科技部補助專題研究計畫成果報告
期末報告

探討Gamma次亞麻油酸調控癌症惡病質誘發骨骼肌肉耗損之功效及相關機制

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 103-2320-B-040-009-
執 行 期 間 ： 103年08月01日至104年07月31日
執 行 單 位 ： 中山醫學大學營養學系（所）

計 畫 主 持 人 ： 劉凱莉

計畫參與人員： 碩士班研究生-兼任助理人員：蘇芸瑩
碩士班研究生-兼任助理人員：施玉惠
碩士班研究生-兼任助理人員：吳昀軒
碩士班研究生-兼任助理人員：顏玉佩
碩士班研究生-兼任助理人員：蔡佩珊
博士班研究生-兼任助理人員：陳姵吟
博士班研究生-兼任助理人員：巫玉琳

處 理 方 式 ：
1.公開資訊：本計畫涉及專利或其他智慧財產權，2年後可公開查詢
2.「本研究」是否已有嚴重損及公共利益之發現：否
3.「本報告」是否建議提供政府單位施政參考：否

中 華 民 國 104 年 10 月 31 日

中 文 摘 要 ： 統計資料顯示，約 50~80%癌症病患會發生癌症惡病質，且約20%癌症病
患會因癌症惡病質相關併發症而死亡。癌症惡病質誘發的骨骼肌肉耗損會降低癌症病患
對治療的耐受性及存活率。癌細胞分泌的促蛋白質異化物質及誘發大量促發炎細胞激素
分泌可降低肌肉蛋白質合成及增加蛋白質降解是造成癌症惡病質病患骨骼肌肉耗損的
主因。癌症惡病質病患及癌症惡病質鼠類皆發骨骼肌蛋白質合成減少與經泛素-蛋白酶
體途徑 (ubiquitin-proteasome pathway, UPP)和自噬作用-溶酶體途徑
(autophagy-lysosome pathway, ALP)的蛋白質降解增加。癌細胞分泌物經由誘發轉錄因子
nuclear factor-B (NF-B), the forkhead type transcription factors (FoxOs),及
CCAAT/enhancer binding protein (C/EBP)的轉錄活性，增加UPP及ALP 路徑中與肌
肉蛋白質降解相關atrophy-specific genes (統稱為atrogenes)蛋白質表現量，導致骨骼肌肉
耗損。Gamma 次亞麻油酸 (gamma linolenic acid, GLA)，雖可經由6 desaturase 代謝亞
麻油酸 (linoleic acid, LA)生成，但許多生理及病理因素會降低6 desaturase 酵素活性，
導致體內無法自行合成GLA，需由飲食中補充，故GLA 被認為是條件必需脂肪酸
(conditional essential fatty acid)。研究證實GLA 優於LA，具有抗發炎的及抑制
lipopolysaccharide 誘發C2C12 骨骼肌纖維細胞及C57BL/6 小鼠骨骼肌耗損的功效。本
計畫將以癌症惡病質小鼠及含癌細胞培養基的Conditioned media及TNF- 處理已分化
的C2C12 骨骼肌纖維細胞為研究模式，探討GLA 調控癌症惡病質誘發骨骼肌肉耗損之
功效及相關機制。本計畫假說，GLA 可經由抑制癌細胞誘發骨骼肌轉錄因子NF-B，
FoxOs 及C/EBP 轉錄活性，抑制UPP 及ALP 相關atrogenes 表現，而減緩骨骼肌肉蛋
白質降解及增加蛋白質合成，且GLA 調控癌症惡病質肌肉耗損的功效優於LA。本研究
計畫結果有助於了解GLA 對抑制癌症惡病質誘發骨骼肌肉耗損的保健功效，並提供了
解飲食中添加富含GLA 油脂作為改善癌症惡病質病患肌肉耗損輔助治療的可行性，以
作為研發富含GLA 相關保健食品的參考依據

中 文 關 鍵 詞 ： Gamma 次亞麻油酸，癌症惡病質，骨骼肌耗損

英 文 摘 要 ： Skeletal muscle wasting is present in about 50% of cancer patients and accounts for 20% of all cancer deaths. The 18-carbon polyunsaturated fatty acids gamma-linolenic acid (GLA) and its precursor linoleic acid (LA) have shown that they have anti-inflammation and anti-tumor effects. In this study, we investigates the effects of GLA and LA on the anti-muscle wasting events of Lewis lung carcinoma (LLC) conditioned media treated C2C12 myotubes and LLC tumor-bearing C57BL/6 mice and the possible mechanisms underlying. The results showed that GLA inhibited LLC-induced body weight and muscle weight loss as well as circulating tumor necrosis factor-α (TNF-α) secretion. GLA has more potent effect than LA in the attenuation the expression of LLC-induced TNF-α, myostatin (Mstn) and its downstream atrophy-related gene such as muscle RING-finger1, muscle atrophy F-box, and microtubule-associated protein 1 light chain 3B as well as the accumulation of ubiquitin proteins via protein kinase B (Akt)-forkhead box O1 (FoxO1) and p38 MAPK-CCAAT enhancer binding proteins β (C/EBPβ) pathways to reduce myosin heavy chain protein degradation in both C2C12 myotubes and GA muscles. The transient expression of dominant negative Akt (K179M) in C2C12 myotubes can abolish the protective events of GLA and LA. In conclusion, these results suggest that GLA has the ability to attenuate skeletal muscle protein degradation in vitro and in vivo via the downregulation of Akt-FoxO1-dependent and p38 MAPK-C/EBPβ pathways as well as reduce the expression of TNF-α, Mstn, and atrophy-related gene. It suggests that GLA is a potential therapeutic choice to prevent cancer cachexia.

英 文 關 鍵 詞 ： gamma-linolenic acid, Lewis lung carcinoma, muscle wasting, Akt-FoxO1 pathway, p38 MAPK-C/EBPβ pathway

OXFORD UNIVERSITY PRESS | Carcinogenesis

Gamma-linolenic acid ameliorate Lewis lung carcinoma-induced muscle wasting via Akt and p38 MAPK pathways in both C2C12 myotubes and C57BL/6 mice

Journal:	*Carcinogenesis*
Manuscript ID:	CARCIN-2015-00076
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	13-Feb-2015
Complete List of Authors:	Chen, Pei-Yin; Chung Shan Medical University, Department of Nutrition Sun, Hai-Lun; Chung Shan Medical University, School of Medicine; Chung Shan Medical University Hospital, Division of Allergy, Asthma and Rheumatology, Department of Pediatrics Chen, Shyh-Chang; Taichung Veterans General Hospital, Department of Pathology and Laboratory Medicine Liu, Te-Chung; Chung Shan Medical University Hospital, Department of Dietitian; Chung Shan Medical University, Department of Nutrition Yang, Ya-Chen; Asia University, Department of Health and Nutrition Biotechnology Lii, Chong-Kuei; Asia University, Department of Health and Nutrition Biotechnology; China Medical University, Department of Nutrition Chen, Haw-Wen; China Medical University, Department of Nutrition Liu, Kai-Li; Chung Shan Medical University, Department of Nutrition; Chung Shan Medical University Hospital, Department of Dietitian
Keywords:	gamma-linolenic acid, Lewis lung carcinoma, muscle wasting, Akt-FoxO1 pathway, p38 MAPK-C/EBPβ pathway

Gamma-linolenic acid ameliorates Lewis lung carcinoma-induced muscle wasting via Akt and p38 MAPK pathways in C2C12 myotubes and C57BL/6 mice

Pei-Yin Chen[1], Hai-Lun Sun[2,3], Shyh-Chang Chen[4], Te-Chung Liu[1,5], Ya-Chen Yang[6], Chong-Kuei Lii[6,7], Haw-Wen Chen[7,*], Kai-Li Liu[1,5,*]

[1] Department of Nutrition, Chung Shan Medical University, No. 110, Sec. 1, Chien-Kuo N. Rd., Taichung 40203, Taiwan

[2] School of Medicine, Chung Shan Medical University, Taichung, Taiwan

[3] Division of Allergy, Asthma and Rheumatology, Department of Pediatrics, Chung Shan Medical University Hospital, Taichung, Taiwan

[4] Department of Pathology and Laboratory Medicine, Taichung Veterans General Hospital, No. 1650, Sec. 4, Taiwan Boulevard, Taichung 40705, Taiwan

[5] Department of Dietitian, Chung Shan Medical University Hospital, Taichung, Taiwan

[6] Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan

[7] Department of Nutrition, China Medical University, Taichung, Taiwan

Running title: GLA ameliorates cancer cachexia-induced muscle wasting

***Corresponding Author:** Haw-Wen Chen; Tel.: +886 4 22053366x7519; Fax: +886 4 22062891.

E-mail: chenhw@mail.cmu.edu.tw (C.-W. Chen).

***Corresponding Author:** Kai-Li Liu; Tel.: +886 4 24730022x12136; Fax: +886 4 23248175.

E-mail: kaililiu@csmu.eud.tw (K.-L. Liu).

Disclosure of Potential Conflicts of Interest: All authors indicated “no conflict of interest.”

These authors contributed equally to this work.

