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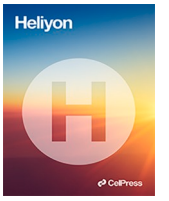

Research article

Effect of *Glycyrrhiza uralensis* crude water extract on the expression of Nitric Oxide Synthase 2 gene during myogenesis

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ABSTRACT

Glycyrrhiza uralensis is a traditional herbal medicine with significant bioactivity. This study investigated the effect of *G. uralensis* crude water extract (GU-CWE) on nitric oxide synthase 2 (NOS2) expression during myogenesis. GU-CWE treatment increased myoblast differentiation by downregulating NOS2 and upregulating myogenic regulatory factors (MYOD, MYOG, and MYH). Notably, this effect was supported by an observed decrease in NOS2 expression in the gastrocnemius tissues of mice treated with GU-CWE. In addition, GU-CWE treatment and NOS2 knockdown were associated with reductions in reactive oxygen species levels. We further elucidate the role of the NOS2 gene in myoblast differentiation, demonstrating that its role was expression dependent, being beneficial at low expression but detrimental at high expression. High NOS2 gene expression induced oxidative stress, whereas its low expression impaired myotube formation. These findings highlight that the modulation of NOS2 expression by *G. uralensis* can potentially be use for managing muscle wasting disorders.

1. Introduction

Skeletal muscle (SM) is consists of multinucleated myofibers that can regenerate and repair themselves after injury, but this ability declines with age and reduces the ability to conduct daily activities. The regenerative potential of SM is primarily dependent on muscle stem cells (MSCs), which are important for directing myofiber development. Myogenic transcription factors like Pax3, Pax7, MYOD, and MYOG regulate MSC progression [1,2], and after injury, quiescent MSCs expressing $Pax7^{+}$ become activated and participate in the activations of MYOD, Myf5, and MYOG, which lead to the formation of myotubes [3]. Differentiation of MSC is critical for SM regeneration and is regulated by many signaling pathways and interactions between various extracellular matrix components and MSCs [4–6]. Furthermore, loss of SM mass is a hallmark of diabetes, obesity, cancer, and aging. Over the years, our group has sought to identify novel inhibitory peptides and small molecules with therapeutic potential in this area [7–14].

Muscle wasting is a pathological condition commonly encountered in the elderly caused by the combined effects of muscular atrophy and myocyte death, which reduce SM mass and muscular strength [15] and leads to incapacitation and an enhanced risk of

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death. Inducible nitric oxide synthase (iNOS or NOS2) is an enzyme that produces nitric oxide (NO), which is associated with muscle atrophy, and is produced by various cellular components, including muscle fibers, in response to TNF-α [16]. Furthermore, accumulating evidence demonstrates that the iNOS/NO pathway is involved in the progression of sarcopenia and muscle wasting caused by cachexia [16–18].

Glycyrrhiza uralensis (licorice) is a popular herbal medicine in Asia, southern Europe and Russia, where it is used to treat coughs, spasms, pain, and inflammation and reduce immune reactions [19–21]. *G. uralensis* research has primarily focused on the pharmacological properties of its constituents, which include polysaccharides, saponins, and flavonoids. These constituents have been extensively studied in animal models and cells for their anti-inflammatory, immunomodulatory, and anti-cancer properties [22,23]. Recently, we reported that *G. uralensis* extracts and three components, namely, licochalcone B, tetrahydroxymethoxychalcone, and liquiritigenin, promote myogenesis [24]. This study aimed to evaluate the effect of *G. uralensis* crude water extract (GU-CWE) on NOS2 gene expression during myogenesis. Furthermore, the antioxidant characteristics of *G. uralensis* was also examined.

2. Materials and methods

2.1. Cell culture

Murine C2C12 myoblasts were acquired from the Korean Cell Line Bank (Seoul, South Korea) and cultured as previously described [24].

2.2. Cell differentiation

The growth media, consisting of DMEM supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (P/S), was substituted with differentiation media. The differentiation media comprised DMEM supplemented with 2 % FBS and 1 % P/S. This substitution occurred once the cells had reached complete confluency or 100 % coverage of the growth surface. Cells were grown in differentiation media supplemented with 100 μg/ml of GU-CWE for 6 days.

2.3. Preparation of G. uralensis CWE

The desiccated roots of *G. uralensis* were acquired from the Kwangmyeong Herbal Store (KM Herb Co., Ltd., Busan, Korea). The voucher specimens were preserved in an herbal bank at the KM Application Center of the Korea Institute of Oriental Medicine [24]. A water extract was prepared from 50 g of these roots, as we previously described [24]. Briefly, 1000 ml of distilled water (DW) was added to dried *G. uralensis* roots (50 g) and extraction was performed by heating at 115 °C for 3 h. The mixture was then filtered through 150 μm standard sieves, and lyophilized. The lyophilized powder was dissolved in DW, chilled at 4 °C for 24 h, and centrifuged at 5000×*g* for 5 min. Supernatant was stored at −20 °C.

