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Original Paper

Butein Activates Autophagy Through AMPK/TSC2/ULK1/mTOR Pathway to Inhibit IL-6 Expression in IL-1β Stimulated Human Chondrocytes

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Key Words
Butein • Osteoarthritis • Autophagy • Inflammation • AMPK • mTOR • TSC2 • ULK1

Abstract
Background/Aims: Butein (2',3,4,4'-Tetrahydroxychalcone), a polyphenol produced by several plants including *Butea monoserpma*, has been reported to exert potent anti-inflammatory activity but the mechanism remains unknown. In the present work we investigated the mechanism of Butein-mediated suppression of IL-6 expression in normal and human osteoarthritis (OA) chondrocytes under pathological conditions. ***Methods:*** Expression level of interleukin-6 (IL-6) protein in OA cartilage was analyzed by immunohistochemistry using a validated antibody. Chondrocytes derived from normal or OA cartilage by enzymatic digestion were pretreated with Butein followed by stimulation with interleukin-1β (IL-1β) and the levels of IL-6 mRNA were quantified by TaqMan assay and the protein levels were measured by Western immunoblotting. Autophagy activation was determined by Western blotting and confocal microscopy. Autophagy was inhibited by siRNA mediated knockdown of ATG5. ***Results:*** Expression of IL-6 protein was high in the OA cartilage compared to smooth cartilage from the same patient. OA chondrocytes and cartilage explants stimulated with IL-1β showed high level expression of IL-6 mRNA and protein. Butein increased the phosphorylation of AMPKα$^{Thr-172}$, TSC2$^{Ser-1387}$ and ULK1$^{Ser-317}$ and inhibited the phosphorylation of mTOR$^{Ser-2448}$ and its downstream target p70S6K and increased autophagy flux that correlated with the suppression of the IL-1β mediated expression of IL-6 in normal and OA chondrocytes. In OA chondrocytes with siRNA-mediated knockdown of ATG5 expression, treatment with Butein failed to activate autophagy and abrogated the suppression of IL-1β induced IL-6 expression. ***Conclusion:*** Our findings demonstrate for the first time that Butein activate autophagy in OA chondrocytes via AMPK/TSC2/ULK1/mTOR pathway. Additionally, activation of autophagy

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was essential to block the IL-1β-induced expression of IL-6 in OA chondrocytes. These data support further studies to evaluate the use of Butein or compounds derived from it for the management of OA.

Introduction

Osteoarthritis (OA) occurs due to irreversible degeneration of articular cartilage, remodeling of subchondral bone, and progressive pain and joint dysfunction. OA is a leading cause of disability in adults resulting in a severe burden on the affected individuals, their families and the society. Development of OA depends on genetic predisposition, physical activity, health status and age. Aging-related OA is symptomatic in at least 30% of senior citizens, and major trauma to joints leads to the development of post-traumatic OA relatively quickly, even in young adults. The prevalence of OA is on the rise worldwide due to steady increase in life expectancy and obesity rates. Recent studies have shown that inflammation plays a key role in the development and pathogenesis of osteoarthritis wherein both the chondrocytes and synovium produce proinflammatory mediators in the joint [1] including the high level expression of IL-6 [2]. Increased expression of inflammatory cytokines and chemokines cause aberrant changes in differentiated chondrocytes function which leads to an excess of chondrocyte catabolic activity, mediated by factors including matrix metalloproteinases (MMPs) and aggrecanases [3]. No current medical treatments are available to prevent progressive joint deterioration and disability characteristic of OA leaving total joint arthroplasty, which requires major surgery and involves artificial implants that seldom lasts for >15 years, as the only viable option. It is thus crucial to find and test compounds with little or no toxicity to prevent and block joint deterioration in people with symptomatic OA or at high risk for the development of OA, e.g. due to trauma.

Autophagy, a defensive cellular pathway involved in the degradation and recycling of molecular aggregates and dysfunctional cellular organelles, is a key player in the maintenance of cellular homeostasis [4]. Autophagy is upregulated during stress conditions (starvation, oxidative stress, inflammation, etc) and functions as a critical regulator of cellular metabolism [4]. Dysregulation of autophagy has been associated with the pathogenesis of multiple human diseases [5-7]. We and others have shown that autophagy is downregulated in OA cartilage and in IL-1β treated chondrocytes [8, 9]. Decline in autophagy disrupts the clearance of dysfunctional mitochondria and enhances oxidative stress, inflammation and apoptosis [10]. Autophagy inhibition in chondrocytes resulted in upregulation of cartilage extracellular matrix degrading proteases leading to the development of OA [11, 12]. Studies have demonstrated that inhibition of mammalian target of rapamycin (mTOR) pathway acts as a protective mechanism against the development and progression of OA [13, 14]. Activation of AMP activated protein kinase α (AMPKα), a positive regulator of autophagy, by metformin has been found to suppress the progression of inflammatory arthritis [15].

Recent studies on traditional medicines have shown that many nutraceuticals have anti-inflammatory activities and are good candidate for the prevention and or treatment of OA [16-18]. Butein (2',3, 4,4'-Tetrahydroxychalcone), a polyphenol produced by several plant species, has been reported to have anti-inflammatory activity in several systems [19-21]. Butein increased the activity of SIRT1 and enhanced the life span of *Saccharomyces cerevisiae* [22]. Butein has been shown to have anti-cancer activity through the inhibition of Akt/mTOR activity [23]. Butein has also been shown to subdue the proinflammatory effects of IL-1β on chondrocytes [20] however, the mechanism was not reported and largely remains unknown. We hypothesized that Butein exert the anti-inflammatory effects via autophagy activation in OA chondrocytes. To test our hypothesis, we treated human chondrocytes with Butein and studied autophagy activation, mechanism of autophagy activation and its effect on the regulation of IL-6 expression under pathological conditions. Our results demonstrated that Butein activated autophagy in human chondrocytes through AMPKα/TSC2/ULK1/mTOR pathway. Furthermore, suppression of IL-1β-induced expression of IL-6 by Butein was

dependent on the activation of autophagy as the suppressive effect was completely lost with the inhibition of autophagy. These results identify activation of autophagy via AMPKα/TSC2/ULK1/mTOR pathway by Butein as a critical event in the suppression of IL-6 expression in human chondrocytes under pathological conditions. These data also support further studies on the evaluation of Butein or compounds derived from it as potential therapeutic agents for the treatment and/or prevention of OA.

