Glycogen Synthase Kinase-3 β Inhibitor Lithium Chloride Protects Against Inflammation-Mediated Skeletal Muscle Wasting

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Abstract

Inflammation-mediated skeletal muscle wasting is induced by inflammatory cytokines. It occurs in critically ill patients with sepsis (termed intensive care unit acquired weakness) and patients with advanced metastasis (termed cancer cachexia). Both conditions severely impact on patient morbidity and mortality. Lithium chloride has been investigated as a drug repurposing candidate for numerous diseases. In this study, we assessed whether lithium chloride affects inflammation-mediated muscle wasting, using *in vitro* and *in vivo* models of cancer cachexia and sepsis. Lithium chloride prevented wasting in myotubes cultured with cancer cell conditioned media, maintained expression of the muscle fiber contractile protein, myosin heavy chain 2 and blocked upregulation of the E3 ubiquitin ligase, *Atrogin-1*. Glycogen synthase kinase-3 β inhibition was indicated as the target mechanism, due to the following observations: 1) β -catenin was upregulated in the myotubes and 2) inhibition of IMPA1, the secondary biological target of lithium chloride, did not inhibit the effects of cancer conditioned media. Lithium chloride inhibited upregulation of the inflammation-associated cytokines *Il-1 β* , *Il-6* and *inos* in macrophages treated with lipopolysaccharide. Lithium chloride treatment in an animal model of sepsis improved body weight, increased muscle mass, preserved the survival of larger fibers and decreased expression of the wasting effector genes, *Atrogin-1* and *Murf-1*. In a model of cancer cachexia, lithium chloride increased muscle mass, enhanced muscle strength and increased fiber cross sectional area, with no significant effect on tumorigenesis. These results indicate that lithium chloride could be repurposed as a drug to treat patients with inflammation-mediated skeletal muscle wasting.

Introduction

Inflammation-associated skeletal muscle wasting involves activation of the transcriptional complex NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) via increases in circulating cytokines, such as interleukin-1 β (IL-1 β)¹. Examples include cancer cachexia and intensive care unit acquired weakness (ICUAW) produced by sepsis¹⁻³. Both conditions result from acute-phase responses and systemic inflammation^{4,5}. Cancer cachexia affects around 50% of all cancer patients and can be an immediate cause of death by increasing the side effects of chemotherapy⁶. ICUAW occurs in approximately 90% patients with severe sepsis and increases morbidity and mortality⁷. For patients with cancer cachexia, there are only limited treatment options and exercise programs are not always feasible, due to issues such as chronic fatigue and anemia^{8,9}. ICUAW places a high financial burden on healthcare systems^{5,10}. Therefore, the development of effective therapeutics for inflammation-associated muscle wasting is a research priority.

Lithium has been termed an “Oldie but Goodie” drug that was first used to treat psychiatric disorders in the nineteenth century^{11,12}. It is a commonly prescribed treatment for bipolar disorder and is included in the World Health Organization's list of essential medicines¹³. Lithium chloride is also widely used in basic research as a glycogen synthase kinase-3 β (GSK-3 β) inhibitor¹⁴. Due to its multiple effects in cells,

lithium has been studied as a drug repurposing candidate for numerous diseases, such as enterovirus infection, Parkinson's disease, radiation injury and rheumatoid arthritis¹⁵⁻¹⁸.

In this study, we investigated the effects of lithium chloride on inflammation-associated skeletal muscle wasting using *in vitro* and *in vivo* models of cancer cachexia and sepsis. Our results suggest that lithium chloride has potential for further development as a repurposing drug to treat inflammation-associated muscle wasting.

Methods

Reagents

Lipopolysaccharide (LPS) was purchased from Invitrogen, Thermo Fisher Scientific, Waltham, USA. Lithium chloride (LiCl) was purchased from Sigma-Aldrich, St. Louis, USA. Ebselen was purchased from Tokyo Chemical Industry, Japan. Antibodies for myosin heavy chain 2 (MyHC; catalogue number sc-53095) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; catalogue number sc-365062) were purchased from Santa Cruz Biotechnology, Dallas, USA. A β -catenin antibody (catalogue number CST #9562) was purchased from Cell Signaling Technology, Danvers, USA.

