行政院國家科學委員會專題研究計畫 成果報告

Berberine 抑制癌瘤細胞 migration/invasion、增生及誘導細胞凋亡之研究及保護心血管之活性探討(第 2 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 96-2311-B-040-005-MY2
執 行 期 間 ：97 年 08 月 01 日至 98 年 07 月 31 日
執 行 單 位 ：中山醫學大學醫學系生物化學科

計 畫 主 持 人：謝易修

計畫參與人員：博士班研究生-兼任助理人員：謝明儒
博士班研究生-兼任助理人員：林巧雯

處 理 方 式 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 98 年 10 月 29 日

行政院國家科學委員會補助專題研究計畫 ■成果報告 □期中進度報告

Berberine 抑制癌瘤細胞migration/invasion、增生及誘導細胞凋亡之研究及保護心血管之活性探討

計畫類別：■ 個別型計畫 □ 整合型計畫
計畫編號：**NSC 96-2311-B-040-005-MY2**
執行期間：96 年 8 月 1 日至 98 年 7 月 31 日

計畫主持人：謝易修 中山醫學大學 生化所
共同主持人：
計畫參與人員： 蕭永晉, 陳霈霓, 謝明儒, 林巧雯 中山醫學大學 生化所

成果報告類型(依經費核定清單規定繳交)：□精簡報告 ■完整報告

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年■二年後可公開查詢

執行單位：中山醫學大學

中 華 民 國 98 年 7 月 31 日

中英文摘要

黃連素(berberine; 又稱小蘗鹼) 是一種廣泛存在於許多藥用的草本植物中的生物鹼，其療效包括減緩腹瀉、腸炎以及發炎反應以及抗氧化。多年來在台灣癌症一直為十大死因之首，居高不下，此外，腦血管與心臟疾病在近幾年來則是分居台灣地區十大死因之第二及第三名，僅次於惡性腫瘤之後，因此心血管疾病的預防與治療，是一個急待解決與重視的課題。由實驗結果證實，berberine 能十分有效地抑制子宮頸癌及乳癌細胞的侵襲能力，其中作用機制與抑制 MMP-2, MMP-9, u-PA 的活性有關。其分子機制是透過抑制磷酸化 p-38 與磷酸化 JNK 以及下游的 transcription factor NF–κB。利用活體由尾靜脈注射子宮頸癌細胞 SiHa，證實其在動物體內能有效抑制癌細胞轉移到肺臟的能力。另外，無論是在 in vitro 或 in vivo 實驗中也發現 berberine 具有抑制血管新生作用的活性，與抑制癌細胞侵襲的活性可達到相輔相成的效果。然而，在口腔癌細胞及肺癌細胞則具有降低細胞存活的效果並同時會誘導細胞走向凋亡路徑。而另一方面，針對抑制 LDL 氧化實驗中，利用 relative electrophoretic mobility (REM) shift assays、electrophoresis of ApoB fragmentation、lipid peroxidation assay (malondialdehyde production)，證實黃連素可有效抑制硫酸銅所誘導 LDL 的氧化。在氧化型低密度脂蛋白對人類血管內皮細胞所造成的傷害實驗中，黃連素同時也可抑制氧化型低密度脂蛋白誘發人類臍靜脈內皮細胞的損傷，主要是透過抑制氧化型低密度脂蛋白所破壞粒腺體膜電位 (mitochondrial membrane potential) 及抑制細胞凋亡過程中所釋放出的 cytochrome C 和下游基因 caspase 3 之活化，此外，黃連素同時也可降低氧化型低密度脂蛋白誘發人類臍靜脈內皮細胞的細胞內導致 ROS 的產生。綜合以上結果，berberine 具有保護 LDL 氧化及防止 oxLDL 傷害內皮細胞的效果，黃連素或許可應用於臨床上保護氧化型低密度脂蛋白所造成內皮細胞的功能失調及傷害。且對於乳癌及子宮頸癌的侵襲轉移以及對於口腔癌及肺癌的增生也有顯著抑制效果。

關鍵詞：小蘗鹼; 癌細胞轉移; 動脈硬化; 氧化型低密度脂蛋白; 內皮細胞; 細胞凋亡; 基質金屬水解酶; 胞漿酶原活化劑

Berberine, a natural constituent of plants of the genera Coptis and Berberis, has several anti-inflammation and anti-cancer biological effects. Cancer is the most common cause of death in Taiwan, and followed by atherogenesis-related disease. In this study, we found that berberine could significantly inhibit cell invasion/migration in cervical and breast cancer. It may through the inhibition of matrix degrading enzymes, MMPs and u-PA. Further, a treatment of berberine also resulted in an inhibition of the activation of p-JNK1/2 and p-p-38. In order to confirm whether the activation of above proteins regulates SiHa invasion ability, we used specific inhibitors: SB253080 and SP600125 to clarify that JNK1/2 and p38 signaling regulate expression of MMP-2, cell motility and cell invasion of Si Ha, and JNK1/2 signaling regulate MMP-2, u-PA expression and cell invasion. Finally, an *in vivo* anti-tumor study using nude mice (BALB/c *nu/nu*) xenograft model by a subcutaneous inoculation of SiHa cells was performed. The average tumor volume of treatment groups was statistically lower than that of the control group. Berberine was

also evidenced by its inhibition on the lung metastasis of SiHa cells by tail vein injection *in vivo*. Moreover, berberine exerted anti-angiogenesis activity, such as inhibiting tube formation, and cell migration of HUVEC cells, and it may combined with anti-invasion activity to prevent cancer metastasis. Additionally, berberine decreased cell viability of oral carcinoma cells and lung cancer cells via anti-proliferaion and inducing apoptosis. In the other subject, we evaluated the anti-oxidative activity of berberine and how berberine rescues human umbilical vein endothelial cells (HUVECs) from oxidized LDL (oxLDL)-mediated dysfunction. The anti-oxidative activity of berberine was defined by the relative electrophoretic mobility of oxLDL, fragmentation of Apo B, and malondialdehyde production of the Cu^{2+}-mediated oxidation of LDL. Berberine also inhibited the generation of ROS, and the subsequent mitochondrial membrane potential collapse, chromosome condensation, cytochrome c release, and caspase-3 activation induced by oxLDL in HUVECs. Our results suggest that berberine may protect LDL oxidation and prevent oxLDL-induced cellular dysfunction. In conclusion, we have demonstrated the prevention and its mechanism of berberine on LDL oxidation and oxLDL-induced endothelial cell dysfunction. Otherwise, berberine also exerted a fine anti-cancer activity, such as anti-invasion, anti-angiogenesis, and anti-proliferation.

Keyword：berberine; metastasis; atherogenesis; oxLDL; endothelial cell; apoptosis; matrix metalloproteinase (MMP); urokinase type plasminogen activator (u-PA)

報告內容

一、前言及研究目的

根據衛生署公佈之台灣十大死亡原因顯示癌症仍為十大死因之首，居高不下，如何治療癌症病人，尋找更有效的抗癌藥物，是一個迫切的問題。癌症的形成是透過許多複雜且多重的過程，其中形成的原因雖然被了解，但治療的效果仍然有限，且化療對人體具有相當大之副作用與傷害，因此目前許多研究趨向以天然物成份如多酚類或黃酮類等天然物合併抗癌藥物的使用，期望能加強抗癌藥物的療效，降低抗癌藥物的濃度，以減少化療對人體造成之傷害，達到輔助治療的效果，抑制癌細胞的惡化[1-5]。癌症的生長及轉移過程大概可分為三個階段：1.原位生長、2.局部轉移、3.遠距離轉移。癌細胞與正常細胞差別在於無法進行一般細胞凋亡(apoptosis)且具有異常的增生(proliferation)能力。另外，在轉移擴散的癌細胞中，癌細胞藉由分泌蛋白質水解酵素來分解細胞外基質，導致細胞基質的降解與細胞和細胞間基質的分離，促使癌細胞具有移動性。與胞外基質分解有關的酵素，包括有serine proteinase、matrix metalloproteinases (MMPs)、cathepsins、plasminogen activator (PA)，其中 MMP-9、MMP-2 及 u-PA 在基底膜的破壞扮演著重要角色，與癌症的侵襲及轉移最有關係。惡性腫瘤細胞本身會製造 MMPs，在癌症越末期時某些 MMPs 亦會相對的增加，而 MMPs 具有蛋白分解活性可分解膠原蛋白，在腫瘤破裂的基底膜處可測得大量的 MMPs，現已證實是用來作局部侵襲和遠處轉移，主要在於助長癌細胞穿透基底膜，由血管滲入組織而達轉移之目的[6-19]。

腦血管與心臟疾病在近幾年來則是分居台灣地區十大死因之第二及第三名，僅次於惡性腫瘤之後，因此心血管疾病的預防與治療，也是一個急待解決的課題。動脈硬化(atherogenesis)是一種特殊型態的慢性發炎反應，特徵為脂質沉積、慢性發炎及慢性傷口癒合的過程，最後造成血管引起血栓(thrombosis)，造成局部組織缺血、缺氧而壞死，可能會引起中風(stroke)及心肌梗塞(myocardial infarction)而致死[20,21]。動脈硬化的致病機轉的相關研究已證實：「內皮細胞功能喪失(endothelial dysfunction)」與「低密度脂蛋白氧化修飾(LDL oxidative modification)」是其中的兩大關鍵。而氧化型低密度脂蛋白(oxidized low density lipoprotein, oxLDL)的堆積不僅會誘發免疫反應更會使得內皮細胞功能受損。而功能受損的內皮細胞使得大分子容易通過，並在血管壁上堆積，進而導致許多趨化物質釋出[22]，終使病灶更加惡化[23-25]。

黃連素(berberine; 又稱小蘗鹼) 是一種 *isoquinoline*（異喹啉）衍生物 benzyl isoquinoline proberberine 型的生物鹼，有關小蘗鹼的發現與研究至今已有數十年之久，根據研究發現在許多藥用的草本植物中都能粹取出此成分，例如黃連屬(*Coptis*) 的黃連(*Coptic Chinese*)及日本黃連(*Coptic Japonica*)與小蘗屬(Berberis) 中的上川氏小蘗(*Berberis kawakamii*) [26-28]。Berberine 在歐美地區已經被廣泛的應用在健康食品添加物裡面，其療效包括減緩腹瀉、腸炎以及發炎反應，調控免疫系統作用、對抗細菌、黴菌以及腸內的寄生蟲，另外 berberine 還具有抗氧化[29]、降血糖及減低膽固醇中的低密度膽固醇[30,31]。近來更有研究指出黃連素具有抑制癌瘤細胞增生及生長作用，使細胞週期停滯並造成細胞的凋謝死亡，但是 berberine 對於抑制癌瘤細胞轉移能力的相關研究則尚未清楚。此外，berberine 普遍被認為具抗氧化與抗發炎之功效，但其作用在血管內皮細胞與巨噬細胞之保護作用及其分子機制卻尚未明瞭，本研究利用體外培養人類臍帶靜脈內皮細胞(human

umbilical vein endothelial cells; HUVECs)與 macrophage (RAW 264.7)，以氧化型低密度脂蛋白(oxLDL)誘導血管內皮細胞與 macrophage 之細胞凋亡，用來模擬人體內粥狀動脈硬化的形成，探討 berberine 是否具有保護血管內皮細胞與 macrophage 之作用，並釐清其訊息傳遞路徑與作用機轉。

二、研究方法

1. 分離人類血管內皮細胞 (HUVECs) [32]

利用 PBS (內含 1% penicillin 及 streptomycin) 清洗臍帶後，將 1% trypsin 灌滿臍帶內壁，作用約 6 分鐘，來回沖洗臍帶內壁，再以 M199 培養液沖洗臍帶內壁約 20 次，離心 1200 rpm 5 分鐘，去掉上清，以含 10% FBS 的 M199 培養液打散細胞，將細胞培養在 37℃，5%CO_2 overnight，換新鮮的 M199 培養液 (含 15% FBS、150 μg/ml endothelial cell growth supplement、5 units/ml heparin sodium、100 units/ml penicillin-streptomycin，及 50 μg/ml gentamycin) 持續培養。

2. 細胞培養及處理

乳癌細胞株 HS578T, MDA-MB231，子宮頸癌細胞株 HeLa, SiHa, Caski,以 DMEM 培養基培養, 加入適量 antibiotics 及 10% heat-inactivated FCS；人類血管內皮細胞 human umbilical vein endothelial cells (HUVECs) 以含 15% FBS、150 g/ml endothelial cell growth supplement、5 units/ml heparin sodium、100 units/ml penicillin-streptomycin，及 50 g/ml gentamycin 的 M199 培養液初代培養，持續培養兩週，期間進行所需實驗。

3. 低密度之蛋白的分離及氧化 [33]

將購自於台中捐血中之正常人類血漿 2ml，加入 0.7ml 的 o.15M NaCl 及 0.3 mM EDTA (pH7.4)離心 90000 rpm，10℃，10 分鐘，取下層液 2 ml 後，重複上述步驟後再離心 3.5 小時，再取下層液 2 ml，加入 KBr 離心 3.5 小時，取上層液中淡黃色油層即為 LDL。LDL 先過 PD-10 desalting column 後以去除 EDTA，加入 10 μM $CuSO_4$ 於 37℃反應 16 小時，再過一次 PD-10 desalting column 以去除銅離子。

4. LDL electrophoretic motility assay [34]

利用 lipoprotein 氧化後其帶電荷會由正電性轉為負電性的特性，將氧化後的 oxLDL 90 μl 加入 0.1% sudden black 染劑 10 μl，在 30℃混合作用 20 分鐘後，與 10 μl 50%之甘油混和後，loading 到 1% agarose 電泳膠片上，通電 100 伏特，30 min 後，依電泳結果以 band 移動的距離（electrophoretic mobility；EM）判定 LDL 氧化程度，以未氧化之 LDL 之 EM 為 1。

5. ApoB 蛋白片段化 (fragmentation) 的測試.[34]

在反應結束之後，樣本須以 denaturing bugger (3% SDS, 10% glycerol 及 5% 2-mercaptoethanol) 在 95℃ 加熱 5 分鐘。接著配製3-15% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE)，取樣本 40 μl 注入膠的孔洞 中，然後以 48 V 進行電泳 150 分鐘。在電泳結束之後以 Coomassie Brilliant blue R250 染色2 小時後再進行乾膠

6. TBARS assay [37]

LDL 氧化會生成過氧化物 malondialdehyde (MDA)，一分子的 MDA 與二分子的 thiobarbituric acid (TBA)會生成具螢光性質的聚合物 TBARS (thiobarbituric acid reactive

substance)，此物質於激發波長為 532 nm，發射波長為 600 nm 時可被螢光光讀盤儀(Labsystem, CA)計偵測到。以 1,1,3,3-tetramethoxypropane（TMP）為標準液做標準曲線而可定量 LDL 氧化程度，單位為 nM MDA/mg protein。

7. DPPH 自由基清除試驗

新鮮配製 500μM DPPH 試液 (98.75mg/50ml methanol)，取 0.5ml 的 berberine (0, 25,50, 75, 100 μM)及 Trolox (10, 50, 100 μM)，分別加入 1ml 的 DPPH 試液，混合均勻後，避光靜置室溫反應 30 分鐘，測定 517nm 的吸光值，以下列公式計算其自由基清除率：(1-$A_{Sample}/A_{Control}$)×100%。

8. MTT(Microculture Tetrazolium) 分析 [37]

將內皮細胞以 3.5×10^4 細胞數分至 24 well 中，37 ℃培養 16 小時後，處理不同濃度(0, 25, 50, 75, 100 μM)的 berberine，同時處理 oxLDL (200 μg/ml)，培養 24 小時後，去除加藥的細胞培養液，再加入 1 ml 的 MTT reagent (0.5 mg/ml)，作用 4 小時之後，以異丙醇將結晶溶出，於 O.D. 565 nm 下測定溶液吸光，由吸光強度可得知存活的細胞數多寡。

9. DAPI stain [35]

內皮細胞處理不同濃度(0, 25, 50, 75, 100 μM)的 berberine，同時處理 oxLDL (200 μg/ml)，培養 24 小時後，以 PBS 清洗兩次，然後以 4% para-formaldehyde 固定作用 30 min 後，以 PBS 清洗，加入染劑 (DAPI) 染 30min，以 PBS 清洗，於螢光顯微鏡下觀察 (UV 461nm)。

10. Cell cycle 分析 [37]

以 60 mm 培養皿培養內皮細胞處理不同濃度(0, 25, 50, 75, 100 μM)的 berberine，同時處理 oxLDL (200 μg/ml)，培養 24 小時後，將細胞打下，加入 1 ml 的 70 % cold ethanol 以固定細胞，置於 4℃ 中隔夜。移除上清液並分別加入 1 ml 之 propidium iodide mixture(PI stain) 避光靜置室溫 30 分鐘。用 40 μm nylon mesh 過濾，使用流式細胞儀(FACSCalibur，BECTON DICKINSON)作分析。

11. ROS含量測定 [33]

60 mm 培養皿培養內皮細胞處理不同濃度(0, 25, 50, 75, 100 μM)的 berberine，同時處理 oxLDL (200 μg/ml)，培養 24 小時後，以 PBS 清洗兩次，將細胞打下，加入 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA)螢光劑處理細胞一小時，使用流式細胞儀(FACSCalibur，BECTON DICKINSON)作分析。

