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Ferulic acid attenuates osteoporosis induced by glucocorticoid through regulating the GSK-3β/Lrp-5/ERK signalling pathways

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ABSTRACT

Objective: To evaluate *in-vivo* and *in-vitro* effects of ferulic acid (FA) on glucocorticoid-induced osteoarthritis (GIO) to establish its possible underlying mechanisms. *Methods:* The effects of FA on cell proliferation, cell viability (MTT assay), ALP activity, and mineralization assay, and oxidative stress markers (ROS, SOD, GSH LDH and MDA levels) were investigated by MC3T3-E1 cell line. Wistar rats received standard saline (control group) or dexamethasone (GC, 2 mg^{-1} kg) or DEX+FA (50 and 100 mg^{-1} kg) orally for 8 weeks. Bone density, micro-architecture, bio-mechanics, bone turnover markers and histo-morphology were determined. The expression of OPG, RANKL, osteogenic markers, and other signalling proteins was assessed employing quantitative RT-PCR and Western blotting. *Results:* The findings indicated the elevation of ALP mRNA expressions, osteogenic markers (Runx-2, OSX, Col-I, and OSN), and the β-Catenin, Lrp-5 and GSK-3β protein expressions. FA showed the potential to increase MC3T3-E1 cell differentiation, proliferation, and mineralization. FA increased oxidative stress markers (SOD, MDA, and GSH) while decreasing ROS levels and lactate dehydrogenase release in GIO rats. The OPG/RANKL mRNA expression ratio was increased by FA, followed by improved GSK-3β and ERK phosphorylation with enhanced mRNA expressions of Lrp-5 and β-catenin. *Conclusion:* These findings showed that FA improved osteoblasts proliferation with oxidative stress suppression by controlling the Lrp-5/GSK-3β/ERK pathway in GIO, demonstrating the potential pathways involved in the mechanism of actions of FA in GIO therapy.

KEYWORDS

ferulic acid, osteoporosis, glucocorticoids, osteoblast, oxidative injury

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INTRODUCTION

Osteoporosis is a condition of weakened and brittle bones that may be caused due to extended use of glucocorticoids (GC) and called as GC-caused osteoporosis (GCO) [1]. The disruption between the development and resorption of osteoblastic and osteoclastic bone, respectively, is known to cause Osteoporosis. Ovariectomy-induced osteoporosis was the result of the augmented bone resorption, which is the primary model for osteoporosis [2]. Therefore, suppressing bone formation was the main factor in GCO [3].

Osteoblastic proliferation and differentiation occurs as a result of surplus use of GCs, which causes the suppression of bone formation. Multiple previous studies have established that exogenous GCs promote oxidative stress, osteoblast atrophy, and Wnt pathway suppression in order to inhibit osteoblast development [4]. Additionally, it is known that osteoblastic apoptosis can occur as a result of GC-induced oxidative stress [5], which occurs as a consequence of the increased generation of reactive oxygen species (ROS), within the intracellular spaces [6]. Furthermore, an increase in ROS production can accelerate osteoblastic apoptosis, which is a primary mechanism involved in the inhibition of osteoblast proliferation [7]. Thus, antioxidant drugs can be used to prevent the development of GCO.

It is reported that ERK-Mitogen-activated protein kinase (MAPK) pathways (specifically, kinases regulating with extra-cellular signals) exert an essential role in the osteoblasts differentiation. The ERK-MAPK pathways are intended to enhance bone formation by enhancing osteoblastic differentiation. The ERK phosphorylation was found to decrease in osteoporosis relating to the Wnt signalling pathway activation [8]. The canonical Wnt/Lrp-5/β-catenin signalling cascade, as a major regulator, performs a prominent role in osteoblasts differentiation and mineralisation. The members of the Wnt family bind the co-receptor LDL – receptor – linked protein β-catenin -5 (Lrp-5) to block phosphorylation by glycogen synthase kinase 3β [9]. The Wnt/Lrp-5/β-catenin signalling cascade was modified to several functions in osteoblasts and this alteration is attributed to osteoprotegerin (OPG) and osteogénesis-related proteins Runx2 expression [10] regulation.

Considerable progress has been made in our knowledge of the pathogenesis of GC-induced osteoporosis, but effective treatment remains challenging. Natural products therefore form the basis of many existing medicines. Ferulic acid (FA) is a naturally occurring polyphenol that causes a therapeutic impact on hypertension and diabetes similar to that of other constituents found in different fruits [11]. The study [12] stated that cisplatin-induced nephrotoxicity in rats was strengthened by the anti-oxidant and renal protective functions of FA. Another study [13] documented that FA exerts protective action on bone loss in rats following ovariectomy. The therapeutic effectiveness of FA for persistent cerebral hypoperfusion-induced swallowing impairment was shown by Asano et al. [14] in animals. Indeed, adequate supplementation of FA suppresses many long-term diseases [15]. A study [16] reported that supplementation of ferulic acid in rats with streptozotocin-induced diabetes prevented lipid peroxidation. In addition, it was [17] found that ferulic acid inhibited mature osteoclasts' fusion and apoptosis. Finally, bone degradation in ovariectomized rats was inhibited by ferulic acid.

Therefore, the purpose of the existing investigation was to evaluate the functions of FA on prevention of GCO *in-vitro* and *in-vivo* to explain the promising mechanisms of FA in the promotion of osteoblastic progression in MC3T3-E1 cells treated with DEX and in bone loss protection of GCO-rats.

MATERIALS AND METHODS

Materials

The MC3T3-E1 cell line was purchased from Research Institute (China). Ferulic acid was procured from Sigma Aldrich (Beijing, China). Penicillin, streptomycin, ascorbic acid, fetal bovin serum, β-glycerophosphate were collected from Beijing Zoman Biotechnology Co., Ltd, (Beijing, China). BCA protein and alkaline phosphatase (ALP) activity kits were procured from Nanjing Jiancheng Bioengineering Inc. (Nanjing, China). Analytical grade reagents were used for all experimental purposes.

Cell culture

The MC3T3-E1 cell line was developed with 10 percent FBS, penicillin (120 U mL^{-1}) and streptomycin (80 µg mL^{-1}) enriched with α-MEM. The cells were placed at room temperature in a humidity chamber with a 5 percent CO_2 supply. The cells were extracted using 0.5 percent trypsin comprising 0.05 percent Ethylenediaminetetraacetic acid (EDTA) after reaching 90 percent confluence and were implanted in 6-well or 12-well culture plates. A modified alpha-MEM medium, containing 40 µg mL^{-1} ascorbic acid and 5 mM β-glycerophosphate, was used to stimulate osteoblastic differentiation and the medium was altered each day.

Cell proliferation

The cells (1×10^5 cells per well) were implanted in 96-well plates followed by incubation for 24 h. Cells were loaded with varying amounts of ferulic acid (0.5, 1.5 and 3 µM) and DEX (30 µM) prepared in DMSO (dimethyl sulfoxide) for 24 and 48 h respectively. Cells that are treated with only medium without test samples were regarded as a control. Consequently, each well was added with CCK-8 (10 µL) and incubated for 4 h. A microplate reader was used to measure the absorbance at 450 nm. The activity of cell proliferation (percent) was computed as follows:

Cell proliferation activity (percent) = (As − Ab)/(Ac − Ab) × 100; where the absorbance of the test, the blank and the control groups are defined by As, Ab and Ac respectively.

Cell viability

The cell viability was evaluated using the MTT test. The reduction of the yellowish MTT stain into insoluble formazan crystals is a distinguishing feature of metabolically active cells. The amount of viable cells thus implies the degree of MTT reduction. Each well of the 96-well plate added 100 µL of media comprising differentiated MC3T3 cells at the concentration of 1 x 10^4 cells per well. The ferulic acid (0.5, 1.5, and 3 M) and DEX (30 M) concentrations in dimethyl sulfoxide were incubated for 72 h with cells at 70% confluence. Each well received 0.5 mg mL^{-1} MTT and was incubated for 4–5 h. The resulting insoluble formazan crystals were dissolved in 100 µL of DMSO and a microplate reader was used to test absorbance at 550 nm [18].

