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私立中山醫學院生物化學研究所
碩士論文

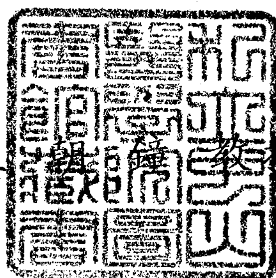
Master Thesis,Institute of Biochemistry, Chung- Shan Medical and Dental College

I.鼠體內亞硝基反應對 AAF 致肝癌作用之影響以及活化致癌基因的角色

Potential effect of sodium nitrite on the expression of nuclear proto-oncogenes during 2-acetylamino-fluorene-induced hepatocarcinogenesis in rats

II.原兒茶酸對鼠體內 LPS 所誘發內生性 NO 產生和 NO-AAF 形成的抑制作用

Inhibition by *Hibiscus* protocatechuic acid of Lipopolysaccharide-induced endogenous Nitric Oxide production and NO-AAF formation in rats



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授權書

（博碩士論文）

本授權書所授權之論文為本人在 中山醫學院 生物化學研究所
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論文名稱：I.鼠體內亞硝基反應對AAF致肝癌作用之影響以及活化致癌基因的角色
II.原兒茶酸對鼠體內LPS所誘發內生性NO產生和NO-AAF形成的抑制作用

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誌　謝

在未進入實驗室之前常常聽老師說 :"今年的Nobel prize 醫學獎在xx期的Science發表了,xx教授在Nature上發表了一篇文章,Cell在SCI的排名是前幾名......................等等 ",眞的是滿頭霧水,不知所云。等到自己進了實驗室之後才發現原來研究的工作並不是那麼容易進行,往往十次的結果,八九次並非我所預期的,此刻在我內心眞正的佩服整天待在實驗室從事研究的學者專家,要有好文章才能被好雜誌刊登,這期間不知經過多少次的失敗與挫折?感觸多多。

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第一部份

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縮寫字

AAF: 2-acetylaminofluorene.

N-NO-AAF: N-nitroso-2-acetylaminofluorene.

DAB: diaminobenzidine.

索引字

2-acetylaminofluorene.

Sodium nitrite.

Hepatocarcinogenesis.

c-Fos.

c-Jun.

c-Myc.

中文摘要

乙醯胺基二苯駢伍圜(2-Acetylaminofluorene簡稱2-AAF) 加上 sodium nitrite, 在酸性狀態下，經nitrosation會產生亞硝基乙醯胺基二苯駢伍圜(N-nitroso-2-acetyl-aminofluorene 簡稱 N-NO-AAF), 過去已經證實其基因毒性和致突變性比 2-AAF 更強。在本篇研究,爲了証明 $NaNO_2$可能促進AAF致肝癌之作用,利用大白鼠同時餵AAF與$NaNO_2$, 來觀察$NaNO_2$對AAF導致肝腫瘤生成的影響。因此本研究，將Wistar雄性大白鼠分成5組:第1組做對照組(control),第2組餵予含0.3%亞硝酸鈉($NaNO_2$)飼料，第3組餵予含0.02% AAF之飼料，第4組餵予含0.02 % AAF加上0.2% $NaNO_2$ 之飼料，第5組餵予含 0.02% AAF加上0.3% $NaNO_2$之飼料，爲期12週。

實驗結果發現第3,4,5組的大白鼠皆發生早期肝癌,包括大小不同病灶的肝腫大和新生的結節。大白鼠餵食AAF加$NaNO_2$會誘發肝細胞癌之生成，但是不同劑量的$NaNO_2$對AAF致肝癌生成有不同的影響，較高劑量之$NaNO_2$有顯著的促進AAF致肝癌之作用，但$NaNO_2$本身並不會有致肝癌作用。

當大白鼠餵食 0.02% AAF三個月後，在鼠肝内的c-Fos,c-Jun, c-Myc蛋白量會上升。 然而由AAF誘發的c-Fos,c-Jun,c-Myc的表現，會因加入$NaNO_2$而有意義的增高($p<0.001$)。這樣的結果證實了 $NaNO_2$ 加強 AAF誘發肝癌生成的初期變化是經由促進誘發c-Jun, c-Fos, c-Myc表現量的增加。

Abstract

2-acetylaminofluorene (AAF) reacts in acidic conditions with nitrous fume yielding N-nitroso-AAF (N-NO-AAF),as previously described,that exerts more toxic and mutagenic effects than its parental compound. In this study,the effect of sodium nitrite ($NaNO_2$) on the tumorgenicity of AAF in rats fed with AAF and $NaNO_2$ was observed. Wistar rats were divided into five groups: group I served as control; group II treated with $NaNO_2$ (0.3%), group III was given 0.02% AAF alone; group IV and group V received both AAF and $NaNO_2$ (0.2 and 0.3% respectively) in diet for 12 weeks. At the end of experimental, all rats in group III, IV and V developed early stage phenomena of hepatocellular carcinoma, including hepatomegaly with variable-size foci and neoplastic nodules. Severe damages were observed in the rats treated with AAF and $NaNO_2$. Feeding with AAF (0.02%) for 3 months elevated the levels of c-Jun,c-Fos and c-Myc proteins in the rats livers.The AAF-induced c-Jun,c-Fos and c-Myc expressions were significantly magnified ($p<0.001$) by $NaNO_2$. These data confirmed that the strengthening of AAF-induced hepatocarcinogenesis by $NaNO_2$ should be associated with its enhancing effect on the AAF-induced increases in the expressions of c-Jun,c-Fos and c-Myc.

Key Words: 2-acetylaminofluorene,
Hepatocarcinogenesis,
Sodium nitrite,
c-Jun, c-Fos, c-Myc.

Abbreviations:AAF, 2-acetylaminofluorene;
N-NO-AAF,N-nitroso-2-acetylaminofluorene
DAB, diaminobenzidine.

實驗的動機與目的

硝酸鹽是一種普遍存在蔬菜(如甘藍,萵苣,菠菜)的化合物。當這些蔬菜食品在人類的上消化道吸收以後, 硝酸鹽便由血液到達唾液腺(salivary glands) , 並且在唾液(saliva)中被還原成爲亞硝酸鹽。另外在許多肉類加工的製品(如肉干,臘肉,香腸…),都會以亞硝酸鹽當做防腐劑,避免肉品腐敗。而且這些蔬菜以及肉類製品又是日常生活中天天都會吃到的東西,就像人類三餐吃飯一樣。然而2-acetylaminofluorene(2-AAF)是一種殺蟲劑(insecticide),如果蔬菜水果噴灑了這種農藥殺蟲劑,而且沒有半衰期結束後就採收上市,如此造成農藥殘留。當亞硝酸鹽進入人體的胃中後,在胃液的強酸環境中和蔬菜水果殘留的殺蟲劑(AAF)可以產生亞硝基化反應(nitrosation),而形成N-NO-AAF。由1988年 Lin & Kuo所發表的論文可以知道N-NO-AAF是direct-acting mutagen and teratogen,因此人類在這種長期慢性的暴露下(即所謂chronic exprosure),是否會引起肝癌的病變?正是本篇論文所要研究的動機與目的。

壹. 前 言

許多具有致癌作用的亞硝基化合物,在周圍環境中包括食物。已經在過去的研究報告中發現食物中的亞硝酸鹽在酸性的胃液環境內會生成內生性致癌物,(1,2)可能是某些特定癌症的危險因子,特別是胃癌和食道癌(3,4)。事實上,在體內形成亞硝酸胺曾經證明會對實驗動物造成癌症(5,6)。以及在食物中加入亞硝酸鹽和許多致癌物,對腫瘤的形成有加強作用(7-10)。另一方面,幾個亞硝酸化合物,對各種不同屬動物的癌症扮演著致癌原因的角色(11,12)。

AAF最常用來當作模式化合物(model compounds)研究芳香醯胺和胺(arylamides and amines)的代謝及致癌作用(13,14). AAF發現有代謝活化作用,包括各種不同的生化反應諸如:氮-氫氧化(N-hydroxylation),硫酸鹽轉換(sulfate transfer) ,氮-氧-乙醯基轉換(N-O-acetyl transfer),去乙醯化作用(deacetylation),或單一電子氧化步驟(1-electron oxidation step)會形成終極致癌物的中間產物(15-19)。相對地,不穩定的氮-亞硝基醯胺化合物(N-nitrosoamides),不論在生物體內或體外實驗都是強有力的烷基化劑(alkylating agents),而且因pH改變而自然水解產生碳離子(carbonium ion),就像in vivo實驗,alkylnitrosourethane和alkylnitrosoguanidine會被硫醇(thiol)所催化(20-21). 如同所預期的, N-NO-AAF合成時會有好的產出率,而且很快的與不同親核子(nucleophile) 反應,包括:amino acid,nucleosides,DNA.所以N-NO-AAF是很強的致突變原(mutagen),致畸胎原(teratogen) (22),而且在正常的生理情況之下就會發生。

許多證據顯示一些已知的oncogenes在不同的生

理情況之下，會在老鼠的肝臟表現,包括出生前與出生之後的發展(23,24),再生(25,26),贅生.雖然有development,regeneration,neoplasia之不同,但是當化學品所誘發的囓齒類動物肝癌時就可以測出c-myc,c-fos,c-jun致癌基因(27-33)。並且會出現某些致癌基因的表現與新生物的轉型步驟有關,以及致癌基因的產物可以當作癌症的指標。

AAF代謝活化會induce hepatocellular carcinomas (HCCs), p53 suppressor gene mutation and allelic loss與人類的肝細胞癌有相當密切的關係。除了p53 gene以外尚未有其它致癌基因參予的文獻報導,因此有必要針對 AAF 以及 NO-AAF 所誘發的HCCs,在carcinomas 內 p53 gene 突變的機率(mutation frequency)加以明瞭(57)。Mutation frequencies在兩種不同的chemical: NO-AAF與AAF分別是19.23% (20/104), 31.1% (33/106)。而K-ras,H-ras之突變機率爲0%。在HCCs內p53 gene mutation spectrum有幾個值得注意的特性: (a) 無論是由NO-AAF或AAF引起的p53 gene mutation ,好發的地方都是在exon 5-8。 (b) 幾乎所有的mutation都是G→A transition,而且guanosine是主要被攻擊的base。(c) p53 gene mutation的發生率與cancer cell differentiation 有很重要的相關性。在poorly-differentiated HCCs其 p53 gene mutation incidence是 41.1%; well-differentiated HCCs其p53 gene mutation incidence是 10-17%。 (d) p53 gene mutations incidence 和mutation sites與cancer cell的分化程度有相當密切的關聯,在分化不良的肝癌細胞,其p53 gene mutation都集中在exon 7-8;然而分化良好的肝癌細胞,p53 gene mutation sites則落在exon 5。 (e) 由 NO-AAF或 AAF所引起的突變幾乎全部(98%)都是點突變(point mutation)。 (f) p53 gene mutation發生頻率較高的是轉換(transition)突變,而

transition / transversion ratio 分別是13/6 (NO-AAF group), 21/12 (AAF group)。

本篇論文爲了更進一步探討$NaNO_2$對AAF所誘發的肝癌之影響,將大白鼠以餵食的方法給予AAF和$NaNO_2$,然後觀察$NaNO_2$對AAF所誘發的肝腫瘤之作用和鼠肝內c-jun,c-fos,c-myc致癌基因表現量增加的情況。

貳.實驗材料與方法(Materials& Methods)

一.材料與儀器

動物飼料選購purina Lab Chow, AAF購自Sigma St Louis, MO,$NaNO_2$- E.Merck,Primary Ab.(rabbit anti-c-fos and c-jun polyclonal antibodies)購自Oncogene Science, Monoclonal anti-c-myc antibody 購自Santa Cruz Biotechnology,Inc., Biotinylated secondary Ab. (Biotinylated anti-rabbit or anti-mouse immunoglobin), streptavidin peroxidase,LSAB kit 購自Dako company. DAB(diaminobenzidine)購自Sigma St Louis,MO. Immunohistochemical staining slides scanner 選購自 Leica Q500 MC image processing & analysis system.

二.實驗動物之分組

雄性Wistar大白鼠(體重120-150公克)購自國立台大醫學院實驗動物中心。每三隻大白鼠一個籠子,然後飼養於有溫度控制的動物房。以Purina Lab Chow爲飼料,蒸餾水不限制飲用。先飼養一星期,然後觀察大白鼠的健康情況,選取健康者供實驗使用,飼養 3 個月。

第1組: 6 隻, 對照組,只餵普通飼料。
第2組: 6 隻, 餵0.3% $NaNO_2$的飼料。
第3組: 6 隻, 餵0.02% 2-AAF的飼料。
第4組: 6 隻, 餵0.02% 2-AAF加0.2% $NaNO_2$飼料。
第5組: 6 隻, 餵0.02% 2-AAF加0.3% $NaNO_2$飼料。

二:屍體解剖與組織學檢查

當大白鼠斷頭犧牲之後,將屍體進行解剖,打開腹腔取出肝臟,以生理食鹽水清洗,先以肉眼進行檢查新生腫瘤的數目,然後將肝臟以10% formalin固定,之後用傳統的方法進行病理組織切片,每一個埋有肝臟之石蠟連續切五片,再將組織切片以Hematoxylin-eosin做染色.將所看到的腫瘤形狀,數目,所在的位置記錄下來。

肝臟組織切片與染色方法:

1.固定(fixation):

解剖之後,取出大白鼠之肝臟,放入10%中性福馬林固定24小時以上,然後取出固定好的肝臟,切成0.5 X 0.5 X 0.3 mm大小,置入包埋盒中,在連續水流中沖水2小時,以去除福馬林。

2.脫水(dehydration):

將沖掉福馬林的肝臟取出,先用吸水紙將多餘水份吸走再進行脫水步驟:(1)70%酒精20分(2)80%酒精20分(3)90%酒精20分(4)95%酒精I 20分(5)95%酒精II 20分(6)100%酒精I 20分(7)100%酒精II 20分

3.透明(clearing):

將完全脫水的肝臟組織,浸於二甲苯(xylene)中,一直到組織浸至透明爲止。步驟:(1)二甲苯I 10分(2)二甲苯II 10分。

4.浸潤(infiltration):

二甲苯與石蠟(paraffin)可以互溶,所以先以二甲苯透明後,才能夠使浸潤得到完全,所以浸潤的目的是要讓paraffin完全進入肝臟組織。步驟:(1)浸潤

於(二甲苯:石蠟 = 1:1)溶液中,置於烘箱中40℃, 1小時(2)石蠟I, 55℃,30分(3)石蠟II, 55 C,30分(4) 石蠟III,55 C,30分

5.包埋(embedding):

將浸潤完全的肝臟組織放在模子中央,然後將溶化的 石蠟加入模子中把肝臟組織完全覆蓋,接著將包埋台放在模子上,再加入少許石蠟使肝臟組織固定在包埋台.然後將包埋好的石蠟塊置於-20℃冰箱,可以使石蠟結晶較細,等石蠟凝固後,除去模子,包埋工作完成。

6.連續切片(serial section):

將包埋好的肝臟組織石蠟塊固定於連續切片機(microtome)調整好刀片角度及距離,先以 20 μm 粗切至所要觀察的肝組織面,然後再調整至 5 μm 切出連續的組織切片,接下來就用毛筆取下切出的連續切片,將它置放在38℃的溫水中,使切片能完全展開來.然後以上面塗有(蛋白:甘油= 1 : 1)的混合液的載玻片,將已展開的切片撈上來,並以毛筆將切片調至載玻片中間,然後放在38℃的乾燥臺上烘乾,以便染色之用。

7.染色(staining):

以蘇木素-依紅(hematoxylin-eosin簡稱H & E)染色

詳細步驟如下:

(1) 二甲苯I 6 分鐘
(2) 二甲苯II 6 分鐘
(3) 100% 酒精 I 3 分鐘

(4) 100% 酒精 II 3 分鐘
(5) 95% 酒精 3 分鐘
(6) 80% 酒精 3 分鐘
(7) 沖水 30 分鐘
(8) 蘇木素染色液 10 分鐘
(9) 以酸性酒精行辨色至核清楚爲止
(10) 沖水 30 分鐘
(11) 依紅染色液 5 分鐘
(12) 70% 酒精 30 秒鐘
(13) 80% 酒精 30 秒鐘
(14) 95% 酒精 30 秒鐘
(15)100% 酒精 I 3 分鐘
(16)100% 酒精 II 3 分鐘
(17) 二甲苯 I 6 分鐘
(18) 二甲苯 I 6 分鐘

8.封片(mounting):

將封片膠滴於已染色的組織切片上，然後以45度角慢慢蓋上蓋玻片，避免氣泡產生，待封片膠凝固後，即完成封片，如此一來切片即可長期保存。

三.用Immunocytochemical stain觀察proto-oncogenes的表現

測定$NaNO_2$加強AAF誘發proto-oncogenes表現之方法是依據 Lu等數位作者於1994年發表的論文(34)加上一些修改的。從大白鼠切下的肝臟用10%中性福馬林固定24小時，脫水，包埋，再以連續切片機切成5μm 厚度的切片。然後將切片做免疫組織化學染色。步驟如下:

(1)脫蠟(56℃,20-30 min.oven)
(2)Xylene (3-5 min.pure,repeat again)
(3)Absolute ethanol.(5 min.repeat again)
(4)95% Ethanol(3-5 min.repeat again)
(5)以二次水沖洗(5-10 min.repeat again)
(6)TBS (Tris buffer,5 min.)
(0.05M, PH7.8,NaCl 0.15M, Add 1ml Triton X-100/L.)
(7)將citrate buffer微波加熱至沸騰(大約要五分鐘
(citric acid monohydrate 2.1g溶於900 ml.二次水)
+
(2M NaOH 13ml.)
+
(二次水至 1000 ml.)
(** Add 1 ml. MP40/1000ml.)
(8)置入已脫蠟完全的玻片(citrate buffer still keep in boiling situation) 繼續加熱 5 min。
(9)取出玻片,然後讓它自動降溫至室溫。
(10)取出後用二次水沖洗。
(11)Tris buffer (5 min.)
(12)3% H_2O_2 (5-10 min.)
(13)加入二次水(5 min.)
(14)Tris buffer.(5 min.)
(15)Primary antibody.(1:50,20-40 min用diluent buffer稀釋)
(16)加入二次水(5 min.)
(17)Tris buffer .(5 min.)
(18)Secondary antibody(The yellow bottle,30 min.)
(19)加入二次水(5 min.)
(20)Tris buffer (5 min.)
(21)Add biotin ligand (The red bottle, 30 min.)

(22)加入二次水 (5 min.)
(23)Tris buffer (5 min.)
(24)DAB (5 min.at room temperature)(DAB 1顆配
10ml.分裝成2ml.加15μl 3%H_2O_2/2ml DAB
(25)加入二次水(5 min.)
(26)Hematoxylin (不超過1 min.) (染background)
(27)加入二次水清洗(15 min.)
(28)95% Ethanol (5 min.)
(29)Absolute ethanol (5 ml.)
(30)Xylene
(31)mounting(封片)
(32)blue color爲negative control,計算brown color的面積(positive areas)
(Leica Q500 MC image processing & analysis system)

四.病理切片鏡檢分類(52-55)

鼠肝內specific hepatocellular lesions 的分類主要的根據 paper(52) Report of a workshop on classification of specific hepatocellular lesions in rats.

