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行政院國家科學委員會補助專題研究計畫成果報告

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一、中文摘要

血清中高濃度的膽固醇與低密度脂蛋白已經被證實與粥狀動脈硬化之形成有關，此心血管疾病之人數在台灣,甚至於全世界已躍居所有疾病之冠。近年來有許多報導指出，低密度脂蛋白經氧化後產生的氧化性低密度脂蛋白(oxidized low density lipoprotein; ox-LDL) 在粥狀動脈硬化形成中參與了重要的角色。Cholesterol oxides，為膽固醇的氧化產物，經證實是導致粥狀動脈硬化之氧化性低密度脂蛋白的主要致毒成份。此類化合物在膽固醇分子上有 hydroxy- 或 keto-group，常見的有 7-OH-, 7-keto, 19-OH-, 22-OH- 和 25-OH-cholesterol 等.

Cholesterol oxides 已知在血管平滑肌與內皮細胞可造成程序性死亡 (programmed cell death; PCD)。在初步結果中我們也看到了 cholesterol oxides 可在培養之血管內皮細胞造成毒性作用，然而此種死亡模式與機制至今並不明瞭。 MAP kinase (Mitogen-Activated Protein kinases) 在程序性死亡是很重要的角色之一， MAP kinase pathway 包含了三種平行的路徑。ERK (p42/44, Extracellular signal-Regulated Kinase)，SAPK/JNK (p46, Stress-Activated Protein Kinase/c-Jun NH2-terminal Kinase) 與 p38 pathway。活化以上的每一條路徑都會牽涉到一系列上游以及下游物質的磷酸化過程。在此計劃中我們推測 MAP kinase 可能參與了這些 cholesterol oxides 在血管細胞所造成的死亡機制。我們將利用培養的血管平滑肌細胞以及內皮細胞來研究一些常見的膽固醇氧化物 7-OH-cholesterol 與 7-keto-cholesterol, 3β,5α,6β-trihydroxycholesterol 、5α,6α-epoxycholestan-3β-ol 等在這些細胞所導致的程序性死亡是否與 MAP kinase 之失調有關，同時釐清粒線體與 cytochrome c 的釋放是否參與在此路徑中，以及鈣離子在此所扮演的角色。

這些實驗可以讓我們進一步的瞭解氧化性膽固醇在血管細胞的致毒機轉，以及藥理性的改變是否能影響氧化性膽固醇在這些細胞所造成的死亡結果。期待能提供一些新的醫療方向用以避免氧化性膽固醇所導致的粥狀動脈硬化症。

關鍵詞：膽固醇氧化物、程序性死亡、MAP kinase、平滑肌細胞、內皮細胞，粥狀動脈硬化

Abstract

High level of serum cholesterol and low density lipoprotein (LDL) have been associated with the genesis of atherosclerosis, a leading cause of cardiovascular disease that affect a large number of people all over the world. It has became clear that the pathological conditions associated with escess level of LDL are actually caused by the oxidized products of LDL. Cholesterol oxides, the oxygenated derivatives of cholesterol, appear to be the major cytotoxic compound in oxidized LDL. These compounds have a hydroxy- or a keto-group on the cholesterol molecule. Example of cholesterol oxides include: 7-OH-, 7-keto-, 19-OH-, 22-OH- , 25-OH-cholesterol, 3 ,5 ,6 - trihydroxycholesterol 、5 ,6 -epoxycholestan-3 -ol.

There is evidence showing that programmed cell death (PCD) occurs in smooth muscle cells and endothelial cells

treated with cholesterol oxides. In our prelminary data also show cytotoxicity effect in cholesterol treated human unbilical endothelial cells. The detail mechanism of PCD undergoing the pathway is still unclear.

MAP kinase have very important role in PCD. The MAP kinase pathway consists 3 major parallel pathways designated as the ERK, SAPK/JNK and p38 pathways. Activation of each above involves phosphorylation of a number of upstream and dowmstream family members of the pathway. We hypothesize that a dysregulation of MAP kinases is responsible for the vascular cell death caused by cholesterol oxides. This studies is designed to determine the relationship between cholesterol induced PCD and MAP kinase activities. We will used 7-OH-cholesterol and 7-keto-cholesterol , 3 ,5 ,6 -trihydroxycholesterol,5 ,6 -e poxycholestan-3 -ol for this project because these compounds are the most abundant in animal tissues, and also are common among the most extensively studied cholesterol oxides in the field. We will used cultured human aortic smooth muscle cells and endothelial cells to test this hypothesis. The studies can clearlify the mechanism of cholesterol oxides induced apoptosis on vascular cells, and whether the pharmacological modification can change this pathway, providing a new way for preventing atherosclerosis formation in cardiovascular diseases.

