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A Balanced Amino Acid Supplementation Enhances Myogenic Differentiation, Energy Metabolism, and Antioxidant Capacity in C2C12 Cells

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Abstract

Conventional nutritional strategies for supporting muscle health have largely focused on increasing the intake of branched-chain amino acids (BCAAs), particularly leucine. However, simply increasing the amount of BCAAs or total amino acids may not be sufficient to elicit optimal physiological effects. Given that individual amino acids exert distinct metabolic and physiological functions, functionally complementary combinations administered at appropriate concentrations may provide a more effective strategy for promoting muscle health. This study investigated whether a combination of leucine, cysteine, glycine, glutamine, and taurine could coordinately enhance myogenic differentiation, cellular energy metabolism, and antioxidant defense in C2C12 muscle cells. After determining appropriate treatment concentrations for each amino acid, C2C12 myoblasts were differentiated for 7 days in the presence of leucine alone or various amino acid combinations. Myotube morphology, the expression of genes associated with muscle anabolism, myogenic differentiation, catabolism, energy metabolism, and antioxidant defense, and intracellular ATP, glutathione (GSH), and reactive oxygen species (ROS) levels were evaluated. Leucine treatment enhanced myotube formation and increased Akt1 and Igf1 expression. However, combined amino acid treatments generally promoted greater myotube formation than leucine alone. Notably, the half-strength five-amino-acid formulation (1/2 LCGQT) produced the greatest increases in myotube length and thickness and markedly enhanced the expression of myogenic regulatory genes, including Myod1, Myf5, Myf6, and Mef2. Combined amino acid treatments also favorably regulated genes associated with mitochondrial function and energy metabolism, including Cycs, Ucp2, Ampk, and Pgc1a, and specific combinations increased intracellular ATP levels. In addition, the 1/2 LCGQT formulation elicited the highest Gpx1

expression and intracellular GSH concentration, while several combinations significantly reduced intracellular ROS levels. The expression of the muscle catabolism-related genes Mstn and Foxo3 was not significantly altered. Collectively, these findings indicate that combining amino acids according to their complementary physiological and metabolic functions may more effectively promote myogenic differentiation and support cellular energy and redox homeostasis than leucine supplementation alone. Moreover, the superior and more consistent effects of the half-strength formulation demonstrate that optimal amino acid composition and concentration are more important than simply increasing the total amount supplied. This study therefore provides a basis for a function-guided nutritional strategy that shifts from quantity-oriented BCAA supplementation toward the selective combination of physiologically relevant amino acids at appropriately optimized levels.

Keywords: amino acid combination; myogenic differentiation; energy metabolism; glutathione; oxidative stress

Introduction

Growing interest in maintaining health and physical function has led to an increase in the number of individuals who participate regularly in exercise. This trend has also heightened the demand for nutritional strategies that can effectively improve exercise performance, muscle strength, and muscle function. In particular, proteins and amino acids are increasingly recognized as important nutrients for maintaining muscle mass and supporting post-exercise recovery. Consequently, they have been widely incorporated into dietary supplements and sports nutrition products, and the market for these products continuaes to expand.

Amino acids have traditionally been valued primarily as the fundamental building blocks of body proteins. However, increasing evidence indicates that individual amino acids function not only as substrates for protein synthesis but also as metabolic and signaling regulators involved in cellular signaling, energy metabolism, redox homeostasis, and tissue function (Hu and Guo 2021). Amino acid–based nutritional strategies for muscle health have largely focused on supplementation with branched-chain amino acids (BCAAs), particularly leucine, to stimulate muscle protein synthesis (Kaspy et al. 2024). Arginine has also been used as a sports nutrition ingredient because of its physiological roles in nitric oxide production and blood flow regulation (Park et al. 2023).

Leucine is a potent anabolic amino acid that stimulates muscle protein synthesis primarily through activation of the mTORC1 signaling pathway (Ely et al. 2023). However, the maintenance and development of skeletal muscle cannot be achieved solely by activating anabolic signaling or increasing the supply of a single amino acid. Muscle protein synthesis requires a balanced supply of amino acid substrates, while muscle cell growth and differentiation also depend on adequate energy production and the maintenance of cellular

redox homeostasis (White 2021; Lian et al. 2022). Moreover, the indiscriminate consumption of amino acids in amounts exceeding physiological requirements may disrupt amino acid balance and increase the metabolic burden associated with amino acid catabolism and nitrogen disposal (Torres et al. 2023). Therefore, nutritional strategies for muscle health should move beyond the simple provision of high doses of individual amino acids and instead consider combinations and concentrations based on the distinct physiological and metabolic functions of each amino acid.

Efficient muscle cell growth and differentiation require not only anabolic signaling and substrates for protein synthesis but also sufficient energy to meet increased metabolic demands and an appropriate intracellular environment for controlling oxidative stress (Atherton et al. 2010; Pala et al. 2018). Because dietary protein and amino acids are already consumed in sufficient quantities by many individuals, increasing total amino acid intake may not necessarily provide additional benefits. A more effective strategy may be to optimize the balance of metabolically complementary amino acids so that available nutrients can be used more efficiently by muscle cells. Accordingly, the present study focused on leucine, cysteine, glycine, glutamine, and taurine, which may contribute to muscle protein synthesis, cellular energy metabolism, and antioxidant defense through distinct but complementary mechanisms.

Cysteine is a sulfur-containing amino acid that can be synthesized through the methionine transsulfuration pathway and serves as a major limiting substrate for the synthesis of glutathione, a principal intracellular antioxidant (Zhu et al. 2019). Glycine is also a constituent of glutathione, which is synthesized from glutamate, cysteine, and glycine. In addition, glycine participates in serine and one-carbon metabolism and serves as a substrate

for creatine synthesis (Alves et al. 2019), suggesting that it may support cellular biosynthesis, antioxidant capacity, and the energy-buffering system of skeletal muscle. Glutamine plays an important role in nitrogen transport and amino acid metabolism (Cruzat et al. 2018). Through glutaminolysis, glutamine can be converted to glutamate and subsequently to α-ketoglutarate, thereby replenishing tricarboxylic acid cycle intermediates and supporting cellular energy metabolism (Yoo et al. 2020). Glutamate derived from glutamine can also be used for glutathione synthesis, indicating that glutamine may contribute to both energy metabolism and antioxidant defense.

