CORDYCEPIN ENHANCES *SIRT1* EXPRESSION AND MAINTAINS STEMNESS OF HUMAN MESENCHYMAL STEM CELLS

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คอร์ไดเซปินกระตุ้นการแสดงออกยีนเซอทูอีนวันและรักษาคุณสมบัติเซลล์ต้นกำเนิดในเซลล์ต้นกำเนิดชนิดมีเซนไคม์ของมนุษย์

พงศกร เชื้อพรมศรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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คำสำคัญ: คอร์ไดเซปิน/เซลล์ต้นกำเนิดชนิดมีเซนไคม์/ยีนเซอทูอีนวัน/การต้านความชรา

ปัจจุบันเซลล์ต้นกำเนิดชนิดมีเซนไคม์ถูกใช้ในการปลูกถ่ายเซลล์ต้นกำเนิดและรักษาโรคความเสื่อมต่างๆ ในร่างกายมนุษย์ เช่น โรคกระดูก โรคกราฟท์ต่อกรกับโฮสต์ โรคหัวใจและหลอดเลือด โรคตับแข็ง โรคไขข้ออักเสบ โรคปลอกประสาทเสื่อมแข็ง โรคพาร์กินสัน และโรคอัลไซเมอร์ เนื่องจากสามารถสร้างอวัยวะจำเพาะและซ่อมแซมเนื้อเยื่อที่เสียหายโดยไม่ต้องกังวลเรื่องจริยธรรมและการปฏิเสธภูมิคุ้มกัน อย่างไรก็ตามปัญหาที่สำคัญ คือ เซลล์ต้นกำเนิดชราทำให้คุณสมบัติการสร้างและซ่อมแซมเนื้อเยื่อต่างๆลดลง ดังนั้นวิธีการชะลอการชราของเซลล์ต้นกำเนิดจึงมีความจำเป็นในการรักษาโรคความเสื่อมด้วยเซลล์ต้นกำเนิด วัตถุประสงค์ของงานวิจัยนี้คือ การหาความเข้มข้นที่เหมาะสมของสารออกฤทธิ์ทางชีวภาพจากเชื้อรา *Cordyceps militaris* คือ คอร์ไดเซปินในการกระตุ้นยีนเซอทูอีนวันในเซลล์ต้นกำเนิดชนิดมีเซนไคม์ของมนุษย์ ซึ่งเป็นยีนที่ควบคุมความชรา และใช้คอร์ไดเซปินในการรักษาคุณสมบัติเซลล์ต้นกำเนิดและต้านความชราโดยนำไปผสมกับอาหารเลี้ยงเซลล์ การหาความเข้มที่เหมาะสมของคอร์ไดเซปินและการวัดประสิทธิภาพในการต้านความชราถูกทดสอบด้วยวิธีดังนี้ คือ การทดสอบความเป็นพิษต่อเซลล์ ระยะเวลาในการเพิ่มสองเท่าของเซลล์ การแสดงออกของยีนและโปรตีนที่เกี่ยวข้อง การวัดปริมาณเอนไซม์ บีตา-กาแล็กโทสิเดส การวัดความยาวของเทโลเมียร์ และการสร้างเอนไซม์เทโลเมียร์เรส ผลการทดลองพบว่า คอร์ไดเซปิน ปริมาณ 0.25 ไมโครโมลาร์ สามารถกระตุ้นการแสดงออกของยีนเซอทูอีนวันในเซลล์ต้นกำเนิดชนิดมีเซนไคม์ของมนุษย์ได้อย่างมีนัยสำคัญผ่านการส่งสัญญาณ AMPK-SIRT1 และสามารถรักษาคุณสมบัติเซลล์ต้นกำเนิดผ่านการบวนการ deacetylating ของ SOX2 ด้วยเซอทูอีนวัน นอกจากนั้นยังพบว่าคอร์ไดเซปินสามารถต้านความชราได้โดยกระตุ้นกระบวนการกลืนกินตัวเองของเซลล์ (autophagy) ลดการสร้างเอนไซม์ บีตา-กาแล็กโทสิเดส ช่วยรักษาอัตราการแบ่งตัวของเซลล์ และช่วยรักษาความยาวของเทโลเมียร์ ดังนั้นคอร์ไดเซปินมีความสามารถในการเป็นตัวกระตุ้นเซอทูอีนวันในเซลล์ต้นกำเนิดชนิดมีเซนไคม์ของมนุษย์เพื่อการต้านความชรา งานวิจัยนี้ทำให้การเข้าถึงวิธีรักษาโรคความเสื่อมด้วยเซลล์ต้นกำเนิดของผู้ป่วยเพิ่มขึ้น และจะช่วยลดจำนวนผู้ป่วยโรคความเสื่อม นอกจากนั้นยังประยุกต์ใช้คอร์ไดเซปินในการเป็นผลิตภัณฑ์เสริมอาหารและผลิตภัณฑ์เครื่องสำอางสำหรับต้านความชรา

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา พงศกร

ปีการศึกษา 2565 ลายมือชื่ออาจารย์ที่ปรึกษา

PHONGSAKORN CHUEAPHROMSRI : CORDYCEPIN ENHANCES *SIRT1* EXPRESSION AND MAINTAINS STEMNESS OF HUMAN MESENCHYMAL STEM CELLS. THESIS ADVISOR : ASSOC. PROF. DR. PARINYA NOISA, Ph.D. 49 PP.

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Recently, mesenchymal stem cells (MSCs) have been used for stem cell transplantation and the treatment of degenerative diseases, such as bone and cartilage diseases, graft versus host disease, cardiovascular disease, cirrhosis, rheumatoid arthritis, multiple sclerosis, Parkinson's disease, and Alzheimer's disease because MSCs can regenerate a specific organ and repair damaged tissue without ethical concern and immune rejection. However, the major problem is that aging stem cells have reduced ability to build and repair various tissues, thus the need to establish a method to delay MSC ageing is significant. The objective of this research is to find the optimal concentration of cordycepin, a bioactive component from the *Cordyceps militaris* for activating the *SIRT1* gene in human mesenchymal stem cells which *SIRT1* controls cellular senescence. Moreover, cordycepin was used to maintain self-renewal and multipotency of MSCs by adding cordycepin into a standard culture media. The methods were tested to determine the optimal cordycepin concentration and evaluate the anti-aging efficiency: cell viability, doubling time, key gene/protein expression, galactosidase-associated senescence assay, relative telomere length, and telomerase expression. It was discovered that cordycepin significantly activated *SIRT1* in MSCs by triggering the AMPK-SIRT1 signaling pathway. Cordycepin maintains the stemness of MSCs by deacetylating SOX2 through SIRT1, and cordycepin can prevent aging of MSCs by promoting autophagy, decreasing the activity of senescence-associated-galactosidase, maintaining proliferation rate, and enhancing telomere activity. Cordycepin may activate *SIRT1* in MSCs as an anti-aging therapy. The use of MSCs as a culture medium can improve patient access to cell therapy and regenerative medicine, reducing the number of people suffering from degenerative diseases. Furthermore, optimal cordycepin dosages can be used in anti-aging supplements and cosmetics.

School of Biotechnology Student's Signature พงศกร

Academic Year 2022 Advisor's Signature

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Phongsakorn Chueaphromsri

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LIST OF ABBREVIATIONS

μM	=	Micromolar
36B4	=	Ribosomal protein lateral stalk subunit P0
AMP	=	Adenosine monophosphate
AMPK	=	Adenosine monophosphate-activated protein kinase
ATG	=	Autophagy related
ATG5	=	Autophagy related 5
ATG7	=	Autophagy related 7
ATG8	=	Autophagy related 8
ATG12	=	Autophagy related 12
ATP	=	Adenosine triphosphate
BM-MSCs	=	Bone marrow mesenchymal stem cells
BSA	=	Bovine serum albumin
cDNA	=	Complementary deoxyribonucleic acid
CO2	=	Carbon dioxide
DAPI	=	4′,6-diamidino-2-phenylindole
DNA	=	Deoxyribonucleic acid
DT	=	Doubling time
ESCs	=	Embryonic stem cells
FBS	=	Fetal bovine serum
FOXO	=	Forkhead box O
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	=	Genomic deoxyribonucleic acid
hESCs	=	Human embryonic stem cells
hTERT	=	Human telomerase reverse transcriptase
IC50	=	Half maximal inhibitory concentration
IFA	=	Immunofluorescent antibody assays
IGF	=	Insulin like growth factor
IgG	=	Immunoglobulin G

LIST OF ABBREVIATIONS (Continued)

Jak2	=	Janus kinase 2
LC3	=	Microtuble-associated protein light chain 3
LKB1	=	Liver kinase B1
MDC	=	Monodansylcadaverine
mRNAs	=	Messenger ribonucleic acids
MSCs	=	Mesenchymal stem cells
m-TOR	=	Mammalian target of rapamycin
m-TORC1	=	Mammalian target of rapamycin complex 1
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD+	=	Nicotinamide adenine dinucleotide
NADH	=	Nicotinamide adenine dinucleotide
NANOG	=	Nanog homeobox
NF-**K**B	=	Nuclear factor kappa B
NRF2	=	Nuclear factor erythroid 2–related factor 2
OCT4	=	POU class 5 homeobox 1
$p^{16INK4\alpha}$	=	Cyclin-dependent kinase inhibitor 2A, CDKN2A
p53	=	Tumor protein p53
p65	=	REL-associated protein
PBS	=	Phosphate buffered saline
PCR	=	Polymerase chain reaction
poly(A)	=	The poly-A tail
PSCs	=	Pluripotent stem cells
p-SIRT1	=	Phosphorylated sirtuin 1
qPCR	=	Quantitative polymerase chain reaction
RNA	=	Ribonucleic acid
RT-PCR	=	Reverse transcription-polymerase chain reaction
SA-β-gal	=	Senescence-associated β-galactosidase
SD	=	Standard deviation
shRNA	=	Short hairpin RNA

LIST OF ABBREVIATIONS (Continued)

SIRT1	=	Sirtuin 1
SOX2	=	SRY-box transcription factor 2
STAT3	=	Signal transducer and activator of transcription 3
TEL	=	Telomere
TERT	=	Telomerase reverse transcriptase
TNF-α	=	Tumor necrosis factor-alpha
TSC2	=	Tuberous sclerosis complex 2
Ulk1	=	unc-51 like autophagy activating kinase 1
USA	=	United states of america

CHAPTER I
INTRODUCTION

1.1 Significance of study

Regenerative medicine and cell therapy are modern medical advancements that are gaining a lot of attention as novel methods for treating severe diseases (Chosa and Ishisaki, 2018). MSCs are used to treat a variety of degenerative diseases, such as cardiovascular diseases, neuro degenerative diseases, bone and cartilage diseases, cancers, liver diseases, kidney diseases, and autoimmune diseases [including: graft versus-host diseases, multiple sclerosis, Crohn's disease, type1 diabetes, systemic lupus erythematous, rheumatoid arthritis] (Saeedi, Halabian, and Imani Fooladi, 2019). MSCs are adult stem cells that can be obtained from the bone marrow, adipose tissue, umbilical cord tissue, and umbilical cord blood (Nguyen et al., 2022). MSCs can differentiate into a variety of cells, including adipocytes, osteoblasts, chondrocytes, endothelial cells, and cardiomyocytes (Ishikane, Ikushima, Igawa, Tomooka, and Takahashi-Yanaga, 2021). MSCs have the capacity for self-renewal and multipotency (Y. K. Yang, Ogando, Wang See, Chang, and Barabino, 2018), and play a significant role in the development of specific organs and tissues with special functions (Lavenus, Rozé, Hoornaert, Louarn, and Layrolle, 2012). However, MSC aging is a critical problem that contributes to the loss of self-renewal, stemness and differentiation potential (Zainabadi, 2018). MSCs are ineligible to be used in regenerative medicine treatments after prolonged in vitro cultivation because their self-renewal and multipotency declines (Raggi and Berardi, 2012). Additionally, replicative senescence influences long-term changes in phenotype, differentiation potential, whole-map gene expression patterns, and microRNA profiles, all of which should be taken into consideration as therapeutic targets for MSC rejuvenation (X. Zhou, Hong, Zhang, and Li, 2020). Therefore, an appropriate method to maintain the self-renewal and multipotency of MSCs is very important for their use in therapeutic applications.

