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AMPK activation by Licochalcone D and Camphorquinone modulates the cellular senescence and energy metabolism

Graduate School of Chosun University

Department of Integrative Biological Science

Nagarajan Maharajan

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Licochalcone D 와 Camphorquinone 에 의한 AMPK 활성화에 따른

세포 노화 및 에너지 대사 조절에 관한 연구

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지도교수 조광원

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글로벌바이오융합학과

Nagarajan Maharajan

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Nagarajan Maharajan

Approved:

Committee chair _____
Prof. Taeck Joong Jeon
Chosun University

Committee _____
Prof. Jun Sik Lee
Chosun University

Committee _____
Prof. Jeong Kyu Bang
University of Science & Technology (UST)

Committee _____
Prof. A. K. Munirajan
University of Madras

Committee _____
Prof. Gwang Won Cho
Chosun University

2022 년 1 월

Chosun University Graduate School

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ABSTRACT*

AMPK activation by Licochalcone D and Camphorquinone modulates the cellular senescence and energy metabolism

Nagarajan Maharajan

Advisor: Prof. Gwang-Won Cho, Ph.D.

Department of Integrative Biological Science

Graduate school of Chosun University

Aging is characterized by a progressive loss of tissue homeostasis and physiological integrity, increasing the likelihood of disease development and death. Chronic non-communicable diseases such as cancer, neurological diseases, cardiovascular diseases, articular injury, and metabolic diseases are all associated with aging. Accumulation of senescent cells with age is one of the important hallmarks of aging and aging-related diseases. In this study, I have focused on the beneficial effects of Licochalcone D (Lico D) and Camphorquinone (CQ) on cellular senescence and common aging-related diseases such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D).

In part one, I investigated the effects of Lico D and CQ on oxidative stress-induced senescence, *in vitro* and *in vivo*. Increased oxidative stress is a crucial factor for the progression of cellular senescence and aging. Hydrogen peroxide (H_2O_2) (200 μM for double-time) and D-galactose (D-Gal) (150 mg/kg) were used to induce oxidative stress

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in human bone marrow-mesenchymal stem cells (hBM-MSCs) and mouse, respectively. To study the mechanism of cellular senescence, I performed the SA-β-gal assay, evaluated the senescence markers, activation of AMP-activated protein kinase (AMPK) and autophagy. First, I used Lico D to evaluate its effect on the senescence model. I found that Lico D potentially reduced the oxidative stress-induced senescence by upregulating AMPK-mediated activation of autophagy in hBM-MSCs. D-Gal treatment significantly increased the expression level of senescence markers, such as p53 and p21 in the heart and hippocampal tissues, while this effect was reversed in the Lico D treated animals. Furthermore, a significant increase in AMPK activation was observed in both tissues, while the activation of autophagy was only observed in the heart tissue. Interestingly, I found that Lico D significantly reduced the expression level of receptors for advanced glycation end products (RAGE) in the hippocampal tissue. Collectively, the findings highlight the antioxidant, anti-senescent, and cardioprotective effects of Lico D and suggest that the activation of AMPK and autophagy could ameliorate the oxidative stress-induced senescence. Second, I used CQ to evaluate its role on the oxidative stress-induced senescence model. I found that CQ potentially reduced the senescence in hBM-MSCs and mouse heart tissue. In addition to that, CQ boosted AMPK/SIRT1 activation as well as autophagy in both *in vitro* and *in vivo*. The results were subsequently verified by using compound C (an AMPK inhibitor) in hBM-MSCs. Furthermore, CQ treatment decreased mRNA level of inflammation markers such as *Il-1α, Il-1β, Il-6*, and *Rage* in D-Gal induced aging mice heart tissue. Collectively, the findings show that CQ possesses anti-senescence and cardioprotective properties, implying that oxidative stress-induced senescence could be suppressed by AMPK/SIRT1 and autophagy mechanisms.

In part two, I investigated the effect of Lico D and CQ in a high-fat diet (HFD)/streptozotocin (STZ)-induced hyperglycemic mouse model and a free fatty acid-induced hepatosteatosis cell model. NAFLD and T2D are two pathological conditions that are known to frequently coexist and act synergistically to increase the risk of simple steatosis to hepatocellular carcinoma, cardiovascular and kidney diseases. In HFD/STZ mice model, either Lico D (1 mg/kg/day) or CQ (10 or 30 mg/kg/day) was injected intraperitoneally (i.p.) for 3 weeks after the onset of T2D and fasting blood glucose (FBG),

glucose tolerance, and liver lipid metabolism were assessed. The administration of Lico D and CQ to HFD/STZ mice reduced body and liver weight gain and improved glucose tolerance. Lico D and CQ also significantly reduced the gene expression level of lipid biosynthesis and inflammation markers and increased the fatty acid oxidation markers in liver and HepG2 cells. Additionally, Lico D or CQ treatment increased the activation of AMPK/SIRT1 pathway *in vitro* and *in vivo*.

Overall, the findings imply that Lico D and CQ can reduce the oxidative stress-induced senescence, regulate blood glucose level and improve the lipid metabolism. The stimulation of AMPK/SIRT1 pathway could be responsible for all of these favorable effects. However, AMPK/SIRT1 pathway can be activated mainly through the upstream kinases such as liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) or alteration in the metabolic sensors such as AMP and NAD^+. Therefore, it is crucial to determine the upstream activator of AMPK/SIRT1 pathway upon Lico D or CQ treatment.

초록*

Licochalcone D 와 Camphorquinone 에 의한 AMPK 활성화에 따른 세포 노화 및 에너지 대사 조절에 관한 연구

마하라잔 나가라잔

지도교수: 조광원

글로벌바이오융합학과

조선대학교 대학원

노화는 조직 항상성과 생리학적 완전성의 점진적인 상실을 특징으로 하며, 질병 발병 및 사망의 가능성을 증가시킨다. 암, 신경계 질환, 심혈관 질환, 관절 손상, 대사 질환과 같은 만성 비전염성 질환은 모두 노화와 관련이 있다. 노화에 따른 노화 세포의 축적은 노화 및 노화 관련 질병의 중요한 특징 중 하나이다. 본 연구에서는 세포노화 및 비알코올성 지방간질환 (NAFLD) 및 제 2 형 당뇨병 (T2D)과 같은 일반적인 노화 관련 질병에 대한 Licochalcone D (Lico D) 및

* A thesis submitted to the committee of Graduate School, Chosun University in partial fulfillment of the requirements for the degree on Doctor in Philosophy conferred in December 2021.

Camphorquinone (CQ)의 유익한 효과에 초점을 맞추었다. 증가된 산화 스트레스는 세포 노화 및 노화의 진행에 중요한 요소이다.

제 1 부에서는 생체 외 및 생체 내 산화 스트레스 유발 노화에 대한 Lico D 와 CQ 의 효과를 조사했다. 과산화수소 (H_2O_2) 및 D-갈락토오스 (D-Gal)를 사용하여 인간 골수 중간엽 줄기 세포 (hBM-MSC) 및 마우스에서 산화 스트레스를 유도했다. 세포 노화의 기전을 연구하기 위해 SA-β-gal assay 를 수행하여 노화 마커, AMP-activated protein kinase (AMPK) 활성화 및 자가포식을 평가했다. 먼저 Lico D 를 사용하여 노화 모델에 미치는 영향을 평가했다. Lico D 가 hBM-MSC 에서 자가포식의 AMPK 매개 활성화를 상향 조절함으로써 산화 스트레스로 유발된 노화를 잠재적으로 감소시킨다는 것을 발견했다. D-Gal 처리는 심장 및 해마 조직에서 p53 및 p21 과 같은 노화 표지자의 발현 수준을 유의하게 증가시켰지만 이 효과는 Lico D 처리 동물에서 역전됐다. 또한, AMPK 활성화의 상당한 증가가 두 조직 모두에서 관찰된 반면, 자가포식의 활성화는 심장 조직에서만 관찰되었다. 흥미롭게도 Lico D 가 해마 조직에서 최종 당화 생성물 (RAGE)에 대한 수용체의 발현 수준을 유의하게 감소시킨다는 것을 발견했다. 종합적으로, 연구 결과는 Lico D 의 항산화, 항노화 및 심장 보호 효과를 강조하고 AMPK 및 자가포식의 활성화가 산화 스트레스로 유발된 노화를 개선할 수 있음을 시사한다. 다음으로 CQ 를 사용하여 산화 스트레스로 유발된 노화 모델에 대한 CQ 의 역할을 평가했다. 나는 CQ 가 hBM-MSC 와 마우스 심장 조직의 노화를 잠재적으로 감소시킨다는 것을 발견했다. 그 외에도 CQ 는 시험관 내 및 생체 내

모두에서 AMPK/SIRT1 활성화와 자가포식을 촉진했다. 결과는 hBM-MSC 에서 Compound C (AMPK 억제제)를 사용하여 후속적으로 확인되었다. 또한, CQ 처리는 D-Gal 유도 노화 마우스 심장 조직에서 Il-1α, Il-1β, Il-6 및 Rage 와 같은 염증 마커의 mRNA 수준을 감소시켰다. 종합적으로, 이 발견은 CQ 가 노화 방지 및 심장 보호 특성을 가지고 있음을 보여주며, 이는 산화 스트레스로 유발된 노화가 AMPK/SIRT1 및 자가포식 기전에 의해 억제될 수 있음을 암시한다.

제 2 부에서는 저용량 스트렙토조토신 (streptozotocin, STZ)과 고지방식이 (HFD)를 함께 사용하여 유도된 고혈당 마우스 모델과 유리지방산 유도 간지방증 세포모델을 사용하여 노화 관련 질병에서 Lico D 와 CQ 의 효과를 조사했다. NAFLD 및 T2D 는 간세포 암종, 심혈관 및 신장 질환에 대한 단순 지방증의 위험을 증가시키기 위해 빈번하게 공존하고 상승작용하는 것으로 알려진 두 가지 병리학적 상태이다. T2D 발병 후 3 주 동안 Lico D (1 mg/kg/day) 또는 CQ (10 또는 30 mg/kg/day)를 복강 내 주사하고 공복 혈당 (FBG), 내당능, 및 간 지질 대사를 평가했다. HFD/STZ 마우스에 Lico D 와 CQ 를 투여하면 신체 및 간의 체중 증가가 감소하고 내당능을 개선했다. Lico D 와 CQ 는 또한 간 및 HepG2 세포에서 지질 생합성 및 염증 마커의 유전자 발현 수준을 유의하게 감소시켰고 지방산 산화 마커를 증가시켰다. 또한 Lico D 또는 CQ 처리는 시험관 내 및 생체 내에서 AMPK/SIRT1 경로의 활성화를 증가시켰다.

결론적으로 연구 결과는 Lico D 와 CQ 가 산화 스트레스로 인한 노화를 줄이고 혈당 수준을 조절하며 지질 대사를 개선할 수 있음을 의미한다.

AMPK/SIRT1 경로의 자극은 이러한 모든 유리한 효과의 원인이 될 수 있다. 그러나 AMPK/SIRT1 경로는 주로 간 키나제 B1 (LKB1) 및 칼슘/칼모듈린 의존성 단백질 키나제 2 (CaMKK2)와 같은 업스트림 키나제와 AMP 및 NAD+와 같은 대사 센서의 변경을 통해 활성화될 수 있다. 따라서 Lico D 또는 CQ 처리 시 AMPK/SIRT1 경로의 업스트림 활성제를 결정하는 것이 중요하다.

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ABBREVIATIONS

hBM-MSCs	Human bone marrow mesenchymal stem cells
HepG2	Hepatoma-derived cell line
HSCs	Hematopoietic stem cells
IGF-1	Insulin-like growth factor-1
IIS	Insulin and IGF-1 signaling
ISCs	Intestinal stem cells
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor-kappa B
NAFLD	Non-alcoholic fatty liver disease
T2D	Type 2 Diabetes
OXPHOS	Oxidative phosphorylation
PGC1	Peroxisome proliferator-activated receptor gamma coactivator-1α
ROS	Reactive oxygen species
SIRT1	Sirtuin 1
AMPK	AMP-activated protein kinase
HFD	High fat diet
STZ	Streptozotocin
FFAs	Free fatty acids
SA-β-gal	Senescence-Associated β-Galactosidase
GTT	Glucose tolerance test

I. INTRODUCTION

1. Aging

Aging is characterized by a constant deterioration of tissue homeostasis and loss of physiological integrity of tissues after reaching sexual maturity, which lead to increase the risk for disease development and death (Barzilai et al., 2012; Lopez-Otin et al., 2013; Sen et al., 2016). Increased age is one of the strongest risk factor for the development of chronic non-communicable diseases including cancer, neurological diseases, cardiovascular diseases, articular damages and metabolic diseases (Barbé-Tuana et al., 2020). The rate of aging is inversely proportional to the average lifespan of most organisms and the term healthspan refers to how long an organism may go without developing a chronic disease (Sen et al., 2016). Biogerontology study is mainly focused on finding ways to extend lifespan and healthspan. Various animal models have been used to uncovering key pathways related to aging process. Classic model animals such as laboratory mouse (*Mus musculus*), rat (*Rattus norvegicus domestica*), roundworm (*Caenorhabditis elegans*), and common fruit fly (*Drosophila melanogaster*) have been used to investigate aging and aging-related disorders (Holtze et al., 2021).

Due to the complexity of the aging process, it is still unclear how organism's age and what factors can speed up the aging process. López-Otín et al., reported nine potential hallmarks including i. genomic instability, ii. telomere attrition, iii. epigenetic alterations, iv. loss of proteostasis, v. deregulated nutrient sensing, vi. mitochondrial dysfunction, vii. cellular senescence, viii. stem cell exhaustion, and ix. altered intercellular communication that are widely regarded to contribute to the aging process and together determine the aging phenotype in order to investigate the mechanism of aging (López-Otín et al., 2013). Therefore, researchers classified the hallmarks of aging using the following criteria: 1. It must appear as part of the natural aging process; 2. Its experimental exacerbation must hasten the aging process; and 3. Its experimental amelioration must slow the aging process and extend healthy lifespan.

1.1. Hallmarks of aging

Each of the hallmarks plays an essential role in the aging processes. Briefly, i. Genomic instability - the accumulation of DNA damages throughout the life is one of the common dominators of aging and premature aging diseases such as Werner syndrome

and Bloom syndrome (Burtner and Kennedy, 2010; Moskalev et al., 2013). Both endogenous (DNA replication error and ROS) and exogenous (physical, chemical, and biological) factors can stimuate excessive DNA damages or insufficient DNA repair mechanism, favouring the aging process (Hoeijmakers, 2009). ii. Telomere shortening or telomere exhausion - can be explained by the process of replicative senescence or Hayflick limit, which is limited proliferative capacity of some types of *in vitro* cultured cells (Hayflick and Moorhead, 1961; Olovnikov, 1996). Pathological telomere dysfunction promotes aging in mice and humans, whereas genetic activation of telomerase, (an enzyme that can maintain the length of telomere) can reverse the premature aging in telomerase-deficient mice (Jaskelioff et al., 2011). iii. Epigenetic modification - including DNA methylation, histone methylation or acetylation, chromatin remodeling as well as trascriptional alteration can affect all cells and tissues throughout life, contributing to aging (Lopez-Otin et al., 2013). iv. Loss of proteostasis – proteostais is a quality control mechanism that preserves the protein's stability and functionality. Exogenous and endogenous stresses that cause the accumulation of unfolded or misfolded proteins, leading to the impairment in protein hemostasis. Consequently, many investigations have revealed that proteostasis can be changed with age (Koga et al., 2011). v. Deregulated nutrient sensing or metabolic alterations - the evolutionary conserved aging control pathway such as growth factor (GF) and insulin and insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway and its downstream targets such as AKT, mTOR and FOXO have also been linked to longevity in both human and animal models (Barzilai et al., 2012; Fontana et al., 2010; Kenyon, 2010). Alterations in other nutrient sensing (particularly glucose sensing) pathway such as mTOR, AMPK and sirtuin, contribute to the progression of aging. vi. Mitochondrial dysfunction - the respiratory chain's efficiency deteriorates as cells and organisms age, resulting in increased electron leakage and decreased ATP production. Apart from this, mitochodrial DNA mutations, reduced mitochodrial biogenesis, electron chain destabilization, alterations in mitochondrial dynamics and defects in mitophagy also affect the mitochondrial function and contribute to aging (Green et al., 2011; Kujoth et al., 2005; Trifunovic et al., 2004; Vermulst et al., 2008). vii. Cellular senescence is defined as a cell cycle arrest that is accompanied by predictable phenotypic alterations. (Campisi and d'Adda di Fagagna, 2007; Collado et al.,

2007; Kuilman et al., 2010). Cellular senescence protects against cancer and contributes to tissue homeostasis in young organisms by preventing the growth of damaged cells. The accumulation or deficiency in the clearence of these senescent cells accelerates the aging process in old organisms. viii. Stem cell exhausion - stem cells are speciallized cells that can able to regulate the tissue homeostasis by regenerating or replacing damaged cells. The loss of stem cells including HSCs, MSCs, satellite cells, intestinal epithelial stem cells contributes to the development of deleterious changes within the tissue and development of diseases such as anemia and osteoporesis (Lopez-Otin et al., 2013). ix. Altered intracellular communications - neuroendocrine dysfunction (imbalance in hormone production linked to the human brain), inflammaging (a proinflammatory phenotype during aging), immunosenescence (a process of immune dysfunction that occurs with age) and bystander effect or contagious aging (senescent cells induce senescence in neighboring cells) are also involved in the aging process (Lopez-Otin et al., 2013). Therefore, all of these indicators must be considered in the context of aging process.

1.2. Stem cell aging

Stem cell aging is defined as a progressive decline of overall stem cell properties including self-renewal, differentiation, and regenerative capability. Conserved cellular processes such as intrinsic (DNA damages, accumulation of reactive oxygen species (ROS), alteration of protein homeostasis, mitochondrial dysfunction, and epigenetic remodeling) and extrinsic (alteration in the stem cell niche, modulation of local and systemic factors, and depletion of stem cells) changes severely affect the functions of stem and progenitor cells as well as lifespan of organisms (Maharajan et al., 2020; J. Oh et al., 2014) (Figure 1). However, alterations of both cell intrinsic and extrinsic changes may reduce the number of skeletal muscle stem cells, function, and regenerative capacity. These reductions increase the risk for the development of age-related diseases. Therefore, modulation of the stem cell niche could preserve or restore the youthful condition of stem cells and improve health during organismal aging (Sousa-Victor et al., 2015).

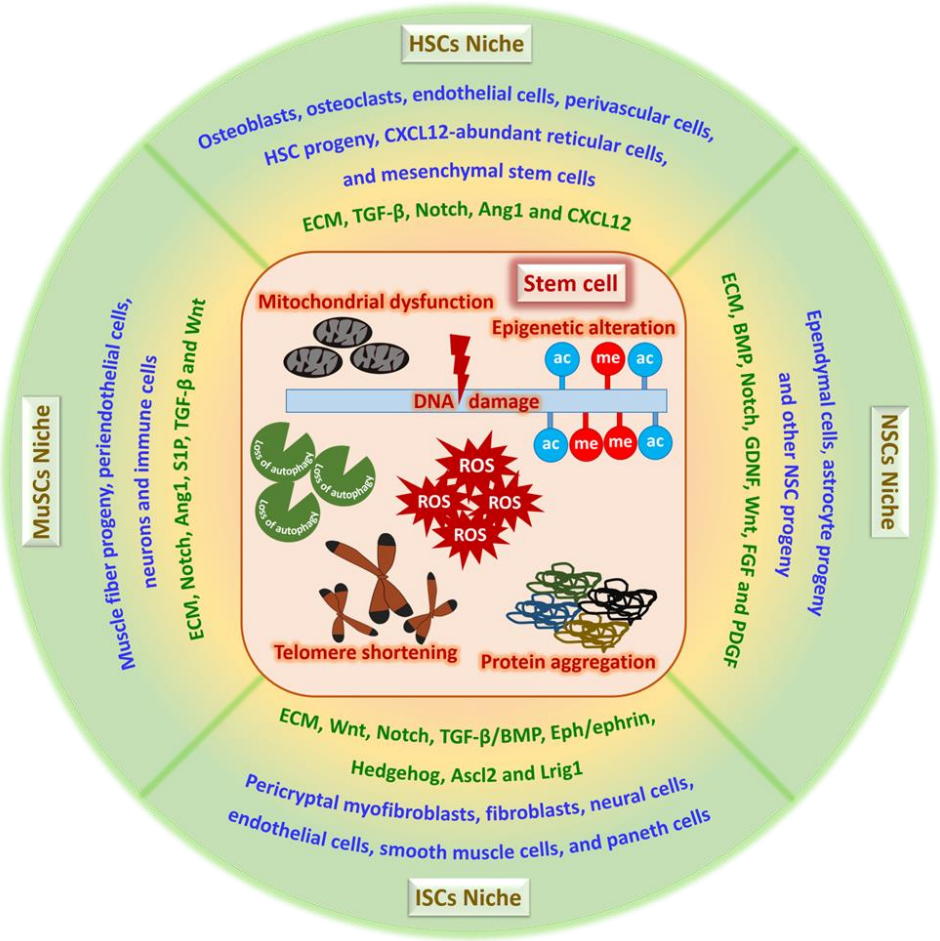

Figure 1. Major influences of stem cell aging. The stem cell niche is composed of both cellular (blue) and acellular (green) components. The alteration in the stem cell niche and/or intrinsic influences like DNA damage, epigenetic alteration, telomere shortening, mitochondrial dysfunction, loss of autophagy, protein aggregation, and increased ROS levels lead to loss of stem cell functions and stem cell aging (Maharajan et al., 2020).

2. Senescence

Aging phenotypes can be determined by a variety of cellular and molecular hallmarks, including cellular senescence, a state of permanent cell cycle arrest. (A. Hernandez-Segura et al., 2018; Lopez-Otin et al., 2013; Lozano-Torres et al., 2019). Leonard Hayflick and Paul Moorhead discovered that primary human cells have a finite lifespan in culture, defining the phenomenon as "cellular senescence" (Hayflick and Moorhead, 1961). Since then, significant progress has been made in understanding how damaged cells establish a stable proliferation arrest, now known as cellular senescence, with research focusing on identifying molecular triggers, mechanisms, phenotypes, and functional impact of engaging the senescence program. Biologically, senescence is classified into two type's acute and chronic senescence. Acute senescent cells have several beneficial roles during development, regeneration, wound healing, tissue repair and act as a strong safeguard against tumorigenesis, but they can also induced by a variety of stressors such as DNA damage, hypoxia, oxidative and metabolic stress and anticancer drugs, all of which may cause the deleterious effects (Lunyak et al., 2017). Chronic accumulation of senescent cells has negative consequences in life, including aging and aging-related diseases (Lunyak et al., 2017; van Deursen, 2014).

2.1 Senescence inducing stimuli and its pathway

A variety of cell intrinsic and extrinsic stresses can activate the cellular program, including DNA damage, oxidative stress, telomere shortening, chronic mitogen signaling, oncogene activation, tumor suppressor loss, oncogene inactivation, nucleolar stress, epigenetic stress, and spindle stress (Childs et al., 2015; van Deursen, 2014). These stresses can activate various cellular signaling cascades but ultimately activate p53, p16 or both. For example, DNA damage response (DDR) can activate p53/p21 signaling pathway, which inhibits cyclin E-Cdk2 and causes a temporary cell-cycle arrest. However, p16 can also inhibits cell cycle progression by targeting cyclin D–Cdk4 and cyclin D–Cdk6 complex. Both p21 and p16 act by preventing the inactivation of retinoblastoma protein, thus resulting in continued repression of E2F target genes required for S-phase

onset. If cells are exposed to mild damage, they can be successfully repaired and may resume to their normal cell-cycle progression. However, if cells are severely damaged they can undergo a permanent cell cycle arrest or senescence via p53/p21 pathway, which can either antagonize or synergize with p16, depending on the type of stresses or damages.

2.2 Induction and characterization of senescence

To better understand the mechanism of cellular senescence, researchers are using a variety of senescence models and detection methods. In vitro senescence can be induced by various stimuli, which are summarized in the Table 1. Identification and characterization of senescent cells are very critical because senescent cells are highly variable and heterogeneous. Therefore, a combination of different techniques is often used and encouraged for the detection of senescent cells including formation of senescence-associated heterochromatic foci (SAHF), DNA damage response, cell cycle arrest, secretory phenotype, apoptosis resistance, increased cell size and lysosomal content and accumulation of damaged mitochondria.

Table 1. In vitro senescence cell model

Senescence model	**Stress**
Replicative senescence	Long-term culture (telomere shortening)
DNA damage-induced senescence	Ionizing and UV radiation
Oncogene-induced senescence (OIS)	Activation of oncogenes Ras or BRAF, inactivation of tumor suppressor PTEN
Oxidative stress-induced senescence	Either oxidizing products of the cell metabolism or oxidative agents H_2O_2
Chemotherapy-induced senescence	Anticancer drugs (bleomycin or doxorubicin) induce DNA damage and inhibition of CDKs (e.g., abemaciclib and palbociclib)
Mitochondrial dysfunction-associated senescence (MiDAS)	Secretes senescence associated secretory phenotype (SASP)
Epigenetically induced senescence	Inhibitors of DNA methylases (e.g., 5-aza-20-deoxycytidine) or histone deacetylases (e.g., suberoylanilide hydroxamic acid and sodium butyrate)
Paracrine senescence	SASP produced by a primary senescent cell

Note. Types of senescence adopted from (Alejandra Hernandez-Segura et al., 2018).

3. Oxidative stress

Initially, reactive oxygen species (ROS) were assumed to be a detrimental consequence of intracellular aerobic metabolism in the mitochondria. However, recent research suggest that ROS regulate numerous physiological and biological functions including growth factor signaling, autophagy, hypoxic signal transduction, immune responses, metabolic adaptation and stem cell proliferation and differentiation (Holmström and Finkel, 2014; Reczek and Chandel, 2015). Various antioxidant enzymes and modulators under normal physiological conditions tightly regulate ROS. In some circumstances, excessive ROS accumulation occurs, making detoxification beyond the capacity of the antioxidant cellular defense system challenging. Excessive production of mitochondrial ROS or a depleted antioxidant system can cause DNA damage in both mitochondria and nuclei, alter protein structure, and activate stress-activated kinases like JNK and p38, all of which can hasten the aging process (Balaban et al., 2005). Even cellular redox state determines the function and fate decision of stem cells. For example, quiescent and self-renewing stem cells have a low level of reactive oxygen species (ROS) and live in a hypoxic environment. A moderate rise in ROS causes lineage differentiation in stem cells; however, acute or severe ROS causes stem cell senescence or aging, as well as cell death (J. Lee et al., 2018). Therefore, researchers are focusing on the role of oxidative stress in stem cell senescence and aging.

