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

指導教授: 謝易修 博士 (Yih-Shou Hsieh)

第一部份:

G6PD 病人紅血球中 Carbonic Anhydrase Isoenzyme 變化之分析探討

Determination of Erythrocyte CA I and CA II Isoenzyme in G6PD Deficiency.

第二部份:

台灣地區正常人血液中抗氧化酵素活性(SOD, GSH)及 Total Antioxidative Status 關係之研究

Determination of Antioxidative Enzyme Activity and Variation of Superoxide Dismutase, Glutathione Peroxidase, and Total Antioxidant Status in Healthy Chinese.

研 究 生: 江蕙玲 (Whei-Ling Chiang)

中華民國八十八年六月

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 大學(學院) 生物化學研究所 系所

_____組 八十七 學年度第 二 學期取得 碩 士學位之論文。

論文名稱：1:G6PD 病人紅血球中 Carbonic Anhydrase Isoenzyme 變化之分析探討

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指導教授姓名: 謝 易 修 博士

研究生簽名: 江萬珍 學號: R86201

(親筆正楷) (務必填寫)

日期:民國 88 年 8 月 16 日

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1. 本授權書請以黑筆撰寫並影印裝訂於書名頁之次頁。
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本論文為中山醫學院授與理學碩士學位之必備條件之一，經中山醫學院生物化學研究所碩士論文考試委員會審查合格及口試通過。

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(論文指導教授)

中華民國八十八年六月

學生江蕙玲論文題目為第一部份: G6PD 病人紅血球中 Carbonic Anhydrase Isoenzyme 變化之分析探討；第二部份:台灣地區正常人血液中抗氧化酵素活性(SOD, GSH)及 Total Antioxidative Status 關係之研究，其論文已經中山醫學院生物化學研究所碩士論文考試委員會審查合格及口試通過，並由其指導教授核閱後無誤。

指導教授　　謝易修　博士　　簽名: 謝易修

中華民國八十八年六月

誌　謝

兩年的碩士班生活在忙碌的學習中匆匆劃下句點。感謝我的指導教授謝易修博士在學業研究上不厭其煩的指導，在實驗遇到困難及瓶頸時給予最大的協助，並在論文撰寫期間悉心加以指導及修改使本論文得以順利完成，學生衷心感謝。文稿初成，蒙私立中台醫護技術學院食品衛生系朱淑珍博士及中山醫學院生物化學研究所劉哲育博士對本論文細心審查並詳加斧正使得本論文更臻完善，在此謹表致謝。

研究過程中感謝臺中榮民總院生化科賴志昌副技師及林淑雯小姐在檢體提供上給予協助，也感謝醫技系的同仁的鼓勵與幫忙，更感謝劉哲育博士及陳凌雲博士在課業及實驗上的指正，實驗室內研究伙伴玉燕、惠珍、意偉，在實驗上的切磋與討論，使本論文順利完稿。

最後更要感謝我的父母、大姐麗雲及姐夫純宇在兩年來總是適時伸出援手，解我燃眉之急，使我在家庭、學業及工作得以兼顧。而我的最愛亦辰、光泓及先生志昌更是我在研究所研讀期間最大的精神支柱，你們無怨無悔的付出與支持，使我得以順利完成學業。

目　錄

缩寫表

G6PD: glucose-6-phosphate dehydrogenase

NADP: nicotinamide adenine dinucleotide phosphate

CA: carbonic anhydrase

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

HMP: hexose monophosphate shunt

PCR: polymerase chain reaction

AE 1: anion exchanger 1

CARP: CA-related protein

SOD: superoxide dismutase

GSHPx: glutathione peroxidase

TAS: total antioxidant status

OFR: oxygen free radical

Se: selenium

DMPO: 5,5-dimethyl-1-pyrroline-N-oxide

MDA: malondialdehyde

1-NP: nitropyrene

第一部份:
G6PD 缺乏病人紅血球中 Carbonic Anhydrase Isoenzyme 變化之分析

Determination of Erythrocyte CA Ⅰ and CA Ⅱ Isoenzyme in G6PD Deficiency.

SUMMARY

The aim of the present study was to determine the level of erythrocyte carbonic anhydrase isoenzyme of G6PD deficiency patient in Taiwan . Carbonic anhydrase (CA) is a zinc-containing enzyme, it reversibly catalyzes the hydration of carbon dioxide to bicarbonate and hydrogen ions. CA II is believed to be a chloride/bicarbonate exchanger in red blood cells, and also directly coupled to band 3 protein. Human erythrocyte CA I and CAII were measured in normal subjects (n=30) and G6PD deficiency patients (n=30), by using Western blot assay (with 12.5 % SDS-PAGE- 0.8 % bisacrylamide). In the groups of G6PD deficiency patients, there was a tendency for CA I / CA II ratio (mean= 2.3) to be lower than normal subjects (mean= 5.2)($P<0.001$). The band 3 protein were resol-ved by 8 % SDS-PAGE, normal subjects was also significantly higher than the G6PD deficiency patients. We may speculate that in G6PD deficiency might be to a change in CA II and band 3 protein expression and stability. The cytoskeletal proteins which bind spectrin, and that this change makes the affected protein more susceptible to oxidative damage, band 3 protein loss and increased levels of CAII in G6PD deficiency patients may lead to hemolytic anemia. Detailed mechanism is unclear.

中 文 摘 要

本實驗主要是研究臺灣地區 G6PD 缺乏的病人紅血球內 carbonic anhydrase isoenzyme 變化的情形。carbonic anhydrase(CA)是一種含鋅離子的金屬酵素，為紅血球中除了血紅素外主要的蛋白，在紅血球中扮演著 $Cl^-/HCO3^-$ 轉換代謝的重要角色，已證實會與 band 3 蛋白結合。我們使用 12.5 % SDS PAGE (0.8 % bisacrylamide)電泳分析，並以專一的 carbonic anhydrase II 抗體進行西方墨點法分析正常個人(n=30)及 G6PD 缺乏病人(n=30)紅血球內 CA I 及 CAII 的蛋白變化。結果顯示 G6PD 缺乏病人(n=30)紅血球中 CA II 明顯上升。所得數值以 student's test 及 stepwise regression 進行分析後發現在 G6PD 缺乏病人組別中 CA I / CA II 的平均比值為 2.3，明顯低於正常個體的平均比值 5.2 ($p<0.05$)。正常個體樣本中 band 3 的蛋白表現明顯高於 G6PD 缺乏病人。由結果我們得知當 G6PD 缺乏可能會影響 CA II 及 band 3 等蛋白的基因表現或蛋白穩定度，當有氧化性傷害出現更加強改變 spectrin 與 cytoskeleton 的結合，使 cytoskeleton 自 spectrin 蛋白中流失，導致 band 3 蛋白減少及 CA II 蛋白合成也會跟著上升，造成溶血之臨床症狀，而其詳細機轉則需深入探討。

緒　　言

全球的人口約二億的人有 glucose-6-phosphate dehydrogenase (G6PD)缺乏的問題[1]，尤其是地中海沿岸國家的住民(希臘、土耳其、義大利、以色列等)，非洲人，亞洲人(東南亞，中國南部)較多，而日本的病例較少[2]。臺灣有 2～3%，美國黑人有 10～20%的發生率。由於 G6PD 本身的物理及生化學不同至今已發現有 400 種以上不同的變異型(variants)，而不同的地理區域也有不同的 G6PD variants 分佈。也因 G6PD variants 與人類生活環境之不同，所引起的溶血性疾病也有不盡相同之處[3-4]，大多數 G6PD 患者的貧血都是陣發的，而奎寧類及蠶豆等氧化物會誘導急性溶血的發作。在台灣 G6PD variants 有 50%為 G1376T; 21.3 %為 G1388A; 7.4 %為 A 493G; 7.4%為 A 95G; 4.2%為 C1024 T[5-6]。病人表現出來的臨床症狀以新生兒高膽紅素血症(neonatal hyperbilirubinemia)、急性溶血性貧血(acute hemolytic anemia)及蠶豆症(Favism)為主。

一、 Glucose-6-phosphate dehydrogenase (G6PD)

1、G6PD 的特性與構造

G6PD 是一種看家酵素(house keeping enzyme)，G6PD 的單體由 515 個胺基酸所組成，分子量為 59,256 daltons。活化態的酵素是由兩個單體 G6PD 與 NADP 緊密結合所組成[7-9]。NADP 結合的位置是在第 386 及 387 胺基酸間(lysine 和 arginine)[10]而第 205 胺基酸(lysine)是葡萄糖-6-磷酸的結合位，G6PD 是六碳糖單磷酸分路(Hexose Monophosphate Shunt; HMP)的第一個酵素，它催化葡萄糖-6-磷酸氧化成 6-磷酸葡萄糖酸，同時使 NADP 還原成 NADPH，NADPH 是氫過氧化物分解時最終電子供給者。HMP 是紅血球中唯一的 NADPH 來源，並可提供五碳糖合成核甘酸。NADPH 之 H 為 glutathione reductase (GSHPx)之 H 的主要來源，可保護紅血球對抗氧化物的破壞[11]。因為 G6PD 主要存在胞質中，而其他會產生 NADPH 的酵素如 isocitrate dehydrogenase 是存在細胞粒腺體。因此 G6PD 缺乏是現今最常見可影響人類紅血球 NADPH 產生的主要因素。而且 G6PD 活性會影響 cell attachment(細胞附著)及 growth factor stimulated tyrosine phosphorylation 在細胞生長過程中扮演重要的角色[12]。

G6PD 缺乏為性聯性遺傳，其基因位於 X 染色體長臂區 28 帶(Xq28)，男性 G6PD 缺乏常為極度或完全缺乏，為 homozygote，而女性 G6PD 缺乏大都是屬於輕度缺乏[13-14]，為 heterozygote 很少有 homozygote，這就是為什麼蠶豆症患者男性遠多於女性的原因。在這段區域的基因產生的疾病尚包括 fragile A, hemophilia A (A 型血友病), color vision (色盲), Bornholm eye disease, clasped-thumb mental retardation (MASA) syndrome 和 dyskeratosis congenita (先天性角化不良)。

2、G6PD 突變型之特性

1986 年 Persico 等人已將 G6PD 基因找出並定出它的序列[15-16]，這段基因長度超過 20 Kb 包括 13 個 exons，第一個 exon 不含 coding seguence，exon 2 和 exon 3 之間的 intron 特別長，約 9857 bp，5'端序列為 71 bp 及 3' 端序列為 608 bp，依其生化特性不同，至今已有 442 個不同的 G6PD 突變型被發現，因為大多數的 G6PD 突變型皆具有不正常的電泳特性及酵素活性，例如 G6PD A 突變型主要發現於非洲，當 G6PD 突變型其電泳的速度比正常快，稱為 G6PD A^-。目前藉由 polymerase chain reaction (PCR)的技術可鑑定出的 G6PD 突變型已有 60 個突變型[4, 17-18] (Table 1)。G6PD 缺乏也是一種基因多態性(polymorphism)，根

據流行病學上的調查發現 G6PD 缺乏病人可抵抗 falciparum malaria (鐮刀狀瘧疾)的感染[19-21]。可能是因為 G6PD 缺乏病人體內的 oxidative stress(氧化壓迫)可使瘧疾寄生蟲無法生存。相對的 G6PD 缺乏病人是 AIDS 的高危險群，理由為 1、體內氧化狀態不平衡在淋巴組織中會造成 macrophage 的活化而誘導細胞的增生[71]。2、G6PD 的轉錄作用會受 HIV 調節蛋白的影響。G6PD 缺乏可分為① class Ⅰ:與 nonspherocytic hemolytic anemia (非球形溶血性貧血)有關，G6PD 主要突變點在 NADP 或葡萄糖-6-磷酸的結合位。② classⅡ:G6PD 缺乏非常嚴重，G6PD 的活性低於正常之 10 %。③ classⅢ:G6PD 中度缺乏，G6PD 的活性介於正常之 10～60%間。④classⅣ:G6PD 的活性表現正常(60～150%)。⑤classⅤ:G6PD 活性表現增加[22]。

3、G6PD 缺乏之臨床症狀

臺灣常見為 classⅡ及 classⅢ G6PD deficiency，除貧血外，茶色尿、腹痛、嘔吐等為其特色。

①hemolytic anemia:包括 drug-induced hemolysis (Table 2);

因感染所誘導的溶血及 diabetes mellitus 所誘導的溶血反應，其中 8-aminoquinoline 及抗瘧疾藥物(primaquine)等會使血紅素構形改變，形成 Heinz body，此變性的蛋白聚集在紅血球膜

上引發溶血[23]。

② Favism(蠶豆症): 較常發生在 class Ⅱ G6PD deficiency，因豆類中的 L-dopa、vicine、 convice 等成份可產生 semiquinoid 自由基而使紅血球變性，造成溶血[24]。

③ neonatal jaundice: 原因極複雜至今仍無定論，G6PD 缺乏為其中因素之一，主要發生於亞洲及地中海沿岸住民，膽紅素值常超過換血之標準(20 mg/dL)，若黃疸持續二週，常會發生核黃疸，嚴重者造成智力障礙甚至死亡。

④nonspherocytic hemolytic anemia: 屬 class I G6PD deficiency 在臺灣較罕見。

4、G6PD 之實驗室診斷

① G6PD deficiency 之定性

1961 年 Motulsky 及 Campbell-Kraut[25]利用 brilliant cresyl blue 的褪色反應。當 G6PD 缺乏造成時 NADPH 量減少，使 methemoglobin 還原速率減緩，除了 brilliant cresyl blue，methylene blue 也廣泛被使用。

②紅血球內 G6PD 活性之定量

一種簡單的定量法，當有葡萄糖-6-磷酸及經溶血處理的紅血球存在下可將 NADP 轉變成 NADPH，可由紫外光吸光或螢

光變化得知 G6PD 活性[26]。

③ G6PD 突變型的鑑定

早期方法須先將酵素經由 DEAE cellulose 等步驟純化再進行活性、電泳及熱穩定性之分析[27]。現在則改用 polymerase chain reaction (PCR)的技術鑑定[28]。