ALP enzyme assay

MC3T3-E1 cells (6×10^4 cells per well) were implanted onto 12-well plates. The cells were cultivated in the presence of osteogenic differentiation medium with ferulic acid (0.5, 1.5 and 3 µM) and DEX (30 µM) concentrations at 80 percent confluence for 7 and 14 days. Cells that

treated with osteogenic differentiation medium without test samples were regarded as a control. Cells were rinsed three times with PBS after specified treatment and lysed on ice with 0.2 M Tris buffer comprising 0.2 percent Triton- X as a lysis buffer. The lysates were removed and sonicated for 10 min and centrifugated for 20 min at 12,000 rpm (4 °C). The intracellular ALP activity and total protein was calculated employing bicinchoninic acid (BCA) assay kits as per the manufacturer instructions. Intracellular alkaline phosphatase activity, thus determined was standardized to the protein concentration.

Mineralization assay

MC3T3-E1 cells were implanted onto six-well plates (2×10^5 cells per well) and cultivated to attain 80 percent confluence. The culture (osteogenic differentiation) medium was altered each day in the presence of ferulic acid (0.5, 1.5, and 3 μM) and DEX (30 μM). The cells were rinsed two times with phosphate buffer saline after 21 days of cultivation and positioned with paraformaldehyde (5%) for 10 min. After washing with distilled water, alizarin Red S (pH 5.1) was used to stain the cells at 37 °C for 20 min. Unbound staining was removed by rinsing the wells after staining and visualized through microscope. The cells were detained in the dark for 1 h with 10% cetylpyridinium chloride to determine the bound stain. A microplate reader was used to determine the levels of the soluble form Alizarin Red S at 570 nm.

Measurement of ROS generation

Ferulic acid (0.5, 1.5 and 3 μM) and DEX (30 μM) concentrations for 24 h were treated with MC3T3-E1 cells. After incubation, by using fluorescent probe, DCFH-DA2 (7-dichlorofluorescein diacetate) the amount of intracellular ROS was examined. A flow cytometer was used to measure the relative levels of fluorescence.

Measurement of oxidative stress markers

The cellular SOD and LDH activities, cellular GSH and MDA content of the cell culture solution; the serum SOD and LDH activities and serum MDA and GSH content were calculated as per instructions indicated by the manufacturer using appropriate assay kits.

Experimental design

Animals. Wistar rats (males, 220 ± 30 g) were acquired from the Animal Research Centre (Beijing, China). Rats were held under 12 h of light and dark periods at 30 °C with access to food and water. Before the trial day [19], they were accustomed to the conditions of the study room. The study trials were approved by the university hospital's ethics board in accordance with the standards for the safety and use of research animals.

Induction of osteoporosis. Glucocorticoid induced osteoporosis was planned with modifications based on the Shi-Yu Lu method [20]. Ferulic acid was suspended in 2% of sodium carboxymethyl cellulose and administered once every day for 8 weeks to rats exposed to osteoporosis. The animals were divided into four classes of seven rats per category, as follows:

I. Group of control (0.2 mL normal saline) p.o. on a normal basis (7 rats)

II. DEX group – 2.5 mg per kg per day two times a week for eight weeks intramuscularly (0.2 mL normal saline)
III. DEX intramuscularly – 2.5 mg per kg per day two times a week + FA 50 mg^{-1} kg^{-1} day p.o. daily
IV. DEX intramuscularly – 2.5 mg per kg per day two times a week + FA 100 mg^{-1} kg^{-1} day p.o. daily

The animals were anaesthetized with xylazine (20 mg^{-1} kg) and ketamine (60 mg^{-1} kg) on the last day of the drug administration (after 8 weeks), and blood was obtained by cardiac puncture for serum biomarker analyses such as phosphorus (P), calcium (Ca), osteocalcin (OCN), RANKL, bone specific alkaline-phosphatase (B-ALP) and osteoprotegerin (OPG). The rats were then killed and biomechanical and histomorphometric evaluations of the femoral and tibia bones of the right and left were carried out.

Assessment of bone mineral density. As described earlier, the animals were exposed to double-energy X-ray dependent absorptiometry [21]. In brief, the anterior portion of the tibiae's bone mineral density (BMD) was assessed over a scan speed of 60 mm s^{-1} at a scan area of 1.5 mm. The whole femur, along with the femoral shaft and distal femoral epiphysis, was split into 3 identical portions for X-ray examination.

Measurement of bone metabolic and biochemical markers. Colorimetric methods were used to examine serum samples for calcium (Ca) and phosphorus (P) levels utilising specialized kits and a sophisticated autoanalyzer. The B-ALP (serum bone alkaline phosphatase) levels were measured using the Luminex Osteocalcin kit. The osteocalcin serum levels, RANKL and OPG were assessed by an ELISA assay kits as directed by the manufacturer's instructions.

Bio-mechanical evaluation of the bone. Specimens from osteoporotic (tibia) rats were employed for biomechanical tests to assess quality of bone. To evaluate the midpoint, the length of the extracted left tibia was calculated using a varnier calliper. Tibiae were then placed at a distance of 15 mm in a measuring unit. The load was added to the midpoint of the tibiae at a rate of displacement of 1 mm per second before the specimen (bone) failed and the breaking strength (maximum load) was determined [22].

Study of bone histomorphometry and histo-chemistry (TRAP staining). Femur bones were extracted and placed in formalin (10%) accompanied by immersion before decalcification in the EDTA (ethylenediamine tetraacetic acid) solution. Four (micrometer) slices were cut, followed by staining using H and E (hematoxylin/eosin) and TRAP (tartrate-resistant acid phosphatase) after regular histological processing. Besides cortical thickness and area, to evaluate trabecular bone thickness and number, histomorphometric assessment was conducted on hematoxylin/eosin-stained sections. Osteoclasts were counted at a magnification of 400 × on slides stained with TRAP. In twenty fields selected from the metaphysis cancellous bone, both measurements were taken at a maximum of 200 µm from the growth plate [23]. The samples placed on a microscope (Olympus) fitted with a digital camera and evaluation software were evaluated by different examiners without information on the study groups.

Assessment of reverse transcription polymerase chain reaction. To evaluate the mRNA expressions of osteoblast differentiation markers (Runx2, Col I, OPN, OCN and ALP) utilizing

GAPDH as an internal control, real-time PCR was carried out. The total RNA was isolated from MC3T3-E1 cells or femur tissue utilising Trizol reagents. Two microgram of total RNA was employed to produce cDNA with the Reverse Aid First Strand cDNA Synthesis Kit (Cell Signaling Technology, China). The Real Time-PCR was done as follows: 60 °C for 3 min, 85 °C for 4 min, 30 cycles for 20 s at 85 °C and 50 °C for 60 s. Using the 2-Δct method, the levels of the expression of the sample genes were determined.

Western blot analysis. MC3T3-E1 cells or femur were isolated using a cold lysis buffer containing phenlymethylsulfonly fluoride. After evaluation of total protein content by means of protein (bicinchoninic acid) assay kit, the total content was operated for electrophoresis with 10 percent sodium dodecyl sulphate polyacrylamide gel and exchanged onto the membrane. Through Trisbuffered saline plus Tween 20, the blots were blocked for 1 h with 5 percent skimmed milk and the membrane was allowed for incubation for 12 h (4 °C) with the primary antibodies. Subsequently, the membrane was allowed to incubate for 2 h with secondary antibodies at 37 °C. Blots were observed by means of improved chemiluminescence method. In order to determine protein expression, optical blot density was calculated. The findings were standardized against the internal control (GAPDH).

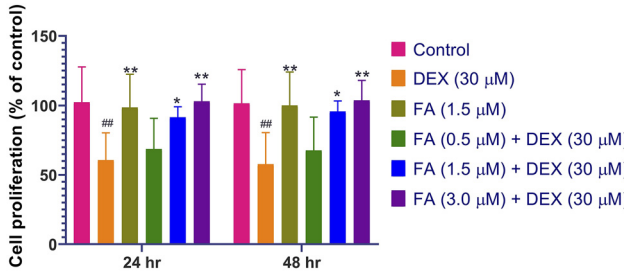
Statistical analyses

All results were recorded as mean ± SD for all experiments conducted in triplicate. Graphpad Prism ver. 8.0.2 was used to perform all statistical analyses. One-way ANOVA was employed to compare the data in multi-groups, followed by post-test analysis. P <0.05 was considered statistically significant.

