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Abstract: Osteoporosis as well as cardiovascular disease and Diabetes may share some common pathophysiological mechanisms of disease such as the effects of accumulated advanced glycation end products (AGE) and the increase of intracellular oxidative stress that takes place after AGE interacts with its receptor RAGE. AGE have been demonstrated to induce the osteogenic trans-differentiation of vascular smooth muscle cells (VSMC). Strontium ranelate (SR) has been developed as an innovative treatment for osteoporosis since it has both anti-catabolic and anabolic actions on bone tissue. However, in the last years SR has been associated with an increase of cardiovascular risk, peripheral artery disease, non-fatal myocardial infarction and venous thromboembolism. Since strontium can replace extracellular calcium causing similar biological effects, we hypothesized that SR could increase the osteoblastic trans-differentiation of VSMC and thus the induction of extracellular calcifications, an effect that could be potentiated in the presence of AGE and inhibited by simultaneous administration of vitamin D. The present results of our in vitro experiments demonstrate that AGE and SR alone or in combination, can stimulate L-type calcium channels, causing an increase in reactive oxygen species that in turn activate NFkB, generating a vicious cycle with the final effect of promoting the osteogenic shift of VSMC. Importantly, these in vitro effects of AGE and/or SR are prevented by co-incubation with vitamin D.

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Dear Dr. C.M. Klinge,

We are submitting our manuscript entitled “Advanced Glycation End Products and Strontium Ranelate Promote Osteogenic Differentiation of Vascular Smooth Muscle Cells In Vitro: Preventive Role of Vitamin D”, by Juan Manuel Fernandez, María Silvina Molinuevo, Ana María Cortizo, Antonio Desmond McCarthy, Leon Schurman and Claudia Sedlinsky, to be considered for publication in Molecular and Cellular Endocrinology.

It is widely known that osteoporosis as well as cardiovascular disease and Diabetes may share some common pathophysiological mechanisms of disease such as the effects of accumulated advanced glycation end products (AGE) and the increase of intracellular oxidative stress that takes place after AGE interacts with its receptor RAGE. AGE have been demonstrated to induce the osteogenic trans-differentiation of vascular smooth muscle cells (VSMC).

Strontium ranelate (SR) has been developed as an innovative treatment for osteoporosis since it has both anti-catabolic and anabolic actions on bone tissue. This agent is particularly valuable due to the lack of approved pharmacological anabolic agents for bone, out of teriparatide. However, in the last years SR has been associated with an increase of cardiovascular risk, peripheral artery disease, non-fatal myocardial infarction and venous thromboembolism.

Since strontium can replace extracellular calcium causing similar biological effects, we hypothesized that SR could increase the osteoblastic trans-differentiation of VSMC and thus the induction of extracellular calcifications, an effect that could be potentiated in the presence of AGE and inhibited by simultaneous administration of vitamin D. The present results of our in vitro experiments demonstrate that AGE and SR alone or in combination, can

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stimulate L-type calcium channels, causing an increase in reactive oxygen species that in turn activate NFkB, generating a vicious cycle with the final effect of promoting the osteogenic shift of VSMC. Importantly, these in vitro effects of AGE and/or SR are prevented by co-incubation with vitamin D. Although in vitro studies cannot be extrapolated to the clinical situation, we believe that our results will encourage researchers to analyze the adverse effects of SR in the context of vitamin D deficit or repletion.

Best regards,

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Title

Advanced Glycation End Products and Strontium Ranelate Promote Osteogenic Differentiation of Vascular Smooth Muscle Cells In Vitro: Preventive Role of Vitamin D.

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Abstract

Osteoporosis as well as cardiovascular disease and Diabetes may share some common pathophysiological mechanisms of disease such as the effects of accumulated advanced glycation end products (AGE) and the increase of intracellular oxidative stress that takes place after AGE interacts with its receptor RAGE. AGE have been demonstrated to induce the osteogenic trans-differentiation of vascular smooth muscle cells (VSMC). Strontium ranelate (SR) has been developed as an innovative treatment for osteoporosis since it has both anti-catabolic and anabolic actions on bone tissue. However, in the last years SR has been associated with an increase of cardiovascular risk, peripheral artery disease, non-fatal myocardial infarction and venous thromboembolism. Since strontium can replace extracellular calcium causing similar biological effects, we hypothesized that SR could increase the osteoblastic trans-differentiation of VSMC and thus the induction of extracellular calcifications, an effect that could be potentiated in the presence of AGE and inhibited by simultaneous administration of vitamin D. The present results of our in vitro experiments demonstrate that AGE and SR alone or in combination, can stimulate L-type calcium channels, causing an increase in reactive oxygen species that in turn activate NFkB, generating a vicious cycle with the final effect of promoting the osteogenic shift of VSMC. Importantly, these in vitro effects of AGE and/or SR are prevented by co-incubation with vitamin D.

Key words: advanced glycation end products; vascular smooth muscle cells; strontium ranelate; osteoblasts; vitamin D

Introduction

Osteoporosis is a highly prevalent condition, closely related to the aging process. The most common treatments for osteoporosis include oral supplementation with calcium and vitamin D, and/or anti-osteoporotic drugs such as bisphosphonates, strontium ranelate (SR) or intermittent administration of rhPTH. In particular, SR is an interesting therapeutic option since it shows dual in vitro and in vivo anti-osteoporotic action: it decreases osteoclastic bone resorption while stimulating osteoblastic bone formation [1].

In order to select the best treatment option for an individual patient, it is important to investigate possible non-skeletal actions of these drugs or of their possible therapeutic combinations. For example, calcium supplementation on its own in patients with a negative balance of this cation (such as older adults) is controversial: most studies have found it to increase cardiovascular risk, although others have not [2-4]. On the other hand, low serum levels of vitamin D that are prevalent in older adults have been associated with an increase both in cardiovascular risk and in postural instability (and thus of bone fractures). For these reasons, simultaneous administration of calcium and vitamin D is currently recommended [5].

Osteoporosis as well as cardiovascular disease and Diabetes may share some common pathophysiological mechanisms of disease such as the effects of accumulated advanced glycation end products (AGE) and the increase of intracellular oxidative stress that takes place after AGE interaction with its cognate receptor RAGE [6-8]. We have previously demonstrated that AGE can induce deleterious effects on osteoblasts proliferation, differentiation and survival, and that these effects were prevented in vitro by SR [9]. Regarding cardiovascular disease, AGE has been demonstrated to increase the proliferation and migration as well as osteogenic trans-differentiation of vascular smooth

muscle cells (VSMC) following AGE-RAGE interaction [10-11]. On the other hand, physiological concentrations of vitamin D induce protective effects on vascular calcifications, whereas vitamin D deficiency is associated with an increase in vascular calcifications [12-13].

Recently, SR has been claimed to increase cardiovascular risk, peripheral arterial disease, non-fatal myocardial infarction and venous thromboembolism, and its use was restricted by the European Medicine Agency (EMA 2013). Nevertheless, other communications have shown conflicting data regarding this issue. Svanström and coworkers showed in a nationwide cohort study of postmenopausal women in Denmark, using as a primary outcome acute coronary syndrome and as a secondary outcome any-cause mortality, that compared to the use of alendronate or risedronate, strontium ranelate was not associated with an increased risk of both end-points [14]. Another study by Cooper and coworkers using myocardial infarction, hospitalization with myocardial infarction, or cardiovascular death as primary outcome, was unable to find evidence for a higher risk for cardiac events associated with the use of strontium ranelate in postmenopausal osteoporosis [15].

Since the actions of strontium may have similarities to those of extracellular calcium, we propose the following working hypothesis: Strontium ranelate could increase the osteoblastic transdifferentiation of VSMC and thus the induction of extracellular calcifications, an effect that could be potentiated in the presence of AGE and inhibited by simultaneous administration of vitamin D. The aim of this study was to evaluate the in vitro effects of strontium ranelate on VSMC proliferation, migration and transdifferentiation to the osteoblastic phenotype, with or without the influence of AGE, and to determine if vitamin D is able to modulate strontium ranelate and/or AGE effects on VSMC.

Materials and Methods

2.1 Preparation of advanced glycation end products

Advanced glycation end product-modified bovine serum albumin (AGE) was prepared by incubation of 10 mg/ml bovine serum albumin (Sigma, Argentina) with 33 mM d-glycolaldehyde in 150 mM phosphate-buffered saline pH 7.4 at 37ºC for 3 days under sterile conditions after which unincorporated sugar was removed [16]. Control bovine serum albumin (BSA) was incubated in the same conditions without sugar. The formation of AGE was assessed with a Shimadzu spectrofluorometer by their characteristic fluorescence - emission maximum at 420nm upon excitation at 340nm. The estimated level of AGE obtained by this in vitro incubation was 18.5% relative fluorescence units/mg protein, in contrast to 3.2% for control BSA.

2.2 Isolation of vascular smooth muscle cells (VSMC)

Adult male Sprague – Dawley rats (190 – 210 g) were maintained in a temperature - controlled room at 23 °C, with a fixed 12h light: 12h darkness cycle, and fed standard rat laboratory chow and water ad libitum. All experiments with animals were performed in conformity with the Guidelines on Handling and Training of Laboratory Animals published by the Universities Federation for Animals Welfare [17]. Approval for animal studies was obtained from the institutional animal care committee (CICUAL approval number: 001-05-15). Animals were sacrificed by cervical dislocation under anesthesia and aortas were dissected.

VSMC were isolated from aorta rings as previously described [18]. Briefly, tunica adventitia was dissected and the aorta cut into 1mm rings. Individual rings were cultured in

25 cm^2 flasks at 37 ºC in a humidified incubator with an atmosphere of 95% air-5% CO_2. Tissue explants were further cultured in DMEM containing 10% fetal bovine serum (FBS) (Natocor, Cordoba, Argentina) and antibiotic-antimycotic products (10,000 U/ml penicillin G sodium, 10,000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B in 0.85% saline). The medium was changed initially after 24 h and then every 2–3 d. After about 2-3 weeks, when cells had formed a confluent monolayer, they were harvested by addition of 0.05% trypsin, and the culture was continued using DMEM containing 10% FBS. VSMC were characterized by expression of α-actin.

For migration, proliferation and differentiation experiments, cells were seeded on multi-well plates and incubated in DMEM and 100 µg/ml of either BSA or AGE, with or without 0.1 mM of strontium ranelate (SR, kindly provided by Servier Argentina).

2.3 Cell proliferation

A mitogenic bioassay was carried out as previously described [19] with modifications. Briefly, VSMC were cultured in 24-well plates for 24 or 48 hours submitted to different experimental conditions, after which they were washed with phosphate buffered saline (PBS) and fixed with 5% glutaraldehyde/PBS for 10 min. Cells were stained with 0.5% crystal violet (25% methanol) for 10 min. Excess dye was discarded and the plate was extensively washed with water. The dye taken up by cells was extracted using 0.5 ml/well 0.1 M glycine/HCl buffer, pH 3.0 /30% methanol, which was transferred to test tubes and its absorbance read at 540 nm. Correlation between the cell number and the absorbance at 540 nm has been previously established [20].

2.4 Cell migration

VSMC migration was assessed by an in vitro wound assay as previously described [21]. Confluent monolayers of VSMC were scratched (wounded) with a pipette tip. After washing with culture media, cells were incubated for an additional 12 hours in DMEM—10% FBS with either BSA or AGE, in the presence or absence of SR. After this incubation period, the monolayers were fixed and stained with Giemsa. Cell migration distance from the edge of the wound was assessed using the freeware Image J program (www.macbiophotonics.ca/imagej).

2.5 Osteogenic differentiation

Osteogenic induction of VSMC was performed by incubating confluent cell monolayers in DMEM-10% FBS supplemented with 5 mM b-glycerolphosphate and 25 µg/ml ascorbic acid. Under these culture conditions cells begin to secrete type 1 collagen, express alkaline phosphatase activity (ALP) and accumulate extracellular mineral deposits after 1 week, reaching a maximum after 2 weeks. For ALP determination, cells were washed with phosphate-buffered saline (PBS) and solubilized in 0.5 ml 0.1% Triton X-100. Aliquots of this total cell extract were used for protein determination [22] and for measurement of ALP by spectrophotometric determination of initial rates of hydrolysis of p-nitrophenyl-phosphate (p-NPP) to p-nitrophenol (p-NP) at 37ºC for 10 min. For evaluation of type 1 collagen production, cells were fixed with Bouin's solution and stained with Sirius red dye for 1 h. The stained material was dissolved in 1 ml 0.1 N sodium hydroxide and the absorbance of the solution was recorded at 550 nm [23]. Accumulation of extracellular mineral deposits was evaluated by staining with Alizarin S Red, and

quantitated spectrophotometrically at 540 nm after solubilization with 0.1N sodium hydroxide [23].