二、 Human band 3

在細胞膜 lipid bilayer 的結構中穿插著膜蛋白及細胞骨架，這些細胞骨架包括 α, β spectrin；ankyrin；protein 4.1 及 actin。當 α 和 β spectrin 形成四聚體(tetramer)會接上 ankyrin 再與 band 3 形成巨大的分子，而 band 4.2 則可加強 ankyrin 與 band 3 間的交互作用[29] (Fig. 1)。因此分析 band 3 蛋白時，band 4.2 會與 band 3 平行升降，其它蛋白則無此現象。band 3 蛋白為紅血球膜蛋白中含量最豐富的蛋白(1.2×10^6 copies per cell，約佔紅血球膜蛋白的 25 %)[67]。

band 3 也稱為 AE 1(anion exchanger 1)，主要表現在紅血球膜上及腎小管與尿道的細胞膜上，是一種含有 911 個胺基酸的 glycoprotein[30]，是紅血球上的 anion exchanger，它可快速的催化著碳酸根離子及氯離子間的交換(chloride shift)[31]，band 3 包含兩個不同的構造及功能單位：① 43 kDa amino-terminal cytoplasmic domain 在這段區域的結合位包括多種的 glycolytic enzymes、血紅素及 tyrosine kinase 等。變性血紅素會與 band 3 的 amino-terminal domain 結合會導致 band 3 clusters 的形成，在循環血流中會被體內抗體(IgG)所認識，進而被 phagocytes 及 macrophage 排出體外[32]。②55 kDa carboxyl-terminal membrane

domain 由 12～14 個 transmembrane helics 形成生理上重要的離子通道。在 band 3 蛋白之 C 端上 $Tyr^{359}Lys^{360}$ 的位置上很容易發生 proteolysis 的現象而使 43 kD 與 55 kD 兩個 domain 分開[61]。

1998 年有研究論文指出 carbonic anhydrase 會與 band 3 的 carboxyl-terminal region 以相等比例緊密的結合，是紅血球中離子代謝的主要酵素(68)。當紅血球中的 CA II 水解二氧化碳形成碳酸根離子時，紅血球胞膜可透過 band 3 將氯離子及碳酸根離子進行轉運，使二氧化碳順利的轉運到循環血流中經由肺部排泄出去，此活性並可調節細胞內的 pH 值。這是紅血球維持細胞穩定性及細胞膜上架構蛋白完整的重要機轉[62]。band 3 蛋白亦可提供 hemoglobin、hemichrome 及醣化酵素的結合位。另外可作為老舊及受傷細胞的 senescence antigen 紅血球發生病變時，血紅素會與膜上之 band 3 結合，也就是形成陰離子通道(anion channel)，相聚而形成膜表面上之新抗原。此新產生的抗原會與體內之 IgG 相結合，改變膜的抗原性而導致血球被清除，這是目前研究已知溶血現象發生的原因之一[29]。

band 3 在紅血球及腎臟疾病中扮演重要角色。美洲及歐洲罹患有 hereditary spherocytosis(遺傳性圓球血症)的病人中有 20 % 出現 band 3 缺乏[33]。研究因蠶豆等氧化物會誘導急性溶血的 G6PD

缺乏患者中發現 Ca^{2+}-ATPase 失去活性；在 SDS-PAGE 中可發現 band 3、band 4.1 蛋白減少，band 4.5 及 band 4.9 蛋白增加，並形成大分子的凝集。在 23 到 29 kDa 中出現新的 polypeptide 化合物及明顯的 membrane-bound globin[34-37]。而在 G6PD Seattle(E282H)及 G6PD Mediterranean(S188F)的病人中也發現 spectrin 及 band 3 蛋白減少[38]。band 3 在腎皮質集尿管細胞中亦有明顯的表現，會增加尿液中碳酸根離子的排泄，使尿液偏酸[39]，研究中發現 band 3 缺乏會引發中度的酸中毒，血液中 pH 值約會下降 0.15 unit[63]。而在骨細胞中發現 band 3 缺乏亦會使碳酸根離子無法順利排泄，導致骨膠質在酸性中會被分解，造成 osteopetrosis(骨質疏鬆症)[40]。

三、carbonic anhydrase (carbonic hydrolase)

CA 是一種含鋅離子的金屬酵素，其分子量約為 30,000 左右，於 1935 年首先由 Roughton 在紅血球中發現，1940 年以後 Davenport 發現腎小管中亦含有高濃度的 CA，CA 廣泛分佈於動、植物，主要是催化 $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ 可逆性水解反應，在二氧化碳的輸送或分泌過程、鈣化及光合作用(photo-synthesis）方面非常重要，在哺乳動物中的生理功能包括維持離子輸送(Na^+/H^+，Cl^-/HCO_3^-)及調節 pH 值及離子及水份的平衡。在代謝上參與肝醣生成、尿素生成反應及脂質生成反應，幾乎存在於所有器官中，目前有 7 種 isozyme 在哺乳動物被分離出來，包括：①存於細胞質的 CA I，II，ⅢA，ⅢB，Ⅶ：負責離子交換(Na^+/H^+，Cl^-/HCO_3^-)。②與細胞膜相關的 CA IV：含量較少，是負責幫助二氧化碳排出細胞外。有一半的人類肺臟 CA IV，80%老鼠肺臟 CA IV 及大部份老鼠骨骼肌的細胞膜相關 CA 被發現是透過 phosphatidylinositol glycan anchors 連結在細胞膜上[41]。③存於粒線體的 CA V：是負責幫助二氧化碳排出粒線體外。④分泌型 CA VI。

1、carbonic anhydrase families 個論

①carbonic anhydrase I 位於第 8 染色體長臂區 22 帶(8q22)，分

子量 29 kDa，是人類紅血球中除了血紅素外主要的蛋白質，含量約 12 mg CA I / gHb，在紅血球中的含量為 CA II 的 5～6 倍，但活性確僅為 CA II 的 15 %，在 25℃ 下約 2×10^5 sec^{-1} [43]，容易被 Cl^- 或鹵化物所抑制。CA I 的分佈不如 CA II 廣泛，在大腸、直腸等器官有明顯表現[44]。在人類胎兒紅血中無法測得 CA I，一般在正常懷孕 40 週後的 新生兒可測得 CA I，慢慢升高約一年後與成人的量相同。

② carbonic anhydrase II 為研究最多的 CA isoenzyme，位於第 8 染色體長臂區 22 帶(8q22)，分子量 29 kDa，水解 CO_2 的活性非常強，在 25℃，pH 9 的環境下約 1×10^6 sec^{-1}[42]會被 iodide，sulfanilamide 和 bromopyruvic acid 所抑制。一般 CA II 常由紅血球中純化得到，含量約 2 mg CA II / gHb。CA II 在組織中的分佈很廣包括骨組織的噬骨細胞(osteoclass)、腦的少突神經膠質細胞(oligodendrocyte)、眼睛的膜絡叢(choroid plexus)上皮細胞和睫狀體(ciliary body)、肝細胞、腎臟細胞、胃壁細胞、唾液腺、胰臟、紅血球、白血球、血小板等 CA II 皆有表現。CA II deficiency 與 osteopetrosis(骨質疏鬆症)；renal tubular acidosis (腎小管酸中毒)，cerebral calcification (脊髓鈣化)等疾病有關。在 7 種 carbonic anhydrase isozyme 中 CA II 對二氧化碳轉換成碳酸

根離子的活性最強，紅血球膜上有大量的 anion transporter 亦可加強 HCO_3^-/Cl^-交換。

③ carbonic anhydraseⅢ 也是位於第 8 染色體長臂區 22 帶(8q22)，分子量 29 kDa，含有 2 個 isoform(CAⅢA, CAⅢB)水解 CO_2 的活性非常強，在 25℃的環境下約 8×10^3 sec^{-1}[45]。CAⅢ主要在骨骼肌中有明顯表現，佔 slow-twitch(type I fiber) red skeletal musce 中可溶性蛋白質 8 %之多，可幫助 CO_2 順利闊散到組織微血管中[46]。含有 type I fibers 的器官包括脾臟、腎臟、心臟及肺臟會有 CAⅢ高度表現。

④ carbonic anhydraseⅣ 位於第 17 染色體長臂區 23(17q23)，分子量 39～52 kDa，包含 1-5N-linked oligosaccharide chains，為 membrane-bound 之 glycoprotein，具有穩定的雙硫鍵，而人類 CAⅣ缺乏碳水化合物部份因此分子量較其它哺乳動物的 CAⅣ小。CAⅣ是透過 phosphatidylinositol glycan anchors 連結在細胞膜 Ser-284 上，在其結構上有一段 signal sequence，當 CAⅣ到達作用位置時，signal peptidase 會在 C 端分裂成片段[49]。以 phosphatidylinositol-specific phospholipase C 處理可使 CAⅣ由細 胞膜釋放出來。CAⅣ主要位於肺臟的微血管上皮細胞的表層及腎小管上皮細胞的刷狀緣(brush border)[44]。

⑤ carbonic anhydrase V 位於第 16 染色體，成熟的酵素位於 mitochondria matrix[52,53]，在人類肝細胞 mitochondria 中測得的分子量約為 30 kD，在鹼性的環境下(pH 9.2)之活性最強(3×10^5 sec^{-1})。體內新陳代謝反應及輸送過程須要碳酸根離子，碳酸根離子要往細胞內移動需轉變成二氧化碳後再穿過細胞膜並移動進入粒線體基質，此過程除了 CAV 外也須要粒線體外面的 CA 幫忙(CA II & CAIII)。CAV 在肝臟粒腺體之肝醣生成及尿素生成反應中也扮演重要的角色，可以 提供酸根離子給 pyruvate carboxylase 及 carbamyl phosphate synthetase[54,55]，也有研究指出 CAV 可能參與脂質生成反應[56]。

⑥ carbonic anhydrase VI 位於第 1 染色體 p 區 36, 22, 23 帶 (1p36.22-23)，分子量 42 kDa，也是自唾液腺中分離出來[50]，在 Cys-42 和 Cys-224 位置上有雙硫鍵結構，Asn-67 和 Asn-256 上有 N-linked carbohydrate[50,51]。CAVI與 CA II 一樣具有水解 CO_2 的活性，但約祇有 CA II 的 2～3 %，一般相信 CAVI可調節唾液中的 pH 值。

⑦carbonic anhydrase VII 位於第 16 染色體長臂區 22 帶(16q22)，主要表現在唾液腺，包括唾液中碳酸根離子的分泌[47]。近來將 mouse CAVII送入 *E-coli* 表現，得知其水解 CO_2 的活性約為

CA II 的 40 %[48]。

⑧ carbonic anhydraseIX(MN protein)及 CA-related protein 在這類蛋白已確定的包括 CAVIII(CARP)、receptor type protein tyrosine phosphatase(CA)RPTP-β和 RPTP-α。CAIX是位於細胞表面上的 transmembrane 蛋白，以 Western blotting 分析得知其分子量為 58/54 kD，且在許多癌症組織中均有發現[57]。

2、CAs 的金屬離子結合位

已知在存於動物界中 7 種 CA isozyme 皆為α type，多數 CA 金屬離子結合位之研究對象是人類及牛的 CA II。在酵素的四面結構中 Zn^{2+}位於中心點，三面為含 His 的 imidazole，另一面則是透過 Thr 199 的 OH^-與 Zn^{2+}連接，Thr 199 的另一個 OH^-可與 Glu106 連接形成 Zn-OH^-/Thr199/Glu106 的網狀構造，這個網狀構造非常重要，是 CO_2、各種抑制劑 (sulfonamide、anionic)的結合位(Fig. 2)。早期研究曾使用螯合劑移去 Zn^{2+}，此時 CA 酵素失去活性，但再加入 Zn^{2+}則可恢復活性[58]。許多的金屬離子(Co^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+})皆可與 CA 結合，祇有 Co^{2+}才會產生與 Zn^{2+}相同的活性[59]。

3、CAs 的催化機轉

CAs 有足夠的能力可催化水解二氧化碳為碳酸根離子及質

子的轉移，其中已知活性最強的酵素為 CAⅡ(1×10^6 sec^{-1})，CAs 並可水解芳香族及脂肪族等酯類。作用機轉分述如下[60]：

①水解二氧化碳為碳酸根離子

$$EZn\text{-}OH^- + CO_2 \longleftrightarrow EZn\text{-}HCO_3^- \overset{+H2O}{\longleftrightarrow} EZn\text{-}H_2O + HCO_3^-$$

CO_2 與 CAs 是以非共價鍵的方式結合，因此結合力較弱。在細胞內 CO_2 不須透過水解，可靠著擴散的方式自由通過生物膜，到胞外體液 (ECF)中，而碳酸根離子無法以擴散的方式通過，必須與 CO_2 結合，而 CAs 的功能則可維持細胞內二氧化碳與碳酸根離子量的平衡。

②質子的轉移

$$His64\text{-}EZn\text{-}H_2O \longleftrightarrow H^+\text{-}His64\text{-}EZn\text{-}OH^- \overset{B}{\underset{BH+}{\longleftrightarrow}} His64\text{-}EZn\text{-}OH^-$$

分子內質子的轉移已知須在 B/BH^+(buffer system)中進行。在解離常數(pKa)為 7 時，將酸基質子轉移給水之最大反應常數為 10^3 ～10^4 sec^{-1}。高濃度 buffer system 中質子的轉移為 rate-limiting，而在低濃度 buffer system 中則為 buffer-dependent step。

研 究 動 機

G6PD 是六碳糖單磷酸分路(Hexose Monophosphate Shunt; HMP)的第一個酵素，HMP 也是紅血球中唯一的 NADPH 來源。NADPH 之 H 為 glutathione reductase (GSHPx)之 H 的主要來源，可保護紅血球對抗氧化物的破壞[11]。如果此一抗氧化物系統缺乏，無法抗衡血球要承受的氧化壓力(oxidative stress)時被氧化之變性血紅素會與膜上之 protein band 3 結合，也就是形成陰離子通道(anion channel)，相聚而形成膜表面上之新抗原。此新產生的抗原會與體內之 IgG 相結合，改變膜的抗原性而導致血球被清除。而 carbonic anhydrase 的生理功能廣泛包括 acid-base balance、carbon fixation 及 control of ventilation，所以血液中二氧化碳及碳酸根離子轉換及通過細胞膜須要 CA 活性催化。1998 年 John W.V.等學者之研究論文指出 carbonic anhydrase 會與 band 3 的 carboxyl-terminal region 以相等比例緊密的結合，是紅血球中離子代謝的主要酵素。在臺灣 G6PD deficiency 病患的變異型多屬於 class Ⅱ&Ⅲ，會有溶血症狀發生，因此推論 CA isoenzyme 的變化會影響 band 3 蛋白及使溶血症狀更明顯，所以在本研究中希望探討 G6PD deficiency 的病人紅血球中 G6PD 酵素活性與 band-3、 carbonic anhydrase 蛋白表現之關係。

實驗材料與方法

一、藥品與試劑：

1、acrylamide，Tris base 購自 Sigma chemical Co.（USA）

2、protein assay dye reagent，TEMED 購自 Bio-Rad laboratory (USA)

3、Coomassie brilliant blue R-250，sodium dodecyl sulfate，ammonium persulphate 購自 Serva chemical Co.(Germany)

4、Triton X-100 購自 American Biorganics Inc. (USA)

5、$KH_2PO_4.H_2O$，Na_2HPO_4，glycerol 購自 E. Merck chemical Co. (Germany)

6、NaN_3 購自 United States biochemical Co. (USA)

7、glycine，NaOH，acetic acid 購自聯工化學廠股份有限公司（Taiwan）

8、methanol 購自皓峰企業股份有限公司(Taiwan)

9、G6PDH reagent (No.345) 購自 Sigma chemical Co.

