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Berberine Suppresses Hepatocellular Carcinoma Proliferation via Autophagy-mediated Apoptosis

Yun Kyu Kim[1] and Myeong Gu Yeo[2]*

[1]*Department of Convergence Medicine, Asan Institute for Life Sciences, University of Ulsan College of Medicine, Asan Medical Center, Seoul 05505, Korea*
[2]*Department of Integrative Medical Sciences, Nambu University, Gwangju 62271, Korea*

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Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related mortality worldwide, necessitating novel therapeutic strategies. The chemotherapeutic agents used to treat HCC patients are toxic and have serious side effects. Therefore, we investigated the efficacy of anticancer drugs that reduce side effects by targeting tumor cells without causing cytotoxicity in healthy hepatocytes. Berberine, an isoquinoline alkaloid derived from plant compounds, has emerged as a potential candidate for cancer treatment due to its diverse pharmacological properties. The effect of berberine on HepG2 cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay. HepG2 cell proliferation was determined through a colony-forming assay. The effects of berberine on HepG2 cell migration were evaluated using a wound-healing assay. Berberine inhibited the proliferation of HepG2 cells, as well as colony formation and migration. Berberine treatment increased the expression of autophagy-related genes and proteins, including Beclin-1 and LC3-II, and elevated the activities and mRNA expression of Caspase-9 and Caspase-3. Additionally, in experiments utilizing the Cell-Derived Xenograft animal model, berberine treatment reduced tumor size and weight in a concentration-dependent manner. These results demonstrate the potential of berberine as a versatile anticancer agent with efficacy in both cellular and animal models of hepatocellular carcinoma. The findings herein shed light on berberine's efficacy against HCC, presenting opportunities for targeted and personalized therapeutic interventions.

Key words : Apoptosis, autophagy, berberine, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver and stands as a major global health concern due to its high incidence and poor prognosis [5]. Despite significant advances in cancer research and treatment, the intricate molecular mechanisms underlying hepatocarcinogenesis remain partially understood. The need for innovative therapeutic strategies has driven the exploration of natural compounds as potential agents for HCC prevention and treatment. The risk factors of HCC include chronic exposure of the liver to damage from the hepatitis C virus, hepatitis B virus, non-alcoholic fatty liver disease, and non-alcoholic steatohepatitis with metabolic liver disease. The high recurrence rate of HCC is the second cause of cancer-related deaths [12]. HCC is resistant to cytotoxic chemotherapy and more likely to metastasize due to the abundant blood flow to the liver, patients with HCC have a low long-term survival rate. General methods for HCC cancer therapy have conducted surgical elimination of tumors, liver transplantation, radiotherapy, and chemotherapy [23]. Though a variety of treatments is available for HCC, chemotherapy is used to treat patients who are thought to be unsuited for surgical elimination of tumors or liver transplantation [6, 7]. Several chemotherapy agents are used for HCC patients including atezolizumab plus bevacizumab, sorafenib, lenvatinib, regorafenib, cabozantinib, and ramucirumab. Among chemotherapy agents, Sorafenib has been widely used as a chemotherapy agent in the treatment of HCC. Sorafenib, a multiple-target tyrosine kinase inhibitor, can target vascular endothelial growth factor receptor2, platelet-derived growth factor receptor, *c-KIT*

***Corresponding author**
Tel : +82-62-970-0169, Fax : +82-62-970-0169
E-mail : mgy11@nambu.ac.kr
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(hepatocyte factor receptor), and other proteins to prevent tumor angiogenesis [18]. Although Sorafenib has been used in clinical settings to prolong survival, it is expensive. It has serious side effects such as hypertension, cardiovascular events, arterial thromboembolic events, bleeding, hand-foot skin reaction, diarrhea, and renal toxicity [9].

Recent studies have suggested that berberine, an isoquinoline alkaloid derived from rhizomes of *Coptis Chinensis* and *Hydrastis Canadensis,* possesses anti-inflammatory, anti-oxidative, and anti-proliferative properties, making it a promising candidate for cancer therapy [13]. Berberine hinders inflammation by regulating *AMPK/mTOR*, signaling pathways, and cytokines of TNFα, IL-1β, and IL-6 [22].

Berberine triggers apoptosis in HCC in several different ways. It induces apoptosis via the mitochondrial route, AMPK-pathway, and suppresses the *iPLA2/LOX-5/LTB4* pathway in HepG2, Huh7, H22, and Bel-7404 [8, 20]. Berberine induces autophagic cell death in HepG2 and MHCC97-L cells through *Beclin-1* up-regulation and activation of mTOR down-regulation by suppressing the activation of Akt and up-regulating P38 MAPK signaling [17]. Berberine induced Beclin-1 and LC3-II (microtubule-associated protein light chain 3) upregulation, by inhibiting mTORC1 via AMPK activation in HepG2 cells [21].