Keywords: Cholesterol oxides, Low-Density-Lipoprotein, MAP kinase, Smooth Muscle Cells, Endothelial Cells, Atherosclerosis.

二、緣由與目的

研究目的

Cholesterol oxides，為膽固醇的氧化產物，經證實是導致粥狀動脈硬化之氧化性低密度脂蛋白(oxidized low density lipoprotein; ox-LDL) 的主要致毒成份。Cholesterol oxides 已知在血管平滑肌與內皮細胞可造成程序性死亡 (programmed cell death; PCD)。在初步結果中我們也看到了 cholesterol oxides 可在培養之血管內皮細胞造成毒性作用，然而此種死亡模式與機制至今並不明瞭，在此計劃中我們推測 MAP kinase 可能參與了這些 cholesterol oxides 在血管細胞所造成的死亡機制，為了證實我們的假設，設計了以下的實驗。

1. 我們將測試 cholesterol oxides 在培養之血管平滑肌細胞與內皮細胞所造成的毒性作用是否與活化 JNK、p38 MAP kinase 或抑制 ERK kinase 之活性有關。
2. 我們選擇幾種在動物體內常見且含量多的 cholesterol oxides, 7-keto-cholesterol 與 3β,5α,6β-trihydroxycholesterol 、5α,6α-epoxycholestan-3β-ol 為測試化合物。
3. Cholesterol oxides 處理後導致 MAP kinase 活化的結果，我們將會在每個不同的時間點做詳盡的觀察。
4. 我們亦將測試藥理性的干擾是否會改變細胞死亡的結果。
5. 許多證據顯示 cholesterol oxides 會在多種細胞導致外鈣的內流，在此我們也要討論鈣離子在此死亡途徑中所扮演的角色。

這些實驗可以讓我們進一步的瞭解氧化性膽固醇在血管細胞的致毒機轉，以及藥理性的改變是否能影響氧化性膽固醇在這些細胞所造成的死亡結果。這些發現或許能提供一些新的醫療方向用以避免氧化性膽固醇所導致的粥狀動脈硬化症。

研究背景

(1) 粥狀動脈硬化與低密度脂蛋白

血清中高濃度的膽固醇與低密度脂蛋白已經被證實與 (Atherosclerosis)之形成有關，此心血管疾病之人數在台灣,甚至於全世界已躍居所有疾病之冠。近年來有許多報導指出，低密度脂蛋白經氧化後產生的氧化性低密度脂蛋白(ox-LDL) 在粥狀

動脈硬化形成中參與了重要的角色[1,2]。在此複雜的過程中 ox-LDL 的生成可能是由於 LDL 沉積在血管壁，傷害內皮細胞層與巨噬細胞 (Macrophage)。Macrophage 的活化可以產生很多種自由基，氧化 LDL，生成許多具毒性的脂質過氧化產物 (主要為 ox-LDL 與 cholesterol oxides)。Ox-LDL 會誘發細胞膜的損傷、降低細胞的存活率、抑制血管內皮細胞對於受傷部位的修復能力以及增加細胞內鈣離子濃度的上升等。這些影響皆造成了細胞不可回復的損傷。

另外，cholesterol oxides，如前所述為 ox-LDL 的主要毒性產物 [3,4]。此類化合物在膽固醇分子上有 hydroxy- 或 keto-group。常見的有 7-OH-, 7-kito, 19-OH-, 22-OH- 和 25 OH-cholesterol、3β,5α,6β-trihydroxycholesterol、5α,6α-epoxycholestan-3β-ol [5] 等.

(2) 膽固醇氧化物 (Cholesterol oxides)