二、儀器：

1、Beckman DU 640 spectrophotometer

2、Beckman GS－6R centrifuge

3、DASG－250 slab gel unit

4、Bio-Rad model 200/2.0 power supply

5、Alpha Imager 2000 documentation and analysis system

6、Beckman J2-21 refrigeratory centrifuge

7、Eppendorf centrifuge 5415C

8、Hybrid HB-SHK 1 shaker

9、Orbital shaker

10、Fargo 旋轉動力器

11、Hitachi model 7170 autoanalyzer

三，緩衝液及溶劑：

1、PBS buffer 配製：

取 8 g NaCl，0.2 g KCl，0.2 g KH_2P0_4 H_2O，1.95 g Na_2HPO_4，加水至 1000 ml，調 pH 至 7.2。

2、5X SDS－PAGE loading buffer 配製：

取 5 g SDS，加入 10. 4 ml l.5 M Tris－HCl pH 6.8 ，加入 25 ml glycerol，及 6.25 mg bromophenol blue，加水至 50 ml。

3、Tray buffer 配製：

取 3 g Tris base，l4.7 g glycine，10 ml 10% SDS，再加水至 1000 ml。

4、Stain solution 配製：

取 227 ml dH_2O，46 ml acetic acid，1.25 g Coomassie brilliant blue R-250，混合均勻，直至染色劑全部溶解再加入 methanol 227 ml

5、Destain solution 配製：

取 875 ml dH_2O，加入 50 ml methanol，75 ml acetic acid 混合

均勻。

6、Electrotransfer buffer 配製：

取 6 g Tris base、28.6 g glycine、800 ml methanol，再加水至 4000 ml。

7、TBS buffer (pH 7.4)

取 2.66 g Tris base、18 g NaCl ，加水至 1000 ml 再加 0.5 ml Tween 20，調 pH 值至 7.4。

8、Blocking buffer 配製：

9、Substrate buffer(pH 7.4)配製：

取 2.66 g Tris base、18 g NaCl，加水至 1000 ml，調 pH 值至 7.4。

四、12.5 % SDS-PAGE 電泳膠之配製：

1、 Resolving gel (12 ml) :

3.0 ml 1.5M Tris-HCl (pH 8.8)，120 μl 10% SDS，5.1 ml 30% acrylamide – 0.8 % bisacrylamide，0.6 ml APS (10 mg/ml)，3.2 ml dH_2O，10 μl TEMED.

2、Stacking gel (5 ml) :

1.3 ml 0.5M Tris-HCl (pH6.8)，50 μl 10 % SDS，0.7 ml 30 % acrylamide-1.2 % bisacrylamide，0.25 ml APS (10 mg/ml)，2.7 ml dH_2O，20 μl TEMED.

五、8.0 % SDS-PAGE 電泳膠之配製：

1、 Resolving gel (12 ml) :

3.0 ml 1.5M Tris-HCl (pH 8.8)，120 μl 10% SDS，3.2 ml 30% acrylamide – 0.8 % bisacrylamide，0.6 ml APS (10 mg/ml)，5.1 ml dH_2O，10 μl TEMED.

2、Stacking gel (5 ml) :

1.3 ml 0.5M Tris-HCl (pH6.8)，50 μl 10 % SDS，0.7 ml 30 % acrylamide-1.2 % bisacrylamide，0.25 ml APS (10 mg/ml)，2.7 ml dH_2O，20 μl TEMED.

六、檢體收集：

正常人及 G6PD 缺乏患者檢體來自台中榮民總醫院新生兒篩檢實驗之新生兒患者父母，檢體以 EDTA 抗凝固劑收集，離心 1000 xg 10 分鐘，取得之紅血球以生理食鹽水洗滌三次，加 lysis buffer，於 4℃下靜置 10 分鐘，再以 11600 xg 離心 5 分鐘。取出上層液體進行 CA 分析，下層沉澱物以 PBS 洗滌，取 ghost cell 進行 band 3 分析。

七、G6PD 活性的測定：

血液樣本取得後與 ACD 抗凝固劑以 4:1 比例混合，測定時先以 lysis buffer 處理樣本。測定原理為利用 G6P 與 $NADP^+$ 在 G6PD 存在下會轉變為 6PG 及 NADPH。NADPH 形成的量與 G6PD 活性成正比，而 NADPH 在 340 nm 有吸光，可以分光光度計測定。G6PD 活性的測定目前已普遍使用生化自動分析儀取代。

八、蛋白質之定量：

Bradford 蛋白質測定法：

試管分別加入 0、4、8、12、16、20、24、28、32 *ul* 蛋白質標準品（BSA，0.4 *ug/ul*），再加水至 700 *ul*，分別加入 PBS 緩衝液 100 *ul*，Coomassie bri11liant blue G－250 染料 200 *ul*，混合均勻，置室溫 15 分鐘後，測 OD_{595} 吸光值，再依據其吸光值畫出標準曲線。取適當量的血球萃取液，以 PBS 緩衝液補足到 100 *ul* 之檢體量，加水 700 *ul*，Coomassie brilliant blue G-250 染料 200 *ul*，混合均勻，置室溫 15 分鐘後，測 OD595 吸光值，再與標準曲線比對，計算出所含蛋白量。

九、西方墨點分析法(Western blot):

配製 12.5 % SDS-PAGE 電泳膠片，將已聚合之板膠固定到電泳裝置上，於電泳槽內注滿電泳緩衝液 (Tray buffer)，取 30 μl 檢體(總蛋白量 20 μg)，加入 6 μl 5X loading buffer(不含還原劑)，混合後於 90℃加熱 5 分鐘，loading 至板膠槽中，進行電泳分離，電壓為 150 伏特，待染料泳動至距膠體底部約二公分。電泳結束後進行蛋白轉移，將膠體浸入 electrotransfer buffer，,再放置在兩張浸濕過 transfer buffer 的濾紙上面，膠體上面覆蓋與膠體同樣大小的硝化纖維紙，此硝化纖維紙也須先浸濕過 transfer buffer，最後再放置兩張浸濕過 transfer buffer 的

濾紙，並擠壓方式趕走夾在其中的氣泡，裝入 transfer holder 然後置於 electrotransfer tank 於 4℃下，進行 100 伏特電轉移 1 小時，之後將硝化纖維紙取出，用 PBS-T buffer 洗滌約 5 分鐘，5% blocking buffer 於室溫下溫和作用 1 小時。倒掉 blocking buffer，再用 PBS-T buffer 快速洗滌三次，每次 5 分鐘。換上一級抗體 (polyclonal antibody) carbonic anhydrase Ⅱ (1000 倍稀釋) ，於 4℃下作用 24 小時，再用 PBS-T buffer 快速洗滌三次，每次 5 分鐘。再換上接有 peroxidase 的二級抗體 anti-rabbit IgG (5000 倍稀釋) 於室溫下溫和作用一小時，再用 PBS-T buffer 快速洗滌三次，每次 5 分鐘。最後將硝化纖維紙浸入 50ml 的 substrate buffer (50 mM Tris-HCl pH 7.4 50 ml，1 ml DAB，1ml 4CN，5 μl H_2O_2)中進行呈色反應，待硝化纖維紙上有明顯的 band 出現，即可以去離子水終止呈色反應，清洗乾淨後晾乾。

十、Human band 3 蛋白分析

以含 0.5 M EDTA 之 lysis buffer 將 ghost cell 洗滌數次，目的為將血紅素完全洗去，最後加入 100 μl lysis buffer 備用。配製 8 % SDS-PAGE 電泳膠片，將已聚合之板膠固定到電泳裝置上，於電泳槽內注滿電泳緩衝液 (Tray buffer)，取 10 μl 檢體，加入 5 μl 5X loading buffer(不含還原劑)，混合後於 90℃加熱 5

分鐘，loading 至板膠槽中，進行電泳分離，電壓為 150 伏特，待染料泳動至距膠體底部約二公分。取下膠片以 Coomassie bri11liant blue 進行染色。

十一：統計方法：

硝化纖維紙上的 band 利用 Alpha Imager 2000 數位化影像分析儀作定量分析，每片硝化纖維紙上皆以 control sample 校正後作為原始數據。所有數據輸入電腦軟體 SigmaPlot 分析相關數據間的差異。平均值及標準偏差的計算用以瞭解正常個體各項分析值的正常分佈關係；並利用 student's test 及 stepwise regression 分析以瞭解不同組別中各項分析值之差異及相關性。

實 驗 結 果

所有樣本均先以生化自動分析儀進行 G6PD 酵素活性分析，正常成人參考值為 6.4～12.9 U/gHb。再根據 G6PD 活性數值將所有樣本區分為正常個體及 G6PD 缺乏兩組，進行紅血球細胞膜上 band 3 蛋白分析及 carbonic anhydrase 酵素分析。

一、紅血球膜上 band 3 蛋白分析

本實驗先將兩組樣本在加與還原劑(β-MSH)的情形下使用 8 % SDS-PAGE 之電泳膠片進行 band 3 蛋白分析，結果在 95 kD 附近均可發現 band 3。不加β-MSH 之電泳膠片上的 band 3 呈擴散狀(Fig. 3)，但將檢體加β-MSH 處理後再跑電泳，發現相同的位置上原本呈擴散狀的 band 3 變為帶狀(Fig. 4)。在正常個體樣本中 band 3 的蛋白表現明顯高於 G6PD 缺乏病人樣本，但在 G6PD 缺乏病人樣本中仍有個體上的差異存在。

二、紅血球中 carbonic anhydrase 酵素分析

本實驗使用西方墨點的方法分析比較 carbonic anhydrase 酵素在正常個體及 G6PD 缺乏病人紅血球中的表現。最初使用 12.5 % SDS-PAGE 含 1.2 % bisacrylamide 之電泳膠片，使用專一 CA II 抗體之西方墨點法分析證實在 30 kD 的位置附近有 CA 的蛋白表

現，但並無法使 CA I 及 CA II 順利的分開(Fig.5)。將電泳膠片中 1.2 % bisacrylamide 改為 0.8 % bisacrylamide 後，CA I 及 CA II 則可明顯的分開。再以西方墨點的方法分析證實在 30 kD 的位置附近有 CA I 及 CA II 的蛋白存在(Fig.6 & Fig.7)，兩個不同組別硝化纖維紙上的 band 利用 Alpha Imager 2000 數位化影像分析儀作定量分析並進行量化。正常個體(n=30)中 CA I 的量約為 CA II 的 4～6 倍(Fig.6)，而 G6PD 缺乏病人(n=30)紅血球中 CA II 明顯上升(Fig.7、Fig.8 & Fig.9)。所得數值以 student's test 及 stepwise regression 進行分析後發現在 G6PD 缺乏病人組別中 CA I / CA II 的平均比值為 2.3，明顯低於正常個體的平均比值 5.2 ($p<0.001$) (Table 3)。

討　　論

carbonic anhydrase 是一種含鋅離子的金屬酵素，為紅血球中除了血紅素外主要的蛋白，在紅血球中扮演著氣體轉運的重要角色，CA II 已證實會與 band 3 蛋白 Ct region 在紅血球膜上結合，藉由此結合方式可將碳酸根離子以擠壓方式排出細胞外，而 band 3 蛋白具有醣化酵素的結合位，這些膜上的醣化酵素可產生 ATP，並可作為 Na^+-K^+ ATPase 的能量來源[68]。一般相信紅血球膜是靠著 band 3 與細胞內的蛋白架構連接，而 band 3 亦提供多種蛋白結合位。先前有多篇研究報告指出在血液性疾病發生時 band 3 蛋白有不正常減少的現象[33,38]。由本實驗亦觀察到 ghost 細胞經 8 % SDS-PAGE 電泳膠片分析在 95 kD 附近有擴散狀的 band 3 蛋白，而 G6PD 缺乏的病人這片區域較淡(Fig. 3)。樣本經還原劑(β-MSH)處理後的電泳膠片在 95 kD 附近擴散狀的 band 3 蛋白轉為帶狀(Fig. 4)。

本實驗中以 12.5 % SDS-PAGE (含 1.2 % bisacryl-amide)之電泳膠片分析正常個體與 G6PD 缺乏病人紅血球中 CA 蛋白總量，數據顯示並無明顯差異(Fig.5)。進一步改用含 0.8 % bisacrylamide 之電泳膠片分析正常個體(n=30)之 CA I 與 CA II，顯示之間的比