In this study, we elucidate the interplay between berberine and the HepG2 cell line. Exploring berberine's efficacy against HCC opens new avenues for targeted and personalized therapeutic interventions.

Materials and Methods

Cell culture

Human hepatoma cell line HepG2 cells were obtained from the Korean Cell Line Bank. HepG2 cells were cultured in medium RPMI 1640 with 5% fetal bovine serum (FBS) at 37°C in a humidified chamber with 95% air and 5% CO_2.

MTT assay

Cell proliferation rates were assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, HepG2 cells were seeded into 48-well plates at a density of 0.7×10^4 cells/well. The experimental groups were treated with different concentrations of berberine ranging from 5, 10, and 15 μM. After 6 hr and 12 hr, MTT was added into each well at a concentration of 100 μl of 2 mg/ml per well, incubated for 2 hr, and then replaced with 100 μl of dimethyl sulfoxide (DMSO). The number of viable cells was evaluated by measuring the absorbance at an optical density (OD) of 595 nm using enzyme-linked immunosorbent assay (ELISA).

Wound-healing assay

We assessed cell migration using a wound-healing assay. Seeding the cells at 8×10^5 cells/well in a 12-well plate and cultured confluently at 37°C, 5% CO_2 incubator. Then, after making a scratch line on the cells using a 200 μl sterile pipette tip, the plates with FBS 2% media were incubated at 37°C in 5% CO_2. Wound healing was observed at 0 and 24 hr using an inverted microscope system (Olympus, Japan).

Colony formation assay

Human HepG2 cells were made into single-cell suspensions with trypsin and then incubated in six-well plates at a density of 5×10^2 cells per well. Cells were treated with berberine for 10 days. Then cells were washed with phosphate-buffered saline (PBS) twice, fixed in 4% paraformaldehyde, and stained with 0.5% crystal violet for 15 min. After being washed by PBS, images were captured.

RT-PCR experiments

Total RNA was isolated from cultured cells using Trizol (Cellconic, Shanghai, China) reagent and reverse transcribed using the Maxime™ RT PreMix Kit (Oligo dT15 Primer, iNtRON, Seong-Nam, Korea) according to the manufacturer’s protocol. Real-time PCR was carried out using Luna ® Universal qPCR Master Mix Kit under standard reaction conditions: 45 cycles at 95°C for 5 min, 95°C for 10 sec, and 60°C for 30 sec with the 7500 Real-Time PCR Detection System (Bio Molecular Systems Queensland, Australia) and lists of primers summarized in Table 1.

Western blot assay

Cells were harvested, washed twice with cold PBS, and extracted using a NE-PER extraction kit according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL, USA). The lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The proteins were detected by the specific primary antibody (Beclin-1: sc-48381, LC3A/LC3B: sc-398822, Santa Cruz, Dallas) and secondary antibody (Anti-mouse IgG, HRP-linked Antibody #7076, Cell Signaling Technology, Danvers) conjugated to horseradish peroxidase and visualized using an enhanced chemiluminescence western blot detection system.

Table 1. List of primer sequences used for RT-PCR analysis

Gene	Primer Sequence (5' – 3')
S18	F: TTT GCG AGT ACT CAA CAC CAA CA R: CCT CTT GGT GAG GTC AAT GTC TG
LC3-II	F: GAG AAG CAG CTT CCT GTT CTG G R: GTG TCC GTT CAC CAA CAG GAA G
Beclin-1	F: GGC TGA GAG ACT GGA TCA GG R: CTG CGT CTG GGC ATA ACG
Caspase-3	F: AGG ACT CTA GAC GGC ATC CA R: CAG TGA GAC TTG GTG CAG TG
Caspase-9	F: AAC CCT AGA AAA CCT TAC CCC R: CAT CAC CAA ATC CTC CAG AAC

Caspase-3 and caspase-9 activity assay

Caspase-3 and Caspase-9 in culture medium were determined using ELISA kits (AbCAM, Boston, MA, USA) according to the manufacturer's instructions. Briefly, standards and samples are added to appropriate wells and incubated. Then, the Biotin-conjugated antibody is added, followed by additional washing steps. SABC working solution is added next, followed by further washing. TMB substrate is added, and after incubation, a Stop Solution is added to terminate the reaction, and a standard curve is generated for each assay. Color changes were determined at 450 nm.

