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Research Article

Synergistic Effects of Cordycepin and Genistein on Preosteoblast MC3T3-E1 Cells with Endoplasmic Reticulum Stress

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Abstract

Endoplasmic reticulum (ER) stress activation disturbs bone homeostasis and plays an important role in osteolytic bone diseases development. Cordycepin (3'-deoxyadenosine), a natural structural analog of adenosine, possess multiple pharmacological activities and exerts osteoprotective effect. Genistein can be used as protectors or synergists of adenosine drugs. The aim of the present study was to investigate whether the combination of cordycepin (Cor) and genistein (Gen) regulates proliferation, migration and differentiation of preosteoblast MC3T3-E1 cells in a tunicamycin (TM)-induced acute ER stress model. Cell viability were assessed using cell counting kit-8 (CCK-8) assay and osteoblast migration were evaluated by transwell chamber assay. The differentiation was assessed by alkaline phosphatase (ALP) staining assay. qRT-PCR assay was performed to confirm the establishment of ER stress model and to measure the mRNA level of key molecules involved in osteogenic differentiation. Exposure to the combination of 10μM cordycepin and 10μM genistein had no cytotoxicity in MC3T3-E1 cells, but increased the number of proliferative cells after pretreatment with 2 μg/ml TM. Analogously, ER stress-induced suppression on MC3T3-E1 cells migration was restored to control levels by the administration of cordycepin and genistein combination. Cordycepin and genistein combination significantly up-regulated ALP activity of MC3T3-E1 cells in TM-induced ER stress model. The qRT-PCR data demonstrated that the combined treatment effectively accelerated osteogenic differentiation by enhancing the mRNA level of differentiation markers including collagen type I, ALP, BSP and OPN. These results reveal that the co-administration of cordycepin and genistein exerts synergistic osteoprotective effects and has the potential to serve as a host risk reduction strategy for osteolytic bone diseases associated with ER stress activation.

Keywords: *Cordycepin; Genistein; Osteoblast; Endoplasmic Reticulum Stress*

Introduction

The endoplasmic reticulum (ER) is the cellular organelle that is critical for protein folding and secretion, calcium homeostasis, and lipid biosynthesis. Under various conditions, ER stress is recognized as the accumulation of misfolded proteins in the ER leading to the impaired biosynthesis, folding, assembly and modification of numerous soluble proteins and membrane proteins [1]. The association between ER stress and the pathogenesis of many metabolic

conditions have been well characterized using both *in vivo* and *in vitro* models [2]. It has been reported that ER stress activation disturbs bone homeostasis and plays an important role in osteolytic bone diseases development [3,4].

Osteoporosis, a common osteolytic bone disease, and associated fractures are a noteworthy public health problem and the disease has severe consequences if untreated. It is characterized by low bone mineral density and loss of the structural and biomechanical properties that are required to maintain bone homeostasis. Despite current treatment options that include vitamin D, hormone, and bisphosphonates therapy, the outcomes are often discouraging due to significant morbidity and mortality. Researches during the last decade have demonstrated that natural products have been a rich source of biocompatible substances with effective osteoprotective applications, and without the unexpected side effects on the immune system and on the activity of osteoblasts.

Cordycepin (3'-deoxyadenosine), is the major bioactive component of *Cordyceps militaris* [5] and is a natural structural analog of adenosine [6,7]. Cordycepin exerts multiple physiological and pharmacological activities, such as tumor metastasis, vascular disorder, hypolipidemic, antioxidant, and anti-inflammatory activities [8]. Cordycepin exerts osteoprotective effect and acts as an anti-inflammatory agent in chemical-induced inflammation in osteoporosis [9]. It has been reported that oral administration of cordycepin can counteract the bone loss in an experimental rat model of estrogen deficiency-induced osteoporosis [10]. However, being degraded easily by adenosine deaminase in human tissues, cordycepin has a short half-life and high rates of clearance, and its efficiency has been limited [7,11].

Phytoestrogen genistein, the most prominent isoflavone from soy, has high estrogenic activity and shows beneficial effects of hormone replacement therapy on osteoporosis in previous studies. Furthermore, it was indicated that genistein is identified as an adenosine deaminase inhibitor and improve the efficacy of adenosine analogue drugs such as cordycepin by inhibiting their degradation [12]. In a recent study genistein showed synergistic anticancer and antimicrobial activities with cordycepin [13].

