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Targeting myostatin using quercetin as a media supplement to improve myogenesis for cultured meat production: An *in silico* and *in vitro* study

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ABSTRACT

Cultured meat (CM) is an alternative protein food and is produced by cultivating muscle satellite (stem) cells (MSCs) derived from livestock animals (bovine, chickens, and porcine) through myogenesis leading to generate muscle mass. Myostatin (MSTN) is well well-known negative regulator of myogenesis, and in the present study, *in silico* screening of natural compounds was performed to identify MSTN inhibitors. Interestingly, quercetin was found to inhibit MSTN (binding energy −7.40 kcal/mol), and this was further validated by a 100 ns molecular dynamics simulation. Quercetin was added to culture media to boost myogenesis, and its potent antioxidant property helped maintain media pH. Furthermore, quercetin increased the myotube thickness and length, increased MSC differentiation, and upregulated the gene and protein expressions of myoblast determination protein 1 (MYOD), Myogenin (MYOG), and Myosin heavy chains (MYH) *in vitro*. In addition, quercetin inhibited the activities of MSTN, activin receptor type-2B (ACVR2B), and SMAD2 and 3, and thus significantly enhanced MSC differentiation and myotube formation. Overall, this study shows that quercetin might be useful for enhancing large-scale CM production. It is hoped that this study provides a starting point for research in the CM area aimed to enhancing product quality, nutritional values, and the efficacy of large-scale production.

1. Introduction

Cultured meat (CM), also known as lab-grown meat or cell-based meat, was first produced by primary bovine skeletal muscle (SM) cells but can also be produced by the *in vitro* cultivation of variously sourced muscle satellite (stem) cells (MSCs) (Post, 2012). MSCs are a type of stem cell found in SM and are responsible for muscle growth/regeneration. Meat from industrial animals (bovine, porcine, poultry, and fish) is largely composed of SM, which contains ~90% muscle fiber, ~10% connective and fat tissues, and ~1% blood. In addition, meat provides vitamin B12 and heme iron, which are essential for human nutrition (Dodson et al., 2015; Laumonier and Menetrey, 2016; Listrat et al., 2016; Warriss and Rhodes, 1977). CM provides an excellent alternative source of protein that is both economical and ecologically beneficial (Jalil et al., 2023; Shaikh et al., 2021). However, proteinaceous food is crucial for the next 30 years because it is expected that the world population is going to be ~9 billion by 2050, so sustainability is an essential motivation for CM production (Mattick et al., 2015; Tuomisto and de Mattos, 2011) as a main protein source. Additionally, CM has environmental advantages over regular meat such as ethical issues, water and environmental pollution, and land use (Tuomisto and de Mattos, 2011). Further, it is very difficult to raise enough livestock for protein sources in the future. CM will rise as a food alternative. It is likely to be said that CM can feed our increasing population in the future.

MSCs are primary generators of new myonuclei and require a nutrient-rich culture medium to produce muscle tissue through myogenesis (Coleman et al., 1995; Florini et al., 1991). This process involves MSC activation, proliferation, and the fusion of differentiating myoblasts into mature myofibers under the direction of muscle regulatory factors (Relaix et al., 2005; Zammit and Beauchamp, 2001), growth factors (Allen and Boxhorn, 1989), and cytokines (Spangenburg and Booth, 2002) to produce SM tissue. Many studies have described the cell

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types (Shaikh et al., 2021) and the roles of growth factors (Ahmad et al., 2023) and extracellular matrix (ECM) (Lim et al., 2021) in CM production. However, large-scale CM production depends upon the *in vitro* production and differentiation of MSCs, as it was concluded in the first report issued on the cultivation of muscle-based beef burgers (Post, 2012).

Myostatin (MSTN) is a myokine and negative regulator of myogenesis that is highly expressed in animal and human SM (Lee et al., 2021) and inhibits MSC proliferation, differentiation, and muscle development (Rios et al., 2002). The active form of MSTN binds to its receptor (activin receptor type-2B (ACVR2B)) to activate signaling for protein degradation via SMAD2/3-mediated transcription (Ahmad et al., 2021). The inhibitory effects of several ECM proteins, such as decorin, fibronectin, laminins (Miura et al., 2006; Miura et al., 2010), and fibromodulin, on MSTN and their abilities to regulate myogenesis have been investigated in efforts to counteract the inhibitory effects of MSTN and regulate myogenesis (Lee et al., 2021). Moreover, it was reported recently that *Glycyrrhiza uralensis* (Shaikh et al., 2021), dithymoquinone (Ahmad et al., 2021), and some other natural compounds from Chinese traditional medicine (Ali et al., 2022) inhibit MSTN expression and promote myogenesis.

In the present study, we screened food-grade supplements and edible, safe, and cost-effective natural compounds for MSTN inhibitory activity *in silico*. Interestingly, quercetin, a polyphenol (flavonoids) present in vegetables (garlic, onions, and broccoli), fruits (apples, berry crops, and grapes), and some herbs (tea) (Mlcek et al., 2016), was found to be a potent MSTN inhibitor. Subsequently, quercetin was subjected to an *in vitro* study, in which it was used as a media supplement at different concentrations to check its effects on MSC morphology, proliferation, and differentiation, and inhibitory effects on MSTN, ACVR2B, and SMAD2/3, and its antioxidant properties. MSCs obtained from livestock animals (bovine, porcine, and chickens) and C2C12 cells were used to investigate the effects of quercetin on myotube formation.

