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Inhibitory Effect of Gossypol on Aldose Reductase Activity

조선대학교 대학원

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글로벌바이오융합학과

정 주 호

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지도교수 조광원

이 논문을 이학석사학위 신청 논문으로 제출함

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조선대학교 대학원

글로벌바이오융합학과

정 주 호

정주호의 석사학위논문을 인준함

위원장 조선대학교 교 수 이 준 식 (인)

위 원 조선대학교 교 수 전 택 중 (인)

위 원 조선대학교 교 수 조 광 원 (인)

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조선대학교 대학원

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ABBREVIATIONS

AGEs	Advanced glycation end products
AMPK	AMP-activated protein kinase
AR	Aldose reductase
ARI	Aldose reductase inhibitors
D-gal	D-galactose
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GP	Gossypol
GSH	Glutathione
mTOR	Mammalian target of rapamycin
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PVDF	Polyvinylidene difluoride
PMSF	Phenylmethylsulfonyl fluoride
RAGE	Receptor of Advanced glycation end products
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SDH	Sorbitol dehydrogenase
SDS	Sodium Dodecyl Sulfate
SFM	Serum Free Media
SIRT1	Sirtuin 1
SM	Serum Media

국문초록

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알도스환원효소 (AR; AKR1B1)와 소르비톨 탈수소효소 (SDH)는 당 대사 경로 중 하나인 폴리올 경로에 관여한다. 이 경로는 에너지원인 과당을 생성하는데 관여한다. AR은 이 경로에서 초기 효소로 작용하며, NADPH를 필요로 한다. 그러나 고혈당 상태에서 AR 활성이 증가하면 NADPH/NAD^+/글루타치온 (GSH)/글리옥살라제 (GLO)/시르투인1 (SIRT1)의 수준이 감소하는데, 이들은 항산화 및 항노화 관련이 있다. 게다가 증가된 AR 활성은 최종당화산물 (AGEs) 생성을 촉진하여 단백질 기능을 손상시키거나 최종당화산물 수용체 (RAGE)에 결합하여 활성산소종 (ROS)의 수준을 증가시키고, 이로 인해 노화나 질병이 발생할 수 있다.

AR의 활성을 조절하기 위해 다양한 유형의 알도스 환원효소 억제제 (ARI)가 개발되었지만, 대부분은 부작용을 유발하여, 개발이 중단되었다. 따라서 현재는 ARI 개발하기위해 천연화합물에 초점을 맞추고 있고, 연구가 진행되

고 있다. 목화에서 추출한 천연 폴리페놀 화합물인 고시폴 (GP)은 과거 중국에서 남성용 피임약으로 사용되었지만, 현재 항암 또는 항종양에 대해 연구가 되고 있다. 하지만, 구조적 측면에서 항산화, 항노화, AR을 표적하는 것으로 추측되지만, 아직 연구가 진행되지 않았다. 흥미롭게도, GP는 당뇨병 모델에서 연구가 되었는데, 당뇨병은 AR의 활성을 높이는데 관여한다. 그러나 AR에 대한 연구는 조사되지 않았다. 따라서 본 연구는 GP가 D-galactose (D-gal)에 의해 증가된 AR을 억제하여, AGEs/RAGE의 수준을 감소시키는지 분석하기 위해 연구를 진행하였다.

본 연구에서는 섬유아세포를 이용하여, D-gal 처리가 용량 의존적으로 AGEs 및 RAGE 수준을 증가시키고, AMPK/SIRT1/ACC 수준을 상대적으로 감소시키는 것을 확인하였다. 중요한 점은 0.1~2 μM 농도의 GP는 세포에게 독성 효과를 나타내지 않고 세포 생존율을 향상시키는 사실을 확인하였다. 이러한 사전 연구 결과를 바탕으로, GP가 D-gal에 의해 증가된 AR/AGEs/RAGE 수준을 효과적으로 감소시킬 수 있는지 조사하기위해 GP를 후처리 하여 조사했다. 결과는 GP가 D-gal에 의해 상승된 AR/AGEs/RAGE 수준을 효과적으로 감소시키고 손상된 AMPK/SIRT1/ACC 수준을 회복시켰음을 보여주었다. 이러한 결과는 GP가 AR를 억제할 수 있는 능력을 가지고 있으며, 알도스 환원효소 억제제로서 유망한 후보물질로 기능할 수 있다는 것을 시사한다.

결론적으로, 본 연구는 GP가 D-gal에 의해 유발된 AR/AGEs/RAGE 수준을 효과적으로 감소시키고 손상된 AMPK/SIRT1/ACC 수 준을 회복시키는 것을 입증하고 있다. 이러한 결과는 GP가 AR 억제제로서의 잠재력을 강조

하며, 따라서 그 치료적 응용 분야에 대한 추가적인 연구가 필요함을 보여준다.

ABSTRACT

Inhibitory Effect of Gossypol on Aldose Reductase Activity

Juho Jeong

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

Department of Integrative Biological Sciences,

Graduate School of Chosun University

Aldose reductase (AR; AKR1B1) and sorbitol dehydrogenase (SDH) are involved in the polyol pathway, a metabolic pathway related to sugar metabolism. This pathway is involved in the production of fructose for energy source. AR, an NADPH-dependent enzyme, serves as the initial enzyme in this pathway. However, elevated AR activity under conditions of high blood glucose levels leads to a reduction in the levels of NADPH/NAD^+/Glutathione (GSH)/Glyoxalase (GLO)/Sirtuin1 (SIRT1), which are associated with antioxidant and antiaging processes. Moreover, heightened AR activity promotes the generation of Advanced glycation end products (AGEs), which can impair protein function or bind to the receptor for AGEs (RAGE), resulting in increased levels of reactive oxygen species (ROS) and subsequent aging or disease.

Various types of aldose reductase inhibitors (ARI) have been developed to regulate excessive AR activity. However, many of these inhibitors exhibit toxicity, resulting in the suspension of their development. Consequently, researchers are currently exploring the potential of developing ARI through natural compounds. Gossypol (GP), a natural polyphenolic compound extracted from cotton, has been primarily investigated for its

anticancer or antitumor properties, although its effects on antioxidant activity and antiaging processes are also noteworthy. GP has been found to inhibit AR, although specific research on this aspect is lacking. Interestingly, GP has demonstrated inhibitory effects on diabetes, a condition characterized by increased AR activity; however, its impact on AR inhibition has not yet been investigated. Therefore, the present study aims to assess whether GP reduces the levels of AR/AGEs/RAGE induced by D-galactose (D-gal).

Experiments using fibroblasts revealed that D-gal treatment led to a dose-dependent increase in AGEs and RAGE levels, accompanied by a relative decrease in the levels of AMPK/SIRT1/ACC at 5 mg/ml of D-gal. The important point is that GP at concentrations ranging from 0.1 to 2 μM was found to have no toxic effects on cells and improved cell viability. Based on these preliminary findings, a post-treatment approach was employed to examine whether GP could effectively reduce the levels of AR/AGEs/RAGE induced by D-gal. The results demonstrated that GP effectively lowered the levels of AR/AGEs/RAGE induced by D-gal and restored the diminished levels of AMPK/SIRT1/ACC. These findings suggest that GP possesses the ability to inhibit AR and could serve as a promising candidate for ARI

In conclusion, this study provides evidence that GP effectively reduces the levels of AR/AGEs/RAGE induced by D-gal and restores the compromised levels of AMPK/SIRT1/ACC. These findings highlight the potential of GP as an AR inhibitor, thus warranting further investigation into its therapeutic applications

I. INTRODUCTION

I-1. Pathophysiological role of aldose reductase

The polyol pathway is one of the pathways responsible for metabolizing sugar. Aldose reductase (AR) and sorbitol dehydrogenase (SDH) are involved in the polyol pathway. AR is the first enzyme to react in this pathway and utilizes nicotinamide adenine dinucleotide phosphate (NADPH) to convert glucose into sorbitol and SDH consumes nicotinamide adenine dinucleotide (NAD^+) to break down sorbitol into fructose (Fig. 1) (Ramana, 2011). Fructose produced in the polyol pathway can be used as an energy source in the brain, seminal vesicles and other organs (Abdillahi & Ramasamy, 2014). However, AR has a weak affinity with sugar, typically less than 5% of the sugar breaks down through the polyol pathway to produce fructose (Abdillahi & Ramasamy, 2014). AR belongs to the aldo-keto reductase family, and its gene name is AKR1B1.

AR plays three additional roles. First, sorbitol, which has low cell membrane permeability, serves to protect kidney cells from osmotic stress that occurs after urine excretion. Moreover, small amounts of urine excretion do not significantly increase the levels of sorbitol, due to the low osmotic pressure changes (Liu et al., 2011). Second, AR is involved in the detoxification of toxic aldehyde intermediates formed during hormone metabolism such as steroids and norepinephrine (Kawamura et al., 2002; Petrash et al., 1997). Lastly, 4-hydroxy-2-nonenal (4-HNE) is a toxic aldehyde that, when its levels increase, activates nuclear factor kappa B (NF-κB) and Jun N-terminal kinase (JNK), resulting in inflammation. However, AR functions to detoxify 4-HNE, mitigating its detrimental effects (Li et al., 2005; Ye et al., 2016).

