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Insights into the antiosteoporotic mechanism of the soy-derived isoflavone genistein: Modulation of the Wnt/beta-catenin signaling

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Abstract

Bone remodeling is a process that involves osteoblasts, osteoclasts, and osteocytes, and different intracellular signaling, such as the canonical Wnt/β-catenin pathway. Dysregulations of this pathway may also occur during secondary osteoporosis, as in the case of glucocorticoid-induced osteoporosis (GIO), which accelerates osteoblast and osteocyte apoptosis by reducing bone formation, osteoblast differentiation and function, accelerates in turn osteoblast, and osteocyte apoptosis. Genistein is a soy-derived nutrient belonging to the class of isoflavones that reduces bone loss in osteopenic menopausal women, inhibiting bone resorption; however, genistein may also favor bone formation. The aim of this study was to investigate whether estrogen receptor stimulation by genistein might promote osteoblast and osteocyte function during glucocorticoid challenge. Primary osteoblasts, collected from C57BL6/J mice, and MLO-A5 osteocyte cell line were used to reproduce an in vitro model of GIO by adding dexamethasone (1 μM) for 24 h. Cells were then treated with genistein for 24 h and quantitative Polymerase Chain Reaction (qPCR) and western blot were performed to study whether genistein activated the Wnt/β-catenin pathway. Dexamethasone challenge reduced bone formation in primary osteoblasts and bone mineralization in osteocytes; moreover, canonical Wnt/β-catenin pathway was reduced following incubation with dexamethasone in both osteoblasts and osteocytes. Genistein reverted these changes and this effect was mediated by both estrogen receptors α and β. These data suggest that genistein could induce bone remodeling through Wnt/β-catenin pathway activation.

KEYWORDS

genistein, glucocorticoid-induced osteoporosis, osteocytes, primary osteoblasts, Wnt/β-catenin pathway

Federica Mannino and Chiara Imbesi contributed equally to this work.

1 | INTRODUCTION

Osteoporosis causes an exaggerated fragility of bones that favors the development of fractures, especially during post-menopause, but considering the several causes of secondary osteoporosis men are also affected at all ages.[1–5] The suppression of osteoblast activity, and the enhanced activity of osteoclasts may result from conditions different from aging, such as lack of vitamin D, chronic inflammatory diseases, and oxidative stress[6]; thus, several drug approaches have been proposed over the years.[7]

Bone remodeling, in which osteoblasts play an important role, is characterized by a sophisticated series of events involving a number of cells, and intracellular signaling molecules.[8,9] One of the main pathways involved in remodeling is the canonical Wnt/β-catenin pathway, deeply involved in the intracellular events leading to osteoblast maturation and bone formation.[10] Also during secondary osteoporosis, as glucocorticoid-induced osteoporosis (GIO), the Wnt/β-catenin pathway is affected causing, in turn, a reduction of osteoblast differentiation and activity, with accelerated osteoblast and osteocyte apoptosis.[11]

During GIO, the Wnt/β-Catenin signaling can be affected by two different pathways: (1) the PI3K/Akt where activation of GSK3ß through glucocorticoids (GCs) causes ß-catenin degradation[12]; (2) the stimulation of Wnt inhibitors, such as DKK proteins, which increase ß-catenin degradation rate.[13]

Several articles show that high-dose of GCs not only decrease bone formation, but also can compromise bone quality[14]; this phenomenon could be due to osteocytes apoptosis caused by the inhibition of several survival mechanisms, such as Wnt/β-catenin signaling and Akt pathway, and may alter the postosteoblast mineralization process interfering with osteocyte regulation of bone matrix.[15]

Genistein has been proposed as a metabolic treatment for osteoporosis following several clinical evidence in postmenopausal women.[16–18] The positive effects of genistein on bone metabolism have been primarily linked to the inhibition of bone resorption and osteoclast activity,[19] while no conclusive data are available on its effects on osteoblast activity and bone formation.[20]

Therefore, this research is aimed at clarify the role of genistein in secondary osteoporosis induced by GC and its ability to modulate the Wnt pathway in either osteoblasts and osteocytes.

2 | MATERIALS AND METHODS

2.1 | Osteoblast culture

Primary osteoblasts were collected from long bones of 5–6-week-old female C57BL6/J mice killed through an overdose of Ketamine/xylazine. Limbs were disarticulated, soft tissues were removed, and clean bones were subjected to epiphyses removal using a scalpel. Bone marrow was flushed out using a syringe loaded with cold PBS until the bone appeared transparent. Diaphysis was cut into little pieces of 1–2 mm, washed several times with PBS, and incubated in a solution containing 2 mg/mL of collagenase II at 37°C for 2 h on a shaker to remove all tissue residues. Bone pieces were washed with PBS, transferred into flasks at a density of about 20–30 fragments per flask, and cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin–streptomycin (Sigma–Aldrich, Milan, Italy) at 37°C in a humidified atmosphere containing 5% CO_2. Cell migration was observed from bone pieces starting at day 2 and the medium was replaced every 3 days to remove non-adherent cells. At days 10–12 following bone collection, the fragments were removed, and osteoblasts were cultured for additional 10 days to obtain an adequate number of cells; medium was replaced every 2–3 days.

2.2 | Osteocyte culture

Preosteocytes cell line was provided by Kerafast (EKC003, Kerafast, Boston, USA). MLO-A5 cells were cultured in alpha-MEM containing L-glutamine and deoxyribonucleosides and supplemented with 5% FBS and 5% calf serum and penicillin–streptomycin at 100 U/mL–100 μg/mL in a humidified incubator at 37°C with 5% of CO_2. For mineralization studies, cells were plated in collagen-coated plates at a density of 3.5×10^4 cells/cm^2; at confluence (day 0), cells were cultured in Alpha-MEM (L-glutamine and deoxyribonucleosides) supplemented with 10% FBS, penicillin–streptomycin at 100 U/mL–100 μg/mL, 100 μg/mL of ascorbic acid and 4 mM of β-glycerophosphate (β-GP). The differentiation medium was replaced every 2–3 days. The collagen fibrils started forming into a swirling honeycomb pattern 4–6 days after the addition of β-GP and ascorbic acid; optimal mineralization was usually found around days 10–14. Osteocytes were cultured for additional 10 days to obtain an adequate number of cells; medium was replaced every 2–3 days.