Materials and Methods

Reagents and antibodies

Butein was procured from Extrasynthese (#1103 S, Extrasynthese, France). The culture media (DMEM-F12) was procured from Lonza (#12-7192, Walkersville, MD). Pronase and collagenase were from Roche Diagnostics (#11459643001 and #11088793001 respectively). TaqMan assays for IL-6 and β-Actin were from Integrated DNA Technologies (IDT, Coralville, Iowa). Validated antibodies against human IL-6 (#SC-130326) and β-Actin (#SC-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against MAP1LC3B (#2775S), P-mTOR (#5536S), mTOR (#2983S), P-AMPKα (#2535S) AMPKα (#2603S), P-ULK1 (#12753) and ULK1 (#8054) were from Cell Signaling Technology (CST, Beverly, MA). Lysotracker Red (#L7528) for lysosomes staining was obtained from Life Technologies.

Chondrocytes preparation and maintenance

The study protocol to use discarded, de-identified human cartilage tissue was reviewed and approved by the Institutional Review Board (IRB) of Northeast Ohio Medical University, Rootstown, Ohio as a "non-human subject study under 45 CFR". OA cartilage was from donors who underwent total knee arthroplasty (TKA) and normal cartilage was from donors with no known history of arthritic disease and was obtained from NDRI (National Disease Research Interchange, PA). Chondrocytes were prepared by sequential digestion with Pronase and Collagenase and maintained in DMEM-F12 as described previously [10].

Histopathological analysis of cartilage tissue

Cartilage tissue was obtained from donors after the knee replacement surgery. The damaged and undamaged areas of cartilage were identified by India ink staining, full thickness cartilage pieces were resected and fixed with 4% paraformaldehyde. The fixed tissues were dehydrated by passing through a graded series of ethanol (50%, 70%, 90% and 100%) followed by Xylene wash and embedded in paraffin and 5μM thick sections were cut and stained with Safranin O/Fast Green protocol. For immunohistochemistry, the tissue sections were deparaffinized in Xylene for 5 minutes and rehydrated by passing through a series of graded ethanol (100%, 90%, 70% and 50%) as above and finally washed with 1X-TBS for 10 minutes, and antigen retrieval was performed in 10mM citrate buffer (pH 6.0) using a microwave oven for 3 minutes at 700 W. After cooling, sections were washed with 1XTBS and endogenous peroxidase activity was neutralized by 3% hydrogen peroxide in 1XTBS. Sections were washed again with TBS and blocked in 5% Goat serum for 30 minutes at room temperature and incubated in primary antibody for overnight at 4 °C. Sections were washed with 1XTBS and incubated with HRP conjugated secondary antibody for 1 hr followed by washing and developing with DAB substrate kit (#34002, Pierce).

Treatment of chondrocytes with IL-1β and Butein

Normal and OA Chondrocytes were seeded in 6 wells plates ($1x10^6$ per well) in complete media (DMEM-F12 supplemented with 10% fetal calf serum) and cultured for 48-72 hr and were then pretreated with 10μg/ml (36μM) or different concentrations of Butein for 2 hr (or for the indicated time) followed by stimulation with IL-1β (1ng/ml). 0.1% DMSO alone treatment of chondrocytes served as control.

Chondrocyte viability assay

OA chondrocytes were seeded in 96 well plates (20, 000 cells/well) and treated with different concentration of Butein for 24 hr or with 10μg/ml (36μM) of Butein for up to 72 hr. The viability of Butein treated chondrocytes was determined by MTT assay as described previously [24].

Measurement of autophagy and autophagy flux

Autophagy activation in Butein treated OA chondrocytes was measured by immunoblot analysis of the levels of MAP1LCB-II and the formation of autophagosomes by immunofluorescence microscopy as described previously [8]. Autophagy activation by Butein was determined by taking the ratio of MAP1LC3B-II to β-actin using Image J, as the ratio of MAP1LC3B-II to MAPLC3B-I may not be appropriate measurement of autophagy activation [25]. The number of autophagosomes were quantified by counting the LC3 positive (green) puncta in around 50 chondrocytes in control and Butein treated chondrocytes using Imgae J. Autophagy flux was measured by treating the OA chondrocytes with Butein overnight followed by treatment with Bafilomycin for 2hr before lysate preparation and Western blotting to measure the levels of MAP1LCB-II protein. The fusion of the autophagosomes with lysosomes represents the last step of the autophagy pathway. We confirmed the fusion of autophagosomes with lysosomes in Butein treated OA chondrocytes by immunofluorescent staining and confocal microscopy using the Olympus FV1000 confocal microscope as described previously [10]. In brief, chondrocytes were seeded in 8 well chamber slides and treated with Butein or DMSO alone as control and fixed with 4% paraformaldehyde and stained for MAP1LC3B. For lysosomal staining, Lysotracker Red was used to stain the cells for 30 minutes before the end of the experiment.

Determination of ROS levels

ROS levels in OA chondrocytes were measured by DHR123 or DCFDA staining followed by flow cytometry as described previously [8]. In brief, OA chondrocytes were stained with DCFDA or DHR123 for 30 minutes followed by treatment with Butein for 5 minutes, washed with PBS and analyzed for DCFDA or DHR123 fluorescence by flow cytometer (BD Accuri C6) and the data was analyzed by FlowJo software.

Western blotting and ELISA of IL-6

Normal or OA chondrocytes treated with Butein or IL-1β were lysed in RIPA buffer supplemented with phosphatase and protease inhibitors cocktail. The lysate (20 μg) was resolved on 10% or 12% SDS-PAGE and Western blotting with validated antibodies was done as described previously [26]. Chondrocytes stimulated with IL-1β secrete very high amount of IL-6 in the culture supernatant that can be analyzed by ELISA as well as Western blotting [2]. So, we collected the culture supernatants from Butein and/or IL-1β treated chondrocytes and determined the levels of IL-6 by ELISA or equal volume (30 μl) was used for Western blotting to determine the secreted levels of IL-6.

RNA isolation and TaqMan Assay

Normal or OA chondrocytes seeded in 6 well plate ($1x10^6$ cells/well) were treated with IL-1β (1ng/ml) or Butein (10μg/ml or 36μM) for 2 hrs followed by IL-1β (1ng/ml) treatment for overnight. Total RNA was isolated using RNeasy kit (#74104, Qiagen) and gene expression was quantified using TaqMan assay as described [10].

siRNA transfection

siRNA for ATG5 knockdown was procured from Qiagen (#GS9474). OA chondrocytes (70% confluent) were transfected with siATG5 or Control siRNA using X-tremeGENE siRNA transfection reagent (#4476093001, Roche). siATG5 or siControl transfected chondrocytes were pretreated with Butein for 2 hrs followed by stimulation with IL-1β. siRNA-mediated knockdown of ATG5 expression was confirmed by Western blotting using a validated antibody (#12994, CST). Effect of ATG5 depletion on autophagy was assessed by immunofluorescence staining of autophagosomes with MAP1LC3B antibody (#2775S, CST) or the levels of MAP1LC3B-II formation by Western blotting. The effect of ATG5 knockdown on Butein mediated suppression of IL-6 was assessed by pretreating siControl or siATG5 transfected chondrocytes with Butein followed by stimulation with IL-1β. The chondrocytes were harvested for total RNA preparation using a commercially available kit (#74104, Qiagen) that was used subsequently for cDNA preparation and quantification of IL-6 mRNA levels by TaqMan assay.

