Evaluation of the effects of ursolic acid/γ-cyclodextrin or cyclodextrin-based metal-organic framework-1 complexes on reducing cytotoxicity and improving muscle atrophy in C2C12 cells

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Abstract. The present study aimed to evaluate the effects of ursolic acid (UA), a compound found in apple peels and other fruits and known for its biological activities on the improvement of muscle atrophy. The present study also examined γ-cyclodextrin (γ-CD) and cyclodextrin-based metal-organic framework-1 (CD-MOF-1) complexes for their effects on the proliferation of C2C12 myotube cells, which are derived from mouse skeletal muscle. Cell viability under proliferative and differentiation conditions was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability was reduced at 20 μM in intact UA, at 40 μM in UA/γ-CD, and at 20 μM in UA/CD-MOF-1 cells under proliferative conditions. Under differentiation conditions, cell viability was decreased at 20 μM in intact UA, at 40 μM in UA/γ-CD, and at 10 μM in UA/CD-MOF-1. The reduced viability of C2C12 cells under proliferative and differentiation conditions was not observed in UA/γ-CD concentrations up to and higher than those of intact UA, suggesting that the cytotoxic effect of UA was mitigated. Under both proliferative and differentiation conditions, encapsulation with CD-MOF-1 did not reduce the cytotoxic effect of UA, suggesting that UA/CD-MOF-1 enhanced the intracellular release of UA. The myotube diameters of the C2C12 cells were measured to evaluate muscle atrophy. The addition of dexamethasone (DEX) decreased the myotube diameter; however, UA + DEX, UA/γ-CD + DEX, and UA/CD-MOF-1 + DEX exhibited no difference compared with the control, suggesting that UA ameliorated muscle atrophy induced by DEX. On the whole, the present study confirms that UA myotubular cells can act even when UA/γ-CD and UA/CD-MOF-1 form inclusion complexes, and that muscle atrophy is sustained in UA myotubular cells.

Introduction

In contemporary societies with aging populations, the potential of extracting ursolic acid (UA) from apple peels to utilize biomass, support sustainable resource management and promote health is attracting attention (1,2). Such efforts provide a promising novel approach for treating frailty (3). Typically, apples are peeled from their pulp, and the peels are often discarded as waste. However, the peels are a valuable source of reusable components, particularly UA, a natural triterpene compound and one of the most prevalent components in apple peels. UA has a C-30 chemical structure derived from isoprenoid units (4), exhibits low toxicity and demonstrates anti-inflammatory effects. These effects result from the inhibition of the inflammatory cytokines, cyclooxygenase and inducible nitric oxide synthase (5). Additional effects include the activation of p53, promoting the production of reactive oxygen species, inhibiting cell proliferation (6) and exhibiting anticancer action (6). Moreover, UA inhibits α-glucosidase and α-amylase enzymes (7), inhibits myostatin, a key regulator of skeletal muscle, and prevents muscle atrophy by downregulating muscle atrophy-related genes [such as atrogin-1, muscle ring-finger protein-1 (MuRF1)] (8).

Notably, UA has the ability to activate muscle stem cells and myoblasts, which are involved in maintaining and regenerating muscle health. Thus, it is considered very promising for preventing and improving frailty characterized by muscle

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Abbreviations: UA, ursolic acid; γ-CD, γ-cyclodextrin; CD-MOF-1, cyclodextrin-based metal-organic frameworks-1; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; DMSO, dimethyl sulfoxide

Key words: ursolic acid, γ-cyclodextrin, C2C12 cell, muscle atrophy

weakness, physical debilitation (9), and loss of physical strength (10). However, the clinical applications of UA are limited due to its poor solubility (11). Improving solubility could pave the way for expanding its applications in nutritional therapies.

γ-cyclodextrin (γ-CD) is an oligosaccharide consisting of a glucose molecule ring joined by α-1,4 linkages and composed of eight glucose units. CDs form inclusion complexes when one molecule is internalized by another guest molecule. This property is expected to improve drug stability, solubilization and sustained release (12-15). Metal-organic frameworks (MOFs) are porous materials composed of organic ligands and metals joined through coordination bonds and are characterized by very high pore volumes and surface areas compared to activated carbon and zeolites (16,17). Among these, the most notable, γ-CD-MOF, can be prepared using bioelectrolyte salts [potassium hydroxide (KOH)], alcohol (ethanol) and γ-CD (16). Previous studies have suggested that CD-MOF can encapsulate drugs within the MOF cavity, not only enhancing the water solubility and stability of the drug, but possibly also improving the efficiency of drug delivery through the biomembrane (18,19). Consequently, CD-MOFs are promising and innovative materials for drug delivery systems. In summary, CD-MOF, a combination of γ-CD and MOF, can markedly improve drug properties and is expected to be widely used in drug development and biological applications.