2.4. DNA microarray

DNA hybridization was performed using An Agilent Technologies mouse GE 4 × 44 K (V2) chip (Agilent Technologies, Santa Clara, CA, USA). Microarray analysis was performed on C2C12 cells treated with GU-CWE (100 μg/ml) for 4 days to identify differentially expressed genes, as described previously [25]. Briefly, C2C12 cells were grown in a differentiation medium for four days before being used to create cDNA probes using an Agilent Low RNA Input Linear Amplification kit.

2.5. Gene knockdown

C2C12 cells were cultured in growth media until 30 % confluent and then transfected using 1 ng of iNOS2 shRNA or a scrambled vector containing green fluorescent protein gene with reagents and OPTIMEM media. Cells that had undergone transfection were selected using a concentration of 2 μg/ml puromycin (Sigma Aldrich, St. Louis, MO, USA) for four days, and knockdown efficiencies were confirmed by comparing iNOS2 mRNA levels in knocked-down cells with those in scrambled vector-transfected cells. Table S1 lists the shRNA sequence information.

2.6. Mouse experiment

C57BL/6 male mice (6 weeks old) were purchased from Hyochang Science (Daegu, Korea) and housed in a temperature-controlled room at four mice per cage. Animal experiments were conducted per the guidelines issued by the Institutional Animal Care and Use Committee of Yeungnam University (YU-IACUC-2022-013). Mice were given GU-CWE (100 mg/kg/daily) orally for 14 consecutive days. Mice were then subjected to avertin anesthesia (Sigma Aldrich), and gastrocnemius muscle tissues were collected for analysis.

2.7. Real-time RT-PCR

Total RNA was isolated from cells using Trizol® reagent (Invitrogen), and Real-time RT-PCR was conducted as we previously described [24]. Table S2 lists the primer information.

2.8. Western Blot (WB) analysis

WB analysis was conducted using the method described earlier [24]. Briefly, electrophoresed proteins (40 μg–70 μg) were transferred to PVDF membranes (Millipore, Billerica, MA, USA), which were then blocked with 3 % skim milk in tris buffer saline (TBS) for 1 h and incubated with primary antibodies [iNOS2 (1:250), MYOD (1:400), MYH (1:400) MYOG (1:400), and β-actin (1:2000)] in TBS overnight at 4 °C. Membranes were washed, then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature (RT) and detected using Super Signal West Pico Chemiluminescent Substrate. An Azure 300 chemiluminescent imager (Azure biosystem, Dublin, CA, USA) was used to quantify band chemiluminescence.

2.9. Immunocytochemistry

Immunocytochemistry was conducted using antibodies against iNOS2, as previously described [12]. A Nikon Eclipse TE2000-U inverted fluorescent phase-contrast microscope equipped with a Nikon Eclipse Ts2R objective (Nikon, Melville, NY, USA) and a ProgRes C3 CCD Digital Microscope Camera operated by NIS System software was used for fluorescence imaging.

2.10. Immunohistochemistry

Paraffin-embedded gastrocnemius tissues were deparaffinized and hydrated using a xylene and ethanol series (Millipore, Billerica, MA, USA). Endogenous peroxidase activity was inhibited with 0.3 % H_2O_2/methanol, which was then blocked with 1 % normal goat serum. The sections were incubated with iNOS2 antibodies (1:250) overnight at 4 °C, and then with HRP-conjugated secondary antibody (1:100) or stained with hematoxylin and eosin. Signals were visualized using diamino-benzidine and H_2O_2. A light microscope (Leica, Wetzlar, Germany) was used to examine the stained sections.

2.11. Creatine Kinase (CK) activity assay

CK activity was assayed using a CK Activity Assay Kit (EnzyChrom). Briefly, 100 μL of reconstituted reagent was added to cell samples (10 μL) in a 96-well microplate, which was incubated for 20 or 40 min at RT. Absorbance was subsequently quantified at a wavelength of 340 nm using a microplate reader (SYNERGY HT, BioTek).

2.12. Determination of intracellular reactive oxygen species (ROS) levels during myogenesis

After removing the differentiation media, cells were rinsed twice with DMEM (a medium without serum). Then, cells were exposed to 10 μM 2′,7′-Dichlorodihydrofluorescein diacetate in DMEM for 30 min at 37 °C. Finally, the cells were washed twice with phosphate buffer saline. Fluorescence intensities were then measured by a fluorescence microscope equipped with a digital camera (Nikon, Japan) and fluorescence microplate reader (SYNERGY, BioTek) at excitation/emission wavelengths of 485/530 nm. The ROS levels in $NOS2_{kd}$ cells were also measured.