值約為 5.2 (Fig.6)，與文獻的研究結果相符[44,64]。而在 G6PD 缺乏病人(n=30)的分析中發現 CA I 與 CA II 間的比值約 2.3 (Fig.7)，與正常個體比值較明顯的下降。此結果在目前的文獻中並無描述。但就目前已了解 CA I 與 CA II 間的比值會隨年齡的增長而有不同的變化，在胎兒期 CAI 通常無法偵測到，懷孕 40 週是一個轉換期，正常分娩的新生兒此時 CA I 蛋白開始有表現，到 1 歲後 CA I 的量漸漸上升到與成人相似，CA II 的表現與 CA I 恰好相反[44]。1996 年 Akbar S.A.等人針對不同懷孕週期、新生兒及成人紅血球中 CA I 及 CA II 蛋白分析，結果顯示懷孕 20 週 CA I 與 CA II 間的比值約為 0.9，懷孕 38 週為 2.5，成人則上升至 8.8[65]。而我們的結果顯示 G6PD 缺乏病人的紅血球中 CA I 與與 CA II 比值約 2.3 顯示 CA II 的蛋白表現增加。目前已有報告指出 uremic anemia、cancer anemia 及 megaloblastic anemia 紅血球中的 CA II 的蛋白表現會增加，可能反應著 CA 蛋白的不正常合成，而 catalase 及 glutathione peroxidase 活性也伴隨增加[66]，表示血球中有氧化傷害產生。1994 年 Alfinito F.的研究中更指出在 G6PD Mediterranean (S188F)的病人中也發現 spectrin 及 band 3 蛋白減少[38]，原因為 G6PD 是六碳醣單磷酸分路中第一個也是最重要的

一個調節酵素，所以當發生 G6PD 缺乏時會影響六碳醣單磷酸分路，使 NADPH 的來源降低而造成過氧化現象，此氧化性傷害出現時會改變 spectrin 與 cytoskeleton 的結合，使 cytoskeleton 自 spectrin 蛋白中流失，導致 band 3 蛋白減少，而 G6PD Seattle (E282H)則無此現象。我們由結果我們得知當 G6PD 缺乏可能會影響 CA II 及 band 3 等蛋白的基因表現或蛋白穩定度，當胞內 band 3 蛋白異常，則會使得碳酸根離子上升，為了調節細胞內的酸鹼平衡及氣體的轉運而 CA II 蛋白合成也跟著上升，這種現象在以上多種血液疾病中均可見到[69]。

1987 年 Meloni 等學者指出溶血發生的程度與 G6PD 酵素活性有關，而在 G6PD 缺乏的病人的紅血球中含有較高量的變性血紅素(如 carboxyhemoglobin; COHb 等)[72]，變性血紅素會與膜上之 protein band 3 結合，相聚而形成膜表面上之新抗原。此新產生的抗原會與體內之 IgG 相結合，改變膜的抗原性而導致血球被清除，這是目前研究已知溶血現象發生的原因之一[29]，而且推論在此病理現象產生時因 CA II 在生理功能上與 band 3 間會形成代償作用，而使得 CA II 蛋白合成會跟著上升。實際上當面對 G6PD 缺乏病人所產生的慢性溶血的現象有兩個可能必須考慮 1、G6PD 缺乏病人屬於會產生溶血的 class II 及 class III。2、G6PD 缺乏病

人伴隨著其他的病理現象。而臺灣、東南亞地區的 G6PD 缺乏病人多屬於會產生溶血的 class II 及 class III，而在本實驗中發現 G6PD 酵素活性缺乏的病人紅血球中 band 3 及 CA I、CA II 的蛋白表現分析出現正常之個別差異現象，而此個別差異是否與不同的 G6PD 變異型有關，而 band 3 蛋白減少及 CA II 蛋白合成上升，造成溶血之臨床症狀，皆是必須深入探討的課題。

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圖表與圖表說明

Table 1 G6PD 變異株

Variant	Nucleotide Substitution	WHO Class	Amino acid Substitution
Gaohe,Gaozhou**	95 A → G	2	32 His → Arg
Sunderland	105 – 107 del	1	35 Ile → del
Aures	143 T → C	2	48 Ile → Thr
Metaponto	172 G → A	3	58 Asp → Asn
Castilla	202 G → A	3	68 Val → Met
Alabama	376 A → G		126 Asn → Asp
Ube	241 C → T	3	81 Arg → Cys
Lagosanto	242 G → A	3	81 Arg → His
Vancouver	317 C → G	1	106 Ser → cys
	544 C → T		182 Arg → Trp
	592 C → T		198 Arg → Cys
Sao Borga	337 G → A	4	113 Asp → Asn
A	376 A → G	4	126 Asn → Asp
Chinese-4**	392 G → T	?	131 Gly → Val
Ilesha	466 G → A	3	156 Glu → Lys
Mahidol**	487 G → A	3	163 Gly → Ser
Plymouth	488 G → A	1	163 Gly → Asp
Chinese-3**	493 A → G	2	165 Asn → Asp
Shinshu	527 A → G	1	176 Asp → Gly
Santamaria	542 A → T	2	181 Asp → Val
	376 A → G		126 Asn → Asp
Birmingham	563 C → T	2	188 Ser → Phe

Table 1 G6PD 變異株(續)

Variant	Nucleotide Substitution	WHO Class	Amino acid Substitution
Coimbra	592 C → T	2	198 Arg → Cys
Santiago	593 G → C	1	198 Arg → Pro
Sibari	634 A → G	3	212 Met → Val
Marion	637 G → T	1	213 Val → Leu
Harilaou	648 T → G	1	216 Phe → Leu
Mexico city	680 G → A	3	227 Arg → Gln
A-	680 G → T	3	227 Arg → Leu
	376 A → G		126 Asn → Asp
Stonybrook	742 - 729 GGC del	1	242-243 Gly & Thr
Wayne	769 G → C	1	257 Arg → Gly
Cleveland	820 G → A	1	274 Glu → Lys
Chinese-1	835 A → T	2	279 Thr → Ser
Montalbano	854 G → A	3	285 Arg → His
Viangchan	871 G → A	2	291 Val → Met
West Virginia	910 G → T	1	303 Val → Phe
Kalyam	949 G → A	3	317 Glu → Lys
Nara	953-976 del	1	319-326 del
Chatham	1003 G → A	3	335 Ala → Thr
Chinese-5**	1024 C → T	?	342 Leu → Phe
Ierepetra	1057 C → T	2	353 Pro → Ser
Loma Linda	1089 C → A	1	363 Asn → Lys
Tomah	1153 T → C	1	385 Cys → Arg

Table 1 G6PD 變異株(續)

Variant	Nucleotide Substitution	WHO Class	Amino acid Substitution
Walter Reed	1156 A → G	1	386 Lys → Glu
Guadalajara	1159 C → T	1	387 Arg → Cys
Genova	1160 G → A	1	387 Arg → His
Anaheim	1178 G → A	1	393 Arg → His
Alhambra	1180 G → C	1	394 Val → Leu
Riverside	1228 G → T	1	410 Gly → Cys
Japan	1229 G → A	1	410 Gly → Asp
Tokyo	1246 G → A	1	416 Glu → Lys
Georgia	1284 C → A	1	428 Tyr → End
Pawnee**	1316 G → C	2	439 Arg → Pro
Telti	1318 C → T	1	440 Leu → Phe
Union	1360 C → T	2	454 Arg → Cys
Andalus	1361 G → A	1	454 Arg → His
Cosenza	1376 G → C	2	459 Arg → pro
Taiwan-Hakka*	1376 G → T	2	459 Arg → Leu
Anant	1388 G → A	2	463 Arg → His
Campinas	1463 G → T	1	488 Gly → Val

**臺灣已發現之變異株

Table 2　G6PD 缺乏病人需避免之藥物

Acetanilid	Primaquine
Furazolidone (Furoxone)	Sulfacetamide
Methylene blue	Sulfamethoxazole (Gantanol)
Nalidixic acid (NegGram)	Sulfanilamide
Naphthalene	Sulfapyridine
Niridazole (Ambilhar)	Thiazolesulfone
Isobutyl nitrite	Toluidine blue
Nitrofurantoin (Furadantina)	Trinitrotoluene (TNT)
Phenazopyridine (Pyridium)	Urate oxidase
	Phenylhydrazine

Table 3 正常個體與 G6PD 缺乏病人紅血球中各項分析值之比較

		正常個體	G6PD 缺乏病人
No.		30	30
G6PD (U/g Hb)	mean	9.4	3.5**
	S.D	2.3	2.0
	S.E.	0.38	0.41
CA I	mean	30138	21974**
	S.D	14905	5345
	S.E	2721	976
CA II	mean	5801	9960**
	S.D.	2853	2631
	S.E	520	480
CA I/ CA II ratio**		5.2	2.3
	S.D	1.38	0.55
	S.E	0.25	0.10