Taurine is a sulfur-containing amino sulfonic acid derived from cysteine metabolism and has multiple physiological functions, including membrane stabilization, osmoregulation, calcium homeostasis, and antioxidant defense (Seidel et al. 2019). When the physiological demand for taurine increases, cysteine may be directed toward endogenous taurine synthesis. Therefore, exogenous taurine supplementation may reduce the metabolic requirement for taurine synthesis from cysteine and potentially preserve cysteine availability for glutathione production. On this basis, the combination of cysteine, glycine, glutamine, and taurine was expected to provide complementary support for glutathione synthesis, cellular energy metabolism, and redox homeostasis, thereby creating a metabolic environment favorable for leucine-induced muscle cell growth and differentiation.

In the present study, C2C12 myoblasts were treated with various combinations and concentrations of leucine, cysteine, glycine, glutamine, and taurine. Their effects on myotube formation and morphology were evaluated together with changes in markers related to myogenic synthesis and differentiation, energy metabolism, and antioxidant defense. This study aimed to determine whether amino acid combinations designed according to the

metabolic functions of their individual components could promote myogenic differentiation and improve the metabolic environment of muscle cells more effectively than supplementation with a single amino acid or BCAAs alone. Furthermore, by examining the importance of both amino acid composition and concentration, this study sought to provide a scientific basis for developing precisely balanced amino acid formulations to support muscle formation and muscle cell metabolism.

Material and methods

Cell culture and amino acid treatments

C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, SH30243.01) supplemented with 10% fetal bovine serum (FBS; Corning, 35-015-CV) and 1% penicillin-streptomycin-glutamine (Gibco, 10378-016) at 36°C in a humidified atmosphere containing 5% CO_2.

For individual amino acid treatments, cells were seeded in 24-well plates at a density of 5×10^4 cells/well. When the cells reached approximately 60–70% confluence, they were treated with the indicated amino acids. Each amino acid was dissolved in phosphate-buffered saline (PBS) to prepare a 500 mM stock solution. cells were treated for 24 h with leucine, cysteine with glycine or glutamine or taurine and amino acids mixture. The amino acid formulation used in this study was developed and supplied by Samyang Foods Co., Ltd. (Seoul, Republic of Korea). The formulation consisted of leucine, glutamine, cysteine, glycine, and taurine. The relative proportions of the amino acid formulation are currently withheld because the formulation is the subject of an ongoing patent application. The detailed information of the formulation will be disclosed upon completion of the patent process in accordance with the journal's data-sharing policies. In addition to the standard-concentration mixture, a half-dose formulation was prepared by reducing the concentration of each amino acid to 50% while maintaining the same relative proportions.

Myotube differentiation and combined amino acid treatment

C2C12 myoblasts were seeded in 24-well plates at a density of 5×10^4 cells/well. When the cells reached approximately 90% confluence, the growth medium was replaced with

differentiation medium to induce myogenic differentiation. The differentiation medium consisted of DMEM (HyClone, SH30243.01) supplemented with 2% heat-inactivated horse serum (Gibco, 26050070) and 1% penicillin-streptomycin-glutamine (Gibco, 10378-016).

Amino acids were added to the differentiation medium at the concentrations specified for each treatment group. The differentiation medium containing the corresponding amino acid combinations was replaced every 24 h. Cells were differentiated for 7 days, and the progression of myotube formation was monitored throughout the differentiation period.

Analysis of myotube morphology

Myotube morphology was evaluated by measuring myotube length and thickness. Three images were acquired from each well at 100× magnification. The lengths and thicknesses of the myotubes in each image were measured, and the mean values obtained from the three images were used as representative values for each well. These values were subsequently compared among the experimental groups.

RNA extraction and quantitative real-time PCR

After completion of the experiments, total RNA was extracted using TRIzol reagent (Invitrogen, 15596026). Complementary DNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1621) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qPCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, K0222) to determine the relative mRNA expression levels of the target genes. Gene expression levels were normalized to those of *Gapdh* and expressed relative to the control group. The primer sequences used for qPCR

are listed in Table 1.

Measurement of intracellular ATP levels

Intracellular ATP levels were measured using an ATP Assay Kit (Abcam, ab83355) according to the manufacturer's instructions.

Measurement of intracellular glutathione concentrations

Intracellular glutathione concentrations were measured using a GSH+GSSG/GSH Assay Kit (Abcam, ab239709) according to the manufacturer's instructions.

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA was added to the cell culture medium and taken up by the cells. Intracellular oxidation of the probe by ROS generated the fluorescent compound 2′,7′-dichlorofluorescein. Fluorescence intensity was measured and used as an indicator of intracellular ROS levels.

Statistical analysis

Statistical analyses were performed using ordinary one-way analysis of variance (ANOVA) in GraphPad Prism version 10.1.1 (GraphPad Software). Differences among groups were evaluated using Tukey's multiple-comparisons test. Differences were considered statistically significant at $p < 0.05$.

Results

Amino acid mixtures enhance myotube formation and growth

To evaluate the effects of amino acid mixtures on myogenic differentiation, C2C12 myoblasts were treated with leucine or defined amino acid combinations and subsequently induced to differentiate into myotubes. Leucine treatment increased myotube formation relative to the untreated control (Figure 1). The combination of leucine and cysteine (LC) further enhanced myotube formation compared with leucine alone. The addition of glycine (LCG), glutamine (LCQ), or taurine (LCT) to LC produced a further increase in myotube formation (Figure 1). Treatment with the five-amino-acid mixture containing leucine, cysteine, glycine, glutamine, and taurine (LCGQT) also enhanced myotube formation compared with leucine alone. Notably, the half-dose mixture (1/2 LCGQT) produced the most pronounced morphological response (Figure 1).

Quantitative measurements of myotube length and diameter were consistent with the morphological observations. Myotube length was significantly greater in the three-amino-acid mixture groups than in the control, leucine, and LC groups ($p < 0.05$). Significant increases in myotube length were also observed in the LCGQT and 1/2 LCGQT groups (Figure 2a). Myotube diameter was significantly greater in the LCQ and 1/2 LCGQT groups than in the control and leucine groups ($p < 0.05$; Figure 2b).