2.6 Western blot assays

Cell monolayers were lysed with Laemmli's buffer [24]. Total protein content of the cell lysates was evaluated by a micro-method [25]. Lysates were heated to 100 ◦C for 3 min, after which aliquots containing 40µg of protein were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were then transferred to PVDF membranes (Millipore, Bedford, MD), which were blocked in 3% non-fat dry milk in Tris-buffered saline (TBS) for 2 h at room temperature. They were then incubated at 4ºC for 24 hours with anti-Runx2 (Santa Cruz Biotechnologies, sc-10758, Lot # H1909), anti-α-actin (Santa Cruz Biotechnologies, sc-53142, Lot # H1909), or anti-β-actin polyclonal antibodies (Novus Biologicals, NB600-503, Lot # A5) diluted 1:2000 in PBS with 0.5% bovine serum albumin. After four washes in PBS with 0.1% Tween 20, the membranes were incubated with a secondary goat anti-rabbit antibody, followed by staining with the peroxidase-biotin reagent and diaminobenzidine from the Vectastain kit. The intensity of the Runx2, α-actin and β-actin specific bands was quantified by densitometry after scanning the PVDF membrane with a Hewlett-Packard Scanjet 4C. Images were analysed using the gel plugin of Image J program (www.macbiophotonics.ca/imagej).

2.7 Reactive oxygen species

Stock solutions of dihydrorhodamine (DHR) (25 mM) and rhodamine123 (0.5 mM) were prepared in nitrogen-purged dimethylformamide and stored in the dark at -20°C until

used [26]. A fresh 10 mM solution of DHR in DMEM was prepared every day. VSMC were incubated with AGEs, BSA and/or SR for 12 hours, after which the culture media was replaced by DHR in DMEM and cells were additionally incubated for an additional 4h at 37 °C. VSMC monolayers were then washed twice with PBS and lysed with 1 ml of 0.1 % Triton X100. In the lysate, fluorescence intensity of the oxidized product rhodamine was determined (excitation wavelengh, 495 nm; emission wavelengh, 532 nm), using an Aminco-Bowman SPF100 spectrofluorometer equipped with a Hamamatsu R928 photomultiplier tube. Fluorescence intensity was corrected for protein content [22]. A standard curve of rhodamine123 was constructed from 0.1 to 5 mM.

2.8 Statistical analysis

Three independent experiments were run for each experimental condition. Results are expressed as the mean ± S.E.M. One Way ANOVA with Dunnett's post test was performed using GraphPad InStat version 3.05 GraphPad Software, San Diego, California USA. $P<0.05$ was considered to be statistically significant.

Results

Effects of AGE and/or SR treatment on VSMC proliferation and migration

In a first series of experiments we evaluated the effect of 100 μg/ml AGE or BSA and/or 0.1mM SR on VSMC proliferation. We found a significant increase in cell proliferation after 48h of AGE treatment (Fig 1A, $p<0.01$ vs. BSA). On its own, SR also increased VSMC proliferation compared to BSA ($p<0.05$). Co-incubation of SR and AGE induced an additive increase in VSMC proliferation (Fig 1A). This increase in VSMC

proliferation caused by AGE and/or SR was completely prevented by treatment with either 10 μM nifedipine (L-type calcium channel inhibitor), a mixture of vitamins C and E (50 μM of each), 100 μM sulfasalazine (IKKS inhibitor, thus preventing NFκB nuclear translocation) or 50 ng/ml of vitamin D (Fig 2 A-D).

The migration ability of VSMC was evaluated using the scratch wound assay. We found that either AGE or SR significantly increased VSMC migration compared to BSA ($p<0.01$), and that co-incubation of AGE with SR induced an additive effect (Fig 1B). Again, the effect of both AGE and SR alone or in combination could be prevented by co-treatment with either nifedipine, a mixture of vitamins E and C, sulfasalazine or vitamin D (Fig 3 A-D).

Effect of AGE and/or SR on VSMC osteogenic differentiation

In additional experiments, we evaluated the effect of AGE and/or SR on the osteoblastic trans-differentiation of VSMC. We found that the addition of AGE to the osteogenic media caused an increase in collagen type I production (Fig. 4 A), alkaline phosphatase activity (Fig 4 B) and extracellular mineral deposits (Fig 4 C) while it increased the expression of the osteogenic transcription factor Runx2 (Fig 5 A) and inhibited the expression of α-actin, a marker of VSMC phenotype (Fig 5 B). Similarly, SR on its own also caused VSMC to increase their production of collagen and extracellular mineral deposits, to stimulate alkaline phosphatase activity (Fig 4) and to increase Runx2 expression while decreasing α-actin expression (Fig 5). SR co-incubated with AGE caused an additive effect on the induction of osteogenic parameters and in the decrease of VSMC phenotypic markers (Fig 4 and 5). Pro-osteogenic effects induced by AGE and/or SR could

be prevented by co-incubation with either nifedipine, a mixture of vitamins E and C, sulfasalazine or vitamin D (Fig 6 A-D shows their effect on type 1 collagen secretion, as a representative example). Additionally, vitamin D also prevented the stimulation of Runx2 expression and extracellular mineral deposits induced by AGE and/or SR (Fig 6 E-F).

Role of intracellular oxidative stress on the osteogenic actions of AGE and/or SR

In order to investigate the role of oxidative stress on the actions of AGE, SR or their combination, we measured their effect on production by VSMC of intracellular reactive oxygen species (ROS). We found that 100 μg/ml of AGE caused a significant increase in ROS production compared to BSA (Fig 7A, $p<0.01$). SR also increased ROS production, while co-incubation of SR with AGE induced an additive effect (Fig 7A). Nifedipine, vitamin D or a mixture of vitamins C and E completely prevented the increase in ROS production induced by AGE and/or SR (Fig 7 B-D). On the other hand, co-incubation with sulfasalazine did not prevent this effect (Fig 7).

Discussion

In the present work we evaluated the osteogenic trans-differentiation of VSMC after co-incubation with BSA or AGE and/or SR, and its possible modulation with vitamin D. One of the first steps for transition from myocytic to osteogenic phenotype is an increase in cellular secretory, proliferative and migratory capacity (Owens et al., 2004). In our experiments with VSMC, we found an AGE-induced increase in cell proliferation and migration (compared to BSA). Additionally, and in agreement with the previous research of other authors, we found an AGE-induced increase in the expression of osteogenic

phenotype markers (alkaline phosphatase activity, type 1 collagen secretion, mineral nodule formation, Runx2/β-actin ratio) [27-29]. Although those authors described pro-osteogenic actions of AGE on VSMC, none evaluated the decrease of α-actin as a marker of smooth muscle phenotype. In our present work, we demonstrate that exposure of VSMC to AGE induces loss of α-actin and acquisition of osteoblastic genes. Other authors previously demonstrated that VSMC lose their myocytic phenotype as they trans-differentiate to osteoblasts [30]. In particular they showed that induction of vascular calcifications was concomitant with loss of smooth muscle α-actin and smooth muscle 22alpha expression, and with gain of osteopontin expression both in vitro and in vivo. On the other hand, we have previously demonstrated that bone formation is diminished in vitro and in vivo in the presence of AGE, as well as in pathological conditions where AGE accumulate such as Diabetes or the metabolic syndrome [31-38]. These apparently opposing effects of AGE (anti-osteogenic for bone cells, pro-osteogenic for VSMC), are in fact supported by several population studies that point out the coexistence of bone loss with calcifications of the aortic tunica media. Although the molecular mechanisms that regulate this phenomenon are incompletely known, it has been suggested that AGE plays an important role in this process [39-40]. Previous studies have demonstrated that both in VSMC and in osteoblasts AGE interact with its receptor RAGE which in turns activates intracellular signals that activate pro-inflammatory genes responsible of its effects on these cells [10,12,31,35,37,41].

Strontium ranelate (SR) has been approved for treatment of postmenopausal osteoporosis. However, in 2014 the European Medicine Agency (EMA) restricted the use of SR because its concern about associated cardiovascular risk (peripheral thrombosis, cerebral and myocardial infarction). Two retrospective studies have proposed to restrict the

use of SR in patients with previous cardiovascular events of any type [42-43]. To date, there are no reports evaluating possible mechanisms for the incidence of cardiovascular events in patients treated with SR.

Our present results demonstrate that SR induces VSMC to accumulate extracellular calcifications in vitro, and that SR also causes an additive effect on calcifications when it is co-incubated with AGE. Furthermore we show that the actions of SR on VSMC can be prevented by sulfasalazine and nifedipine, suggesting that in this cell type SR stimulates the pro-inflammatory pathway of NFkB by activating the L-type calcium channels. However it is not clear from our present study if SR enters VSMC via L-type calcium channels, or simply activates them. In any case, we show that activation of these channels increases intracellular ROS and thus promotes NFkB, which finally leads to an increase in the proliferative, migratory and pro-osteogenic capacity of these cells. It has been pointed out that oxidative stress drives the osteoblastic trans-differentiation of VSMC [12]. Intracellular ROS production can also be increased by binding of AGE to its cognate receptor RAGE [34]: thus it is not unexpected to find that both AGE and SR promote the osteogenic induction of VSMC, showing an additive effect when they are co-incubated. Both agents could have the final result of potentiating activation of the NFkB pathway (Figure 8).

Our present results with VSMC differ from those previously reported by our group with osteoblasts in culture [9]. In our previous work, we found that SR prevented the deleterious actions of AGE on osteoblasts in culture by activation of L-type calcium channels and a decrease in the secretion of pro-inflammatory cytokines. We have yet to elucidate the molecular reasons for these divergent effects; however, these results highlight the fact that the specific effect of SR in the context of AGE accumulation depends on the cell system under study.

In our present study, we have also demonstrated that vitamin D (cholecalciferol) modulates the osteogenic trans-differentiation of VSMC in response to AGE and/or SR. Indeed, we found that the osteogenic differentiation of VSMC was blunted when the cells exposed to AGE and/or SR were co-incubated with vitamin D. Moreover, we found that vitamin D exerted an antioxidant effect preventing the up-regulation of Runx2. VSMC have been previously demonstrated to express 25- and 1α-hydroxylases, suggesting that these cells can metabolize either cholecalciferol or calcidiol [44-45]. Additionally, anti-inflammatory properties of 1,25-vitamin D (calcitriol) have been reported in VSMC [46]. This evidence points to a significant and protective role of vitamin D on the vasculature.

Additionally, our results show in an in vitro model a reversion of SR pro-osteogenic effects on VSMC differentiation by co-treatment with vitamin D. However, we cannot extrapolate these results to humans. Further clinical investigations are needed to prove that this drug association has similar effects in human patients.

In conclusion, we demonstrated in vitro that AGE and SR alone or in combination, stimulate L-type calcium channels, causing an increase on reactive oxygen species, which in turns activate NFkB generating a vicious cycle with the final effect to promote the osteogenic shift of VSMC (Figure 8). In addition, that these effects of AGE and/or SR can be prevented by co-incubation with vitamin D.

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Conflict of Interest

All authors declare that they have no conflict of interest.

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469 1,25(OH)2D3 Induces Placental Vascular Smooth Muscle Cell Relaxation by
470 Phosphorylation of Myosin Phosphatase Target Subunit 1Ser507: Potential Beneficial
471 Effects of Vitamin D on Placental Vasculature in Humans. Biol Reprod. 2016
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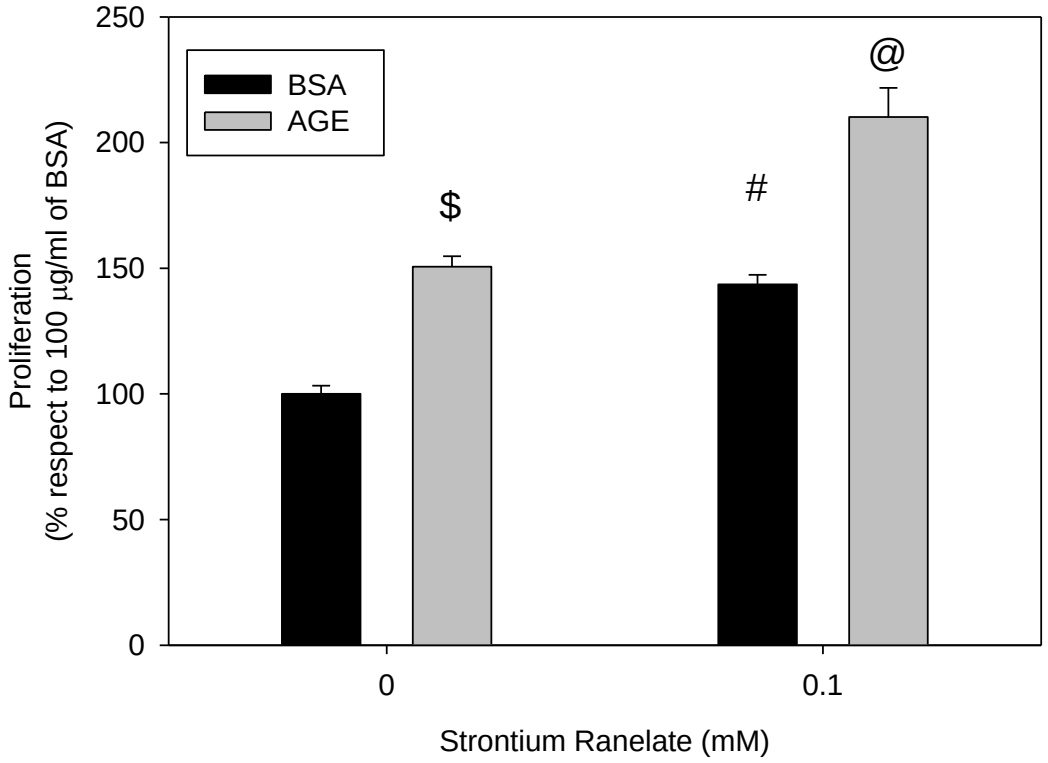
474 vascular smooth muscle cells. Acta Biochim Pol 2012;59:395-400.
475
476
477

