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A Combination of Lithium and Glycyrrhizic Acid has Anti-aging Effects *via* Increase of Intracellular pH. Hypothesis of Cytosol Acidification as One of the Driving Forces of Aging

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Abstract

Optimal intracellular pH (pHi) provides a necessary environment for the activity of key biochemical processes, including glycolysis, mitochondrial function, and genomic function; it also affects membrane potential, cell growth, and polymerization of the cytoskeleton. The aging process is accompanied by gradual acidification of pHi, affecting these and numerous other intercellular processes. In this study, we investigate the potential role of a combined treatment of lithium and glycyrrhizic acid (CRX) to counteract pHi acidification. We observe concomitant reversal of many of the generally accepted physiological attributes of the aging process. In cell culture, treatment with the combination of lithium and glycyrrhizic acid caused decreased expression of the senescence marker beta-galactosidase, as well as altered expression of other genes associated with aging and enhanced mitochondrial respiration. Our results provide preliminary data supporting the hypothesis that increasing pHi may reverse some of the markers of age-related physiological processes.

Keywords: Aging; Intracellular pH; Lithium; Cytosol

Introduction

Intracellular pH (pHi) affects nearly every aspect of cell function. Proteins depend on pHi to maintain their structure and function, and protonation–deprotonation events dictate the charge of biological surfaces and are an integral part of most metabolic reactions [1]. Endocytic and secretory pathways in intracellular organelles require an acidic environment [2]. Multiple metabolic reactions lead to proton production, which results from CO2 production, glycolysis, formation of creatinine phosphate, ATP and triglyceride hydrolysis, lipolysis, and the generation of superoxide and hexose monophosphate [3]. Passive influx of H+ across the cell membrane and its concomitant leakage from acidic internal compartments contribute to cytoplasmic acidification [4,5].

Cells have many biochemical pathways to expel these acid by-products into the extracellular space and keep intracellular pH near neutral, which is optimal for most cells to maintain their proper biological functions [1,6]. Most cells expel metabolic waste products by coordinated actions of transporters and exchangers for protons, sodium, potassium, chloride and bicarbonate, with the sodium/proton exchanger having a central role in intracellular alkalization. Intracellular pH is therefore stringently regulated, though it still varies greatly among the different organelles [7]. The predominant pH-regulatory transporters responsible for alkalization are the plasma membrane Na+ –H+ exchangers (NHEs) and Na+–HCO3– co-transporters (NBCs). The alkalinizing mechanisms of these transporters are counterbalanced by the actions of plasma membrane Cl-–HCO3- exchangers, or Anion Exchangers (AEs), which acidify the cell. These dynamic and sustained mechanisms are required to ensure long-term pH homeostasis, and different mechanisms regulate pH in individual cellular compartments [8].

Published data shows that older cells and tissues have slightly more acidic baseline pHi than their younger counterparts [9,10]. In addition, older cells experience slower recovery from acid load compared to younger cells [11]. These observations point to diminished capacity of older cells to effectively remove the acidic by-products of metabolism from their intracellular domain. Given the significant influence of pHi on multiple biochemical processes necessary for cell survival, the control and shift of pHi to create a more alkaline environment may provide for an environment leading to a reversal of many of the markers of aging and ultimately the aging process itself.

In addition to, and perhaps in tandem with changes in pHi, aging is characterized by changes in gene expression and mitochondrial respiration. A large-scale, meta-analysis of gene expression profiles in mice, rats, and humans identified at least 56 genes that are upregulated with age and 17 genes that are downregulated [12]. Genes upregulated with age were often associated with inflammation and immune responses, while genes downregulated with age were often associated with tissue structure (e.g., collagen) and energy metabolism.

One of the most well-known biomarkers of aging is beta-galactosidase, as its expression increases in many human cell cultures as they become senescent [13]. Staining for beta-galactosidase has become a routine procedure to measure cellular senescence and aging [14]. Other reports have found that decreased intracellular pH is associated with senescence and that senescent cells exhibit slower recovery from pHi changes compared to younger cells [15,16].