2.13. Statistical analysis

The significant gene expression differences versus mean normalized expressions were determined using Tukey's Studentized Range (HSD) test. Real-time RT-PCR, CK, and ROS levels were analyzed using one and two-way ANOVA in GraphPad Prism ver. 9.0, and statistical significance was accepted for P values < 0.05.

3. Results

3.1. Expression of myogenic marker genes in C2C12 cells

Myogenic marker genes and protein expression were analyzed after differentiating cells for different times (0, 2, 4, and 6 days). During differentiation, mononucleated myoblasts fuse to form elongated myotubes (Suppl. Fig. S1A). CK activity significantly increased over time from D0 to D6 (Suppl. Fig. S1B). mRNA and protein expression of myogenic markers were analyzed. MYOD and MYOG were significantly expressed on D2 and D4 respectively, while MYH expression increased gradually with time and peaked on D6 (Suppl. Fig. S1C). Furthermore, the protein expression levels of these myogenic markers were consistent with the RT-PCR results (Suppl. Fig. S1D).

3.2. Effect of G. uralensis on C2C12 cell differentiation

To study the effect of *G. uralensis* on C2C12 myoblast differentiation, cells were treated with 100 μg/ml of GU-CWE and cultured in differentiation media. Cell differentiation, myotube formation, and CK activity were greater for GU-CWE-treated cells than for non-treated cells (Fig. 1A and B). Further, GU-CWE increased the myogenic regulatory factors' mRNA and protein expressions (Fig. 1C and D). These observations show that GU-CWE promotes myoblast differentiation.

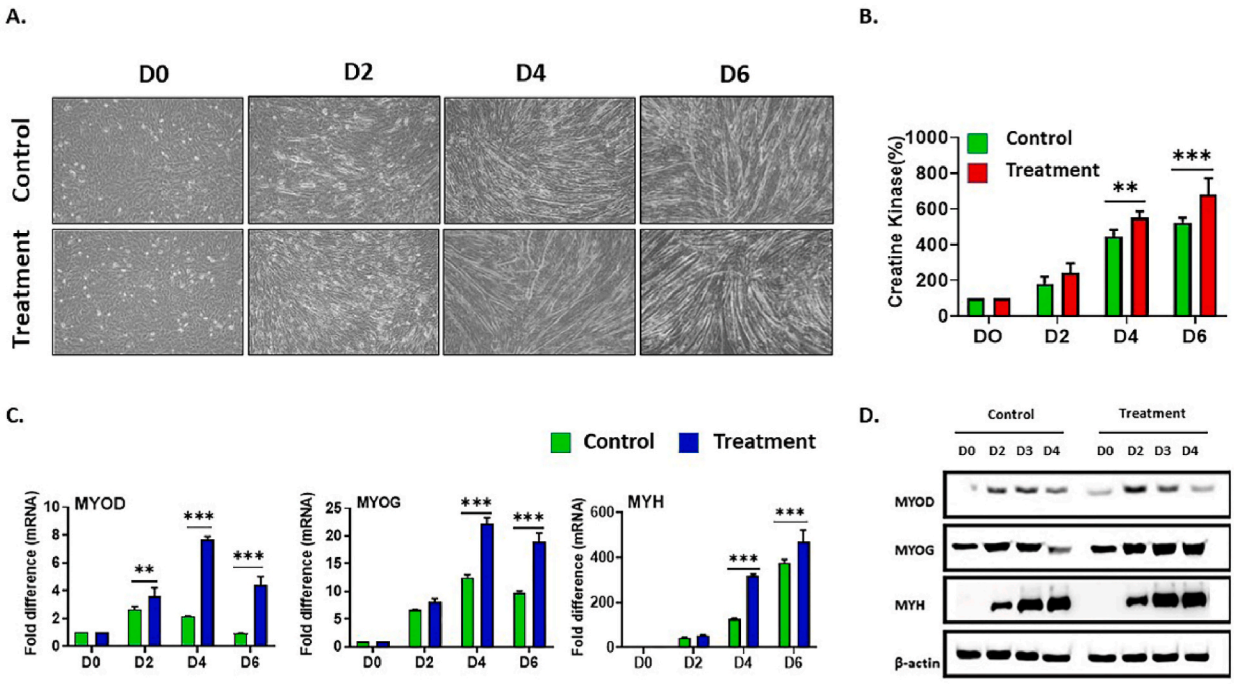

Fig. 1. Effect of *G. uralensis* CWE on C2C12 cell differentiation. (A) Myotube formation of C2C12 cells cultured in differentiation media supplemented with 100 μg/ml GU-CWE for 6 days. (B) Cells were treated with 100 μg/ml of GU-CWE. Creatine Kinase activity in treated and untreated (control) groups. (C) MYOD, MYOG, and MYH mRNA expression with 100 μg/ml of GU-CWE treated and non-treated control groups, as determined by real-time RT-PCR. (D) MYOD, MYOG, and MYH protein expressions as analyzed by Western Blot. Cells were treated with 100 μg/ml of GU-CWE. Day 0 sample was considered as control. Results shown are means ± SD ($n \geq 3$). D = Day. ** $p \leq 0.01$, *** $p \leq 0.001$.