The present study focused on UA and the findings presented herein demonstrate that its solubility can be improved by forming inclusion complexes, such as UA/γ-CD and UA/CD-MOF-1 (20). The UA inclusion complex has the potential to activate muscle stem cells, which play crucial roles in maintaining muscle health and facilitating muscle regeneration. This suggests that it may be useful in preventing or mitigating frailty, a condition characterized by muscle weakness and reduced physical strength. Furthermore, investigating the effects of UA with enhanced solubility will likely contribute to the development of a wide range of pharmaceutical formulations.

The present study used UA/γ-CD and UA/CD-MOF-1 inclusion complexes to improve the handling and availability of UA and explore its potential for pharmaceutical applications. Myotube cell proliferation was evaluated using C2C12 cells derived from mouse skeletal muscles.

Materials and methods

Cells and culture conditions. C2C12 cells (cat. no. RCB0987) were purchased from RIKEN BioResource Center. They were cultured in Dulbecco's modified Eagle's medium (DMEM) (high glucose), purchased from FUJIFILM Wako Pure Chemical Corporation. The DMEM was supplemented with 10% fetal bovine serum (FBS) containing 1% penicillin (100 U/ml) and 1% streptomycin (100 μg/ml). Penicillin-streptomycin solution (100X) was purchased from FUJIFILM Wako Pure Chemical Corporation. Trypsin was purchased from Nacalai Tesque, Inc. FBS was purchased from BioWest. The cells were incubated at 37˚C and 5% CO_2/95% air. The supernatant was then removed, the cells was washed well with 4 ml phosphate-buffered saline (PBS), 1 ml of 0.05% trypsin was added and incubated for a further 5 min at 37˚C. Following trypsinization, the cells were collected into 15-ml tubes (AS ONE Corporation), diluted 10-fold with 9 ml PBS, and centrifuged at 400 x g for 5 min at 20˚C. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of 10% FBS-DMEM. The cells were counted using a blood cell counter, seeded at a density of $6.0x10^5$ cells per dish, and incubated for 3 days at 37˚C in 100-mm culture dishes (SARSTEDT, Nümbrecht, Germany). C2C12 myoblast differentiation into myotubes was induced by switching to high-glucose DMEM containing 2% horse serum (HS-DMEM) after the cells reached confluency (>90%). The cell culture medium was replaced every two days. HS was purchased from MilliporeSigma.

Materials and reagents. The PBS components were as follows: NaCl, 137 mM; $Na_2HPO_4{\cdot}12H_2O$, 8.1 mM; KCl: 2.68 mM; and KH_2PO_4, 1.47 mM. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Tokyo Chemical Industry Co. γ-CD (lot 801005) was supplied by CycloChem Bio Co., Ltd. (chemical structure illustrated in Fig. 1). UA (lot VH5LE-HL) was purchased from Tokyo Chemical Industry Co. May-Grünwald Giemsa stain solution and Giemsa stain solution were purchased from Nacalai Tesque, Inc. All other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation.

Sample preparation of UA/γ-CD and UA/CD-MOF-1. CD-MOF-1 was prepared as described in the study by Sarabia-Vallejo *et al* (15). Briefly, γ-CD (1 mmol) and KOH (8 mmol) were dissolved in distilled water (~20 ml) and allowed to stand for 1 week at room temperature with ethanol (50 ml) for vapor diffusion. The precipitated crystals were filtered, washed with ethanol, and air-dried at room temperature. The CD-MOF-1 was stored with desiccant beads at room temperature in a desiccator. To prepare three-dimensional mixed granulates (3DGM), a method previously reported by the authors' laboratory was used (20). As previously described, the 3DGM (UA/γ-CD=1/1 molar ratio) and 3DGM (UA/CD-MOF-1=1/1 molar ratio) were prepared by grinding in a three-dimension ball mill with a Φ5 mm 200 g ball for 60 min.

Preparation of UA, UA/γ-CD and UA/CD-MOF-1 cell-test solutions. UA was dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration of 2.5-40 μM. UA/γ-CD and γ-CD were dissolved in 10% FBS-DMEM or 2% HS-DMEM (high glucose). UA/CD-MOF-1 and CD-MOF-1 were dissolved in 10% FBS-DMEM or 2% HS-DMEM (high glucose) containing 20 mM HEPES. Each sample was diluted with added medium to reach a final concentration of 2.5-40 μM. Herein, the 'control' group refers to the control configured vehicle (CCV: UA at 0 μM).