Cell culture

Cell culture was carried out as previously described^{19,20}. C2C12 murine skeletal muscle precursor cells (myoblasts) were purchased from Koram Biotech Corp., Seoul, Republic of Korea, and maintained in growth media (GM), consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 mg/mL streptomycin (PenStrep). Myoblasts were induced to differentiate into myotube by treatment with differentiation media (DM: DMEM supplemented with 2% horse serum (HS) and PenStrep for 3 days). The RAW 264.7 murine macrophage and CT-26 murine colon carcinoma cell line were purchased from the Korean Cell Line Bank, Seoul, Republic of Korea, and cultured in GM.

Collection of conditioned media

To prepare cancer conditioned media (CCM), the previously described protocol was followed²¹. CT26 colon carcinoma cells were seeded in 100 mm culture plates at a density of 2×10^6 cells/plate. 48 h later, the culture media was changed to serum-free media. After 24 hours, the CCM were harvested, and debris was removed by centrifugation (1,500 rpm for 3 min at 4°C) and filtering with a 0.22 μ m disk filter (Corning, New York, USA).

MTT assay for cell proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay was used for analyzing cell proliferation as previously described^{19,22}. Myoblasts were seeded in a 96-well plate at a density of

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1.5x10³ cells per well. After 24 hours stabilization, compound of interest and/or CCM was applied for additional 48 hours. After, the medium was changed to MTT solution (0.5 mg/mL; final concentration), and incubated in a 37°C, 5% CO₂ incubator for 60 minutes. 50 µL of DMSO was added to solubilize and optical density was measured at 570 nm using a microplate reader (VersaMax, Molecular Devices, San Jose, USA).

Real-time quantitative PCR (qPCR)

qPCR was carried out as previously described ²¹. The RNAs were extracted by using TRizol reagent following manufacturer’s instruction (Thermo Fisher Scientific, USA), and reverse transcribed with the AccuPower® RT PreMix (Bioneer, Daejeon, Republic of Korea). 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, UK) was used for real-time PCR with specific primers. For amplification and detection, StepOnePlus Real Time PCR System (Applied Biosystems, UK) was used according to the manufacturer’s instructions. The expression level of *Gapdh* was used for normalization while calculating the expression levels of all of the other genes (as indicated in the text). Primer details are shown in Table 1.

Table 1: qPCR primers used in this study

Gene	Locus	Source		Primer sequence	Size
Myosin heavy chain 2 (<i>Myh2</i>)	NM_001039545	<i>Mus musculus</i>	Forward	GAAGAGCCGGGAGGTTTAC	113 bp
			Reverse	ACACAGGCGCATGACCAAA	
<i>Pax-7</i>	NM_011039	<i>Mus musculus</i>	Forward	CCCTTTCAAAGACCAAATGCA	198 bp
			Reverse	CCCTCACGGGCAGATCATTA	
<i>Atrogin-1</i>	NM_026346	<i>Mus musculus</i>	Forward	CAGAGAGCTGCTCCGTCTCA	178 bp
			Reverse	ACGTATCCCCCGCAGTTTC	
<i>MuRF-1</i>	NM_001039048	<i>Mus musculus</i>	Forward	CCGAGTGCAGACGATCATCTC	198 bp
			Reverse	TGGAGGATCAGAGCCTCGAT	
<i>Il-6</i>	NM_031168	<i>Mus musculus</i>	Forward	GAGGATACCACTCCCAAC	141 bp
			Reverse	AAGTGCATCATCGTTGTT	
<i>inos</i>	NM_010927	<i>Mus musculus</i>	Forward	CCCCTTCAATGGCTGGTACA	64 bp
			Reverse	GCGCTGGACGTCACAGAA	
<i>Il-1β</i>	NM_008361	<i>Mus musculus</i>	Forward	TGCCACCTTTTGACAGTGATG	135 bp
			Reverse	TGTGCTGCTGCGAGATTTGA	
<i>Gapdh</i>	NM_001289726	<i>Mus musculus</i>	Forward	CTCCACTCACGGCAAATTCA	120 bp
			Reverse	GCCTCACCCCATTTGATGTT	