SIRT1 is a nicotinamide adenine dinucleotide (NAD+)-dependent lysine

deacetylase that participates in numerous biological processes, including gene silencing, DNA repair, metabolic regulation, cell cycle regulation, apoptosis, inflammation, autophagy, cellular senescence (Y. Wang et al., 2022). SIRT1 participates in the regulation of the aged-related signaling pathways (FoXO1, NF-**K**B, AMPK, m-TOR, p53, and PGC-1**α**) directly or indirectly by deacetylating a number of key proteins to delay cellular senescence (C. Chen, Zhou, Ge, and Wang, 2020). A growing body of research indicates that increased SIRT1 activity may have positive effect on aging and aging-related diseases in mammals by regulating DNA and metabolic damage (H. Chen, Liu, Zhu, et al., 2014). Additionally, SIRT1 may play a significant role in maintaining the stemness of both embryonic stem cells (ESCs) and MSCs due to its capacity to control the expression of pluripotent transcription factors such as NANOG, and OCT4 (D. S. Yoon et al., 2014). Moreover, SIRT1 deactivation has also been shown to decrease cellular proliferation and accelerate senescence in MSCs (Imperatore et al., 2017). Therefore, effective strategies for enhancing *SIRT1* expression may contribute to the maintenance of MSC properties.

Cordycepin also known as 3'-deoxyadenosine, is a derivative of the nucleoside adenosine and a bioactive compound derived from *Cordyceps militaris*, a fungus of the phylum Ascomycota (Alam et al., 2023; He, Park, and Cho, 2021). Cordycepin has been demonstrated to have numerous pharmacological properties, including anticancer, antiviral, antioxidant, anti-aging, and anti-inflammatory activities (Ashraf et al., 2020). Cordycepin is a natural nucleoside analogue compound that has been shown to effectively reduce cell senescence and radiation-induced ulcer (Z. Wang et al., 2019). Additionally, it has been discovered that cordycepin increases expression of SIRT1 through the AMPK-SIRT1 signaling pathway by increasing NAD+ to prevent age-related testicular dysfunction in rats (Kopalli et al., 2019; Takahashi et al., 2012). According to studies, cordycepin improved the antioxidant capacity and reduced age-related oxidative stress in old rats (Ramesh et al., 2012). Moreover, cordycepin has been reported to maintain the pluripotency of ESCs by activating the janus kinase 2 (Jak2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway (C. H. Wang et al., 2020). Therefore, cordycepin is an attractive bioactive compound for promoting *SIRT1* to maintain the MSC properties.

1.2 Research objective

1.2.1 To obtain the optimal cordycepin concentration for upregulation *SIRT1* and stemness transcription factors in MSCs.

1.2.1 To obtain the knowledge of the relationship between the upregulate of *SIRT1* and stemness transcription factors.

1.2.3 To demonstrate that cordycepin can prevent MSCs against aging and cellular senescence.

1.3 Hypotheses

1.3.1 Cordycepin is capable of the maintenance of self-renewal and multipotency of MSCs because cordycepin is an interesting candidate that able to upregulate *SIRT1* a transcription factor regulating cell senescence in MSCs.

1.3.2 The upregulation of *SIRT1* will affect stemness transcription factors (*SOX2*, *NANOG*, and *OCT4*) and delay cellular senescence and aging of MSCs.

1.4 Scope of limitations

In this research, we optimized the concentration of cordycepin for up-regulation of *SIRT1* in MSCs using cytotoxicity assay and determining key gene/protein expression. Moreover, we also investigated how cordycepin affected MSC aging using quantitative polymerase chain reaction (qPCR) to assess relative telomere length and telomerase activity. To understand the interaction between the expression of *SIRT1* and stemness transcription factors in MSCs, sirtinol (a *SIRT1* inhibitor) was employed to block *SIRT1* expression. In addition, we also investigated how cordycepin affects the activation of autophagy in MSCs by examining key gene/protein expression. Furthermore, we developed cordycepin-containing cultural media for the maintenance of the self-renewal and multipotency of MSCs by combining an appropriate concentration of cordycepin with the standard medium. The anti-aging effects of the developed cultural media were determined by assaying for doubling time, senescence-associated β-galactosidase activity, relative telomere length, and telomerase activity.

1.5 Expected results

1.5.1 Cordycepin able to upregulate *SIRT1* and stemness transcription factors (*SOX2*, *NANOG*, and *OCT4*) in MSCs.

1.5.2 *SIRT1* is important in preventing the degradation of stemness transcription factors (*SOX2*, *NANOG*, and *OCT4*) in MSCs.

1.5.3 Cordycepin able to delay cellular senescence and aging of MSCs.

CHAPTER II

LITERATURE REVIEW

2.1 Mesenchymal stem cells (MSCs)

MSCs are adult stem cells that can be obtained from the bone marrow, umbilical cord, and cord blood which MSCs can be differentiated into different types of cells in the body, such as adipocytes, osteoblasts, chondrocytes, and myocytes (Backesjo, Li, Lindgren, and Haldosen, 2006). MSCs also play an important role in the generation of specific organs and tissue, as well as having the ability of self-renewal and multipotency (Fox et al., 2014). In cell therapy, human MSCs are cultured in vitro and subsequently auto-implant to a patient for the treatment that MSCs are able to repair damaged tissue through processes of self-renewal, migration, and differentiation without immune rejection (Chosa and Ishisaki, 2018).

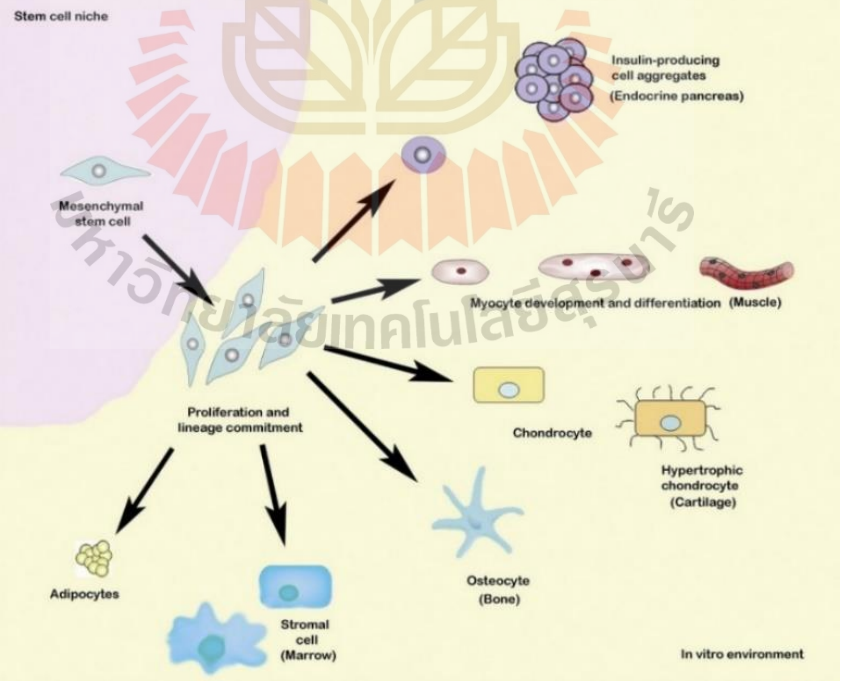

Figure 2.1 MSCs and MSC differentiation (Kode, Mukherjee, Joglekar, and Hardikar, 2009)

2.2 Mesenchymal stem cell in regenerative medicine

Today, a medical innovation known as regenerative medicine and cell therapy is gaining great attention as a new approach to the treatment of serious diseases caused by disorders or body defects (Chosa and Ishisaki, 2018). Currently, MSCs are being used in the treatment of degenerative diseases including bone and cartilage diseases, graft versus host disease, cardiovascular disease, autoimmune disease, cirrhosis, rheumatoid arthritis, diabetes, multiple sclerosis, Parkinson's disease, and Alzheimer's disease due to its unique healing properties (S. Wang, Qu, and Zhao, 2012).

2.3 The aging of mesenchymal stem cells

The aging of MSCs is a major problem that causes the decrease of stem cell properties. When long-term culture MSCs *in vitro*, the ability of self-renewal and multipotency is attenuated and cannot be used for therapies in regenerative medicine. (Muraglia, Cancedda, and Quarto, 2000; Raggi and Berardi, 2012). The population of senescent cells shows an age-dependent increase in the stem cell pool cause the aging-induced loss of MSCs number and function (H. Chen, Liu, Zhu, et al., 2014). Long-term culture MSCs in vitro leads to the slow accumulation of senescent cells (Wagner et al., 2008), telomere erosion (Baxter et al., 2004), and phenotypic alteration (Halfon, Abramov, Grinblat, and Ginis, 2011; Jones et al., 2010). Moreover, long-term cultured murine MSCs was reported that lose their ability to be recruited into the bone marrow and spleen (Rombouts and Ploemacher, 2003).

2.4 The functions and beneficial effect of cordycepin on stem cells

Cordycepin (3'-deoxyadenosine) is the derivative of the nucleoside adenosine and a bioactive compound from *Cordyceps militaris*, a fungus of the phylum Ascomycota (Cho, Cho, Rhee, and Park, 2007; Ramesh et al., 2012). Cordycepin has been reported about various biological benefits such as anti-tumor, antiviral, antioxidant, and anti-inflammatory (Z. Li, Gu, Lin, Ma, and Zhang, 2020). Cordycepin demonstrates inhibitory effects on cell proliferation among several cancer types by acting as a polyadenylation inhibitor (Noh et al., 2010). Cordycepin promotes the generation efficiency of induced pluripotent stem cells and maintain the self-renewal

and pluripotency of embryonic stem cells (ESCs) by activating Jak2/STAT3 signaling (C. H. Wang et al., 2020). Cordycepin able to inhibit m-TOR signaling that Leukemia inhibitory factor maintain the self-renewal and pluripotency of mouse ESCs by suppressing mTOR signaling (Cherepkova, Sineva, and Pospelov, 2016; Wong et al., 2010). Cordycepin exhibits the beneficial properties in osteogenesis of mesenchymal stem cells (MSCs). For example, the TNF-α-elicited inhibition of osteogenic differentiation of human adipose-derived MSCs was antagonized by cordycepin (J. Yang et al., 2015), oxidative stress-induced inhibition of osteogenesis of human bone marrow MSCs was prevented by cordycepin (F. Wang et al., 2015), and chondrocyte hypertrophy of C3H10T1/2 MSCs was inhibited by cordycepin (Cao et al., 2016).

2.5 The beneficial effect of cordycepin on anti-aging

Cordycepin has been used in traditional Chinese medicine to treat patient related to aging and senescence (Ji et al., 2009). Cordycepin has been demonstrated to have numerous pharmacological properties, including anticancer, antiviral, antioxidant, anti-aging, and anti-inflammatory activities (Ashraf et al., 2020). Cordycepin is a natural nucleoside analogue compound that has been shown to effectively reduce cell senescence and radiation-induced ulcer (Z. Wang et al., 2019). Cordycepin qualified to stimulate SIRT1 a regulator of cellular senescence through the AMPK-SIRT1 signaling pathway by increasing NAD+ (Takahashi et al., 2012). Cordycepin has been reported that ameliorates age-related testicular dysfunction in rats by ameliorating aging-induced gene expression associated with acetylation (SIRT1), and autophagy-related (m-TORC1) activity (Kopalli et al., 2019). Cordycepin attenuates aging by reducing the intensity of lipid peroxidation and by enhancing the activities of enzymatic and non-enzymatic antioxidants (Ramesh et al., 2012). Cordycepin inhibits cell senescence via NRF2 and AMPK for preventing radiation ulcer in rodents (Z. Wang et al., 2019).