Livestock is already a main contributor to antibiotic resistance worldwide, and the use of sub-therapeutic medicines are expected to rise further in the future. While, CM does not necessitate the use of antibiotics in large quantities, which decreases the public health issue. When compared to livestock, the hazards of pandemics and antibiotic resistance from CM are small. This study aimed to identify food-grade supplements or natural compounds that inhibit MSTN activity and it is suitable for incorporation in media for the large-scale production of CM. Additionally, selected supplements should be helpful for bovine, chicken, and porcine-derived MSC proliferation and differentiation for CM production.

2. Materials and methods

2.1. Molecular docking and molecular dynamics simulation study (MDS)

The selected target, MSTN, was retrieved from the RCSB-PDB (https://www.rcsb.org/), and the structures of potential natural inhibitors were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Heteroatoms were removed from MSTN using Discovery Studio, and molecular docking was performed using PyRx (Dallakyan and Olson, 2015). The MSTN pocket was set at X = −29.37, Y = −20.90, and Z = 21.61 to check the affinities of selected compounds. In addition, MDS was performed using GROMACS 2019.6 (Van Der Spoel et al., 2005) and the GROMOS96 43a1 force field (Pol-Fachin et al., 2009) to check the stability of the selected complex obtained by docking analysis. A dodecahedron box of extended simple point charge water was used to immerse the complexes. Simulation boxes were neutralized using the gmx_genion module, and bond lengths were constrained using the LINCS algorithm (Hess et al., 1997). Finally, root mean square deviations (RMSDs), root mean square fluctuations (RMSFs), radii of gyration (Rg), numbers of H-bonds, and solvent-accessible surface areas (SASAs) of complexes were analyzed.

2.2. Cell culture

2.2.1. C2C12 myoblasts

Murine C2C12 myoblasts (Korean Cell Line Bank, Seoul, South Korea) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Cytiva, Marlborough, MA, USA) supplemented with 10% FBS (Fetal Bovine Serum, Cytiva, Marlborough, MA, USA) and 1% P&S (penicillin and streptomycin, Cytiva, Marlborough, MA, USA) in a humidified 5% CO_2 incubator at 37 °C. When cells reached 90% confluence, growth media was replaced with differentiation medium (DMEM supplemented with 2% FBS and 1% P&S) containing 0, 1, 10, 100, or 1000 nM of quercetin and cultured for 0, 2, 4, or 6 days with daily media changes. The C2C12 culture method was adopted from our previous experiment (Lim et al., 2021).

2.2.2. Bovine, porcine, and chicken MSC cultures

Bovine, porcine, and chicken MSCs were cultured in Ham's F-10 growth medium (F-10 Nutrient Mixture, Cytiva, Marlborough, MA, USA) supplemented with 20% FBS, 1% P/S, and 5 ng/mL of FGF2 (fibroblast growth factor 2, Miltenyi Biotec, Auburn, CA, USA) in a humidified 5% CO_2 incubator at 37 °C. When cells reached 90% confluence, the growth medium was replaced with a differentiation medium containing 0, 1, 10, 100, or 1000 nM of quercetin and cultured for 0, 2, 4, or 6 days with daily media changes. The bovine, porcine, and chicken MSCs culture method was the same as our previous experiment (Lim et al., 2022).

2.2.3. Bovine, porcine, and chicken MSC isolation

Bovine top-round muscles, porcine hindshank, and chicken whole leg muscles were collected from female calves (17 weeks old), male porcine (3 days after birth), and embryonic chickens (16 days after fertilization), minced, digested with 1% pronase (Roche, Mannheim, Germany) for 1 h at 37 °C, and centrifuged at 1000×*g* for 3 min. Digested tissue was passed through a 100 mm cell vacuum strainer (Millipore, Darmstadt, Germany), and the filtrate was centrifuged at 1000×*g* for 5 min. Pellets were suspended in Ham's F-10 + 20% FBS +1% P&S + 5 ng/mL FGF2, seeded on collagen-coated plates (Corning, Brooklyn, NY, USA), and incubated in a 5% CO2 humidified incubator at 37 °C. The bovine, porcine, and chicken MSCs isolation method was adopted from our previous report (Lim et al., 2022).

2.3. The animal experiment

All experiments were conducted via the guidelines issued by the Institutional Animal Care and Use Committee of Yeungnam University (AEC2022-022).

2.4. MTS assay

MTS assay was performed to compare cell proliferation. For MTS assays, cells were incubated in CellTiter 96® AQueous One Solution Reagent (Promega, Madison, WI, USA) for 1 h in a humidified 5% CO_2 incubator at 37 °C. Absorbance was measured at 490 nm using a microplate reader (Biotek Synergy H1, Winooski, VT, USA).

2.5. Measurement of creatine kinase levels

Cellular creatine kinase levels were analyzed using the EnzyChrom™ Creatine Kinase Assay Kit (BioAssay Systems, Hayward, CA, USA), according to manufacturers' instructions.

2.6. Total RNA extraction, cDNA synthesis, and real-time RT-PCR

Total RNA was extracted from cells using Trizol® reagent (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions, and 2 μg was used to synthesize cDNA using a high-capacity

cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Briefly, 2 μg of total RNA in a 20 μL reaction mixture containing random hexamers and reverse transcriptase was used to synthesize cDNA using the following schedule; 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. Gene expressions were analyzed using cDNA (2 μL), target gene-specific primers (10 pmole, 2 μL), and a 7500 real-time PCR system using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). All reactions were performed in triplicate, and relative gene expressions were normalized vs. non-treated controls and calculated using $2-\Delta Ct$, where $\Delta Ct = Ct\ gene - Ct\ control$. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control. PCR primers are detailed in Table S1.