Animals

Specific pathogen-free NOD-SCID (Nonobese diabetic/severe combined immunodeficiency) male mice (weight 20–22 g) were purchased from Central Laboratory Animal Inc. (Seoul, Korea). The animals were housed and maintained under controlled specific pathogen-free conditions at 21–24°C and 40–60% relative humidity under a 12 hr light/dark cycle with free access to food and water. The mice were provided with veterinary/supportive care when they began to show signs of illness. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Asan Medical Center and performed in compliance with the institutional guidelines (IACUC approval number 2022-12-078).

In vivo tumor model

NOD-SCID mouse was implanted with Hep3B cells at a density of 2×10^6 cells/each. After making a Cell-Derived Xenograft (CDX) model, the cell was transferred to another NOD-SCID mouse and transplanted. When the tumor volume reached 100 mm^3, mice were randomly divided into three groups. The experimental group was intraperitoneally injected with berberine (5 mg/kg and 10 mg/kg) daily for 11 days. The control group received daily intraperitoneal injections with an equal volume of saline. The long axis (D) and the short axis (d) of the tumor were measured daily. Tumor volume (Tv) was calculated by the formula: $Tv = 0.5 \times D \times d^2$. On day 11 post-administration, all mice were sacrificed, tumor tissues were weighed, and fixed with paraffin blocks for subsequent experiments.

Statistics analyses

Values are expressed as the mean ± standard error of the mean (SEM). Data were statistically analyzed using the independent samples t-test and an analysis of variance; *p-values* <0.05 were considered statistically significant. All experiments were performed in triplicate.

Results

Berberine suppressed cell proliferation in the HepG2 cell line

To determine the efficiency of berberine in cell proliferation *in vitro*, we examine cell proliferation in the HepG2 by MTT assay. The cell proliferation was inhibited by the berberine's concentration and time dependence (Fig. 1). Berberine suppressed cell viability in HepG2 cells by 55.4% at 24 hr, 27.7% at 48 hr, and 15% at 72 hr at 15 μM, respectively.

Berberine suppressed cell migration

We investigated the effect of berberine on HepG2 cell migration by wound-healing assay. The region of the wounded area, between cell layers after generating a scratch was at 76.07% occupied by migrating cells after 24 hr in the control group in HepG2 cells (Fig. 2A). The HepG2 cells were treat-

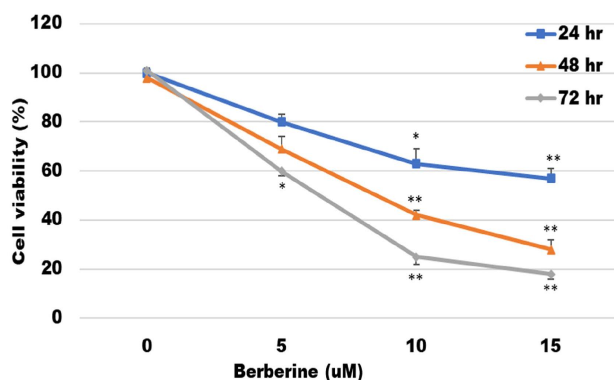

Fig. 1. Berberine suppressed cell proliferation in the HepG2 cell line. HepG2 cells were cultured in the 48-well plates 3 hr and then treated with different concentrations of berberine (5 μM, 10 μM, 15 μM) for 24, 48, and 72 hr. The cell viability was analyzed by MTT assay. The percentage was calculated by comparing the O.D. *: *p<0.05*. **: *p<0.01* versus control (untreated berberine). All experiments were performed in triplicate.

ed with 10, 20, and 30 μM of berberine, on the other hand, did not occupy 44.78%, 25.92%, or 24.62% of the vacant area of the cells. The region of the wounded area was at 52.41% occupied by migrating cells after 48 hr in the control group (Fig. 2A).

Berberine suppressed colony formation

We also examined the effect of berberine on colony cell formation in HepG2 by colony formation assay. Berberine treatment showed a clear reduction in colony formation in a dose-dependent manner. In comparison to the control group, colony formation was reduced in berberine-treated HepG2 cells at 1 μM (72.15%), 2 μM (31.81%), 3 μM (23.29%), 4 μM (22.15%), and 5 μM (13.63%) (Fig. 2B).