2.6 The role of SIRT1 for maintenance of stemness in mesenchymal stem cells

2.6.1 SIRT1 induce cell proliferation and differentiation of MSCs

Sirtuin 1 (SIRT1) is an NAD+-dependent lysine deacetylase that

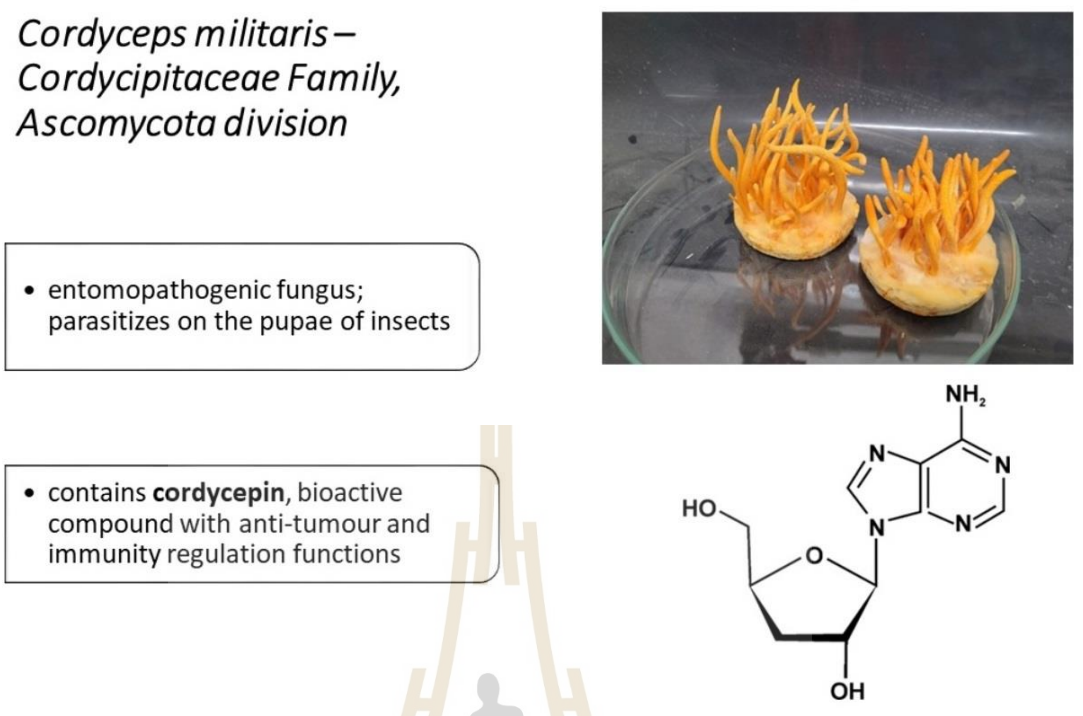

Figure 2.2 Cordycepin, a bioactive compound from the fungus Cordyceps militaris and structure (Jedrejko, Lazur, and Muszynska, 2021)

is involved in the regulation of cellular processes such as cellular senescence and metabolic process (D. S. Yoon et al., 2014). Aging of MSCs is negatively affected to cell proliferation and differentiation including osteogenesis and chondrogenesis (Alt et al., 2012) that SIRT1 is down-regulated during aging. Knockdown of SIRT1 in young MSCs inhibited cell proliferation whereas overexpression of SIRT1 in aged MSCs stimulated cell proliferation (H. Chen, Liu, Zhu, et al., 2014). The upregulation of SIRT1 induced cell proliferation and promoted the multilineage differentiation in MSCs (H. Chen, Liu, Chen, et al., 2014) but the reduction of SIRT1 in MSCs declines the self-renewal and multipotency (D. S. Yoon et al., 2014).

2.6.2 SIRT1 protects cellular senescence of MSCs

Increasing evidence suggests that increased SIRT1 activity may have a beneficial effect on aging and aging-related diseases in mammals (Herranz and Serrano, 2010). The upregulation of SIRT1 protected MSCs from cellular senescence caused by oxidative stress by downregulating $p16^{INK4\alpha}$ expression (L. Zhou et al., 2015). SIRT1 was

reported that could mitigate the replicative senescence of young MSCs in vitro (Yuan et al., 2012). SIRT1 contributes to long-term growth through the delay of senescence during prolonged cell passages in bone marrow MSCs (Yuan et al., 2012) Knockdown of Sirt1, early-passage bone marrow MSCs lose their self-renewal capacity and exhibit increased expression of cell cycle inhibitors, leading to the acceleration of cellular senescence (D. S. Yoon et al., 2014). Moreover, SIRT1 has been reported that prevent endothelial cells from replicative senescence or stress-induced premature senescence (J. Liu et al., 2010). SIRT1 associates anti-aging effects by deacetylating LKB1 or p53 (J. Liu et al., 2010; Ota et al., 2007). SIRT1 promotes transcription of telomerase reverse transcriptase to protect human umbilical cord fibroblasts from replicative senescence (Yamashita et al., 2012). Various proteins related to cellular senescence and the cell cycle including p53 and FOXO were suppressed by SIRT1 (Brooks and Gu, 2009; Finkel, Deng, and Mostoslavsky, 2009).

2.6.3 SIRT1 regulate the expression of pluripotent transcription factors

SIRT1 play a crucial role in maintaining the stemness of both ESCs and MSCs because SIRT1 able to regulate the expression of pluripotent transcription factors including SOX2, NANOG and OCT4 (D. S. Yoon et al., 2014). SOX2, NANOG and OCT4 a transcription factor that is well known as an important regulator for the maintenance of the self-renewal and pluripotency in ESCs (Boiani and Scholer, 2005; Boyer et al., 2005). In MSCs, SOX2, NANOG and OCT4 have also been identified as important regulators in maintaining self-renewal and multipotency (T. M. Liu et al., 2009; Pierantozzi et al., 2011; Seo et al., 2013; Tsai, Su, Huang, Yew, and Hung, 2012). SOX2 is considered to be essential for maintaining the self-renewal and multipotency of bone marrow MSCs (D. S. Yoon, Kim, Jung, Paik, and Lee, 2011). It has recently been shown that recombinant p300 acetylated SOX2 at lysine 75 induces nuclear export and proteasomal degradation in hESCs (Baltus et al., 2009). SOX2 at the protein level in BM-MSCs and SOX2-overexpressing HeLa cells were directly regulated by SIRT1 deacetylase through post-translational modification (D. S. Yoon et al., 2014). The knockdown of SIRT1 also leads to the acetylation and nuclear export of SOX2 protein, which reduces SOX2 level and stemness of bone marrow MSCs (D. S. Yoon et al., 2014). Moreover, the p53-mediated decrease of NANOG expression was suppressed by SIRT1, resulting in the

maintenance of self-renewal and pluripotent capacities in mouse ESCs (Han et al., 2008). The suppressed pluripotency in shRNA-mediated OCT4-silenced hESCs was rescued by SIRT1 overexpression via preventing p53 activation (Z. N. Zhang, Chung, Xu, and Xu, 2014).

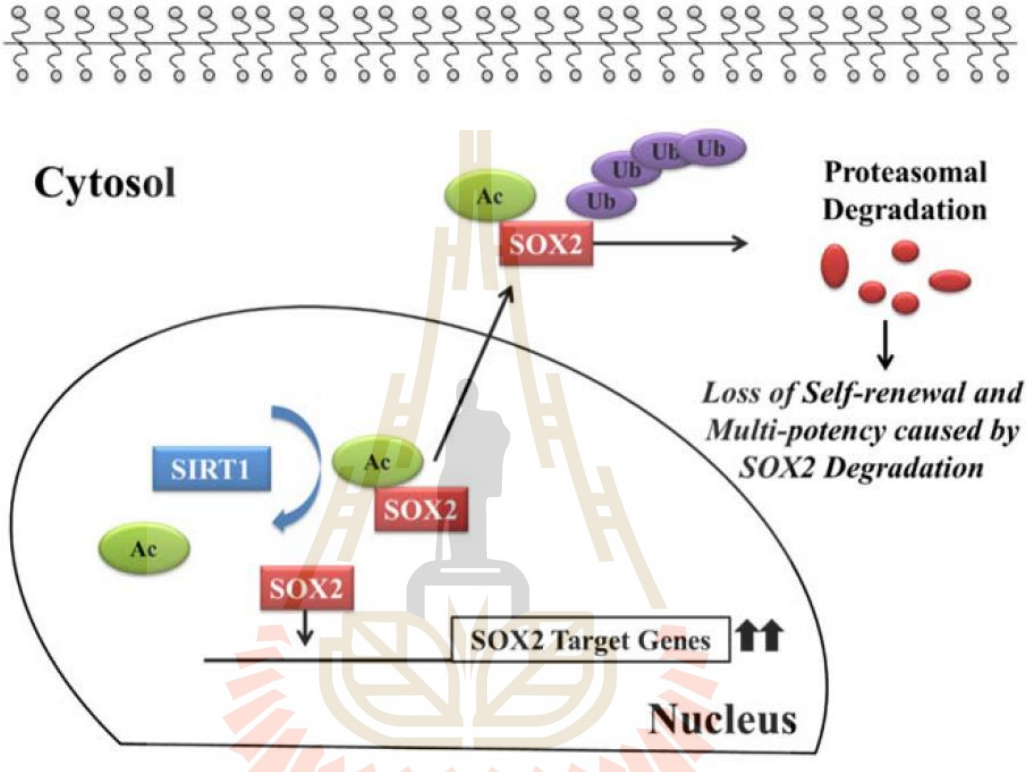

Figure 2.3 SIRT1 maintain stemness of MSCs by preventing the degradation of SOX2 (D. S. Yoon et al., 2014)

2.7 Aging-related genes in mesenchymal stem cells

2.7.1 p53 (cellular tumor antigen p53)

The tumor suppressor p53 has been studied almost exclusively as a transcription factor that functions by transactivating downstream targets involved in cell-cycle arrest, apoptosis, and DNA repair (Jin and Levine, 2001). p53 regulates polarity of cell division in mammary stem cells and suggest that loss of p53 favors symmetric divisions of cancer stem cells, contributing to tumor growth (Cicalese et al., 2009). The activation of p53 in ESCs leads to NANOG downregulation and ESC differentiation

(Yoshida-Koide et al., 2004). A primary role of p53 in pluripotent stem cells (PSCs) is to induce the differentiation of PSCs and inhibit pluripotency, providing mechanisms to maintain the genomic stability of the self-renewing PSCs (Fu, Wu, Li, Xu, and Liu, 2020)

2.7.2 m-TOR (mammalian target of rapamycin)

The mammalian target of rapamycin (m-TOR) is a crucial regulatory factor in the aging process of many different animals. Several different animals, including Caenorhabditis elegans, Drosophila melanogaster, Mus musculus, and Saccharomyces cerevisiae have shown that suppressing m-TOR activity increases lifespan (Deprez, Eskes, Winderickx, and Wilms, 2018; Vellai et al., 2003). According to research, SIRT1 and mTOR regulate autophagy function to help control aging (P. Chen, Chen, Lei, Li, and Zhou, 2019). SIRT1 restored autophagy impairment brought on by oxidative stress and increased embryonic stem cell survival by inhibiting the m-TOR pathway (Ou, Lee, Huang, Messina-Graham, and Broxmeyer, 2014). Additionally, it has been discovered that SIRT1 inhibition activates the m-TOR pathway, resulting in autophagy damage (Takeda-Watanabe, Kitada, Kanasaki, and Koya, 2012).

2.7.3 NF-**K**B (Nuclear factor kappa B)

A key transcription factor known as NF-**K**B is involved in a variety of inflammatory and aging-related disorders, including Alzheimer's, Parkinson's, diabetes, atherosclerosis, and arthritis. The aging process is accelerated by persistent NF-**K**B activation, which is also related to cellular and physical aging (H. Zhang et al., 2019). SIRT1 is associated with longevity and inhibits NF-**K**B signaling (Edwards et al., 2013).

2.8 Role of telomere length and telomerase activity in aging

Telomeres in young humans are approximately 8,000-10,000 nucleotides long. They do, however, shorten with each cell division, and when they reach a critical length, the cell either stops dividing or dies (Conger, 2015). Telomeres are repeating non-coding DNA sequences that are found at the ends of chromosomes in eukaryotic cells. Their primary function is to prevent chromosomal ends from being mistaken for DNA damage (Kosebent, Uysal, and Ozturk, 2018). Telomeres can only replicate for a limited amount of time before they become too short, which could result in chromosome instability or cell death. Each time a cell replicates, these chromosomal

ends are shortened. Chromosomes may suffer telomere end fusions, abnormal recombination, and degradation if they become too short (Axelrad, Budagov, and Atzmon, 2013). Therefore, maintaining telomere length is crucial for chromosome stability and cell survival (Maser and DePinho, 2004). According to numerous studies, an organism's longevity and pathological state, such as cancer, diabetes, and cardiovascular disease are related to telomere length (Bekaert et al., 2007; Monickaraj et al., 2012; X. Wu et al., 2003). Additionally, shortened telomeres have been linked to unhealthy lifestyles or high levels of stress, possibly via accelerating cell aging and degeneration (Pont, Sadri, Hsiao, Smith, and Schneider, 2012; Sun et al., 2012). The telomere-lengthening process is assisted by the telomerase enzyme. Telomerase has been found to be exclusively expressed in germ cells, granulosa cells, early embryos, stem cells, and many cancerous cell types (Kosebent et al., 2018). The activation of the cellular enzyme telomerase reverse transcriptase (TERT) is one of the cell's natural defenses against telomere shortening (Axelrad et al., 2013).