However, excessive consumption of sugar results in the metabolism of approximately 30% of the total sugar by AR, leading to adverse consequences within the body (Abdillahi

& Ramasamy, 2014; Jannapureddy et al., 2021). The reason for this is as follows. The production of glutathione (GSH) from glutathione disulfide (GSSG), which acts as an antioxidant capable of decomposing H_2O_2 or generating glyoxalase (GLO), requires nicotinamide adenine dinucleotide phosphate (NADPH) (He et al., 2020; Ju et al., 2020). GLO plays a role in detoxifying toxic methylglyoxal (MGO), and increased levels of MGO reduce levels of AMP-activated protein kinase (AMPK), which is involved in delaying aging and extending lifespan (Gugliucci, 2017; Stancu, 2015; Yang et al., 2022). Accumulation of sorbitol, caused by elevated levels of AR, can result in osmotic stress due to its low cell membrane permeability. However, sorbitol is converted into fructose by SDH, an NAD^+ dependent enzyme (Jannapureddy et al., 2021). Increased sorbitol levels decrease NAD^+ levels, which are involved in the expression of Sirtuin1 (SIRT1), a regulator of p53 expression associated with apoptosis or senescence. (Amjad et al., 2021; Covarrubias et al., 2021). Here, reduced levels of NAD^+ and NADPH are believed to lead to aging and aging-related diseases. And, SDH used here breaks down sorbitol to produce fructose, but high levels of SDH expressed by high levels of sorbitol deplete ATP, leading to inflammation and reactive oxygen species (ROS) (Spagnuolo et al., 2020; Yan, 2018). Elevated fructose levels resulting from SDH activity contribute to the development of non-alcoholic fatty liver disease and decrease AMPK activity Moreover, fructose breakdown produces advanced glycation end products (AGEs), which can cause tissue and cellular problems. The receptor for advanced glycation end products (RAGE), an immunoglobulin, increases with AGE levels and interacts with AGEs to induce the generation of ROS, inflammatory signals, and apoptosis (Ott et al., 2014; Shen et al., 2020). Furthermore, AGEs bind to cell membranes and proteins, leading to glycosylation modifications that impair their function, especially in long-lifespan proteins (Ott et al., 2014). Accumulation of AGEs in collagen leads to reduced elasticity, which is detrimental to long-lived collagen (Li et al., 2013). Ultimately, elevated AGE levels disrupt the

function and structure of proteins and peptides, activating NF-kB, MAPK, and AKT pathways, thereby inducing oxidative stress and apoptosis (Shen et al., 2020; Song et al., 2021). Unfortunately, elevated AR expression decreases sugar consumption through glycolysis while increasing sugar consumption through the polyol pathway (Tigchelaar et al., 2022). In conclusion, increased AR expression induces senescence by reducing NAD^+/NADPH/GSH levels and elevating AGEs/RAGE/ROS levels, resulting in the deterioration of cellular and tissue function and contributing to aging-related diseases (Fig. 2). Factors that increase this AR include diabetes (Tang et al., 2012), ischemic disease (Kaneko et al., 2005), obesity (Obrosova et al., 2007) and aging (Hallam et al., 2010).

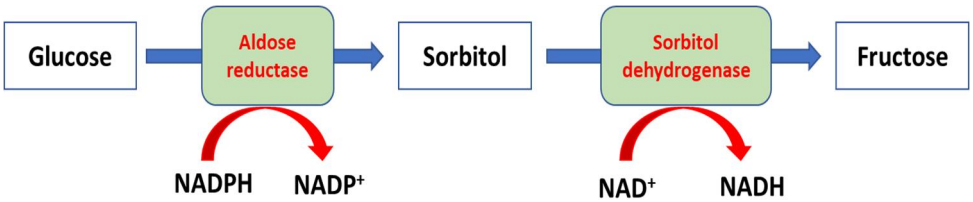

Fig. 1. Polyol pathway.

Under normoglycemic conditions, aldose reductase converts glucose to sorbitol utilizing NADPH as a cofactor, while sorbitol dehydrogenase utilizes NAD^+ to convert sorbitol back to fructose, which serves as an energy source. These enzymatic reactions represent the respective roles of aldose reductase and sorbitol dehydrogenase in sugar metabolism.

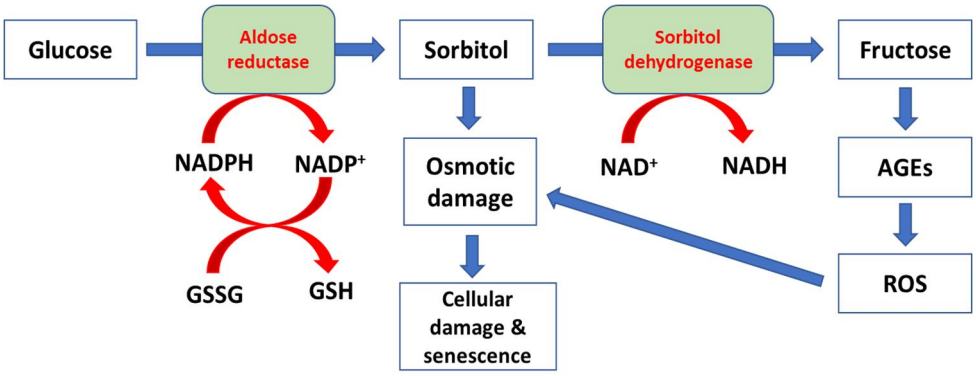

Fig. 2. High sugar concentration causes defective effect in the polyol pathway.

A substantial quantity of sugar during the polyol pathway activates aldose reductase, leading to a reduction in the glutathione level and the production of sorbitol, thereby inducing osmotic stress. In addition, when the levels of sorbitol are elevating, the level of AGEs by sorbitol dehydrogenase increases, and as a result, the level of ROS increases.

I-2. Relationship between aging and aldose reductase

AR has traditionally been extensively studied in the context of diabetic complications, but emerging research has also highlighted its involvement in cardiovascular disease and cancer (Khayami et al., 2020; Vedantham et al., 2012). The accumulation of AGEs induced by AR contributes to aging by promoting the generation of ROS, and it has been observed that AR levels are higher in the old people compared to young people. Based on that, two papers confirmed the level of AR in the elderly. *Hallam et al*., confirmed the levels of AR/Sorbitol/Fructose in the aorta in two types of rats, old and young. In old rats, these levels were significantly higher, but the levels decreased after ARI injection. Also, the level of GSH in old rats was reduced compared with that in young rats (Hallam et al., 2010). In addition, the level of AR in red blood cells increased proportionally with age. *Funasako et al.,* investigated in red blood cells of people between the ages of 16 and 91, regardless of gender, and found that the levels of AR and SDH increased in proportion to age (Funasako et al., 1994). Collectively, these studies support the association between AR and aging.

NADPH serves as an electron donor and regulates redox and antioxidant capacities in organisms. Elevated levels of AR impair the electron transport process, thus affecting DNA replication, cholesterol synthesis, and telomere maintenance (Ju et al., 2020). Next is about NADH and NAD^+. NADH, primarily derived from glucose, accumulates with high glucose levels, leading to increased mitochondrial ROS production and activation of the polyol pathway (Choi et al., 2017). SIRT1, regulated by NAD^+, plays a crucial role in extending nuclear lifespan by deacetylating transcription factors. It reduces insulin resistance and enhances glucose uptake, while low levels of SIRT1 inhibit acetylation of proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), influencing telomere maintenance and epigenetics (Aman et al., 2018). PGC-1α is a master factor that increases mitochondrial DNA replication. Deacetylated PGC-1α reduces mitochondrial

levels of transcription factor A mitochondrial (TFAM), eventually leading to DNA damage and mitochondrial dysfunction (Spagnuolo et al., 2020). There is an experiment conducted to determine whether AR is related to AMPK and SIRT1. A study by *Pal et al.,* demonstrated that high blood glucose levels increased mTOR and decreased AMPK/SIRT1 levels, while treatment with the Fidarestat, an ARI, restored the levels of AMPK/SIRT1 and suppressed mTOR levels (Pal et al., 2019). This indicates the involvement of AR in the regulation of AMPK and SIRT1.

