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行政院國家科學委員會專題研究計畫成果報告

數種抗氧化劑影響氧化低密度脂蛋白及發炎細胞激素對內皮與巨噬細胞誘發 Matrix metalloproteinase 活性之探討

The studies of various antioxidants influence on oxidized LDL- or inflammatory cytokines-induced the activation of matrix metalloproteinase in endothelial cells and macrophages

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*主持人：蕭哲志　台北醫學大學醫學系藥理科

共同主持人：許準榕　台北醫學大學醫學系藥理科

*E-mail address: geohsiao@tmu.edu.tw

一、　中文摘要

基質金屬酵素在粥狀動脈硬化症所引發之血管內斑塊剝離上扮演相當重要的角色。本計畫之目的在探討抗氧化劑是否能影響細胞激素或氧化型人類低密度脂蛋白所誘發細胞之基質金屬酵素的表現與活化。從實驗結果發現，的確細胞激素（如 TNFα）能使單核球細胞引發大量基質金屬酵素的表現與活化。尤其第九型基質金屬酵素的表現特別明顯。在分析篩選許多抗氧化劑後，我們發現兩個有效成分，其中 Trolox 不僅能抑制此基質金屬酵素的分解作用，也能減低其蛋白表現。另外，Brazilin 僅能抑制此基質金屬酵素之分解活性。而這些抑制作用不是源自於細胞傷害。特別的是，未刺激之人類臍帶靜脈內皮細胞能持續釋放多量的第二型基質金屬酵素。細胞激素(TNFα)或氧化型人類低密度脂蛋白僅些微增加此第二型基質金屬酵素之釋放與活化。然而抗氧化劑中只有 N-acetylcysteine 能部分抑制此細胞基質金屬酵素分解作用。從這些結果可提供有關抗氧化劑對不同血管性細胞基質金屬酵素之作用性，以利臨床上穩定血管(vascular stability)之應用。

關鍵詞：基質金屬蛋白酵素、抗氧化劑、單核球、內皮細胞

Abstract

Matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of atherosclerotic plaque destabilization, a disorder characterized by vascular chronic inflammation and destruction of connective tissues. The purpose of this study was to determine if antioxidants might inhibit the cytokine- or oxLDL-induced MMPs activation, and thus represents an attractive therapeutic target. Exposure monocyte (THP-1) to TNFα (10 ng/ml) increased MMP-9 protein expression as measured by

ELISA and gelatinolytic activity as determined by zymography. We found that treatment with Trolox (50 μM), decreased not only MMP-9 gelatinolytic activity but also gelatinase expression. However, Brazilin merely inhibited gelatinolytic activity. These inhibitory actions of antioxidants were not mediated by reduction of cellular viability. Differently, non-stimulated HUVEC could expressed much amount of MMP-2. Such constitutve expression of MMP-2 was slightly enhanced by TNFα or oxLDL. However, only N-acetylcysteine could partially reduced inducible MMP-2 expression. These data demonstrate that even same stimulator might induce different MMPs from different cells. Furthermore, some antioxidants abrogate MMP-9 or MMP-2 expression. This newly described action of antioxidant therapy might prove useful to inhibit matrix degradation and to improve vascular stability.

Keywords: matrix metalloproteinse, antioxidant, monocyte, endothelial cell

二、緣由與目的

Matrix metalloproteinses (MMPs)為一群結構類似含鋅(Zinc ion)金屬離子之蛋白酵素, 它們能夠催化分解維持組織結構之細胞外基質蛋白(extracellular matrix proteins, ECM)，如collagen、elastin 及 lamin 等。因此對於組織之結構重組(remodeling)、修補(repairing)與破壞(destroy)都具有相當重要之角色(1)。同時 MMP 的含量與活性表現均受到許多方式嚴密地調節控制(2)。

近年許多文獻指出氧化變性之低密度脂蛋白(oxidized low-density lipoprotein)或發炎性細胞激素(inflammatory cytokines)均能刺激活化單核球或巨噬相關細胞局部釋放分解結締基質之 matrix metalloproteinase (MMPs), 使得纖維帽(fibrous cap)內纖維結構易於鬆脫(weaken), 呈現不穩定(instability)狀態, 進而容易剝離而造成血栓(3,4)。另外血管斑塊表面內皮細胞之剝蝕脫落(endothelial erosion)隨後血栓形成, 同樣地也可藉由不正常增加 MMPs 之釋放與活化所造成(5,6)。其他如癌細胞轉移(tumor cell invasion)、關節炎(arthritis) 等致病機理皆與 MMP 的生成(production) 及活化(activation)關係密切。