12. 粒線體膜電位測定 [37]

60 mm 培養皿培養內皮細胞處理不同濃度(0, 25, 50, 75, 100 μM)的 berberine，同時處理 oxLDL (200 μg/ml)，培養 24 小時後，以 PBS 清洗兩次，加入染劑 JC-1(1uM)染 30 min，再以 PBS 清洗，於螢光顯微鏡下觀察 (red fluorescence light at 590 nm; green fluorescence light at 527 nm)。在低膜電位的狀態下，JC-1 染劑會以 monomer 的形式發出綠色螢光，而高膜電位下，則會形成 J-aggregates 發出紅色螢光。

13. 西方墨點法 (Western blot) [36]

首先製備 10 % SDS-PAGE 電泳膠片，置於電泳槽中, 並加入電泳緩衝液，取 16μl sample (蛋白總量 30μg)，加入 4μl loading buffer，將 sample denature (95℃;10 min)之後進行電泳分離。在將蛋白轉漬到 NC paper 上，加入 blocking buffer，在室溫下搖動一個小時。然後加入一級抗體於 TBS buffer，在 4℃ 下反應 overnight，之後以 washing buffer (TBS+0.05% Tween

20)清洗三次，每一次 10 分鐘。接著再加入二級抗體於 TBS buffer, 於室溫作用二個小時後以 washing buffer 清洗三次，每一次 10 分鐘。最後加入 25ml substrate buffer 進行呈色反應(或以 ECL 冷光系統紀錄)，並以 densitometer (Alphalmage 2000, AlphaImage comp)量化結果。

14. 細胞移動性分析(Motility) [36]

利用 48 well Boyden chamber 的分析方法，lower chamber 為含有 10% FBS 的 DMEM，將癌細胞處理 berberine 後，計算細胞數 (10^4-1.5×10^4) 注入於 upper chamber，待細胞移動 8 小時以後，取下薄膜，以甲醇固定細胞 10 分鐘，風乾 5 分鐘之後，以 Giemsa (1：20)染色 1 小時，最後固定住薄膜，擦拭掉薄膜之上層細胞，在顯微鏡底下隨機選取視野，作移動細胞數之統計。

15. 細胞侵入性分析(Invasion) [36]

利用 48 well Boyden chamber 的分析方法，將 cellulose nitrate filters coating 上 100μg/cm^2 matrigel，在 Laminar flow 風乾，其他操作與細胞移動性分析相同。

16. Gelatin Zymography [36]

以不含胎牛血的培養液培養細胞 24 小時後與 5×loading dye 均勻混和後，注入 0.1% Gelatin-8% SDS-PAGE 的電泳膠片中，進行電泳分離。膠片以 washing buffer (2.5% Triton X-100)在室溫下沖洗 30 分鐘 2 次，然後加入 reaction buffer (10 mM Tris-HCl, 10 mM $CaCl_2$, 0.1% NaN_3, pH 8.0)在 37℃恆溫箱中反應 12 小時，最後反應完的膠片以染色液染色 30 分鐘，再以退色液退染，觀看結果。

17. Casein Zymography [36]

以不含胎牛血的培養液培養細胞 24 小時後與 5×loading dye 均勻混和後，注入 8% SDS-PAGE (含有 48mg casein, 0.55mg plasminogen)的電泳膠片中，進行電泳分離。後續操作與 gelatin zymography 相同。

18. DNA 斷裂分析(DNA fragmentation)

將細胞以 1×10^6 細胞數分盤至 10 公分的培養皿中，加入含有 10%胎牛血的培養液並且添加不同濃度的補骨脂，處理 48 小時，將細胞培養液移至 15 mL 離心管中，離心 1500 rpm 10 分鐘，去上清液，離心下來的細胞以 50μL lysis buffer (1% NP40，20mM EDTA，50mM Tris-HC1；pH7.5)作用 30 分鐘，之後以 12000×rpm 的速度離心 5 分鐘，接著取上清液至新的微量離心管，加入 20% SDS 及 10mg/mL 的 RNase A，於 56℃作用 4 小時，接著加入 25mg/mL 的 proteinase K，於 37℃作用 4 小時，加入 1/2 倍體積的 3M Sodium acetate (pH5.2)，以及 2.5 倍體積的 95%以上的酒精，放置於-20℃中 overnight 使 DNA 沉澱，沉澱完的產物以 12000 rpm 於 4℃下離心 15 分鐘，去除上清液後，將離心下來的 DNA 放置於室溫下使其自然風乾，加入 20 μL 二次水將其回溶，以 50V 的電壓以及 2%的 agarose 電泳膠片進行電泳分析，接著以 EtBr 染劑反應 10 分鐘之後，照相觀察結果。

19. 分析berberine對人類癌細胞在活體內(*in vivo*)轉移情形

免疫缺陷的小鼠(BALB/c *nu/nu* mice)接種人類癌細胞，將 $1x10^6$/100 μl PBS 洗淨的人類癌細胞，打入老鼠的尾靜脈，將接種癌瘤細胞的老鼠逢機分組，進一步注射或餵食不同濃度葉下珠，犧牲老鼠，取出肺臟以觀察其腫瘤數目及轉移至肺部的能力，並作組織免疫染色與 HE stain。

20. 分析berberine對人類癌細胞在活體內(*in vivo*)生長情形

免疫缺陷的小鼠(BALB/c *nu/nu* mice)接種人類癌細胞，將 1x10^7/100 μl PBS 洗淨的人類癌細胞，打入老鼠的右後腿動脈肱附近皮下(right hind limb)，7 天後將接種癌瘤細胞的老鼠逢機分組，進一步注射或餵食不同濃度補骨脂，每天以游標閥尺測量腫瘤長寬，以 1/2x長x寬x寬 計算腫瘤體積，5 週後犧牲老鼠，取出腫瘤及肺臟以觀察其腫瘤大小、重量，以評估應用的可行性，並作組織免疫染色與 HE stain。

21. 統計分析

所有數據以電腦統計軟體SigmaStat (Jandel Scientific Software, USA)進行one-way analysis of variance(one-way ANOVA)分析。

三、結果與討論

第一部分 探討 berberine 抑制癌細胞轉移之能力

癌瘤細胞的侵入(invasion)及轉移(metastasis)亦是癌症導致死亡以及治療複雜度提昇的主要原因。因此，我們進行了 berberine (化學結構如 **Fig. 1** 所示) 抑制癌瘤細胞侵襲轉移的相關研究。以不同濃度(0, 5, 10, 15, 20 μM)的 berberine 處理子宮頸癌及乳癌細胞，培養 24 小時後，進行細胞侵襲(invasion)、移動(migration)、細胞存活(cell viability)以及 MMP-2, MMP-9, u-PA 的活性分析。結果顯示，在子宮頸癌細胞(SiHa, CaSki)中，berberine 在不影響細胞存活的濃度下(0, 5, 10, 15, 20 μM)，對於 cell invasion/migration 有顯著的抑制效果，且對於 MMP-2, MMP-9 以及 u-PA 的活性也有顯著抑制效果 **(Fig. 2, Fig. 3)**。然而，在乳癌細胞(MDA-MB231)中，berberine 同樣對於 cell invasion/migration 有顯著的抑制效果，但對於 MMP-2, MMP-9 以及 u-PA 的活性並無抑制效果 **(Fig. 4)**。berberine 對 CaSki 細胞中 PAI-1 與 TIMP-2 卻是沒有影響**(Fig. 5)**。但是 berberine 卻會增加 SiHa 細胞中 PAI-1 與 TIMP-2 的蛋白表達量**(Fig. 6)**。為了要探討 berberine 抑制 SiHa 細胞 MMPs 與 u-PA 表現、細胞移動及侵入能力與訊息蛋白表現之間的相關性，利用西方墨點法，以 0, 5, 10, 15, 20 μM 的 berberine 處理細胞 24 小時，收集 cell lysates，並且利用 anti-Pi-ERK1/2 與 anti-ERK1/2、anti-Pi-P38 與 anti-P38、anti-Pi-JNK 與 anti-JNK、和 anti-Pi-Akt 與 anti-Akt、PI3K 抗體進行 western blot。實驗結果發現，ERK1/2、P38、JNK、Akt 與 PI3K 的蛋白表現，並不會受 berberine 的影響**(Fig. 7 & Fig. 8)**；但 P38 與 JNK1/2 的 phosphorylation，卻會隨著 berberine 濃度增加而逐漸遞減**(Fig. 7)**。利用 EMSA 實驗方法，觀察 berberine 對 SiHa 細胞核內轉錄因子 AP-1 與 NF-κB 與 DNA 結合能力，發現當細胞處理 berberine 之後，NF-κB 和 AP-1 與 DNA 結合能力有顯著的抑制效果(Fig. 9B & Fig. 9C)。利用西方墨點法，將 SiHa 細胞處理 berberine，萃取核蛋白，進行 Western blot，結果發現，當處理 berberine，NF-κB 的蛋白表現量會隨著 berberine 濃度增加而顯著的下降(Fig. 9A)。利用專一性的磷酸化抑制劑：SB203580 與 SP600125 証實 berberine 的確是透過抑制 p38 與 p-JNK1/2 來抑制細胞的侵襲(Fig. 10)、轉移(Fig. 11)與蛋白(Fig. 12& Fig. 13)的分泌。將 5-6 週 BALB/c *nu/nu* mice 老鼠於皮下打入 1×10^7 個 SiHa 細胞，之後餵食 PBS 或 berberine (5 mg/kg/day 或 10 mg/kg/day)，於期間每天測量腫瘤的大小(Fig. 14A)與老鼠的體重(Fig. 14B)，並於 33 天的時候犧牲老鼠，並將腫瘤割下秤重，此外於老鼠體內癌細胞轉移實驗，將 5-6 週 BALB/c *nu/nu* mice 由尾靜脈打入 1×10^6 個 SiHa 細胞，之後餵食 PBS 或 berberine (20 mg/kg/day)，並於 21 天的時候犧牲老鼠，並將肺臟割下且將轉移至肺臟的 colony 計數拍照(Fig. 15)，並做組織切片做 H&E stain(Fig. 16A)與秤重(Fig. 16B)。目前發現 berberine 在子宮頸癌及乳癌細胞能十分有效地抑制癌細胞的侵

襲能力，berberine 抑制癌細胞的侵襲轉移也許是與抑制 MMP-2, MMP-9, u-PA 的活性有關，而相關的分子機制的探討正在進行中。其中，在 MDA-MB231 細胞中，berberine 並無抑制 MMP-2, MMP-9, u-PA 的活性，但仍十分有效地抑制癌細胞的侵襲能力，因此，認為 berberine 除了抑制 MMP-2, MMP-9, u-PA 的活性外，尚能透過其他機制進而抑制癌細胞的侵襲轉移。並再活體中也看到很好的抑制效果。

第二部分 探討 berberine 抑制血管新生之能力

血管新生(angiogenesis)作用對腫瘤而言，不僅是提供血液養分共癌細胞增生、腫瘤壯大，更是提供了癌細胞轉移的管道。因此，進一步探討 berberine 對血管新生的抑制效果。以不同濃度(0, 10, 20, 30, 40, 50 μM)的 berberine 處理人類臍帶靜脈血管內皮細胞(HUVECs)。結果發現，berberine 對內皮細胞的成管作用(tube formation)具有明顯抑制效果**(Fig. 17)**，且由活體 Matrigel blood plug assay 發現餵食 berberine 的組別,在活體中也可抑制血管的形成**(Fig. 18)** 。並且進一步分析發現，berberine 並不會影響內皮細胞的生長**(Fig. 19A)**，但可以抑制內皮細胞的移動能力**(Fig. 19B)**。証實 berberine 可以藉由抑制內皮細胞的移動及成管作用，進而抑制血管的新生作用。而更進一步的分子機制的探討也正在進行中。

第三部分 探討 berberine 抑制癌細胞增生並誘導癌細胞凋亡之能力

我們發現 berberine 除了抑制乳癌及子宮頸癌的侵襲轉移的效果之外，對於口腔癌細胞及肺炎細胞都具有相當不錯的抗增生及誘導凋亡的效果。其中在口腔癌細胞(SAS)的實驗，發現 berberine 在 0.5 μM 的濃度下即能有效降低細胞存活率**(Fig. 20)**。而在肺癌的實驗，發現 berberine 對於實驗中所選四株非小細胞肺癌細胞株(NSCLCs)：A549、H1299、H460、H1355，都具有降低細胞存活的效果**(Fig. 21)**。選用 A549 進行實驗，也發現 berberine 確實可以造成細胞 sub-G1 phase 的增加**(Fig. 22A)**以及 DNA 片段化的現象產生**(Fig. 22B)**。証實 berberine 在非小細胞肺癌誘導細胞凋亡的效果，其中相關機制尚待進一步分析。由於 berberine 在口腔癌細胞的效果顯著，甚至有效濃度僅需 0.5 μM，十分具有應用價值。因此，進一步的研究重點將著重在口腔癌的相關探討。

第四部分 探討 berberine 降低動脈硬化與保護血管內皮細胞之能力

氧化型低密度脂蛋白(oxLDL)在血管內的堆積會使得內皮細胞功能受損甚至死亡。我們取純化之LDL (100μg/ml)，加入10μM $CuSO_4$使其氧化，同時處理不同濃度(0, 25, 50, 75, 100 μM)的berberine或維他命E類似物Trolox (10, 20, 50 μM)，在37℃下共同反應4小時(ApoB fragmentation assay)、16小時(LDL electrophoretic motility, TBARS assay)，以Trolox作為positive control，分析berberine保護LDL氧化的效果。結果顯示，25μM的berberine對於LDL泳動率 **(Fig. 23)**、ApoB斷裂化 **(Fig. 24)**及MDA的生成 **(Fig. 25A)**皆有部分的保護效果，而50μM的berberine其保護效果更是超過50%。証實berberine具有保護LDL不受氧化的效果。而一般認為天然物抗氧化的效果與其化學結構上所具有的自由基清除能力有關。然而，在DPPH自由基清除試驗中發現，Trolox的確具相當不錯的清除效果，但berberine則並無明顯的清除效果 **(Fig. 25B)**。這樣的結果，說明berberine本身化學結構上並無自由基清除能力，但卻具有保護LDL不受氧化的效果。以不同濃度(0, 25, 50, 75, 100 μM)的berberine處理HUVEC細胞，同時再加入200 μg/ml的氧化型低密度脂蛋白(oxLDL)處理，共同培養24小時。利用MTT assay偵測細胞的存活率，發現berberine可以保護由oxLDL所造成的內皮細胞死亡**(Fig. 26A)**。再以Trypan blue exclusion assay證實berberine的確可以保護oxLDL處理下內皮細胞的存活細胞以及降低死亡細胞數 **(Fig. 26B)**。而內皮細胞的型態及其細胞的完整性會影

響內皮細胞控制分子通透的功能，且這個功能的缺失與動脈粥狀硬化的形成有關。因此，我們同時也以相位差顯微鏡觀察細胞型態，證實berberine不僅有效提高oxLDL處理下內皮細胞的存活，且存活細胞的細胞型態與control組相比並無明顯不同 **(Fig. 27)**。進一步以DAPI染色及Flow cytometry分析oxLDL的處理是否造成HUVEC細胞的細胞凋亡。證實oxLDL的處理會造成chromosome condensation，而berberine可以明顯降低這個現象 **(Fig. 28)**。也證實oxLDL的處理會造成subG1 phase (hypodiploid cell)的大量增加，而berberine可以有效的減少sub G1 phase，甚至完全回復 **(Fig. 29)**。針對oxLDL造成HUVEC細胞的細胞凋亡及berberine的保護效果，探討其中的分子機制。首先，以DCFH-DA染色再以Flow cytometry進行分析細胞內ROS的產生，發現oxLDL會造成HUVEC細胞內的ROS增加，而berberine則會抑制ROS的增加 **(Fig. 30)**。另外，以JC-1染色再以螢光顯微鏡觀察HUVEC細胞的粒線體膜電位，發現oxLDL會造成HUVEC細胞的粒線體膜電位降低，而berberine則會保護粒線體膜電位的降低 **(Fig. 31)**。進一步以Western Blot分析，証實oxLDL會造成粒線體內cytochrome C 釋放到細胞質中，進而促使caspase 3的活化以及PARP的切割，同時，也發現Bcl-2減少而BAX增加的現象，而berberine對於osLDL所造成的apopotosis相關蛋白的變化都有保護的效果 **(Fig. 32)**。綜合以上結果，berberine不僅可以有效保護LDL不受氧化，且對於oxLDL所造成的內皮細胞的apoptosis也有相當好的保護效果，而其中機制，可能是透過降低oxLDL所造成細胞內ROS的增加，進而保護粒線體不受傷害且防止細胞的apoptosis。

四、計畫成果及自評

關於探討 berberine 保護 LDL 的氧化及保護內皮細胞不受 oxLDL 傷害的效果及機制，已整理發表在 **J Agric Food Chem.**。而且，已證實 berberine 抑制子宮頸癌及乳癌的侵襲轉移的效果，且有效濃度低於 20μM，認為 berberine 除了抑制 MMP-2, MMP-9, u-PA 的活性外，尚能透過其他機制進而抑制癌細胞的侵襲轉移。並再活體中也看到很好的抑制效果；此外 berberine 除了降低口腔癌細胞及肺癌細胞的細胞存活有很好的效果，甚至在口腔癌細胞的有效濃度僅需 0.5 μM。因此，認為 berberine 在這些癌症應是具有應用的價值。其中作用機制的探討也已在進行中了。最後，也發現 berberine 對於血管新生作用無論是 *in vivo* 及 *in vitro* 也有抑制效果，這也大大增加 berberine 在抑制癌細胞侵襲轉移上的應用價值。

已發表論文:

Hsieh YS, Kuo WH, Lin TW, Chang HR, Lin TH, Chen PN, Chu SC. Protective effects of berberine against low-density lipoprotein (LDL) oxidation and oxidized LDL-induced cytotoxicity on endothelial cells. 2007 55(25):10437-45.