In the elderly, heightened levels of AR have detrimental effects. The reduced proliferation of pancreatic β-cells due to aging leads to a decline in insulin secretion, resulting in elevated blood glucose levels in non-diabetic elderly people and, as a result, the levels of AR would increase (Kalyani & Egan, 2013). The increased AGEs due to elevated AR levels contribute to oxidative stress by promoting ROS generation, subsequently activating ERK, JNK, and mTOR pathways, which induce cytokine production (Shen et al., 2020). Decreased GSH levels caused by increased AR activity fail to eliminate ROS, and downregulated GLO activity is insufficient to detoxify AGEs, ultimately leading to mitochondrial damage in blood vessels and tissues (Shields et al., 2021). Furthermore, in elderly people, there may be limitations in reducing the levels of AGEs, which can contribute to disease, as autophagy activity deteriorates. Although autophagy, as though GLO, can decrease AGEs levels, compromised autophagy function in aged tissues may not sufficiently lower AGEs levels, potentially contributing to disease development. This phenomenon could be associated with the decline in cellular metabolic capacity due to aging (Barbosa et al., 2018; Takahashi et al., 2017). In addition, the activity of Autophagy is related to the activity of AMPK, and the activity of AR lowers the activity of Autophagy by lowering the level of AMPK (Stancu, 2015).

Taken together, these research findings support the notion that AR contributes to aging and that elevated AR levels in old people contribute to age-related diseases. Figure

3 provides an illustrative representation of how high sugar levels induce disease and aging through the polyol pathway (Fig. 3).

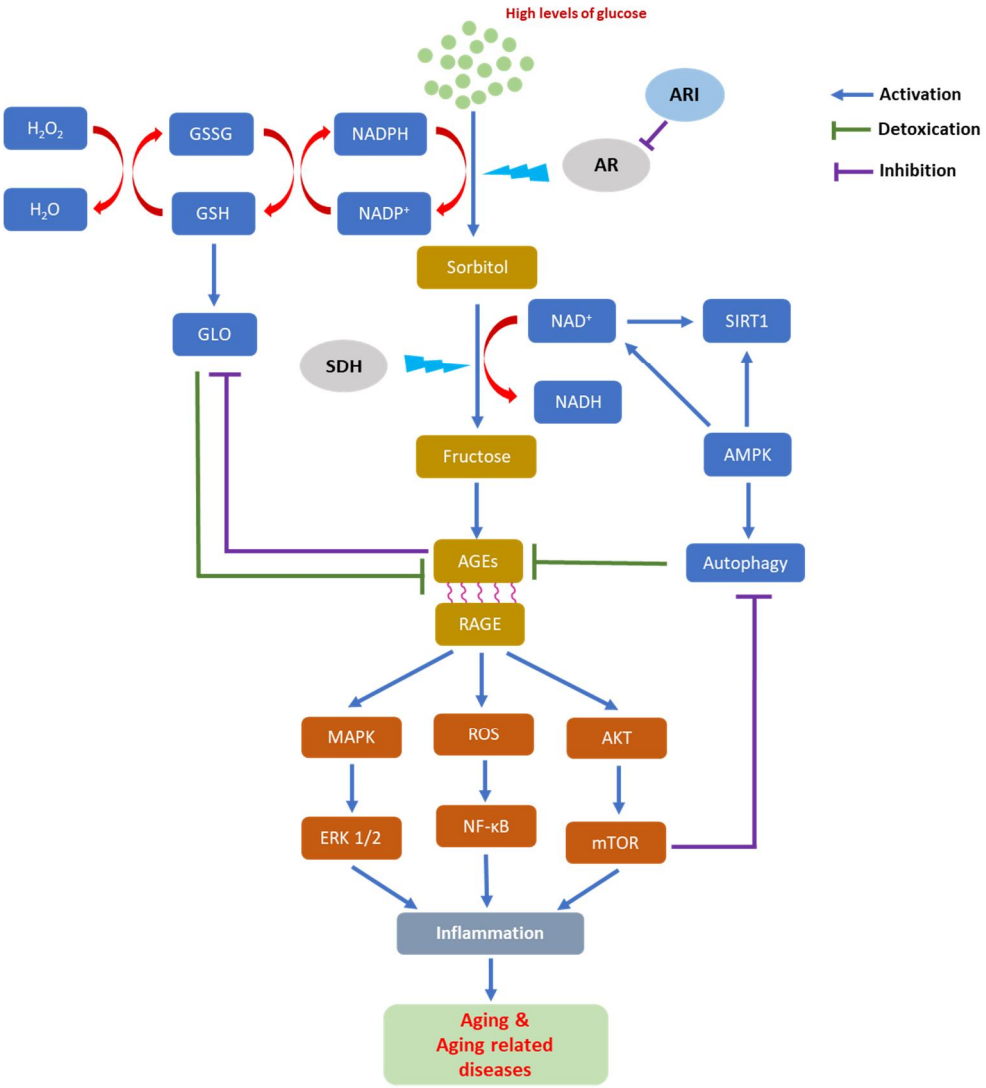

Fig. 3. The mechanism by which aldose reductase contributes to the process of aging.

The activity of aldose reductase leads to a decrease in the level of NADPH, resulting in an increase in H_2O_2 levels within the body, thereby impeding the detoxification of AGEs by GLO. Additionally, the sorbitol generated by aldose reductase is metabolized by sorbitol dehydrogenase, which subsequently reduces the levels of SIRT1 and AMPK due to its dependency on NAD^+. Consequently, the excessive production of AGEs

combines with the RAGE, ultimately triggering the generation of ROS and inflammatory responses, thereby contributing to the development of age-related diseases and the aging process.

I-3. Gossypol

Gossypol (GP) is a natural compound with a polyphenolic structure extracted from cotton, it is named after the scientific name *Gossypium* (Šudomová & Hassan, 2022) (Fig. 4). While it was previously utilized as a male contraceptive in ancient China, current research is investigating its potential as an anticancer and antitumor treatment (Zhao et al., 2020). Furthermore, GP is speculated to possess antioxidant, antiviral, antimalarial, and antibacterial properties (Pal et al., 2022). However, research on this is not yet in progress. Additionally, there is speculation regarding the inhibitory effects of GP on AR (Kawanishi et al., 2003). Despite the characterization that GP can inhibit AKR1B1, this has not been investigated yet. However, *Alam et al*., demonstrated that GP treatment improved glucose uptake by increasing the expression of glucose transporter 4 (GLUT4) in a diabetic rat model. Moreover, they observed an increase in GLUT4 levels in C2C12 cells through enhancement of insulin receptor substrate 1 (IRS-1) levels, mimicking the effect of insulin (Alam et al., 2018). These findings suggest the potential of GP to target AR. Furthermore, studies have indicated that GP improved fibrosis in the liver and lungs induced by diabetes and bleomycin (Chen et al., 2016; Judge et al., 2018). These findings not only support the anticancer and antitumor properties of GP but also highlight its potential in improving conditions such as diabetes and fibrosis.

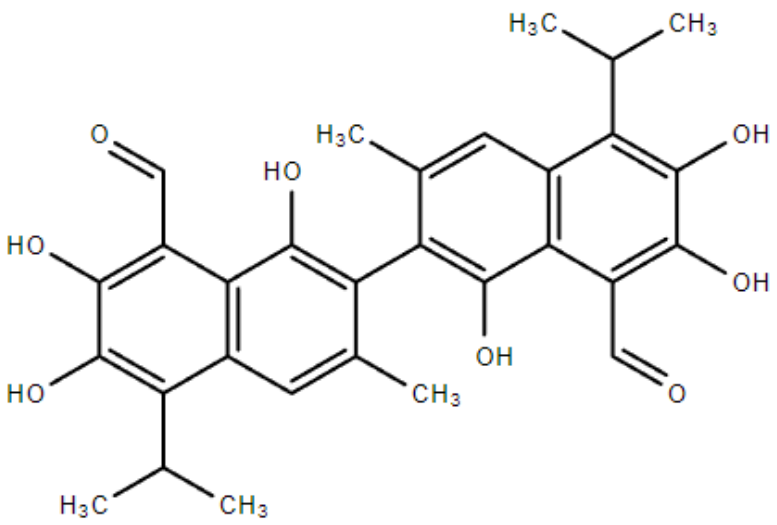

Fig. 4. Structure of Gossypol.

Gossypol has a polyphenol structure. Chemical formula is $C_{30}H_{30}O_8$. Chemical structure formula is (±)-2, 2'-bis(8-formyl-1, 6, 7-trihydroxy-5-isopropyl-3-methylnaphthalene).

II. MATERIALS AND METHODS

II-1. Cell culture and compound treatment

NIH/3T3 cells, the fibroblast derived from mouse embryos, were cultured in high-glucose DMEM media (Gibco, NY, USA) supplemented with 10% FBS (Gibco, ON, Canada), 1% Penicillin-Streptomycin (Lonza, Walkersville, MD, USA) and 1% Sodium Pyruvate (Gibco, NY, USA) at 37 incubator with 5% CO_2. Cells were subcultured in 100 mm dishes at a density of 2×10^5 cells every 3 days.