Up to now only few studies focus on the effects of the combination of cordycepin and genistein on bone related-cells under ER stress condition. Therefore, we hypothesized that cordycepin in the presence of genistein regulated synergistic protective activity in preosteoblast MC3T3-E1 cells with tunicamycin (TM)-induced ER stress. In the present study these hypotheses were elucidated by focusing on cell viability assay, migration analysis, differentiation alterations and signaling cascades.

Materials and methods

Reagents

Cordycepin and genistein were obtained from m Selleck Chemicals (Houston, TX, USA). They were prepared as stock solution in double-distilled water and diluted in culture medium at concentration of 10 μM prior to experiments. FBS, α-MEM, penicillin-streptomycin, trypsin-EDTA and TRIzol were purchased from GIBCO (Grand Island, NY, USA). L-ascorbic acid, β-glycerophosphate disodium salt hydrate and tunicamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Counting Kit-8 and BCIP/NBT Alkaline Phosphatase Color Development Kit were purchased from Beyotime Biotechnology (Shanghai, China). TB Green™ Premix Ex Taq™ II and PrimeScript™ RT reagent Kit with gDNA Eraser were obtained from TaKaRa Bio (Shiga, Japan). PCR primers were designed by Sangon Biotech (Shanghai, China). All reagents were of analytical grade, unless otherwise specified.

Cell culture and ER stress osteoblast model establishment

The murine calvaria-derived preosteoblast MC3T3-E1 Subclone 4 was obtained from America Type Culture Collection (Manassas, VA, USA) and maintained in α-MEM with 10% FBS and 1% penicillin-streptomycin at 37˚C in a 5% CO2. For osteoblast differentiation, the culture medium was replaced with an osteogenic differentiation medium (α-MEM modification containing 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate). The osteogenic differentiation medium was changed every two days. Here tunicamycin was used to model acute ER stress in osteoblast [14]. Briefly MC3T3-E1 cells were kept quiescent in serum free medium for 12 hours before treated with various concentrations of tunicamycin (0.5 μg/ml, 1 μg/ml, 2 μg/ml) for 24 hours.

Cell viability assay

Cell proliferation was assessed by CCK-8 assay according to the manufacturer's instructions. Briefly, MC3T3-E1 cells were seeded at 5,000 cells/well into a 96-well plate. Confluent cells were treated with 10 μM cordycepin and 10 μM genistein, with or without

tunicamycin for 1, 3, 5 and 7 days. At the designed time points, 10 µl of CCK-8 was added to each well and the plate was incubated for 3h. The mixture was quantified by determining the absorbance at 405nm using a microplate reader (BioRad, Hercules, CA).

Transwell chamber assay

A Transwell chamber containing an 8-µm pore polycarbonate membrane filter was inserted in a 24-well culture plate. MC3T3-E1 cells were pretreated with 2 µg/ml tunicamycin for 24h. The cells were then detached with trypsin-EDTA and resuspended in serum-free α-MEM. After filling the lower chamber with 10 µM cordycepin and 10 µM genistein as a chemoattractant medium, 10^5 cells/well were loaded in the upper chambers. After 12h incubation, cells in the upper chamber that did not migrate were scraped away. Then the transmembrane cells were fixed with 4% paraformaldehyde, and stained with 0.1% crystal violet, 0.1M borate (pH 9.0), and 2% ethanol for 5 minutes at 25°C. The migrated cells were captured using an inverted microscope and analyzed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Alkaline phosphatase staining assay

Upon the termination of treatment (7 or 14 days), the cells were washed twice with PBS and fixed in 4% paraformaldehyde at room temperature. ALP staining was performed with the BCIP/NBT Alkaline Phosphatase Color Development Kit following the standard protocol.

Quantitative Real-Time reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed to characterize and establish which TM concentration was optimal for *in vitro* use to model acute ER stress, and to assess the mRNA level of osteogenic differentiation markers in various groups. On the designed timepoints, the total RNA was isolated and reversely transcribed into cDNA using a reverse transcription kit, then qRT-PCR was performed using LightCycler® 480 Instrument II Real-Time PCR System (Roche Molecular Systems, Inc.) according to the manufacturer's instructions. β-actin was used as a housekeeping gene. The primer sequences of osteogenic differentiation markers are provided in Table 1.