2.9 Autophagy and aging

Autophagy is a fundamental cellular mechanism that degrades molecules and subcellular components, such as nucleic acids, proteins, lipids, and organelles, through lysosome-mediated degradation in order to support homeostasis, differentiation, development, and survival (Aman et al., 2021). Numerous reports suggest that Sirtuin 1 or other proteins necessary for triggering autophagy, such as ATG proteins, have decreased expression in aging tissues and that autophagy declines with aging (Rubinsztein, Marino, and Kroemer, 2011). During autophagy, carboxy-terminal lipid modification of LC3 is a well-known process necessary for autophagosome formation (Klionsky et al., 2021). The finding that improved autophagy might increase lifespan and the reduction of autophagy caused by critical ATG gene mutations inhibits the gain of longevity in C. elegans (Melendez et al., 2003). Caloric restriction triggers autophagy by turning on either of the two energy sensors AMPK or Sirtuin 1 (Canto et al., 2010; Elgendy, Sheridan, Brumatti, and Martin, 2011). Additionally, caloric restriction can cause autophagy by suppressing insulin/insulin-like growth (IGF) factor signaling, which also inhibits mTOR (Kenyon, 2010). Moreover, adenosine monophosphate-activated protein

kinase (AMPK) is another factor that can directly trigger autophagy, this is brought on when nutrients are scarce or when AMP/ATP ratios increase, resulting in immediate Ulk1 activation (Egan et al., 2011; J. Kim, Kundu, Viollet, and Guan, 2011).

2.10 The senescence-associated β-galactosidase assay

Senescent cells can be identified from healthy cells by failing to synthesize DNA under optimal culture conditions or by having unique gene expression patterns when they are aging (Itahana, Campisi, and Dimri, 2007). The discovery that senescent cells exhibit β-galactosidase activity, which is histochemically observable at pH 6.0 (Dimri et al., 1995). The discovery named this activity the senescence-associated β-galactosidase, or SA-β-gal, and hypothesized that it might be a useful biomarker to detect senescent cells in culture and in vivo (Dimri et al., 1995). SA-β-gal also demonstrated an age-dependent increase in dermal fibroblasts and epidermal keratinocytes in skin samples from human donors of various ages (Dimri et al., 1995). Despite the fact that results demonstrated that this marker was not a perfect senescence- or age-dependent marker (for example, it was also expressed when cells were maintained at confluence for prolonged periods), the discovery demonstrated that it was closely related to the senescent phenotype and that its frequency increased with aging in tissues, which is consistent with the accumulation of senescent cells in vivo (Itahana et al., 2007). The hypothesis that SA-β-gal is a helpful biomarker for the detection of senescent cells in vitro as well as in vivo in rats and primates has been supported by a number of subsequent studies (Itahana, Campisi, and Dimri, 2004; Krishnamurthy et al., 2004).

2.11 Cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the cell viability assay that converted MTT into a purple-colored formazan product by viable cells with active metabolism (Riss et al., 2016). To determine the level of cell viability, the amount of formazan is measured using a spectrophotometer in absorbance at 570 nm (Mashyakhy et al., 2021). Since dying cells are incapable of converting MTT into formazan, color formation serves as a

useful and valuable indicator of only the living cells. Although the exact mechanisms by which MTT is reduced into formazan in cells is unknown, it is most likely a result of an interaction with NADH or other reducing molecules that help MTT receive electrons (Marshall, Goodwin, and Holt, 1995). The MTT tetrazolium assay can be used to test for drug sensitivity, cytotoxicity, cell stimulation, and growth factor responsiveness.

CHAPTER III

MATERIALS AND METHODS

3.1 MSCs and cell culture

MSCs were obtained from I Wellness co., Ltd. (Nakhon Ratchasima, Thailand) under ethical approved conditions. MSCs were cultured in the standard cell culture medium, which includes 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, and 1% (v/v) penicillin-streptomycin. MSCs were incubated at 37°C in a humidified incubator with 5% carbon dioxide (CO_2).

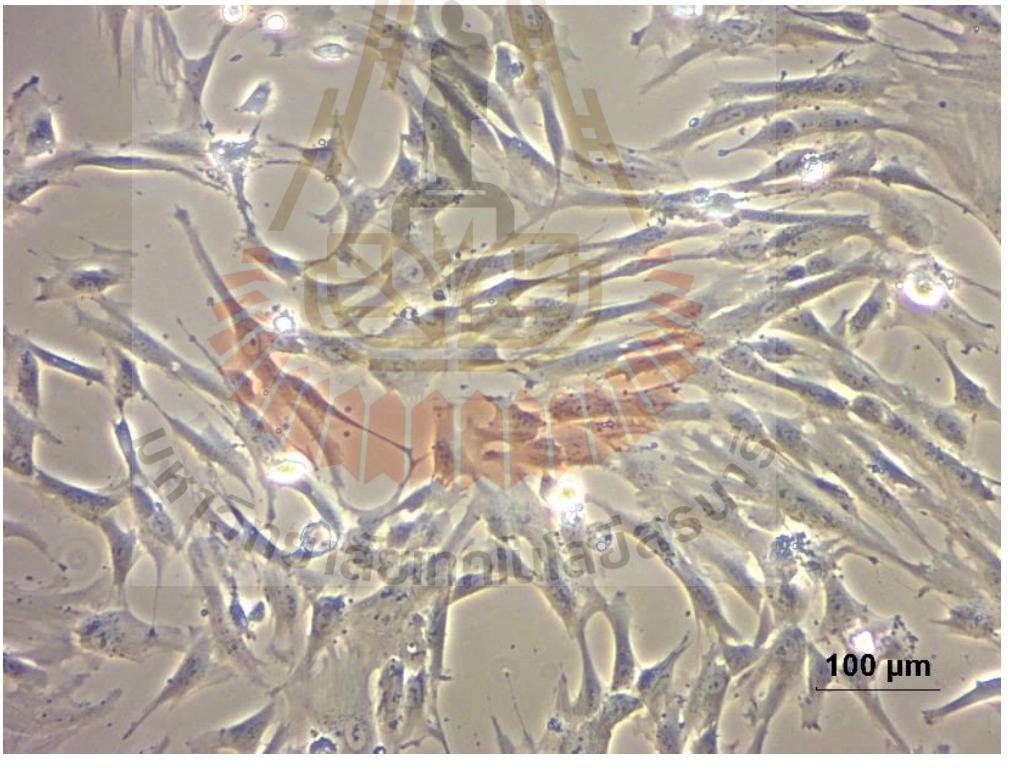

Figure 3.1 MSCs culture in standard culture medium and morphology

3.2 Cell viability assays

MSCs were cultured in a 96-well plate with 2,000 cells per well and incubated for 24 h. After that, cells were treated with various doses of cordycepin standard or left

untreated for 24 h. The cordycepin standard was purchased from Sigma-Aldrich (Darmstadt, Germany), which was dissolved in dimethyl sulfoxide (Sigma-Aldrich) at a stock concentration of 25 mM. Then, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) colorimetric technique was used to determine cytotoxicity (Van Meerloo, Kaspers, and Cloos, 2011). The absorbance was determined at 570 nm with the use of a microplate reader (BMG Labtech, Ortenberg, Germany). It was assumed that the control group represented 100% viability. Additionally, cordycepin's half-maximal inhibitory concentration (IC50) was calculated using equation 1. The evaluate of IC50 is to plot x-y and fit the data with a straight line (linear regression). The x is a series of cordycepin concentrations and the y is a series of %cell viability.

$$IC50 = (0.5-b)/a,\ Y=a*X+b \quad (1)$$

3.3 Effects of cordycepin on messenger ribonucleic acid (mRNA) expression in MSCs by reverse transcription-polymerase chain reaction (RT-PCR)

MSCs at passage 8 (late passage) were seeded at 30,000 cells per well in a 6-well plate and incubated for 24 h. After that, cells were treated with doses of cordycepin standard in 0.25, 1, and 4 μM for 24 h, Cells left untreated were used as a negative control and young MSCs at passage 3 (early passage) as a positive control group. To investigate the interaction between the expression of SIRT1 and stemness transcription factors, MSCs were treated with 100 μM sirtinol (Sigma-Aldrich) and 0.25 μM cordycepin for 24 h. The MSCs were collected using 0.025% trypsin (Sigma-Aldrich) and total ribonucleic acid (RNA) was extracted using NucleoSpin®, MACHEREY-NAGEL, Dueren, Germany. Complementary deoxyribonucleic acid (cDNA) was synthesized from 1 μg of RNA by ReverTra AceTM qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). PCR reactions were prepared using 2X Taq Master Mix (Vivantis, Shah Alam, Malaysia), cDNA, nuclease-free water, and specific primers for SIRT1, SOX2, OCT4, NANOG, p53, m-TOR, NF-**K**B, LC3, ATG5, and ATG12. GAPDH was used as a control (Table I). PCR reactions were amplified using C1000 Touch PCR thermal cycler (BIO-RAD, Singapore) according to Molaee's protocol (Molaee, Abtahi, Ghannadzadeh, Karimi, and

Ghaznavi-Rad, 2015). The products were separated by electrophoresis on 1.5% agarose gel (Vivantis) in a buffer solution of Tris base-boric acid-EDTA (TBE, pH = 8) (Sigma-Aldrich). 6X loading dye (Vivantis) was used to track deoxyribonucleic acid (DNA) migration and 0.005% RedSafe™ (iNtRON Biotechnology, Gyeonggi-do, Korea) was used to stained DNA during electrophoresis. Agarose gel electrophoresis was performed using PowerPac™ Basic Power Supply (BIO-RAD) according to Abid's protocol (Abid et al., 2022). PCR amplicons were visualized using ImageQuant™ LAS 500, GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

3.4 Immunofluorescent antibody assays (IFA)

MSCs were seeded at 15,000 cells per well on slides in a 24-well plate and incubated for 24 h. After that, cells were treated with 0, 0.25, 1, and 4 μM cordycepin standard for 24 h. Fixing solution I (4% paraformaldehyde, 400 mM sucrose in phosphate buffered saline (PBS)) was applied to cells that were cultured on slides, and the cells were then incubated at 37°C for 30 min. Fixing solution II (fixing solution I with 0.5% Triton X-100) was applied to cell cultures on slides for 15 min at room temperature. Slides were washed with PBS, then treated with blocking buffer (0.5% bovine serum albumin (BSA) in PBS) and left at room temperature for 1 h. They were then washed three times with PBS before being incubated with anti-phospho-SIRT1 (Affinity Biosciences, Jiangsu Sheng, China) or anti-LC3-I/II (Merck, Darmstadt, Germany) antibodies at a 1:500 dilution at 4°C overnight. Slides were washed five times with cold PBS before reacting with 488 goat anti-rabbit IgG (H+L) (Sigma-Aldrich) at a dilution of 1:500. After washing five-time with cold PBS, slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Waltham, Massachusetts, USA) to identify the nucleus and mounted. Then, slides were observed using a fluorescence microscope (ZOETM Fluorescent Cell Imager, BIO-RAD).

3.5 Monodansylcadaverine (MDC) staining

MSCs were seeded at 15,000 cells per well on slides in a 24-well plate and incubated for 24 h. After that, cells were treated with doses of cordycepin standard in 0.25, 1, and 4 μM or left untreated (control) for 24 h. Fixing solution I (4%

paraformaldehyde, 400 mM sucrose in PBS) was applied to cells that were cultured on slides, and the cells were then left at 37°C for 30 min. Fixing solution II (fixing solution I with 0.5% Triton X-100) was applied to cell cultures on slides for 15 min at room temperature. Slides were washed with PBS, then treated with blocking buffer (0.5% BSA in PBS) and left at room temperature for 1 h. They were then washed three more times with PBS before being incubated with 0.05 mM MDC (Sigma-Aldrich) at 37°C for 30 min. Then, slides were observed using a fluorescence microscope (ZOETM Fluorescent Cell Imager, BIO-RAD).

3.6 Senescence-associated β-galactosidase assay

MSCs were cultivated in 96-well plates from passages 3 to 9 using culture medium-containing 0.25 μM cordycepin or culture medium without cordycepin. Cells at passages 3, 5, 7, and 9 of continuous cell cultures were used to examine senescence-associated β-galactosidase. After cells reached 80% confluency, they were washed twice with PBS. Then, cells were fixed for 5 min at room temperature with freshly made 3.7% formaldehyde in PBS and washed twice with PBS. Then, 100 μl of the X-gal staining solution was added per well, according to Itahana's protocol (Itahana et al., 2007) and cells were incubated at 37°C (not in a CO2 incubator) for 15 h. A Microplate reader (BMG Labtech, Ortenberg, Germany) was used to measure absorbance at 420 nm (Kolev et al., 2022).