Figure 1

(A)


250
200
150
100
50
0
Proliferation
(% respect to 100 μg/ml of BSA)
BSA
AGE
$
#
@
0
0.1
Strontium Ranelate (mM)

(B)

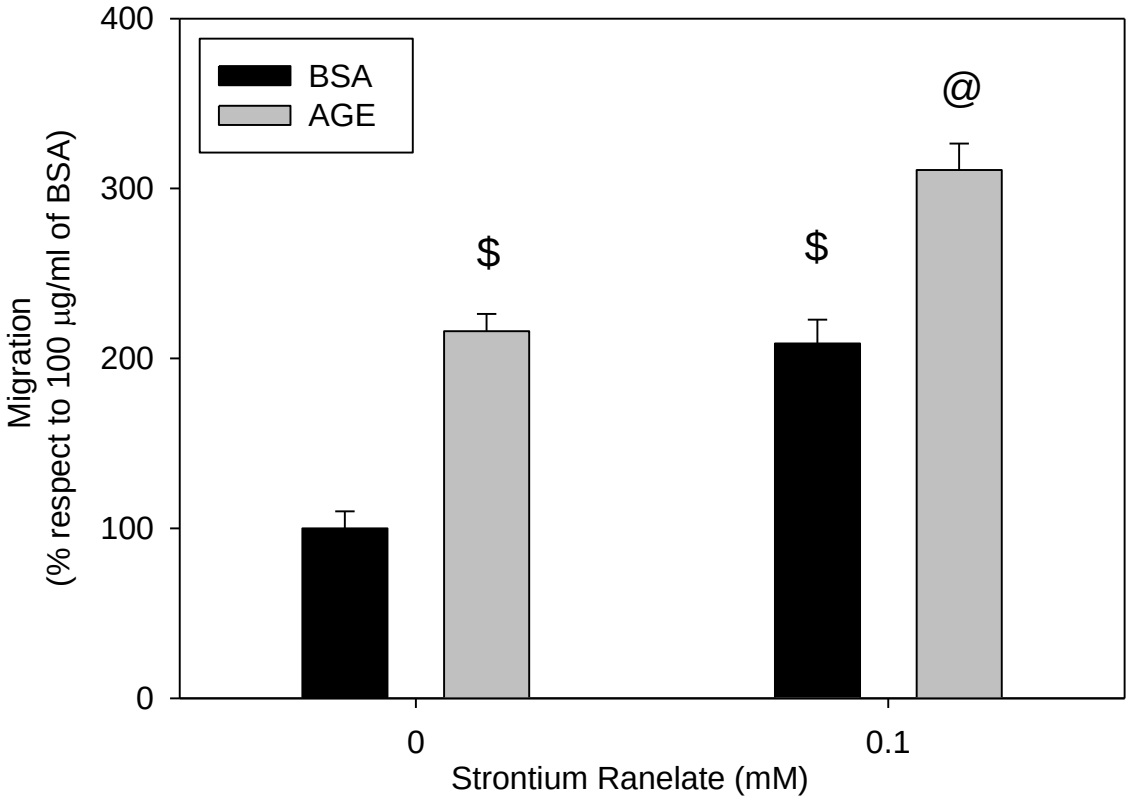
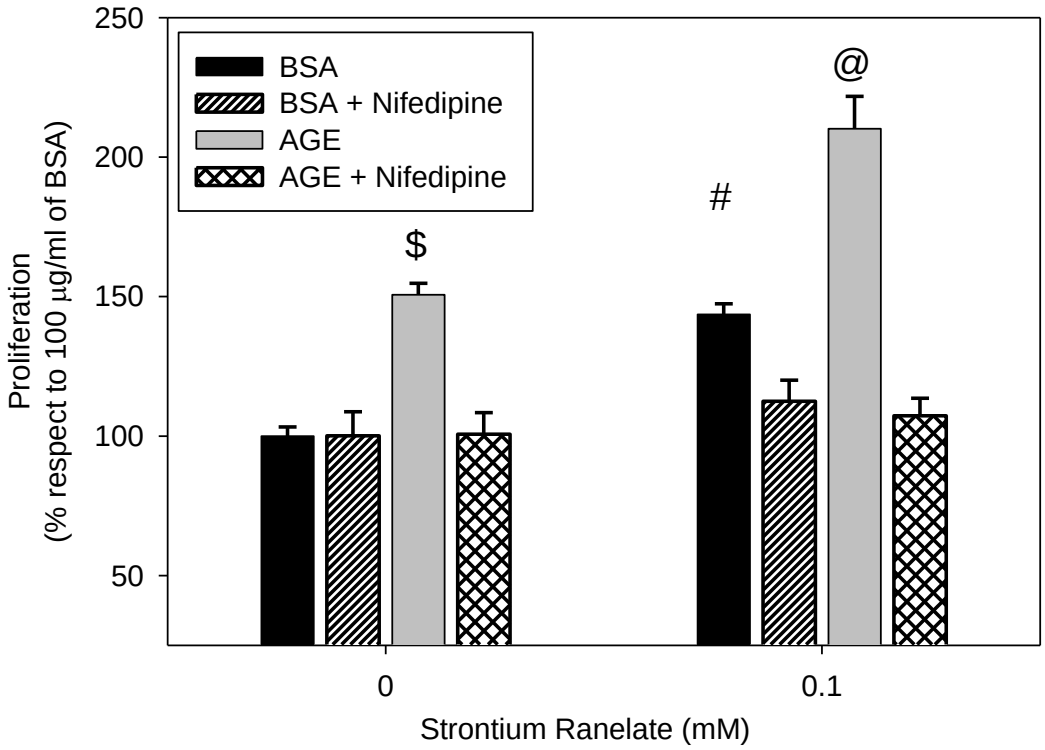
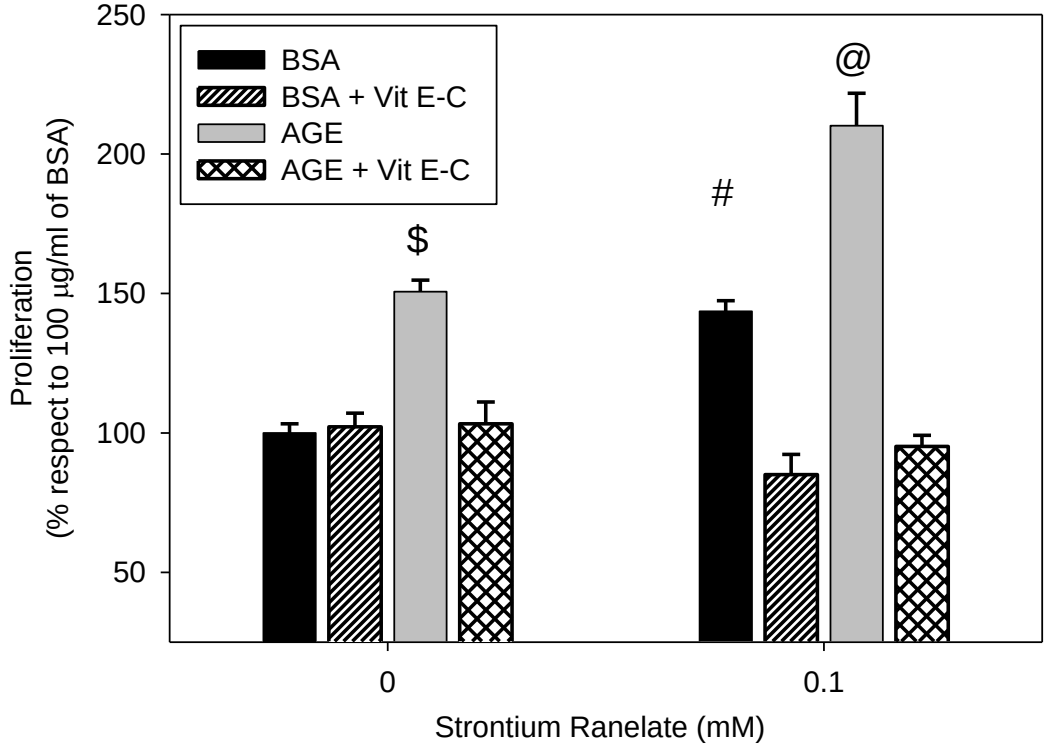

400
300
200
100
0
Migration
(% respect to 100 μg/ml of BSA)
BSA
AGE
$
$
@
0
0.1
Strontium Ranelate (mM)

Figure 2

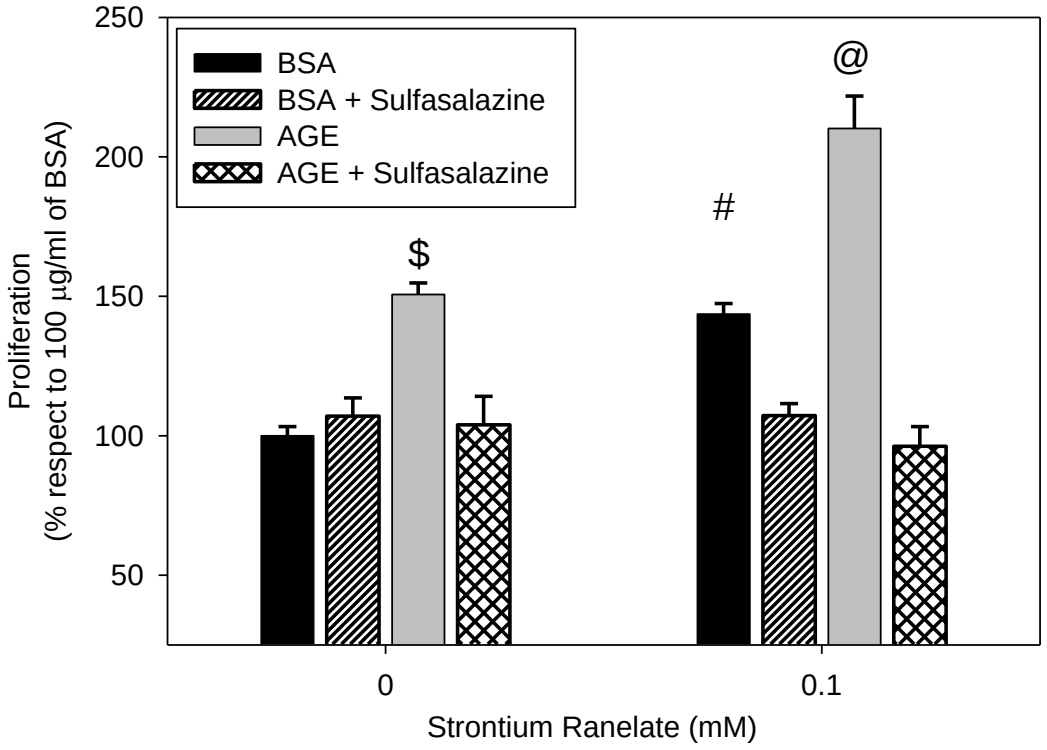
(A)


250
200
150
100
50
Proliferation
(% respect to 100 µg/ml of BSA)
BSA
BSA + Nifedipine
AGE
AGE + Nifedipine
$
#
@
0
0.1
Strontium Ranelate (mM)

(B)


250
200
150
100
50
Proliferation
(% respect to 100 µg/ml of BSA)
BSA
BSA + Vit E-C
AGE
AGE + Vit E-C
$
#
@
0
0.1
Strontium Ranelate (mM)

(C)


BSA
BSA + Sulfasalazine
AGE
AGE + Sulfasalazine
Proliferation
(% respect to 100 µg/ml of BSA)
250
200
150
100
50
$
#
@
0
0.1
Strontium Ranelate (mM)

(D)

