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Original Article

Biphasic Response to Luteolin in MG-63 Osteoblast-Like Cells under High Glucose-Induced Oxidative Stress

Naser Abbasi[1], PharmD;
Afra Khosravi[2], PhD;
Ali Aidy[2], MS;
Massoumeh Shafiei[1], PhD

[1]Department of Pharmacology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran;
[2]Biotechnology and Medicinal Plants Research Center, Ilam University of Medical Sciences, Ilam, Iran

Correspondence:
Massoumeh Shafiei, PhD;
Department of Pharmacology, School of Medicine, Iran University of Medical Sciences, Hemmat Highway, Tehran, Iran
Tel: +98 21 88622573
Fax: +98 21 88622696
Email: shafiei.m@iums.ac.ir
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What's Known

- Diabetes induces the impairment of osteogenesis through a decrease in osteoblast activity.
- Flavonoids increase the differentiation and mineralization of osteoblasts in hyperglycemia.
- Luteolin has shown an anabolic property in mouse osteoblastic MC3T3-E1 cells. Most flavonoids, including luteolin, also demonstrate dose-dependent cytotoxicity.

What's New

- In the present study, luteolin showed modulatory effects on the osteogenic characteristics of human osteoblast-like MG-63 cells under normal and high glucose conditions *in vitro*.
- Low concentrations of luteolin had a protective action against hyperglycemic insult in MG-63 cells. High concentrations of luteolin demonstrated cytotoxicity in both normal and high glucose states.
- Cytoprotective levels of luteolin were lower, by a factor of approximately 20-26, than its cytotoxic concentrations.

Abstract

Background: Clinical evidence indicates the diabetes-induced impairment of osteogenesis caused by a decrease in osteoblast activity. Flavonoids can increase the differentiation and mineralization of osteoblasts in a high-glucose state. However, some flavonoids such as luteolin may have the potential to induce cytotoxicity in osteoblast-like cells. This study was performed to investigate whether a cytoprotective concentration range of luteolin could be separated from a cytotoxic concentration range in human MG-63 osteoblast-like cells in high-glucose condition.

Methods: Cells were cultured in a normal- or high-glucose medium. Cell viability was determined with the MTT assay. The formation of intracellular reactive oxygen species (ROS) was measured using probe 2',7' -dichlorofluorescein diacetate, and osteogenic differentiation was evaluated with an alkaline phosphatase bioassay.

Results: ROS generation, reduction in alkaline phosphatase activity, and cell death induced by high glucose were inhibited by lower concentrations of luteolin (EC_{50}, 1.29±0.23 µM). Oxidative stress mediated by high glucose was also overcome by N-acetyl-L-cysteine. At high concentrations, luteolin caused osteoblast cell death in normal- and high-glucose states (IC_{50}, 34±2.33 and 27±2.42 µM, respectively), as represented by increased ROS and decreased alkaline phosphatase activity.

Conclusion: Our results indicated that the cytoprotective action of luteolin in glucotoxic condition was manifested in much lower concentrations, by a factor of approximately 26 and 20, than was its cytotoxic activity, which occurred under normal or glucotoxic condition, respectively.

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Keywords ● Luteolin ● Human osteoblast-like MG-63 cells ● High glucose ● Oxidative stress ● Alkaline phosphatase

Introduction

Diabetes mellitus is a common metabolic disease that affects multiple organs and tissues, including bone.[1] *In vivo* studies in diabetic rodent models indicate that impaired bone formation appears to be a major contributing factor to diabetes-mellitus-related osteopenia.[2] Although it is known that diabetes mellitus affects bone, the exact mechanism is not yet clear. One of the possible underlying processes, in addition to advanced glycation end products, has been linked to the inhibition of osteoblast

function and the reduced formation of a mineralized matrix.[3] One of the major molecular changes in diabetes mellitus is the formation of reactive oxygen species (ROS) as a result of high glucose and/or insulin insufficiency or resistance.[4] In osteoblasts, ROS has been reported to inhibit differentiation as evidenced by core-binding factor α 1 (Cbfal)/runt-related transcription factor 2 (RUNX2), which is a key transcription factor associated with osteoblast differentiation. The differentiation markers, including alkaline phosphatase (ALP) and type I collagen, decrease in the presence of oxidative stress.[5] In one study on subjects with type 1 diabetes, serum ALP and osteocalcin levels were significantly lower than those of the control subjects, suggesting reduced bone formation.[6]

