類胡蘿蔔衍生物對黃麴毒素誘發DNA傷害及細胞毒性之抑制作用

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利用C3H小白鼠的胚胎纖維母細胞C3H10T½細胞株，來分析Carotenoid衍生物（包括β-carotene, BCT; Lycopene, LCP; Crocetin, CCT及Crocin heptacetate, CH）對黃麴毒素B_1（AflatoxinB$_1$, AFB$_1$）誘發細胞毒性之保護作用。實驗於有老鼠肝細胞微粒體酶活化系統及沒有酶活化系統下（S-9 activation system），分別加入0.5μg／mℓ AFB$_1$及不同濃度的Carotenoid衍生物處理一天或連續處理一星期後，以C3H10T½細胞株的種植效率（plating efficiency）為細胞毒性指標。結果顯示在Carotenoid衍生物本身於10 μg／mℓ之環境下不會造成細胞毒性，因此選擇此濃度以下的Carotenoid衍生物同時加入含AFB$_1$處理之C3H10T½細胞中，結果在沒有S-9活化下，0.5 μg／mℓ之AFB$_1$的細胞毒性不太，Carotinoid衍生物沒有顯著的影響；若在有S-9活化系統下，Carotenoid衍生物能抑制AFB$_1$降低C3H10T½的種植效率，而在連續處理一週之Carotenoid衍生物之效果較顯著；以處理AFB$_1$及Carotinoid衍生物4hrs後也顯著降低了AFB$_1$對DNA損害之程度。大部份的Carotenoid衍生物也能減少因AFB$_1$引起的DNA合成之抑制作用。這些結果顯示Carotenoids及其衍生物有抑制AFB$_1$對C3H10T½之細胞毒性，其抑制AFB$_1$毒性的作用，可能經由Carotenoid衍生物增加DNA被AFB$_1$攻擊後的修補系統（repair system），致使DNA的合成增加，減少AFB$_1$產生的細胞的毒性。

Key words: Carotenoid derivatives, C3H10T1/2 cells, Cytotoxicity, DNA damage, Alflatoxin B_1

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黃麴毒素B_1（Aflatoxin B_1；AFB$_1$）在各種動物實驗中均被證明為一種很強的致癌物質[1]，從流行病學上的研究也顯示與人類肝癌有關[2]，其致癌的機轉為AFB$_1$先經肝臟酵素系統代謝為AFB$_1$-8，9-epoxide[3]，此親核代謝物（electrophilic metabolite）能夠攻擊DNA[3,4]形成共價加成物（adduct），此AFB$_1$-DNA adduct被認為是形成癌化的起始[4]原因。

許多研究均顯示維生素A及它的衍生物

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Retinoids有抑制癌化的產生[5-10]，而胡蘿蔔素（β-carotene）也被證實能抑制由7，12-dimethyl-benz(a) anthracene誘導老鼠產生之腫腺癌[11]，及紫外線引起的皮膚癌[12]，從流行病學上調查提出攝食較多的胡蘿蔔素地區產生癌症之機率較低[13-14]，此種抑制癌症的作用，一般認為它具有維生素A的活性，並能捕獲（Scavenger）游離的自由基及抗氧化之作用[15-17]。

雖然Retinokds及β-carotene之抗癌作用研究很多，但Carotenoids對於AFB_1引起的毒性及致癌作用則沒有，由於台灣地區潮濕氣候屬於AFB_1易污染地區，與肝癌發生率有極大的相關[18]，因此發現一些天然物來抑制AFB_1之致癌作用及抗癌機轉，實屬重要，本研究初步想以in vitro的實驗中比較各種具維生素A活性的β-corotene（BCT）及不具維生素A活性的Lycopene（LCP），Crocetene（CCT）及其乙醯化衍生物Crocin heptacetate（CH）對AFB_1誘導之細胞毒性及DNA損害之抑制作用，並探討與其作用之機轉。結果顯示無論具或不具維生素A活性之Carotenoid，均有抑制AFB_1對C3H10T½細胞的損害，其機轉可能為Carotenoid衍生物增加AFB_1對DNA損害之修補作用，或減少AFB_1對DNA之傷害作用。