其它成果也在陸續整理準備投稿中

參考文獻

[1] 1. Alexandrakis, M., Letourneau, R., Kempuraj, D., Kandere-Grzybowska, K., Huang, M., Christodoulou, S., Boucher, W., Seretakis, D., and Theoharides, T. C. (2003). Flavones inhibit proliferation and increase mediator content in human leukemic mast cells (HMC-1). Eur J Haematol *71*, 448-454.

[2] Booth, N. L., Overk, C. R., Yao, P., Totura, S., Deng, Y., Hedayat, A. S., Bolton, J. L., Pauli, G. F., and Farnsworth, N. R. (2006). Seasonal variation of red clover (Trifolium pratense L., Fabaceae) isoflavones and estrogenic activity. J Agric Food Chem *54*, 1277-1282.

[3] Chakraborti, S., Mandal, M., Das, S., Mandal, A., and Chakraborti, T. (2003). Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem *253*, 269-285.

[4] Chen, Y. C., Shen, S. C., Chow, J. M., Ko, C. H., and Tseng, S. W. (2004). Flavone inhibition of tumor growth via apoptosis in vitro and in vivo. Int J Oncol *25*, 661-670.

[5] Dhanalakshmi, S., Agarwal, P., Glode, L. M., and Agarwal, R. (2003). Silibinin sensitizes human prostate carcinoma DU145 cells to cisplatin- and carboplatin-induced growth inhibition and apoptotic death. Int J Cancer *106*, 699-705.

[6] Jeanes A, Gottardi CJ, Yap AS. (2008) Cadherins and cancer: how does cadherin dysfunction promote tumor progression? Oncogene;27:6920-9

[7] Yanamoto S, Kawasaki G, Yamada SI, Yoshitomi I, Yoshida H, Mizuno A (2008). Ribonucleotide reductase small subunit p53R2 promotes oral cancer invasion via the E-cadherin/beta-catenin pathway. Oral Oncol

[8] Nahari D, Satchi-Fainaro R, Chen M, Mitchell I, Task LB, Liu Z, et al. (2007) Tumor cytotoxicity and endothelial Rac inhibition induced by TNP-470 in anaplastic thyroid cancer. Mol Cancer Ther;6:1329-37

[9] Song J, Bai J, Yang W, Gabrielson EW, Chan DW, Zhang Z. (2007) Expression and clinicopathological significance of oestrogen-responsive ezrin-radixin-moesin-binding phosphoprotein 50 in breast cancer. Histopathology;51:40-53

[10] Sossey-Alaoui K, Safina A, Li X, Vaughan MM, Hicks DG, Bakin AV, et al. (2007) Down-regulation of WAVE3, a metastasis promoter gene, inhibits invasion and metastasis of breast cancer cells. Am J Pathol;170:2112-21

[11] Yamaguchi H, Condeelis J. (2007) Regulation of the actin cytoskeleton in cancer cell migration and invasion. Biochim Biophys Acta;1773:642-52

[12] Borstnar S, Vrhovec I, Svetic B, Cufer T. (2002) Prognostic value of the urokinase-type plasminogen activator, and its inhibitors and receptor in breast cancer patients. Clin Breast Cancer;3:138-46

[13] Duffy MJ. (2002) Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level 1 evidence studies. Clin Chem;48:1194-7

[14] Evans DM, Sloan-Stakleff KD. (1998) Maximum effect of urokinase plasminogen activator inhibitors in the control of invasion and metastasis of rat mammary cancer. Invasion Metastasis;18:252-60

[15] Foekens JA, Ries C, Look MP, Gippner-Steppert C, Klijn JG, Jochum M. (2003) The prognostic value of polymorphonuclear leukocyte elastase in patients with primary breast cancer. Cancer Res;63:337-41

[16] Hildenbrand R, Arens N. (2004) Protein and mRNA expression of uPAR and PAI-1 in myoepithelial cells of early breast cancer lesions and normal breast tissue. Br J Cancer;91:564-71

[17] Kobayashi H, Suzuki M, Tanaka Y, Kanayama N, Terao T. (2003) A Kunitz-type protease inhibitor, bikunin, inhibits ovarian cancer cell invasion by blocking the calcium-dependent transforming growth factor-beta 1 signaling cascade. J Biol Chem;278:7790-9

[18] Robert C, Bolon I, Gazzeri S, Veyrenc S, Brambilla C, Brambilla E. (1999) Expression of plasminogen activator inhibitors 1 and 2 in lung cancer and their role in tumor progression. Clin Cancer Res;5:2094-102

[19] Suzuki M, Kobayashi H, Tanaka Y, Hirashima Y, Kanayama N, Takei Y, et al. (2003) Suppression of invasion and peritoneal carcinomatosis of ovarian cancer cell line by overexpression of bikunin. Int J Cancer;104:289-302

[20] Navab, M., Berliner, J.A., Watson, A.D., Hama, S.Y., Territo, M.C., Lusis, A.J., Shih, D.M., Van Lenten, B.J., Frank, J.S., Demer, L.L., Edwards, P.A., Fogelman, A.M., (1996) The Yin and Yang of oxidation in the development of the fatty streak. A review based on the 1994 George Lyman Duff Memorial Lecture. Arterioscler Thromb Vasc Biol, 16; 831-842.

[21] Ross, R., (1999) Atherosclerosis--an inflammatory disease. N Engl J Med, 340; 115-126.

[22] Wang,D.L., Wung,B.S., Shyy,Y.J., Lin,C.F., Chao,Y.J., Usami,S., and Chien,S. (1995). Mechanical strain induces monocyte chemotactic protein-1 gene expression in endothelial cells. Effects of mechanical strain on monocyte adhesion to endothelial cells. Circulation Research 77, 294-302.

[23] Ross,R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362, 801-809.

[24] Ross,R. (1986). The pathogenesis of atherosclerosis--an update. New England Journal of Medicine 314, 488-500.

[25] Libby,P., Warner,S.J., Salomon,R.N., and Birinyi,L.K. (1988). Production of platelet-derived growth factor-like mitogen by smooth-muscle cells from human atheroma. New England Journal of Medicine 318, 1493-1498.

[26] Molero, M. L., and Stockert, J. C. (1981). Fluorescence reaction of chromatin and basophilic cytoplasm by berberine sulfate. Cell Mol Biol Incl Cyto Enzymol 27, 523-525.

[27] Munshi, H. G., and Stack, M. S. (2006). Reciprocal interactions between adhesion receptor signaling and MMP regulation. Cancer Metastasis Rev 25, 45-56.

[28] Nachtigal, P., Semecky, V., Kopecky, M., Gojova, A., Solichova, D., Zdansky, P., and Zadak, Z. (2004). Application of stereological methods for the quantification of VCAM-1 and ICAM-1 expression in early stages of rabbit atherogenesis. Pathol Res Pract 200, 219-229.

[29] Okegawa, T., Pong, R. C., Li, Y., and Hsieh, J. T. (2004). The role of cell adhesion molecule in cancer progression and its application in cancer therapy. Acta Biochim Pol 51, 445-457.

[30] Palinski, W., and Napoli, C. (2002). The fetal origins of atherosclerosis: maternal hypercholesterolemia, and cholesterol-lowering or antioxidant treatment during pregnancy influence in utero programming and postnatal susceptibility to atherogenesis. Faseb J 16, 1348-1360.

[31] Park, C. H., Hahm, E. R., Lee, J. H., Jung, K. C., and Yang, C. H. (2005). Inhibition of

beta-catenin-mediated transactivation by flavanone in AGS gastric cancer cells. Biochem Biophys Res Commun 331, 1222-1228.

[32] Genis, L., Galvez, B. G., Gonzalo, P., and Arroyo, A. G. (2006). MT1-MMP: universal or particular player in angiogenesis? Cancer Metastasis Rev 25, 77-86.

[33] Giatromanolaki, A., Sivridis, E., and Koukourakis, M. I. (2006). Angiogenesis in colorectal cancer: prognostic and therapeutic implications. Am J Clin Oncol 29, 408-417.

[34] Glazunov, I. S. (1987). [Practical implications of the results of recent Soviet research concerning the prevention of cardiovascular diseases]. Kardiologiia 27, 5-8.

[35] Fingleton, B. (2006). Matrix metalloproteinases: roles in cancer and metastasis. Front Biosci 11, 479-491.

[36] Chen, P. N., Kuo, W. H., Chiang, C. L., Chiou, H. L., Hsieh, Y. S., and Chu, S. C. (2006). Black rice anthocyanins inhibit cancer cells invasion via repressions of MMPs and u-PA expression. Chem Biol Interact *163*, 218-229.

[37] Hsieh YS, Kuo WH, Lin TW, Chang HR, Lin TH, Chen PN, Chu SC (2007) Protective effects of berberine against low-density lipoprotein (ldl) oxidation and oxidized ldl-induced cytotoxicity on endothelial cells. J Agric Food Chem;55:10437-10445.

附表及附圖

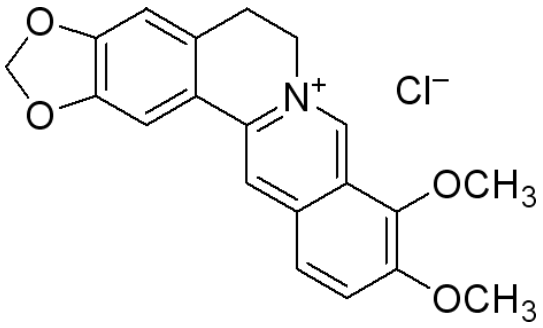


Fig. 1. The chemical structure of berberine.

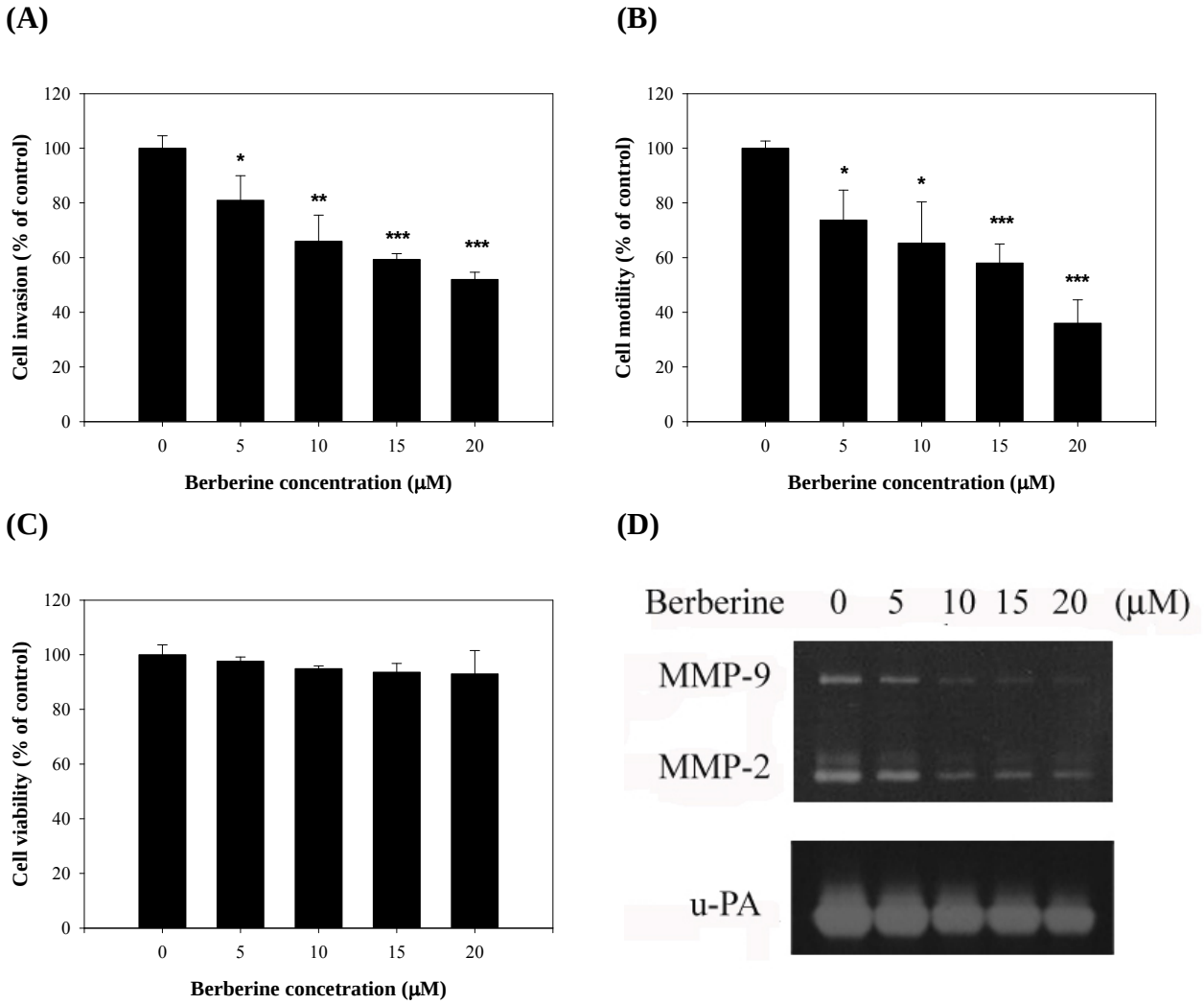


Fig. 2. Effects of berberine on cell invasion/migration, viability and matrix degrading enzymes activities in cervical cancer SiHa cells. SiHa cells were treated with berberine (0, 5, 10, 15, 20 μM) for 24 h, and capacities of cell invasion (A) and migration (B) were detected by Boyden chamber chemotaxis assay. Meanwhile, the cell viability was analyzed by MTT assay (C), and the activities of MMP-2, MMP-9, and u-PA were detected by gelatin and casein zymography (D). (*, P<0.05; **, P<0.01; ***, P<0.001)

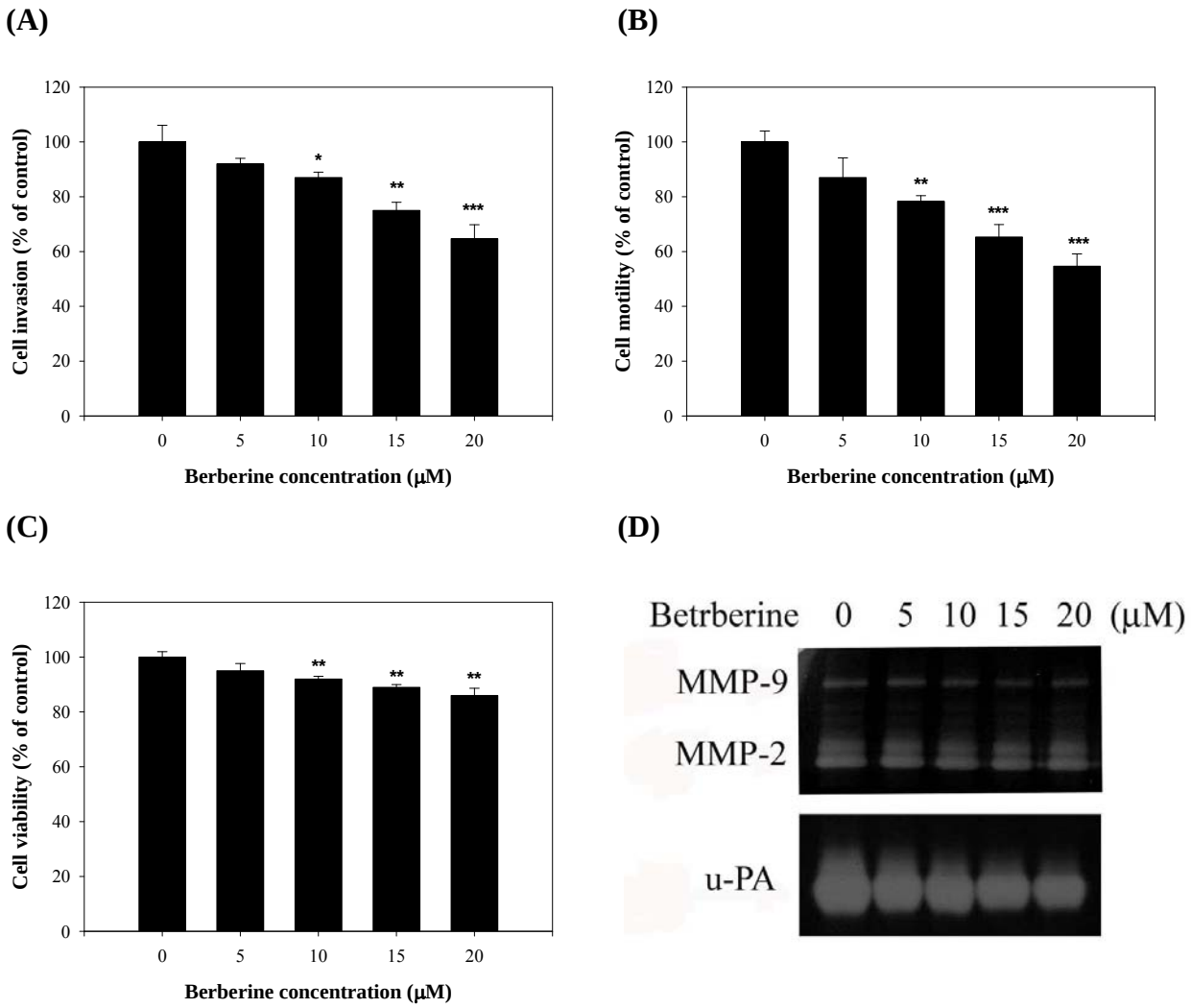


Fig. 3. Effects of berberine on cell invasion/migration, viability and matrix degrading enzymes activities in cervical cancer CaSki cells. CaSki cells were treated with berberine (0, 5, 10, 15, 20 μM) for 24 h, and capacities of cell invasion (A) and migration (B) were detected by Boyden chamber chemotaxis assay. Meanwhile, the cell viability was analyzed by MTT assay (C), and the activities of MMP-2, MMP-9, and u-PA were detected by gelatin and casein zymography (D). (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$)