1.肝細胞變異性的病灶(Foci of cellular alteration)

(1) 亮細胞病灶(Clear cell foci)
(2) 嗜酸性細胞或毛玻璃樣病灶 (Eosinophilic or ground glass foci)
(3) 嗜鹼性細胞病灶(Basophilic foci)
(4) 混合形細胞病灶(Mixed cell foci)

所謂foci是指肝臟的病變小於一個肝小葉(liver lobule),foci剛開始主要是肝細胞質著色性質以及構造顯現的改變;肝臟的結構並沒有明顯的分裂開,而受影響的肝細胞板與周圍的肝臟組織是合併的,而沒有明顯的界限存在。受到影響的肝細胞可能比正

常的肝細胞大或小,而某些細胞核可能變大,有泡泡,或濃染,而且核仁變大。嗜酸性細胞病灶通常是細胞質增加而造成整個細胞的增大;嗜鹼性細胞病灶含有擴散的嗜鹼性細胞質,細胞大小比正常的肝細胞大或小;亮細胞病灶細胞質有空空的外觀,細胞是正常的大小或稍微腫大。一般認爲嗜鹼性細胞病灶和腫瘤之生成比其它的變異性細胞有比較明顯的特異性。同時也認爲foci在細胞學的表現與新生性結節(neoplastic nodules)細胞的組成相同,因此foci很可能進一步形成neoplastic nodules。

2.新生性結節(Neoplastic nodules)

一般新生性結節爲球形的病變,經常大於好幾個肝小葉,在結節內正常的肝臟結構會消失,其內的肝細胞由嗜酸性細胞或亮細胞所組成,而嗜鹼性細胞較少見;細胞大小不一,細胞核腫大且濃染,數目加倍,並且有明顯的核仁,有時可出現有絲分裂的現象。肝竇可能變窄或變寬,肝細胞其排列可能是立體的,雜亂的一片,或是不規則的盤狀(一層或多層細胞的厚度),同時會壓迫到周圍的肝組織而有明顯的界限,肝竇(sinusoids)可能受到腫大的肝細胞壓迫或顯示不同程度的擴張或膨大。雖然有很少的case可以發現portal areas會停留在nodules內,但是大部份的portal areas是看不見的。最重要的特徵是構造的扭曲,以及與包圍在nodules周圍的肝組織界限明顯。結節的肝細胞板(plate)與不受影響的細胞是不連續的。在動物腫瘤生成的實驗中,這種新生性結節是增殖性病變,它的出現顯示肝細胞癌生成的可能性會增加。

參. 結 果(Results)

本篇論文主旨是在探討$NaNO_2$影響AAF所誘發的大白鼠肝癌。結果顯示當飼養的大白鼠在飼料內加入AAF (0.02%),經過三個月的飼養觀察,其相對的肝臟重量(肝/體重)較只飼養普通飼料的對照組爲重(圖1)。餵予普通飼料的第1組相對的肝臟重量是3.25%;飼料加入$NaNO_2$的第2組相對的肝臟重量是3.00%;飼料內加入AAF的第3組相對的肝臟重量是11.3%;而飼料內加入AAF以及不同濃度$NaNO_2$的第4組(0.2%)與第5組(0.3%)相對的肝臟重量是17.6%和19.0%。所以很清楚的發現第4組與第5組的大白鼠其肝臟腫大的情況與飼料內只加入AAF(0.02%)的第3組比較($p<0.001$),顯然是有意義的增加。(Fig.1)

組織病理學分析檢查呈現出對照組與飼料內只加入$NaNO_2$的第2組,不論以肉眼觀察或以組織病理切片檢查,並沒有值得注意的變化(圖 2A , 2B).所有飼料內加入AAF 飼養的大白鼠都可以發現許多病灶(foci)以及在門脈區周圍有大小不一的新生結節(neoplastic nodules) (圖2C).在第3,4,5 組的大白鼠發現肝臟有明顯的扭曲(distortion)以及廣泛的壓迫到周圍的間質(parenchyma)。在結節內的肝細胞,主要是由排列成小柱形狀的嗜酸性細胞所構成,而且是被薄薄的結締組織線分開的(圖2C)。新生的結節內是增殖的病變,並且知道增殖是由化學致癌物所誘發的.至少暗示可能增加發展成爲肝癌的機率。第4組及第5組的所有大白鼠在飼養三個月後都發生相同的病變,而且可以見到許多肝臟的損傷有發展成爲肝癌的可能。

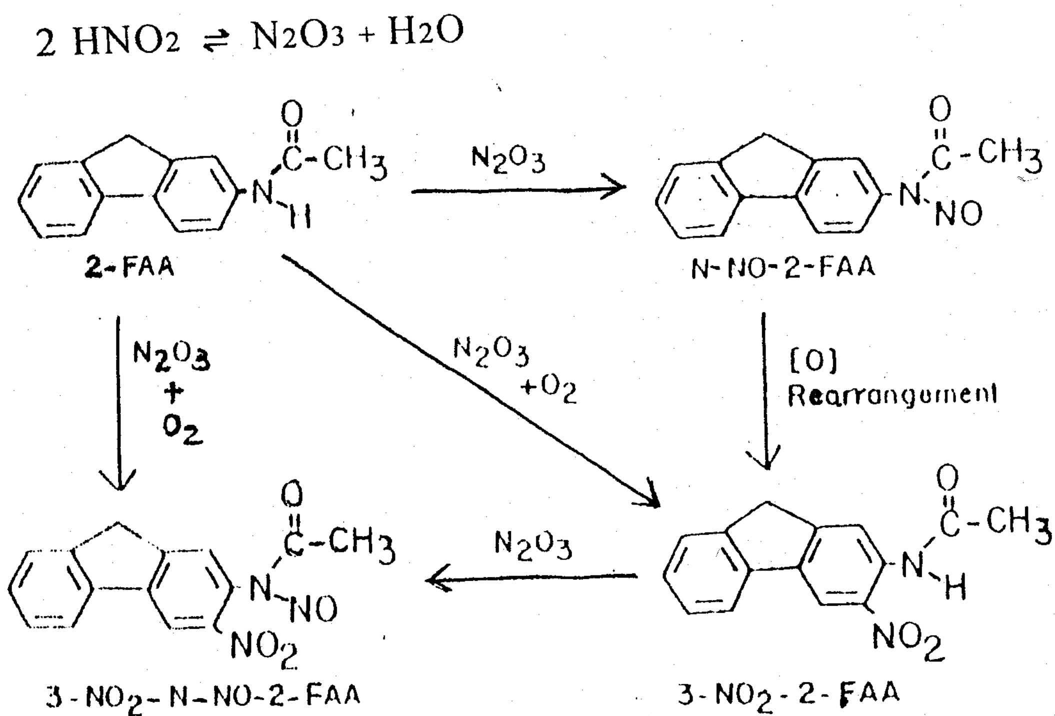
爲了要瞭解c-Jun, c-Fos, c-Myc等致癌基因會停留在那裡表現?必須以免疫組織化學染色法將所有的組織

切片染色，所使用的試劑是biotin-avidin peroxidase system。如圖三所示，如果是c-Jun有表現可以看到肝臟的切片染成棕色(brown)。c-Jun protein level由Table 1可以清楚：control的foci area(mm^2)分別是 0.32, 0.35, 0.40, 0.39, 0.42, 0.31Mean ± SD是 0.37± 0.05；group I 的foci area分別是0.46, 0.41, 0.41, 0.50, 0.48, 0.43 。Mean ± SD 是 0.47 ± 0.05; group III 的 foci area 分別是0.61, 0.55, 0.60, 0.59, 0.63, 0.65。Mean ± SD 是 0.61 ± 0.04; group IV 的 foci area分別是 0.76, 0.75, 0.71, 0.75, 0.70, 0.72 。 Mean ± SD是0.73 ± 0.03; group V的 foci area分別是0.86, 0.83,0.84, 0.91, 0.85, 0.88。Mean ± SD 是 0.86 ± 0.03 。 所以用 AAF(0.02%)混合飼料飼養的第3組大白鼠，可見到鼠肝內c-Jun的表現有明顯的增加(約爲對照組的1.65倍)；以AAF (0.02%)加上 0.2%或 0.3% $NaNO_2$混合飼料飼養的第4組和第5組大白鼠可以發現$NaNO_2$會有意義的增加AAF所誘導的c-Jun表現($P<0.001$)(約爲對照組的1.97與2.32倍）(表一，圖3D，圖3E) 。以上用來計算面積的儀器是 Leica image processing and analysis system 利用相同的儀器來偵測 c-Fos的表現，由Table 2就可以明白：c-Fos foci area (mm^2) control組分別是0.36, 0.37, 0.40, 0.43, 0.48, 0.57。Mean ± SD 是 0.43 ± 0.08; group II 的 foci area分別是0.39, 0.47, 0.47, 0.48, 0.58, 0.62 。Mean ± SD 是 0.52± 0.08; group III的foci area分別是0.63, 0.64, 0.62, 0.64, 0.64, 0.65。Mean ± SD 是 0.64 ± 0.01; group IV的fociarea 分別是0.76, 0.75, 0.74, 0.73, 0.77, 0.80 。 Mean ± SD 是 0.76 ± 0.03; group V的 foci area分別是 0.86, 0.85,0.87, 0.90, 0.92, 0.88 。Mean ± SD是 0.88 ± 0.03 。可以明白用AAF(0.02%)混合飼料飼養的第3組大白鼠其 c-Fos的表現量是對照組的1.5倍(圖4C)；在第4組與第5組的大白鼠因爲加入 $NaNO_2$而有意義的增加AAF所誘導的 c-Fos表現($P<0.001$) (約爲對照組的1.77與2.05倍) (表二，圖4D，圖

4E)。相同的結果也出現在c-Myc的表現，c-Myc protein level由 Table 3可明白：control 組的 foci area (mm^2) 分別是 0.14, 0.21, 0.23, 0.31, 0.27, 0.26。Mean ± SD是 0.24 ± 0.06; group II的foci area分別是0.32,0.34, 0.44, 0.31, 0.41, 0.32。Mean ± SD 是0.36 ± 0.05; group III的foci area分別是0.41, 0.56, 0.50, 0.59, 0.58, 0.63。Mean ± SD 是 0.55 ± 0.08 ; group IV的foci area分別是 0.68, 0.69, 0.64, 0.67, 0.69, 0.67 。Mean ± SD是 0.67 ± 0.02; group V 的 foci area 分別是0.85, 0.81, 0.82, 0.79, 0.80, 0.82。Mean ± SD 是 0.82 ± 0.02 。其表現量在加入AAF (0.02%)的第3組是對照組的2.3倍。 (表三,圖5C)；在加入不同濃度$NaNO_2$的第4組與第5組也可以發現會有意義的增加AAF所誘導的c-Myc表現(P值<0.001) (約爲對照組的2.8與3.5倍) (表三,圖5D,圖5E)。

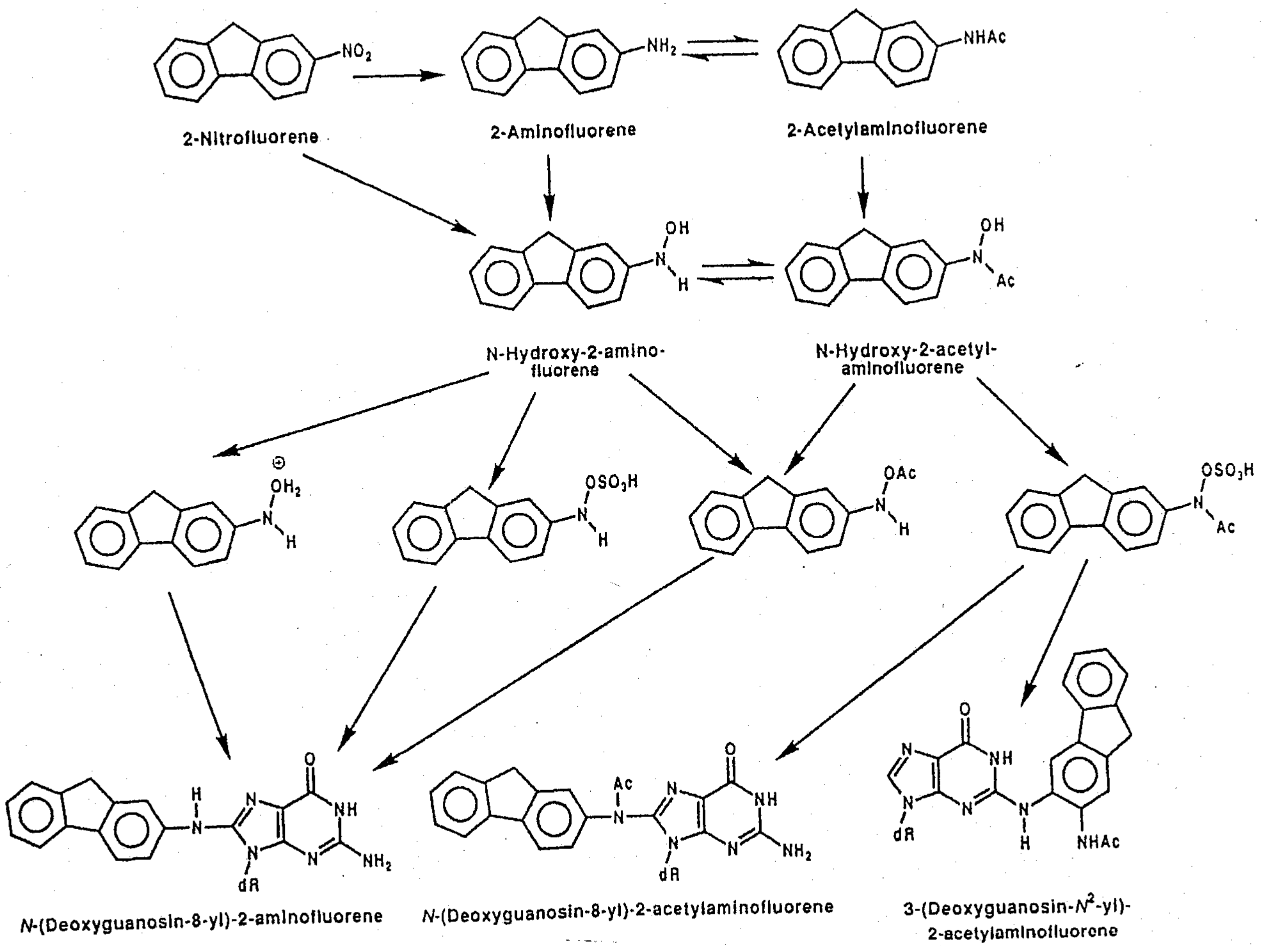
附圖一: AAF 在酸性狀態下亞硝基化反應

$2\ HNO_2 \rightleftharpoons N_2O_3 + H_2O$

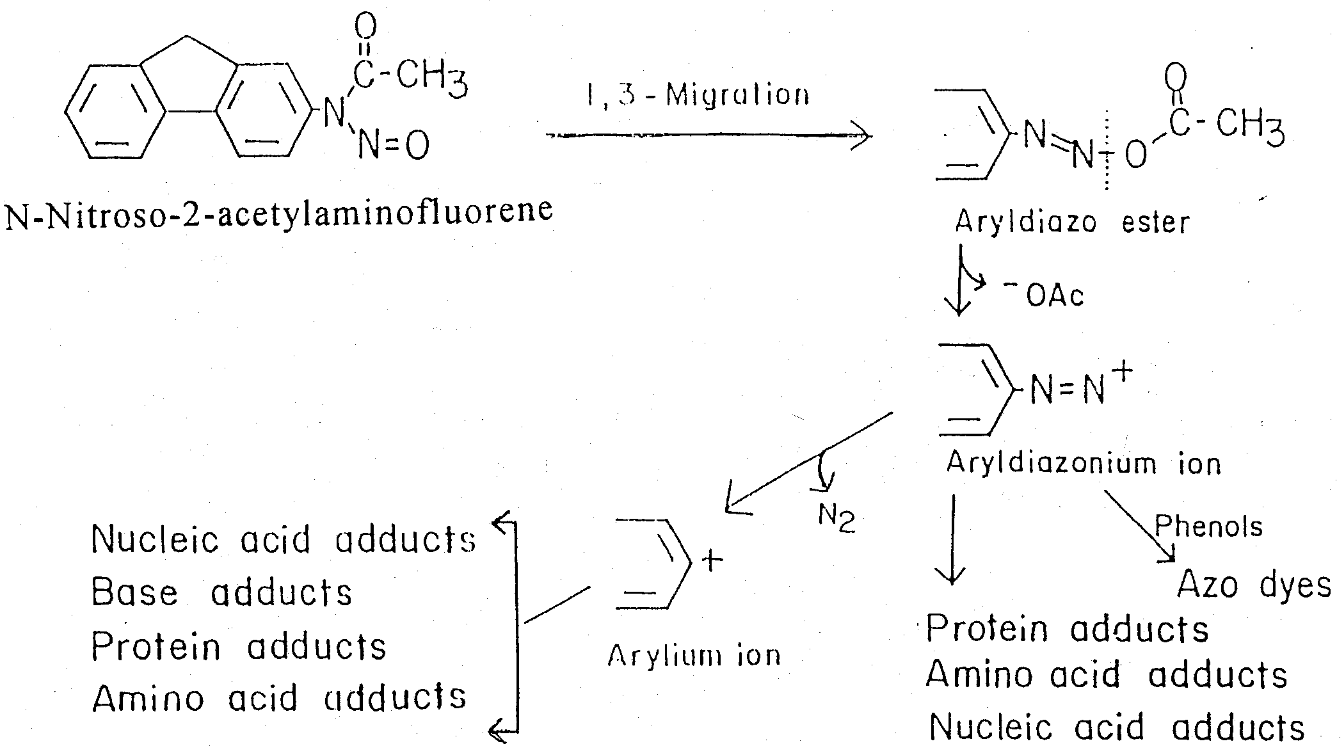


附圖一. Nitrosation of N-2-fluorenylacetamide in acidic conditions with N_2O_3 yielding N-nitroso-AAF and 3-nitro derivatives.(22)

附圖二. AAF 致癌途徑(56)



附圖三. NO-AAF 致癌途徑(22)



Nitrosocompound can develop its mutagenicity by spontaneous conversion to diazonium ion which can attack macromolecules in living cells electrophilically.

Fig.1 NaNO2 對 AAF 處理的大白鼠相對肝臟重量之影響

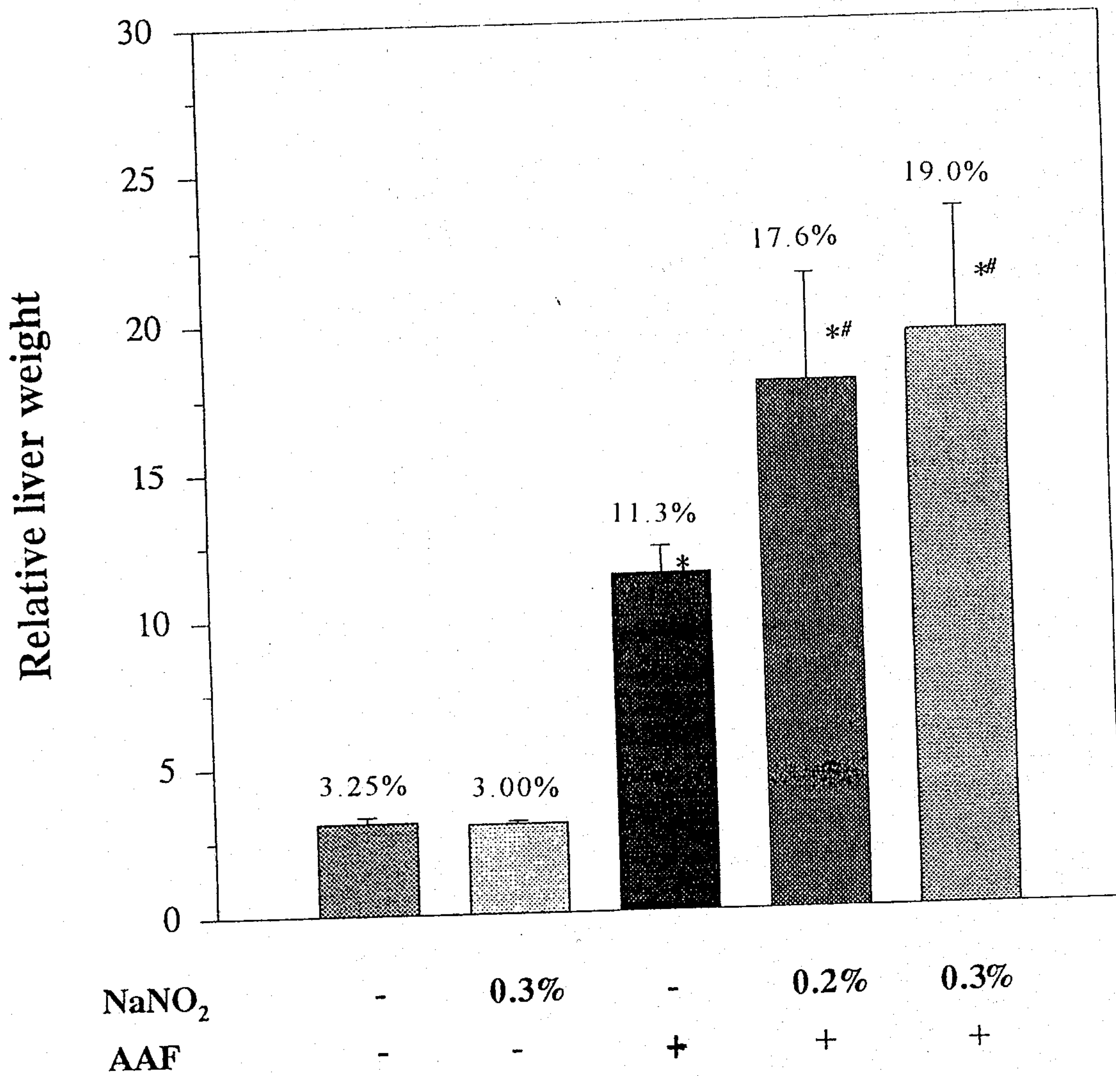


Fig.1 Effects of NaNO2 on the relative liver weight of AAF- treated rats. Rats received AAF with or without NaNO2 in diet for 12 weeks. Relative liver weight=(liver weight/body weight) x 100,*p<0.001,compared with control;# p<0.001 compared with AAF-treated groups.