Statistical analysis

All the data were analyzed for statistical significance using the software SigmaPlot version 12.3 (Systat Software Inc.) Values are represented as Mean±SD. The statistical significance between two groups were analyzed by t-test whereas statistical significance between experimental and control groups were determined by using one-way analysis of variance (ANOVA) followed by Tukey's test for post hoc analysis. Each experiment was repeated at least three times using chondrocytes from three independent patient samples. P value < 0.05 was considered as statistically significant.

Results

IL-6 was highly expressed in the damaged areas of OA cartilage and in IL-1β treated chondrocytes

OA joints have high levels of proinflammatory cytokines, chemokines and prostaglandin levels [1, 27]. To measure the levels of IL-6, we isolated cartilage tissue pieces from smooth (unfibrillated or undamaged) and damaged (fibrillated) areas of cartilage from OA donors and analyzed for proteoglycan levels by Safranin O-fast green staining. In comparison to smooth cartilage, the cartilage from damaged area showed significant loss of proteoglycan levels (Fig. 1A, left panel). Immunohistochemistry of IL-6 on cartilage sections showed high levels of IL-6 expression in the damaged areas, in comparison to undamaged areas (Fig. 1A, right panel). Proinflammatory cytokine IL-1β is expressed at high levels in OA joints and treatment of chondrocytes with recombinant human IL-1β induces the expression of OA signature genes [28, 29]. Analysis of IL-6 mRNA and protein levels showed upregulation of IL-6 expression in IL-1β stimulated OA chondrocytes (Fig. 1B and 1C, respectively). These results showed that OA joints have high levels of IL-6 expression and IL-1β treatment of chondrocytes increased the expression of IL-6 mimicking the pathological conditions of OA.

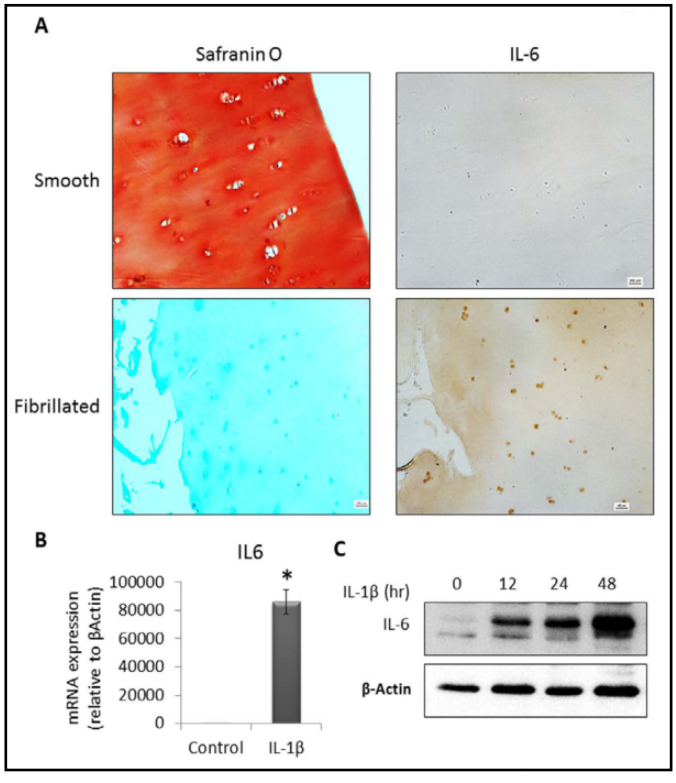

Fig. 1. IL-6 is highly expressed in the damaged areas of OA joints. (A) Cartilage pieces were taken from the damaged and smooth areas of cartilage obtained from OA donors and processed for histopathology. The cartilage sections were analyzed by Safranin O-fast green staining to determine the level of degradation of proteoglycan (left panel) and immunohistochemistry to determine the levels of IL-6 (right panel). (B) Chondrocytes were isolated from OA donors and maintained in DMEM/F12 supplemented with 10% serum. Chondrocytes were left untreated (control) or treated with IL-1β (1 ng/ml) for overnight (16 hrs) and cells were harvested for total RNA preparation and the mRNA levels of IL-6 was analyzed by RT-qPCR using TaqMan assays. β-Actin was used as normalization control, (*P<0.005). (C) Chondrocytes were treated with IL-1β (1 ng/ml) for indicated time and the lysate was prepared and analyzed for the IL-6 expression levels by Western blotting.

Butein did not affect the viability of OA chondrocytes in vitro

There are reports that have shown that Butein reduces cell viability and enhances apoptosis in multiple cancer cells including ovarian cancer cells [30] cervical cancer cells [23], neuroblastoma [31] and T-cell lymphoma [32]. In order to study the effects of Butein on the viability of OA chondrocytes, OA chondrocytes were treated with different concentrations of Butein [0.6 μg/ml -10 μg/ml (or 2.25 μM -36 μM)] for 24 hr or with 10 μg/ml Butein for up to 72 hr and the viability was analyzed by MTT assay. The results showed no significant toxic effects of Butein on the viability of primary human OA chondrocytes *in vitro* (Fig. 2A and 2B). Based on this data we conclude that Butein was non-toxic to human OA chondrocytes *in vitro* in the dose range tested.