根據最近研究報告指出在 ultraviolet B 與鐵離子(7)、過氧化氫或脂過氧化物(8)處理下所造成之氧化迫傷(oxidative stress), 均引起纖維母細胞(fibroblast)增加某類 MMP 產生。相同地，巨噬細胞衍生之泡沫細胞釋出的過氧化氫的確也能活化血管內 proMMP-2 與-9 (9), 並也可提高纖維母細胞之 MMP-1 產生及釋出。抗氧化劑(antioxidant)如 *N*-acetylcysteine 可抑制軟骨結締組織之基質異常性崩解(10)。另外對於巨噬細胞衍生之泡沫細胞或血管組織所增加之基質崩解作用(包括 MMP 之產量與活化程度), 此抗氧化劑都具有相當明顯之抑制程度(11)。

因此開發並了解 antioxidant 影響發炎性細胞激素或氧化低密度脂蛋白

對內皮或單核球（巨噬細胞）產生與活化 MMP 之作用機轉，可能為粥狀動脈硬化血管剝離預防及治療上(plaque stabilization therapy)之新方向。

三、結果與討論

首先為設立電泳酵素分析法(Zymography)與刺激物（如 TNFα）引發 MMP 之實驗標準條件。因此最初實驗，以不同濃度之 TNFα（5, 10 及 20 ng/ml）刺激不同細胞濃度之人類單核球細胞（THP-1）。由於巨噬細胞源自於單核球細胞，而人類單核球細胞（THP-1）目前廣泛為學界研究 MMP 所應用。隨後在不同時間下，離心收集培養液，並進行電泳酵素分析法。我們發現以 TNFα 刺激 24 小時後，隨 TNFα 濃度提升，培養液分解 gelatin 之效果愈強，但 TNFα刺激濃度 10 與 20 ng/ml 並無明顯差異。而且在 TNFα 刺激下，細胞濃度以 2×10^6/ml 效果最適切。另外實驗也進行 12 小時及 48 小時，結果顯示實驗均以 24 小時的刺激為佳。由電泳酵素分析圖更顯示單核球(THP-1)分解 gelatin 主要以 92 kDa 之 MMP-9 為主，而 72 kDa 之 MMP-2 為次要（圖一）。

關於抗氧化劑或相關成分對於細胞 MMP 等之作用，我們先以多種成分（PDTC, Trolox, N-acetylcysteine, Brazilin 等）進行篩選實驗。另外以 PMA（positive control）及 Doxycycline 或 Minocycline（negative control）進行相關實驗。在測試成分相同濃度下（50 μM），實驗發現 TNFα 刺激單核球細胞所引起之 gelatin 崩解作用，尤其是 MMP-9 之分解反應，可明顯且有意義被 Trolox 或 Brazilin 所部分抑制且完全被 Doxycycline 所抑制（圖一、二）。另外，在同樣實驗條件下，PMA 的確可引起大量 MMP-9 崩解反應，尤其活化型(active form)明顯出現。測試成分 PDTC 與 Doxycycline 均可部分抑制 MMP-2 引起的 gelatin 崩解反應(圖一)。

由人類臍帶靜脈內皮細胞（HUVEC）的電泳酵素分析實驗結果知，HUVEC 在未受刺激時，便會釋放大量 72 kDa 之 MMP-2 與少量 92 kDa 之 MMP-9（圖三、四），此結果與其它相關文獻一致。當以 PMA 刺激下，除了 MMP-2 會增加反應與其活化型(active form)出現外，MMP-9 也同時會明顯產生作用，但仍以 MMP-2 為主（圖三、四）。若以 TNFα刺激 HUVEC 時，並不會明顯促進 MMP-9 或 MMP-2 之產生與作用。Bazilin, Trolox, PDTC 及 Minocycline 不影響 MMP-2 之作用，而 NAC (50 μM)會部分減低其作用（圖三）。另外以 Cu^+氧化 LDL 生成的 oxLDL，不論氧化時間是 12 小時或 24 小時均僅些微增加 MMP-2 與 MMP-9 之崩解作用。且其中僅 NAC (50 μM)有些微抑制作用，其它成分都沒明顯作用（圖四）。