Gossypol (Tocris, Bristol, UK) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, USA) at a concentration of 1 mg/ml and stored at -20 ℃ fridge. 0~6 μM dose of GP was treated for 24 hours. D-galactose (D-gal) (Sigma-Aldrich, Saint Louis, USA) was dissolved in Serum Media (SM) at 1mg/ml and stored at 4 ℃ fridge. 0~20 mg/ml of D-gal was treated for 72 hours. D-gal and GP were dissolved in SM and treated in NIH/3T3. In the treatment of D-gal and GP, D-gal was first treated for 72 hours and D-gal was removed, and then GP was treated for 24 hours.

II-2. Cell viability assay

The viability of cell was assessed by MTT assay (Invitrogen, USA). The 96-well plate was used, and 200 μl of MTT solution, dissolved in SFM at a ratio of 9:1, was added to each well. The 96-well plate and incubated under specific conditions (e.g., CO_2-free incubator, temperature, duration) for 2 hours. Following incubation, 200 μl of DMSO (DAEJUNG, Gyeonggi province, South Korea) was added to each well to measure the absorbance of cells that had absorbed MTT. Cell viability was assessed by measuring the

absorbance at 570 nm using the SpectraMax ABS Plus Feature Demonstration (Molecular Devices, San Jose, USA).

The cell count of NIH/3T3 cells may vary depending on the compound used. The number of cells in the group treated with GP was 3×10^3 / well. For the group treated with D-gal, the cell count was 1×10^3 / well. In the post-treated group where GP was applied, the count was 5×10^2 / well.

II-3. Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from NIH/3T3 cultured in 6-wells plates using RNAiso Plus regent (TAKARA, Japan) according to the manufacturer's instructions. The extracted total RNA was synthesized into cDNA using the PrimeScrip™ 1st strand cDNA Synthesis kit (TAKARA, Japan). The protocol for synthesizing cDNA followed the Product Manual provided in the kit, and Random 6 mers (50 μM) was used. The information about primers for RT-PCR analysis was summarized in Table 1. Power SYBR™ Green PCR Master Mix (Applied Biosystems™, USA) was used to formulate the amplified RNA. The RT-PCR cycling conditions were as follows: an initial holding stage at 95 for 10 minutes, followed by 40 cycles of a cycling stage at 95 for 15 seconds and 60 for 1 minute. Subsequently, a melt curve stage was performed at 95 for 15 seconds, 60 for 1 minute, and 95 for 15 seconds to obtain information on the amplified products. The RT-PCR was carried out using the StepOnePlus™ Real-Time PCR System (Bio Systems).

Table 1. PCR primers used for RT-PCR

Gene	Species	Forward & Reverse	Sequence (5'-3')
AGER	**Human/Mouse**	**Sn**	AGG TGG GGA CAT GTG TGT C
		An	TCT CAG GGT GTC TCC TGG TC
ß-Actin	**Mouse**	**Sn**	CCA CCA TGT ACC CAG GCA TT
		An	CGG ACT CAT CGT ACT CCT GC

II-4. Aldose reductase assay

Aldose reductase activity was measured using an aldose reductase assay kit (Biovision, Milpitas, USA). Aldose reductase assay followed the protocol provided by the product. Referring to the product protocol, to match the number of cells, the cells were cultured in a 100 mm dish, and the cells were collected using 0.25% Trypsin-EDTA (1X; Gibco, Canada). To homogenize the collected cells, AR Assay Buffer containing 10 μM DTT was added and BioMasher-II Sterile (Optima INC, Japan) was used. Only AR was extracted from the homogenized cells, and samples containing AR Assay Buffer, AR substrate, and NADPH were duplicated in 96 wells, incubated in an incubator without CO_2, and absorbance at 340 nm was measured by ELISA over time. The measured activity is the activity for aldose reductase for 120 minutes.

II-5. Immunoblot analysis

A total protein extract was obtained from NIH/3T3 cells cultured in a 60mm dish using 30 ul RIPA lysis buffer (Sigma-Aldrich, Saint Louis, USA) containing phenylmethylsulfonyl fluoride (PMSF; Enzo life sciences, NY, USA), protease inhibitor cocktail (Thermo Fisher scientific, MA, USA) and sodium orthovanadate (Na_3VO_4) in an ice box at 4 for 30 minutes and then the proteins were centrifuged at 16000 × g for 20 minutes. The dose of protein was quantified using the BCA Protein Assay kit (Thermo Fisher Scientific, MA, USA). The sample proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Germany). The membrane was blocked in 1% blocking solution. Then, proteins were analyzed by immunoblot analysis using antibodies against ACC, p-ACC, AGEs, AMPK, p-AMPK, SIRT1 at 4

overnight. The secondary antibody was processed according to the species of the primary antibody, and anti-mouse IgG (Cell signaling, Saint Louis, USA) and mouse anti-rabbit IgG (Santa Cruz Biotechnology, CA, USA) were used. Detailed information on the antibody is listed in Table 2. Blots were detected in response to enhanced chemiluminescence (ECL) (GE Healthcare, Buckinghamshire, UK) treatment and exposed as X-ray films in a space without light.

Table 2. Primary and secondary antibodies used for immunoblot analysis

1st antibody	Titer	2nd antibody	Titer	Company (Cat.NO.)
AGEs	1:1000	Rabbit	1:5000	Abcam (ab23722)
ACC	1:1000	Rabbit	1:3000	Cell Signaling (#3662)
p-ACC	1:1000	Rabbit	1:3000	Cell Signaling (#3661)
AMPKα	1:1000	Rabbit	1:3000	Cell Signaling (#2535)
p-AMPK	1:1000	Rabbit	1:3000	Cell Signaling (#5832)
SIRT1	1:500	Mouse	1:3000	Santa Cruz (sc-74465)
GAPDH	1:1000	Mouse	1:5000	Santa Cruz (sc-365062)

II-6. Statistical analysis

All data are presented as mean ± standard deviation (SD) of the indicated number of experiments and significance was estimated using a Student's t-test. Statistical comparisons between groups were analyzed using an independent *t*-test. Statistical significance levels are indicated in the figures using asterisks as follow: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

III. RESULTS

III-1. D-galactose increased the levels of AGEs/RAGE.

To evaluate the impact of different doses of D-galactose on cell viability in NIH/3T3 cells, the cells were treated with D-galactose for a duration of 72 hours. Subsequently, cell viability was determined using the MTT assay (Fig. 4A). D-galactose at a dose of 20 mg/ml was considered unsuitable due to its significantly decreased cell viability. Immunoblot analysis was performed to evaluate whether the level of AGEs increase due to D-gal (Fig. 4B). The protein levels of AGEs were assessed by performing immunoblot analysis four times to ensure statistical significance (Fig. 4C). To evaluate whether the levels of RAGE increase in response to changes in AGEs levels, the mRNA levels of RAGE were evaluated using RT-PCR (Fig. 4D). In a dose dependent manner, D-galactose increased the levels of AGEs and RAGE in NIH/3T3 cell.