Cell viability under cell-growth conditions. Cell viability was evaluated using an MTT assay. The C2C12 cells were prepared at $1.0x10^5$ cells/ml and seeded at 50 μl in 96-well plates (flat bottom) (AS ONE Corporation), and incubated in 10% FBS-DMEM for 24 h at 37˚C. Subsequently, without removing the supernatant, 50 μl of each sample were diluted in 10% FBS-DMEM to twice the desired concentration, bringing the total volume to 100 μl; each sample was then

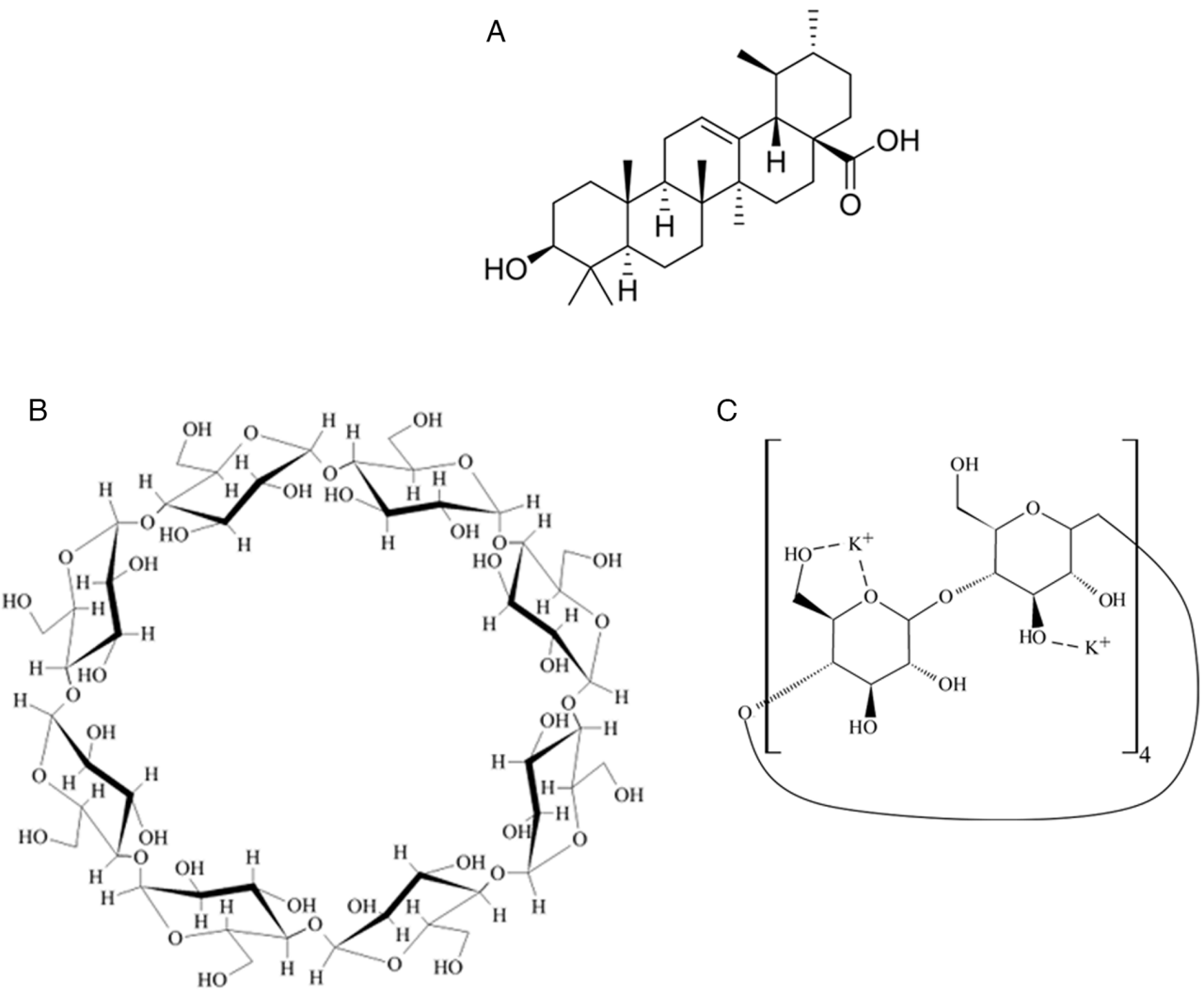

Figure 1. Chemical structures of (A) UA, (B) γ-CD, and (C) γ-CD(KOH)$_2$:(CD-MOF-1). UA, ursolic acid; γ-CD, γ-cyclodextrin; CD-MOF-1, cyclodextrin-based metal-organic frameworks-1.