Abstract

Skeletal muscle wasting is present in about 50% of cancer patients and accounts for 20% of all cancer deaths. The 18-carbon polyunsaturated fatty acids gamma-linolenic acid (GLA) and its precursor linoleic acid (LA) have shown that they have anti-inflammation and anti-tumor effects. In this study, we investigates the effects of GLA and LA on the anti-muscle wasting events of Lewis lung carcinoma (LLC) conditioned media treated C2C12 myotubes and LLC tumor-bearing C57BL/6 mice and the possible mechanisms underlying. The results showed that GLA inhibited LLC-induced body weight and muscle weight loss as well as circulating tumor necrosis factor-α (TNF-α) secretion. GLA has more potent effect than LA in the attenuation the expression of LLC-induced TNF-α, myostatin (Mstn) and its downstream atrophy-related gene such as muscle RING-finger1, muscle atrophy F-box, and microtubule-associated protein 1 light chain 3B as well as the accumulation of ubiquitin proteins via protein kinase B (Akt)-forkhead box O1 (FoxO1) and p38 MAPK-CCAAT enhancer binding proteins β (C/EBPβ) pathways to reduce myosin heavy chain protein degradation in both C2C12 myotubes and GA muscles. The transient expression of dominant negative Akt (K179M) in C2C12 myotubes can abolish the protective events of GLA and LA. In conclusion, these results suggest that GLA has the ability to attenuate skeletal muscle protein degradation *in vitro* and *in vivo* via the downregulation of Akt-FoxO1-dependent and p38 MAPK-C/EBPβ pathways as well as reduce the expression of TNF-α, Mstn, and atrophy-related gene. It suggests that GLA is a potential therapeutic choice to prevent cancer cachexia.

Keywords: gamma-linolenic acid, Lewis lung carcinoma, muscle wasting, Akt-FoxO1 pathway, p38 MAPK-C/EBPβ pathway

Summary

Gamma-linolenic acid (C18:3 n-6) attenuates Lewis lung carcinoma-induced tumor necrosis factor-α and myostatin levels as well as atrophy-related genes expression and skeletal muscle protein degradation via downregulating the Akt-FoxO-dependent and p38 MAPK-C/EBPβ pathways in C2C12 myotubes and C57BL/6 mice.

Abbreviations

Akt, protein kinase B; ALP, autophagy-lysosome pathway; BO, borage oil; BW, body weight; C/EBPβ, CCAAT enhancer binding proteins β; EDL, extensor digitorum longus; FBS, fetal bovine serum; FoxO, forkhead box O; GA, gastrocnemius; GLA, gamma-linolenic acid; HS, horse serum; i.p., intraperitoneal; IL-6, interlekine-6; LA, linoleic acid; LC3B, microtubule-associated protein 1 light chain 3B; LCM, Lewis lung carcinoma conditioned media; LLC, Lewis lung carcinoma; LPS, lipopolysaccharides; MAFbx, muscle atrophy F-box; MAPKs, mitogen-activated protein kinases; MHC, myosin heavy chain; Mstn, myostatin; MuRF1, muscle RING-finger 1; NF-κB, nuclear factor κB; PA, palmitate; PBS, phosphate-buffered saline; SO, soybean oil; TNF-α, tumor necrosis-α; UPP, ubiquitin-proteasome pathway.

Introduction

Cachexia is a complex syndrome in lung, stomach, pancreas, and colon cancers [1]. It is characterized by progressive depletion of body weight and skeletal muscle mass with or without the loss of fat mass [2]. Cancer cachexia induces 75% muscle loss, which causes weakness, immobility, and heart and lung failure, as well as enhances chemotherapy toxicity that leads to subsequent decrease in survival rate. Therefore, reducing cancer cachexia-induced muscle wasting is important to maintain life quality and survival rate of patients [3, 4].

Maintenance of muscle mass is controlled by the balance between protein synthesis and degradation. In cachexia, the muscle protein breakdown is higher than the protein synthesis probably because of the release of pro-inflammatory cytokines and tumoral factors, such as tumor necrosis-α (TNF-α) and myostatin (Mstn) from the tumor. In these conditions, two muscle-specific E3 ubiquitin ligases, namely, muscle RING-finger1 (MuRF1) and muscle atrophy F-box (MAFbx), are upregulated by the pro-inflammatory cytokines and tumoral factors. They advance the ubiquitination of myofibrillar proteins, such as myosin heavy chain (MHC), for subsequent degradation. This protein degradation pathway is termed as ubiquitin-proteasome pathway (UPP) [5, 6]. Microtubule-associated protein 1 light chain 3B (LC3B), a specific element of the autophagic process, is involved in the formation of autophagosomes and is implicated in the degradation of proteins; this pathway is termed as autophagy-lysosome pathway (ALP), which can coordinate with UPP to enhance muscle wasting [7]. These atrophy-related genes, namely, MuRF1, MAFbx, and LC3B (also called atrogenes), are regulated by activating transcription factors, such as the forkhead box O (FoxO) and CCAAT enhancer binding proteins β [8, 9]. In response to TNF-α and Mstn, the phosphorylation of protein kinase B (Akt) and FoxO1/3 is reduced, which increases the activity of transcription factor FoxO1 and elevates the gene expression of MuRF1 and MAFbx to enhance muscle wasting. This process can be further confirmed by injection of Mstn antibody (PF-354) and treatment of Mstn inhibitor (sActRIIB) as well as knockout of *tnf-α* with tumor-bearing mice to prevent muscle wasting [10–15]. In addition, treatment of Akt inhibitor (API-2) or expression of constitutively active FoxO3 with myotubes can induce MAFbx and LC3B expression to enhance protein degradation via the Akt-FoxO3-dependent pathway. Protein degradation is increased through inhibiting Akt activity and stimulating FoxO

transcription factor activity, following the expression of MuRF1, MAFbx, and LC3B, thereby leading to muscle wasting [16].

Cancer cachexia-induced muscle wasting is also involved in other signal transduction pathways, such as p38 MAPK-C/EBPβ pathway [17]. TNF-α upregulates MuRF1 and MAFbx via p38 MAPK. This effect can be blocked by a p38 MAPK inhibitor (SB20219) [11, 17]. In Lewis lung carcinoma (LLC)-induced muscle wasting model, treatment with SB20219 and *cebpb*-deficient mice can block MAFbx expression and reverse wasting events [17]. Therefore, treatment of anti-inflammatory or anti-tumor drugs to repress Akt-FoxO and p38 MAPK-C/EBPβ pathways, as well as reduce UPP and ALP systems, may be a therapeutic alternative to prevent muscle protein degradation in cancer cachexia-associated muscle wasting.

Linoleic acid (18:2 n-6, LA) is an essential fatty acid in humans. LA is available in regular diets and can convert into gamma-linolenic acid (18:3 n-6, GLA) by delta-6-desaturase enzyme. In cases of aging, nutrient deficiency, inflammatory diseases, and cancer, delta-6-desaturase activity decreases and leads to reduced GLA level. Supplementation with GLA, such as borage oil, evening primrose and/or black currant, can overcome the disadvantage of delta-6-desaturase deficiency and relieve the signs and symptoms of cancer and inflammatory diseases, such as sepsis, atherosclerosis, rheumatoid arthritis, and acute respiratory distress syndrome [18]. Our previous studies showed that GLA is more potent than LA in reducing lipopolysaccharide (LPS)-induced pro-inflammatory mediator production via mitogen-activated protein kinases (MAPKs; including extracellular regulated protein kinase1/2, c-Jun N-terminal kinases, and p38 MAPK) and nuclear factor-κB (NF-κB) signalling pathway in RAW264.7 macrophages [19]. GLA can also improve pro-inflammatory cytokines, including interleukine-6 (IL-6) and TNF-α, expression, and insulin resistance via MAPks, IκB kinase (IKK)-NF-κB, and Akt signalling pathways in palmitate (PA)-stimulated C2C12 myotubes. Accumulating evidences also showed that treatment with GLA and eicosapentaenoic acid (20: 5n-3, EPA) inhibited tumor growth in human lung, mammary, prostatic, and gastric carcinoma cells, whereas LA exhibited no effects [20–23]. These studies show that GLA possesses anti-inflammatory and anti-tumor effects, and LA only has anti-inflammatory effect. However, the ability of GLA and LA to restore skeletal muscle from cancer cachexia-induced muscle wasting remains unclear. The LLC conditioned media (LCM)-induced C2C12 myotubes, which provide a well established *in vitro* model

system, and the LLC tumor-bearing C57BL/6 mice, which exhibit rapid and progressive loss of body weight and tissue wastage, were used to determine the effect of GLA and LA on skeletal muscle wasting [17]. The result showed that GLA could restore cancer-induced skeletal muscle wasting via downregulation of TNF-α, Mstn, and atrogenes by Akt-FoxO and p38 MAPK-C/EBPβ pathways.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), lipofectamine$^{TM\ 2000}$, and Tri-Reagent^{TM} were obtained from Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS), horse serum (HS), and penicillin–streptomycin solution for cell culture were purchased from HyClone (Logan, UT, USA). LA and GLA were obtained from NuChek Prep, Inc. (Elysian, MN, USA), and borage oil was provided by Sigma Chemical Co. (St. Louis, MO, USA). Reagents for synthesizing complementary DNA and TaqMan® Universal PCR Master Mix were purchased from Promega Corp. (Madison, WI, USA) and Applied Biosystems (Foster City, CA, USA), respectively. Antibodies against C/EBPβ, Mstn, MuRF1, MAFbx, Ub, actin, and histone1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against LC3 and MF20 were provided by MBL (San Diego, CA, USA) and eBioscience (San Diego, CA, USA), respectively. Antibodies against TNF-α as well as native and phosphorylated forms of p38 MAPK, Akt, FoxO1a/3a, and FoxO1 were obtained from Cell Signalling Technology Inc. (Beverly, MA, USA). Antibody against PARP was purchased from Roche (Basel, Switzerland). Oligonucleotide primer sequences of TNF-α, MuRF1, MAFbx, and delta-6 desaturase for real-time PCR were synthesized by MDBio Inc. (Taipei, Taiwan). All other chemicals and reagents were commercially obtained.