As these changes in gene expression occur with aging, mitochondrial function declines. The respiratory capacity, or ability to consume oxygen, of mitochondria is known to decrease with age [17]. In turn, cells become less able to produce ATP by oxidative phosphorylation. These effects are due to decreased rates of electron transfer by proteins in the mitochondrial inner membrane. Mitochondrial proteins that experience decreased activity with age in mammalian tissues include NADH dehydrogenase, cytochrome oxidase, and mitochondrial nitric oxide synthase [18]. When mitochondrial function becomes severely impaired, reactive oxygen species build up inside both the mitochondrial matrix and cytosol, causing oxidative stress that can trigger apoptosis [19].

In this study we investigate the anti-aging effects of a combination of lithium and glycyrrhizic acid *via* its effects on inducing a shift in the intracellular pH towards a more alkaline environment that characterizes younger cells.

Published data show that the Na+-H+ exchanger, which regulates pHi by mediating extrusion of H+ and entry of Na+ into cells, has affinity for lithium [20,21]. This affinity has been directly measured in human blood platelets, where treatment with Li+ was able to raise the pHi of pre-acidified cells [22]. This raise in pHi may be a result of increased activation, as well as expression of the Na+-H+ exchanger in cells treated with Li+ [23,24]. Intriguingly, lithium can also reduce apoptosis. Glycyrrhizic also impacts pHi, though indirectly. This compound inhibits the enzyme 11 beta-hydroxysteroid dehydrogenase and leads to increased cortisol production. Both cortisol and aldosterone bind the Mineralocorticoid Receptor (MR), and in doing so, can cause a rapid increase in pHi [25-27].

Materials and Methods

Cell culture

CCD 841 CoN normal human colon cells (CRL-1790; ATCC, Manassas, VA) were grown in EMEM complete medium (Cat. No. 30-2003; ATCC, Manassas, VA) in an incubator at 37°C and 5% CO2. Cells were passaged every 2-3 days.

For lithium and glycyrrhizic acid treatments, CCD 841 CoN cells were grown in EMEM complete medium and kept in an incubator at 37°C and 5% CO2 until 100% confluence was reached. The flasks were kept in the incubator for an additional 4 days. After the 4 days, the EMEM medium in each flask was changed to fresh medium with or without lithium carbonate (Cat. No. 62472; Sigma-Aldrich, St. Louis, MO) and or glycyrrhizic acid (Cat. No. G2137; Sigma-Aldrich, St. Louis, MO). The flasks were divided into 4 groups consisting of 3 flasks each. Each group was given one of the following treatments in the medium change: No lithium carbonate or glycyrrhizic acid (control), 0.15 µM lithium carbonate, 0.25 mM glycyrrhizic acid, or 0.15 µM lithium carbonate and 0.25 mM glycyrrhizic acid. All flasks were kept in the incubator at 37°C and 5% CO2 for additional 48 hours of treatment.

qPCR analysis

qPCR was conducted to measure the expression levels of genes associated with aging in cell cultures. For age-related gene expression analysis in cell culture CCD 841 CoN cells were treated with lithium carbonate and or glycyrrhizic acid as described above. After the treatment period, the cells were detached from flasks using trypsin and re-suspended in 5 mL of fresh media. This cell suspension was then divided into Eppendorf PCR-clean tubes. In each tube, 100 µL of cells was dissolved in 0.5 mL of TRIzol reagent (Cat. No. 15596026; Thermo Fisher Scientific, Waltham, MA). qPCR was performed for each sample to evaluate the gene expression profile of cells in the control and treatment groups [Table 1].