3.7 Doubling time calculation

MSCs were cultured at a cell density of 10,000 cells per well (initial cells) in 6-well plates using culture medium containing 0.25 μM cordycepin or culture medium without cordycepin. After 72 h, cells were collected using 0.025% trypsin (Sigma-Aldrich). The number of cells was counted using Bright-Line™ Haemocytometer (Sigma-Aldrich). The number of cells was converted to doubling time using equation 2 (V., 2006). Cells at passages 3, 5, 7, and 9 of continuous cell cultures were obtained to examine doubling time.

$$\text{Doubling time} = (\text{Duration}*\log 2)/(\log(\text{Final cells})-\log(\text{Initial cells})) \quad (2)$$

3.8 Relative telomere length by qPCR

Genomic DNA was extracted from MSCs using Mouse Direct PCR Kit (Selleck Chemicals LLC, Houston, USA). qPCRBIO SyGreen Mix (PCR Biosystems, Wayne, Pennsylvania, USA) and QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were used for quantitative analyses. The 36B4 gene was employed as a single-copy gene and the TEL gene was used to determine the relative telomere length (Table I) according to Vasilishina's protocol (Vasilishina, Kropotov, Spivak, and Bernadotte, 2019). Cells at passages 3, 5, 7, and 9 of continuous cell cultures were taken to examine relative telomere length.

3.9 Telomere activity measurement by qPCR

Total RNA was extracted by NucleoSpin® (MACHEREY-NAGEL) and cDNA was synthesized using ReverTra AceTM qPCR RT Master Mix with gDNA Remover (TOYOBO). qPCRBIO SyGreen Mix (PCR Biosystems) and QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific) were used for quantitative analyses. The GAPDH gene was employed as a housekeeping gene and hTERT (human telomerase reverse transcriptase) primers were used to analyze telomerase activity (Table I) (X. Liu et al., 2017) according to Vasilishina's method (Vasilishina et al., 2019). Cells at passages 3, 5, 7, and 9 of continuous cell cultures were taken to examine telomerase activity.

3.10 Statistical analysis

Statistical analysis was conducted using the IBM SPSS Statistics (IBM®, Armonk, New York, USA). Image J software (National Institute of Mental Health, Rockville, MD, USA) was used to analyze images of RT-PCR bands, IFA, and MDC staining. The results are shown as mean ± standard deviation (SD). The independent-samples t-test was used to analyze group differences. Statistical significance is indicated by $*p<0.05$ ($*p=0.01$-0.05; $**p=0.001$-0.01).

Table 3.1 PCR primer sets used for gene expression analysis.

Genes	Primers	Sequence (5'-3')
Glyceraldehyde	GAPDH-F	5'-CTCTGCTCCTCCTGTTCGAC-3'
3-phosphate dehydrogenase	GAPDH-R	5'-TTAAAAGCAGCCCTGGTGAC-3'
Sirtuin 1	SIRT1-F	5'-GAATACCTCCACCTGAGTTG-3'
	SIRT1-R	5'-GGCGAGCATAAATACCATCC-3'
Nanog homeobox	NANOG-F	5'-TCAATGATAGATTTCAGAGACAG-3'
	NANOG-R	5'-GGGTAGGTAGGTGCTGAGGC-3'
POU class 5	OCT4-F	5'-CTGAAGCAGAAGAGGATCAC-3'
homeobox 1	OCT4-R	5'-GGCCGCAGCTTACACATGTT-3'
SRY-box transcription factor 2	SOX2-F	5'-CACCTACAGCATGTCCTACTCG-3'
	SOX2-R	5'-GGTTTTCTCCATGCTGTTTCTT-3'
Tumor protein p53	p53-F	5'-CCCCTCCTGGCCCCTGTCATCTTC-3'
	p53-R	5'-GCAGCGCCTCACAACCTCCGTCAT-3'
Mammalian target of	m-TOR-F	5'-CTGGGACTCAAATGTGTGCAGTTC-3'
rapamycin kinase	m-TOR-R	5'-GAACAATAGGGTGAATGATCCGGG-3'
Nuclear factor kappa B	NF-**K**B-F	5'-GGTCTCTGGGGGTACAGTCA-3'
subunit 1	NF-**K**B-R	5'-GTCCTTCCTGCCCATAATCA-3'
Microtubule associated	LC3-F	5'-GATGTCCGACTTATTCGAGAGC-3'
protein 1 light chain 3 beta 2	LC3-R	5'-TTGAGCTGTAAGCGCCTTCTA-3'
Autophagy related 5	ATG5-F	5'-TGGCTGAGTGAACATCTGAG-3'
	ATG5-R	5'-AAGTAAGACCAGCCCAGTTG-3'
Autophagy related 12	ATG12-F	5'-TGCTGGAGGGGAAGGACTTA-3'
	ATG12-R	5'-CACGCCTGAGACTTGCAGTA-3'
Ribosomal protein lateral	36B4-F	5'-CGTCCTCGTGGAAGTGACAT-3'
stalk subunit P0	36B4-R	5'-ATCTGCTTGGAGCCCACATT-3'
Telomere	TEL-F	5'CGGTTTGTTTGGGTTTGGGTTTGGGT TTGGGTTTGGGTT-3'
	TEL-R	5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTA CCCT-3'
Telomerase reverse	hTERT-F	5'-GAGAACAAGCTGTTTGCGGG-3'
transcriptase	hTERT-R	5'-AAGTTCACCACGCAGCCATA-3'

CHAPTER IV
RESULTS AND DISCUSSION

4.1 The cytotoxicity of cordycepin on MSCs

To demonstrate cell survival and cytotoxicity, MSCs were treated with cordycepin at concentrations of 0.25, 1, 4, 8, and 16 μM and untreated MSCs served as the control (Figure 4.1 A). Our results indicated that, %cell viability following treatment with cordycepin at concentrations between 0.25 and 4.0 μM was not significantly different from the control group; however, concentrations between 8.0 and 16.0 μM differed significantly from the control group. After treatment with 0.25, 1, and 4 μM cordycepin for 24 h, the cell morphology of MSCs was similar to that of the control group in terms of size and flatness (Figure 4.1 B). Furthermore, the cordycepin IC50 was 27.6 μM.

Cordycepin has been demonstrated to inhibit mRNA polyadenylation by incorporating into RNA (Liao et al., 2015). Our results revealed that proliferation of MSCs is decreased when cordycepin concentration is raised. Moreover, the results revealed that cordycepin significantly reduced percent viability of MSCs at concentrations greater than or equal to 8.0 μM and percent viability of MSCs is decreased to 50% at 27.6 μM cordycepin. Therefore, the appropriate concentrations to investigate the effect of cordycepin in MSCs is less than or equal to 4 μM.

4.2 The effect of cordycepin on the expression of SIRT1 and aged-related mRNAs in MSCs

The expression levels of SIRT1, SOX2, OCT4, NANOG, p53, m-TOR, and NF-**K**B mRNAs in the cordycepin-treated MSCs were assessed using RT-PCR (Figure 4.2). In comparison to the aged MSC group, cordycepin significantly up-regulated SIRT1 at 0.25 μM, insignificantly up-regulated SIRT1 at 0.1 μM, and significantly down-regulated SIRT1 at 4.0 μM. In comparison to the aged MSCs group, cordycepin significantly up-regulated SOX2 at 0.25 μM, insignificantly up-regulated SOX2 at 0.1 μM, and significantly

down-regulated SOX2 at 4.0 μM. Compared to the aged MSCs group, cordycepin significantly up-regulated OCT4 at 0.25 μM and significantly down-regulated OCT4 at 0.1 and 4.0 μM. Compared to the aged MSCs group, cordycepin significantly up-regulated NANOG at 0.25 μM and significantly down-regulated NANOG at 1.0 and 4.0 μM. Compared to the aged MSCs group, cordycepin significantly up-regulated p53 at 0.1 μM and significantly down-regulated p53 at 0.25 and 4.0 μM. Compared to the aged MSCs group, cordycepin significantly up-regulated m-TOR at 0.1 μM and significantly down-regulated m-TOR at 0.25 and 4.0 μM. Compared to the aged MSCs group, cordycepin significantly down-regulated NF-**K**B at concentrations of 0.25, 1.0, and 4.0 μM.

Cordycepin, also known as 3'-deoxyadenosine, is a derivative of the nucleoside adenosine, which is a bioactive compound derived from the Cordyceps militaris (S. Y. Yoon, Lindroth, Kwon, Park, and Park, 2022). The AMPK pathway has also been observed to be triggered by cordycepin (Guo et al., 2010; Wong et al., 2010). Cordycepin activates AMPK in cells by mimicking the effects of its natural activator, adenosine monophosphate (AMP) (Hawley et al., 2020). Cordycepin uptake to cells by adenosine transporter, and adenosine kinase converts it to cordycepin 5'-monophosphate, which then functions as an AMP analogue (Hawley et al., 2020). Cordycepin-induced activation of AMPK enhanced the activity of SIRT1 by increasing cellular NAD+ levels (Canto et al., 2009; Takahashi et al., 2012). Therefore, cordycepin significantly enhances SIRT1 expression in MSCs via the activation of AMPK-SIRT1 signaling pathway (Figure 4.8). Furthermore, cordycepin at concentrations of 1 and 4 μM showed neutral or negative effects on the expression of SIRT1, aged-related mRNAs (SOX2, OCT4, and NANOG), autophagy-related mRNAs (LC3, ATG5, and ATG12) and LC3 protein in MSCs; this could be because the cordycepin concentration was too high, which inhibits mRNA synthesis. Moreover, reduced Sirt1 expression resulted in increased expression of p53, m-TOR, and NF-**K**B at cordycepin concentrations 1 and 4 μM, but because cordycepin concentrations were high, it decreased expression of p53, m-TOR, and NF-**K**B at 4 μM and 1 μM of NF-**K**B by inhibiting mRNA synthesis while the reduced expression of p53, m-TOR, and NF-**K**B at 0.25 μM cordycepin caused by deacetylation of SIRT1.

Aging is a natural process in which structural integrity of an organism steadily

deteriorates over time resulting in impaired function and raising the possibility of death (Lopez-Otin, Blasco, Partridge, Serrano, and Kroemer, 2013). Cellular senescence is a state of persistent cell-cycle arrest that results in a stable and long-term loss of proliferative capability, despite continued cell viability and metabolic activity (Zurgil et al., 2014). During the cellular aging process, autophagy, energy metabolism regulation, stress tolerance, and metabolic state gradually deteriorate (Y. Liu, Weng, Gao, and Liu, 2019). SIRT1 integrates a variety of signaling and transcriptional pathways such as, p53, m-TOR, and NF-**K**B, which are known to control aging, and therefore is a target for increasing a healthy lifespan (C. Chen et al., 2020). Our results indicated that cordycepin increases the expression of SIRT1 and reduces the expression of p53 in MSCs, suggesting that cordycepin protects MSCs from DNA damage and stress-induced cellular senescence (M. Li et al., 2012). Our study showed that cordycepin inhibits m-TOR expression in MSCs through SIRT1-mediated deacetylation of tuberous sclerosis complex 2 (TSC2), which activates autophagy and extends lifespan (P. Chen et al., 2019; Garcia-Aguilar, Guillen, Nellist, Bartolome, and Benito, 2016). Moreover, our results showed that cordycepin suppresses NF-**K**B expression in MSCs via SIRT1-mediated deacetylation of p65, which acts against inflammation and aging (B.-W. Wu et al., 2021). Therefore, cordycepin inhibits cellular senescence and slows the aging process via SIRT1 and age-related signaling pathways (Figure 4.8).

4.3 The effect of cordycepin on the protein levels of phosphorylated SIRT1 (p-SIRT1) in MSCs

Immunofluorescence staining was used to measure the protein levels of p-SIRT1 in cordycepin-treated MSCs (Figure 4.3). Compared with the control group, cordycepin treatment significantly up-regulated the protein levels of p-SIRT1 at 0.25 μM; however, at 1.0 and 4.0 μM significantly down-regulated the levels of p-SIRT1.

4.4 The interaction between the expression of SIRT1 and stemness transcription factors in MSCs

To examine the interaction between SIRT1 expression and stemness transcription factors, MSCs were treated with 100 μM sirtinol to suppress SIRT1 activity.