RESULTS

Defensive effect of ferulic acid on osteoblast in-vitro

Effects of FA and DEX on cell proliferation of MC3T3- E1. As is noticeable from Fig. 1 relative to the control group, the MC3T3-E1 cell-viability was greatly enhanced after ferulic acid culture



Data are represented as mean ± SD. ##P < 0.01 when compared to control group.
*P < 0.05 and **P < 0.01 when compared to DEX group

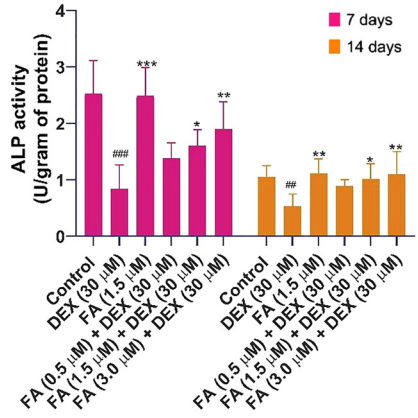
Fig. 1. Effects of FA and DEX on MC3T3- E1 cell proliferation at 24 and 48 h

(0.5, 1.5 and 3 μM) and significantly ($P < 0.01$) decreased with DEX (30 μM) concentrations at 24 and 48 h respectively. These findings showed that DEX impaired oestoblast differentiation, while FA was able to facilitate dose-dependent osteoblasts cell proliferation.

Effects of FA and DEX on cellular ALP activity of MC3T3-E1. After treatment with FA (0.5, 1.5 and 3 μM) and DEX (30 μM) for seven days and fourteen days, the ALP activity of MC3T3-E1 cells was assessed by an assay kit for ALP and the findings were seen in Fig. 2. Subsequent to seven days of differentiation, the dose-dependent activity of ALP was remarkably elevated in MC3T3-E1 cells treated with FA than in the DEX group. It can be noticed that the ALP activity after treatment with FA was slightly ($P < 0.05$) increased at seven days and subsequently declined at fourteen days (Fig. 2) suggested that FA was capable of promoting the activity of ALP in early-stage MC3T3-E1 cells. However, the DEX group demonstrated a decline in ALP activity in DEX mediated MC3T3-E1 cells relative to the control group.

Effects of FA and DEX on cellular mineralization of MC3T3-E1. The MC3T3-E1 cellular mineralization was assessed at twenty one days through Alizarin Red S staining. The amount of mineralized nodules in the DEX (30 μM) treated group was the lowest in contrast to the control group and dramatically improved in DEX-induced MC3T3-E1 cells following treatment with FA (0.5, 1.5 and 3 μM) and the amount of alizarin red stain was remarkably improved in a dose-dependent approach (Fig. 3). The amount of bound dye (alizarin red S) within the cells was measured by determining the absorbance at 570 nm using a microplate reader.

Action of FA on MC3T3-E1 cells in preventing the oxidative injury caused by DEX. The intracellular ROS level was assessed to evaluate whether FA (0.5, 1.5 and 3 μM) influences ROS production. As seen in Fig. 4, DEX treated (30 μM) alone greatly increased the production of



Data are represented as mean ±SD. ###P < 0.01 when compared to control group. *P < 0.05; **P < 0.01 and ***P < 0.001 when compared to DEX group

Fig. 2. Effects of FA and DEX on MC3T3-E1 cellular ALP activity at 7 and 14 days

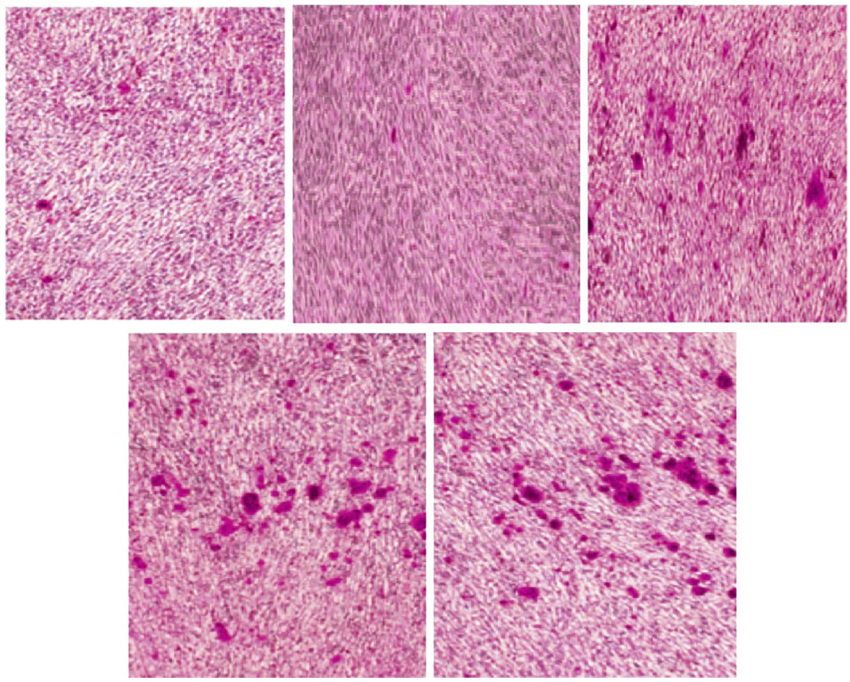
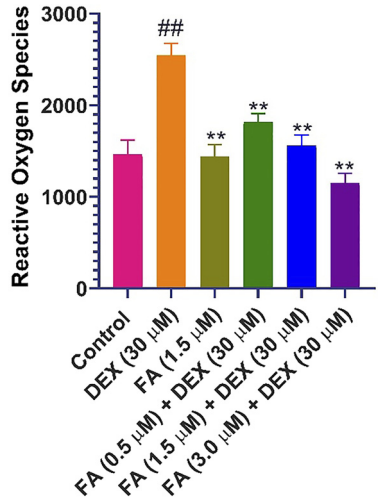


Fig. 3. Effects of FA and DEX on MC3T3- E1 cellular mineralization at 21 days through Alizarin Red S staining showing the lowest mineralized nodules in the DEX (30 μM) group compared to the control group, followed by dramatically ($P < 0.05$) improved with FA (0.5, 1.5 and 3 μM) treatments. The intensity of alizarin red stain was remarkably ($P < 0.05$) improved in a dose-dependent manner

ROS relative to the control group. FA has remarkably reduced DEX induced intracellular generation of ROS relative to DEX treatment alone.

Action of FA on MC3T3-E1 cells in preventing oxidative damage triggered by DEX. In order to determine if FA prevents MC3T3-E1 cells from DEX caused oxidative damage (30 μM), the effects of FA (0.5, 1.5 and 3 μM) on biochemically involved oxidative stress markers such as MDA, SOD, LDH, and GSH were studied. The findings revealed that DEX greatly lowered the amounts of SOD and GSH relative to the control group. In comparison, relative to the DEX treatment, FA greatly improved the amount of SOD and GSH (Table 1). The increased amount of MDA and LDH caused by DEX, on the other side, was apparently decreased by FA.

FA enhanced the OPG/RANKL ratio and enabled the signalling pathway of Lrp5/β-catenin/GSK-3β in MC3T3-E1 cells treated with DEX. The OPG/RANKL ratio stands as an essential to bone resorption and bone formation pairing and was embraced for bone remodelling evaluation. DEX (30 μM) treated alone greatly declined OPG's mRNA level and improved RANKL's mRNA level. The FA (0.5, 1.5 and 3 μM) groups, however, demonstrated an increase in the OPG mRNA level and decline in the RANKL mRNA level (Fig. 5A and B). The reduced OPG/RANKL ratio caused by DEX was, however, remarkably improved by FA (Fig. 5C).



Data are represented as mean ± SD. ##P < 0.01 when compared to control group. **P < 0.01 when compared to DEX group

Fig. 4. MC3T3-E1 cells were preserved by FA from oxidative injury due to intercellular ROS levels caused by DEX

The LRP5/β-catenin pathway mRNA levels were also observed to determine if it is implicated in the actions of FA on MC3T3-E1 cells treated with DEX. It can be noticed from Fig. 5D and E, DEX substantially lowered the β-catenin and LRP5 mRNA expressions relative to the control group, while FA specifically upregulated the β-catenin and LRP5 mRNA expressions relative to DEX alone. In addition, the western blotting finding reveals that the degree of expression of Ser9-phosphorylated GSK-3β declined dramatically in the DXM alone treatment relative to the control group and treatment with FA significantly improved (Fig. 5F) the protein expression level of Ser9-phosphorylated GSK-3β relative to DXM-treated group. In order to facilitate bone development, these findings revealed that FA may activate the Wnt signalling pathway.