2.3 | Cell treatments

Osteoblasts (21–25 days following collection) and osteocytes (10 days after differentiation) were plated in six-well plates at a density of 1.5×10^6 cells/well; the day after cells were stimulated with 1 μM dexamethasone (Dex) for 24 h to reproduce an in vitro model of secondary osteoporosis. At

the end of Dex induction cells were treated with different concentrations of genistein (10, 50, 100, and 200 μM) for 24 h alone or in combination with two different antagonists of estrogen receptors: the specific ERα inhibitor (MPP; 1,3-Bis (4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride), and the selective ERβ antagonist (PHTPP; 2-Phenyl-3-(4-hydroxyphenyl)-5,7-bis(trifluoromethyl)-pyrazolo[1,5-a]pyrimidine,4-[2-phenyl-5,7 bis (trifluoromethyl) pyrazolo [1,5-a]-pyrimidin-3-yl] phenol) at the dose of 1 and 10 μM, respectively.

2.4 | Alizarin red S staining

At the end of the treatments, primary osteoblasts were stained with Alizarin red S staining (ARS) as previously reported.[21]

2.5 | PCR assay

Total mRNA was extracted from osteoblasts using Trizol LS reagent (Thermo Fisher Scientific, Waltham, MA), as previously described.[22] First-strand DNA (1 μL) was added to the BrightGreen qPCR Master Mix (Applied Biological Materials Inc., Richmond, Canada) in a total volume of 20 μL per well to evaluate gene expression of target genes: BMP6 (bone morphogenetic protein 6), RUNX2 (runt-related transcription factor 2), Wnt5a, Wnt10b, β-catenin, osteocalcin, and osteoprotegerin (OPG), Collagen1a1, ALP, Destrin, DKK1 and SOST using specific mouse primers (Table 1). Samples were loaded in duplicate and GADPH was used as housekeeping gene; the reaction was performed using the two-step thermal protocol suggested by the manufacturer (Applied Biosystems, Foster City, California, Stati Uniti). Results were quantified using the $2^{\Delta\Delta Ct}$ method and expressed as *n*-fold increase of gene expression by using untreated osteoblasts as calibrator.

2.6 | Western blot

After 24 h, cells were collected and protein extracted for analysis. Protein extraction and western blot analysis were performed as previously described.[23–25]

Briefly, a total of 30 μg of proteins was loaded in each lane. In order to avoid non-specific binding, membranes were incubated with 5% non-fat dry milk and specific antibodies were used to detect RUNX2 (Rabbit monoclonal, IgG, Cell Signaling, 12,556, anti-RUNX2, 1:1000 in

TABLE 1 The forward and reverse primers used for q-PCR evaluation.

GADPH	Primer F CTC ATG ACC ACA GTC CAT GC Primer R TTC AGC TCT GGG ATG ACC TT
β-catenin	Primer F GCC GGC TAT TGT AGA AGC TG Primer R GAG TCC CAA GGA GAC CTT CC
Col1a1	Primer F GTG CTA AAG GTG CCA ATG Primer R CTC CTC GCT TTC CTT CCT CT
DKK1	Primer F GAG GGG AAA TTG AGG AAA GC Primer R GGT GCA CAC CTG ACC TTC TT
Wnt-5a	Primer F CCA TGA AGAAGC CCA TTG GAA TA Primer R GGC CAA AGC CAT TAG GAA GAA
Wnt-10b	Primer F CAG AAC CAC CCG TGA GTT AG Primer R GGG AGG GAG TGA TCC AGA TA
Osteocalcin	Primer F AAG CAG GAG GGC AAT AAG GT Primer R TGC CAG AGT TTG GCT TTA GG
RUNX2	Primer F GCC GGG AAT GAT GAC AAC TA Primer R GGA CCG TCC ACT GTC ACT TT
Osteoprotegerin	Primer F CTG CCT GGG AAG AAG ATC AG Primer R TTG TGA AGC TGT GCA GGA AC
ALP	Primer F ATT GCC CTA AAA CTC CAA AAC C Primer R CCT CTG GTG GCA TCT CGT TAT C
SOST	Primer F GCC GGA CCT ATA CAG GAC AA Primer R CAC GTA GCC CAA CAT CAC AC
BMP6	Primer F CTC TTC GGG CTT CCT CTA TC Primer R CCA ACA CCG ACA GGA TCT
DESTRIN	Primer F TGT ATG ACG CCA GCT TTG AG Primer R AGC CAC CTA GCT TTT CAG CA

1X TBST with 5% BSA), BMP6 (Rabbit monoclonal, IgG, Abcam, ab 155,963, anti-BMP6, 1:1000 in TBS 0.1% Tween), OPG (Rabbit polyclonal, IgG, abcam, ab183910, anti-osteoprotegerin, 1:1000 in TBS 0.1% Tween), phospho (Active) β-catenin (Rabbit Policlonal, Cell Signaling, 9561, phospho β-catenin ser33,37 thr41, in 1:1000 in 1X TBST with 5% BSA), Wnt10b (Rabbit polyclonal, IgG, Abcam ab70816, 2 μg/mL in in TBS 0.1% Tween) and Sclerostin (Rabbit polyclonal, IgG, abcam ab63097, anti-Sclerostin, 1: 1000 in TBS 0.1% Tween), β-actin (Rabbit, Cell Signaling, 4967, β-actin 1:1000 in 1X TBST with 5% BSA) was used on stripped blots as loading control. A secondary antibody conjugated with peroxidases was used to bind and detect the target proteins (Goat anti-rabbit, IgG, Genetex GTX213110-01 1:5000 in TBS 0.15% Tween). All primary and the secondary antibodies were prepared freshly before the use. The images have been acquired using a dedicated software and densitometric data were expressed as integrated intensity, and reported as means and SD.

2.7 | Data and statistical analysis

All the results are expressed as mean ± SD. The reported values are the result of at least three experiments. All assays were performed in duplicate to ensure reproducibility. The differences between the groups were evaluated by one-way ANOVA with Tukey's post-test. A *p*-value less than 0.05 was considered significant. Graphs were prepared using GraphPad Prism Version 8.0 for macOS (GraphPad Software Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | Genistein improves bone formation and osteoblast differentiation

Osteoblasts challenged with Dex showed a significant reduction in the expression of genes involved in bone formation and osteoblast differentiation (BMP6, Osteocalcin, OPG, and RUNX2); thus, demonstrating that in vitro Dex, under these experimental conditions, induces an impairment of osteoblast function that resembles some of the features observed in GIO (Figure 1).