Gene		Sequence (5' - 3')	Length (bp)	Accession number
Dyskerin (DKC1)	For	TGCCGAAGCAGCAAAAACTG	20	NM_001363
	Rev	TGATCAACTGAGGAGCTGCTG	21	
DNA 36B4	For	CAGCAAGTGGGAAGGTGTAATCC	21	NG_009952
	Rev	CCCATTCTATCATCAACGGGTACAA	21	
Apolipoprotein D (APOD)	For	TTGAGAATGGACGCTGCATC	20	NM_001647
	Rev	TTCCATCAGCTCTCAACTCCTG	22	
Fc Fragment of IgG (FCGR2B)	For	AGGCCAACAACAATGACAGC	20	NM_201563
	Rev	AAGCACAGTCAGATGCACAG	20	
Complement component 3 (C3)	For	ACAAAACTGTGGCTGTTCGC	20	NM_000064
	Rev	TGGGATGTCCTCTTTCTGCAC	21	
Clusterin (CLU)	For	TGAACTTCCACGCCATGTTC	20	NM_001831
	Rev	TCATCGTCGCCTTCTCGTATG	21	

Transferrin receptor (TRFC)	For	CGTGAGGCTGGATCTCAAAAAG	22	NM_003234
	Rev	TTGATCACGCCAGACTTTGC	20	
Collagen type 3 (COL3A1)	For	CTGGTAAGAATGGTGCCAAAGG	22	NM_000090
	Rev	TTGCCATCTTCGCCTTTAGC	20	
ATP synthase (ATP5G3)	For	TTATGCCAGAAACCCTTCGC	20	NM_001689
	Rev	ACCCATAGCTTCAGACAAGGC	21	
NADH dehydrogenase (NDUFB11)	For	AACCCGAGGACGAAAACTTG	20	NM_019056
	Rev	ACAAGTCGCATGTTCCAGAC	20	

Table 1: Forward (For) and reverse (Rev) primers for each gene analyzed by qPCR in this study.

Beta-Galactosidase staining

CCD 841 CoN cells were cultured in flasks and treated with lithium carbonate and or glycyrrhizic acid as described above. After the treatment period, all flasks were stained using the senescence beta-galactosidase staining kit (Cat. No. 9860; Cell Signaling Technology, Beverly, MA) according to the manufacturer's protocol.

After staining, the flasks were examined with an Olympus BX51 light microscope under 100 x magnifications. Images were collected with an Olympus DP73 digital camera.

Mitochondrial activity measurements

Four 25 cm2 Nunclon delta surface flasks with EMEM complete medium were seeded with cultures of CCD 841 CoN cells and kept in an incubator at 37°C and 5% CO2 until 70 % confluence was reached.

Next, in 2 flasks (control group), the medium was replaced with fresh medium. For another 2 flasks (treatment group), the medium was replaced with fresh medium containing 0.15 μM Lithium carbonate and 0.25 mM glycyrrhizic acid. All flasks were kept in the incubator for an additional 12 hours. All flasks were then stained with mito tracker deep red FM (Cat. No. M22426, Thermo Fisher Scientific, Waltham, MA), which highlights active mitochondria, according to the manufacturer's protocol. Cells were counterstained with DAPI and analyzed with a fluorescence microscope.

Result

The combination of lithium and glycyrrhizic acid decreases expression of the senescence marker beta-galactosidase in human cell cultures

Cells treated with either lithium carbonate or glycyrrhizic acid alone stained positively for beta-galactosidase (Figure 1, B and C). The untreated control cells also stained positively for beta-galactosidase (Figure 1, D). However, cells treated with the combination of lithium carbonate and glycyrrhizic acid showed little to no positive staining for beta-galactosidase (Figure 1, A).

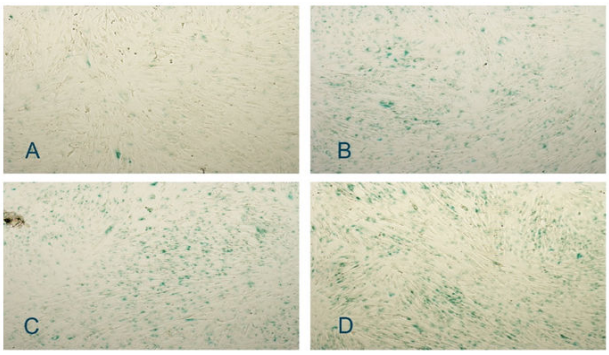

Figure 1: Representative images of samples treated with lithium alone (B), glycyrrhizic acid alone (C), lithium and glycyrrhizic acid (A), and neither lithium nor glycyrrhizic acid (D).