Cell culture and LCM preparation

Two cell lines, murine C2C12 skeletal myoblasts and murine LL/2 (LLC1) cells, were characterized and authenticated by BCRC (Bioresource Collection and Research Center, Taiwan, http://www.bcrc.firdi.org.tw) which were originally got from the American Type Culture Collection (ATCC, Manassas, VA). After received/purchased, all cell lines were immediately expanded and frozen down such that they could be restarted from the same batch of cells. Cell lines used throughout this study were not used for more than 1 months and/or 9 passages. They have been

confirmed negative for mycoplasma in 2013 by using a MycoAlert mycoplasma detection kit (Lonza, Rockland, ME, USA). The C2C12 myoblasts were cultured in growth medium (DMEM supplemented with 10% FBS) at 37 °C under 5% CO2. At 80% confluence, the myoblasts were switched to differentiation medium (DMEM supplemented with 2% HS) for 6 days to differentiate into myotubes. The collection of LCM is shown as follows. The LLC cells were plated at 50% confluence and grown in DMEM with 10% FBS after 72 h incubation. The resulting LCM was collected by centrifugation and either stored at −80° or used immediately. The C2C12 myotubes in DMEM with 5% HS supplement were pretreated with 100 μM GLA, LA, or methanol for 12 h and then incubated with or without LCM for another 48 h (diluted to a ratio of 1:2 with 5% HS-DMEM).

Transient transfection

The Akt-empty, Akt (K179M) and C/EBPβ-Luciferase plasmids were purchased from Upstate (Lake Placid, NY) and Stratagene Inc. (La Jolla, CA). At 50-60% confluence, the C2C12 myoblasts were used for transfection with lipofectamine$^{TM\ 2000}$ reagent as described by the manufacturer. The transfected myoblasts were cultured in differentiation media for 6 days before the treatment. The transcriptional activity of C/EBPβ was determined by the Luciferase Assay System and β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer from Promega Co., respectively.

Animal studies

Five-week-old male C57BL/6JNarl male mice were provided by the National Laboratory Animal Center (Taipei, Taiwan) and were housed in standard laboratory conditions (22 ± 2 °C and 60% to 80% relative humidity, 12-h light–dark cycle) with free access to food and water. The food intake was measured daily. All animal study protocols were approved by the Institutional Animal Care and Use Committee. For LLC-induced cachexia model, animals were randomly divided into three groups, namely, control (C, n = 5), LLC tumor-bearers with soybean oil (LSO, n = 5), and LLC tumor-bearers with borage oil (LBO, n = 5). The LLC tumor-bearing mice were implanted with 150 μl of LLC cells (2 × 10^5) or an equal volume of phosphate-buffered saline (PBS) (control) intraperitoneally (i.p.) injected into the left flank at day 0. The LLC tumor-bearing mice were subsequently treated with soybean oil (SO, 150 μl/mice by intragastric gavage), borage oil (BO, 150 μl/mice by intragastric gavage), or an equal volume of SO (control) every other day from day 1 to day 21. At day 21, body weight, organ weight, tumor weight, gastrocnemius (GA), extensor digitorum longus (EDL), and soleus muscles

were dissected, weighed, and collected from mice immediately after rapid euthanization. The plasma and muscle specimens were frozen at −80° or fixed in 10% buffered formalin for histochemistry studies.

Real-time reverse transcriptase-PCR (real-time PCR)

Total RNA was extracted from GA muscles and C2C12 myotubes by using Tri-Reagent™ as described in the manufacturer's protocol. RNA extracts were suspended in RNase-free water and were frozen at −80° before use. RNA was reverse transcribed with M-MMLV reverse transcriptase to synthesize complementary DNA. Real-time PCR was performed using the Step One Plus Real Time PCR Instrument (Foster City, CA, USA) with the TaqMan Gene Expression Assay, Roche Universal Probe Library, or SYBR GREEN. TaqMan probe used β-actin: Mm01205647_gl. Roche UPL primer probe and SYBR GREEN primer sets are listed in Table S1. Relative expression compared with the internal control β-actin was determined by using the $2^{-\Delta\Delta Ct}$ method [19].

Extraction of total protein and cytoplasmic and nuclear proteins

Total protein extracts were prepared by homogenization of GA muscles and C2C12 myotubes in PBS and RIPA buffer, respectively. Cytoplasmic and nuclear proteins of GA muscles and C2C12 myotubes were prepared by following the procedures of Chen et al. and Chang et al., respectively [19, 24]. The protein contents were quantified by modified Lowry assay [25].

Western blot analysis

Equal amounts of total protein, cytoplasmic, and nuclear protein extracts (10 μg–20 μg) derived from the C2C12 myotubes and GA muscles were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (New Life Science Product, Inc., Boston, MA, USA). The blots were sequentially incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA, USA). Immunoreactive protein bands were developed with an enhanced chemiluminescence kit and visualized by a luminescent image analyzer (LAS-1000 plus; Fuji Photo Film Company, Japan). The bands were quantified by AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA, USA).

Serum TNF-α

Serum TNF-α concentrations obtained from control and tumor-bearing mice were determined using a commercial ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Histology and measurement of myofiber diameter

Muscle tissues were removed and fixed in 10% formalin solution at room temperature for 24 h. The samples were processed with a tissue processor (Leica, ASP300 S). The tissues were serially dehydrated in alcohol, cleared in xylene, and impregnated with liquid paraffin wax at 56 °C. Tissue blocks were sectioned with a microtome to 3 μm thickness (Leica, 2235). The sections were floated in a water bath at 40 °C and then placed on special coated glass slides (Superfrost plus; Menzel Glasser, Germany). Sections were deparaffinized and rehydrated prior to hematoxylin and eosin (H&E) staining. The myofiber diameter was measured as described in Menconi et al. [26]. Images were acquired using an upright fluorescence microscope (200× magnification, scale bar = 100 μm) and analyzed with AlphaImager 2200 (Alpha Innotech Corporation). Approximately 100 muscle fibers from each muscle were analyzed for longitudinal-sectional area quantification. The results were expressed as a percentage of the diameter in relation to the control group.

Immunohistochemistry (IHC)

IHC was performed with a BenchMark IHC staining system (Vision BioSystems, San Francisco, CA, USA). Sections were stained with an antibody against the FKHR (C-9), a mouse monoclonal antibody against the FKHR (Santa Cruz, CA, USA), and a rabbit polyclonal antibody against the C/EBPβ (C-19) (Santa Cruz, CA, USA). Anti-mouse and anti-rabbit Envision (DAKO, Santa Barbara, CA, USA) were used as secondary antibodies. The slides were counterstained with hematoxylin (Ventana Medical Systems). IHC staining was analyzed under an upright fluorescence microscope at the Instrument Center of Chung Shan Medical University, which is supported by the National Science Council, Ministry of Education, and Chung Shan Medical University.

Statistical analysis

Data are expressed as mean ± SD of at least three independent experiments. One-way ANOVA and Tukey's multiple-range test were carried out using Statistical Analysis System (Cary, NC, USA) to evaluate statistical significance. $P < 0.05$ was considered statistically significant.

Results

GLA and LA prevent LCM-induced C2C12 myotubes wasting

To mimic the aspect of cancer cachexia-induced muscle wasting model *in vitro*. We performed the LCM to induce the C2C12 myotubes wasting event. Following the exposure of C2C12 myotubes with 100 μM GLA and LA for 12 h and/or incubating with or without LCM for further 48 h. Western blot analysis was performed, the results showed that pretreatment with GLA dramatically decreased LCM-induced TNF-α and Mstn protein as well as increased LCM-inhibited MHC protein in C2C12 myotubes as compared with LCM treatment. The pretreatment with LA also inhibited LCM-induced Mstn protein and increased LCM-inhibited MHC protein, but did not reduce the TNF-α level in C2C12 myotubes ($P<0.05$, Figure 1A and 1B).

Cancer cachexia can stimulate TNF-α and Mstn expression and increase atrogenes such as MuRF1, MAFbx, and LC3B expression and ubiquitin proteins accumulation which are the significant causative of skeletal muscle wasting [12, 27]. Therefore, the real-time PCR and Western blot analysis were performed to determine whether GLA and LA can downregulate the atrogenes expression in LCM-stimulated C2C12 myotubes wasting. As shown in 1C-E, LCM upregulated atrogenes expression and ubiquitin proteins accumulation, and these effects were significantly blocked by GLA pretreatment. In addition, LCM-induced MAFbx expression as well as ubiquitin proteins accumulation and LC3B protein were also inhibited by LA. These results represented that GLA and LA, especially GLA, can significantly avoid LCM-stimulated C2C12 myotubes wasting by the inhibition of TNF-α, Mstn, and atrogenes expression.

