辣椒成分對自由基及肝癌細胞終端酵素之影響

獲獎榮譽榜

參加美國第五十二屆國際科技展覽會獲
大會獎化學科二等獎

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我叫袁于婷，今年剛畢業於國立高師大附中，並推甄上陽明醫學系。高中三年的科展經驗是最讓我難忘的一部分，使我了解了科學的美妙並奠定了我對基礎研究的興趣，也使我成長了不少。尤其在出國前的集訓期間，承蒙各教授的指導，讓我學會嚴謹的研究方法及論文撰寫。非常感謝這段時間陪我一路走來的師長、家人及同學們，他們的支持和鼓勵給了我無比的勇氣，使我能在每次遭遇困難時，繼續堅持下去。

研究動機

近年來，不論在世界各地，人民罹患癌症的機率都有不斷攀高的趨勢，其成長之快速令人擔憂。因此，尋找具抗癌、防癌效力的天然食物便成為許多人感興趣的事。我曾在之前的科展實驗中，發現辣椒是一種良好的抗氧化劑，也知道抗氧化反應與自由基（free radicals）的反應有關，而癌症的發生，又與自由基的過量有密切的關係，因此便決定深入探討辣椒素對自由基及癌細胞的影響。

研究目的

本研究分為兩大部分，以探討辣椒重要成分辣椒素（Capsaicin）抑癌的能力。

（一）、試樣（辣椒素 &Vitamin E）清除 DPPH 自由基及超氧陰離子的能力
（二）、終端酵素活性之偵測及 hTERT mRNA 的表現
（三）、 MTT 細胞毒殺檢驗
（四）、固著非依賴性生長分析

研究設備器材

水浴恆溫槽 離心機 無菌操作台 無菌培養箱 微離心機 微量天秤 電泳槽
分光光度計 真空乾燥機 紫外燈照相設備 PCR 機器 ELISA Plate Reader
細胞株（Cell lines）：
人類肝癌細胞株 J5（Human J5 hepatoma cell line）
人類非癌肝細胞株 NTL003（Non-tumor liver cell）
（NTL003 由高雄長庚醫院提供組織檢體，由國立中山大學建立細胞株。）

文獻探討

自由基與癌症

自由基（free radicals）和活性氧（active oxygen）近年來廣受注目的原因是，許多研究報導指出，自由基、活性氧在許多疾病（如老化、癌症、心血管疾病）的發展上扮演極重要的角色。

人體在正常代謝過程中會產生自由基與活性氧，本實驗使用之DPPH即為自由基，而超氧陰離子($O_2^{-}\cdot$)既是自由基亦為活性氧的一種；一些外來的物質在體內代謝過程中也會產生自由基與活性氧，這些物質會進而攻擊細胞膜、細胞組織並危害細胞核內基因物質，並傷害細胞引發病變、癌化，甚至死亡[1]。雖然自由基與活性氧會造成生物體細胞的損害，甚至導致死亡，然在正常狀況下，生物體本身具有抗氧化防禦系統，產生抗氧化酵素及抗氧化物來移除自由基與活性氧，並由修護系統修補所引發的傷害；但若外在的刺激過大，使自由基與活性氧多過人體內防禦系統所能負荷時，便極有可能產生如上述之細胞的癌化或病變，對人體形成危害[2]。

終端酵素與癌症

一般而言，正常人類體細胞的染色體終端長度，會隨著細胞分裂變短，而使細胞的分裂能力受限，但學者發現，人類生殖細胞及癌細胞卻可維持染色體終端的長度，使細胞具不斷分裂的能力[3]。

正常細胞增殖到一定階段會達到一分隔點，然後細胞便停止生長，然而此障礙會被某些病毒性致癌基因（viral oncogenes）所克服，使得細胞能持續再增殖，當然此時其染色體終端也會持續縮短，到最後，細胞達到另一個關鍵點，大部份細胞會死亡，但能通過此關鍵期的細胞，便會成為不朽化細胞，終端酵素之活化被認為是通過關鍵點的關鍵因子[4]。 從1994年首先發現在人類的卵巢癌其終端酵素被活化[5]後，陸陸續續在這幾年的研究中發現許多人類的腫瘤組織都可以測得終端酵素活性，例如：肝癌[6]。

人類染色體終端酵素目前已知主要由三個次單位所組成：（1）hTR, human telomerase RNA；（2）hTERT, human telomerase reverase transcriptase；（3）TP1, telomerase-associated protein 1。其中hTR及TP1幾乎在所有正常及腫瘤組織都有其mRNA的表現，hTERT的基因表現卻與telomerase活性有密切關係，在一些惡性腫瘤組織都可以偵測到其mRNA的表現，被認為是人類telomerase的催化次單位[7]。

研究過程或方法

（一）、清除DPPH自由基能力測定

取4 mL之試樣甲醇溶液（濃度為 2.5 ~ 20 mg/mL），分別加入新鮮配製之0.4 mM DPPH甲醇溶液1 mL，反應10分鐘。以分光光度計，在517 nm波長下，測定其吸光值。

（二）、清除超氧陰離子能力測定

取1 mL之試樣甲醇溶液（濃度為 1 ~ 5 mg/mL），依序分別加入等體積的300mM NBT、120mM PMS、936mM NADH溶液。室溫下靜置5分鐘，再以分光光度計，在560 nm波長下，測定其吸光值。

（三）、Total Protein的萃取及定量

取適量的細胞置於1.5 mL eppendorf tube中，各加入適量lysis buffer（Intergen）混合，置冰上45分鐘，再以4℃ 12,000 rpm 離心15分鐘，使用BCA Protein Assay Reagent Kit(PIERCE)測定蛋白質濃度，以ELISA Plate Reader定量。