Berberine initiated the *Beclin-1* and *LC3-II* mRNA and protein expression

To examine the berberine efficacy in autophagy pathways, mRNA expression of *Beclin-1*, and *LC3-II,* which are associated with autophagy, was identified in HepG2 cells. Compared to *Beclin-1* and *LC3-II* mRNA expressions, *Beclin-1* mRNA expression was higher at both 6 hr and 12 hr, but *LC3-II* mRNA expression was increased at 12 hr in HepG2

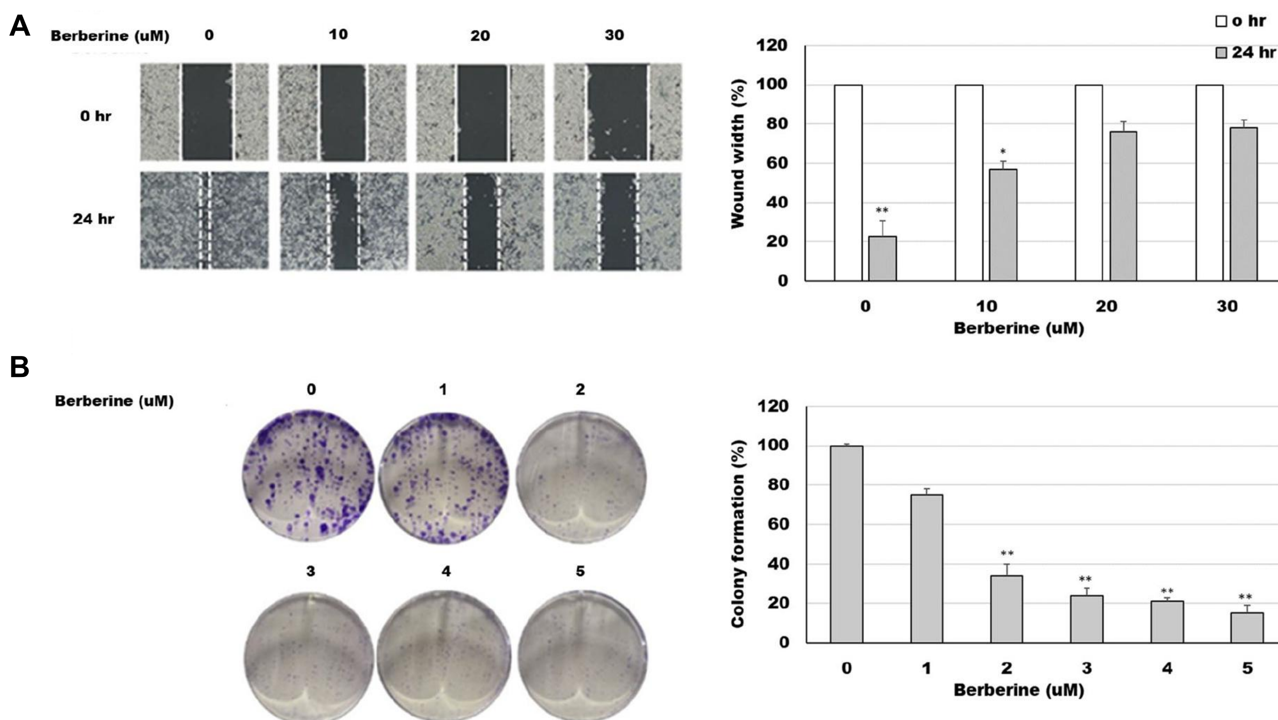

Fig. 2. Berberine inhibited HepG2 cell migration and proliferation in the HepG2 cell line. (A) HepG2 cells were treated with berberine, and cell migration was measured by wound healing assay. HepG2 cells were scratched and treated with different concentrations of berberine (10 μM, 20 μM, 30 μM) for 24 hr (left panels). (B) Cells were cultured in the 6-well plates at a density of 5×10^{2} cells per well and then treated with different concentrations of berberine (1 μM – 5 μM) for 10 days. Cells are fixed in 4% paraformaldehyde and stained with 0.5% crystal violet for 15 min. *: *p<0.05*. **: *p<0.01* versus control (0 hr). All experiments were performed in triplicate.

cells that were treated with berberine (Fig. 3A). Additionally, the Beclin-1 protein expression was increased at 6 hr and 12 hr. The LC3-II protein expression was increased at 6 hr in HepG2 cells (Fig. 3B). These results imply that berberine treatment initiates the *Beclin-1* mRNA stimulation, thereafter *LC3-II* mRNA, and is associated with autophagy pathways.

Berberine induced mRNA and activities of caspase-3 and caspase-9 in HepG2 cells

Otherwise, to examine the berberine effectiveness on autophagy-mediated apoptosis signaling pathways, the *Caspase-3*, and *Caspase-9* mRNA expression was identified in HepG2 cells (Fig. 4A). The *Caspase-9* mRNA expressions were higher in HepG2 cells treated with berberine for 6 hr than control. Otherwise, the *Caspase-3* mRNA expressions were higher in HepG2 cells treated with berberine for 12 hr than in control but were lower in HepG2 cells treated with berberine for 24 hr than in controls (Fig. 4A). Additionally, caspase-3 and caspase-9 activity was increased in HepG2 cells treated berberine (Fig. 4B). From these results, we confirmed that berberine treatment affects Caspase-mediated apoptosis pathways.