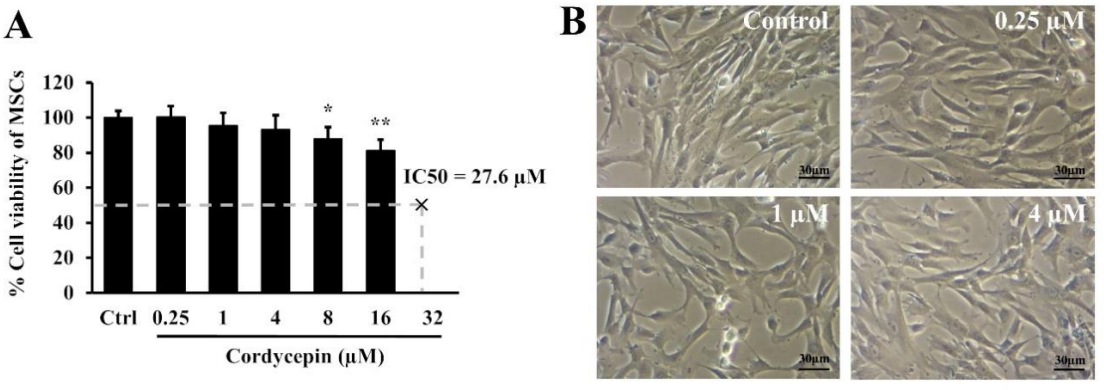

Figure 4.1 The effect of cordycepin on cell proliferation in MSCs. (A) The cytotoxicity of cordycepin on MSCs was evaluated by using the MTT assay. IC50 was calculated using equation 1. (B) The morphology of MSCs treated with or without cordycepin. 10X magnification was used to digitally capture the images (scale bar: 30 µm). Data is presented as mean ± SD. Independent-samples t-test was used to analyze group differences (n = 3). Statistical significance is indicated by *p<0.05 (*p=0.01-0.05; **p=0.001-0.01).

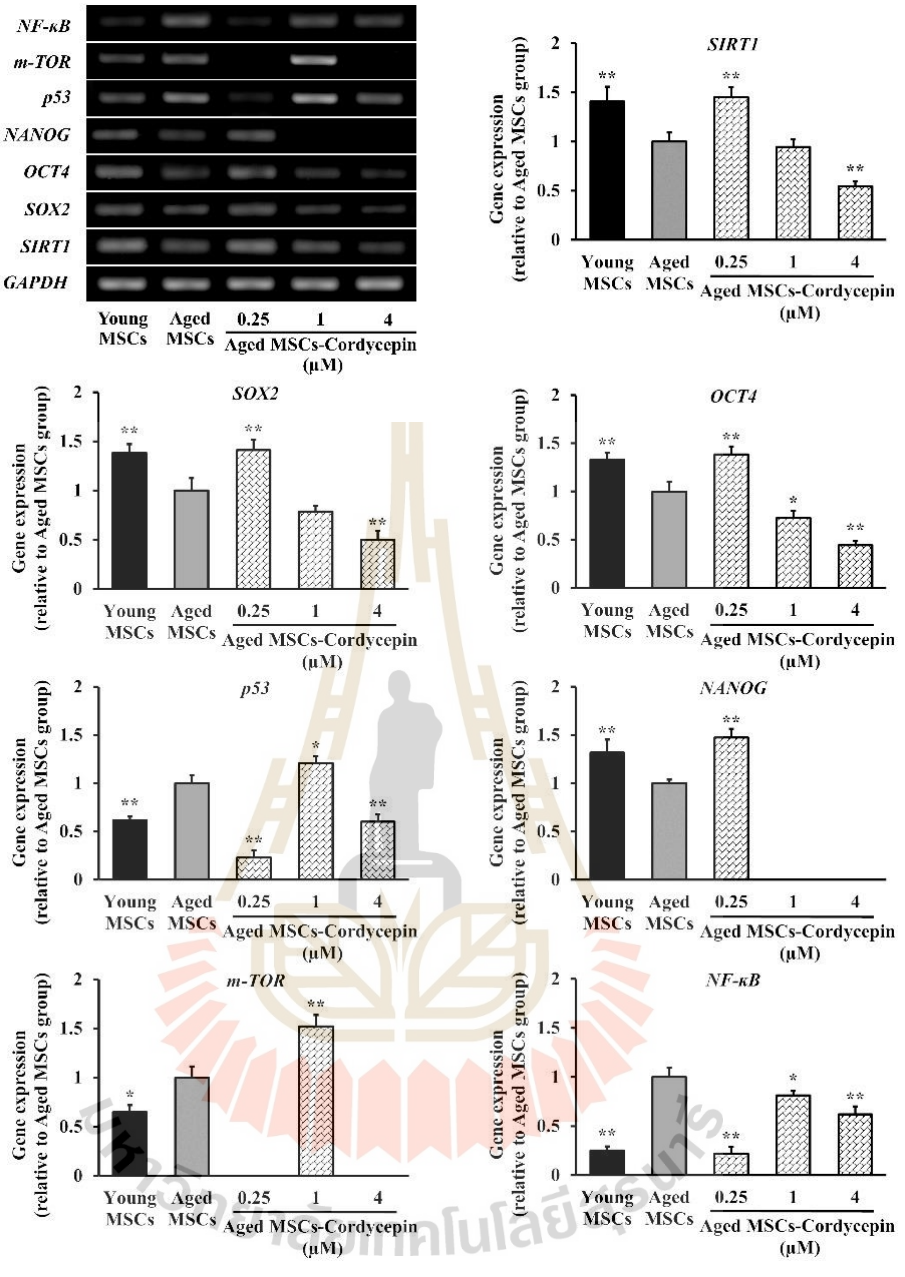

Figure 4.2 The effect of cordycepin on mRNA expression in MSCs. The mRNA expression levels of SIRT1, SOX2, OCT4, NANOG, p53, m-TOR, and NF-**K**B were examined using RT-PCR in aged MSCs treated with cordycepin for 24 hours; young MSCs were used as a positive control and aged MSCs as a negative control. Image j was used to analyze images of RT-PCR bands. Data is presented as mean ± SD. Independent-samples t-test was used to analyze group differences (n = 3). Statistical significance is indicated by *p<0.05 (*p=0.01-0.05; **p=0.001-0.01).

MSCs received four different treatments: treatment 1, standard medium (control group); treatment 2, standard medium containing 0.25 μM cordycepin; treatment 3, standard medium containing 100 μM sirtinol; and treatment 4, standard medium containing 0.25 μM cordycepin and 100 μM sirtinol. The results showed that in comparison to the control group treatment 2 significantly increased the expression of SIRT1, SOX2, OCT4, and NANOG, treatment 3 significantly decreased the expression of SIRT1, SOX2, OCT4, and NANOG, and treatment 4 significantly decreased the expression of SIRT1, SOX2, OCT4, and NANOG (Figure 4.4).

According to the results, cordycepin can activate the stemness transcription factors SOX2, OCT4, and NANOG in MSCs when used at a concentration of 0.25 μM. Additionally, the transcription factors SOX2, NANOG, and OCT4 have also been discovered as being essential for maintaining the self-renewal and multipotency of MSCs (T. M. Liu et al., 2009; Pierantozzi et al., 2011; Seo et al., 2013; Tsai et al., 2012). Yoon et al. have suggested that SIRT1 may be a crucial regulator in maintaining the stemness of MSCs due to its capacity to control the expression of transcription factors including SOX2, OCT4 and NANOG through deacetylation, which prevents nuclear export of and its subsequent ubiquitination and degradation in the cytoplasm (D. S. Yoon et al., 2014). When SOX2 is in the nucleus, it regulates the expression of OCT4 and NANOG for maintaining self-renewal and multipotency in MSCs (Lakatos, Travis, Pierson, Vivian, and Czirok, 2014). Our results revealed that sirtinol-induced down-regulation of SIRT1 expression also decreased the expression of SOX2, OCT4, and NANOG. Therefore, SIRT1 is important in preventing the degradation of stemness transcription factors SOX2, OCT4 and NANOG in MSCs, and cordycepin has the potential to maintain the stemness of MSCs by interacting with SIRT1 and SOX2 (Figure 8).

4.5 The effect of cordycepin on autophagy in MSCs.

The expression levels of LC3, ATG5, and ATG12 mRNAs in cordycepin-treated MSCs were assessed using RT-PCR (Figure 4.5). Compared to the aged MSCs group, cordycepin significantly up-regulated LC3 at 0.25 μM, insignificantly up-regulated LC3 at 0.1 μM, and significantly down-regulated LC3 at 4.0 μM. Compared to the aged MSCs group, cordycepin significantly up-regulated ATG5 at 0.25 μM, but insignificantly up-regulated ATG5 at 0.1 and 4.0 μM. Compared to the aged MSCs group, cordycepin

significantly up-regulated ATG12 at 0.25 μM and significantly down-regulated ATG12 at 0.1 and 4.0 μM. Additionally, the protein levels of LC3 in MSCs were determined using immunofluorescence staining, and the autophagic vacuoles of MSCs were identified using MDC staining (Figure 4.6). Compared to the control group, treatment with cordycepin significantly increased the protein levels of LC3 at 0.25 μM, insignificantly up-regulated the protein levels of LC3 at 0.1 μM. However, the protein levels of LC3 were significantly down-regulated following treatment with 4.0 μM cordycepin. Moreover, in compared to the control group, treatment with cordycepin significantly increased the levels of autophagic vacuoles at 0.25 μM, insignificantly up-regulated the levels of autophagic vacuoles at 0.1 μM, and significantly down-regulated autophagic vacuoles at 4.0 μM.

Autophagy is a crucial degradative mechanism for adaptive responses to metabolic stress, such as nutrient starvation; it functions to remove potentially harmful components including protein aggregates and dysfunctional subcellular organelles from cells to maintain cellular homeostasis (Ha, Guan, and Kim, 2015). Reduced autophagy has been linked to accelerated aging, whereas increased autophagy may have powerful anti-aging effects (Madeo, Tavernarakis, and Kroemer, 2010). The results indicate that 0.25 μM cordycepin can promote autophagy in MSCs by increasing the expression of proteins and genes involved in autophagy, including LC3, ATG5, and ATG12. Cordycepin has the potential to induce autophagy by activating AMPK (Figure 4.8), which is increased when nutrients are limited or when AMP/adenosine triphosphate (ATP) ratios rise, and results in the direct activation of Ulk1 (Egan et al., 2011; J. Kim et al., 2011). Additionally, AMPK activation can potentially cause autophagy through blocking m-TOR (Rubinsztein et al., 2011). Moreover, SIRT1 promotes the formation of the ATG16-ATG5-ATG12 complex by directly deacetylating ATG5, ATG7, and ATG12, thereby promoting autophagic vesicle elongation (J. Y. Kim, Mondaca-Ruff, Singh, and Wang, 2022).

4.6 The anti-aging effects of cordycepin-containing culture media

To examine anti-aging effects of cordycepin, MSCs were cultured in medium containing 0.25 μM cordycepin standard medium without cordycepin as a control. Senescence-associated β-galactosidase assay was used to determine the aging of MSCs. The optical density of MSCs at passages 3 and 9 were not significantly different between