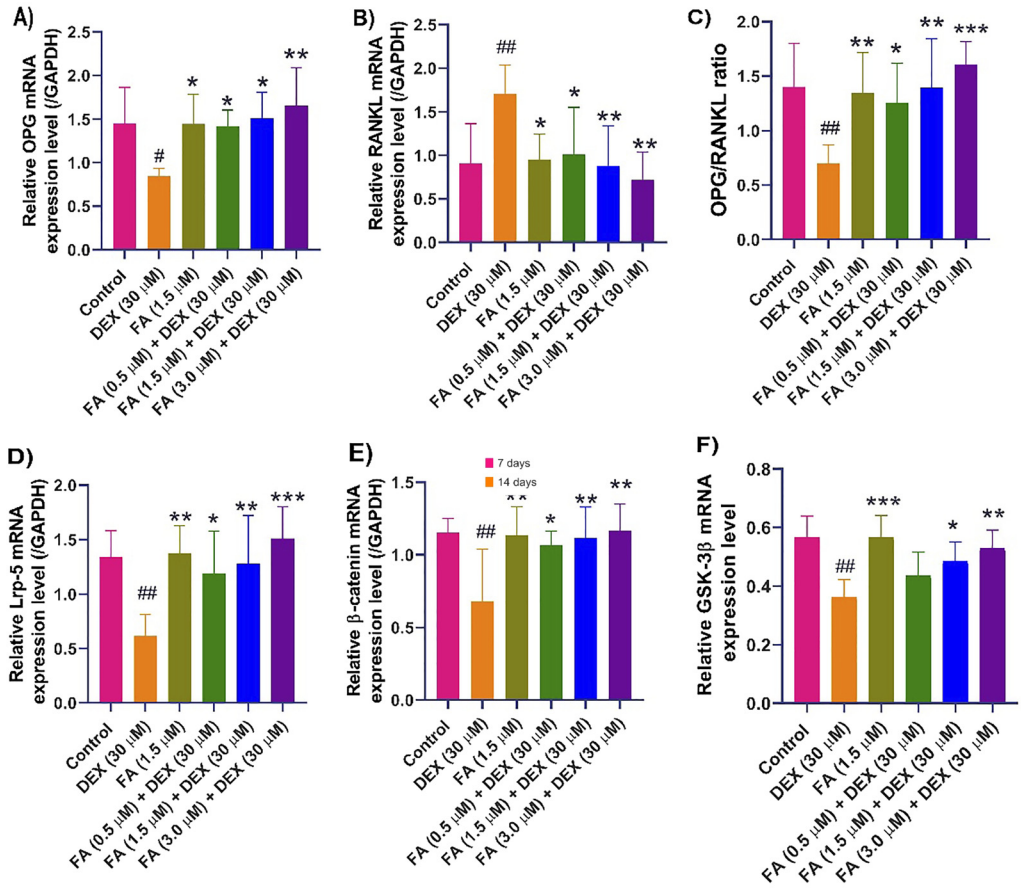
Effects of FA on MC3T3-E1 cells treated with DXM on ERK-phosphorylation. We then investigated the effect of FA (0.5, 1.5 and 3 μM) on Wnt signalling and ERK-phosphorylation pathways in MC3T3-E1 cells treated with DXM to unravel the probable mechanism. The findings of western blotting (Fig. 6) revealed that ERK-phosphorylation was significantly reduced in the DXM group compared to the control group and the declined ERK-phosphorylation in the FA groups was obviously increased.

FA improved the expression of osteoblastic development indicators in MC3T3-E1 cells treated with DEX. OCN, Col I, Runx2 and OPN in MC3T3-E1 cells, Real Time-PCR was employed to determine the OCN, Col I, Runx2 and OPN mRNA expressions in MC3T3-E1 cells treated with DEX (30 μM) and FA (0.5, 1.5 and 3 μM) for seven and fourteen days, respectively (Fig. 7).

Table 1. Effects of Ferulic acid on MC3T3-E1 cells from oxidative damage triggered by DEX showing the biochemical oxidative stress markers

Biochemical parameters	Control	DEX (30 μM)	FA (1.5 μM)	FA (0.5 μM) + DEX (30 μM)	FA (1.5 μM) + DEX (30 μM)	FA (3.0 μM) + DEX (30 μM)
SOD (Umg^{-1} of gram of protein)	45.09 ± 13.95	23.65 ± 7.79$^{\#}$	43.44 ± 8.97*	45.36 ± 14.48*	43.99 ± 11.16*	43.99 ± 13.89*
GSH (μmol L^{-1})	74.17 ± 18.26	44.32 ± 8.95$^{\#\#}$	71.95 ± 16.01*	68.60 ± 11.50*	78.52 ± 19.49**	85.53 ± 11.85**
MDA (nmol mg^{-1} of gram of protein)	0.83 ± 0.36	1.78 ± 0.48$^{\#\#}$	0.91 ± 0.65*	0.86 ± 0.39**	0.67 ± 0.51**	0.49 ± 0.30***
LDH (UL^{-1})	243.7 ± 43.84	342.2 ± 58.74$^{\#\#}$	248.0 ± 42.54**	262.1 ± 45.51*	236.8 ± 27.96***	219.7 ± 29.62***

Data are presented as mean ± SD. $^{\#}P < 0.05$ and $^{\#\#}P<0.01$ compared with control group; *$P< 0.05$; **$P < 0.01$ and ** $P< 0.01$ compared with DEX group.

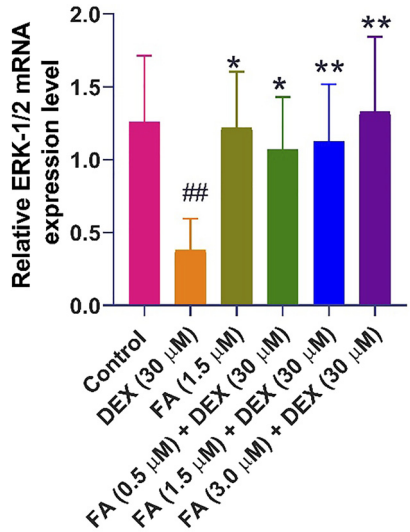


(A) OPG, (B)RANKL, (C) OPG/RANKL ratio, (D) Lrp-5 (E) β-Catenin and (F) β-GSK-3β. Data are represented as mean ± SD. $^{\#}P < 0.01$ and $^{\#\#}P < 0.01$ when compared to control group. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to DEX group

Fig. 5. Effect of FA on DEX-exposed MC3T3- E1 cells that enhanced the OPG/RANKL ratio and enabled the signalling pathway of Lrp5/*β*-catenin/GSK-3*β*

Osteogenic markers (OCN, Col-I, Runx2 and OPN) were noticeably declined in DEX-treated MC3T3-E1 cells relative to the control group.

In the osteoblast-synthesized bone matrix and active in differentiation, Col I is the main protein [24]. Following 14 days of FA culture, Col-I in MC3T3-E1 cells was significantly elevated (Fig. 7A). OPN and OCN are the major indicators which are expressed for the period of differentiation later on. OCN is essential for attaching and accumulation of hydroxyapatite in the extra-cellular bone matrix, and OPN is a bone matrix multifunctional protein recognized as an essential factor in bone remodelling. The OCN and OPN mRNA expressions were also improved by FA and the rate of expression were higher on day fourteenth day than on day seven (Fig. 7B and C). The study also observed that FA increased Runx2 expression, a main osteoblast



Relative mRNA expression of ERK-1/2. Data are represented as mean ± SD. ##P < 0.01 when compared to control group. *P < 0.05 and **P < 0.01 when compared to DEX group