Effects of amino acid mixtures on the expression of genes associated with muscle synthesis and myogenic differentiation

We next examined the effects of the amino acid mixtures on the expression of genes involved in muscle protein synthesis and myogenic differentiation. mTOR mRNA expression did not

differ significantly among the groups (Figure 3a). Akt1 mRNA expression tended to be higher in amino-acid-treated cells than in untreated controls, although most differences did not reach statistical significance. In contrast, Akt1 expression was significantly increased in the 1/2 LCGQT group compared with the other groups (Figure 3b).

Igf1 mRNA expression was significantly increased in the leucine, LC, and LCG groups relative to the control group ($p < 0.05$). A significant increase was also observed in the 1/2 LCGQT group (Figure 3c). Myod1, a key regulator of myoblast differentiation, was significantly upregulated in the 1/2 LCGQT group compared with the control and leucine groups ($p < 0.05$; Figure 3d). Myf5 mRNA expression was significantly increased in the leucine, LC, and LCG groups relative to the control group ($p < 0.05$). Myf5 expression was also significantly higher in the LCGQT and 1/2 LCGQT groups than in the control group (Figure 3e). Mef2 mRNA expression was significantly increased in the LCG group compared with the control group ($p < 0.05$), with the highest expression observed in the 1/2 LCGQT group (Figure 3f).

Effects of amino acid mixtures on genes associated with muscle protein degradation

The mRNA expression levels of Mstn and Foxo3a, which are associated with negative regulation of muscle growth and muscle protein degradation, respectively, were not significantly altered by treatment with leucine or any of the amino acid mixtures (Figure 4a, b).

Effects of amino acid mixtures on energy metabolism in muscle cells

We next assessed the expression of genes involved in mitochondrial energy metabolism and measured intracellular ATP levels. Cycs mRNA expression was generally higher in the amino acid mixture groups than in the leucine group and was significantly increased in the LCT group ($p < 0.05$; Figure 5a). The highest Cycs expression was observed in the 1/2 LCGQT group

(Figure 5a).

Ucp2 mRNA expression tended to be higher in the LC, LCG, LCQ, and LCT groups than in the leucine group, although these differences were not statistically significant (Figure 5b). Ucp2 expression was significantly increased in the LCGQT group, and the greatest increase was observed in the 1/2 LCGQT group (Figure 5b). Ampk mRNA expression did not differ significantly between the control and leucine groups but was significantly increased in the 1/2 LCGQT group ($p < 0.05$; Figure 5c). Pgc1a mRNA expression tended to increase following leucine treatment, although the difference from the control was not significant. In contrast, Pgc1a expression was significantly increased by the amino acid mixtures, with the highest level observed in the 1/2 LCGQT group (Figure 5d).

Consistent with these transcriptional changes, intracellular ATP levels were unchanged by leucine alone but were significantly increased in the LCG and LCQ groups compared with the control group (Figure 5e).

Effects of amino acid mixtures on antioxidant-related gene expression, glutathione levels, and intracellular reactive oxygen species

Finally, we evaluated antioxidant-related gene expression, intracellular glutathione (GSH) levels, and reactive oxygen species (ROS) levels. Sod1 mRNA expression tended to be higher in the LC group than in the control group, although the difference was not statistically significant (Figure 6a). In contrast, Sod1 expression was significantly increased in the 1/2 LCGQT group compared with the control group ($p < 0.05$; Figure 6a). Cat mRNA expression was also significantly increased in the 1/2 LCGQT group relative to the control group (Figure 6b).

Gpx1 mRNA expression was significantly increased in the LCT group compared with the control group ($p < 0.05$) and was also significantly elevated in the LCGQT group. The highest Gpx1 expression was observed in the 1/2 LCGQT group (Figure 6c). Intracellular GSH levels were significantly increased by leucine and by all amino acid mixtures compared with the control treatment ($p < 0.05$; Figure 6d). The amino acid mixture groups also exhibited significantly higher GSH levels than the leucine group ($p < 0.05$), with the highest concentration detected in the 1/2 LCGQT group (Figure 6d). Although intracellular ROS levels did not differ significantly among the groups, a decreasing trend was observed following treatment with the amino acid mixtures (Figure 6e).

Discussion

Amino acids serve not only as substrates for protein synthesis but also as metabolic and signaling regulators that influence cellular differentiation, energy production, and redox homeostasis (Ling et al. 2023). Nevertheless, nutritional strategies intended to support skeletal muscle have largely focused on increasing the intake of branched-chain amino acids, particularly leucine, because of its well-established anabolic effects (Li et al. 2024). Myogenic differentiation, however, is a metabolically demanding process that requires the coordinated activation of anabolic pathways, sufficient energy supply (Pala et al. 2018), and maintenance of intracellular redox balance (Rajasekaran et al. 2020). On this basis, we hypothesized that combining amino acids with distinct but complementary metabolic functions would enhance myotube formation more effectively than simply increasing the supply of a single anabolic amino acid.

The amino acid combination used in this study was designed according to these complementary functions. Leucine was included as the principal anabolic stimulus, whereas glycine and glutamine were selected to support cellular and energy metabolism (Kamei et al. 2020). Cysteine and taurine were included to reinforce glutathione-dependent antioxidant capacity and redox homeostasis (Gould and Pazdro 2019). Leucine alone enhanced myotube formation relative to the untreated control, and the addition of cysteine produced a further increase. The three-amino-acid mixtures containing glycine, glutamine, or taurine generally enhanced differentiation beyond that observed with leucine alone, while the complete LCGQT mixture and its half-dose formulation markedly increased myotube length and diameter. Thus, the overall pattern of the morphological results supports the concept that amino acids selected for complementary metabolic roles can act cooperatively to promote myogenic differentiation.

The expression patterns of myogenesis-related genes were consistent with the morphological findings. Leucine and several amino acid combinations increased Igf1 and Myf5 expression, whereas the 1/2 LCGQT mixture produced prominent increases in Akt1, Igf1, Myod1, Myf5, and Mef2 expression. In contrast, Mstn and Foxo3a expression was not significantly altered. These results suggest that the amino acid mixtures primarily promoted the transcriptional program associated with myogenic differentiation and growth rather than producing a broad suppression of genes related to negative regulation or protein degradation. Although mTOR mRNA expression was unchanged, this does not conflict with the observed myogenic response because mTOR signaling is regulated mainly at the post-translational level (Yin et al. 2021). Taken together, the enhanced myotube morphology and coordinated expression of myogenic factors indicate that the mixtures provided a more favorable cellular environment for differentiation than leucine alone.