Flavonoids are known to have multiple beneficial biological effects owing to their anti-inflammatory,[7] antioxidant,[8] and antidiabetic[9] activities. Available literature suggests that flavone apigenin, identified as the main flavonoid in soybean, may prevent bone loss in ovariectomized animal models and that it may inhibit osteoclast differentiation.[10] However, quercetin, one of the main dietary flavonoids happening in foods of plant origin, is reported to induce apoptosis in osteoblastic cells.[11] Luteolin, a flavone rich in many plants, has anti-inflammatory effects both *in vivo* and *in vitro*.[12] Moreover, luteolin has an anabolic property in that not only does it raise the synthesis of collagen, activity of ALP, and secretion of osteocalcin but also it hinders 3-morpholinosydnonimine-triggered generation of pro-inflammatory mediators in osteoblastic MC3T3-E1 cells *in vitro*.[13] Nonetheless, it has been reported that luteolin markedly diminishes the viability of osteoblastic MC3T3-E1 cells, demonstrating the cytotoxic activity of this flavoniod.[14]

Thus, we designed the present study to collect data on the concentration-response relation between the protective and cytotoxic effects of luteolin on human MG-63 osteoblast-like cells under high-glucose versus normal-glucose states to distinguish between luteolin cytoprotective and cytotoxic concentration ranges.

Materials and Methods

Cell Culture

Human osteoblast-like MG-63 cell line was obtained from the National Cell Bank of Iran (NCBI) (c-555) at Pasteur Institute. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 IU/mL of penicillin, and 100 mg/mL of streptomycin at 37°C in 95% humidified air with 5% CO2. The medium was replaced every 3 days, and the confluent cells were digested with 0.25% trypsin. The cells between the second and fifth passages were used in the following studies. Cell viability was assessed via the trypan blue exclusion method. The MG-63 cells were divided into 6 groups: control group (original DMEM medium), normal-glucose group (5.5 mM of glucose), high-mannitol group (30 mM of mannitol as osmotic control), high-glucose group (30 mM of glucose),[15] and 2 luteolin (Sigma) treatment groups in normal- and high-glucose states. The concentrations of luteolin used for the cell viability test were 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, and 100 μM. The experiments were conducted in media with either normal or high glucose or high mannitol for 24, 48, and 72 hours. The time point of 48 hours was selected for the treatment exposure.

Cell Viability Assay

The cytotoxic effects of luteolin on the human MG-63 osteoblast-like cells were evaluated via the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-based experiment.[16] The MG-63 cells were plated at a density of 3×10^4 cells/well in 96-well plates. After overnight incubation in a humidified incubator with 5% CO2 at 37°C, the cultures were continued for 48 hours in the medium. The MTT (20 μL, 5 mg/mL) was then added to each well, and the cultures were continued for 4 hours at 37°C. The medium was subsequently aspirated, and 150 μL of dimethyl sulfoxide was added to each well. The supernatant was transferred to microplate wells, and colorimetric changes were quantified in a microplate reader (Dynex MRX) at a wavelength of 570 nm. The MTT colorimetric assay was performed in triplicate. The percentage of cell viability was calculated using the following formula: %cell viability=[(Mean Abs. of the samples-Mean Abs. of the blank)/(Mean Abs. of the control-Mean Abs. of the blank)]×100.

The half maximal effective concentration (EC_{50}) and the half maximal inhibitory concentration (IC_{50}) were calculated in normal- and high-glucose conditions.

