in vivo *37*: 596-610 (2023)
doi:10.21873/invivo.13118

Cordycepin Enhances *SIRT1* Expression and Maintains Stemness of Human Mesenchymal Stem Cells

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Abstract. *Background/Aim: Mesenchymal stem cells (MSCs) have been employed for therapeutic applications of various degenerative diseases. However, the major concern is MSC aging during the in vitro cultivation. Thus, the approach to delay MSC aging was examined in this research by focusing on the expression of Sirtuin 1 (SIRT1), a key anti-aging marker. Materials and Methods: Cordycepin, a bioactive compound derived from Cordyceps militaris, was used to up-regulate SIRT1 and maintain stemness of MSCs. Upon treatment with cordycepin, MSCs were investigated for cell viability, doubling time, key gene/protein expression, galactosidase-associated senescence assay, relative telomere length, and telomerase expression. Results: 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. Conclusion: Cordycepin could be used to increase SIRT1 expression in MSCs for anti-aging applications.*

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Key Words: Cordycepin, mesenchymal stem cells, SIRT1, anti-aging.

Regenerative medicine and cell therapy are modern medical advancements that are gaining a lot of attention as novel methods for treating severe diseases (1). 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] (2). MSCs are adult stem cells that can be obtained from the bone marrow, adipose tissue, umbilical cord tissue, and umbilical cord blood (3). MSCs can differentiate into a variety of cells, including adipocytes, osteoblasts, chondrocytes, endothelial cells, and cardiomyocytes (4). MSCs have the capacity for self-renewal and multipotency (5), and play a significant role in the development of specific organs and tissues with special functions (6). However, MSC aging is a critical problem that contributes to the loss of self-renewal, stemness and differentiation potential (7). MSCs are ineligible to be used in regenerative medicine treatments after prolonged *in vitro* cultivation because their self-renewal and multipotency declines (8). 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 (9). 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 (10). SIRT1 participates in the regulation of the aged-related signaling pathways (FoXO1, NF-κB, AMPK, m-TOR, p53, and PGC-1α) directly or indirectly by deacetylating a number of key proteins to delay cellular senescence (11). 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 (12). 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 (13). Moreover, SIRT1 deactivation has also been shown to decrease cellular proliferation and accelerate senescence in MSCs (14). 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 (15, 16). Cordycepin has been demonstrated to have numerous pharmacological properties, including anticancer, antiviral, antioxidant, anti-aging, and anti-inflammatory activities (17). Cordycepin is a natural nucleoside analogue compound that has been shown to effectively reduce cell senescence and radiation-induced ulcer (18). 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 (19, 20). According to studies, cordycepin improved the antioxidant capacity and reduced age-related oxidative stress in old rats (21). 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 (22). Therefore, cordycepin is an attractive bioactive compound for promoting *SIRT1* to maintain the MSC properties.

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.

Materials and Methods

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).

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 (23). 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 (IC_{50}) was calculated using equation 1. The evaluate of IC_{50} 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.

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

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 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-κB*, *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 (24). 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, Republic of 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 (25). PCR amplicons were visualized using ImageQuant™ LAS 500, GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Table I. *PCR primer sets used for gene expression analysis.*

Genes	Primers	Sequence (5'-3')
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH-F	5'-CTCTGCTCCTCCTGTTCGAC-3'
	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 homeobox 1	OCT4-F	5'-CTGAAGCAGAAGAGGATCAC-3'
	OCT4-R	5'-GGCCGCAGCTTACACATGTT-3'
SRY-box transcription factor 2	SOX2-F	5'-CACCTACAGCATGTCCTACTCG-3'
	SOX2-R	5'-GGTTTTCTCCATGCTGTTTCTT-3'
Tumour protein p53	p53-F	5'-CCCCTCCTGGCCCCTGTCATCTTC-3'
	p53-R	5'-GCAGCGCCTCACAACCTCCGTCAT-3'
Mammalian target of rapamycin kinase	m-TOR-F	5'-CTGGGACTCAAATGTGTGCAGTTC-3'
	m-TOR-R	5'-GAACAATAGGGTGAATGATCCGGG-3'
Nuclear factor kappa B subunit 1	NF-κB-F	5'-GGTCTCTGGGGGTACAGTCA-3'
	NF-κB-R	5'-GTCCTTCCTGCCCATAATCA-3'
Microtubule associated protein 1	LC3-F	5'-GATGTCCGACTTATTCGAGAGC-3'
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 stalk subunit P0	36B4-F	5'-CGTCCTCGTGGAAGTGACAT-3'
	36B4-R	5'-ATCTGCTTGGAGCCCACATT-3'
Telomere	TEL-F	5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'
	TEL-R	5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'
Telomerase reverse transcriptase	hTERT-F	5'-GAGAACAAGCTGTTTGCGGG-3'
	hTERT-R	5'-AAGTTCACCACGCAGCCATA-3'

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, PR 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, MA, USA) to identify the nucleus and mounted. Then, slides were observed using a fluorescence microscope (ZOE™ Fluorescent Cell Imager, BIO-RAD).

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 (ZOE™ Fluorescent Cell Imager, BIO-RAD).