2.7. Western blot analysis

Bovine, porcine, and chicken MSCs and C2C12 cells were lysed using RIPA buffer containing 1% protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), and total protein concentrations were quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). Proteins (50 μg) were subjected to 8% or 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Burlington, MA, USA). Membranes were then blocked with BSA (Bovine serum albumin) or 3% skim milk in TBS (Tris-buffered saline) containing 0.1% Tween 20 for 1 h and incubated with target protein-specific primary antibodies [MYOD (1:400), MYOG (1:400), MYH (1:400), MTSN (1:400), SMAD2 (1:400), phosphorylated SMAD2 (1:400), ACVR2B (1:400), or β-actin (1:400)] in TBS containing 1% skim milk or BSA overnight at 4 °C. Blots were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse or anti-rabbit; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 h and detected using the Dyne ECL Pico Plus Western Blotting Detection Kit (Dyne Bio, Seongnam, South Korea). Band images were analyzed using an Azure 300 chemiluminescent imager (Azure Biosystems, Dublin, CA, USA).

2.8. Cell culture medium pH measurement

The pH values of bovine, porcine, and chicken MSC spent differentiation media were measured on days 0–2 and 2–4 in the presence of an antioxidant supplement (Sigma-Aldrich, St. Louis, MO, USA) or quercetin using a pH meter (Mettler Toledo, Columbus, OH, USA). The antioxidant supplement is a commercialized product used in different types of cell cultures as a cocktail containing various antioxidants (Luo et al., 2014; Tai et al., 2012). Sigma-Aldrich's proprietary antioxidant supplement (A1345) can be used in the diversity of cell culture including primary cell culture. Therefore, the antioxidant supplement (Sigma-Aldrich, A1345) is already in the market and is considered suitable for use as a positive control to confirm the antioxidant effect that is used in this study.

2.9. Reactive oxygen species (ROS) level analysis

The media of bovine, porcine, and chicken MSCs differentiated for 4 days in the presence of antioxidant supplement (Sigma-Aldrich, A1345) or quercetin was removed and treated with 10 μM 2',7'-dichlorofluorescein (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. After washing twice with PBS, fluorescence was measured (excitation 498 nm and emission 522 nm) using a microplate reader (BioTek, SynergyTM H1 Hybrid Multi-Mode Microplate Reader, Winooski, VT, USA).

2.10. Statistical analysis

Statistical analysis was performed based on the results of at least three repeated experiments. The significance of differences between mean normalized expression was determined using Tukey's test. Real-time RT-PCR, proliferation, and creatine kinase levels were analyzed by one-way ANOVA using PROC GLM in SAS ver. 9.0 (SAS Institute, Cary, NC, USA). Statistical significance was accepted for p values < 0.05.

3. Results

3.1. In silico analysis

Quercetin binds to the Val50, Phe51, Leu52, Gln53, Tyr55, Pro56, His57, Thr58, His59, and Leu60 amino acid residues MSTN with a free energy −7.40 kcal/mol (Fig. 1A). The docking study showed three H-bonds, namely, Leu52:HN- quercetin:O6, His59:HN- quercetin:O5, and quercetin:H30-Pro56:O during quercetin binding (Fig. 1B and Table 1). Furthermore, molecular dynamics simulation for up to 100ns suggested the MSTN-quercetin complex was stable after 10ns. The RMSD values of MSTN and MSTN-quercetin were 0.35 and 0.29 nm, respectively (Fig. 1C), and RMSF values were lower at interacting sites (from amino acids 50 to 70) (Fig. 1D). Rg and SASA values for MSTN-quercetin were 1.75 nm and 71.19 nm^2, respectively (Fig. 1E&F). Though four H-bonds were present by molecular dynamics simulation, only two were present throughout the simulation period (Fig. 1G).

3.2. In vitro analysis

3.2.1. Effects of quercetin on the proliferation of C2C12 cells and bovine, porcine, and chicken-derived MSCs

An *in vitro* study was performed to investigate the effect of quercetin-supplemented media on the proliferation and differentiation of C2C12 cells and MSCs obtained from different livestock animals (bovine, porcine, and chickens). Quercetin at 1, 10, 100, or 1000 nM for up to 4 days had no significant effect on cell proliferation as determined by MTS analysis (Supplementary Figs. S1, S2, S3, and S4).

3.2.2. Quercetin for C2C12 cell line differentiation

Microscopic observations showed that quercetin supplementation promoted myotube formation and thickness (Fig. 2A) and significantly increased creatine kinase activity in C2C12 cells by 4, 16, and 7% at 1, 10, and 100 nM, respectively (Fig. 2B), and significantly increased the expressions of gene and protein markers of muscle differentiation (MYOD, MYOG, MYH) (Fig. 2C). Collectively, C2C12 differentiation was also found to significantly increase in quercetin-supplemented media.

3.2.3. Effect of quercetin on the MSTN/ACVR2B-SMAD2/3 pathway

Quercetin treatment reduced the gene and protein expressions of SMAD2 and 3, which are signal transducers that inhibit muscle development and growth, which suggests that MSTN-quercetin complex formation promotes muscle differentiation by interfering with MSTN signals that inhibit muscle development. Quercetin did not affect the mRNA expression of ACVR2B (MSTN receptor) but reduced its protein levels, which showed quercetin supplementation blocked MSTN-ACVR2B binding and reduced ACVR2B levels (Fig. 3A).

3.2.4. Quercetin as an antioxidant in culture media

The pH values of cell cultures reduce with time because of the acid by-products generated during metabolic processes. When myoblasts were differentiated in quercetin-supplemented media, pH (6.77–6.99) was maintained for up to 4 days, whereas the pH value of control media fell to under pH 6.53. These results were similar to those obtained for commercially available antioxidant media supplements (Fig. 3B). Furthermore, treatment with quercetin or antioxidant supplements (Fig. 3C) reduced ROS (reactive oxygen species) levels.

