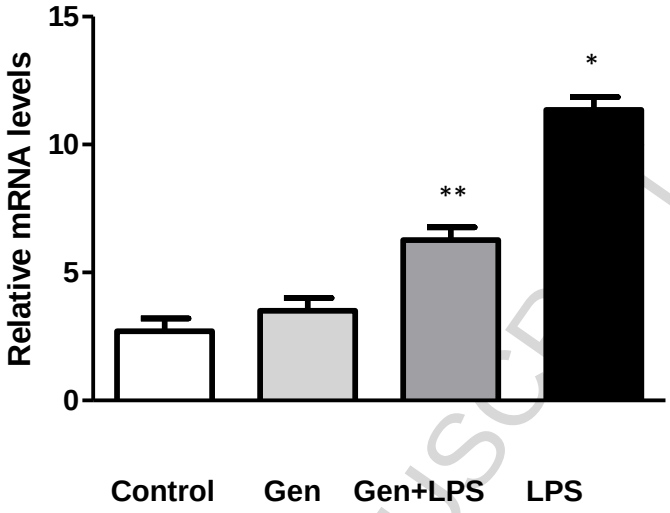
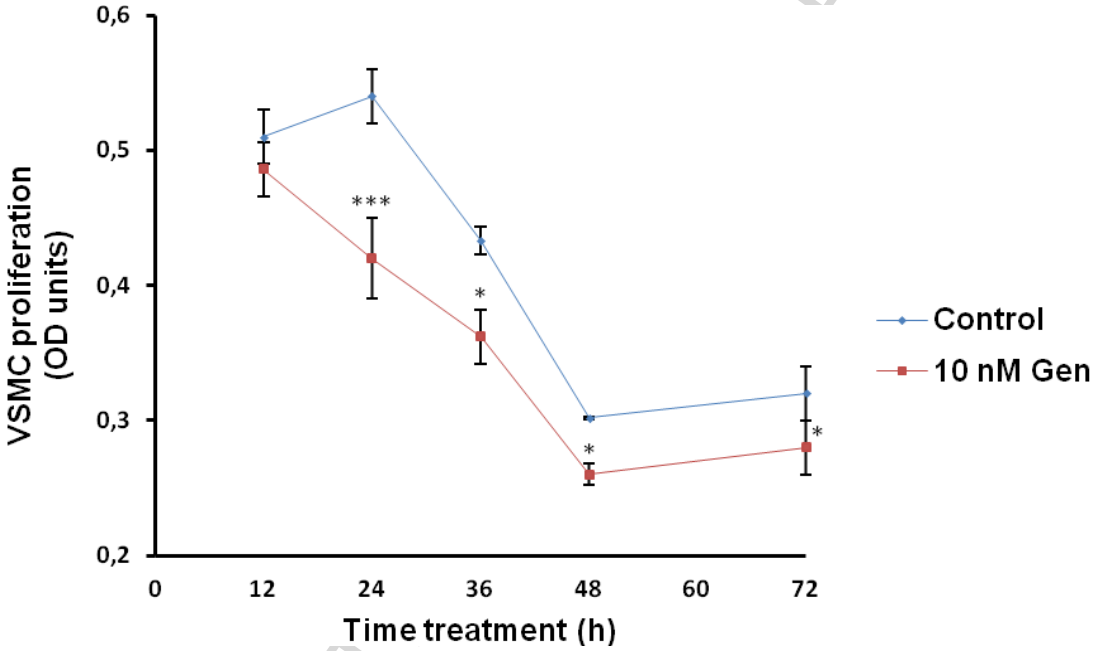


Figure 2

(A)



(B)