The effects of the mixtures on energy metabolism further support this interpretation. Glycine and glutamine were incorporated because their metabolic pathways can contribute to the biosynthetic and energetic requirements of differentiating muscle cells. Amino acid mixture treatment increased the expression of Cycs, Ucp2, Ampk, and Pgc1a in selected groups, and intracellular ATP levels were higher in specific three-amino-acid mixture groups than in the control group. The complete mixture and particularly the 1/2 LCGQT formulation elicited strong responses in several energy-metabolism-related genes. These findings suggest that adding metabolically supportive amino acids to leucine may facilitate myotube formation by improving the cellular capacity to meet the increased energy demand associated with differentiation. Therefore, the beneficial effect of the mixtures appears to involve not only the provision of amino acid substrates for muscle protein synthesis but also coordinated support of

the metabolic processes required for muscle cell growth.

Cysteine and taurine provided a complementary contribution through the antioxidant system. Cysteine is a major precursor for glutathione synthesis and can also be metabolized to taurine (Yin et al. 2016). We therefore proposed that the combined provision of cysteine and taurine would support glutathione synthesis while reducing the metabolic demand for conversion of cysteine to taurine. Consistent with this rationale, mixtures containing cysteine and taurine increased Gpx1 expression and intracellular GSH levels compared with leucine alone. The LCGQT and 1/2 LCGQT groups showed particularly strong increases, with the highest GSH level observed in the half-dose group. Intracellular ROS levels also tended to decrease following treatment with the mixtures, although the differences were not statistically significant. These results indicate that reinforcement of antioxidant capacity may be another important component of the enhanced myogenic response, as appropriate redox control is required to sustain the metabolic activity of differentiating muscle cells.

The combined results demonstrate the value of integrating anabolic, energetic, and antioxidant functions within a single amino acid formulation. Leucine provided a direct stimulus for muscle formation, while glycine and glutamine supported energy-related metabolism and cysteine and taurine reinforced glutathione-dependent antioxidant capacity. The greater effects of the mixtures than of leucine alone suggest that these functional roles complemented one another and collectively created metabolic conditions favorable for myotube formation. Although the present experimental design does not formally quantify synergy among the individual components, the consistent improvement across myotube morphology, myogenic gene expression, ATP production, and GSH status supports a cooperative effect of the combined amino acids.

An especially important finding was that the half-dose LCGQT mixture produced stronger effects than the full-dose mixture across most measured outcomes. The 1/2 LCGQT group exhibited the greatest increases in myotube length and diameter, myogenesis-related factors, energy-metabolism-related genes, and antioxidant markers. This non-linear response indicates that the efficacy of an amino acid formulation is determined not simply by the total amount supplied but by the balance between its components and the concentration delivered to the cells. Excessive amino acid availability may disturb intracellular amino acid balance or increase the metabolic burden associated with amino acid catabolism (Bröer and Bröer 2017), whereas an appropriately balanced dose may allow each amino acid to perform its complementary role more efficiently. Accordingly, these findings emphasize that a metabolically optimized combination at an appropriate concentration may be more effective than a larger, non-selective supply of amino acids.

This study was limited to C2C12 cells, and most mechanistic outcomes were assessed at the mRNA level. Further studies examining protein-level signaling, metabolic changes, and muscle-related functional outcomes in primary cells and in vivo models will be useful for confirming the proposed complementary actions of the mixture. Nevertheless, the present findings provide clear evidence at the cellular level that the rational combination of amino acids according to their metabolic functions can enhance myogenic differentiation more effectively than leucine alone.

In conclusion, a mixture of leucine, cysteine, glycine, glutamine, and taurine promoted myotube formation by coordinately supporting myogenic regulation, energy metabolism, and antioxidant capacity. The superior efficacy of the half-dose formulation further demonstrates that the biological effects of amino acids depend on appropriate composition and dosage rather

than simply on the total quantity supplied. This metabolically targeted approach may provide a useful basis for developing precisely balanced amino acid formulations to support muscle formation and muscle cell metabolism.

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Table 1. Primer sequences used for quantitative real-time PCR

Gene name	NCBI ID	Primer sequence	
Gapdh	NM_001289726.2	Forward	CATCACTGCCACCCAGAAGACTG
		Reverse	ATGCCAGTGAGCTTCCCGTTCAG
Myod1	NM_010866.2	Forward	CTCTTCCCAACTGTCCTTTC
		Reverse	GGACTATGTCCTTTCTTTGGG
Myf6	NM_008657.3	Forward	CAGCTACATTGAGCGTCTAC
		Reverse	CCGCACCCTCAAGAATTT
Myf5	NM_008656.5	Forward	CTCCAGCTCCAGACTTATCTA
		Reverse	CAGCTTCAGGGCTTCTTT
Mef2	NM_001347564.2	Forward	CCAGCACTGACATGGATAAG
		Reverse	CATTGAGGCCCTTCTTTCTC
Mstn	NM_010834.3	Forward	CAGCAGTGATGGCTCTTT
		Reverse	CCATCCGCTTGCATTAGA
Foxo3	NM_001376967.1	Forward	GATCTCCTTGTCTGGCAATC
		Reverse	CCAATTCTTCTGGTCTCCTTC
Cycs	NM_007808.5	Forward	GGCAGGGAAATGAGTGATAG
		Reverse	ACCTGAGGCCCAGTTAAT
Ucp2	NM_001417452.1	Forward	CTAATGGCTGCCTACCAATC
		Reverse	TGGGCAGAGGATGAAGAA
Ampk	NM_001013367.3	Forward	GAAGAGGGCCGCAATAAA
		Reverse	GGGTCCTCAGGAAAGAGATA
Pgc1a	NM_008904.3	Forward	GACAATCCCGAAGACACTAC
		Reverse	AGAGAGGAGAGAGAGAGAGA