Genistein induced a significant increase in the expression of BMP6 (at the dose of 200 μM) and osteocalcin (at the dose of 10 μM), suggesting a clear involvement of either the α and the β estrogen receptor, respectively. Moreover, genistein significantly increased RUNX2 expression, except at the concentration of 50 μM and with the most impressive results at 200 μM. Osteoprotegerin expression was significantly enhanced with 10 and 50 μM of genistein (Figure 1).

To dissect out the role of each estrogen receptor in this specific experimental model, the specific inhibitors of ERα (MPP) and ERβ (PHTPP) alone or in combination were added to the Dex-challenged osteoblasts. As a result, cells that received MPP showed a significant reduction in BMP6, osteocalcin, OPG, and RUNX2 expression only when genistein was used at 100 or 200 μM (Figure 1A–D). The use of the selective ERβ antagonist PHTPP produced a net reduction in gene expression only in cells receiving 10 or 50 μM (Figure 1A–D).

On the basis of the effect observed at 200 μM on BMP6 and RUNX2 which are involved in the maturation of osteoblasts and in matrix production, Alizarin Red S staining was performed to evaluate mineralization nodules in mature osteoblasts. As compared to control, Dex stimulation resulted in red color attenuation and reduced nodule formation (Figure 1F). On the other hand, genistein restored osteoblast matrix formation (Figure 1G) except when co-cultured with ERα or ERβ inhibitors. When MPP and PHTPP were administered together the strongest reduction in alizarin red staining was observed (Figure 1H–L).

To confirm that genistein at 200 μM promotes bone formation and osteoblast differentiation, BMP6, RUNX2, and OPG protein expressions were evaluated by western blot analysis. Osteoblasts challenged with Dex showed a significant reduction in BMP6, RUNX2, and OPG protein levels compared to untreated cells (Figure 2A–C). Genistein treatment markedly increased BMP6, RUNX2, and OPG protein expressions following Dex challenge; on the contrary, when osteoblasts were treated with PHTTP, MPP, and even more with their combination, the expression of BMP6 and RUNX2 was reverted to that observed in Dex-stimulated cells (Figure 2A–C). In addition, OPG expression was significantly reduced following PHTTP and MPP co-incubation compared to genistein-treated cells (Figure 2B).

3.2 | Genistein improves mineralization in osteocytes

Alizarin red staining was also performed in the osteocyte cell line (murine MLO-A5) to confirm the effect of genistein on bone matrix deposition. As already demonstrated in osteoblasts, Dex challenge was able to significantly reduce matrix formation compared to untreated cells (Figure 3A,B, G). Instead, bone matrix deposition was markedly restored when genistein was added to the system (Figure 3C, G); co-incubation of genistein with