** $P<0.001$

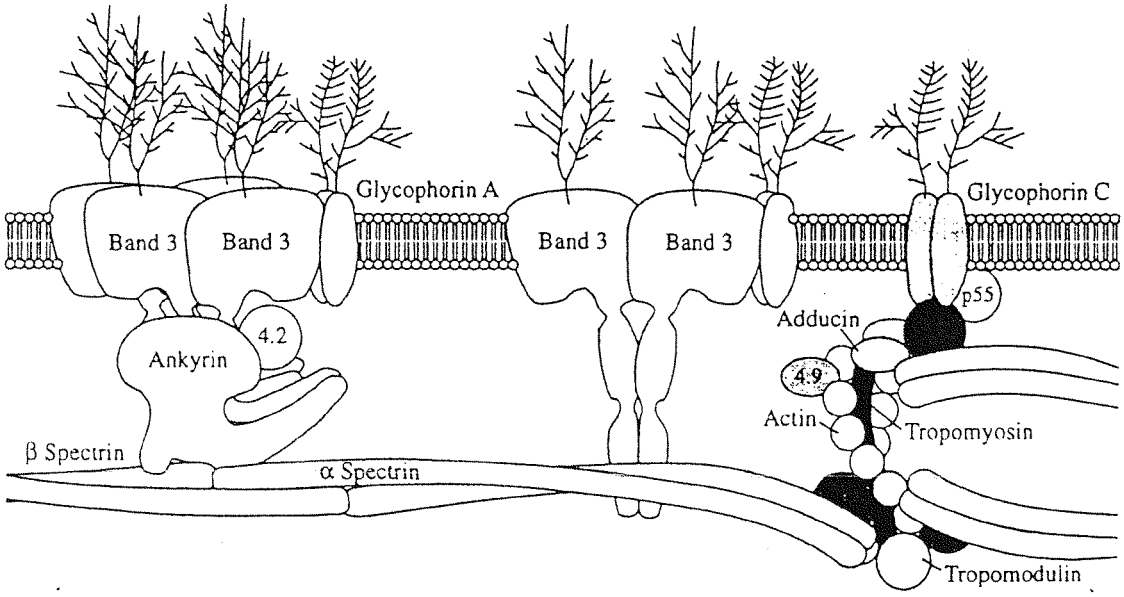


Fig.1　紅血球膜上蛋白的架構

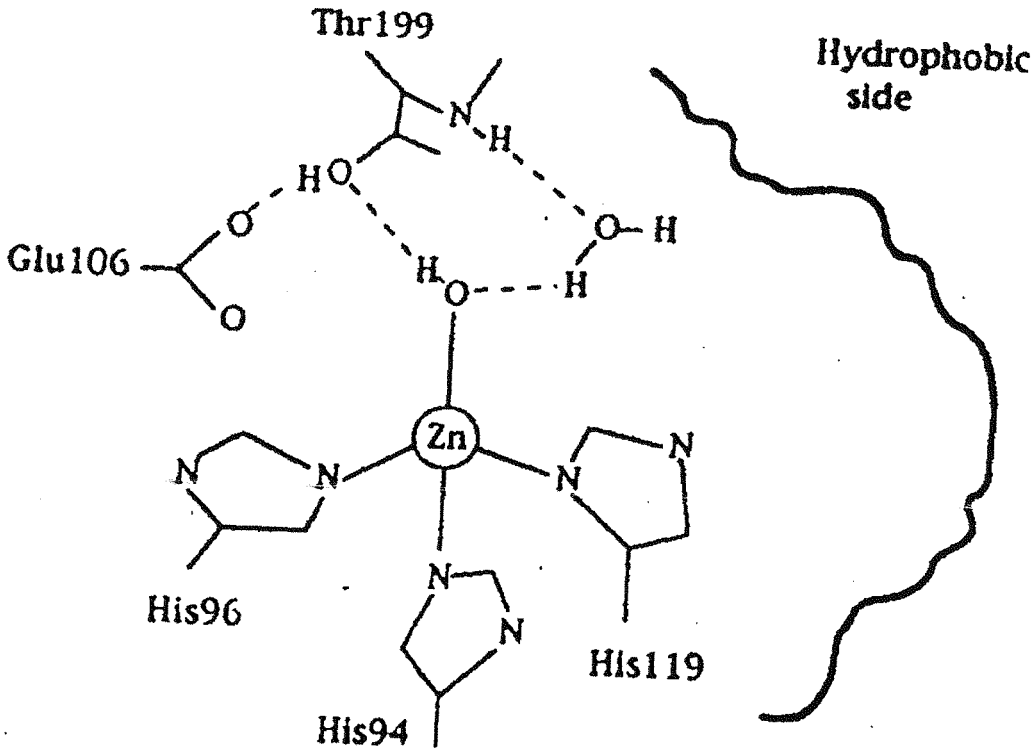


Fig. 2　Carbonic anhydrase II 之催化位置

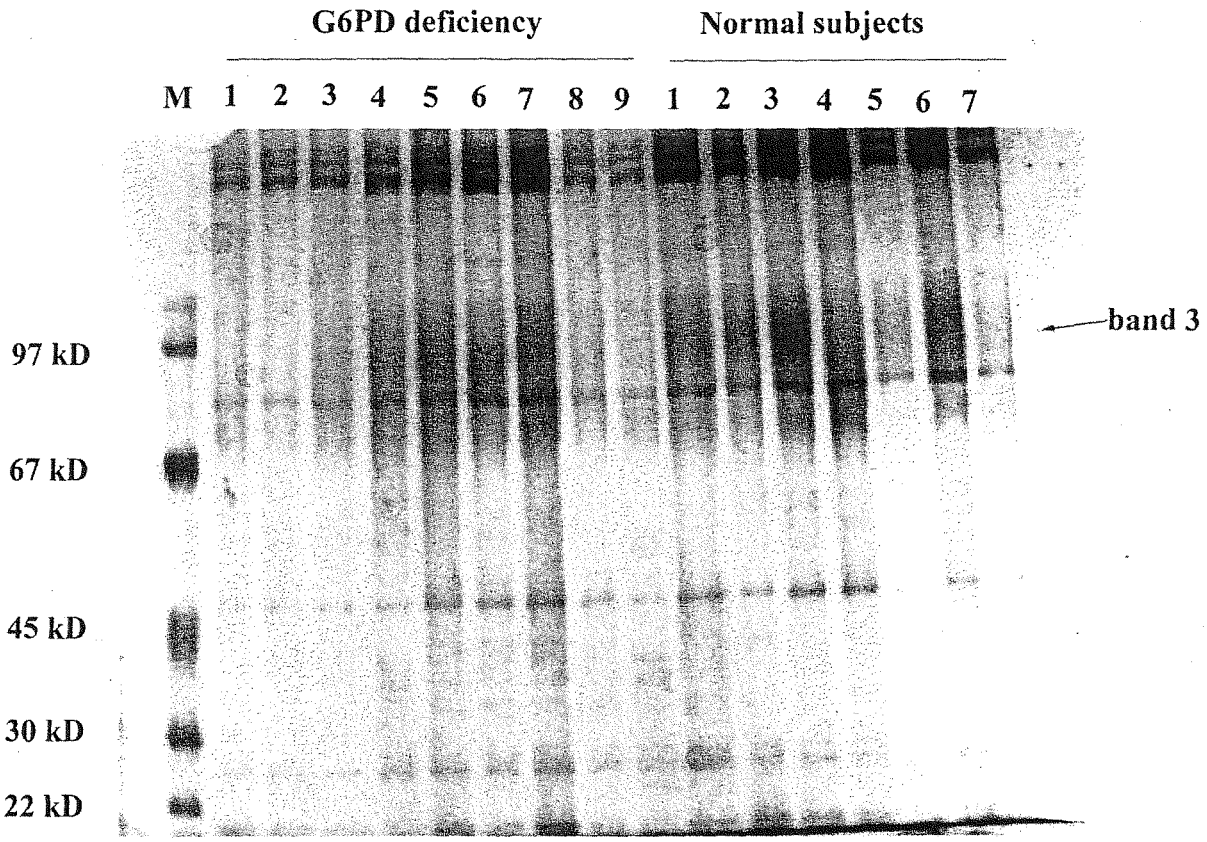


Fig. 3 紅血球膜細胞上的蛋白表現

100 *ul* 之全血經洗滌並溶血後取 ghost cell，以 8 % SDS-PAGE 進行電泳分析，以 Coomassie brilliant 染色。

M: M.W.marker

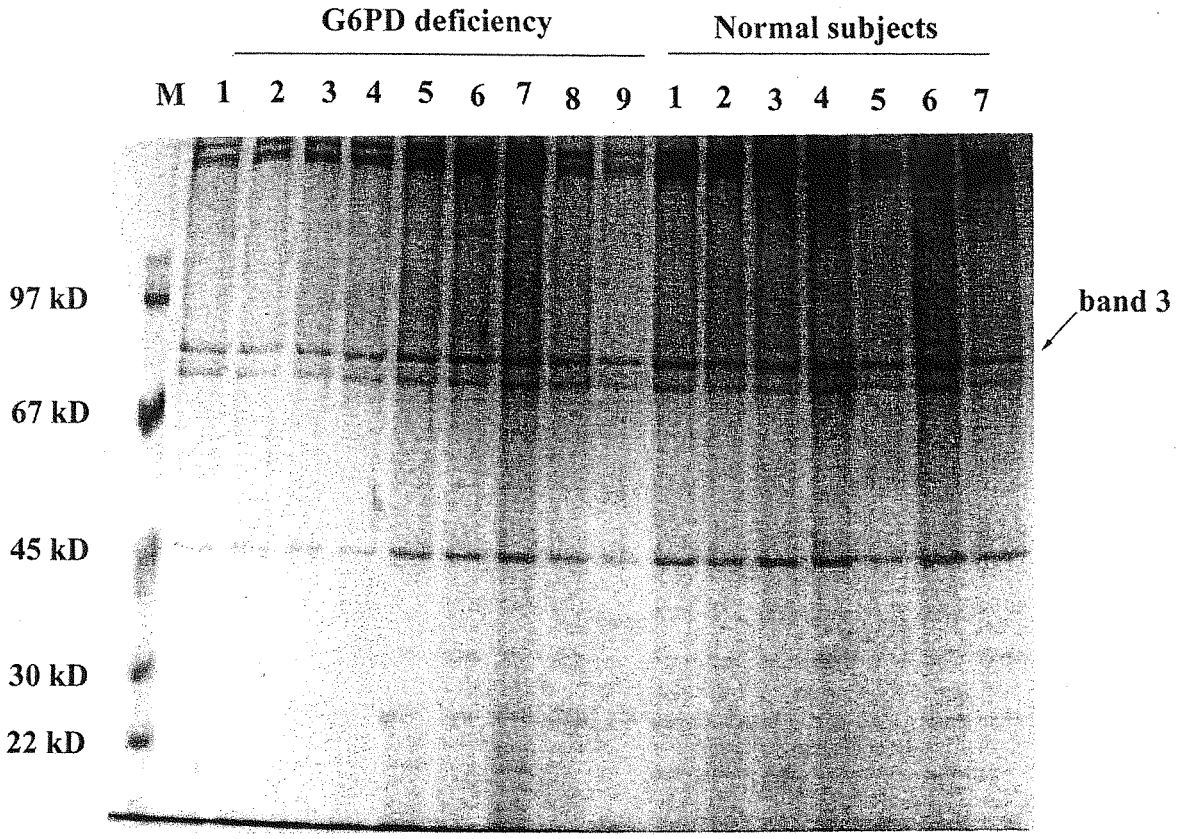
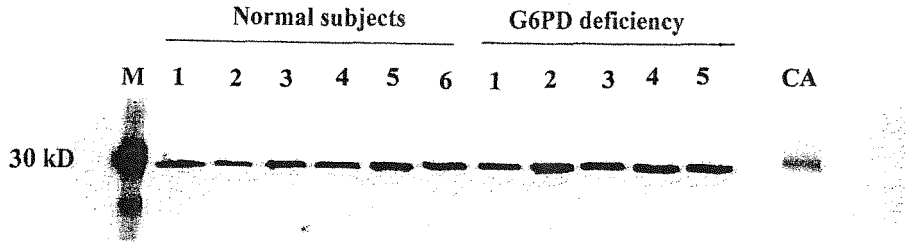


Fig. 4 紅血球膜細胞經還原劑處理後的蛋白表現

100 *u*l 之全血經洗滌並溶血後取 ghost cell，加 β-MSH 處理後以 8 % SDS-PAGE 進行電泳分析，以 Coomassie brilliant 染色。

M: M.W.marker



	Normal subjects	G6PD deficiency
1	90168	92378
2	99008	93096
3	92820	88842
4	93704	93538
5	88400	93980
6	89726	
mean	9230.4	9236.7
S.D.	3837.2	2057.3
S.E.	1566.5	920.1
T value	*0.2188*	
P value	*0.8375*	

Fig. 5 西方墨點法分析紅血球中 carbonic anhydrase 之蛋白表現

溶血後的血球高速離心後取細胞質部份，以 12.5 % SDS PAGE (1.2 % bisacrylamide)電泳分析，並轉移至硝化纖維紙上，並以專一的 carbonic anhydrase II 抗體進行反應。利用數位影像分析儀定量後並以 Sigma Plot 分析。

M: M.W.marker CA: CA II control

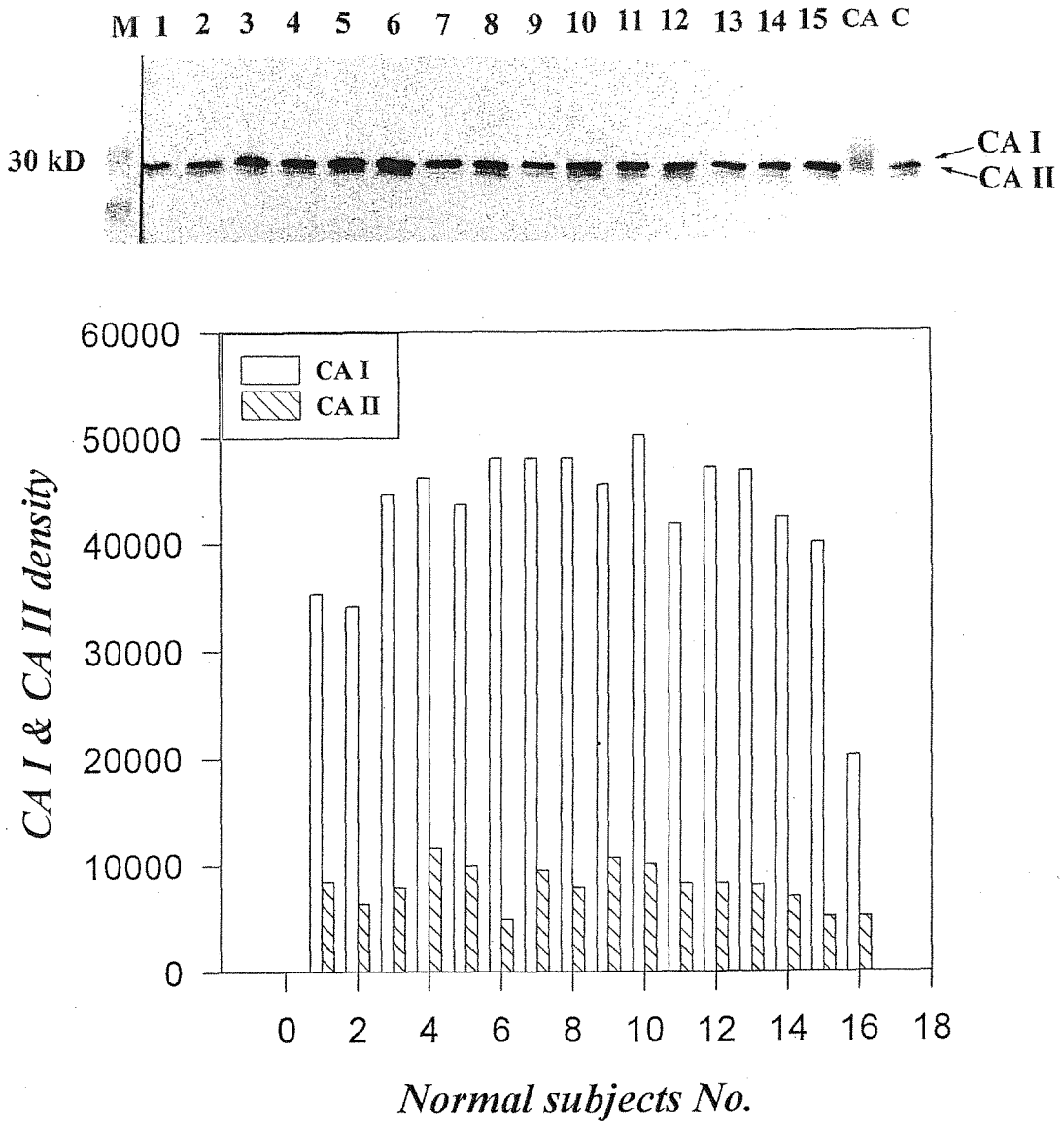


Fig. 6 西方墨點法分析正常個體紅血球中 carbonic anhydrase 之蛋白表現。

溶血後的血球高速離心後取細胞質部份，以 12.5 % SDS PAGE (0.8 % bisacrylamide)電泳分析，並轉移至硝化纖維紙上，並以專一的 carbonic anhydrase II 抗體進行反應。利用數位影像分析儀定量後製成柱狀圖。

M: M.W.marker CA: CA II control C: patient control

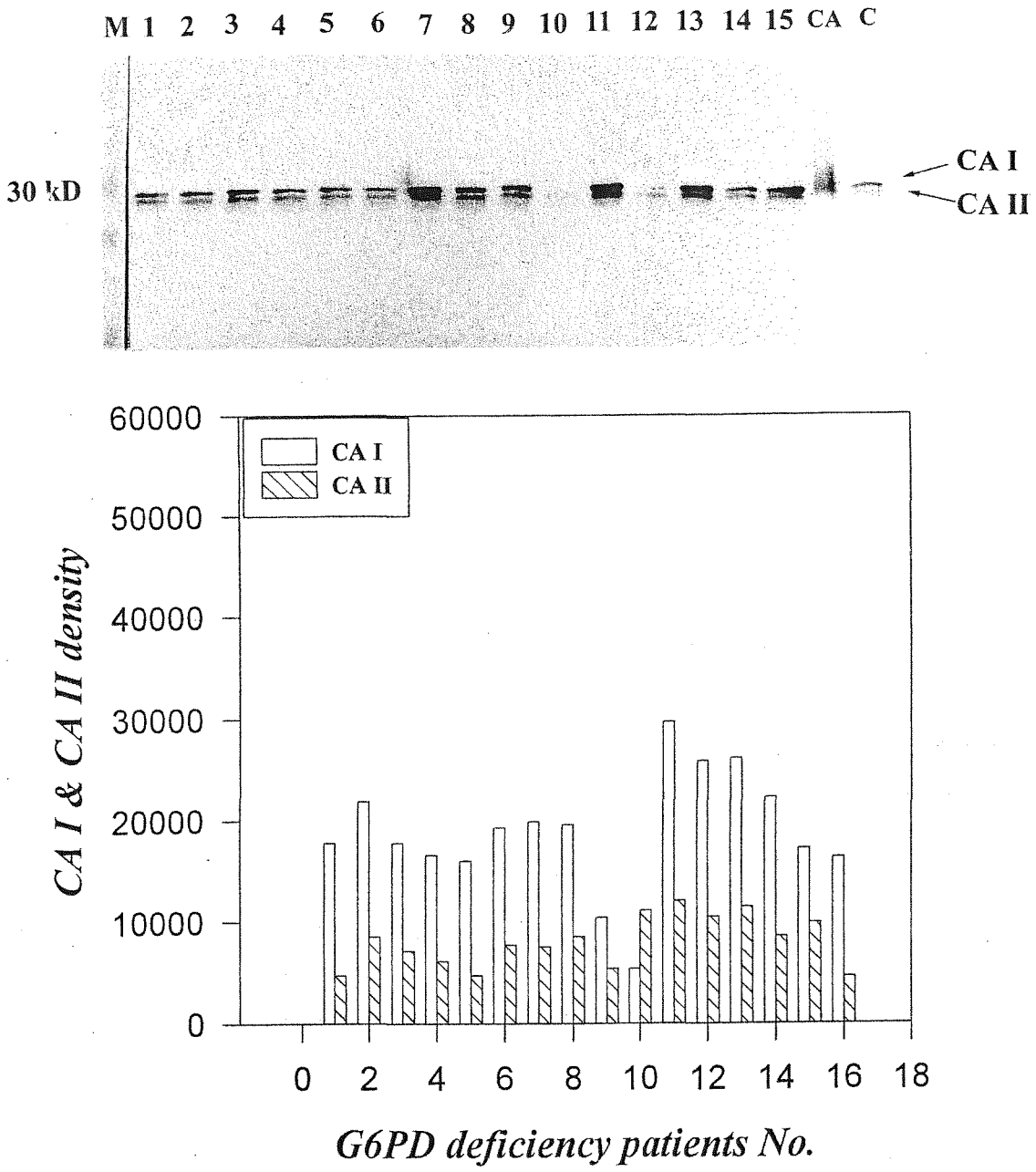


Fig. 7 西方墨點法分析 G6PD 缺乏病人紅血球中 carbonic anhydrase 之蛋白表現。

溶血後的血球高速離心後取細胞質部份，以 12.5 % SDS PAGE (0.8 % bisacrylamide)電泳分析，並轉移至硝化纖維紙上，並以專一的 Carbonic anhydrase II 抗體進行反應。利用數位影像分析儀定量後製成柱狀圖。