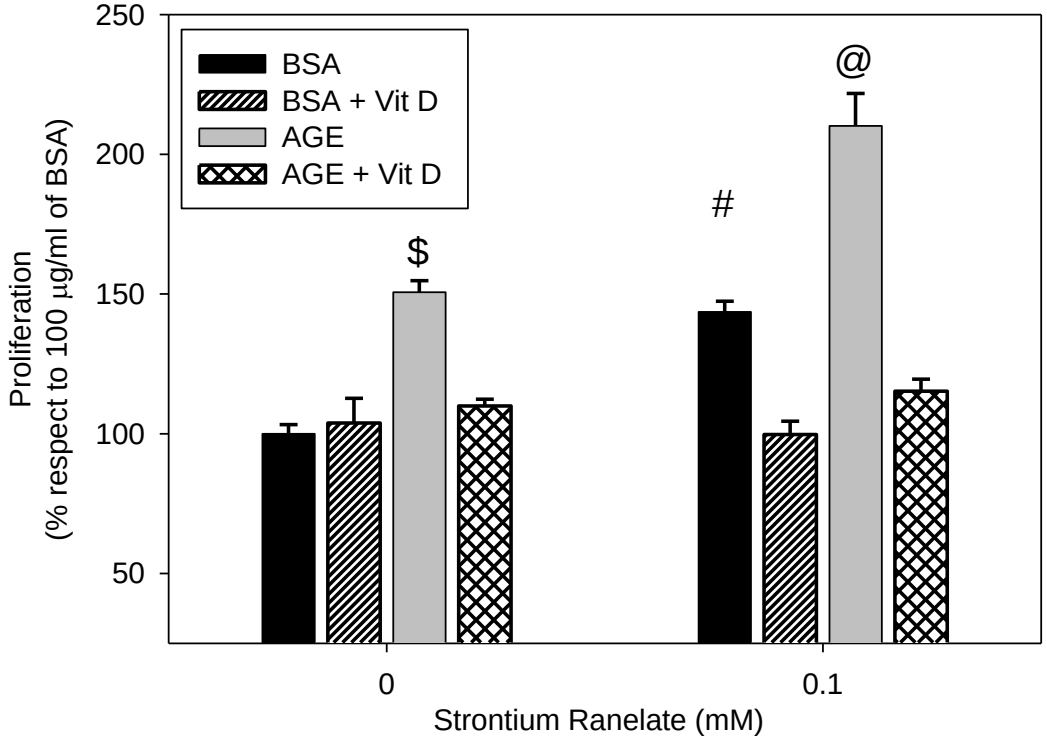
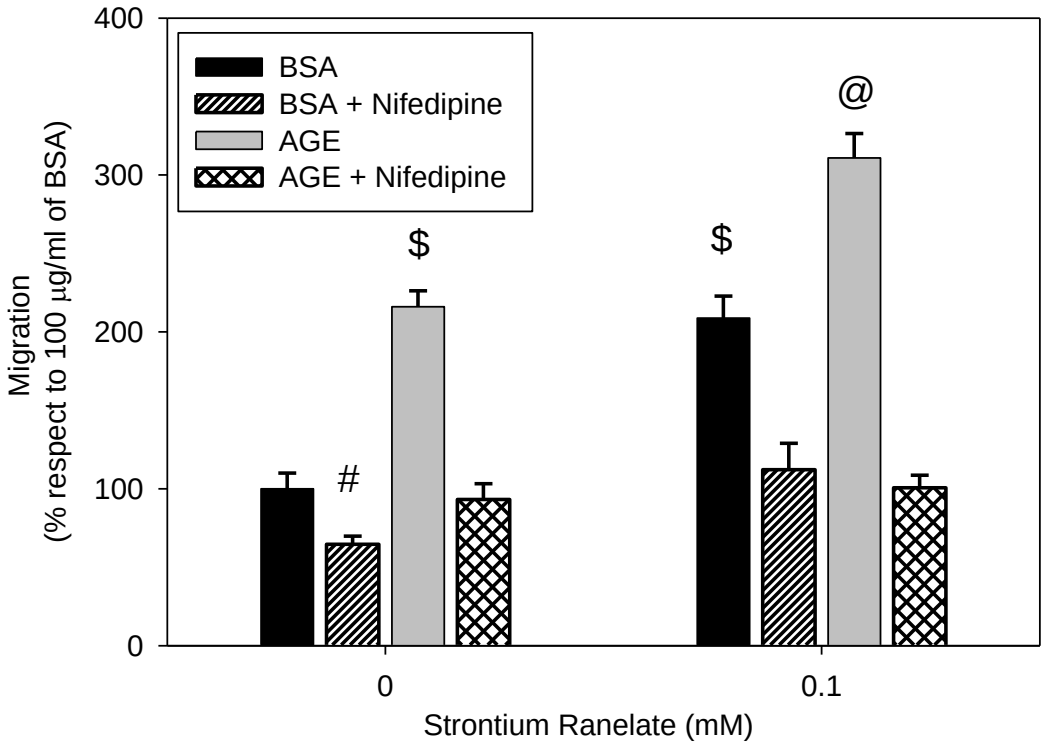
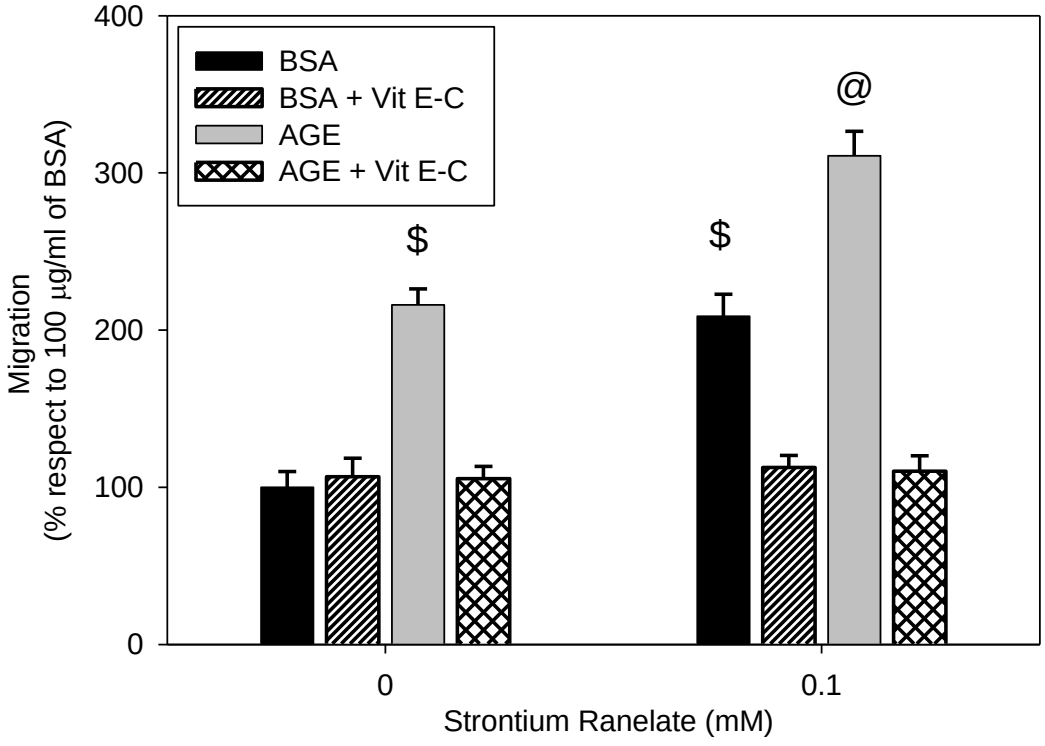

BSA
BSA + Vit D
AGE
AGE + Vit D
Proliferation
(% respect to 100 µg/ml of BSA)
250
200
150
100
50
$
#
@
0
0.1
Strontium Ranelate (mM)

Figure 3

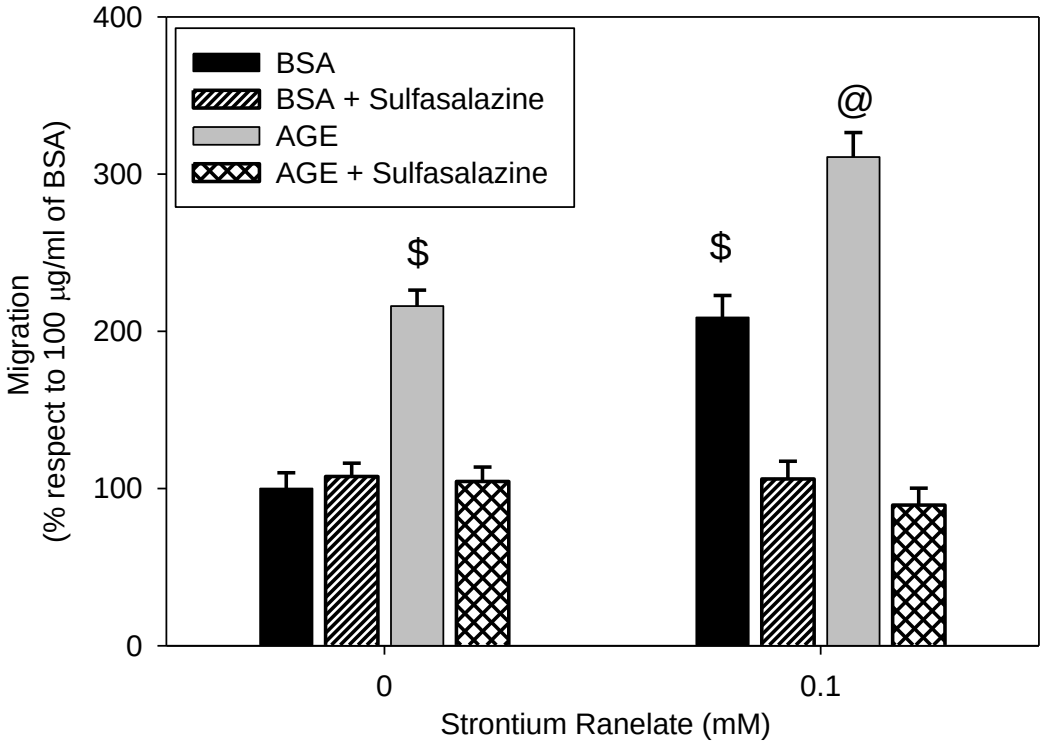
(A)


400
300
200
100
0
Migration
(% respect to 100 µg/ml of BSA)
BSA
BSA + Nifedipine
AGE
AGE + Nifedipine
$
$
@
#
0
0.1
Strontium Ranelate (mM)

(B)


400
300
200
100
0
Migration
(% respect to 100 µg/ml of BSA)
BSA
BSA + Vit E-C
AGE
AGE + Vit E-C
$
$
@
0
0.1
Strontium Ranelate (mM)

(C)


400
300
200
100
0
Migration
(% respect to 100 μg/ml of BSA)
BSA
BSA + Sulfasalazine
AGE
AGE + Sulfasalazine
$
$
@
0
0.1
Strontium Ranelate (mM)

(D)