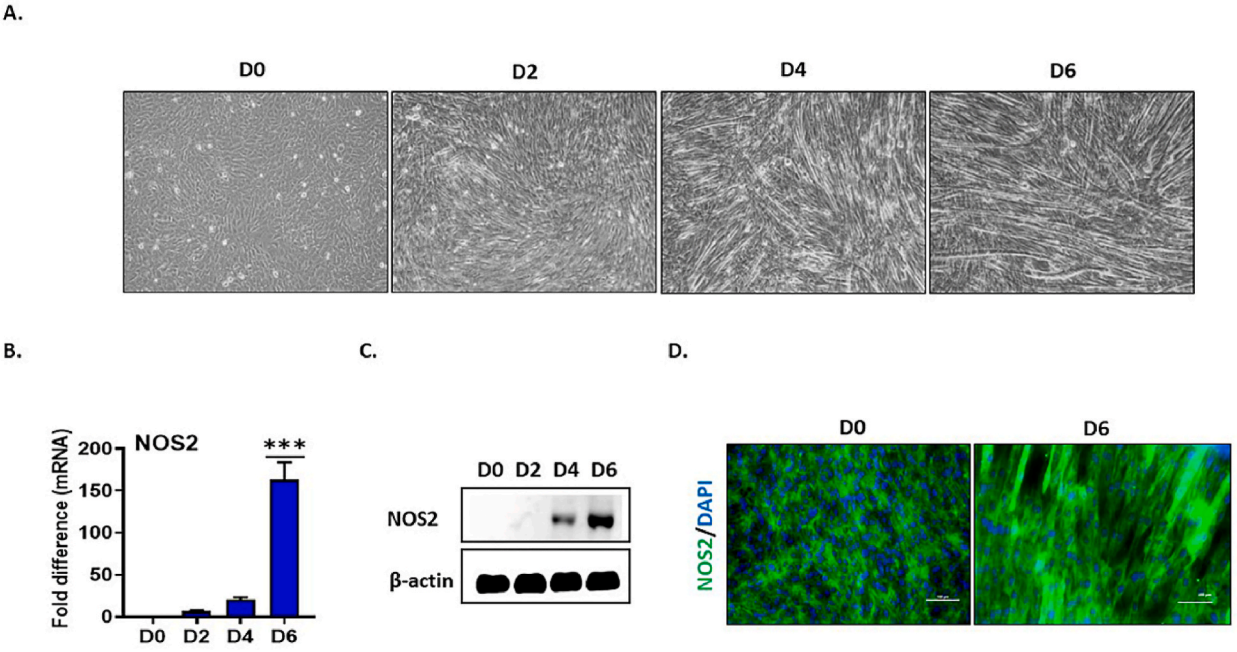

Fig. 2. Expression of NOS2 in C2C12 cells. (A) C2C12 cells were cultured in differentiation media for 6 days. Myotube formation by C2C12 cells incubated in differentiation media for different times (0, 2, 4 and 6 days). (B) NOS2 mRNA expression as assessed by real-time RT-PCR at these time points. (C) NOS2 protein expression as analyzed by Western Blot. (D) NOS2 protein localization as determined by Immunocytochemistry. Day 0 samples were used as a control. Results shown are means ± SD ($n \geq 3$). D = Day. *** $p \leq 0.001$.

3.3. Microarray analysis of gene expressions after G. uralensis treatment

After treating C2C12 cells with GU-CWE for 4 days in differentiation media, microarray analysis revealed significant changes in gene expression. Fifty-three genes were downregulated and 49 were upregulated when a 2-fold cutoff was applied, and 2 were downregulated and 8 upregulated when a 4-fold cutoff was applied (Suppl. Fig. S2) (Suppl. Tables S3 and S4). Notably, the NOS2 gene was among the top downregulated genes. Thus, we conducted an *in vitro* study to investigate the role played by the NOS2 gene in myogenesis and to determine the effect of GU-CWE on NOS2 expression.

3.4. Expression of NOS2 gene during C2C12 cell differentiation

C2C12 cells were grown in differentiation media and the expression of NOS2 gene was analyzed at various time points. NOS2 mRNA and protein expressions peaked on D6 (Fig. 2A–2C). In addition, immunocytochemistry assessment of NOS2 protein localization demonstrated a strong correlation with RT-PCR and WB results (Fig. 2D).