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 (26) and cells were incubated at 37˚C (not in a CO_2 incubator) for 15 h. A Microplate reader (BMG Labtech, Ortenberg, Germany) was used to measure absorbance at 420 nm (27).

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

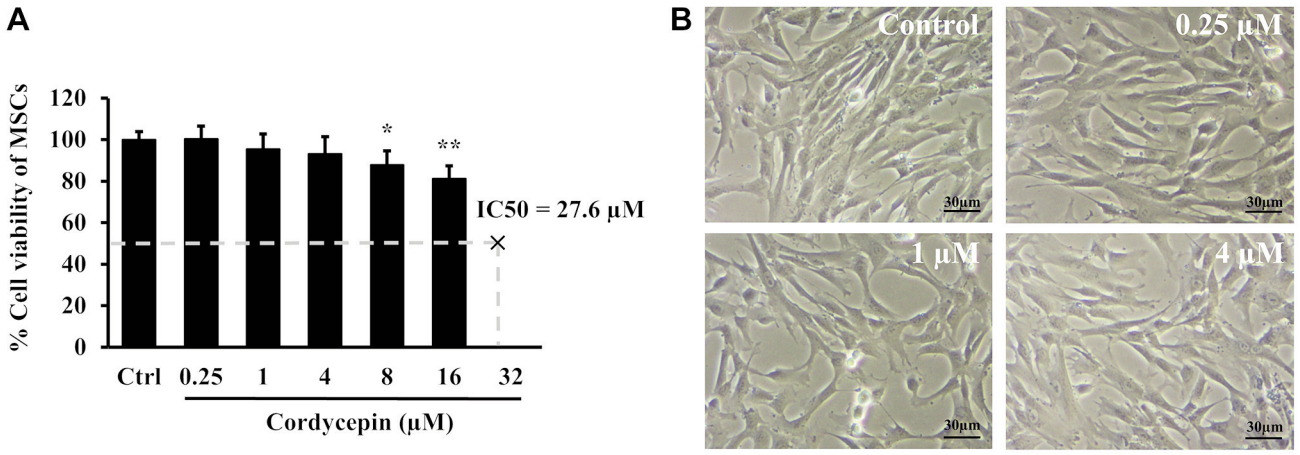

Figure 1. *The effect of cordycepin on cell proliferation in MSCs. (A) The cytotoxicity of cordycepin on MSCs was evaluated by using the MTT assay. IC_{50} was calculated using equation 1. (B) The morphology of MSCs treated with or without cordycepin. 10X magnification was used to digitally capture the 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).*

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 (28). Cells at passages 3, 5, 7, and 9 of continuous cell cultures were obtained to examine doubling time.

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

Relative telomere length by qPCR. DNA was isolated from mesenchymal stem cells with the genomic DNA extraction kit (Macherey-Nagel) for telomere length measurement. qPCRBIO SyGreen Mix (PCR Biosystems, Wayne, PA, USA) and QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) were used for quantitative analyses. The *36B4* gene was employed as a single-copy gene and the *TEL* primers were designed to hybridize to the TTAGGG and CCCTAA repeats of telomere sequence (Table I) according to Vasilishina's protocol (29). Cells at passages 3, 5, 7, and 9 of continuous cell cultures were taken to examine relative telomere length.

Telomere activity measurement by qPCR. Total RNA was extracted by NucleoSpin® (MACHEREY-NAGEL) and cDNA was synthesized using ReverTra Ace™ 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) (30) according to Vasilishina's method (29). Cells at passages 3, 5, 7, and 9 of continuous cell cultures were taken to examine telomerase activity.

Statistical analysis. Statistical analysis was conducted using the IBM SPSS Statistics (IBM®, Armonk, NY, 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).

Results

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 1). 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 1). Furthermore, the cordycepin IC_{50} was 27.6 μM.

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-κB* mRNAs in the cordycepin-treated MSCs were assessed using RT-PCR (Figure 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