4. Diabetes mellitus

Diabetes is a chronic metabolic disease, characterized by hyperglycemia which is caused by problem with insulin secretion, insulin action or both. Diabetes is associated with long-term damage and dysfunction of different organs, especially the eyes, nerves, heart, kidneys and blood vessels. The symptoms marked by diabetes including fatigue, significant weight loss, polyuria, polydipsia, occasionally with polyphagia. Retinopathy, nephropathy, neuropathy, cardiovascular disease, and sexual dysfunctions are long-term complications of diabetes. The vast majority of diabetes cases fall into one of the two broad etiopathogenetic types, such as Type 1 and Type 2 diabetes. Apart from these, there are other types of diabetes called gestational diabetes and specific type of diabetes.

Type 1 diabetes (T1D) is also known as insulin dependent diabetes or juvenile-onset diabetes. T1D is an absolute deficiency of insulin secretion caused by cellular-mediated autoimmune destruction of β-cells in the pancreas and affects about 5-10% of diabetes patients. Both genetic and environmental factors have been linked to the susceptibility of this diabetes. Type 2 Diabetes (T2D), commonly known as non-insulin diabetes or adult-onset diabetes, is characterized by insulin resistance and mild insulin deficiency and accounts for approximately 90-95% of diabetes patients (Khan et al., 2020). Obesity, stress, and metabolic abnormalities are common causes of T2D, which can lead to macro and microvascular problems. Gestational diabetes mellitus (GDM) is defined as glucose intolerance with on-set or first recognition during pregnancy. Although, this type of diabetes resolves at the end of pregnancy, certain complications may arise which may be irreversible. Specific type of diabetes such as genetic defect in the β-cells or insulin action, disease of the exocrine pancreas, endocrinopathies, drug or chemical induced diabetes, and genetic syndrome sometimes associated with diabetes ("Diagnosis and classification of diabetes mellitus," 2011).

5. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a condition in which triglycerides build up in the liver without being caused by excessive alcohol intake or other attributable cause. NAFLD has become the world's most common liver disease, with an estimated quarter of the world's population suffering from it, and still there is no approved pharmaceutical treatment or prevention for this illness (Ferguson and Finck, 2021). NAFLD refers to a range of liver diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis, as well as end-stage liver failure. Histopathologically, simple steatosis is defined as >5% with minimal inflammation or hepatocellular injury, whereas NASH characterized by severe inflammation and hepatocellular injury. Simple steatosis has been linked to the development of other metabolic diseases including insulin resistance, T2D, dyslipidemia and hypertension (Ferguson and Finck, 2021; Smith and Adams, 2011). NAFLD affects 50-60% of people with T2D and up to 45% of people with T1D. In patients with NAFLD, T2DM raises the risk of cirrhosis and liver death, whereas NAFLD raises the risk of cardiovascular disease and total mortality in T2DM patients.

Therefore, medications that are used to treat T2D are also beneficial in the treatment of NASH (Tilg et al., 2017).

6. AMP-activated protein kinase (AMPK)

Cells must constantly control their energy consumption based on food availability and ATP production capacity. ATP is primarily produced by catabolic process such as the breakdown of glucose and lipids in the mitochondria at a site of OXPHOS. Cells utilize these ATP molecules for their cellular processes by breakdown of ATP into ADP and AMP and cells must maintain their cellular energy state in order to function. Therefore, cells adapt their metabolism to encounter their energy needs and respond to nutrient availability on a continuous basis. In higher eukaryotes, a sophisticated system to sense low cellular ATP levels through the serine/threonine kinase AMP-activated protein kinase (AMPK) complex. Under low-energy conditions, AMPK is activated by an allosteric mechanism that stimulates its kinase activity, that increases the catabolic (ATP generation) as well as reduces the anabolic (ATP consumption) processes (Herzig and Shaw, 2018).

6.1 Structure and function of AMPK

AMPK is a heterotrimeric complex protein composed of one catalytic α-subunit and two regulatory subunits called β and γ. In humans, there are two α-subunits, α1 and α2; two β-subunits, β1 and β2; and three γ-subunits, γ1, γ2 and γ3 have been identified (Cheung et al., 2000; Herzig and Shaw, 2018; Stapleton et al., 1996; Thornton et al., 1998). Each AMPK complex composed of one α-subunit, one β-subunit and one γ-subunit, and thus potentially creating 12 distinct AMPK complexes (Ross et al., 2016). The activation of AMPK is mediated by two upstream kinases named calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) and liver kinase B1 (LKB1). Energy status modifiers such as exercise, nutrient starvation, and mitochondrial poisons (metformin) can activate AMPK pathway. Drugs such as AMP mimetic AICAR and several small-molecules or allosteric activators including A-769662, 991, salicylate, PF-739, MT63-78, GSK621, MK-8722 can activate the AMPK pathway (Herzig and Shaw, 2018). AMPK activation regulates the metabolic processes by upregulating catabolic processes such as glucose metabolism, autophagy, and lipid oxidation and downregulating the anabolic

processes such as lipid and protein synthesis, gluconeogenesis, and glycogen storage. Therefore, activated AMPK can control a wide range of cellular processes, which are grouped into four categories such as glucose metabolism, protein metabolism, lipid metabolism and autophagy, and mitochondrial homeostasis.

7. Sirtuins

Sirtuins are evolutionary conserved from bacteria to human and are nicotinamide adenine dinucleotide-dependent (NAD^+) deacetylases or adenosine diphosphate-ribosyltransferases (Nakagawa and Guarente, 2011; X. Ye et al., 2017). Sirtuins have gained a lot of attention since they can extend the lifespan in budding yeast and regulate the longevity in lower organisms including flies and worms (Imai et al., 2000; Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). The mammalian sirtuin family consists of seven members (SIRT1-7), which are found in many cellular compartments and that can modify a wide range of proteins, including histone and non-histone proteins, through a variety of enzymatic activities. SIRT1, SIRT6, SIRT7 are predominantly localized in the nucleus, SIRT2 is cytoplasmically localized and SIRT3, SIRT4, SIRT5 are localized in the mitochondria (Park et al., 2013). Under certain conditions, the localization of SIRT1 and SIRT2 may shift between the nucleus and the cytosol (Houtkooper et al., 2012). Sirtuins are involved in many biological processes including genomic stability, cell survival, proliferation, senescence, metabolism and stress response (X. Ye et al., 2017) and regulate the aging and age-related diseases including cardiovascular disease, cancer and neurodegenerative diseases (Nakagawa and Guarente, 2011; Yamamoto et al., 2007). SIRT1 is the most studied member of the mammalian sirtuin family, which can deacetylate both histone (H3K9, H4K16 and H1K26) and non-histone proteins (Tumor suppressor protein p53, PGC1α, FOXO1, NF-κB, SREBP1c and others) and is involved in chromatin/epigenetic modification, mitochondrial biogenesis, hypoxia, angiogenesis, cancer, cardiovascular function, metabolism, neuronal function and inflammation or stress response (Nakagawa and Guarente, 2011). SIRT2 can function as a deacetylase and regulate cell cycle, adipocyte, and oligodendroglial cell differentiation. SIRT3, SIRT4 and SIRT5 are mitochondrial sirtuins that promote the production of energy in the cells, protect the cells

from ROS, control insulin secretion in pancreatic β-cells, and regulate urea cycle. SIRT6 is an ADP-ribosylase and NAD^+-dependent deacetylase, involved in DNA repair, telomere maintenance and regulate the glucose and lipid metabolism, similar to SIRT1. SIRT7 is a nuclear protein, which has been shown to be a positive regulator of RNA pol I transcription and required for cell viability (Ford et al., 2006).

8. Autophagy

Autophagy is a self-degradative process that is crucial for balancing energy sources during development and in response to dietary stress. Autophagy also removes misfolded or aggregated proteins, clears damaged organelles including mitochondria, endoplasmic reticulum, and peroxisomes, and eliminates intracellular infections (Glick et al., 2010). Micro-autophagy, macro-autophagy, and chaperone-mediated autophagy are the three kinds of autophagy that all induce proteolytic destruction of cytosolic components at the lysosome. Macro-autophagy transports cytoplasmic cargo to the lysosome via a double membrane-bound vesicle called an autophagosome, which merges with the lysosome to produce an autolysosome. In micro-autophagy by contrast, cytosolic components are directly collected by lysosome itself via invagination of the lysosomal membrane. In chaperone-mediated autophagy, targeted proteins are translocated across the lysosomal membrane in a complex with chaperone proteins, resulting in their unfolding and degradation (Saftig et al., 2008). Autophagy consists of several sequential steps including autophagosome formation and maturation, autophagolysosome formation, degradation and utilization of degraded products. The term autophagic flux refers to the measurement of autophagic degradation activity that encompasses the entire process, starting with autophagosome formation and ending with the release of degraded macromolecules back into the cytosol (X. J. Zhang et al., 2013). Multiple assessments of macroautophagy markers across time are required to establish the rate of autophagic flow (Loos et al., 2014). Proteins encoded by Atg5 and Atg12, mTOR, Beclin 1, SQSTM1 and LC3-II/LC3-I ratio have all been used to quantify macroautophagy. During autophagy, all LC3 isoforms undergo post-translational changes, particularly phosphatidylethanolamine (PE) conjugation (lipidation). The cytosolic form of LC3 (LC3-I) is conjugated to PE to generate LC3-PE conjugate (LC3-II), which is attracted to the autophagosomal

membranes in response to an autophagic signal. Beclin-1 is an autophagy-specific protein that controls the development of autophagosomes. Other macroautophagy marker Sequestosome-1/autophagic adaptor protein p62 (SQSTM1/p62), which has several binding motifs and assembling autophagic protein complex. Autophagy induction is regulated by the kinase mTOR, with active mTOR (by Akt, MAPK) reducing it and negative regulation of mTOR (by AMPK) encouraging it.

9. Licochalcone D

Flavonoids are polyphenolic compounds produced by plants as secondary metabolites (Van De Wier et al., 2017). Flavonoids have been shown to have positive effects on insulin resistance, lipid metabolism, oxidative stress and inflammation (Fang et al., 2019; Van De Wier et al., 2017; Y. Wang et al., 2011). Licochalcone D (Lico D) is flavonoid compound, which has been isolated as a prenylated retrochalcone in the root of licorice (*Glycyrrhiza inflata*) in 1992. Licorice has been used as an integral part of Chinese traditional medicine and Ayurveda for centuries (Kajiyama et al., 1992; Okada et al., 1989). The chemical structure was elucidated as 2-methoxy-3′prenyl-3,4,4′-trihydroxychalcone (Kajiyama et al., 1992). Lico D has been found to have various biological effects including anti-oxidant, anti-allergic, anti-inflammation, anti-cancer, anti-viral, anti-senescence and cardioprotective properties (Furusawa et al., 2009; Haraguchi et al., 1998; Kajiyama et al., 1992; Maharajan et al., 2021; Mitra et al., 2021; Si et al., 2018; Tanifuji et al., 2010; Yuan et al., 2015). However, the effect of Lico D on senescence and aging remains unknown.

10. Camphorquinone

Terpenes are a vast class of plant secondary metabolites with a broad range of pharmacological and physiological properties, as well as the ability to act as geroprotectors (Brahmkshatriya and Brahmkshatriya, 2013; Jaeger and Cuny, 2016; Proshkina et al., 2020). Camphor is a bicyclic monoterpene (1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one) that can be found in essential oils from various herbs, including rosemary, lavender, and sage, and used in traditional Chinese medicine to treat sprains, swelling, and inflammation (Al-Qudsi et al., 2012; T. A. Tran et al., 2015).

Camphorquinone (CQ) is a bicyclic monoterpene (1,7,7-Trimethylbicyclo[2.2.1]heptan-2,3-dione) that can be synthesized (~93%) from camphor using a two-step process that includes camphor bromination and the oxidation of 3-brominated camphor, catalyzed by FE-porphyrins, in the presence of air (Noirbent and Dumur, 2021; J. Wang et al., 2013). CQ is widely used as a photoinitiator to induce polymerization of light-curing materials such as dental adhesive and composites (Van Landuyt et al., 2007). However, the biological effects of CQ on senescence and aging are yet to be investigated.

II. MATERIALS AND METHODS

1. Chemicals and reagents

Methylthiazolyldiphenyl-tetrazolium bromide (#M2128 MTT) assay; 2′,7′-dichlorofluorescin diacetate (#D6883), ascorbic acid (#A8960), H_2O_2 (#H1009), Camphorquinone (#124893; purity 97%), Oil red O stain (#O0625), D-Glucose (#G7021) and D-Galactose (#G0750) were purchased from Sigma-Aldrich (USA). S-β-gal staining assay kit (#9860) was obtained from Cell Signaling Technology (Danvers, MA, USA). Licochalcone D (#CFN99805; purity ≥ 98%) was purchased from ChemFaces (430056; Wuhan, Hubei) and Compound C was purchased from Calbiochem (Darmstadt, Germany). Radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) and Pierce BCA protein assay kit and Hoechst 33342 Trihydrochloride Trihydrate (#H3570) were purchased from Thermo Fisher Scientific, USA, respectively. RNAiso Plus (#9109; Total RNA extraction reagent) and PrimescriptTM II 1st strand cDNA synthesis kits (#6210A) were purchased from Takara Bio Inc. (Japan). Primary antibodies were purchased from following companies Santa Cruz Biotechnology INC. (TX, USA), Merck Millipore (Germany), Cell Signaling Technology (USA), Enzo Life Sciences (Lausen, Switzerland) and Abcam (Cambridge, UK). The appropriate HRP-conjugated secondary antibodies, mouse anti-rabbit (#sc-2357), and mouse anti-goat (#sc-2354) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and the horse anti-mouse (#7076) antibody was from Cell Signaling Technology (USA). ECL Western blotting detection reagents (RPN2209) were purchased from GE Healthcare (Buckinghamshire, UK).

2. hBM-MSCs cell culture

hBM-MSCs were acquired from Cell Engineering for Origin (CEFO), Seoul, Korea. The cells were free from bacterial, viral, and mycoplasmal contamination. The cells were characterized by flow cytometry analysis, which revealed the $CD73^+$, $CD105^+$, and $CD31^-$ phenotype. The cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Life Technologies, USA), L-glutamine, and 1% penicillin/streptomycin solution (Lonza, Walkersville, MD, USA). The cells were manipulated under sterile conditions in a humidified incubator at 37 °C with 5% CO_2. The cells were subcultured as soon as they

reached confluence. The cells were serially monitored under bright field microscopy (Nikon Eclipse TS100; Tokyo, Japan), and the media was changed every 3 days.

3. HepG2 cell culture

The HepG2 hepatocyte cells were cultured in low glucose Dulbecco's modified Eagle medium (DMEM) (Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (Gibco, Life Technologies, USA), L-glutamine, and 1% penicillin/streptomycin solution (Lonza, Walkersville, MD, USA). The cells were manipulated under sterile conditions in a humidified incubator at 37 °C with 5% CO_2. The cells were subcultured as soon as they reached confluence. The cells were serially monitored under bright field microscopy (Nikon Eclipse TS100; Tokyo, Japan), and the media was changed every 3 days.

4. Senescence-Associated β-Galactosidase staining

Senescence-associated β-galactosidase positive cells were identified using a SA-β-gal assay kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions. Briefly, after 72 h of treatment, cells were washed with phosphate buffered saline (PBS) and fixed with 1x fixative for 10-15 min at room temperature. The β-galactosidase staining solution (pH 6.0) was added to the plates and incubated overnight in a dry incubator at 37 °C without CO_2. The next day, the β-Gal positive cells were observed under a light microscope (Nikon Eclipse TS100; Tokyo, Japan) and images were captured using an IMTcam3 cameras (IMT i-Solution Inc., Brook Anco Corporation, Cicero, NY, USA).

5. Free fatty acids (FFAs) preparation and treatment

Free fatty acids (FFA) were prepared by a combination of palmitic acid (PA) and oleic acids (OA). Briefly, 100 mM of palmitic acid and 100 mM of oleic acids stock were prepared separately in sodium hydroxide solution. These stock solutions were conjugated with 10% BSA (1:9). All stock solutions were stored at -20 °C. The cells were seeded on the six well plate and after attachment; the cells were treated with a mixture of 0.2 mM of PA and 0.4 mM of OA (1:2) used for the culture or equal volume of 10% BSA solutions

for 24 h. The following day, FFA was removed and either 10 μM of Lico D or 500 μM of CQ was added and leave it for another 24 h. Then the plates were used for protein and Oil red O staining.

6. Oil red O stain preparation and staining

Stock and working solution of Oil Red O staining solutions were prepared according to Mehlem *et al.* (Mehlem et al., 2013) and the manufacture instructions with some modifications. Briefly, Oil Red O stock solution was prepared in isopropyl alcohol by mixing the solution with magnetic stirring for 2 h at room temperature and the solution was stored at room temperature. For Oil Red O working solution, the stock solution mixed with water (3:2) and incubated at 4 ℃ for 10 min. The solution was filtered through 0.22 μm filters and the solution was used within 6 h.

HepG2 cells were treated as described above. After 24 h, the cells were washed with sterile PBS, fixed with 4% formaldehyde for 20 min, and stained with 0.5% Oil red O in isopropanol for 3 min at room temperature. The Oil Red O stained cells were then rinsed with PBS few times to remove the excess stain. The stained lipid droplets within cells were visualized by light microscope and captured. The stained lipid droplets were dissolved in isopropyl alcohol and the absorbance was measured at 490 nm to quantify lipid accumulation.

7. Glucose tolerance test

After an overnight fast, 1.5 g/kg of d-glucose was administered intraperitoneally. To measure the fasting blood glucose level, the blood samples were collected from the tail of each mouse at 0, 15, 30, 60, 90 and 120 min post-glucose injection using Green Doctor blood glucose monitoring system (G400; Green Doctor, GC Pharma, Yongin-si, South-Korea).

8. Immunoblot analysis

Total proteins were extracted with RIPA lysis buffer system containing phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na_3VO_4), and protease inhibitor cocktail (Santa Cruz Biotechnology) at 4 ℃ for 30 min, and the samples were

centrifuged at 16,000× g for 20 min. The total protein concentration was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins (30-50 μg) were loaded and separated via sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, followed by blotting on a PVDF membrane (GE Healthcare, Germany). The membranes were blocked with 1% blocking solution containing non-fat dry milk in TBST for 1 h 30 min at RT to prevent non-specific binding of primary antibodies. Subsequently, the membranes were incubated overnight at 4 °C with the corresponding primary antibodies. Primary antibodies were listed in the Table 2. Secondary antibodies were conjugated with horseradish peroxidase and visualized using an enhanced chemiluminescence detection kit (GE Healthcare). Densitometry analysis was performed using the ImageJ software.

Table 2. List of primary and secondary antibodies used for Western blot

Primary antibody	Primary antibody Dilutions	Secondary antibody
ACC	1:1000	Rabbit
AMPKα	1:1000	Rabbit
BECN1	1:500	Mouse
Caspase 3 active form	1:500	Rabbit
Caspase-3	1:500	Rabbit
Catalase	1:500	Goat
GAPDH	1:500	Goat/mouse
MAP LC3α/β	1:500	Mouse
p16	1:500	Mouse
p21	1:500	Rabbit
p53	1:500	Rabbit
p-ACC	1:1000	Rabbit
p-AMPK	1:1000	Rabbit
SIRT1	1:500	Mouse
SOD1	1:500	Rabbit
SOD2	1:500	Rabbit
SQSTM1	1:500	Mouse

9. RNA extraction and quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

RNAisoPlus (Takara) was used to isolate total RNA from the HepG2 cells, hippocampus or heart tissues. Total RNA (2.5 μg) was then reverse-transcribed using PrimescriptTM II 1st strand cDNA synthesis kit (Takara) and quantified using the Power SYBR Green PCR Master mix (Applied BioSystems). The list of primer pairs was summarized in the Table 3. Real-time PCR reactions were performed using the StepOneTM Real-Time PCR system (Applied Bio Systems) and the primer pairs were synthesized by GenoTech (Daejeon, Korea) or IDT (Integrated DNA Technologies, Coralville, IA, USA).

Table 3. List of primers used for RT-PCR

Species	Gene	Forward / Reverse	Sequence (5'->3')
Human	*ACTB*	F	ATCCGCAAAGACCTGTACGC
		R	TCTTCATTGTGCTGGGTGCC
Human	*CES1*	F	GACCCCAGAGAGAGTCAACC
		R	CTCCTGCTTGTTAATTCCGACC
Human	*CES2*	F	CTTTCAGCCTGTCCCTAGCA
		R	TGTAGGAGGCAACATCAGCA
Human	*ChREBP*	F	CCTCTTCGAGTGCTTGAGCC
		R	CTCTTCCTCCGCTTCACATACTG
Human	*CPT1A*	F	GGGCTACAAATTACGTGAGCGA
		R	CTTGCTGCCTGAATGTGAGT
Human	*IL-1α*	F	GTGCTGCTGAAGGAGATGCC
		R	CTTAGTGCCGTGAGTTTCCCAG
Human	*IL-6*	F	AATGAGGAGACTTGCCTGGTG
		R	CTCTGGCTTGTTCCTCACTACTC
Human	*IL-8*	F	TGCAGCTCTGTGTGAAGGTG
		R	GGTCCACTCTCAATCACTCTCA
Mouse	*Actb*	F	CCACCATGTACCCAGGCATT
		R	CGGACTCATCGTACTCCTGC
Mouse	*Ager*	F	AGGTGGGGACATGTGTGTC
		R	TCTCAGGGTGTCTCCTGGTC
Mouse	*Cpt1a*	F	ATGAGGCTTCCATGACTCGG
		R	AACCTCTGCTCTGCCGTTG
Mouse	*Fasn*	F	GACTCGGCTACTGACACGAC
		R	CGAGTTGAGCTGGGTTAGGG
Mouse	*Il-1α*	F	CCACCAAAGAACAAAGTCGGG
		R	CAGACTGTCAGCACTTCCCAA

Mouse	*Il-1β*	F	AAGAGCCCATCCTCTGTGACT
		R	GGAGCCTGTAGTGCAGTTGT
Mouse	*Il-6*	F	AGACAAAGCCAGAGTCCTTCAG
		R	GAGCATTGGAAATTGGGGTAGG
Mouse	*Srebf1*	F	TACAGCGTGGCTGGGAAC
		R	GCATCTGAGAACTCCCTGTCT
Mouse	*Tnf-α*	F	GTGCCTATGTCTCAGCCTCTTC
		R	GAGGCCATTTGGGAACTTCTCATC
Mouse	*Trp53*	F	GTATTTCACCCTCAAGATCCGC
		R	CTGCTGTCTCCAGACTCCTCT

10. Statistical analyses

All data are presented as the mean ± standard deviation from at least three or more biological replicates. The differences between data sets were assessed by Student's t-test and analysis of variance (ANOVA) with Holm-Sidak's multiple comparison test using GraphPad Prism (GraphPad Software). Statistical significance levels are indicated in the figures using asterisks as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

III. RESULTS AND DISCUSSION

PART 1. EFFECTS OF LICOCHALCONE D AND CAMPHORQUINONE ON OXIDATIVE STRESS-INDUCED SENESCENCE

CHAPTER 1. Licochalcone D ameliorates oxidative stress-induced senescence via AMPK activation*

1. Introduction

Cellular senescence is a permanent condition of cell cycle arrest that promotes tissue remodeling during development, wound healing, host immunity, and tumor protection, but can also affect tissue homeostasis, tissue regenerative capacity, and inflammation, even leading to cancer (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014). Different types of cellular senescence have been identified based on its mechanism of activation and classified as either replicative senescence, physiological senescence, or stress-induced premature senescence (Lozano-Torres et al., 2019). Accumulation of senescent cells with age, one of the hallmarks of aging, contributes to the development of aging and aging-related diseases, including cancer, cardiovascular disease, and neurodegenerative diseases (Biran et al., 2017; Burd et al., 2013; Lopez-Otin et al., 2013; Soto-Gamez and Demaria, 2017; M. Xu et al., 2018). Conversely, clearance of these senescent cells can increase the health and lifespan of old mice (M. Xu et al., 2018). Cellular senescence can occur naturally in vitro and in vivo and can be induced by cells or animals subjected to oxidative stress from substances such as hydrogen peroxide (H_2O_2) (Q. Chen and Ames, 1994; Q. M. Chen et al., 1998; Crowe et al., 2016; Han et al., 2016; Z. Wang et al., 2013b) and D-galactose (D-Gal) (Q. Li et al., 2018; Nam et al., 2018; Sun et al., 2020; Sun et al., 2018). This type of senescence is called stress-induced premature senescence (SIPS).

Hydrogen peroxide (H_2O_2) has been widely employed *in vitro* to generate oxidative stress and senescence (Han et al., 2016; Huang et al., 2018). D-Gal treatment increases senescence in various organs including heart and brain senescence, receiving the most attention in aging research (Bo-Htay et al., 2018; Shwe et al., 2018). Additionally, chronic administration of D-Galactose undergoes non-enzymatic glycation and react with amines

* Modified from the published article: Maharajan, N., Ganesan, C. D., Moon, C., Jang, C. H., Oh, W. K., & Cho, G. W. (2021). Licochalcone D Ameliorates Oxidative Stress-Induced Senescence via AMPK Activation. *Int J Mol Sci, 22*(14).

to form Schiff's base composite and Amadori products, which results in the increased level of AGE, RAGE and NADPH oxidase (Shwe et al., 2018). These factors cause oxidative stress and inflammatory pathway activation, which leads to cellular death and degenerative alterations, eventually leading to aging and age-related illnesses (Azman and Zakaria, 2019). Therefore, it is necessary to develop a therapeutic strategy to either remove or inhibit the activation of these products under oxidative stress conditions.