3.5. Effect of G. uralensis CWE on NOS2 expression

To examine the effect of GU-CWE on NOS2 expression, C2C12 cells were treated with GU-CWE and cultured for six days. GU-CWE increased cell differentiation and myotube formation (Fig. 3A) and caused NOS2 mRNA and protein expression to decrease gradually versus non-treated cells (Fig. 3B and C). In addition, immunocytochemistry showed that NOS2 protein expression concurred with RT-PCR and WB results (Fig. 3D). Furthermore, consistent with our *in vitro* findings, GU-CWE reduced NOS2 expression in mouse gastrocnemius tissues (Fig. 3E and F). Altogether, GU-CWE downregulated NOS2 expression during myoblast differentiation.

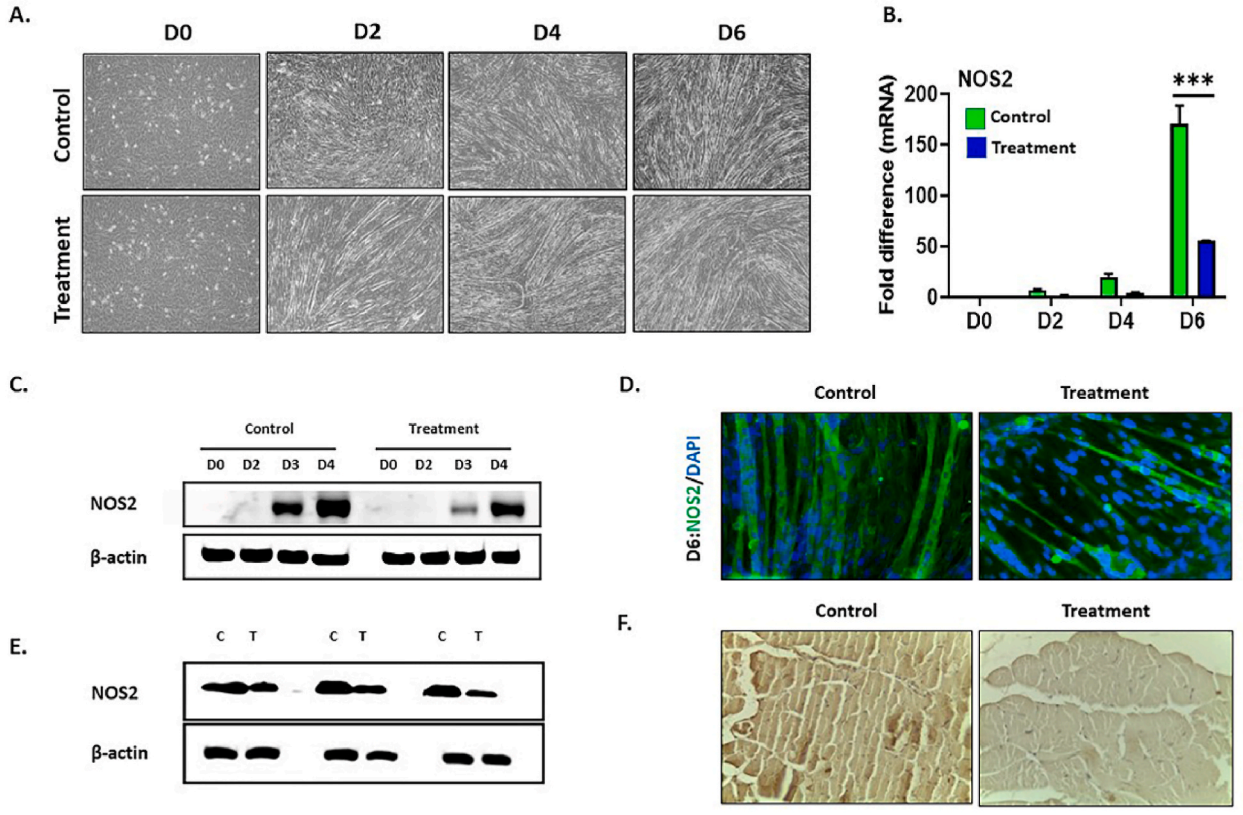

Fig. 3. Effect of *G. uralensis* CWE on NOS2 gene expression. (A) Myotube formation by C2C12 cells treated or not with 100 μg/ml of GU-CWE for 6 days. (B) NOS2 mRNA expression in C2C12 cells treated or not with 100 μg/ml of GU-CWE as determined by real-time RT-PCR. (C) NOS2 protein expression as determined by Western Blot in control and treated groups. (D) NOS2 protein localization by immunocytochemistry on differentiation day 6. (E) NOS2 protein expression was determined by Western Blot. (F) NOS2 expression in muscle tissues was determined by immunohistochemistry. Mouse were orally administered with GU-CWE (100 mg/kg) for fourteen days. Results shown are means ± SD ($n \geq 3$). D = Day. *** $p \leq 0.001$.