GLA and LA protect LCM-induced C2C12 myotubes wasting through the Akt-FoxO dependent pathway

Promoting myotubes wasting using TNF-α and Mstn-induced atrogenes depends on the activation of the Akt-FoxO pathway [10, 11, 28]. The levels of native and phosphorylated forms of Akt and FoxO protein in these cells were examined upon challenge with LCM treatment to determine whether GLA and LA against LCM-induced changes are through the regulation of Akt-FoxO pathway. Although LCM treatment increased the native FoxO1 protein level in the nucleus, the phosphorylated Akt and FoxO1a/3a protein levels in the cytoplasm of the C2C12 myotubes exhibited no difference between the treatment of control and LCM ($P < 0.05$; Figure 2A). GLA and LA treatment increased the phosphorylated Akt and FoxO1/3a protein levels in the cytoplasm and decreased the native FoxO1 protein level in the nucleus ($P < 0.05$; Figure 2A). These data indicate that GLA and LA could ameliorate the cachexia-induced skeletal muscle wasting by regulating the Akt-FoxO pathway. The Akt dominant-negative mutant plasmid-Akt (K179M) was used to investigate whether Akt-FoxO pathway controls atrogenes expression. As shown in Figure 2B–E, transfection of C2C12 myotubes with Akt (K179M) enhanced MHC protein loss, increased atrogenes protein levels, and blocked the protective effects of GLA and LA on LCM-induced C2C12 myotubes wasting. These results suggest that Akt-FoxO pathway played a pivotal role of muscle wasting in C2C12 myotubes.

GLA prevents LCM-induced muscle wasting via additional p38 MAPK-C/EBPβ pathway in C2C12 myotubes

Accumulating evidence has shown that TNF-α and LLC-induced muscle wasting are also involved in activating p38 MAPK-C/EBPβ pathway and atrogenes expression in cancer cachexia [17, 29]. The effects of GLA and LA on the activation of p38 MAPK-C/EBPβ pathway in LCM-stimulated C2C12 myotubes wasting were also measured. When C2C12 myotubes were treated with LCM, the phosphorylation level of p38 MAPK and the nuclear C/EBPβ protein, as well as the C/EBPβ reporter gene activity, were increased with or without LA. By contrast, LCM-stimulated upregulation of p38 MAPK-C/EBPβ pathway was significantly reduced in cultures, which were pretreated with GLA ($P < 0.05$; Figure 3). These data suggest that the p38 MAPK-C/EBPβ pathway implicates reduced LCM-induced C2C12 myotubes wasting in GLA.

Borage oil ameliorates body weight loss and muscle wasting in LLC tumor-bearing mice

To determine whether GLA and LA protect muscle wasting *in vitro*, we established the LLC tumor-bearing mice to evaluate the effectiveness of GLA and LA on cancer cachexia-induced skeletal muscle wasting. SO or BO was administered to mice 1 day after LLC treatment, and changes in body weight (BW) and body composition were analyzed. Three weeks after i.p. injection of PBS or LLC, difference was not observed in food intake and weights of tumor, heart, liver, and epididymis adipose tissues among groups. The lung weight of the LSO group was lower than that of the control; the lung weight of the LBO group was the same as that of the control (Figure 4A and 4B). Moreover, the LSO group significantly decreased the final BW, tumor-free BW, and percentage change of tumor-free BW from the initial BW, as well as muscle weight, such as GA, soleus, and EDL muscles, as compared with the control. By contrast, BO treatment increased the weight as previously mentioned (Figure 4). BO's ability to reduce TNF-α, Mstn, and atrogenes change in response to muscle wasting of LLC tumor-bearing mice was tested. As shown in Figure 5A and 5B, the LSO group enhanced circulating TNF-α secretion, as well as TNF-α and Mstn expression in GA muscle, as compared with the control. These disadvantages were diminished by BO treatment. Similarly, the LBO group also dramatically suppressed atrogenes expression and ubiquitin protein accumulation, as well as reversed MHC protein level, as compared with the LSO group (P <0.05; Figure 5C–F). These data indicate that BO treatment did not only inhibit TNF-α and Mstn expression but also reduced atrogenes expression and ubiquitin protein accumulation to restore LLC-induced skeletal muscle wasting.

Borage oil suppresses Akt-FoxO and p38 MAPK-C/EBPβ pathways in GA muscles of LLC tumor-bearing mice

Western blot analysis and IHC staining were performed to measure the levels of native and phosphorylated forms of Akt, FoxO1a/3a, FoxO1, p38 MAPK, and C/EBPβ in the GA muscle of LLC tumor-bearing mice. As shown in Figure 6A–D, the LBO group increased the phosphorylated Akt and FoxO1a/3a and decreased the nuclear FoxO1 protein level, as well as reduced the phosphorylated p38 MAPK and nuclear C/EBPβ protein levels, as compared with the LSO group. These data suggest that BO ameliorated LLC-induced skeletal muscle wasting by downregulating the Akt-FoxO and p38 MAPK-C/EBPβ pathways.

Discussion

Epidemiologic and clinical reports indicate that cancer cachexia-induced body weight loss and muscle wasting lead to surgical and radiotherapy complications and intolerance of chemotherapy and eventually cause the reduction of survival rate [30–32]. Therefore, the improvement of body weight and muscle mass may limit the adverse effect of cancer cachexia. In the present study, GLA, not LA, restored LLC-induced muscle wasting events *in vitro* and *in vivo*, as well as prevented body weight loss, in mice. Although GLA (5.5% in diet or 50 μM–100 μM) and LA [8% (w/w) in diet or 300 μM] have the ability to inhibit tumor growth in animals and cultures [21, 33–37], the amount of GLA (0.625% in diet) and LA (2.5% in diet) showed a minor effect on the inhibition of LLC tumor growth in the present study (Figure 4A). This contradiction may be due to the different cell types used in the experiments. In the case of the PF-354 and SB202190 treatments or the use of *cebpb*-deficient mice, muscle wasting events are restored without inhibiting the LLC tumor growth [15, 17]. These data are consistent with our results, which indicated that the treatments that inhibited cancer cachexia-induced muscle wasting exhibited no relationship with tumor growth.

LA can convert into GLA by delta-6 desaturase in liver, smooth, and skeletal muscles. However, the activity of delta-6 desaturase is impaired in inflammatory conditions, such as diabetes and cancer, which is consistent with our result [38–40]. Our data indicated that the mRNA expression of delta-6 desaturase significantly reduced in cachectic muscle compared with the control (Figure 5D). Although delta-6 desaturase mRNA expression was impaired, supplementation with GLA decreased circulating TNF-α as well as TNF-α and Mstn levels in LCM-induced C2C12 myotubes and GA muscles of LLC tumor-bearing mice (Figures 1A and 5B). Consistent with our previous studies, GLA treatment can inhibit LPS-induced inducible nitric oxide synthase, pro-interleukin-1β, and cyclooxygenase-2 as well as nitric oxide production in RAW264.7 macrophages. GLA can also reduce IL-6 and TNF-α expression and cause insulin resistance in PA-stimulated C2C12 myotubes and streptozotocin-induced diabetes mice. LA can only inhibit LPS-induced inflammatory mediators and reduce IL-6 and TNF-α expression as well as insulin resistance in cultures [19]. However, excessive intake with LA (e.g., 15 g–20 g/day/person or up to 10% of a person's daily calorie intake) can enhance pro-inflammatory cytokine secretions [41]. These results suggest that GLA had more potent anti-inflammation effect than LA.

Cancer cachexia-induced muscle wasting is involved in upregulating pro-inflammatory cytokines and tumor-derived factors, as well as atrogenes, to enhance muscle protein degradation through several major intracellular signal transduction systems, such as PI3 K-Akt-FoxO pathway [28, 42]. Mstn is a TGFβ superfamily member obtained from tumor secretion; Mstn can negative regulate skeletal muscle mass by inducing downstream gene expression, such as MAFbx, MuRF1, and LC3B, depending on Akt-FoxO1 pathway in LLC-induced muscle wasting model [13, 43]. In addition, TNF-α is a pro-inflammatory cytokine that can also induce MAFbx and MuRF1 expression through the inactivation of Akt-FoxO1 pathway in L6 myotubes [11]. Therefore, we measured the expression of Mstn- and TNF-α-mediated Akt-FoxO pathway and then treated with GLA and LA in LLC-induced muscle wasting. The results show that GLA could inhibit both LLC-induced Mstn and TNF-α expression, as well as increase phosphorylated Akt-FoxO and downregulate nuclear FoxO1, to reduce atrogenes expression and reverse MHC expression *in vitro* and *in vivo*. However, LA can only inhibit Mstn expression, increase phosphorylated Akt-FoxO, and reduce nuclear FoxO1 to reduce MAFbx and LC3 and reverse MHC protein expression in C2C12 myotubes (Figures 1, 2A, 4A, and 4B). The inhibition of LLC-induced atrogenes expression and the reversion of muscle wasting via GLA and LA were abolished by transit transfection of dominant negative Akt (K179M) in C2C12 myotubes (Figure 2B to 2E). These results indicate that GLA had more potent effect than LA to downregulate Mstn and TNF-α, as well as activate Akt-FoxO1-dependent pathway, to reverse muscle wasting.