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical analysis. All data were evaluated for statistical significance using oneway ANOVA and post hoc analysis was done by Tukey's test for multiple comparisons. Data were expressed as the mean ± SD (standard deviation) and *p* values < 0.05 was considered to indicate a statistically significant difference.

βactin-mouse-F	GTGACGTTGACATCCGTAAAGA
βactin-mouse-R	GCCGGACTCATCGTACTCC
PERK-mouse-F	CCGTGACCCATCTGCACTAAT
PERK-mouse-R	CATAAATGGCGACCCAGCTT
eIF-2α-mouse-F	CCCCTCATTTGTTCGACTGTATC
eIF-2α-mouse-R	CCAGCACAGCAGTAGCTTTTT
ATF4-mouse-F	CCTGAACAGCGAAGTGTTGG
ATF4-mouse-R	TGGAGAACCCATGAGGTTTCAA
CHOP-mouse-F	CCAGAATAACAGCCGGAACC
CHOP-mouse-R	TCCTGCAGATCCTCATACCA
Collagen I-mouse-F	GGAGAGAGCATGACCGATGG
Collagen I-mouse-R	GGGACTTCTTGAGGTTGCCA
ALP-mouse-F	CCCCATGTGATGGCGTAT
ALP-mouse-R	CGGTAGGGAGAGCACAGC
BSP-mouse-F	ATGGAGACGGCGATAGTTCC
BSP-mouse-R	CTAGCTGTTACACCCGAGAGT
OPN-mouse-F	CCGAGGTGATAGCTTGGCTTATG
OPN-mouse-R	TGGCTGCCCTTTCCGTTGTT

Table 1: The primer sequences of osteogenic differentiation markers.

Results

Characterization of tunicamycin (TM) inducing ER stress in MC3T3-E1 cells

To assess the effective TM concentration at inducing ER stress in osteoblast model, we treated MC3T3-E1 cells with TM (0.5 µg/ml, 1 µg/ml, 2 µg/ml) diluted in medium. Treatment of MC3T3-E1 cells with 2 µg/ml TM yielded a consistent heightened ER stress response as compared to the other concentrations. Key acute ER stress markers PERK, eIF-2α, ATF4 transcript were consistently induced with TM relative to control (Figure 1A-1C). Up-regulation of C/EBP homology protein (CHOP) gene expression was seen with TM doses tested (Figure 1D). To eliminate the possibility of cytotoxicity effects of different concentrations, we performed a cell viability assay to further assess their efficacy at the preferred doses. Viability was quantifiably reduced with all TM doses relative to

control (Figure 1E). In fact, TM at a dose of 2 μg/ml yielded robust and stable ER stress induction though slight adverse effects on cell viability relative to control and other doses tested.

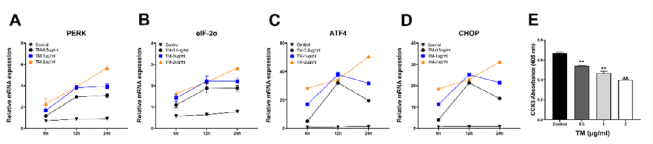

Figure 1: Tunicamycin induces ER stress in MC3T3-E1 cells. (A-D) MC3T3-E1 cells were exposed to TM (0.5 μg/ml, 1 μg/ml, 2 μg/ml) for 24 hours, and total RNA was extracted and transcribed to cDNA. Relative mRNA levels of the indicated ER stress genes were quantified using qRT-PCR. (E) Cell viability assay was performed after MC3T3-E1 cells were incubated with various TM doses. TM: tunicamycin; PERK: Protein kinase R-like endoplasmic reticulum kinase; eIF-2α: eukaryotic initiation factor2; ATF4: Activating transcription factor 4; CHOP: C/EBP homology protein. Each value is presented as the mean ± SD of three independent experiments. *P < 0.05 and **P < 0.01 vs. the control group.