（四）、終端酵素活性分析

使用TRAP（Telomeric Repeat Amplification Protocol）非放射性方法分析終端酵素活性。

PCR condition：

30℃ 30 min

95℃ 5 min

94℃ 30 sec ； 50℃ 30 sec ；72℃ 1.5min （32 cycles）

92℃ 10 min

配製10%非變性聚丙烯醯胺凝膠（Nondenaturing polyacrylamide gel），各取18 μL PCR產物以100伏特電壓進行電泳。

（五）、Total RNA的萃取

在各個收有細胞的1.5mL eppendorf tube中，加入TRIzol Reagent（GIBCO BRL, USA）800 μL，混合均勻，靜置於室溫下5~15分鐘。各管內加入160 μL 氯仿混勻，置於室溫下3分鐘，再於4℃離心15分鐘後，將最上層無色水層吸至新的tube中。各管內加入400 μL Isopropyl alcohol混勻，置於室溫下10分鐘，接著於4℃ 9,000 rpm離心5分鐘，然後吸去上清液，並以800 μL 75%酒精清洗沉澱物，再於4℃ 9,000 rpm離心5分鐘。吸去各管上清液，乾燥沉澱物，再加入10~20 μL DEPC-H_2O將之溶解。使用分光光度計測各管Total RNA的 $A_{280/260}$。

（六）、反轉錄聚合酵素連鎖反應

各取5 μg Total RNA，加入Oligo dT（500 μg/μL）2 μL，1 μL Random Primer（20 μM），以DEPC-H_2O補足體積12.5 μL，混合後於70℃下作用10分鐘。分別加入5 x 1st strand buffer 5 μL，RNasin 1 μL，0.1 M DTT 2 μL，dNTPs（2.5 mM淤）2.5 μL，於37℃作用2分鐘，再加入M-MLV RT（Moloney Murine Leukemia Virus Reverse Transcriptase）200 units，於37℃作用60分鐘，再於70℃下作用15分鐘，產物即為cDNA。各取 0.5 μL cDNA與適當的primers及PCR Reagent進行PCR反應。各取PCR產物10 μL跑1.5% agarose gel，再以EtBr染色，並照相紀錄之。

（七）、MTT細胞毒殺（Cytotoxity）檢驗

將細胞（J5、NTL003）在medium中打散，平均分到96-well plate中（500~1,000 cells/well），將plate置於37℃培養箱中培養1~3天。先備妥含8種不同濃度藥品的medium。分別將含藥品的medium加入columns 3~10，在培養箱培養1天。換新的medium，繼續培養2~3 個細胞倍增時間。最後，全部換新的medium，各再加入50 μL 3-(4,5-dimethylthiazol- 2-yl, 5-diphenyltetrazolium bromide（MTT, Merk, Germany）溶液（5mg/mL, filter sterile）於well中，以鋁箔包覆plate，培養4~8小時。抽掉medium & MTT混合液，加入200 μL DMSO溶解細胞代謝生成的formazan crystals及25 μL Soresen glycine buffer（pH = 10.5）調整pH值，再用ELISA Reader讀波長570 nm的吸光值。

討論及應用

（一）試樣在高濃度（500 μ g/mL）時， Vit. E 及辣椒素與 DPPH 自由基作用十分鐘後，反應趨於穩定，比較 Vit. E 及辣椒素兩者清除 DPPH 自由基能力不相上下。

（二）低濃度時，辣椒素有較 Vit. E 高的自由基及活性氧清除效力。

（三）由於辣椒素不能直接溶解在培養液中，故先將辣椒素溶解在少量100% 酒精中，再取需要量溶在 DMEM growth medium 中。而每次加入辣椒素反應時，都加入一組酒精處理的細胞作為對照，而在後面各實驗的結果中，也發現酒精的確不會對實驗結果造成影響，因此，以酒精萃取辣椒成分或溶解辣椒素的方法可行。

（四）運用 RT-PCR 的方法，偵測 hTERT 之 mRNA 在細胞中的表現，雖然無法定量，但仍可以比較出表現量的強弱。經過酒精、 1.0 mM、 0.8 mM、 0.6 mM 辣椒素處理 1 小時後，細胞 hTERT 基因的 mRNA 表現量，卻似乎有升高的趨勢。這可能是因為加入外來並非癌細胞生長所需的物質後，對細胞造成了衝擊，挑起其抵抗能力（而使上游基因有增強趨勢），但也許因接觸時間太短，只有一小時，尚未能促成終端酵素的合成，所以這個部份終端酵素的活性並沒有如 hTERT 基因有增強的情況。

（五）另外，由結果可以看到，經 0.6 mM、 0.8 mM 辣椒素處理 1 天的肝癌細胞，雖皆偵測不出終端酵素活性，但除了1.0 mM辣椒素該組外，其餘各組都尚有hTERT基因的mRNA 表現。對於這樣的現象可能的解釋是：這些試樣進入細胞後，直接破壞了染色體終端酵素此複合物的構造，但對於其上游基因 hTERT 的影響（破壞）較慢。所以，若較長時期以辣椒素培養癌細胞，除了抑制了終端酵素的活性，應該也能徹底關閉hTERT基因，或許能使癌細胞走向凋亡的途徑。不過，這部份仍有待實驗證明。

（六）由於非癌肝細胞測不出終端酵素活性，無法藉終端酵素活性偵測法探討試樣對其活性的影響，故進行 MTT 細胞毒殺檢驗以探討試樣對非肝癌細胞的衝擊。

（七）肝癌細胞分裂能力極強，不具固著依賴性，能在 soft agar 中長成 foci，但非癌肝細胞卻不具此能力，在 soft agar 中無法分裂，因此，此方法可作為癌細胞的檢驗方式。實驗結果顯示，所採試樣皆有降低癌細胞活性的能力，只是強弱不同罷了。其中，0.8 mM、 1.0 mM 辣椒素處理一天的癌細胞已完全不具癌細胞活性，此部份結果與終端酵素活性偵測的實驗結果十分相符，再次證明了終端酵素的活化對癌細胞分裂能力的重要。