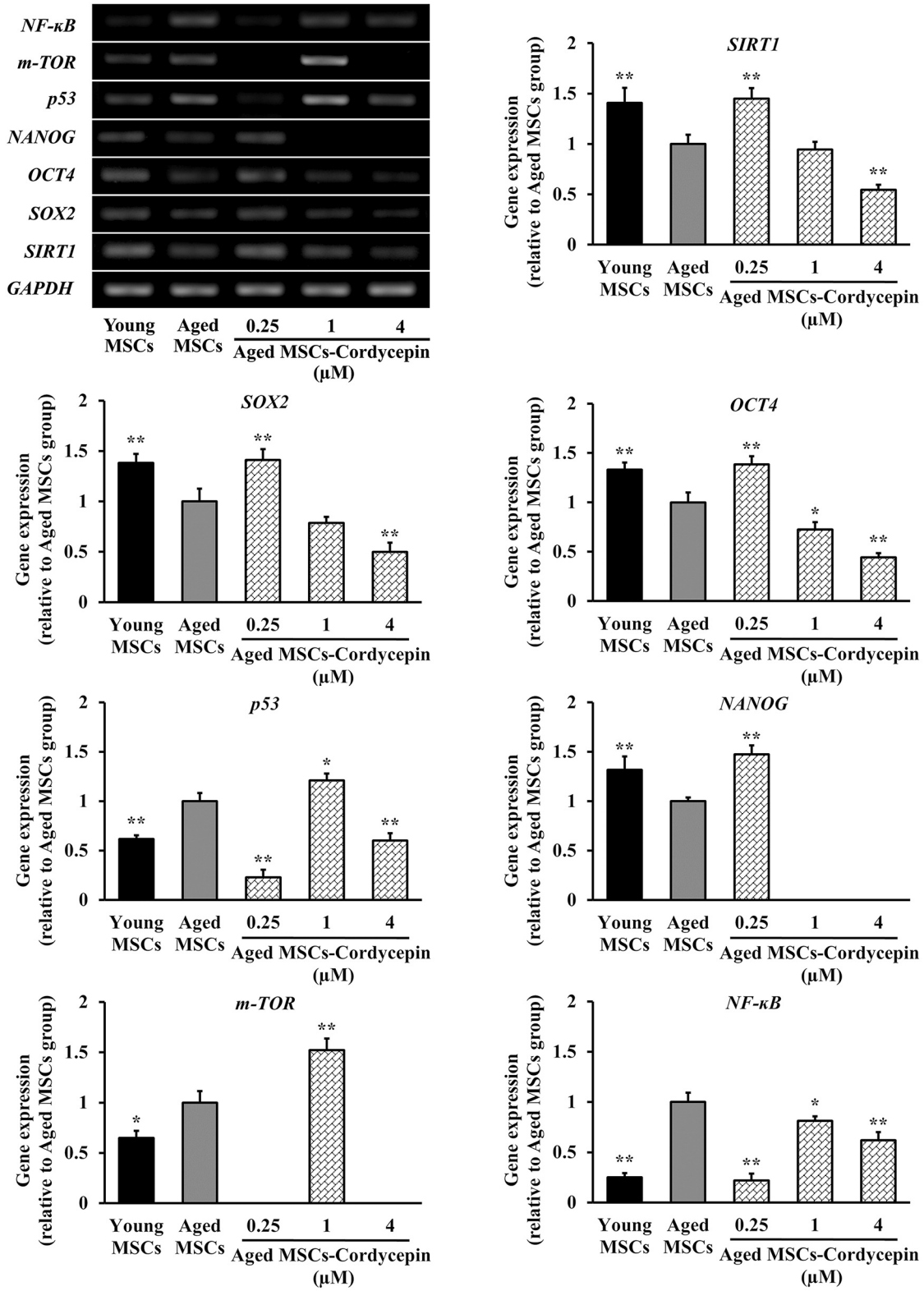

Figure 2. *The effect of cordycepin on mRNA expression in MSCs. The mRNA expression levels of SIRT1, SOX2, OCT4, NANOG, p53, m-TOR, and NF-κB were examined using RT-PCR in aged MSCs treated with cordycepin for 24 h; young MSCs were used as a positive control and aged MSCs as a negative control. Image 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).*