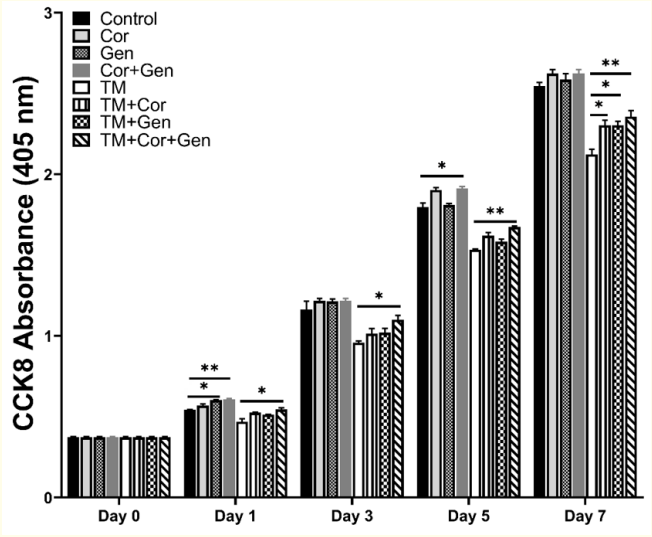

Figure 2: The ameliorated effects of combined cordycepin and genistein treatment on MC3T3-E1 cells viability against the ER stress. Each value is presented as the mean ± SD of three independent experiments. TM: tunicamycin; Cor: cordycepin; Gen: genistein. *P < 0.05 and **P < 0.01 vs. the control group.

The administration of cordycepin and genistein combination ameliorated cell viability in a TM-induced ER stress osteoblast model

As discussed above, a TM-induced ER stress osteoblast model was established in this study to explore the effects of cordycepin and genistein combination on MC3T3-E1 cells viability. Initially, the influence on cell proliferation after exposure to 10 μM cordycepin and 10 μM genistein for 1, 3, 5 and 7 days, either separately or in combination, was tested via CCK8 assay. MC3T3-E1 cells viability was not significantly altered after exposure to cordycepin or genistein alone, but after treatment with the combination a trend towards slight proliferation induction was noted (P < 0.05). The addition of 2 μg/ml TM reduced the proliferation of MC3T3-E1 cells (~25% reduction). After application of cordycepin or genistein alone, proliferation was not different from TM induced controls. Treatment with the combined cordycepin and genistein only revealed partial recovery of proliferation induction (~118% induction). Collectively the administration of cordycepin and genistein combination ameliorated ER stress-induced proliferation suppression in MC3T3-E1 cells (Figure 2).

The administration of cordycepin and genistein combination accelerated cell migration in ER stress osteoblast model

To assess which of drug, cordycepin or genistein is more effective at stimulating migration in ER stress osteoblast model, by using transwell chamber assay we treated MC3T3-E1 cells with both drugs, either alone or in combination, and performed a comparative analysis. Thus, compared to the control, the combination treatment seemed to have a larger influence on the migrating abilities of the investigated samples than the treatment with cordycepin or genistein alone (P < 0.01, Figure 3A). The addition of 2 μg/ml TM suppressed the migrating abilities of MC3T3-E1 cells (~ 20% reduction).

Cordycepin showed accelerated effect on MC3T3-E1 cells migration under ER stress condition (P < 0.05), but less effective as that of genistein and the combined treatment (P < 0.01, Figure 3B). Collectively, ER stress-induced suppression on MC3T3-E1 cells migration was restored to control levels by the administration of cordycepin and genistein combination.

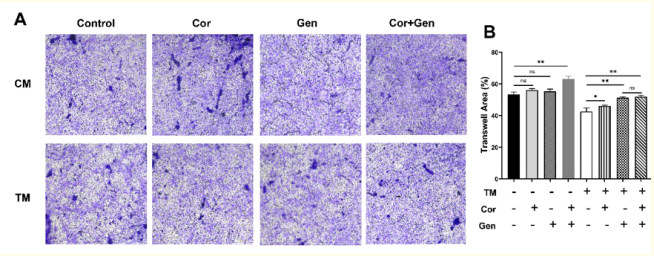

Figure 3: The accelerated effects of combined cordycepin and genistein treatment on MC3T3-E1 cells migration against the ER stress. The migrating ability of MC3T3-E1 cells was examined by transwell chamber assay. (A) MC3T3-E1 cells were chemically induced with cordycepin, genistein or their combination. Migrated cells were imaged under an inverted microscope (A x100; scale bar, 250 μm) and the data were digitally recorded. (B) The migrating area per five randomly selected fields from every transwell filter was shown as the percentage compared to control, bar graph. TM: tunicamycin; Cor: cordycepin; Gen: genistein. Each value is presented as the mean ± SD of three independent experiments. *P < 0.05 and **P < 0.01 vs. the control group.