Myoblasts were differentiated by treating them with antioxidant supplements or quercetin, respectively, and degrees of myotube formation were compared using a creatine kinase activity assay. Both the antioxidant supplement and quercetin treatment groups showed a significant increase in creatine kinase activity versus the non-treated control group. In addition, creatine kinase activity was higher in the

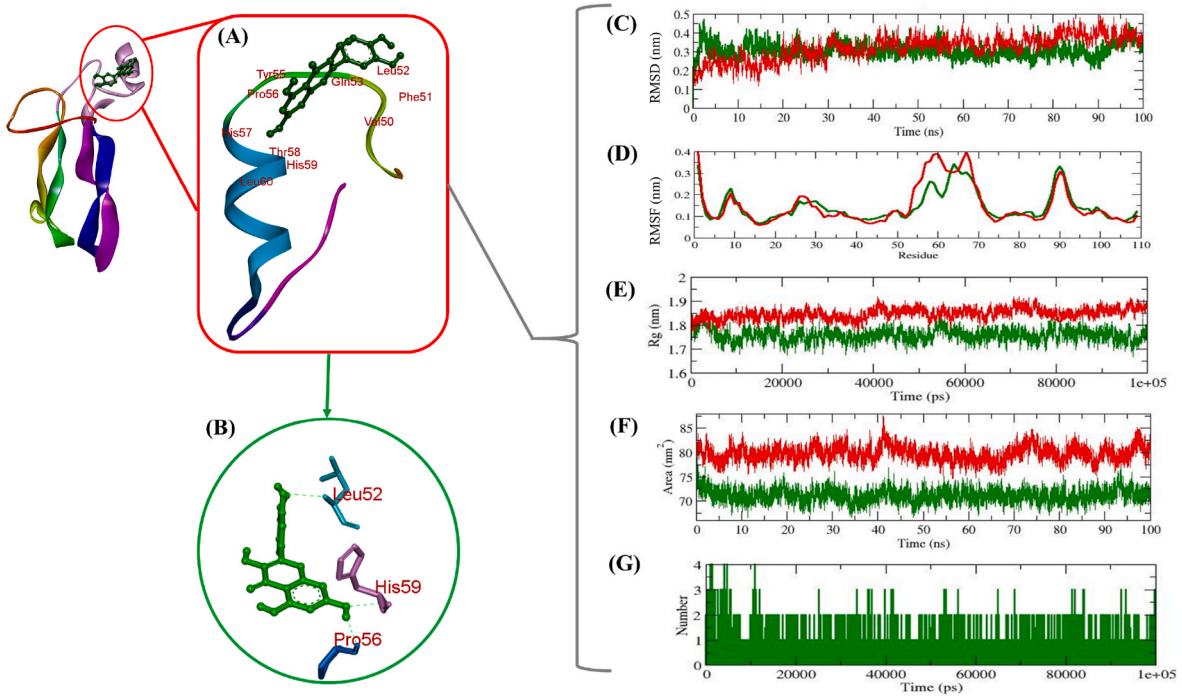

Fig. 1. Structure of MSTN-quercetin complex. **A)** Amino acid residues of MSTN that interact with quercetin. **B)** H-bond formation between MSTN and Quercetin. **C)** The RMSD of MSTN (red) and MSTN-Quercetin (green). **D)** The RMSF of MSTN (red) and MSTN-Quercetin (green). **E)** The Rg of MSTN (red) and MSTN-Quercetin (green). **F)** The SASA of MSTN (red) and MSTN-Quercetin (green). **G)** H-bond formation in MSTN-Quercetin.

Table 1
Amino acid residues and H-bonds involved in MSTN-quercetin complex formation.

Binding energy for MSTN-quercetin	H-bonds interaction	H-bonds distance (Å)	Total amino acid residues in MSTN+Quercetin interaction
−7.40 kcal/mol	LEU52:HN-quercetin:O6	1.84	Val50, Phe51, Leu52, Gln53, Tyr55, Pro56, His57, Thr58, His59, and Leu60
	HIS59:HN-quercetin:O5	1.85	
	quercetin: H30-PRO56: O	2.04	

quercetin-supplemented group than in the antioxidant-treated group, indicating muscle differentiation was promoted more by quercetin-supplemented media (Fig. 3D). These results confirmed that quercetin promotes myogenesis in C2C12 cells by regulating the pH of the cell culture medium and inhibiting MTSN, ACVR2B, and SMAD and 3.

3.2.5. *Quercetin and bovine MSC differentiation*

Bovine MSCs were grown in quercetin-supplemented media at different concentrations (1, 10, 100, and 1000 nM). Microscopic observations confirmed that quercetin at 10 and 100 nM effectively promoted the differentiation of bovine MSCs and increased myotube thickness (Fig. 4A). Similarly, creatine kinase activity (an indicator of muscle formation/differentiation) was significantly increased by 6, 13, and 10% at 1, 10, and 100 nM, respectively (Fig. 4B). Further, treatment with 10 nM quercetin significantly increased the mRNA and protein expressions of MYOD (early muscle differentiation marker), MYOG (middle muscle differentiation marker), and MYH (late/terminal muscle differentiation marker) (Fig. 4C). These findings suggested quercetin might be used at 10 nM to enhance the differentiation of bovine MSCs for CM production.

3.2.6. *Quercetin and porcine MSC differentiation*

Quercetin did not induce any significant morphological change in porcine MSCs (Fig. 5A). However, creatine kinase activity assays showed quercetin supplementation (1, 10, or 100 nM) significantly increased creatine kinase activity by 27, 29, and 40%, respectively, versus non-treated controls (Fig. 5B). In addition, 10 nM of quercetin supplementation increased myogenic marker gene expressions, viz. MYOD by 1.5-fold and MYOG and MYH by 2-fold, and their protein expressions (Fig. 5C).