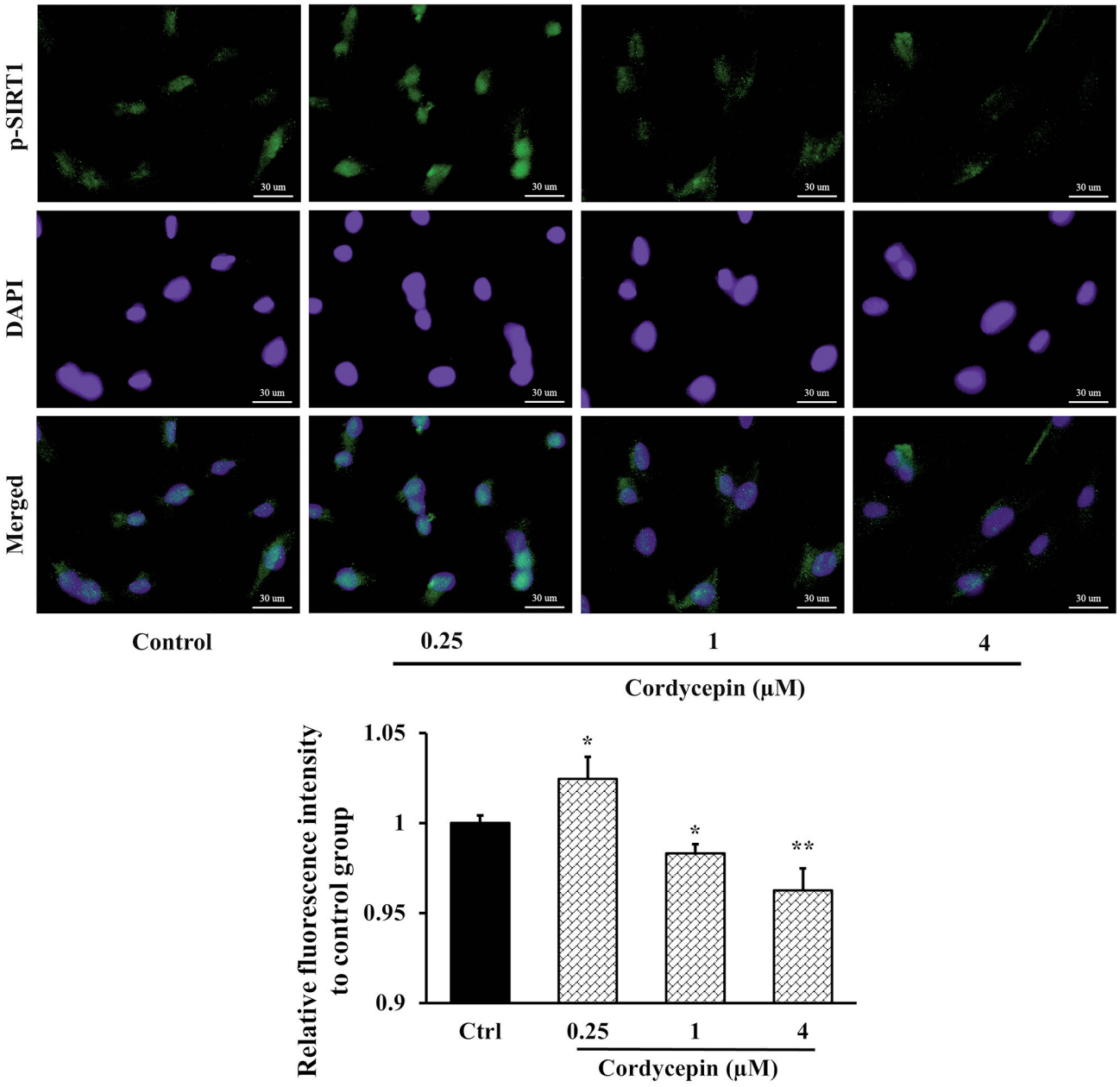

Figure 3. *The effect of cordycepin on the protein expression levels of p-SIRT1 in MSCs. The protein levels of p-SIRT1 were assessed using immunofluorescent staining and ImageJ was used for staining intensity evaluation. DAPI was used to counterstain the cell nuclei. Fluorescence microscope was used 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).*

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-κB* at concentrations of 0.25, 1.0, and 4.0 μM.

The effect of cordycepin on the protein levels of phosphorylated SIRT1 (p-SIRT1) in MSCs. Immunofluorescence staining was