來源與吸收

Cholesterol oxides 可在含 cholesterol 食物 (奶粉、起司，蛋製品) 之儲存中形成 [6,7]，或是在動物體內經代謝產生 [8]。肉類製品之 cholesterol oxides 含量明顯的隨著烹調的情況而增加。例如，烹煮前後，雞腿肉內所含的 cholesterol oxides 可由 ~ 0.25 μg /g 增加至 ~5 μg/g [7]。食入 100 g (~ 1/4 pound) 之雞肉相當於食入 500 μg 之 cholesterol oxides。動物亦可經由與吸收 cholesterol 相同的途徑吸收 cholesterol oxides。外來之 cholesterol oxides 食入體內可鑲嵌入血漿脂蛋白中[9]，之後隨著血液循環至全身[10]。Cholesterol oxides 與 cholesterol 在細胞皆透過相同的機制輸送，只是 cholesterol 運送的效率較高 [11]。高濃度的 cholesterol oxides 亦可在高膽固醇血脂動物身上發現。例如，餵食高膽固醇食物六周之兔子，其血漿中 7-OH-cholesterol 之量會由 15 μM 增加至 200 μM [12]。高濃度之 cholesterol oxides 已被發現在實驗動物損傷血管組織 [13]。而此類化合物在血管細胞之致毒機制將是本計劃未來探討的重點。

體內合成與代謝

除了經食物攝取外，動物組織亦會產生內生性之 cholesterol oxides，例如，神經組織有轉化 cholesterol oxides 之能力。突觸體及粒線體皆可將 cholesterol 轉化成 cholesterol oxides，而其主要合成物 7-OH-與 7-Keto-cholesterol，在腦組織受到氧化性迫傷 (oxidative stress) 所導致之傷害上扮演了很重要的角色 [14]。中樞神經系統的神經與神經膠質亦含有酵素，可將 cholesterol oxides 代謝成其它的代謝物 [15]。另一方面，cholesterol oxides 是很強的 HMG-CoA 抑制劑，此為合成膽固醇之主要酵素 [16]。Cholesterol oxides 可以與細胞內接受體結合，形成 "oxysterol binding protein"，此種結合程度亦與抑制 cholesterol 之合成有關[17,18]。最近，於生理濃度下的 cholesterol oxides (1-10 μM 或 0.4-4 μg/ml) 發現可活化一些細胞核內接受體(nuclear receptor) 如 steroidogenic factor [19] 或 LXR 等 (可能與 cholesterol oxides 之生理活化有關) [20,21]。

Cholesterol oxides 與細胞死亡

In vitro

Cholesterol oxides 在很多細胞皆有毒性作用 [22]，此類化合物可傷害血管內皮細胞 [23-25], 血管平滑肌細胞 [26-28], 神經細胞 [29], 纖維組織母細胞[30]等，以上有些為構築血管壁的主成份。在這些細胞中, cholesterol oxides 所造成的毒性包括了 apoptosis [31-33]，降低內皮細胞的屏障功能 [34,35]、抑制內皮細胞 NO 的釋出 [36]，動脈血管的放鬆作用[37]以及活化 acyl Co A: cholesterol acyltransferase (ACAT) 之活性[38,39]。Cholesterol oxides 亦可抑制 trypan blue uptake[40], [^{3}H]thymidine incorportation into DNA 以及 DNA content [41], 降低膽固醇的合成、利用與輸送 [42,43]。加強白蛋白運輸至培養的內皮細胞[42,44]以及刺激鈣離子內流至細胞內等[45,46]。除此之外, oxysterols 也會影響細胞膜的通透性和穩定度 [47,48], 鑲嵌入細胞 [45,49]或血小板的膜上[50], 造成細胞膜內部的膨脹。抑制 $\triangle^5$ 與 $\triangle^9$ 非飽合脂肪酸之去飽和作用以及延長作用 [50]，促進 phosphatidylinositol 之合成與代謝 [51]，之後增加 sphingomylin

的含量等 [50,52]。比較特別的是, oxysterol 這些所有的作用皆伴隨著鈣離子內流至細胞的現象,最後導致細胞死亡 [49-52]。在細胞增殖的情況之下, 須要大量的cholesterol來構築細胞膜,因此缺乏 cholesterol 會導致細胞周期停滯在 G1 phase, 抑制 DNA 合成 [53]。從 cholesterol oxides 抑制 [^{3}H]mevalonolactone incorporation 至內皮細胞的結果亦證實了 oxysterols 抑制膽固醇合成的位置在 HMG-CoA reductase 之後 [54-56]