材料與方法

AFB_1及Carotenoid衍生物之製備：

將AFB_1及Carotenoid derivatives（LCP、CCT、BCT均購自美國Sigma公司；CH從梔子中抽取後乙醯化[19]）溶於DMSO中，濃度分別為AFB_1(2 mg／mℓ)；Carotenoid衍生物400，40，4，0.4，0.04 mg $mℓ^{-1}$）置於0℃貯存，使用時分別以MEM培養基（Gibco）稀釋100倍，各取100 μℓ放入培養皿中，最終濃度AFB_1為0.5 μg／mℓ；Carotenoid衍生物為100，10，1，0.1，0.01 μg／mℓ（LCP為10，1.0，0.1，2.5 μg／mℓ），DMSO最終濃度為0.025%。

細胞培養：

C3H10T½細胞株來自於C3H mice embryo cell分離培養的正常纖維母細胞[20]，本實驗所用之C3H10T½ cell均在8～15代之間，培養於Eagle MEM（購自Gibco）中，含10%胎牛血清，100 IU $mℓ^{-1}$ penicillin及100 μg $mℓ^{-1}$Streptomycin，且使25或75 cm^2角瓶（Nunc，Denmark）培養，置於37℃及5%CO_2之恆溫箱中，使用時再以trypsin-EDTA（Sigma美國）處理為密度均勻之懸浮液。

細胞毒性分析：

均勻懸浮之細胞調整密度為500 cells $mℓ^{-1}$，取0.5 mℓ放入培養皿中培養24 hrs後取上述稀釋好之Carotenoid衍生物100 μℓ加入，以0.05%DMSO當控制組，培養24 hrs後，移去所有培養基，換入新的培養基，繼續培養7天後，以甲醇固定，Gimsa染色，計算直徑大於0.05 cm的colonies數目，以DMSO之plating efficiency為100，計算Relative plating efficiency（RPE），計算公式如下：

$$\%\,RPE=\frac{PE\ of\ test\ compound\ treated}{PE\ of\ DMSO\ control}\times 100\%$$

由上述結果，選擇Carotenoid衍生物對C3H10T½細胞非毒性劑量（由表一選擇最終濃度為1，0.1，及0.01 μg／mℓ），同時加入AFB_1（0.5 μg／$mℓ^{-1}$）在存在與不存在S-9活化系統下（每mℓ含由Aroclor-1254誘導分離之Rat liver S-9 0.1 mℓ；$Mgcl_2$-Kcl Salts 0.02 mℓ；1M glucose-6-phosphate 0.005 mℓ；0.1M NADP 0.04 mℓ；0.2M phosphate buffer pH 7.4 0.5 mℓ；Sterile distilled H_2O 0.335 mℓ）分別測定RPE值，另以同劑量之Carotensid衍生物在長期作用下（7天）觀察其對AFB_1之細胞毒性的影響。

DNA damage分析：

C3H10T½ cell培養在25 cm^2角瓶中，經過24 hrs後，加入0.2 μCi methyl-^{3}H-Thymidine（購自New England Nuclear；Specific activity 6.7 ci／nmole），培養24 hrs後，移去^{3}H-thymidine，再加入新的MEM培養基，24小時後，加入上述濃度之Carotenoid衍生物及AFB_1（0.5 μg $mℓ^{-1}$），培養4～6小時後，以Trypsin-EDTA收集細胞，在2000 rpm下離心10分，倒去上清液，加1 mℓ PBS，再均勻化，置於－70℃至少48小時。

DNA damage分析，根據Kohn等人[21]修飾的alkaline elution method，簡單說明如下：

將polyvinyl chloride filter裝在syringe filter holder上，下接peristaltic pump（0.5 mℓ／mℓ），先以1mℓ PBS潤濕holder，再加上

Table 1. Effect of carotenoid derivatives on cytotoxicity in cultured C3H10T1/2 cell.

Treatment*	Dose (μ g/ml)	Plating efficiency	% RPE**
Normal		29 ± 3**	
DMSO	0.025 %	28 ± 3	100
BCT	1.0	31 ± 5	115
	10.0	3 ± 1[b]	11
	100.0	0 ± 0[b]	0
LCP	0.1	26 ± 6	95
	1.0	21 ± 3[a]	78
	2.5	24 ± 6	86
CH	1.0	27 ± 5	99
	10.0	31 ± 4	112
	100.0	24 ± 5	88
CCT	1.0	24 ± 9	89
	10.0	27 ± 2	99
	100.0	6 ± 0[b]	0

* Cells were seeded at 250 cells/dish and treated with indicated concentration of carotenoid derivatives in DMSO for 1 week.