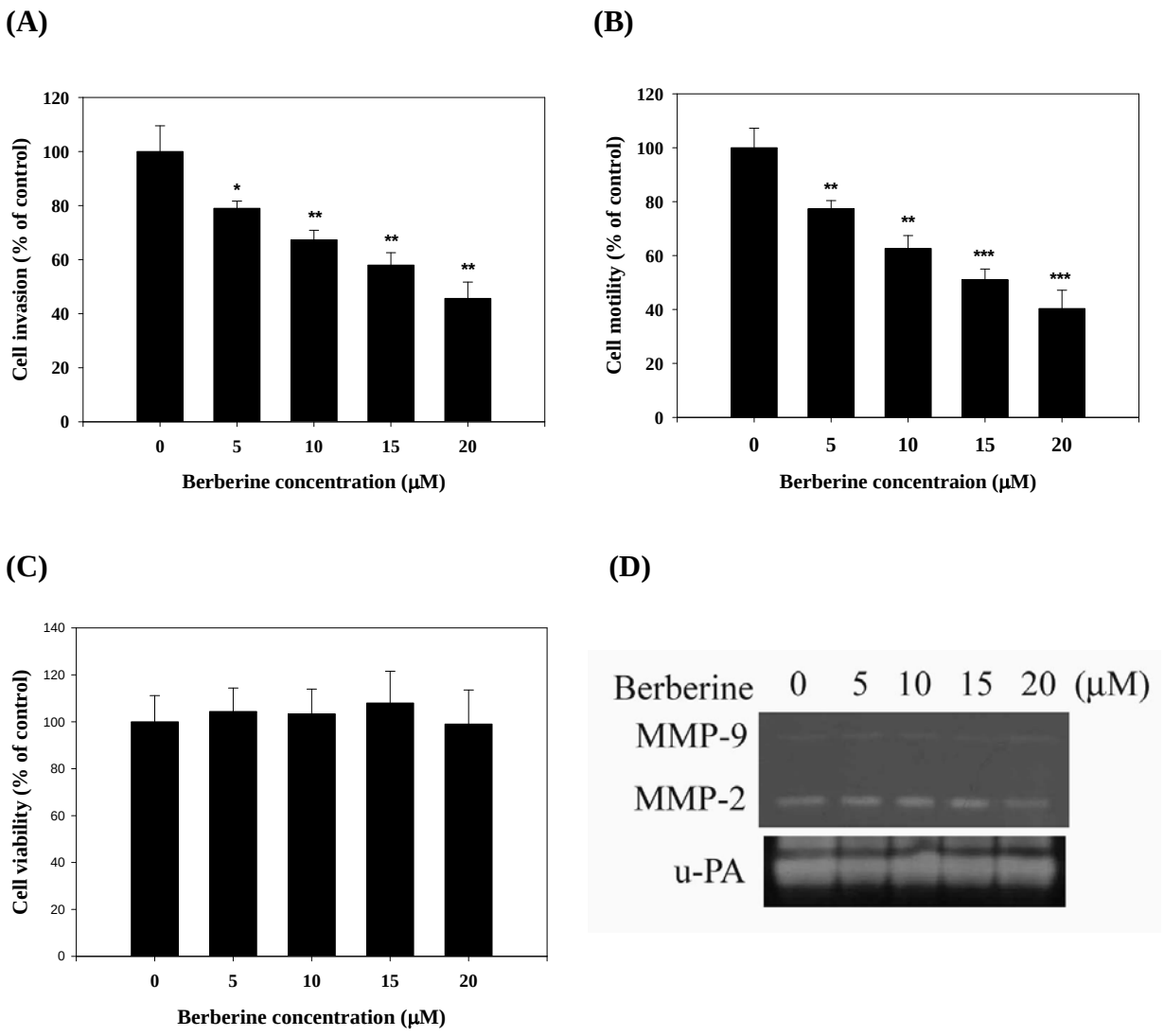


Fig. 4. Effects of berberine on cell invasion/migration, viability and matrix degrading enzymes activities in breast cancer MDA-MB231 cells. MDA-MB231 cells were treated with berberine (0, 5, 10, 15, 20 μM) for 24 h, and capacities of cell invasion (A) and migration (B) were detected by Boyden chamber chemotaxis assay. Meanwhile, the cell viability was analyzed by MTT assay (C), and the activities of MMP-2, MMP-9, and u-PA were detected by gelatin and casein zymography (D). (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$)

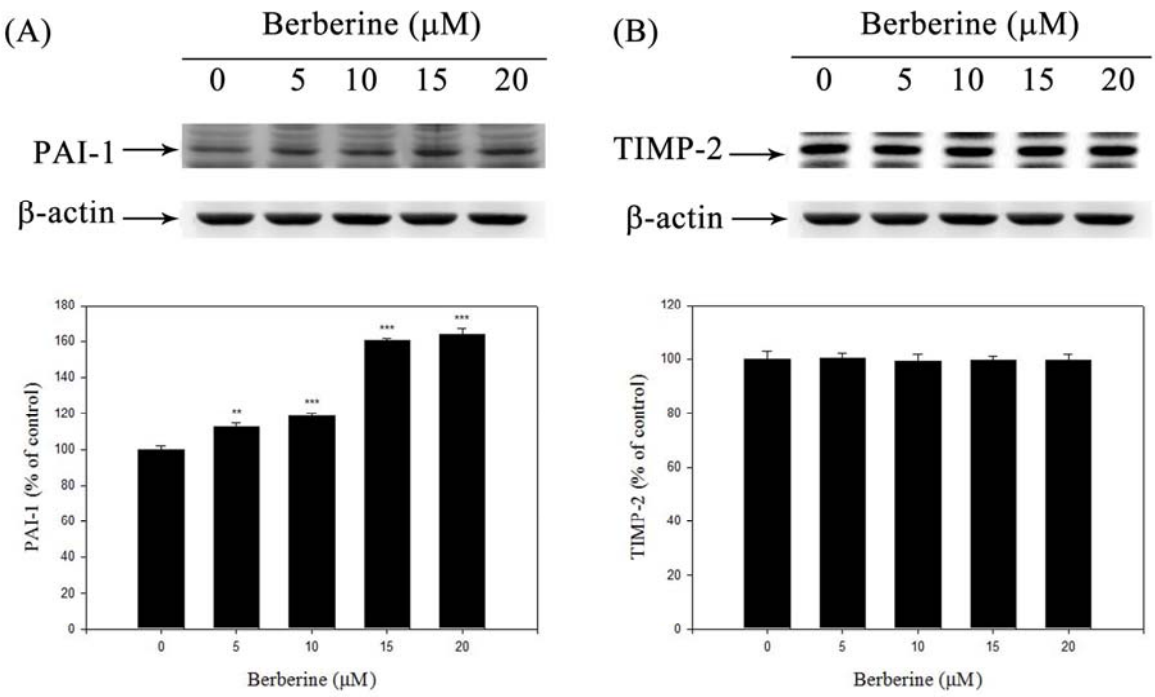


Fig. 5. Effects of berberine on proteases endogenous inhibitors. Ca Ski cells were treated with 0, 5, 10, or 15 μM of berberine for 24 h. Cells were subjected to western blotting to analyze the expression of (A) PAI-1 and (B) TIMP-2 as described in Materials and Methods. Determined activities of these proteins were subsequently quantified by densitometric analysis with that of control being 100% as shown just below the gel data. Data represented the mean ± SD of at least 3 independent experiments. Statistical significance was determined by Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

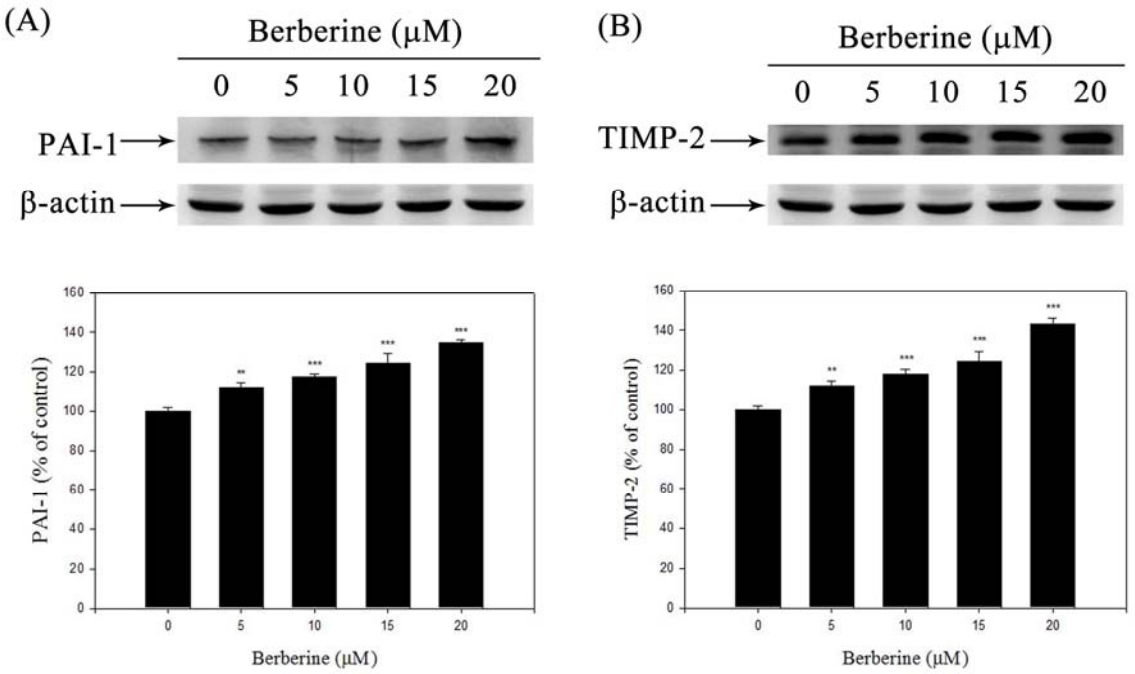


Fig. 6. Effects of berberine on proteases endogenous inhibitors. Si Ha cells were treated with 0, 5, 10, or 15 μM of berberine for 24 h. Cells were subjected to western blotting to analyze the expression of (A) PAI-1 and (B) TIMP-2 as described in Materials and Methods. Determined activities of these proteins were subsequently quantified by densitometric analysis with that of control being 100% as shown just below the gel data. Data represented the mean ± SD of at least 3 independent experiments. Statistical significance was determined by Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

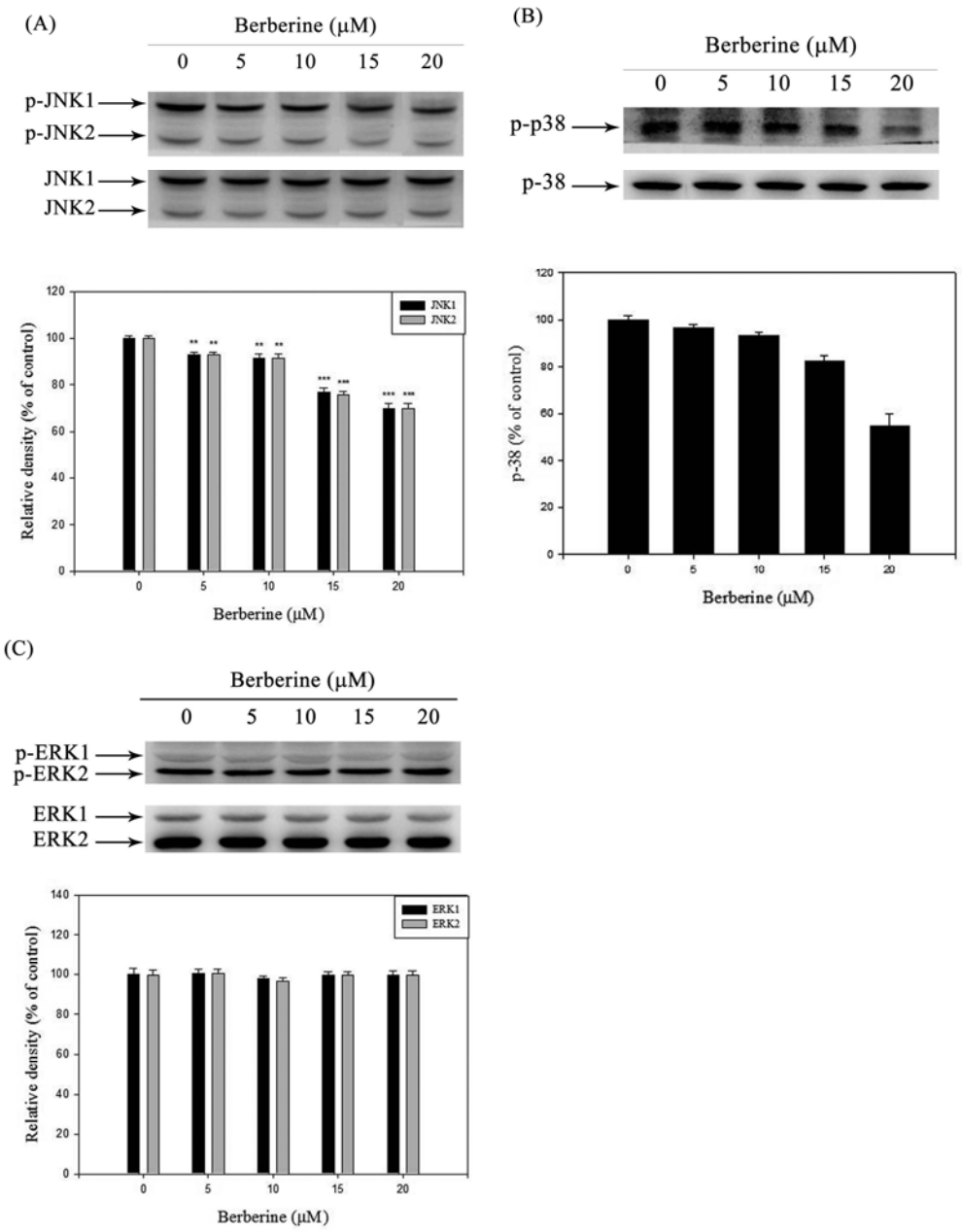


Fig. 7. Inhibitory effect of berberine on the phosphorylation of p38. Si Ha cells were cultured in various concentrations of berberine for 24 h, and then cell lysates were subjected to SDS-PAGE followed by Western blotting with anti-phospho-JNK1/2 (A), anti-phospho-p38 (B), or anti-phospho-ERK1/2 (C) antibodies. Signals of proteins were visualized with an ECL detection system. Determined activities of these proteins were subsequently quantified by densitometric analysis with that of control being 100% as shown just below the gel data. Results from 3 repeated and separated experiments were similar. Statistical significance was determined by Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

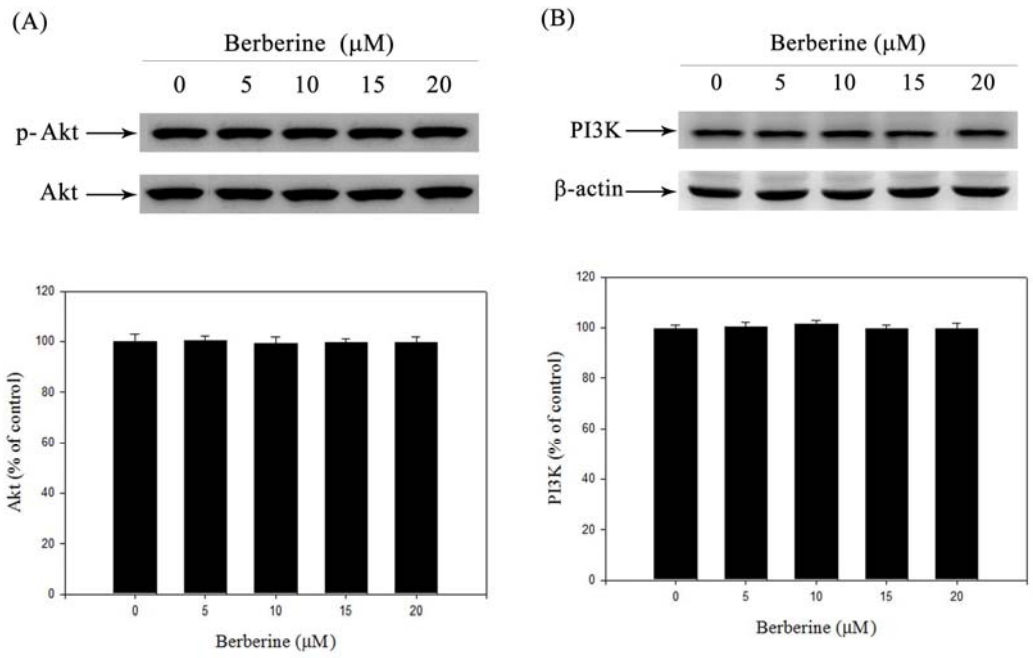


Fig. 8. Effects of berberine on the p-Akt and PI3K. Si Ha cells were cultured in various concentrations of berberine for 24 h, and then cell lysates were subjected to SDS-PAGE followed by Western blotting with anti-phospho-Akt (A) and PI3K antibodies (B). Signals of proteins were visualized with an ECL detection system. Determined activities of these proteins were subsequently quantified by densitometric analysis with that of control being 100% as shown just below the gel data. Results from 3 repeated and separated experiments were similar.