***In vitro* model of cancer cachexia and morphological analysis of myotubes.**

An *in vitro* model of cancer cachexia was established using the previously published protocol ²³. 3×10^5 myoblasts were seeded in each well of a 12 well plate and stabilized for 24 h in GM. The media was changed into DM for 72 h to produce myotube. Myotubes were treated with 1:1 ratio of DM and CCM for 72 hours to induce myotube wasting. Compounds of interest were added into CCM mixture before administration. Myotubes were then fixed with 4% formaldehyde and permeabilized with PBS containing 0.5% Triton X-100 (Sigma Aldrich, USA). Myosin Heavy chain 2 (MyHC) antibody (Santa Cruz, USA) was treated in 1% BSA in PBST (0.02% Tween 20 in PBS) for overnight at 4°C. The secondary antibody, Alexa fluor 488 (ThermoFisher, USA), was applied for 1 hours at room temperature. Fluorescence images were taken in 5 different area by using DMI 3000 B (Leica, Germany) and analyzed with ImageJ 1.52 software (National institutes of Health, Bethesda, MD, USA). MyHC positive myotube containing more than 3 nuclei were considered as myotube and measured diameter.

Immunoblotting

Immunoblotting was performed as previously described²¹. Briefly, the concentration of protein lysate was quantified using the Bradford reagent (Bio-Rad, Hercules, USA). After electrophoresis, separated proteins were transferred on to PVDF membranes, blocked with 5% non-fat powdered milk in TBST (0.02% Tween 20 in TBS), and subsequently incubated overnight at 4°C with the primary antibody. The secondary antibody was used at a 1: 10000 dilution (For mouse: Abcam, cat. no. ab6789, For rabbit : Cell Signaling Technology, cat. No. #7004). Detection was performed with ImageQuant™ LAS 500 (GE Healthcare, USA) with manufacturer's instruction, quantified by using ImageJ 1.52 software (National Institutes of Health). Full-length blots are included as Supplementary Information Figure 1 and 2.

Assessment of pro-cachexia cytokine induction in macrophages

RAW 264.7 macrophages were seeded in 6 well culture plates at a density of 5×10^5 cells/well. 24 h later, macrophages were pre-treated with compound of interest for 1 h, followed by 200 ng/mL LPS and compound for 24 h. RNA was then isolated using the Trizol reagent and cytokine gene expression measured using qPCR, as described above.

Animal studies

Animal experiments were carried out in accordance with the ethical guidelines established by the Animal Care and Use Committee of the Gwangju Institute of Science and Technology, Republic of Korea (study approval number: GIST-2019-042). The experimental protocols were approved by the Animal Care and Use Committee of the Gwangju Institute of Science and Technology, Republic of Korea. In addition to the guidelines established by the ACUC, the study was carried out in compliance with the ARRIVE guidelines. Animals were supplied by Damool Science, Republic of Korea.

Animal model of sepsis-induced muscle wasting

Studies of the therapeutic effect of LiCl on sepsis-induced muscle wasting *in vivo* were based on the previously published protocols^{24,25}. 10 weeks old male C57BL/6 mice were randomly assigned into the following treatment groups: 1) 4 d intraperitoneal (IP) injection of saline vehicle every 24 h, 2) 4 d IP delivery of 40 mg/kg LiCl every 24 h, 3) Untreated. On the fourth day, 1 mg/kg LPS was treated to the saline and LiCl groups by IP, and the untreated group received saline by IP. 18 h after LPS injection, mice were anaesthetized using ketamine (22 mg/kg; Yuhan, Republic of Korea) and xylazine (10 mg/kg; Bayer, Republic of Korea) and sacrificed. Mouse limb muscles were dissected, weighed and frozen at -80 °C.

Animal model of cancer cachexia model

The murine model of cancer cachexia was based on a previous study²³. 10 weeks old male BALB/c mice were randomly assigned into the following treatment groups: 1) 1×10^6 of CT26 colon cancer cells inoculated subcutaneously (SC) in the right flank, 2) Equal volume of saline inoculated SC in the right flank. Tumor growth was monitored twice weekly, and tumor volume was calculated using a caliper and the following formula: $V(\text{volume}) = (\text{longitudinal} \times \text{transverse}^2)/2$. 10 d after tumor injection, daily ip

administration of LiCl was conducted with specific concentration for 21 d. Grip strength was measured using the BIO-GS3 strength meter (Bioseb, France). Each mouse was tested 4 times with a 30 sec interval and the maximum value force was used to represent muscle force. 28 d after CT26 cell or saline injection, mice were anaesthetized and sacrificed for further analysis.