結論

（一）、辣椒素具有清除自由基的效力，且效能並不遜於一般常用的抗氧化劑 － 維他命 E。

（二）、辣椒素具有抑制肝癌細胞中染色體終端酵素之功效，對其催化次單位 － hTERT基因的表現也具抑制效果，而其功效會隨濃度及處理時間的的增加而增強。且經辣椒素處理一天的肝癌細胞，在軟瓊脂凝膠中的分裂情形，已大不如未處理的細胞，即其腫瘤特性降低。

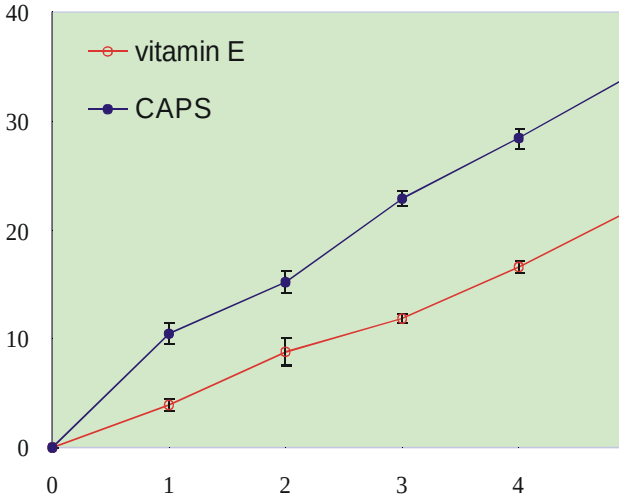

Fig. 1A. DPPH free radical scavenging effect

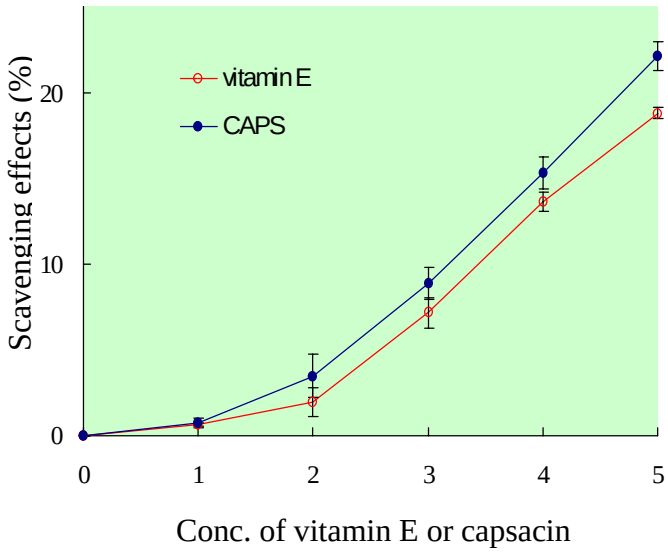

Fig. 1B. Superoxide anion scavenging effect

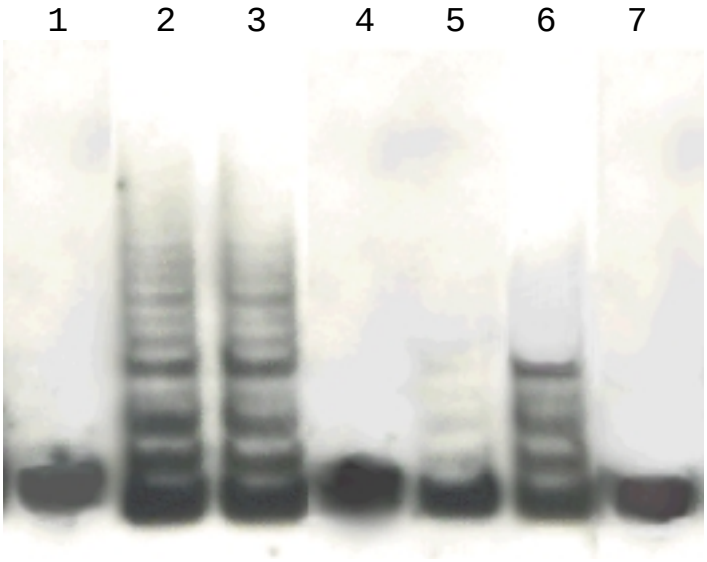

Fig. 2A. Inhibitory effects of CAPS on the telomerase activity of J5 cells as shown by PAGE

Lane 1: Control, without cells
Lane 2: Control, J5 cells only
Lane 3: Cells treated with ethanol
Lane 4: Cells treated with 800 ìM CAPS
Lane 5: Cells treated with 600 ìM CAPS
Lane 6: Cells treated with 400 ìM CAPS
Lane 7: NTL 003 cells

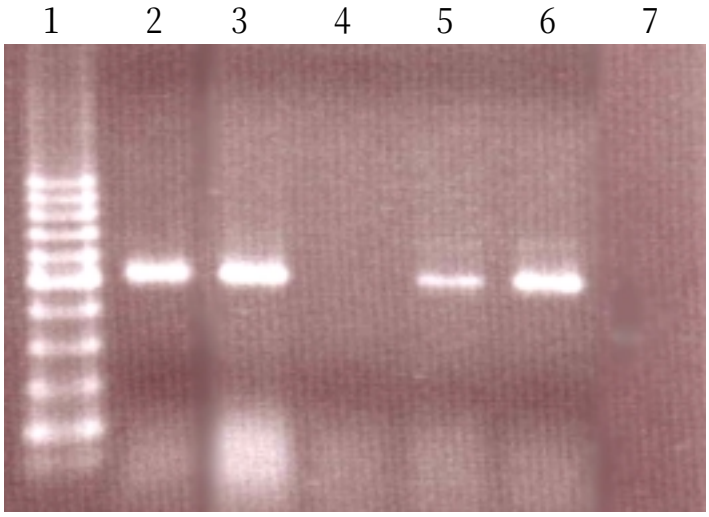

Fig. 2B. Inhibitory effects of CAPS on the expression of hTERT mRNA in J5 cells as shown by agarose gel electrophoresis