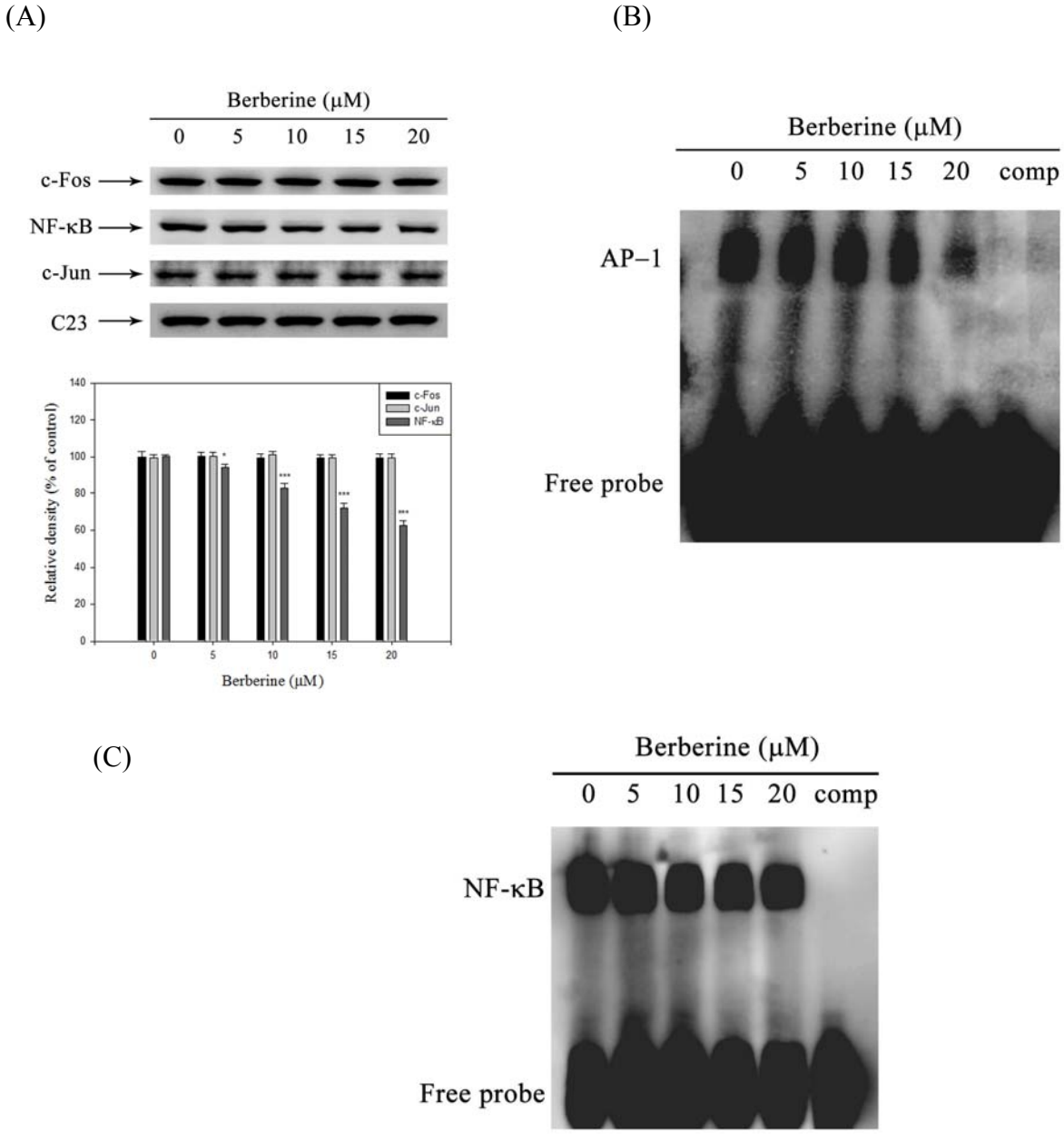


Fig. 9. Effects of berberine on the activation of NF-κB, c-Jun, and c-Fos. (A) Cells were treated with berberine and then nuclear extracts were subjected to SDS-PAGE followed by western blotting with anti-NF-κB, c-Fos, c-Jun, or C23 antibodies. Signals of proteins were visualized with an ECL detection system. Cells were treated with berberine and then nuclear extracts were analysed for DNA binding activity of AP-1 (B) and NF-κB (C) using biotin labeled NF-κB and AP-1 specific oligonucleotide in EMSA. The last lane represented nuclear extracts incubated with unlabeled oligonucleotide (Comp) to confirm the specificity of binding. Determined activities of these proteins were subsequently quantified by densitometric analysis with that of control being 100% as shown just below the gel data. The experiments were repeated three times with similar results. Statistical significance was determined by Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

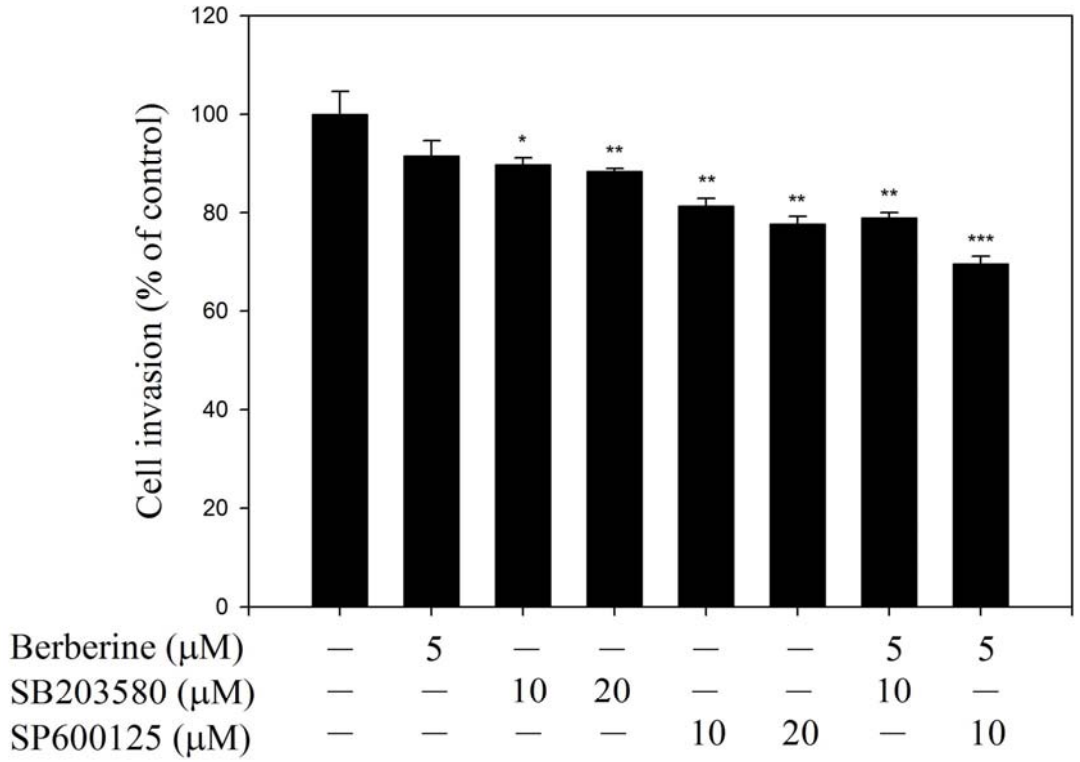


Fig. 10. Berberine-induced decreases in the invasiveness of Si Ha cells related to p38 and JNK pathways. Si Ha cells were seeded onto 6-well plates and pre-treated with SB203580 (10 or 20 μM) or SP600125 (10 or 20 μM) for 30 min and then incubated in the presence or absence of berberine (5 μM) for 24 h. Afterwards, cells were then subjected to analyses for invasion ability. Data represented the mean ± SD of at least 3 independent experiments. Statistical significance was determined by using Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

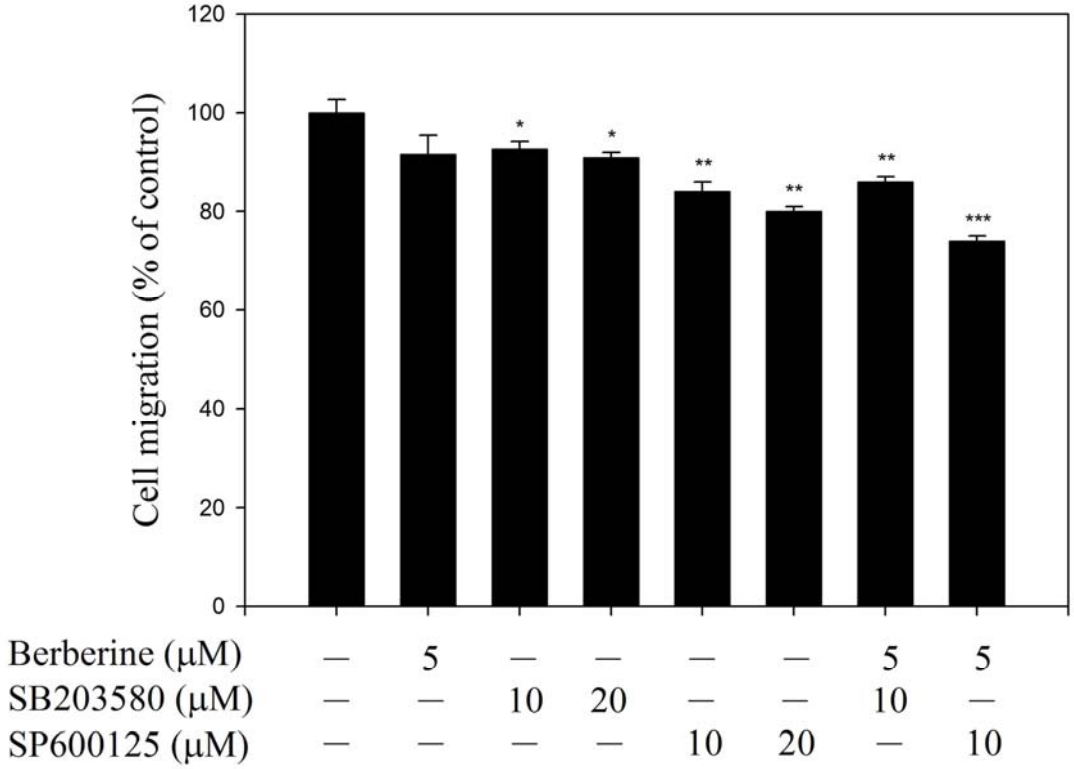


Fig. 11. Berberine-induced decreases in the migration of Si Ha cells related to p38 and JNK pathways. Si Ha cells were seeded onto 6-well plates and pre-treated with SB203580 (10 or 20 μM) or SP600125 (10 or 20 μM) for 30 min and then incubated in the presence or absence of berberine (5 μM) for 24 h. Afterwards, cells were then subjected to analyses for migration ability. Data represented the mean ± SD of at least 3 independent experiments. Statistical significance was determined by using Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

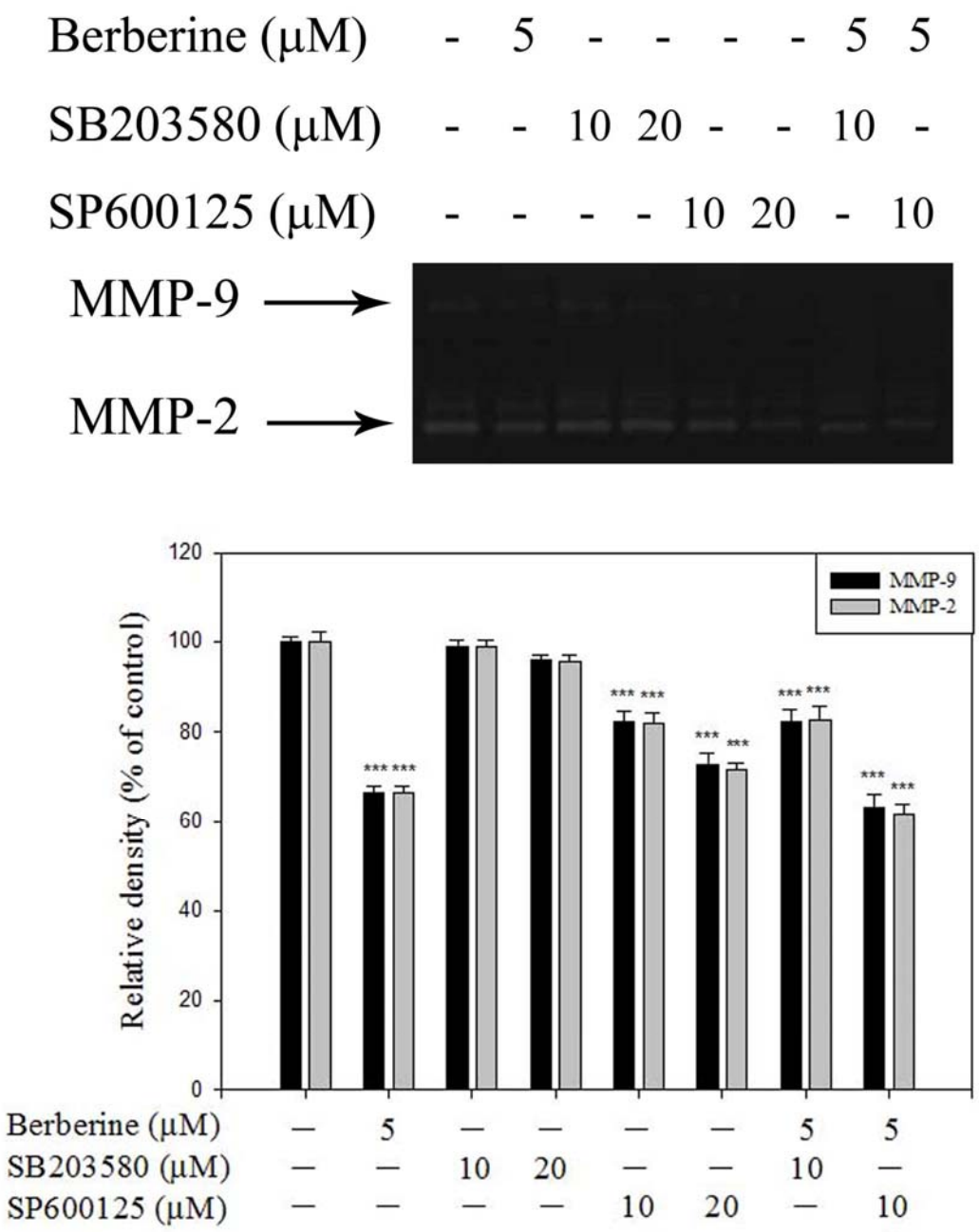


Fig. 12. Berberine-induced decreases in the migration of Si Ha cells related to p38 and JNK pathways. Si Ha cells were seeded onto 24-well plates and pre-treated with SB203580 (10 or 20 μM) or SP600125 (10 or 20 μM) for 30 min and then incubated in the presence or absence of berberine (5 μM) for 24 h. Afterwards, cells were then subjected to gelatin zymography to analyze the activities of MMPs. Data represented the mean ± SD of at least 3 independent experiments. Statistical significance was determined by using Student's *t* test (*, $P<0.05$; **, $P<0.01$;***,$P<0.001$).