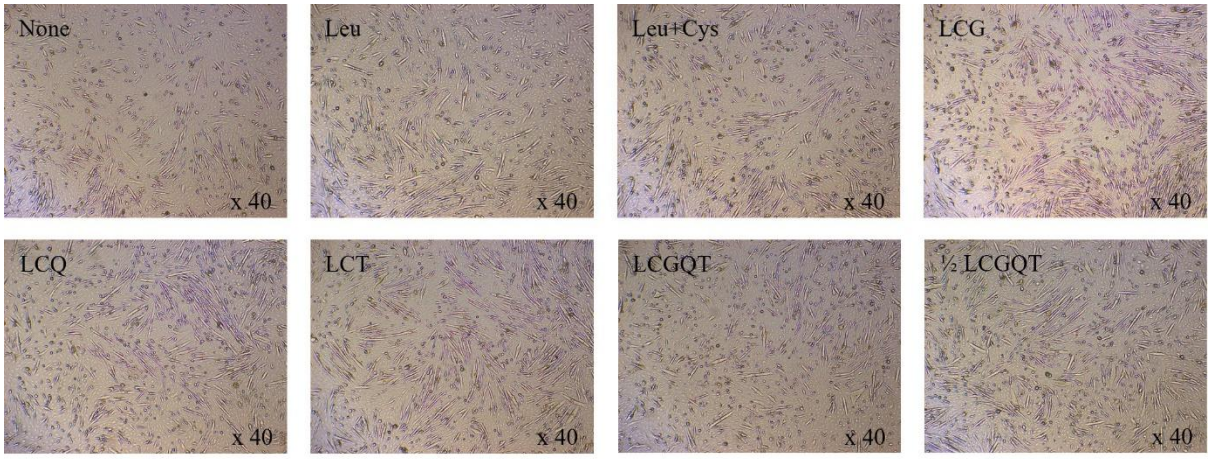


Figure 1. Effects of combined amino acid treatment on myotube formation in C2C12 cells. C2C12 myoblasts were cultured to approximately 90% confluence and subsequently differentiated for 7 days in differentiation medium containing the indicated amino acids. Representative phase-contrast microscopic images show myotube morphology in the untreated control (None), leucine-treated group (Leu), leucine and cysteine-treated group (Leu+Cys), and groups treated with leucine and cysteine in combination with glycine (LCG), glutamine (LCQ), or taurine (LCT). LCGQT represents treatment with all five amino acids—leucine, cysteine, glycine, glutamine, and taurine—whereas 1/2 LCGQT represents treatment with the same combination at half the concentration of each amino acid. Images were acquired at 40× magnification.

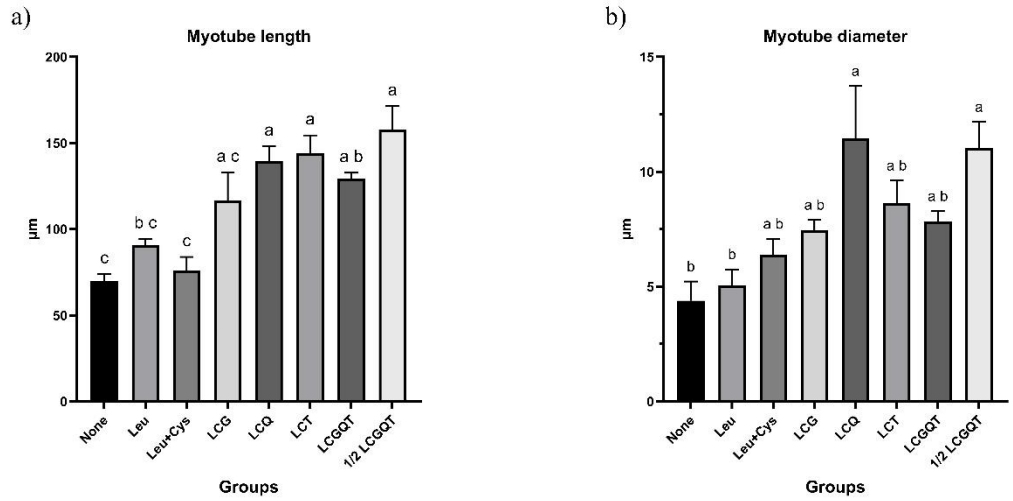
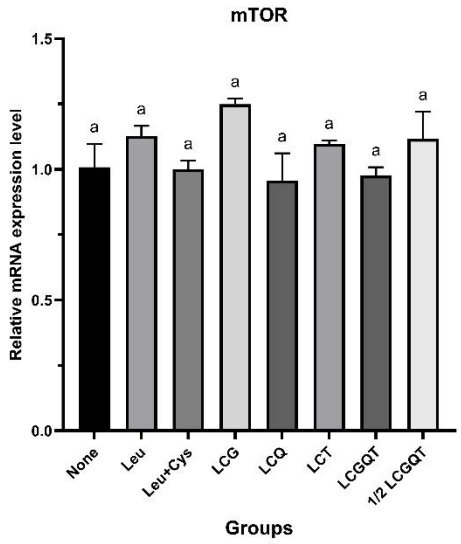
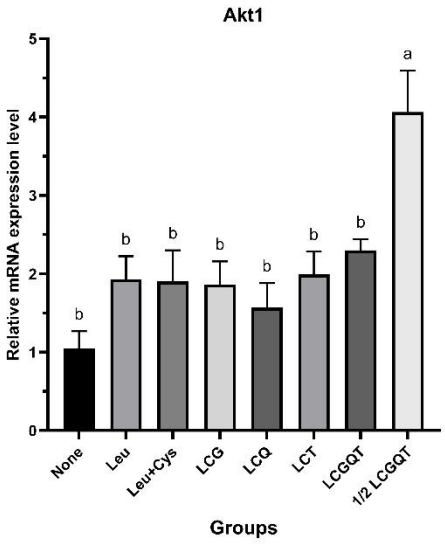