Butein inhibited the IL-1β induced expression of IL-6 in normal and OA chondrocytes

In order to study the anti-inflammatory effect of Butein on chondrocytes under pathological conditions, we used chondrocytes isolated from donors with no known history of OA and treated with IL-1β to mimic pathological conditions which has been shown by us and others to induce the expression of OA signature genes [8, 28]. We pretreated the chondrocytes with Butein (10 μg/ml) for 2 hr (0.1% DMSO alone was used as control) followed by IL-1β (1ng/ml) treatment for 16 hr and harvested for either RNA isolation or cell lysate preparation to analyze the levels of IL-6 mRNA and protein expression respectively. Stimulation of normal human chondrocytes with IL-1β (1 ng/ml) increased the expression of IL-6 mRNA (Fig. 2C) and protein levels (Fig. 2D) significantly compared to controls ($P<0.005$). Importantly, pretreatment of chondrocytes with Butein dramatically suppressed the IL-1β induced expression of IL-6 at mRNA and protein levels (Fig. 2C and 2D respectively, $P<0.05$). We observed similar results with chondrocytes isolated from donors with OA (Fig. 2E and 2F). To rule out that this effect was due to the possible dedifferentiation of chondrocytes in monolayer culture, we prepared cartilage explants from the undamaged areas of OA cartilage and treated them with Butein and IL-1β as above. The level of IL-6 protein in the cartilage explants was then analyzed by immunohistochemistry and in the culture supernatant by ELISA and immunoblotting. IL-1β stimulation resulted in increased expression of IL-6 protein in human cartilage explants which was suppressed by pre-treatment with Butein (Fig. 2G, 2H and 2I). These results demonstrated that Butein has anti-inflammatory property and suppresses the IL-1β induced expression of IL-6 in human normal and OA chondrocytes and OA cartilage explants under pathological conditions.

Butein suppressed the IL-6 expression by inducing autophagy

Autophagy is a protective mechanism against cellular stress and inflammation and OA pathogenesis [4, 11]. We investigated whether (1) Butein activates autophagy in chondrocytes; and (2) whether Butein-mediated suppression of IL-1β induced expression of IL-6 was via autophagy activation in human chondrocytes. To investigate the first question, OA chondrocytes were treated with Butein for overnight and analyzed for the expression levels of MAP1LC3B-II protein, a known marker of autophagy activation [25]. Treatment of OA chondrocytes with Butein increased the levels of MAP1LC3B-II protein in a dose dependent manner indicating the activation of autophagy (Fig. 3A and 3B). We further confirmed the activation of autophagy by immunofluorescence staining of endogenous MAP1LC3B protein followed by quantification of autophagosomes. This analysis demonstrated that the number of autophagosomes was significantly increased in OA chondrocytes treated with Butein (Fig. 3C and 3D). Furthermore, we measured the autophagy flux by treating OA chondrocytes with Butein for overnight followed by treatment with Bafilomycin-A1 for 2 hr [33] and measured the levels of MAP1LC3B-II protein by immunoblotting. The increase in MAP1LC3B-II levels by Butein treatment in OA chondrocytes was further increased by Bafilomycin-A1 treatment of OA chondrocytes (Fig. 3E) indicating that the treatment of OA chondrocytes with Butein increased the autophagy flux. We also found that the colocalization of autophagosomes (stained with MAP1LC3B antibody, green color) with lysosomes (stained with Lysotracker