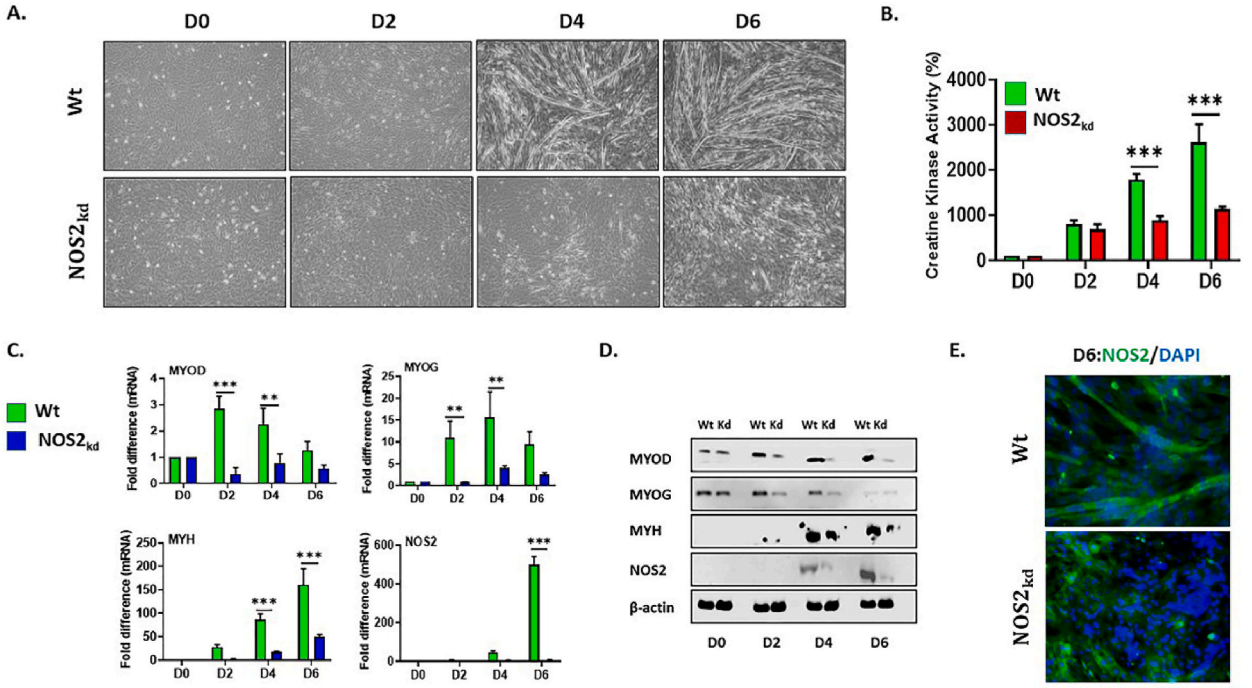

Fig. 4. NOS2 knockdown performed in C2C12 cells. (A) NOS2 was knockdown in C2C12 cells, and cells were then cultured in differentiation media for 6 days. Myotube morphologies of wildtype (Wt) and $NOS2_{kd}$ cells are shown. (B) Creatine kinase activity in Wt and $NOS2_{kd}$ cells. (C) MYOD, MYOG, MYH, and NOS2 mRNA expressions in Wt and $NOS2_{kd}$ cells as determined by real-time RT-PCR. (D) Myogenic markers and NOS2 protein expressions as determined by Western blot. (E) NOS2 protein localization in Wt and $NOS2_{kd}$ cells as determined by immunocytochemistry. Results shown are mean ± SD ($n \geq 3$). D = Day. *** $p \leq 0.001$.