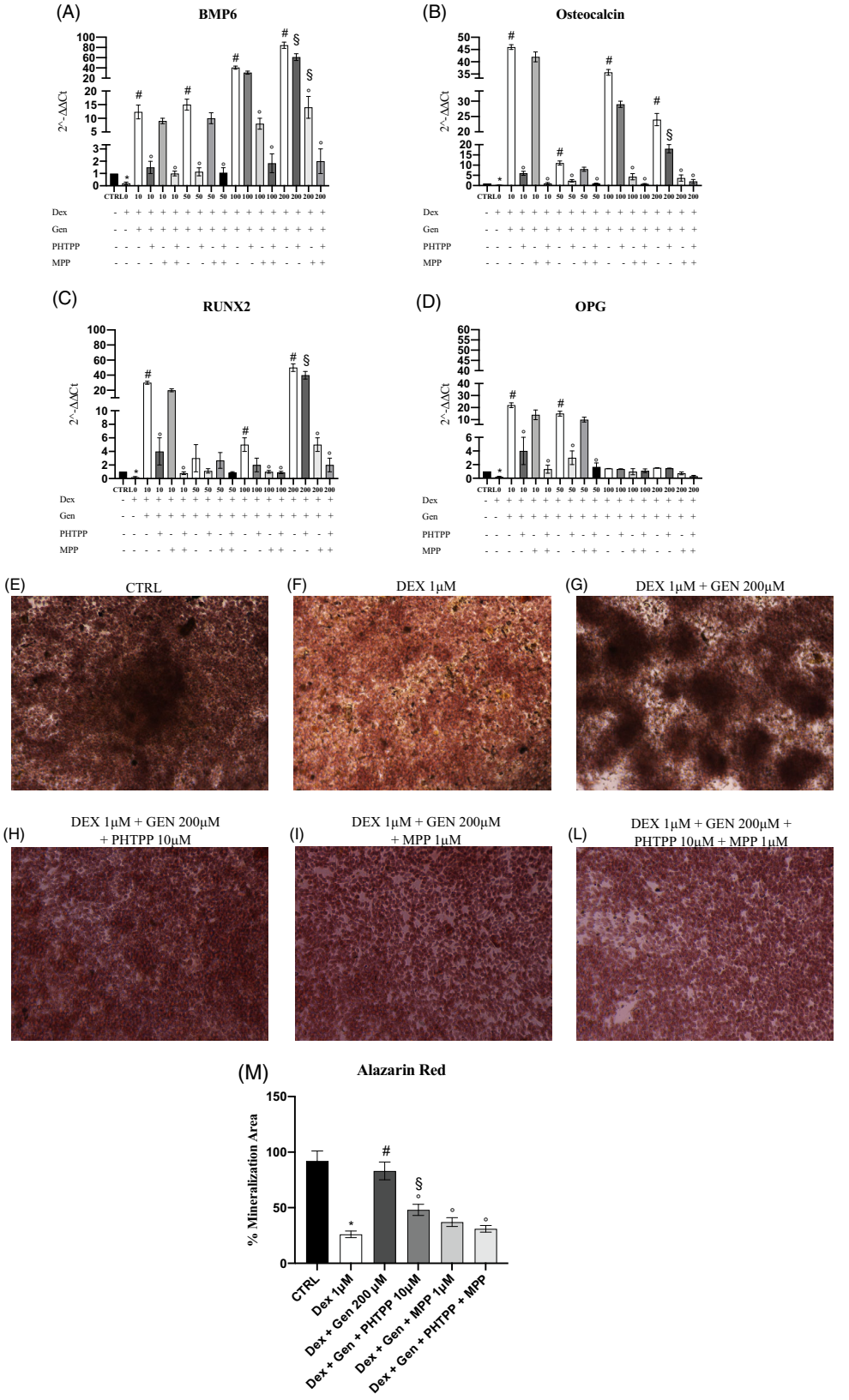

FIGURE 1 The graphs represent mRNA expression of BMP6 (A), osteocalcin (B), RUNX2 (C), and OPG (D) evaluated by qPCR in primary osteoblasts treated with genistein (10, 50, 100, and 200 μM), alone or in combination with PHTPP (10 μM), MPP (1 μM) and PHTPP + MPP for 24 h following stimulation with Dex for 24 h. The figures (E–L) show Alizarin red S staining in primary osteoblasts. Panel M shows the % of mineralization area. The data are expressed as the means and SD of 3 experiments. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus Dex; °$p < 0.05$ versus genistein; §$p < 0.05$ versus PHTPP and MPP.

PHTPP or MPP, and even more their combination, caused a significant reduction of matrix formation, thus suggesting that genistein effect is strictly dependent on estrogen receptors binding (Figure 3D–G).

Collagen1a1, ALP, and destrin mRNA expression was investigated as hallmarks of bone matrix deposition. MLO-A5 cells challenged with Dex for 24 h exhibited a marked down-regulation of these markers compared to

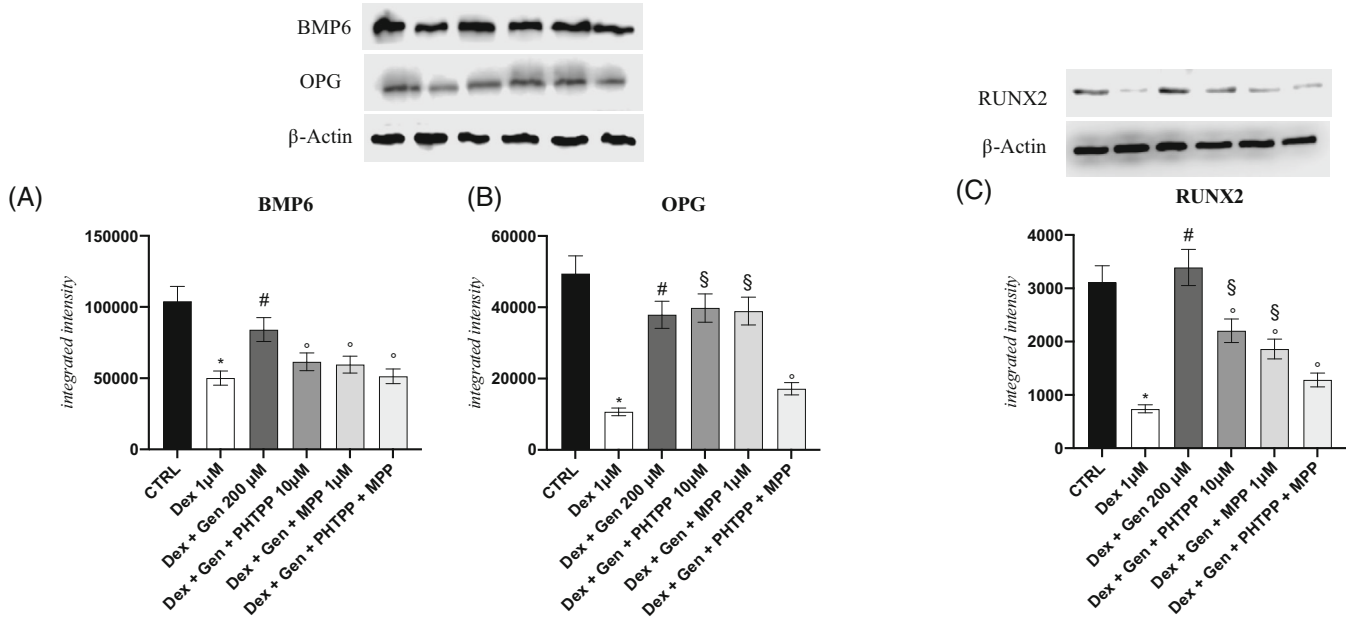

FIGURE 2 The graphs show protein levels of BMP6 (A), RUNX2 (B), and OPG (C) evaluated by western blot in primary osteoblasts treated with genistein (200 μM) alone or in combination with PHTPP (10 μM), MPP (1 μM) and PHTPP + MPP for 24 h following dexamethasone (Dex) challenge for 24 h. The data are expressed as the means and SD of three experiments. *$p < 0.05$ vs Ctrl; #$p < 0.05$ versus Dex; °$p < 0.05$ versus genistein; §$p < 0.05$ versus PHTPP and MPP.

control cells. On the other hand, genistein treatment completely restored their mRNA expression compared to Dex-challenged cells; PHTPP and MPP, alone but especially in combination, blocked the effects of genistein, confirming the role of both estrogen receptors on genistein mechanism of action (Figure 4).

3.3 | Genistein stimulates Wnt/β-catenin signaling pathway

Considering the wide variability in the expression of the Wnt/β-catenin pathway during the stages of bone formation/maturation/degradation a multiple-dosing experiment on primary femur osteoblasts was set to evaluate genistein effects under Dex challenge. The GC reduced the expression of Wnt5a, 10b, and β-catenin expression in osteoblasts as could be expected due to its anti-anabolic activity. Genistein treatment restored and even prompted (at certain doses), the expression of Wnt5a and 10b promoting the expression and nuclear translocation of β-catenin (Figure 5). The most effective dose in inducing this effect was observed at the concentration of 200 μM.

Co-incubation of genistein with MPP or PHTPP, alone or in association, demonstrated that the stimulation of both the canonical (10b) and noncanonical (5) Wnt/β-catenin pathway was predominantly dependent on estrogen receptor alpha stimulation (Figure 5).