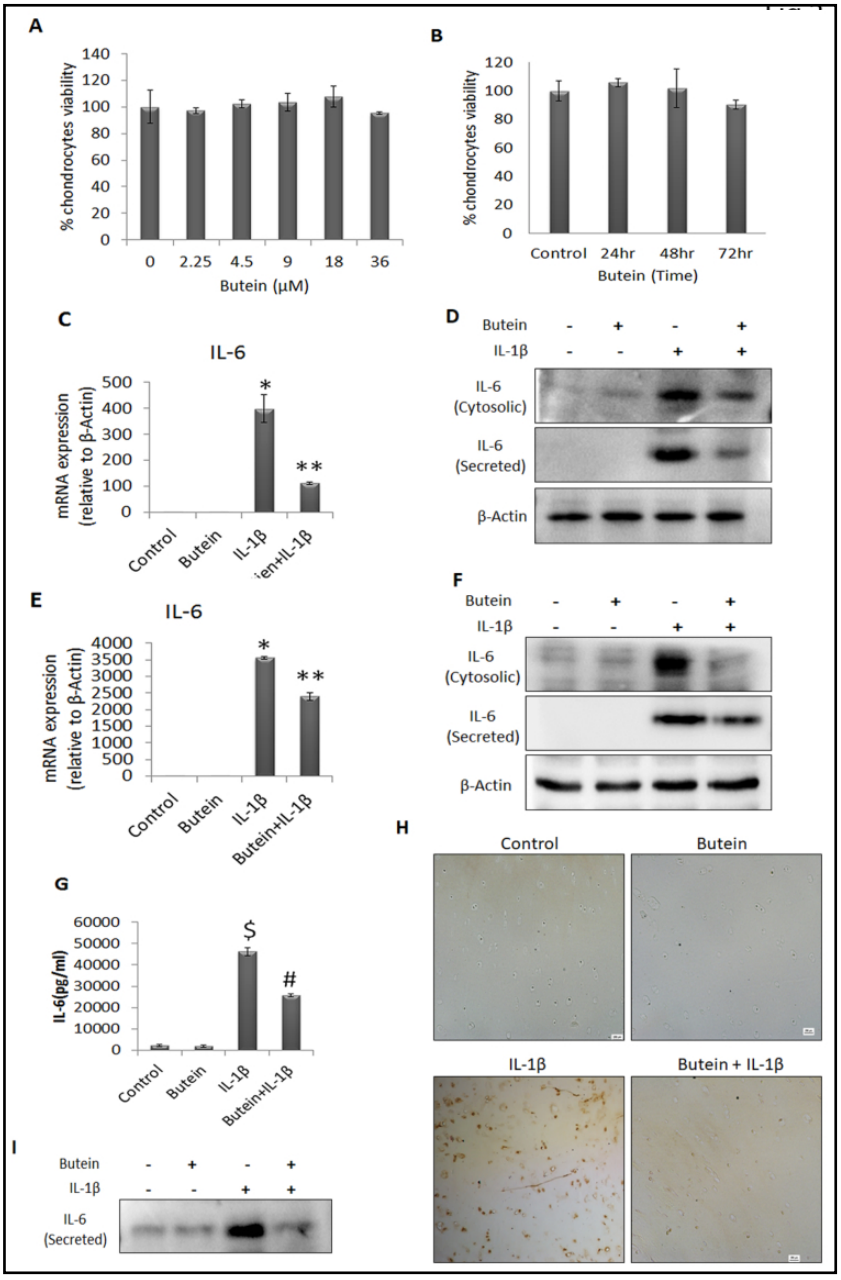

Fig. 2. Butein is not toxic and inhibits IL-1β induced expression of IL-6 in normal and OA chondrocytes. Chondrocytes were seeded in 6-well plates ($1x10^6$ cells/well) and treated with (A) different concentrations of Butein (36 to 2.25 μM or 10 to 0.6μg/ml) for 24 hours and (B) 10μg/ml (or 36μM) for 24, 48 and 72 hrs. DMSO alone (0.1%) was used as control. Chondrocytes viability was measured by MTT assay. (C) and (D) Chondrocytes prepared from normal donors (no known history of any rheumatic disease) were pretreated with Butein (10μg/ml or 36μM) for 2 hrs followed by addition of IL-1β (1 ng/ml) for overnight. Chondrocytes were harvested either for RNA isolation to determine the expression levels of IL-6 mRNA or cell lysate preparation and immunoblotting of IL-6 (*P<0.005, **P<0.05). Culture supernatants were also collected and equal volume of it was loaded on 12% SDS-PAGE to determine the levels of IL-6 secreted in the culture supernatant. β-Actin was used as loading or normalization control. (E) and (F) Chondrocytes were isolated from OA donors and treated as above with Butein and IL-1β to measure the levels of IL-6 mRNA and protein by qPCR and Western blotting. (G) The IL-6 levels in the culture supernatant were determined by ELISA ($P<0.05 vs Control, #P<0.05 vs IL-1β). (H) Cartilage explants were prepared from the undamaged areas of cartilage obtained from OA donors and maintained in DMEM/F12 supplemented with 10% serum. The explants were treated with IL-1β (10ng/ml) for 72 hrs in the presence or absence of Butein (10μg/ml or 36μM). The explants were fixed with 4% PFA and processed for histological analysis of IL-6 expression levels. (I) The culture supernatant from the above experiment was analyzed for secretory levels of IL-6 by Western blotting.

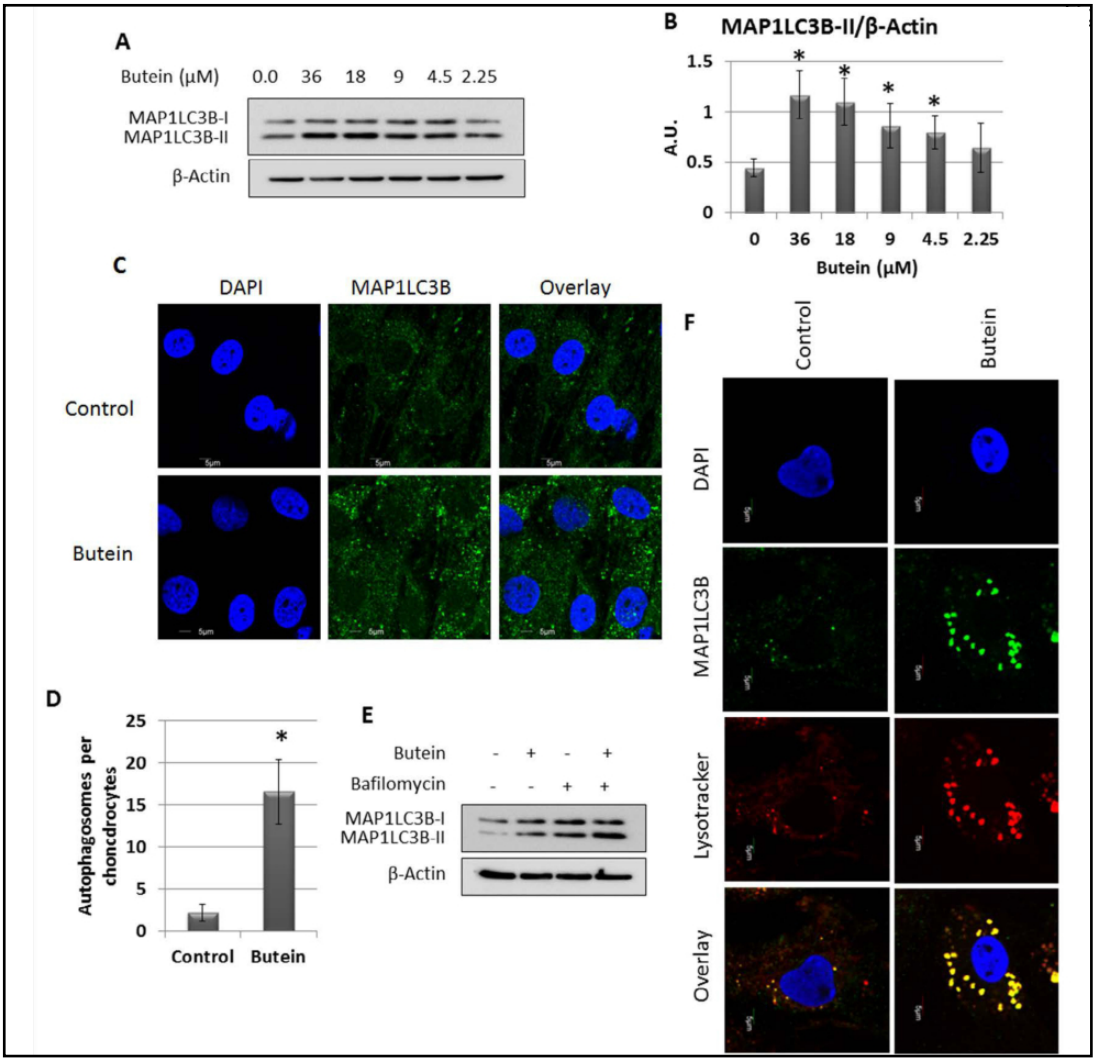

Fig. 3. Butein induced autophagy in chondrocytes. (A) Chondrocytes were treated with different concentrations of Butein for overnight and harvested for cell lysate preparation for immunoblot analysis of MAP1LC3B-II levels. β-Actin was used as loading control. (B) Bar graph represents the quantification of endogenous MAP1LC3B-II with respect to β-Actin. (C) OA chondrocytes seeded in 8 well chamber slides were treated with Butein (10μg/ml or 36μM) for overnight and fixed with 4% paraformaldehyde and probed for autophagosomes using anti-MAP1LC3B antibody followed by anti-rabbit Alexa-fluor-488 secondary antibody. Chondrocytes treated with 0.1% DMSO were taken as control. (D) Bar graph represents the quantification of autophagosomes. (E) OA chondrocytes were treated with Butein for overnight (16 hrs) followed by Bafilomycin A1 for 2 hrs to analyze the autophagy flux. Chondrocytes were harvested for cell lysate preparation and measured the levels of MAP1LC3B-II by immunoblotting. β-Actin was used as loading control. (F) OA chondrocytes seeded in 8 well chamber slides were treated with Butein for overnight followed by the addition of Lysotracer Red for 30 minutes. Chondrocytes were fixed with 4% paraformaldehyde and probed for autophagosomes using anti-MAP1LC3B antibody followed by anti-rabbit Alexa-fluor-488 secondary antibody. Chondrocytes treated with DMSO were taken as control. DAPI was used to counterstain the nuclei (*P<0.05).

Red, red color) was increased in Butein treated OA chondrocytes (Fig. 3F) demonstrating the maturation of the response.