Adenosine 5′ monophosphate-activated protein kinase (AMPK) is a cellular energy sensor that regulates a variety of functions including glucose, protein and lipid metabolism as well as autophagy and mitochondrial homeostasis (Herzig and Shaw, 2018). It is well known that increasing oxidative stress inhibits the expression of AMPK, whereas activation of AMPK has an anti-senescence impact and is implicated in autophagy induction (Han et al., 2016; Y. F. Li et al., 2018; Moreno-Blas et al., 2019; Tai et al., 2017). Autophagy is a highly conserved catabolic process that maintains cellular homeostasis by degrading long-lived or dysfunctional proteins and organelle lysosomes of all living cells (Mizushima et al., 2008; Mizushima et al., 2010). Previous studies have reported that AMPK activates autophagy directly (by phosphorylating ULK1 on at least four residues, Ser467, Ser555, Thr574, and Ser637) and indirectly (phosphorylates the mTOR upstream regulator TSC2 on Thr1227 and Ser1345 and the mTOR subunit RAPTOR on Ser722 and Ser792 by inhibiting mTORC1, which phosphorylates and inhibits ULK1) activating ULK1 (García-Prat et al., 2016; Herzig and Shaw, 2018; Ma, Qi, et al., 2018). Recent studies have demonstrated that autophagy is required to maintain stem cell properties, including proliferation, differentiation, and stem cell fate and to prevent senescence both in vitro and in vivo under oxidative stress conditions (García-Prat et al., 2016; Han et al., 2016; Ho et al., 2017; Y. F. Li et al., 2018; Ma, Qi, et al., 2018; Moreno-Blas et al., 2019; Revuelta and Matheu, 2017). Therefore, activation of AMPK could serve as a critical mediator of autophagy activation in response to protection from oxidative stress-induced senescence.

Licorice, also known as *Radix Glycyrrhizae*, is a perennial herb that has been used as an integral part of Chinese traditional medicine and Ayurveda for centuries. *Glycyrrhiza inflata* (*G. inflata*) is a major botanical source of licorice-containing species. *G. inflata* is chemically characterized and identified by the presence of retrochalcones, which are part

of an important class of natural chalcones (Okada et al., 1989) and are precursors of flavonoids. The lack of oxygen functionality on C-2′ and C-6′structurally distinguishes these retrochalcones from other chalcones (Kajiyama et al., 1992). Based on their chemical structure, licochalcones are classified as licochalcone A, B, C, D, E, F, and G. Many biological activities have been reported for these chalcones, including anticancer, anti-inflammatory, antimicrobial, antiviral, antiallergic, antioxidant, osteogenic, and antidiabetic properties (Maria Pia et al., 2019). It has also been observed that licorice extract diminishes brain aging by inhibiting oxidative stress and neuronal apoptosis (Y.-Z. Zhou et al., 2017). Although considerable evidence has shown that licorice has a remarkable anti-aging effect in senescence related models, little research has revealed which components from the licorice provide these beneficial effects.

In this study, I chose Licochalcone D (Lico D) to explore its role in anti-aging properties. Lico D was first isolated as a prenylated retrochalcone from *Glycyrrhiza inflata* (*G. inflata*) in 1992 and its chemical structure was elucidated as 2-methoxy-3′prenyl-3,4,4′-trihydroxychalcone (Kajiyama et al., 1992). It possesses antioxidant, anti-inflammatory, anticancer, antiviral, and cardioprotective activities (Furusawa et al., 2009; Haraguchi et al., 1998; S. J. Kim et al., 2014; Maria Pia et al., 2019; Mitra et al., 2020; H. N. Oh et al., 2020; Seo et al., 2019; Si et al., 2018; Tanifuji et al., 2010; Yuan et al., 2015). However, the effect of Lico D on senescence and aging remains unknown. Therefore, I investigated the effects of Lico D on senescence and aging by (1) testing the protective effects of Lico D against H_2O_2 induced oxidative stress in human bone marrow mesenchymal stem cells (hBM-MSCs); (2) confirming the effect of Lico D on oxidative stress-induced senescence *in vitro* and *in vivo*; (3) monitoring the mechanism of action of Lico D in the senescence model. The results indicate that Lico D protects cells and tissues from oxidative stress-induced senescence via activating AMPK.

2. Materials and Methods

Cell Viability Assay

The cytotoxic and protective effects of Lico D in hBM-MSCs were determined using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) test, according to the manufacturer's instructions. To check the cytotoxic effect, hBM-MSCs were grown in a 96-well plate at a density of 8×10^3 cells per well. The cells were treated with different concentrations of Lico D (1-8 μg/mL) for 12 h, then incubated with MTT solution for 2 h and the formazan crystals were dissolved using dimethyl sulfoxide (DMSO). Cell viability was assessed using a spectrophotometer (Multiskan FC, Thermo Fisher Scientific). For the protective effect of Lico D, cells were pre-treated with 1 μg/mL of Lico D for 12 h followed by incubation with different concentrations of H_2O_2 (0.5-1 mM) for 1 h. Following H_2O_2 treatment, cells were treated with MTT solution for 2 h and cell viability was evaluated.

Detection of Intracellular ROS

To quantify the production of intracellular ROS, I used cell permeable substrate 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which can be converted to highly detectable fluorescent 2′,7′-dichlorofluorescein upon oxidation. The cells were cultured in 12-well plates and 96-well plates for 24 h. After 24 h of culture, the cells were treated with 1 μg/mL of Lico D for 12 h and incubated with 20 μM DCFH-DA for 30 min. The cells were then washed and incubated with H_2O_2 (0.7 mM) for 1 h at 37 ℃. The cells were then fixed with 4% paraformaldehyde at room temperature for 15 min and the nuclei were stained with Hoechst. Then, the cells were observed under fluorescent microscope (Nikon Eclipse Ti2; Japan) and the images were captured (Nikon DS-Ri2; Japan) and analyzed using the Image J software. To analyze the apoptotic markers, the cells were pretreated with 1 μg/mL of Lico D followed by incubation with H_2O_2 (0.7 mM) for 1 h at 37 °C, and then the proteins were isolated as described below.

Oxidative Stress-Induced Senescence

H_2O_2 was used to induce oxidative stress-induced cell cycle arrest and senescence as described previously (J. H. Chen et al., 2004; Z. Wang et al., 2013a) with some

modifications (Figure 3A). Briefly, the cells were exposed to 200 μM H_2O_2 for 2 h and cultured for 2 days without H_2O_2. In the second treatment with H_2O_2, the cells were split 1:3, exposed to 200 μM H_2O_2 for 2 h, and cultured with either normal media (control), Lico D (1 μg/mL), ascorbic acid (500 μM AA; a positive control), or the compound C (0.5 μM; AMPK inhibitor) for 72 h. Cellular senescence was confirmed by SA-β-gal assay and immunoblot analysis.

Animals and Administration of Drugs

Six-week-old male C57BL/6 mice (weighing 22 ± 2 g) were purchased from Samtako Bio Korea Co., Ltd. (Osan, Gyeonggi, Korea) and maintained at 23-25 °C under a 12-h light and 12-h dark cycle with free access to food and water in a pathogen-free facility. All animal experiments were approved by the Institutional Animal Care and Use Committee of Chosun University (CIACUC2020-A0009). After a week of acclimation, the animals were randomly divided into three groups (four mice per group): normal control group (PBS alone), D-Gal model group (150 mg/kg/day), and D-Gal + Lico D (0.5 mg/kg/day). The mice were intraperitoneally injected with either PBS or D-Gal for 10 weeks and from the third week onwards, the mice were intraperitoneally injected with Lico D for 8 weeks (Figure 6A). Body weight was measured once a week till the end of the experiments.

3. Results

3.1. Lico D Protects the hBM-MSCs against Oxidative Stress

Initially, in order to determine the effect of Lico D on hBM-MSCs viability, I treated hBM-MSCs with different concentrations of Lico D (0-8 μg/mL) for 12 h and cell viability was measured. As shown in Figure 2A, there were no significant changes in cell viability upon Lico D treatment. However, previous reports highlighted that high concentration of Lico D possesses anti-cancer activity in different cancer cell lines (H. N. Oh et al., 2020; Seo et al., 2019; Si et al., 2018). Therefore, I chose the lowest concentration of Lico D (1 μg/mL) for further *in vitro* experiments. The antioxidant properties of Lico D were evaluated against H_2O_2 in hBM-MSCs. Lico D-pretreated hBM-MSCs were exposed to different concentrations of H_2O_2 (0-1 mM) and cell viability was measured. The results indicated that cell viability was significantly increased in Lico D-pretreated hBM-MSCs (Figure 2B). Next, I examined the effect of Lico D on ROS generation and cell death. As expected, intracellular ROS levels were significantly increased upon H_2O_2 treatment, whereas they were significantly reduced in Lico D-pretreated hBM-MSCs (Figure 2C,D). Furthermore, these protective effects of Lico D were also confirmed by the expression levels of apoptotic proteins, such as p53 and cleaved caspase 3 (Figure 2E). Both markers were increased upon treatment with H_2O_2, whereas the expression of these markers were reduced in Lico D-pretreated hBM-MSCs. Collectively, these results suggest that Lico D protects hBM-MSCs against oxidative stress by inhibiting excess intracellular ROS production and cell death.

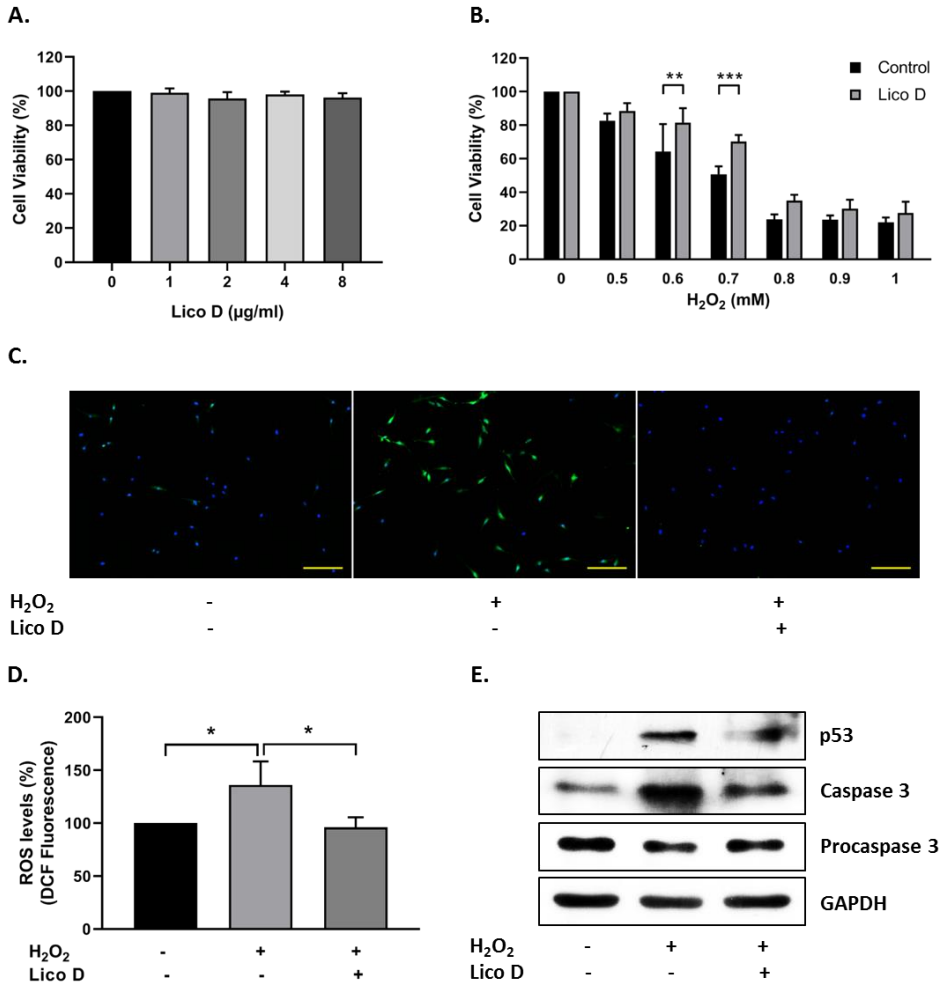

Figure 2. Protective effects of Lico D against hydrogen peroxide (H_2O_2). (A) Effect of Lico D on the cell viability was determined by MTT assay. (B) Protective effect of Lico D against H_2O_2 was confirmed by MTT assay. (C) ROS scavenging activity of Lico D was determined by DCFH-DA fluorescence assay and (D) fluorescence intensity was quantified using Image J software. (E) Representative images from immunoblot analysis against apoptotic markers such as procaspase 3, Caspase 3 (cleaved form) and p53. GAPDH was used as an internal control. The scale bar represents 100 μm. All data are represented as the mean ± standard deviation (SD) (n = 3) * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.2. Lico D Reduces the Oxidative Stress Induced Senescence in hBM-MSCs

To investigate the protective effect of Lico D in hBM-MSCs senescence, the cells were subjected to stress-induced premature senescence (SIPS), particularly the oxidative stress-induced senescence model (Figure 3A), as described in the Methods section. As shown in Figure 3B, the oxidative stress-induced senescence cells became enlarged, flattened, and highly SA-β-gal-positive cells. The accumulation of SA-β-gal positive cells was significantly augmented in the double time H_2O_2 treated hBM-MSCs (40%) when compared with single time treated hBM-MSCs (12%) (Figure 3C). Therefore, I chose a double-time H_2O_2 treatment for further studies. As expected, the number of SA-β-gal-positive cells was significantly reduced by treatment with Lico D (13%) and ascorbic acid (17%, a positive control). Furthermore, the anti-senescence effects of Lico D were evaluated by the expression of well-known senescence markers, including p53, p16, and p21. As shown in Figure 4A-D, the expression of senescence markers was increased in double-time H_2O_2 treated hBM-MSCs, while it was reduced in Lico D-treated hBM-MSCs. These results suggest that Lico D not only protects hBM-MCSs from oxidative stress, but also reduces oxidative stress-induced senescence in hBM-MSCs.

3.3. Lico D Reduces the Oxidative Stress-Induced Senescence via Activation of AMPK and Autophagy

Next, I assessed the role of AMPK in the senescence model, as AMPK is a well-established mediator in the prevention of oxidative stress. The results showed that AMPK activation was significantly decreased in the oxidative stress-induced senescence model (Figure 4E,F), while AMPK activation was significantly restored in Lico D-treated senescent cells. Furthermore, this effect was confirmed using compound C (CC), a potential AMPK inhibitor. As expected, Lico D-mediated AMPK activation was abolished by treatment with CC (Figure 4E,F). These data suggest that AMPK activation by Lico D can reduce oxidative stress-induced senescence in hBM-MSCs and this reduction could be prevented by CC.

Further, I extended the study to explore the role of Lico D in autophagy activation. The results showed that Lico D treatment significantly increased the expression level of LC3-II/LC3-I ratio and BECN1 and decreased the expression of SQSTM1 (Figure 5A-

D). To verify the involvement of AMPK in autophagy, I used the CC. Interestingly, I found that AMPK-mediated autophagy was inhibited by treatment with CC. Taken together, the findings indicate that Lico D activates autophagy through AMPK and reduces oxidative stress-induced senescence.

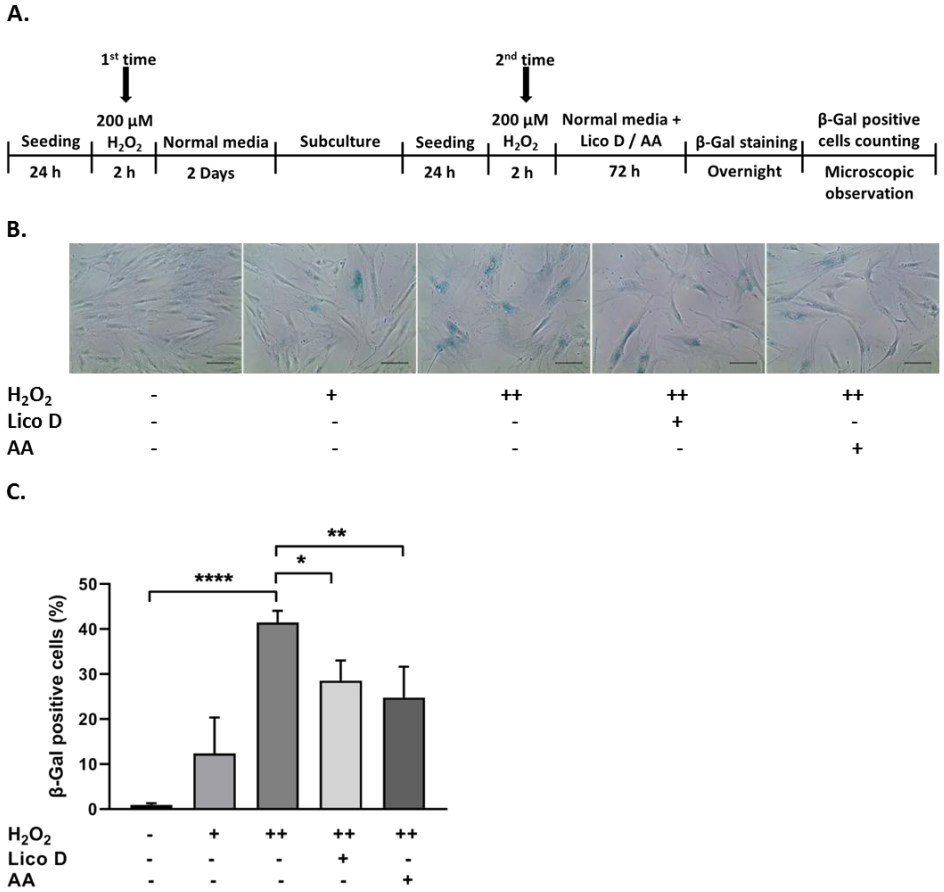

Figure 3. Effect of Lico D in oxidative stress-induced senescent hBM-MSCs. (A) Graphical representation of the oxidative stress-induced senescence in hBM-MSCs. (B) Representative image of SA-β-gal positive cells and (C) percentage of SA-β-gal positive cells in oxidative stress-induced senescence model. H_2O_2 (+) represents one-time treatment and H_2O_2 (++) represents double-time treatment. The scale bar represents 100 μm. All data are represented as mean ± SD (n = 3) * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

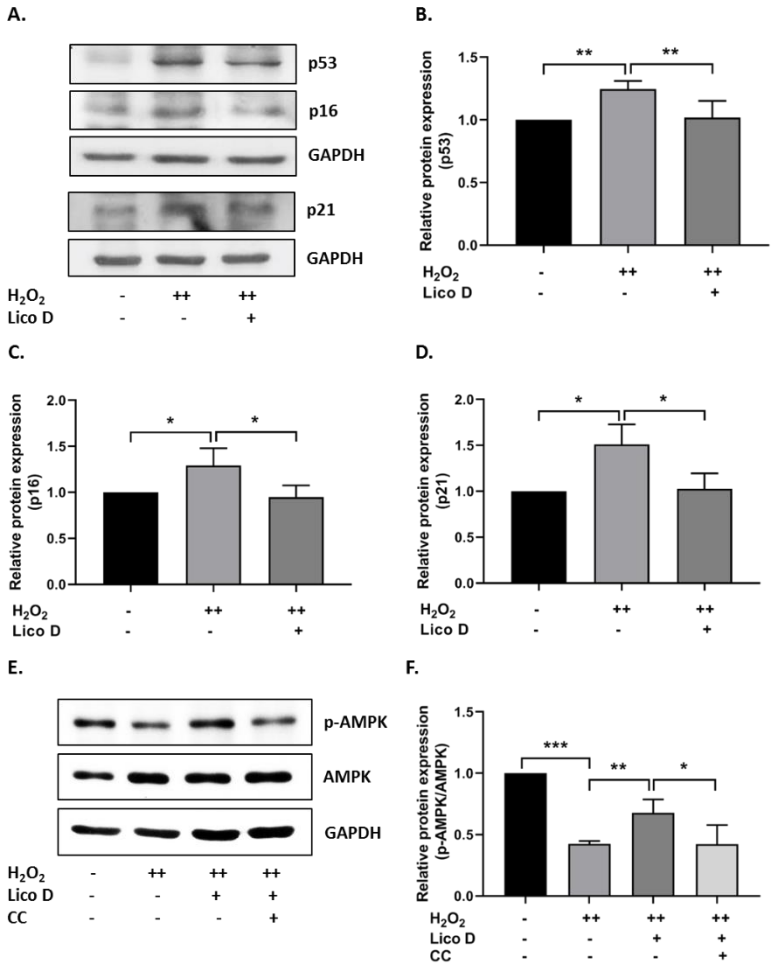

Figure 4. Lico D reduces senescence via AMPK activation in hBM-MSCs. (A) Representative images from immunoblot against senescence markers p53, p16, and p21 and (B-D) the expression level of target proteins was quantified using Image J software. (E) Representative images from immunoblot assay against AMPK and phosphorylated AMPK and (F) the expression level of target proteins was quantified using Image J software. GAPDH was used as an internal control. Compound C (CC), an AMPK inhibitor was used. H_2O_2 (+) represents one-time treatment and H_2O_2 (++) represents double-time treatment. The scale bar represents 100 μm. All data are represented as mean ± SD (n = 3) * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

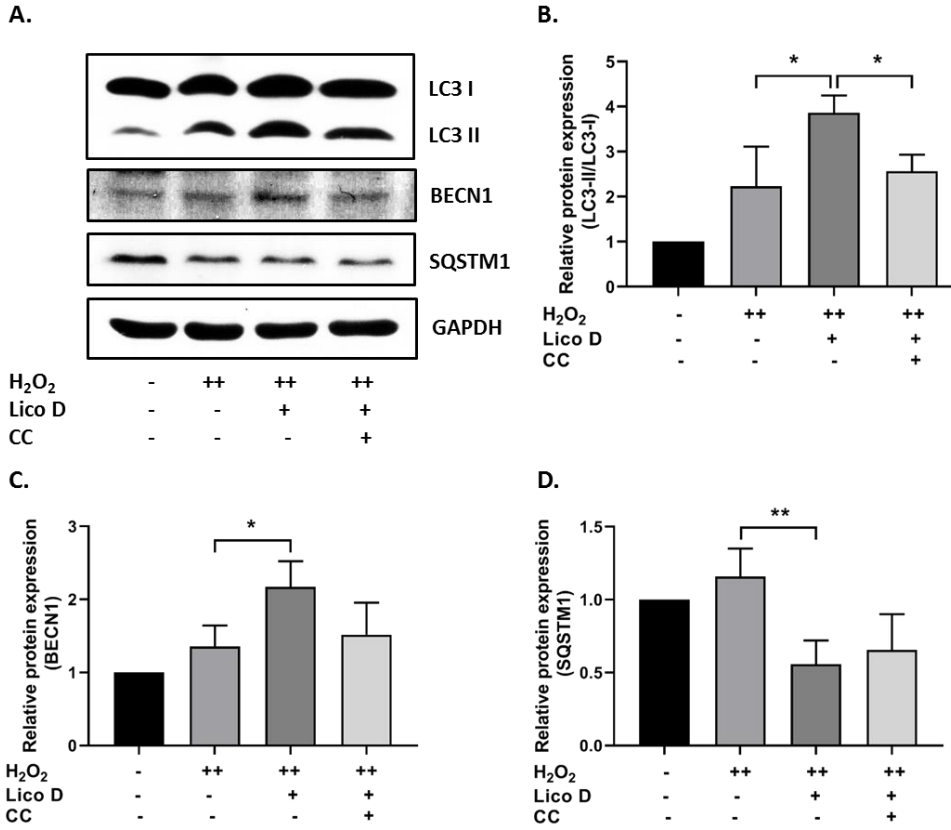

Figure 5. AMPK activation increases the autophagy in oxidative stress-induced senescent hBM-MSCs. (A) Representative images from immunoblot against autophagy markers including LC3, BECN1, and SQSTM1. (B-D) The expression level of target proteins was quantified using Image J software. GAPDH was used as an internal control. Compound C (CC), an AMPK inhibitor was used. The results are represented as mean ± SD (n = 3). * $p < 0.05$ and ** $p < 0.01$.

3.4. Effect of Lico D on Body Weight in D-Gal Induced Aging Mice

The animal's body weight was measured every week until the experiment ended. No significant differences were observed in either control, D-Gal or Lico D-injected animals (Figure 6B).

3.5. Lico D Reduces RAGE Expression in D-Gal Induced Aging Mice

Long-term D-Gal treatment can cause cognitive dysfunction by causing oxidative stress and neuro-inflammation, both of which are linked to signs of aging in the brain. To identify the role of Lico D in hippocampal inflammation, I screened the aging-related inflammation marker RAGE. The data showed that D-Gal treatment increased the expression of *Rage* at the mRNA level compared to that in the control group (Figure 6C). Treatment with Lico D significantly reduced *Rage* expression compared to that in D-Gal treated mice. The results suggest that Lico D can reduce D-Gal-induced hippocampal inflammation by reducing RAGE expression.

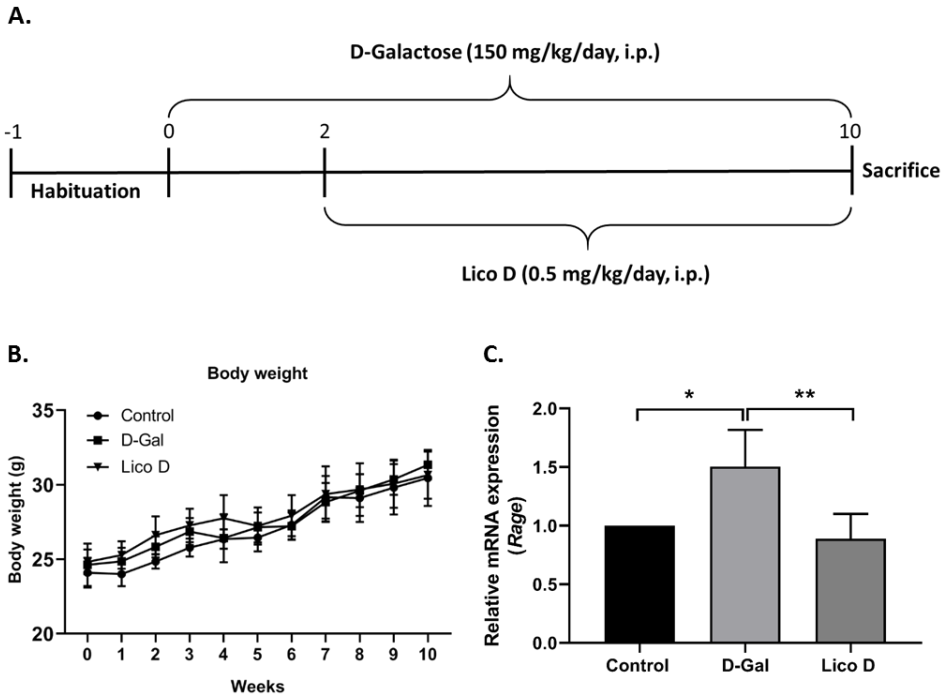

Figure 6. Lico D reduces receptors for advanced glycation end products (RAGE) expression in D-Galactose treated mice. (A) Schematic representation of the experimental plan and (B) body weights of the mice. (C) Relative fold changes in mRNA of *Rage* was quantified by RT-PCR. All data are represented as mean ± SD (n = 4). * p < 0.05 and ** p < 0.01.