Lane 1: Control, without cells
Lane 2: Control, J5 cells only
Lane 3: Cells treate ethnola d with
Lane 4: Cells treated with 800 ìM CAPS
Lane 5: Cells treated with 600 ìM CAPS
Lane 6: Cells treated with 400 ìM CAPS
Lane 7: NTL 003 cell

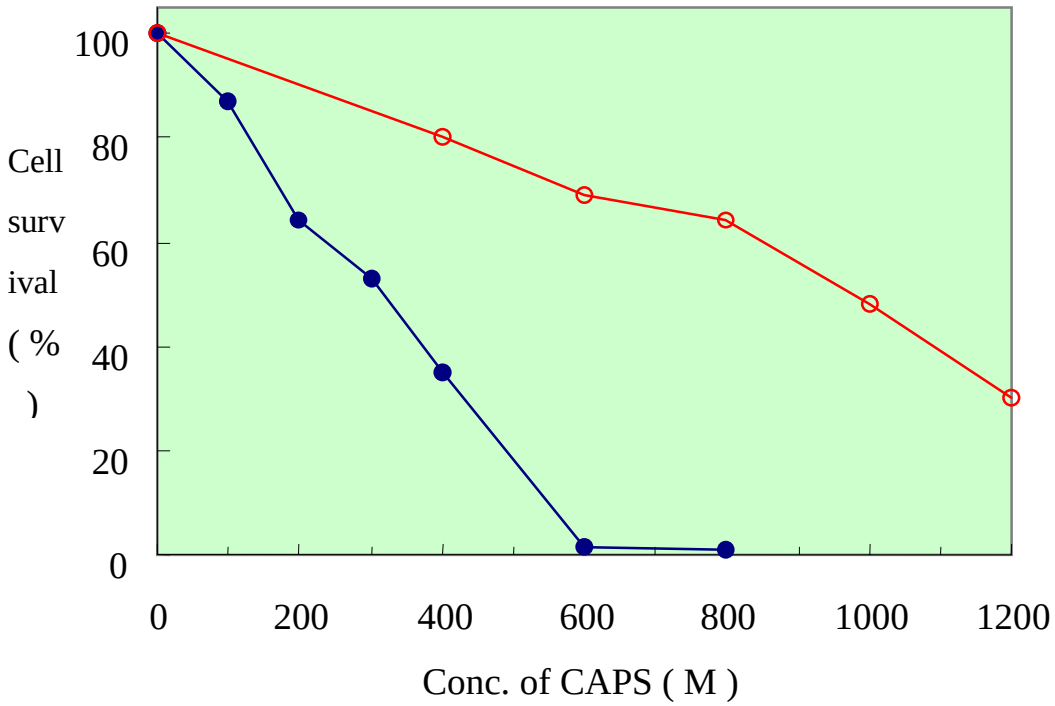

Fig. 6. Cytotoxicity of CAPS on J5 cells and NTL 003 cells

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Effects of Capsaicin on Free Radicals and the Telomerase Activity of J5 Hepatoma Cell Line

Abstract

One of the major constitutions, capsaicin in chili (Capsicum annum L. var. acuminatum Fingerh) was shown to be an antioxidant agent in this study. It had free radical scavenging effects to reduce α, α-diphenyl, β-picrylhydrazyl free radicals, superoxide anions and hydrogen peroxide, and the effects were better than those of vitamin E, one of the most commonly used antioxidants. Moreover, the telomerase activity and human telomerase reverse transcriptase gene expression were significantly inhibited in J5 cells treated with capsaicin. Capsaicin was able to induce apoptosis in J5 cells. IC 50 of capsaicin of hepatoma cells was 300mM and was lower than that of NTL003 cells (non-tumor human liver cells, fine needle biopsy specimen) (1000mM). The anchorage independence of J5 cells was lost after the cells were treated with capsaicin. Taken together these findings, it indicates that capsaicin could be a potential chemotherapeutic agent for the treatment of tumor.

Introduction

Capsacin(CAPS), the major pungent principle in different species of hot peppers, is known for its hot and burning taste. Since CAPS has a phenolic hydroxyl group, it could be used as an antioxidant agent. It is known that the antioxidant reaction is related to the reaction of free radicals, and that the over-production of free radicals in the human body may induce diseases such as cancer.

Free radicals and active oxygen species(ROS)are produced daily by the metabolic process in human bodies. Under normal conditions, the defense system of the human body is able to scavenge the free radicals and active oxygen species. However, if the human body is exposed to certain harmful substances such as chemical toxins, smoke, radioactive rays or subjected to great mental or physical stress, this may cause the over-production of free radicals and ROS that are too numerous for the body's defense system to scavenge. These free radicals are likely to attack cell membranes, or have a bad impact on genes in the cell nuclei of human bodies [1], [2].

Present studies were carried out to investigate the scavenging effects of CAPS on the1)free radical formed by α, α-dyphenyl, β-picrylhydrazyl (DPPH); 2)superoxide anion produced by nitro blue tetrazolium(NBT); phenazine methosulphate(PMS); and dihydromicotineamidadenibe dinucleolide(NADH) and 3)hydrogen peroxide. Moreover, the IC_{50} of CAPS of hepatoma cells and non-tumor human liver cells(NTL 003)were determined.

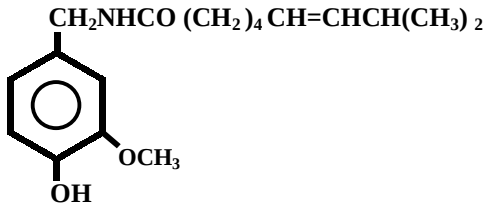


Figure 1. The structure of CAPS

Materials and Methods

DPPH, NBT, PMS, NADH were purchased from Sigma.