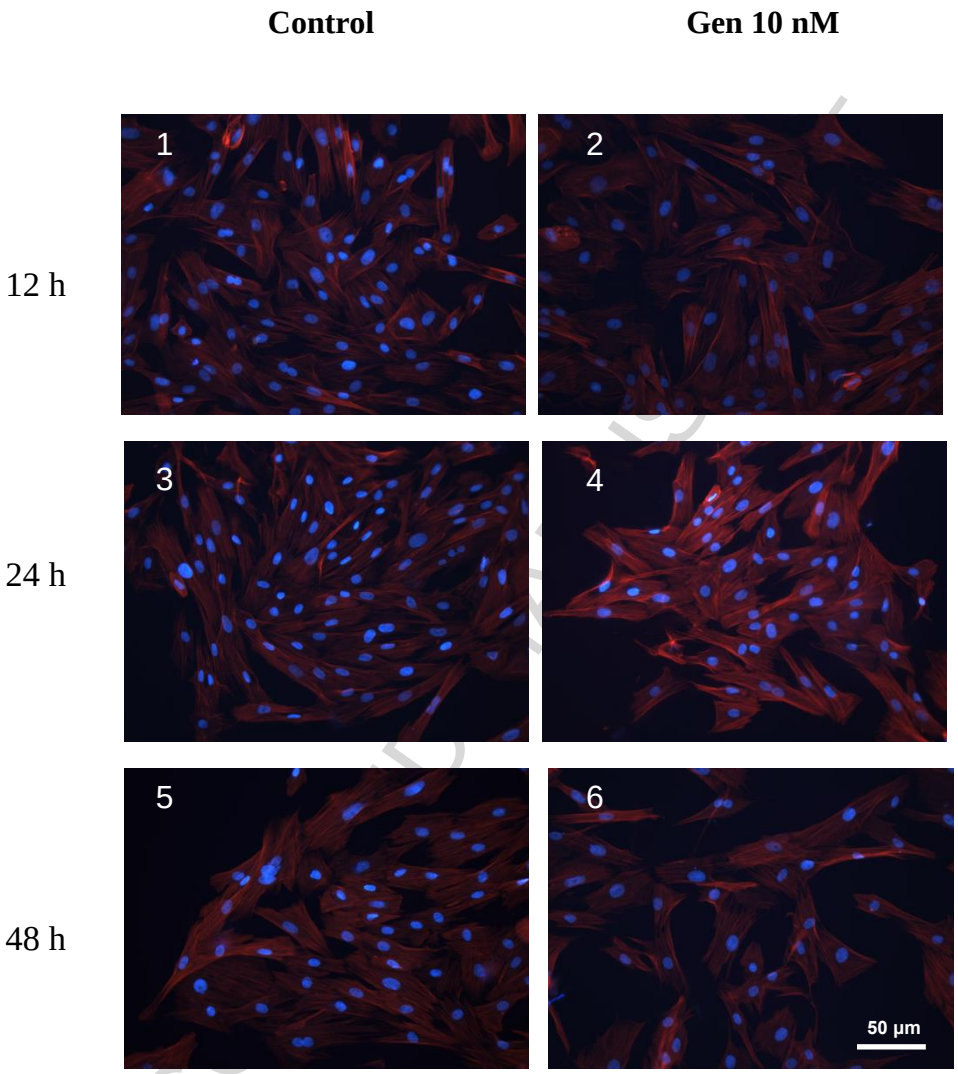
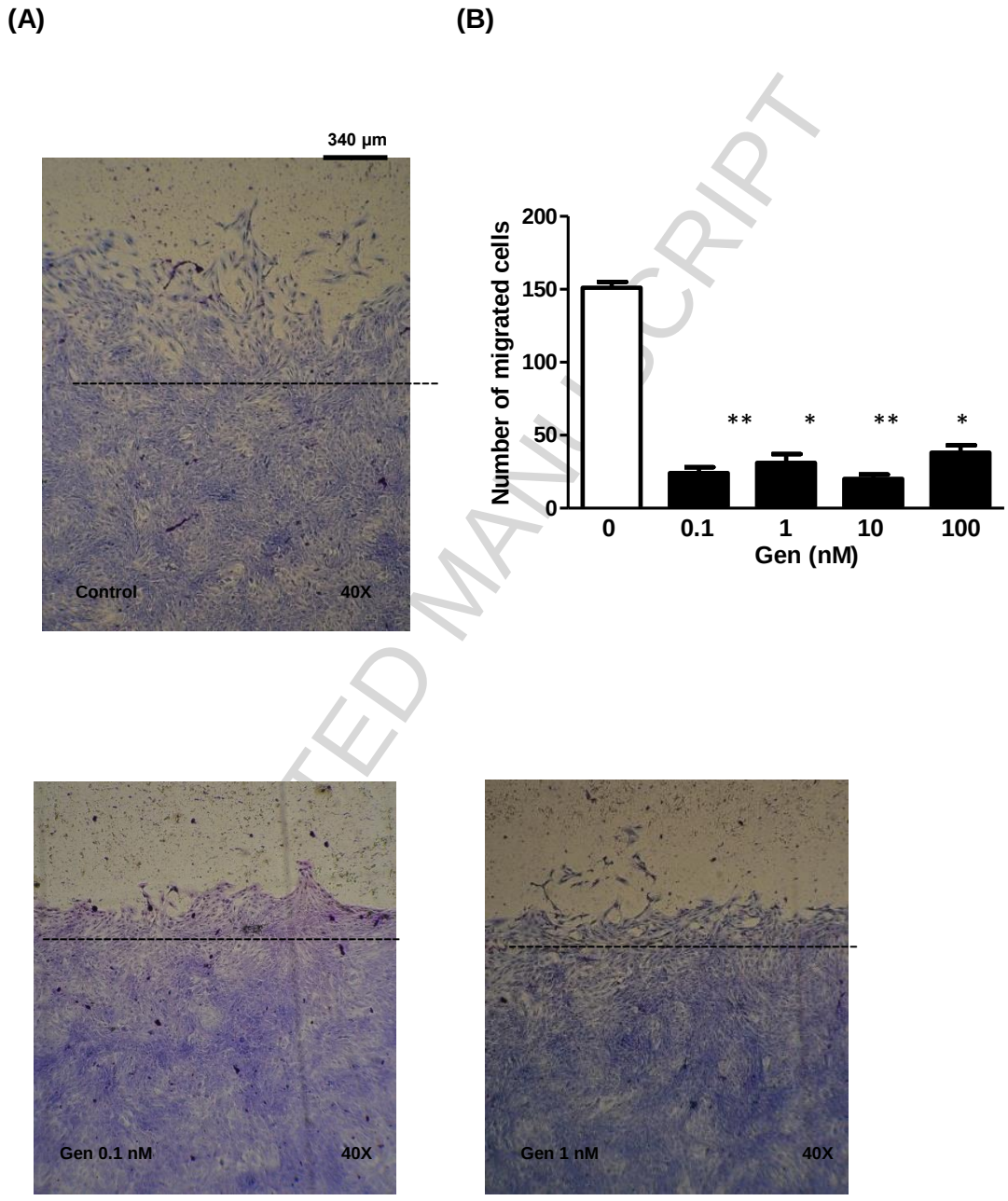
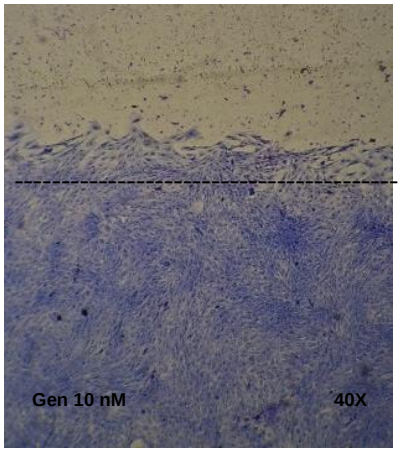