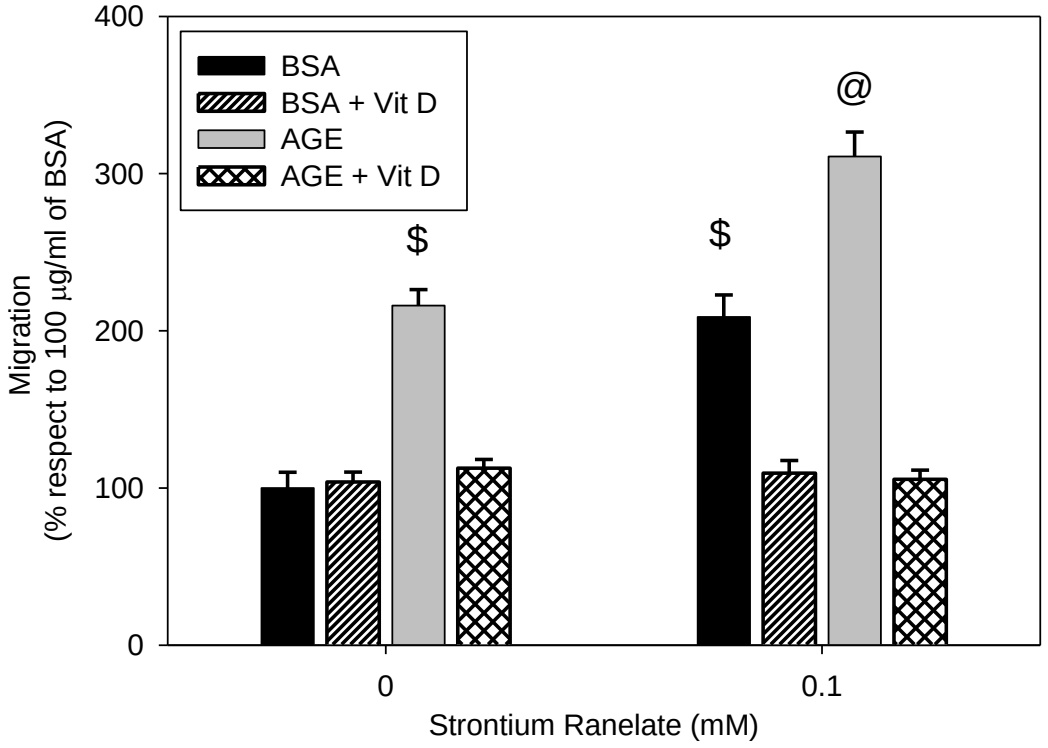
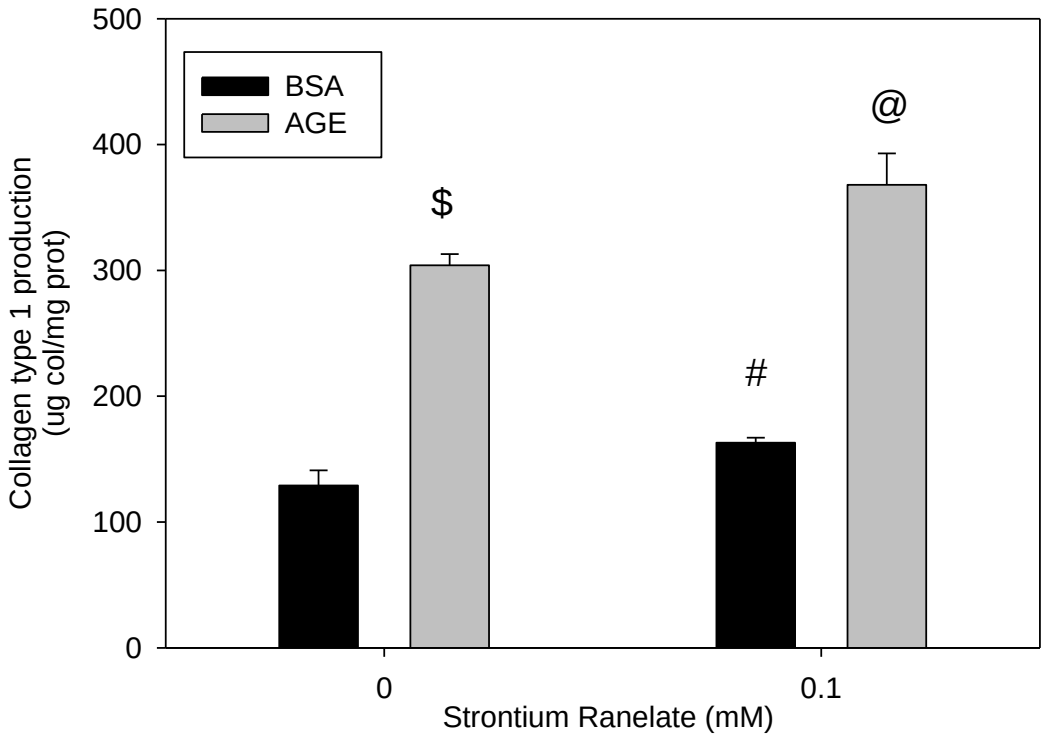
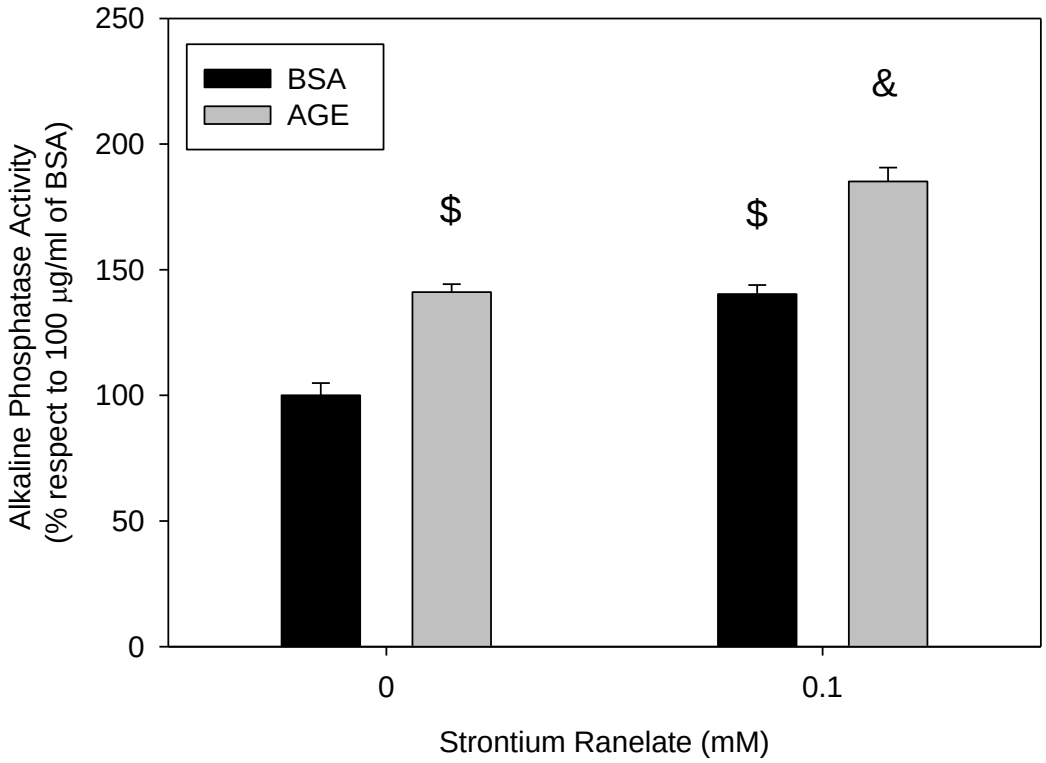

400
300
200
100
0
Migration
(% respect to 100 μg/ml of BSA)
BSA
BSA + Vit D
AGE
AGE + Vit D
$
$
@
0
0.1
Strontium Ranelate (mM)

Figure 4

(A)


500
400
300
200
100
0
Collagen type 1 production
(ug col/mg prot)
BSA
AGE
$
#
@
0
0.1
Strontium Ranelate (mM)

(B)


250
200
150
100
50
0
Alkaline Phosphatase Activity
(% respect to 100 µg/ml of BSA)
BSA
AGE
$
$
&
0
0.1
Strontium Ranelate (mM)

(C)

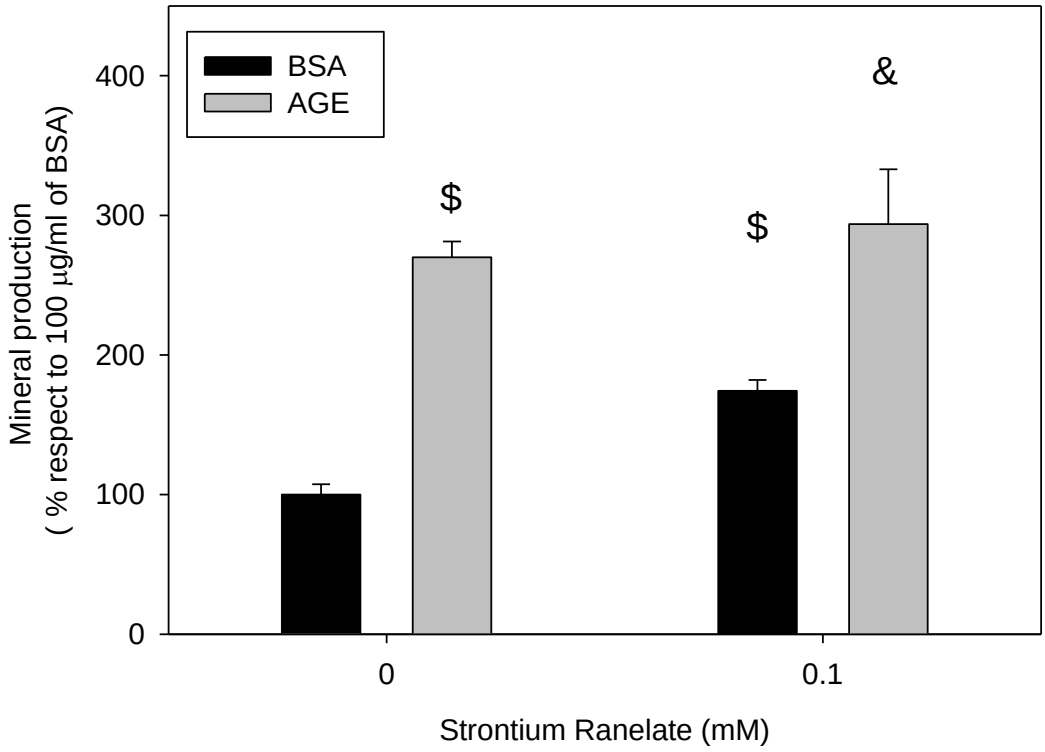
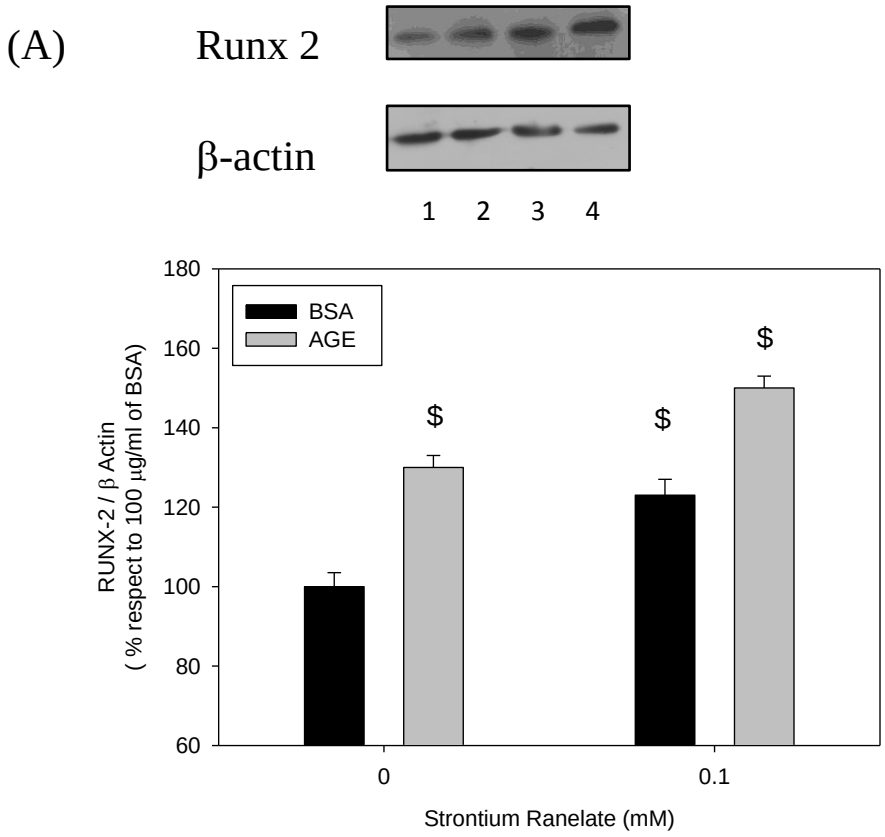

BSA
AGE
Mineral production
(% respect to 100 µg/ml of BSA)
400
300
200
100
0
$
$
&
0
0.1
Strontium Ranelate (mM)

Figure 5


(A)
Runx 2
β-actin
1 2 3 4
BSA
AGE
RUNX-2 / β Actin
(% respect to 100 μg/ml of BSA)
180
160
140
120
100
80
60
$
$
$
0
0.1
Strontium Ranelate (mM)