另外利用 ELISA 方法，直接測定 proMMP-9 之含量。由初步實驗成果顯示以 TNFα 或 PMA 刺激單核球細胞皆可促使 proMMP-9 增加。多種成分（如 Trolox 與 Doxycycline）在 50 μM 均可抑制 proMMP-9 之釋放量，但其它成分卻無抑制效果（圖五；初步實驗，n 數不足，未加以統計分析）。為確定測試成分抑制之效果非源自於細胞傷害，所以也利用 MTT 方法進行細胞存活實驗。不論是單核球或內皮細

胞，從結果顯示測試成分在高濃度（100 μM）及 PMA（16 nM）時，僅 PDTC 降低還原量，而其它成分不具有細胞致毒作用（圖六）。但有趣的是 PDTC 處理之內皮細胞幾乎緊縮未懸浮，是否造成細胞死亡，仍須再以 LDH 方式確認，因為 PDTC 卻不影響 MMPs 之作用。

由實驗結果了解，以相同成分 TNFα 刺激，不同細胞則有不同的 MMP 表現反應。我們目前實驗正進行藥效成分的作用-濃度曲線(如 Trolox、Brazilin 等)，也篩選其它是否有如 Doxycycline 般的強效成分，同時也進行有效成分機轉之探討。至少由 gelatin 分解與初步 proMMP-9 含量之結果，我們推測 Doxycycline 與 Trolox 可能直接影響 MMP-9 生成，而 Brazilin 僅影響 MMP-9 之活化過程。當然下一步有關 TIMP 之定量也將進行。另外我們也正嘗試其它 Cytokine 以了解對 HUVEC 的誘發 MMP 作用。重要的是有關 oxLDL 誘發 HUVEC 之 MMP 表現，此結果不儘理想。我們將嘗試其它氧化方式(如 methemoglobin 氧化方式)，以獲得較佳之 MMP 表現。

四、計畫成果自評

由於此研究計劃為本人第一次取得之國科會研究計劃（共九個月），相關之研究設備比較缺乏。因此藉用其它單位的儀器進行實驗時較為不便。加上本計劃唯一購置之儀器（倒立顯微鏡附相機）開標與到達的時間較預期晚，造成細胞相關實驗不便並影響實驗進度。然而本實驗室仍努力設立了兩種細胞的培養，其中包括人類初級細胞與細胞株（內皮細胞與單核球細胞）。同時也設立了電泳酵素分析法與 MMP 之 ELISA 定量法。篩選許多的成分，也將進行有效成分機轉之探討。但對於 oxLDL 誘發 HUVEC 之 MMP 表現，此結果不儘理想。目前將嘗試其它氧化方式(如 methemoglobin 氧化方式)，以獲得較佳之 MMP 表現，以進行藥物或相關機轉的探討。

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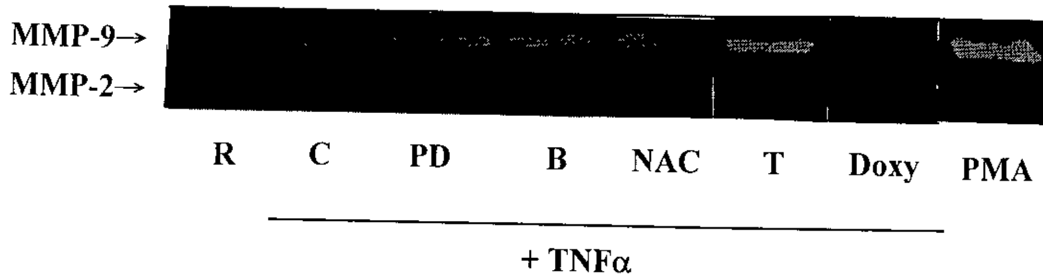