Measurement of Reactive Oxygen Species Formation

For the measurement of ROS, all the cells were incubated with 10 μM of 2', 7'-dichlorfluorescein diacetate ($DCFH_2$-DA) (Sigma)[17] in a serum-free culture medium for 45 minutes at 37°C and 5% CO2. During oxidative stress, the added chemical

compound (i.e., $DCFH_2$-DA) was catalyzed to 2`7`-dichlorofluorescein (DCFH) and analyzed in a bioassay multi-detection fluorescent plate reader (Biotek-FLx800) at excitation/emissions of 485/520 nm. The production of ROS was determined from an H2O2 standard curve (10 to 200 nM).

Assay of Alkaline Phosphatase Activity

The human MG-63 osteoblast-like cells were seeded in a 96-well plate, with 3,000 cells per well, and placed in 5% CO2 at 37°C for 48 hours.[18] After being rinsed with phosphate-buffered saline, the cells were treated with various concentrations (i.e., 1, 5, 50, and 100 μM) of luteolin. After the removal of the medium, the cells of each well were fixed with 100 μL of 4% formaldehyde for 10 minutes. After washing, the cells of each well were treated with 30 μL of *p*-nitrophenyl phosphate (pNPP) at 37°C for 30 minutes to determine ALP activity. Afterward, 30 μL of 0.5 N NaOH was added to terminate the reaction. Lastly, pNPP was converted to *p*-nitrophenol utilizing an ELISA reader at a wavelength of 405 nm, and the relative level of ALP activity over control was measured. Moreover, a protein synthesis inhibitor (i.e. cycloheximide [1 μM]), was studied in the MG-63 osteoblast-like cells.

Statistical Analysis

The results are expressed as mean±SEM. The statistical evaluation of the data was performed with the Student *t*-test for simple comparison between 2 values when appropriate. For multiple comparisons, the data were analyzed using the analysis of variance. A $P<0.05$ was considered statistically significant. The terms "EC_{50}" and "IC_{50}" are the statistically derived concentrations of a substance in an environmental medium expected to produce a certain effect in 50% of the test organisms in a given population under a defined set of conditions after a specified exposure time.

Results

Effects of High Glucose on Cell Viability

The human MG-63 osteoblast-like cells were exposed to 30 mM of either glucose or mannitol. On day 1, the MTT assay indicated no significant differences in cell viability between the high- and normal-glucose groups. On days 2 and 3, the MG-63 cells in the high-glucose medium, compared to the normal-glucose medium, showed significantly decreased optical density (data not shown) (P=0.006). Mannitol, which was used to create a high-osmotic pressure mimicking high-glucose condition, did not show significant changes in cell viability. The time point of 48 hours was selected as the treatment exposure time.

Effect of Luteolin on Cell Viability

Luteolin at very low concentrations (i.e. 0.05 and 0.1 μM) did not change high-glucose-induced cell death in the human MG-63 osteoblast-like cells; while at low concentrations (i.e., 0.25, 0.5, 1, and 5 μM), it attenuated high-glucose-induced cell death (P=0.009, P=0.008, P=0.008, and P=0.005, respectively, vs. high-glucose control) with an EC_{50} value of 1.29±0.23 μM. Additionally, 10 μM of luteolin decreased cell viability compared to the previous dilution (i.e., 5 μM) in both normal- and high-glucose states (P=0.031). However, 10 μM of luteolin was still able to partially suppress high-glucose-induced cell death (P=0.007 vs. high-glucose control). In our experiments, the MG-63 cells underwent severe cell death after high concentrations of luteolin (i.e., 25, 50, and 100 μM) treatment for 48 hours with IC_{50} values of 34±2.33 and 27±2.42 μM in normal- and high-glucose states, correspondingly (Figure 1). A near-significant difference (P=0.052) was found between the IC_{50} values obtained in normal- and high-glucose conditions. The reduction in cell viability by the high concentrations of luteolin was greater than that observed by high-glucose control (P=0.039, P=0.009, and P=0.006, respectively). The molecular-level experiments were limited to 1 and 5 μM as low- and 50 and 100 μM as high-luteolin concentrations, which showed cytoprotective and cytotoxic activities, respectively.