Histological analysis

Histological analysis of dissected muscles was carried out as previously described²⁶. Quadriceps were fixed with 4% paraformaldehyde solution overnight at 4 °C and embedded into paraffin. 5 µm of each muscle sections were made stained with hematoxylin and eosin (H&E) using a kit (Merck, Darmstadt, Germany). Images were obtained by light microscopy (Leica, DM 2500) and cross sectional areas were measured by Image J 1.52 software (National Institute of Health).

Statistics

Microsoft Excel 2016 (Redmond, USA) was used to determine statistical significance with the Student's *t*-test in Figures 1-4. ANOVA test was used to determine statistical significance in Figures 5-7 (Tukey's post-hoc comparison of the means using Origin Pro 9.1 software). *p* values of less than 0.05 were deemed to be statistically significant. Unless otherwise stated in the figure legends, experiments were carried out in triplicate and the error bars are standard deviation.

Results

LiCl increases *Myh2* expression and reduces *Pax-7* expression in differentiating myoblasts treated with CCM

LiCl has been reported to induce myogenic differentiation in C2C12 myoblasts^{27,28}. Prior to evaluate the effect on cancer cachexia, we verified the phenotype of LiCl on myogenic differentiation. As expected, treatment of LiCl reduced the expression of paired box protein *Pax-7* (a myoblast marker indicating myotube dedifferentiation^{46,47}) while increasing the expression *Myh2* (a myotube marker²) (Figure 1A-B). CCM has been shown to contain factors that inhibit both myoblast proliferation and myogenic differentiation²⁹. To test the effect of LiCl on proliferation and differentiation, C2C12 myoblasts were treated with CCM, or CCM containing LiCl. CM treatment significantly reduced C2C12 myoblast proliferation. LiCl did not affect this inhibition of myoblast proliferation (Figure 1C-D). To investigate the effect LiCl on myogenic differentiation in the presence of CCM, the expression of *Pax-7* and *Myh2* was measured in differentiating myoblasts treated with CCM or CCM plus LiCl. CCM increased the expression of *Pax-7* and decreased the expression of *Myh2*. LiCl inhibited the effect of CCM on *Pax-7* and *Myh2* expression (Figure 1E-F).

LiCl prevents CCM-induced myotube wasting

Differentiated C2C12 myotubes were treated with CCM with or without LiCl. CCM treated myotubes showed an almost 30% decrease in diameter, which was prevented by co-treatment with LiCl (Figure 2A-B). Measurement of myotube diameter distribution indicated that LiCl preserved larger sized myotubes (Figure 2C). Similar to differentiating myoblasts, LiCl prevented both the upregulation of *Pax-7* and downregulation of MyHC in the myotubes (Figure 2D-E). LiCl is known to function as a GSK-3 β inhibitor that upregulates the expression of β -catenin³⁰. LiCl treated myotubes increased expression of β -catenin in the presence of CCM (Figure 2F). The E3 ubiquitin ligase, atrogin-1 (MAFbx/ FBXO32) is a major effector of the increased muscle proteolysis in cancer cachexia^{31,32}. *Atrogin-1* expression was upregulated in myotubes treated with CCM myotubes and inhibited by treatment with LiCl (Figure 2D).

Lithium mimetic ebselen does not prevent myotube wasting induced by CCM

The synthetic organoselenium drug, ebselen, has been reported as a lithium mimetic by inhibiting the secondary lithium target, IMPase³³. We investigated if the effects of lithium on CCM-treated myotubes can be reproduced by ebselen. It was observed that ebselen did not attenuate the reduction of myotube diameter after CCM treatment (Figure 3A-B). In addition, ebselen did not inhibit the upregulation of *Atrogin-1* and *Pax-7* expression, or the downregulation of *Myh2*, by CCM (Figure 3C-D).