Telomerase PCR ELISA Kit was obtained from Roche.

Moloney murine leukemia virus reverse transcriptase(M-MLV RT)was the product of GIBCO BRL, USA.

DPPH free radical scavenging effect of CAPS

Freshly prepared 0.4 mM DPPH methanol solution was mixed with various concentrations of CAPS. After incubation at room temperature for 10 min, the absorbance of each reaction mixture was determined at 517 nm.

Superoxide anion scavenging effect of CAPS

One mL methanolic solution containing 300 μ M NBT, 120 μ M PMS, and 936 μ M NADH was added to various concentrations of CAPS. The reaction mixtures were incubated at RT for 5 min, and the absorbance of each reaction mixture was measured at 560 nm.

Hydrogen peroxide scavenging effect of CAPS

Two mL of 4 mM hydrogen peroxide(H2O2)in phosphate buffered saline(PBS; pH 7.4)was added to equal volume of various concentrations of CAPS. The reaction mixtures were incubated at RT for 5 min and the absorbance of each reaction mixture was determined at 230 nm.

Inhibitory effects of CAPS on telomerase activity

J5 cells(6 x 106)were treated with various concentrations of CAPS for 24 hr. Cellular proteins were extracted for measuring the telomerase activity with a Telomerase PCR ELISA Kit. The reaction products were subjected to 10 % nondenaturing polyacrylamide gel electrophoresis(PAGE)and the gels were dried under vacuum and then blotted onto a nylon membrane. The biotin-streptavidin complexes were visualized by adding disodium 3-(4-methoxyspiro {1, 2-dioxetane-3, 2'-(5'-chloro)tricyclo- [3.3.1.13,7] decan}-4-yl phenyl phosphate(CSPD)

Inhibitory effects of CAPS on hTERT expression by RT-PCR

hTERT cDNA was obtained by PCR from 5 μ g of RNAs extracted from J5 cells treated with various concentrations of CAPS(5×10^6), and the reaction products were analyzed by 1.5% agarose gel electrophoresis.

MTT cytotoxicity assay

J5 cells were treated with various concentrations of CAPS for 24 hr, and the cells were treated with 5mg/mL MTT for 6 hr. The absorbances of the reaction products were measured at 570 nm with an ELISA reader.

Assessment of apoptosis

J5 cells(1 x 106)were treated with various concentrations of CAPS for 18 hr. DNA was extracted, digested overnight in 0.5 mg/mL of proteinase K, and treated with 10 μ L of RNase A for 1 hr. The cellular DNA was analyzed by 1.5% agarose gel electrophoresis.

Cytochemical staining of apoptosis cells

J5 cells were incubated with various concentrations of CAPS for 24 hr at 37°C. The treated cells were washed twice with PBS and fixed with 3% paraformaldehyde in PBS. The fixed cells were stained with 16 ug/mL Hoest33258 and observed(excitation, 360 nm; emission, 490-505 nm)by fluorescence microscopy.