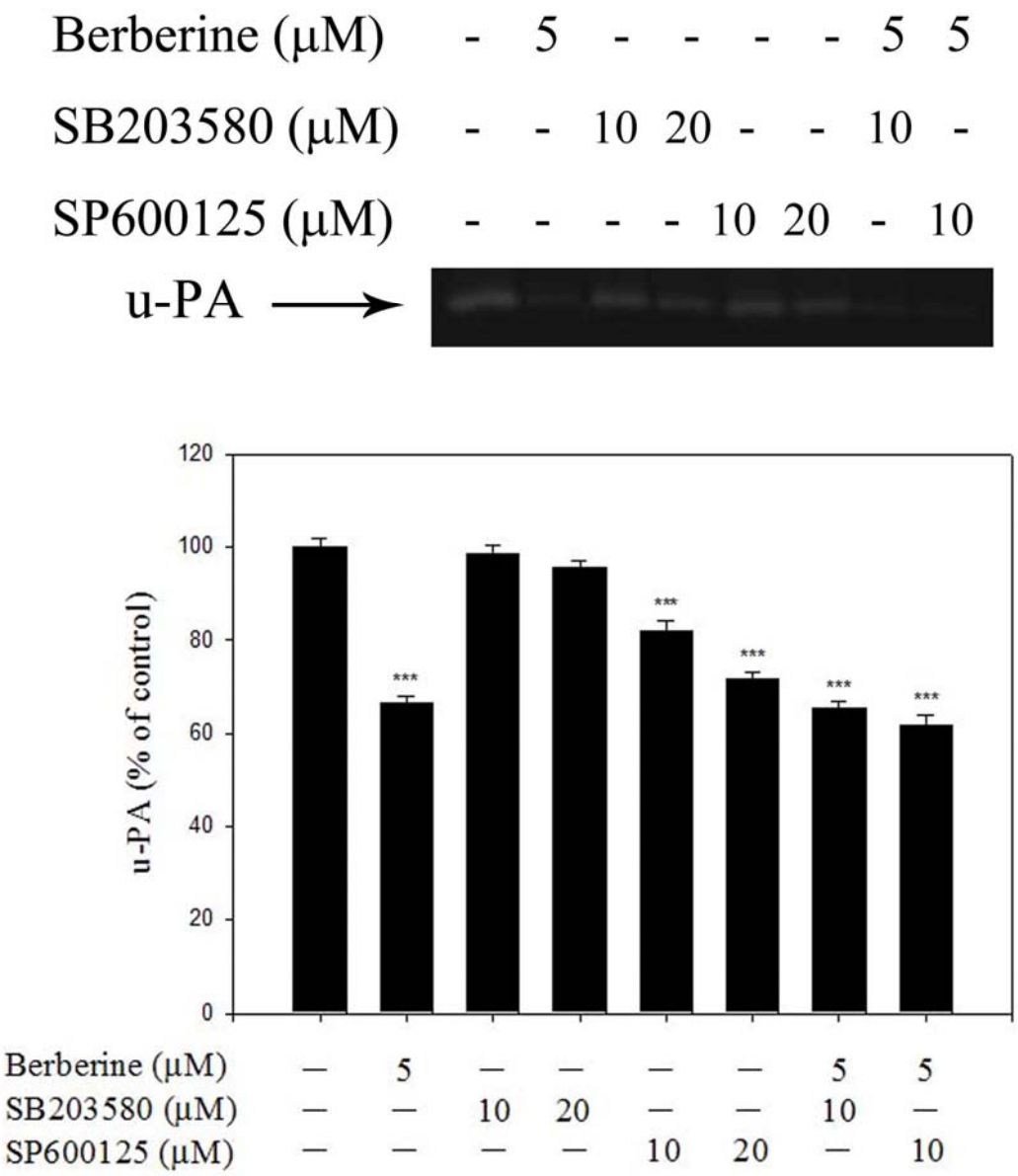
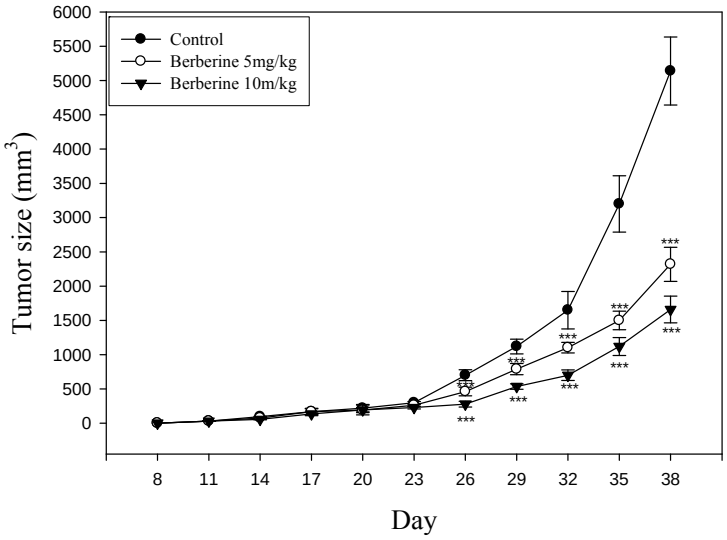


Fig. 13. Berberine-induced decreases in the migration of Si Ha cells related to p38 and JNK pathways. Si Ha cells were seeded onto 24-well plates and pre-treated with SB203580 (10 or 20 μM) or SP600125 (10 or 20 μM) for 30 min and then incubated in the presence or absence of berberine (5 μM) for 24 h. Afterwards, cells were then subjected to casein zymography to analyze the activities of u-PA. Data represented the mean ± SD of at least 3 independent experiments. Statistical significance was determined by using Student's *t* test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

(A)



(B)

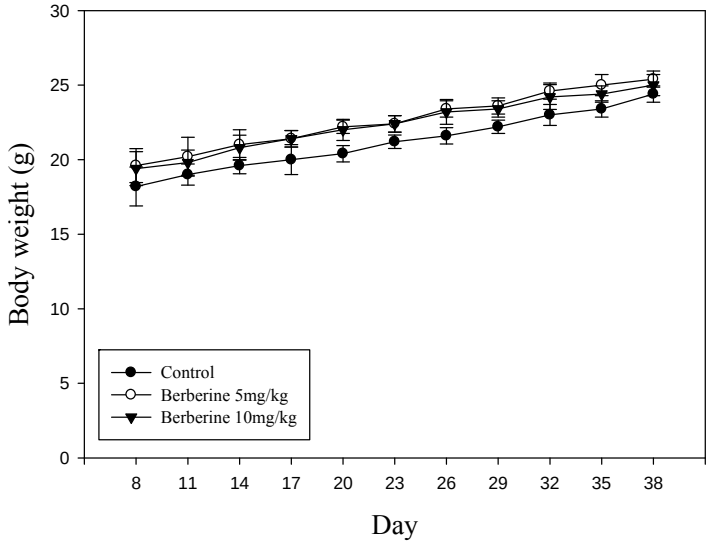


Fig. 14. The *in vivo* anticancer effects of berberine. After subcutaneous implantation of Si Ha cells, BALB/c *nu/nu* mice were treated with saline or berberine as described in Materials and Methods and then analyzed for the growth of tumor (A), and the weight of primary tumor (B). The values represented the means ± SD (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; OAs compared with saline).

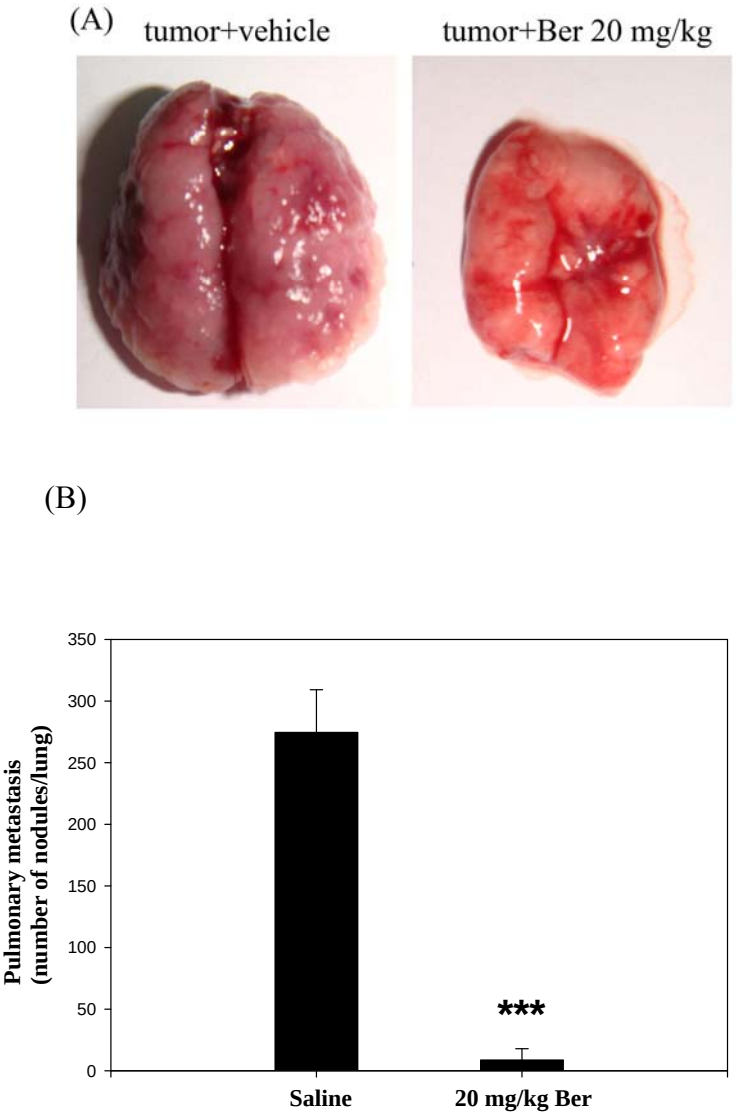
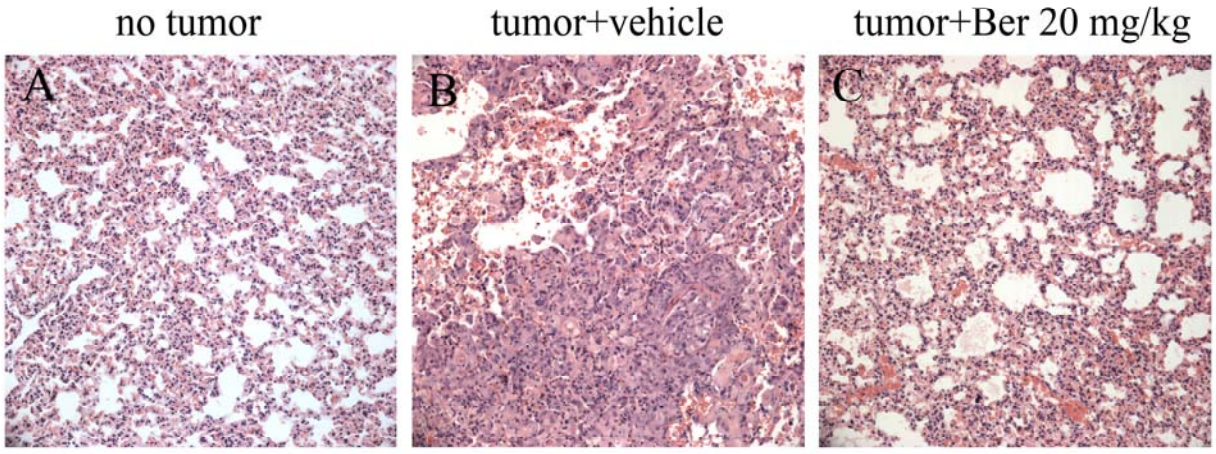


Fig. 15 Suppression of lung metastasis of Si Ha cells by berberine (Ber). (A) Photographs show lungs of Si Ha cell-bearing mice. (B) Si Ha cells were injected into the tail veins of 6-week-old female BALB/c nude mice. After injection of Si Ha cells, berberine (Ber, 20 mg/kg/day) and vehicle (saline) alone were administered oral gavage for 21 days to the berberine-treated groups and the control groups, respectively. Mice were sacrificed and the number of metastasis in the lung surface was counted on the 22 day after the cells were injected.

***, $p < 0.001$. Each value represents the mean ±SE.



D

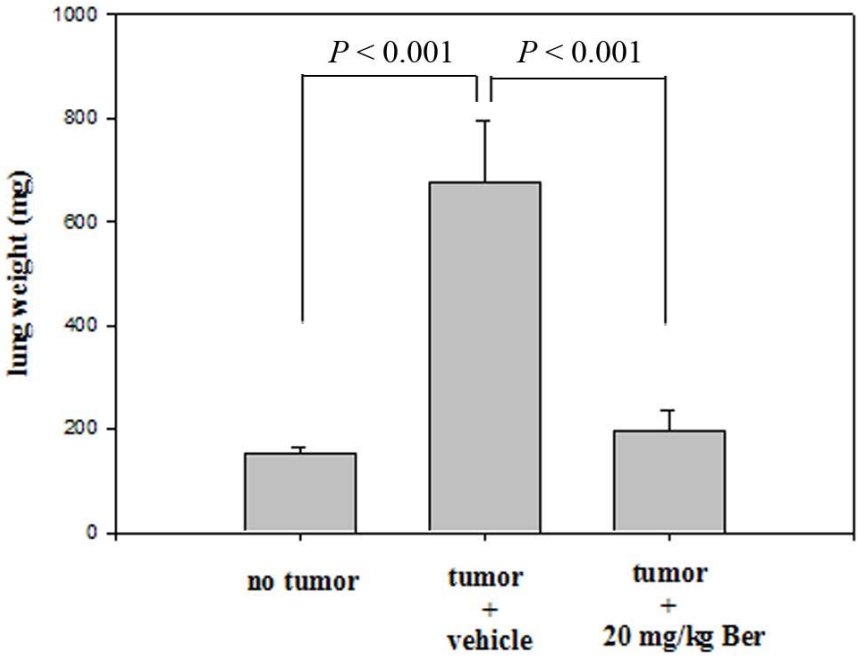


Fig. 16. Histopathology of lung of metastatic tumor bearing animals (×100). Lungs of the metastasis- induced animals were fixed in neutral buffered formalin, and stained with hematoxyline and eosine. (A) Normal lung, (B) Control (Si Ha + saline), (C) berberine (Ber, 20 mg/kg/day). (D) After injection of Si Ha cells, berberine (Ber, 20 mg/kg/day) and vehicle (saline) alone were administered oral gavage for 21 days. Mice were sacrificed and the weight of lung was measured on the 22 day after the cells were injected.

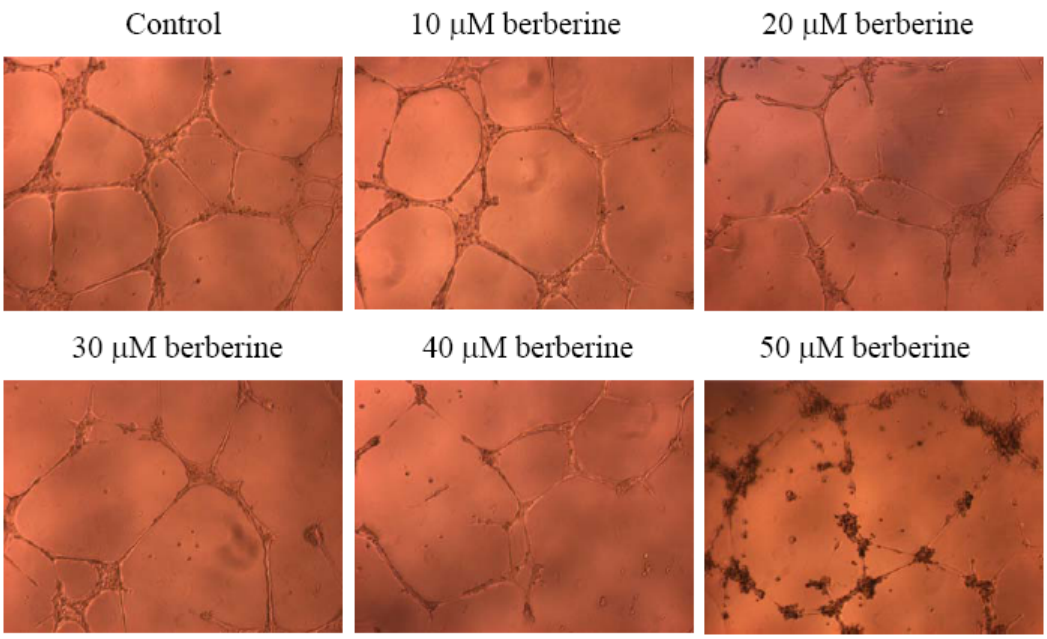


Fig. 17. Effects of berberine on tube formation capacity of HUVEC cells. Phase contrast micrographs illustrating the arrangement of HUVECs into a rich meshwork of capillary-like tubular structures when cultured on Matrigel for 18 h.

(A) (B)

Fig. 18. Berberine inhibits angiogenesis in vivo. Matrigel blood plug assay in C57BL/6 mice (five per group). (A) Berberine (Ber, 20 mg/kg/day) and (B) vehicle (saline) alone were administered oral gavage for 10 days. The mice were killed 10 days after implantation. Gross appearance of the plugs was photographed.