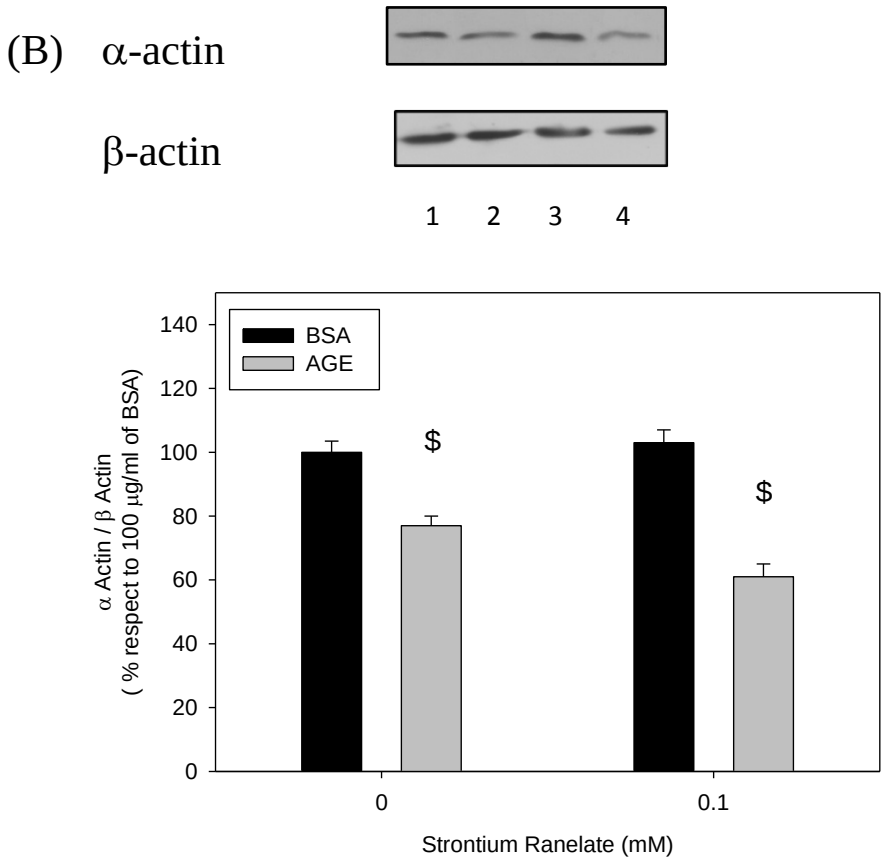
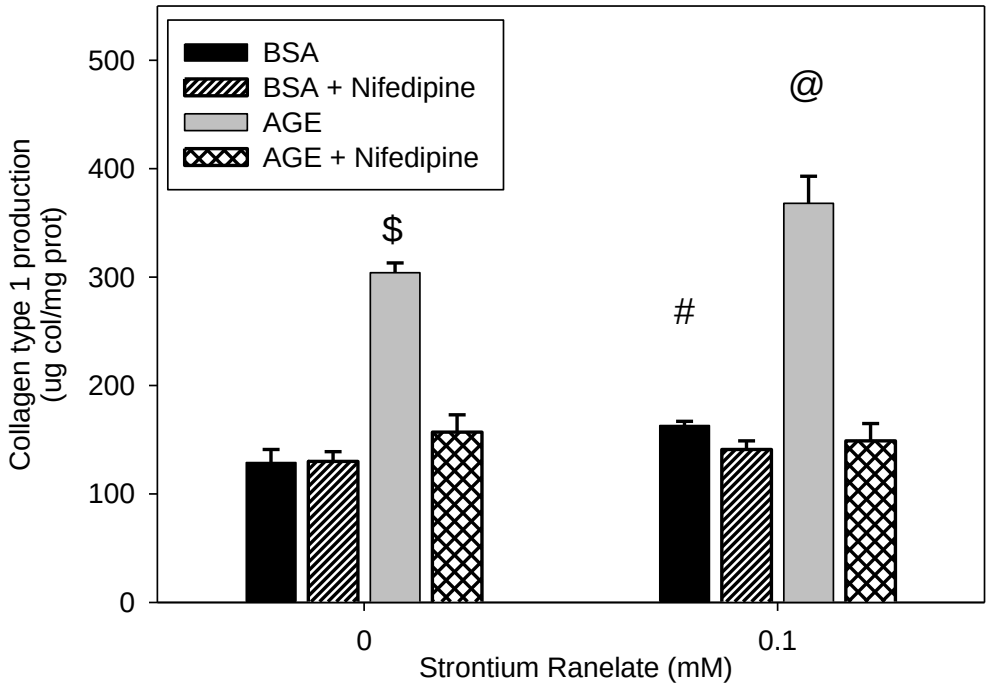
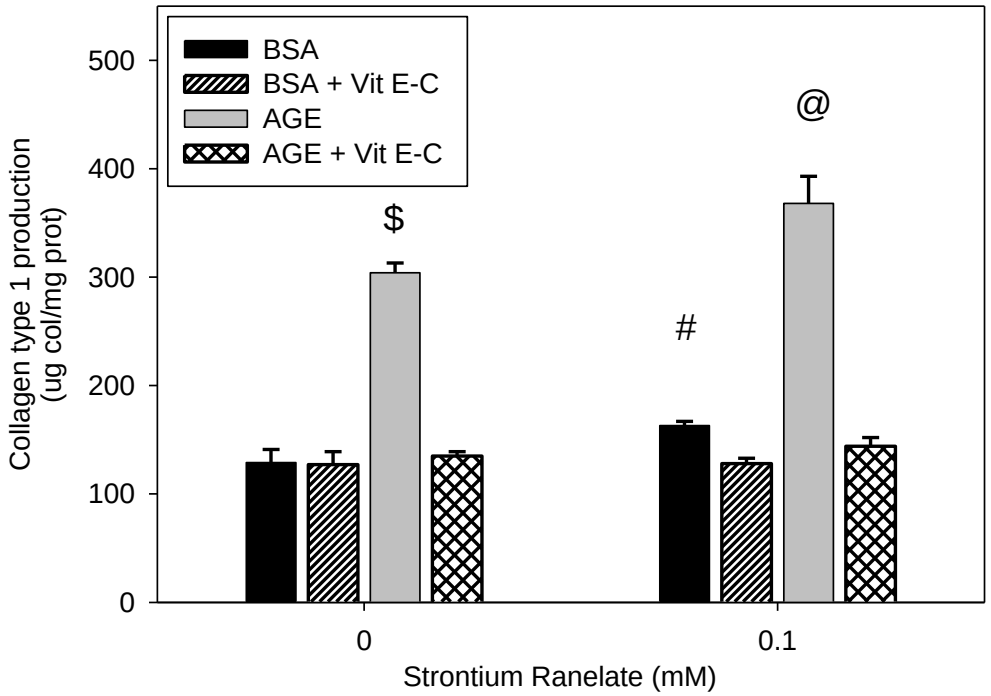

(B)
α-actin
β-actin
1 2 3 4
BSA
AGE
α Actin / β Actin
(% respect to 100 μg/ml of BSA)
140
120
100
80
60
40
20
0
$
$
0
0.1
Strontium Ranelate (mM)

Figure 6

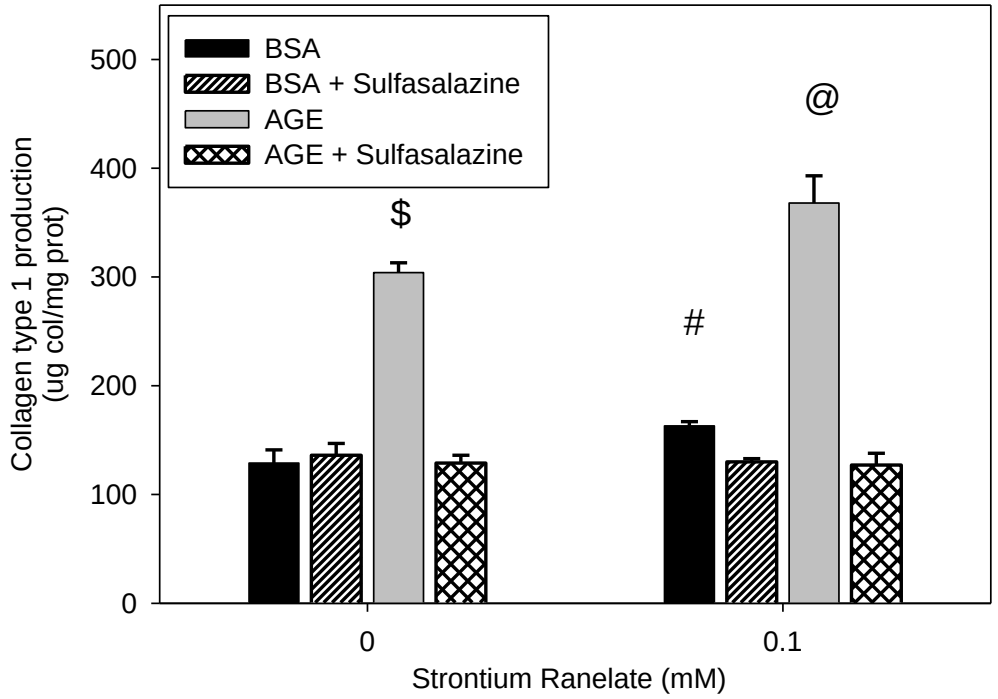
(A)


BSA
BSA + Nifedipine
AGE
AGE + Nifedipine
Collagen type 1 production
(ug col/mg prot)
500
400
300
200
100
0
$
#
@
0
0.1
Strontium Ranelate (mM)

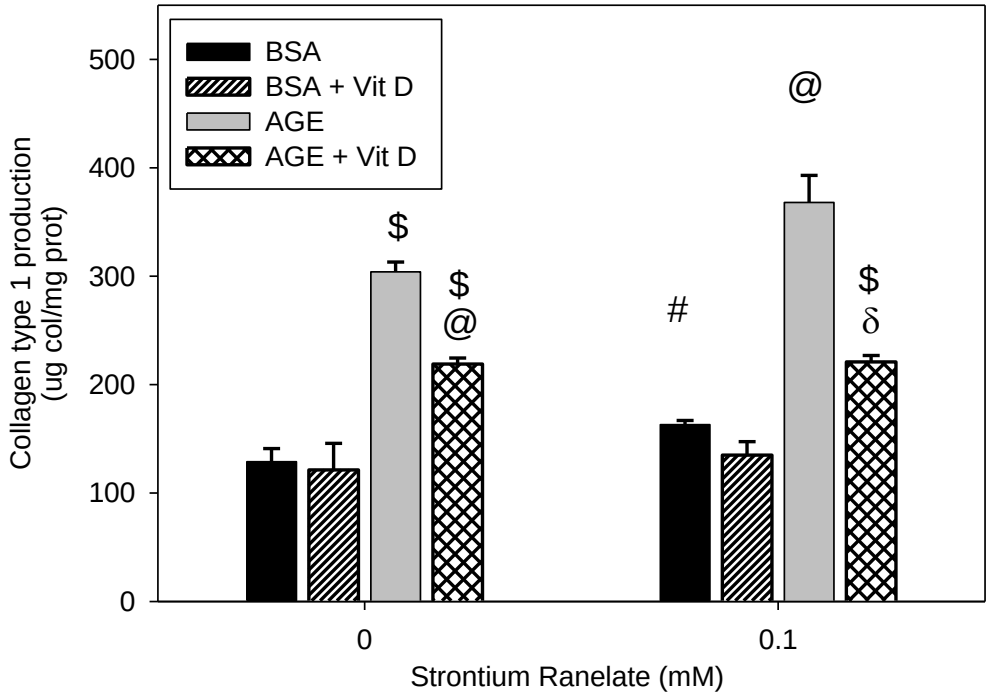
(B)


BSA
BSA + Vit E-C
AGE
AGE + Vit E-C
Collagen type 1 production
(ug col/mg prot)
500
400
300
200
100
0
$
#
@
0
0.1
Strontium Ranelate (mM)

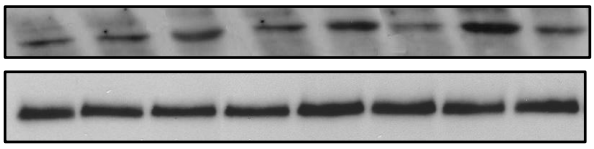
(C)

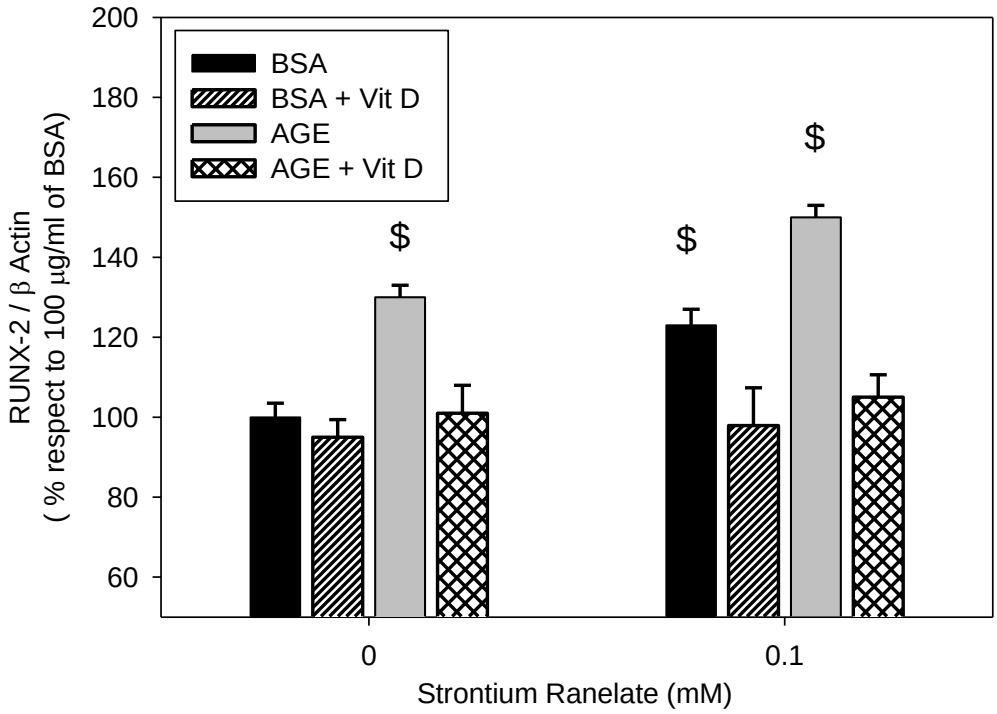

BSA
BSA + Sulfasalazine
AGE
AGE + Sulfasalazine
Collagen type 1 production
(ug col/mg prot)
500
400
300
200
100
0
$
#
@
0
0.1
Strontium Ranelate (mM)

(D)


BSA
BSA + Vit D
AGE
AGE + Vit D
Collagen type 1 production
(ug col/mg prot)
500
400
300
200
100
0
$
$
@
#
@
$
δ
0
0.1
Strontium Ranelate (mM)