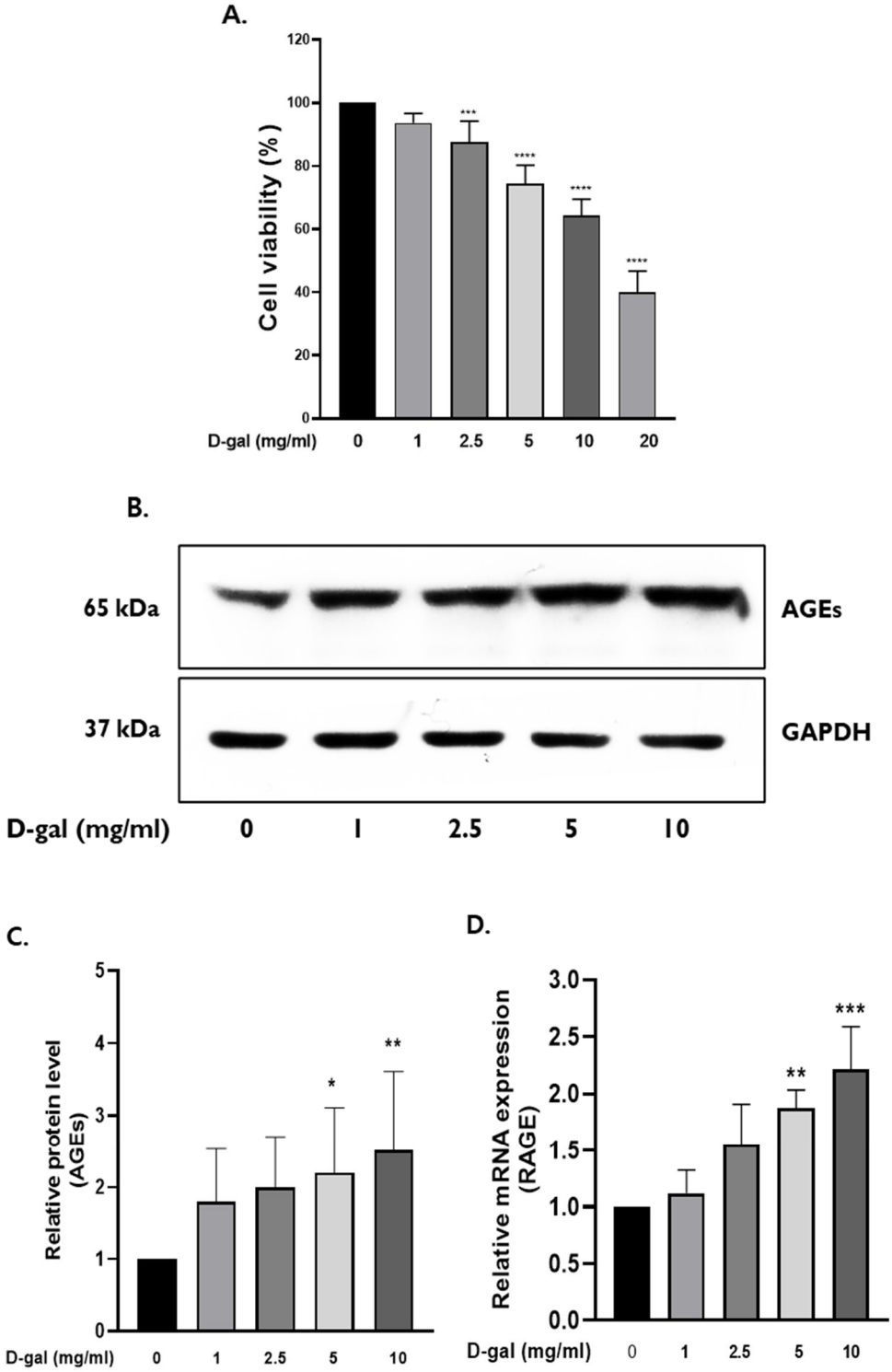

Fig. 5. The increased levels of AGEs/RAGE induced by D-galactose decrease the viability of NIH/3T3 cell.

(A) The cell viability of NIH/3T3 cells treated with different concentration of D-gal was analyzed by MTT assay (t-test, ** $p < 0.01$, **** $p < 0.0001$, mean ± SD, n = 5). (B) Immunoblot analysis shows the degree of increase in the expression of AGEs upon different doses of D-gal treatment. (C) Protein levels of AGEs were measured from immunoblot analysis and quantified using Image J software (*t*-test, * $p < 0.05$, ** $p < 0.01$, mean ± SD, n = 4). (D) The mRNA level of RAGE was analyzed by RT-PCR (*t*-test, * $p < 0.05$, **** $p < 0.0001$, mean ± SD, n = 3). GAPDH was used as the internal standard.

III-2. D-galactose treatment reduced the levels of AMPK, SIRT1, and ACC.

A study was conducted to investigate whether increased levels of AGEs lead to a decrease in the levels of AMPK and SIRT1, which are known indicator of aging. Immunoblot analysis was performed to evaluate the dose dependent effect of D-gal on AMPK/SIRT1/ACC (Fig. 5A). The levels of AMPK/SIRT1/ACC, as measured by immunoblot analysis, exhibited a substantial reduction starting from the dose of 5 mg/ml D-gal (Fig. 5B-D). These findings indicate that the levels of AMPK/SIRT1/ACC began to decrease at the dose of 5 mg/ml D-gal. Upon integrating the previous findings, it was determined that a dose of 5 mg/ml D-gal was optimal. This determination was based on the observed increase in significance starting from the dose of 5 mg/ml D-gal.

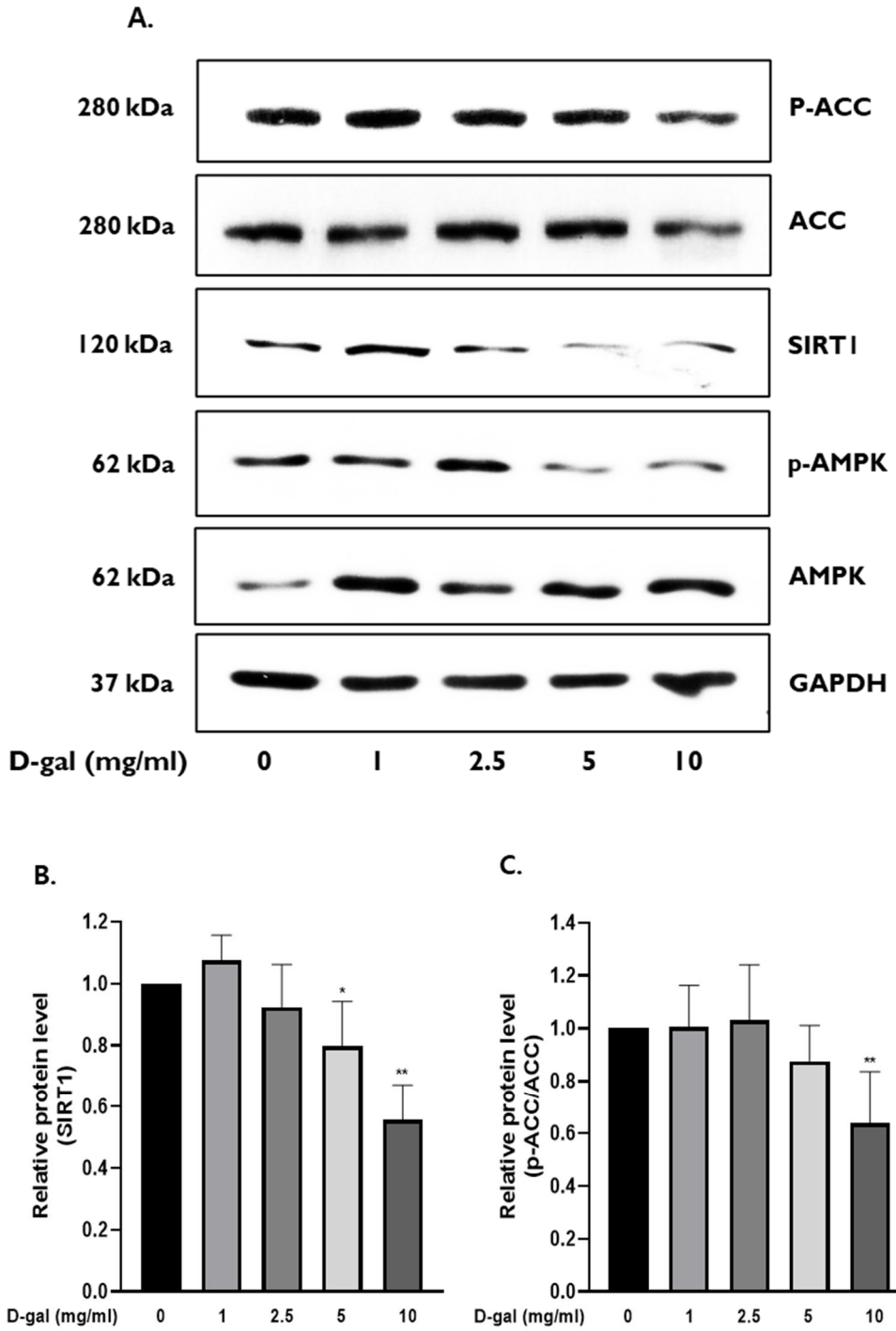

Fig. 6. (Continued)

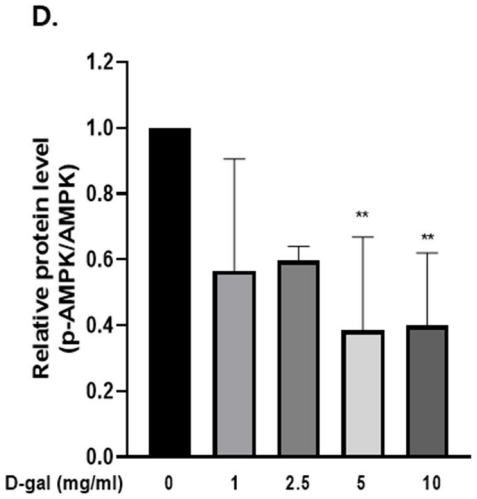

Fig. 6. High dose of D-galactose decreased the levels of AMPK/SIRT1/ACC.

(A) The protein levels of AMPK/SIRT1/ACC, which are known as antiaging indicator, were measured by Immunoblot analysis. (B-D) The levels of AMPK/SIRT1/ACC were measured and quantified from immunoblot analysis using Image J software (*t*-test, * $p<0.05$, ** $p<0.01$, mean ± SD, n=4).

III-3. Efficacy of Gossypol in NIH/3T3 cells.