Considering the role of Wnt10b in maintaining osteoblast function and regulating their maturation, its protein expression was investigated together with β-catenin; the results demonstrated a clear decrease in their expression following Dex stimulation that was reverted by 200 μM genistein. Moreover, co-incubation with MPP and the combination of the estrogen receptor inhibitors reduced the expression of Wnt10b and β-catenin (Figure 6).

To confirm the involvement of genistein in modulating Wnt/β-catenin signaling pathway, the gene expression of Wnt10b, β-catenin, and sclerostin gene expression was evaluated in MLO-A5 cells. Osteocytes stimulated with Dex showed a significant down-regulation of Wnt10b and β-catenin with simultaneous increase of SOST mRNA, whereas genistein treatment was able to invert the effects of Dex effects (Figure 7).

Wnt10b and β-catenin protein levels were significantly reduced when osteocytes were challenged with Dex for 24 h whereas the expression of sclerostin was markedly increased by Dex stimulation. These effects induced by Dex were completely reversed by genistein; co-incubation with PHTPP or MPP alone or in combination abrogated genistein's effects (Figure 8).

4 | DISCUSSION

Osteoporosis is a chronic pathological condition, characterized by alterations in the microarchitecture of bone

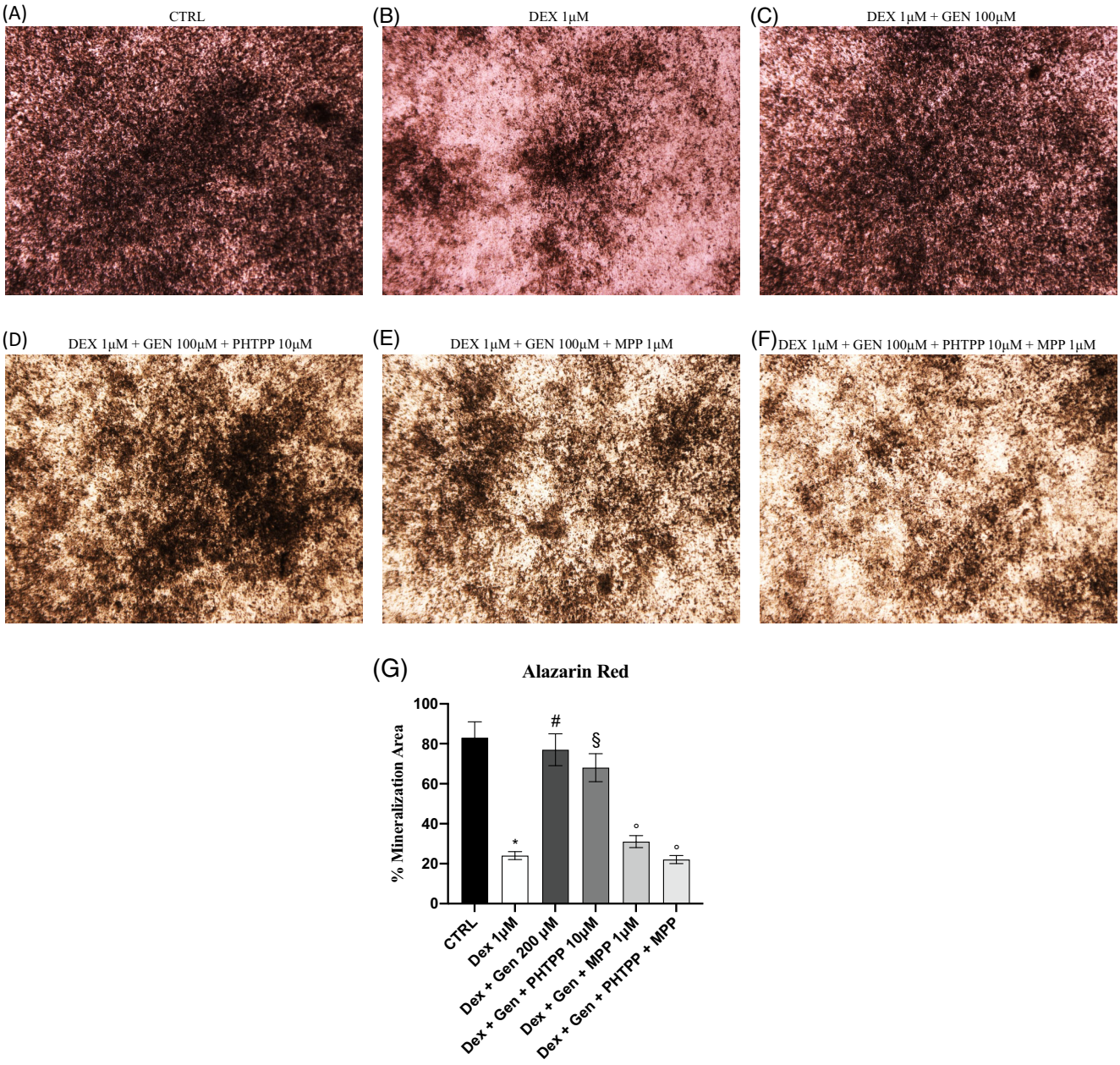

FIGURE 3 The images (A–F) show Alizarin red S staining in MLO-A5 osteocyte cell line. (G) The % of mineralization area. The data are expressed as the means and SD of three experiments. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus Dex; °$p < 0.05$ versus genistein; §$p < 0.05$ versus MPP.

tissue with decreased bone mass and consequent susceptibility to fractures. The pathogenesis of this metabolic bone disease is likely the consequence of an altered balance between osteoblast and osteoclast function.[26] Furthermore, osteocytes dysfunction is mainly involved in bone loss because, these key players modulate bone turnover through their dendritic processes that are in contact with the bone marrow; thus, osteocytes can attract osteoclast precursors and stimulate differentiation of mesenchymal stem cell.[27]

From a mechanistic point of view, the anti-osteoporotic drugs are classically divided into drugs that inhibit bone resorption and agents that prompt bone formation.[28] However, the efficacy of these therapeutics is hampered by the occurrence of several side effects and, very often, they encounter low adherence in patients, especially in the elderly ones.[29]

The protective effect of genistein in preclinical models of osteoporosis,[30–33] as well as in osteopenic postmenopausal women,[34,35] has been mainly ascribed to an anti-