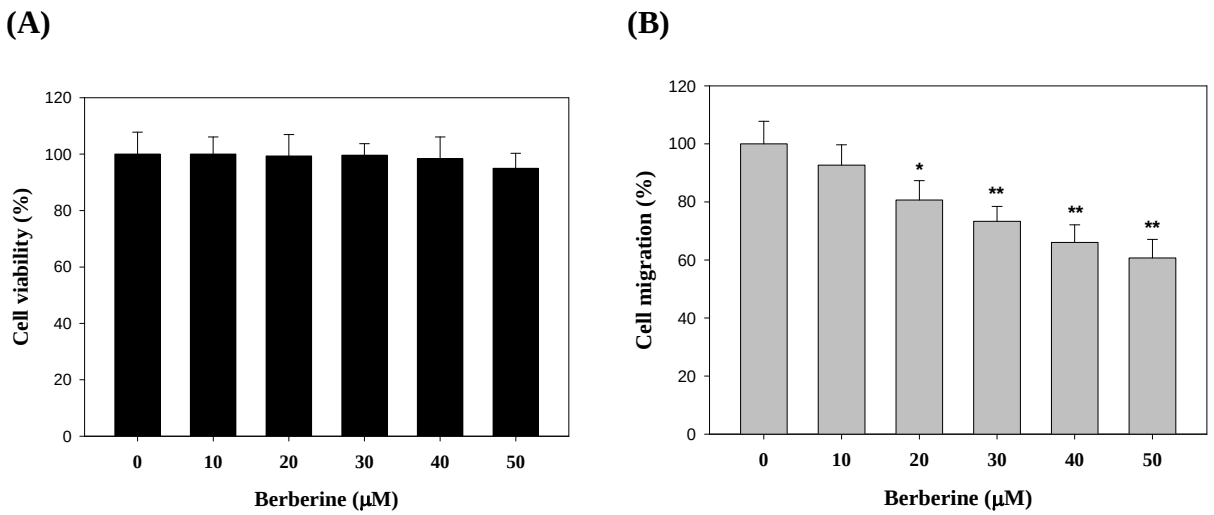


Fig. 19. Effects of berberine on cell viability and migration of HUVEC cells. HUVEC cells were treated with berberine (0, 10, 20, 30, 40, 50 μM) for 24 h, and cell viability (A) and migration (B) were detected by MTT and Boyden chamber chemotaxis assay. (*, P<0.05; **, P<0.01; ***, P<0.001)

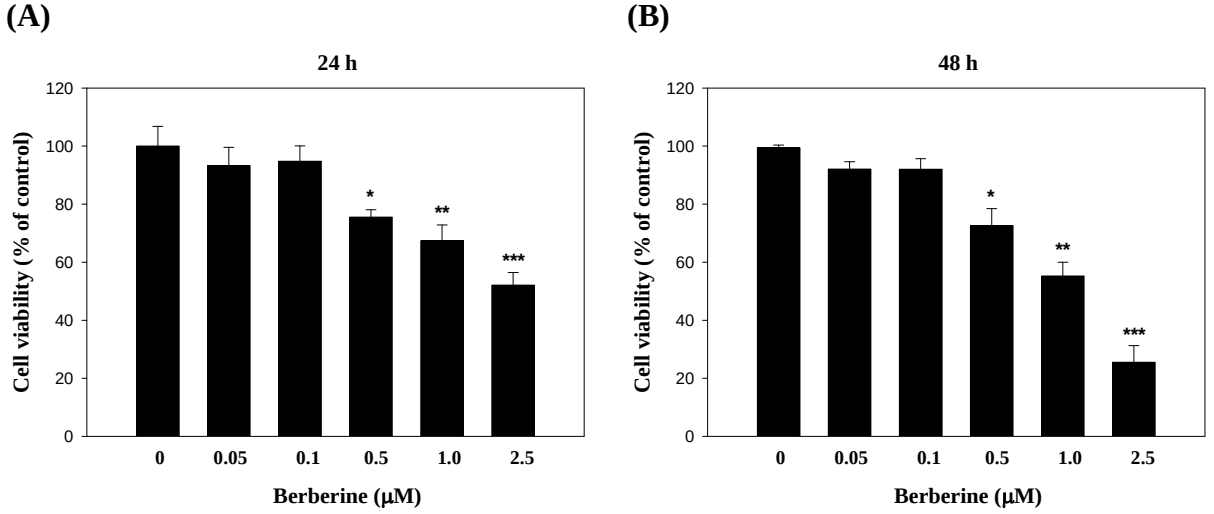


Fig. 20. Effects of berberine on cell viability of oral carcinoma cell. SAS cells were treated with berberine (0, 0.05, 0.1, 0.5, 1, 2.5 μM) for 24 h (A) and 48 h (B), and cell viability were detected by MTT. (*, P<0.05; **, P<0.01; ***, P<0.001)

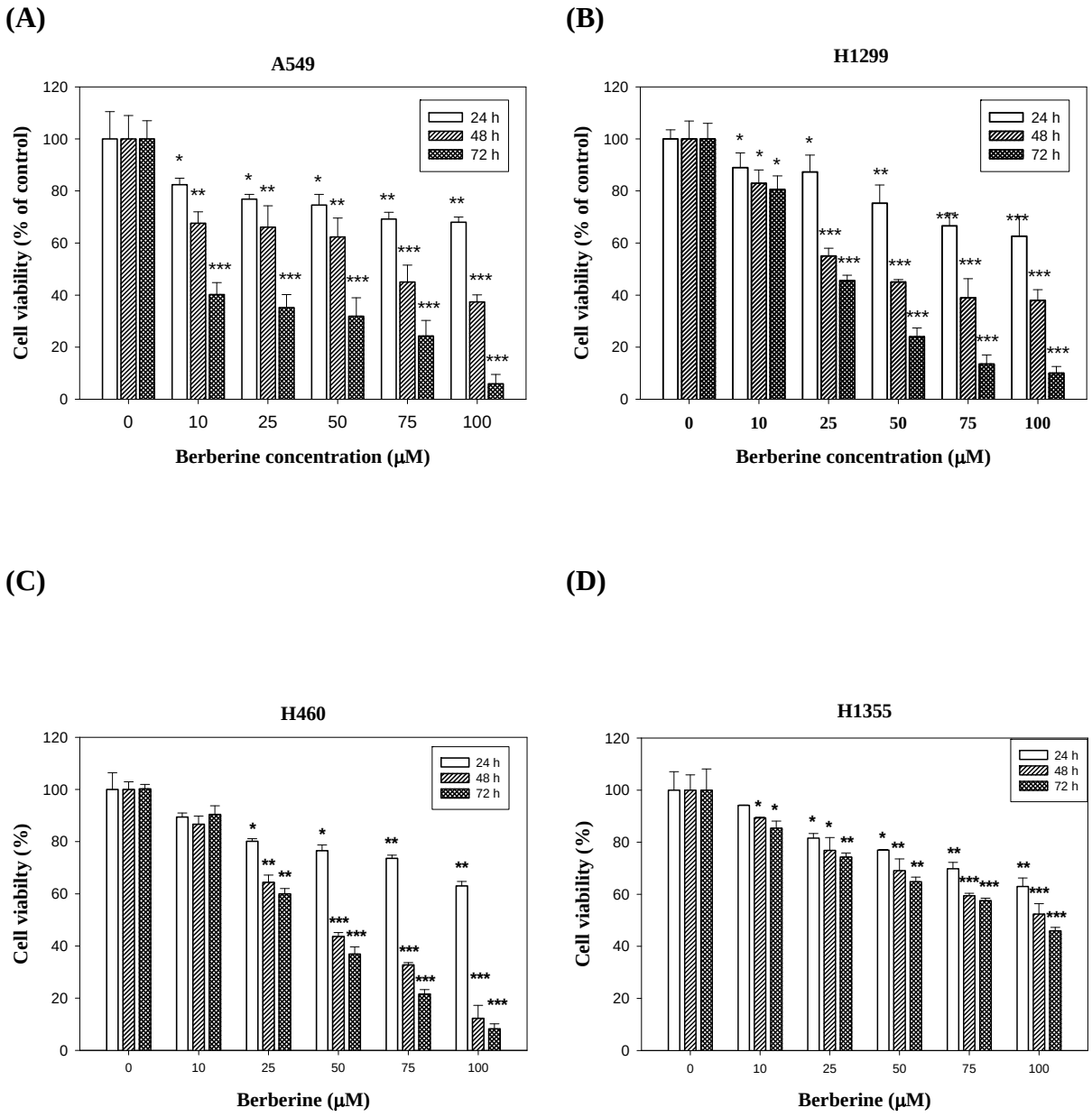


Fig. 21. Effects of berberine on cell viability of non-small cell lung cancer cells. (A) A549 cells, (B) H1299 cells, (C) H460 cells, and (D) H1355 cells were treated with berberine (0, 10, 25, 50, 75, 100 μM) for 24, 48, and 72 h, and cell viability were detected by MTT. (*, P<0.05; **, P<0.01; ***, P<0.001)

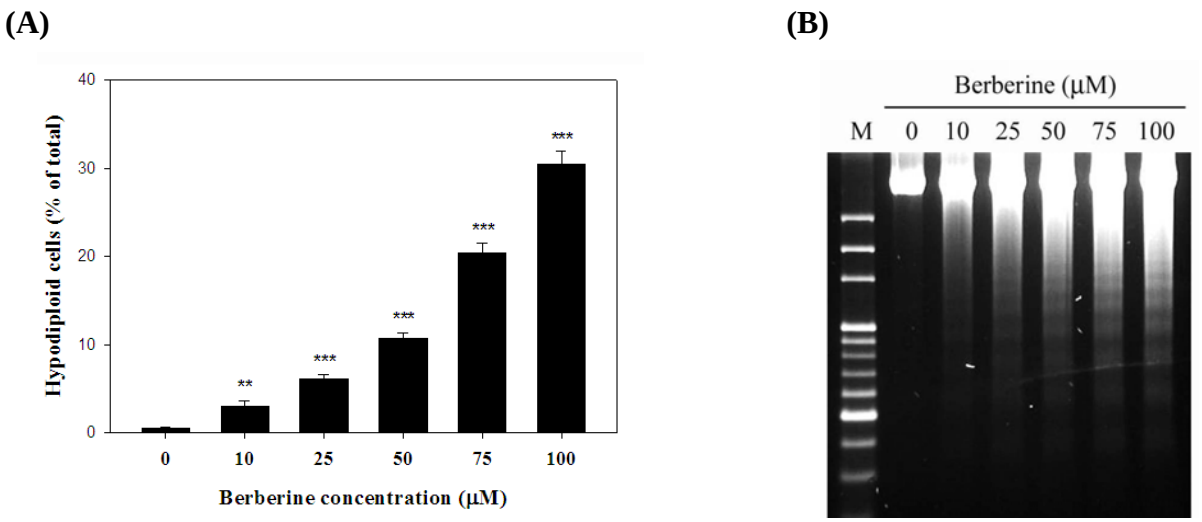


Fig. 22. Apoptotic effects of berberine on lung cancer A549 cells. A549 cells were treated with berberine (0, 10, 25, 50, 75, 100 μM) for 48 h, afterward, sub-G1 phase population was detected by flow cytometry analysis (A), and DNA integrity was analyzed by DNA electrophoresis (B). (*, P<0.05; **, P<0.01; ***, P<0.001)

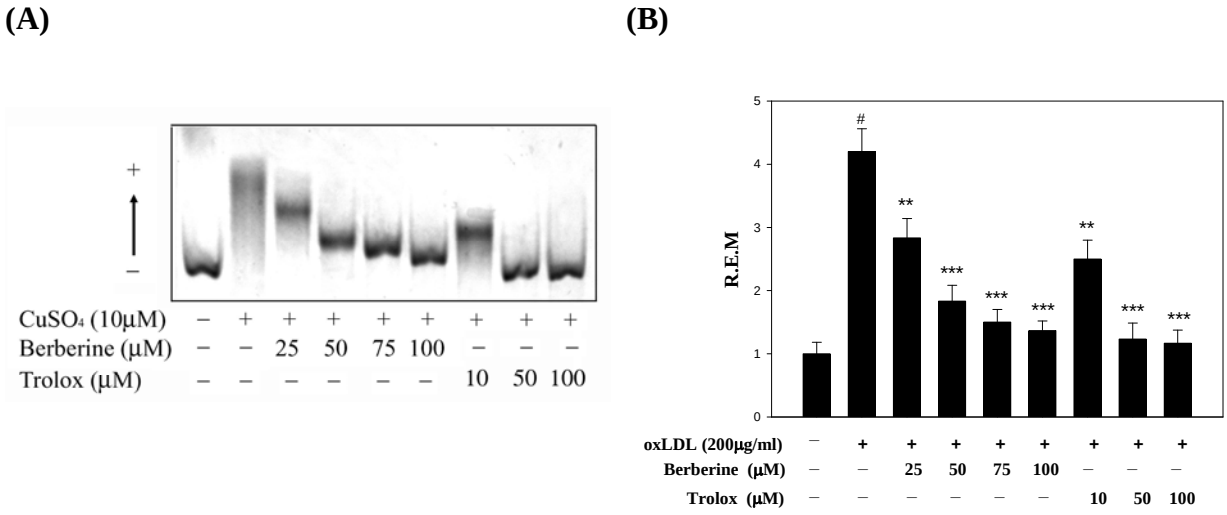


Fig. 23. Effect of berberine on Cu^{2+}-mediated shift of electrophoretic mobility in LDL. (A) LDL was incubated with 10 μM $CuSO_4$ for 16 h in the presence or absence of berberine or Trolox, as positive control, and applied to 0.6% agarose gels. (B) The results were quantified and expressed in the form of relative electrophoretic mobility (REM). (#, P<0.001 compared with control. *, P<0.05; **, P<0.01; ***, P<0.001 compared with oxLDL-treated group.)

(A) **(B)**

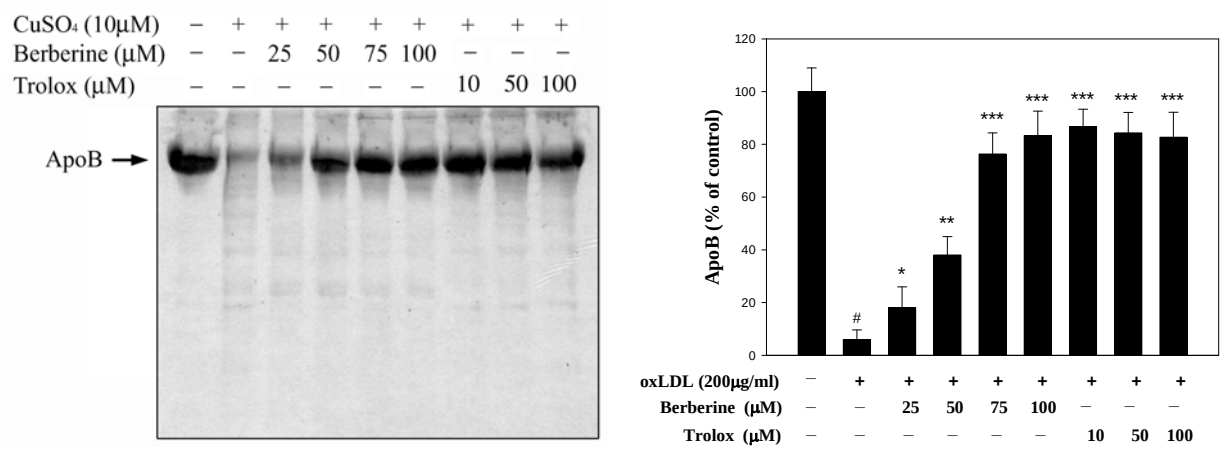


Fig. 24. Effect of berberine on Cu^{2+}-mediated Apo B fragmentation in LDL. (A) LDL (200 μg/ml) was incubated with 10 μM $CuSO_4$ in the absence or presence of berberine or Trolox for 4 h, and applied to 7.5% SDS-PAGE. (B) Quantification of ApoB fragmentation were presented as means ± SD of three independent experiments. ($^{\#}$, P<0.001 compared with control. *, P<0.05; **, P<0.01; ***, P<0.001 compared with oxLDL-treated group.)

(A) **(B)**

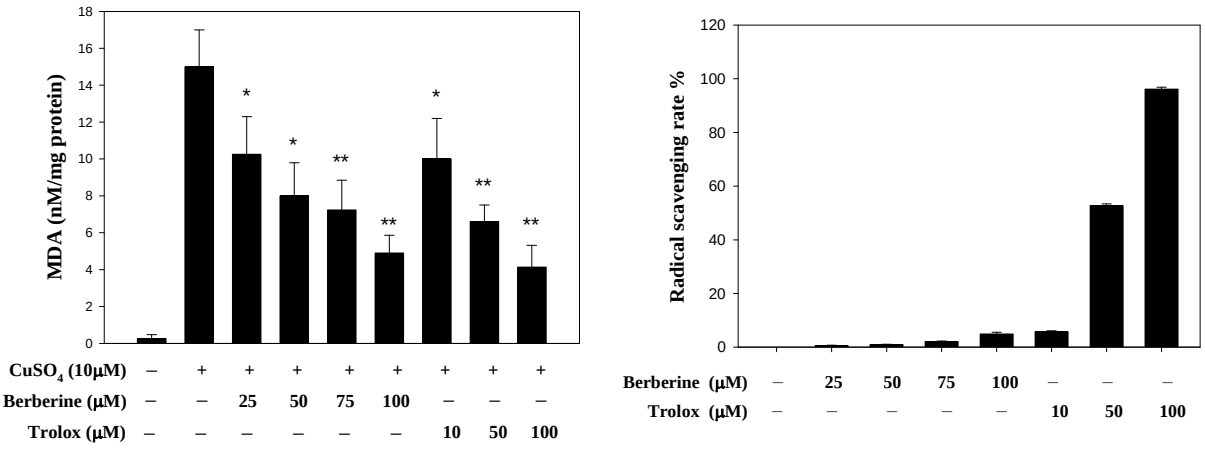


Fig. 25. Effect of berberine on Cu^{2+}-induced MDA formation of LDL and DPPH scavenging capability. (A) LDL (200 μg/ml) was incubated with 10 μM $CuSO_4$ for 16 h in the present or absence of berberine or Trolox and then measure MDA formation. (B) The radical-scavenging activity of berberine and Trolox was evaluated by using the DPPH radical scavenging assay. (*, P<0.05; **, P<0.01; ***, P<0.001 compared with oxidative LDL-treated group.)