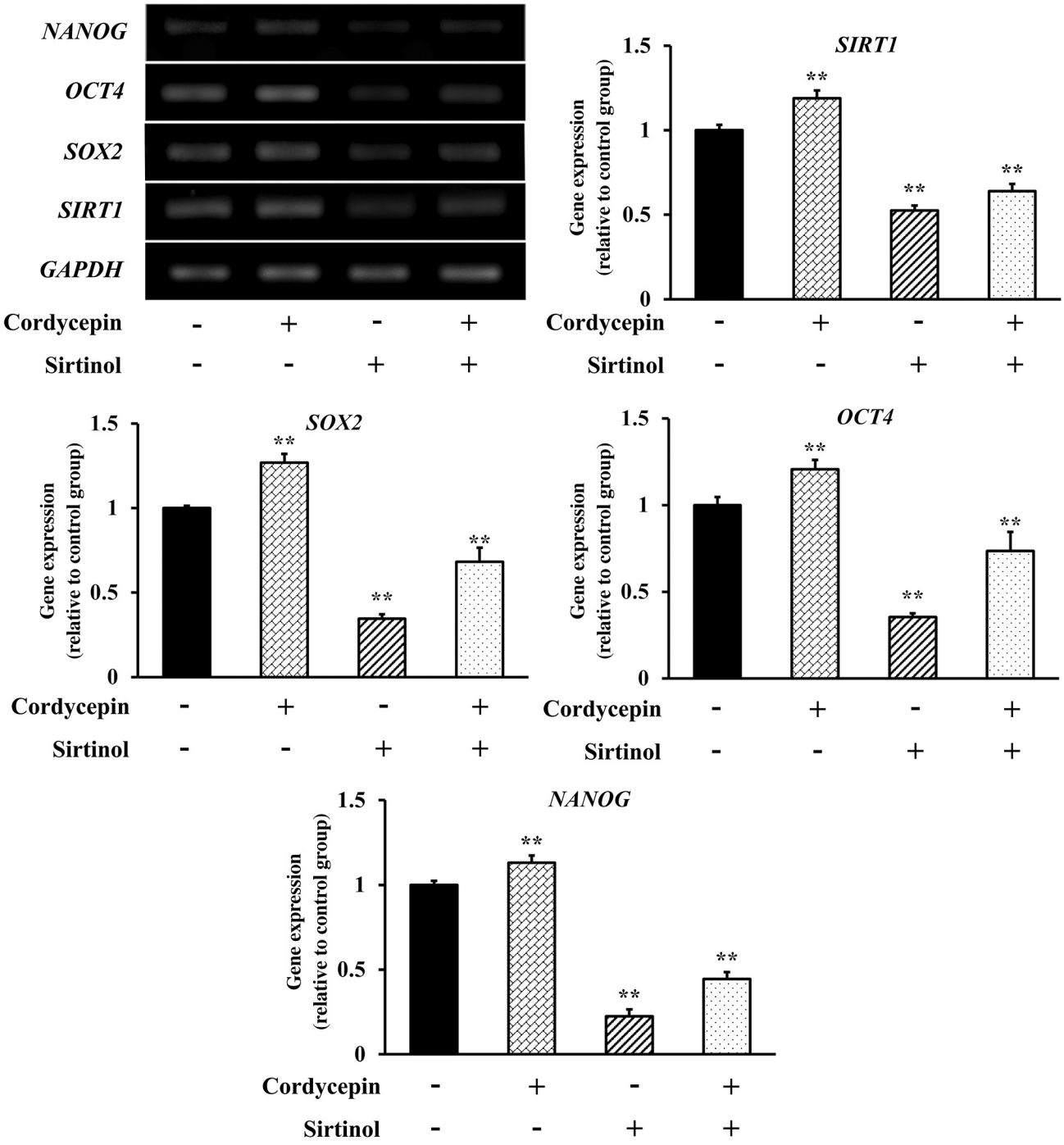

Figure 4. *The effect of cordycepin and sirtinol on mRNA expression in MSCs. The mRNA expression levels of SIRT1, SOX2, OCT4, and NANOG were assessed using RT-PCR after MSCs were treated cordycepin and sirtinol for 24 h. MSCs cultured in standard medium are the control group. Image j was used to analyze 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).*

used to measure the protein levels of p-SIRT1 in cordycepin-treated MSCs (Figure 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.

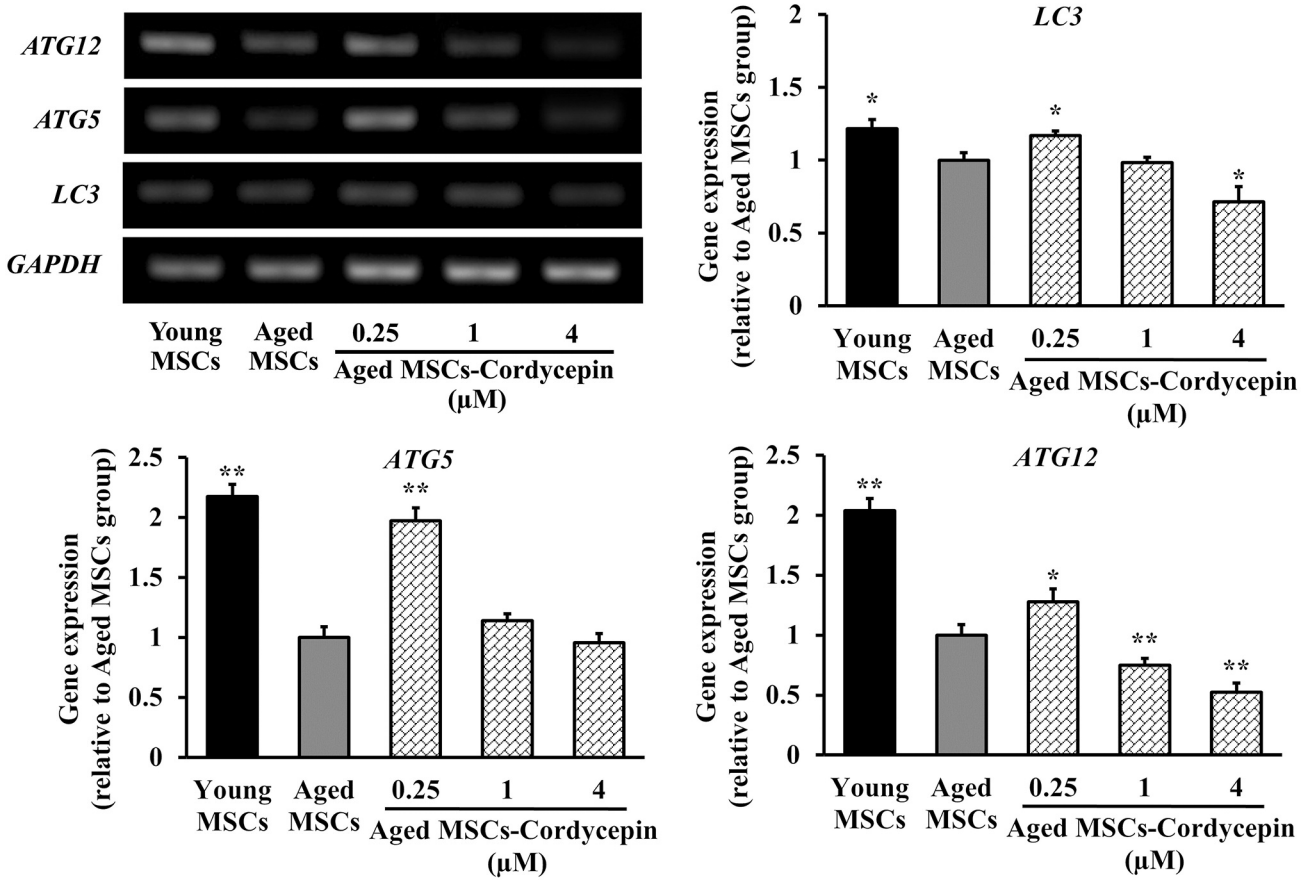

Figure 5. *The effect of cordycepin on autophagy in MSCs. The mRNA expression levels of LC3, ATG5, and ATG12 were examined using RT-PCR in aged MSCs treated with cordycepin for 24 h; young MSCs were used as a positive control and aged MSCs as a negative control. Image j was used to analyze images of 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).*

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. 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).