Effect of Luteolin on the Generation of Reactive Oxygen Species

The effect of luteolin on the generation of ROS in the human MG-63 osteoblast-like cells is shown in Figure 2. We used the fluorescent

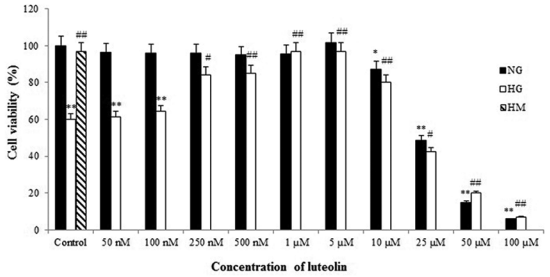

Figure 1: Effect of different concentrations of luteolin on osteoblast-likeMG-63 cell viability. The MG-63 cells were treated with normal glucose (NG), high glucose (HG), high mannitol (HM), NG plus luteolin, and HG plus luteolin at 37°C in 5% CO2. Data are expressed as mean±SEM. * P<0.05 vs. NG and HM. **P<0.01 vs. NG, #P<0.05 vs. HG, ##P<0.01 vs. HG.

probe $DCFH_2$-DA to assess the release of ROS in the MG-63 cells and found that the incubation of these cells in high glucose for 48 hours markedly increased the fluorescence intensity of DCF (P=0.006 vs. normal glucose), indicating significant ROS-induced stress in the model. Treatment with low concentrations of luteolin and N-acetyl-L-cysteine (5 mM)[19] prevented the enhancement of DCF fluorescence intensity. However, high concentrations of luteolin increased ROS generation in both normal- and high-glucose states in the MG-63 cells. High-concentration mannitol had no effects on the level of ROS.

Effect of Luteolin on Alkaline Phosphatase Activity

ALP activity was significantly reduced in the high-glucose-cultured MG-63 cells. Treatment with low concentrations of luteolin restored ALP activity in the cells exposed to high glucose. Nonetheless, the effect of luteolin (5 μM) in increasing ALP activity was completely eliminated in the presence of cycloheximide (1 μM)[13] (P=0.008 vs. normal glucose). Furthermore, ALP activity was reduced after treatment with high concentrations of luteolin in both of the normal- and high-glucose-treated human MG-63 osteoblast-like cells (Figure 3).

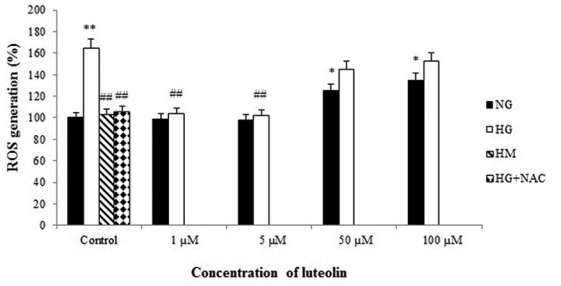

Figure 2: Effect of different concentrations of luteolin on the generation of reactive oxygen species (ROS) in normal- (NG) and high-glucose (HG) states. Bar graphs show ROS production (%) in MG-63 osteoblast-like cells exposed to NG, high mannitol (HM), HG, HG plus N-acetyl-L-cysteine (NAC) (5 mM), NG plus luteolin, and HG plus luteolin. Data are expressed as mean±SEM. *P<0.05 vs. NG and HM, **P<0.01 vs. NG and HM, ##P<0.01 vs. HG.

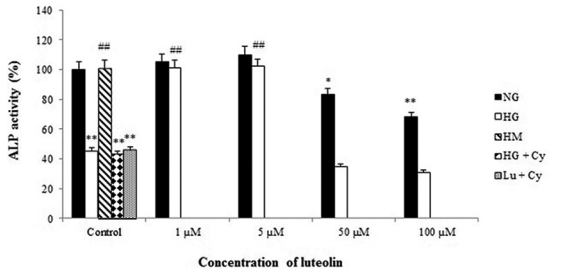

Figure 3: Effect of different concentrations of luteolin (Lu) on alkaline phosphatase (ALP) activity in normal- (NG) and high-glucose (HG) states. Bar graphs show ALP activity (%) in MG-63 osteoblast-like cells exposed to NG, high mannitol (HM), HG, HG plus cycloheximide (Cy) (1 μM), Lu (5 μM) plus Cy (1 μM), NG plus Lu, and HG plus Lu. Data are expressed as mean±SEM. * P<0.05 vs. NG and HM. **P<0.01 vs. NG and HM, ##P<0.01 vs. HG.