(A) **(B)**

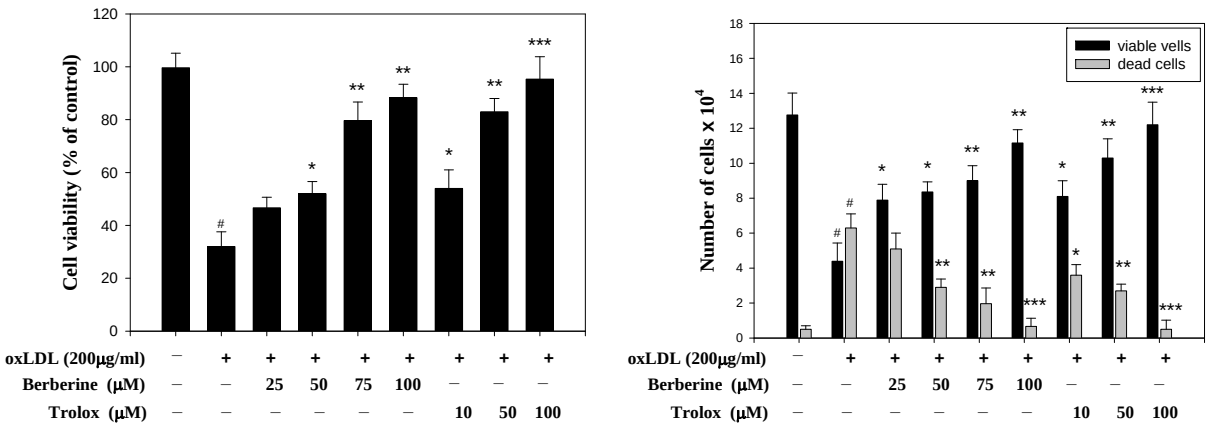


Fig. 26. Effect of berberine on oxLDL-induced endothelial cell death. HUVECs were incubated with oxLDL (200 μg/ml) in the absence and presence of berberine (BER; 25, 50, 75 and 100 μM) or Trolox (10, 50, and 100 μM) for 16 h. (A) The viability of treated HUVEC cells was detected using MTT assay. (B) Viable cells and dead cells were counted using Trypan blue exclusion assay. ($^{\#}$, P<0.001 compared with control. *, P<0.05; **, P<0.01; ***, P<0.001 compared with oxLDL-treated group.)

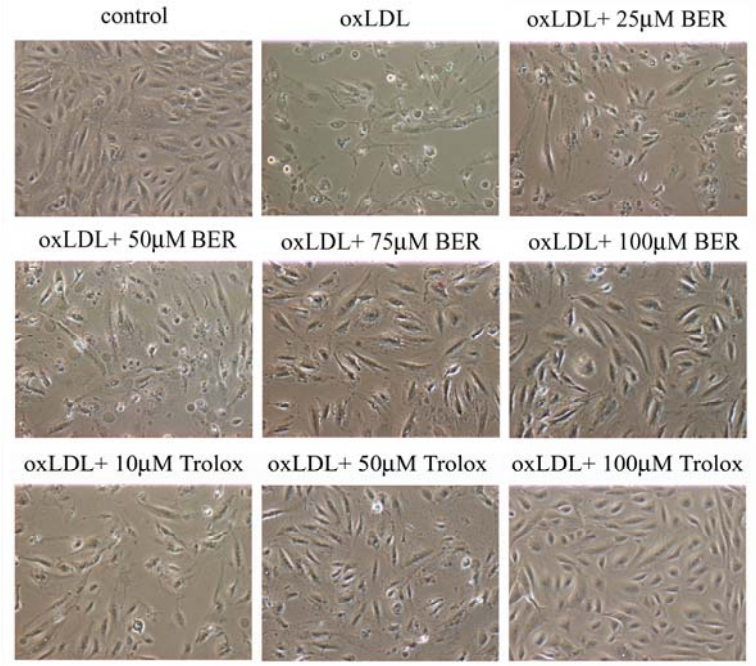


Fig. 27. Effect of berberine on oxLDL-induced endothelial cell morphology change. HUVECs were incubated with oxLDL (200 μg/ml) in the absence and presence of berberine (BER; 25, 50, 75 and 100 μM) or Trolox (10, 50, and 100 μM) for 16 h. Photomicrographs of the treated HUVEC cells were observed by using phase-contrast microscopy.

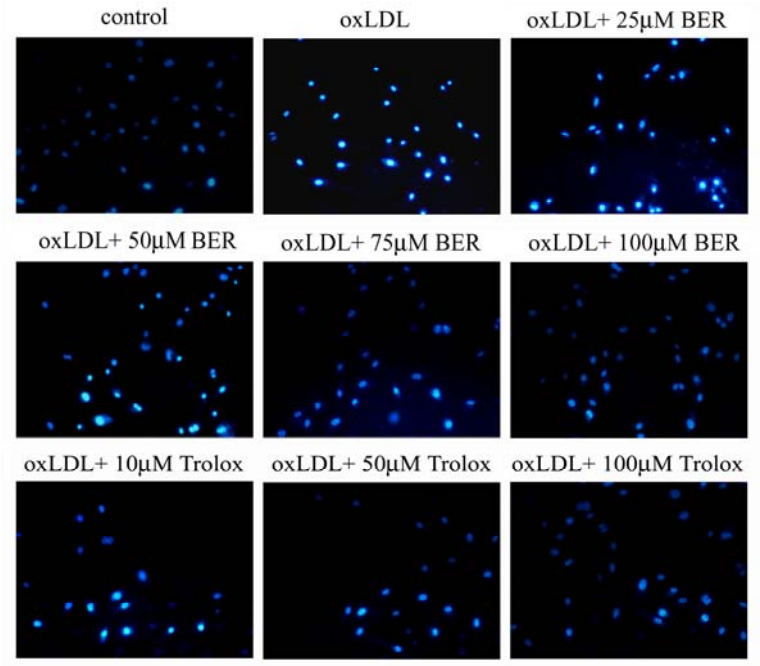


Figure 28. Protective effect of berberine on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL in the present and absence of berberine (BER) or Trolox for 16h. Nuclear morphology of the treated cells was observed by fluorescence microscopy using DAPI stain (at a magnification of 200 ×). Arrows showed areas of intense fluorescence staining with condensed nuclei.

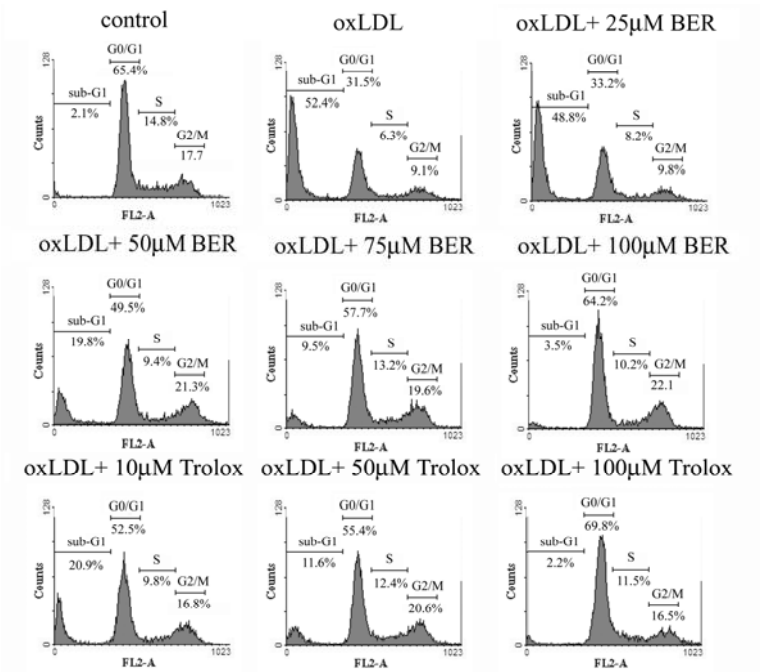


Figure 29. Protective effect of berberine on oxLDL-induced endothelial cell apoptosis. HUVECs were incubated with oxLDL in the present and absence of berberine (BER) or Trolox for 16h. Hypodiploid cells population (sub G1 phase) of the treated HUVEC cells were analyzed by flow cytometry using PI stain and at last 10,000 event of total cells were analyzed for each experimental treatment.

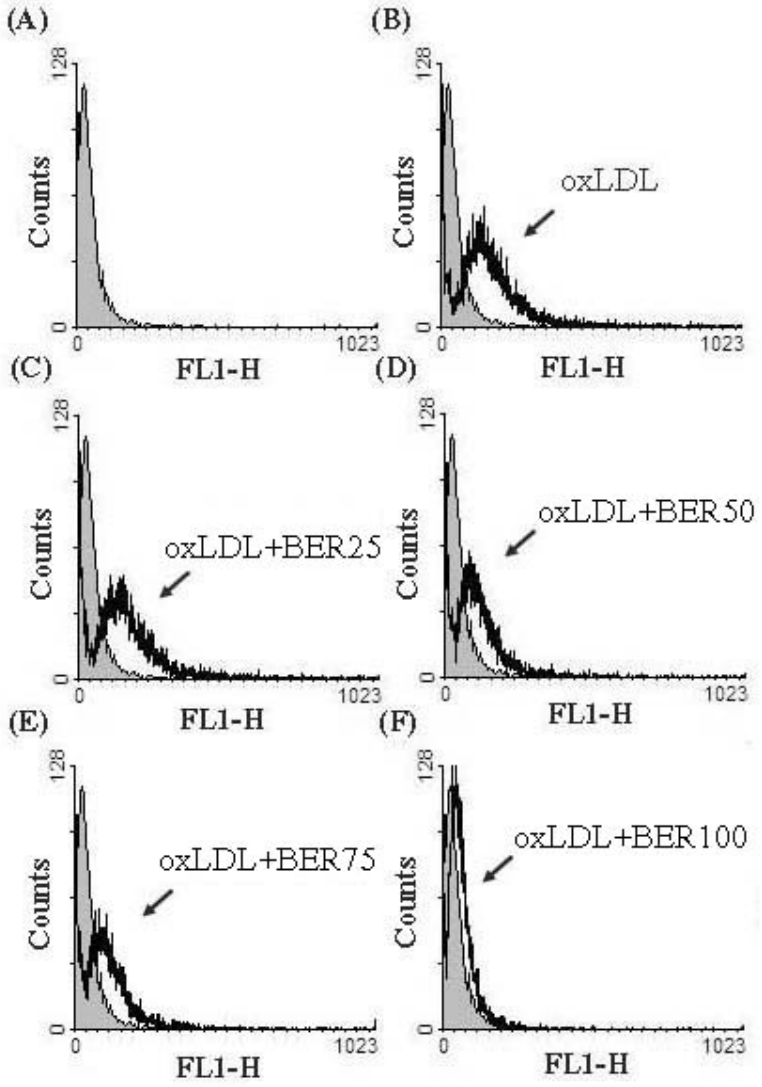


Fig. 30. Effects of berberine on oxLDL-induced ROS production in HUVECs. The ROS levels of (A) the HUVEC cells without treatment, control (gray trace); (B) the HUVEC cells with treatment of 200 μg/ml oxLDL, oxLDL (dark trace); (C) the HUVEC cells with treatment of oxLDL and 25 μM berberine, oxLDL+BER25 (dark trace); (D) the HUVEC cells with treatment of oxLDL and 50 μM berberine, oxLDL+BER50 (dark trace) (E) the HUVEC cells with treatment of oxLDL and 75 μM berberine, oxLDL+BER75 (dark trace); (F) the HUVEC cells with treatment of oxLDL and 100 μM berberine, oxLDL+BER100 (dark trace) were measured by flow cytometry using DCFH-DA staining.

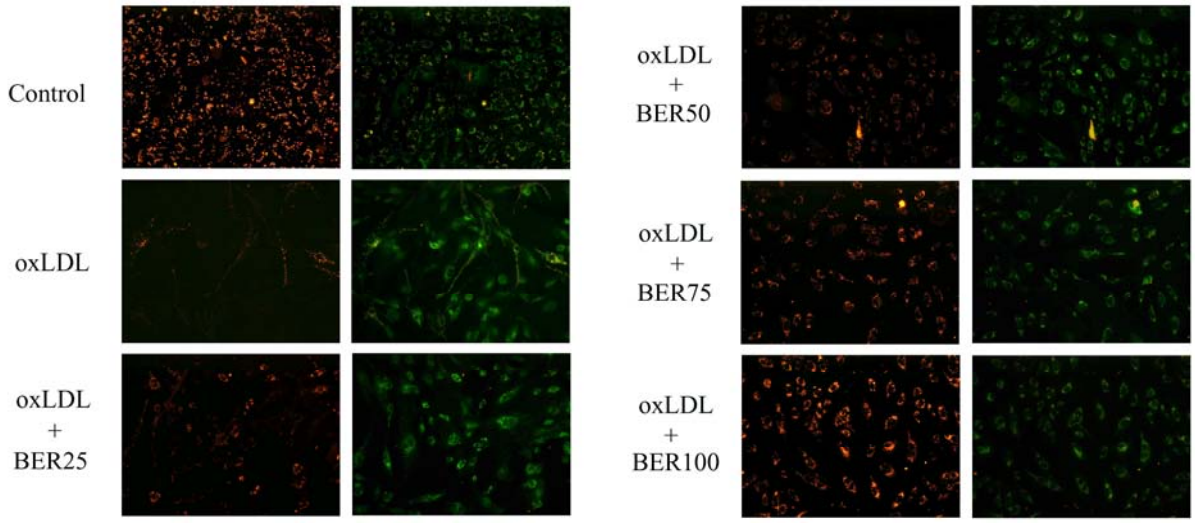


Fig. 31. Effects of berberine on oxLDL-induced changes in mitochondrial membrane potential in endothelial cells. HUVECs were incubated with oxLDL (200 μg/ml) in the absence and presence of berberine (BER; 25, 50, 75 and 100 μM) for 16 h. The changes of mitochondrial membrane potential ($\Delta\Psi m$) were assessed by using fluorescent lipophilic cationic JC-1 dye. JC-1 is selectively accumulated within intact mitochondria to form multimer J-aggregates emitting fluorescence light at 590 nm (red) at a higher membrane potential, *left*, and monomeric JC-1 emits light at 527 nm (green) at a low membrane potential, *right*.

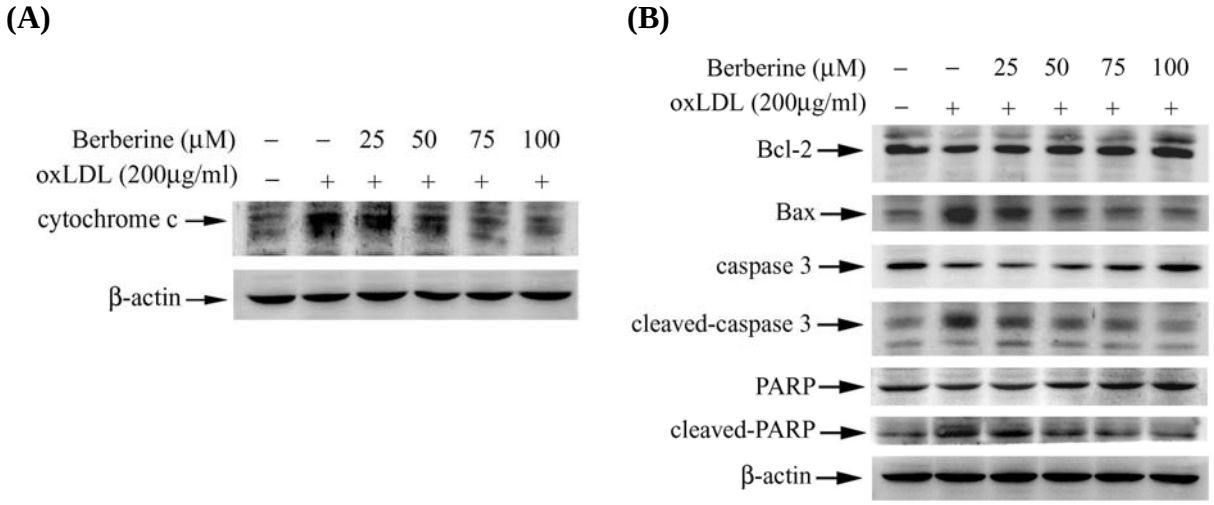


Fig. 32. Effects of berberine on oxLDL-induced caspase-3 and PARP activation. In western blot assay, cell lysates were subjected to SDS-PAGE, and the membranes were probed with antibodies against cytochrome C (A) Bcl-2, Bax, caspase-3, and PARP (B), with β-actin being an internal control. Signals of proteins were visualized with an ECL detection system. The results were representative of three independent experiments.