3.2.7. *Quercetin and chicken MSC differentiation*

Quercetin (10 and 100 nM) increased the length of myotubes produced by chicken MSC differentiation (Fig. 6A). As it was observed for bovine and porcine MSCs, treatment with quercetin at 10 or 100 nM significantly increased creatine kinase activity by 9 and 13%, respectively (Fig. 6B). Furthermore, quercetin supplementation at 10 nM increased the mRNA and protein expressions of MYOG and MYH in chicken MSCs, but unlike that observed for bovine and porcine MSCs, quercetin did not affect the gene and protein expressions of MYOD (early muscle differentiation) (Fig. 6C). Interestingly, the promotion of differentiation during the middle and end of differentiation by quercetin at 10 nM is compatible with the requirements of CM industries.

4. Discussion

The *in silico* and *in vitro* studies demonstrated that quercetin is a strong inhibitor of MSTN and significantly enhances MSC differentiation and myotube thicknesses, presumably due to downregulation of the MSTN-SMAD2/3 pathway and its antioxidant and anti-ROS effects. Quercetin is a major flavonoid in the human diet (3–38 mg is consumed daily (Manach et al., 2005)), and thus quercetin is considered a high-grade food supplement readily available in diets with anti-carcinogenic, anti-inflammatory, and antiviral activities (Li et al., 2016).

MSTN inhibition is an accepted strategy for enhancing myogenesis and CM production because MSTN is a negative regulator of muscle

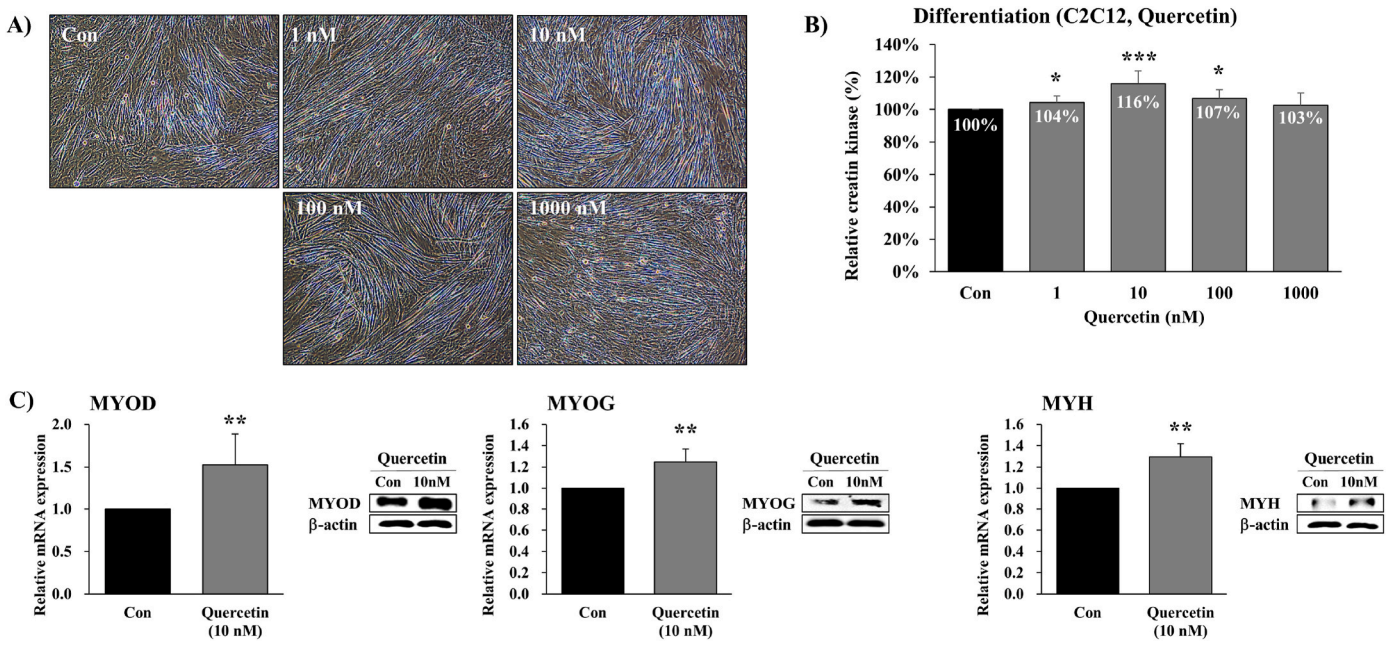

Fig. 2. Differentiation of C2C12 cells treated with different concentrations of quercetin **A)** Cell morphology observed under a microscope **B)** Differentiation was assessed using a creatine kinase activity assay **C)** mRNA and protein levels of myogenic markers were determined by Real-time RT-PCR and western blot.