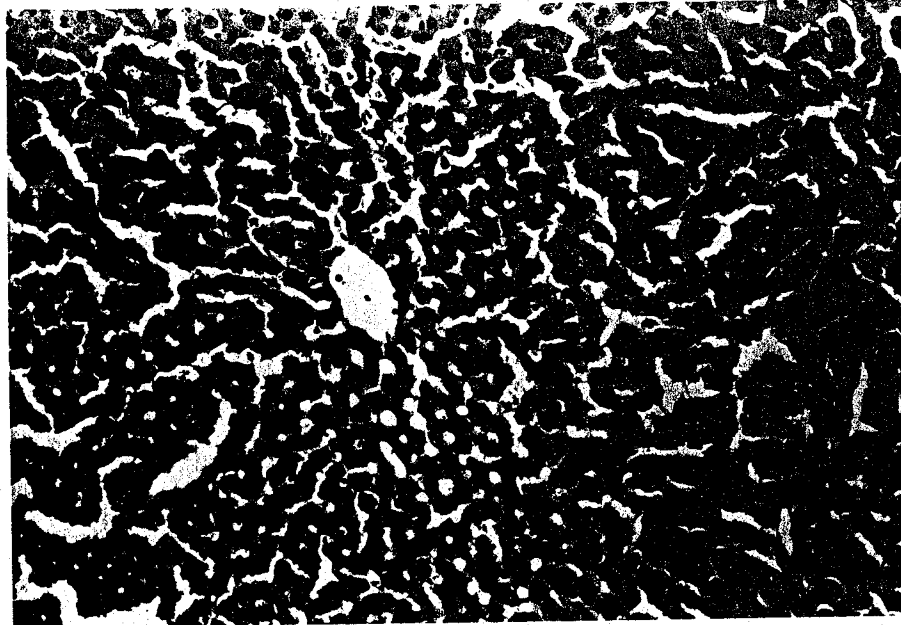


Fig.2A Histopathological examination of the effect of $NaNO_2$ on the liver of AAF-treated rat.
Liver section obtained from the control animal showed no noticeable change. H&E, x100

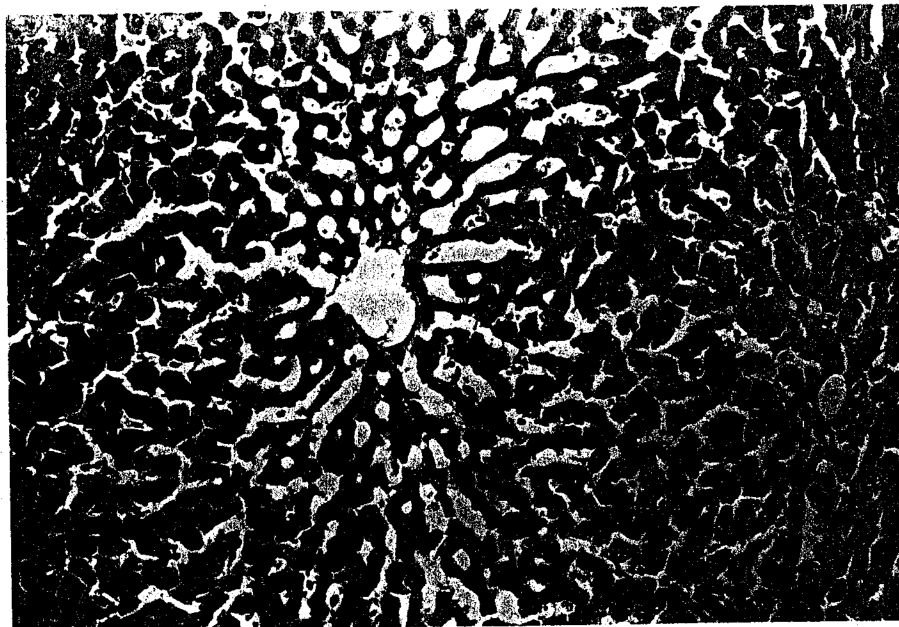


Fig.2B Histopathological examination of the effect of $NaNO_2$ on the liver of AAF-treated rat.
Liver section obtained from the $NaNO_2$(0.3%)-treated animal showed no noticeable change.
H&E, x100

Fig.2C Histopathological examination of the effect of $NaNO_2$ on the liver of AAF-treated rat.
Liver section obtained from the AAF-treated animal showed a focus. H&E, x100

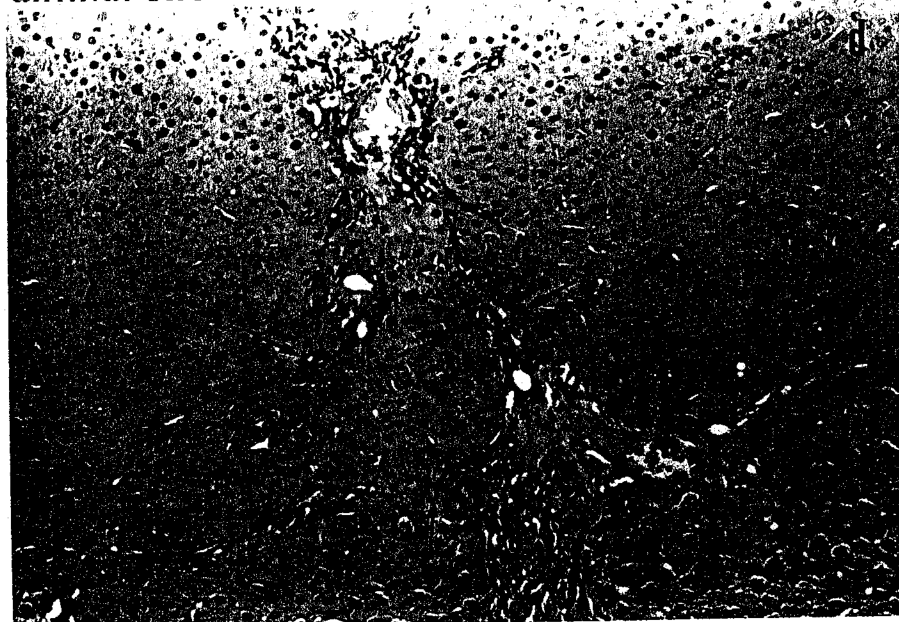


Fig.2D Histopathological examination of the effect of $NaNO_2$ on the liver of AAF-treated rat.
Liver section obtained from rat received both AAF and 0.2% $NaNO_2$ showed variable-sized foci and neoplastic nodules in the periphery of the portal areas. H&E, x100

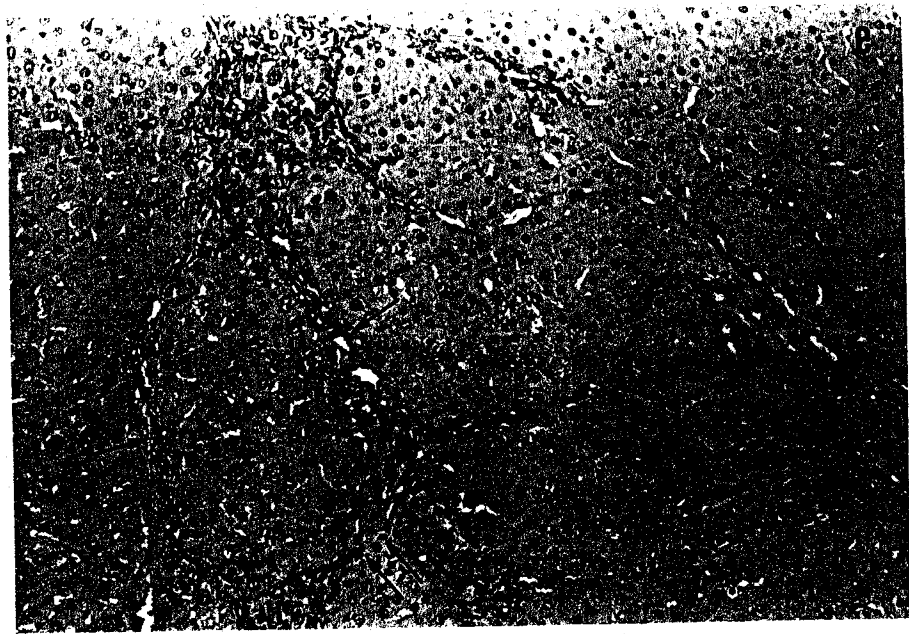


Fig.2E Histopathological examination of the effect of $NaNO_2$ on the liver of AAF-treated rat.
Liver section obtained from rat received both AAF and 0.3% $NaNO_2$ showed variable-sized foci and neoplastic nodules in the periphery of the portal areas. H&E, x100

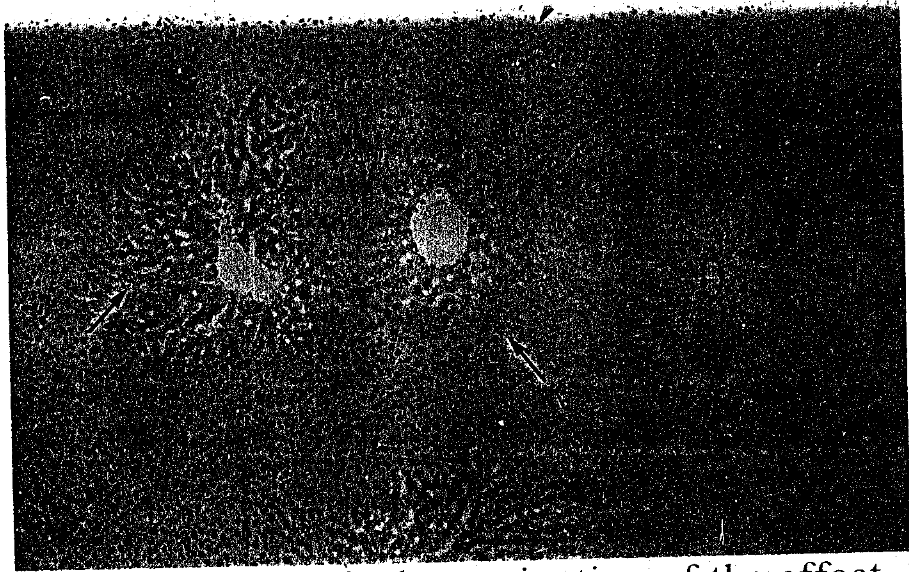


Fig.3A Immunocytochemical examination of the effect of $NaNO_2$ on the c-Jun expression in AAF treated rat. Liver section obtained from the animals of control,were subjected to immunocytochemical stain for c-Jun expression. Arrows indicate representative stain areas. x200.

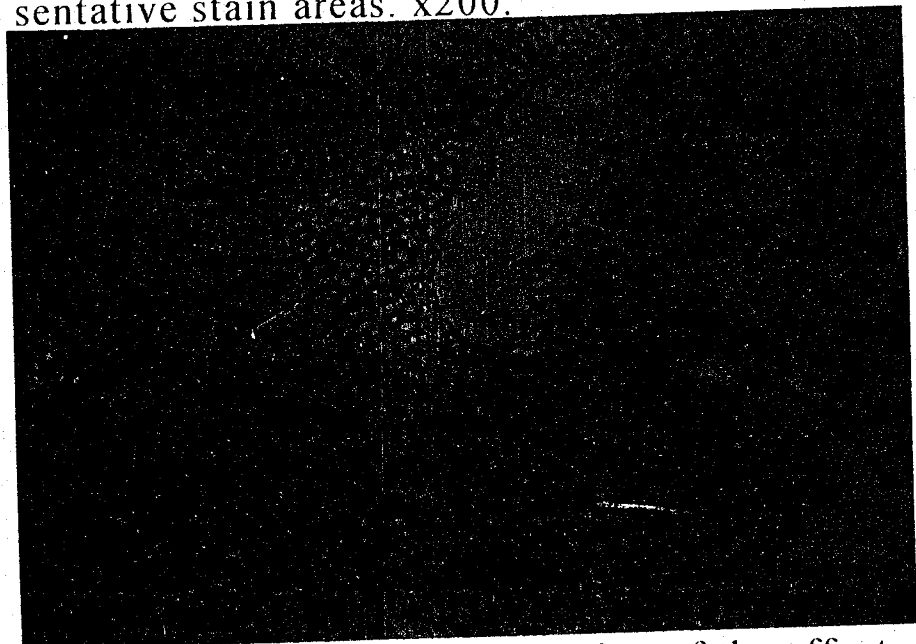


Fig.3B Immunocytochemical examination of the effect of $NaNO_2$ on the c-Jun expression in AAF-treated rat. Liver sections obtained from the animals treated with $NaNO_2$(0.3%) ,were subjected to immunocytochemical stain for c-Jun expression. Arrows indicate representative stain areas. x200.

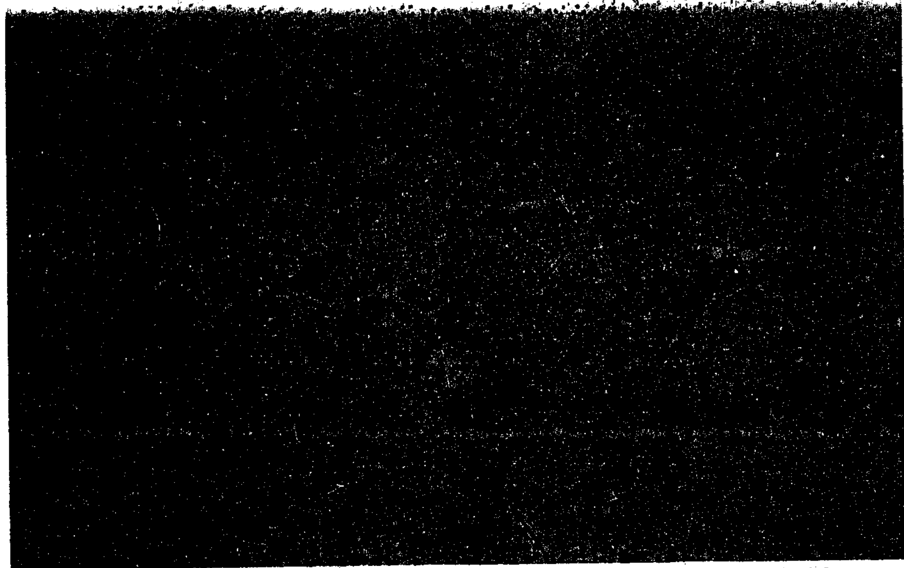


Fig.3C Immunocytochemical examination of the effect of $NaNO_2$ on the c-Jun expression in AAF-treated rat. Liver sections obtained from the animals treated with 0.02% AAF,were subjected to immu -nocytochemical stain for c-Jun expression. Arrows indicate representative stain areas.x200

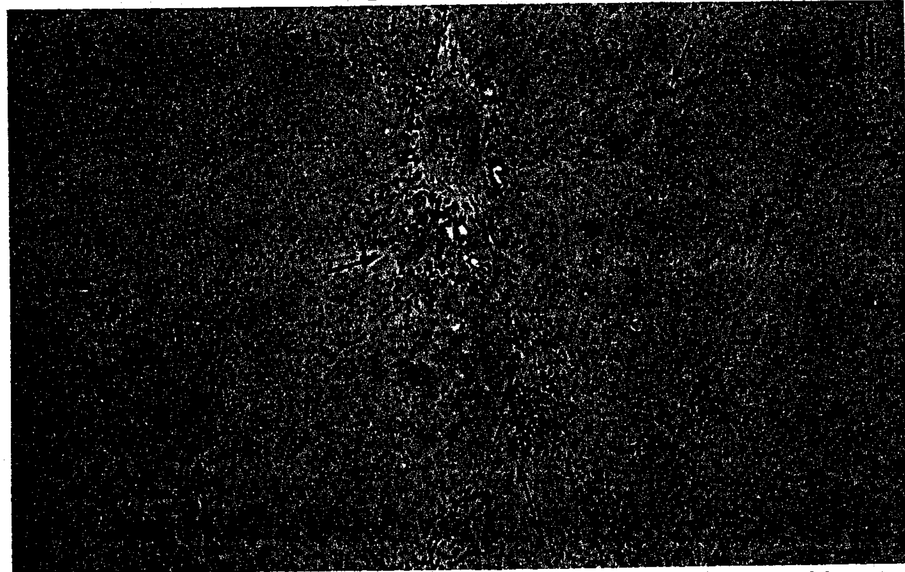


Fig3D Immunocytochemical examination of the effect of $NaNO_2$ on the c-Jun expression in AAF-treated rat. Liver sections obtained from the animals received both AAF and $NaNO_2$ (0.2%),were subjected to immunocytochemical stain for c-Jun expression. Arrows indicate representative stain areas. x200

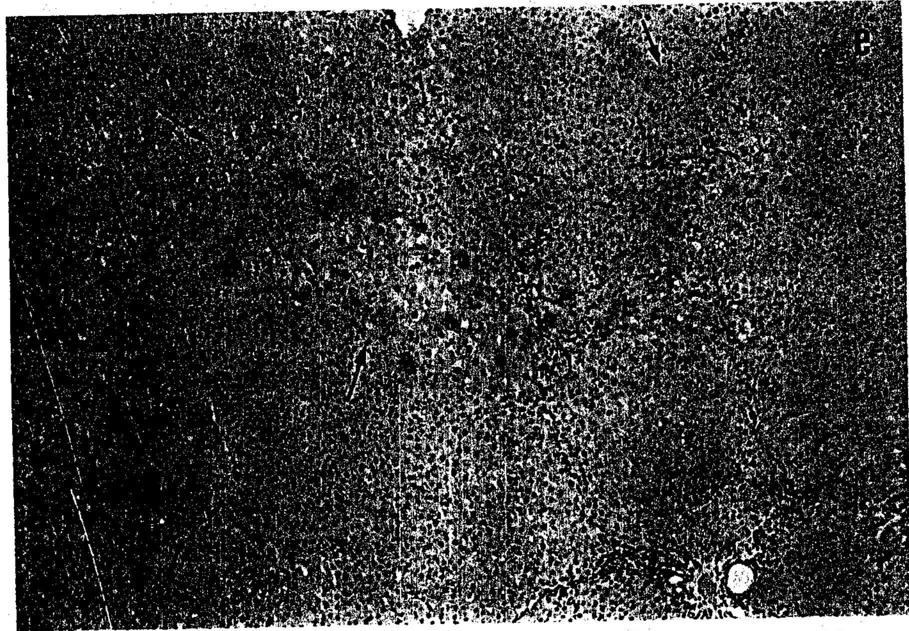


Fig.3E Immunocytochemical examination of the effect of $NaNO_2$ on the c-Jun expression in AAF-treated rat. Liver sections obtained from the animals received both AAF and $NaNO_2$ (0.3%),were subjected to immunocytochemical stain for c-Jun expression. Arrows indicate representative stain areas. x200

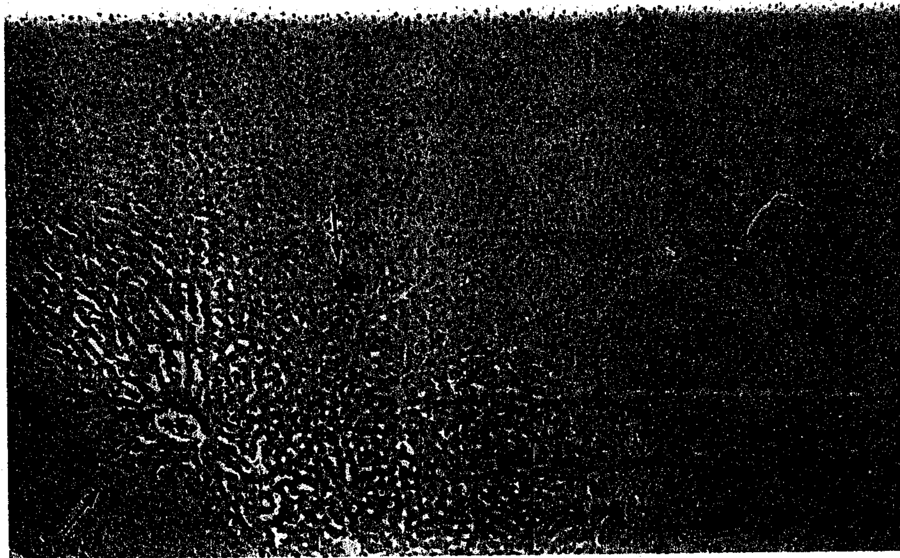


Fig.4A Immunocytochemical examination of the effect of $NaNO_2$ on the c-Fos expression in AAF-treated rat. Liver sections obtained from the animals of control,were subjected to immunocytochemical stain for c-Fos expression. Arrows indicate representative stain areas. x200.

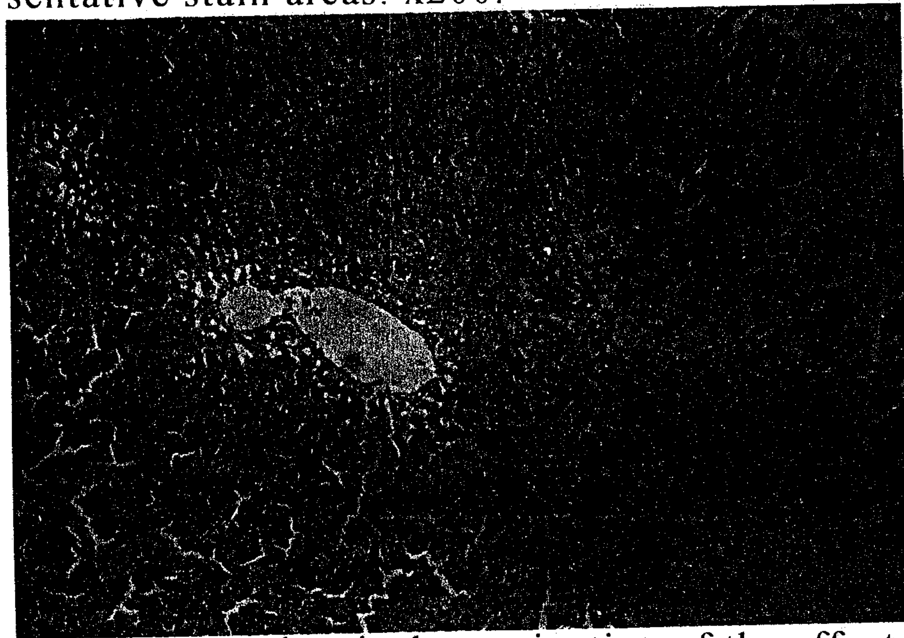


Fig.4B Immunocytochemical examination of the effect of $NaNO_2$ on the c-Fos expression in AAF-treated rat. Liver sections obtained from the animals treated with $NaNO_2$ (0.3%),were subjected to immunocytochemical stain for c-Fos expression Arrows indicate representative stain areas.x200

Fig.4C Immunocytochemical examination of the effect of $NaNO_2$ on the c-Fos expression in AAF-treated rat. Liver sections obtained from the animals treated with 0.02% AAF,were subjected to immunocytochemical stain for c-Fos expression. Arrows indicate representative stain areas. x200

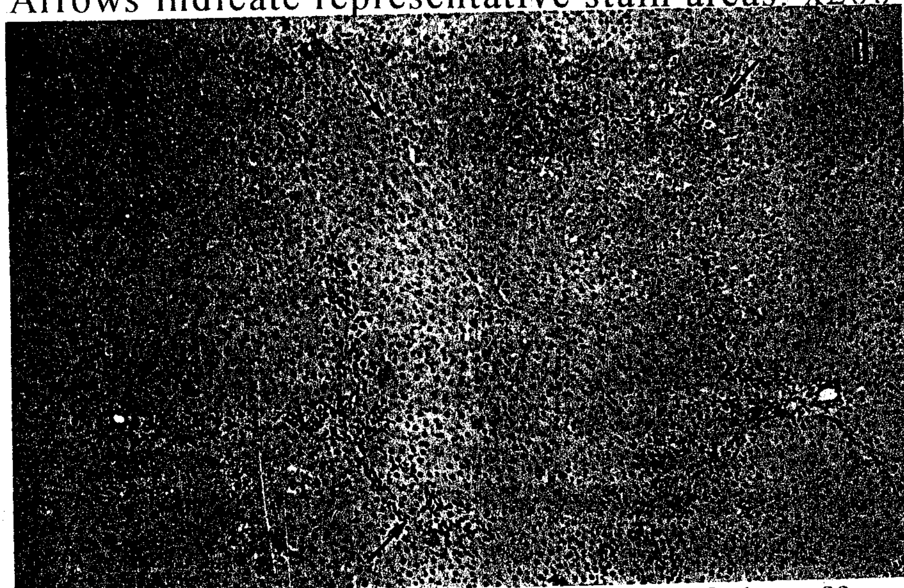


Fig.4D Immunocytochemical examination of the effect of $NaNO_2$ on the c-Fos expression in AAF-treated rat. Liver sections obtained from the animals received both AAF and $NaNO_2$(0.2%), were subjected to immunocytochemical stain for c-Fos expression. Arrows indicate representative stain areas. x200

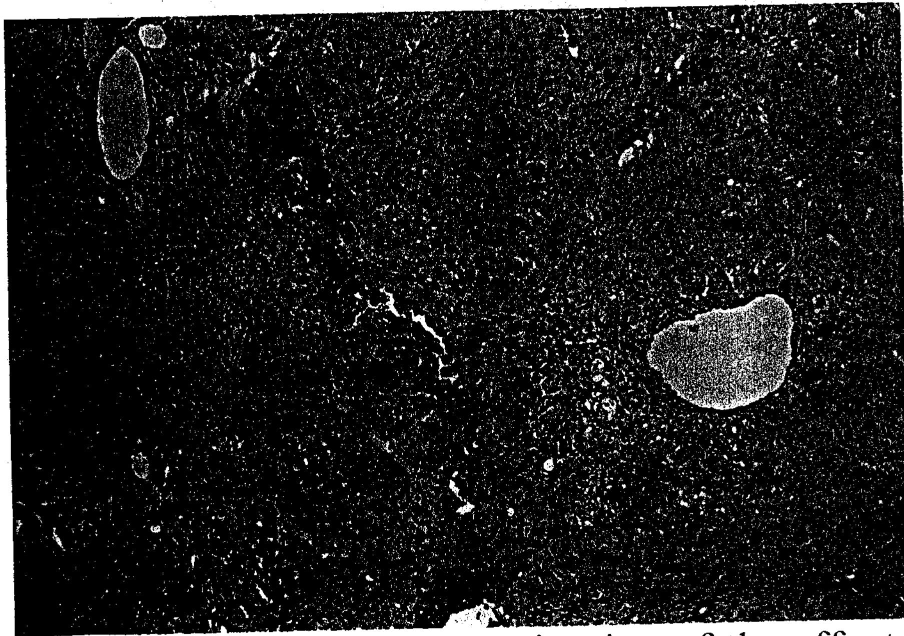


Fig.4E Immunocytochemical examination of the effect of $NaNO_2$ on the c-Fos expression in AAF-treated rat. Liver sections obtained from the animals received both AAF and $NaNO_2$(0.3%),were subjected to immunocytochemical stain for c-Fos expression. Arrows indicate representative stain areas. x200.