M: M.W.marker CA: CA II control C: patient control

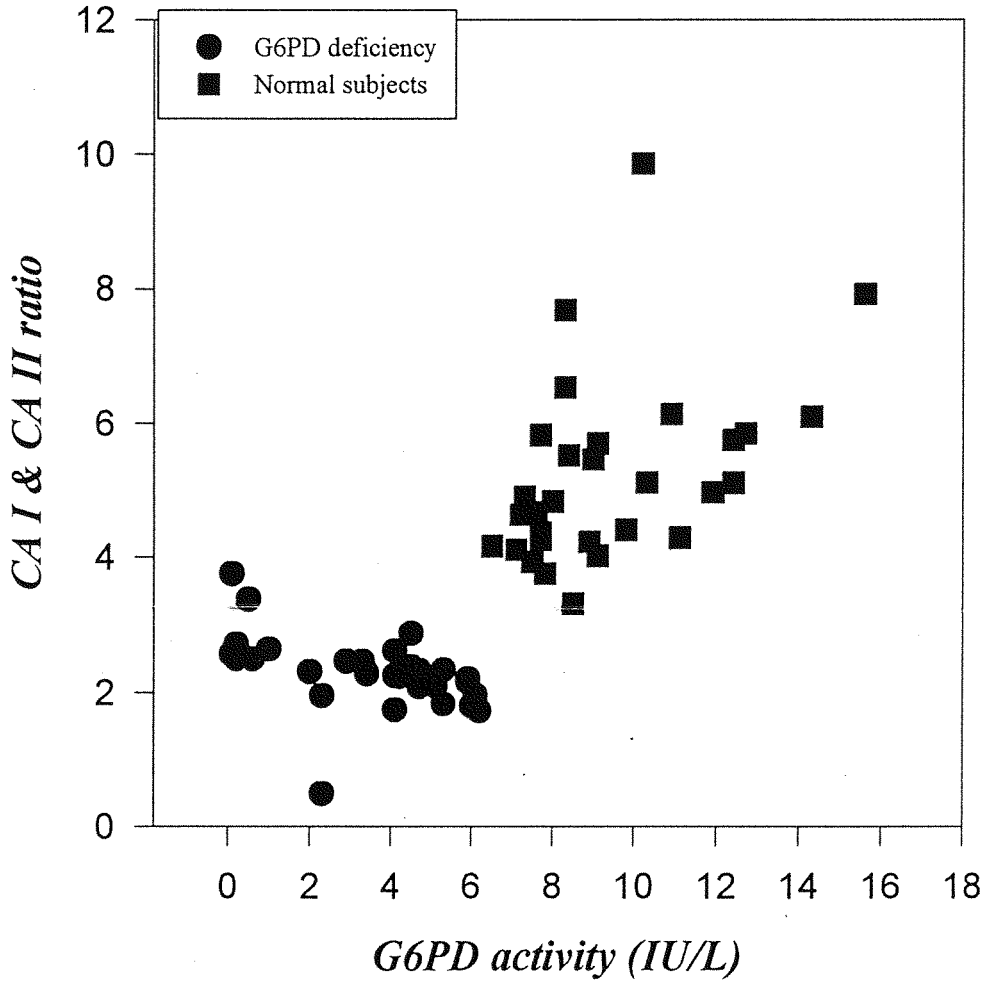


Fig. 8 正常個體與 G6PD 缺乏病人紅血球中 CAI/CA II 比例之分佈圖

● : G6PD deficieny ■ : Normal subjects

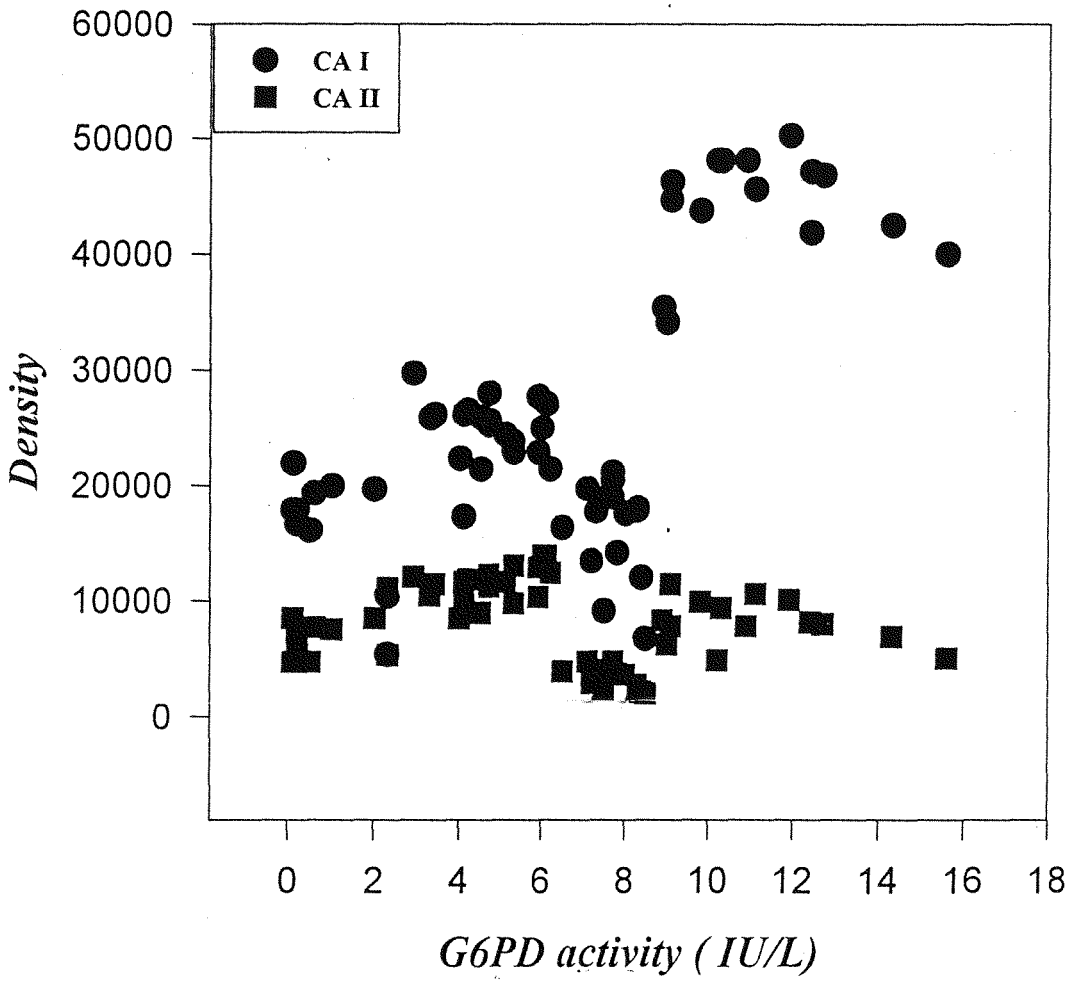


Fig. 9 紅血球中 G6PD 活性與 CAI 及 CA II 蛋白表現之關係

● : G6PD deficieny ■ : Normal subjects

第二部份：

分析台灣地區正常人血液中抗氧化酵素活性(SOD, GSH)及 Total Antioxidative Status 之研究

Determination of Antioxidative Enzyme Activity and Variation of Superoxide Dismutase, Glutathione Peroxidase, and Total Antioxidant Status in Healthy Chinese.

SUMMARY

A rapid automated system for assessing the activities of superoxide dismutase (SOD), total antioxidant status (TAS), and glutathione peroxidase (GSHPx) in blood was developed using a set of commercial kits and the Beckman Synchrom CX-5 automatic analyser. Reproducibility data for assaying SOD, GSHPx, and TAS by our proposed procedure were excellent (CVs < 6.0 %). We then recruited a total of 188 apparently healthy individuals and their antioxidative status were assessed by our proposed method. We found that female (n=90) had a significant higher SOD (1082±261 unit/g Hb) and GSH-Px (91±16 unit/g Hb) than its male counterparts (n=98)[SOD: 989± 196 unit/g Hb; GSH-Px: 79±11 unit/g Hb] ($P < 0.01$). GSH-Px (R = 0.26) and TAS (R = -0.38) was correlated with age. Interestingly, we also found that alcoholics and motor cyclists seemed to have lower GSH-Px ($P < 0.05$). In contrast, we did not find any significant discrepancy in SOD, GSH-Px and TAS between smokers and non-smokers.

中 文 摘 要

本研究使用商業化試劑及 Beckman Synchrom CX-5 自動化分析系統，提供快速準確的分析方法評估血液中 superoxide dismutase (SOD)、 total antioxidant status (TAS)和 glutathione peroxidase (GSHPx)等活性分析。以本方法分析 SOD、TAS 和 GSHPx 所得再現性非常好，CVs 小於 6。我們收集了 188 個健康個體的樣本(90 個女性及 98 個男性)進行分析。發現女性的 SOD 與 GSHPx (SOD:1082±261 unit/g Hb; GSH-Px: 91±16 unit/g Hb)較男性高(SOD: 989±196 unit/g Hb; GSH-Px: 79±11 unit/gHb)。令人感興趣的是在以上樣本中具有飲酒及以機車作為交通工具者，其血中 GSHPx 的活性較對照組低($P < 0.05$)。而在分析抽煙及不抽煙樣本中， SOD、TAS 和 GSHPx 的數據並沒有發現明顯的差異。

緒　言

當生物體開始利用氧的那一刻起，有氧呼吸生物就承受氧毒性的損傷，在生物體內常見的自由基多半和氧分子參與的新陳代謝有關。自由基是能夠單獨存在且具有一個或一個以上未成對電子的物質。生物體內常見的含氧自由基包括 superoxide anion ($O_2^{-\cdot}$)、hydroxyl radical($OH^{\cdot}$)、hydrogen peroxide(H_2O_2)以及 single oxygen($^1O_2$)。生物體為了對抗氧毒性的損傷，演化出兩級保護系統：1、酵素性抗氧化物（antioxidants enzymes），如 SOD，catalase，glutathione peroxides 等，主要在清除 $O_2^{-\cdot}$ 和 H_2O_2 等損傷因子。2、小分子(<1000 M.W.)抗氧化物：如胡蘿蔔素、硒、Vit. C 及 Vit. E、bilirubin、uric acid 等，主要在阻斷自由基的有害連鎖反應。已知 SOD[1]存在紅血球中，是一個很重要的抗氧化物(anti-oxidant)，抗氧化物又叫抗自由基(anti-free radical)，它會將 oxygen free radical (OFR)捕捉，也會抑制 OFR 引起的氧化作用，以防止 OFR 造成的傷害，包括：細胞膜崩潰，酵素蛋白質損毀，及對細胞核內物質造成突變性的傷害[2]。對人體造成的疾病包括癌症[3] neurodegenerative[4]、atherosclerosis[26-27]、巴金氏症[28-29]和阿茲海默症[30-31]等。

一、抗氧化物個論

1、superoxide dismutase (SOD: E.C.1.15.1.6.)[1]

SOD 是一種金屬酵素（metalloenzymes），廣泛存在於自然界一切須氧及耐氧的生物體各組織細胞中[48]，須有銅、鋅、錳等微量元素作為 cofactor 才能發揮效用，SOD 是一種細胞內酵素依其與結合的金屬種類不同，可將 SOD 分為 MnSOD、CuZnSOD 及 FeSOD，FeSOD 只有一些低等動植物和某些微生物中才存在。所有人類及動物的活細胞都含有 MnSOD 及 CuZnSOD，如粒線體中含有四聚體的 MnSOD；細胞漿中含有二聚體的 CuZnSOD 等[33]。人類組織中 CuZnSOD 含量以肝臟最多。SOD 可防止體內組織因 OFR 氧化引起的自體破壞，催化 $O_2^{-}\cdot$ 的歧化作用並產生 H_2O_2[34]。

$$O_2^{-}\cdot + O_2^{-}\cdot + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2$$

所以 SOD 可以清除 $O_2^{-}\cdot$，因此對細胞有保護作用。SOD 具有種族特異性，黑人的 SOD 比黃種人及白種人高，且會隨生長及年齡增加而變化。SOD 對熱穩定，是目前人們發現對熱穩定性最高的蛋白之一。SOD 不足在體內有二種狀況，(1)含 SOD 但沒活性，有些癌症患者體內可測到很多 SOD，但都不具活性。(2)有活性的 SOD，測老人紅血球中 CuZnSOD 總含量得知，老

人的 CuZnSOD 比年青人少 2/3[5]；很多老人出現多器官衰竭 (multiple organ failure）症狀時，CuZuSOD 會比同年齡層老人少 1/2；老人常見的疾病如高脂血症、糖尿病、腦血管疾病、冠狀動脈疾病[16,17]、體內 SOD 都降低。有些人在三、四十歲左右，紅血球 SOD 活性即開始偏低，因為人在四十歲左右即慢慢老化，若暴露於自由基環境下會老化更快[5]。根據世界衛生組織（W.H.O.）統計的結果，真正最有意義偏低的是四十歲。糖尿病病人其有活性的 SOD 比常人少，且有較多的氧自由基活動；得糖尿病的時間越久 SOD 也越低。非胰島素依賴型糖尿病病人紅血球中 CuZnSOD 的量也減少[35]。隨著年齡的遞增，抗氧化物活性也相對遞減，而削減了對自由基的防禦能力，也加速了老化。早老症患者(progeria)體內 SOD 生產不夠，所以壽命很短。烏龜體內含有大量的 SOD，可能與烏龜長壽有關。