Discussion

We used human MG-63 osteosarcoma cells to investigate the modulatory effects of luteolin on osteogenic characteristics under normal- and high-glucose conditions. These cells display osteoblast-like properties with rapid cell growth and no interspecies differences.[20] ALP activity and osteocalcin are examples of the phenotypic markers that MG-63cells can exhibit.[21] In the present study, the exposure of human MG-63 osteoblast-like cells to high glucose for 48 and 72 hours resulted in a significant decrease in cell viability, as compared with cells cultured in a normal-glucose or a high-mannitol concentration. Previously reported *in vitro* studies have indicated that oxidative stress inhibits osteoblast differentiation[5] and induces osteoblastic insults and apoptosis.[22] An imbalance between bone-forming osteoblasts and bone-resorbing osteoclasts leads to the pathogenesis and etiology of certain bone metabolic diseases, including osteoporosis and osteopetrosis.[23] It is presumed that diabetes-induced adverse sequelae are largely due to oxidative stress, which leads to the augmentation of ROS production under hyperglycemic state, capable of inducing disturbances in cellular functioning in various cell types. Oxidative stress is induced by a variety of mechanisms, including formation of increased advanced glycation end products, increased polyol pathway flux, activation of protein kinase C isoforms, glucose autoxidation, and mitochondrial overproduction of superoxide under diabetic conditions.[4]

In the present study, treatment with luteolin at low concentrations (i.e., 0.25, 0.5, 1, and 5 μM) prevented high-glucose-mediated cell death with an EC_{50} value of 1.29±0.23 μM and at high concentrations (i.e., 25, 50, and 100 μM) reduced cell viability with an IC_{50} value of 27±2.42 μM. Both cytoprotective and cytotoxic effects of luteolin were exhibited in a concentration-dependent manner. Under normal-glucose condition, low concentrations of luteolin had no effects on cell viability in the MG-63 cells. Nevertheless, treatment with high concentrations reduced cell viability with an IC_{50} value of 34±2.33 μM. A comparison of the toxicity of luteolin between the normal- and high-glucose groups revealed a near-significant difference (P=0.052) between the IC_{50} values obtained

in normal- and high-glucose conditions. We suggest that future studies investigate whether hyperglycemia can potentiate the toxicity of luteolin in human osteoblastic cells.

Studies on osteoblasts have shown that the total flavonoids from *Herba Epimedii* have osteoblastic proliferation-stimulating activity toward osteoblast-like UMR106 cells.[24] Moreover, myricetin, a naturally occurring flavonoid, was reported to protect osteoblasts from 2-deoxy-D-ribose-induced dysfunction and oxidative damage in MC3T3-E1 cells, mouse pre-osteoblast cell line.[25] It was also reported that luteolin, under normal-glucose condition, inhibited osteoclast bone resorption with an EC_{50} value of approximately 2.5 μM.[26] However, consistent with our results, a study demonstrated that 25 and 50 μM of luteolin significantly reduced the number of viable MC3T3-E1 cells compared with untreated cells.[14] Furthermore, quercetin may exert both protective and deleterious effects in bone repair.[11] In a study, quercetin inhibited osteoblast proliferation, differentiation, and mineralization.[27] Quercetin also induced apoptosis through a mitochondria-dependent mechanism.[11] Concentration-dependent cytoprotective and cytotoxic activity of luteolin has been previously demonstrated in human umbilical vein endothelial cells.[28]