To determine whether GP affects the viability of NIH/3T3 under normal conditions, it was measured by MTT assay (Fig. 6A). Cell viability was decreased from the dose of 2μM GP. To confirm whether GP affects AMPK/SIRT1/ACC, protein levels were measured by immunoblot analysis (Fig. 6B). The low dose of GP significantly increased the levels of SIRT1 and AMPK (Fig. 6C, D). The level of ACC was reduced since the dose of 2 μM GP (Fig. 6E). In contrast to D-galactose, GP enhanced the levels of AMPK and SIRT1. The effect was significant at dose of 0.5 and 1μM GP. The levels of AMPK/SIRT1/ACC were decreased since 2μM as shown in the MTT assay. A low dose of GP increased cell activity.

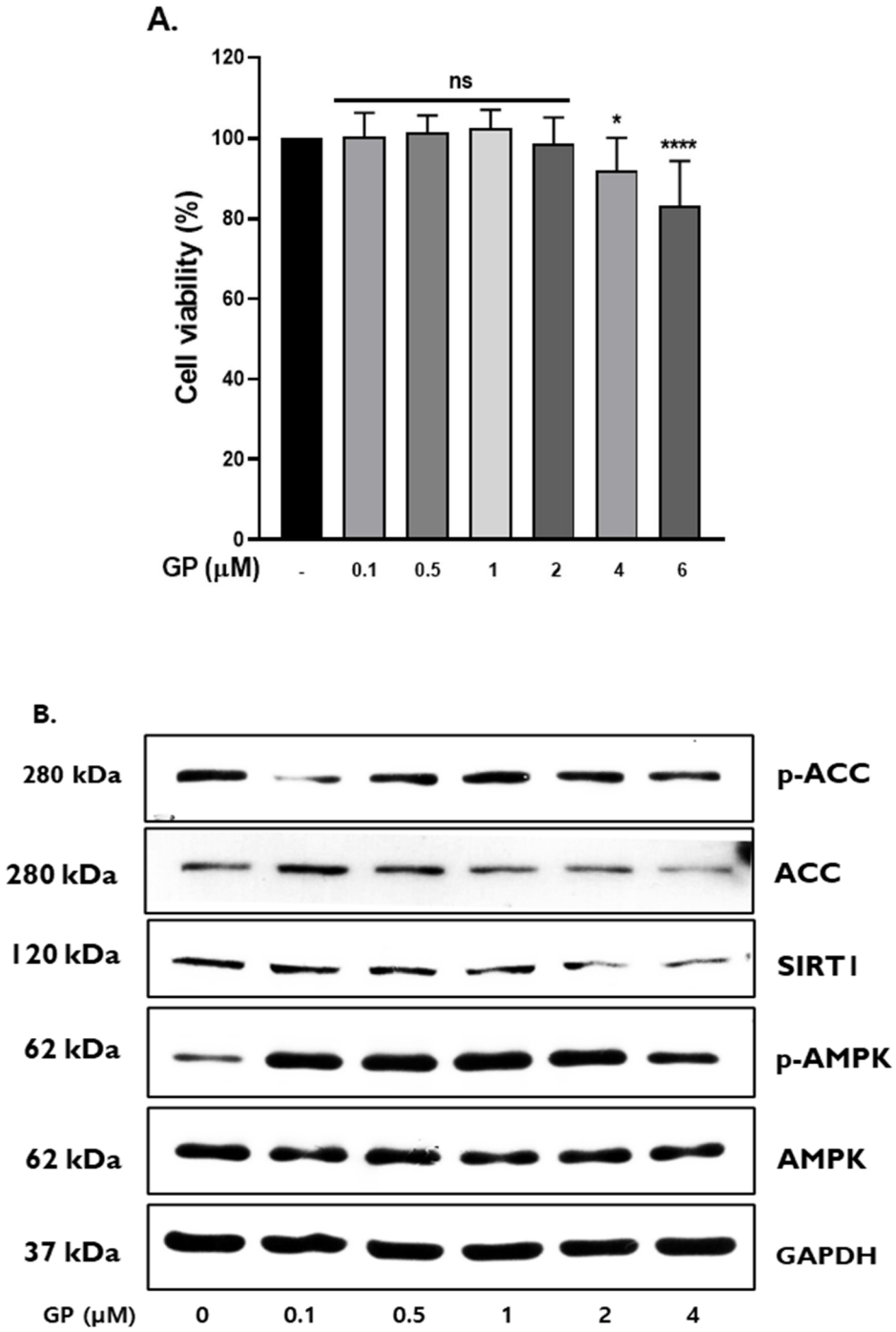

Fig. 7. (Continued)

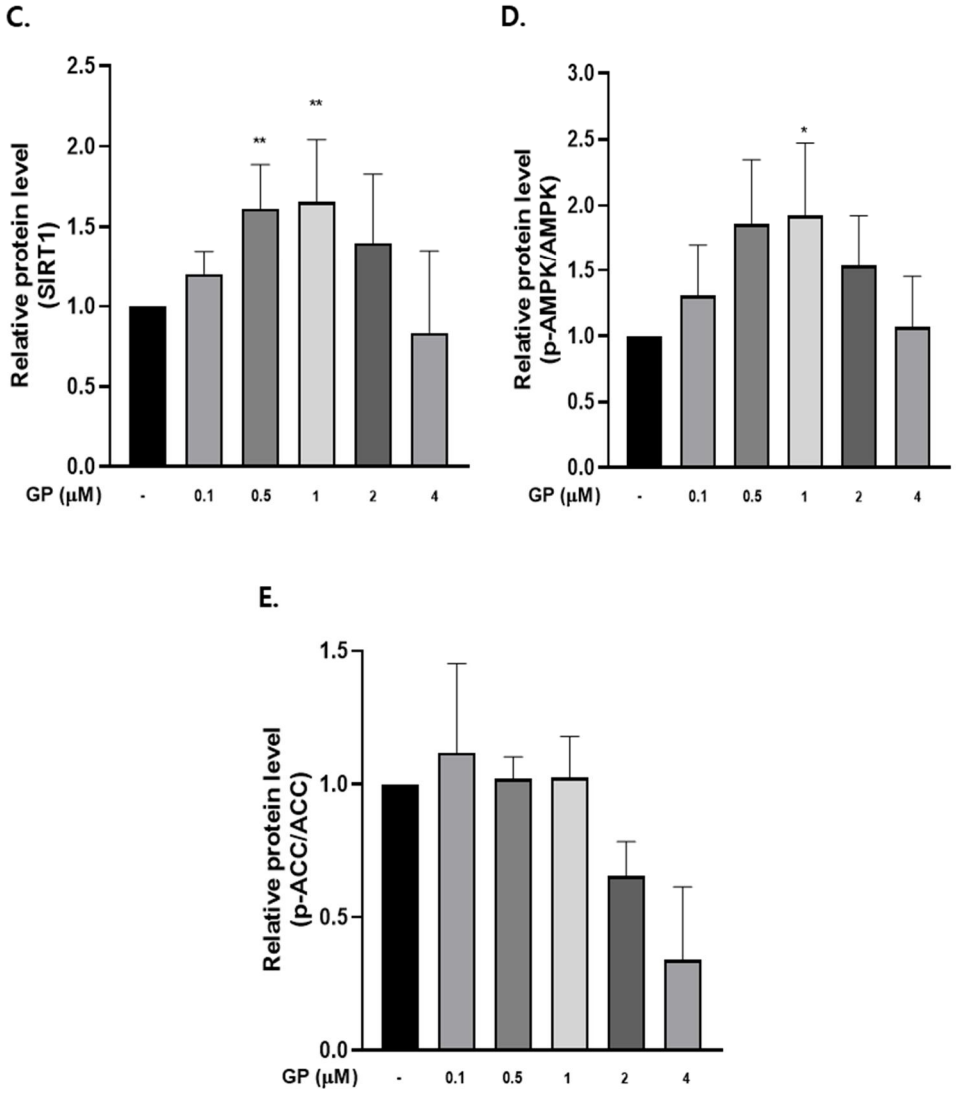

Fig. 7. A low dose of Gossypol treatment enhanced the levels of AMPK and SIRT1.

(A) The cell viability treated of NIH/3T3 cells treated with different concentration of GP was analyzed by MTT assay (*t*-test, ** $p < 0.01$, **** $p < 0.0001$, mean ± SD, n = 5). (B) Protein levels of AMPK/SIRT1/ACC were measured by immunoblot analysis. (C-E) The levels of AMPK/SIRT1/ACC were measured from immunoblot analysis and quantified using Image J software (*t*-test, * $p < 0.05$, ** $p < 0.01$, mean ± SD, n = 4).

III-4. Gossypol reduced the level of AGEs/RAGE by targeting aldose reductase.