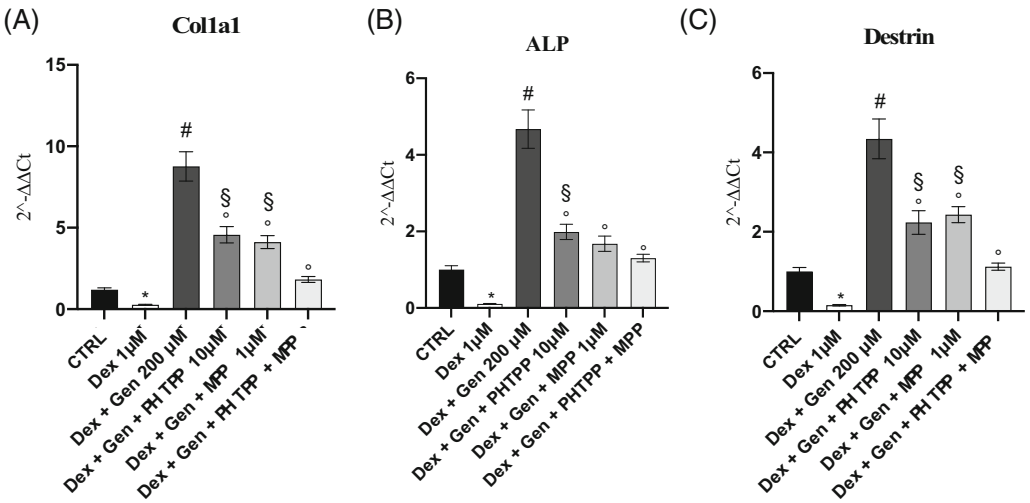

FIGURE 4 The graphs represent mRNA level of Collagen1a1 (A), ALP (B), and destrin (C) evaluated by qPCR in osteocytes treated with genistein (200 μM), alone or in combination with PHTPP (10 μM), MPP (1 μM) and PHTPP + MPP for 24 h following stimulation with dexamethasone (Dex) for 24 h. The data are expressed as the means and SD of three experiments. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus Dex; °$p < 0.05$ versus genistein; §$p < 0.05$ versus PHTPP and MPP.

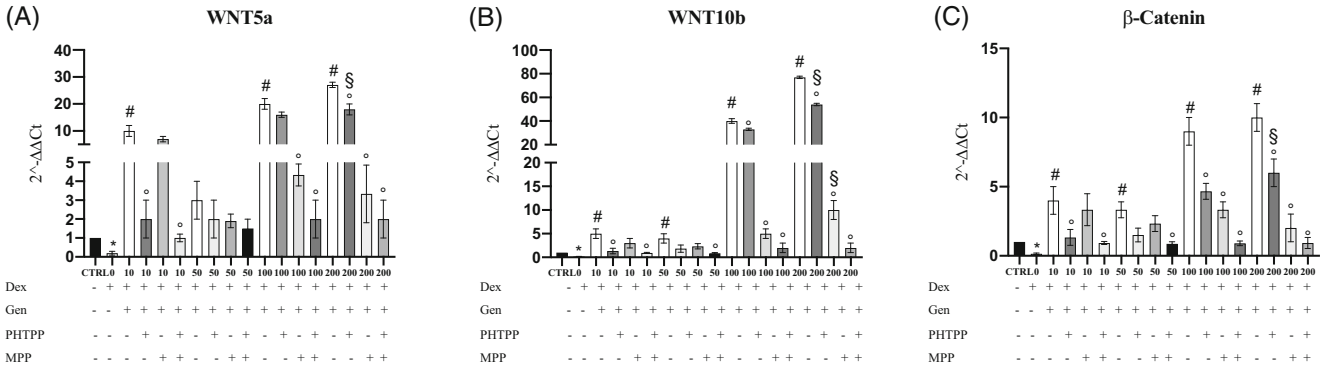

FIGURE 5 The graphs represent mRNA level of Wnt5a (A), Wnt10b (B), and β-catenin (C) evaluated by qPCR in primary osteoblasts treated with genistein (10, 50, 100, and 200 μM), alone or in combination with PHTPP (10 μM), MPP (1 μM), and PHTPP + MPP for 24 h following stimulation with Dex for 24 h. The data are expressed as the means and SD of three experiments. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus Dex; °$p < 0.05$ versus genistein; §$p < 0.05$ versus PHTPP and MPP.

resorption mechanism in analogy to the classical estrogen replacement therapy.[17] Moreover, Ming et al. reported that genistein is able to stimulate osteoblasts proliferation and differentiation and inhibit osteoclast formation and activity in vitro.[36]

In the present experimental model, osteoblasts challenged with Dex showed a marked reduction in matrix deposition, as well as in RUNX2, BMP6, osteoprotegerin, and osteocalcin expression. On the other hand, genistein administration completely restored and even increased the expression of these osteogenic markers, in accordance with other paper.[37] In addition, Dex stimulation affects bone mineralization also in osteocyte cell line, as demonstrated by the reduction of the three main markers, namely Collagen1a1, ALP, and destrin. Under our experimental conditions, genistein improved bone matrix deposition increasing calcium deposits, Collagen1a1, ALP, and destrin gene expression. This is the first report that suggests a role for this isoflavone also on mineralization, following GC challenge.

Although previous data demonstrated that genistein improves bone remodeling binding to ERβ,[38,39] Hertrampf et al. demonstrated that genistein could prevent bone healing in ovariectomized rats through ERα modulation.[40,41] All considered, the specificity of genistein remains debatable, thus, the specific inhibitors of ERα and ERβ were used to prove that genistein binds to the ERs in a dose-dependent manner. In particular,