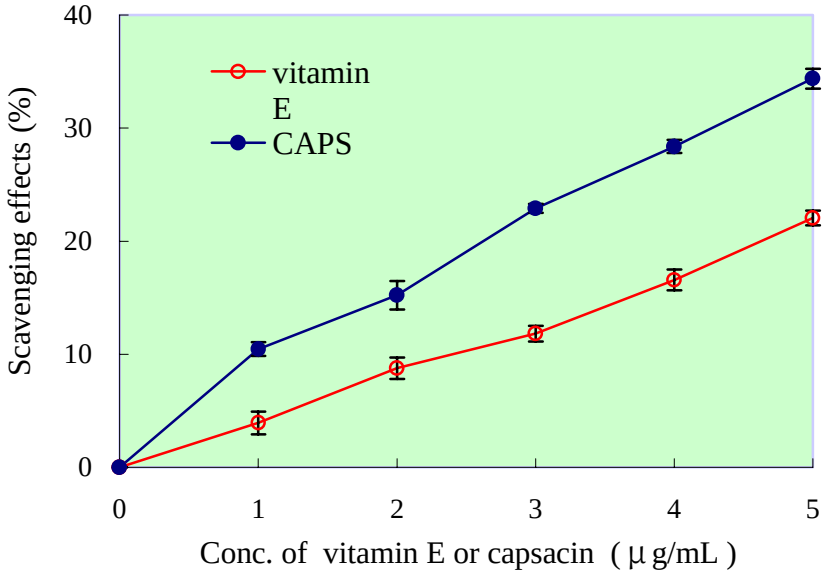


Fig.3A.DPPH free radical scavenging effects

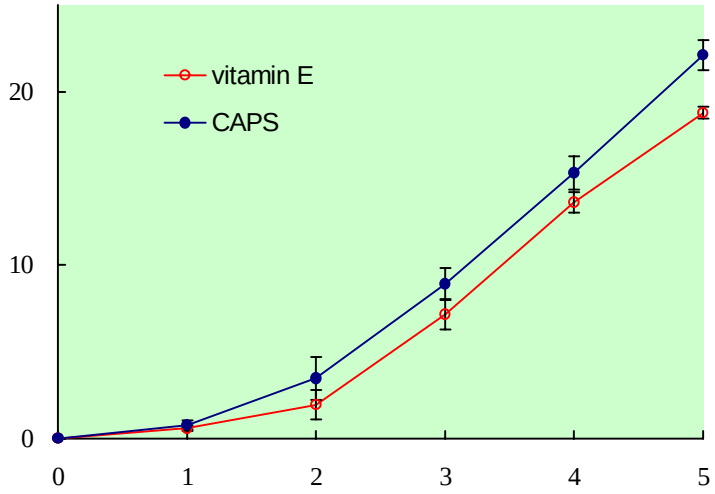


Fig. 3B. Superoxide anion scavenging effects

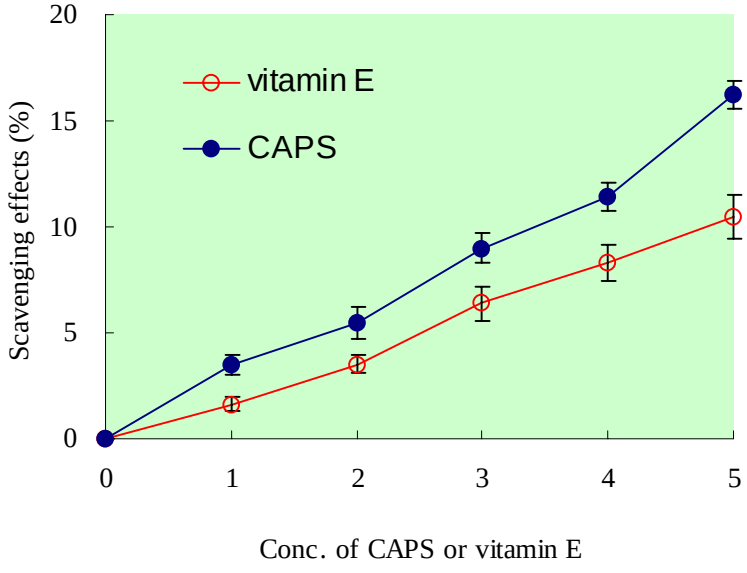
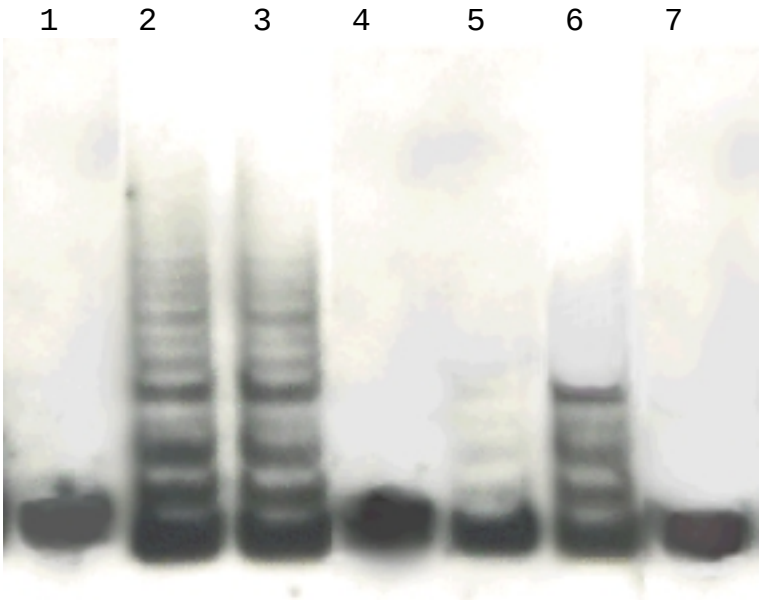


Fig. 3C. Hydrogen peroxide scavenging effects



Lane 1: Control, without cells
Lane 2: Control, J5 cells only
Lane 3: Cells treated with ethanol
Lane 4: Cells treated with 800 ìM CAPS
Lane 5: Cells treated with 600 ìM CAPS
Lane 6: Cells treated with 400 ìM CAPS
Lane 7: NTL 003 cells

Fig. 4A. Inhibitory effects of CAPS on the telomerase activity of J5 cells as shown by PAGE

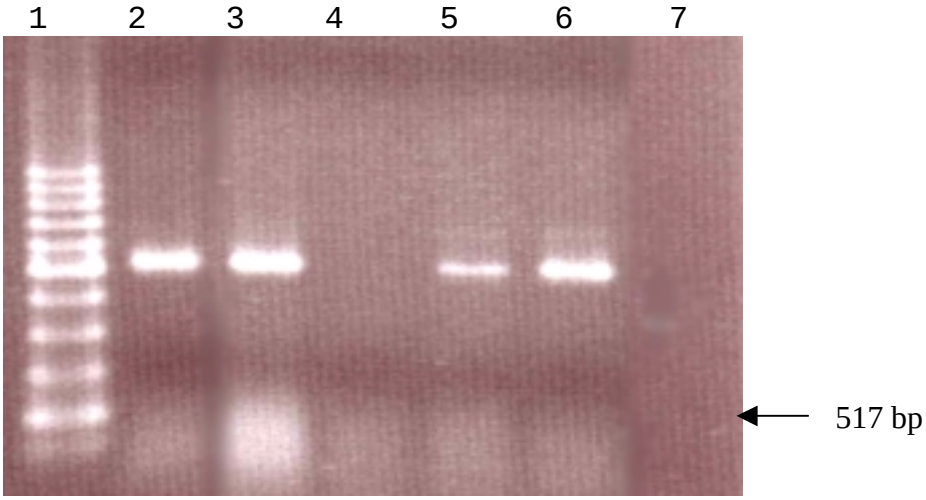
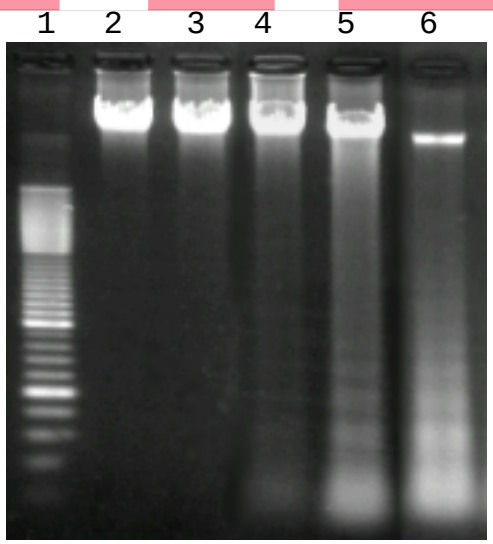