3.6. AMPK Activation by Lico D Ameliorates Heart and Hippocampus Senescence in D-Gal Induced Aging Mice

Oxidative stress can activate cellular senescence through various signaling cascades, but ultimately activate p53, p16, or both, and mediate cell cycle arrest (Campisi and d'Adda di Fagagna, 2007; van Deursen, 2014). Therefore, I first examined the expression of senescence markers p53 and p21 in the heart and hippocampus tissues. As expected, the expression level of p53 and p21 were upregulated in D-Gal-injected mice compared to those in the vehicle (Figure 7A-C heart, and F-H hippocampus). More importantly, their expression was significantly reduced in the Lico D treated group. These data suggest that Lico D ameliorates heart and hippocampal senescence in D-Gal-induced aging mice. Next, I analyzed the effect of Lico D on AMPK activation in the heart and hippocampus. There were no significant changes observed in the AMPK level of heart and hippocampus tissues of D-Gal-treated mice (Figure 7D,E hearts; and I, J hippocampus). However, when compared to the D-Gal treatment, AMPK activation was significantly increased in both tissues of Lico D. These results suggest that Lico D reduces senescence by activating AMPK in D-Gal-induced aging mice.

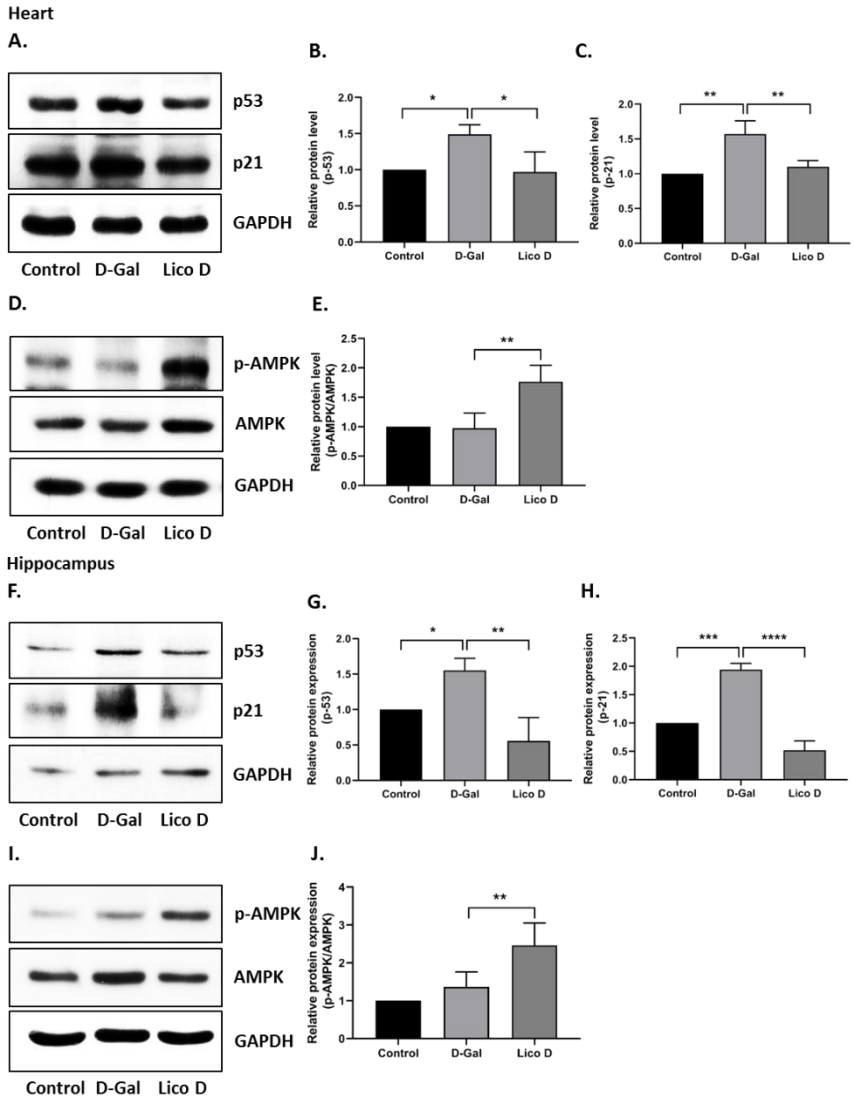

Figure 7. AMPK activation ameliorates the senescence in D-Gal-induced aging mice heart and hippocampal tissues. Representative images from immunoblot against p53, p21 and GAPDH from heart (A-C) and hippocampus (F-H) tissues. The representative images from immunoblot against AMPK, p-AMPK and GAPDH from heart (D,E) and hippocampus (I,J) tissues. The expression levels of proteins were quantified using Images J software. All data are represented as mean ± SD (n = 3 or 4) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

3.7. Lico D Activates Autophagy in D-Gal Induced Aging Mice Heart Tissue

To confirm the effect of Lico D on autophagy activation, I analyzed the expression levels of autophagy markers in the mouse model. As shown in the Figure 8A-D autophagy activation was significantly decreased in D-Gal-treated mice heart tissue compared to that in the control group. Lico D administration markedly increased the expression of these autophagy markers in heart tissue. Collectively, these results reinforce that Lico D reduces heart senescence by activating AMPK and autophagy in a D-Gal-induced aging mouse model.

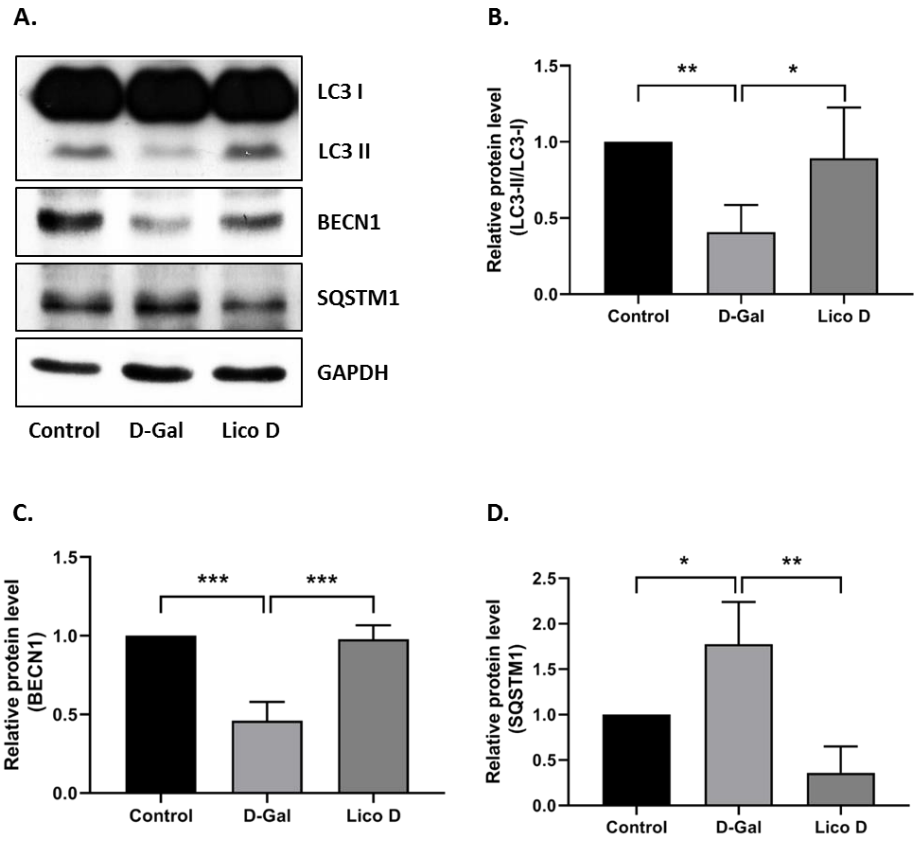

Figure 8. AMPK activation improves autophagy in D-Gal induced aging mice heart tissue. Representative images from immunoblot against LC3, BECN1, SQSTM1 and GAPDH from heart (A-D) tissue. The expression levels of proteins were quantified using Images J software. All data are represented as mean ± SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

4. Discussion

In this study, I found that Lico D can ameliorate senescence and aging, and obtained interesting findings: (i) Lico D reduced oxidative stress-induced premature senescence by AMPK activation both *in vitro* and *in vivo*; (ii) AMPK activation by Lico D can restore impaired autophagy under oxidative stress conditions; (iii) Lico D reduced RAGE expression in the hippocampal tissue. As AMPK is a master regulator of cellular metabolic homeostasis, the findings will be useful for further research on the role of AMPK activation in oxidative stress-induced senescence, which will hopefully contribute to reducing the burden of oxidative stress-induced cellular senescence during aging.

4.1. Lico D Ameliorated the Oxidative Stress Induced Premature Senescence via AMPK Activation

Owing to their multipotency, MSCs can be readily expanded and differentiated under appropriate condition *in vitro* and virtue of their trophic and growth factor-rich secretome, it can attract other cells necessary for tissue repair and help to create a regenerative environment *in vivo* (García-Prat et al., 2016; Ma, Qi, et al., 2018). Thus, MSCs are promising cell therapy candidates for regenerative medicine. In particular, the number of clinical trials has been growing in autologous stem cell therapy compared to allogenic applications (Revuelta and Matheu, 2017). However, the functionality and longevity of MSCs are reduced during ex vivo expansion, which is caused by increased levels of oxidative stress. Furthermore, these increased oxidative stresses may inhibit the proliferative and differentiation capacity of MSCs and direct them into senescence, resulting in reduced function and engraftment (Ho et al., 2017). Therefore, finding a way to reduce the accumulation of oxidative stress may enhance the benefits of MSCs in regenerative medicine.

As reported earlier, Lico D possesses antioxidant effects against various oxidative stress conditions both *in vitro* and *in vivo* (Haraguchi et al., 1998; Yuan et al., 2015). Therefore, in this study first I confirmed the antioxidant effects of Lico D against H_2O_2 in hBM-MSCs. Interestingly, I found that Lico D pretreatment increased the hBM-MSCs viability and reduced the ROS production as well as apoptotic cell death under oxidative stress conditions (Figure 2B-E). Oxidative stress is a type of SIPS that can develop

senescence faster than replicative senescence and has been extensively used to study the influence of extracellular and intracellular stress on the aging process. H_2O_2 and D-Gal are well-known inducers of oxidative stress *in vitro* and *in vivo* respectively (J. H. Chen et al., 2004; Han et al., 2016; Q. Li et al., 2018; Nam et al., 2018; Sun et al., 2020; Sun et al., 2018; Z. Wang et al., 2013b). The present study was designed to assess the anti-senescence effect of Lico D in H_2O_2 induced senescence model *in vitro* and a D-Gal-induced senescence model *in vivo*. Due to the heterogeneous nature of senescent cells and the lack of specific markers, a combination of multiple techniques has been used to identify the senescent cells. In the study, SA-β-Gal staining and senescence markers, such as p21 and p53 were evaluated. The findings confirmed that Lico D significantly reduced the accumulation of SA-β-Gal positive cells in vitro, as well as the reduction of the senescence markers p21 and p53 observed in both models (Figures 4 and 7).

AMPK activation plays an important role in cellular senescence and aging (Yu et al., 2021). The AMPK pathway is downregulated in oxidative stress-induced senescence, whereas pharmacological activation of AMPK or swimming exercise training may increase AMPK and inhibit senescence-associated pathways (Han et al., 2016; Lin et al., 2020). Therefore, in this study, I attempted to determine the importance of AMPK under oxidative stress conditions. In the *in vitro* senescence model, AMPK activation was significantly reduced, whereas the reduction was greatly restored upon Lico D treatment (Figure 4E,F). To confirm the above finding, I used Compound C, a potent inhibitor of AMPK, and obtained consistent data with a previous report (Han et al., 2016). I used the D-Gal-induced aging mice model, which is a widely used artificial aging model to obtain a better understanding of senescence and aging *in vivo*. Chronic administration of D-Gal induces degenerative changes in many tissues and organs, particularly in the heart and brain tissues were deeply focused (Azman and Zakaria, 2019; Bo-Htay et al., 2018; Shwe et al., 2018). Hence, I chose the heart and hippocampus tissues for the molecular experiments. The results demonstrated that Lico D administration significantly upregulated AMPK in these tissues, whereas no changes were observed in D-Gal-treated mice (Figure 7D,I). Therefore, the findings strongly support the anti-senescence activity of Lico D against oxidative stress via AMPK activation. In general, AMPK activation is thought to be mediated by two distinct kinases, liver kinase B1 (LKB1) and calcium-

sensitive kinase CAMKK2, as well as other factors such as allosteric activators (e.g., A-769662, salicylate, etc.), energy status modifiers (starvation, exercise, mitochondrial poisons like metformin), and AMP mimetic (AICAR) (Herzig and Shaw, 2018). However, it remains unknown whether Lico D activates AMPK by one of the mechanisms mentioned above or by directly enhancing ROS scavenging activity and thus preventing AMPK inactivation. Considering this, activation of AMPK by Lico D requires further investigation.

4.2. Lico D Restored the Impaired Autophagy via AMPK Activation under Oxidative Stress

The anti-senescence effect of AMPK was closely associated with the induction of autophagy, which was determined by autophagic flux. Impairment of autophagic flux with lysosomal dysfunction was also observed in oxidative stress-induced senescence or H_2O_2 induced senescence (Han et al., 2016; Tai et al., 2017). In the results, I showed that Lico D treatment improved autophagy activation in the senescence model, which was detected by elevated level of LC3-II/LC3-I ratio and BECN1 and reduction of SQSTM1. Furthermore, the importance of AMPK in autophagy was also evaluated using Compound C, which supported the hypothesis that Lico D activates autophagy by activating AMPK in the senescence model. Most importantly, I observed a similar effect in D-Gal-induced aging mouse heart tissue (Figure 8), but not in the hippocampus. One of the possible mechanisms behind this effect is that AMPK-mediated RAGE inhibition may trigger the anti-senescence effect of Lico D in hippocampal tissue. Collectively, AMPK facilitated autophagy may be one of the mechanisms by Lico D, which can reduce oxidative stress-induced premature senescence *in vitro* and *in vivo*.

4.3. Lico D Reduced the RAGE Expression in Hippocampus

The receptor for advanced glycation end product (RAGE) was first characterized as a transmembrane receptor of the immunoglobulin superfamily in 1992 (Brett et al., 1993; Neeper et al., 1992). AGE activation is considered as a major mediator of AGE pathogenicity, and AGE-RAGE association is the most studied phenomenon for the induction of oxidation and inflammation (Ramasamy et al., 2005). Therefore, targeted

pharmacological interventions that can either inhibit or modulate the expression levels of AGE and RAGE, and their signaling could be a promising therapeutic strategy to slow down the development of aging and age-related diseases, including Alzheimer's disease (Pinkas and Aschner, 2016; Srikanth et al., 2011). Given the importance of RAGE in this process, I evaluated RAGE expression in hippocampal tissues. Surprisingly, the expression level of RAGE was reduced in the Lico D-treated mice (Figure 6C). Consistent with previous reports, activation of AMPK could inhibit the AGE-induced inflammatory response and suppress RAGE/NF-κB signaling (Ali et al., 2015; J. Lu et al., 2010; Ma, Ma, et al., 2018; Z. Zhou et al., 2016). Therefore, the findings suggest that Lico D-mediated AMPK activation is sufficient to reduce D-Gal-induced hippocampal RAGE expression. Overall, these results indicate that Lico D diminishes RAGE-mediated inflammation in D-Gal induced aging mouse hippocampal tissue.

In summary, using an oxidative stress-induced senescence model, I can able to provide evidence that the effect of Lico D on senescence and aging relies on both the activation of AMPK and autophagy as well as inhibition of RAGE-mediated inflammation. AMPK activation may involve multiple mechanisms in the cells to prevent oxidative stress-induced senescence and aging. This is the first report that Lico D activates AMPK and autophagy to reduce oxidative stress-induced senescence (Figure 9). These findings suggest that Lico D may be a possible therapeutic candidate for the prevention or treatment of age-related diseases.

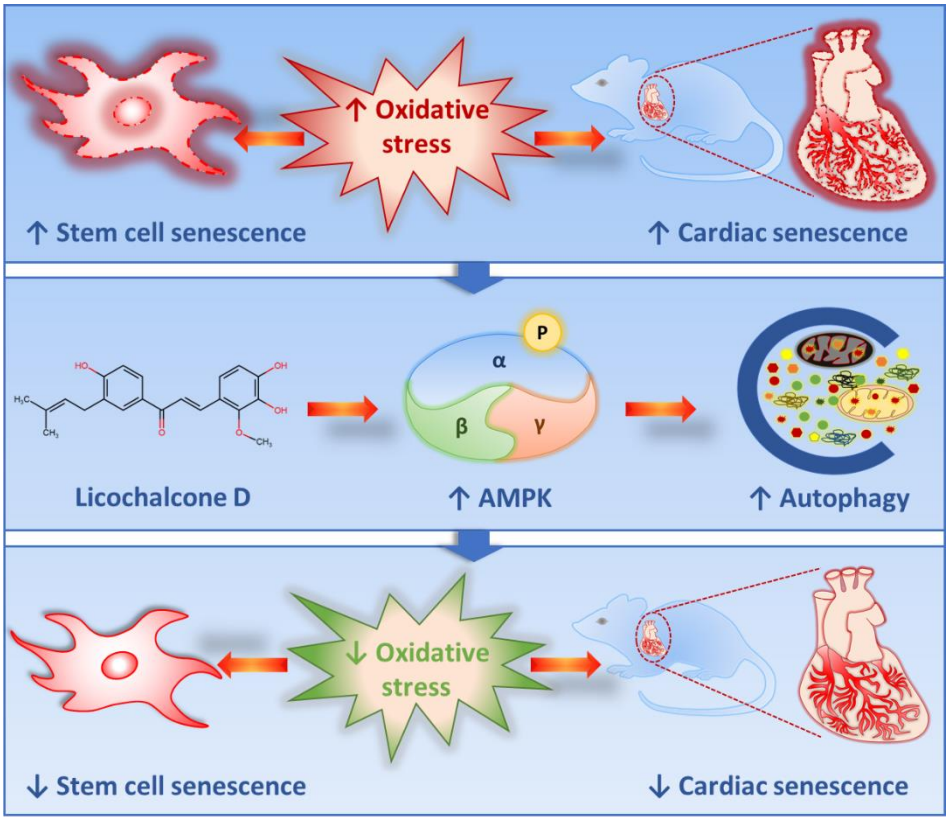

Figure 9. Lico D ameliorates oxidative stress induced senescence in stem cells and aging mice via AMPK/autophagy. An increased oxidative stress can promotes the senescence in stem cells and mice heart tissues. Lico D mediated AMPK and autophagy activation may reduce the oxidative stress as well as senescence in stem cells and heart tissues.

CHAPTER 2. Camphorquinone promotes the anti-senescence effect via activating AMPK/SIRT1 in stem cell and D-Galactose-induced aging mice*

1. Introduction

Cellular senescence is a state of persistent cell cycle arrest that is considered to be a hallmark of aging (A. Hernandez-Segura et al., 2018; Lopez-Otin et al., 2013; Lozano-Torres et al., 2019). Senescent cells are important in maintaining tissue homeostasis and act as a powerful defense against cancer. Pathological conditions in senescent cells, on the other hand, increase aging and aging-related illnesses (Lunyak et al., 2017; van Deursen, 2014). Therefore, researchers are searching for new therapeutic medications or targets to eradicate or reduce the negative consequences of these senescent cells while animal's age (Di Micco et al., 2021; M. Xu et al., 2018). A variety of senescence models, including oxidative stress-induced senescence model have been adopted to better understand the molecular mechanisms underlying senescence. Oxidative stress is a type of stress-induced premature senescence (SIPS) that can be utilized to investigate the mechanism of cellular senescence both *in vitro* and *in vivo* (Bo-Htay et al., 2018; Maharajan et al., 2021; Sun et al., 2018; Z. Wang et al., 2013b). Activation of AMPK, SIRT1, and autophagy mechanisms has been shown to prevent oxidative stress-induced senescence (Y. F. Li et al., 2018; Maharajan et al., 2021; Tai et al., 2017). To alleviate oxidative stress-induced senescence and aging, a novel medication or compound that can activate AMPK, SIRT1, and autophagy is required.

Terpenes are a large class of plant secondary metabolites, accounting for about 55% of total secondary metabolites (Brahmkshatriya and Brahmkshatriya, 2013). Terpenoids and their derivatives have a wide range of pharmacological and physiological properties and act as potential geroprotectors (Brahmkshatriya and Brahmkshatriya, 2013; Jaeger

* Modified from the published article: Maharajan, N.; Cho, G.-W. Camphorquinone Promotes the Antisenescence Effect via Activating AMPK/SIRT1 in Stem Cells and D-Galactose-Induced Aging Mice. Antioxidants 2021, 10, 1916. https://doi.org/10.3390/antiox10121916.

and Cuny, 2016; Proshkina et al., 2020). Based on the number of carbon units, they can be classified as hemiterpenes, monoterpenes, sesquiterpenes, or diterpenes. Monoterpenes are isoprene dimers ($C_{10}H_{16}$) that are structurally classified as either acyclic, mono, or bicyclic. Camphor is a bicyclic monoterpene (1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one) found in essential oils from various herbs, including rosemary, lavender, and sage, and is used in traditional Chinese medicine to treat sprains, swelling, and inflammation (Al-Qudsi et al., 2012; T. A. Tran et al., 2015). The anti-aging property of camphor has been shown to boost the proliferation and anti-senescence effects of human primary dermal fibroblast cells, as well as block UV-induced wrinkles in mouse skin (T. A. Tran et al., 2015). Camphorquinone (CQ) is a bicyclic monoterpene (1,7,7-Trimethylbicyclo[2.2.1]heptan-2,3-dione) (Figure 10A) that can be efficiently synthesized (~93%) from camphor using a two-step process that includes camphor bromination and the oxidation of 3-brominated camphor, catalyzed by FE-porphyrins in the presence of air (Noirbent and Dumur, 2021; J. Wang et al., 2013). Therefore, I used CQ in this study to investigate the mechanisms underlying senescence and aging.

2. Materials and Methods

Cell Viability Assay

The cytotoxic and protective effects of CQ in hBM-MSCs were determined using the MTT assay according to the manufacturer's instructions. To determine the cytotoxic effect, the hBM-MSCs (8×10^3 cells per well) were grown in a 96-well plate. The cells were treated with different concentrations of CQ (1-8 μg/mL) for 12 h, followed by incubation with the MTT solution for 2 h, and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Cell viability was assessed using a spectrophotometer (Multiskan FC, Thermo Fisher Scientific). For assessing the protective effect of CQ, cells were pre-treated with 1 μg/mL (6.016 μM) CQ for 12 h, followed by incubation with H_2O_2 (0.7 mM) for 1 h. Following H_2O_2 treatment, cells were treated with MTT solution for 2 h, and the cell viability was evaluated.

Oxidative Stress-Induced Senescence

H_2O_2 was used to instigate oxidative stress-induced cell cycle arrest and senescence, as described previously (Maharajan et al., 2021). Briefly, the cells were exposed to 200 μM H_2O_2 for 2 h and cultured for 2 days in normal media. For the second round of H_2O_2 treatment, the cells were split in a ratio of 1:3, exposed to 200 μM H_2O_2 for 2 h, and cultured with either normal media (control), CQ (1 μg/mL), ascorbic acid (500 μM AA; a positive control), or compound C (0.5 μM; AMPK inhibitor) for 72 h. The SA-β-gal assay and immunoblot analysis were used to identify cellular senescence.

Animals and Administration of Drugs

Six-week-old male mice (C57BL/6; 22 ± 2 g) were obtained from Samtako Bio Korea Co., Ltd. (Osan, Gyeonggi, Korea) and housed in a pathogen-free animal facility center maintained at 23-25 ℃ with a 12 hour light/dark cycle and free access to drink water and food. The Institutional Animal Care and Use Committee of Chosun University (CIACUC2020-A0009) approved the animal experiments. The mice were randomly assigned into three groups (n = 4) after a week of acclimation: control group (PBS alone), D-Gal model group (administered D-Gal at 150 mg/kg/day), and D-Gal + CQ group (administered D-Gal + CQ at 5 mg/kg/day). The mice were administered either a PBS or D-Gal intraperitoneal injection for 10 weeks or were injected with CQ for 8 weeks starting

from the 3rd week of D-Gal injection (Figure 12A). Mice were sacrificed at the end of the experiments and heart tissues were isolated, snap-frozen in liquid nitrogen, and maintained at -80 °C until further use.

Statistical Analyses

All data are presented as the mean ± standard deviation of at least three or more biological replicates. The differences between data sets were assessed via Student's t-test and analysis of variance (ANOVA) with Holm-Sidak's multiple comparison test using GraphPad Prism (GraphPad Software). Statistical significance levels are indicated by asterisks as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3. Results

3.1. CQ Protects hBM-MSCs Against Oxidative Stress-induced Senescence

To investigate the effect of CQ on cell viability, hBM-MSCs were treated with 0-8 μg/mL (0-48.13 μM) CQ for 12 h and cell viability was evaluated using the MTT assay. As shown in Figure 10B, there were no significant changes in cell viability up to 4 μg/mL but viability was reduced at 8 μg/mL. I selected 1 μg/mL concentration (6.016 μM) for further experiments. Next, I evaluated the antioxidant properties of CQ against H_2O_2 (0.7 mM). CQ increased the viability of H_2O_2-treated hBM-MSCs (Figure 10C). Furthermore, I evaluated the effect of CQ on oxidative stress-induced senescence, as described previously (Maharajan et al., 2021). As shown in Figure 10D and E, the senescent cells became enlarged, flattened, and highly SA-β-gal-positive (48%) when compared with single-treated hBM-MSCs (11%). Therefore, I chose double-time H_2O_2 treatment for further studies. The number of SA-β-gal-positive cells was considerably reduced upon treatment with either CQ (16%) or ascorbic acid (19%, a positive control). Furthermore, the anti-senescence effects of CQ were evaluated on the basis of the expression of well-known senescence markers, including p53, p16, and p21 in the senescence model. As shown in Figure 10F-I, the expression of senescence markers was increased in double-time H_2O_2-treated hBM-MSCs, while it was reduced in CQ-treated hBM-MSCs. These results suggest that CQ protects hBM-MSCs from oxidative stress-induced senescence.