Berberine inhibits HCC growth in the CDX mouse model

The Hep3B cells are implanted in NOD-SCID mice and

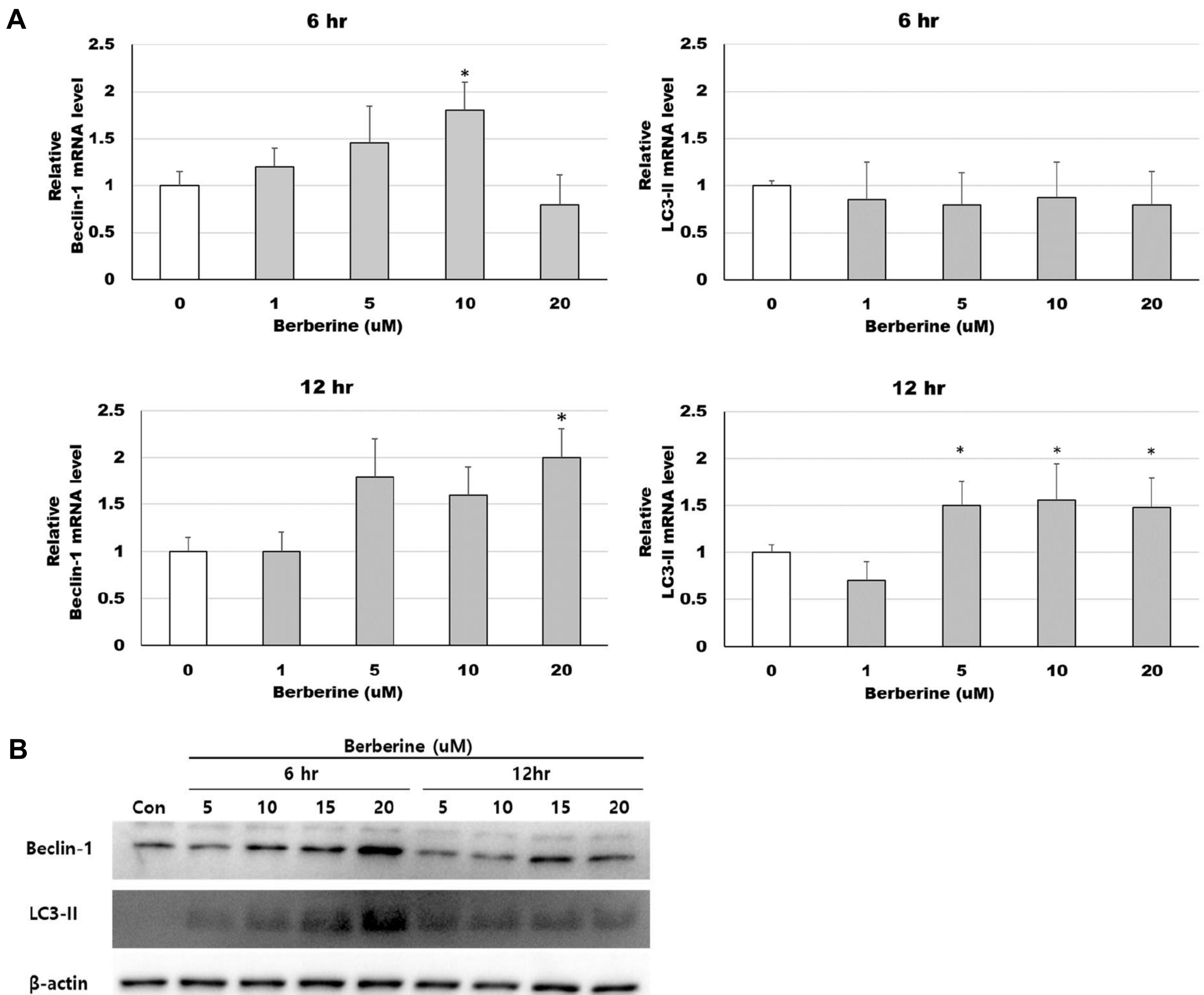

Fig. 3. Quantitative RT-PCR and western blot assay determined. The mRNA and protein expression levels of autophagy-related genes, Beclin-1 and LC3-II. HepG2 cells are treated with different concentrations of berberine (1 μM, 5 μM, 10 μM, 20 μM) for 6 and 12 hr, respectively. (A) mRNA expression of *Beclin-1* and *LC3-II*. *: $p<0.05$ versus control (untreated berberine). (B) Relative protein expression of Beclin-1 and LC3-II. All experiments were performed in triplicate.