adjusted to the desired concentration. The cells were incubated for 48 h at 37˚C, following which, the medium was removed. MTT reagent (MTT dissolved in PBS 5 mg/ml) was diluted 10-fold in 10% FBS-DMEM, 100 μl/well was added, and the cells were incubated for 3 h at 37˚C. Following this, 200 μl/well of PBS were added and the cells were washed with PBS. Subsequently, the supernatant was removed, 200 μl/well of PBS were added, the cells were washed with PBS, 100 μl/well of 2-propanol containing 0.029% hydrochloric acid (as solubilizing solution) was added, and the cells were allowed to stand at room temperature for 30 min under light-shielded conditions. Absorbance was measured at a wavelength of 570 nm and a reference wavelength of 650 nm using a Spectra MaxM2 microplate reader (Molecular Devices, LLC). Cell viability was calculated by comparison with 0 μM (UA, UA/γ-CD, or UA/CD-MOF-1).

MTT cell viability assay under differentiation conditions. Cell viability was calculated using MTT assay. C2C12 cells were seeded into 96-well plates at 4.0x10^4 cells/well and incubated at at 37˚C for 48 h after cell confluency (>90%) was confirmed. UA, UA/γ-CD, γ-CD, UA/CD-MOF-1 and CD-MOF-1 were added in 2% HS-DMEM (high glucose) diluted to the desired concentration, and the medium was changed every 2 days for 6 days to induce differentiation. The supernatant was removed following incubation at 37˚C. MTT reagent was then diluted 10-fold in 2% HS-DMEM (high glucose) and 100 μl/well were added to the plates followed by 3 h of incubation at 37˚C. Subsequently, 200 μl/well PBS were added, the cells were washed with PBS, 100 μl/well of solubilizing solution (2-propanol containing 0.029% hydrochloric acid) was added, and the cells were allowed to stand for 30 min under light-shielded, room-temperature conditions. The absorbance was measured using a Spectra MaxM2 microplate reader at a wavelength of 570 nm and a reference wavelength of 650 nm. Cell viability was calculated by comparison with 0 μM (UA, UA/γ-CD, or UA/CD-MOF-1).

Measurement of C2C12 cell myotube diameters under differentiation conditions. The C2C12 cells were stained with May-Grünwald Giemsa stain, and the myotube diameters were measured. A total of 3-15 passages were completed as the cells were less likely to differentiate as the number of passages increased. The C2C12 cells were prepared at 5.0x10^5 cells/

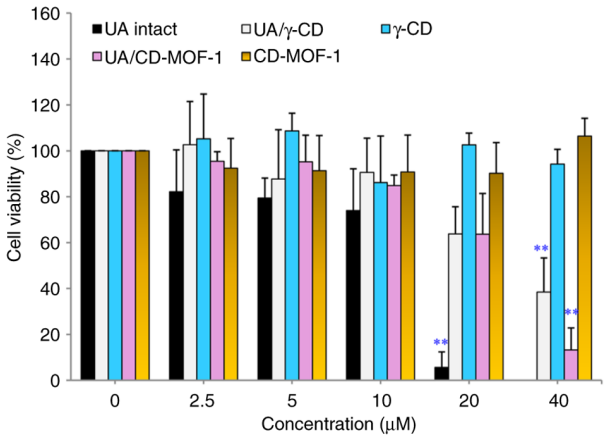

Figure 2. Effects of UA, γ-CD, UA/γ-CD, CD-MOF-1 and UA/CD-MOF-1 on the proliferation of C2C12 cells. The 'control' represents the control configured vehicle (CCV: UA at 0 μM). Data are expressed as the mean ± SD. **P<0.01 (n=3) in the control vs. sample. Data were analyzed using one-way ANOVA with Dunnett's post hoc test. UA, ursolic acid; γ-CD, γ-cyclodextrin; CD-MOF-1, cyclodextrin-based metal-organic frameworks-1.