The combination of lithium and glycyrrhizic acid alters the expression of genes associated with aging in human cell cultures

Other than beta-galactosidase, a standard for measuring cellular aging, we next examined the effect of the lithium and glycyrrhizic acid, alone or in combination, alter the expression of other genes associated with aging. We chose to investigate 8 signature aging biomarkers. Four of these genes are known to be upregulated with age, and the other four are downregulated. These genes were previously identified by Magalhaes and colleagues [12].

Cells from were then collected and processed for qPCR analysis of 8 age-related genes: Apolipoprotein D (APOD), Fc Fragment of IgG (FCGR2B), Complement component 3 (C3), Clusterin (CLU), Transferrin receptor (TFRC), Collagen Type 3 (COL3A1), ATP synthase (ATP5G3), and NADH dehydrogenase (NDUFB11).

The combination treatment of lithium and glycyrrhizic acid significantly reduced expression of C3 and CLU, which are

consistently elevated with aging. The treatment also increased the expression of ATP5G3 and NDUFB11, which are consistently reduced with aging. Other genes did not show significantly altered expression. These results indicate that lithium and glycyrrhizic may cause decreased inflammation and cellular stress, as C3 is normally expressed to promote inflammation in response to immune challenges, and CLU is normally expressed to prevent apoptosis induced by cellular stress [28,29]. In addition, lithium and glycyrrhizic acid may enhance cellular respiration [30,31]. ATP5G3 is a component of the mitochondrial ATP synthase and catalyzes ATP production, and NDUFB11 is a component of the mitochondrial complex I, which transfers electrons from NADH to the respiratory chain [Figure 2].

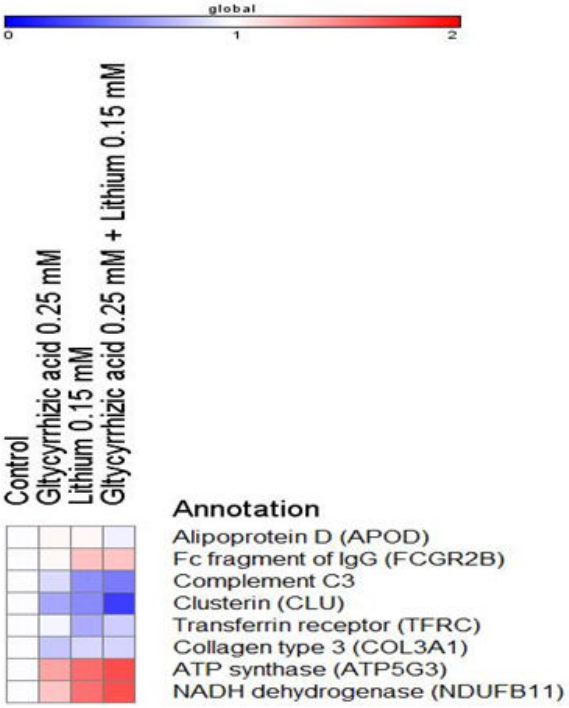

Figure 2: Changes in gene expression of aging biomarkers in human colon cells when treated with a combination of lithium and glycyrrhizic acid. Expression levels were normalized to control cells with no treatment. The heat scale represents relative mRNA expression values. Blue indicates lower gene expression and red indicates higher gene expression than the control.

The combination of lithium and glycyrrhizic acid increases mitochondrial respiration in human cell cultures

In addition to changes in gene expression, altered mitochondrial respiration is associated with the aging process. We thus tested if lithium and glycyrrhizic acid also impact mitochondrial activity in our normal human colon cell cultures.

We first treated aged cells (grown to 100% confluence and then kept in culture for an additional 4 days) with lithium and glycyrrhizic acid and then stained the cells with Mito Tracker Deep red, which labels active mitochondria. We observed that cells treated with lithium and glycyrrhizic acid had a noticeable increase in staining compared to cells that were left untreated [Figure 3].