C/EBPβ, a transcriptional factor required for LLC-induced muscle wasting through upregulation of MAFbx, is directly activated by phosphorylated p38 MAPK. SB202190 treatment or knockout of *cebpb* prevent MAFbx expression and muscle wasting; thus, p38 MAPK-C/EBPβ pathway is necessary for cachectic muscle wasting [17]. Moreover, TNF-α induction by injecting LPS (1 mg/kg) and dexamethasone (25 mg/kg) upregulates atrogenes through the p38 MAPK-dependent pathway with or without the activation of NF-κB and FoxO3 transcriptional activity in the cachectic muscle [11, 17, 29]. Our results are consistent with these findings. GLA also downregulates the TNF-α expression and the circulation of TNF-α level to reduce atrogenes and p38 MAPK-C/EBPβ pathway in vitro and in vivo. Interestingly, the LA treatment was neither accompanied with reduced TNF-α in circulation and GA muscle, or reduced p38 MAPK-C/EBPβ pathway in the GA muscle (Figures 3, 5C, and 5D). These findings clearly demonstrate that GLA had more potent effect than LA in modulating the TNF-α-mediated p38 MAPK-C/EBPβ pathway to restore LLC-induced muscle wasting.

Conclusion

In C2C12 myotubes and GA muscles of LLC tumor-bearing mice, GLA had more potent effect than LA to counteract muscle wasting events. The anti-muscle wasting effect of GLA was potentially driven by the inhibition of tumoral factors (Mstn) and proinflammatory cytokine (TNF-α) expression and downregulation of Akt-FoxO-dependent and p38 MAPK-C/EBPβ pathways, eventually inhibiting the downstream atrogenes expression and decreasing the process of ubiquitinization to reduce the protein degradation. Therefore, GLA can be used as an alternative for the amelioration of a cachectic syndrome in humans.

Funding

This work was supported by grants from the grant NSC 103-2320-B-040-009 and CMU102-ASIA-11.

References

1. Dodson, S. *et al*. (2011) Muscle wasting in cancer cachexia: clinical implications,diagnosis, and emerging treatment strategies. *Annu Rev Med*., **62**, 265-279.

2. Fearon, K. *et al*. (2011) Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol*., **12**(5), 489-495.

3. Siddiqui, R. *et al*. (2006) Pandya D, Harvey K, Zaloga GP. Nutrition modulation of cachexia/proteolysis. *Nutr Clin Pract*., **21**(2), 155-167.

4. Tisdale, M. J. (2002) Cachexia in cancer patients. *Nat Rev Cancer*., **2**(11), 862-871.

5. Clavel, S. *et al*. (2006) Atrophy-related ubiquitin ligases, atrogin-1 and MuRF1 are up-regulated in aged rat Tibialis Anterior muscle. *Mech Ageing Dev*., **127**(10), 794-801.

6. Edstrom, E. *et al*. (2006) Atrogin-1/MAFbx and MuRF1 are downregulated in aging-related loss of skeletal muscle. *J Gerontol A Biol Sci Med Sci*., **61**(7), 663-674.

7. Sandri, M. (2010) Autophagy in health and disease. 3. Involvement of autophagy in muscle atrophy. *Am J Physiol Cell Physiol*., **298**(6), C1291-1297.

8. Kamei, Y. *et al*. (2004) Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. *J Biol Chem*., **279**(39), 41114-41123.

9. Yang, H. *et al*. (2005) Expression and activity of C/EBPbeta and delta are upregulated by

dexamethasone in skeletal muscle. *J Cell Physiol*., **204**(1), 219-226.

10. Lokireddy, S. *et al*. (2012) Myostatin is a novel tumoral factor that induces cancer cachexia. *Biochem J*., **446**(1), 23-36.

11. Sishi, B. J. *et al*. (2011) Engelbrecht AM. Tumor necrosis factor alpha (TNF-alpha) inactivates the PI3-kinase/PKB pathway and induces atrophy and apoptosis in L6 myotubes. *Cytokine*., **54**(2), 173-184.

12. Llovera, M. *et al*. (1998) Role of TNF receptor 1 in protein turnover during cancer cachexia using gene knockout mice. *Mol Cell Endocrinol*., **142**(1-2), 183-189.

13. McFarlane, C. *et al*. (2006) Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent mechanism. *J Cell Physiol*., **209**(2), 501-514.

14. Zhou, X. *et al*. (2010) Reversal of cancer cachexia and muscle wasting by ActRIIB antagonism leads to prolonged survival. *Cell*., **142**(4), 531-543.

15. Murphy, K. T. *et al*. (2011) Antibody-directed myostatin inhibition enhances muscle mass and function in tumor-bearing mice. *Am J Physiol Regul Integr Comp Physiol*., **301**(3), R716-726.

16. Zhao, J. *et al*. (2007) FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. *Cell Metab*., **6**(6), 472-483.

17. Zhang, G. *et al*. (2011) C/EBPbeta mediates tumour-induced ubiquitin ligase atrogin1/MAFbx upregulation and muscle wasting. *EMBO J*., **30**(20), 4323-4335.

18. Kapoor, R. *et al*. (2006) Gamma linolenic acid: an antiinflammatory omega-6 fatty acid. *Curr Pharm Biotechnol*., **7**(6), 531-534.

19. Chang, C. S. *et al*. (2010) Gamma-linolenic acid inhibits inflammatory responses by regulating NF-kappaB and AP-1 activation in lipopolysaccharide-induced RAW 264.7 macrophages. *Inflammation*., **33**(1), 46-57.

20. Begin, M. E. *et al*. (1985) Selective killing of human cancer cells by polyunsaturated fatty acids. *Prostaglandins Leukot Med*., **19**(2), 177-186.

21. Matsuoka, T. *et al*. (2010) Elevated dietary linoleic acid increases gastric carcinoma cell invasion and metastasis in mice. *Br J Cancer*., **103**(8), 1182-1191.

22. Rose, D. P. *et al*. (1990) Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth

of a human breast cancer cell line in culture. *Cancer Res*., **50**(22), 7139-7144.

23. Gabor, H. *et al*. (1985) Effect of dietary fat on growth kinetics of transplantable mammary adenocarcinoma in BALB/c mice. *J Natl Cancer Inst*., **74**(6), 1299-1305.

24. Chen, H. W. *et al*. (2013) Andrographis paniculata Extract and Andrographolide Modulate the Hepatic Drug Metabolism System and Plasma Tolbutamide Concentrations in Rats. *Evid Based Complement Alternat Med*., 982689.

25. Lowry, O. H. *et al*. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem*., **193**(1), 265-275.

26. Menconi, M. *et al*. (2008) Dexamethasone and corticosterone induce similar, but not identical, muscle wasting responses in cultured L6 and C2C12 myotubes. *J Cell Biochem*., **105**(2), 353-364.

27. Lee, J. Y. *et al*. (2011) Myostatin induces autophagy in skeletal muscle in vitro. *Biochem Biophys Res Commun*., **415**(4), 632-636.

28. Reed, S. A. *et al*. (2012) Inhibition of FoxO transcriptional activity prevents muscle fiber atrophy during cachexia and induces hypertrophy. *FASEB J*., **26**(3), 987-1000.

29. Adams, V. *et al*. (2008) Induction of MuRF1 is essential for TNF-alpha-induced loss of muscle function in mice. *J Mol Biol*., **384**(1), 48-59.

30. Andreyev, H. J. *et al*. (1998) Why do patients with weight loss have a worse outcome when undergoing chemotherapy for gastrointestinal malignancies? *Eur J Cancer*., **34**(4), 503-509.

31. Donohoe, C. L. *et al*. (2011) Cancer cachexia: mechanisms and clinical implications. *Gastroenterol Res Pract*., **2011**, 601434.

32. Bachmann, J. *et al*. (2008) Cachexia worsens prognosis in patients with resectable pancreatic cancer. *J Gastrointest Surg*., **12**(7), 1193-1201.

33. Fujiwara, F. *et al*. (1986) Antitumor effect of gamma-linolenic acid on cultured human neuroblastoma cells. *Prostaglandins Leukot Med*., **23**(2-3), 311-320.

34. Leaver, H. A. *et al*. (2002) Antitumour and pro-apoptotic actions of highly unsaturated fatty acids in glioma. Prostaglandins Leukot Essent Fatty Acids., **66**(1), 19-29.

35. Colquhoun, A. (2002) Gamma-linolenic acid alters the composition of mitochondrial membrane subfractions, decreases outer mitochondrial membrane binding of hexokinase and alters carnitine palmitoyltransferase I properties in the Walker 256 rat tumour. *Biochim Biophys Acta*., **1583**(1), 74-84.

36. Rose, D. P. *et al*. (1993) Effect of diets containing different levels of linoleic acid on human breast

cancer growth and lung metastasis in nude mice. *Cancer Res.*, **53**(19), 4686-4690.

37. Lu, X. F. *et al*. (2010) Colorectal cancer cell growth inhibition by linoleic acid is related to fatty acid composition changes. *J Zhejiang Univ Sci B*., **11**(12), 923-930.