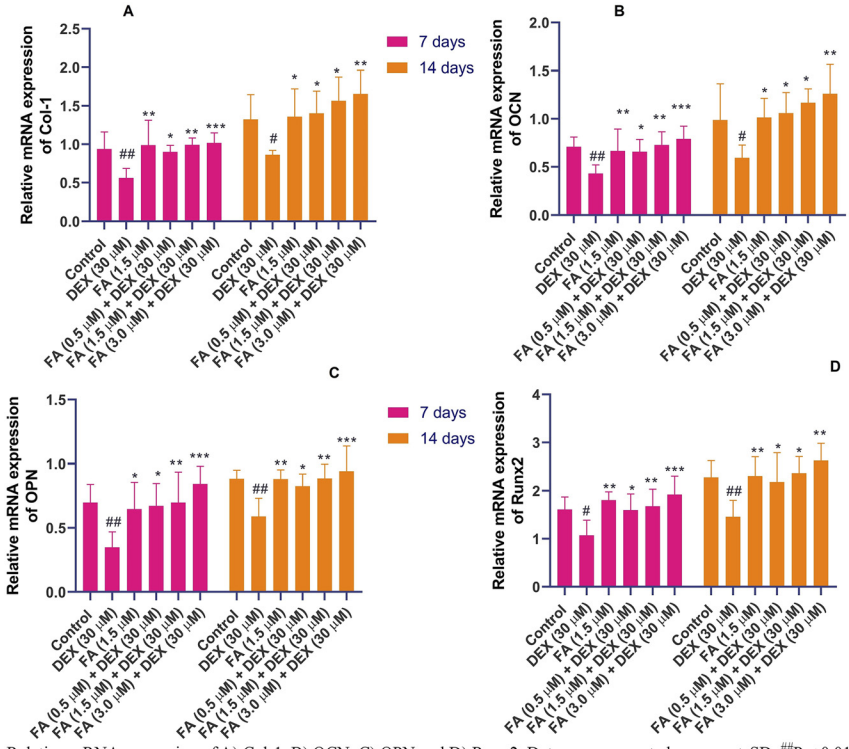
Fig. 6. Effects of FA in DXM treated MC3T3-E1 cells on ERK- phosphorylation

differentiation-related transcription factor (Fig. 7D). These findings suggested that FA was capable of fostering osteoblast differentiation substantially ($P < 0.05$).

Bone protective effects of LA in GIO rats (*in-vivo*)

Determination of bone mineral density (BMD). The effects of treatment with ferulic acid (50 and 100 mg kg^{-1}) on osteoporosis caused by glucocorticoids in rats were determined. In osteoporotic rats caused by DEX, BMD was significantly lower relative to the control rats (Table 2). Treatment with ferulic acid (50 and 100 mg kg^{-1}), however, improved BMD in a dose dependent fashion respectively ($P < 0.05$).

Effect of FA on the bone metabolic, biochemical indicators, the OPG/RANKL ratio and enabled signalling pathway of Lrp5/β-catenin/GSK-3β. The results on biochemical serum markers of the treatment of osteoporotic rats with FA (50 and 100 mg kg^{-1}) have been shown in Table 3. The findings revealed that the amount of Ca was slightly declined in the DEX group relative to control group. On comparison with the DEX group, FA treatment dramatically ($P < 0.05$) elevated the Calcium concentrations. In the DEX group, the measured osteocalcin level was substantially reduced relative to the control group and FA treatment was remarkably effective in restoring to its normal levels. The serum phosphorus (P) levels in DEX rats were remarkably reduced than in control rats ($P < 0.05$). The result revealed that only FA treatment dramatically ($P < 0.01$) improved the amount of serum phosphorus. In the DEX group, the B-



Relative mRNA expression of A) Col-1, B) OCN, C) OPN and D) Runx2. Data are represented as mean ± SD. ##P < 0.01 when compared to control group. *P < 0.05; **P <0.01 and ***P< 0.001 when compared to DEX group

Fig. 7. Effect of FA on DEX treated MC3T3-E1 cells, thus improving the expression of osteoblastic development markers on 7 and 14 days

ALP serum levels, the bone formation marker, was substantially ($P < 0.01$) diminished relative to control group. Compared with DEX treated rats, serum B-ALP levels were substantially elevated in the control groups ($P < 0.001$).

In osteoporotic rats caused by DEX, the amount of Receptor activator of nuclear factor kappa-B ligand (RANKL) was additionally enhanced relative to the control group (Fig. 8A). FA treatments could bring the levels of RANKL down to its normal value. The same pattern was

Table 2. Determination of Bone Mineral Density

Bone Mineral Density	Control	DEX	FA (50 mg kg^{-1}) + DEX	FA (100 mg kg^{-1}) + DEX
Femur	0.26 ± 0.09	0.16 ± 0.03#	0.27 ± 0.07*	0.30 ± 0.06**
Tibia	0.23 ± 0.07	0.14 ± 0.03#	0.24 ± 0.06*	0.28 ± 0.05**

Data are presented as mean ± SD. ##$P < 0.01$ compared with control group; *$P < 0.05$ and **$P < 0.01$ compared with DEX group.

Table 3. Effect of FA on the bone metabolic and biochemical markers

Parameters	Control	DEX	FA (50 mg kg^{-1}) + DEX	FA (100 mg kg^{-1}) + DEX
Serum Calcium (mg dL^{-1})	10.38 ± 1.42	7.97 ± 1.13$^{\#\#}$	10.09 ± 0.63*	10.75 ± 1.41**
Serum Osteocalcin (ng mL^{-1})	19.31 ± 1.20	13.70 ± 2.84$^{\#\#}$	17.56 ± 1.80**	20.01 ± 1.71***
Serum Phosphorus (mg dL^{-1})	6.54 ± 0.99	5.16 ± 0.65$^{\#}$	6.53 ± 0.81*	6.98 ± 0.39**
B-ALP (U mL^{-1})	294.8 ± 79.44	186.1 ± 29.76$^{\#}$	299.3 ± 89.28*	343.7 ± 63.47**

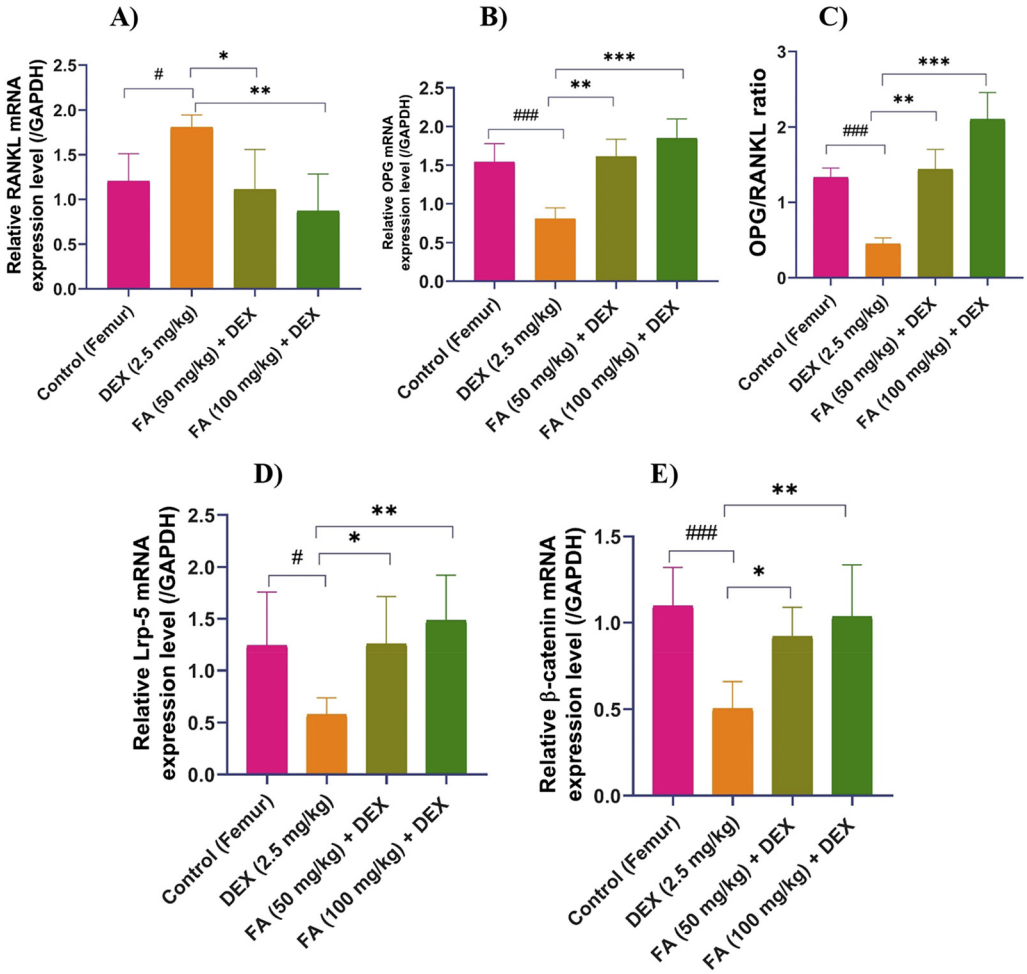
Data are presented as mean ± SD. ##$P < 0.01$ compared with control group; *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ compared with DEX group.

found for OPG levels (Fig. 8B), which decline remarkably ($P < 0.05$) in the DEX group and increased noticeably with FA treatment to the normal level respectively. These findings showed the slightly higher OPG/RANKL ratio in the treatment and control groups with respect to the DEX group (Fig. 8C). It is important to remember that the successful treatment with respect to the enhancement of serum bone metabolic biochemical markers was shown by FA.