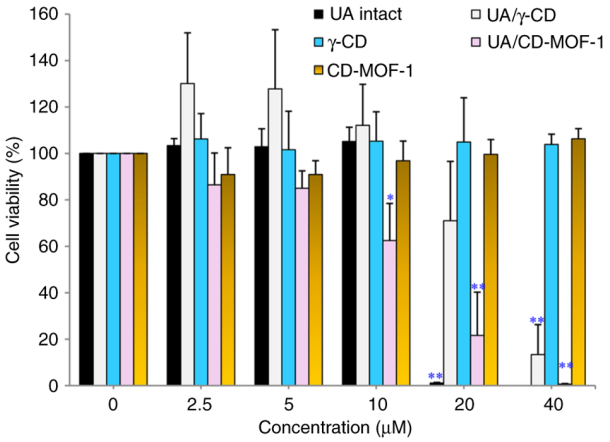

Figure 3. Effects of UA, γ-CD, UA/γ-CD, CD-MOF-1, and UA/CD-MOF-1 on the differentiation of C2C12 cells. The 'control' represents the control configured vehicle (CCV: UA at 0 μM). The results are expressed as the mean ± SD (n=3). *P<0.05 and **P<0.01, compared with the control. Data were analyzed using one-way ANOVA with Dunnett's post hoc test. UA, ursolic acid; γ-CD, γ-cyclodextrin; CD-MOF-1, cyclodextrin-based metal-organic frameworks-1.

ml and seeded at 500 μl in 24-well plates. After confirming that the cells were confluent, the medium was changed to 2% HS-DMEM, and differentiation was induced by changing the medium every 2 days for 6 days. Following the induction of differentiation, DMSO, UA, UA/γ-CD, and UA/CD-MOF-1 were diluted in 2% HS-DMEM and 500 μl/well were added. Of note, 1 h of incubation was followed by incubation with dexamethasone (DEX) (FUJIFILM Wako Pure Chemical Corporation) for a further 24 h at 37˚C, and May-Grünwald Giemsa staining was performed. The cells were fixed with methanol (500 μl/well) for 5 min and air-dried for 10 min at room temperature. May-Grünwald stain solution/phosphate buffer (1/3) was added, and the cells were stained for 5 min at room temperature. The cells were then washed twice with Milli-Q (MilliporeSigma) water (300 μl/well). The cells were then stained with Giemsa stain solution/Milli-Q water (1/10) solution for 20 min at room temperature and washed three times with Milli-Q water. Microscopic observation (CKX53, Olympus Corporation) of the stained cells was performed with a random-field selection of stained cells from the wells, and the diameter of myotube cells with at least three cell nuclei was measured. At least 50 myotubes were analyzed under each condition.

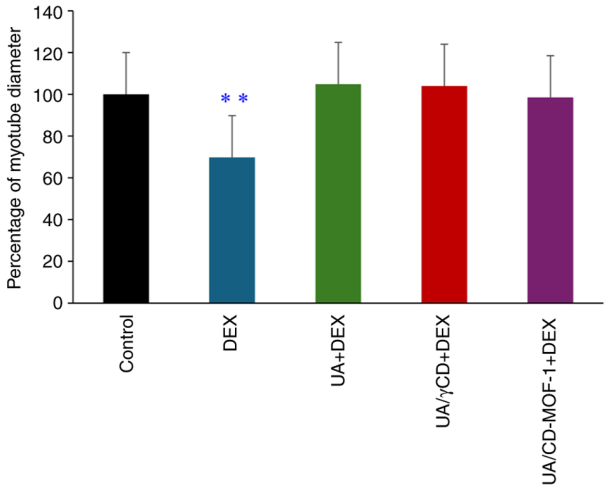

Figure 4. Effect of UA, γ-CD, UA/γ-CD, CD-MOF-1 and UA/CD-MOF-1 on DEX-induced atrophy in C2C12 myotubes. The results are expressed as the mean ± SD (n=5). **P<0.01, compared with the control. Data were analyzed using one-way ANOVA with Dunnett's post hoc test. UA, ursolic acid; γ-CD, γ-cyclodextrin; CD-MOF-1, cyclodextrin-based metal-organic frameworks-1; DEX, dexamethasone.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). The analysis of MTT assay and measurement of myotube diameters were performed using one-way ANOVA with Dunnett's test for multiple comparisons. Comparisons between experimental groups were also assessed using one-way ANOVA followed by Tukey's test. Values of P<0.05 or P<0.011 were considered to indicate statistically significant or highly statistically significant differences, respectively. Data were analyzed using Statcel - the Useful Addin Forms on Excel, 4th edition.