38. Horrobin, D. F. (1992) Nutritional and medical importance of gamma-linolenic acid. *Prog Lipid Res*., **31**(2), 163-194.

39. Das, U. N. (2010) A defect in Delta6 and Delta5 desaturases may be a factor in the initiation and progression of insulin resistance, the metabolic syndrome and ischemic heart disease in South Asians. *Lipids Health Dis*., **9,** 130.

40. Pham, H. *et al*. (2006) Dietary gamma-linolenate attenuates tumor growth in a rodent model of prostatic adenocarcinoma via suppression of elevated generation of PGE(2) and 5S-HETE. *Prostaglandins Leukot Essent Fatty Acids*., **74**(4), 271-282.

41. Kris-Etherton, P. M. *et al*. (2000) Polyunsaturated fatty acids in the food chain in the United States. *Am J Clin Nutr*., **71**(1 Suppl), 179S-188S.

42. Stitt, T. N. *et al*. (2004) The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell*., **14**(3), 395-403.

43. Gallot, Y. S. *et al*. (2014) Myostatin gene inactivation prevents skeletal muscle wasting in cancer. *Cancer Res*., **74**(24), 7344-7356.

Table and figures legens

Fig. 1. GLA and LA attenuate LCM-induced TNF-α and Mstn protein levels and prevent C2C12 myotubes wasting events

C2C12 myotubes were preincubated with 100 μM GLA or LA for 12 h and then treated with or without LCM for 48 h. **(A)** and **(B)** TNF-α, Mstn, and MHC protein levels were measured by Western blot analysis. **(C)** MuRF1 and MAFbx mRNA were measured by real-time PCR. **(D** and **E)** MuRF1, MAFbx, Ub, and LC3A into LC3B conversion protein levels were measured by Western blot analysis. Data are the mean ± SD of at least four separate experiments and are expressed as a percentage of LCM alone. Values not sharing the same letter are significantly different ($P < 0.05$).

Fig. 2. GLA and LA avoid LCM-induced C2C12 myotubes wasting via the Akt-FoxO1-dependent pathway

C2C12 myotubes were pretreated with 100 μM GLA or LA for 12 h and then treated with or without LCM for 48 h. **(A)** The phosphorylated and native forms of Akt protein and phosphorylated FoxO1a/3a protein levels in cytosolic fraction and FoxO1 in nuclear extracts were measured by Western blot analysis. The C2C12 myoblasts were transiently transfected with Akt-empty and Akt (K179M) for 24 h and switched to 2% HS-DMEM for 6 days. The myoblasts were then treated as mentioned in Figure 1. **(B)** The phosphorylated and native forms of Akt protein and phosphorylated FoxO1a/3a protein levels in cytosolic fraction and FoxO1a protein in nuclear extracts and **(C** to **E)** MuRF1, MAFbx, LC3B, and MHC protein levels were evaluated by Western blot analysis. Data are the mean ± SD of at least three separate experiments and are expressed as a percentage of LCM alone or Akt-empty control alone. Within treatments with the same plasmid transfection, values not sharing the same letter are significantly different ($P < 0.05$).

Fig. 3. GLA inhibits LCM-induced p38 MAPK-C/EBPβ pathway in C2C12 myotubes

C2C12 myotubes were pre-cultured with 100 μM GLA or LA for 12 h and then treated with or without LCM for 48 h. **(A** and **B)** The phosphorylated and native forms of p38 MAPK protein and nuclear C/EBPβ protein levels were determined by Western blot analysis. The PARP protein served as nuclear control. **(C)** To confirm the C/EBPβ transcription activity, the C2C12 myoblasts were transiently transfected with pSV-β-galactosidase and pC/EBPβ-Luc report genes for 24 h and switched to 2% HS-DMEM for 6 days. The myoblasts were then treated as mentioned in Figure 1, and the cells were harvested and determined by Luciferase Assay System. Data are the mean ± SD of at least four separate experiments and are expressed as a percentage of LCM alone. Values not sharing the same letter are significantly different ($P < 0.05$).

Fig. 4. Borage oil reverses body weight and muscle weight loss in LLC tumor-bearing C57BL/6 mice

C57BL/6 mice were randomly distributed into three groups, including control (C), LLC tumor-bearing with soybean oil (LSO), and LLC tumor-bearing with borage oil (LBO). LLC cells or PBS (control) were i.p. injected into the left flank of mice as described in Materials and methods. **(A)** Upon inoculation,

food intake and body weight were recorded before euthanasia was performed. Mice in the LSO and LBO groups were killed, and tumor weight was calculated. **(B** and **C)** Quantification of organ and hind limb muscle weights. **(D)** H&E staining of the longitudinal sections and area of myofiber. Data are the mean ± SD of at least five mice per group and are expressed as a percentage of expression in the LSO group. Values not sharing the same letter are significantly different ($P < 0.05$). EAT, GA, and EDL are abbreviations of epicardial adipose tissue, gastrocnemius, and extensor digitorum longus, respectively.

Fig. 5. Borage oil ameliorates muscle wasting in LLC tumor-bearing C57BL/6 mice

(A) After 21 days, plasma was collected from mice after death and measured by TNF-α kit, and TNF-α mRNA from GA muscles was measured by real-time PCR. **(B** and **C)** The GA muscles of TNF-α, Mstn, and MHC protein levels were measured by Western blot analysis. **(D)** The GA muscles of delta-6 desaturase, MuRF1, and MAFbx mRNA expression were assessed by real-time PCR. **(E** and **F)** MuRF1, MAFbx, Ub, and LC3A into LC3B conversion protein levels were determined by Western blot analysis. Data are the mean ± SD of at least three to five mice per group and are expressed as a percentage of expression in the LSO group. Values not sharing the same letter are significantly different ($P < 0.05$).

Fig. 6. Borage oil regulates Akt-FoxO and p38 MAPK-C/EBPβ pathways in LLC tumor-bearing C57BL/6 mice

(A) GA muscle lysates were performed to detect phosphorylated and native forms of Akt protein, and phosphorylated FoxO1a/3a protein levels in cytosolic fraction as well as FoxO1 protein in nuclear extracts were determined by Western blot analysis. **(B)** Immunohistochemistry staining with the FKHR (FoxO1) antibody in GA muscles. **(C)** GA muscle lysates were performed to detect phosphorylated and native forms of p38 MAPK protein levels, and C/EBPβ protein in nuclear extracts was determined by Western blot analysis. **(D)** Immunohistochemistry analysis with the C/EBPβ antibody in GA muscles. **(E)** GLA ameliorated LLC-induced atrogene expression via Akt-FoxO-dependent and p38 MAPK-C/EBPβ pathways and reduced Mstn and TNF-α expression in C2C12 myotubes and GA muscles. (Dashed line indicates the indirect effect within the pathway)

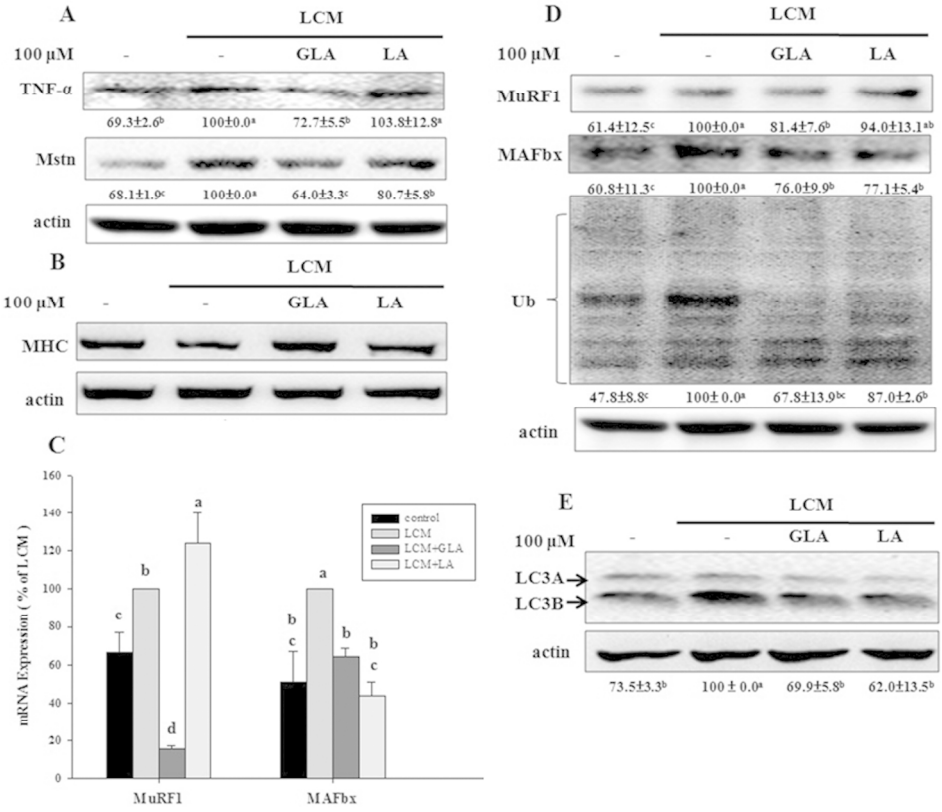

Fig. 1. GLA and LA attenuate LCM-induced TNF-α and Mstn protein levels and prevent C2C12 myotubes wasting events

C2C12 myotubes were preincubated with 100 μM GLA or LA for 12 h and then treated with or without LCM for 48 h. (A) and (B) TNF-α, Mstn, and MHC protein levels were measured by Western blot analysis. (C) MuRF1 and MAFbx mRNA were measured by real-time PCR. (D and E) MuRF1, MAFbx, Ub, and LC3A into LC3B conversion protein levels were measured by Western blot analysis. Data are the mean ± SD of at least four separate experiments and are expressed as a percentage of LCM alone. Values not sharing the same letter are significantly different ($P < 0.05$).