Figure 3





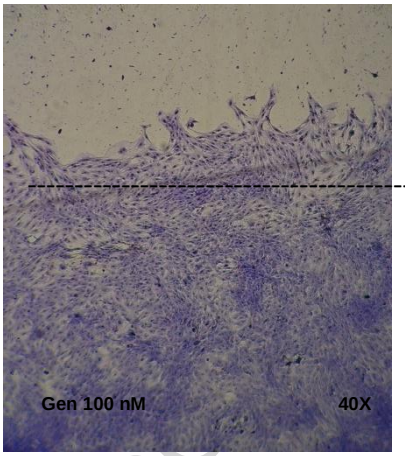


Figure 4

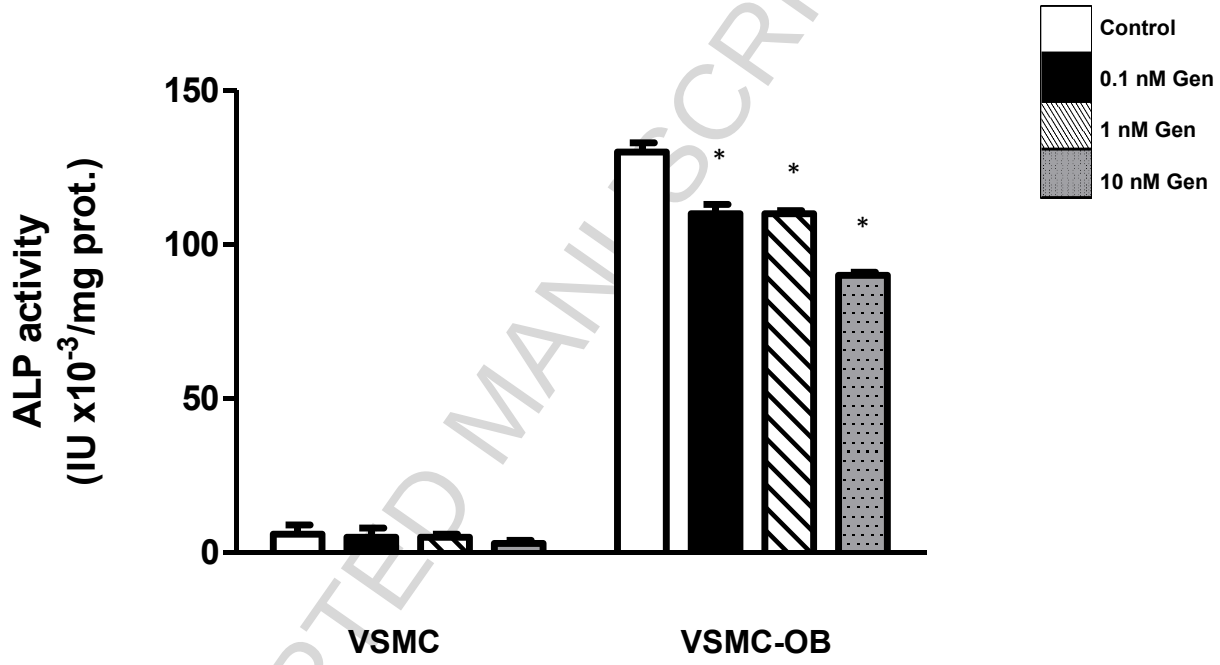
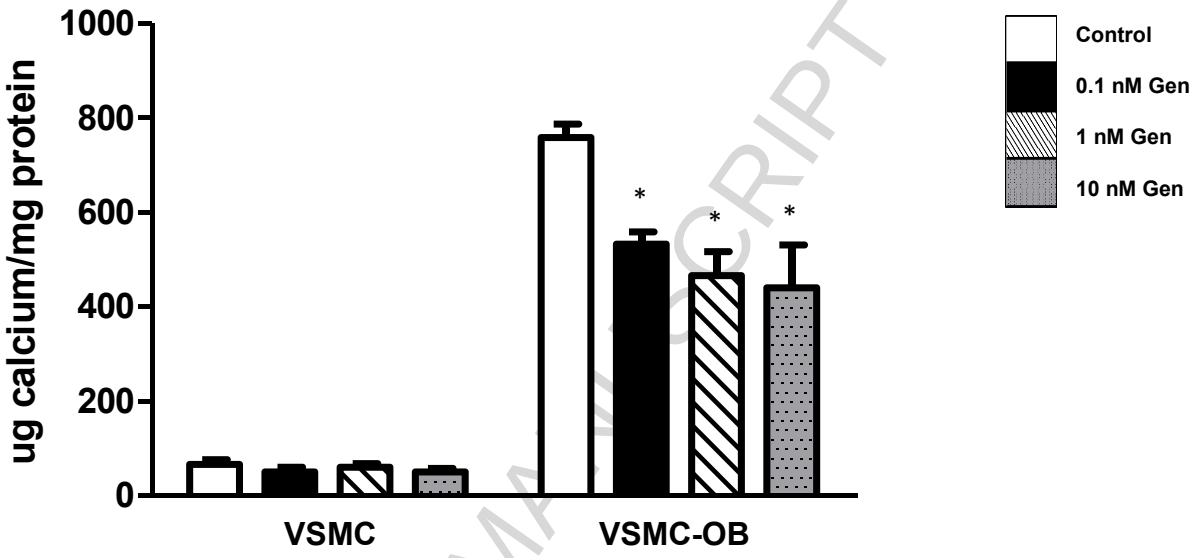
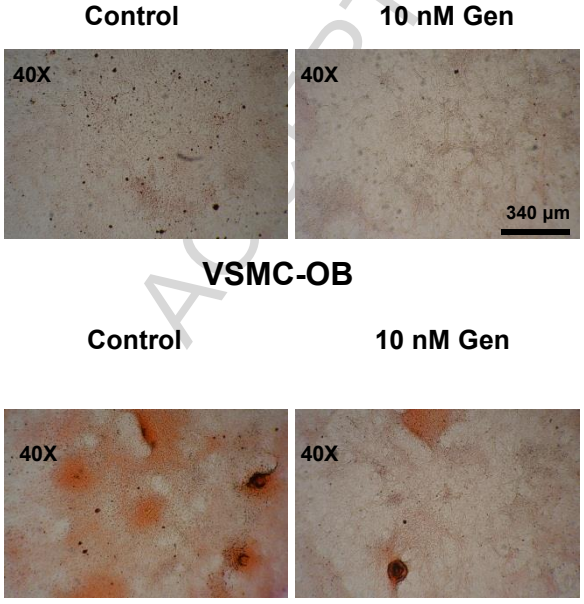