Results and Discussion

Cell viability under proliferative condition, examined using MTT assay. The results of cell viability assay with intact UA, UA/γ-CD, γ-CD, UA/CD-MOF-1 and CD-MOF-1 under growth conditions using the MTT assay test are presented in Fig. 2. For intact UA, the results revealed that the concentrations of 0-10 μM had no effect on cell viability; however, a significant difference was observed between 0 and 20 μM, confirming cytotoxicity. For UA/γ-CD, cell viability was not affected at 0-20 μM; however, a significant difference was observed between 0 and 40 μM, confirming cytotoxicity. For UA/CD-MOF-1, cell viability was not affected at 0-20 μM; however, a significant difference was observed between 0 and 40 μM, confirming cytotoxicity. No effect on cell viability was observed for γ-CD and CD-MOF-1, as no significant difference was observed at 0-40 μM. The cell viability assay revealed that UA/γ-CD was not cytotoxic to the C2C12

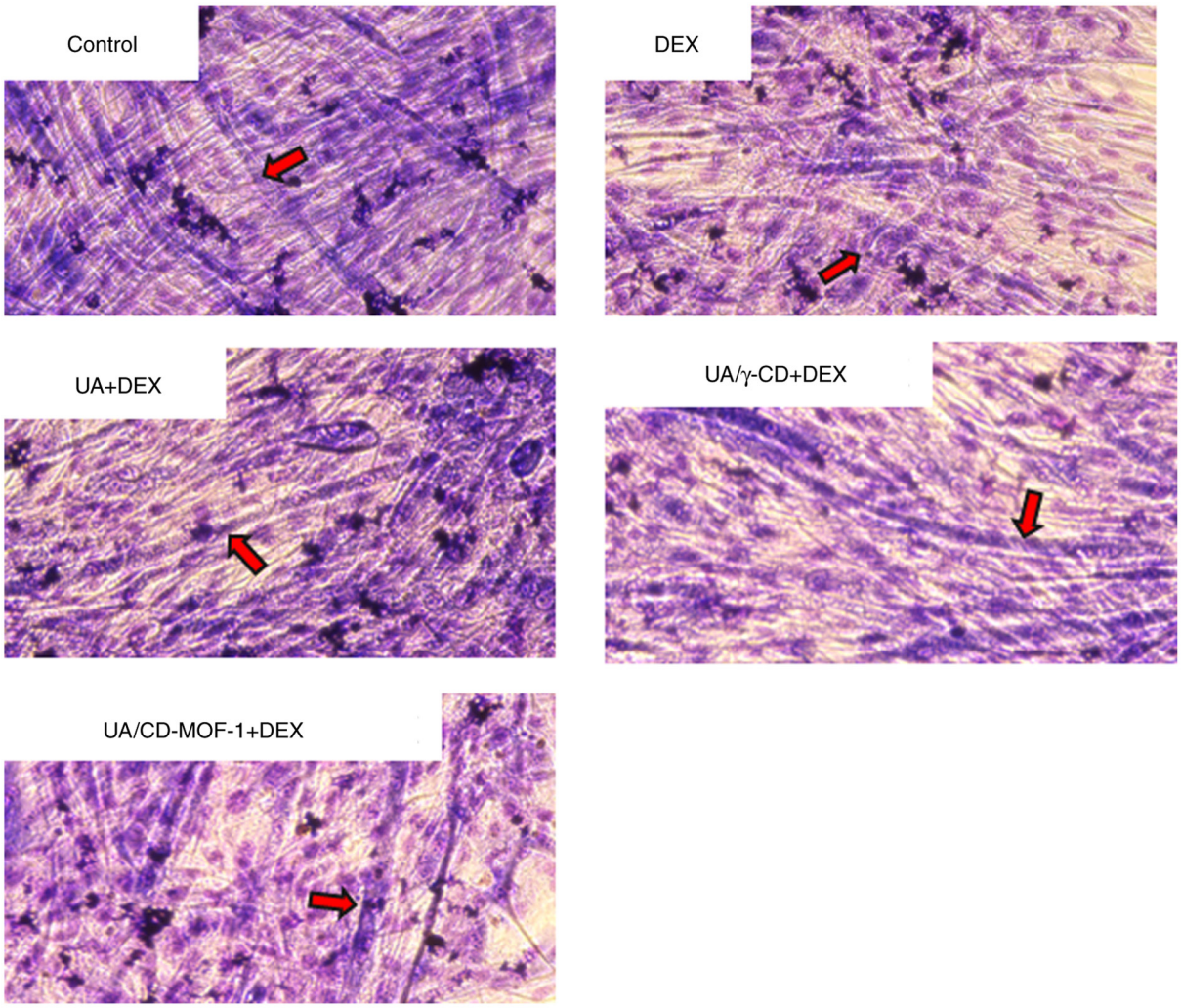

Figure 5. Morphological observation of the control, DEX, UA + DEX, UA/γ-CD + DEX and UA/CD-MOF-1 + DEX under differentiation conditions of C2C12 cells. Magnification, x20. UA, ursolic acid; γ-CD, γ-cyclodextrin; CD-MOF-1, cyclodextrin-based metal-organic frameworks-1; DEX, dexamethasone. Red arrows indicate myotube diameter.

cells under proliferative conditions at concentrations up to and higher than those of intact UA, suggesting that the cytotoxic effect of UA was reduced. The cytotoxic effects of UA alone were comparable with those of the UACD-MOF-1 inclusion product. This finding may be related to the difference in the inclusion modes of UA/γ-CD and UA/CD-MOF-1.