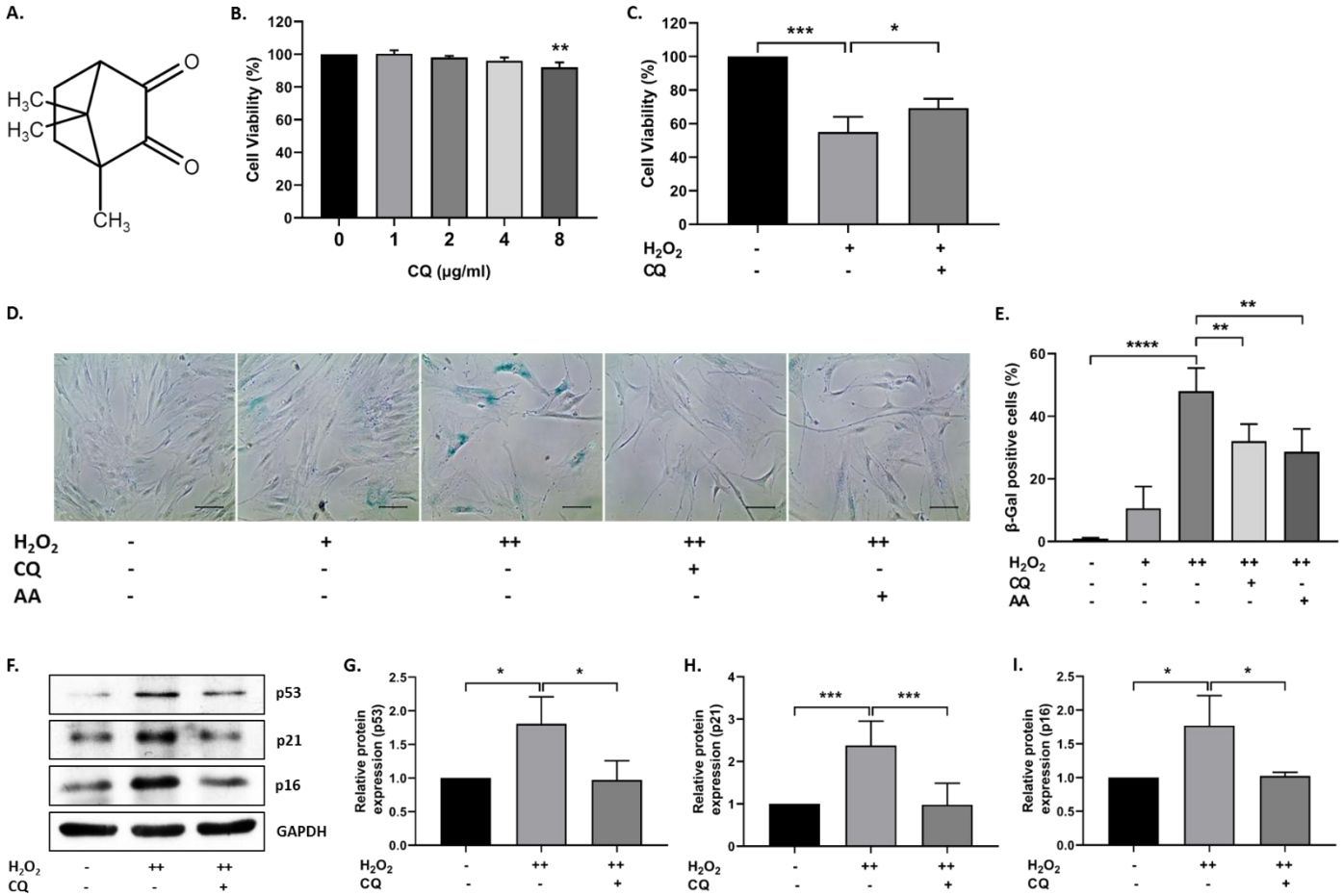

Figure 10. Effects of CQ on oxidative stress-induced senescence. (A) Chemical structure of camphorquinone. (B) Effect of CQ on cell viability was measured using the MTT assay (n = 3). (C) Protective effect of CQ (1 μg/mL) against H_2O_2 (0.7 mM) was confirmed via the MTT assay. (D) Representative image of SA-β-gal-positive cells in either single- or double-time H_2O_2 (200 μM) treatment and (E) percentage of SA-β-gal-positive cells in the oxidative stress-induced senescence model (n = 4). (F) Representative images from the immunoblot analysis against the senescence markers p53, p21, and p16. (G-I) The expression levels of target proteins were quantified using the ImageJ software. GAPDH was used as an internal control. H_2O_2 (+) represents a one-time treatment and H_2O_2 (++) represents double-time treatment. The scale bar represents 100 μm. All data are presented as mean ± standard deviation (SD) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3.2. Activation of AMPK, SIRT1, and Autophagy Reduces Oxidative Stress-induced Senescence

Previously, it was reported that AMPK-mediated autophagy could protect cells from oxidative stress-induced senescence (Han et al., 2016; Maharajan et al., 2021). In the senescence cell model, I tested whether CQ can activate AMPK and autophagy. As shown in Figure 11A-C, AMPK and SIRT1 activation was increased in CQ-treated hBM-MSCs, while AMPK activation was significantly inhibited by compound C (an AMPK inhibitor) but not SIRT1. Next, I evaluated the autophagy mechanisms in the senescence cell model. As shown in Figure 11D-G, CQ increased the LC3-II/LC3-I ratio and BECN1 expression, while it reduced the SQSTM1 expression in our senescence model. Collectively, these data indicate that CQ activates AMPK and SIRT1 mediates autophagy in the oxidative-stress-induced senescent cells, while AMPK inhibition by CC slightly reduced the autophagy markers.

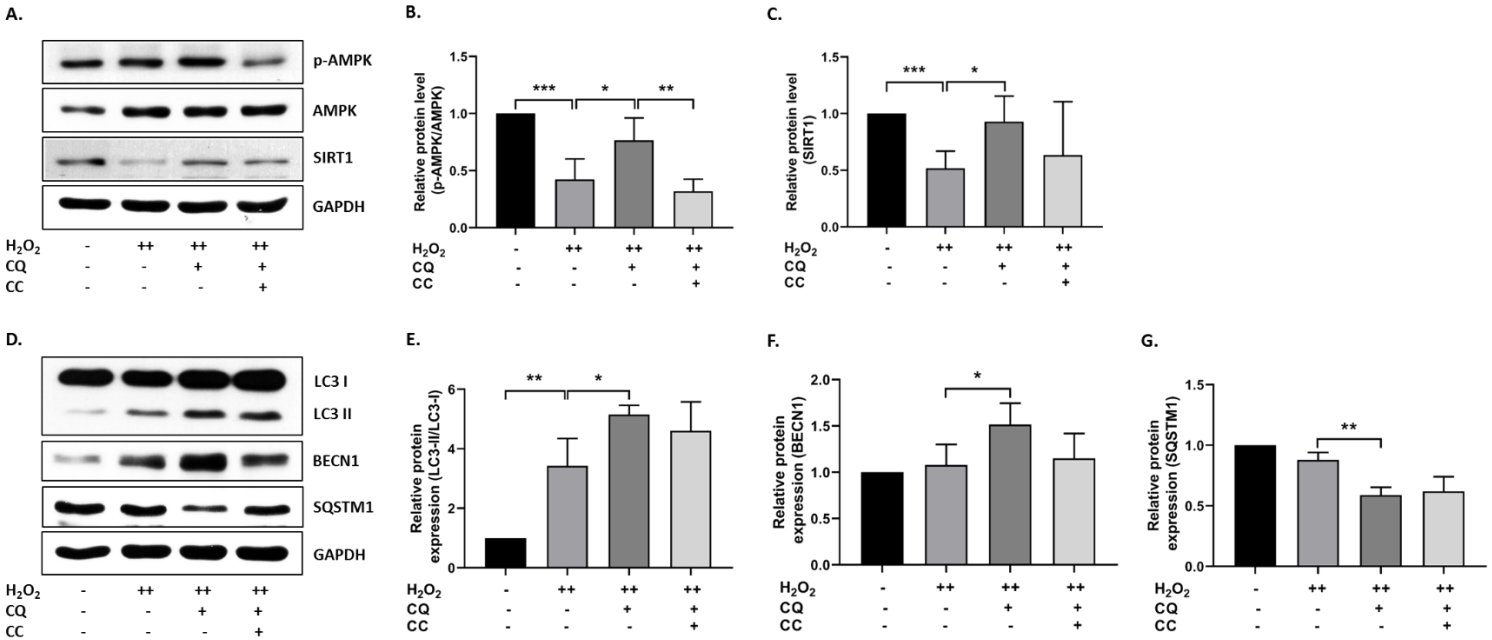

Figure 11. CQ increases autophagy in oxidative stress-induced senescent hBM-MSCs. (A-C) Representative images from the immunoblot analysis against AMPK, phosphorylated AMPK and SIRT1. (D-G) Representative images from the immunoblot analysis against autophagy markers including LC3, BECN1, and SQSTM1. Compound C (CC), an AMPK inhibitor was used. H_2O_2 (++) represents double time treatment. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

3.3. CQ Diminishes Cardiac Senescence in D-Gal-induced Aging Mice

First, I evaluated the antioxidant effect of CQ in D-Gal-induced aging mouse heart tissue. As shown in Figure 12B-E, SOD1 and SOD2 expressions did not change considerably, while catalase expression increased significantly in the D-Gal-induced aging mouse heart tissue. Next, I evaluated the senescence markers in mouse heart tissue, because long-term administration of D-Gal increased oxidative stress and influenced cardiac senescence in mice (Hong et al., 2021; Maharajan et al., 2021). As expected, the expression of senescence markers p53 and p21 was increased in D-Gal-treated mouse heart tissue, while it was considerably reduced in CQ-treated mice (Figure 12F-H). These results suggest that CQ exhibits an anti-senescence effect and partially increases antioxidant level in D-Gal-treated mice.

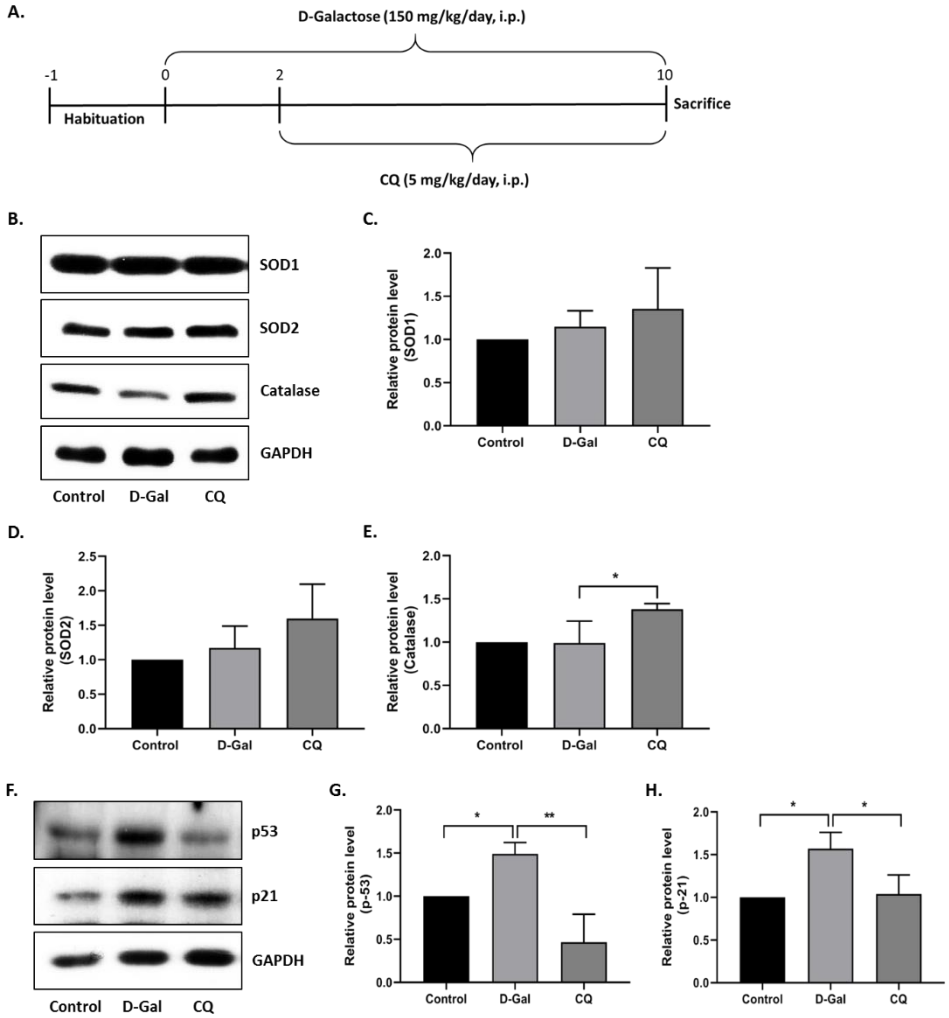

Figure 12. CQ ameliorates senescence in heart tissue. (A) Schematic representation of the experimental plan. (B-E) Representative images from the immunoblot analysis against the antioxidant enzymes SOD1, SOD2, and Catalase from heart tissue. (F-H) Representative images from the immunoblot assay against p53, p21, and GAPDH from heart tissue. Expression levels of proteins were quantified using the ImageJ software. All data are presented as mean SD (n = 3 or 4). * $p < 0.05$ and ** $p < 0.01$.

3.4. CQ Activates the AMPK/SIRT1 Autophagy Pathway in D-Gal-induced Aging Mouse Heart Tissue

Next, I examined the activation of AMPK, SIRT1, and autophagy pathways in heart tissue. AMPK and SIRT1 expression was remarkably elevated in CQ-treated mice, but no changes or a slight decrease was observed in the D-Gal-induced aging model mice (Figure 13A-C). Furthermore, I examined autophagy markers in the aging mouse model. As shown in Figure 13D-G, the CQ treatment increased LC3-II/LC3-I ratio and BECN1 expression and decreased SQSTM1 expression compared to that in the D-Gal-treated group. These findings indicate that CQ activates AMPK/SIRT1-mediated autophagy in cardiac tissues of D-Gal-induced aged mice.

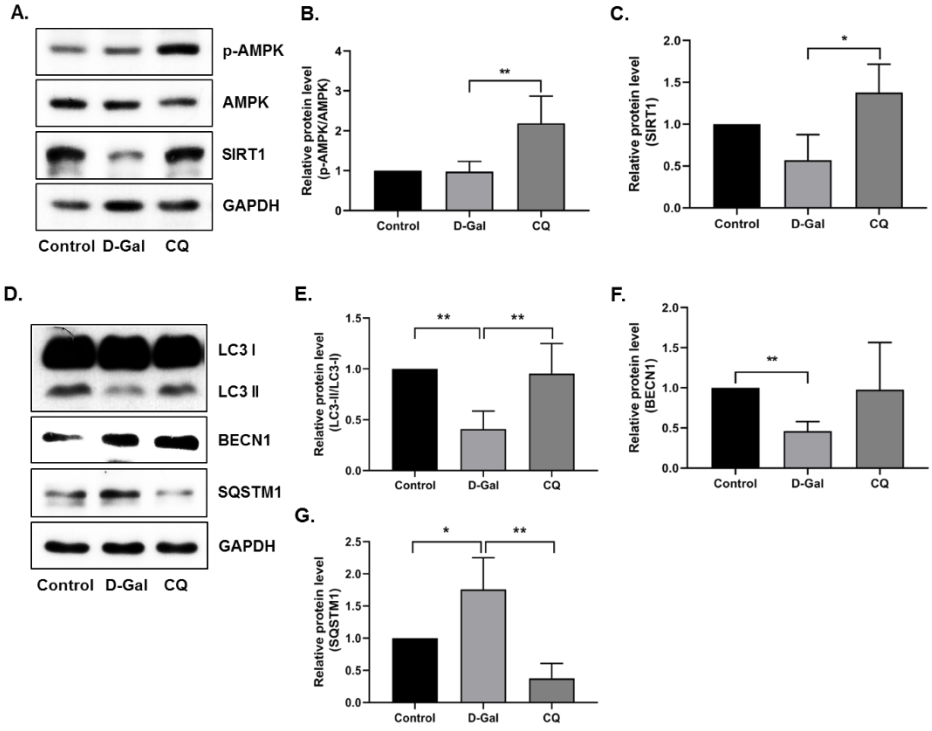

Figure 13. AMPK/SIRT1 activation improves autophagy in D-Gal-induced aging mouse heart tissue. (A-G) Representative images from the immunoblot analysis against LC3, BECN1, SQSTM1, and GAPDH from heart tissue. Expression levels of proteins were quantified using the ImageJ software. All data are presented as mean ± SD (n = 3). * $p < 0.05$; ** $p < 0.01$.

3.5. CQ Reduces Cardiac Inflammation in D-Gal-induced Aging Mice

Exogenous administration of D-Gal increases the accumulation of senescent cells in cardiac tissue and low-grade inflammation while reducing regenerative capacity, subsequently inducing aging and age-related diseases (Bo-Htay et al., 2018). With this in mind, I investigated cardiac inflammation markers in the aging mouse model. Results demonstrated that D-Gal treatment enhanced aging marker such as receptor for advanced glycation end product (RAGE) and the levels of inflammation markers (IL-1α, IL-1β, and IL-6) in D-Gal-induced aging model (Figure 14A-D), whereas CQ treatment markedly reduced the expression of these markers.

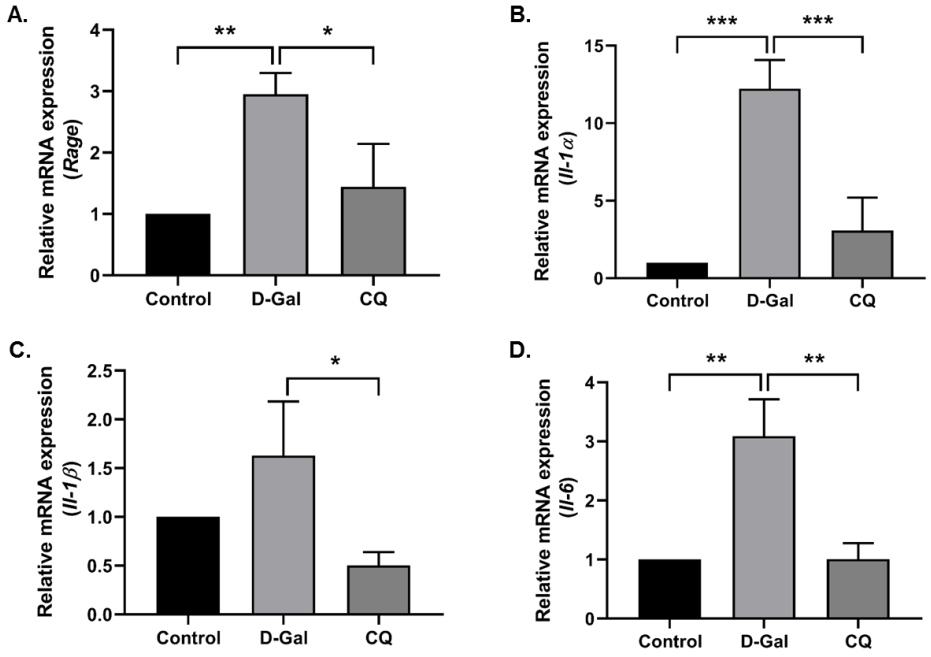

Figure 14. CQ reduces cardiac inflammation in D-Gal-induced aging mice. (A) RT-PCR for *Rage* and (B–D) inflammatory markers (*IL-1α, IL-1β, and IL-6*) in D-Gal-induced aging mouse heart tissue. All data are represented as mean ± SD (n = 3 or 4). * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$.

4. Discussion

In this study, I evaluated the effects of the monoterpenoid bicyclic compound camphorquinone on senescence and aging. The findings from this study were as follows: i. CQ reduced oxidative stress-induced senescence in stem cells and cardiac tissue and ii. CQ activated AMPK/SIRT1-mediated autophagy in both stem cells and D-Gal-induced aging mouse heart tissue.

4.1. CQ Improved Oxidative Stress-induced Senescence in Stem Cells

Adult stem cells are a promising tool for treating a variety of diseases, particularly in regenerative research. The main disadvantage of stem cells is that they may experience replicative senescence after a long period of replication (Jeong and Cho, 2016). Various intrinsic and extrinsic factors can accelerate stem cell aging (Maharajan et al., 2020). Recent studies have shown that low and moderate levels of oxidative stress influence stem cell fate, including quiescence, proliferation, and differentiation. In contrast, excessive ROS levels can damage nucleic acids, proteins, and lipids, ultimately causing stem cell senescence and accelerating aging and aging-related diseases (Chaudhari et al., 2014; Davalli et al., 2016; J. Lee et al., 2018). Antioxidant enzymes, including superoxide dismutase (SOD1 and SOD2), glutathione peroxidase (GPx), and catalase (CAT), play a crucial role in the defense system to regulate cellular redox balance under normal and pathological conditions (S. E. Lee and Park, 2021; Moret-Tatay et al., 2016). Defects in the antioxidant system or persistent accumulation of damage lead to the activation of p53/p21, which induces temporal cell cycle arrest by inhibiting cyclin E-Cdk2 (Pole et al., 2016; van Deursen, 2014). Cells may resume normal cell cycle progression if the damage is successfully repaired; otherwise, they may undergo permanent cell cycle arrest or senescence, as well as the release of pro-inflammatory cytokines (van Deursen, 2014).

Camphor has been reported to have anti-skin aging effect (T. A. Tran et al., 2015), anti-genotoxic effect at low concentrations (Nikolić et al., 2015) and anti-tumor activity (Moayedi et al., 2019). In this study, I used CQ, a camphor derivative that is widely used as a photoinitiator to trigger the polymerization process during dental composite preparation (Ikemura and Endo, 2010). CQ, at concentrations of 100-2000 μM, has been shown to enhance oxidative stress, inhibit cell cycle progression and differentiation, and

induce apoptosis and inflammatory cytokine production in human dental pulp cells (Chang et al., 2015; R. H. Kim et al., 2013). In general, several monoterpenes have cytoprotective and antimutagenic properties at low concentrations, whereas the opposite effect is observed at higher concentrations (Proshkina et al., 2020). With this in mind, in this study low concentration of CQ was used (Figure 10B; 1 μg/ml i.e., 6.016 μM), which do not have any toxic effect on cell viability, to investigate the role of CQ in senescence during aging. I employed an oxidative stress-induced senescence (H_2O_2) paradigm in hBM-MSCs to examine the mechanism of stress-induced premature senescence (SIPS) *in vitro* (Han et al., 2016; Maharajan et al., 2021). In the senescence model, the findings implied that CQ reduced the number of SA-β-Gal-positive cells and the expression of senescence markers, such as p16, p21, and p53 (Figure 10) (González-Gualda et al., 2021; A. Hernandez-Segura et al., 2018). Further, I investigated AMPK-mediated autophagy activation as a preventive mechanism that might diminish oxidative stress-induced senescence (Han et al., 2016; Maharajan et al., 2021). It has been proposed that AMPK activation induces autophagosome formation through Unc-51-like autophagy activating kinase (ULK1), while activation of both AMPK/SIRT1 upregulates the expression of autophagy-related genes (Atgs) through FOXO and PGC1α and downregulates mTORC1 (Escobar et al., 2019). Under caloric restriction conditions, reciprocal activation of the energy sensor molecules AMPK and SIRT1 is thought to inhibit mTOR and enhance autophagy activation (Cantó et al., 2010; Rubinsztein et al., 2011). Activation of the AMPK/SIRT1-FOXA3-Beclin1 pathway has been shown to not only activate autophagy but also prevent senescence and promote the proliferation of human umbilical vein endothelial cells (Q. Liu et al., 2020). Therefore, I analyzed AMPK/SIRT1 and autophagy in the senescence model. I found that activation of AMPK/SIRT1 and autophagy markers was increased upon treatment with CQ in the senescence model, while inhibition of AMPK slightly lowered the autophagy activation (Figure 11). SIRT1 activation, on the other hand, activates autophagy directly (Sacitharan et al., 2020). Therefore, activation of AMPK and SIRT1 may regulate the autophagy mechanism. However, in this study, we only utilized AMPK inhibitor but not SIRT1 inhibitor, which could explain why autophagy was not significantly lowered. It should be noted that SIRT1 activation might enhance autophagy and prevent apoptosis partly by deacetylation and degradation of p53

under oxidative stress conditions (T. Liu et al., 2018). This indicates that SIRT1 activation increases autophagy and reduces p53/p21-mediated senescence. Overall, these findings suggest that CQ activates AMPK/SIRT1-mediated autophagy and reduces oxidative stress-induced premature senescence in stem cells.

4.2. CQ Reduced the Senescence of D-Gal-induced Aging Mouse Heart Tissue

To better understand the action mechanism of CQ *in vivo*, I used D-galactose (D-Gal)-induced aging mouse model (Bo-Htay et al., 2018; Maharajan et al., 2021; Shwe et al., 2018). D-Gal is a monosaccharide molecule found in dairy products, peanuts, honey, cherries, and kiwi fruit. For healthy adults, the daily limit for D-galactose is 50 g. D-Gal is metabolized by galactose-metabolizing enzymes, such as galactose mutarotase, galactokinase, galactose1-phosphate uridyltransferase, and UDP-galactose epimerase. Excessive accumulation of D-Gal or alterations in the metabolizing enzymes might have a negative impact on the organism, leading to the onset of aging and age-related disorders (Bo-Htay et al., 2018; Bosch, 2006; Shwe et al., 2018). Herein, I used D-Gal-induced aging mouse model to investigate the effect of CQ on cardiac aging and senescence.