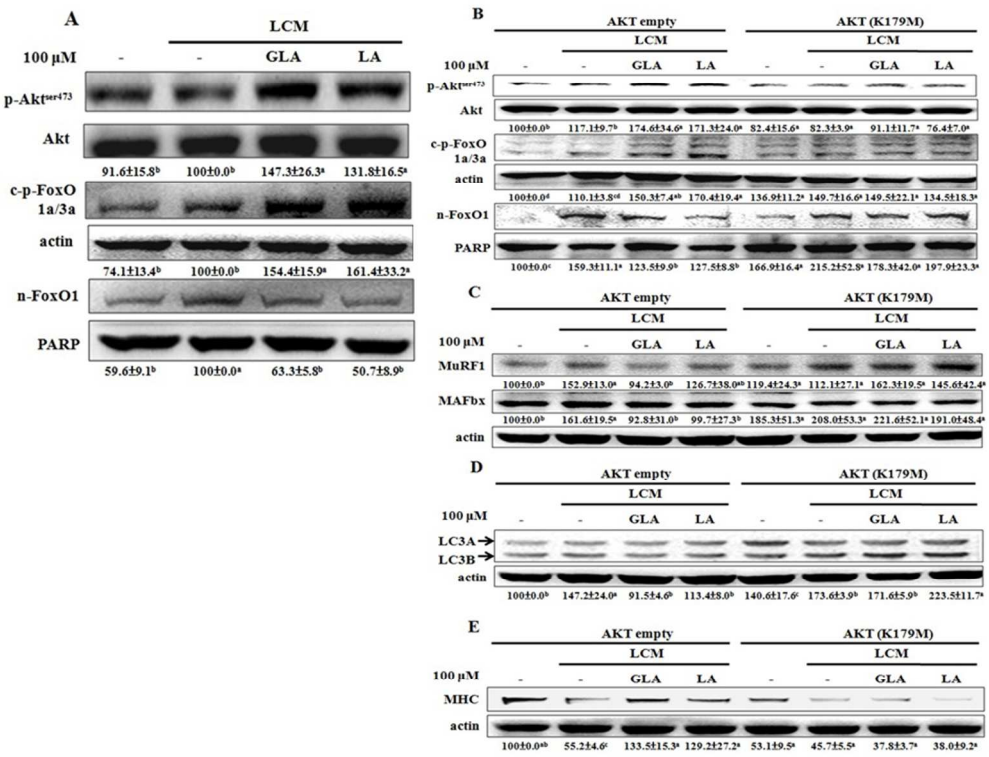

Fig. 2. GLA and LA avoid LCM-induced C2C12 myotubes wasting via the Akt-FoxO1-dependent pathway C2C12 myotubes were pretreated with 100 μM GLA or LA for 12 h and then treated with or without LCM for 48 h. (A) The phosphorylated and native forms of Akt protein and phosphorylated FoxO1a/3a protein levels in cytosolic fraction and FoxO1 in nuclear extracts were measured by Western blot analysis. The C2C12 myoblasts were transiently transfected with Akt-empty and Akt (K179M) for 24 h and switched to 2% HS-DMEM for 6 days. The myoblasts were then treated as mentioned in Figure 1. (B) The phosphorylated and native forms of Akt protein and phosphorylated FoxO1a/3a protein levels in cytosolic fraction and FoxO1a protein in nuclear extracts and (C to E) MuRF1, MAFbx, LC3B, and MHC protein levels were evaluated by Western blot analysis. Data are the mean ± SD of at least three separate experiments and are expressed as a percentage of LCM alone or Akt-empty control alone. Within treatments with the same plasmid transfection, values not sharing the same letter are significantly different ($P < 0.05$).

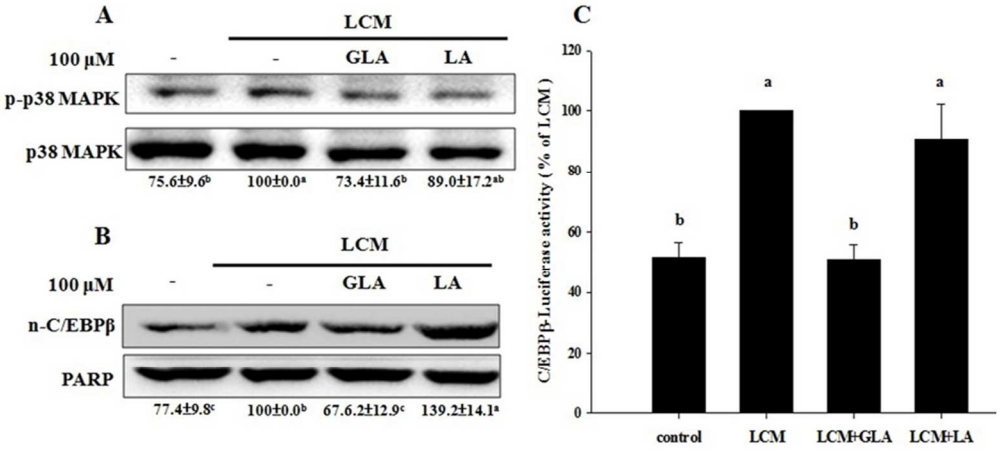

Fig. 3. GLA inhibits LCM-induced p38 MAPK-C/EBPβ pathway in C2C12 myotubes
C2C12 myotubes were pre-cultured with 100 μM GLA or LA for 12 h and then treated with or without LCM for 48 h. (A and B) The phosphorylated and native forms of p38 MAPK protein and nuclear C/EBPβ protein levels were determined by Western blot analysis. The PARP protein served as nuclear control. (C) To confirm the C/EBPβ transcription activity, the C2C12 myoblasts were transiently transfected with pSV-β-galactosidase and pC/EBPβ-Luc report genes for 24 h and switched to 2% HS-DMEM for 6 days. The myoblasts were then treated as mentioned in Figure 1, and the cells were harvested and determined by Luciferase Assay System. Data are the mean ± SD of at least four separate experiments and are expressed as a percentage of LCM alone. Values not sharing the same letter are significantly different ($P < 0.05$).

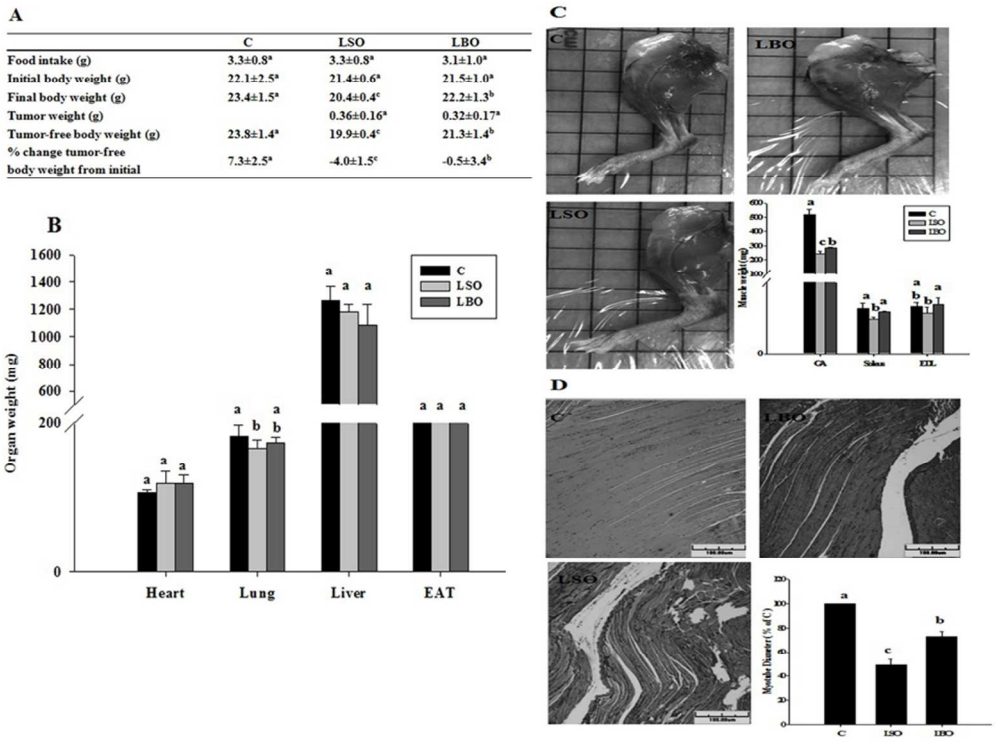

Fig. 4. Borage oil reverses body weight and muscle weight loss in LLC tumor-bearing C57BL/6 mice C57BL/6 mice were randomly distributed into three groups, including control (C), LLC tumor-bearing with soybean oil (LSO), and LLC tumor-bearing with borage oil (LBO). LLC cells or PBS (control) were i.p. injected into the left flank of mice as described in Materials and methods. (A) Upon inoculation, food intake and body weight were recorded before euthanasia was performed. Mice in the LSO and LBO groups were killed, and tumor weight was calculated. (B and C) Quantification of organ and hind limb muscle weights. (D) H&E staining of the longitudinal sections and area of myofiber. Data are the mean ± SD of at least five mice per group and are expressed as a percentage of expression in the LSO group. Values not sharing the same letter are significantly different ($P < 0.05$). EAT, GA, and EDL are abbreviations of epicardial adipose tissue, gastrocnemius, and extensor digitorum longus, respectively.