LiCl inhibits LPS-induced inflammatory cytokine production

Measuring the induction of inflammatory cytokines in macrophages treated with LPS has been used as a model for sepsis³⁴. RAW264.7 macrophages were treated with LPS in the presence or absence of LiCl, and induction of the cachexia-related cytokines *Il-1 β* ³⁵, *Il-6*³⁶ and *inos*³⁷ was measured by qPCR. It was observed that LiCl treatment significantly reduced the induction of *Il-1 β* , *Il-6* and *inos* by LPS (Figure 4).

LiCl prevents muscle wasting in a mouse model of septic cachexia

In order to investigate the protective effect of LiCl on inflammation-induced muscle wasting, we first employed a mouse model of septic cachexia using LPS²⁵. 18 h post-LPS injection, there was significant reduction in body weight compared to mice with saline treatment. Administration of LiCl attenuated this decline in body weight (Figure 5A). Quadriceps muscle weight was measured to determine the effect of LiCl on muscle wasting. LPS treatment decreased muscle mass and LiCl treatment attenuated muscle loss (Figure 5B). The effect of LiCl on sepsis-induced fiber wasting was assessed by measuring cross sectional area in the quadriceps. LPS produced a significant reduction in muscle fiber CSA, which was ameliorated by LiCl treatment (Figure 5C-D). Additionally, the proportion of relatively large fibers was maintained in the LiCl group (Figure 5E). Skeletal muscle E3 ligases are known to mediate LPS induced muscle wasting²⁵. As expected, LPS treatment upregulated the expression of the E3 ligases, *Atrogin-1* and *Murf-1*. LiCl inhibited the induction of atrogin-1 and MuRF-1 (Figure 5F). Since *inos* is known to be an important regulator of cytokine-mediated muscle wasting^{38,39}, the expression of this gene was also measured. LiCl completely inhibited the induction of *inos* gene after LPS administration (Figure 5F).

To further validate the anti-atrophic effect of LiCl on inflammation induced muscle wasting, we used a syngeneic mouse model of cancer cachexia based on the transplantation of CT26 colon carcinoma cells into BALB/c mice, as previously described⁴⁰. Treatment with LiCl did not significantly affect the overall body weight of CT26 tumor bearing mouse (Figure 6A and D). In addition, tumor growth was not altered by LiCl (Figure 6B-C). Skeletal muscle and adipose tissue are among the most affected tissues in cancer cachexia⁴¹. Tumor bearing mice showed a significant reduction in quadriceps muscle, tibialis anterior muscle and gonadal adipose tissue weight. LiCl treatment significantly ameliorated quadriceps and tibialis anterior muscle loss (Figure 6E). In contrast, adipose tissue mass was not affected by LiCl (Figure 6F). Grip strength was measured to assess whether the improvement in muscle mass after LiCl treatment influenced muscle function. LiCl increased muscle function at the 80 mg/kg treatment dose (Figure 6G). In line with the grip strength data, the 80 mg/kg dose preserved average fiber cross sectional area, with a higher proportion of fibers in the 4000-5000 μm^2 range (Figure 6H-I). The increased expression of *Atrogin-1* and *Murf-1* in tumor bearing mice was attenuated in both the 40 and 80 mg/kg LiCl treatment groups (Figure 7A). In addition to E3 ligases, previous studies have demonstrated the role of interleukin-6 on the progression of cancer mediated muscle wasting^{42,43}. Significant upregulation of *Il-6* was observed in the skeletal muscle tissue in tumor bearing mice, and administration of LiCl completely inhibited the tumor-induced increase of *Il-6* (Figure 7A).

Discussion

Inflammation-associated skeletal muscle wasting is produced by cytokines, such as IL-1 β and IL-6. It occurs in cancer cachexia and ICUAW and significantly affects patient morbidity and mortality. Due to a lack of effective treatment options, this study investigated the potential of LiCl to treat these disorders. It was observed that LiCl was effective at inhibiting wasting in cell-based and animal models of inflammation-mediated skeletal muscle wasting.