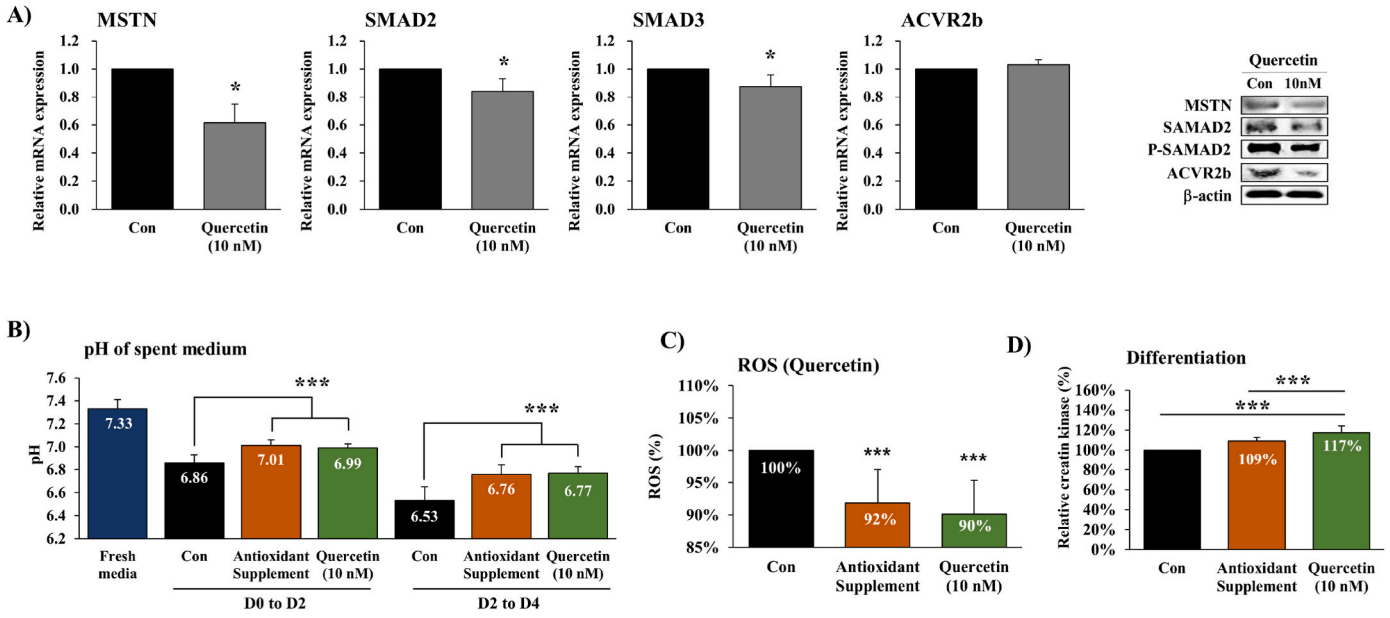

Fig. 3. Inhibitory effect of quercetin on myostatin.
A) mRNA and protein expressions of myostatin and myostatin-related markers as determined by Real-time RT-PCR and western blot.
B) pH of spent media containing antioxidant or quercetin after 0–2 days or 2–4 days. **C)** ROS levels measurement with the treatment of an antioxidant supplement or quercetin **D)** Differentiation in the presence of the antioxidant supplement or quercetin was assessed by measuring creatine kinase activities.

development and growth (Ahmad et al., 2021; Lee et al., 2021). Recently, it was demonstrated that curcumin and gingerol suppress MSTN to ACVR2B binding and improve SM growth (Baig et al., 2017). In the present study, quercetin-supplemented media promoted myotube formation and thickness. At 100 and 10 nM, quercetin increased porcine MSC differentiation by 40% and 29%, respectively.

The presence of quercetin in media increased the expressions of MYOD and MYOG while MYH is significantly enhanced along with protein expression means quercetin-supplemented media is highly recommended for the porcine MSC differentiation specifically terminal differentiation for large-scale production of CM. Consistently, a 13% increment was observed in chicken and bovine. It has been reported that MYOD and MYOG are responsible for MSC progression in chicken muscles (Day et al., 2009).

The presence of quercetin reduces the expression of MSTN, ACVR2B, and SMAD2 and 3 in growth media, which favors MSC differentiation and CM production. In a previous study, MSTN null mice weighed 2–3 times more and had more SM mass than wild-type mice (McPherron et al., 1997), and in humans, mice, dogs, sheep, and cattle, a naturally occurring mutation in the MSTN gene results in a hypermuscular phenotype (Rodriguez et al., 2014). Overexpression of activin receptor-like kinase 5 (which is triggered by MSTN binding to ACVR2B