Figure 1. Effects of antioxidants on TNFα (10 ng/ml)-induced MMP-9 secretion by monocytes (THP-1 cells). THP-1 cells (2 106/ml) were incubated with antioxidants for 30 min, then stimulated with TNFα for 24 hours. The cell supernatants were then removed, added to the SDS sample buffer and incubated for 10 min at room temperature, then analyzed by zymography on 10 % acrylamide-0.1 % gelatin gels. The top row of bands represents the 92-kDa MMP-9, and the bottom row is the 72-kDa MMP-2. Lane 1, untreated THP-1 cells (R); lane 2, THP-1 cells treated with TNFα (C); lane 3, lane 4, lane 5, lane 6 and lane 7, THP-1 cells treated with PDTC (PD, 50 μM), Brazilin (B, 50 μM), N-acetylcysteine (NAC, 50 μM), Trolox (T, 50 μM) and Doxycycline (Doxy, 50 μM), before TNFα treatment, respectively; lane 8, THP-1 cells treated with PMA. The zymogram shown is from one experiment that is representative of three separate experiments.

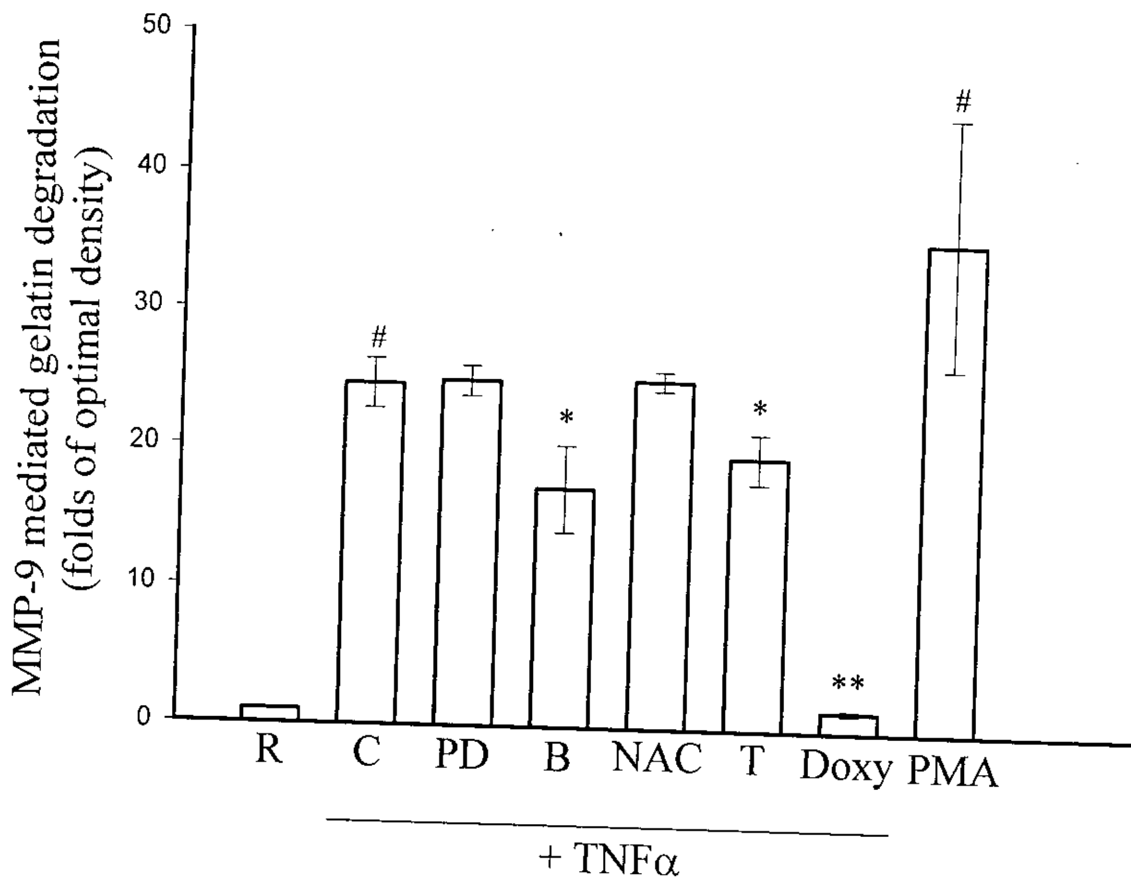

Figure 2. Effects of antioxidants on gelatinolytic activity in culture media of TNFα (10 ng/ml)-induced THP-1 activation. Densitometric analysis of gelatinolytic activity (MMP-9) released by TNFα-induced THP-1 cell activation for 24 hours after treatment