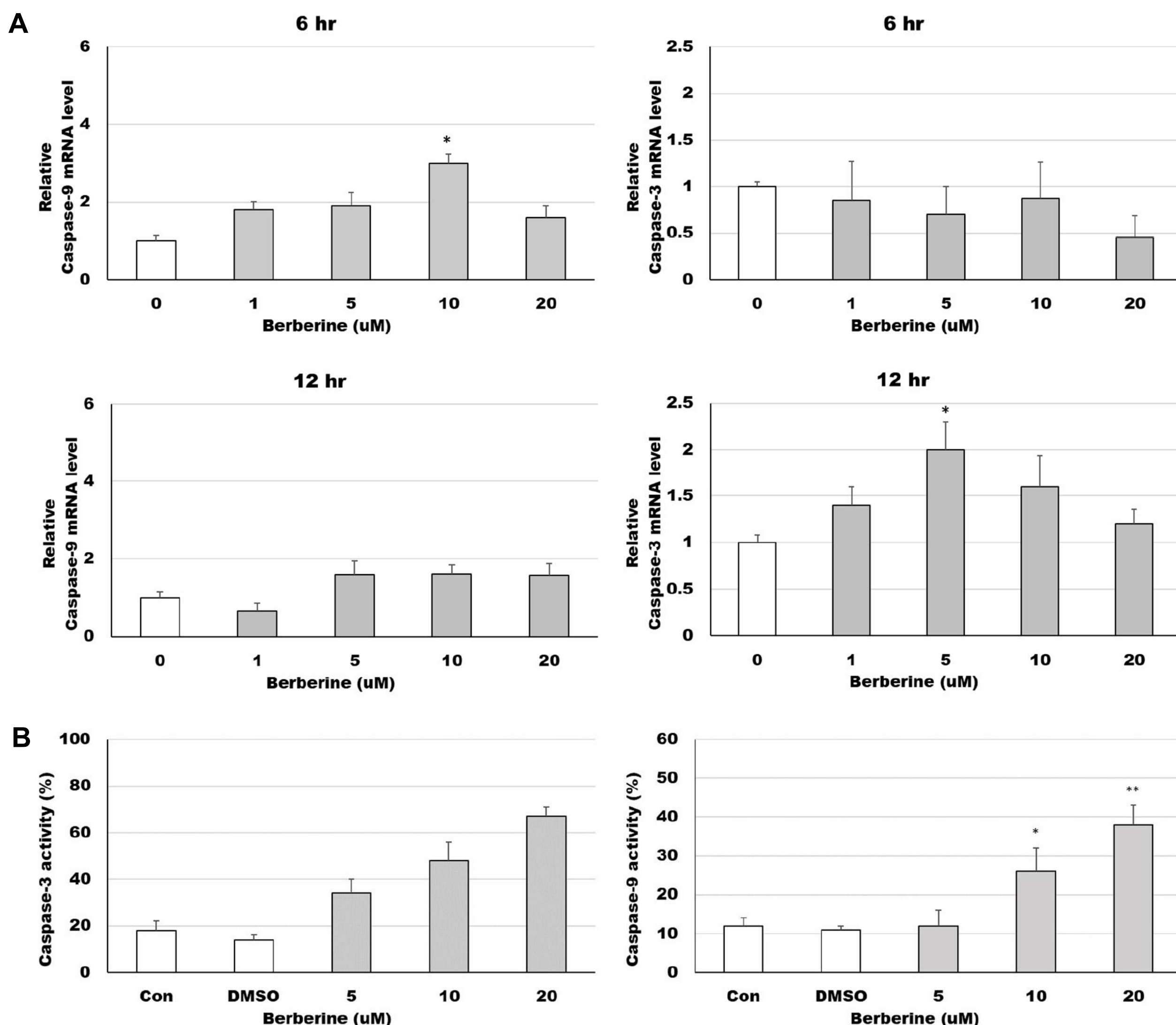

Fig. 4. Relative activities and mRNA expression of *Capase-9* and *Capase-3* in HepG2 cell line. (A) HepG2 cells were treated at indicated berberine concentrations and *Caspase-3* and *Caspase-9* mRNA levels were examined. (B) Active assay of Caspase-3 and Caspase-9. *: $p<0.05$. **: $p<0.01$ versus control. All experiments were performed in triplicate.

treated with indicated concentrations of berberine, respectively. After 11 days, the animals were sacrificed, and the 10 mg/kg berberine caused a 67.37% decrease in tumor volume as compared with the control group, whereas 5 mg/kg berberine caused a 39.72% decrease respectively (Fig. 5A, 5B). Similarly, the tumor weight also significantly decreased tumor weight when 10 mg/kg berberine treatment, but mouse body weight was not changed during the experiments (Fig. 5C, 5D, 5E). This result implies that berberine inhibited the Hep3B growth *in vivo*.