We demonstrated that luteolin at low concentrations, as well as N-acetyl-L-cysteine, decreased intracellular ROS in high-glucose condition. Nonetheless, treatment with high concentrations of luteolin resulted in augmented intracellular ROS in both normal- and high-glucose states in a concentration-dependent manner. Different flavonoids have been shown to augment antioxidant enzyme activities in osteoblastic cells. The results of a study demonstrated that quercetin diminished oxidative damage in human osteoblastic cell by scavenging radicals.[29] An extract of total flavonoids from persimmon leaves was shown to significantly decrease the level of ROS in MC3T3-E1 cells.[30] Moreover, an investigation reported that the total flavonoids in *Chrysanthemum zawadskii* decreased 2-deoxy-D-ribose-induced cell damage in MC3T3-E1 osteoblastic cells due to its antioxidant activity and positive effect on differentiation and suggested that it might promote bone recovery in diabetes-associated bone diseases.[21] Another study demonstrated that glabridin, a flavonoid, attenuated 2-deoxy-D-ribose-induced cell damage in osteoblastic cells and suggested that it might be useful for the treatment of diabetes-related bone disease.[31] Elsewhere, a study showed that morin, a member of flavonols, alleviated diabetic-induced osteopenia and postulated that this effect was through its both anti-inflammatory and antioxidant properties.[32]

ALP has been generally considered a well-known early marker of differentiation in osteoblasts.[33] A study found that high concentrations of glucose inhibited ALP activity, collagen formation, and mineralization in osteoblastic cells.[6] In the present study, the activity of ALP was utilized as an osteoblast differentiation and maturation indicator. Our results showed that luteolin at low concentrations increased ALP activity in human MG-63 osteoblast-like cells, indicating its anabolic effect. The enhancing effect of luteolin at 5 μM on ALP activity in MG-63 cells was blocked completely by the presence of cycloheximide, an inhibitor of protein synthesis. Thus, the anabolic effect of luteolin may be based partly on a newly synthesized protein component. However, we demonstrated that luteolin at high concentrations decreased ALP activity in both normal- and high-glucose states in a concentration-dependent manner. A previous investigation reported that a low concentration of luteolin (1 μM) increased ALP activity in osteoblastic MC3T3-E1 cells *in vitro*.[13] Another study demonstrated that hesperetin, which is classified in flavanone subgroup and is largely specific to citrus fruits, ameliorated the suppression of osteogenesis caused by high glucose in stem cells obtained from periodontal ligaments (as assessed via the measurement of ALP activity).[34] The results of another research indicated that a high concentration of luteolin (16 μM) decreased ALP activity in MC3T3-E1 cells.[14]

Based on our findings, careful attention should be paid to the amount of luteolin in foods or supplements available for consumption. Flavonoid supplements are aggressively marketed by the nutraceutical industry for various purposes.[35] In addition, luteolin, as a common dietary flavonoid, is found in many medicinal herbs, fruits, and vegetables such as green pepper, chamomile tea, perilla leaf,[36] parsley, and artichoke.[37] Also, high amounts of luteolin can be found in peanut hulls.[37]

We demonstrated the cytoprotective and cytotoxic activity of low and high concentrations, respectively, of luteolin in osteoblast-like cells in normal- and high-glucose states; nonetheless, there are some limitations in our study. First and foremost among these limitations is that we considered only 1 time point (i.e., 48 h) as the treatment exposure in the experiments. Although this time point was the optimal duration of incubation in our previous studies, using longer exposure times in the case of osteoblastic

cells may provide more precise results. Another shortcoming of note is that we determined the activity of only 1 osteoblast differentiation marker (i.e., ALP), although there are some other osteoblast markers whose assessment would be helpful. Currently, the present study is being continued in our laboratory and some experiments are underway to provide supplementary data.

Conclusion

Taken together, our results showed that low concentrations of luteolin had a protective effect against hyperglycemic stress in human MG-63 osteoblast-like cells by reducing intracellular ROS overproduction, thereby restoring ALP activity. In contrast, high concentrations totally reversed the above-mentioned effects. Luteolin at much lower concentrations conferred protection against high-glucose-induced cell death, by a factor of approximately 26 and 20, compared to its cytotoxic activity under both normal- and high-glucose conditions, respectively. Thus, it is safest to avoid taking high doses of luteolin in food supplements when its protective actions are desired.

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Conflict of Interest: None declared.

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