** % of relative plating efficiency = $\frac{\text{Colonies of treatment}}{\text{Colonies of DMSO control}} \times 100\ \%$

*** mean ± SD, values are the average of triplicate determinations.

[a] $p < 0.05$; [b] $p < 0.001$; compared with DMSO control.

2mℓ PBS及6mℓ lysing solution（含2M Nacl，0.02M EDTA，及0.2%triton X-100 pH 8.2）後，將細胞均勻液慢慢滴入，待syringe內溶液流完後，加5mℓ 1mM EDTA pH 7.8，流完後再加入13.5mℓElution Soln（含0.02 M TEAH，0.02M EDTA，pH 12.2）後，開始收集總量以0.5mℓ／min之速度收集。

取全量1.5mℓ加13.5mℓ Scientillation liquid（每1000mℓ toluene含30%triton X-100，0.4% pop及0.01% popop（Sigma，美國）測Single strand DNA，用Aloka L-Sc-900閃爍計數儀，測得cpm值，所得之結果均爲三次重覆之平均值。

將membrane自然乾燥後，剪成小塊狀，盛裝在瓶內，先吸5c.c Elution Soln（含0.02 M TEAH，0.02M EDTA，pH 12.2）振盪1分後，吸到另一瓶，再加5mℓ Elution Soln振盪1分鐘，吸到瓶內共10c.c，取1c.c加2c.c Elution Soln，再加13.5 mℓ Scientillation liquid-33258測cpm值，計算Elution ratio之公式如下：

$$\text{Elution ratio} = \frac{(\text{Eluted cpm}) \times 9}{\text{Eluted cpm} \times 9 + \text{residue cpm} \times 30} \times 100\%$$

對巨分子（DNA）生合成之影響：

取已稀釋好之細胞（2×10^5 cells／mℓ）0.1 mℓ，放入microtiter plate（Nunc，96 wells，Denmark）中培養24小時後，將預先溶於DMSO之Carotenoids調配成每mℓ含0.02 μg，0.2 μg，2.0 μg後，取100 $\mu\ell$及$AFB_1$0.5 μg／mℓ，10 $\mu\ell$放入每個well中，最終濃度為AFB_1 0.48 μg／mℓ，Carotenoid衍生物0.01，0.1，1.0 μg／mℓ，DMSO 0.25%及10 $\mu\ell$ S-9共0.2 c.c及label^{3}H-Thymidine，

處理6小時，以Bellco harvester收集細胞予Bellco glass filter strip上，加Scientillation liquid 3.5 c.c後分別測cpm值。

活化系統S-9製備：

以已知致突變物多氯聯苯混合物Aroclor 1254，以500 mg／kg注入Sprague-Dawley品系雄性老鼠體內，五天後，將老鼠肝臟取出，以鐵弗龍均質器均勻化後，在9000 xg離心10分鐘後，取其上層液，貯存於－80℃冰箱。使用時以輔助因子配成10%混合液包括：S-9 1mℓ，$Mgcl_2$-Kcl Salts 0.2mℓ，1M Glucose-6-phosphate 0.05mℓ，0.1MNADP 0.4mℓ，0.2 M Phosphate buffer（pH 7.4）5mℓ及二次水 3.35mℓ，每個試驗取250 μℓ加入。

結果

由於高濃度的Carotenoid derivatives本身可能也會造成細胞毒性，因此利用各種不同的Carotenoid derivatives在不同濃度下處理C3H10T½細胞，顯示濃度在1.0 μg／mℓ以下之Carotenoid derivatives對C3H10T½細胞不會造成毒性(如表一)，因此選擇1.0 μg／mℓ以下之Carotenoid derivatives研究其對AFB_1毒性之影響。在沒有活化系統（S-9 activation system）下，0.5 μg／mℓ之AFB_1產生細胞毒性很小（表二），但若以S-9活化作用後其PE值降為2%（表三），顯見AFB_1須經代謝活化才對細胞作用；若同時以不同之Carotenoid derivatives在各種濃度同時處理24小時

Table 2. Inhibitory effect of carotenoid derivatives on cytotoxicity induced by AFB_1 in culture C3H10T1/2 fibroblast cells without Sq activation system.