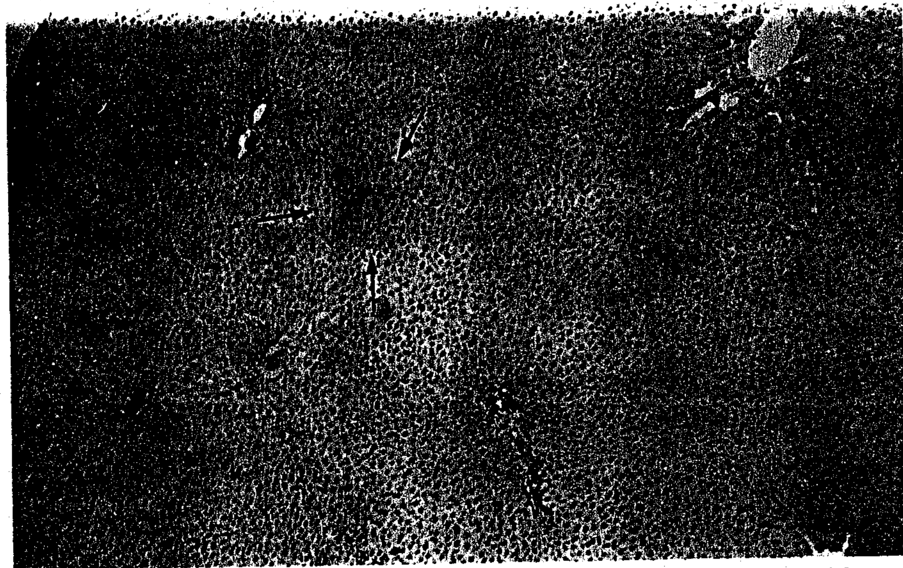


Fig.5A Immunocytochemical examination of the effect of $NaNO_2$ on the c-Myc expression in AAF-treated rat. Liver sections obtained from the animals of control,were subjected to immunocytochemical stain for c-Myc expression. Arrows indicate representative stain areas. x200.

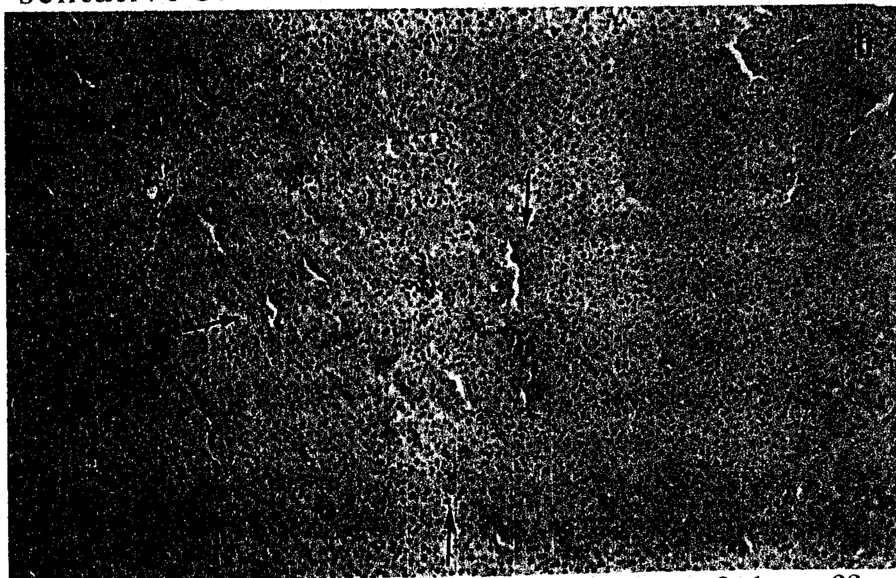


Fig.5B Immunocytochemical examination of the effect of $NaNO_2$ on the c-Myc expression in AAF-treated rat. Liver sections obtained from the animals treated with $NaNO_2$(0.3%),were subjected to immunocytochemical stain for c-Myc expression. Arrows indicate representative stain areas. x200.

Fig.5C Immunocytochemical examination of the effect of $NaNO_2$ on the c-Myc expression in AAF-treated rat. Liver sections obtained from the animals treated with 0.02% AAF,were subjected to immunocytochemical stain for c-Myc expression. Arrows indicate representative stain areas. x200 .

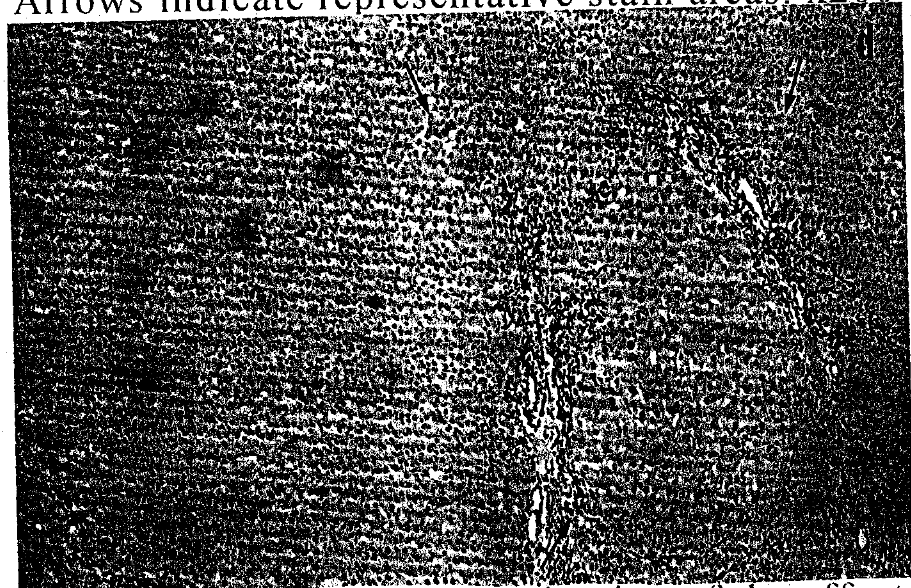


Fig.5D Immunocytochemical examination of the effect of $NaNO_2$ on the c-Myc expression in AAF-treated rat. Liver sections obtained from the animals received both AAF and $NaNO_2$(0.2%), were subjected to immunocytochemical stain for c-Myc expression. Arrows indicate representative stain areas. x200

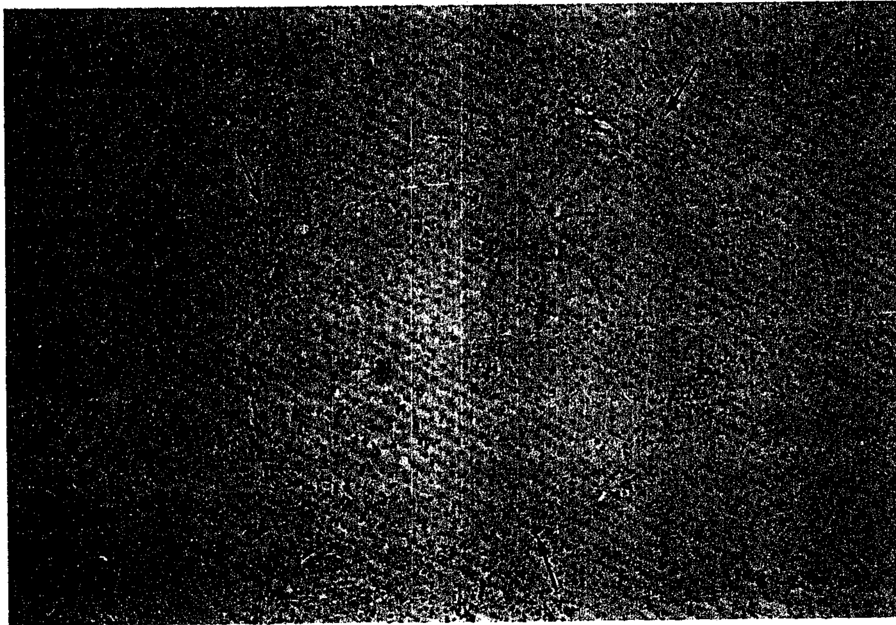


Fig.5E Immunocytochemical examination of the effect of $NaNO_2$ on the c-Myc expression in AAF-treated rat. Liver sections obtained from the animals received both AAF and $NaNO_2$(0.3%), were subjected to immunocytochemical stain for c-Myc expression.Arrows indicate representative stain areas. x200.

Table 1. Effect of $NaNO_2$ on c-Jun protein level in AAF-treated liver

Treatment [a]	c-Jun foci area [b] (mm^2)	Mean ± SD [c] (mm^2)	Fold of control
Control	0.32, 0.35, 0.40 0.39, 0.42, 0.31	0.37 ± 0.05	—
$NaNO_2$ (0.3%)	0.46, 0.41, 0.41 0.50, 0.48, 0.43	0.47 ± 0.05	1.27
AAF (0.02%)	0.61, 0.55, 0.60 0.59, 0.63, 0.65	0.61 ± 0.04*	1.65
AAF + $NaNO_2$ (0.2%)	0.76, 0.75, 0.71 0.75, 0.70, 0.72	0.73 ± 0.03*#	1.97
AAF + $NaNO_2$ (0.3%)	0.86, 0.83, 0.84 0.91, 0.85, 0.88	0.86 ± 0.03*#	2.32

[a] Control rats were on synthetic basal diet while experimental rats received AAF with or without $NaNO_2$ in the diet for 3 months.

[b] The foci area was determined in five randomly selected fields from six liver tissues of every group by Leica image processing and analysis system.

[c] Mean ± SD of six animals

* $p<0.001$ compared with control group.

# $p<0.001$ compared with AAF group.

Table 2. Effect of $NaNO_2$ on c-Fos protein level in AAF-treated liver

Treatment [a]	c-Fos foci area [b] (mm^2)	Mean ± SD [c] (mm^2)	Fold of control
Control	0.36, 0.37, 0.40 0.43, 0.48, 0.57	0.43 ± 0.08	—
$NaNO_2$ (0.3%)	0.39, 0.47, 0.47 0.48, 0.58, 0.62	0.52 ± 0.08	1.21
AAF (0.02%)	0.63, 0.64, 0.62 0.64, 0.64, 0.65	0.64 ± 0.01*	1.49
AAF + $NaNO_2$ (0.2%)	0.76, 0.75, 0.74 0.73, 0.77, 0.80	0.76 ± 0.03*#	1.77
AAF + $NaNO_2$ (0.3%)	0.86, 0.85, 0.87 0.90, 0.92, 0.88	0.88 ± 0.03*#	2.05

[a] Control rats were on synthetic basal diet while experimental rats received AAF with or without $NaNO_2$ in the diet for 3 months.

[b] The foci area was determined in five randomly selected fields from six liver tissues of every group by Leica image processing and analysis system.

[c] Mean ± SD of six animals

* $p<0.001$ compared with control group.

# $p<0.001$ compared with AAF group.

Table 3. Effect of $NaNO_2$ on c-Myc protein level in AAF-treated liver

Treatment [a]	c-Myc foci area [b] (mm^2)	Mean ± SD [c] (mm^2)	Fold of control
Control	0.14, 0.21, 0.23 0.31, 0.27, 0.26	0.24 ± 0.06	—
$NaNO_2$ (0.3%)	0.32, 0.34, 0.44 0.31, 0.41, 0.32	0.36 ± 0.05	1.50
AAF (0.02%)	0.41, 0.56, 0.50 0.59, 0.58, 0.63	0.55 ± 0.08*	2.29
AAF + $NaNO_2$ (0.2%)	0.68, 0.69, 0.64 0.67, 0.69, 0.67	0.67 ± 0.02*#	2.79
AAF + $NaNO_2$ (0.3%)	0.85, 0.81, 0.82 0.79, 0.80, 0.82	0.82 ± 0.02*#	3.42

[a] Control rats were on synthetic basal diet while experimental rats received AAF with or without $NaNO_2$ in the diet for 3 months.

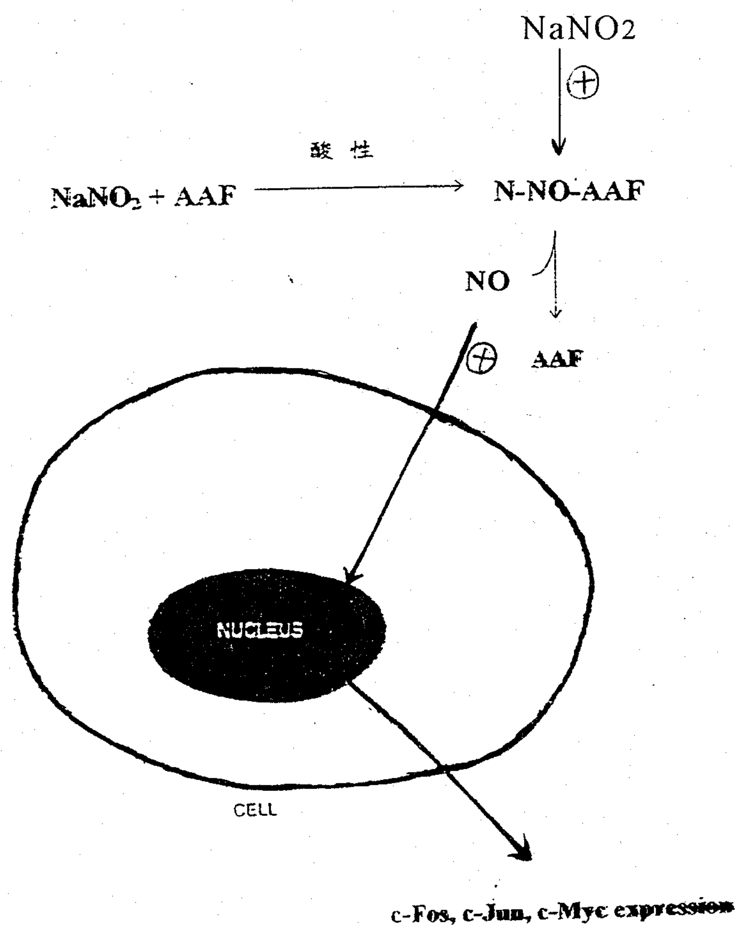
[b] The foci area was determined in five randomly selected fields from six liver tissues of every group by Leica image processing and analysis system.

[c] Mean ± SD of six animals

* $p<0.001$ compared with control group.

# $p<0.005$ compared with AAF group.

Conclusion:



肆. 討 論

本篇論文旨在研究$NaNO_2$使AAF所誘發的肝癌形成機率產生增加的作用。當大白鼠給予AAF加上$NaNO_2$混合的飼料飼養三個月與只給予AAF混合飼料的第3組做比較時,可以發現會產生較大的不規則病灶和新生的結並且結節有可能發展成肝癌。因爲$NaNO_2$ 在低濃度時本身並沒有毒性,而且與AAF代謝過程無關。$NaNO_2$會增加AAF所誘發的癌症機率是眞實的,這是因爲發生內生性AAF nitrosation的結果,而nitrosation的致癌性比AAF代謝路徑的調控更強。本篇的研究結果與以前的報告是一致的,表示經由內生性的nitrosation所產生的亞硝基化合物(nitroso-compounds)比單獨給予$NaNO_2$和許多致癌物,可能更具致癌性(5,6)。AAF與N_2O_3 (nitrous fume) 在酸性狀態下已經證明會形成N-NO-AAF (22)。N-NO-AAF在正常生理誘發下與其他化合物一樣都可以生成(5,6)。以Salmonella typhimurium TA 98做突變性實驗,發現N-NO-AAF 比 N-methyi-N'-nitro-N-nitrosoguanidine(MNNG) 或是 N-acetoxy-N-2-fluorenylacetamide (N-AcO-AAF)更強。N-NO-AAF所引起的致癌機制被認爲是經由 fluorenyl-2-diazonium ion與細胞內的大分子像DNA, RNA, protein產生交互作用(如附圖三)。最近研究指出N-NO-AAF具有很強的親電子性(electrophile)而且在中性時,不需要代謝活化很容易與histidine,lysine,cysteine,glutathione,tryptophan, adenosine,cytidine產生反應。N-NO-AAF對C3H10T1/2老鼠纖維母細胞與中國倉鼠卵巢(CHO)細胞產生直接DNA傷害遠比原來的化合物還強(35)。Lin and Kuo (1990)發表的文章得到N-NO-AAF會誘發C3H10T1/2細胞ouabain-resistancemutation與cycle-dependent轉型作用,也證明了N-NO-AAF是一種新的直接作用的致突變劑,而且較原來的化合物毒性更強(36)。依據文獻報導(47),顯示C3H10T1/2細胞在S期特別容易受到NO-AAF的攻擊而產生惡性的轉

型作用,甚至產生染色體的異常,譬如姐妹染色分體互換(Sister Chromatid Exchange簡稱S.C.E.)。依據相同文獻報導指出以NO-AAF在大白鼠皮下注射時,產生明顯的皮下組織病變(腫塊),在顯微鏡下可看出位於皮下有發炎組織,伴隨著鈣化(calcification),壞死(necrosis)產生,甚至有長出fibroadenoma的大白鼠,所以 NO-AAF是一種很強的刺激性化合物。另外在肝細胞的毒性方面(包括壞死,硬化等),不論是經皮下或腹腔注射,NO-AAF組所引起的肝細胞毒性較AAF組明顯。另外,NO-AAF在試管內可攻擊含有p53 cDNA的質體(php53B)。利用DNA 聚合酵素足跡試驗(DNA polymerase footprinting analysis, 簡稱DPFA)的技術可以偵測出p53的exon 6-8較易爲NO-AAF所攻擊(57)。因此,可以假設當NO-AAF攻擊p53基因的exon 6-8所形成的NO-AAF-DNA adduct使得 p53基因的抑癌功能大受影響,這一點或許可以做爲癌症初期(initiation stage) 的分子機制。當這些攻擊行爲發生在p53基因時,造成癌症的初期病變。反之,假使這些病變未被修復,則分化良好型(Well differentiated)HCC於焉產生。若此時又加上他種致癌基因(如K-ras, H-ras)則癌細胞將更形惡化而成爲惡性分化(Poorly-differentiated)HCC。根據日人 Tatsuya Oda等人所建立的人類致肝癌(hepatocarcinogenesis)模式實驗顯,不同位置和型態的p53基因和人類肝癌的分化和生成有密切關聯。由 Tatsuya的報告結果可以看出,在惡性分化的HCC,p53致突變的機率很高(54%),同時大部分哆都集中在exon 7和8之間。反之,在分化較爲良好的HCC,其p53突變的比例就比較氐(21%),而且平均分散到exon 5-8之間(48)。且 NO-AAF 釋放 NO 造成 aorta relaxation, DNA basehydroxylation,deamination(adenine→hypoxanthine,guan-ine→xanthine,cytosine→uracil)(49)。由以上的證明綜合而言,AAF加上亞硝酸鈉($NaNO_2$),可能AAF的毒性以及與AAF內生性的亞硝基化產生的N-NO-AAF變成比較強的致癌作用。