In view of the significance of the LRP5/β-catenin pathway in in-vitro [25] osteoblastic development, the actions of FA on the signalling pathway of osteogenesis involving LRP5/β-catenin were evaluated. As indicated in Fig. 8D and E, the β-catenin mRNA expressions in the DEX group were dramatically declined relative to the control group, nonetheless FA dramatically increased the LRP5 and β-catenin mRNA expression levels relative to the DEX group.

Effect of FA on bone bio-mechanical enhancement. The findings of eight weeks treatment in DEX rats illustrated a substantial improvement in bone stiffness relative to the DEX group in the three point bending test of the tibia (Table 4). The findings revealed a substantial decline in bone stiffness as relative to control in the DEX group. In contrast to the DEX group, the FA (50 and 100 mg kg^{-1}) treated groups greatly increased bone strength ($P < 0.001$). Biomechanical properties were identified in our study as indicators of osteoporosis.

Outcomes of bone histomorphometry through FA. As represented in Table 5 and Fig. 9. Oral administration of FA demonstrated a substantial increase in histomorphometric (bone) indicators relative to the DEX and control group. Upon comparison between DEX and control groups, thickness of the trabeculae and number of osteocytes were substantially increased in the FA treatment groups. In our findings, analysis of histomorphometric parameters also showed a reduction in the thickness of cortical and trabecular followed by a substantial decline in osteoblasts number in DEX rats, although such indicators progressed in a dose-dependent manner at (50 and 100 mg kg^{-1}) respectively following treatment with FA. As seen in Table 5, an improvement in diaphysis suggests that in FA treated groups, bone development may occur, ensuing in a reduction of bone resorption in osteoporosis. The outcomes of TRAP staining (Fig. 9) also indicated that the osteoblasts number per mm of trabecular surface increased dramatically in the DEX group relative to the control group. Significantly, FA treatment (50 and 100 mg kg^{-1}) was affected by a substantial reduction in this parameter.



Relative mRNA expression of (A) OPG, (B) RANKL, (C) OPG/ RANKL ratio, (D) Lrp-5 and (E) β-Catenin. Data are represented as mean ± SD. $^{\#}P < 0.05$ and $^{\#\#\#}P < .001$ when compared to control group. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to DEX group

Fig. 8. Effect of FA on the OPG/RANKL ratio and enabled the signalling pathway of Lrp5/β-catenin/GSK-3β n GIO- rats

Table 4. Effect of FA on Bone bio-mechanical enhancement

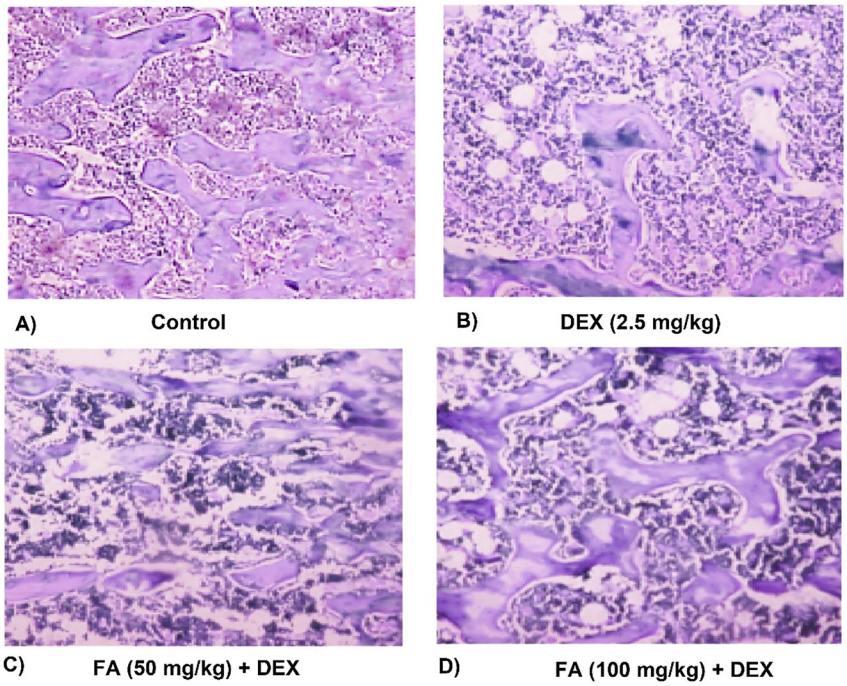
Groups	Control	DEX	FA (50 mg kg^{-1}) + DEX	FA (100 mg kg^{-1}) + DEX
Biomechanical force	100.0 ± 13.84	65.45 ± 21.29	93.55 ± 11.71	104.5 ± 16.74

Data are presented as mean ± SD. $^{\#\#}P < 0.01$ compared with control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with DEX group.

Table 5. Bone histomorphometric analysis of control, DEX and FA (50 and 100 mg kg^{-1}) groups respectively

Groups	Cortical Thickness (μm)	Cortical area (μm^2)	Trabecular thickness (μm)	Trabecular number per mm	Diaphysis thickness	Osteocyte number	Bone marrow cavity
Control	512.7 ± 67.3	728,123 ± 6,821	167.4 ± 7.2	57.42 ± 8.1	181.8 ± 7.1	43.9 ± 3.7	156.8 ± 2.7
DEX	281.8 ± 51.2#	342,517 ± 2,461#	78.9 ± 5.9#	27.91 ± 6.7#	123.5 ± 11.3#	13.6 ± 1.9#	105.9 ± 3.6#
FA (50 mg kg^{-1}) + DEX	389.7 ± 45.7*	528,196 ± 7,213*	162.8 ± 9.1*	45.82 ± 9.4*	173.2 ± 8.3*	33.8 ± 2.3*	123.7 ± 6.3*
FA (100 mg kg^{-1}) + DEX	489 ± 42.9**	772,891 ± 8,923**	172.7 ± 11.3**	61.9 ± 7.2**	194.5 ± 8.8*	38.5 ± 3.2*	145.9 ± 7.6*

Data are presented as mean ± SD. #$P < 0.01$ compared with control group; *$P < 0.05$ and ** $P < 0.01$ compared with DEX group.



A) Control, B) DEX, C) FA (50 mg/kg) + DEX and D) FA (100 mg/kg) + DEX

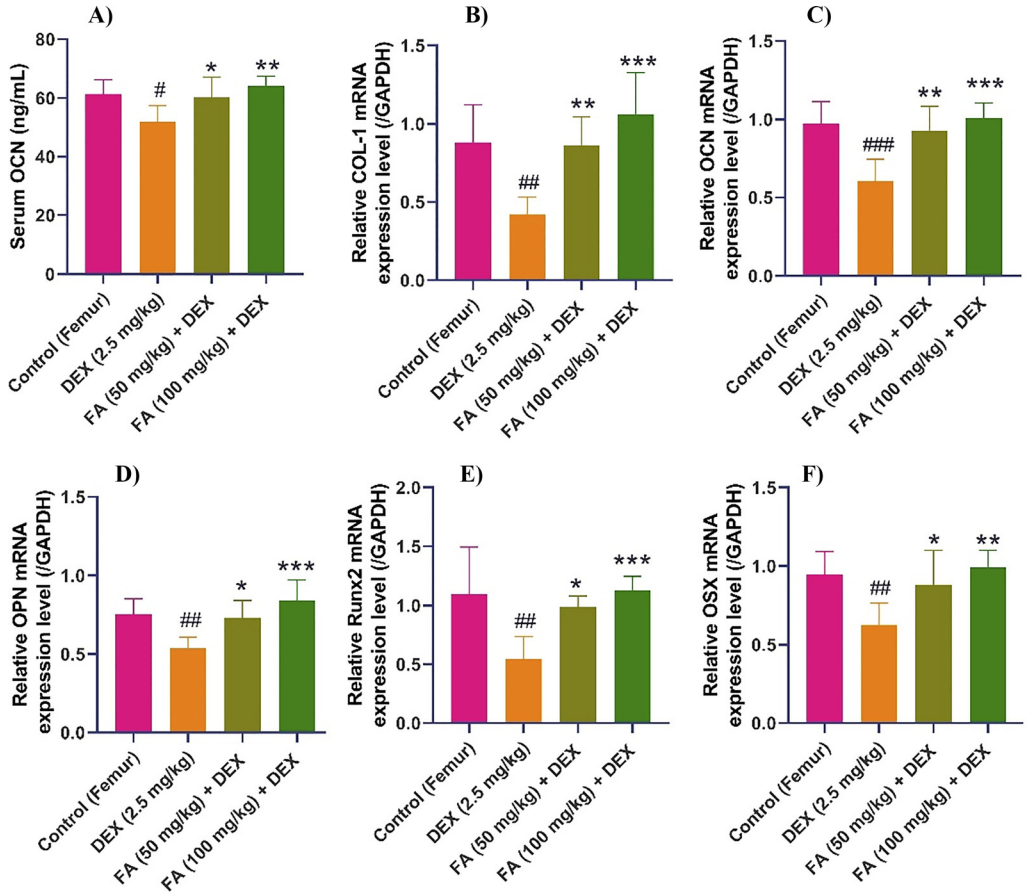
Fig. 9. Histomorphological images (x 200) of a longitudinal cross-sectional area of the distal portion of femoral trabeculae showing trabeculae thickness and osteocyte number of the rat femur after 8 weeks administration of DEX (2.5) mg kg^{-1} p.o. and the FA treatments (50 and 100 mg kg^{-1})