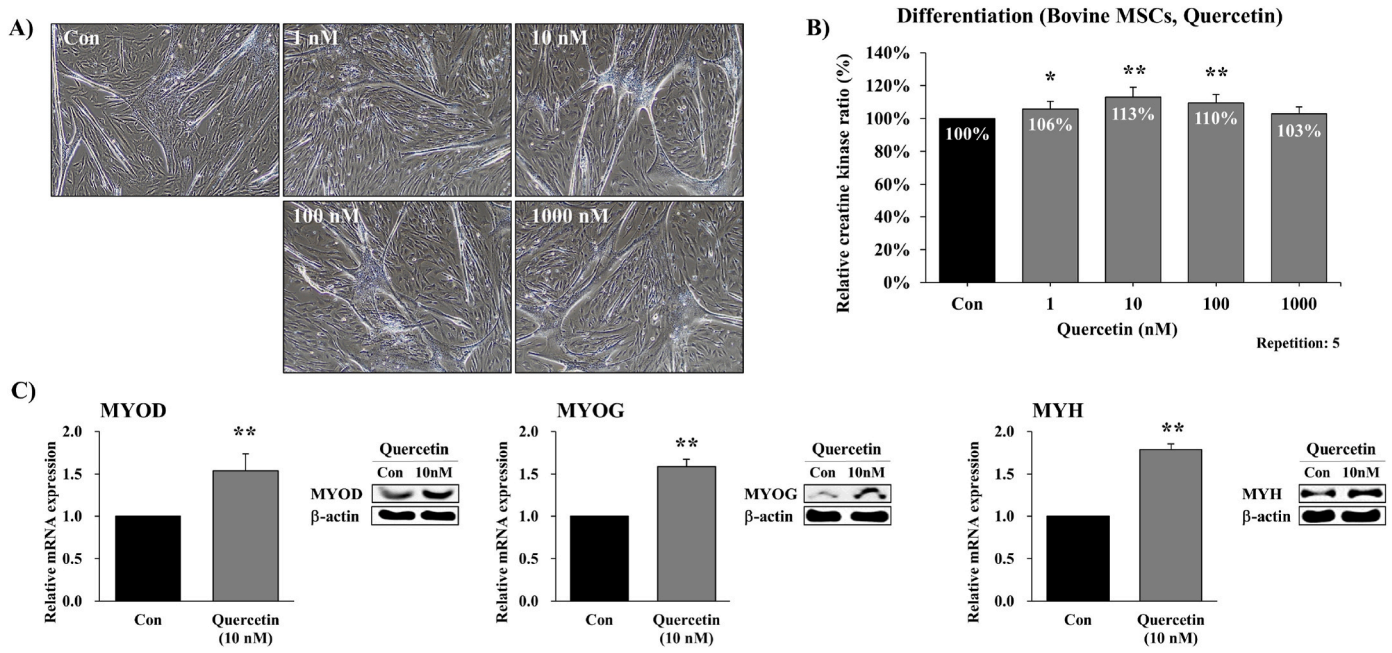

Fig. 4. Bovine MSC differentiation in the presence of different concentrations of quercetin. **A)** Cell morphology observed under a microscope. **B)** Creatine kinase activity results. **C)** The mRNA and protein levels of myogenic markers were determined by Real-time RT-PCR and western blot.

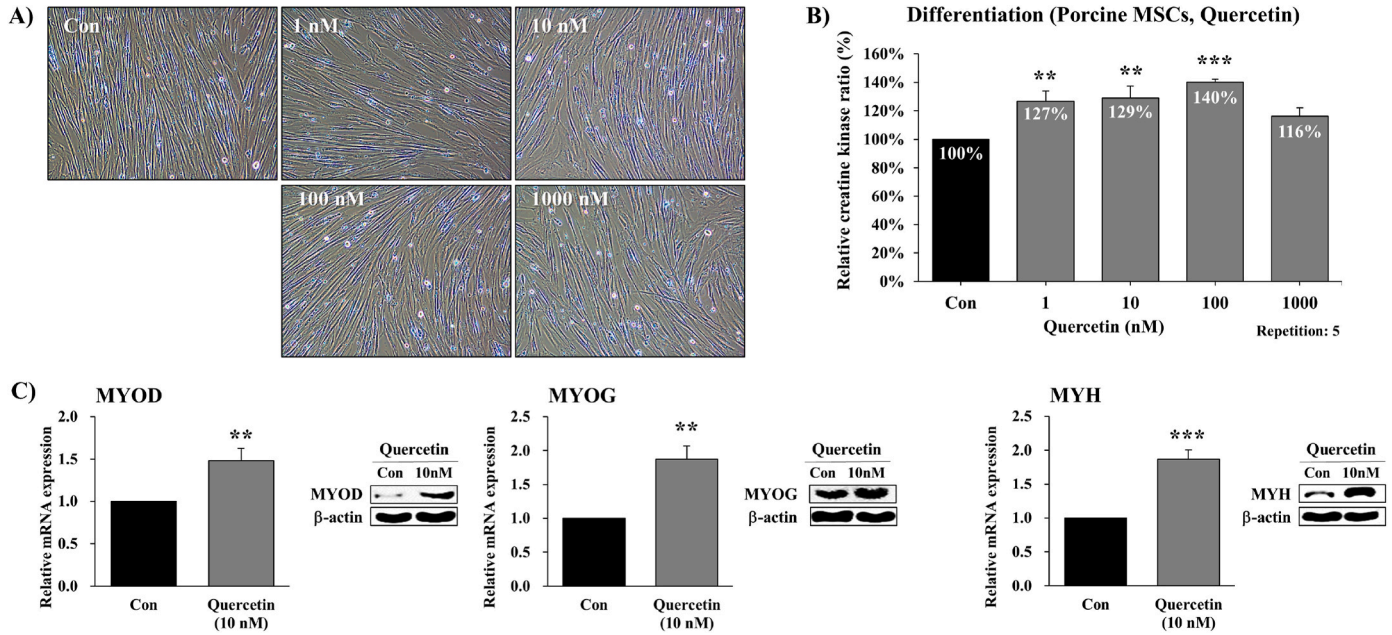

Fig. 5. Porcine MSC differentiation in the presence of different concentrations of quercetin. **A)** Cell morphology observed under a microscope. **B)** Creatine kinase activity results. **C)** The mRNA and protein levels of myogenic markers were determined by Real-time RT-PCR and western blot.

and subsequently phosphorylates SMADs) or SMAD3 enhances atrogin-1 activity in murine SM (Goodman et al., 2013; Sartori et al., 2009), and it has also been reported that SMAD2 or SMAD3 knockdowns increased the cross-sectional area of the murine muscle fiber by 10% and 22%, respectively (Saneyasu et al., 2019). Further, SMAD2 or SMAD3 inhibition promoted muscle hypertrophy (Sartori et al., 2009). In another study, berberine, which binds to MSTN, was found to reduce MSTN expression and increase SM mass (Chen et al., 2020). Thus, it appears that inhibition of MSTN and SMADs offers a means of enhancing muscle mass.