Discussion

Primary treatments for HCC include surgery for early stages, ablative procedures for small tumors, transarterial chemoembolization to block the tumor blood supply, targeted therapy, immunotherapy, and often a combination including chemotherapeutic agents. The chemotherapeutic agents are used to treat HCC patients which are unsuited for surgical removal of tumors or liver transplantation, however, has serious side effects and side effects [1, 2, 4, 9, 10, 14, 15]. Berberine is a plant alkaloid used for cancer treatment due to its high antitumor activity [1]. Previous studies reported that berberine represses tumor progression by cell proliferation in various cancers and has little to no cytotoxic impact on normal liver cells [11, 19]. However, the precise mechanisms are not elucidated. In the present study, the anti-cancer effects of berberine were examined by the biochemical meth-

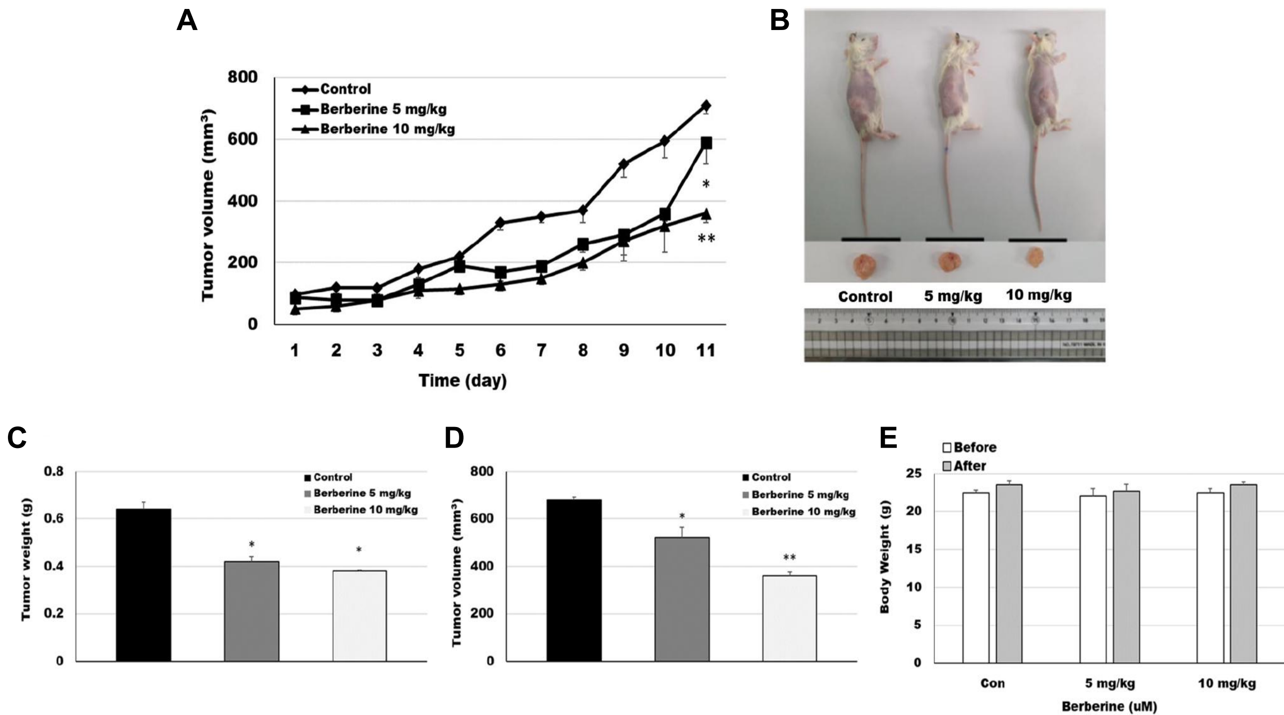

Fig. 5. Berberine inhibits HCC growth in CDX Hep3B mouse. Hep3B cells were implanted into NOD-SCID mice, and the tumor volume was measured in CDX Hep3B for 11 days with or without berberine treatment. (A) Berberine inhibits HCC growth *in vivo.* (B) Mice were sacrificed 11 days of berberine treatment, and the tumor weight, volume, and body weight were measured. Berberine-treated CDX Hep3B suppressed the tumor weight and volume compared with the control group (C, D). *: $p<0.05$. **: $p<0.01$ versus control. All experiments were performed in triplicate.