LiCl treatment was shown to upregulate β -catenin expression in myotubes incubated with CCM. β -catenin protein levels are known to be down-regulated in muscle wasting and activation of the Wnt signaling pathway by GSK-3 β inhibition increases β -catenin expression⁴⁴. In addition, therapy with the Wnt family member, Wnt7a, has been shown to increase β -catenin levels and inhibit cancer cachexia⁴⁵. These findings indicate that GSK-3 β inhibition by LiCl could be the mechanism by which this compound prevents cancer cachexia. This is also supported by our data showing that targeting the secondary biological target of LiCl, IMPase, using the chemical inhibitor, ebselen, did not prevent CCM-induced myotube wasting *in vitro*^{33,46}. There are numerous reports of role of GSK-3 β inhibition in myotube differentiation and atrophy *in vivo* and *in vitro*⁴⁷⁻⁵⁰. We believe that the significance of our study is the demonstration that a clinically approved GSK-3 β inhibitor (LiCl) can prevent inflammation mediated myotube atrophy in animal models. The small molecule GSK-3 β inhibitor, 6-bromoindirubin-3'-oxime (BIO) has been reported to enhance skeletal muscle regeneration *in vivo*⁵¹. However, BIO not yet approved for clinical use and is known to inhibit JAK/STAT3 signaling, which can also enhance muscle regeneration

The animal studies revealed a difference in the LiCl dosage required for effectiveness in LPS-induced muscle wasting and cancer cachexia. The 40 mg/kg dose was effective in the LPS model, while 80 mg/kg was needed for effectiveness in the cancer cachexia model. This may be due to differences in the treatment regime between the two models. In the cancer cachexia model, LiCl was treated 10 d after the transplantation of cancer cells. At this time point the inflammatory environment is already established. Thus, a higher dosage of LiCl may be needed to inhibit the progression of wasting in this model.

LiCl was effective at preserving muscle mass and function in tumor bearing mice. However, gonadal adipose was unaffected by LiCl treatment. GSK-3 β activity is known to increase in animal models with greater adiposity⁵⁴. In addition, repressing Wnt signaling has been shown to enhance adipogenesis⁵⁵. Therefore, LiCl-mediated GSK-3 β inhibition and increased Wnt signaling may explain the lack of effect on adipose tissue mass. It should also be noted that skeletal muscle wasting rather than adipose tissue wasting is used in the formal definition of cancer cachexia⁵⁶. Thus, treatments for cancer cachexia should ideally target skeletal muscle mass rather than adipose tissue.

It was previously reported that lithium carbonate (Li₂CO₃) therapy was ineffective at treating cancer cachexia⁵⁷. These different results compared to the current study could be explained by the models used to assess cancer cachexia. The Li₂CO₃ study utilized a model of hepatoma. However, unlike colon carcinoma, hepatoma is not commonly associated with cancer cachexia⁵⁶. In addition, there was no *in vitro* demonstration of myotube wasting after treatment with hepatoma CCM or its prevention by Li₂CO₃.

Lithium has been previously investigated as a treatment for sepsis-induced myotube wasting⁵⁸. It was concluded that LiCl may be partially effective at preventing sepsis-induced muscle wasting. However, this previous study was carried out in a different animal model (Sprague-Dawley rats) and only reported *ex vivo* data, in which dissected muscles from septic animals were incubated with LiCl-supplemented buffer. Our results build upon this report by demonstrating that direct treatment with LiCl can prevent sepsis-induced muscle wasting *in vivo*.

Lithium has been used as a neuroprotective agent in patients with cancer⁵⁹. Protective effects of lithium on motor neurons may contribute to the reduced muscle wasting observed in the current study. Our *in vitro* data indicated that LiCl also has a direct effect on preventing muscle wasting, because treated myotubes showed reduced wasting and lower expression of atrogin-1 in the presence of CCM.

The *in vitro* and *in vivo* experiments in this study utilized the CT26 colon carcinoma cell line, because cancer cachexia is commonly associated with this tumor type⁶⁰. Cancer cachexia is also a common complication of lung, breast and pancreatic carcinoma⁶¹. As a future study, it may be interesting to test the effectiveness of LiCl to treat cachexia in models of these tumor types.

It is interesting to note that the effect of LiCl was rapid in this study (96 h treatment for the sepsis model). In contrast, lithium takes longer to become effective in bipolar patients, usually around 6–8 weeks¹¹.