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 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 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

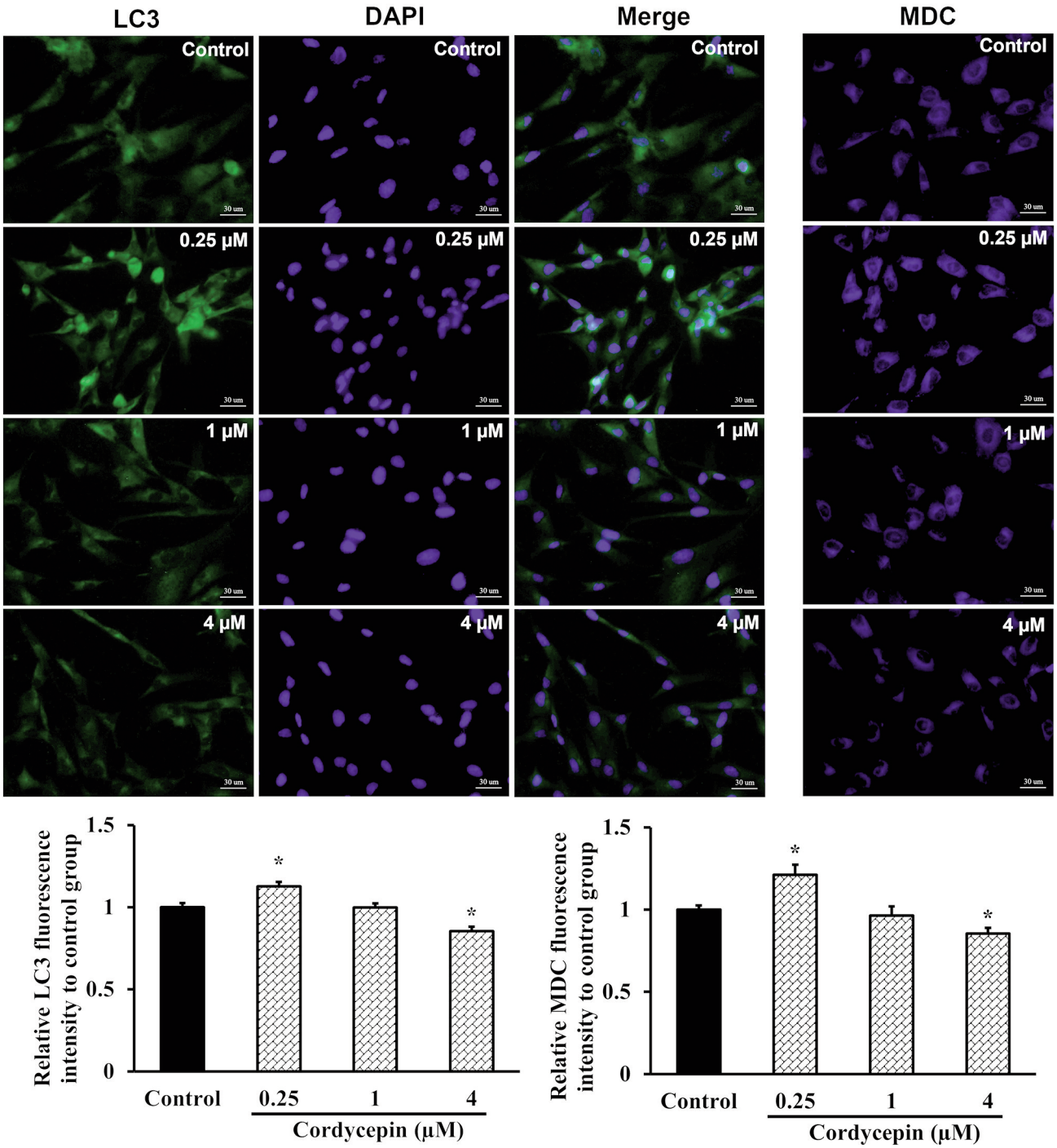

Figure 6. *The effect of cordycepin on autophagy in MSCs. The protein levels of LC3 were measured using immunofluorescent staining and the levels of autophagic vacuoles were measured using MDC staining. ImageJ was utilized to evaluated staining intensity. The cell nuclei were stained with DAPI. The photos 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).*

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.