In order to test the hypothesis that autophagy activation by Butein is required to suppress the IL-1β induced expression of IL-6 in human OA chondrocytes, we suppressed autophagy using siRNA mediated knockdown of autophagy related protein 5 (ATG5) and autophagy

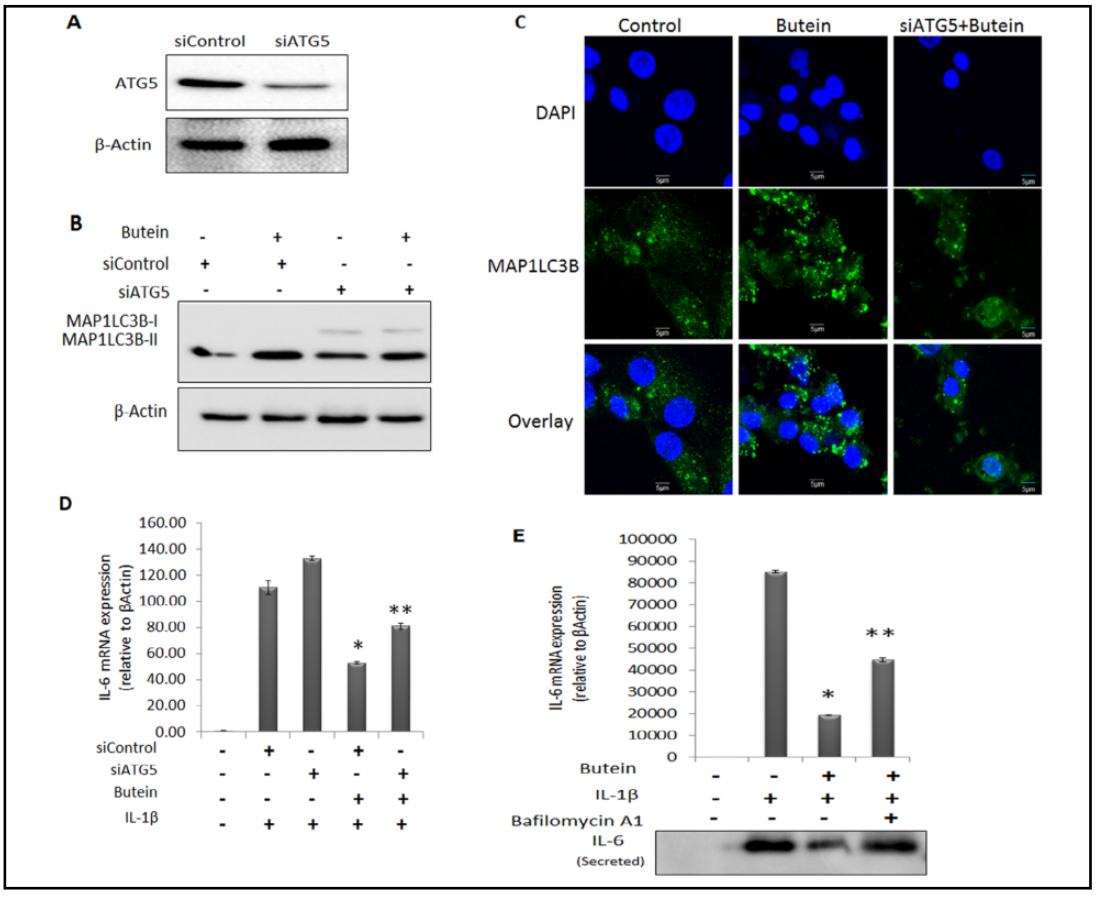

Fig. 4. Butein mediated suppression of IL-6 expression was dependent on autophagy. (A) Chondrocytes were transfected with siRNA targetting ATG5 (siATG5) or scrambled control (siControl) for 48 hrs. ATG5 knockdown was confirmed by Western blotting. (B) siATG5 or siControl transfected chondrocytes were treated with Butein (10μg/ml or 36μM) and harvested to prepare lysate and MAP1LC3B-II levels were determined by immunoblotting. β-Actin was used as loading control. (C) Chondrocytes were transfected as above and treated with Butein to analyze the formation of autophagosomes by immunofluorescence staining of MAP1LC3B. DAPI was used to counterstain the nuclei. (D) The chondrocytes were transfected as above and pretreated with Butein followed by IL-1β. The chondrocytes were harvested for RNA preparation and IL-6 mRNA levels were determined by qPCR. β-Actin was used as normalization control (*P<0.05, **P<0.01). (E) chondrocytes were pretreated with autophagy inhibitor Bafilomycin for 2 hrs followed by Butein (10μg/ml or 36μM) and IL-1β. The chondrocytes were used to prepare total RNA for the analysis of IL-6 mRNA and culture supernatant was used to determine the levels of IL-6 protein. β-Actin was used as normalization control (*P<0.05, **P<0.01).

inhibitor Bafilomycin-A1. ATG5 is essential for autophagy process and is required for the formation of autophagosomes [34]. We knocked down the ATG5 expression in chondrocytes using siRNA (Fig. 4A) and analyzed the effect of Butein on autophagy activation. ATG5 depletion markedly reduced the levels of Butein induced MAP1LC3B-II in OA chondrocytes (Fig. 4B). Additionally the numbers of autophagosomes in ATG5 depleted chondrocytes were also reduced (Fig. 4C). Of importance is our finding that the Butein mediated suppression of IL-1β induced IL-6 expression was inhibited by ATG5 depletion (Fig. 4D) further confirming that active autophagy was required for the anti-inflammatory activity of Butein in human OA chondrocytes. Further, human OA chondrocytes were pretreated with Bafilomycin-A1 to block autophagy and then treated with Butein followed by IL-1β treatment and the expression of IL-6 was analyzed. Butein mediated suppression of IL-1β induced IL-6 expression in OA chondrocytes was abrogated by pre-treatment with Bafilomycin-A1 (Fig. 4E) indicating that