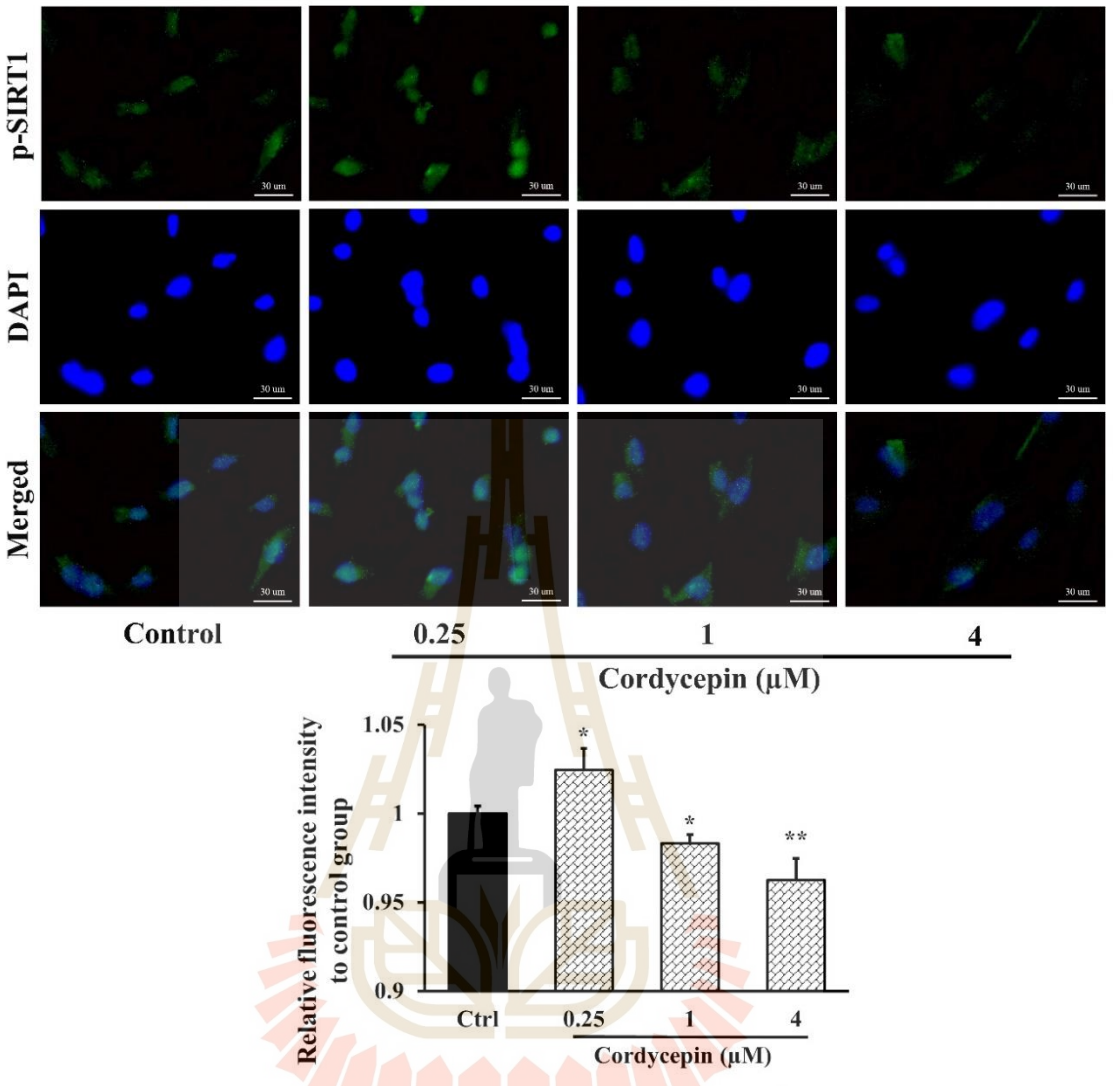

Figure 4.3 The effect of cordycepin on the protein expression levels of p-SIRT1 in MSCs. The protein levels of p-SIRT1 were assessed using immunofluorescent staining and ImageJ was used for staining intensity evaluation. DAPI was used to counterstain the cell nuclei. Fluorescence microscope was used to digitally capture the images (scale bar: 30 μm). Data is presented as mean ± SD. Independent-samples t-test was used to analyze group differences (n = 3). Statistical significance is indicated by *p<0.05 (*p=0.01-0.05; **p=0.001-0.01).

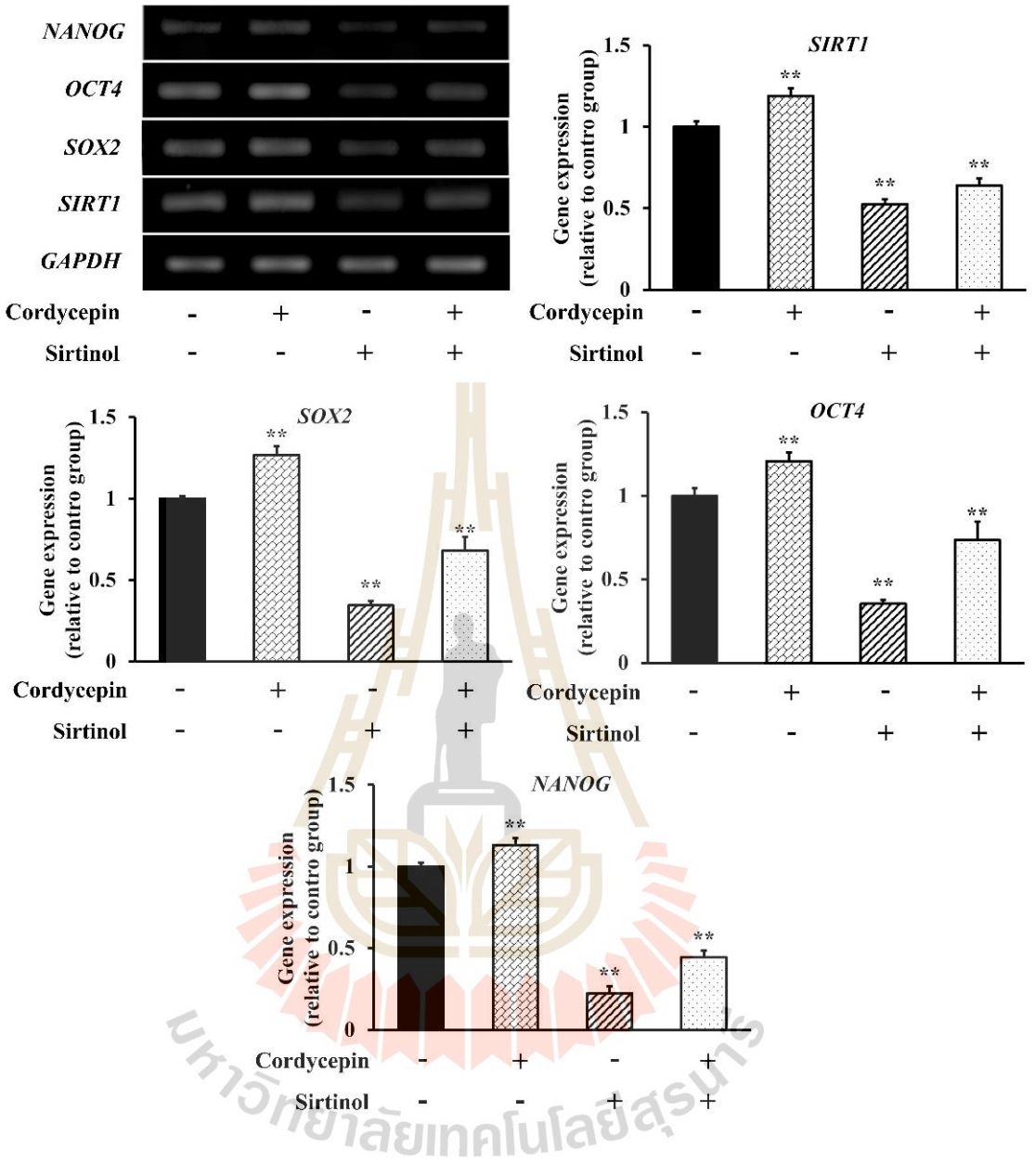

Figure 4.4 The effect of cordycepin and sirtinol on mRNA expression in MSCs. The mRNA expression levels of SIRT1, SOX2, OCT4, and NANOG were assessed using RT-PCR after MSCs were treated cordycepin and sirtinol for 24 hours. MSCs cultured in standard medium are the control group. Image j was used to analyze images of RT-PCR bands. Data is presented as mean ± SD. Independent-samples t-test was used to analyze group differences (n = 3). Statistical significance is indicated by *p<0.05 (*p=0.01-0.05; **p=0.001-0.01).

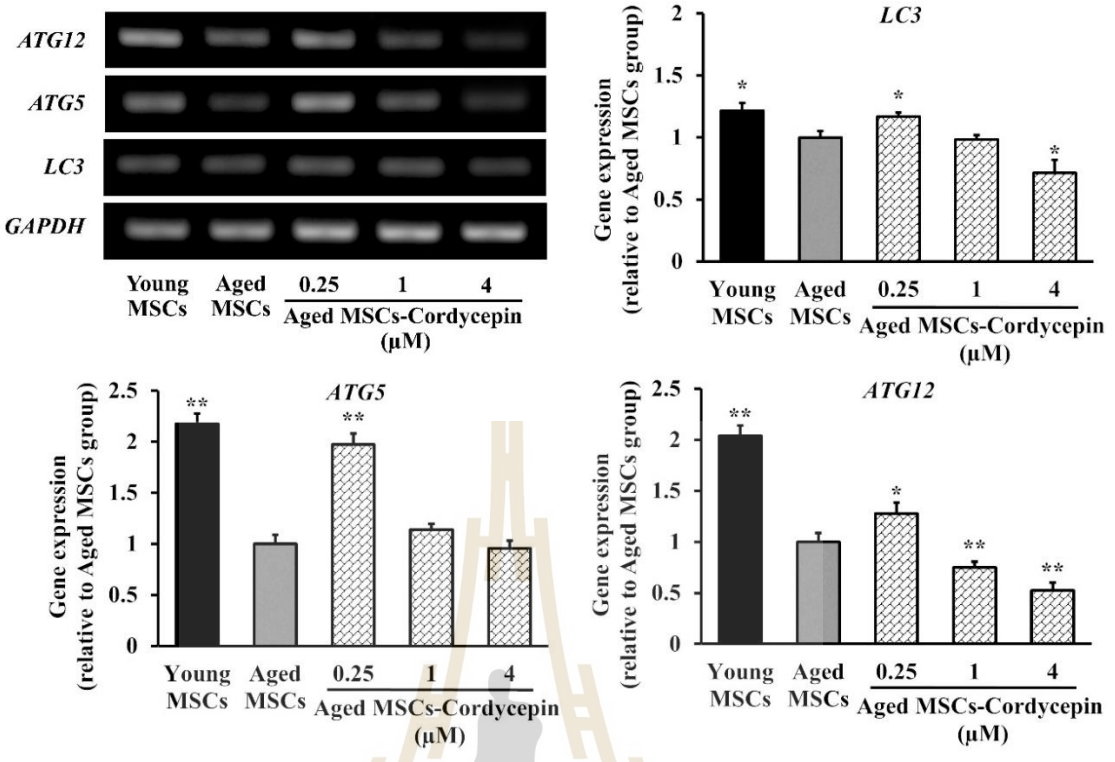

Figure 4.5 The effect of cordycepin on autophagy in MSCs. The mRNA expression levels of LC3, ATG5, and ATG12 were examined using RT-PCR in aged MSCs treated with cordycepin for 24 hours; young MSCs were used as a positive control and aged MSCs as a negative control. Image j was used to analyze images of RT-PCR bands. Data is presented as mean ± SD. Independent-samples t-test was used to analyze group differences (n = 3). Statistical significance is indicated by *p<0.05 (*p=0.01-0.05; **p=0.001-0.01).