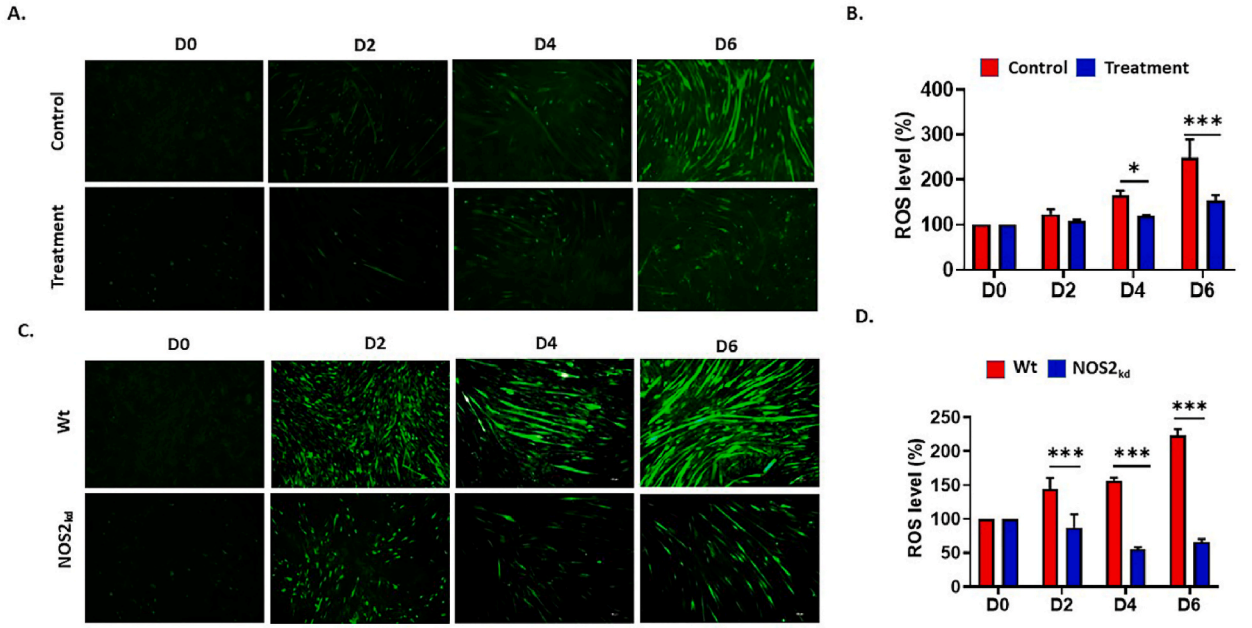

Fig. 5. ROS level in C2C12 cells. (A) ROS was assessed to investigate H_2O_2 involvement using a fluorescence microscope in non-treated controls and cells treated with GU-CWE. (B) ROS levels in cells were determined using a fluorescence microplate reader. (C) ROS levels were measured to determine H_2O_2 involvement using a fluorescence microscope in non-treated controls and $NOS2_{kd}$ cells. (D) ROS level as determined using a fluorescence microplate reader. Results shown are mean ± SD ($n \geq 3$). D = Day. *$p \leq 0.05$, *** $p \leq 0.001$.

3.6. Role of NOS2 in myoblast differentiation

To determine the role played by NOS2 during myoblast differentiation, NOS2 was knocked down in C2C12 cells, and then cells were cultured in differentiation media for six days. Myotube formation and CK activity were significantly lower in $NOS2_{kd}$ cells than in wild-type (Wt) controls (Fig. 4A and B). Furthermore, MYOD, MYOG, and MYH and NOS2 mRNA and protein expressions were downregulated in $NOS2_{kd}$ cells (Fig. 4C and D), and immunocytochemistry results for NOS2 protein in $NOS2_{kd}$ cells were consistent with RT-PCR and WB findings (Fig. 4E). Thus, NOS2 knockdown was found to reduce myoblast differentiation.

3.7. Effect of G. uralensis CWE on ROS level in C2C12 cells

ROS levels in C2C12 cells were reduced by GU-CWE treatment, indicating *G. uralensis* has antioxidative properties (Fig. 5A and B). In addition, ROS levels were lower in $NOS2_{kd}$ cells than in Wt controls (Fig. 5C and D). These findings suggest that GU-CWE treatment and NOS2 knockdown reduce ROS levels.

4. Discussion

Skeletal muscle improves the quality of life by providing body movement, energy metabolism, and postural support, and declines in SM-related functions are associated with aging, chronic diseases, metabolic problems, muscle atrophy, and reduced muscle mass and strength [26]. Unfortunately, the mechanisms responsible for SM mass loss are poorly understood, and no effective pharmacological intervention has yet been developed for muscle atrophy. An investigation of muscle tissue from young and old mice revealed significantly higher levels of iNOS in the muscles of aged mice and associations between this increase and age-associated increases in caspase-2 and JNK signaling activities, which suggested a link between increased iNOS expression and age-associated SM apoptosis [18]. In addition, iNOS has been implicated in cytokine-stimulated cachexia [16,17,27]. Sarcopenia and cachexia are the most common and deadly muscle loss-associated diseases. However, no approved treatment is available [28], although natural products, such as polyphenols, terpenoids, flavonoids, alkaloids, and vitamin D, have been reported to have potential use for regulating SM health and preventing muscle atrophy [29]. Therefore, the current study was conducted to determine whether GU-CWE can down-regulate NOS2 and promote myogenesis.