Cell viability under differentiation conditions. The results of cell viability assay with intact UA, UA/γ-CD, γ-CD, UA/CD-MOF-1 and CD-MOF-1 under differentiation conditions are illustrated in Fig. 3. For UA intact, cell viability was unaffected at 0-10 μM, although a significant difference was observed between 0 and 20 μM, confirming cytotoxicity. For UA/γ-CD, cell viability was not affected at 0-20 μM; however, a significant difference was observed between 0 and 40 μM, confirming cytotoxicity. For UA/CD-MOF-1, no effect on cell viability was observed at 0-5 μM, although a significant difference was observed between 0 and 10-40 μM, confirming cytotoxicity. For γ-CD and CD-MOF-1, no significant difference was observed between 0 and 40 μM, demonstrating no effect on cell viability. The cell viability assay revealed that UA/γ-CD (40 μM) was not cytotoxic to C2C12 cells under differentiated conditions up to or at higher concentrations than intact UA (20 μM), suggesting that the inclusion of UA into γ-CD reduced the cytotoxicity of UA. Nevertheless, the cytotoxic effects of UA under differentiation conditions were not reduced by the inclusion of UA in CD-MOF-1. These findings suggest that the exposure of UA/CD-MOF-1 complexes to unincorporated UA under differentiation conditions may affect the cytotoxicity of the complexes. In other words, the mode of inclusion of UA with γ-CD or CD-MOF-1 under both proliferative and differentiation conditions may affect cytotoxicity. Based on these results, the authors decided to use a UA concentration of 5 μM for the C2C12 cell myotube study to match the concentration at which cytotoxicity was not observed with UA/γ-CD and UA/CD-MOF-1. UA is toxic to normal cells at high concentrations. On the other hand, the results of MTT assay revealed that the UA/γCD complex was less cytotoxic than UA alone. The myotube diameter measurement results suggested that the addition of UA, UA/γ-CD, or UA/CD-MOF-1 may alleviate the DEX-induced atrophy. However, the UA/CD-MOF-1 is different from the UA/γCD inclusion mode, suggesting that even if UA is included in CD-MOF-1, the amount of UA released may not be sufficient to suppress the toxicity to cells.