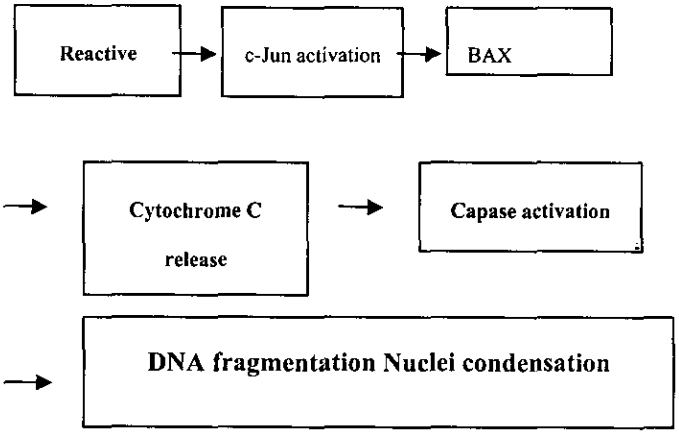
Cholesterol oxides 對於一些免疫系統衍生細胞亦有毒性作用，如 macrophage、thymocytes、lymphoma 細胞與 leukemic T-cell 等[32]。

In vivo

在活體動物實驗上, 亦證實了 oxysterol 在血管壁會有毒性作用, 高膽固醇酯血症病人、兔子之血漿中有高含量的氧化性膽固醇 [57-59]。直到最近才有人開始研究 oxysterol 在血管細胞造成 apoptosis 與 necrosis 的分子機制 [60-62]。Cholesterol oxides 在這些細胞導致毒性的作用模式目前正積極的研究中。有證據顯示在許多細胞上處理 cholesterol oxides 會導致細胞程續性死亡(programmed cell death; PCD)。目前大多數之研究仍然專注於心血管系統上的高血脂症與粥狀動脈硬化症。

(3) PCD and MAP kinase

PCD 即所謂的 "apoptosis"，是一種經由生長因子或毒物誘發出來的特殊細胞死亡型態[63-65]。以血管平滑肌或內皮細胞經 oxysterol 刺激導致 apoptosis 之作用為模式--細胞死亡發生過程從活性氧化物(reactive oxygen species)的生成開始，之後抑制了 DNA、RNA 與 protein 的生成，活化 immediate early protein, c-jun 和 cytochrome c 從粒線體釋出。活化了一系列的 protease 即"caspases"，如 caspase-3 (CPP32) 在很多類型的細胞反應了 apoptosis。形態學上，PCD bisbenzimide 或 propidium iodide 染色呈現或以 TUNEL 方法來 assay 透過 PCD 死亡的細胞，將其 DNA 抽取出來在瓊膠電泳片上通常會呈現出片段或 ladder 的現象。以下的簡圖歸納了血管細胞在行 PCD 時可能參與的重要因子。(Adapted from [32])



MAP kinase (Mitogen-Activated Protein kinases) 在 PCD 是很重要的角色之一，MAP kinase pathway 包含了三種平行的路徑。ERK (p42/44, Extracellular signal-Regulated Kinase)，SAPK/JNK (p46, Stress-Activated Protein Kinase/c-Jun NH2-terminal Kinase) 與 p38 pathway。活化以上的每一條路徑都會牽涉到一系列上游以及下游物質的磷酸化過程(phosphorylation)。例如，活化 JNK 路徑會造成 JNK 與 JNK 上游物質(如, SEK-1) 或下游物質 (如 c-jun 或 ATF-2)的磷酸化。這些下游物質屬於一個可起動轉譯活性轉譯因子家族(AP-1)[66,67]。

(4) PCD and cholesterol oxides toxicity

PCD 在許多類型的細胞處理 cholesterol oxides 時皆可看到，有研究指出將 7-keto-cholesterol (12 μg/ml, 30 μM) 處理培養之血管平滑肌細胞 24 小時後會造成 DNA fragmentation 之現象。當加入 caspase 抑制劑時則可避免 DNA fragmentation。基於這些觀察，研究者認為此細胞之死亡是行 PCD 路徑。除了血管平滑肌細胞，血管內皮細胞在 cholesterol oxides 之處理下亦行 apoptosis [68]。綜合言之，cholesterol oxides 造成細胞死亡的機制仍需進一步探討。此外，必需留意的是 cholesterol oxides 在 cholesterol 生成具有抑制作用，此時即

使外加 cholesterol 也無法抑制細胞的死亡，此結果告訴我們抑制 cholesterol 之生成與造成 PCD 無關[69]。可能是由於 ERK、JNK 與 p38 之不協調所造成。**本計劃的目的便是要證明這個假說。**

以下是目前已知有關 cholesterol oxides 造成細胞毒性與 atherosclerosis 間之關係

1. Cholesterol oxides 為 cholesterol 之代謝物，且含量隨著食物烹煮之程度而增加，也會內生性的由動物組織產生。
2. Cholesterol oxides 與 ox-LDL 影響 atherosclerogenic 之過程有關，他們可在血管壁細胞造成 PCD。
3. 雖然在神經細胞已有些證據指向 cholesterol oxides 可能影響 MAP kinase fanmily [32]，但至目前為止並沒有任何證據可證實 MAP kinase 與 cholesterol oxides 在血管細胞 (smooth muscle cell and endothelial cell) 造成之毒性作用之關聯。