Effects of FA on mRNA expression of bone development indicator in GIO-rats

Inhibition of the bone development indicated by DEX plays a main role in GIO rats and serum OCN levels are broadly employed as markers of bone developement [26, 27]. Hence, in an in vivo evaluation, serum OCN was evaluated. It can be noticed from Fig. 10A, a substantial decline in serum OCN levels in the DEX group was found relative to the control group. However, relative to the DEX group, treatment with FA increased the amount of OCN. The effects of FA on indicators of bone development, such as RUNX2, COL-I, OSX, OPN and OCN were determined. The outcomes revealed that in the DEX group, the mRNA levels of the bone development genes RUNX2, COL-I, OSX, OPN and OCN were apparently reduced relative to the control group, while FA remarkably improved the mRNA levels of bone development indicators when compared to the DEX group (Fig. 10B–F).

Effects of FA on the ERK/Lrp-5/GSK-3β signalling pathways in GIO-rats

Quantitative Real Time-PCR and Western Blot analysis found that the DEX group found down-regulation of the expression of ERK/β-catenin/Lrp-5 protein relative to the control group, while the ERK/β-catenin/Lrp-5 protein over-expression caused by DEX was noticeably up-regulated

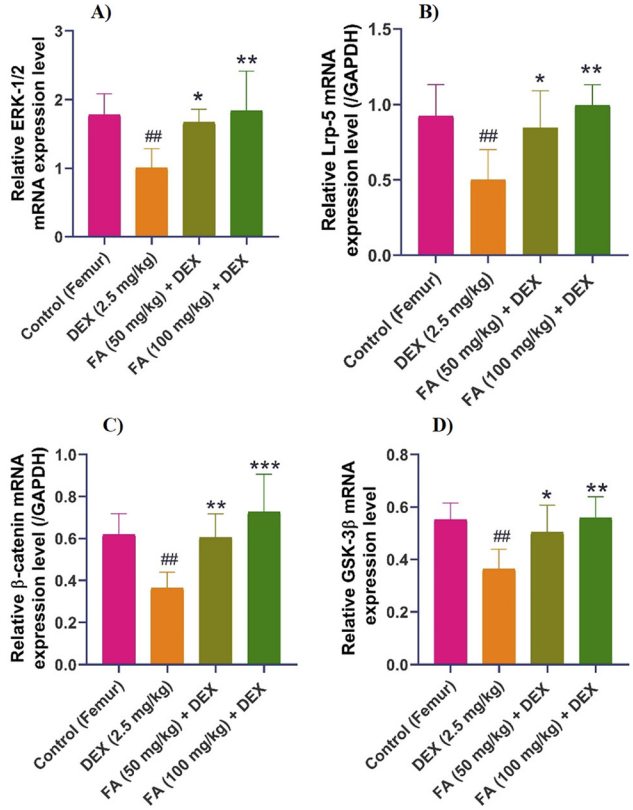


Relative mRNA expression of B) Col-, C) OCN, D) OPN, E) Runx2 and F) OSX. Data are represented as mean ± SD ##P < 0.01 and ##P < 0.001 when compared to control group. *P < 0.05; **P < 0.01 and ***P < 0.001 when compared to DEX group

Fig. 10. Effects of FA on serum OCN levels (A) and mRNA expression off bone development markers in GIO- rats

by FA (Fig. 11). Figure 11A indicates that ERK-phosphorylation was substantially diminished in the DEX group relative to the control group, accompanied by a rise in the treatment groups of FA. Furthermore, relative to the control group, DEX greatly declined Lrp-5 and β-catenin protein expression levels. However, FA treatment at 50 and 100 mg kg^{-1} greatly increased the dose-dependent expressions of Lrp-5 and β-catenin protein (Fig. 11B and C).

In comparison, the expression level of GSK-3β was found to have declined in the DEX group relative to the control group. Upon comparison to the DEX-induced group, FA (50 and 100 mg kg^{-1}) treatment at substantially improved GSK-3β protein expression levels (Fig. 11D). Our results revealed that the ERK MAPK and Wnt signalling pathways could be triggered by FA to facilitate bone development.



mRNA expression of A) ERK-1/2, B) Lrp- 5, C) β- catenin and D) GSK-3β. Data are represented as mean ± SD. ##P < 0.01 when compared to control group. *P < 0.05; **P < 0.01 and ***P < 0.001 when compared to DE group

Fig. 11. Effects of FA on the ERK/Lrp- 5/GSK-3β signalling pathways in GIO- RATS

Discussion

Reduced bone growth appears to be a prominent aspect of glucocorticoid induced osteoporosis (GIO), according to well-documented literature. However, drug development is steadily improving for GIO therapy. In our study, we investigated on MC3T3-E1 cells after treatment for 48 h with 30 µM DEX and *in-vivo* rats receiving intramuscular DXM (2.5 mg kg^{-1}) injections two times a week for eight weeks to ascertain *in-vitro* (MC3T3-E1 cells) and *in-vivo* GIO animal model. In addition, as a prospective natural agent for anti-osteoporotic activity, we studied ferulic acid (FA) suggesting that FA exerts a protective impact on GIO by boosting osteogenesis and noticed that the mechanisms underlying bone loss suppression requires modulation of the cascade pathways of Lrp-5/GSK-3β/ERK. The outcomes of our study indicated that GC-induced disruption to the bone strength and its micro-architecture, which was attenuated by FA, leading to osteoporosis prevention (Fig. 9 and Table 5). Furthermore, through activation of osteoblastic

activities, osteoblast differentiation and ossification, FA inhibited GC-induced impairment of bone development. The rationale of this research was to study the promising *in-vitro* and *in-vivo* protective effects of FA on glucocorticoid induced osteroporosis and to explain the prospective pharmacological pathways of osteoblastic activity of FA.

Our *in-vitro* results on MC3T3-E1 cell proliferation suggested that FA at different concentrations of 0.5, 1.5 and 3 μM could enable osteoblast proliferation in a dose dependent approach. The ALP function of MC3T3-E1 cells after seven days and fourteen days of FA treatment was determined by the ALP assay shown in Fig. 2. Both *in-vitro* and *in-vivo* evaluations showed reduced ALP activity on the fourteenth day as compared to seventh day. This may be attributed to the assumption that ALP during osteoblast differentiation seems to be a premature phenotypic indicator, and ALP might catalyse monoester bone phosphate hydrolysis, producing inorganic phosphates that adhere to calcium to influence bone mineralization and metabolism [28]. Mineralization is the final phase of osteoblast differentiation and may represent the degree of bone development visually. Calcium ion complexation in mineralized alizarin red S nodules deepens red colour and absorption is equal to the magnitude of calcium deposited. The enhanced accumulation of alizarin red S colour was found in the dose-dependent FA treatment groups, suggesting that FA had a significant effect on the mineralization of MC3T3-E1 cells (Fig. 3).