細胞核的致癌基因,如c-Jun, c-Fos, c-Ha-ras,當老鼠產生肝癌時,曾經報告過這些基因都會被活化,而且可以在再生的肝臟和許多實驗的系統觀察到c-Myc表現量增加與細胞增殖有很強的相關性(37-40)。因此以化學品(chemical)誘發老鼠產生肝癌是最能表現實驗腫瘤模式特徵之一(41,42)。這些由tumor promoters(如phorbol esters)所誘發的早期反應基因(primary response genes)牽涉到細胞生長和分化(43)。這些已經被接受的基因特別受到注意,因爲這些基因在許多人類的癌症也會過度表現,尤其是肝癌(44)。然而,卻很少將焦點集中在老鼠發生肝癌期間致癌前基因(proto-oncogenes)像c-Fos的活化(45,46)。在本篇的研究報告,所有以AAF處理過的老鼠,在早期階段(early stage), c-Myc, c-Fos, c-Jun蛋白會增加,這樣的結果和別人的發現是一致的(44,47)。當大白鼠餵食AAF加上$NaNO_2$之後,鼠體內肝臟所表現的 c-Jun, c-Fos, c-Myc蛋白量與只餵食AAF的大白鼠做比較是有意義的增高,而$NaNO_2$會增強AAF所誘發細胞核內proto-oncogenes的表現是因爲AAF的內生性亞硝基化形成 N-NO-AAF的結果。

動物體內生成亞硝基化合物的方式(50)。依照pH值分成二類:一種是在酸性的pH下,化合物和亞硝酸鹽起反應;另一種是在生理的pH(中性)情況下,化合物和NO或硝酸鹽反應,以下詳述:

二級或三級amine在酸性水溶液中和HNO_2或在有機溶液中和 NOCl, N_2O_3, N_2O_4, $NOBF_4$起反應會形成劇毒的N-亞硝基二級或三級amine,亞硝基反應的開始是亞硝酸鹽會先轉變爲Pka 3.37的nitrous acid (HNO_2,這可以解釋爲何亞硝基反應可由酸催化), HNO_2接著轉化成active nitrosating species 如 nitrous anhydride (N_2O_3),nitrosyl thiocyanate(ON-NCS),nitrosyl halide(NOX), or nitrous acidium ion($H_2NO_2^+$) $2HNO_2 = N_2O_3 + H_2O$, 2-AAF在酸性情況下與 N_2O_3形成 N-NO-AAF(詳見附圖一)。

AAF的致癌途徑如附圖二: AAF會先在肝臟代謝爲N-hydroxy-2-AAF(51),且在肝臟的soluble sulfotransferase作用之下轉變爲主要的終極致癌物 N-sulfonoxy-2-acetylaminofluorene,以及 N-acetoxy-2-aminofluorene,再去攻擊DNA 形成 DNA adducts,導致基因突變,癌化產生。

N-NO-AAF的致癌路徑如附圖三(22): N-NO-AAF並不需要代謝活化,只有電子的轉移(1,3-migration),產生diazonium ion 或 arylium ion 再去攻擊體內的大分子如DNA,RNA,protein 形成adducts,造成mutagenicity,tumor產生。

結論:當大白鼠餵予$NaNO_2$加上AAF的飼料時,會增加AAF所誘發的鼠肝內c-Jun, c-Fos, c-Myc蛋白的表現量,這樣的結果暗示著由於是增加AAF活化細胞核內的致癌前基因,可能是增強AAF所誘發的早期肝癌化的部份機轉。

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第二部份

原兒茶酸對鼠體內 LPS 所誘發內生性 NO 產生和 NO-AAF 形成的抑制作用

Inhibition by *Hibiscus* protocatechuic acid of Lipopolysaccharide-induced endogenous Nitric Oxide production and NO-AAF formation in rats

目 錄

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縮寫字

PCA, protocatechuic acid

NO, nitric oxide

LPS, lipopolysaccharide

中文摘要

亞硝基化合物由於直接攻擊DNA形成DNA adducts,所以會造成癌症,而且在 in vivo 也會產生,表示內生性的 NO 可能扮演著重要的角色。NO,不管是內生性或外生性,都可以將二級 amine 亞硝基化,如此會造成癌症。內生性的 NO 在微生物感染時會 induce macrophage 內的 iNOS 活化,然後 release NO,所以 NO 量增加。在肝臟內也有 iNOS 存在,當受到感染時 Kupffer cell 會產生 cytokine 來活化 iNOS。因此可以假設減少感染時期的內生性 NO 產生可以避免像 nitroso compound 所引起的化學致癌作用。*Hibiscus* PCA 本身帶有 catechol structure,是 oxygen free radical 捕捉劑,即抗氧化劑。假設 PCA 藉由 free radical 捕捉的能力,抑制 LPS 所誘發的內生性 NO,就可能預防亞硝基化進行。

當大白鼠事先處理 PCA,可以証明會特異性的降低內生性NO的產生(表一)。當大白鼠用 LPS 處理三天後,NO 的量增加約八倍。當大白鼠用 LPS和 AAF處理三天後,NO的量是745.18±17.45,如果先以各種不同濃度的 PCA(50mg/kg. 100mg/kg. 250mg/kg.)處理的大白鼠,再處理 LPS 和 AAF,第七組的 NO 量是 453.33±50.48; 第八組的 NO 量是 403.13±30.40; 第九組的量是 320.00±37.37,顯然PCA會有意義的降低NO的量 ($p<0.005$)。

除此之外,PCA 還會稍微抑制由 AAF 和 LPS

所引起的肝炎(表二),LPS 與 AAF 都會引起肝功能指數 ALT 升高(89.2±28.5; 99.15±19.3 比 59.93±16.3)($p<0.01$)。如果大白鼠同時以 LPS 與 AAF 處理,其肝功能指數明顯上升 212.50 ± 14.8。如果先以 PCA(50mg/kg.;100mg/kg.;250mg/kg.)處理,再處理 LPS,AAF,則肝功能指數可以降至 138.98 ± 27.1;116.44 ± 16.9;128.86 ± 24.6,顯然 PCA 會有意義的降低肝功能指數($p<0.005$)。

以 LPS 活化 inducible NO synthase (iNOS) 使內生性 NO 增加,在生理情況之下,內生性的 NO 有其攻擊的靶分子(像 Heme proteins, Fe-S proteins, SH groups, Metalloproteins, Oxygen, DNA, Amines...........)所以 NO 要讓它在生理狀況與外來的 AAF 結合並不容易。本篇實驗以光電比色法(spectrophotometer)與高效率液相層析法去分析血清內的 N-NO-AAF,不論那一組的老鼠血清都無法偵測到 N-NO-AAF。

以 LPS 與 AAF 處理過的大白鼠可以看到各種不同程度的壞死現象: 第三組(LPS)可見到局部性壞死; 第四組(AAF)可以見到凝固性壞死; 第五組(LPS + AAF)顯現出嚴重的液化性壞死; 第七組(PCA 50 mg/kg.+ LPS+ AAF),第八組(PCA 100 mg/kg.+ LPS+ AAF),第九組(PCA 250mg/kg.+ LPS+ AAF)都可以見到液化性的壞死。

// Abstract

Nitrosocompound,which can cause cancer by directly attacking DNA to form DNA adducts,can be produced in vivo , indicating that endogenous nitric oxide (NO) plays an important role. NO,either exogenous or endogenous,can nitrosate secondary amines,thus causing cancer. Endogenous NO would increase in infections by macrophage secretion in response to infection via inducible NO synthase which could be activated by various microorganisms. Inducible NO synthase also exists in hepatocyte and is activated by cytokines from Kupffer cells in infection. Therefore, it was hypothesized that reduced endogenous NO in infection may contribute to prevention in chemical carcinogenesis caused by nitroso compounds. *Hibiscus* protocatechuic acid (PCA),an antioxidant, can scavenge oxygen free radicals with the catechol structure. It was hypothesized if PCA could inhibit the elevation of endogenous NO induced by lipopolysaccharides(LPS), via scavenging free radicals, it would prevent nitrosation reaction progression.

Significantly reduced endogenous NO production was demonstrated in rats treated with PCA. In addition,rats pretreated with PCA inhibited slightly hepatitis caused by AAF. Thus,PCA can be used as an efficient chemopreventor in carcinogenesis caused by nitrosation of endogenous NO via free radical scavenging action.

Endogenous NO production , induced by Lipopolysaccharide,in physiological conditions,reacts with target molecules (Heme proteins , Fe-S proteins , SH groups , Other non-heme Fe and metalloproteins , Amines , DNA, Oxygen, Superoxide anion, Hydrogen peroxide) , so that

NO reacted with AAF may be difficult to detect.

Detect N-NO-AAF by Spectrophotometer method and by HPLC method showed not detectable.

Rats treated with LPS and AAF showed variable necrosis: in group III(LPS only) showed focal necrosis ; in group IV (AAF only) showed coagulative necrosis; in group V (LPS and AAF) showed severe liquefactive necrosis; in group VII (PCA 50mg/kg. and LPS and AAF), in group VIII (PCA 100mg/kg. and LPS and AAF) , in group IX (PCA 250mg/kg. and LPS,and AAF) showed liquefactive necrosis in these groups.

Key words: PCA ,protocatechuic acid
NO,nitric oxide
LPS,lipopolysaccharide

實驗動機與目的

當人體遭受細菌,病毒,寄生蟲等慢性感染時會誘發 macrophage 或 hepatocytes 内的 inducuble NO synthase,release NO 。由於這種内生性的 NO 量很大(非 constitutive NOS 所 release 的 NO),所以會有病理性產生,包括 nitrosation,DNA deamination,oxidation........等。如果能將 NO 捕捉掉,那麼 nitroso compound 減少,carcinogenesis 就會被抑制掉。Protocatechuic acid(PCA)是從洛神花萃取的,其構造式具有 catechol,以 o-dihydroxy 來捕捉 free radical,是 antioxidant 。因此本篇論文實驗嘗試著先用 PCA 以口服方式灌大白鼠,等 30 分鐘,再用 LPS(Lipopolysaccharide) ip.injection,來誘發 inducible NO synthase release NO,再隔 30 分鐘,最後再將 AAF 用胃管灌入 rats 的胃内,實驗時間爲 3 天,然後觀察 PCA 是否會降低 NO 的產生和 N-NO-AAF 的形成。

壹. 前　言

內生性的 nitric oxide (NO)可以在受到任何感染時由 macrophage 所分泌(4), 它有正常的生理功能包括 antimicrobial ,antitumor,因此在 host defense system 中扮演一個重要的角色(1)(附圖一)。由於有學者不斷研究報告發表出來, 讓我們對NO的釋放更加清楚,當有任何發炎的刺激時就會活化 inducible-NO synthase(iNOS) release NO 。譬如以 Interferon-γ 與 Tumor necrosis factor,interleukin-1, or endotoxin 就可以使 endothelial cell 產生 NO (5);以 cytokines 可以誘發 血管平滑肌細胞產NO (6)。NO的生理功能還包括 blood vessels relaxation 而增加局部血流。已經有研究報告証明NO對 tumor cell 的毒殺作用是抑制 DNA 的合成 (附圖二),(38)。無論如何,有許多報告指出不管內生性的或是外生性的 NO, 如果量太多時就會有病理性產生,包括致癌作用—secondary amine nitrosation (nitrosamine)(2,3) primary amine deamination(引起突變) , production of free radical($OH^{\bullet}$, $O_2^{\bullet}$直接攻擊 DNA)(附圖三)。

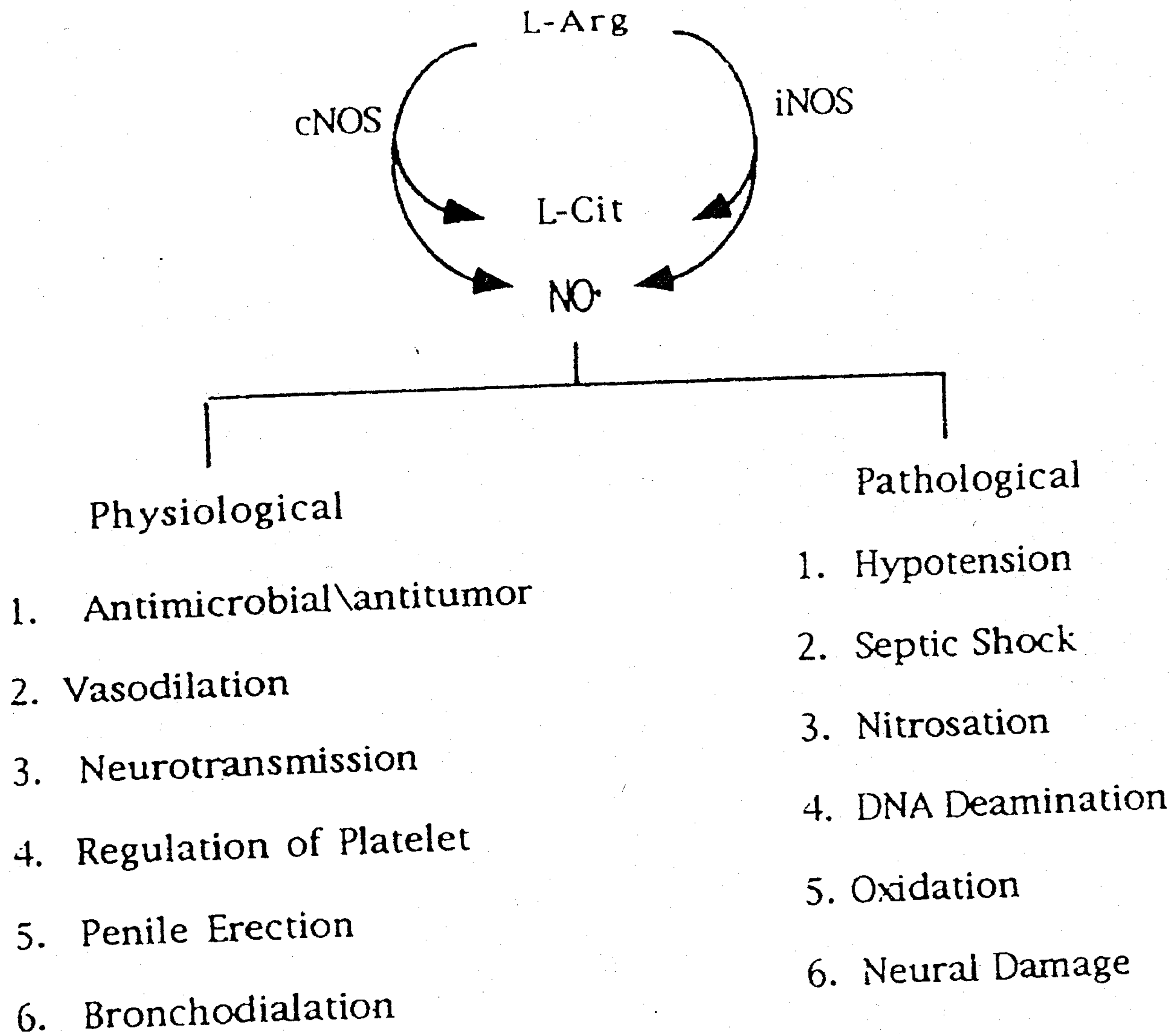
Protocatechuic acid(PCA),簡稱原兒茶酸(附圖四)是由 Chinese herbal medicine(中藥劑)-乾燥的洛神花 (*Hibiscus sabdariffa* L) 萃取的,用來治療高血壓以及肝功能異常。PCA 是 simple phenolic compound 之一具有 oxygen free radical 捕捉的作用。許多研究報告指出 phenolic compound 的抑制作用會減少 oxidative process,特別是降低 atherosclerosis and cancer (8) 。PCA 廣泛存在於水果, 核果以及蔬菜中。越來越多的報告証實 PCA 對化學致癌機轉扮演著抑制的作用,舉例而言:diethylnitrosamine 誘發肝癌,4-nitroquinoline 1-oxide 誘發口腔癌,azoxymethane 誘發 colon cancer, N-methyl-N-nitrosourea 誘發胃腺泡癌

(10,11,12,13)。令人覺得有趣的報導指出除了 PCA 之外的 phenolic compounds(如 caffeic acid),雖然具有比 PCA 更強的過氧化自由基捕捉能力,但是卻會促進胃腺癌的形成。原因是這些 phenolic compound 缺少 electron -withdrawing substituents 的能力(14)。所以選用 PCA 來研究仍然需要留意。

Lipopolysaccharide(LPS),是革蘭氏陰性桿菌(Gram's negative baçilli)細胞壁的成份,會引起 acute hepatitis, sepsis, and shock 。利用 LPS 來活化 macrophage 的方式,在肝臟內 macrophage 會 release 某些 cytokines 來 activate inducible NOS,將 L-arginine 轉換成 L-citrulline 並且 release NO(15,16)。LPS 已經被廣泛使用來誘導內生性 NO 的產生。這種誘發的方式被用在 animal models 和研究內生性 nitrosamine---是一些較容易亞硝基化的 amine(含 Fe,S)與 NO 產生 nitrosation,會有致癌作用。

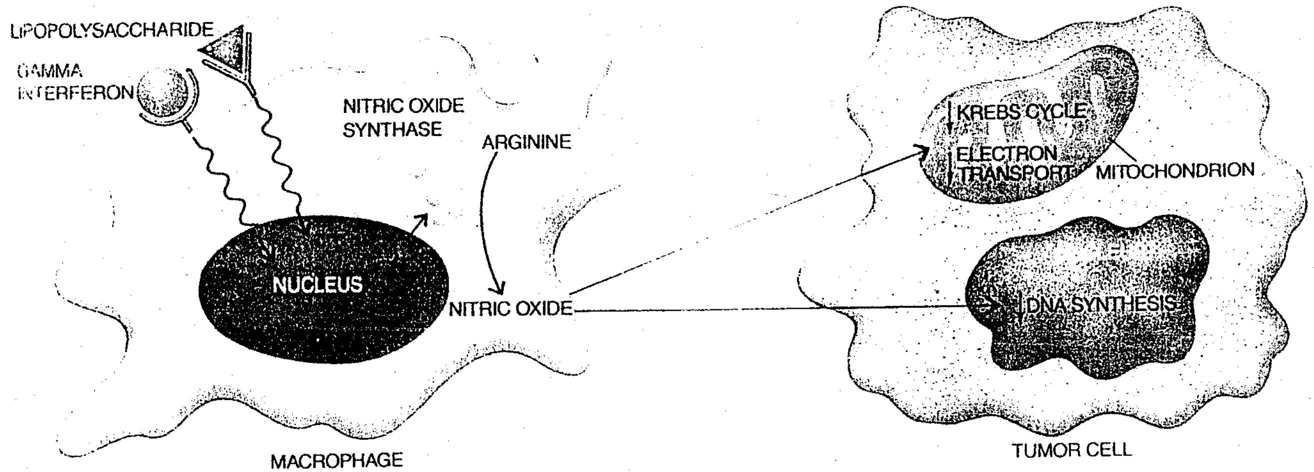
Hepatic carcinogene(AAF),需要肝臟 cytochrome P-450 代謝活化,然後直接攻擊 DNA 形成 DNA adducts(17,18,19)。在最近 1988 年研究報告指出 AAF 的 nitroso-derivative product,N-NO-AAF,是在 in vitro 合成的,已經被證實是較強的 direct-acting mutagen and teratogen(20,21,22)。先前本實驗室曾經將 $NaNO_2$ 與 AAF 混合飼料餵養大白鼠,會在胃液酸性的狀態下形成 NO-AAF。基於這個理論,做成一個臆測(hypothesis):誘發內生性的 NO 增加,可能促成 AAF 與 NO 產生 nitrosation 而形成 NO-AAF(形成的量可能很少)(附圖五)。因此,用 free radical scavenging agent-PCA,來抑制 LPS-induced endogenous NO production 所形成的 nitrosation then carcinogenesis ,可以當作一種化學預防劑(chemopreventor)。

附圖一: NO 的生理與病理角色



(Mutation Research 339,1995)

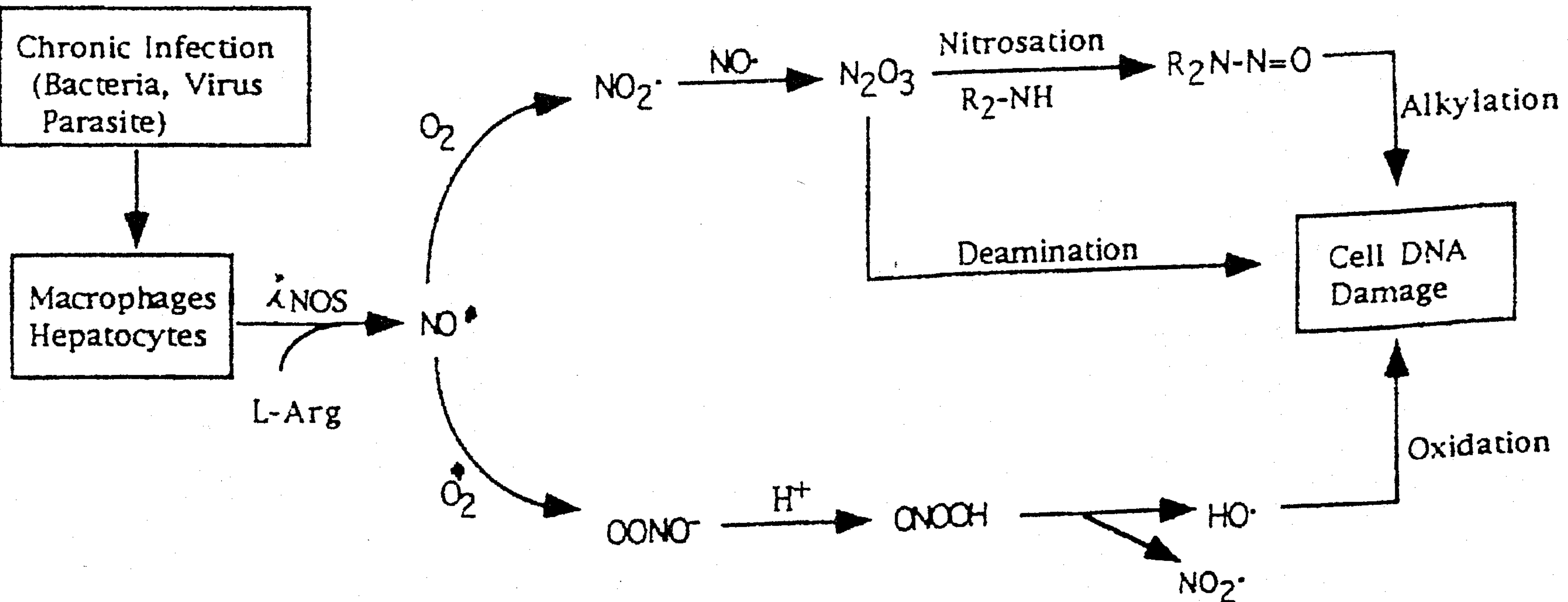
附圖二：巨噬細胞分泌NO與NO消滅癌細胞



附圖二. Immune system stimuli—γ-interferon and lipopolysaccharide transmits signals to macrophage nucleus.The signals cause production of nitric oxide synthase,the enzyme that convert L-arginine to nitric oxide (NO).NO destroys tumor cells by inhibiting the energy producing Krebs cycle and electron transport activities as well as DNA synthesis.(38)