Loading [MathJax]/jax/output/CommonHTML/jax.js with kidney toxicity¹¹. However, subsequent research has

indicated the need to re-evaluate the effect of lithium on kidney function, due to reports of protective outcomes in models of acute and chronic kidney disease⁶². Although Wnt pathway activation is associated with cancer progression, it has been shown that long term lithium therapy does not increase cancer risk⁶³. Moreover, the potentially beneficial effects of lithium therapy was revealed in analyses showing enhanced life expectancy in humans and metazoans⁶⁴. These reports support the potential development of lithium therapy for patients with muscle wasting.

In summary, the results presented herein show that LiCl treatment is effective in two models of skeletal muscle wasting: cancer cachexia produced by tumor-derived factors, and endotoxin-induced muscle weakness resulting from sepsis. A two-fold higher dose was required for effectiveness in the cancer cachexia model compared to the sepsis model. Further studies could assess the potential of LiCl treatment in other types of skeletal muscle wasting, such as aging-related sarcopenia, sarcopenic obesity and immobilization-induced muscle wasting.

Declarations

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Author contributions

J-H Lee designed experiments and carried out the cell-based and animal model experiments, S-W K, H-J Kim and J U contributed to the animal model experiments, J-H Kim carried out cell-based experiments, D-W J and D-R W designed experiments and wrote/edited the manuscript.

Conflict of interest

The authors declare no conflict of interest in relation to this study.

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Figures

Figure 1

Figure 1

LiCl ameliorates the inhibition of myoblast differentiation by CT26 colon carcinoma cell conditioned media (CCM). (A) qPCR analysis of the myotube marker, myosin heavy chain 2 (Myh2) and myoblast marker paired box protein Pax-7 (n=4). (B) Representative western blot image for myosin heavy chain (MyHC) after 72 h treatment in differentiation media (n=4). (C) Cell proliferation assay for C2C12 myoblasts treated for 48 h with the indicated concentrations of LiCl (n=5). (D) C2C12 myoblast proliferation after 48 h culture with the following media: 1) Growth media (GM), 2) CCM, 3) CCM and 5 mM LiCl (n=3). (E) qPCR analysis of the myotube marker, Myh2 and the myoblast marker Pax-7, after 24 h with the following media: 1) GM, 2) Differentiation media (DM), 3) CCM, 4) CCM and 5 mM LiCl (n=5). (F) Representative western blot for myosin heavy chain (MyHC) after 72 h culture with 1) DM, 2) CCM, 3) CCM and 5 mM LiCl (n=3). Significance difference compared to DM was marked with * (*=p-value<0.05, **=p-value<0.01, ***=p-value<0.001). # represents statistical significance compared to the CCM group (value<0.001).

Figure 2

Figure 2

LiCl prevents CCM-mediated wasting in C2C12 myotubes. (A) Immunofluorescence staining for MyHC after 72 h culture as follows: 1) DM, 2) CCM, 3) CCM and 5 mM LiCl. DAPI staining was used to visualize cell nuclei. Scale bar=100 μm (n=4). (B) Quantification of relative myotube diameter (n=4). (C) Frequency distribution of individual myotube diameters. (D) qPCR of Pax-7 and Atrogin-1 expression in myotubes experiment, 2) DM for 24 h, 3) CCM for 24 h, 3) CCM and 5

mM LiCl for 24 h (n=3). (E) Western blot for MyHC and GAPDH in C2C12 myotubes after 72 h culture as follows: 1) DM, 2) CCM, 3) CCM and 5 mM LiCl (n=4). Lower panel shows western blot for β -catenin and GAPDH in C2C12 myotubes after 24 h culture as follows: 1) DM, 2) CCM, 3) CCM and 5 mM LiCl (n=3). (F) Quantification results of western blots for MyHC (n=4) and β -Catenin (n=3). Significance compared to DM treatment was marked with * (*=p-value<0.05, **=p-value<0.01, ***=p-value<0.001). Significance compared to CCM treatment was marked with # (#=p-value<0.05) ##=p-value<0.01, ###=p-value<0.001).