with antioxidants or PMA, such as PDTC (PD, 50 μM), Brazilin (B, 50 μM), N-acetylcysteine (NAC, 50 μM), Trolox (T, 50 μM), Doxycycline (Doxy, 50 μM) and PMA (50 μM), respectively. Data are expressed as mean ± S.E.M. (n = 3), $^{*}p < 0.05$ and $^{**}p < 0.001$ vs. TNFα + DMSO; $^{\#}p < 0.001$ vs. resting (R, DMSO).

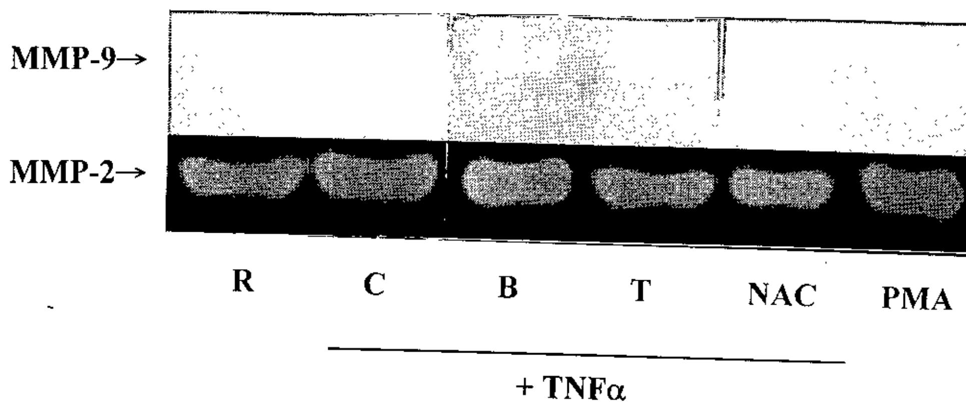

Figure 3. Effects of antioxidants on TNFα (10 ng/ml)-induced MMPs secretion by HUVEC. Almost 80-90 % of confluent cells were incubated with antioxidants for 30 min, then stimulated with TNFα for 24 hours. The cell supernatants were then removed, added to SDS sample buffer and incubated for 10 min at room temperature, then analyzed by zymography on 10 % acrylamide-0.1 % gelatin gels. The top row of bands represents the 92-kDa MMP-9, and the bottom row is the 72-kDa MMP-2. Lane 1, untreated HUVEC cells (R); lane 2, HUVEC cells treated with TNFα (C); lane 3, lane 4 and lane 5, HUVEC cells treated with Brazilin (B, 50 μM), Trolox (T, 50 μM) and N-acetylcysteine (NAC, 50 μM), before TNFα treatment, respectively; lane 6, HUVEC cells treated with PMA. The zymogram shown is from one experiment that is representative of three separate experiments.