附圖三：NO˙的基因毒性

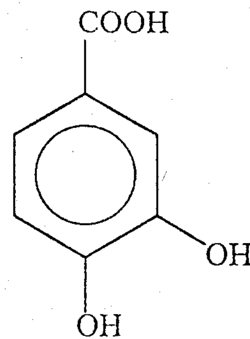
(Mutation Research 339,1995)



附圖四: 原兒茶酸的結構式

PCA 學名: 3,4-dihydroxy benzoic acid

(Biochemical Pharmacol. Vol.43,No.2,1992)



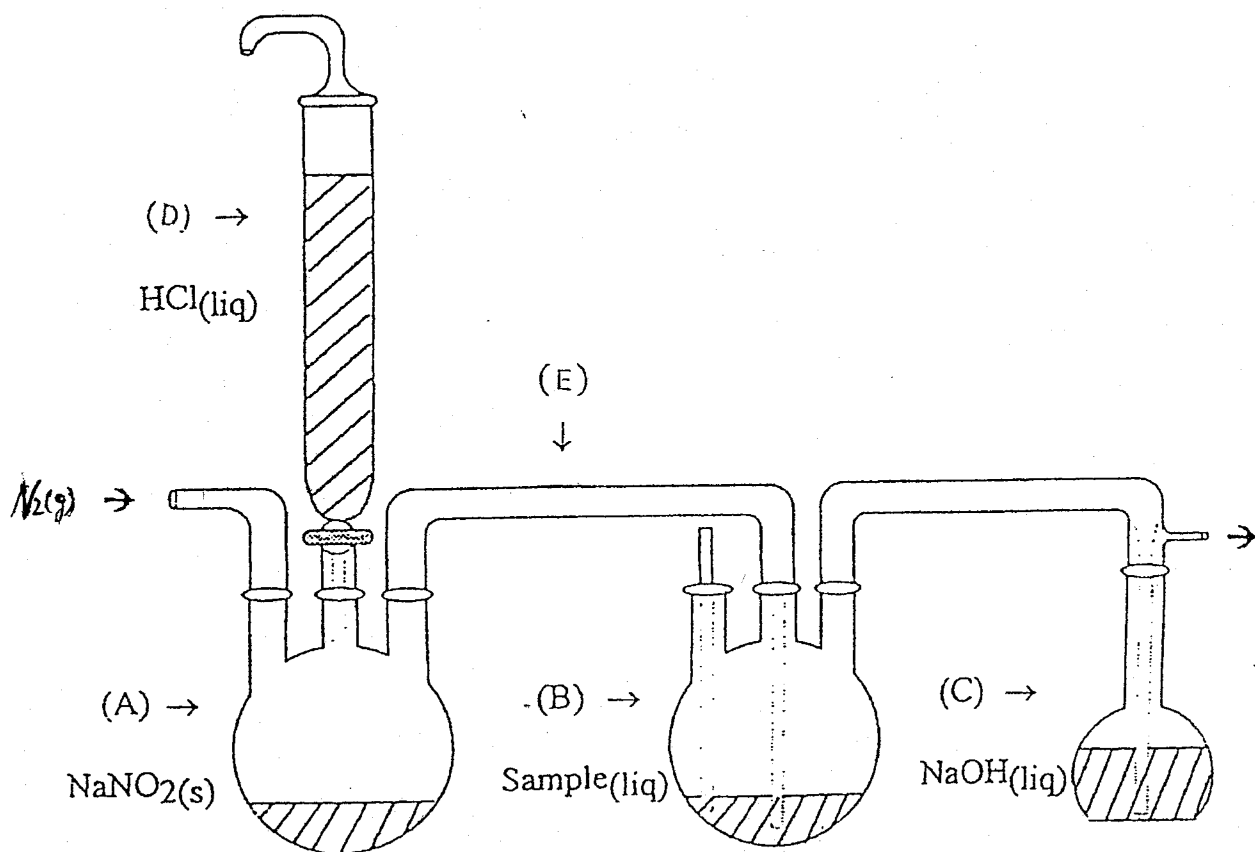
Protocatechuic acid structure

附圖五：NO與靶分子的反應和產生的影響

(Mutation Res.305,1994)

Target molecules	Example	Effect
Heme proteins	Soluble guanylate cyclase	activation
	Cytochrome P-450s	inhibition
	Hemoglobin / myoglobin	inhibition
Fe-S proteins	Mitochondrial complexes I and II	inhibition
	Aconitase	inhibition
Other non-heme Fe and metalloproteins	Ferritin, transferrin	iron loss
	Ribonucleotide reductase	inhibition
Tyrosyl radical	Ribonucleotide reductase	inhibition
SH groups	Glyceryl 3-phosphate dehydrogenase	inhibition
	Cysteine, glutiathione	*S*-nitrosation, oxidation
Unknown	ADP-ribosyl transferase	activation
Amines	Dimethylamine	nitrosation / deamination
DNA		deamination, strand breaks
Oxygen		NO_2, NO_x formation
Superoxide anion		peroxynitrite formation
Hydrogen peroxide		singlet oxygen formation

附圖六：亞硝基化反應構造



貳. 實驗材料與方法

(一)試劑

NADPH, 2-acetylaminofluorene, FAD, nitrate reductase,LDH,pyruvate,$ZnSO_4$,lipopolysaccharide (011:B4), protocatechuic acid (PCA) and Griess reagent 購自 Sigma chemical company (St Louis,Mo U.S.A.) serum SGOT,SGPT kits 購自美商亞培大藥廠台灣分公司(Taipei,Taiwan); NO-AAF 是依據國立台灣大學醫學院生物化學研究所 林仁混 教授所發表的方法(20),由 AAF nitrosation 合成的---詳細構造敬請參閱附圖六;Acetonitrile,Methanol,Ethanol 購自 Sigma Chemical Company (St Louis, Mo U.S.A.)。

(二) 儀器

1.Double-beam spectrophotometer----Hitachi Model U-3210
2.ABBOTT Spectrum CCX biochemical autoanalyzer --ABBOTT laboratories USA,Taiwan limited.
3.高效率液相層析儀(HPLC)--Hitachi Company, Japan.
Pump: L-6200A intelligent pump
Detector: L-4500 Diode Array detector
Column: C_{18}, 4.0 x 250 mm. 5 μm
Mobile phase: ethanol : acetonitrile : water = 3:3:5 (v/v)
Detect wave length: UV 254 nm.
Flow rate: 1.0 ml/min.
Injector: Hamilton 100μl. syringe
Recorder: D-6500 DAD system manager
Injection volume: 50 μl.
4.Microwell Elisa reader--- Bio-Tek Elx 800 Universal

microplate reader

(三) NO-AAF 之合成

NO-AAF 的合成乃是根據 Jen-Kun Lin 和 Ming-Liang Kuo 在 1988 年所發表的方法 (20)：NO-AAF 的前趨物 (precursor)爲知名的致肝癌化合物 2-acetylaminofluorene(簡稱 AAF),經由亞硝基化反應(nitrosation) 之後所產生。亞硝基化反應的裝置如附圖六所示 ,所有反應皆在通風櫥內進行。在反應瓶 A 中加入過量(約 15 g.)的亞硝酸鈉(sodium nitrite)。在磨口滴瓶 D 中加入鹽酸(HCl) 。反應瓶 B 中則加入 4.5 mmole AAF 溶在 30 ml.冰醋酸 (glacial acetic acid)和 18 ml.失水醋酸(acetic anhydride)C 瓶中則加入濃的氫氧化鈉(NaOH) 溶液以捕捉反應進行時產生過量的三氧化二氮(N_2O_3)氣體。在 A 與 B 以及 B 與 C 之間皆以磨口連通管聯接,置於冰塊中冰浴。 在反應前先通氣 5 分鐘,以便將 A 與 B 瓶中的空氣驅離,然後將盛有鹽酸的磨口 D 瓶開關打開,調整流速至每分鐘流出 1 ml.的速度,同時也調整氮氣的流速,以避免B瓶的溶液因壓力過大而溢出。等待反應 30 分鐘後,可見反應瓶 B 的溶液顏色變成墨綠色。待反應結束後,緩緩加入 500 ml. 冰水並搖勻,可以馬上見到淡黃色晶體, 將沉澱物過濾並繼續以冰水清洗,此晶體以冷凍乾燥法去除水份後,再溶於 1%醋酸的甲醇中置於-20 ℃冰箱中進行再結晶即可得到黃色針狀的 NO-AAF 晶體。

(三)實驗動物分組

雄性 S-D rats (體重 200-250 公克)購自台中榮民總醫院實驗動物中心,飼養於有溫度控制的動物房中,飼料與蒸餾水皆充份供應,不加以限制。先飼養一個星期,觀察大白鼠的健康情況,選取健康的 rats 供實驗使用。

第一組: 6 隻,control.

第二組: 6 隻,solvent control(DMSO solvent).

第三組: 6 隻,LPS 1.0mg/kg. body weight. ip.injection for 3 days.

第四組: 6 隻, 2-AAF 20mg/kg. body weight. oral administration by gastric tube for 3 days.

第五組: 6 隻, LPS ip.injection,after 30 min. then 2-AAF per os for 3 days.

第六組: 6 隻,Protocatechuic acid (PCA) 250mg/kg. body weight. per os for 3 days.

第七組: 6 隻,PCA 50mg/kg. per os after 30 min.LPS ip.injection,30 min.2-AAF per os for 3 days.

第八組: 6 隻,PCA 100mg/kg. per os after 30 min. LPS ip.injection,30 min.2-AAF per os for 3 days.

第九組: 6 隻,PCA 250mg/kg. per os after 30 min. LPS ip.injection,30 min.2-AAF per os for 3 days.

(四) NO 的測定方法

依據 NO 的生理機制 ,可以找到數種直接或間接的方法來測量 NO 。由於 NO 會和 oxyhemoglobin ($Hb^{++}O_2$) 起反應, 將它氧化成爲 methemoglobin(Met Hb^{+++}) ,故可利用 oxyhemoglobin 和 methemoglobin 在 PH 7.7 時,不同的 spectrum,直接定出 NO 的量 (401nm. maximum, 411 nm isobestic point)(33)。由於

NO 即爲 EDRF (endothelium- derived relaxing factor), 故可利用 bioassay studies 直接測量 NO 的量, 不同濃度的 NO 可以讓血管呈現不同比例的放鬆效果 (34)。Chemiluminescence 是一種利用含氧自由基氧化能力去氧化 luminol 的偵測方法 ,優點是不破壞被偵測物,敏感度很高而且能持續偵測, 目前也使用來測 NO (35)。也可以利用測 cGMP 的量間接測 NO 的 量, 目前有 kit 可以用 radioimmunoassay 的方式來測 cGMP 量(Amersham Buchler RIA-kit)(36)。NO 會自動氧化成 NO_2^-或 NO_3^-,可以利用 Griess 反應定 NO_2^- 與 NO_3^-的量,也可以用來間接定量 NO(37)。本篇實驗即是用 Griess 反應測定 NO 的量。

Nitrite 測定步驟如下:

Serum

*以 2 ° H_2O 4x dilute

*加入 1/20 volume Zinc sulfate(300g/L)deprotein

*離心 1000g for 15min.(10000g for 5min) at RT.

*supernatant 100 μl.加入平底的 microplate 內。

*加入 100 μl. Griess reagent.(1 g/L sulfanilamide, 25g/L phosphoric acid, 0.1g/L N-1-naphthylethylene-diamine)

*於室溫下放置 10 分,呈色反應。

*Reading with microwell Elisa reader.
(波長 540 nm)

Nitrate 測定方法:

Serum 100 μl.

*以 2 ° H_2O 4x dilute

*加入 NADPH, FAD, nitrate reductase 使成爲最終濃度 50 μmol/L, 5 μmol/L, 200 U/L.

*incubated for 20 min at 37 ℃

*加入 LDH 使成爲最終濃度 10 mg/L.

*加入 sodium pyruvate 使成爲最終濃度 10 mmol/L.

*incubated for 5 min at 37 ℃

*加入 1/20 volume zinc sulfate(300g/L) deprotein
*離心 1000g for 15min. (10000g for 5min) at RT.
*supernatant 100 μl. 加入平底的 microplate 内。
*加入 100 μl. Griess reagent 。
*於室溫下放置 10 分,呈色反應。
*Reading with microwell Elisa reader.(λ 540nm.)
Total NO = NO_2 + NO_3

Standard Calibration Curve:

Nitrate standard 配製是用 500 pmol/μL.KNO_3 (相當於 500 μM NO)以二次水稀釋成 400pmol/μL.; 200pmol/μL.; 100pmol/μL.; 50pmol/μL.; 20pmol/μL.; 10pmol/μL. 然後依照 nitrate 測定方法，設定 x 軸爲 contration of standard(μM); y 軸爲 OD 値(λ 540 nm) 即可劃出標準曲線圖。

(五) 屍體解剖與組織學檢查

請參閱前文

(六) 肝功能檢查

1.血清檢體的處理: 經過三天藥物處理的大白鼠，以斷 頭(decapitation)方式犧牲。收集血液待 30min.血液凝 固後,離心 3000rpm, 4 ℃, 30min.取上層的血清做血清 酵素分析。包括 ALT,AST.

2.血清酵素測定:

a)Alanine Aminotransferase (ALT)測定:

將血清加入含有 alanine 及 alpha-ketoglutarate 的反應溶液中,在 37 ℃作用 30 分鐘, 使反應產生丙酮酸(pyruvate),然後加入呈色劑 2,4-diphenylhydrazine ，均勻混合後靜置 20 分鐘,最後再加入 0.4 N NaOH 溶液使反應終止並呈色。靜置 5 分鐘後,用分光光度計(Hitachi U-3210 spectrophotometer),以 505 nm. 波長

測吸光值，並以丙酮酸作爲標準液稀釋成各種不同濃度依血清測定方法做一標準曲線，經換算即得ALT的酵素活性。

b) Aspartate Aminotransferase (AST)測定：

將血清加入含有L-aspartate 及 α-ketoglutarate 的反應溶液中，在 37 ℃作用 60 分鐘反應產生oxaloacetate，然後加入呈色2,4-diphenylhydrazine，均勻混合後靜置20分鐘，最後再加入0.4N NaOH溶液，使反應終止並呈色。靜置 5 分鐘後，用分光光度計(Hitachi U-3210 Spectrophotometer)，波長505 nm.的吸光值，並對照標準曲線，經換算即可得到AST的酵素活性。

(七) N-nitroso compound (N-NO-AAF)的測定：

1) N-NO-AAF 用 HPLC 測定：

血清加入methanol (1:1 v/v)，然後離心沉澱(3000 rpm 30 min.)，吸取上清液，再以0.22 μm millipore過濾去掉protein，抽取50 μl.打入HPLC column 內進行層析法分析。

Mobile phase:

Ethanol:acetonitrile:2° H_2O = 3:3:5 (v/v)

Flow rate: 1.0 ml/min.

Wave length: UV 254 nm.

Column: C_{18}, 4.0 x 250 mm. ,5μm.

Injection volume: 50μl.

2) N-NO-AAF 用 Spectrophotometer 測定：

(a)原理：N-NO-AAF 加 1-naphthol 形成 diazo-dye (deep red color)，然後用波長 450 nm. 的Spectrophotometer 去測吸光度(OD 值)。就可以間接測出 N-NO-AAF 的量。

(b)方法：

Serum 1 ml.

⇒加入 1-naphthol solution (10mg/ml.ethanol)1 ml pH 7.5

⇒置於室溫下 30 分鐘,呈色

⇒用 Hitachi U-3210 Spectrophotometer 波長 450 nm.去測吸光度。

(c) Standard calibration curve:

N-NO-AAF 稀釋成 10,20,50,100 m mole 各 1ml.

⇒加入 1-naphthol solution 1 ml. pH 7.5

⇒置於室溫下 30 分鐘,呈色

⇒用 Hitachi U-3210 Spectrophotometer 波長 450 nm. 去測每一管標準液的吸光度(OD 值),記錄,然後做成 standard calibration curve 。(x 軸爲 diazo-dye concentration; y 軸爲 absorbance 就可以做出曲線圖)

(八) 統計分析:

所有酵素分析項目皆做兩次分析,結果的表示方法是 mean ± SD 。統計方法是使用 Student's t test 。

參. 結果(Results)

1)以 LPS 1mg/kg.of body weight , ip injection 的大白鼠(group 3)血清內的 NO 濃度 1282±50.24 μM,約爲對照組(group 1)157±28.04 μM 的 8 倍(Table I) ,顯然是有意義的增加 ($p<0.05$)。而且當大白鼠事先灌入各種不同濃度的 PCA ,約 30 分鐘後, ip injection LPS 1mg/kg,再等 30 分鐘,以胃管灌入 AAF 20mg/kg.。由 Table 1 可以明白 group 5 (LPS 1 mg/kg. ＋ AAF 20 mg/kg.) 的大白鼠血清NO濃度爲745.18 ± 17.45 μM , group 7 (PCA 50mg/kg. ＋ LPS ＋ AAF); group 8 (PCA 100mg/kg. ＋ LPS ＋ AAF); group 9 (PCA 250mg/kg. ＋ LPS ＋ AAF)。大白鼠血清內 NO 濃度分別是 453.33 ± 50.48 μM ; 403.13 ± 30.40 μM; 320.00 ± 37.37 μM 。顯然 PCA 會有意義的降低 LPS induced NO elevated($p<0.005$), 並且有 dose response 。

2)肝臟組織切片染色(H&E stain)鏡檢結果:Fig.1 (normal control)顯示正常的肝臟; Fig.2 (solvent control)也是正常的肝臟; Fig.3(LPS only)顯示出局部性壞死(focal necrosis); Fig.4(AAF only)顯示顯示肝臟有壞死現像; Fig.5 (LPS ＋ AAF)顯示肝臟有嚴重的壞死; Fig.6 (PCA only)顯示出正常的肝臟; Fig.7 (PCA50 ＋ LPS ＋ AAF)顯示肝臟有壞死現像;Fig.8 (PCA100 ＋ LPS ＋ AAF)顯示肝臟也有壞死的現像; Fig.9(PCA250 ＋ LPS ＋ AAF)顯示肝臟也是有壞死現像。

3)肝功能(ALT,AST)檢查(Table II) : control 組的 ALT 是 59.93 ± 16.3 IU/L.; group 3 (LPS)與 group 4 (AAF) , ALT 分別是 89.20 ± 28.5 IU/L.; 99.15 ± 19.3 IU/L.可以明顯看出 LPS 和 AAF 都會有意義的昇高肝功能(ALT)($p<0.01$)。在 group 5 (LPS ＋

AAF) ALT 值非常嚴重的增高，然而事先給予不同濃度的 PCA,再給予 LPS ,AAF , group 7 的 ALT 值是 138.98 ± 27.1 IU/L.; group 8 的 ALT 值是 116 ± 16.9 IU/L.; group 9 的 ALT 值是 128.86 ± 24.6 IU/L. 顯然 PCA 會有意義的降低 LPS 與 AAF 所引起的肝炎 ($p<0.01$)。在 AST 值方面也出現相同的情況。

4) N-NO-AAF 的測定:

a) Spectrophotometer method:

Group I ,II,III,IV,V,VI,VII,VIII,IX 皆無法測出 N-NO-AAF 的存在,可想而知 N-NO-AA 的量應該是相當微量。

b) HPLC method:

Fig.10 Chromatogram of group V 無法測到 N-NO-AAF。

Fig.11 Chromatogram of group VII 無法測到 N-NO-AAF。

Fig.12 Chromatogram of group VIII 無法測到 N-NO-AAF。

Fig.13 Chromatogram of group IX 無法測到 N-NO-AAF。

Table I.

以 LPS 誘導 iNOS,和大白鼠用原兒茶酸或不用原兒茶酸處理過的血清 NO 濃度

Experimental group (n=6)	Nitrite and nitrate levels in serum(μM)
Group I (Normal control)	157.00 ± 28.04*
Group II (Solvent control) DMSO	160.20 ± 20.81
Group III (LPS 1mg/kg.)	1282.00 ± 50.24
Group IV (AAF 20mg/kg.)	149.25 ± 8.96
Group V (LPS ＋ AAF)	745.18 ± 17.45
Group VI(PCA 250mg/kg.)	118.33 ± 8.02
Group VII (PCA 50mg/kg. ＋ LPS ＋ AAF)	453.33 ± 50.48**
Group VIII (PCA 100mg/kg. ＋ LPS ＋ AAF)	403.13 ± 30.40**
Group IX (PCA 250mg/kg. ＋ LPS ＋ AAF)	320.00 ± 37.37**

*$p<0.05$, **$p<0.005$,compared with LPS treated group

Table II.