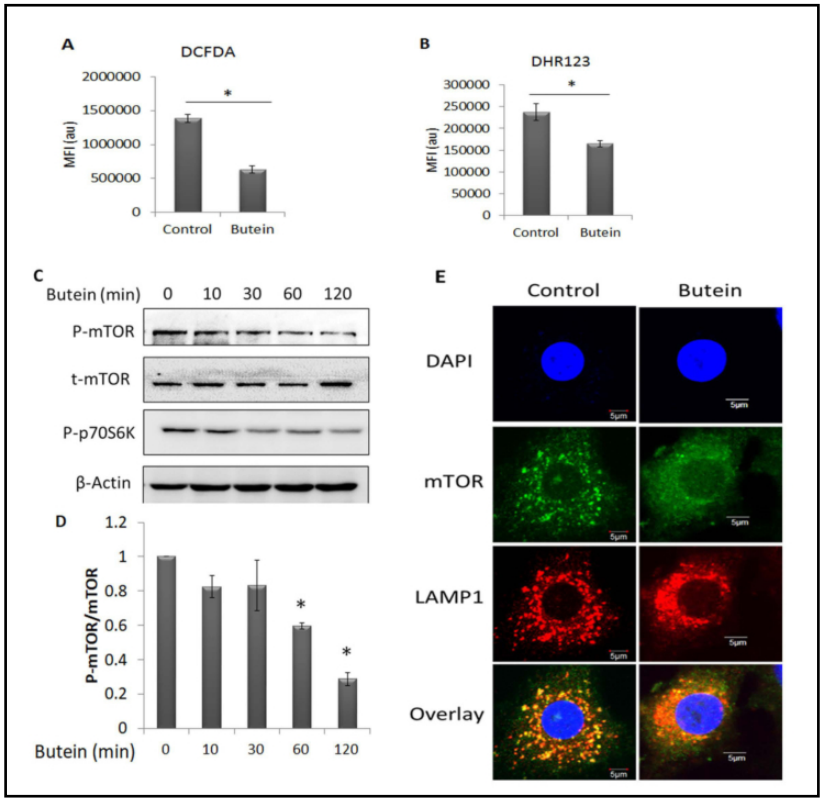

Fig. 5. Butein inhibited mTOR pathway in OA chondrocytes. To measure the levels of ROS in response to Butein treatment, chondrocytes were stained with DCFDA (A) or DHR123 (B) followed by addition of Butein (10µg/ml or 36µM) for 5 minutes. Chondrocytes were analyzed by flow cytometer for DCFDA or DHR123 fluorescence (*P<0.05). (C) Chondrocytes were treated with Butein for indicated time and harvested for lysate preparation followed by immunoblot analysis of Phospho(Ser2448)-mTOR and t-mTOR, Phospho(Ser411)-p70S6K and β-Actin. (D) Represents densitometric analysis of P-mTOR/mTOR (*P<0.05). (E) Chondrocytes were seeded in 8 well chamber slides and treated with Butein for 2 hrs. Chondrocytes were fixed in 4% paraformaldehyde and probed for mTOR and LAMP1 to analyze the colocalization of mTOR with lysosomes. DAPI was used to counterstain nuclei.

the suppression of IL-6 expression by Butein was dependent on active autophagy in human OA chondrocytes. Taken together these results demonstrate that Butein-mediated activation of autophagy is a critical event that is required for the suppression of IL-6 expression in human OA chondrocytes under pathological conditions.

Butein activated autophagy via AMPK/TSC2/ULK1/mTOR pathway in human chondrocytes

Reactive Oxygen Species (ROS) has been reported to activate autophagy in other cell types [35]. Butein has been shown to increase ROS levels in neuroblastoma cells and breast cancer cells [31, 36] and also functions as antioxidant for liver stellate cells and keratinocytes [21, 37]. OA chondrocytes treated with Butein showed no increase in ROS levels as measured by DCFDA (Fig. 5A) and DHR123 staining (Fig. 5B), rather we found a significant decrease in the ROS levels in OA chondrocytes treated suggesting that Butein acts as an antioxidant in primary OA chondrocytes.

Activation of mammalian target of rapamycin (mTOR) signaling pathway is a well-known negative regulator of autophagy activation; therefore, we investigated the effect of Butein treatment on mTOR activation in human OA chondrocytes. The phosphorylation of mTOR $^{Ser-2448}$, which is a marker for its activity [38], was determined by Western immunoblotting and it was found that the phosphorylation of mTOR $^{Ser-2448}$ was decreased in human OA chondrocytes treated with Butein (Fig. 5C and 5D). We further confirmed Butein-mediated inhibition of mTOR activity by measuring the phosphorylation of its downstream target p70S6 kinase. Phosphorylation of p70S6 kinase was negligible (Fig. 5C) in OA chondrocytes treated with Butein confirming suppression of mTOR activity in human OA chondrocytes treated with Butein. Furthermore, as mTOR activity requires the association of mTORC1

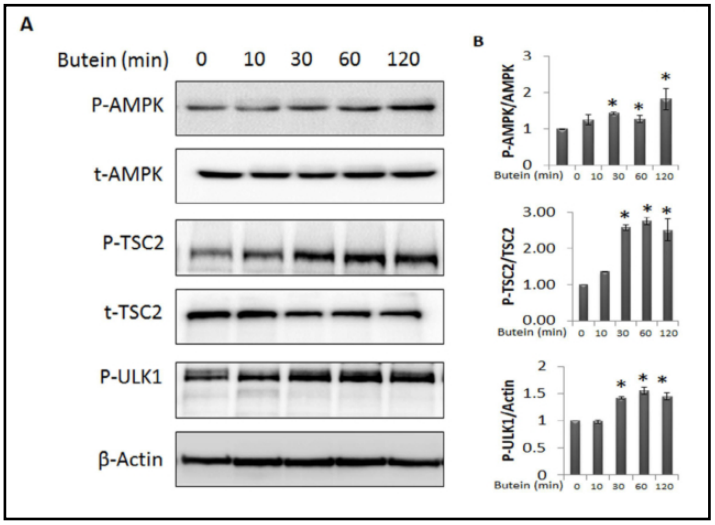

Fig. 6. Butein activated AMPKα/TSC2/ULK1 pathway in chondrocytes to activate autophagy. (A) Chondrocytes were treated with Butein for indicted time and the phosphorylation levels of Phospho(Thr-172)-AMPKα, Phospho(S1387)-TSC2 and Phospho(S317)-ULK1 were determined by Western blotting. β-Actin and respective total levels of proteins were used as normalization control. (B) Bar graph represents the densitometric analysis of Western blots from at least three different samples (*P<0.05).

complex to the lysosomal surface [39], we investigated the colocalization of mTOR with lysosomes by confocal microscopy using LAMP1 as lysosomal marker. In control cultures, mTOR staining showed punctate pattern which were colocalized with lysosomes. However, Butein treated OA chondrocytes showed diffused staining of mTOR suggesting that Butein treatment displaced the mTOR from the surface of lysosomes (Fig. 5E).