Figure 3

The lithium mimetic and IMPase inhibitor, ebselen, does not attenuate CCM-induced wasting in C2C12 myotubes. (A) Immunofluorescence staining for MyHC after 72 h culture as follows: 1) DM, 2) CCM, 3) CCM and 5 mM LiCl, 4) CCM and 10 μ M ebselen. DAPI staining was used to visualize cell nuclei. Scale bar=100 μ m. (n=3). (B) Quantification of relative myotube diameter. (n=3). (C) qPCR analysis for Atrogin-1 after 24 h treatment (n=3). (D) qPCR analysis for myotube marker, Myh2, and myoblast marker, Pax-7, after 24 treatment (n=3). Significance compared to DM was marked with * (*=p-value<0.05, **=p-value<0.01, ***=p-value<0.001). Significance compared to CCM treatment was marked with # (#=p-value<0.05, ##=p-value<0.01, ###=p-value<0.001).

Figure 4

Figure 4

LiCl treatment blocks the LPS-induced upregulation of inflammatory cytokines from RAW264.7 macrophages. (A) qPCR analysis of IL-1 β , iNOS, and IL-6 after treatment with 5 mM LiCl treatment for 24 h, followed by 5 mM LiCl and 200 ng/mL LPS for an additional 24 h. Significance compared to no treatment was marked with * (***=p-value<0.001). Significance compared to LPS treatment was marked with # (###=p-value<0.001).

Figure 5

Figure 5

Therapeutic efficacy of LiCl for skeletal muscle wasting in a model of sepsis. Mice were treated as follows: 1) no treatment, 2) saline for 96 h followed by 1 mg/kg LPS, and 3) 40 mg/kg LiCl for 96 h followed by 1 mg/kg LPS. (A) Relative body weight change after 18 h (n=7). (B) Quadriceps weight/body weight (mg/BW) ratio (n=6). (C) Representative H&E stained images of the quadriceps (scale bar=100 μ m). (D) Cross sectional area of the quadriceps (n=5). (E) Frequency distribution of the

cross sectional area in the quadriceps (n=5). (F) qPCR analysis of inos, Atrogin-1 and Murf-1 in the quadriceps (n=5). Significant difference compared to untreated was marked with * (*=p-value<0.05, **=p-value<0.01, ***=p-value<0.001). Significant difference compared to saline plus LPS (control) was marked with \$ (\$=p-value<0.05,

$$= p - value < 0.01,$$

\$=p-value<0.001).

Figure 6

LiCl ameliorates muscle wasting in a mouse model of cancer cachexia. Mice were treated as follows: 1) no treatment, 2) transplantation with CT26 colon carcinoma cells, 3) transplantation with CT26 cells and treatment with 40 mg/kg LiCl, 4) transplantation with CT26 cells and treatment with 80 mg/kg LiCl. (A) Body weight change (n=5). (B) Time dependent growth of tumor volume (n=5). (C) Dissected tumor mass. (D) Tumor free body weight. (E) Quadriceps and tibialis anterior muscle weight /body weight (mg/BW) ratio. (F) Gonadal adipose tissue mass. For (C-F) n=5 at 28 d post-transplantation. (G) Grip strength at 21d post-transplantation (n=5). (H) Representative H&E stained images of the quadriceps (scale bar=100 μm). (I) Average fiber cross sectional area in the quadriceps (n=4). (J) Frequency distribution of the cross sectional area in the quadriceps (n=4). Significant difference compared to saline was marked with * (*=p-value<0.05, **=p-value<0.01, ***=p-value<0.001). Significant difference compared to CT26 control was marked with # (#=p-value<0.05, ##=p-value<0.01, ###=p-value<0.001).

Figure 7

Figure 7

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LiCl inhibited the upregulation of cachexia related genes in tumor bearing mice. qPCR analysis of Atrogin-1, Murf-1 and Il-6 in quadriceps muscle (n=4). 10 days after tumor inoculation, 40 mg/kg and 80 mg/kg of LiCl were administrated for additional 3 weeks and obtained quadriceps. Significant difference compared to saline was marked with * (*=p-value<0.05, **=p-value<0.01, ***=p-value<0.001). Significant difference compared to CT26 control was marked with # (#=p-value<0.05, ##=p-value<0.01, ###=p-value<0.001).

Supplementary Files

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