First, I examined the antioxidant effect of CQ on the D-Gal-induced aging mouse heart tissue. D-Gal can be oxidized by galactose oxidase to form hydrogen peroxide and impair redox homeostasis, resulting in increased oxidative stress and inflammation in the brain and heart (Bo-Htay et al., 2018; Shwe et al., 2018). I found that CQ significantly increased the level of the antioxidant enzyme catalase in heart tissue; however, no changes were observed in SOD1 and SOD2 levels (Figure 12). One probable mechanism is that a high quantity of D-Gal can be oxidized by galactose oxidase to produce H_2O_2, which can subsequently be reduced or detoxified by GPx and catalase (Arteaga et al., 2017; Bo-Htay et al., 2018); however, SOD converts superoxide radicals into H_2O_2 (Arteaga et al., 2017). It also been proposed that activation of SIRT1 upregulates the antioxidant enzymes SOD2 and catalase via deacetylation of FOXO4 and, as a result, inhibits ROS production (Cheng et al., 2014). Therefore, activation of SIRT1 by CQ is one of the possible reasons why catalase was increased in this study. However, I could not see any changes in the catalase expression in D-Gal treated mice compared with the control group. Next, I evaluated senescence markers in the aging mouse model. Long-

term administration of D-Gal causes cardiac dysfunction in heart tissues by increasing cardiac senescence, oxidative stress, inflammation, apoptosis, and decreasing the levels of antioxidants, as well as altering calcium homeostasis (Bo-Htay et al., 2018; Maharajan et al., 2021). I found that the levels of senescence markers, including p53 and p21 were significantly reduced in the aging mouse model upon CQ administration (Figure 12). I previously observed that AMPK-mediated autophagy could reduce cardiac senescence in D-Gal-induced aging mice (Maharajan et al., 2021). In the present study, I found that CQ activated AMPK/SIRT1 and autophagy in the mouse model (Figure 13). Furthermore, excessive accumulation of D-Gal can increase ROS production via the Maillard reaction, which converts D-Gal to advanced glycation end products (AGEs). The AGE molecules can bind to a receptor called receptor for advanced glycation end product (RAGE), which can enhance ROS production via NADPH oxidase. NADPH increased the activity of p38 MAP kinase, thereby causing NF-κB to translocate to the nucleus and increase the expression of inflammatory genes (Bo-Htay et al., 2018; Frimat et al., 2017). In the D-Gal-induced aging mouse model, I found that CQ remarkably reduced heart aging and inflammatory markers level (Figure 14).

In conclusion, this is the first-time report in which CQ has been shown to diminish senescence and activate antiaging molecular markers AMPK and SIRT1, autophagy mechanism, and reduce cardiac tissue inflammation during oxidative stress (Figure 15). However, various kinases including CaMKKβ and LKB1, and metabolic sensors, such as AMP and NAD^+ have been shown to activate the AMPK/SIRT1 pathway. Therefore, it is crucial to determine how CQ activates the AMPK/SIRT1 pathway under oxidative stress-induced senescence conditions.

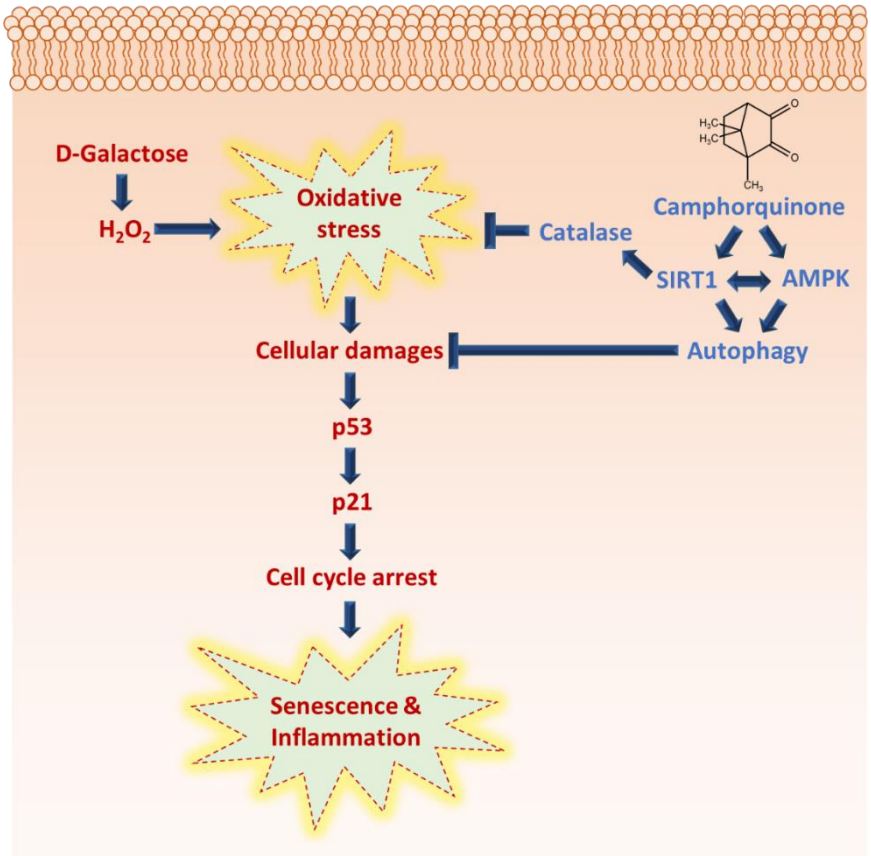

Figure 15. Camphorquinone ameliorates the oxidative stress-induced senescence via activation of AMPK/SIRT1 and autophagy mechanism. D-Galactose and H_2O_2 increase oxidative stress which can inhibit the cell cycle progression and induce cellular senescence via activation of p53/p21 pathway. However, the accumulation of senescent cells with age may induce the further production of senescence-associated secretory phenotype (SASP), which may trigger the neighboring cells to induce senescence. CQ mediated activation of AMPK, SIRT1 and autophagy mechanism may reduce the oxidative stress-induced senescence and SASP productions. Additionally, SIRT1 activation may inhibit the oxidative stress via activating antioxidant enzyme catalase.

PART 2. EFFECTS OF LICOCHALCONE D AND CAMPHORQUINONE ON HIGH FAT DIET AND STREPTOZOTOCIN-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE AND TYPE 2 DIABETES MICE

CHAPTER 1. Licochalcone D regulates blood glucose and ameliorates fatty liver disease via AMPK/SIRT1 signaling in high fat diet/streptozotocin-induced NAFLD/T2D mice

1. Introduction

Type 2 diabetes (T2D) is a chronic metabolic disorder characterized by hyperglycemia caused by impaired insulin secretion, insulin resistance or both. T2D is the most common type of diabetes, accounting for more than 90% of all diabetic patients (DeFronzo et al., 2015). Impaired insulin secretions are primarily caused by pancreatic β-cell dysfunction owing to glucotoxicity, lipotoxicity and resistance to incretins. On the other hand, insulin resistance is caused by a deficiency in the insulin signaling receptor, which leads to resistance in peripheral organs such as liver, muscle, and kidney, resulting in hyperglycemia. In T2D patients, the duration and severity of hyperglycemia determine the risk of both microvascular complications such as retinopathy, nephropathy and neuropathy as well as macro vascular complications including myocardial infarction, peripheral vascular disease and stroke (DeFronzo et al., 2015). Therefore, T2D is one of the world's most serious health problems; one in every eleven people are diagnosed globally (Bellary et al., 2021; Khan et al., 2020), and the number of people with T2D is projected to increase by >50% globally in 2045 (Perreault et al., 2021). Particularly, T2D accounts for about half of all individuals with diabetes in aged people (≥65 years) and heterogeneity in these patients have multiple comorbidities and complexities (Bellary et al., 2021). Over 7,000 trials are registered around the world, with many focusing on 'novel' pharmacological targets (Perreault et al., 2021).

Non-alcoholic fatty liver disease (NAFLD) is defined as ectopic lipid accumulation in the liver, in the absence of excessive alcohol intake. NAFLD has emerged as the most prevalent liver disease in the world, nearly 25.24% of the world's population might have NAFLD with annual direct medical costs of about $103 billion and €35 billion in USA and Europe (Germany, France, Italy, and United Kingdom) respectively (Ferguson and

Finck, 2021; Younossi, Blissett, et al., 2016; Younossi, Koenig, et al., 2016). Despite, the fact that no approved pharmaceutical medications to prevent or treat these illnesses have yet to be developed (Ferguson and Finck, 2021). NAFLD is strongly associated with obesity and T2D and approximate double the risk of T2D (Targher et al., 2021). NAFLD can also promote the development of T2D via exacerbating hepatic and peripheral insulin resistance as well as dyslipidaemia, which can cause the systemic release of pro-inflammatory cytokines and hepatokines (Smith and Adams, 2011; Targher et al., 2021). NAFLD and T2D are known to frequently coexist and act synergistically to increase the risk of adverse clinical outcomes including hepatic and extrahepatic diseases (hepatic cancer, CVD, and chronic kidney disease) (Targher et al., 2021). Therefore, T2D is considered as the most significant risk factor for the progression of NAFLD and the development of non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Ferguson and Finck, 2021; Targher et al., 2021). Hence, medications used in the treatment of T2D are also effective in the therapy of NAFLD.

AMP-activated protein kinase (AMPK) has received a lot of attention as a possible target for diseases associated with metabolic perturbation including diabetes, obesity, NAFLD/NASH, HCC and CVD (Day et al., 2017; DeFronzo et al., 2015; Steinberg and Carling, 2019). In particular, AMPK activation increases fatty acids oxidation, mitophagy and mitochondrial biogenesis in the liver thereby reducing the lipogenesis, cholesterol synthesis, fibrosis and insulin resistance (Steinberg and Carling, 2019). AMPK activation can also regulate the energy expenditure via increasing ATP and NAD^+ production (Cantó et al., 2009; Elhassan et al., 2017; Han et al., 2016). SIRT1 is a highly conserved NAD^+ dependent histone deacetylase that can protect against metabolic diseases such as obesity, insulin resistance, hepatic steatosis and aging-related neuronal diseases by deacetylating many transcription factors as well as cofactors (Nakagawa and Guarente, 2014). The studies have reported that, AMPK/SIRT1 activation can reduce the hepatic inflammation and hepatic steatosis by inhibiting NF-κB, ChREBP and SREBP1 activation as well as enhance the mitochondrial biogenesis and oxidation capacity by increasing the PGC1α activation (M. Foretz and Viollet, 2011; Xie et al., 2020). Therefore, activation of AMPK/SIRT1 pathway could be used to treat or develop drugs for metabolic syndrome, such as diabetes and NAFLD.

Flavonoids are polyphenolic compounds found in fruits, vegetables, and beverages that are produced as secondary metabolites by plants (Van De Wier et al., 2017). Over 9000 flavonoids have been identified in various plants and have been shown to have positive effects on insulin resistance, lipid metabolism, oxidative stress, and inflammation, all of which are common factors for the progression of NAFLD and T2D (Fang et al., 2019; Van De Wier et al., 2017; Y. Wang et al., 2011). A recent study of elderly overweight/obese Chinese population showed that increasing flavonoid intake lowered the risk of NAFLD progression (Zhong et al., 2021). Licochalcone D is a flavonoid compound that was isolated as a prenylated retrochalcone from the root of licorice (*Glycyrrhiza inflata*) in 1992 (Kajiyama et al., 1992; Okada et al., 1989). Lico D possesses the anti-oxidant, anti-inflammation, anti-allergic, anti-cancer, anti-viral, anti-senescence and cardioprotective properties (Furusawa et al., 2009; Haraguchi et al., 1998; Kajiyama et al., 1992; Maharajan et al., 2021; Mitra et al., 2021; Si et al., 2018; Tanifuji et al., 2010; Yuan et al., 2015). Previously, I have reported that Lico D activate AMPK under oxidative stress or D-Galactose-induced aging mice (Maharajan et al., 2021). AMPK/SIRT1 activation may also help to ameliorate the diabetes and NAFLD (S. Li et al., 2020; Liou et al., 2019; Liou et al., 2020). Therefore, in this study I have investigated the effects of Lico D on diabetes and NAFLD as well as explored its mechanisms.

2. Materials and Methods

Animals and Administration of Drugs

Six-week-old male C57BL/6 mice (weighing 22 ± 2 g) were purchased from Samtako Bio Korea Co., Ltd. (Osan, Gyeonggi, Korea) and maintained at 23-25 °C under a 12 h light and 12 h dark cycle with free access to food and water in a pathogen-free facility. The Institutional Animal Care and Use Committee of Chosun University (CIACUC2020-A0009-1) approved all animal experiments. Upon arrival, the animals underwent one week of acclimatization during which all animals were fed with normal chow pellets (ND, SAM #31, Samtako, Inc.). The animals were randomized according to their body weight. The following week, one-third of the animals were allocated to the normal control group, which fed with normal chow pellets throughout the study. The remaining animals were fed with high fat diet (HFD; 60% Kcal energy as fat) (Research diets; #D12492; New Brunswick, NJ 08901 USA) for four weeks to induce insulin resistance; the diet was continued until the end of the study. T2D was induced by administering STZ (30 mg/kg, i.p.) dissolved in 0.05 M (pH 4.5) citrate buffer for five consecutive days starting from fourth week, while normal control animals were given the same volume of citrate buffer. The animals were given a week to develop T2D. At the end of fifth week, blood glucose levels were measured and the animals with FBG ≥ 200 mg/dl (≥11.1 mmol/L) were considered as T2D (Gao et al., 2016; Yan et al., 2018; M. Zhang et al., 2008). The animals were divided into three groups: 1. normal control mice treated with saline; 2. diabetic control mice treated with saline; and 3. diabetic mice treated with Lico D (1 mg/kg/day; i.p.). All the treatments were carried out for three consecutive weeks. Until the end of the study, group 1 was given a normal chow pellet, whereas group 2 and 3 were fed a high fat diet. After three weeks of treatment, blood samples from the mouse tail were used to perform a fasting blood glucose level (FBG) and a glucose tolerance test (GTT). All the animals were sacrificed, and liver tissue was isolated, frozen immediately and stored in liquid nitrogen until needed.

3. Results

3.1. Lico D activates AMPK/SIRT1 in HepG2 cells in a dose-dependent manner

I have shown that Lico D can activate AMPK in oxidative stress-induced senescence model *in vitro* and *in vivo* (Maharajan et al., 2021). In liver tissue, the activation of both AMPK and SIRT1 can protect the hepatocytes from lipotoxicity and improve hepatic steatosis via regulating lipogenesis (S. Li et al., 2020; Liou et al., 2020). Therefore, I investigated the effect of Lico D on AMPK and its downstream target ACC as well as SIRT1 in HepG2 cells. I found that, Lico D increased AMPK and ACC phosphorylation as well as SIRT1 activation in a dose-dependent manner, with 10 μM of Lico D showing significant activation of these markers (Figure 16A-D). Therefore, I have chosen 10 μM of Lico D for further experiments. Additionally, I used compound C (CC; an AMPK inhibitor) to pharmacologically inhibit the AMPK activation. As shown in Figure 16E-H, CC treatment reduced the expression level of p-AMPK, p-ACC and SIRT1 in Lico D treated HepG2 cells. The findings imply that Lico D activate both AMPK and SIRT1 in HepG2 cells.

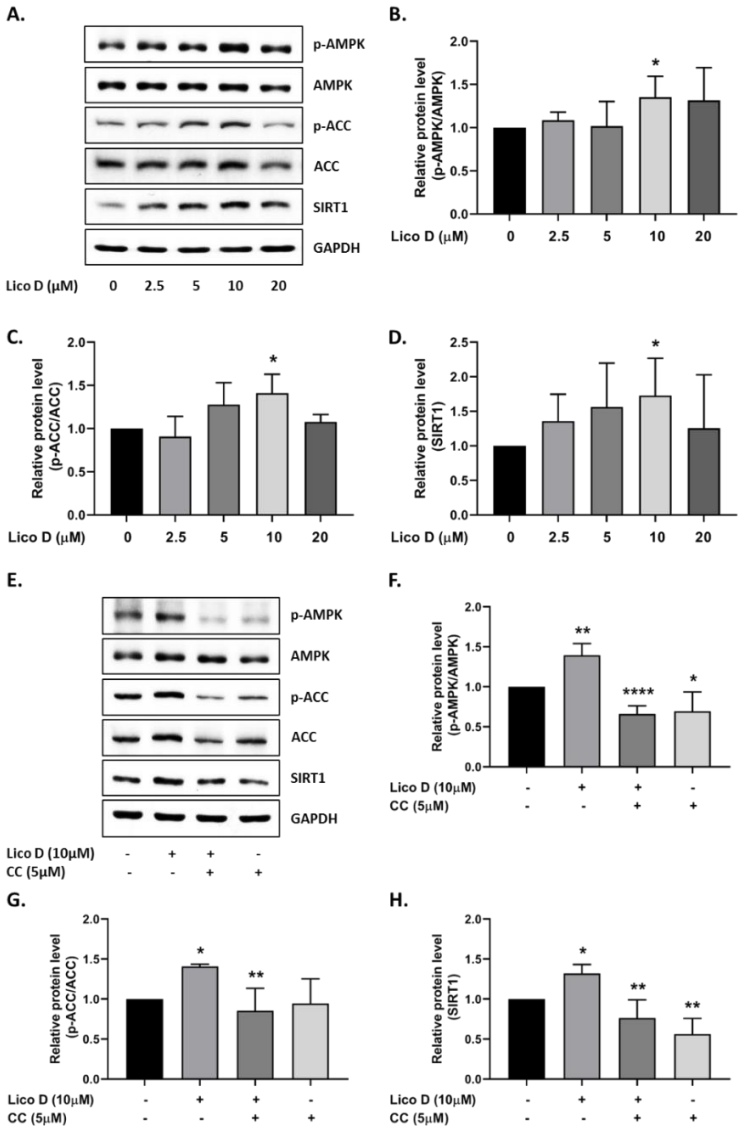

Figure 16. Lico D activates AMPK/SIRT1 in HepG2 cells. (A-D) Representative images from the immunoblot analysis of p-AMPK, p-ACC and SIRT1 activation in HepG2 cells. (E-H) Lico D mediated activation of AMPK, ACC and SIRT1 was reduced upon CC treatment. The expression of protein levels were quantified using Images J software. All data are represented as mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

3.2 Lico D attenuates lipid accumulation and activates AMPK/SIRT1 in FFAs-induced hepatic steatosis cell model

Next, I investigated the effect of Lico D on free fatty acids (FFAs)-induced hepatic lipid accumulation in HepG2 cells. The accumulation of lipid droplets in the FFAs treated cells were visualized and quantified using Oil Red O staining and isopropanol respectively. As shown in Figure 17A and B, FFAs treatment significantly increased the accumulation of lipid in the HepG2 cells, whereas Lico D treatment considerably attenuated the lipid accumulation. Further, I examined the expression of AMPK and SIRT1 protein levels in the FFAs-treated cell model. As shown in Figure 17C, Lico D restored the activation of AMPK, ACC and SIRT1 in FFAs treated cells. Together, the results demonstrate that Lico D effectively reduces the lipid accumulation and enhances the AMPK and SIRT1 activation in the FFAs-induced hepatic steatosis cell model.

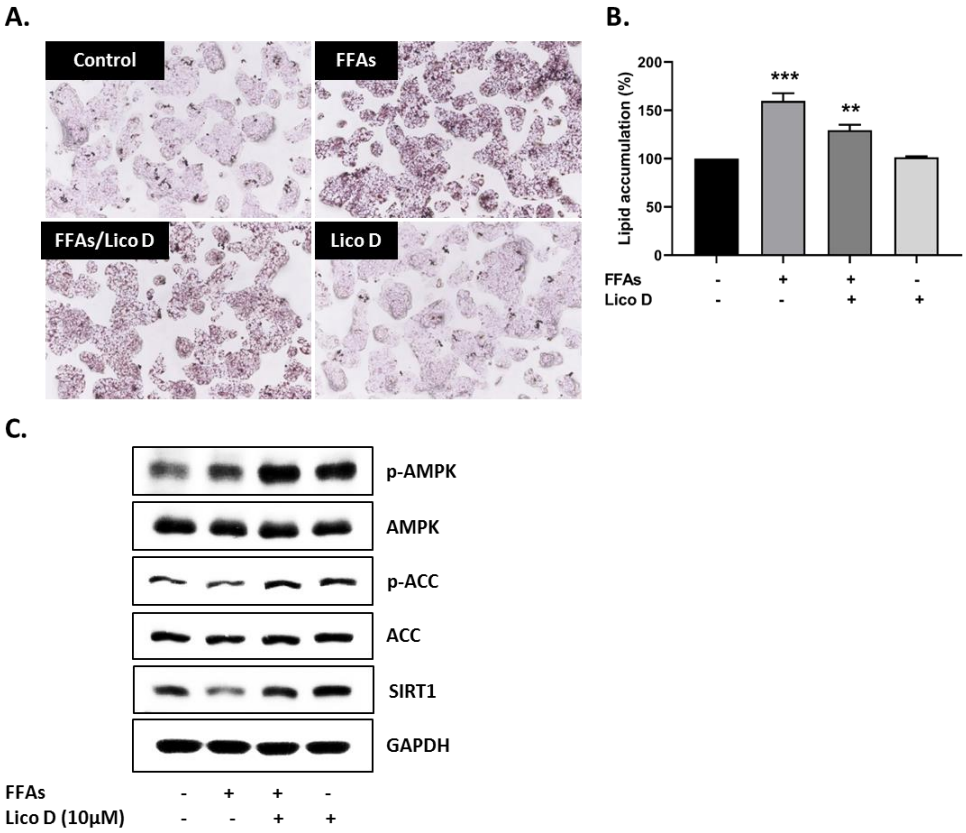

Figure 17. Lico D reduces lipid accumulation in HepG2 cells. (A,B) The accumulation of lipid droplets were visualized by Oil Red O staining and quantified by adding isopropanol to the lipid droplets in the hepatic steatosis cell model. (C) Lico D activates AMPK, SIRT1, and ACC in FFAs-treated HepG2 cells. The expression levels of proteins were quantified using Images J software. All data are represented as mean ± SD (n = 3). $^{**}p < 0.01$ and $^{***}p < 0.001$.

3.3 Lico D alters lipid metabolism in the hepatic steatosis cell model

Further, I examined whether Lico D can alter lipid metabolism in the hepatic steatosis cell model. I treated Lico D to the FFAs-induced hepatic steatosis cell model and evaluated the mRNA expression level of genes involved in the lipid metabolism. In an FFAs-treated cell model, I found that Lico D significantly reduced *ChREBP* mRNA expression (involved in lipid synthesis) while increased *CES1*, *CES2*, and *CPT1A* mRNA expression (involved in lipid oxidation) (Figure 18A). In addition, I evaluated mRNA expression level of inflammatory markers in the hepatic steatosis cell model. I found that Lico D reduced the mRNA level of inflammatory markers including *IL-1α* and *IL-6*, but no changes on *IL-8* expression (Figure 18B). The findings suggest that Lico D enhances the lipid oxidation and attenuates the lipid synthesis in the hepatic steatosis cell model.

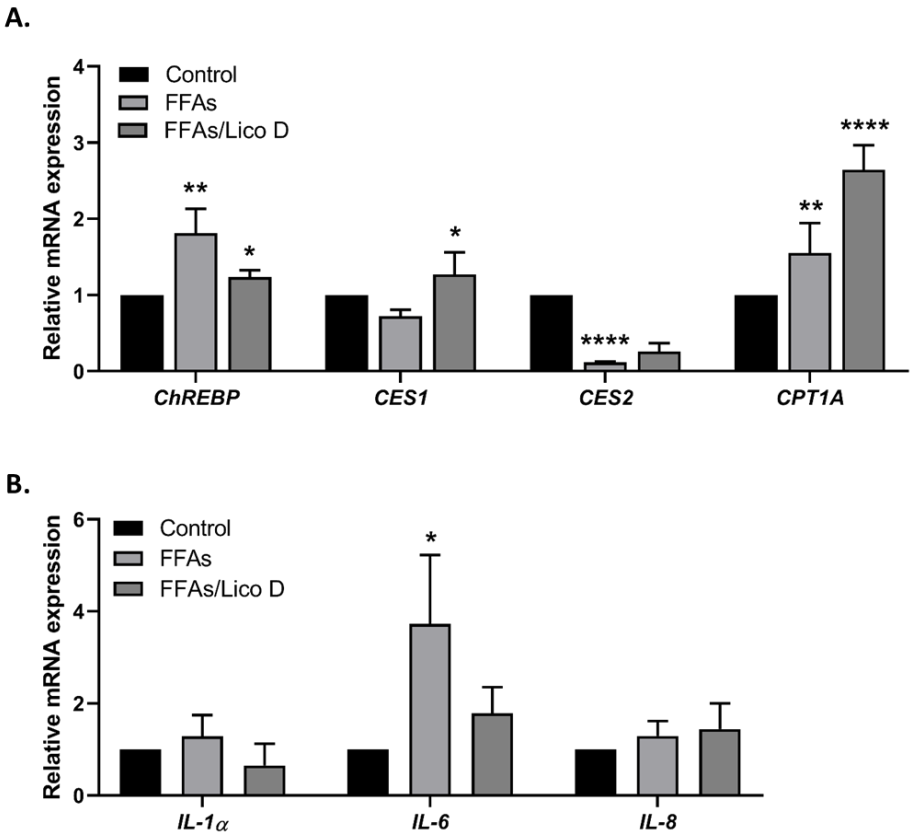

Figure 18. Lico D improves lipid metabolism in FFAs-treated HepG2 cells. (A) RT-PCR for lipid synthesis (*ChREBP*) and fatty acids oxidation (*CES1*, *CES2* and *CPT1A*) and (B) inflammation markers (*IL-1α*, *IL-6* and *IL-8*) in FFAs treated HepG2 cells. All data are represented as mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$.