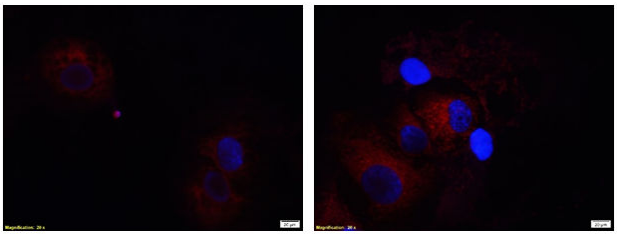

Figure 3: Mitochondrial activity in cells with and without lithium and glycyrrhizic acid treatment. Representative images of Mito Tracker staining of active mitochondria in human colon cells without (left) and treated with (right) the lithium and glycyrrhizic acid. Magnification = 20X. Scale bar = 20 µm.

Discussion

Based on observations that older cells have lower pHi and recover from acidic load at a slower rate than younger cells, we hypothesized that acidification of pHi may be an important factor in processes related to cell, tissue, and overall organism aging [9-11]. We thus sought to test if compounds known to moderately raise pHi would have anti-aging effects. The Na+-H+ exchanger, which regulates pHi by mediating extrusion of H+ and entry of Na+ into cells, has affinity for lithium, which can ultimately lead to increases in pHi [21-23]. Glycyrrhizic can also increase pHi by mimicking the actions of aldosterone, which, like Li+, activates the Na+-H+ exchanger [25].

Since pHi becomes more acidic as cells age, we hypothesized that a combination of these compounds could potentially prevent or reverse symptoms of aging when given to cells.In this study, we investigated how treatment with lithium and glycyrrhizic acid, alone and in combination, may affect cellular processes related to aging.

We found that a combination of lithium and glycyrrhizic acid reduces the expression of the senescence marker beta-galactosidase in aged cell cultures. Interestingly, treatment with either compound alone had little effect on beta-galactosidase expression levels. This result indicates that by increasing pHi with lithium and glycyrrhizic acid, cells were more resistant to senescence.

In addition to beta-galactosidase, we found that aged cells treated with lithium and glycyrrhizic acid showed significantly decreased expression of C3 and CLU, as well as significantly increased expression of ATP5G3 and NDUFB11. C3 is generally upregulated with age, as this gene triggers inflammatory responses to immune challenges [28]. CLU is also upregulated with age in order to prevent apoptosis in response to increased cellular stress that accompanies aging processes [29]. The combination of lithium and glycyrrhizic acid may therefore lead to reduction in inflammation signals associated with aging, thereby requiring lower expression of C3 and CLU. ATP5G3 and NDUFB11 are both involved in cellular respiration. The former catalyzes ATP production, while the latter catalyzes electron transfer [30,31]. Hence, the combination of lithium and glycyrrhizic acid may also lead to reduced or reversal in mitochondrial damage and thus enable respiratory processes that become limited with aging [32]. Other genes associated with aging, including APOD, GCGR2B, TFRC, and COL3A1, did not show significant changes in expression level with lithium and glycyrrhizic acid treatment. This suggests that the treatment alters specific pathways involved in aging rather than the entire process.

Based on the observation of altered expression of mitochondria-associated genes, we sought to test if lithium and glycyrrhizic acid affect the cellular respiration rate, which is known to decline with aging [32]. We observed that aged cells treated with lithium and glycyrrhizic acid had increased levels of active mitochondria, as detected by staining with Mito Tracker.

While we have not directly investigated the biological mechanisms behind the anti-aging effects of lithium and glycyrrhizic acid observed in this study, we hypothesized that these effects stem from an increase in pHi induced by these compounds. Because aging is associated with a decrease in pHi, as well as decreased ability to regulated pHi, our hypothesis was that raising the pHi of cells may reverse or inhibit some aging-related processes in concert. In other words, we seek to change the environment in which proteins operate on the cellular level and thus affect many of the underlying processes characterizing aging instead of focusing on a unique molecular target of the overall aging process.

Conclusion

Hypothesis of cytosol acidification as one of the driving forces of aging is a novel approach of explanation of what is the biological mechanism of aging process. Further studies are warranted to find optimal way of manipulations of intracellular pH to maximize rejuvenation effects.

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