Measurement of myotube diameters in differentiated C2C12 cells. The results of the myotube diameter measurements are shown in Fig. 4. Images of C2C12 cells stained with May-Grünwald Giemsa are shown in Fig. 5. The fusion of nuclei, forming tubular structures, indicated that the C2C12 cells underwent differentiation. Compared with the control group (without UA addition), thinner myotubes were observed in the DEX group, while the UA + DEX, UA/γ-CD and UA/CD-MOF-1 groups did not exhibit such thinning. A significant reduction in myotube diameter was observed in the DEX group when compared with that in the control group (Fig. 4). This indicated that DEX induced atrophy in C2C12 cells, as reflected by the reduced myotube diameter. Moreover, no significant differences were observed between the control group and the UA + DEX, UA/γ-CD + DEX, or UA/CD-MOF-1 + DEX groups (Fig. 4). It has been previously demonstrated that DEX and cortisol suppress the production of IL-6 in RAW264.9 murine macrophages, human monocytes, endothelial cells and fibroblasts (21). By contrast, UA has been shown to inhibit muscle degradation pathways by suppressing the ubiquitin-proteasome system and the expression of atrogenes, such as atrogin-1 and MuRF1 (22). Additionally, UA increased Akt activity in skeletal muscles, leading to muscle hypertrophy. Furthermore, UA enhanced exercise capacity and reduced the resting heart rate (23). In the present study, the absence of changes in myotube diameter in the UA + DEX, UA/γ-CD + DEX, and UA/CD-MOF-1 + DEX groups when compared with the control suggests that UA-containing samples ameliorated DEX-induced atrophy. This indicates that UA contributed to the maintenance of myotube diameter, even when encapsulated in γ-CD or CD-MOF-1 inclusion complexes, thereby confirming its beneficial effects on myotube growth. In C2C12 cells, the activation of the IGF-1/PI3K/Akt pathway promotes muscle protein synthesis and suppresses muscle atrophy (24). UA has also been reported to increase IGF-1 expression, activate the PI3K/Akt pathway and promote Akt phosphorylation (9,25). Consequently, the present study confirmed that UA not only improved solubility by forming a complex with γCD or CD-MOF-1, but also improved muscle atrophy; thus, it was hypothesized that the functional groups of the UA structure that are not encapsulated in the CD cavity affect C2C12 cells. It already has been reported that the inclusion of UA with γ-CD and CD-MOF-1 improves the solubility and stability of UA and reduces its cytotoxicity (26,27). Inclusion complexes of UA and γ-cyclodextrin were prepared and their structural properties and cytotoxicity in C2C12 cells were evaluated in a previous study. The results demonstrated that the inclusion complexation improved the solubility of UA and reduced its cytotoxicity (11). An inclusion complex of UA with CD-MOF-1 has been identified, and potassium has been implicated as a ligand for CD-MOF-1. It has been shown that CD-MOF-1 exhibits no toxicity or effect on cell viability up to 100 μM (28). However, since potassium ions generally play a crucial role in cellular physiology, changes in potassium concentrations may affect cell viability and function in the MTT study. Therefore, it is considered that future studies are required to determine how the combination of UA and potassium affects cytotoxicity. These findings suggest that UA exerts beneficial effects on muscle growth and function, potentially contributing to the maintenance of muscle health and prevention of frailty.

The present study aimed to confirm the effects of UA/γ-CD and UA/CD-MOF-1 complexes on myotubes in C2C12 cells as an initial step and limitation. Therefore, further investigations of muscle atrophy improvement through gene expression and *in vivo* studies using UA/γ-CD and UA/CD-MOF-1 complexes are warranted. In addition, elucidating the biochemical mechanisms underpinning this process remains a challenge for future research. The findings of the present study provide fundamental insighs into the safety of UA/γCD and UA/CD-MOF-1 complexes prepared in the authors' laboratory, as well as their effects on mesenchymal stem cells. Moving forward, the authors aim to conduct *in vivo* studies using UA/γCD and UA/CD-MOF-1 in the future, with the aim of establishing a foundation to enhance the translational nature of the study.

In conclusion, the present study demonstrated that UA/γ-CD reduced the cytotoxic effects of UA based on the results of cell viability assay under proliferative and differentiation conditions. By contrast, UA/CD-MOF-1 did not reduce the cytotoxic effects of UA. The results of the C2C12 cell myotube diameter measurements confirmed that the addition of UA, UA/γ-CD and UA/CD-MOF-1 reduced the atrophy induced by DEX. Moreover, the same muscle atrophy ameliorating effect of UA was confirmed in the experiments using UA/γ-CD and UA/CD-MOF-1 inclusion complexes with improved solubility. UA activated muscle stem cells and myoblasts, which are involved in the maintenance of muscle health and regeneration. It also prevented or ameliorated frailty, the problem of muscle weakness, and the loss of physical strength (23). These findings have expanded the potential for the clinical utilization of UA; it is hoped that these findings may lead to the further applications of UA in pharmaceutical formulations. In the future, it will be necessary to elucidate the mechanisms of muscle atrophy amelioration by gene expression studies, as well as to conduct *in vivo* studies for wider UA applications.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

RK, TT and YIn conceived the study and designed the experiments. RK, MI, TT and YIn designed the study and drafted the manuscript. RK, MI and TT performed the experiments. RK,

MI, TT, YIs, DN, KT and YIn participated in data acquisition, analysis, and interpretation. RK, TT, YIs, DN, KT and YIn provided resources, reviewed, and edited the manuscript, and supervised the study. RK, TT and YI confirm the authenticity of all the raw data. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that the present study received γ-cyclodextrin from CycloChem Co., Ltd.; however, the company was not involved in the study design, collection, analysis, interpretation of data, in the writing of this article, or the decision to submit it for publication.

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