(E)


Runx2
β- actin
1 2 3 4 5 6 7 8


BSA
BSA + Vit D
AGE
AGE + Vit D
RUNX-2 / β Actin
(% respect to 100 µg/ml of BSA)
200
180
160
140
120
100
80
60
$
$
$
0
0.1
Strontium Ranelate (mM)

(F)

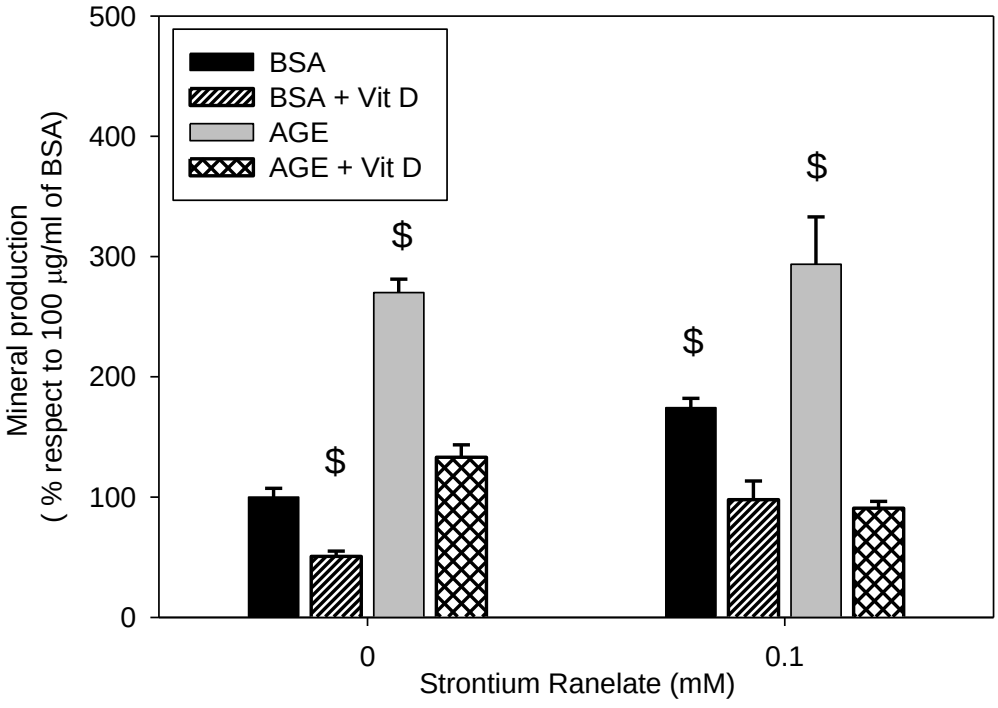
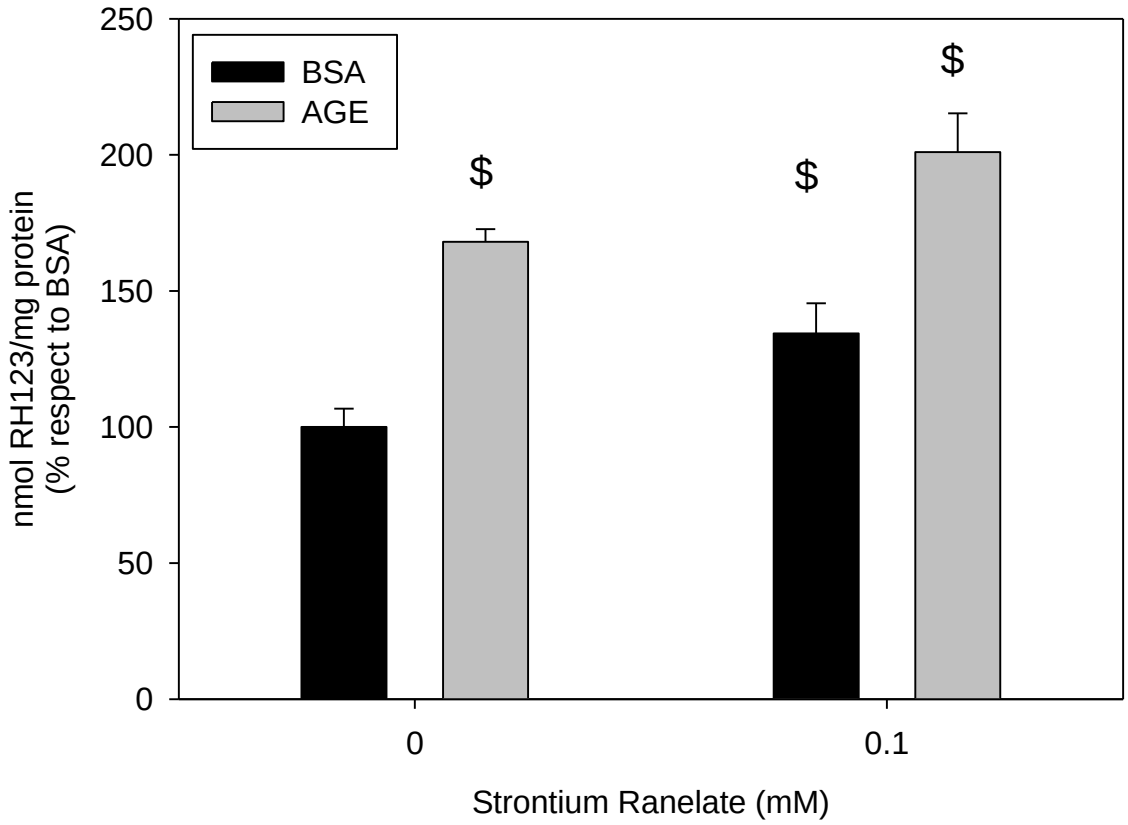
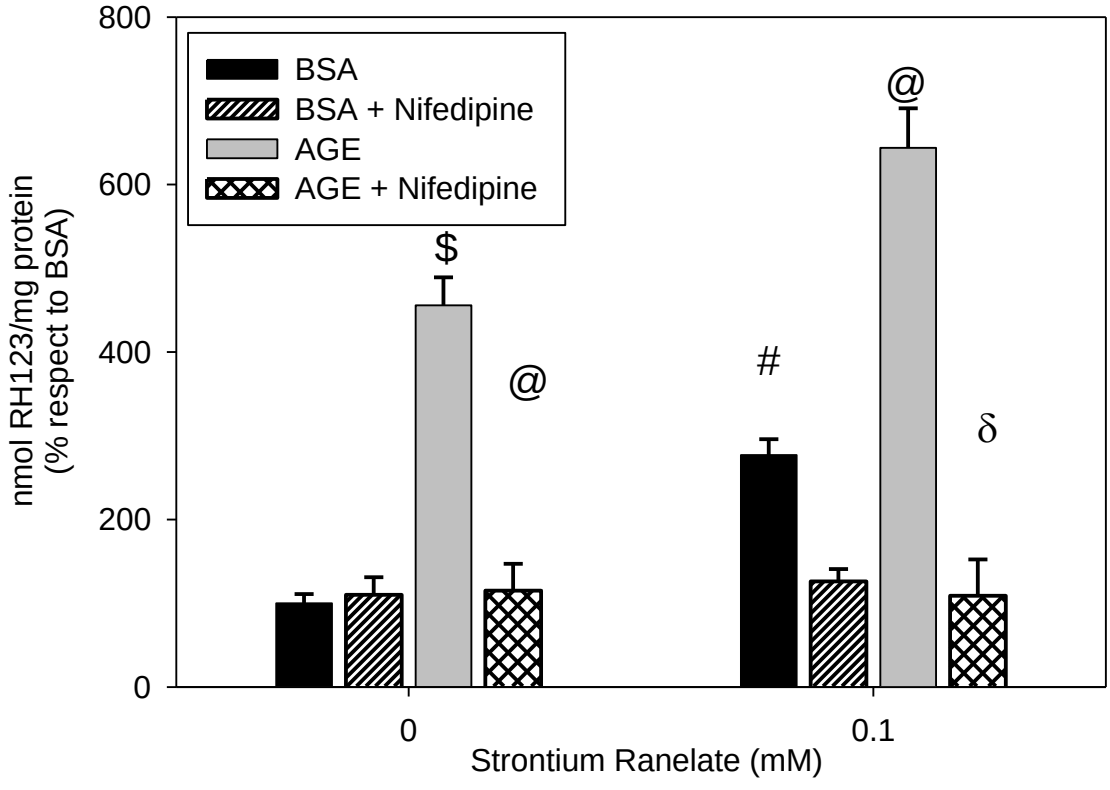

BSA
BSA + Vit D
AGE
AGE + Vit D
Mineral production
(% respect to 100 µg/ml of BSA)
500
400
300
200
100
0
$
$
$
$
0
0.1
Strontium Ranelate (mM)

Figure 7

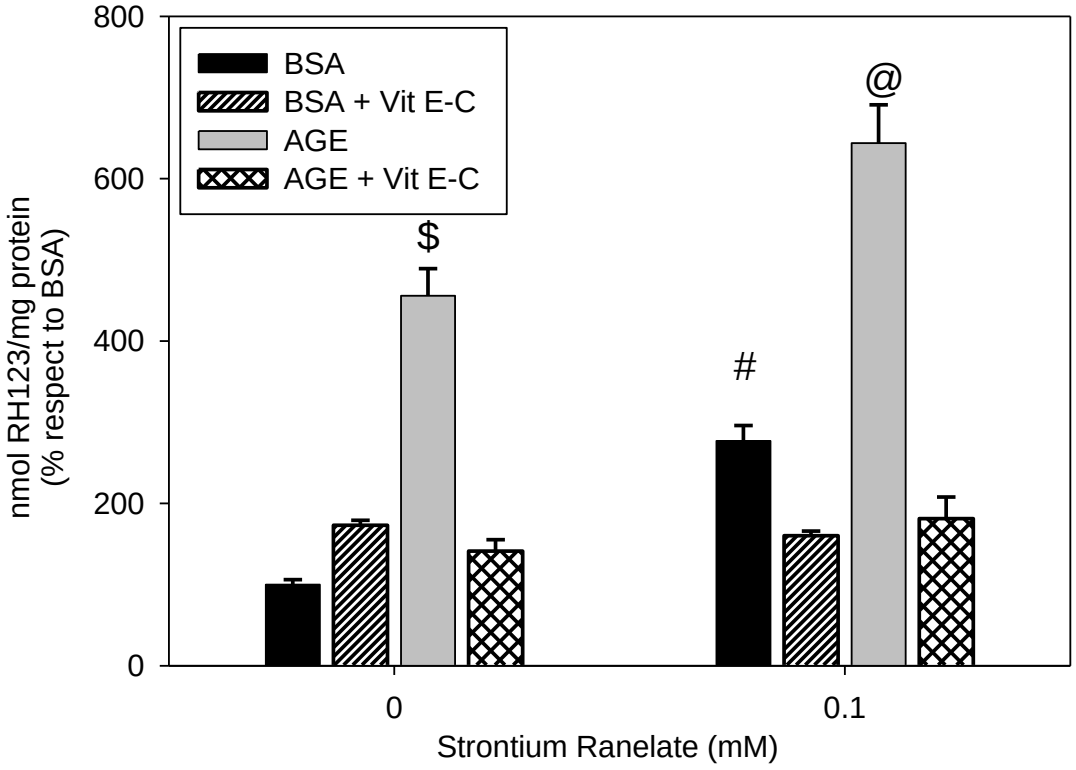
(A)


250
200
150
100
50
0
nmol RH123/mg protein
(% respect to BSA)
BSA
AGE
$
$
$
0
0.1
Strontium Ranelate (mM)