3.4 Lico D administration reduces FBG level and improves glucose tolerance in HFD/STZ mice

To assess whether Lico D exerts *in vivo* therapeutic effects on NAFLD and T2D, I administered Lico D (1 mg/kg/day; i.p; for 3 weeks) to HFD/STZ mice (Figure 19A). Elevated glucose levels are the initial indicators of obesity induced type 2 diabetes mellitus, a promising risk factor of NAFLD (DeFronzo et al., 2015). Therefore, I evaluated the fasting blood glucose levels before and after STZ injection to confirm the mice model. As expected, fasting blood glucose levels were increased significantly before and after STZ injection, which indicate the development of pre-diabetic (≥ 150 mg/dl) and diabetic (≥ 200 mg/dl) conditions of before and after STZ injection respectively (Figure 19B). Additionally, I measured the FBG level and performed the glucose tolerance test (GTT) to evaluate the glucose tolerance and clearance (Rao et al., 2017). The data suggest that FBG levels were reduced significantly in Lico D treated mice at "0 min" (Figure 19C; "0 min") and improved glucose tolerance and clearance in HFD/STZ mice at "15, 30, 60, 90, 120 min" (Fig.19C). As shown in Figure 19D and E, the mouse body and liver weights were increased significantly in HFD/STZ treated mice when compared with control group. Surprisingly, mice treated with Lico D significantly reduced both body and liver weight in HFD/STZ mice. Collectively, the findings suggest that Lico D significantly improves the FBG level, improved glucose tolerance, and reduces body and liver weight in HFD/STZ mice.

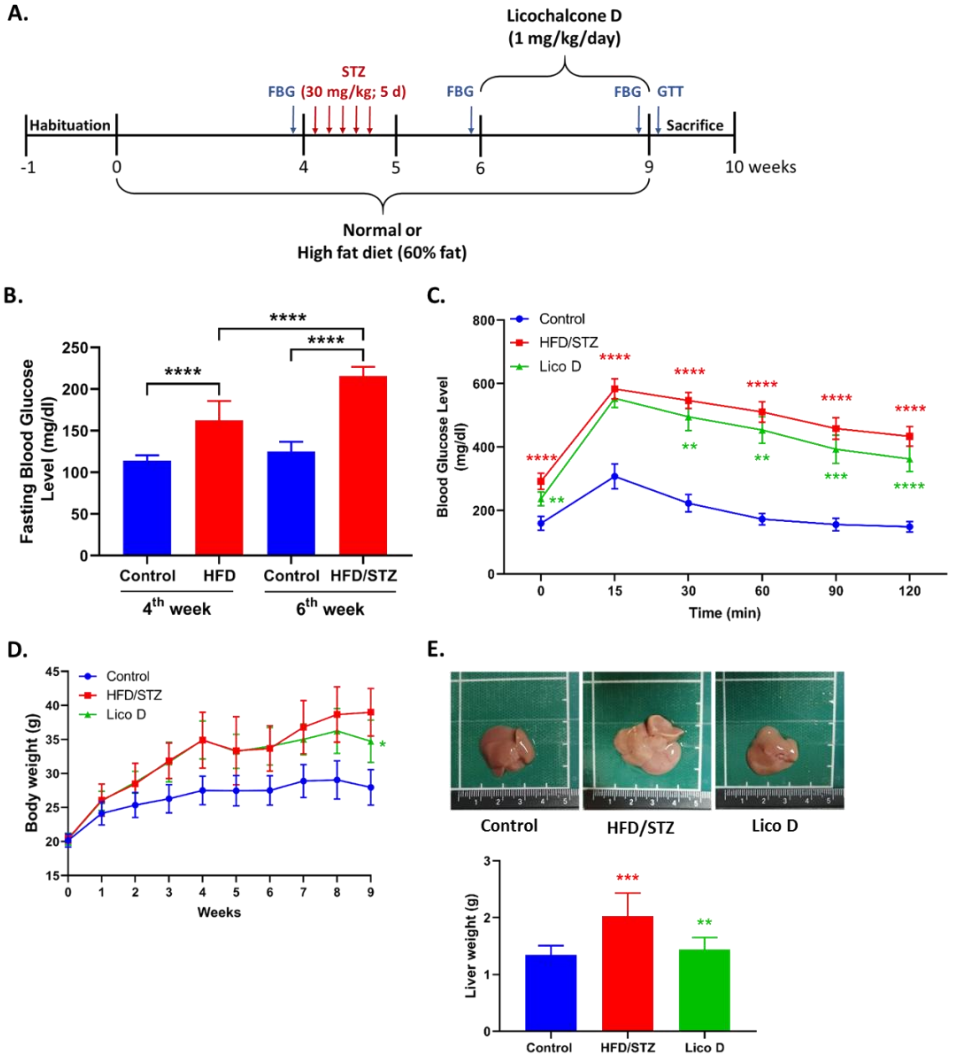

Figure 19. Lico D improves glucose tolerance and reduces body and liver weight. (A) Schematic representation of the experimental plan. (B) Fasting blood glucose level of mice before and after STZ injection and (C) glucose tolerance test (GTT) in HFD/STZ mice. (D,E) Body and liver weight of the mice. All data are represented as mean ± SD (n = 7). *p < 0.05, **p < 0.01, ***p <0.001, and ****p < 0.0001.

3.5 Lico D activates AMPK/SIRT1 pathway in HFD/STZ mice liver tissue

AMPK/SIRT1 activation may reduce the development of hepatic steatosis and regulate lipid and glucose metabolism in HFD induced NAFLD animal model (S. Li et al., 2020; Liou et al., 2020; Rao et al., 2017; Sharma et al., 2021). Next, I have evaluated the expression level of AMPK, SIRT1 and ACC in HFD/STZ mice liver tissue to confirm the *in vitro* findings. The mice treated with Lico D potentially increased the expression level of AMPK, SIRT1 and ACC compared to HFD/STZ mice (Figure 20). The data suggest that Lico D increases AMPK, SIRT1 and ACC activation in HFD/STZ mice.

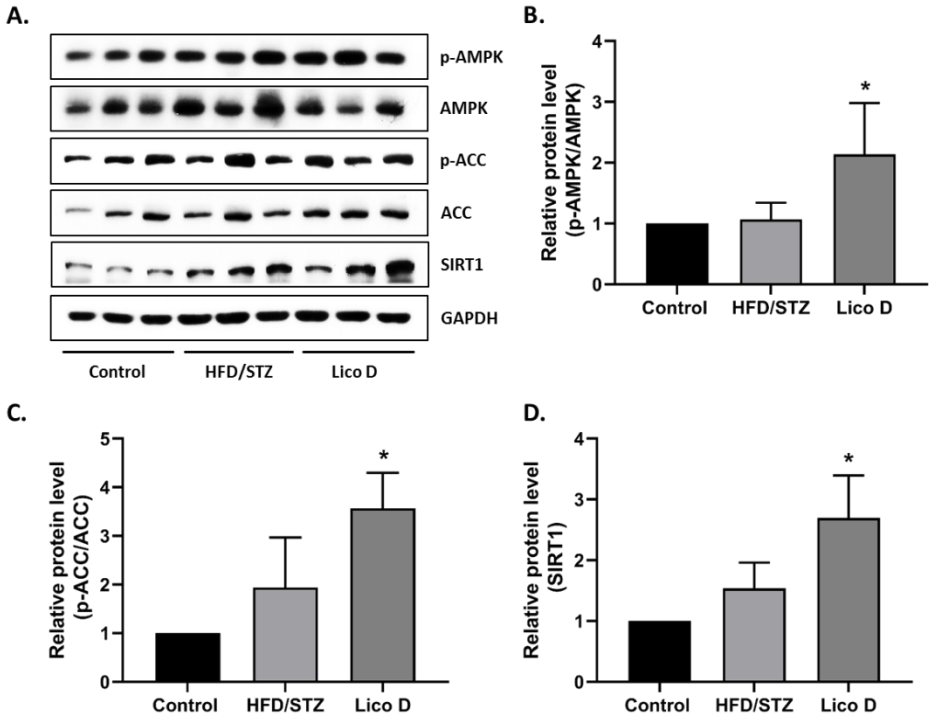

Figure 20. Lico D activates AMPK/SIRT1 pathway in HFD/STZ mice liver tissue. Representative images of immunoblot against AMPK, ACC, SIRT1, and GAPDH from Liver tissue. The expression levels of proteins were quantified using Images J software. All data are represented as mean ± SD (n = 5). *p < 0.05.

3.6 Lico D alters lipid metabolism and reduces inflammation in liver tissue

To investigate the effect of Lico D in liver lipid metabolism, I analyzed the level of gene expression involved in lipogenesis (*Srebp-1* and *Fasn*) and β-oxidation (*Cpt1a* and *Cpt1b*). Lico D administration decreased the expression of lipogenic markers while increased the expression of fatty acid oxidation markers, which was higher in HFD/STZ animals (Figure 21A). Furthermore, I assessed the expression of liver inflammation indicators such as *Il-1α, Il-1β, Il-6, p53, Tnfα,* and *Rage*. When compared to HFD/STZ mice, Lico D treated mice had significantly lower level of all inflammation markers, while *Tnfα* and *Rage* levels were slightly lower (Figure 21B). Collectively, the results suggest that Lico D potentially increases the β-oxidation and reduces lipogenesis and inflammation in HFD/STZ mice.

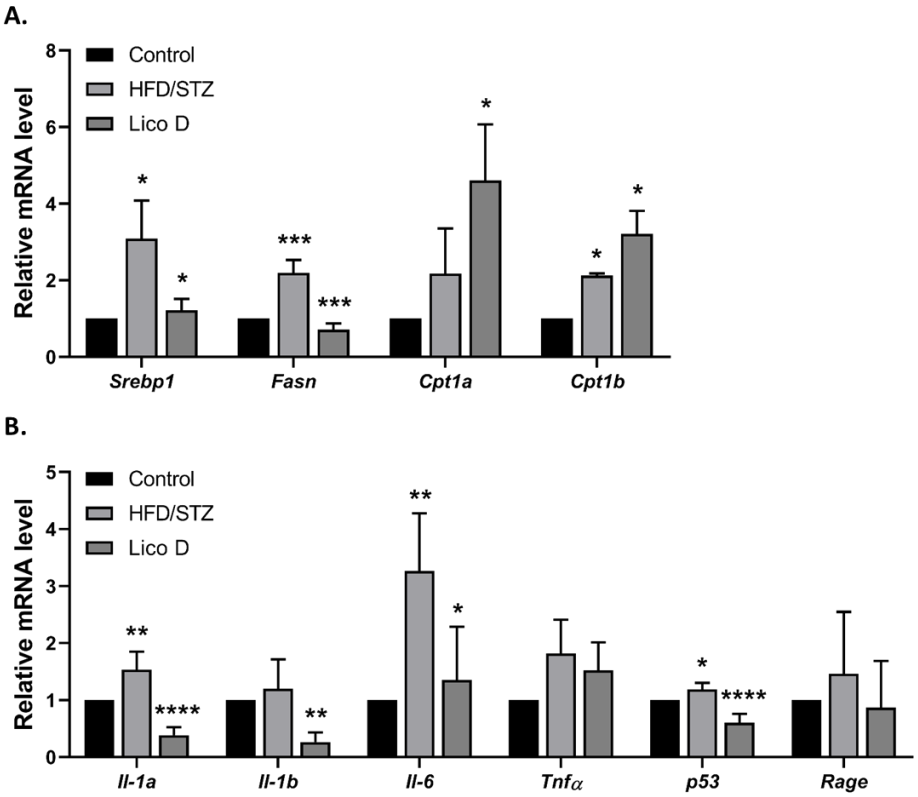

Figure 21. Effects of Lico D in lipid metabolism and inflammation in HFD/STZ mice liver tissue. (A) RT-PCR for lipid synthesis and β-oxidation and (B) inflammation markers in HFD/STZ mice liver tissue. All data are represented as mean ± SD (n = 3 or 4). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p <0.0001.

4. Discussion

T2D is far more common (~90% of all cases) than either type 1 diabetes (T1D) or gestational diabetes (DeFronzo et al., 2015). Moreover, it has been reported that middle-aged obese patients with T2D, the presence of NAFLD is associated with hyperinsulinemia, atherogenic dyslipidaemia and more severe adipose and hepatic insulin resistance compared to patients without NAFLD (Lomonaco et al., 2016). T2D is also considered to be the strongest risk factor for the progression of NAFLD, steatohepatitis, fibrosis, cirrhosis and finally hepatocellular carcinoma. AMPK activator is one of the therapeutic targets for treating or managing T2D. It may act on muscle, liver, and adipose tissue to regulate glucose homeostasis (DeFronzo et al., 2015). Particularly, in liver tissue, AMPK activation can increase the fatty acid oxidation, mitophagy and mitochondrial biogenesis to reduce the hepatic lipogenesis, cholesterol synthesis, fibrosis and insulin resistance (Steinberg and Carling, 2019). Therefore, AMPK modulation has been investigated as a treatment for a variety of metabolic syndromes such as cardiovascular disease (CVD), hepatocellular carcinoma (HCC), non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH) and T2D (Steinberg and Carling, 2019).

To evaluate the effect of Lico D *in vitro* and *in vivo*, I have utilized the FFAs induced hepatic steatosis cell model and high fat diet (HFD)/streptozotocin (STZ) induced hyperglycaemic mice model. The advantage of HDF/STZ mice is that they can develop insulin resistance during HFD and followed by administration of low to moderate dose of STZ can cause mild to moderate insulin deficiency by impairment in insulin secretions (reduction of β-cells in pancreatic tissue) (Furman, 2015). As a result, hyperglycaemia associated with hyperinsulinemia, insulin resistance, and dyslipidaemia, are the major complications in both T2D as well as NAFLD patients (Chao et al., 2020; Fang et al., 2019; Furman, 2015; J. H. Ye et al., 2016). The mouse model was confirmed in combination with Lico D treatment, which improved hyperglycaemia and reduced liver weight (Figure 19). To understand the molecular mechanisms of Lico D, I examined the activation of AMPK/SIRT1 pathway in HepG2, because it has been reported that AMPK/SIRT1 activation can regulate hepatic lipid metabolism, insulin resistance and prevent NAFLD (X. Y. Chen et al., 2019; Jia et al., 2016; Liou et al., 2020). I also reported that Lico D activate AMPK in an oxidative stress-induced *in vitro* and *in vivo* model

(Maharajan et al., 2021). I found the novel mechanism of Lico D in the activation of AMPK/SIRT1 pathway *in vitro* and *in vivo* and the data was also confirmed by using compound C (an AMPK inhibitor) *in vitro* (Figure 16 and 20).

Following that, I investigated the role of Lico D in lipid metabolism since activation of AMPK/SIRT1 regulates hepatic steatosis through regulation of hepatic lipogenesis and fatty acids oxidation in FFA-induced hepatic steatosis cell model and HFD-induced obesity mice model (X. Y. Chen et al., 2019; Gu et al., 2019; Guo et al., 2018; Hou et al., 2008; H. Wang et al., 2010). In HFD mice liver tissue, the activation of AMPK/SIRT1 pathway inhibits lipogenesis by downregulating the acetyl-CoA carboxylase (ACC), sterol regulatory element-binding protein 1 (SREBP1), carbohydrate response element binding protein (ChREBP) and its target genes, including fatty acid synthase while upregulates the fatty acid β-oxidation by carnitine palmitoyltransferase 1α (CPT1α) (X. Y. Chen et al., 2019; M. Foretz and Viollet, 2011). Carboxylesterase 1 (CES1) is a drug metabolizing enzyme, highly expressed in the liver (Ellinghaus et al., 1998) and regulates the hepatic triglyceride metabolism and protects against alcohol or methionine and choline induced liver injury (J. Xu et al., 2016). Overexpression of hepatic CES1 lowered the TG and plasma glucose level in both control and diabetic mice, meanwhile hepatic CES1 knockdown increased the hepatic TG and plasma cholesterol levels (J. Xu et al., 2014). The data suggest that Lico D altered lipid metabolism by reducing lipogenesis markers including SREBP, ChREBP, and FASN and increasing the β-oxidation markers such as CPT1 α, CPT1 β, CES1 and CES2 in either human HepG2 cell line or HFD/STZ induced T2D mice (Figure 18A and 21A). Together, the results indicate that Lico D reduced the hepatic lipid accumulation via activation of AMPK/SIRT1 pathway in both cell and animal model.

In general, the accumulation of lipids >5% is refer to the hepatic steatosis and eventually excess lipid accumulation induces the lipotoxicity, which might produce oxidative stress, endoplasmic reticulum stress and mitochondrial dysfunctions, resulting in inflammation, hepatic degeneration and development of fibrotic lesions which are the important characteristics of NASH (Chalasani et al., 2018; Ferguson and Finck, 2021; Hyysalo et al., 2014). NASH is expected to be present up to 60% of people with NAFLD (Younossi, Koenig, et al., 2016). The anti-inflammatory effect of Lico D has also been

shown to be mediated by suppression of protein kinase A (PKA), which is essential for the phosphorylation and activation of NF-κB and its target genes such as iNOS, TNF, and MCP-1 (Furusawa et al., 2009). Therefore, next I investigated the expression of liver inflammation markers such as, IL-1α, IL-1β, IL-6, P53, TNF-α, and RAGE (Abdelmageed et al., 2021; Derdak et al., 2013; Kamari et al., 2011; Yahagi et al., 2004; Yoo et al., 2017). I found that, inflammation markers were reduced upon Lico D treatment in both hepatic steatosis cell model and HFD/STZ mice (Figure 18B and 21B).

Collectively, Lico D decreases fasting blood glucose level and improves the glucose tolerance and lipid metabolism in HFD/STZ-induced hyperglycaemic mice. Lico D can also activate the AMPK/SIRT1 pathway in human HepG2 cells and mouse hepatic tissue. Therefore, Lico D could be useful therapeutic agent to treat type 2 diabetes and non-alcoholic fatty liver disease.

CHAPTER 2. Camphorquinone ameliorates hepatic lipid accumulation and regulates blood glucose via activating AMPK/SIRT1 in NAFLD/T2D mice

1. Introduction

NAFLD is emerging as the most common chronic liver disease in the world, affecting upto quarter of the world population and serving as a significant risk factor for both hepatic and cardiac metabolic morbidity (Arab et al., 2018; Z. Chen et al., 2019; Eslam and George, 2019; Loomba et al., 2021). The number of people with NAFLD is expected to rise by 83.1 million in 2015 to 100.9 million in 2030. However, the frequency of NAFLD varies widely around the world, with the highest prevalence in the Middle East and South America and the lowest in Africa (Estes et al., 2018; Loomba et al., 2021; Z. Younossi et al., 2019). Currently no approved therapeutic drugs to prevent or treat NAFLD (Z. Chen et al., 2019; Eslam and George, 2019; Ferguson and Finck, 2021). NAFLD encompasses a spectrum of severity, from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma (HCC) and it's also been linked to metabolic risk factors including obesity, insulin resistance, type 2 diabetes (T2D), hypertension and dyslipidaemia (Chalasani et al., 2018; Ferguson and Finck, 2021). In particular, the global prevalence of NAFLD in patients with T2D is estimated to be over 55%, with NASH and advanced fibrosis expected to occur 37% and 17% respectively (Z. M. Younossi et al., 2019). Therefore, NAFLD and advanced liver fibrosis are closely associated and double the risk of T2D. Importantly, T2D stands alone and strongest risk factor for the progression of NAFLD to advanced fibrosis or cirrhosis (Targher et al., 2021). Therefore, medications that are used to treat T2D are also beneficial in the treatment of NASH.

Several antidiabetic drugs are used to prevent or delay the onset and progression of diabetic complications. However, pharmacokinetic features, recurrent failure rate, and side effects of those medications limit their use. The side effects include hypoglycaemia, weight gain or loss, hypersensitivity reactions (Chaudhury et al., 2017). Many natural and synthetic medications with anti-diabetic properties have been identified from plant

sources that contain bioactive components such as terpenoids, flavonoids, carotenoids, glycosides, phenolics, and alkaloids (Kayarohanam and Kavimani, 2015; Panigrahy et al., 2021; N. Tran et al., 2020). Terpenoids are the large group of organic compounds that are modified form of terpenes with various functional groups and oxidized methyl groups (Paduch et al., 2007). Terpenes and terpenoids are important constituents of essential oils from a variety of plant materials such as flowers, seeds, leaves, fruits and roots. Due to their aroma and volatile ingredients, they are widely used in cosmetics, perfumes, and food flavorings as well as can be used to treat human diseases including cardiovascular disease, T2D and hyperlipidemia (Cox-Georgian et al., 2019; Hamedi et al., 2021; Stephane and Juleshttps, 2020). Camphor, a bicyclic monoterpenoid compound (1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one), is one of the major constituents present in the essential oils of several plant species including *Lavandula stoechas, Paederia scandens, and Amomum villosum*. Essential oils from these plant species have been shown to reduce lipid synthesis, TG, TC and FFAs accumulation in HFD-induced NAFLD rat and chicken model as well as attenuate hyperglycaemia in alloxan-induced diabetic rats (S. Lu et al., 2018; Sebai et al., 2013; Wu et al., 2019). Intragastric administration of camphor (30 mg/kg for 21 days) lowered the blood glucose level, TG, TC and LDL-cholesterol, while increased the HDL-cholesterol and antioxidant capacity in alloxan-induced diabetic rats (Drikvandi et al., 2020).

In this study, I choose camphorquinone (CQ) to explore its role in T2D and NAFLD animal model. Camphorquinone (CQ) is a bicyclic monoterpenoid compound (1,7,7-Trimethylbicyclo[2.2.1]heptan-2,3-dione) derived from camphor by continuous reaction of bromination and oxidation process. The oxidation of brominated camphor was catalysed by Fe-porphyrins with air (J. Wang et al., 2013). CQ is widely used as a photoinitiator to induce polymerization of light-curing materials such as dental adhesives and composites (Van Landuyt et al., 2007). However, the biological effect of CQ on diabetes and NAFLD not yet studied. Therefore, in this study, I decided to investigate the possible effects of CQ on the high fat diet (HFD) and low dose of streptozotocin (STZ) induced hyperglycaemia mouse model to mimic concurrent T2D/NAFLD pathological conditions (Chao et al., 2020; D. Y. Kim et al., 2020; J. H. Ye et al., 2016). I also evaluated

the fasting blood glucose level and liver tissue lipid metabolism and molecular pathways to support the findings.

2. Materials and Methods

Animals and Administration of Drugs

Six-week-old male C57BL/6 mice (weighing 22 ± 2 g) were purchased from Samtako Bio Korea Co., Ltd. (Osan, Gyeonggi, Korea) and maintained at 23-25 °C under a 12 h light and 12 h dark cycle with free access to food and water in a pathogen-free facility. The Institutional Animal Care and Use Committee of Chosun University (CIACUC2020-A0009-1) approved all animal experiments. Upon arrival, the animals underwent one week of acclimatization during which all animals were fed with normal chow pellets (ND, SAM #31, Samtako, Inc.). The animals were randomized according to their body weight and one-third of the animals were allocated to the normal control group which fed with normal chow pellets throughout the study. The remaining animals were fed with high fat diet (HFD; 60% Kcal energy as fat) (Research diets; #D12492; New Brunswick, NJ 08901 USA) for four weeks to induce insulin resistance; the diet was continued until end of the study. T2D was induced in animals by administering STZ (30 mg/kg, i.p.) for five consecutive days from fourth week while normal control animals received equal volume of citrate buffer. The animals were allowed to develop T2D for atleast one week. The blood glucose levels were measured at the end fifth week and the animals with FBG ≥ 200 mg/dl were selected for the study. The animals were divided into four groups; group 1: normal control mice treated with saline; group 2: diabetic control mice treated with saline; group 3: diabetic mice treated with CQ-low (CQ-L; 10 mg/kg/day; i.p.) and group 4: diabetic mice treated with CQ-high (CQ-H; 30 mg/kg/day; i.p.). All the treatments were carried out for three consecutive weeks. Until completion of the study, group-1 was fed with normal chow pellet while group 2, 3 and 4 were fed with a high fat diet. Fasting blood glucose level (FBG) and glucose tolerance test (GTT) were performed after 3 weeks of compound treatment. All the animals were sacrificed at the end of the study and liver tissue was isolated, frozen immediately and stored in the liquid nitrogen until further use.

3. Results

3.1. Establishment of T2D/NAFLD mouse model and evaluation of the effects of CQ administration

I used HFD/STZ to establish a combination of NAFLD/T2D mouse model that mimic obesity-related insulin resistance and hyperglycemia in T2D (Figure 22A). As shown in Figure 22B, FBG levels were increased in mice fed with HFD alone at the end of fourth week, indicating the development of obesity-induced insulin resistance and pre-diabetic conditions (FBG level ≥ 150 mg/dl). Furthermore, when compared to the control group or pre-diabetic mice, STZ administration increased FBG levels, suggesting that the HFD/STZ injection completely developed diabetes condition (FBG level ≥ 200 mg/dl) in the animals by the end of the sixth week. After three weeks of CQ administration, I measured FBS levels and glucose tolerance in mice. The results indicate that FBG levels were significantly lower in all CQ-administered animals when compared to HFD/STZ mice, with CQ-L having a higher impact on FBG reduction (Figure 22C). CQ also improved glucose tolerance in HFD/STZ mice, which was confirmed by a time-dependent reduction in blood glucose levels (i.e., 15, 30, 60, 90, and 120 min) (Figure 22 C).

As shown in Figure 22D, the mice body weight gradually increased until four weeks, when it was reduced due to STZ injection, and then increased again in subsequent weeks. STZ causes a diabetic state that include weight loss, polydipsia, polyuria and hyperglycemia (Zafar and Naqvi, 2010). In HFD/STZ treated mice, continuous HFD treatment resulted in a significant rise in liver weight, indicating the onset of NAFLD (Figure 22E). CQ-L injections, on the other hand, significantly reduces both body and liver weight in HFD/STZ animals, whereas mice treated with CQ-H showed no changes (Figure 22D,E).