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

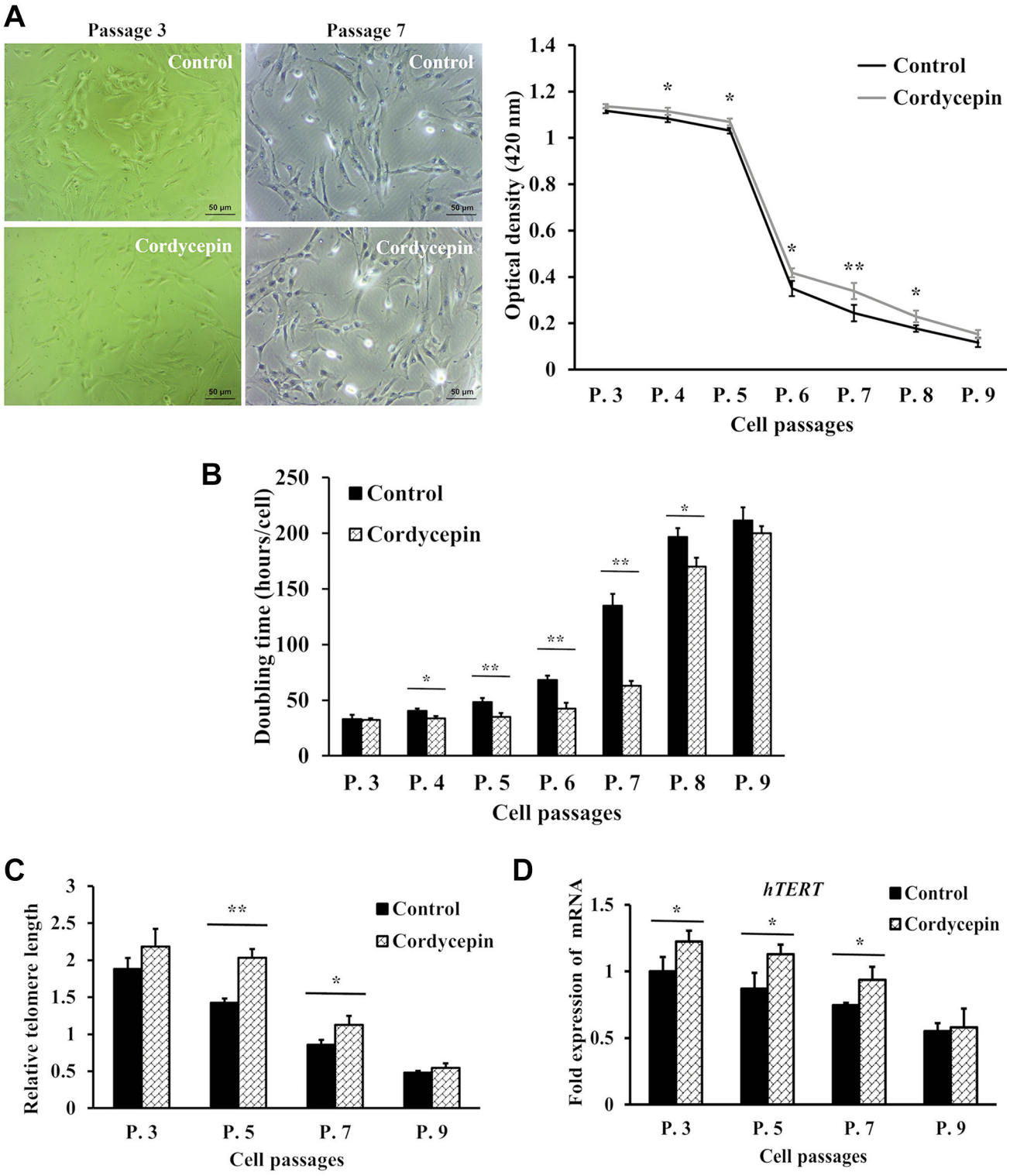

Figure 7. *The anti-aging effects of cordycepin-containing cultural media. (A) The activity of senescence-associated-galactosidase in passage 3 to 9 MSC cultures treated with or without cordycepin by senescence-associated β-galactosidase assay measuring at 420 nm. (B) The rate of proliferation in passages 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).*