The osteoblast differentiation was modulated by oxidative stress and ROS elevated osteoblastic apoptosis and suppressed the development of bone [29]. The levels of MDA, GSH, SOD, ROS and LDH were investigated *in-vitro* and *in-vivo* to assess the possible protective effects of FA on micro-architecture of bone. The findings revealed that by fostering anti-oxidative defending enzymes and suppressing oxidative stress, FA intensively inhibited oxidative damage in DEX treated MC3T3-E1 cells and GIO-rats, suggesting the stimulating impact of FA on bone development. Bone metabolism imbalance contributes to osteopenia, including inhibiton of bone growth and promotion of bone resorption. The signalling system of the OPG/RANKL/RANK displays a fundamental role in the regulation of the bone remodelling mechanism [30]. The osteoblasts [1] secrete OPG and RANKL. To stimulate differentiation, RANKL binds with RANK on osteoclasts. In order to inhibit this mechanism and regulate the remodelling mechanism [31], OPG, a trapping receptor for RANKL, may also adhere to RANK. The OPG/RANKL ratio is thus the cornerstone to bone resorption and bone development pairing and is commonly employed to test bone remodelling [32]. Here, our results indicate that both *in-vivo* and *in-vitro*, FA may substantially increase the OPG/RANKL ratio. FA may also potentially facilitate bone production and prevent bone resorption.

A difference in bone growth and bone resorption has been linked with the reduction of bone mass and the deterioration of bone micro-structure. The biochemical evidence of bone turnover is used widely to measure the impact on bone remodelling of various compounds [33, 34]. In order to facilitate bone remodelling and disruption of calcium homeostasis, acute or chronic treatments with glucocorticoids are recommended [35]. Earlier studies showed that short-term treatment of GC impaired absorption of intestinal Ca [35] and reduced renal Ca and P factor reabsorption [36]. The serum levels of calcium, phosphorus, osteocalcin and B-ALP in GIO-rats recovered after FA treatment at 50 and 100 mg kg^{-1}, which is compatible with the earlier research [34]. Our research also found that treatment of osteoporotic rats with FA at two doses elevated OPG but decreased RANKL secretion from osteoblasts, leading to suppression of osteoclastogenesis and progression of bone development. It was proposed as a main factor in

suppression of bone proliferation to the OPG/RANK/RANKL system and demonstrated that it was directly involved in the bone development and bone resorption processes. A study found that RANKL is a surface-attached ligand tumour necrosis factor molecule that activates the formation of osteoclasts from osteoclastic progenitors [33]. The attachment of RANKL to the RANK receptor identified in osteoclast precursors and mature osteoclasts contributed to osteoclastogenesis and stimulation of mature osteoclasts. Osteoblasts also generate and produce RANKL trap receptor OPG that suppresses interface between RANKL and RANK and thus prevents the development and stimulation of osteoclasts [37, 38]. Therefore, there is a suggestion that FA can control the signalling pathway of OPG/RANK/RANKL and additional osteogenic factors in osteoporotic bone tissue that display an essential role in bone development and resorption suppression.

It has been indicated that osteoporosis caused by glucocorticoids may provide valuable evidence on bone functions [39]. The DEX group showed substantial reduction in the bone rigidity of the femur. In comparison, tibia examination using the three points bending revealed a substantial decline in biomechanical parameters relative to the control group. In the present research, improved tibia's stiffness was observed three weeks after administering FA (50 and 100 mg kg^{-1}) in osteoporotic rats. Increasing the biomechanical properties of the tibia shows that FA was successful in reinstating the power of the bone in GIO-rats [22]. Osteoporosis is a metabolic condition wherein lack of bone mass and strength may result in the damage of bone fracture [40]. The key function of bisphosphonate improvement in loss of bone is attributed to the mineral portion of very high affinity bone binding hydroxyapatite crystals that prevent their breakdown and effectively suppress bone resorption [41].

In this study, DEX administration impaired trabecular and cortical bone degradation, while FA was effectively prevented, thus higher mechanical intensity was noticed in the femora relative to the control group. The long bone diaphysis is constructed of lightweight bone tissue [42]. Based on the increase in histomorphometric parameters and bone rat structure as seen in Table 5 and Fig. 9, FA was more efficient and useful for osteoporosis prevention at both doses [34]. TRAP activity for the marker recognition of osteoclasts and pre-osteoclasts is known to be a significant cytochemical stain. A study showed that less TRAP plus osteoclasts suppressed the activity of bone resorption [43]. In proliferation and differentiation, the ERK-MAPK pathway plays a significant function, and earlier studies have reported that ERK-phosphorylation might progress differentiation of osteoblasts. The ERK-phosphorylation and Lrp-5 relationship, however, remained uncertain. DXM could decrease ERK phosphorylation in this study, while FA could increase both *in-vivo* and *in-vitro* ERK phosphorylation in GIO.

Western blotting was conducted in order to test β-Catenin and Runx2 protein levels utilising MC3T3-E1 cells that were cultivated for seven and fourteen days. Compared to control, FA substantially up-regulated the β-Catenin and Runx2 expression in MC3T3-E1 cells in a time dependent approach. The β-Catenin expression was dramatically higher in FA treated cells. Several recent studies indicated that the MAPK and β-Catenin/Wnt pathways are targets for β-Catenin and Runx2 respectively [44, 45]. In our investigation, the upregulation of Runx2 and β-Catenin expression showed that FA could concurrently stimulate MAPK and Wnt/β-catenin signalling pathways to foster MC3T3-E1 cell proliferation, differentiation and mineralization. It has been documented that several signalling mechanisms are correlated with osteoblast differentiation. A study [46] stated that echinacoside, by up-regulating the OPG/RANKL ratio and implicitly suppressing bone resorption of osteoclasts, facilitated osteoblast proliferation and

differentiation. The studies [47, 48] observed that by triggering the MAPK and Wnt/β-catenin signalling pathways, icariin and orexins facilitated the differentiation and proliferation of osteoblasts, which was compatible with the findings of this paper.

In order to preserve the equilibrium of bone density, the canonical Wnt/β-catenin signalling pathway shows a key function. We demonstrated the *in-vivo* and *in-vitro* β-catenin, p-GSK-3β and Lrp-5 expressions to explain the potential pathways of FA attenuating GIO. DEX lowered the β-catenin, p-GSK-3β and Lrp-5 expressions in the present study, while FA treatment increased the β-catenin, p-GSK-3β and Lrp-5 mRNA expressions, suggesting that DEX could suppress osteoblastic activities by suppressing the Wnt signalling pathway. However, through the β-catenin/Wnt signalling pathway in GIO, FA could facilitate osteoblastic activities. The suppression of GSK-3β and the aggregation of β-catenin may be promoted by Lrp-5. For osteoblastic processes in osteogenesis that can induce transcription of osteoblast associated genes namely Col-I and Runx2 is crucial [49, 50]. Collectively, in regulating osteoblastic differentiation and mineralization, Lrp-5 plays an important function. In addition, through Lrp-5 in GIO, FA may promote bone formation.

CONCLUSION

In brief, this study clearly showed that ferulic acid improved bone development through the promotion of GIO osteoblastic differentiation, mineralization, and proliferation. By inhibiting the over-generation of reactive oxygen species to prevent osteoblastic proliferation, FA attenuated osteoblastic apoptosis, enhanced osteogenesis marker expressions to facilitate osteoblastic differentiation, and improved OPG/RANKL ratio to preserve bone mass. Through cortical and trabecular bone enhancement and avoiding degradation in chemo-mechanical markers of osteoporotic rats, FA induced substantial defence against bone loss. Overall, our findings showed that FA reduced oxidative injury through the Lrp-5/GSK-3β/ERK pathways to advance osteoblastic proliferation and improved osteoblastic activities to retain bone mass and the potential role in the pathophysiology of GIO and suggests that FA may be a new therapeutic compound for GIO treatment.

ABBREVIATIONS

FA	Ferulic acid
GC	Glucocorticoids
DEX	Dexamethasone
ROS	Reactive Oxygen species
MAPK pathway	Mitogen-activated protein kinase
ERK pathway	Extra-cellular signal regulated kinase
OPG	Osteoprotegerin
ALP	Alkaline phosphatase
EDTA	Ethylenediamine tetra-acetic acid
α-MEM	Minimum essential medium
DMSO	Dimethyl sulfoxide

SOD	Superoxide dismutases
DH	Lactate dehydrogenase
GSH	Glutathione
MDA	Malondialdehyde
BMD	Bone mineral density
RANKL	Receptor activator of nuclear factor (NF)-kappaB ligand
TRAP	Tartrate-resistant acid phosphatase
GAPDH	Glyceraldehyde 3-phosphatee dehydrogenase
GSK-β-	Glycogen synthase kinase 3 beta

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