The administration of cordycepin and genistein combination promoted MC3T3-E1 cells differentiation in ER stress osteoblast model

In order to elucidate the combinatory effects of cordycepin and genistein on osteogenic differentiation of MC3T3-E1 cells, qRT-PCR assay in parallel to ALP staining were performed. The data demonstrated that both methods revealed a strong up-regulation of ALP expression after simultaneous stimulation with cordycepin and genistein, in the absence or presence of TM-induced ER stress ($P < 0.01$). Additionally, the single application of 10 μM genistein caused significantly higher ALP expression levels than the treatment with 10 μM cordycepin under ER stress condition ($P < 0.01$). Similar findings were also observed in qRT-PCR, confirming our ALP staining data (Figure 4A). As shown in qRT-PCR array, osteogenic differentiation associated markers such as collagen type I, BSP and OPN were remarkably up-regulated after the combined treatment ($P < 0.01$) but notably down-regulated in the presence of TM stimulation ($P < 0.01$). However, the suppressing expression of these genes was significantly restored to a higher level after the administration of cordycepin and genistein combination ($P < 0.01$, Figure 4B). Collectively, the results of this study further highlighted the fact that combination of cordycepin and genistein restored the suppression, which was induced by TM-stimulated ER stress, on osteogenic differentiation in MC3T3-E1 cells.

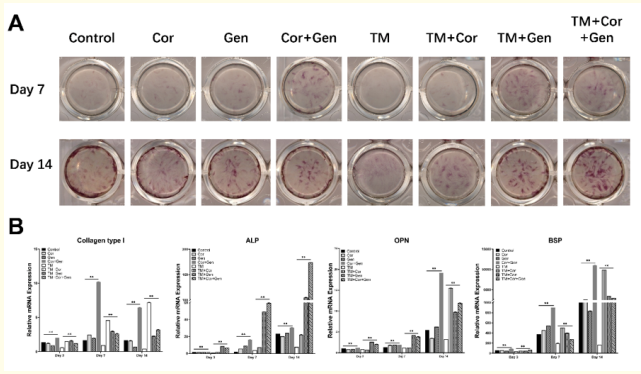

Figure 4: The promoted effects of combined cordycepin and genistein treatment on MC3T3-E1 cells differentiation against the ER stress. (A) After pretreated with or without 2 μg/ml TM for 24 hours, MC3T3-E1 cells were exposed to cordycepin and genistein, either separately or in combination, for 7 and 14 days. ALP was stained by BCIP/NBT. The ALP staining was imaged under an inverted microscope and the data were digitally recorded. (B) Upon the termination of treatment, total RNA was extracted and transcribed to cDNA. Relative mRNA levels of collagen type I, ALP, BSP and OPN were quantified using qRT-PCR and normalized to β-actin level. TM: tunicamycin; Cor: cordycepin; Gen: genistein; ALP: alkaline phosphatase; BSP: bone sialoprotein; OPN: osteopontin. Each value is presented as the mean ± SD of three independent experiments. *P < 0.05 and **P < 0.01 vs. the control group.

Discussion

The present study demonstrated that the administration of cordycepin and genistein combination slightly enhanced MC3T3-E1 cells proliferation, while notably accelerated cell migration and differentiation. Simultaneously, in a TM-induced ER stress osteoblast model, ER stress-induced suppression on MC3T3-E1 cells proliferation, migration and differentiation was significantly restored by the combined treatment. Therefore, cordycepin in the presence of genistein exerts synergistic protective activity on osteoblast and

this effect may contribute, at least in part, to the efficacy of therapy in bone-related diseases associated with ER stress activation.

The ability to accurately model ER stress is critical for unearthing the pathology and development of the conditions of which ER stress has been involved in. Recent studies showed that ER stress played a key role in the pathogenesis of osteolytic bone diseases, and some synthetic chemicals attenuated unloading-induced bone loss by altering proliferation and differentiation of osteoblasts via eIF-2α signaling [3,4]. PERK/eIF2α/ATF4 signaling is one of the three principal signaling pathways in the unfolded protein response in ER stress. When activated, PERK phosphorylates eIF2α, inhibits general translational activities and reduces the flux of proteins entering into the ER. It has been demonstrated that short-term ER stress can protect cell through increasing p-eIF2α and ATF4 level, whereas long-term stress induces apoptosis by inhibiting p-eIF-2α and ATF4. For long-term stress, the expression of CHOP, which regulates expression of pro-apoptotic factors and has the propensity to drive apoptosis, showed a time-dependent increase [3]. Tunicamycin is a strong ER stress inducer. Previous studies revealed that tunicamycin at levels up to 2 μg/ml up-regulated ER stress related genes and had no significant influence on the cell viability of MC3T3-E1 cells [15]. Similar results were observed in our study that tunicamycin worked as a rapid and efficacious inducer of ER stress in MC3T3-E1 cells and consistently upregulated key ER stress markers PERK, eIF-2α, ATF4 and CHOP. This ER stress osteoblast model was considered good model for drug screening in bone-related diseases. However, the slight adverse effects on cell viability in this ER stress osteoblast model prompted that TM treatment *in vitro* could potentially result in the lack of observed efficacy or even toxicity.