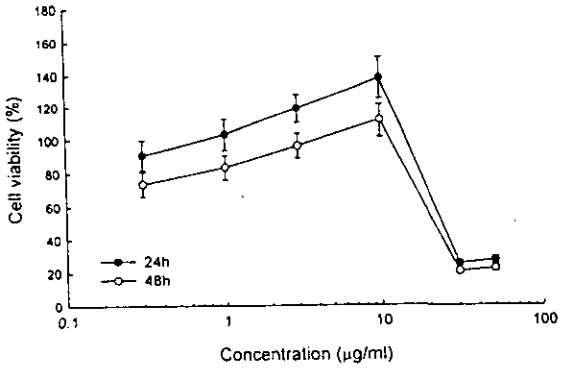
三、結果與討論

實驗結果

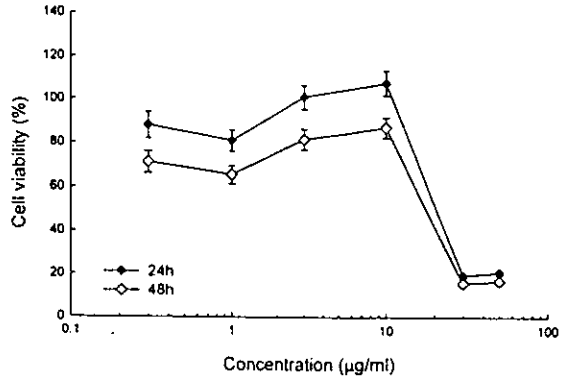
Cholesterol oxides induced cytotoxicity on HUVEC (Human Umbilical Vain Endothelial Cell)

(1) MTT-test

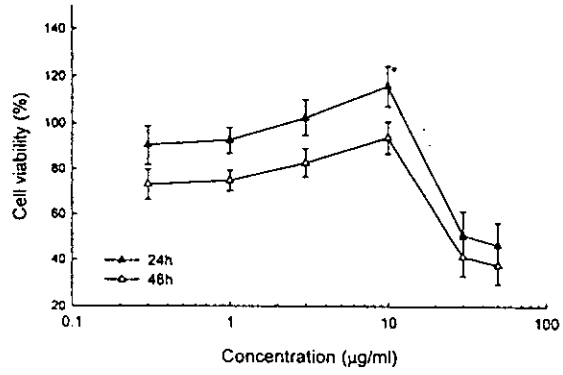
a) Effect of 7-ketocholesterol on HUVEC



b) Effect of 3β,5α,6β-trihydroxycholesterol on HUVEC

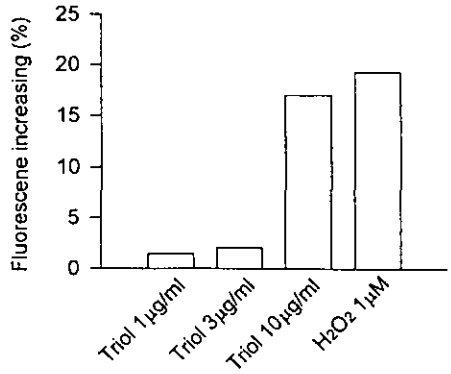


c) Effect of 5α,6α-epoxycholestan-3β-ol on HUVEC



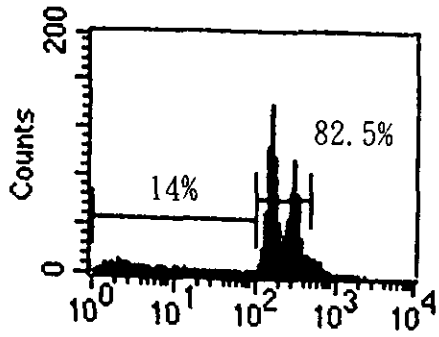
(2) Reactive Oxygen Species

Effect of 3β,5α,6β-trihydroxycholestero induced ROS production on HUVEC (HUVEC were co-treated with DCFH-DA and triol for 2 hrs, detected by flow cytometry)