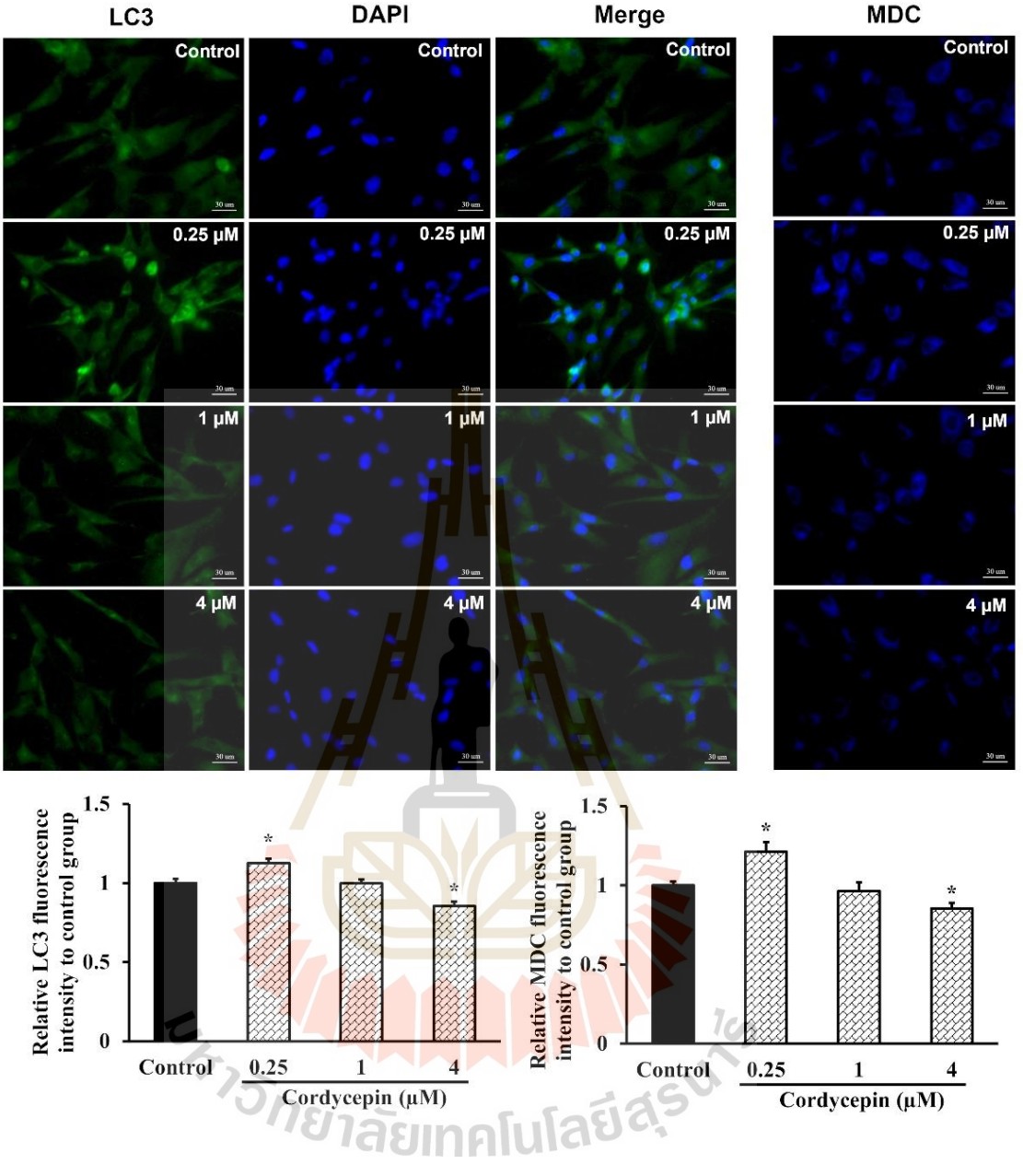

Figure 4.6 The effect of cordycepin on autophagy in MSCs. The protein levels of LC3 were measured using immunofluorescent staining and the levels of autophagic vacuoles were measured using MDC staining. ImageJ was utilized to evaluated staining intensity. The cell nuclei were stained with DAPI. The photos were digitally captured using a fluorescence microscope (scale bar: 30 m). Data is presented as mean ± SD. Independent-samples t-test was used to analyze group differences (n = 3). Statistical significance is indicated by *p<0.05 (*p=0.01-0.05; **p=0.001-0.01).

the control and cordycepin groups; however, at passages 4 to 8 there was a significant difference between the control and the cordycepin groups (Figure 4.7 A). Doubling times were used to measure the rate of cell growth. The doubling times of MSCs at passages 3 and 9 were not significantly different between the control and cordycepin groups, however at passages 4 to 8 were significantly different between the control and cordycepin groups (Figure 4.7 B). The relative telomere length of MSCs at passages 3 and 9 were not significantly different between the control and cordycepin groups, however, at passages 5 and 7 were significantly different between the control and cordycepin groups (Figure 4.7 C). The telomerase activity in MSCs at passages 3 to 7 were significantly different between the control and cordycepin groups, however at passages 9 was not significantly different between the control and cordycepin groups (Figure 4.7 D).

Finally, we developed the cordycepin-containing cultural media by combining 0.25 μM cordycepin with the standard medium to evaluate cordycepin's anti-aging effects in MSCs. MSCs at passages 3 through 9 were cultivated in cordycepin-containing culture media for comparison with the cultivation of MSCs on standard media free of cordycepin as a control. The assay of the activity of senescence-associated-galactosidase, a common marker of cellular senescence, indicated that cordycepin slowed down cellular senescence in MSCs (Piechota et al., 2016). In addition, we found that cordycepin can maintain the proliferation rate of MSCs, as measured by doubling time. The fact that cordycepin is able to maintain the proliferation rate of MSCs indicated that cordycepin can maintain the self-renewal and multipotency of MSCs, because aging is associated with reduced proliferation rate of MSCs (Alt et al., 2012). Moreover, we found that cordycepin prevented telomere length shortening in MSCs by increasing the expression of the hTERT gene (MedlinePlus [Internet], 2020). The health and lifespan of an individual are impacted by the progressive shortening of telomeres, which causes senescence, apoptosis, or oncogenic transformation of somatic cells (Shammas, 2011). However, although cordycepin can maintain stemness and prevent aging in MSCs but the cells will continue to age if culture is continued. Therefore, cordycepin has the ability to delay cellular senescence and aging in MSCs.

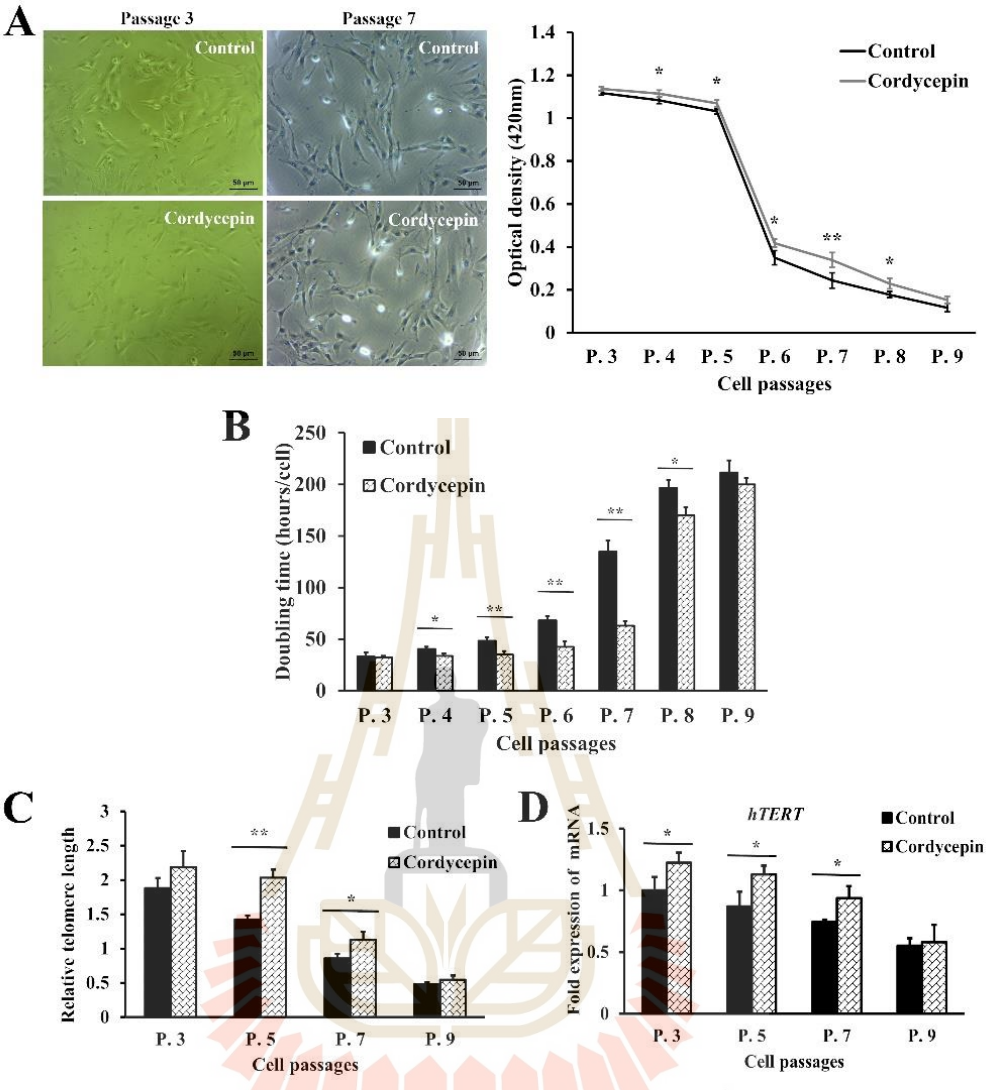

Figure 4.7 The anti-aging effects of cordycepin-containing cultural media. (A) The activity of senescence-associated-galactosidase in passage 3 to 9 MSC cultures treated with or without cordycepin by senescence-associated β-galactosidase assay measuring at 420 nm. (B) The rate of proliferation in passages 3 to 9 MSCs cultured in the presence or absence of cordycepin. (C) The relative telomere length was assessed using qPCR in passages 3 to 9 MSCs cultured with or without cordycepin. (D) The telomerase activity was assessed using qPCR in passages 3 to 9 MSCs cultured with or without cordycepin. Data is presented as mean ± SD. Independent-samples t-test was used to analyze group differences (n = 3). Statistical significance is indicated by *p<0.05 (*p=0.01-0.05; **p=0.001-0.01).

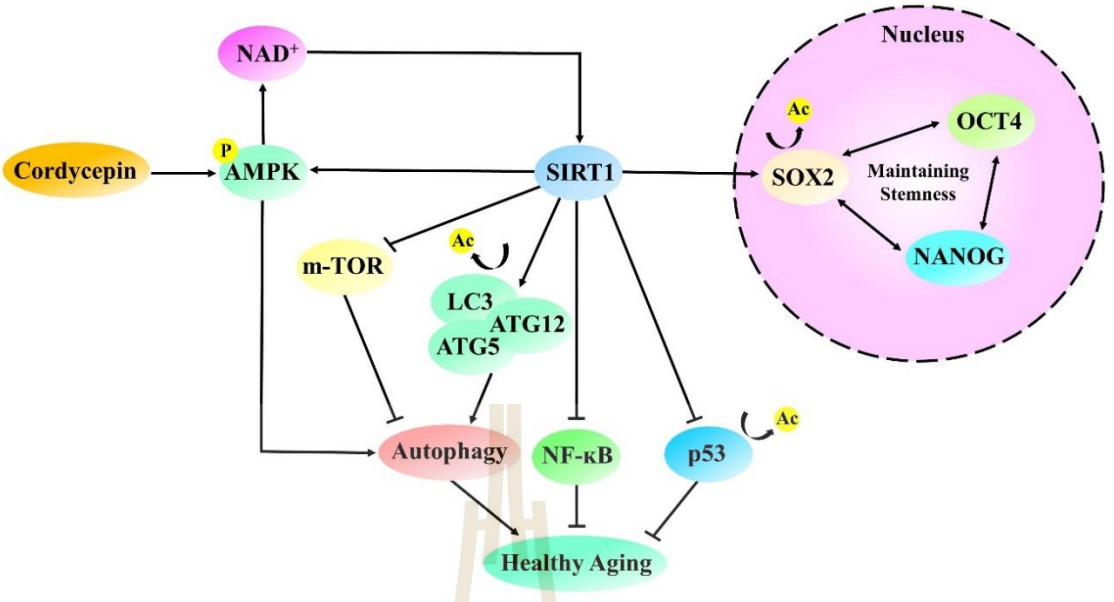

Figure 4.8 The cordycepin pathway promotes stemness maintainance in MSCs and prevents aging. The graphic provides a concise explanation of the signaling mechanisms through which cordycepin activates *SIRT1* for stemness maintenance and anti-aging in MSCs. The arrows represent stimulation, and the whiskered lines represent inhibition.

CHAPTER V
CONCLUSION

Cordycepin significantly increased the expression of *SIRT1* in MSCs by activating the adenosine monophosphate activated protein kinase (AMPK)-SIRT1 signalling pathway. Moreover, cordycepin maintained the stemness of MSCs by deacetylating SRY-box transcription factor 2 (SOX2) *via* SIRT1, and cordycepin delayed cellular senescence and aging of MSCs by enhancing autophagy, inhibiting the activity of senescence-associated-galactosidase, maintaining proliferation rate, and increasing telomere activity. Cordycepin could be used to increase *SIRT1* expression in MSCs for anti-aging applications.

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VITAE

Mr. Phongsakorn Chueaphromsri was born on July 2, 1990, in Chiang Rai, Thailand. In 2013, I graduated with a Bachelor of Science in Microbiology from Chiang Mai University, in Chiang Mai, Thailand. In 2014, I was employed by SGS (Thailand) Ltd as a microbiologist in Bangkok, Thailand. In 2015, I was employed by National Food Institute as a food technologist in Bangkok, Thailand. In 2016, I was a research assistant at King Mongkut's University of Technology Thonburi in Bangkok, Thailand. After that, I began my master's degree in biotechnology at Suranaree University of Technology in 2019 and was received an OROG scholarship. In 2022, I published enhancement of cordycepin production from *Cordyceps militaris* culture by epigenetic modification. The publication is as follows: Kunhorm, P., Chueaphromsri, P., Chaicharoenaudomrung, N., and Noisa, P. (2022). Enhancement of cordycepin production from Cordyceps militaris culture by epigenetic modification. Biotechnol Lett, 44(4), 581-593. In 29 April 2022, I gave an oral presentation entitled: The Effect of Cordycepin on SIRT1 Expression for Anti-aging Effect in Human Mesenchymal Stem Cells at Thai Society for Biotechnology International Conference Online.