Treatment*	Dose (μg/ml)	Plating efficiency	% RPE**
Normal	0	24 ± 8***	98
DMSO	0.025 %	25 ± 4	100
AFB_1	0.5	24 ± 4	84
AFB_1 plus:			
BCT	0.01	21 ± 4	85
	0.1	21 ± 4	85
	1.0	24 ± 2	96
CH	0.01	29 ± 4	116
	0.1	26 ± 1	104
	1.0	32 ± 2[a]	128
LCP	0.001	27 ± 1	110
	0.01	20 ± 0	81
	0.1	28 ± 5	114
CCT	0.01	27 ± 2	111
	0.1	29 ± 1	116
	1.0	24 ± 6	99

* Cells were seeded at 300 cells/dish and treated with 0.5 μg/ml AFB_1 and various concentration of carotenoid derivatives for 24 hrs.

** See Table 1.

*** Mean ± SD, vlaues are the average of triplicate determinations.

[a] $p < 0.05$; compared with AFB_1-treated group.

Table 3. Inhibitory effect of continuous or discontinuous exposure of carotenoid derivatives on cytotoxicity induced by AFB_1 in cultured C3H10T1/2 cells with S-9 activation system.

Treatment*	Dose (μ g/ml)	Carotenoid derivatives (24hr)		Carotenoid derivatives (1wk)	
		colonies	% RPE	colonies	% RPE
Normal	0	19 ± 1**	119	19 ± 1	119
DMSO	0.025 %	16 ± 7	100	16 ± 7	100
AFB_1	0.5	0 ± 1	2	0 ± 1	2
AFB_1 plus:					
BCT	0.01	0 ±	0	6 ± 4	37
	0.1	2 ± 1	13	7 ± 3^a	43
	1.0	7 ± 2^a	44	12 ± 3^b	73
LCP	0.001	1 ± 1	6	6 ± 4	39
	0.01	6 ± 0^c	38	10 ± 1^c	63
	0.1	12 ± 3^b	69	11 ± 3^b	69
CH	0.01	3 ± 2	19	9 ± 2^b	58
	0.1	3 ± 1^a	21	9 ± 2^b	58
	1.0	12 ± 3^b	77	11 ± 4^b	69
CCT	0.01	10 ± 2^b	63	8 ± 2^b	52
	0.1	12 ± 1^c	72	11 ± 1^c	67
	1.0	14 ± 2^c	88	13 ± 4^b	83

* Cells were seeded at 250 cells/dish and treated with AFB_1 and carotenoid derivatives for 24hr in DMSO (0.025%) with activation system, then removed AFB_1. In continuous experiments, carotenoid derivatives were added to culture and keep for one week.

** Mean ± SD, n=3.

$^a p < 0.02$; $^b p < 0.01$; $^c p < 0.001$; compared with AFB_1 treated group.

或一週後，其PE值顯著的增加，其中以CCT效果最好。而連續處理一週者，其降低AFB_1毒性除CCT外均比處理24小時的效果顯著（表三），以上結果顯示除BCT在0.01 μg／mℓ之濃度外，其他Carotenoid derivatives在各種不同之濃度均可降低AFB_1對C3H10T½細胞的毒性。

AFB_1代謝活化性後會對細胞DNA產生損害作用，受損害的DNA在鹼性處理下會斷裂成單鏈的DNA，此單鏈DNA容易透過濾膜被沖洗（Elution）出來，利用此方法，我們研究Carotenoid derivatives（0.01 μg／mℓ～1.0 μg／mℓ）在降低AFB_1對細胞DNA產生傷害的能力，結果顯示被AFB_1傷害的DNA在S-9活化下達76%（無S-9活化下為42%），細胞若同時經Carotenoid derivatives及AFB_1在活化系統下處理24小時後，則DNA傷害度減少，被Eluted的DNA減少，尤其在有S-9活化作用下更顯著，其濃度在0.1 μg／mℓ～1.0 μg／mℓ，與AFB_1組比較P值均小於0.001～0.005，而CH及CCT在無活化系統下也有相同的作用（表四）。因此Carotenoid derivatives可能有抑制AFB_1攻擊DNA的能力，或是增加DNA修補作用。

利用標示的Thymidine（^{3}H-thymidine）為DNA合成的材料，從^{3}H-thymidine嵌入（incorporation）DNA的多寡可預知DNA合成的增加與減少，當AFB_1在S-9及無S-9活化作

Table 4. Protection of carotenoid derivatives on AFB_1 induced single strand DNA breaks in cultured C3H10T1/2 cells.