Figure 5

(A)



(B)



(C)

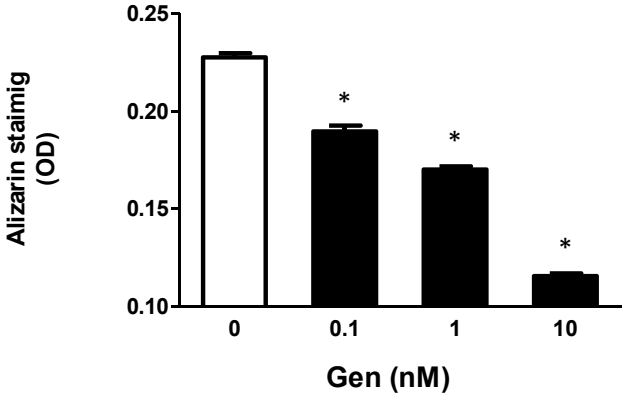


Figure 6

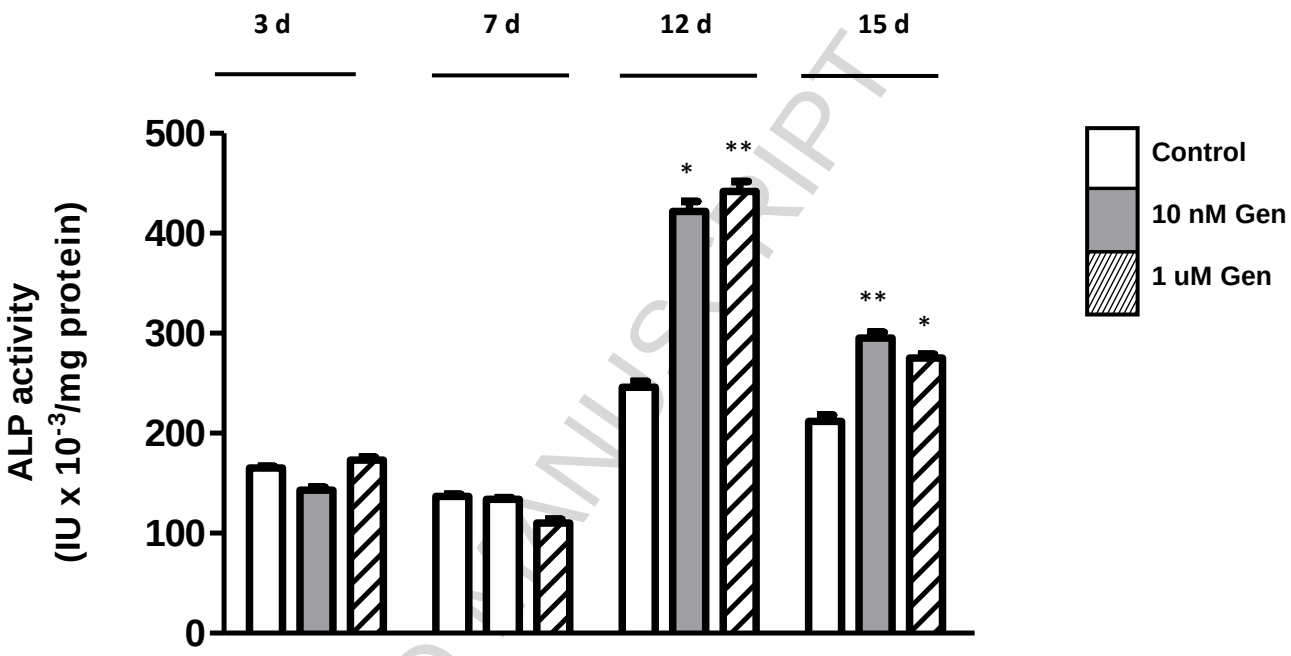
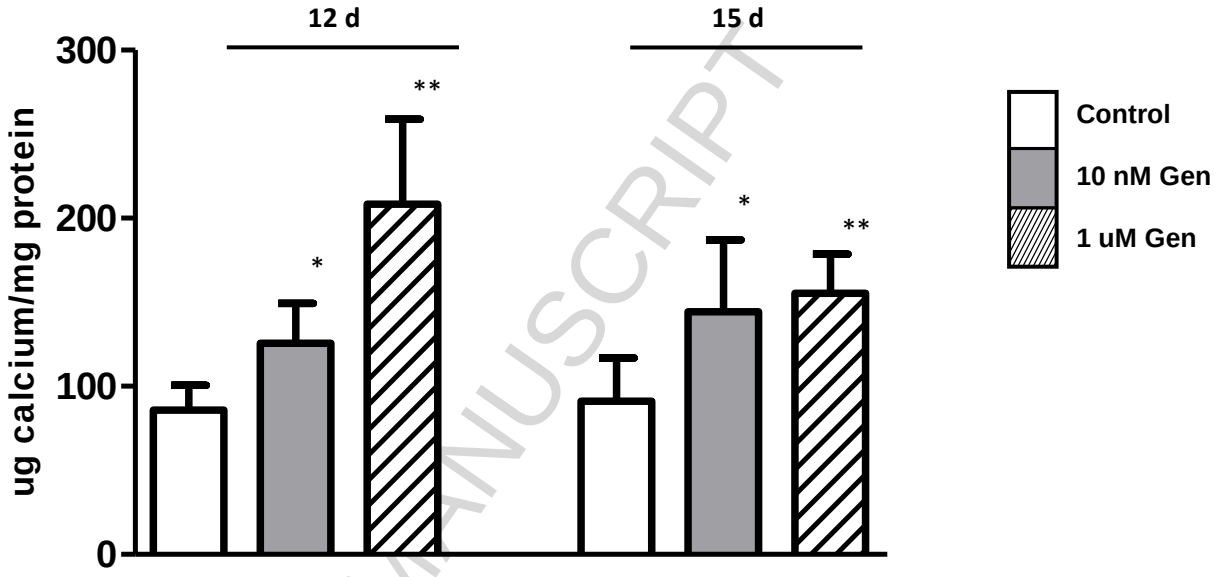