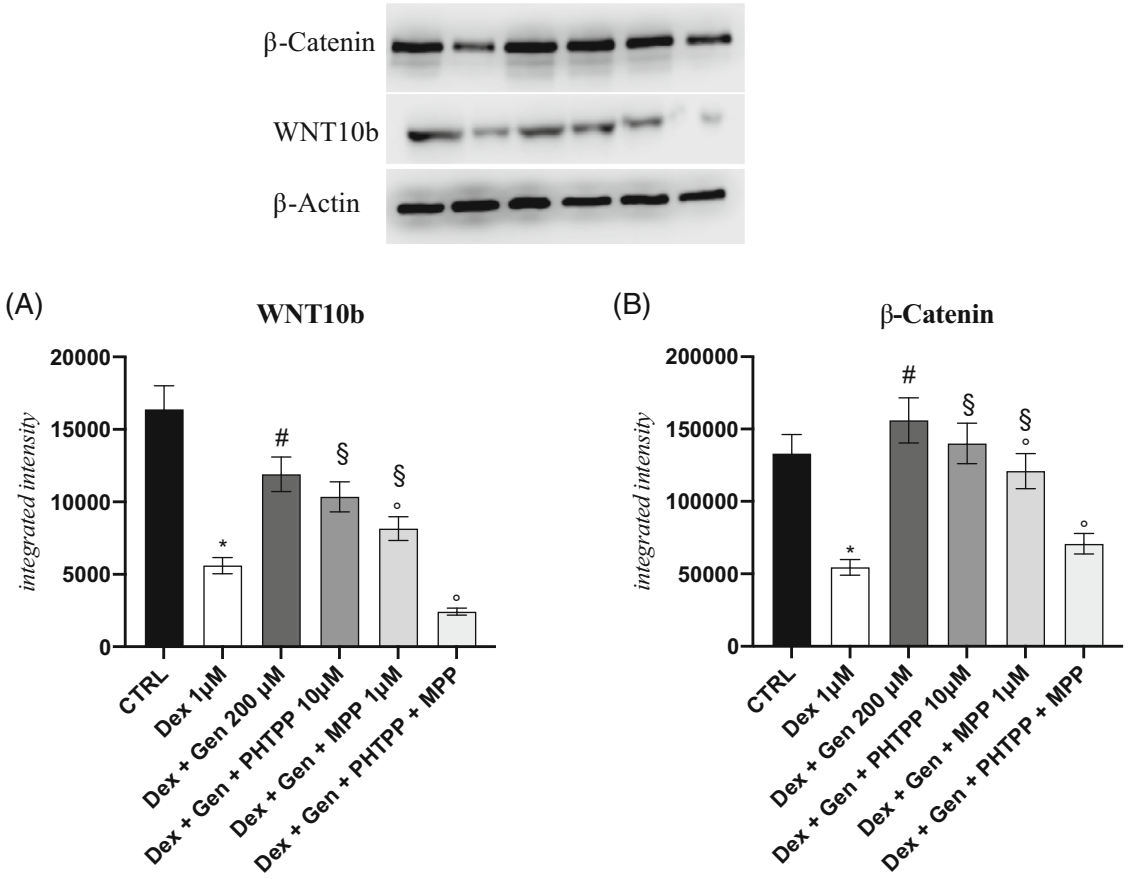

FIGURE 6 The graphs show protein levels of Wnt10b (A) and β-catenin (B) evaluated by Western blot in primary osteoblasts treated with genistein (200 μM) alone or in combination with PHTPP (10 μM), MPP (1 μM), and PHTPP + MPP for 24 h following dexamethasone (Dex) challenge for 24 h. The data are expressed as the means and SD of 3 experiments. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus Dex; °$p < 0.01$ versus genistein; §$p < 0.05$ versus PHTPP and MPP.

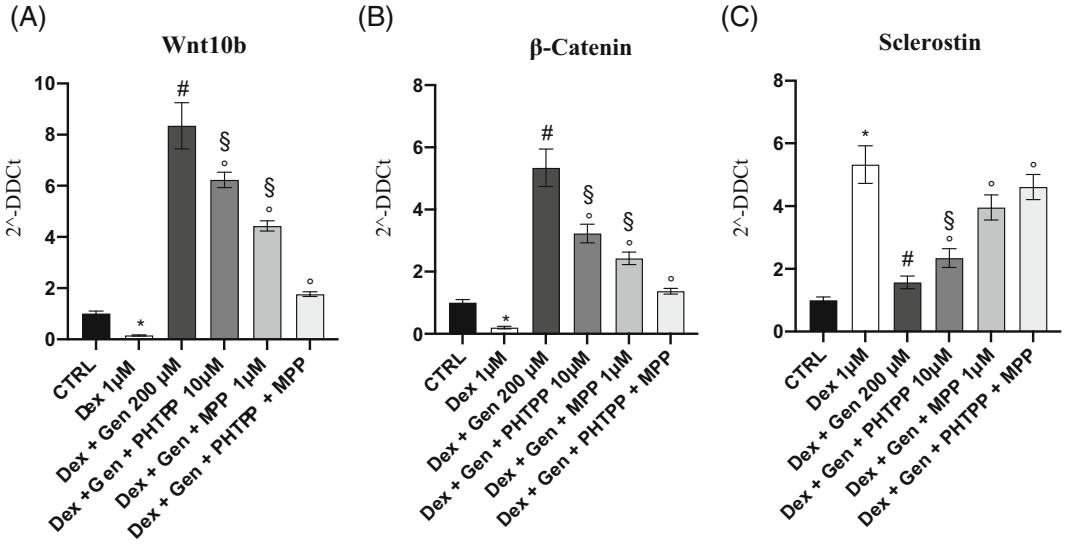

FIGURE 7 The graphs represent mRNA level of WNT10b (A), β-catenin (B) and SOST (C) evaluated by qPCR in MLO-A5 cell line treated with genistein (200 μM), alone or in combination with PHTPP (10 μM), MPP (1 μM) and PHTPP + MPP for 24 h following stimulation with dexamethasone (Dex) for 24 h. The data are expressed as the means and SD of three experiments. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus Dex; °$p < 0.05$ versus genistein; §$p < 0.05$ versus PHTPP and MPP.