Natural products with minimal toxicity and potent anti-inflammatory activity have been discovered to be novel biocompatible substances for the prevention and inhibition of osteolytic bone diseases, such as osteoporosis. Cordycepin, a natural structural analog of adenosine, exerts multiple physiological and pharmacological activities, and mediates many fundamental cell functions including proliferation, adhesion, migration, gene expression and immune reactions [5,16-18]. In the past few years, the regulating effects of cordycepin in inflammatory osteolytic injury aroused increasing attentions. Cordycepin is known to act as an anti-inflammatory and osteoprotective agent in the experimental models of osteoporosis [9,10]. Additionally, the therapeutic effect of cordycepin is not ideal due to its rapid degradation by adenosine deaminase. In order to improve its protective effect and reduce dosage, synergistic effects of cordycepin with other compounds are investigated. Genistein, as a major phytoestrogen of soy, have been proposed as potential alternatives to estrogen replacement therapy for the prevention and treatment of osteoporosis. Genistein is also known to inhibit the activity of adenosine deaminase and function by inhibiting the degradation of cordycepin in cells [12]. It is indicated that cordycepin inhibited ER stress-induced apoptosis via reinforcement of the pro-survival eIF-2a signaling but suppressing downstream induction of ATF4 and expression of CHOP [14]. It is well known that ER stress contributes to genistein-induced apoptosis in different cancer cells. Our results revealed that MC3T3-E1 cells viability was not significantly altered after exposure to cordycepin or genistein alone, however, the administration of cordycepin and genistein combination ameliorated ER stress-induced proliferation suppression in MC3T3-E1 cells.

The recruit of osteoblast precursor cells into the sites of resorption is an early essential process in bone remodeling [19]. Previous studies have shown that cordycepin has osteoprotective effects in bone fracture and osteoporosis treatment [20], but there have been few reports on altering early events, such as migration and adhesion of osteoblast. Genistein is known to stimulate osteoblast migration [21]. Our results revealed that the combined treatment increased the average migration velocity of MC3T3-E1 cells. Furthermore, the administration of cordycepin and genistein combination markedly rehabilitated ER stress-inhibited migratory capacities in osteoblasts.

It has been reported that ER stress occurs during osteoblast differentiation and activates the PERK/eIF2α/ATF4 signaling pathway followed by the promotion of gene expression essential for osteogenesis, such as osteocalcin and BSP [22]. It is shown that tunicamycin inhibited osteoblast differentiation by suppressing ALP and osteocalcin expression [23]. A recent *in vitro* study substantiated that cordycepin stimulated osteoblast differentiation and mineralization in MC3T3-E1cells [24]. Genistein significantly increased osteocalcin expression, extracellular collagen deposition, and alkaline phosphatase activity [21]. Similar results were observed in this study that combination of cordycepin and genistein revealed a strong up-regulation of ALP expression as well as osteogenic markers including collagen type I, BSP and OPN. Collagen type I and ALP,

which affect the morphology and cytoskeleton, are early markers for osteoblastic differentiation. BSP and OPN are expressed at late stages of osteoblastic differentiation and are closely correlated with the appearance of calcium nodules. These results signified that the combined treatment accelerated osteoblast differentiation and maturation at early stage. Meanwhile the combined treatment restored the TM-induced suppression on MC3T3-E1 cells differentiation.

Conclusion

Despite the accurate targets for cordycepin and genistein and modes of actions have not been understood completely yet, these data hinted the clue that the combination of cordycepin and genistein exerts the synergistic osteoprotective activities under ER stress condition. But because there is very scarce evidence on this effect, more researches are needed on this theme. The current experiments also provide the possibility that the co-administration of cordycepin and genistein serve as a host risk reduction strategy for osteolytic bone diseases by suppressing ER stress.

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