Treatment*	Dose (μ g/ ml)	% of DNA-eluted**	
		– (S–9)	+ (S–9)
Normal	0	6 ± 1	10 ± 2
DMSO	0.025 %	12 ± 2	15 ± 3
AFB_1	0.5	42 ± 4	76 ± 8
AFB_1 Plus:			
BCT	0.01	45 ± 5	53 ± 6
	0.1	40 ± 3	47 ± 6^b
	1.0	43 ± 7	32 ± 4^c
LCP	0.001	41 ± 5	65 ± 12
	0.01	44 ± 8	56 ± 8^a
	0.1	40 ± 4	41 ± 7^c
CH	0.01	28 ± 3^a	49 ± 7^b
	0.1	21 ± 4^c	32 ± 5^c
	1.0	17 ± 3^c	23 ± 5^d
CCT	0.01	50 ± 6	51 ± 7^b
	0.1	30 ± 2^c	47 ± 6^c
	1.0	19 ± 4^c	32 ± 6^c

* Cells were seeded $2x10^5$ cells/flask for 24 hr. The DNA labelled by adding 0.2 μ Ci ^{3}H-thymidine for 24hr. and non-radioactive medium incubated for an additional 24 hr., then exposed to carotenoid derivatives and AFB_1 for 4hr. DNA damage assay by alkaine elution method.

** % of DNA damage eluted ratio = $\frac{\text{eluted DNA}}{\text{eluted DNA + residues DNA}}$ x 100 %

$^a p < 0.05$; $^b p < 0.02$; $^c p < 0.01$; $^d p < 0.001$; compared with AFB_1 treated group.

用下，其^{3}H-thymidine嵌入的量比正常組少，表示DNA的合成受阻（表五），若添加Carotenoid derivatives在10.0 μg／mℓ時則^{3}H-thymidine嵌入DNA的量增加，此結果顯示Carotenoid衍生物可以減少AFB_1抑制DNA合成的作用。

上述的結果我們發現具維生素A活性的BCT其抑制AFB_1毒性作用較小，而不具維生素A活性的CCT，LCP，及CH其活性較大。

討論

由以上的結果顯示AFB_1（0.5 μg／mℓ）在S-9活性化系統下形成AFB_1-8，9-epoxide與細胞DNA共價結合，造成對C3H10T½細胞DNA產生傷害造成細胞毒性，而在4種Carotenoid衍生物存在下，均能減少DNA受損的程度，減少AFB_1產生的細胞毒性。但在不具有維生素A活性的CCT，CH及LCP比具維生素A活性的BCT有較強的抑制作用，顯示過去認

Table 5. Increased effect of carotenoid derivatives on DNA synthesis by treatment of AFB_1 in cultured C3H10T1/2 cell.

Treatment*	Dose (μg/ml)	without S–9		with S–9	
		cpm	% of AFB_1	cpm	% of AFB_1
Normal		2409 ± 132	–	2981 ± 251	
DMSO	0.025 %	2367 ± 155	–	2842 ± 201	–
AFB_1	0.5	1888 ± 123	100	2318 ± 367	100
AFB_1 plus:					
BCT	0.01	1823 ± 236	96	2368 ± 489	102
	0.1	2115 ± 107	112	2493 ± 290	107
	1.0	2139 ± 83[a]	113	2678 ± 127	116
LCP	0.001	1907 ± 314[c]	101	2367 ± 128	102
	0.1	2080 ± 185	110	2782 ± 511	119
	1.0	2102 ± 302	111	2838 ± 344	123
CH	0.01	1734 ± 157	91	2190 ± 172	95
	0.1	2019 ± 266	107	2929 ± 171	126
	1.0	2123 ± 120	112	3097 ± 96[a]	134
CCT	0.01	2193 ± 339	116	2246 ± 326	97
	0.1	2129 ± 198	113	2615 ± 510	112
	1.0	2434 ± 41[b]	129	2678 ± 176	116

* Cells were seeded $2x10^4$ cells/well for 24 hr. then 0.5 μ gml^{-1} of AFB_1, 0.01, 0.1, 1.0 μ gml^{-1} of carotenoids, 0.01 μ Ci ^{3}H-thymidine and S–9 with or without were added to the culture and incubated for an additional 6 hr. DNA synthesis is estimated by ^{3}H-thymidine incorporation as described in materials and methods. Values are the average of triplicate determinations.