Figure 2. Effects of combined amino acid treatment on myotube morphology in C2C12 cells. C2C12 myoblasts were differentiated for 7 days in differentiation medium containing the indicated amino acids. Myotube length (a) and diameter (b) were quantified from three microscopic fields per well. None, untreated differentiation control; Leu, leucine; Leu+Cys, leucine and cysteine; LCG, leucine, cysteine, and glycine; LCQ, leucine, cysteine, and glutamine; LCT, leucine, cysteine, and taurine; LCGQT, leucine, cysteine, glycine, glutamine, and taurine; 1/2 LCGQT, the five-amino-acid combination containing half the concentration of each amino acid used in the LCGQT group. Data are presented as the mean ± SEM from 3 independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple-comparisons test. Bars not sharing a common lowercase letter are significantly different ($p < 0.05$).


mTOR
Relative mRNA expression level
1.5
1.0
0.5
0.0
a a a a a a a a
None Leu Leu+Cys LCG LCQ LCT LCGQT 1/2 LCGQT
Groups


Akt1
Relative mRNA expression level
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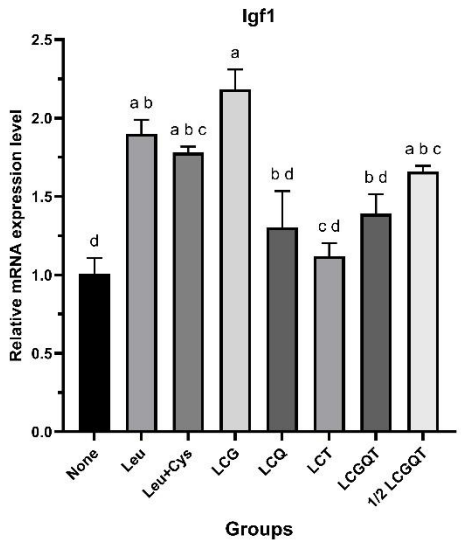
4
3
2
1
0
b b b b b b b a
None Leu Leu+Cys LCG LCQ LCT LCGQT 1/2 LCGQT
Groups


Igf1
Relative mRNA expression level
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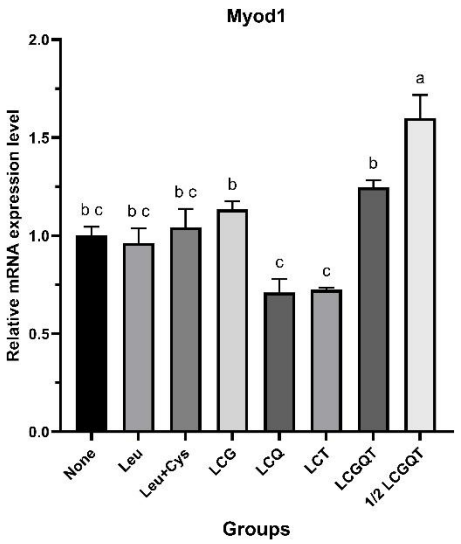
1.5
1.0
0.5
0.0
d a b a b c b d c d b d a b c
None Leu Leu+Cys LCG LCQ LCT LCGQT 1/2 LCGQT
Groups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Relative mRNA expression level
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1.0
0.5
0.0
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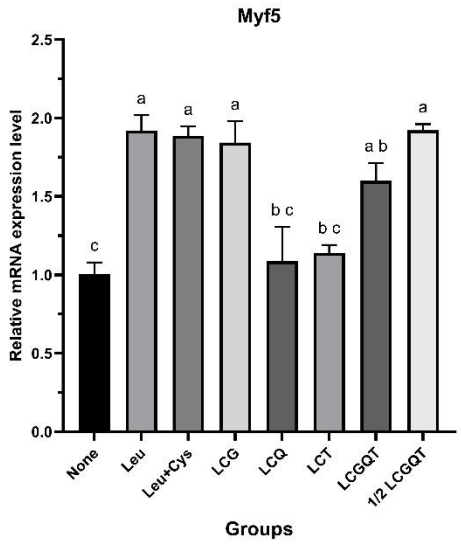
None Leu Leu+Cys LCG LCQ LCT LCGQT 1/2 LCGQT
Groups


Myf5
Relative mRNA expression level
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2.0
1.5
1.0
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0.0
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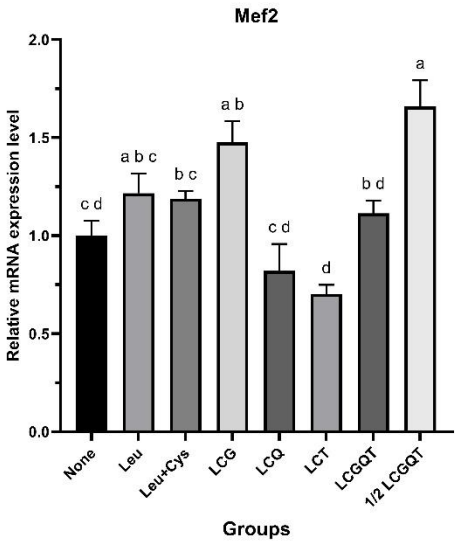
None Leu Leu+Cys LCG LCQ LCT LCGQT 1/2 LCGQT
Groups


Mef2
Relative mRNA expression level
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1.5
1.0
0.5
0.0
c d a b c b c a b c d d b d a
None Leu Leu+Cys LCG LCQ LCT LCGQT 1/2 LCGQT
Groups

Figure 3. Effects of combined amino acid treatment on the expression of muscle anabolic signaling- and myogenic differentiation-related genes in C2C12 cells. C2C12 myoblasts were differentiated for 7 days in the presence of the indicated amino acids. The mRNA expression levels of mTOR (a), Akt1 (b), Igf1 (c), Myod1 (d), Myf5 (e), and Mef2 (f) were determined by quantitative real-time PCR. Gene expression was normalized to Gapdh and expressed relative to the untreated control group. None, untreated differentiation control; Leu, leucine; Leu+Cys, leucine and cysteine; LCG, leucine, cysteine, and glycine; LCQ, leucine, cysteine, and glutamine; LCT, leucine, cysteine, and taurine; LCGQT, leucine, cysteine, glycine, glutamine, and taurine; 1/2 LCGQT, the five-amino-acid combination containing half the concentration of each amino acid used in the LCGQT group. Data are presented as the mean ± SEM from 3 independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple-comparisons test. Bars not sharing a common lowercase letter are significantly different ($p < 0.05$).