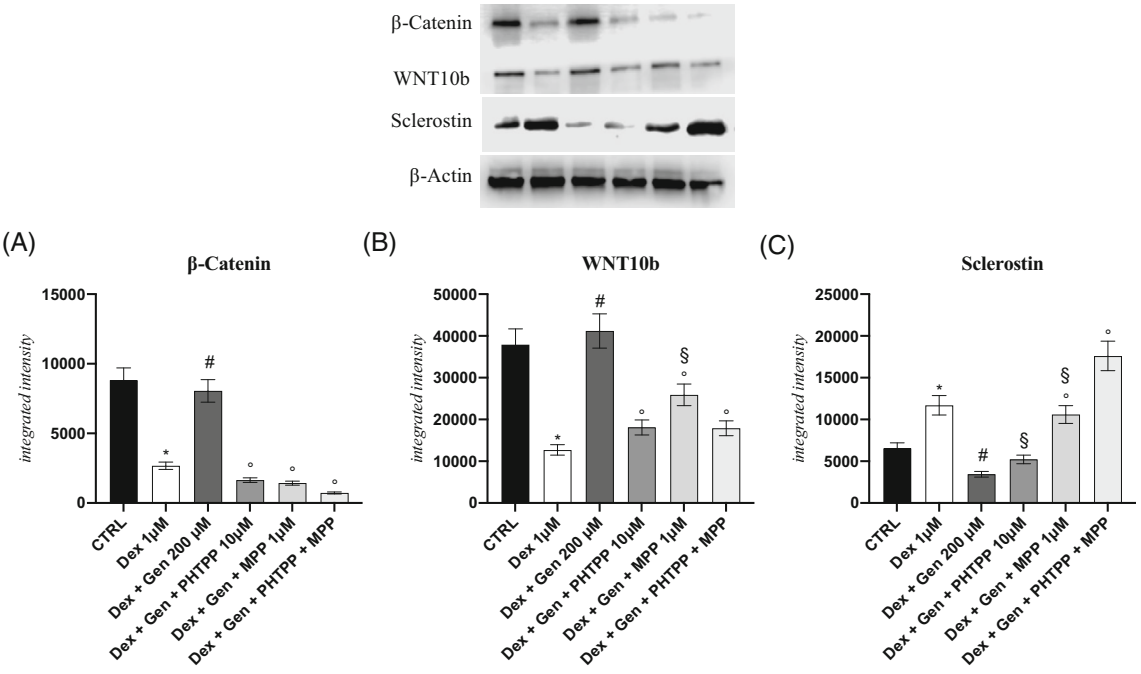

FIGURE 8 The graphs show protein levels of Wnt10b (A), β-catenin (B), and sclerostin (C) evaluated by western blot in MLO-A5 cell line treated with genistein (200 μM) alone or in combination with PHTPP (10 μM), MPP (1 μM), and PHTPP + MPP for 24 h following dexamethasone (Dex) challenge for 24 h. The data are expressed as the means and SD of three experiments. *$p < 0.05$ versus Ctrl; #$p < 0.05$ versus Dex; °$p < 0.05$ versus genistein; §$p < 0.05$ versus PHTPP and MPP.

osteoblasts administered with low doses of genistein produced more osteocalcin and osteoprotegerin following ERβ stimulus; whereas the expression of BMP6 and RUNX2 increased over 40 times with ERα stimulation through 200 μM genistein.

In the last years, the canonical Wnt signaling pathway has emerged as a key and fundamental factor for bone development: in fact, it boosts osteoblast differentiation mainly by stimulating RUNX2,[10] regulates osteocytes function modulating OPG[42,43] and suppresses adipogenesis promoting nuclear β-catenin translocation.[10] WNT10b is a key mediator of Wnt canonical pathway, thus it renews osteoblastic mesenchymal stem cells in the bone marrow, reduces PPARγ, and C/EBPα transcription and regulates osteoblast formation enhancing RUNX2 expression.[44,45] Furthermore, Bennett et al. demonstrated that the mineralized bone volume (BV/TV), bone density level (BMD), and the number of trabeculae (Tb.N) were significantly reduced in WNT10b knockout mice compared to wild-type mice.[46]

Interestingly, the Wnt signaling has been indicated to be of paramount importance in GIO and experimental evidence has highlighted that Dex negatively influences the Wnt/β-catenin signaling.[47] Genistein increased over 60 times Wnt10b indicating a clear proliferative stimulus and a consequent increase in BMP6 and RUNX2 of over 50 times in primary osteoblasts. However, it also stimulated a moderate increase in Wnt5a, which is commonly reported as a stimulator of osteoclast differentiation.[48,49] Moreover, for the first time the present data show that genistein is able to modulate Wnt/β-catenin signaling pathway in osteocyte cell line, stimulating Wnt10b and nuclear translocation of β-catenin, and reducing sclerostin, one of the main Wnt inhibitors expressed by osteocytes. These new results suggest that β-catenin activation by genistein leads to bone formation thanks to the up-regulation of osteogenic markers, such as BMP6 and RUNX2 in osteoblasts; whereas, in agreement with Tu et al.[43] selective modulation of β-catenin signaling causes bone anabolism through sclerostin neutralization and OPG stimulation in osteocytes.

On the other hand, genistein effects are mainly due to an ERα activation of the signaling; moreover, the co-incubation with both ERα and ERβ antagonists abrogated the effects of genistein even more than MPP alone. This result might explain how genistein works in improving bone mass during corticosteroid treatment and gives new insights also on the dual receptor binding action, suggesting that genistein probably acts through the modulation of both estrogen receptors.

This compensatory effect of genistein is extremely important for the safety of this isoflavone when treating patients with osteoporosis; in fact, it might prevent hyperproliferation and possible adverse events. Furthermore,

nuclear translocation of β-catenin has also as downstream target genes LEF1 and DKK1, thus, it seems consequential that an activation of the canonical pathway leads to the increase also in inhibitory proteins.[50,51]

5 | CONCLUSIONS

Our results unmask, for the first time, the ability of genistein to modulate the Wnt/β-catenin signaling, which may explain, at least in part, its ability to stimulate osteogenesis in GIO. Whether this evidence holds true also in a clinical setting, still remains unclear and awaits to be further confirmed, however, a recently accepted paper reports the results on the use genistein in patients with GC osteoporosis.

AUTHOR CONTRIBUTIONS

Conceptualization: Alessandra Bitto. Investigation: Giovanni Pallio and Natasha Irrera. Formal analysis: Giovanni Pallio and Natasha Irrera. Writing original draft: Federica Mannino, Chiara Imbesi. Writing review and editing: Alessandra Bitto; supervision, Francesco Squadrito and Alessandra Bitto. Project administration: Alessandra Bitto.

CONFLICT OF INTEREST STATEMENT

Francesco Squadrito and Alessandra Bitto received research grants from Primus Pharmaceuticals Inc. and are listed as inventors in several patents related to genistein and owned by Primus Pharmaceutical Inc. However, they do not retain economic benefits from the commercial use of these patents.

DATA AVAILABILITY STATEMENT

Data will be made available on request to the corresponding author.

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