AMPKα is the energy sensor and functions as positive regulator of autophagy via phosphorylation based activation of ULK1 and inhibition of mTOR via TSC2 [40]. First we investigated the activation of AMPKα in OA chondrocytes upon Butein treatment. OA chondrocytes treated with Butein showed increased phosphorylation of AMPKα$^{Thr-172}$ suggestive of AMPKα activation (Fig. 6A and 6B). The activation of AMPKα was further confirmed by monitoring the TSC2 and ULK1 phosphorylation. Butein treatment resulted in increased phosphorylation of TSC2$^{Ser-1387}$ and ULK1$^{Ser-317}$ compared to controls (Fig. 6A and 6B) indicating that Butein treatment activated AMPKα in OA chondrocytes. Taken together these results indicate that Butein activates AMPKα/TSC2/ULK1/mTOR pathway to activate autophagy which is critical for the suppression of the expression of inflammatory mediators in OA chondrocytes under pathological conditions.

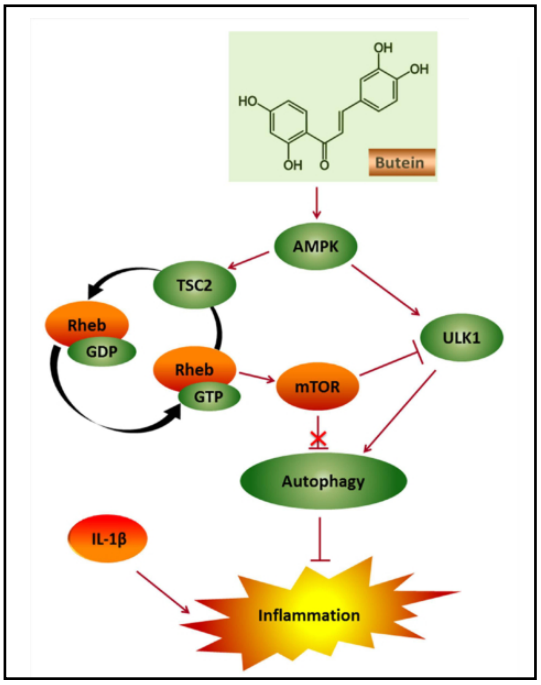

Fig. 7. Schematic diagram depicting the pathway through which Butein activates autophagy and blocks inflammation. Butein activated AMPK which is an activator of ULK1 and TSC2. ULK1 is directly involved in autophagy activation. TSC2 inhibits mTOR via Rheb GTPase eventually leading to autophagy activation.

Discussion

Osteoarthritis is the most common joint disease and a major cause of disability in the affected population. Joint failure caused by arthritis leads to enormous costs to society and, importantly, affects patient quality of life. Recent research has significantly improved our understanding of OA pathogenesis but still there is no disease modifying drug available and the only viable option is the total knee arthroplasty whose incidence is projected to rise by 2050 to 855% by volume compared to the incidence reported in 2012 in the US. TKA is expensive, temporary and not without risk, therefore alternative strategies for treatment are needed urgently. Autophagy is a cellular catabolic process that helps in the clearance of protein aggregates and dysfunctional organelles [41]. Aging is associated with decreased autophagy in cartilage causing increased mitochondrial dysfunction, oxidative stress and inflammation in joints eventually leading to the development of OA. Moreover, autophagy is downregulated in OA cartilage in comparison to normal cartilage tissue [11]. Several studies have demonstrated that activation of autophagy promotes/sustains the activation of anabolic pathways essential for cartilage health and suppresses catabolic pathways involved in OA pathogenesis [8, 12, 14, 41]. Autophagy inhibition was shown to enhance cartilage degradation resulting in increased severity of the disease [12]. Another study demonstrated that cartilage specific deletion of mTOR, which activated autophagy in cartilage chondrocytes, suppressed the progression of OA in a mouse model of experimental OA [42]. These findings suggest that autophagy pathway likely represent a promising therapeutic target for the prevention and progression of OA. In the present study we have demonstrated for the first time that Butein activated autophagy in chondrocytes and suppressed the expression of inflammatory mediators *in vitro*. The fact was further supported by our data that autophagy inhibition by siRNA-mediated depletion of ATG5 abrogated Butein mediated suppression of IL-1β induced expression of IL-6.

Recent work has shown promising effects of Butein against cancer, inflammation and oxidative stress [43]. OA joints have high levels of inflammatory molecules and we show herein that Butein, a plant derived small molecule, suppressed the IL-1β induced expression of inflammatory mediator IL-6 in human OA chondrocytes. This is in accordance to the previous reports that have shown that Butein has anti-inflammatory activity and suppressed inflammation in a variety of cells including human mast cells, macrophages, HUVEC cells etc [19, 44, 45]. The plant extracts containing Butein (for example from *Butea monosperma, Rhus verniciflua, etc.)* have been used traditionally in Indian Ayurvedic and Unani and Chinese medicine system for the treatment of various human ailments [43, 46].

In this study we explored the mechanism of Butein mediated suppression of IL-1β induced expression of IL-6 in human chondrocytes. In previous studies mTOR activity was found to be increased (which suppresses autophagy) in OA cartilage and deletion of mTOR protected mice form the development of OA and this observation has directed the attention towards suppression of mTOR activity as a potential therapeutic target [42, 47]. Our finding that Butein functions as a suppressor of mTOR activity opens up the avenue for its further development as a potential therapeutic for the effective management of OA. Although, earlier studies showed the inhibition of mTOR pathway by Butein in cervical cancer and T cell lymphoma [23, 32] but the mechanism of Butein mediated suppression of mTOR activity was not explored. Thus the data presented in this study is distinct and novel as we demonstrate that Butein activate AMPKα, which phosphorylated its downstream target TSC2 to displace mTOR from the lysosomes resulting in the suppression of mTOR activity in human chondrocytes. We further analyzed the effect of AMPKα activation on its downstream target, ULK1, which is known to phosphorylate Beclin1 and Vps34 complex [48] and activate autophagy. Butein enhanced AMPKα mediated phosphorylation of ULK1 and activated autophagy and autophagy flux and suppressed the expression of IL-6. AMPKα functions as cellular energy sensor and autophagy regulator and has been drawing increased attention of researchers. AMPKα activity is down modulated in aged mouse cartilage [49] and OA

chondrocytes [50] and deletion of AMPKα in chondrocytes resulted in enhanced progression of experimental as well as spontaneous OA [51].

In summary, we demonstrate that Butein activated autophagy via AMPKα/TSC2/ULK1/mTOR pathway suppressed the expression of IL-6 in human chondrocytes under pathological conditions (Fig. 7). These findings suggest that Butein, or compounds derived from it, may be developed for the effective management of OA.

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MYA, NA and TMH conceived and designed the study. MYA and NA performed the experiments. MYA, NA and TMH analyzed and interpreted the data and wrote the manuscript.

Disclosure Statement

No conflict of interests exists.

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