ods using HCC cell lines, such as HepG2 cell lines, and CDX mouse models. In HCC, we also confirmed that berberine inhibited HepG2 cell proliferation. The HepG2 cell lines displayed that emerged to be the most responsive. In the study, we confirmed that berberine significantly inhibited HepG2 cell migration. This result corresponds with what has been previously reported in HepG2 [16]. Berberine effectively suppressed colony formation in HepG2 cells. This result is in line with previously reported [3]. We examined that berberine regulates the autophagy mechanism in HepG2 cells. Previous research found that berberine induces HepG2 and Huh7 [22] cells autophagy at the protein level, but little is known regarding the effects of berberine on autophagy of mRNA level in HepG2 cells. Our results showed that berberine increases the expression of autophagy-related genes *Beclin-1* and *LC3-II* at the mRNA level and protein expression in HepG2 cells, then induces cell death.

Next, we examine how this autophagy affects the Caspase-mediated apoptosis signaling pathways in HepG2 cells. It was reported that berberine decreases the expression of pro-caspase-3 and pro-caspase-9 and increases the expression of cleaved-caspase-3 and cleaved-caspase-9 in protein levels in HepG2 cells [19]. Still, little is known regarding the effects of berberine on the Caspase-mediated apoptosis signaling pathway of mRNA level in HepG2. We found that berberine increases the expression of *caspase-9* and *caspase-3* at the mRNA level in HepG2 cells. This result indicated the berberine treatment to HCC cells induced the caspase-9 activation and then caspase-3 activation, thus involved in apoptosis signaling pathways. From these results, we suggest that berberine regulated the Caspase-mediated apoptosis signaling pathway at the mRNA level in HCC Cells. Finally, we examined the berberine efficacy using an *in vivo* Hep3B CDX mouse model. As shown in Fig. 5, berberine-treated Hep3B CDX mouse showed that the tumor volume and tumor weight are reduced comparing the untreated.

In conclusion, our findings suggest that berberine's impact on HepG2 cells, reveals inhibition of cell proliferation and migration via autophagy and Caspase-mediated apoptotic pathways. Furthermore, berberine-treated Hep3B CDX mice exhibited reduced tumor volume and weight compared to untreated counterparts. These findings highlight berberine's potential as a multifaceted anti-cancer agent, demonstrating efficacy in both cellular and animal models of hepatocellular

carcinoma. This study is valuable as it deepens knowledge of the anti-cancer role of berberine and is a chemotherapeutic method for HCC therapy. Further investigations and clinical trials are required to berberine in cancer patients.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 베르베린을 처리한 간세포암에서 자가포식 경로와 관련된 세포자멸사

김윤규[1] · 여명구[2]
([1]울산대학교 의과대학, [2]남부대학교 통합의료학과)

간세포암종은 전 세계적으로 암 관련 사망의 두 번째 주요 원인으로 새로운 치료 전략을 필요로 한다. 간세포암종 환자를 치료에 사용되는 화학요법제는 독성이 있고 심각한 부작용이 있는 것으로 알려졌다. 본 연구는 건강한 간세포에서 세포독성을 일으키지 않으면서 종양세포를 표적으로 하는 부작용을 감소시키는 항암제의 효능을 조사하였다. Berberine은 이소퀴놀린 알칼로이드의 식물 유래 화합물로 다양한 약리학적 특성으로 인해 암 치료의 잠재적 후보군으로 보고되고 있다. 간암 세포 생존율에 대한 berberine의 효과는 3-[4,5-dimethylthiazol-2–yl]-2,5-diphenyl tetrazolium bromide 분석을 사용하여 측정하였다. HCC 증식은 콜로니 형성 분석을 통해 실시하였다. 세포이동에 대한 berberine의 효과는 상처 치유 분석으로 실시하였다. Berberine은 HepG2 세포와 같은 간암 세포의 증식과 콜로니 형성 및 세포이동을 억제하였다. Berberine 처리는 *Beclin*-1 및 *LC3-II* 등의 자가포식 관련 유전자 발현과 단백질 발현을 증가시켰으며, *Caspase-3* 및 *Caspase-9*등의 세포자멸사의 mRNA 발현 및 활성을 증가시켰다. 또한, 세포유래 이종이식 동물실험에서 berberine 처리에 따른 종양의 크기와 무게가 감소함을 확인하였다. 본 연구 결과는 간세포암에 대한 베르베린의 효능을 조명하여 표적 및 맞춤형 치료의 기회를 제시할 수 있음을 시사한다.