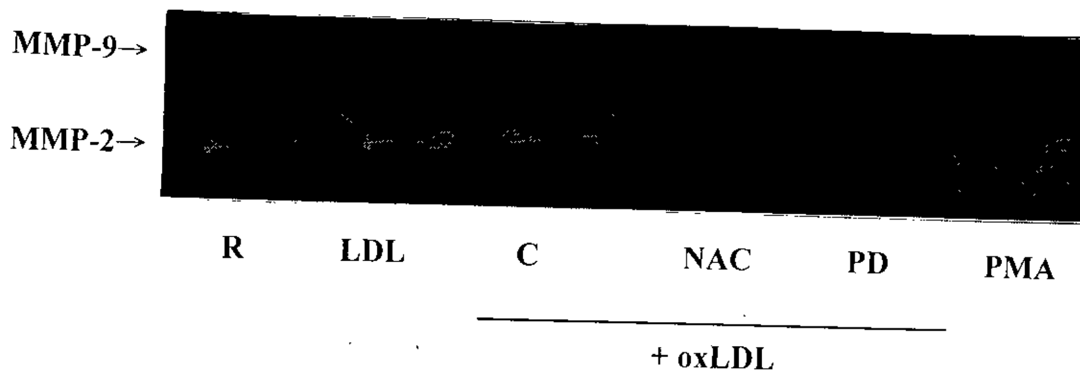

Figure 4. Effects of antioxidants on oxLDL (50 μg/ml)-induced MMPs secretion by HUVEC. Almost 80-90 % of confluent cells were incubated with antioxidants for 30 min, then stimulated with oxLDL for 24 hours. The oxLDLs were freshly prepared from 12 hr-Cu^{+} oxidation. The cell supernatants were then removed, added to SDS sample buffer and incubated for 10 min at room temperature, then analyzed by zymography on 10 % acrylamide-0.1 % gelatin gels. The top row of bands represents the 92-kDa MMP-9, and the bottom row is the 72-kDa MMP-2. Lane 1, untreated HUVEC cells (R); lane 2, HUVEC cells treated with native LDL (LDL); lane 3, HUVEC cells treated with oxLDL (C); lane 4 and lane 5, HUVEC cells treated with N-acetylcysteine (NAC, 50 μM) and PDTC (PD, 50 μM), before oxLDL treatment, respectively; lane 6, HUVEC cells treated with PMA. The zymogram shown is from one experiment that is representative of three separate experiments.