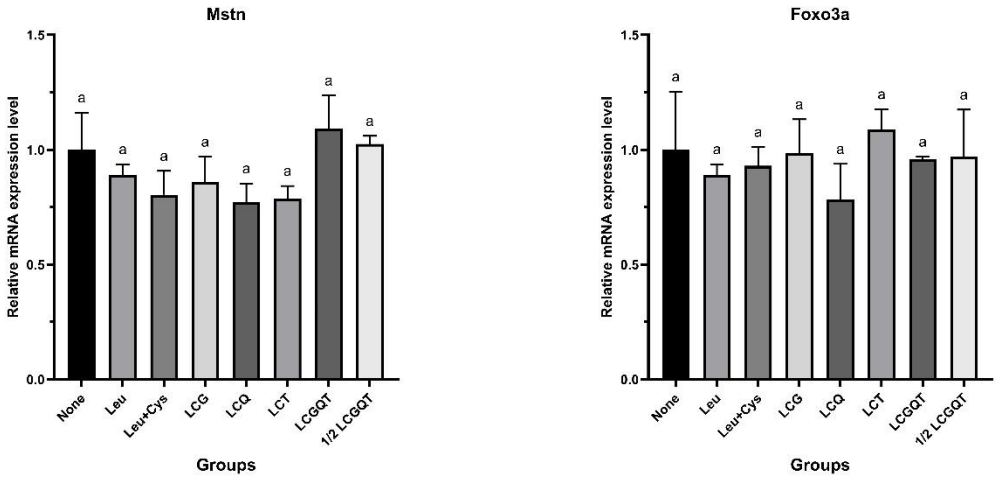

Figure 4. Effects of combined amino acid treatment on the expression of muscle catabolism-related genes in C2C12 cells. C2C12 myoblasts were differentiated for 7 days in differentiation medium containing the indicated amino acids. The mRNA expression levels of Mstn (a) and Foxo3a (b) were determined by quantitative real-time PCR. Gene expression was normalized to Gapdh and expressed relative to the untreated control group. None, untreated differentiation control; Leu, leucine; Leu+Cys, leucine and cysteine; LCG, leucine, cysteine, and glycine; LCQ, leucine, cysteine, and glutamine; LCT, leucine, cysteine, and taurine; LCGQT, leucine, cysteine, glycine, glutamine, and taurine; 1/2 LCGQT, the five-amino-acid combination containing half the concentration of each amino acid used in the LCGQT group. Data are presented as the mean ± SEM from 3 independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple-comparisons test. Bars sharing the same lowercase letter are not significantly different ($p \geq 0.05$).

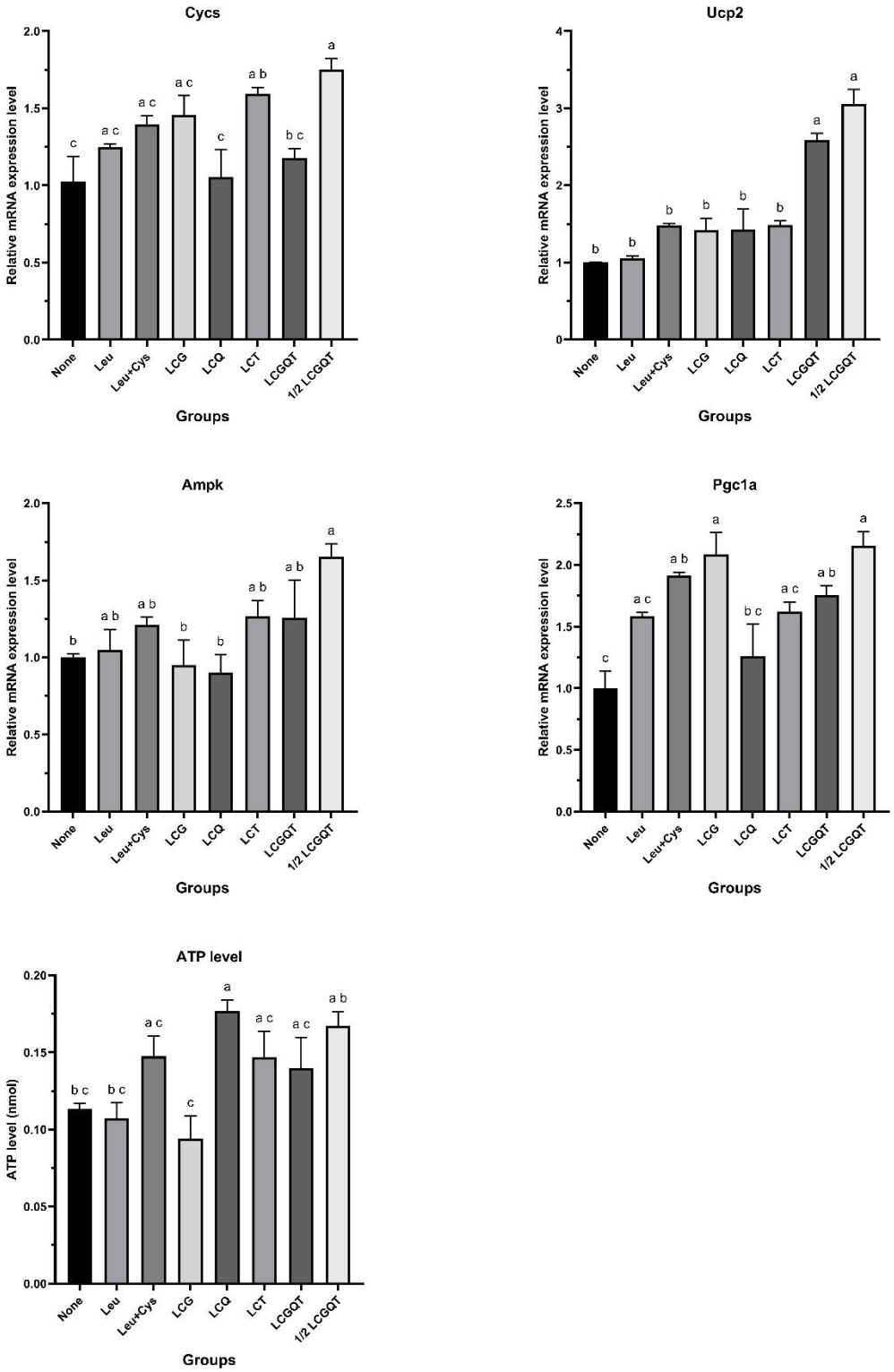

Figure 5. Effects of combined amino acid treatment on cellular energy metabolism in