Fig. 4B. Inhibitory effects of CAPS on the expression of hTERT mRNA in J5 cells as shown by agarose gel electrophoresis

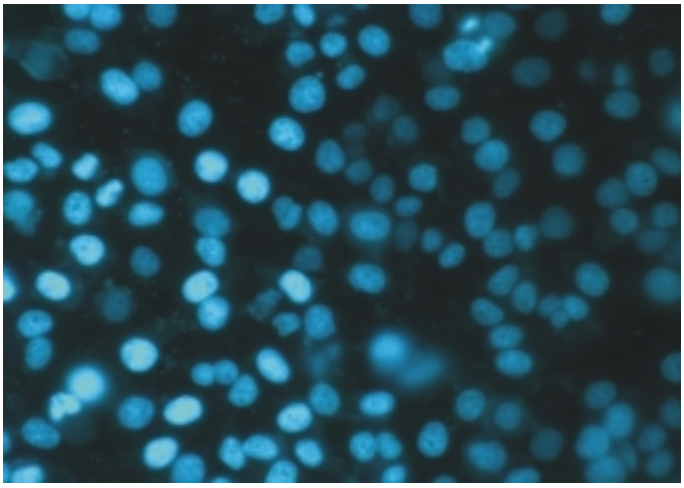
Lane 1: Control, without cells
Lane 2: Control, J5 cells only
Lane 3: Cells treate ethnola d with
Lane 4: Cells treated with 800 ìM CAPS
Lane 5: Cells treated with 600 ìM CAPS
Lane 6: Cells treated with 400 ìM CAPS
Lane 7: NTL 003 cells



Lane 1: 100 bp marker
Lane 2: Control, J5 cells only
Lane 3: Cells treated with ethanol
Lane 4: Cells treated with 200 M CAPS
Lane 5: Cells treated with 400 M CAPS
Lane 6: Cells treated with 600 M CAPS

Fig. 5A. Apoptosis of J5 cells induced by CAPS as shown by agarose gel electrophoresis

(a)



(b)

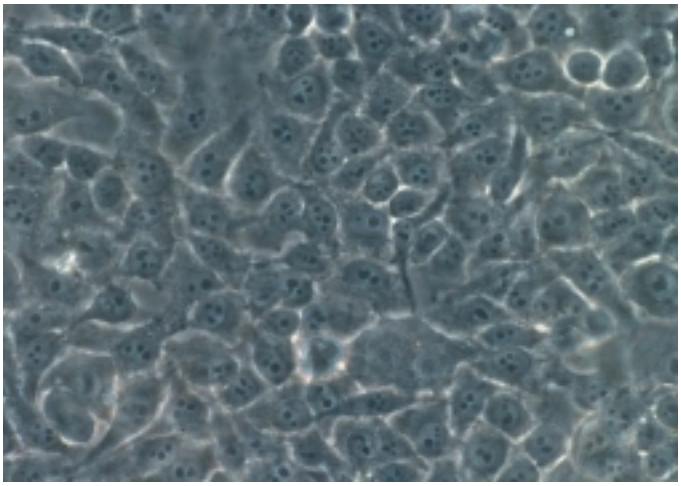
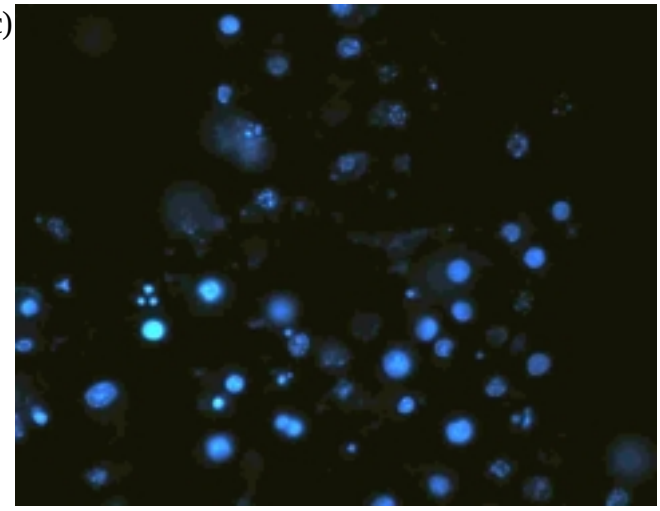


Fig.5B-1.Cytochemical staining of J5 cells treated with CAPS

a. J5 cells treated with ethanol, and observed on a blue filter set.

b. J5 cells treated with ethanol, and observed on a phase contrast filter set.

(c)



(d)

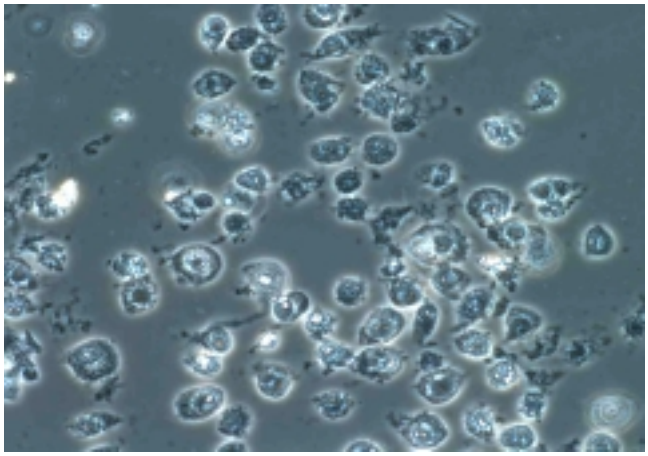


Fig. 5B-2. Cytochemical staining of J5 cells treated with C

c. J5 cells treated with 600 £gM CAPS, and observed on a blue filter set.

d. J5 cells treated with 600 £gM CAPS, and observed on a phase contrast filter set.