2、glutathione peroxidase (GSH-Px; E.C.1.11.1.9)[6]：

GSH-Px 是一種 selenoprotein 在所有組織中均有發現，有四個具有活性的 isoenzyme① cellular glutathione peroxidase, GSH-Px1 ② phospholipid hydroperoxide glutathione peroxidase, GSH-Px ③ plasma glutathione peroxidase, GSH-PxP ④ GSH-PxGI[36]。GSH-Px 可還原 H_2O_2 成水及氧，本身被氧化成 GSSG；glutathione reductase

(GR)會再將 GSSG 還原成 GSH。

$$H_2O_2 + 2GSH \xrightarrow{GSH\text{-}Px} 2\,H_2O + GSSG$$

GSH-Px 已知為四聚體結構，分子量為 22 kD。GSH-Px 存在 cytosol 和 mitochondria 中可將 H_2O_2 及 hydroperoxides 還原為 H_2O 及 alcohol。GSH-Px 必須含有硒(selenium，Se)當做 cofactor，才能完整的發揮效用[37]。GSH-Px 可以去除過氧化氫及超氧自由基。GSH-Px 有兩種①需要依賴硒的 GSH-Px (selenium dependent glutathione peroxidase，Se-GSH-Px)；②不需要依賴硒的 GSH-Px。Se-GSH-Px 是細胞內酵素，它的活性在肝和紅血球中最高，Se-GSH-Px 的活性與血漿或血小板的硒濃度有極密切關係，血漿中 Se-GSH-Px 活性又與食物中硒含量有關。硒是抗氧化(抗自由基)家族成員，也是 Se-GSH-Px 組成成份，若缺乏硒，脂質過氧化物與 OFR 等會傷害細胞膜結構功能。因此硒可經由 Se-GSH-Px 防止細胞膜的脂質過氧化作用[38]。硒有助於生長及生殖，還可增進免疫系統，下列情形硒的需要量增加：老化、大量攝取 PUFA、重金屬污染、暴露於污染的空氣。雖然美國 FDA 尚未定出硒的 RDA，但每天 50～200 *ug* 的硒將是安全的劑量[39]。硒缺乏與心臟疾病有關(如 Keshan disease)[40,41]，這是一種地方性的心肌疾病(endemic cardiomyopathy)，發生於小孩及生育年齡的婦女，嚴重

時會死亡。

3、Catalase：

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2\,H_2O + O_2$$

含有 heme-enzyme，是細胞所必需的酵素，存在細胞的 peroxisome 內，catalase 的活性以肝臟和紅血球的含量較高[42]。需鐵作為 cofactor，才能有效的氧化 H_2O_2 產生氧及水，所以可以去除 H_2O_2。catalase 也可對 LOOH[43]。進行歧化作用代謝掉。紅血球也有抗氧化作用的特性，因為它富含 SOD，catalase 及 glutathione。

4、total antioxidant status (TAS)

①胡蘿蔔素(β-carotene)：

植物中類胡蘿蔔素至少有十種可轉化為身體中的 Vit. A，其中胡蘿蔔素(β-carotene)最為重要，胡蘿蔔素是 Vit. A 的前身，攝取胡蘿蔔素後當身體須要時，一部份會在肝臟轉變為 Vit. A。因為胡蘿蔔素的 Vit. A 活性最高，人類營養所需的 Vit. A，2/3 靠蘿蔔素提供。胡蘿蔔素存在胡蘿蔔、馬鈴薯中，有助於生長和生殖。胡蘿蔔素用來清除高能量激發狀態的單重態氧(excited singlet state oxygen)，同時也是 $OH^{\cdot}$ 及 LOOH 等自由基的清除者，它可保護細胞膜，避免自由基造成的傷害（包括脂質過氧化作用）。因此胡蘿蔔素有預防自由基相關的疾病的效果，還可防止老化及

增強免疫的能力。胡蘿蔔素具有抗腫瘤的作用，及增加巨噬細胞殺死癌細胞的能力，胡蘿蔔素還能預防其他退行性疾病的發生，如衰老和白內障。攝食胡蘿蔔素可防止由自由基傷害 DNA 而來的癌症，在美國研究發現，許多癌症患者體內的胡蘿蔔素的濃度都偏低。例如：子宮頸癌患者血中胡蘿蔔素及 Vit. E 比常人偏低。

②vitamin E：

天然的 Vit. E 成份以 α-tocopherol 為主，活性也最高，也是最有效的疏水性抗氧化物。Vit. E 廣泛存在細胞膜及組織，可以保護細胞膜的 PUFA 及血漿脂蛋白，免於因 OFR 產生脂質過氧化作用而造成傷害，還可阻斷 OFR 的連鎖反應，Vit. E 可消除 O_2，$OH^{\cdot}$ 及 LOOH 等自由基。如果食物中 PUFA 的含量增加，Vit. E 的攝取量也需要跟著增加。Vit. E 的建議每日攝取量(R.D.A.)，美國男性 10 I.U.，女性 8 I.U.。國人的 R.D.A.根據衛生署 82 年修定的標準，男性 12 I.U.，女性 10 I.U.。當自由基攻擊細胞，若細胞膜上有 Vit. E 保護，Vit. E 可以先被 OFR 氧化，此時 Vit. C 再將 Vit. E 還原再生[44]。Vit.C 及 Vit. E 一起使用才有更佳的抗氧化相乘效果(synerg)。Vit. E 若沒 Vit. C 的存在，則功能大打折扣。

③vitamin C (ascorbic acid)：

Vit. C 呈水溶性是細胞外液抗氧化防禦系統的第一道防護線，

也是血漿中最有效的抗氧化物，濃度為 0.9 ± 0.4 mg/100ml。可保護脂肪酸，免於發生脂質過氧化作用。Vit. E 及 C 本身有相乘效果，是亞硝酸鹽(nitrite)的清道夫(scavenger)，因此可防止亞硝酸鹽轉變成 nitrosamines 及 nitrosureas 等致癌物[44]，Vit. C 可以清除 O_2、$OH^{\cdot}$ 等自由基。Johnston 等人的研究發現每日服用 500mg 的 Vit. C 可以使健康成人紅血球中 reduced glutathione (GSH)的量增加[45]。Vit. C 可以促進自然殺手細胞 (natural killer cell, NK cell) 的活性。

二、自由基的分析

自由基由於其化性極不穩定，所以在偵測分析上面有相當的困難度。一般對生物檢體內自由基的分析，常以氧化緊迫(oxidative stress)為主，氧化緊迫定義為含氧自由基的濃度增加對生物體造成的壓迫。而氧化緊迫則可以經由量測三大類的內生性物質來表示。第一類是抗氧化物如：superoxide dismutase、catalase、glutathione peroxidase 的活性；或是維生素 C、維生素 E、β-胡蘿蔔素、麩胱肝胺酸(glutathione)等。第二類是直接測量含氧自由基如 superoxide anion、hydroxyl radical 等。第三類則是組織受自由基攻擊後的代謝產物，包含了脂質過氧化後的產物，DNA 遭 hydroxyl radical 攻擊後的產物，和蛋白質遭自由基攻擊後的產物。

1、酵素性的抗氧化物

superoxide dismutase、glutathione peroxidase、catalase 市面上均有現成的 kit 可以購買。CuZn-SOD 是利用 McCord and Fridovich method[1]，xanthine 及 xanthine oxidase 可以產生 $O_2^{-\cdot}$，$O_2^{-\cdot}$會與 *p*-iodonitrotetrazolium salts(INT)作用產生紅色產物，而 CuZn-SOD 可抑制 $O_2^{-\cdot}$與 INT 作用。GSH-Px 是利用 Paglia and Valentine [19]，當 GSH 與 cumene hydroperoxide 在 GSH-Px 催化下會形成 GSSG，而 GSSG 在 glutathione reductase 作用下可使 NADPH 轉變為 $NADP^+$，此時在 340nm 的吸光波會降低。而 Total Antioxidant Status 是體內抗氧化能力的總評估，體內的自由基(如 H_2O_2)會使血紅素或肌蛋白變性，與 ABTS (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate])結合形成穩定的藍綠色複合物，於 600 nm 有強吸光，如果體內抗氧化能力較強則上述反應則會變弱[12]。此外也可利用 total peroxyl radical trapping assay[7]、enhanced chemiluminescent method[9]或 oxygen radical absorbance capacity assay[10,15]分析體內總抗氧化能力。

2、氫氧自由基的測定

氫氧自由基（hydroxyl radical）對細胞傷害極大。且由於其反應性非常大，故在偵測分析上相當困難。一般而言，可以使用

trapping reagents 先與 hydroxyl radical 反應後形成較為穩定的 radical，再以 electron spin resonance 來檢測。常用的 trapping reagent 包含了 5,5-dimethyl-1-pyrroline-N-oxide(DMPO)等。除了 ESR 之外，trapping reagent 與 hydroxyl radical 反應後的穩定產物亦可以 HPLC 來加以分析。

3、細胞受氫氧自由基攻擊後之代謝物的分析

脂質(lipids)很容易受含氧自由基的攻擊而引發脂質過氧化的連鎖反應。一般有數種方法來評估脂質過氧化的程度[46]。第一種是測定氧氣的消耗量；第二種方法為直接測量過氧化物(peroxides)；第三種方法，也是較為簡單常用的方法：測量不飽和脂肪酸在氧化以後所產生 conjugated diene 的結構。conjugated diene 通常在 230nm 到 250nm 會有吸收。第四種為測量檢體內能和 thiobarbituric acid 反應後顯色的產物。這個方法通常稱為 TBA test，分析脂質過氧化後的產物丙二醛 malondialdehyde (MDA)。因為 MDA 會和 TBA 反應形成 conjugate。但由於生物體內有許多其他物質會與 TBA 反應，因而干擾了 malondialdehyde 的定量。因此，可用 HPLC 系統來加以分離偵測。另外 8-Hydroxydeoxyguanosine(8-OH-dG)為 DNA guanosine residue 受到 hydroxyl radical 攻擊後的斷裂產物。而 8-OH-dG 常被用來評估生物體內 DNA 遭

受氧化傷害(oxidative damage)的指標[47]。在許多有關癌症的相關研究上，有利用 8-OH-dG 的濃度作為病變的參考。8-OH-dG 的測量可以配有電化學檢測器的 HPLC 來進行[48]。有效評估含氧自由基的產生，較為準確的方法應為同時測量抗氧化物質的減少；氫氧自由基的產生；以及生物組織遭自由基攻擊後的產物。

研 究 動 機

近十幾年來，臺灣地區工業成長迅速、各項建設突飛猛進，社會結構及經濟發展均有很大的變化，人口大量湧向城市、郊區工廠林立、市區機動車輛密度大幅成長、能源消耗量大增，導致臺灣居住品質日益惡化。經由流行病學的研究結果顯示空氣污染較嚴重的地區，居民癌症發生的機率均較高。已知空氣污染物的來源相當複雜，包括工廠排放的廢氣、汽、機車引擎排放的廢氣[49]、露天燃燒廢棄物所產生的煙霧[50]，甚至家庭中所使用的燃料、烹調時所產生的油煙、祭祖拜神時所燃燒的拜香煙霧或是抽菸所產生的煙霧等均是造成空氣污染的重要污染來源。其他的環境污染物尚包括水質、輻射污染、農藥以及防腐劑等。這些環境污染物有些本身就是自由基，例如空氣污染帶來的菸煙、NO、CO_2等[51]。但也有一些在未進入人體前雖非自由基，但卻極易變成自由基或能促進自由基的形成，如水質污染帶來的重金屬等。由於臺灣處於開發中的地區，上述的環境污染無可避免圍繞在我們日常生活中。因此本研究希望探討：1、瞭解臺灣地區居民體內抗氧化酵素(SOD; GSH-Px)及抗氧化能力與鄰近地區居民間的差異。2、建立一套快速、準確且實用的檢驗方法，使體內抗氧化酵素(SOD; GSH-Px)及抗氧化能力的檢驗能普及。

實驗材料與方法

一、試劑：

1、Ransod reagent kit；Cat. No. SD 125 購自 Randox Labs.

2、Ransel reagent kit；Cat. No. RS 504 購自 Randox Labs.

3、Randox total antioxidant status kit ；Cat. No. NX 2332 購自 Randox Labs.

4、Ransod control；Cat. No. SD 126 購自 Randox Labs.

5、Ransel control；Cat. No. 099 購自 Randox Labs.

6、Randox total antioxidant status control serum；Cat. No. NX 2331 購自 Randox Labs.

7、Drabkin's reagent

二、儀器：

1、Beckman Synchron CX-7 autoanalyzer.