In order to evaluate whether GP improves the viability of cells that have been reduced by D-gal, cells were treated with different dose of GP, and their viability was assessed using the MTT assay (Fig. 7A). Previous research findings, treatment with 0.5 and 1 μM GP demonstrated the most significant effectiveness under normal condition of cell. Similar to the previous study, treatment with 0.5 and 1 μM GP showed efficacy in improving the viability of cells reduced by D-gal. To investigate whether GP increased cell viability by reducing AGEs level, the levels of AGEs were measured by immunoblot analysis (Fig. 7B). In particular, treatment with 1μM GP restored the elevated level of AGEs increased by D-gal to a level similar to that of the control condition (Fig. 7C). Moreover, the potential of GP to mitigate RAGE levels was confirmed by measuring RAGE mRNA expression using RT-PCR (Fig. 7D). To assess whether GP targets AR and reduces the level of AGEs/RAGE, it was measured with an aldose reductase assay (Fig. 7E). Consistently, Figures 7B-E collectively demonstrated that treatment with 1μM GP resulted in more significant reductions in the levels of AR, AGE and RAGE compared to treatment with 0.5μM GP.

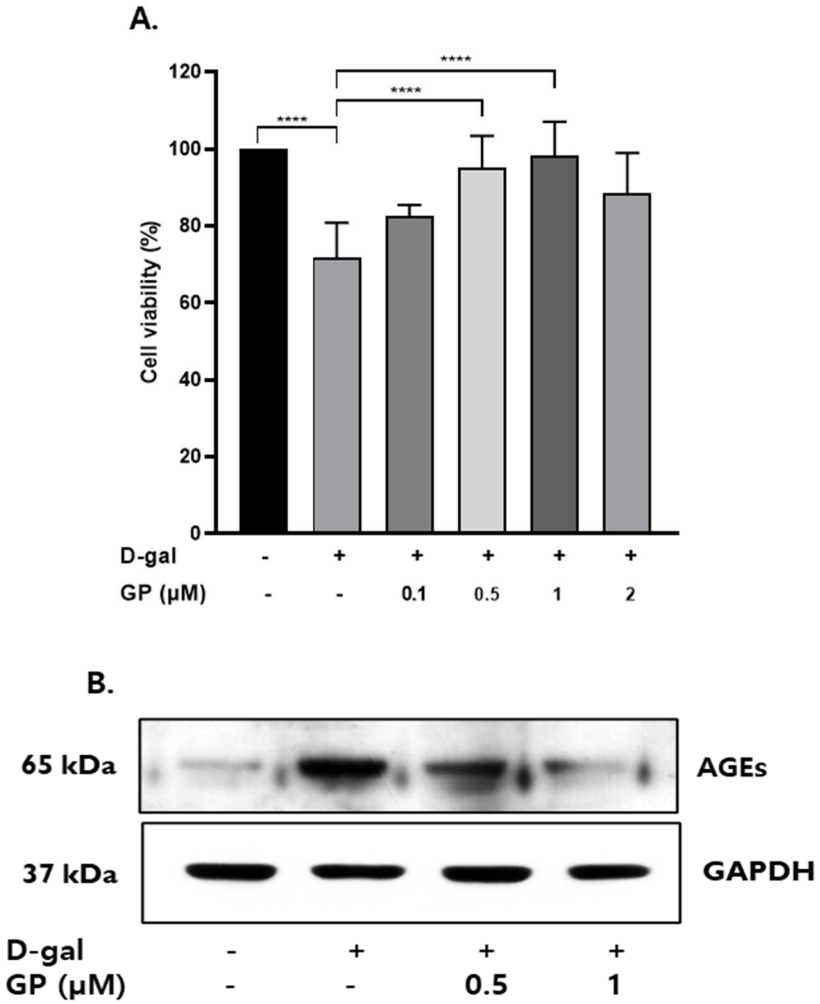

Fig. 8. (Continued)

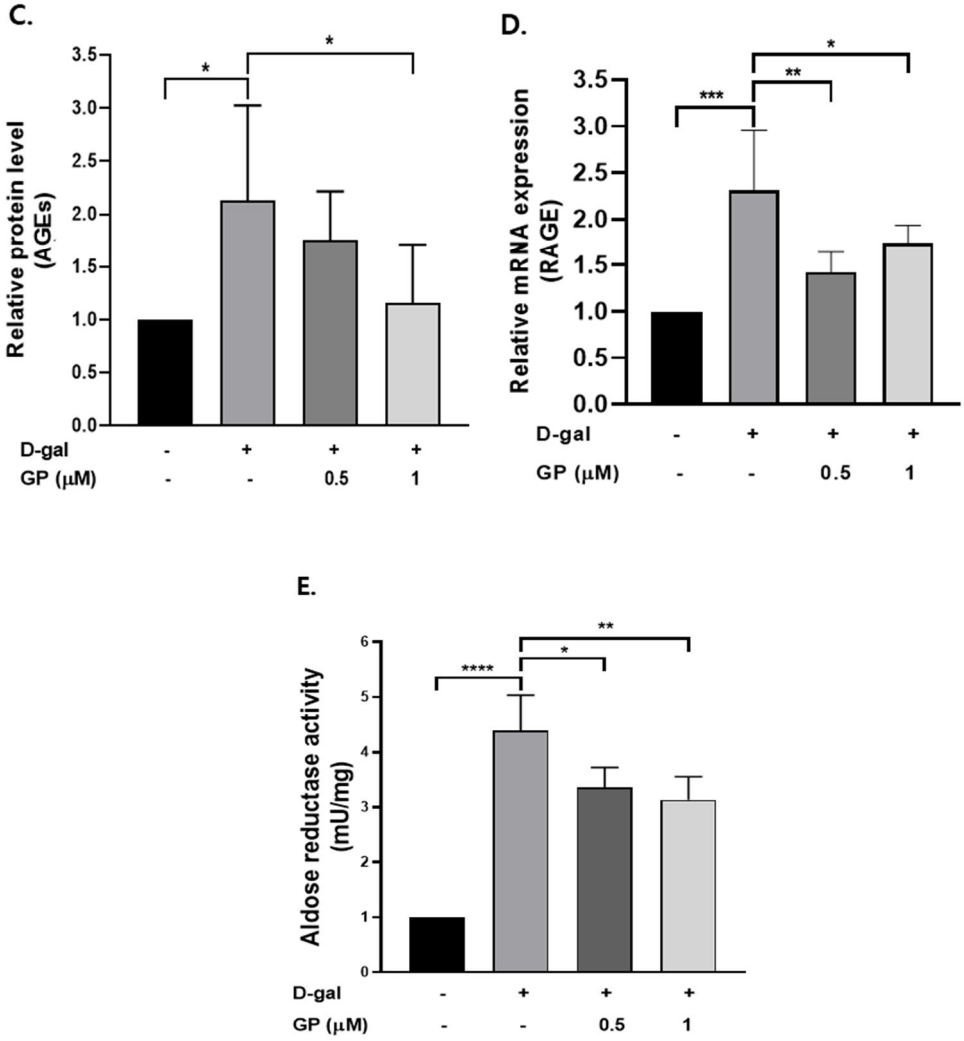

Fig. 8. Gossypol effectively attenuated the elevated levels of AR/AGEs/RAGE induced by D-galactose.

(A) Cell viability was measured by MTT assay. 5 mg/ml D-gal was pre-treated for 72 hours and GP was then treated for 24 hours (*t*-test, **** $p < 0.0001$, mean ± SD, $n = 5$). Treatment of D-gal significantly reduced the cell viability and was recovered on treating GP. (B) The protein level of AGEs was measured by immunoblot analysis. (C) Graph shows that the protein expression level of AGEs was significantly increased in D-gal

treated condition when compared to the control and shows a significant decrease in expression upon the treatment of GP when compared with the D-gal treated cells (*t*-test, * $p < 0.05$, mean ± SD, n = 4). (D) The mRNA expression of RAGE was measured using RT-PCR (*t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, mean ± SD, n = 3). (E) Aldose reductase activity was measured using ELISA after adding aldose reductase assay buffer to the homogenized cells (*t*-test, ** $p < 0.01$, **** $p < 0.0001$, mean ± SD, n = 3).

III-5. Gossypol restored the levels of proteins damaged by D-galactose.

It was confirmed that GP reduced the elevated levels of AGEs/RAGE induced by D-gal. Immunoblot analysis was performed to investigate whether GP restored the decreased levels of AMPK/SIRT1/ACC induced by D-gal (Fig. 8A). Comparison of the levels of AMPK/SIRT1/ACC revealed that treatment with 1 μM GP demonstrated the most significant restorative effect (Fig. 8B). These findings suggest that GP has a significant restorative effect on cells damaged by D-gal.