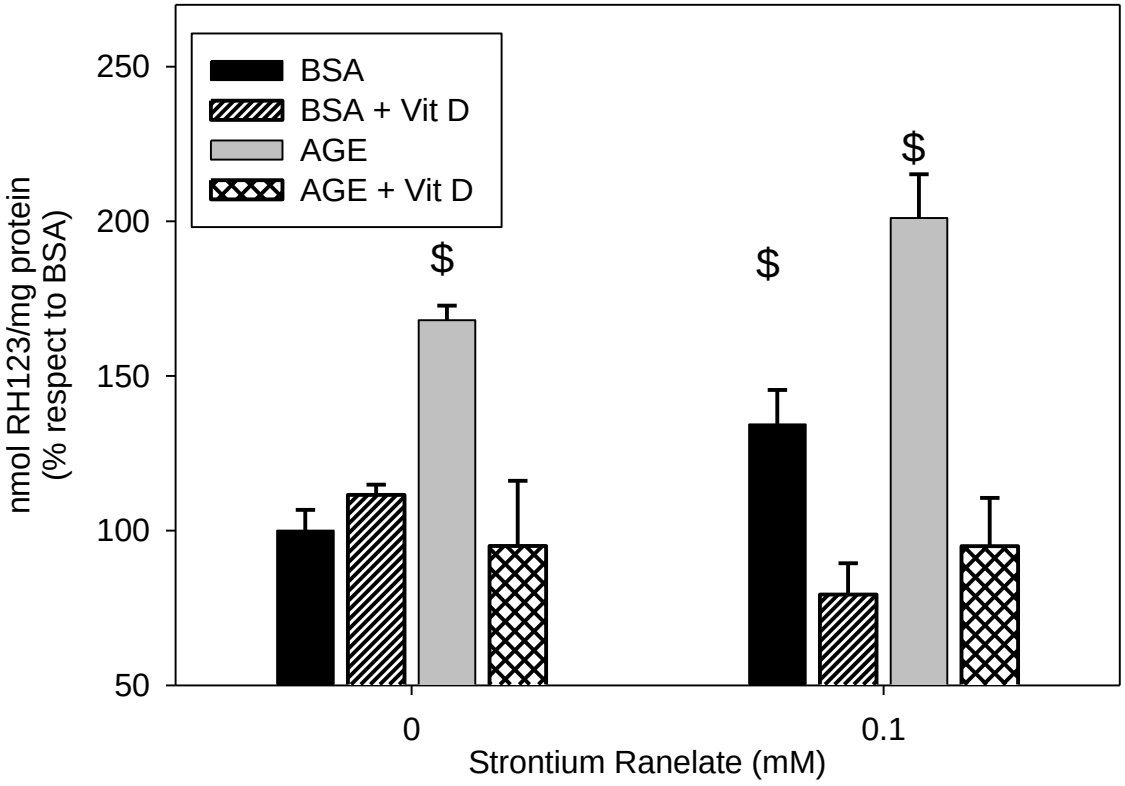
(B)


800
600
400
200
0
nmol RH123/mg protein
(% respect to BSA)
BSA
BSA + Nifedipine
AGE
AGE + Nifedipine
$
@
#
@
δ
0
0.1
Strontium Ranelate (mM)

(C)


800
600
400
200
0
nmol RH123/mg protein
(% respect to BSA)
BSA
BSA + Vit E-C
AGE
AGE + Vit E-C
$
#
@
0
0.1
Strontium Ranelate (mM)

(D)


250
200
150
100
50
nmol RH123/mg protein
(% respect to BSA)
BSA
BSA + Vit D
AGE
AGE + Vit D
$
$
$
0
0.1
Strontium Ranelate (mM)

(E)

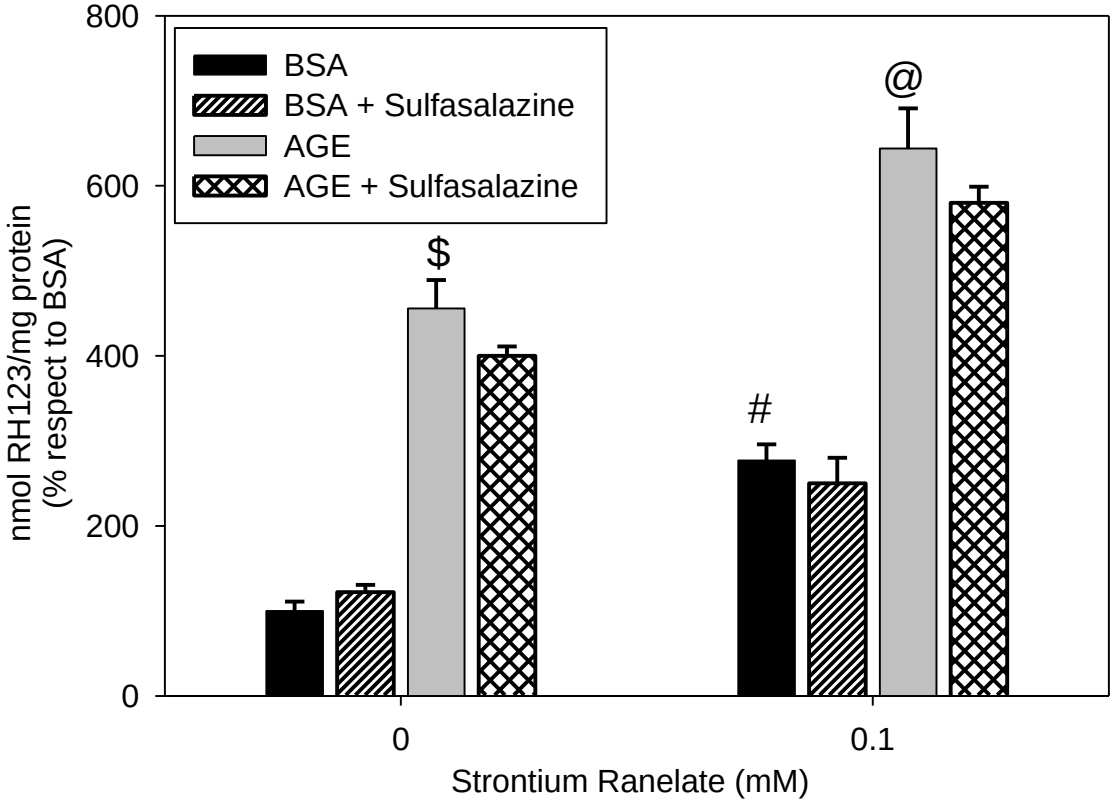
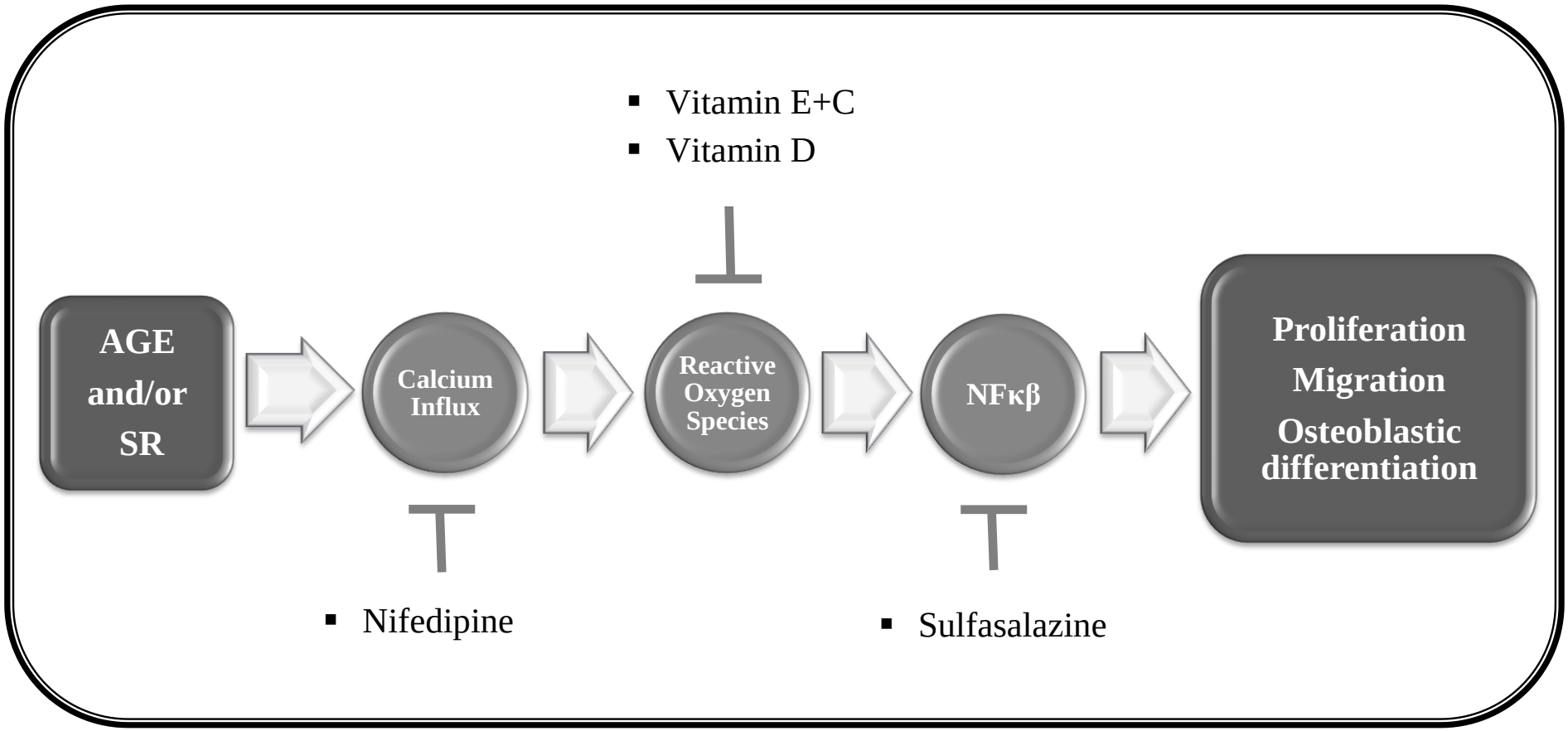

800
600
400
200
0
nmol RH123/mg protein
(% respect to BSA)
BSA
BSA + Sulfasalazine
AGE
AGE + Sulfasalazine
$
#
@
0
0.1
Strontium Ranelate (mM)

Figure 8


Vitamin E+C
Vitamin D
AGE and/or SR
Calcium Influx
Reactive Oxygen Species
NFκβ
Proliferation
Migration
Osteoblastic differentiation
Nifedipine
Sulfasalazine

Legends to the figures

Figure 1: Effect of AGE and/or SR on VSMC. (A) There is a significant increase on VSMC proliferation caused by AGE or SR, while the co-incubation showed an additive stimulatory effect on cell proliferation. (B) AGE or SR promotes VSMC migration, with an additive effect in the co-incubation. Results are expressed as the Media ± SEM, n=8. #: $p<0.05$ vs. BSA; $: $p<0.01$ vs. BSA; @: $p<0.01$ vs AGE.

Figure 2: Effect of 10 μM nifedipine (A), 50 μM vitamin E and C (B), 100 μM sulfasalazine or 50 ng/ml cholecalciferol on cell proliferation. Results are expressed as the Media ± SEM, n=8. #: $p<0.05$ vs. BSA; $: $p<0.01$ vs. BSA; @: $p<0.01$ vs AGE; &: $p<0.05$ vs. AGE.

Figure 3: Effect of 10 μM nifedipine (A), 50 μM vitamin E and C (B), 100 μM sulfasalazine or 50 ng/ml cholecalciferol on VSMC migration. Cell migration was evaluated by the wound assay assessing the cell-free area after 12h. Results are expressed as the Media ± SEM, n=8. #: $p<0.05$ vs. BSA; $: $p<0.01$ vs. BSA; @: $p<0.01$ vs AGE; &: $p<0.05$ vs. AGE.

Figure 4: Effect of AGE and/or SR on osteogenic differentiation of VSMC. Osteoblastic differentiation was evaluated as collagen type 1 expression (A), alkaline phosphatase activity (B) and calcium nodules (mineral, C) . Results are expressed as the Media ± SEM, n=8. #: $p<0.05$ vs. BSA; $: $p<0.01$ vs. BSA; @: $p<0.01$ vs AGE.

Figure 5: Effect of AGE and/or SR on the expression of the osteogenic marker Runx2 (A) and the myogenic marker α-actin (B). Western blot lanes: 1-BSA, 2-AGE, 3-BSA+SR, 4-AGE+SR. Results are expressed as the Media ± SEM, n=8. #: $p<0.05$ vs. BSA; $: $p<0.01$ vs. BSA; @: $p<0.01$ vs AGE.

Figure 6: Effect of 10 μM nifedipine (A), 50 μM vitamin E and C (B), 100 μM sulfasalazine (C) or 50 ng/ml cholecalciferol (D) on collagen type 1 expression. The effect of cholecalciferol on Runx2 expression (E) and mineral production (F) was also evaluated. Western blot lanes: 1- BSA, 2- BSA+VitD, 3- AGE, 4- AGE+VitD, 5- BSA plus SR, 6- BSA+VitD+SR, 7- AGE+SR, 8- AGE+VitD+SR. Results are expressed as the Media ± SEM, n=8. #: $p<0.05$ vs. BSA; $: $p<0.01$ vs. BSA; @: $p<0.01$ vs AGE; &: $p<0.05$ vs. AGE, δ: $p<0.01$ vs. AGE+SR.

Figure 7: Evaluation of the levels of the reactive oxygen species production (A) induced by AGE and/or SR and the action of nifedipine (B), vitamin E and C (C) and vitamin D (D). Results are expressed as the Media ± SEM, n=8. #: $p<0.05$ vs. BSA; $: $p<0.01$ vs. BSA; @: $p<0.01$ vs AGE.

Figure 8: Proposed mechanism of action of AGE on vascular smooth muscle cells osteoblastic trans-differentiation.

Declaration of no conflict of interest

Dear Sirs,

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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Sincerely yours,

Juan Manuel Fernández, December 8, 2016

María Silvina Molinuevo, December 8, 2016

Ana María Cortizo, December 8, 2016

Antonio Desmond McCarthy, December 8, 2016

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Claudia Sedlinsky, Corresponding Author, December 8, 2016