C2C12 cells. C2C12 myoblasts were differentiated for 7 days in differentiation medium containing the indicated amino acids. The mRNA expression levels of Cycs (a), Ucp2 (b), Ampk (c), and Pgc1a (d) were determined by quantitative real-time PCR. Gene expression was normalized to Gapdh and expressed relative to the untreated control group. Intracellular ATP levels were measured using a colorimetric ATP assay (e). None, untreated differentiation control; Leu, leucine; Leu+Cys, leucine and cysteine; LCG, leucine, cysteine, and glycine; LCQ, leucine, cysteine, and glutamine; LCT, leucine, cysteine, and taurine; LCGQT, leucine, cysteine, glycine, glutamine, and taurine; 1/2 LCGQT, the five-amino-acid combination containing half the concentration of each amino acid used in the LCGQT group. Data are presented as the mean ± SEM from 3 independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple-comparisons test. Bars not sharing a common lowercase letter are significantly different ($p < 0.05$).

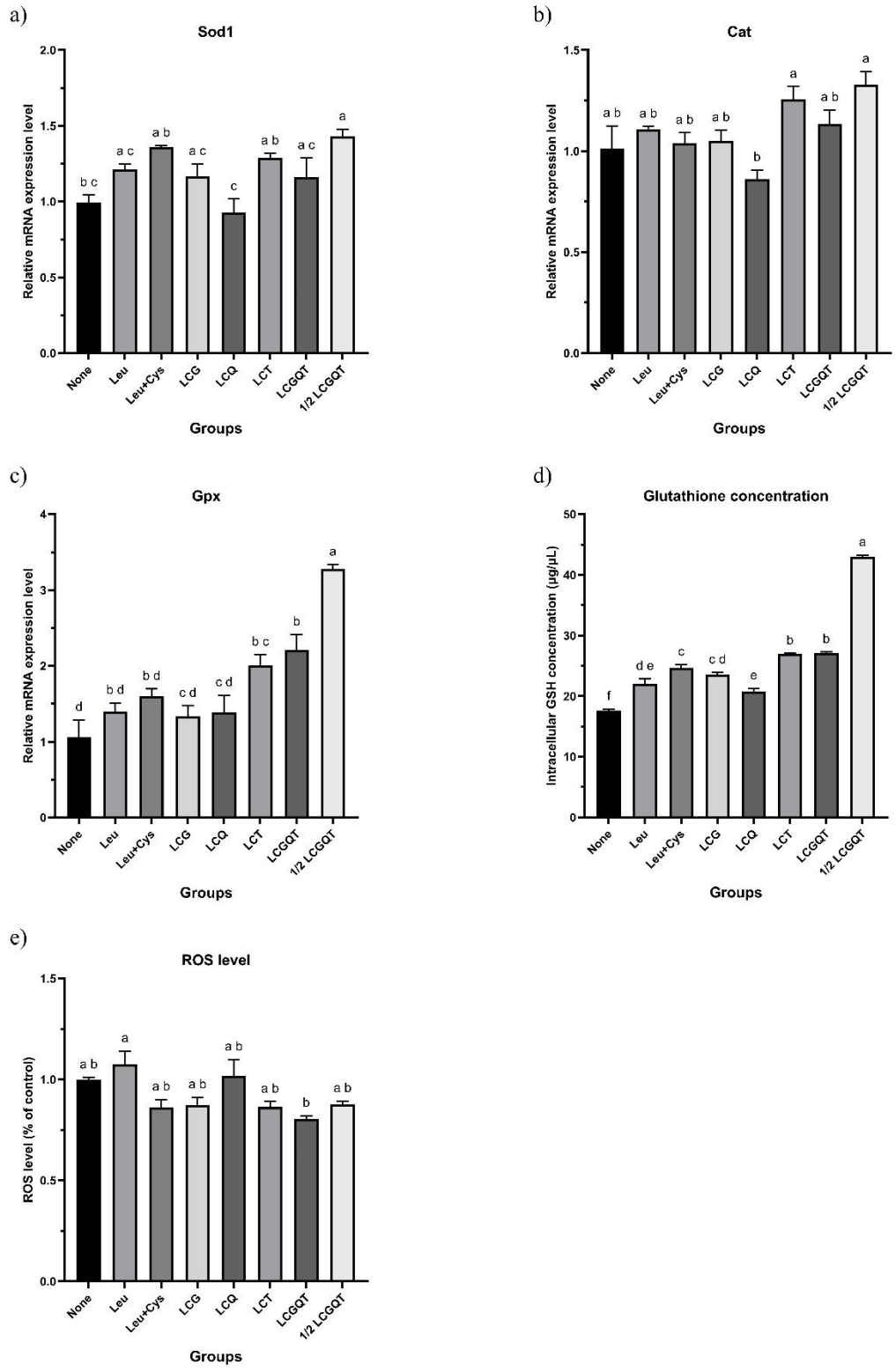

Figure 6. Effects of combined amino acid treatment on antioxidant defense and

intracellular redox status in C2C12 cells. C2C12 myoblasts were differentiated for 7 days in differentiation medium containing the indicated amino acids. The mRNA expression levels of Sod1 (a), Cat (b), and Gpx1 (c) were determined by quantitative real-time PCR, normalized to Gapdh, and expressed relative to the untreated control group. Intracellular glutathione concentration (d) and reactive oxygen species (ROS) levels (e) were measured using a GSH assay kit and DCFH-DA fluorescence assay, respectively. ROS levels were expressed relative to those of the untreated control group. None, untreated differentiation control; Leu, leucine; Leu+Cys, leucine and cysteine; LCG, leucine, cysteine, and glycine; LCQ, leucine, cysteine, and glutamine; LCT, leucine, cysteine, and taurine; LCGQT, leucine, cysteine, glycine, glutamine, and taurine; 1/2 LCGQT, the five-amino-acid combination containing half the concentration of each amino acid used in the LCGQT group. Data are presented as the mean ± SEM from 3 independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple-comparisons test. Bars not sharing a common lowercase letter are significantly different ($p < 0.05$).