原兒茶酸抑制大白鼠由於 AAF 與 LPS 所引起肝臟傷害的作用

Experimental group (n=6)	ALT levels in serum	AST levels in serum
Group I (normal control)	59.93±16.3**	132.0±9.8
Group II(solvent control) (DMSO)	63.72±14.7	139.8±11.5
Group III(LPS 1 mg/kg)	89.20±28.5	168.6±21.5
Group IV(AAF 20mg/kg)	99.15±19.3	199.5±21.6
Group V (LPS + AAF)	212.5±14.8	370.4±32.6
Group VI (PCA 250mg/kg)	59.25±24.8	136.2±20.6
Group VII (PCA 50mg/kg + LPS + AAF	138.98±27.1*	334.0±26.0***
Group VIII(PCA 100mg/kg + LPS + AAF	116.44±16.9*	261.0±28.5***
Group IX (PCA 250mg/kg + LPS + AAF	128.86±24.6*	259.68±26.8***

*p<0.01, ***p<0.005 compared with LPS and AAF treated group

**p<0.01,compared with LPS treated group

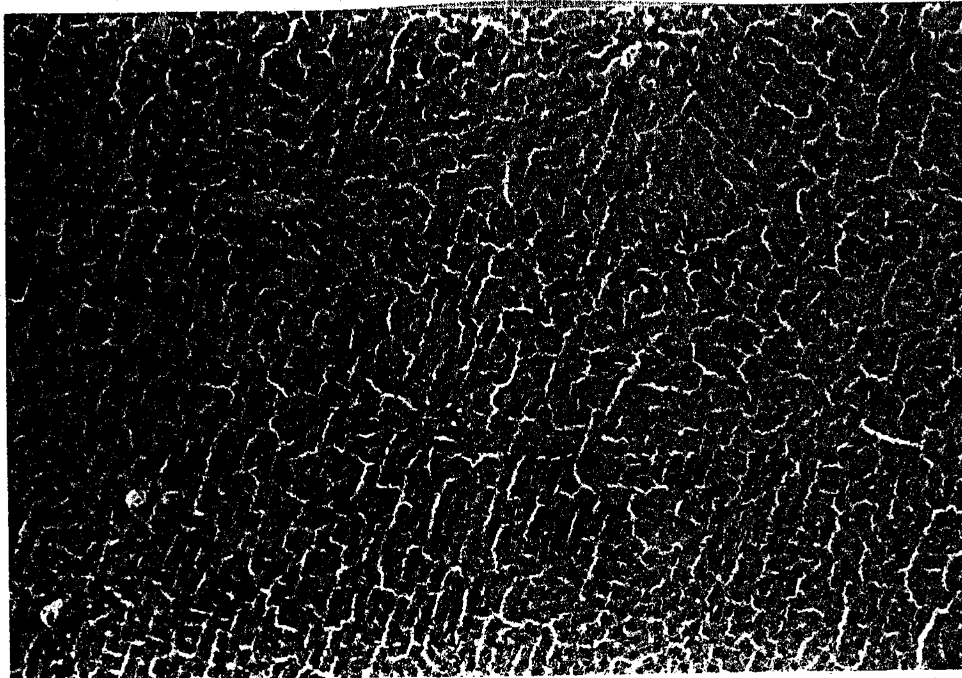


Fig. 1 Liver section obtained from group I rats showed no noticeable leisons (H&E x100)

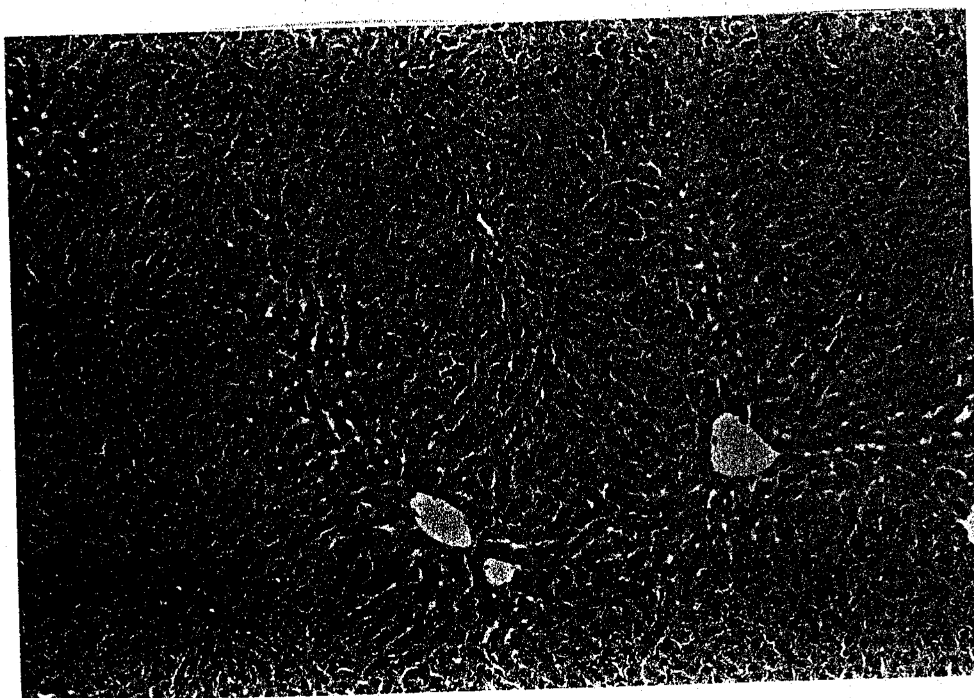


Fig.2 Liver section obtained from group II rats showed no noticeable changes.(H&E x100)

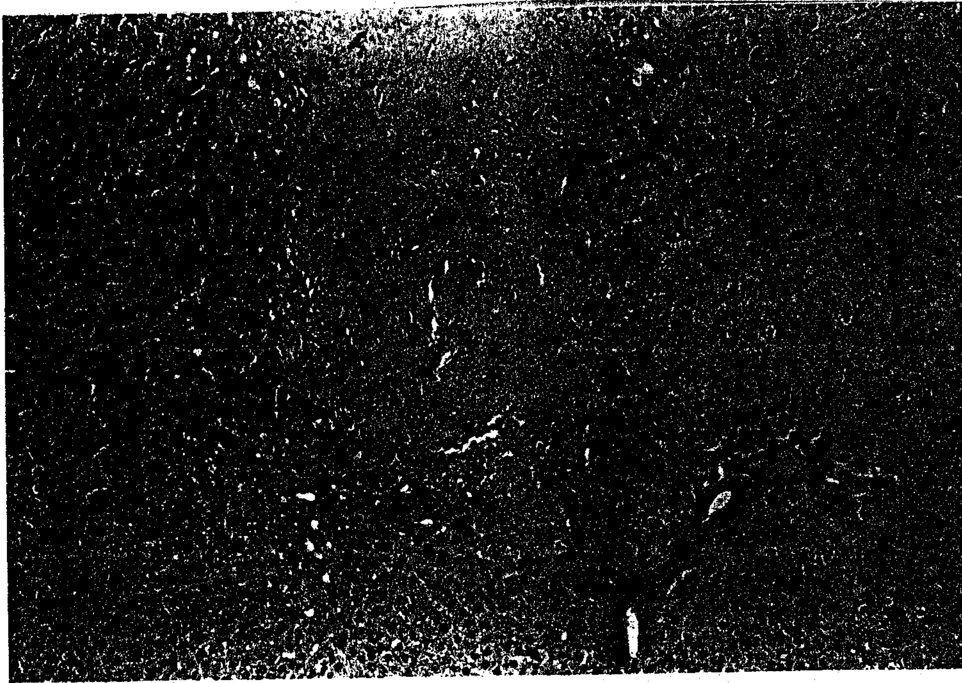


Fig.3 Liver section obtained from group III rats showed focal necrosis.(H&E x100)

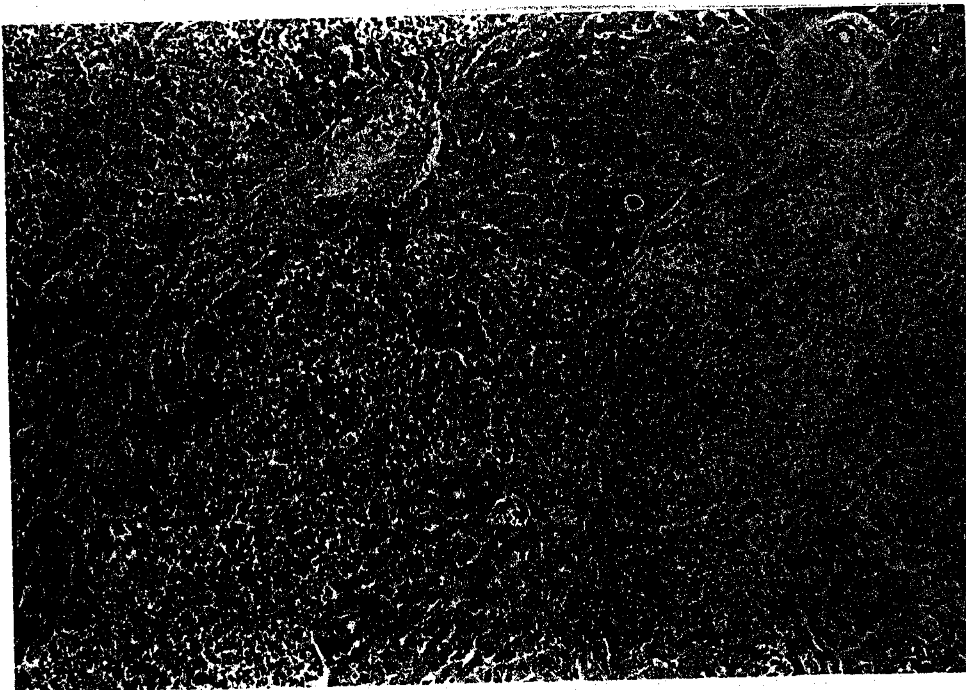


Fig.4 Liver section obtained from group IV rats showed coagulative necrosis.(H&E x100)

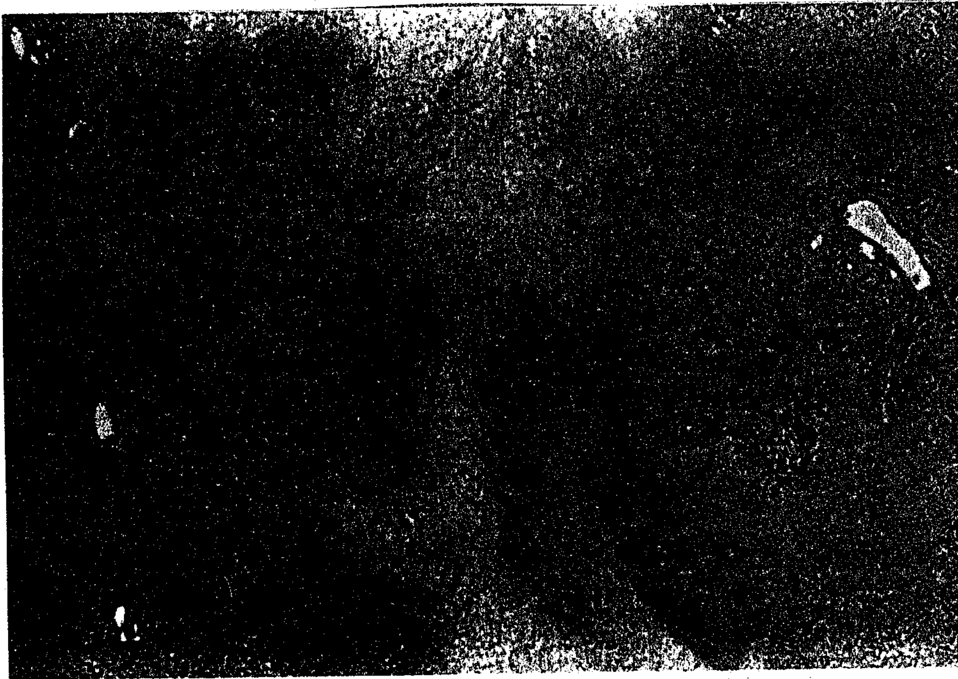


Fig.5 Liver section obtained from group V rats showed severe liquefactive necrosis.(H&E x40)

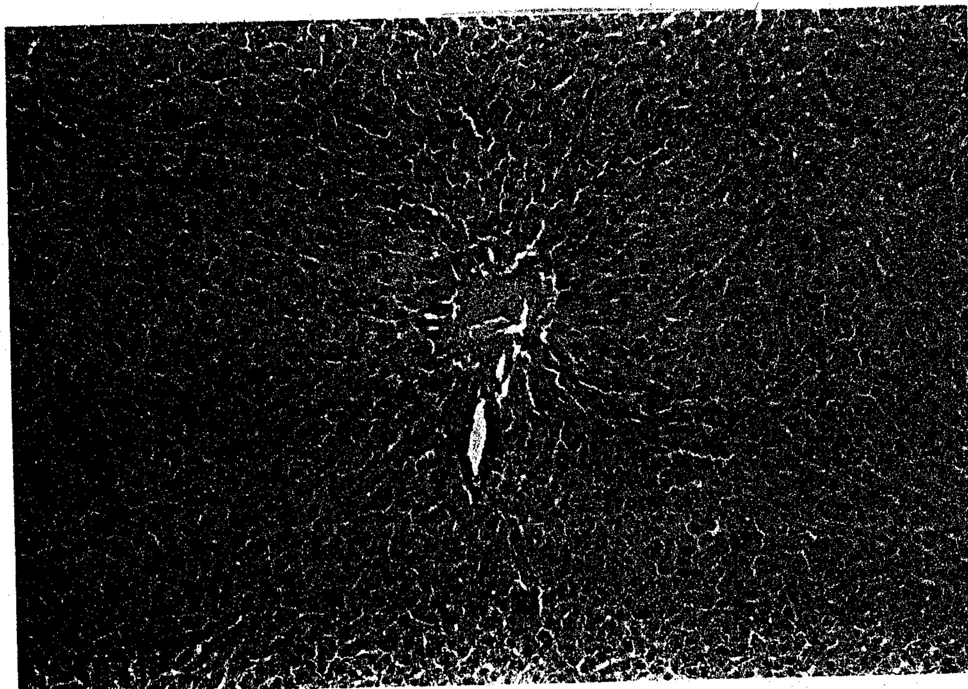


Fig.6 Liver section obtained from group VI rats showed no noticeable changes.(H&E x200)

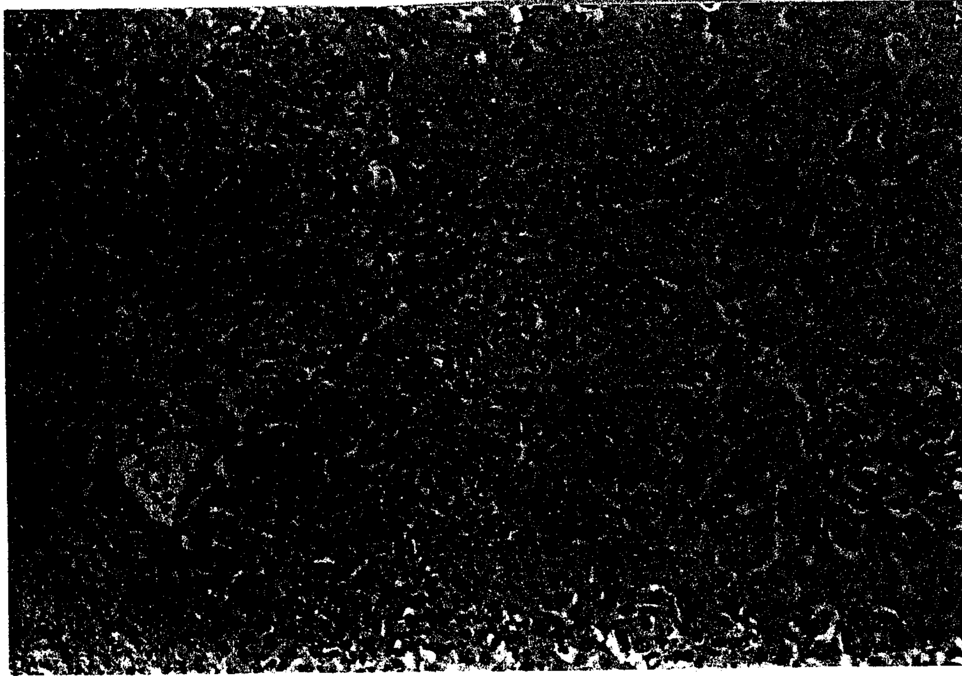


Fig.7 Liver section obtained from group VII rats showed coagulative necrosis. (H&E x200)

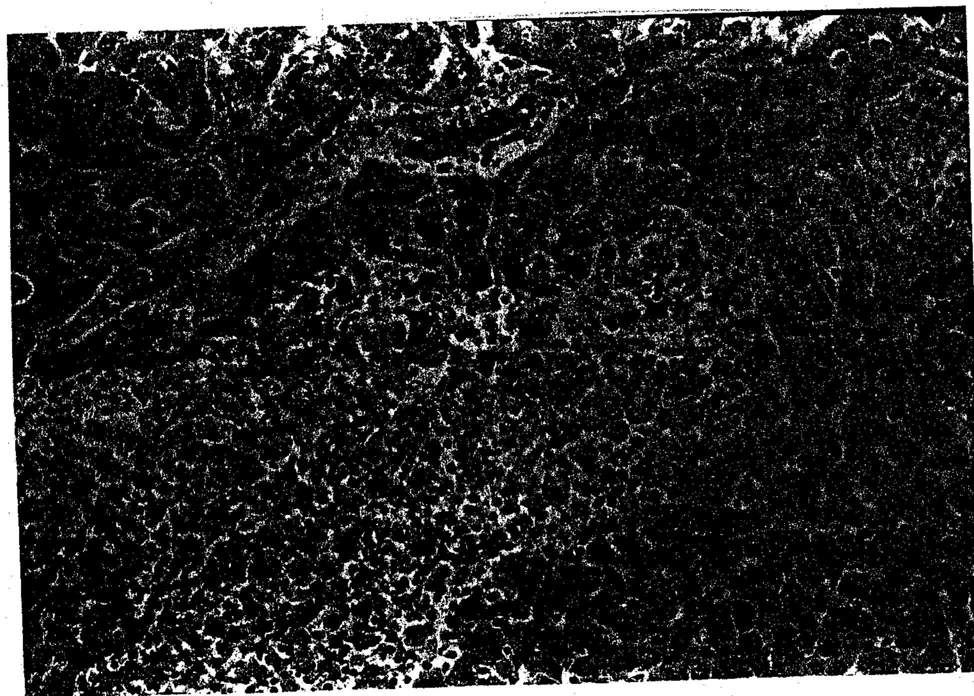


Fig.8 Liver section obtained from group VIII rats showed liquefactive necrosis.(H&E x200)

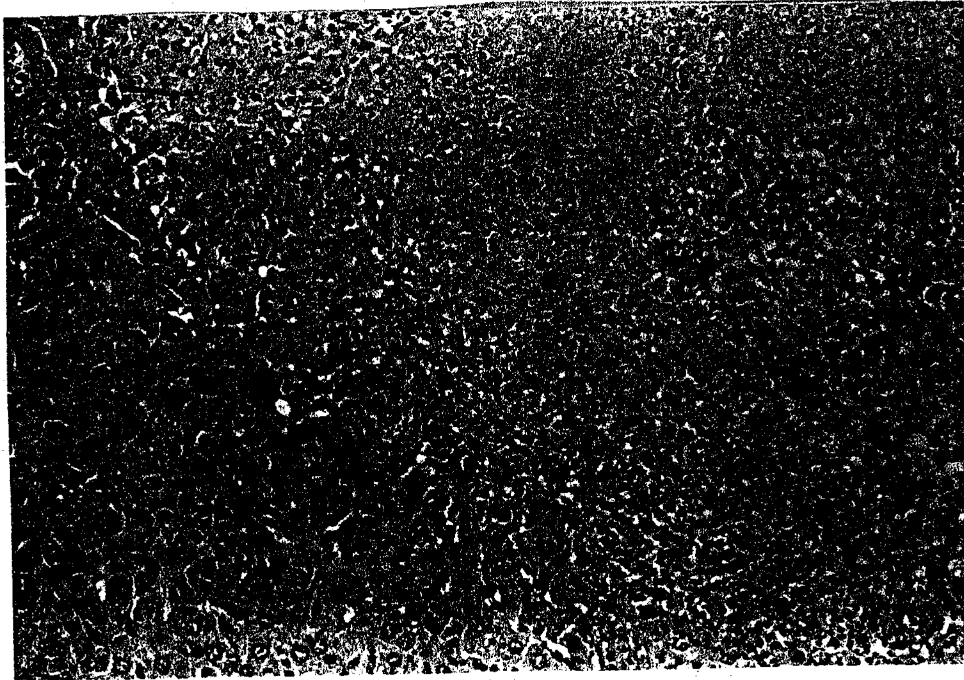


Fig.9 Liver section obtained from group IX rats showed liquefactive necrosis.(H&E x200)