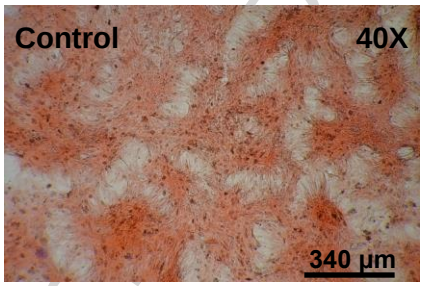


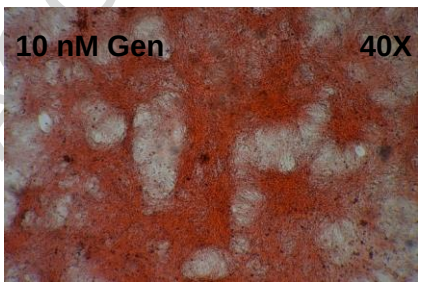
Figure 7

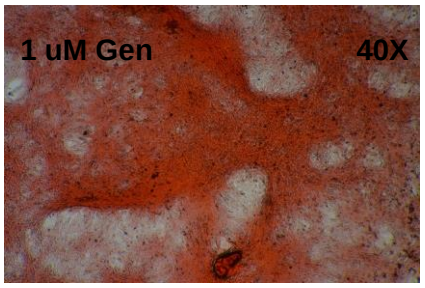
(A)



(B)







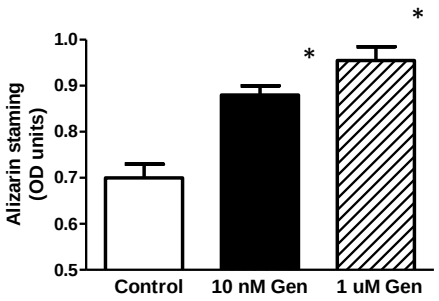


Figure 8