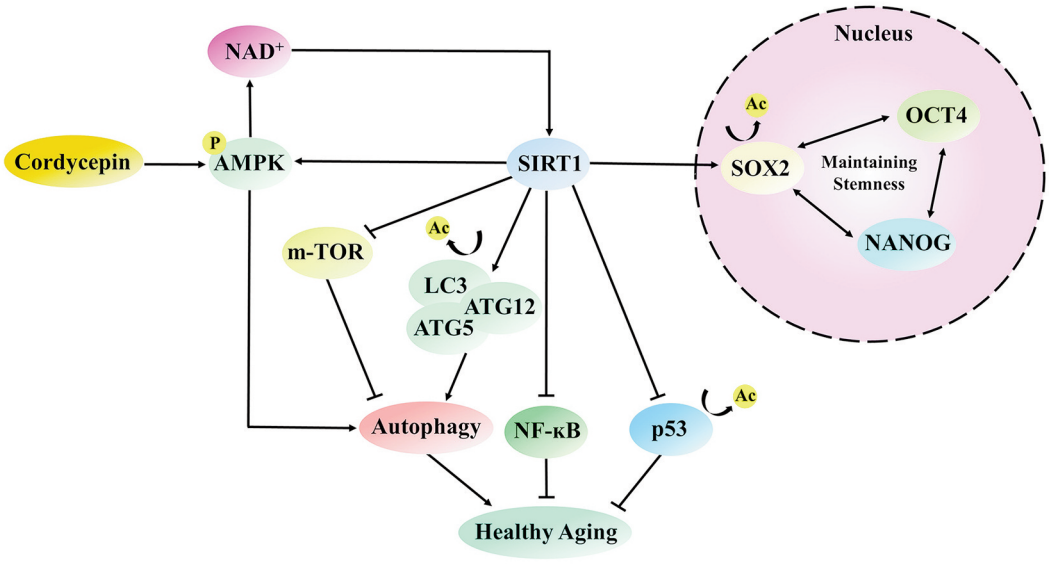

Figure 8. *The cordycepin pathway promotes stemness maintenance in MSCs and prevents aging. The graphic provides a concise explanation of the signaling mechanisms through which cordycepin activates SIRT1 for stemness maintenance and anti-aging in MSCs. The arrows represent stimulation, and the whiskered lines represent inhibition.*

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 the control and cordycepin groups; however, at passages 4 to 8 there was a significant difference between the control and the cordycepin groups (Figure 7). 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 7). 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 7). 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 7).

Discussion

Cordycepin has been demonstrated to inhibit mRNA polyadenylation by incorporating into RNA (31). 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. Furthermore, we found that cordycepin can up-regulate SIRT1 at both the mRNA and protein levels in MSCs when used at a concentration of 0.25 μM.

Cordycepin, also known as 3'-deoxyadenosine, is a derivative of the nucleoside adenosine, which is a bioactive compound derived from the *Cordyceps militaris* (32). The AMPK pathway has also been observed to be triggered by cordycepin (33, 34). Cordycepin activates AMPK in cells by mimicking the effects of its natural activator, adenosine monophosphate (AMP) (35). Cordycepin is transported into cells by the adenosine transporter, and adenosine kinase converts it to cordycepin 5'-monophosphate, which then functions as an AMP analogue (35). Cordycepin-induced activation of AMPK enhanced the activity of SIRT1 by increasing cellular NAD+ levels (20, 36). Therefore, cordycepin significantly enhances *SIRT1* expression in MSCs *via* the activation of AMPK-SIRT1 signaling pathway (Figure 8). Furthermore, cordycepin at concentrations of 1 and 4 μM showed neutral or negative effects on the expression of *SIRT1*, age-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-κB* at cordycepin concentrations 1 and 4 μM, but because cordycepin concentrations were high, it

decreased expression of *p53*, *m-TOR*, and *NF-κB* at 4 μM and 1 μM of *NF-κB* by inhibiting mRNA synthesis while the reduced expression of *p53*, *m-TOR*, and *NF-κB* at 0.25 μM cordycepin caused by deacetylation of SIRT1.

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 (37-40). 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 (13). When SOX2 is in the nucleus, it regulates the expression of OCT4 and NANOG for maintaining self-renewal and multipotency in MSCs (41). 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).

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 (42). 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 (43). During the cellular aging process, autophagy, energy metabolism regulation, stress tolerance, and metabolic state gradually deteriorate (44). SIRT1 integrates a variety of signaling and transcriptional pathways such as, p53, m-TOR, and NF-κB, which are known to control aging, and therefore is a target for increasing a healthy lifespan (11). 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 (45). 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 (46, 47). Moreover, our results showed that cordycepin suppresses *NF-κB* expression in MSCs *via* SIRT1-mediated deacetylation of p65, which acts against inflammation and aging (48). Therefore, cordycepin inhibits cellular senescence and slows the aging process *via* SIRT1 and age-related signaling pathways (Figure 8).

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 (49). Reduced autophagy has been linked to accelerated aging, whereas increased autophagy may have powerful anti-aging effects (50). 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 8), which is increased when nutrients are limited or when AMP/adenosine triphosphate (ATP) ratios rise, and results in the direct activation of Ulk1 (51, 52). Additionally, AMPK activation can potentially cause autophagy through blocking m-TOR (53). Moreover, SIRT1 promotes the formation of the ATG16-ATG5-ATG12 complex by directly deacetylating ATG5, ATG7, and ATG12, thereby promoting autophagic vesicle elongation (54).

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 (55). 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 (56). Moreover, we found that cordycepin prevented telomere length shortening in MSCs by increasing the expression of the *hTERT* gene (57). 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 (58). 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.

Conflicts of Interest

There are no conflicts of interest in relation to this study.

Authors' Contributions

The study was planned by P.C. and P.N. MSCs were provide by R.P. P.K., N.C., and P.N. provided resources and materials. P.C. performed all the experiments. P.C., P.K., and N.C. analysed data. P.C., P.K., and N.C. explained the experimental results. P.C. prepared all figures and wrote the manuscript. P.K., and N.C. contributed to manuscript editing. The paper was revised by P.N.

Acknowledgements

This work was supported by Suranaree University of Technology (SUT), Thailand Science Research and Innovation (TSRI), and National Science, Research, and Innovation Fund (NSRF) (project code 90464).

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Received December 6, 2022
Revised January 29, 2023
Accepted January 30, 2023