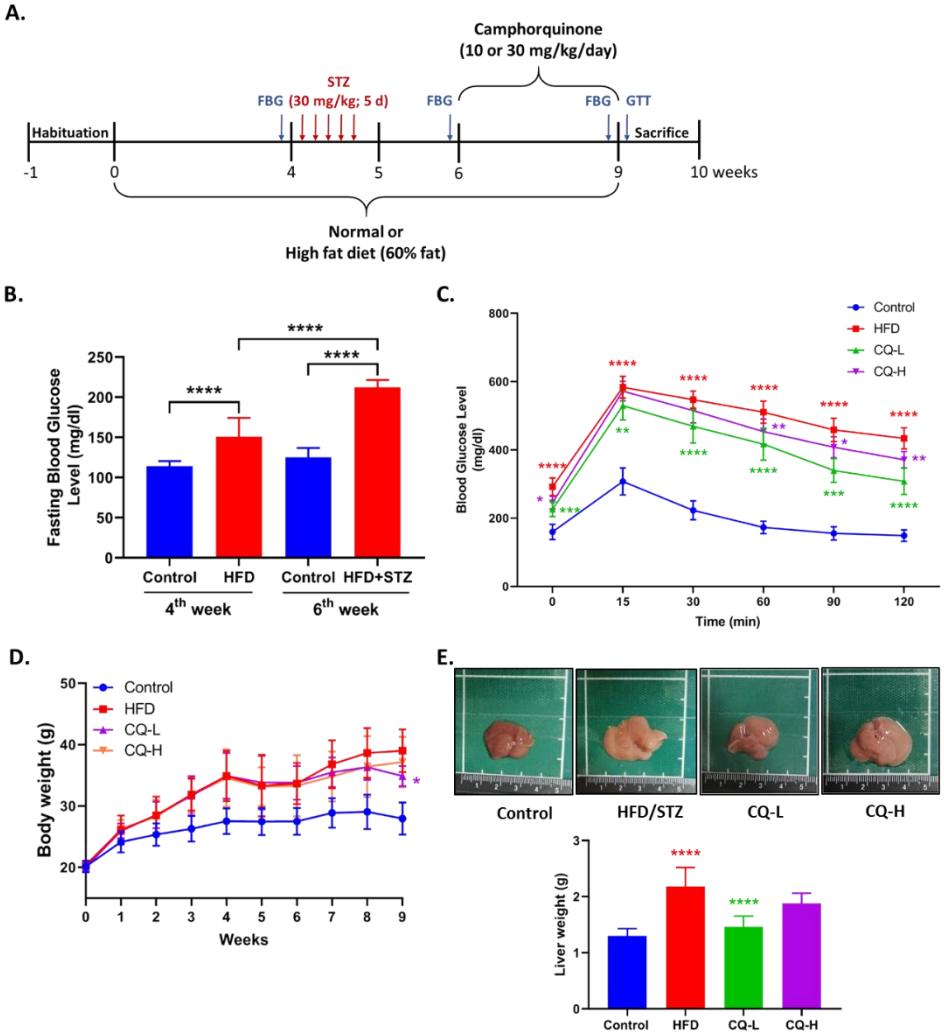

Figure 22. CQ reduces body and liver weight and improves glucose tolerance in HFD/STZ mice. (A) Schematic representation of the experimental plan. (B) Fasting blood glucose level of mice before and after STZ injection and (C) glucose tolerance test in HFD/STZ mice. (D) Body and (E) liver weight of mice. All data are represented as mean ± SD (n = 6). *p < 0.05, **p < 0.01, ***p <0.001, and ****p < 0.0001.

3.2 CQ administration reduces lipid synthesis and inflammation in HFD/STZ mice liver tissue

To investigate the CQ pharmacological mechanism at a molecular level, I examined the expression of genes involved in lipogenesis, fatty acid β-oxidation and inflammation in the liver tissue. The results showed that expression level of lipogenesis-related genes (*Srebp-1, and Fasn*) were reduced in the CQ-L and CQ-H, whereas fatty acid β-oxidation marker (*Cpt1a*) was significantly increased only in CQ-L (Figure 23A). Further, I investigated the expression level of liver inflammation markers including *Il-1α, Il-1β, Il-6, Tnfα,* and *p53*. All the inflammation markers were reduced in both CQ-L and CQ-H, while *Il-1α, Il-1β,* and *Il-6* were reduced significantly compared to the HFD/STZ mice (Figure 23B). Collectively, the data suggest that CQ reduces liver lipid synthesis and inflammation and increases the free fatty acids β-oxidation in HFD/STZ mice.

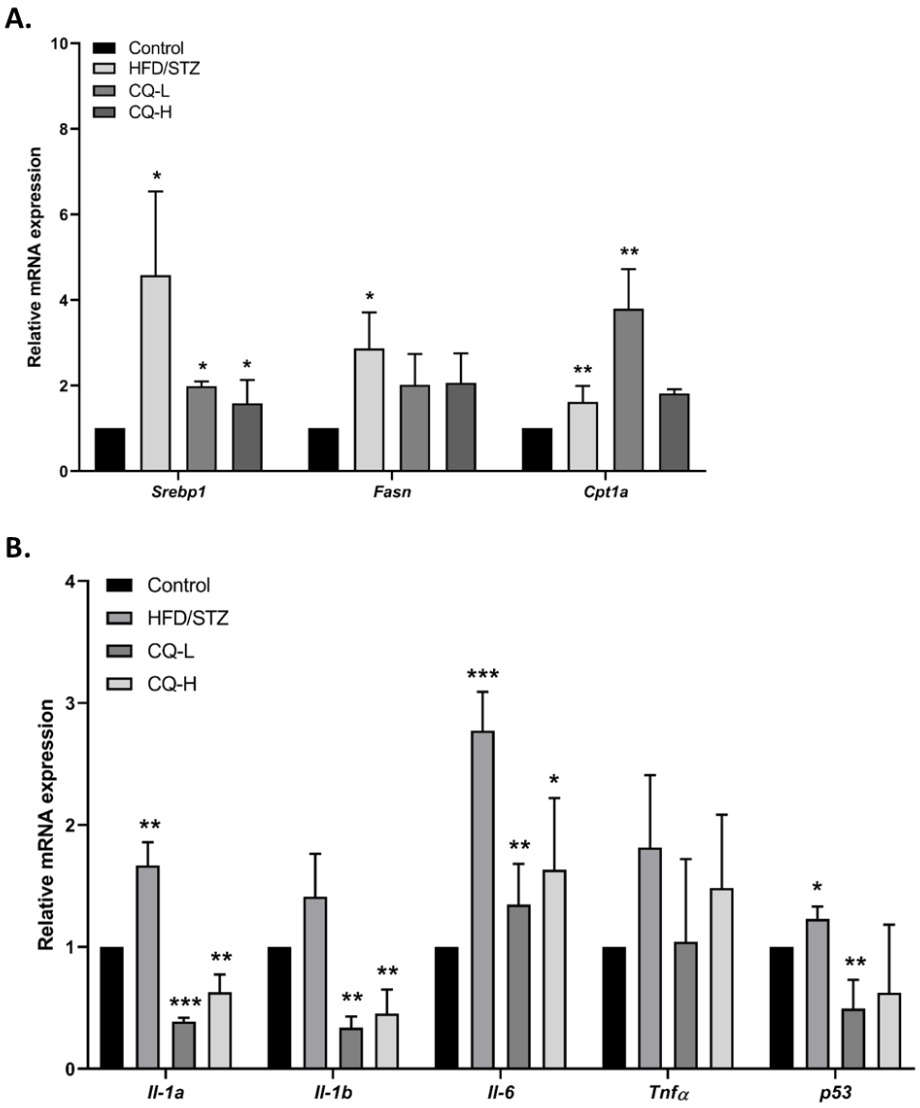

Figure 23. Effects of CQ on lipid metabolism and inflammation in HFD/STZ mice liver tissue. (A) RT-PCR showing lipid synthesis and β-oxidation markers and (B) inflammation markers expression in mice liver tissue. All data are represented as mean ± SD (n = 3 or 4). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

3.3 CQ ameliorates fatty liver by activating AMPK/SIRT1 signaling

To understand the potential mechanism underlying the observed effects, I investigated the signaling pathway, which has been linked to lipid metabolism. It has been reported that activation of AMPK/SIRT1 pathway could ameliorate the lipotoxicity and attenuate the development of hepatic steatosis in an HFD-induced NAFLD animal model (S. Li et al., 2020; Liou et al., 2020; Rao et al., 2017; Sharma et al., 2021). Therefore, I examined the activation of SIRT1, AMPK and its downstream target ACC (involved in fatty acid synthesis). I found that CQ-L significantly increased the protein expression of SIRT1 and p-AMPK and p-ACC than CQ-H (Figure 24). The data suggest that CQ could reduce HFD/STZ induced hepatic steatosis via activating the AMPK/SIRT1 axis.

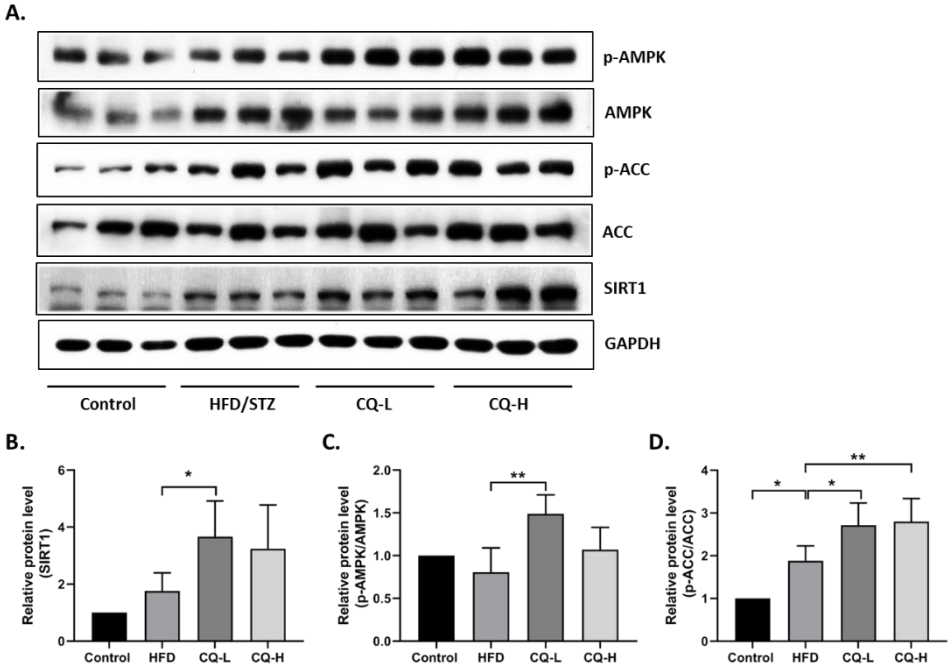

Figure 24. CQ activates AMPK/SIRT1 pathway in T2D mice liver tissue. (A) Representative images from immunoblot against AMPK, ACC, SIRT1, and GAPDH from mice liver tissue. (B-D) The expression levels of proteins were quantified using Images J software. All data are represented as mean ± SD (n = 5). *p < 0.05, **p < 0.01, and ***p < 0.001.

3.4 Dose dependent activation of AMPK/SIRT1 pathway in HepG2

Further, I used HepG2 cells to explore the detailed mechanism of CQ on hepatic lipid metabolism. HepG2 cells were treated with several concentrations of CQ (0-1000 μM) for 24 h and I measured the protein expression levels. As shown in Figure 25A-D, the expression level of SIRT1, p-AMPK and p-ACC were increased in a dose dependent manner, with 500 μM of CQ reaching a maximum protein expression level. Therefore, I have chosen 500μM of CQ for further in vitro experiments. Further, I have confirmed the above AMPK/SIRT1 activation by using compound C (CC; an AMPK inhibitor). As expected, CC treatment significantly reduced CQ mediated AMPK/SIRT1 activation in HepG2 cells (Figure 25E-H). The data suggest that CQ activates AMPK/SIRT1 pathway in HepG2 cells.

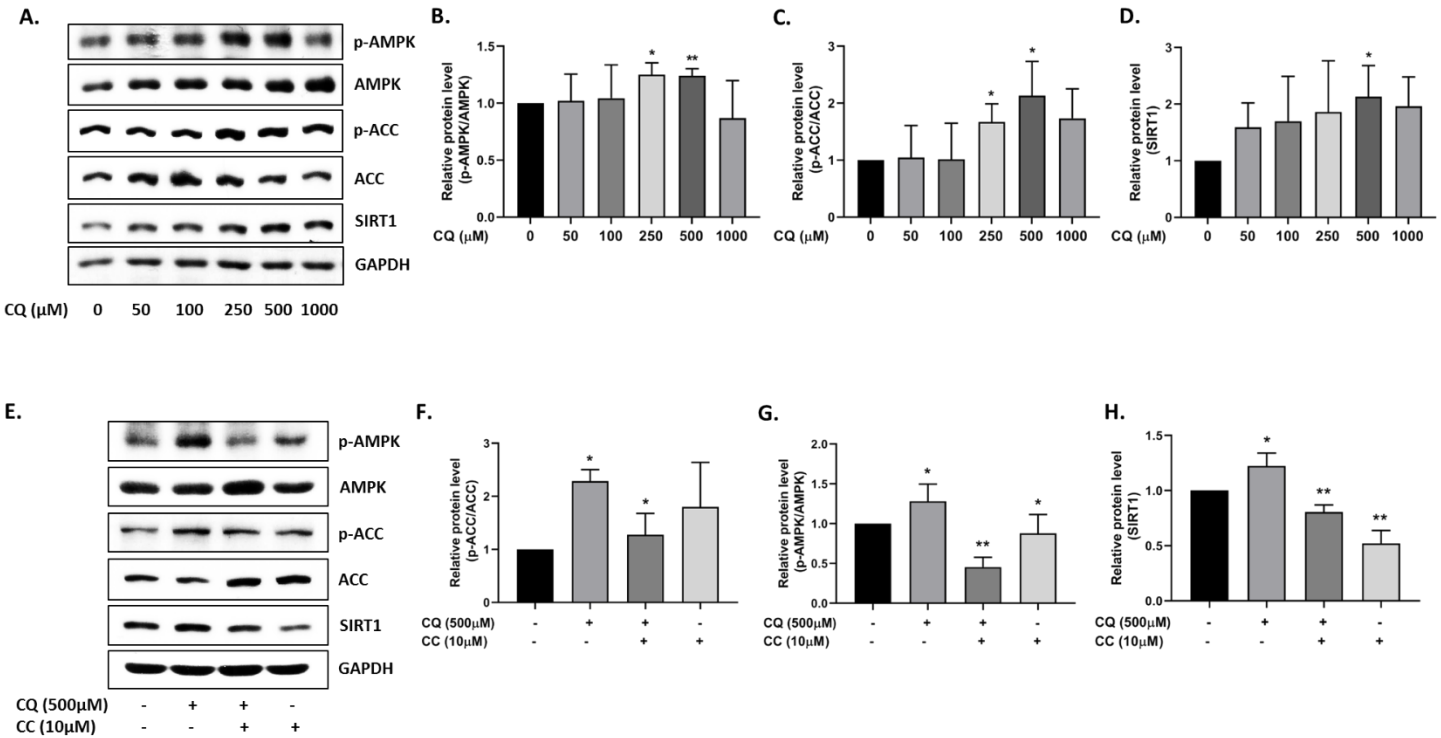

Figure 25. CQ increases the AMPK/SIRT1 in HepG2 cells. (A-D) Immunoblot analysis showing AMPK, ACC, and SIRT1 expression in HepG2 cells treated different concentration of CQ (0-1000 μM). (E-H) Immunoblot analysis showing AMPK, ACC, and SIRT1 expression in HepG2 cells treated with either CQ (500 μM) or CC (10 μM). All data are represented as mean ± SD (n = 3). *p < 0.05 and **p < 0.01.

3.5 AMPK/SIRT1 activation ameliorates FFAs induced hepatic steatosis in HepG2 cells

The effects of CQ on a free fatty acid (FFA)-induced hepatic steatosis cell model were investigated. As shown in Figure 26A, the accumulation of cellular lipid level was significantly increased in the FFAs treated HepG2 cells compared to control. While, CQ reduced the lipid droplets accumulation in FFA-treated cells. Next, I investigated whether CQ activate AMPK/SIRT1 signaling pathway in hepatic steatosis cell model. I found that CQ increased the activation of AMPK/SIRT1 protein expression in FFAs treated cells (Figure 26B). In order to examine the impact of FFAs induced cellular steatosis on HepG2 cells, I investigated the expression of the genes involved in lipid synthesis, fatty acid β-oxidation and inflammation. As shown in Figure 26C, CQ significantly reduced the relative mRNA expression of lipogenesis marker *ChREBP*, and increased the fatty acid oxidation markers such as *CES1*, *CES2* and *CPT1A*. Further, CQ diminished FFAs induced inflammatory markers such as *IL-1α, IL-6* and *IL-8* (Figure 26C). Taken together, the data suggest that CQ potentially inhibits hepatic steatosis development via AMPK/SIRT1 pathway.

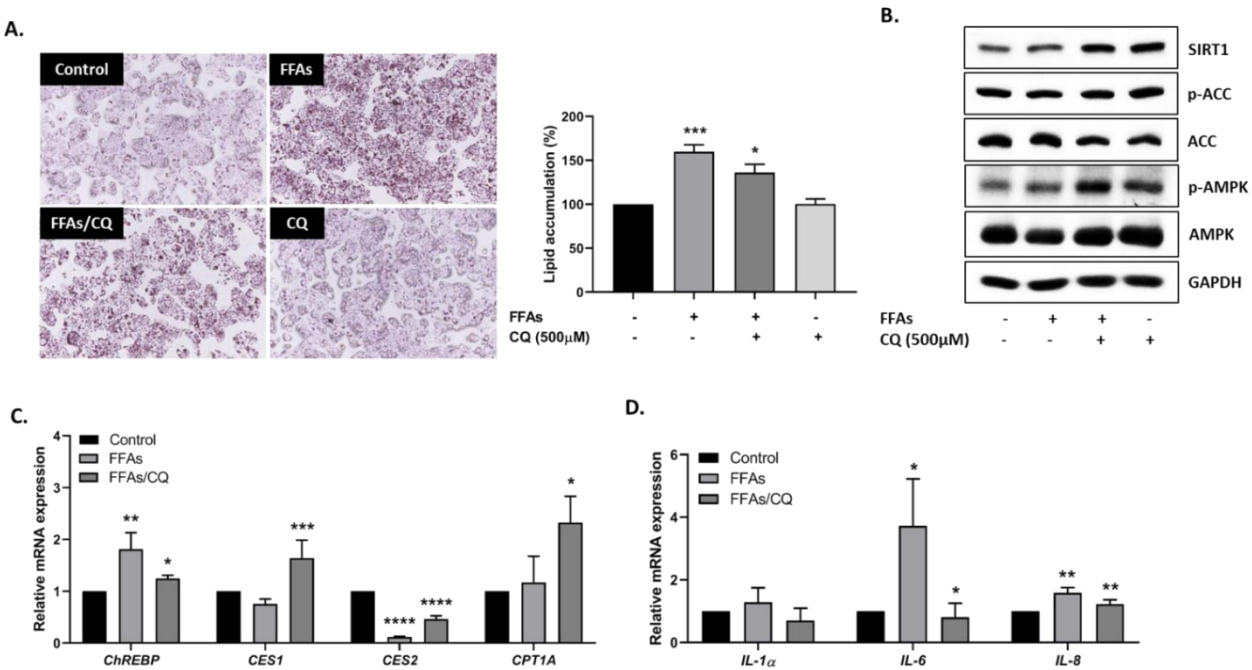

Figure 26. AMPK/SIRT1 activation ameliorates FFAs induced hepatic steatosis in HepG2 cells. (A) Oil Red O staining to visualize the FFAs-induced excess lipid accumulation in HepG2 cells. (B) Immunoblot analysis showing AMPK, ACC and SIRT1 expression in HepG2 cells treated with either FFAs (PA and OA) or CQ (500 μM). (C) RT-PCR showing lipid synthesis (*ChREBP*) and fatty acids oxidation markers (*CES1*, *CES2* and *CPT1A*) and (D) inflammation markers (*IL-1α, IL-6* and *IL-8*) expression in FFAs-induced hepatic steatosis cell model. All data are represented as mean ± SD (n = 3 or 4). *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

4. Discussion

NAFLD is a common chronic liver disease that can range from simple steatosis to the development of hepatocellular carcinoma (a more advanced stage of NAFLD) and is strongly associated to an increased risk of other metabolic disorders including T2D, insulin resistance, hypertension and dyslipidaemia (Ferguson and Finck, 2021; Targher et al., 2021; Tilg et al., 2017). The aim of this present study is to elucidate the role of CQ on NAFLD and T2D. Hepatic steatosis, hepatic inflammation, hyperglycaemia, insulin resistance and glucose intolerance are the key characteristics of NAFLD and T2D (Soret et al., 2020; Tilg et al., 2017). Therefore, I used a high fat diet and streptozotocin-induced hyperglycaemic mice model to stimulate NAFLD and T2D in this investigation (Chao et al., 2020; J. H. Ye et al., 2016). First, I successfully developed a mice model, which was confirmed by the progression of mice body and liver weight, as well as increased FBG level and glucose tolerance impairment (Figure 22). All these negative effects were considerably reduced in mice given CQ, particularly CQ-Low dose.

In T2D, insulin resistance is commonly associated to the progression of NALFD to NAHS, including abnormal lipid metabolism and pro-inflammatory marker secretion (Tian et al., 2016; Trepiana et al., 2018). According to the previous research, pharmacological activation of AMPK/SIRT1 pathway may contribute to inhibit the progression of NAFLD and T2D and serve as a therapeutic target for the treatment of metabolic diseases (S. Li et al., 2020; Liou et al., 2020; Sharma et al., 2021). AMPK is a central regulator of the cellular energy homeostasis and can be activated mainly through the alteration in cellular ATP/AMP ratio as well as two upstream kinases such as LKB1 and CaMKK2 (Herzig and Shaw, 2018). Hepatic AMPK activation inhibits the anabolic process (lipid synthesis) through phosphorylation and inhibition of carbohydrate response element binding protein (ChREBP) and downregulates the expression and stability of sterol regulatory element-binding protein-1c (SREBP-1c) via SIRT1-dependent deacetylation pathway (Marc Foretz et al., 2005; M. Foretz and Viollet, 2011; Viollet et al., 2009). Activated AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), lowering intracellular malonyl-CoA level, a precursor of fatty acid synthesis and an inhibitor of carnitine palmitoyltransferase 1 (CPT1). AMPK/SIRT1 activation also potentially inhibit the over activation of NF-κB and their downstream targets, such as

inflammatory cytokines in HFD induced hepatic steatosis (Tian et al., 2016). I used the SwissTargetPrediction online line tool to predict the target genes that could be triggered by CQ (Daina et al., 2019) and I identified Carboxylesterase 1 and 2 (CES1 and CES2), genes involved in the hydrolysis of various xenobiotic drugs and fatty acid oxidation process, were identified as having a high probability score (Y. Li et al., 2016; J. Xu et al., 2014). Therefore, I screened expression level of genes, which are involved in the lipid biosynthesis (SREBP-1, FASN and ChREBP), fatty acid oxidation (CES1, CES2 and CPT1α) and inflammation (IL1α, IL1β, IL6, TNFα, and, p53) in the HFD/STZ mice as wells as FFAs induced hepatic steatosis cell model. I attained the interesting findings, which supporting the positive role of CQ on lipid metabolism and inflammation (Figure 23 and 26), which was mediated by the molecular activation of AMPK/SIRT1 pathway (Figure 24 and 26). Despite the fact that both SIRT1 and AMPK are thought to regulate each other activation, there is no consensus on which is activated first (Ruderman et al., 2010).

In summary, CQ improves hyperglycemia, glucose intolerance, hepatic steatosis, and inflammation in HFD/STZ-induced NAFLD and T2D mice model, as well as in FFAs-induced hepatic steatosis cell model. The anti-NAFLD effect of CQ was associated with increased fatty acids β-oxidation and reduced lipid biosynthesis and pro-inflammatory cytokine production. Overall, the findings suggest that CQ ameliorates HFD/STZ-induced NAFLD/T2D via activating the AMPK/SIRT1 signaling pathway.

IV. CONCLUDING REMARKS

Aging is characterized by a continual deterioration of tissue homeostasis and loss of physiological integrity of tissues, which raises the risk of disease development and death (Barzilai et al., 2012; Lopez-Otin et al., 2013; Sen et al., 2016). Increased age is the leading cause of chronic noncommunicable diseases such as cancer, neurological diseases, cardiovascular diseases, articular damages, and metabolic diseases (Barbé-Tuana et al., 2020). Due to the complexity of the aging process, it is still unclear how organism's age and what circumstances can accelerate it. To investigate the mechanism of aging, López-Otn et al. identified nine potential hallmarks that are widely thought to contribute to the aging process and collectively determine the aging phenotype (Lopez-Otin et al., 2013). Cellular and molecular hallmarks, such as cellular senescence (an irreversible cell cycle arrest), can be used to predict the aging phenotype (A. Hernandez-Segura et al., 2018; Lopez-Otin et al., 2013; Lozano-Torres et al., 2019). The aim of this study is to explore the role of Lico D and CQ in aging and aging-related diseases. Particularly, I chose "cellular senescence" as one of the factors that can accelerate the aging process and "non-fatty liver disease and type 2 diabetes" as aging-related diseases.

In the first part of the study, I have evaluated the effects of Lico D and CQ in oxidative stress-induced senescence model. To induce oxidative stress *in vitro* and *in vivo*, I utilized hydrogen peroxide (H_2O_2) and D-Galactose, which are well-known oxidative stress inducers (Bo-Htay et al., 2018; Z. Wang et al., 2013b). The data suggest that both compounds potentially ameliorated the oxidative stress induced senescence in hBM-MSCs and mice. Furthermore, the findings suggest that AMPK activation could be a common molecular mechanism for both compounds. In the second part of the study, I investigated the biological benefits of Lico D and CQ in a high-fat diet/streptozotocin-induced hyperglycemic mice model to mimic the pathological circumstances of nonalcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D) in mice as well as Hepatic steatosis cell model (Chao et al., 2020; D. Y. Kim et al., 2020; J. H. Ye et al., 2016). According to the findings of both models, Lico D and CQ reduced hepatic lipid synthesis and hepatic inflammation whereas significantly increased fatty acid oxidation. Furthermore, Lico D and CQ treatment effectively reduced elevated fating blood glucose and glucose intolerance in mice. The activation of AMPK/SIRT1 is a key player in Lico D and CQ's beneficial effects on cellular senescence and NAFLD/T2D. Overall, the

findings suggest that Lico D and CQ could be useful therapeutic agents for reducing the burden of aging and aging-related diseases. Despite the fact that they are structurally two different compounds and produced from different sources, Lico D and CQ exhibit a similar mechanism of action in both oxidative stress-induced senescence and HFD/STZ mice model. Therefore, further studies are required to elucidate the molecular mechanism of Lico D and CQ.

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"கேடில் விழுச்செல்வம் கல்வி யொருவற்கு

மாடல்ல மற்றை யவை"

"Learning is excellence of wealth that none destroy;

To man nought else affords reality of joy"

- Thiruvalluvar