Initially, we examined the expression level of key myogenic markers (MYOD, MYOG, and MYH) in C2C12 cells at various myogenic time points during differentiation. Notably, MYOD expression was significantly increased on Day 2, whereas MYOG expression peaked on Day 4. Furthermore, the expression of MYH increased gradually and peaked on Day 6. This preliminary analysis was conducted to establish a baseline and standardize the experimental conditions before beginning the study on GU-CWE. Subsequent treatment with GU-CWE enhanced the cell differentiation and myotube formation and increased CK activity. Thus, GU-CWE was found to promote myoblast differentiation effectively. Furthermore, these findings were consistent with our recent study [24], and provided additional support for the beneficial effects of GU-CWE on myoblast differentiation. This corroborating evidence enhanced our understanding of the potential benefits of *G. uralensis* in the context of muscle health.

Analysis of gene expression profiles revealed GU-CWE treatment downregulated expression of the NOS2 gene. A series of *in vitro* experiments were conducted to investigate the role played by NOS2 in myogenesis and the effect of GU-CWE on NOS2 expression. NOS2 expression was assessed at the mRNA and protein levels at different time points and found to peak on Day 6. Interestingly, GU-CWE gradually reduced NOS2 expression at the mRNA and protein levels, further supporting the downregulation of NOS2 by GU-CWE. The consistency of these findings was reinforced by an assessment of NOS2 expression in the gastrocnemius tissues of mice treated with GU-CWE. Notably, treating C57BL/6 mice with GU-CWE reduced NOS2 expression in gastrocnemius tissues. NOS2 plays a crucial role in muscle-wasting diseases such as cachexia and sarcopenia [16], and its inhibition was reported to have promising muscle-protective effects in cancer and cachectic models [30]. In another study, isoliquiritigenin obtained from *G. uralensis* exerted anti-inflammatory effects by down-regulating iNOS/NOS2 in RAW 264.7 cells [31]. Our studies showed that GU-CWE downregulates NOS2 during myoblast differentiation. These findings contribute to our understanding of the regulatory effects of GU-CWE on NOS2 and demonstrate its therapeutic potential for combating muscle wasting-associated conditions.

Knockdown of NOS2 in C2C12 cells markedly decreased the myotube formation, indicating that NOS2 plays a critical role in myoblast differentiation. NOS2 has been previously identified as an important player in mitochondrial elongation, a prerequisite for myoblast differentiation [32]. On the other hand, studies have linked NOS2 to muscle wasting [16]. The involvement of NOS2 in muscle wasting is probably associated with its ability to promote excessive NO synthesis and produce excessive ROS levels. During the early differentiation stage, ROS are produced at low levels and serve as physiological intracellular signaling molecules, but at higher concentrations ROS can be cytotoxic. As a result, the overexpression of NOS2 causes oxidative stress, whereas NOS2 deficiency impairs myotube formation.

Increasing evidence supports the involvement of iNOS-stimulated oxidative stress in sarcopenic and cachectic muscle loss. Notably, several studies have reported an age-related rise in protein nitration levels, which suggests increased NO and peroxynitrite productions [30,33–35]. Interestingly, treatment with GU-CWE or NOS2 knockdown lowered intracellular ROS levels, indicating GU-CWE has antioxidant effects. These findings highlight the potential of *G. uralensis* and NOS2 knockdown as antioxidant strategies in managing muscle wasting conditions.

In a previous publication [24], we conducted a comprehensive analysis where we isolated ten individual compounds from the CWE of *G. uralensis*. These compounds were then characterized using GC-MS and NMR, followed by evaluating their effects on myoblast proliferation and differentiation. Specifically, liquiritigenin, one of the isolated compounds, has been previously reported to exhibit

efficacy in reducing ROS activity [36]. These findings suggest a potential ROS relationship between liquiritigenin and NOS2 in the context of GU-CWE treatment.

5. Conclusions

We investigated the effect of GU-CWE on NOS2 during myogenesis, and its potential antioxidant properties. GU-CWE increased myoblast differentiation, and this was attributed to the downregulation of NOS2 and the upregulation of myogenic regulatory factors. Our findings also shed light on the role of the NOS2 gene in myoblast differentiation by demonstrating that its role was expression dependent, being beneficial at low expression but detrimental at high expression. Furthermore, the observations that GU-CWE treatment and NOS2 knockdown reduced ROS levels suggest they may have antioxidative properties on muscle wasting conditions.

Ethics approval and consent to participate

This study did not involve any human samples. Animal experiments were conducted in accordance with the guidelines issued by the Institutional Animal Care and Use Committee of Yeungnam University (YU-IACUC-2022-013).

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Consent for publication

Not applicable.

CRediT authorship contribution statement

Afsha Fatima Qadri: Writing – original draft, Investigation, Methodology, Data curation. **Sibhghatulla Shaikh:** Writing – review & editing, Writing – original draft, Formal analysis. **Ye Chan Hwang:** Writing – review & editing. **Khurshid Ahmad:** Methodology, Formal analysis. **Inho Choi:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Eun Ju Lee:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34747.

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