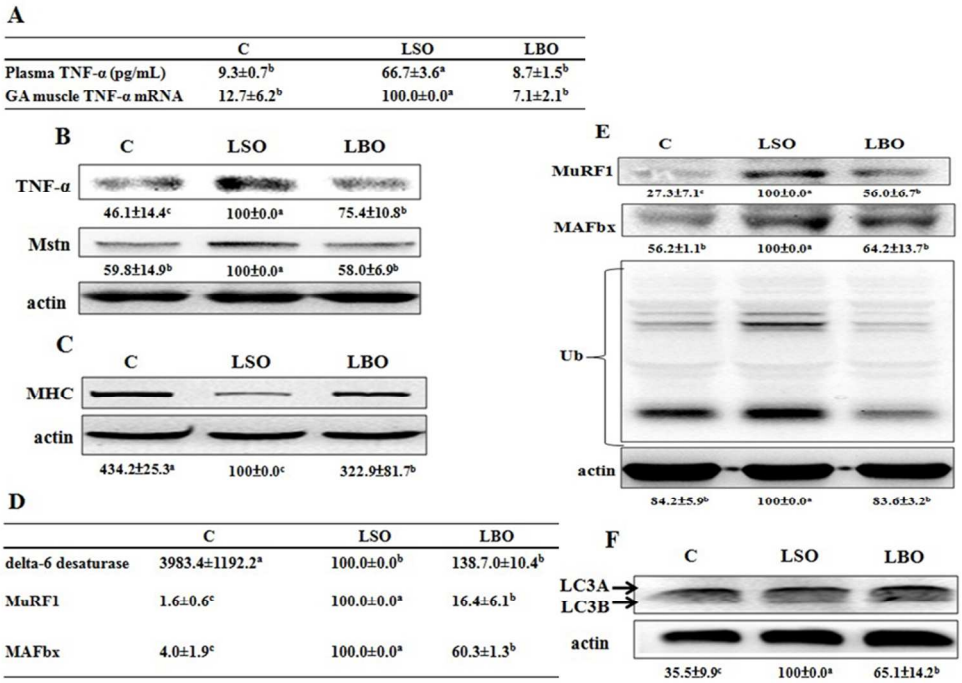

Fig. 5. Borage oil ameliorates muscle wasting in LLC tumor-bearing C57BL/6 mice
(A) After 21 days, plasma was collected from mice after death and measured by TNF-α kit, and TNF-α mRNA from GA muscles was measured by real-time PCR. (B and C) The GA muscles of TNF-α, Mstn, and MHC protein levels were measured by Western blot analysis. (D) The GA muscles of delta-6 desaturase, MuRF1, and MAFbx mRNA expression were assessed by real-time PCR. (E and F) MuRF1, MAFbx, Ub, and LC3A into LC3B conversion protein levels were determined by Western blot analysis. Data are the mean ± SD of at least three to five mice per group and are expressed as a percentage of expression in the LSO group. Values not sharing the same letter are significantly different ($P < 0.05$).

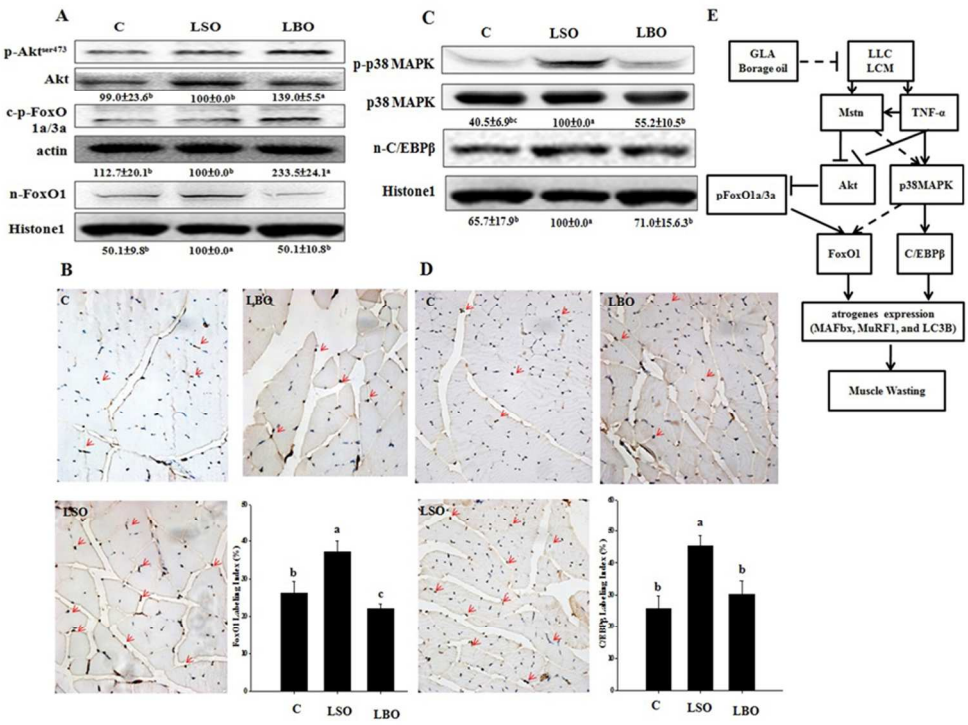

Fig. 6. Borage oil regulates Akt-FoxO and p38 MAPK-C/EBPβ pathways in LLC tumor-bearing C57BL/6 mice (A) GA muscle lysates were performed to detect phosphorylated and native forms of Akt protein, and phosphorylated FoxO1a/3a protein levels in cytosolic fraction as well as FoxO1 protein in nuclear extracts were determined by Western blot analysis. (B) Immunohistochemistry staining with the FKHR (FoxO1) antibody in GA muscles. (C) GA muscle lysates were performed to detect phosphorylated and native forms of p38 MAPK protein levels, and C/EBPβ protein in nuclear extracts was determined by Western blot analysis. (D) Immunohistochemistry analysis with the C/EBPβ antibody in GA muscles. (E) GLA ameliorated LLC-induced atrogene expression via Akt-FoxO-dependent and p38 MAPK-C/EBPβ pathways and reduced Mstn and TNF-α expression in C2C12 myotubes and GA muscles. (Dashed line indicates the indirect effect within the pathway)

Gene Name	Forward Primer (5'-3')	Reverse Primer (3'-5')	
Roche			Probe NO.
TNF-α	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG	102
MuRF1	GTGTACGGCCTGCAGAGG	CTTCGTGTTCCTTGCACATC	31
MAFbx	GGTGGCACTGGTTTAGAGGA	ATCGGCTCTTCCGTTGAAA	31
SYBAR GREEN			
delta-6 desaturase	AGAAGATGCTACGGATGC	CTGAAGTCCTCGGTGATC	

Table S1. Roche and SYBR Green primer and probe sets used for Real-Time PCR

科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/29

科技部補助計畫	計畫名稱: 探討Gamma次亞麻油酸調控癌症惡病質誘發骨骼肌肉耗損之功效及相關機制
	計畫主持人: 劉凱莉
	計畫編號: 103-2320-B-040-009- 學門領域: 保健營養

無研發成果推廣資料

103年度專題研究計畫研究成果彙整表

計畫主持人：劉凱莉	**計畫編號：**103-2320-B-040-009-
計畫名稱：探討Gamma次亞麻油酸調控癌症惡病質誘發骨骼肌肉耗損之功效及相關機制	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	0%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	5	5	50%	人次	
		博士生	2	2	50%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	1	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	1	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）		無					

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：□已發表 □未發表之文稿 ■撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）
   癌症惡病質會導致75%的肌肉流失，使癌症惡病質病患虛弱無力、不活動(immobility)、心肺衰竭，增加對化療毒性敏感性及降低癌症病人存活率(Tisdale, 2002)。本研究計畫結果則有助於了解飲食中添加富含GLA油脂作為改善癌症惡病質病患肌肉耗損輔助治療的可行性，提供未來研發富含GLA相關保健食品的參考依據。本計畫亦以含癌細胞培養基的Conditioned media及TNF-a誘發已分化的C2C12肌細胞蛋白質耗損及肌纖維萎縮的研究模式，探討GLA是否經由抑制轉錄因子NF-kB、FoxOs及C/EBPb轉錄活性，減緩癌症惡病質誘發骨骼肌耗損。此研究結果能更進一步了解GLA抑制癌症惡病質誘發骨骼肌肉耗損的功效及相關分子機制，有助於未來開發富含GLA相關保健食品之依據。