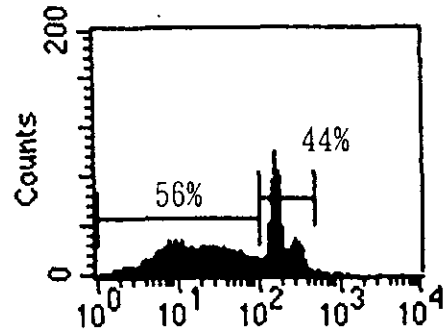


(3) Propidium iodium staining of apoptic nuclei DNA

a) Control

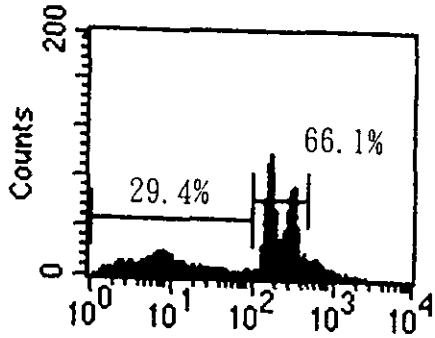


Triol 50μg/ml

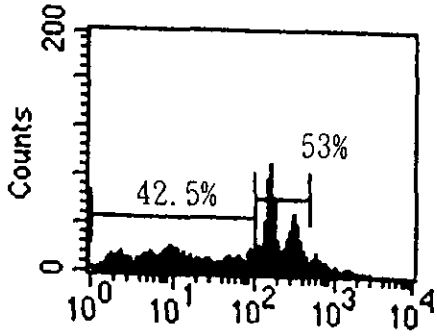


b) 7-ketocholesterol

7-keto 10μg/ml

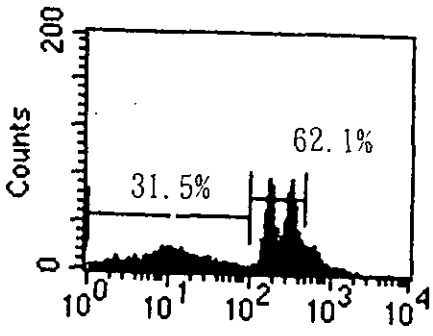


7-keto 50μg/ml



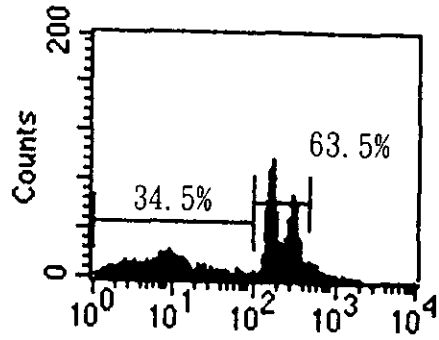
c) 3β,5α,6β-trihydroxycholesterol

Triol 10μg/ml

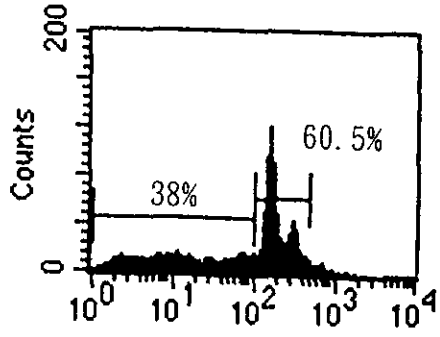


d) 5α,6α-epoxycholestan-3β-ol

α-epoxide 10μg/ml



α-epoxide 50μg/ml



實驗結果

1. 從 MTT data 顯示，三種膽固醇氧化物（7-ketocholesterol, 3β,5α,6β-trihydroxy-cholesterol, 5α,6α-epoxycholestan-3β-ol 皆會直接對內皮細胞造成毒性作用，此實驗結果證實了在血液中自然可生成的氧化性膽固醇的確會對內皮細胞造成毒性作用。

2. 從活性氧自由基生成的實驗結果證實了，三種固醇類氧化物終只有 3β,5α,6β-trihydroxycholesterol, 所導致的細胞毒性作用是透過產生活性氧自由基。

3. 此外，利用 propidium iodium staining of apoptic nuclei DNA 實驗數據顯示此三種固醇類氧化物皆具有程度不同的對內皮細胞造成 apoptosis 之作用，其中以可產生活性氧自由基之 3β,5α,6β- trihydroxy-cholesterol 最明顯。

4. 實驗之結果尚未完成，其中尚有許多蛋白質與酵素量之表現是否受到影響的數據仍在確定中，將繼續探討此方面之結果直至確定的機制出現可合理的推測本計畫的結論。

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