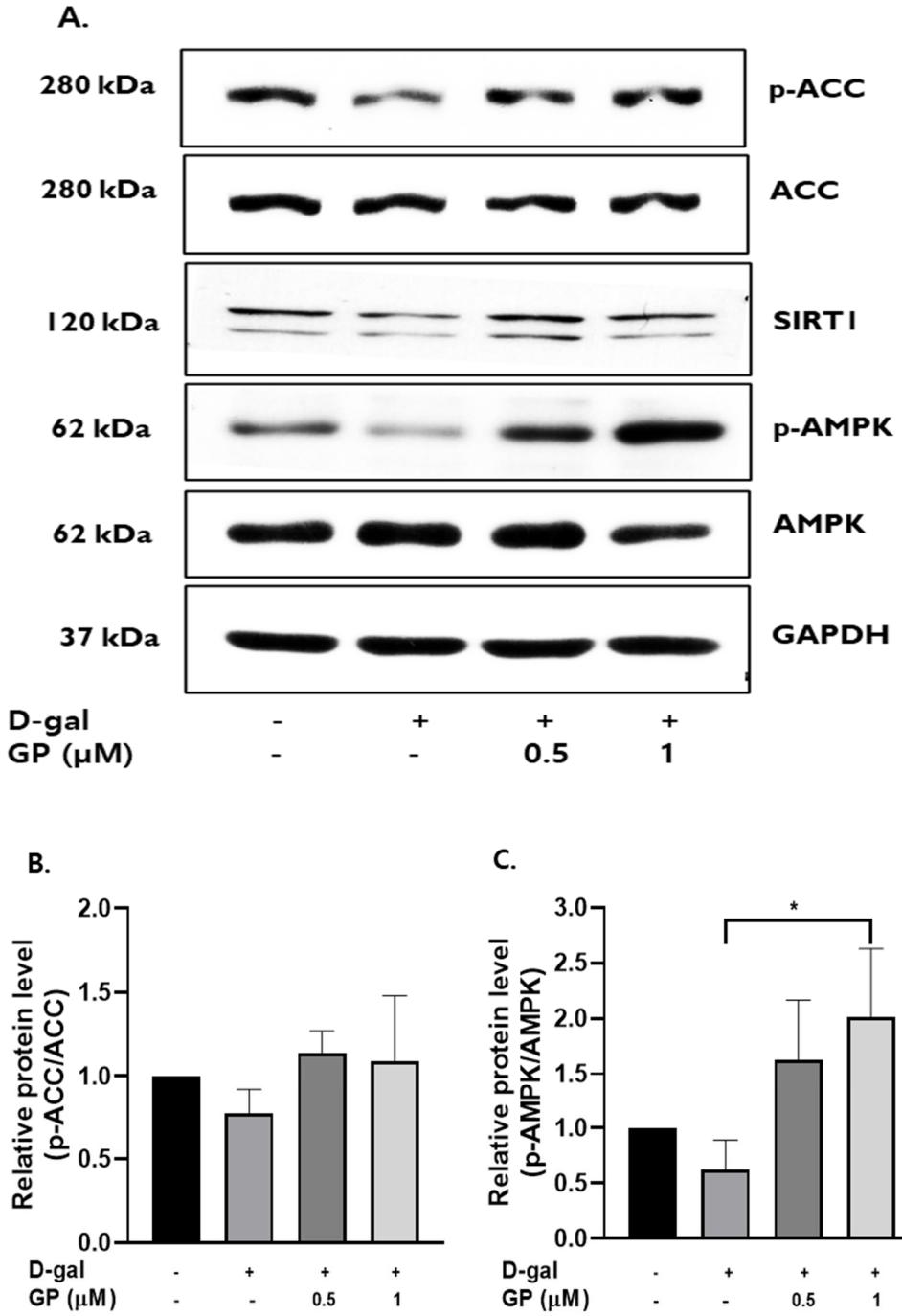

Fig. 9. (Continued)

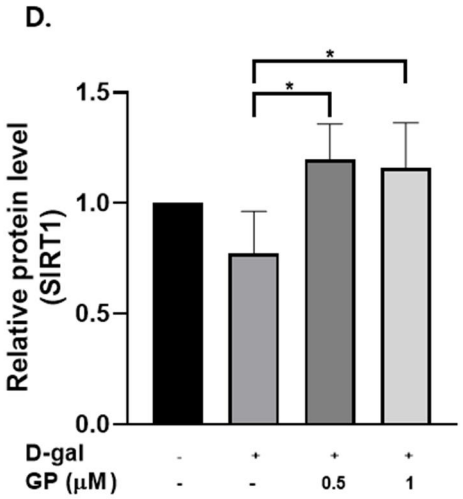

Fig. 9. Gossypol treatment effectively restored the decreased levels of AMPK/SIRT1/ACC induced by D-galactose.

(A) The protein levels of AMPK/SIRT1/ACC were measured by immunoblot analysis. (B-D) The blots for the expression levels of AMPK/SIRT1/ACC were quantified using Image J software shows a significant increase in expression levels during the treatment of GP (*t*-test, * $p<0.05$, mean ± SD, n=4).

IV. DISCUSSION

Previously, the activity of AR mainly has been extensively studied in cardiovascular disease and diabetes models (Vedantham et al., 2012). However, it was confirmed that AR is also associated with obesity and aging (Hallam et al., 2010; Obrosova et al., 2007). The gradual decline of AMPK and SIRT1 levels with aging adversely impacts cellular and tissue viability. In addition, increased AR activity contributes to the reduction of AMPK and SIRT1 levels, leading to decreased cellular activity and impaired function. Consequently, a correlation between AR and aging has been postulated.

Despite the development of variety ARI to prevent complications associated with diabetes and cardiovascular diseases, many have been discontinued due to limited effectiveness or adverse effects (Balestri et al., 2022). Currently, Epalrestat, a carboxylic acid-based ARI, is the only ARI available on the market and distributed in India, Japan, and China (Balestri et al., 2022). The pyrazine-based ARI, Ranirestat, is currently in the phase 3 of clinical trials. Furthermore, Quercetin, a flavonoid polyphenolic compound recognized for its antiaging, antioxidant, and anticancer properties, is undergoing phase 2 clinical trials to assess its potential as an ARI (Grewal et al., 2016). The introduction emphasizes the involvement of AR in aging and age-related diseases, along with the observation of increased AR levels in elderly people. This observation implies that ARIs not only hold promise in preventing or alleviating complications associated with diabetes and cardiovascular diseases but also have the potential to counteract the processes of aging and age-related ailments.

Although reducing AR levels holds promise, it is important to consider the vital metabolic processes and the significance of enzymes for organismal survival, as organisms do not possess enzymes that are unnecessary for their survival. Thorough investigations are required to clarify the specific effects of sustained low AR activity in

this domain. Therefore, it is crucial to administer ARIs appropriately to avoid excessive suppression of AR activity while still achieving the desired therapeutic effects.

GP was utilized as a male contraceptive in China; however, its usage was discontinued upon confirming a decline in sperm count and activity. Nonetheless, studies indicate that the damage to sperm cells caused by GP intake can be reversed upon cessation (Zhao et al., 2020). The observed reversal appears to be attributed to the inhibitory effect of GP on AR activity, thereby hypothesizing that it interferes with the supply of fructose, an energy source, to the seminal vesicles, ultimately affecting sperm function. Also, many natural compounds with a polyphenol structure have antiaging, antioxidant, and anticancer effects, which is the ground for GP to inhibit cancer and tumors (Cháirez-Ramírez et al., 2021). In addition, under normal conditions, low dose of GP enhanced the levels of AMPK and SIRT1, both of which are associated with antiaging (Amjad et al., 2021; Covarrubias et al., 2021; Stancu, 2015). Hence, it is plausible to hypothesize that low-dose GP can serve as an agent to inhibit aging. These findings suggest that GP not only acts as an anticancer and antitumor inhibitor but also possesses antioxidant, antiaging, and ARI capabilities.

V. CONCLUSION

Glucose is a commonly used component in cell culture media. Therefore, D-gal, a different type of sugar, was utilized to determine its specific effects on cellular processes. As anticipated, higher dose of D-gal compared to the control led to increased levels of AGEs and RAGE, thus establishing D-gal as a suitable model (Fig. 5). The reduction of AMPK and SIRT1 levels by D-gal further supported its role in aging induction. However, the optimal dose of D-gal could not be determined solely from the previous figure, prompting an investigation using immunoblotting to assess the levels of AMPK/SIRT1/ACC (Fig. 6). Prior to evaluating the inhibitory effects of GP on the increased levels of AR/AGEs/RAGE induced by D-gal, the efficacy of GP was assessed under normal conditions. Low dose of GP did not exhibit cytotoxicity and instead increased cellular activity (Fig. 7). Notably, a low dose of GP (0.5 μM and 1 μM) improved the viability of cells that had been reduced by D-gal, and simultaneously reduced the levels of AR/AGEs/RAGE (Fig. 8). The levels of AMPK/SIRT1/ACC, which were reduced by D-gal, were restored following GP treatment, consistent with the previous findings (Fig. 9). Based on these results, it can be inferred that GP effectively targeted and inhibited AR, consequently reducing the levels of AGEs/RAGE and restoring cellular conditions. These findings suggest that GP has the potential to serve as a novel ARI.

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