[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$; compared with AFB_1-treated group.

為Carotenoid derivatives的抗癌化作用係因能轉化為維生素A[22]，而有相似於維生素A之作用[23]似乎不是主要的抑癌化途徑。

雖然許多研究顯示Retinoids在抗癌化及抗癌細胞之作用均遠大於Carotenoid derivatives，但值得重視的是Retinoids具有很大的肝毒性及副作用[24]，而在許多動物實驗中均顯示Carotenoid derivatives的低毒性作用[25,26]，因此這些天然存在的Carotenoid derivatives在抑制癌化的角色應更重要。BCT主要存在於綠黃色蔬菜，水果為Carotenoid derivatives的主要成份具有抗氧化作用及抑制癌化之作用[12,13,15,16,17]，CCT為梔子果實（Gardenia jasminodes）的主要成份，過去的研究顯示CCT能夠抑制受病毒感染引起雞腫瘤產生的作用[14]，我們最近的研究也證實其有抗癌細胞在老鼠體內的增生作用[27,28]及抑制化學致癌物誘導之致肝癌作用[29]，其乙醯化衍生物crocin heptacetate（CH）已被我們合成製備，其特性及抗癌活性已被發現[19]；LCP為蕃茄等水果類的主要成份，Petayev等[30]曾證實它能抑制由自由基產生的鏈連鎖反應，減少癌化的作用。

AFB_1代謝活化為AFB_1-8，9-epoxide後與DNA共價結合，雖然沒有直接的證據顯示此種共價結合與DNA受損之程度平行（因為大部份受損之DNA會被修補），但在in vivo及in vitro的試驗中顯示N-hydroxy-2-acetyla-

minofluorene與DNA共價結合後會造成DNA受損的程度是平行的[31]，在我們的研究中也顯示以AFB_1處理C3H10T½細胞，造成DNA的受損（以Alkaline elution測定），此種受損而未修補的DNA可能無法複製，因此我們發現處理AFB_1後，也造成C3H10T½細胞DNA的合成減少，因而造成AFB_1的細胞毒性，而以Carotenoid derivatives添加處理的實驗組則顯示AFB_1引起的DNA受損程度降低，增加了DNA的合成，減少了細胞毒性。

總之，BCT，CCT，LCP及乙醯化衍生物CH，均能抑制AFB_1在活性系統下引起的細胞毒性，其抑制細胞毒性的機制為這些Carotenoid衍生物減少AFB_1引起的DNA損害（或增加DNA修補作用），增加C3H10T½細胞DNA的合成，抑制了AFB_1引起之細胞毒性。

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Carotinoid Derivatives Inhibition of Aflatoxin B_1-induced DNA Damage and Cytotoxicity of C3H10T1/2 Cells in Vitro

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Cytotoxicity and DNA damage of aflatoxin B_1(AFB_1) on C3H10T1/2 embryo cells was suppressed by 4 carotenoid derivatives. Using the alkaline elution method, we found that the DNA damage of AFB_1-treated cells was inhibited by crocetin (CCT), crocin heptacetate (CH), lycopene (LCP) and beta-carotene (BCT).

The decrease in the cellular DNA damage induced by AFB_1 was dependent on the dose of carotenoid derivaties. In the studies to investigate the inhibition of AFB_1-cytotoxicity, we observed that the plating efficiency of C3H10T1/2 cells in S–9 activation system was suppressed effectively with carotenoid derivatives and those compounds also can suppress the inhibition of DNA synthesis caused by AFB_1. From these results, we suggest that the inhibitory effect of carotenoid derivatives on the AFB_1-cytotoxicity in C3-H10T1/2 cells might be due to the cells defense mechanisms that reduced the DNA damage or induced DNA repair system.

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