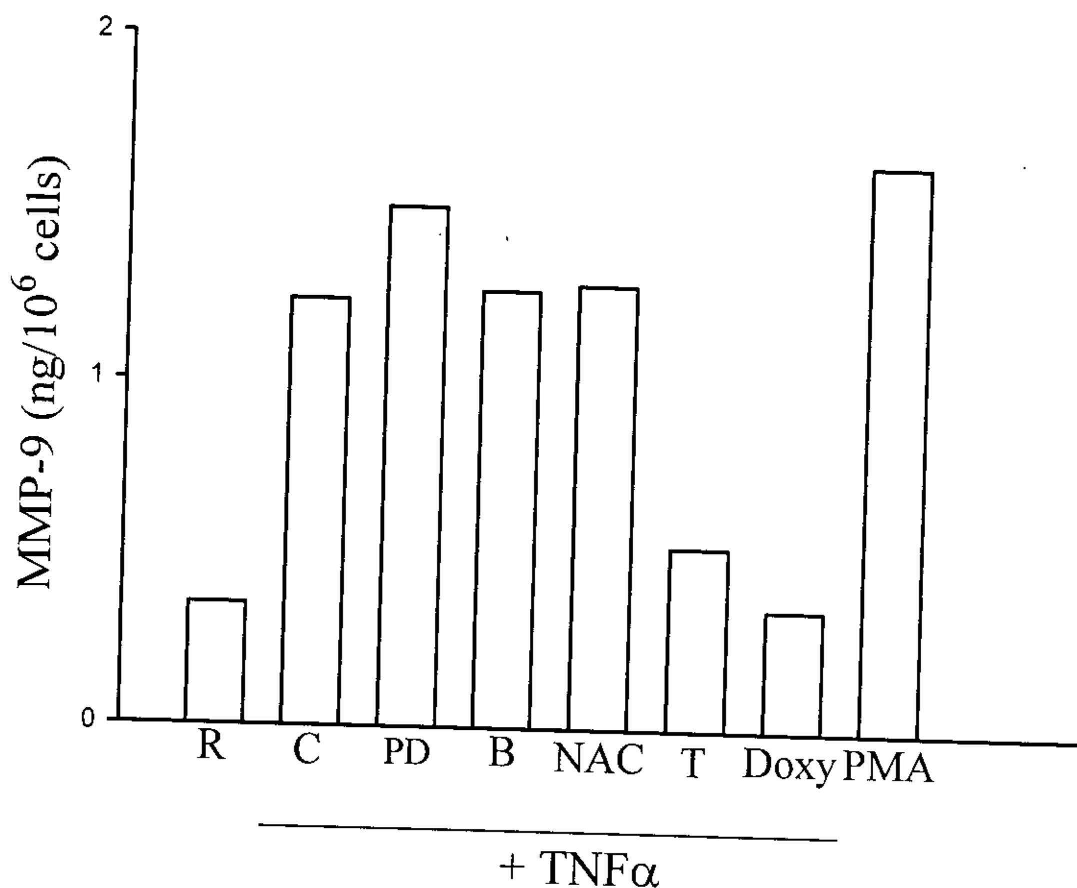

Figure 5. Effects of antioxidants on TNFα (10 ng/ml)-induced MMP-9 expression of THP-1 cells. THP-1 cells were treated with antioxidants for 30 min, then stimulated with TNFα (10 ng/ml). Culture medium was collected at 24 hours for measurement of MMP-9 protein expression by ELISA. Untreated THP-1 cells (R). Before TNFα treatment, THP-1 cells were treated with DMSO (C), PDTC (PD, 50 μM), Brazilin (B, 50 μM), N-acetylcysteine (NAC, 50 μM), Trolox (T, 50 μM) Doxycycline (Doxy, 50 μM) or PMA (50 μM), respectively (n = 2-3).

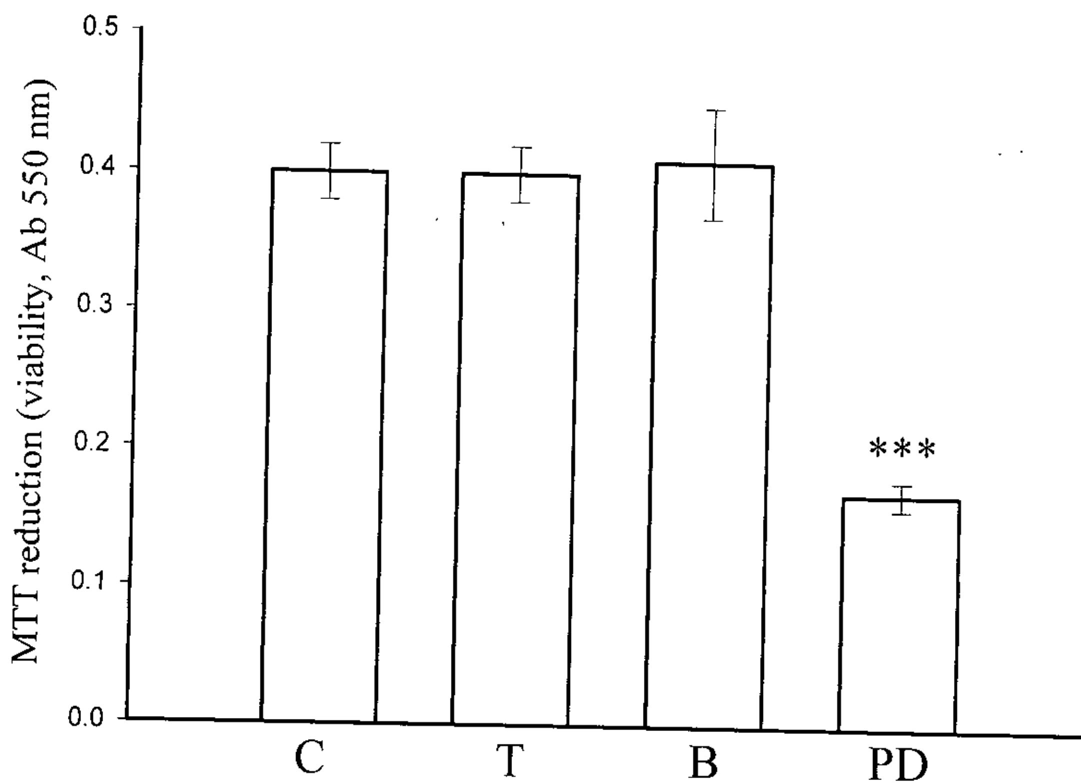

Figure 6. Effects of antioxidants on cellular viability. HUVEC were 80-90 % confluent and treated with antioxidants (Trolox, T; Brazilin, B; PDTC, PD) for 22 hours, then changed medium to MMT (final concentration, 0.5 mg/ml) medium. After 2 hours, cells were washed and dissolved with DMSO. Using the spectrophotometric method to analyze the absorption at 550 nm. Absorption values were expressed as mean ± S.E.M. (n = 3), $^{***}p < 0.001$ vs. treated with DMSO (0.025 %, C).