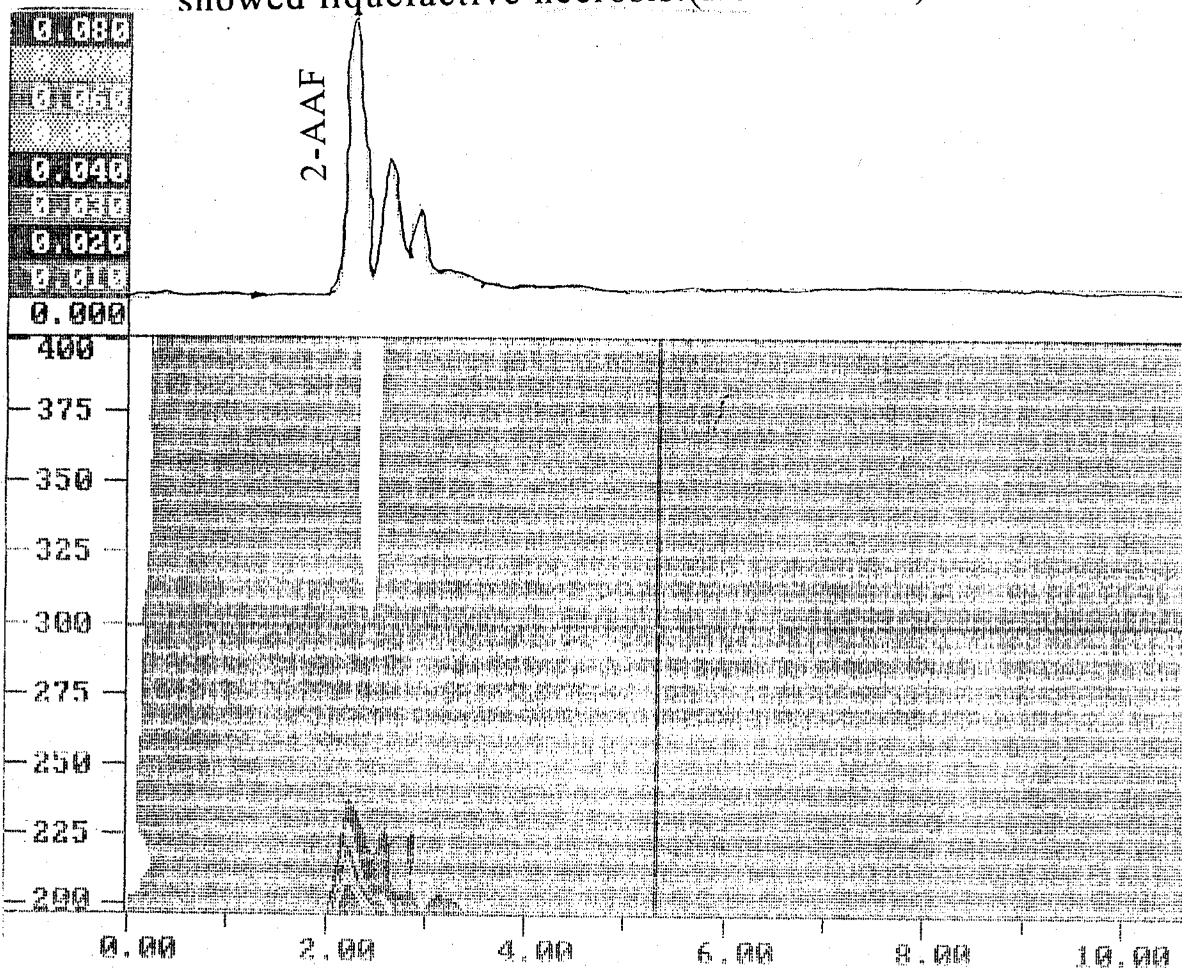


Fig.10 HPLC chromatogram of serum from group V rats

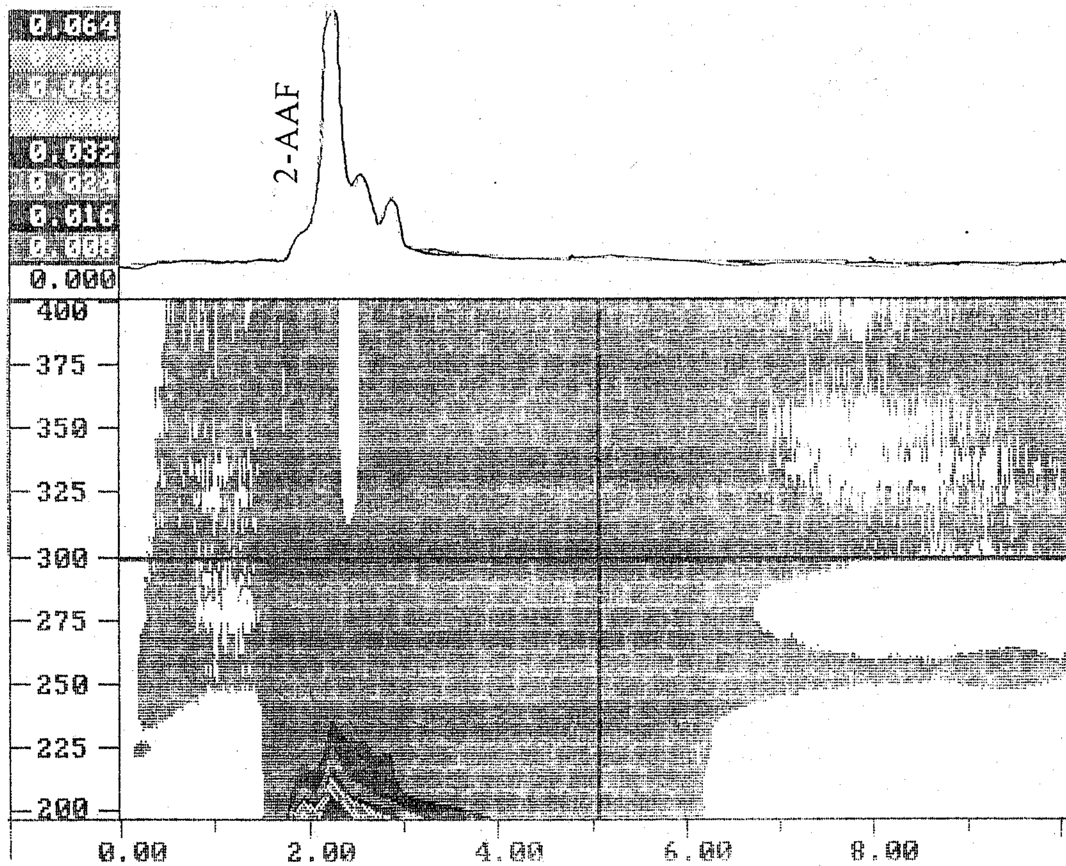


Fig.11 HPLC chromatogram of serum form group VII rats

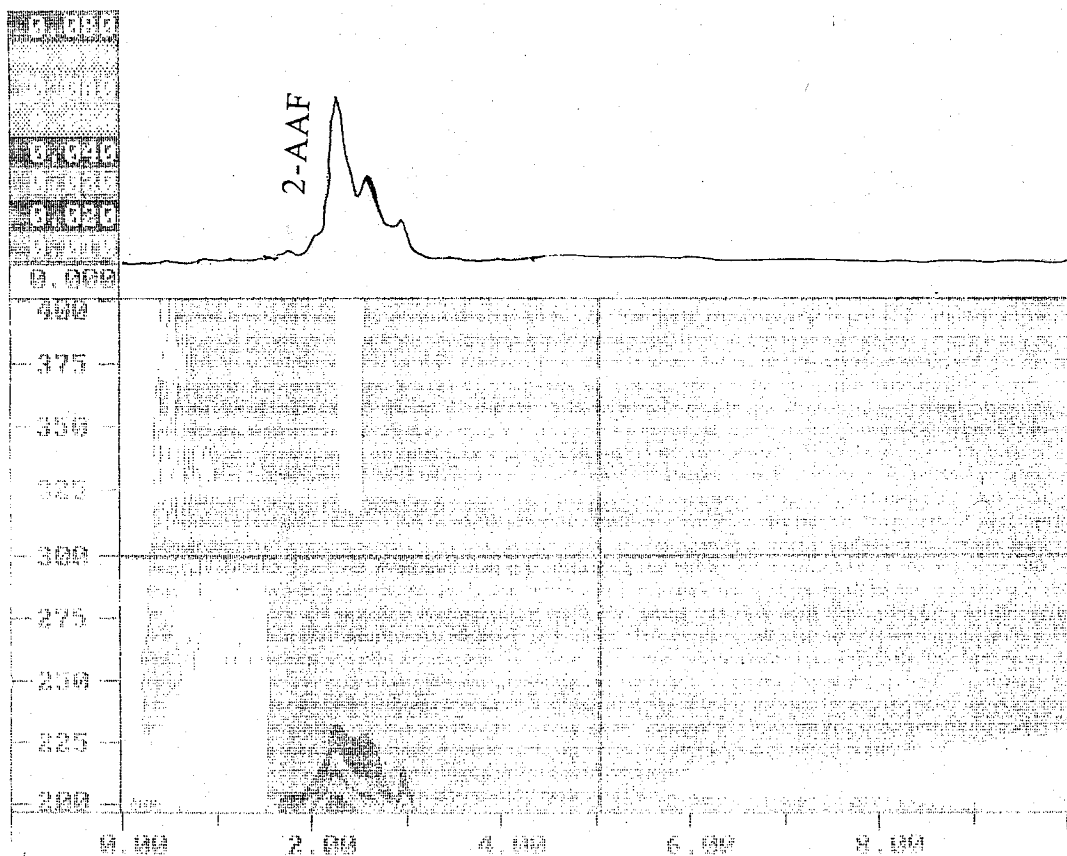


Fig.12 HPLC chromatogram of serum from group VIII rats

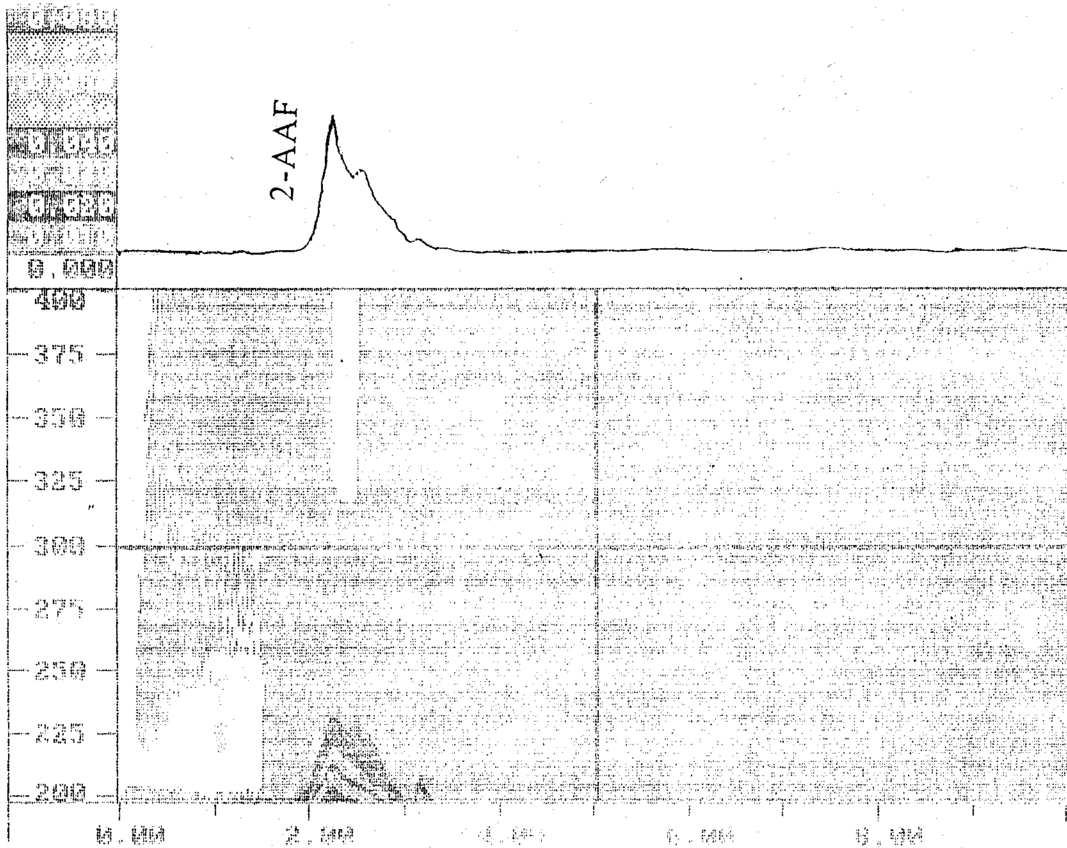


Fig.13 HPLC chromatogram of serum from group IX rats

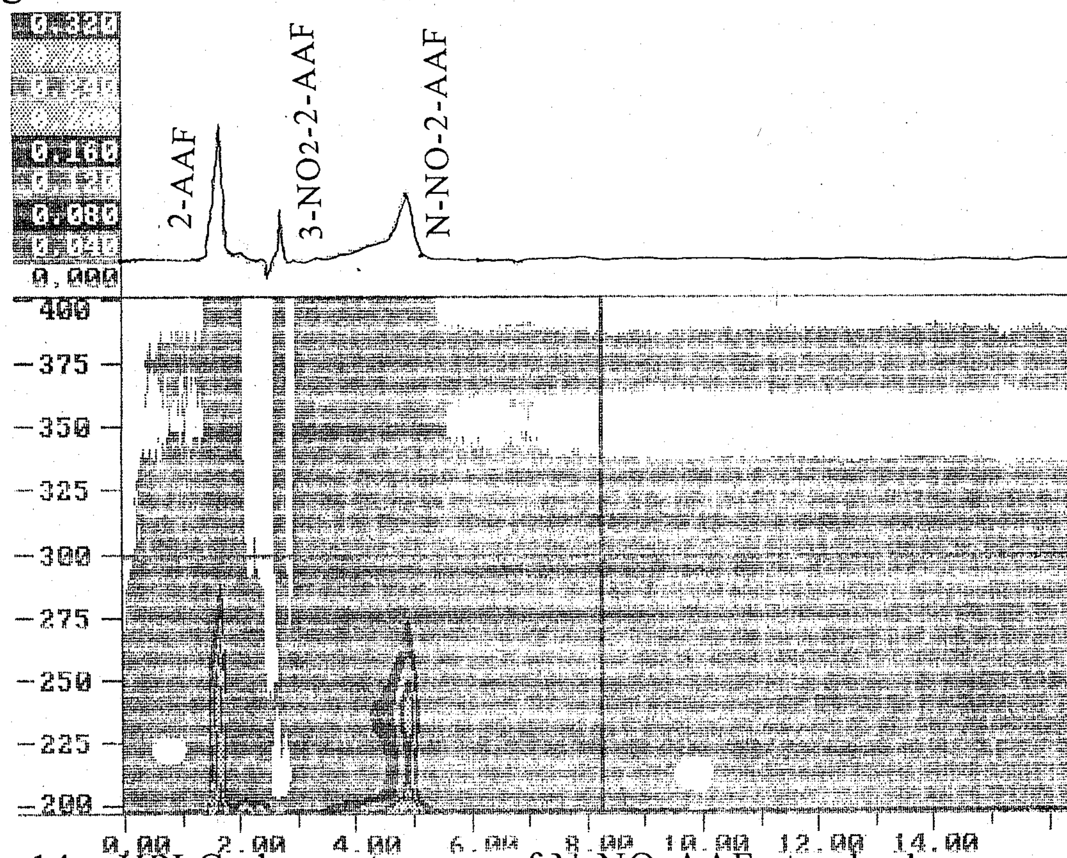
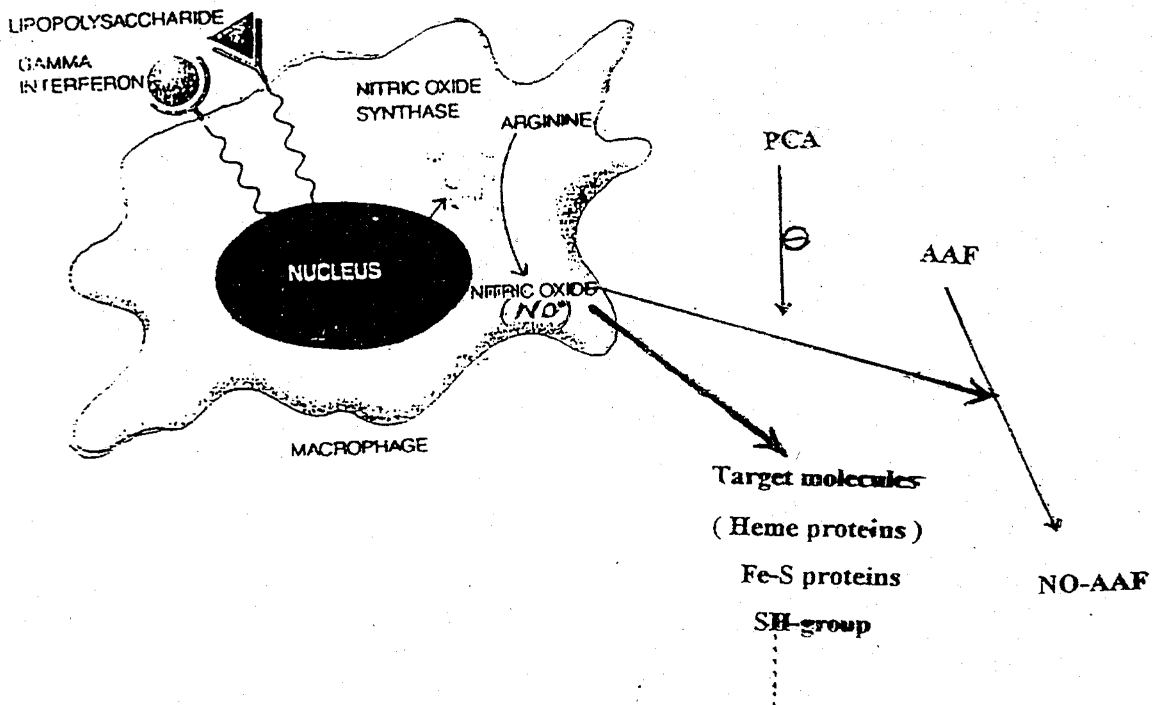


Fig.14 HPLC chromatogram of N-NO-AAF standard.

Conclusion:



肆. 討 論(Discussion)

微生物感染會活化 macrophage 內的 inducible NO synthase,使得內生性 NO 升高,雖然可以當作宿主防禦系統,可是也會引起氧化疾病。特別是 LDL peroxidation 所造成的 atherosclerosis 以及化學品亞硝基化變成有致癌作用(8)。有許多証據顯示 NO 的量增高會導致形成內生性的 nitrosamine 增加,進一步促成癌化(31)。在本篇的研究使用 simple phenolic compound-PCA,具有 free radical scavenging structure-catechol(o-dihydroxy)(8),在 in vivo 實驗時會特異性的降低內生性 NO 的產生。由於 PCA 存在於許多 nuts,fruits,and vegetables,它可以當作 chemopreventive agent 來抑制內生性 nitroso compound 的形成。PCA 可以很容易由天然食物,像 fruit,vegetable and nuts 獲得,所以可藉著日常食物的攝取而達到預防內生性的 nitroso compound 產生(32)。

當實驗的大白鼠事先給與 PCA 時,會降低 AAF 與 LPS 所增加的 ALT 與 NO,所以 PCA 具有抗發炎作用(33)和保護效果。以前的報告証實,用肝細胞培養時,如果加入 PCA 則會捕捉 free radical ,而保護肝細胞免

肆.討　論(Discussion)

微生物感染會活化macrophage內的inducible NO synthase,使得內生性NO升高,雖然可以當作宿主防禦系統,可是也會引起氧化性的疾病。特別是LDL過氧化作用所造成的atherosclerosis,以及chemical nitrosation變成具有致癌性(8)。有許多証據顯示NO的量增高會導致形成內生性的nitrosamine增加,進一步會促成癌化(31)。在本篇的研究使用simple phenolic compound—PCA,具有free radical scavenging structure—catechol(o-dihydroxy) (8),在in vivo實驗時會特異性的降低內生性NO的產生。由於PCA存在於許多nuts,fruits,and vegetables,它可以當作chemopreventive agent來抑制內生性nitroso compound的形成。PCA可以很容易由天然食物像fruit,vegetable and nuts獲得,所以可以藉著日常生活上的飲食攝取而達到預防內生性nitroso compound的產生(32)。

當實驗的大白鼠事先以胃管灌入PCA時,會降低AAF與LPS所增加的ALT(Table II)與NO(Table I),所以PCA有抗發炎的作用(33)和保護效果。以前的報告証實,用肝細胞培養時,如果加入PCA則會捕捉free radical,而保護肝細胞免於受到oxidative damage(28)。有許多研究証明PCA具有保護DNA與降低S-phase細胞數目,所以說PCA是非常有效用的chemopreventive agent。PCA可以藉著減少NO的產生而避免nitroso compounds攻擊DNA,所以PCA對化學致癌物的抑制作用是屬於initial stage。

AAF在1941年美國農業部門使用來當作殺蟲劑(insecticide),當時的描述包括2-aminofluorene是殺蟲

劑的必要成分,"...a material which is relatively non-toxic to man and domestic animals when taken by mouth..."。後來 Wilson et al.(1941)發現 rats 以各種不同濃度 AAF 混合飼料飼養,時間如果超過 100days,在屍體解剖發現鼠體內不同器官與組織有許多腫瘤,這時候才知道 AAF 有致癌性,此後決定了 AAF 永不當作殺蟲劑的命運。然而在台灣仍然有 aminofluorene 成分的殺蟲劑在市面販賣,實在不當。

AAF 首先在 liver ER 與 cofactor-NADPH,O_2 經過 cytochrome p-450 代謝活化形成 N-hydroxy-2-AAF 的中間產物(17,18,19),然後在 cytosol 經 sulfotransferase 活化產生 N-sulfonoxy-2-AAF 的終極致癌物,不管中間產物或終極致癌物都具有更強的親電子性,因此對肝臟的毒性遠較 AAF 爲強。AAF 與 LPS 都能過造成肝炎(27),當大白鼠同時給予 AAF 與 LPS,從病理切片可以觀察到大白鼠的肝臟會有嚴重性的液化性壞死 (liquefactive necrosis)。

由 Table I NO 濃度可以看出 group III(LPS only) 的 NO 是 1282.00±50.24 μM, group IV(LPS + AAF)的 NO 是 745.18±17.45μM.顯然是有意義的降低 NO 的量 ($p<0.001$)。可能的原因是 LPS 所 induce 的 NO 結合了 AAF 所以 NO 量下降;或許是 AAF 會部份抑制 LPS 的 function,使 induce 的效果不理想。然而眞正的原因仍然有待進一步探討。

有許多証據指出單獨給予 LPS 是無法活化肝臟的 inducible NO synthase,除非再給予 cytokine(15,16,32)。當活化肝臟的 inducible NOS 就會 release NO,因爲 NO 產生後有它攻擊的 target molecules(Heme protein, Fe-S proteins,non-heme Fe and metalloproteins, Tyrosyl radical,SH groups,Amines,DNA,Oxygen, Superoxide

anion,hydrogen peroxide),因此在生理狀態下,外加的chemical要與NO大量結合實在是不容易;而且N-NO-AAF本身不穩定,decay時間又短;另外原因是從老鼠取出的血清雖然放在-70℃,不能馬上進行N-NO-AAF測定,都有可能導致誤差,而無法測得N-NO-AAF。

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