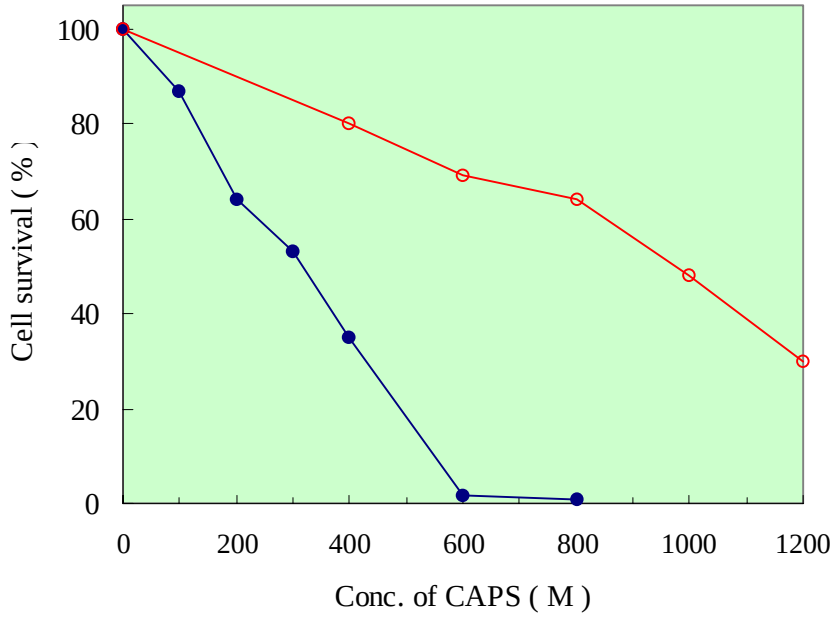


Fig. 6. Cytotoxicity of CAPS on J5 cells and NTL 003 cells

Discussion

1.Present investigation revealed that CAPS had great free radical / ROS scavenging effects in vitro. The scavenging effects of CAPS were found to be in a dose dependent manner. The effects of CAPS on scavenging DPPH free radical (Fig. 3A.), superoxide anion (Fig. 3B.), and hydrogen peroxide (Fig. 3C.) were higher than those of vitamin E, one of the most commonly used antioxidants.

2.CAPS had significant inhibitory effects on telomerase activity (Fig. 4A.) and the expression of hTERT gene mRNA in human hepatoma cell line, J5 cells (Fig. 4B.). The telomerase activity of J5 cells was almost completely inhibited at the concentration of 600 ìM.

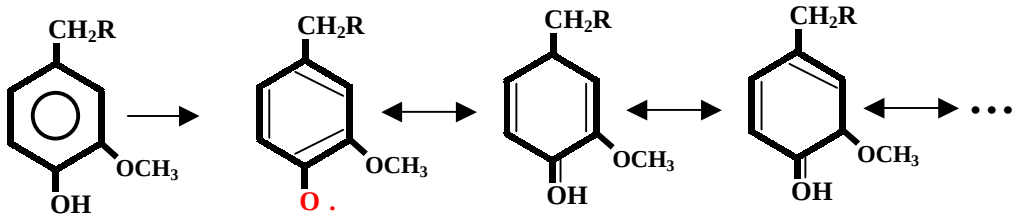
3.Under normal conditions, a drug used for the cancer treatment is able to induce apoptosis in tumor cells. Indeed, CAPS had the effect. CAPS was demonstrated to induce apoptosis in J5 cells as shown by the formation of DNA laddering by agarose gel electrophoresis and DNA fragmentation by cytochemical staining (Fig. 5A. and Fig. 5B.).

4.IC50 of CAPS on hepatoma cell line, J5 cells and non-tumor liver cells, NTL 003 was determined to be 300£gM and 1000 µ M, respectively (Fig. 6). It indicates that the hepatoma cells are more sensitive to CAPS than non-tumor liver cells. Therefore, it suggests that CAPS or its derivatives could be used as potential chemotherapeutic agents.

APS

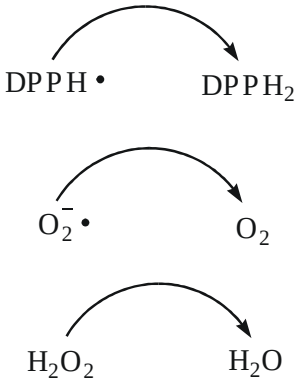
5.The concentrations of CAPS which can either inhibit the telomerase activity, suppress hTERT gene expression, or induce apoptosis in hepatoma cells were all lower then the IC_{50} of NTL 003 cells. This might explain why the IC_{50} of J5 hepatoma cells was three times lower than the IC_{50} of NTL 003 cells.

6.According to the result by Yusaka and Kenzo, the mechanism of the antioxidant activity of CAPS is proposed as follows [3]:



CAPS
(phenolic hydroxyl group)

Resonance structures of CAPS radical



R=NHCO(CH$_2$)$_4$CH=CHCH(CH$_3$)$_2$

During an antioxidant reaction, CAPS acts as an " H-radical donor, " which provides a hydrogen-radical to a free radical to form a stable compound. After the reaction, the CAPS-radical will form resonance structures that it will stop the free radicals from attacking cells in the human body.

Conclusion

CAPS was determined to be a good antioxidant which can scavenge free radicals and can inhibit the viability of J5 hepatoma cells. Hepatoma cells weremore sensitive to it then non-tumor liver cells.

In conclusion, CAPS and its derivatives could be used as potential chemotherapeutic agents.

References

1.Shackelford R.E., Kaufmann W.K., Paules R.S. (2000) Oxidative stress and cell cycle checkpoint function. Free Radic. Biol. Med. 28: 1387-1404.

2.McCord J.M. (2000) The evolution of and oxidative stress. Am. J. Med. 108: 652-659.

3.Yusuke, S. Kanzo, S. (1998) NMR analytical approach to clarify the antioxidative molecular mechanism of catechins using 1,1-diphenyl-2-picrylhydrazyl. J. Agric. Food Chem. 46: 111-114.