Quercetin also has strong antioxidant properties, and antioxidants help to prevent diseases and work as a potential anti-aging agent (Berger et al., 2012). We observed the pH of the control medium and this was found as 6.86 and 6.53 on culture days 2 and 4 respectively, but it was 6.99 and 6.77 for quercetin supplemented medium. Also, the ROS measurement results showed that when treated with Quercetin, ROS decreased by 10% compared to the control group. This is the reason behind selecting quercetin as a media supplement to inhibit MSTN, maintaining the pH and ROS level. Interestingly, quercetin prevented this acidification more than the commercialized antioxidant supplement. Further, quercetin significantly lowered ROS levels; high ROS levels can cause DNA strand breakage, protein and lipid oxidation, protein cross-linking (Uy et al., 2011), and kill cells (Chevallier et al., 2020). Overall, quercetin was found to increase C2C12 cell

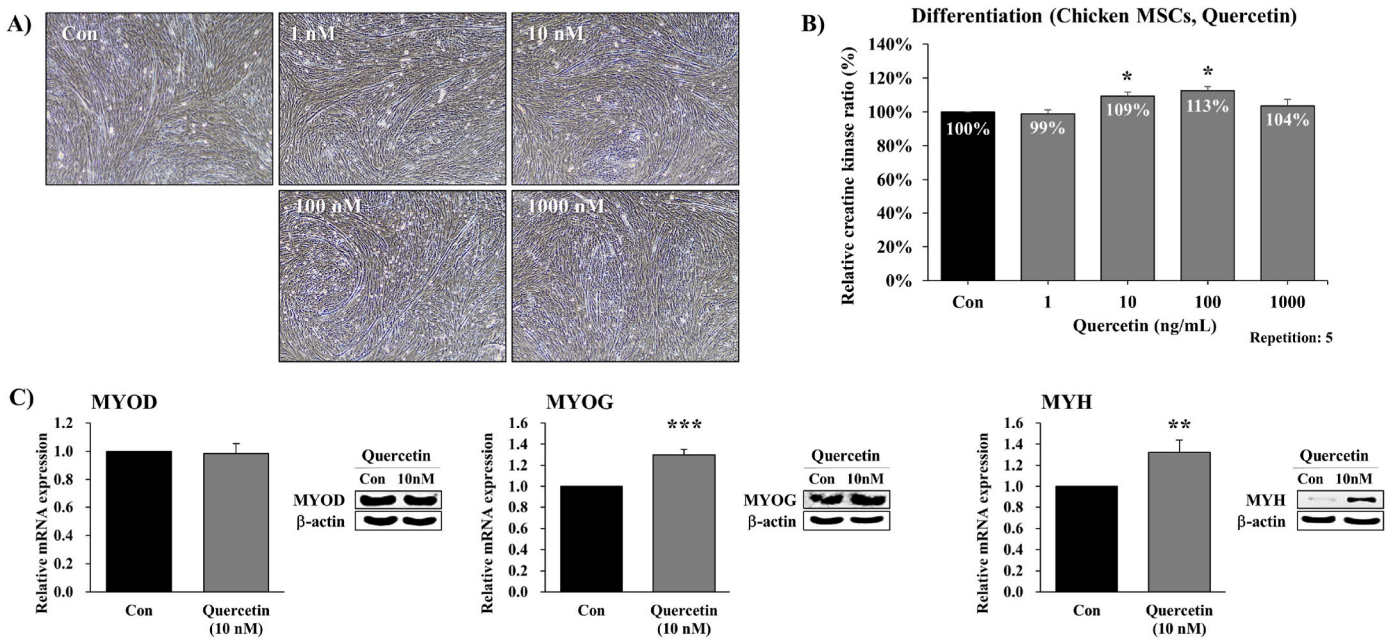

Fig. 6. Chicken MSC differentiation in the presence of different concentrations of quercetin. **A)** Cell morphology observed under a microscope. **B)** Creatine kinase activity results. **C)** The mRNA and protein levels of myogenic markers were determined by Real-time RT-PCR and western blot.

differentiation by 17% versus non-treated controls and by 8% versus antioxidant supplement treatment cells.

CM industries are searching for natural antioxidants that are edible, cost-effective, food-grade supplements or natural compounds. Previous studies have reported that natural plant extracts can be used as antioxidants in meat products (Bellucci et al., 2022), and the present study shows quercetin, a natural antioxidant, has potent anti-oxidative effects and inhibits the negative regulators of myogenesis, which means quercetin is a potential MSC differentiation accelerator for large-scale CM production.

5. Conclusion

The large-scale production of CM requires several improvements in processing conditions. We studied the potential use of quercetin as a medium supplement to improve CM production. Quercetin in media was found to: 1) inhibit MSTN, SMAD2, and SMAD3; 2) improve the differentiation of bovine, porcine, and chicken MSCs; 3) increase the expressions of MYOD, MYOG, and MYH; 4) maintain cell morphology; 5) act as a strong antioxidant; and 6) maintain culture pH. Among the selected livestock species, the differentiation of porcine-derived MSCs was accelerated most by quercetin. It is hoped that this study provides a starting point for research in the CM area aimed at enhancing product quality, nutritional values, and the efficacy of large-scale production.

CRediT authorship contribution statement

Syed Sayeed Ahmad: Methodology, Investigation, Formal analysis, Writing – original draft, Conceptualization, Supervision, Writing – review & editing. **Jeong Ho Lim:** Methodology, Investigation, Formal analysis, Writing – original draft, Methodology, Formal analysis, Writing – review & editing. **Khurshid Ahmad:** Methodology, Investigation, Formal analysis, Writing – original draft. **Hee Jin Chun:** Methodology, Formal analysis, Writing – review & editing. **Sun Jin Hur:** Conceptualization, Supervision, Writing – review & editing. **Eun Ju Lee:** Methodology, Formal analysis, Writing – review & editing. **Inho Choi:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100678.

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