2、Beckman GS－6R centrifuge

三、檢體收集：

本研究共收集 188 個來自中山醫學院附設醫院體檢組健康檢查的檢體，其中包括 98 名男性及 90 名女性，年齡由 22～92 歲，經檢查沒有發現內、外科之病症。並在診間進行問卷調查，除一般基本資料外並包括日常生活習慣：抽煙情形、飲酒狀況及交通工具等。檢體空腹收集以 EDTA 作為抗凝固劑，檢體放置 4

℃環境下，並於 8 小時內完成分析。

四、CuZn-SOD 活性之分析：

取 0.5 ml 全血以 1000 xg 離心 5 分鐘，取紅血球，以 3ml 0.9 % 生理食鹽水洗滌血球三次後，加 2 ml 冷蒸餾水混合並於 4℃下靜置 15 分鐘。溶血完全的檢體以 0.01 M phosphate buffer 稀釋 100 倍，使用 Beckman CX-7 自動分析儀進行分析(Table 1)。

五、GSH-Px 活性之分析：

取 0.05 ml 全血加入 1 ml 稀釋液，靜置 5 分鐘再加入 1 ml double strength Drabkin's reagent，並於 20 分鐘內使用 Beckman CX-7 自動分析儀完成分析(Table 2)。

六、total antioxidant status(TAS)之分析：

取新鮮的血清或血漿，使用 Beckman CX-7 自動分析儀進行分析(Table 3)。檢體置於 2～8℃可保存 18 小時。

七、統計方法：

所有數據輸入電腦軟體 SigmaPlot 分析相關數據間的差異。coefficients of variation (CV)可瞭解分析方法的精密度與準確性；平均值及標準偏差的計算用以瞭解正常個體各項分析值的正常分佈關係；並利用 student's test 及 stepwise regression 分析以瞭解不同組別中各項分析值之差異及相關性。

實 驗 結 果

一、精密度的分析：

我們在 Beckman CX-7 自動化分析儀中使用自行建立之條件進行 CuZn-SOD、GSH-Px、TAS 之 within-run 及 between-run 精密度分析以瞭解方法的再現性。當重覆 20 次品管血清的測試，所得平均值分別為 CuZn-SOD：233 unit/g Hb、GSH-Px：482 unit/g Hb、TAS：1.32 mmol/L，計算其變異系數均在 6 %以下(Table 4)。結果令人滿意。

二、評估正常個體 CuZn-SOD、GSH-Px、TAS 之活性

我們將 188 個正常個體依年齡及性別進行分組，並記錄其日常生活習慣包括抽煙情形、飲酒狀況及是否以機車作為代步工具等。結果發現女性的 CuZn-SOD、GSH-Px 之活性較男性高($p<0.01$)，而 TAS 之並無明顯的差異($p>0.05$)(Table 5)，而在年齡上各項分析值並無明顯的差異(Fig. 2)。

在 188 個正常個體中有 27 人有吸煙(14.4 %)，25 人有飲酒(13.3 %)，57 人每日以機車作為代步工具(30.3 %)。結果發現不論吸煙與否 CuZnSOD 及 TAS 與對照組比較無明顯的差異，而在飲酒及機車代步組別中則發現 GSH-Px 之活性較對照組低($p<0.05$) (Table 6)。

結　論

現代醫學應是預防重於治療，當體內抗氧化系統受到侵害時常會導致細胞破壞及細胞核內物質造成突變性的傷害[2-4]，引起種疾病發生。抗氧化酵素及抗氧化能力評估的檢查，可提供一般健康人在面對因環境污染等致病的危險因子時的預防措施。以往分析紅血球中的 CuZnSOD、GSH-Px 活性及抗氧化能力(TAS)的檢查時，因手工操作法有較多的誤差而產生高度的變異性。基於這些因素在本研究中建立自動化分析系統進行，降低人為誤差，得到極高的再現性及準確度(Table 4)，並且可提供臨床的研究。

環境及種族不同等各項生物變因會使 CuZnSOD、GSH-Px 活性及抗氧化能力(TAS)有不同的表現[18-23]，因此在本研究中我們收集地區性的樣本(臺灣台中地區)建立 CuZnSOD、GSH-Px 活性及 TAS 濃度的參考值(Table 5)。有趣的是相較其它地區結果，女性的 CuZnSOD、GSH-Px 之活性較男性高($p<0.01$)，而 TAS 之並無明顯的差異($p>0.05$) [19-20]，在年齡上各項分析值亦無明顯的差異。在 Ne've[21]及 Ceballos-picot[22]等學者研究中指出在法國及柏林等地區 CuZnSOD、GSH-Px 之活性在年齡上並無差異。總體評估臺灣台中地區的 CuZnSOD 活性的參考值較其他國家

或地區高[18-22]，我們懷疑這項結果與臺灣地區工業成長迅速、各項建設突飛猛進，社會結構及經濟發展均有很大的變化，人口大量湧向城市、郊區工廠林立、市區機動車輛密度大幅成長、能源消耗量大增，導致臺灣居住品質日益惡化有關。

環境污染以空氣污染為主要污染源，室外空氣污染又以工廠廢氣及汽機車排放物為主。氣機車排放物包括一氧化碳、氧化氮(NOx，主要有一氧化氮和二氧化氮)、未燃燒的燃料或部份氧化的碳氫化合物和粒子等。後者屬於未燃燒之碳氫化合物，許多這類存於柴油或汽油車排放物中的化合物大多為致癌物，例如多環芳香烴、硝基多環芳香烴和一些揮發性物質。1、多環芳香烴在生物體內首先會經由微粒體單氧化酵素系統(monooxygenase)或前列腺素 H 合成酵素(prostaglandin H synthase)代謝活化成 epoxide，然後會再水解轉變為 diols 型的活化中間產物，此中間產物可與 DNA 共價結合形成 DNA 鍵結物，造成基因突變而引起癌症發生[52]。2、硝基多環芳香烴是引擎排放物中另一類主要的污染物，是由多環芳香烴經過硝化作用(nitration)而形成的，現已知的硝化機轉有二：①在大氣條件下，當 NO_2 或 N_2O_5 存在時多環芳香烴會經由光化學反應與氫氧自由基($OH^{\cdot}$)反應[53] ②在燃燒過程形成 NO_2^+(nitrinium） 會與親電子性的多環芳香烴

發生硝化作用而形成。在柴油引擎中含有多種硝基多環芳香烴類化合物[54]，Nitropyrene(1-NP)是環境中含量最多的硝基多環芳香烴[55]。3、揮發性有機成分係指低沸點、低分子量之有機化合物。由於具有高揮發性，故常以氣態存於空氣中，車輛常見的揮發性有機物包括苯(benzene)，甲苯(toluene)等。揮發性有機物具高度光化學反應性，在日光之照射下可經由光分解(photolysis)產生自由基。苯是已知之致癌物質，當慢性暴露於低濃度苯中會造成造血系統的為害，引起白血病（leukemia）的發生。室內的空氣污染研究最清楚的是香煙，已知香煙的煙霧懸微粒不但具有細菌致突變性[56]，在體外實驗也會攻擊 DNA 並形成 DNA 鍵結物[57]。

在我們的研究中發現每日以機車作為代步工具者，GSH-Px 的活性較低，原因可能是長期曝曬在污染的空氣中。GSH-Px 與解毒能力有關，當 GSH-Px 的利用增加或分解的機會增加都會造成活性下降。GSH-Px 的活性缺乏導致對毒物的解毒能力下降，進而增加致癌的危險性。相同的 GSH-Px 的活性較低也在有飲酒習慣的組別中發現，此項結果在 Guemouri 等學者的研究報告相同[20]。而在有抽煙習慣的組別中，CuZnSOD、GSH-Px 活性及 TAS 的濃度與對照組並無明顯的差異。結果與 Guemouri

[20]及 Leonard[25]等學者的研究結果一致。

綜合以上的各項結論，我們透過本研究瞭解了臺灣地區居民體內抗氧化酵素(SOD; GSH-Px)及抗氧化能力(TAS)與鄰近地區居民間的差異並建立一套快速、準確且實用的檢驗方法，使體內抗氧化酵素(SOD; GSH-Px)及抗氧化能力(TAS)的檢驗能普及。

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圖表與圖表說明

Table 1 使用 Beckman CX-7 分析 superoxide dismutase activity 之條件

Test Name: SOD	Calculation Factor: 0
Reaction Type: [Rate 1]	Math Model: [1]
Reaction Direction: [positive]	Cal Time Limit: 24 hrs
Units: [IU/mL]	No. of Calibrators: 6
Decimal Precision: [X.XX]	
Primary Wavelength: 520 nm	Secondary Wavelength: 700 nm

	Calibrations	Multipoint Span
Sample Volumn : 6 *ul*	#1: 0.00	1-2: 0.00
Primary Inject Rgt:	#2: 0.17	2-3: 0.00
A: 200 *ul*	#3: 0.34	3-4: 0.00
Secondary Inject Rgt:	#4: 0.68	4-5: 0.00
B: 30 *ul*	#5: 1.35	5-6: 0.00
Add Time: 400 sec	#6: 2.70	6-7: 0.00

Reagent Blank	Reaction
Start Read: 250 sec	Start Read: 90 sec
End Read: 300 sec	End Read: 270 sec
Low ABS Limit: -1.500	Low ABS Limit: -1.500
High ABS Limit: 1.500	High ABS Limit: 1.500
Usable Range	Substrate Depletion
Lower Limit: 0.00	Initial Rate: 99.999
Upper Limit: 2.70	Delta ABS: 1.500

Table 2 使用 Beckman CX-7 分析 glutathione peroxidase activity 之條 件

Test Name: SEL
Reaction Type: [Rate 1]
Reaction Direction: [Negative]
Units: U/L
Decimal Precision: [X.X]

Calculation Factor: 8412
Math Model: [Linear]
Cal Time Limit: 24 hrs
No. of Calibrators: 0

Primary Wavelength: 340 nm
Secondary Wavelength: 410 nm

Sample Volumn : 5 *u*l
Primary Inject Rgt:
A: 250 *u*l
C: 10 *u*l

Calibrations

Multipoint Span

Reagent Blank
Start Read: 250 sec
End Read: 300 sec
Low ABS Limit: -1.500
High ABS Limit: 1.500

Reaction
Start Read: 64 sec
End Read: 192 sec
Low ABS Limit: -1.500
High ABS Limit: 1.500

Usable Range
Lower Limit: 0.0
Upper Limit: 2000.0

Substrate Depletion
Initial Rate: -99.999
Delta ABS: 1.500

Table 3　使用 Beckman CX-7 分析 total antioxidant status 之條件

Test Name: TAS	Calculation Factor: 0
Reaction Type: [Endopoint 2]	Math Model: [Linear]
Reaction Direction: [Negative]	Cal Time Limit: 24 hrs
Units: [mmol/L]	No. of Calibrators: 2
Decimal Precision: [X.XX]	
Primary Wavelength: 600 nm	Secondary Wavelength: 700 nm

Sample Volumn : 4 *ul*	Calibrations	Multipoint Span
Primary Inject Rgt:	#1: 0.00	1-2: 0.00
A: 200 *ul*	#2: 2.50	
Secondary Inject Rgt:		
C: 40 *ul*		
Add Time: 368 sec		

Reagent Blank	Reaction
Start Read: 320 sec	Start Read: 180 sec
End Read: 336 sec	End Read: 212 sec
Low ABS Limit: -1.500	Low ABS Limit:- 1.500
High ABS Limit: 1.500	High ABS Limit: 1.500

Usable Range	Substrate Depletion
Lower Limit: 0.00	Initial Rate: 99.999
Upper Limit: 3.00	Delta ABS: 1.500

Table 4. Accuracy and precision for superoxide dismutase, total antioxidant status, glutathione peroxidase ($N = 20$)

	Within-run precision			Between-run precision		
Item	Mean	SD	CV (%)	Mean	SD	CV (%)
Superoxide dismutase (unit/g Hb)	219	12.3	5.6	222.6	12.2	5.4
Glutathione Peroxidase (unit/g Hb)	484	5.8	1.2	482	10.9	2.3
Total Antioxidant Status (mmol/L)	1.34	0.016	1.2	1.32	0.05	3.8

SD: Standard deviation; CV: Coefficient of variation.

Table 5. superoxide dismutase, glutathione peroxidase, and total antioxidant status in 188 healthy Chinese

Age group (years)	Superoxide dismutase (unit/g Hb)		Glutathione peroxidase (unit/g Hb)		Total antioxidant status (mmol/L)	
	Male	Female	Male	Female	Male	Female
20 - 29	876.98 ± 175.75 *N* = 13	1095.23 ± 299.96* *N* = 15	68.59 ± 7.86 *N* = 13	87.47 ± 8.88*** *N* = 15	1.55 ± 0.07 *N* = 13	1.54 ± 0.05 *N* = 15
30 - 39	961.07 ± 222.59 *N* = 8	973.52 ± 198.61 *N* = 6	72.50 ± 12.34 *N* = 8	82.77 ± 6.08 *N* = 6	1.55 ± 0.06 *N* = 8	1.57 ± 0.06 *N* = 6
40 - 49	1028.85 ± 186.18 *N* = 19	1080.36 ± 241.77 *N* = 20	74.54 ± 8.07 *N* = 19	92.98 ± 18.89** *N* = 20	1.49 ± 0.08 *N* = 19	1.41 ± 0.12 *N* = 20
50 - 59	1054.69 ± 116.80 *N* = 7	1115.37 ± 194.23 *N* = 5	82.45 ± 9.83 *N* = 7	85.32 ± 5.42 *N* = 5	1.39 ± 0.11 *N* = 7	1.41 ± 0.06 *N* = 5
60 - 69	1006.35 ± 239.46 *N* = 25	1038.04 ± 206.72 *N* = 27	80.41 ± 9.88 *N* = 25	87.46 ± 9.50* *N* = 27	1.43 ± 0.10 *N* = 25	1.43 ± 0.10 *N* = 27
70 - 92	991.02 ± 167.51 *N* = 26	1170.72 ± 352.05* *N* = 17	86.38 ± 12.27 *N* = 26	100.30 ± 23.27* *N* = 17	1.45 ± 0.10 *N* = 26	1.42 ± 0.09 *N* = 17
Overall	989.24 ± 196.31 *N* = 98	1082.03 ± 261.32** *N* = 90	78.79 ± 11.73 *N* = 98	90.68 ± 15.66** *N* = 90	1.46 ± 0.10 *N* = 98	1.47 ± 0.10 *N* = 90

Student's t test: *P <0.05, **P < 0.01, ***P < 0.001.

Table 6. Comparison of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and total antioxidant status (TAS) in 188 healthy Chinese by life style

	Cigarette Smoking		Alcohol consumption		Riding motor cycle	
	Yes (N = 27)	No (N = 161)	Yes (N = 25)	No (N = 163)	Yes (N = 57)	No (N = 13
SOD (unit/g Hb)	984.76 ± 138.29	1041.86 ± 245.55	989.18 ± 191.61	1040.48 ± 239.34	1039.01 ± 239.35	1031.34 ± 2
GSH-Px (unit/g Hb)	79.54 ± 11.43	85.31 ± 15.34	77.91 ± 9.21	85.49 ± 15.42*	80.63 ± 10.73	86.16 ± 16.
TAS (mmol/L)	1.44 ± 0.12	1.46 ± 0.10	1.47 ± 0.09	1.46 ± 0.11	1.47 ± 0.11	1.46 ± 0.10

*Student's t-test: *P <0.05.

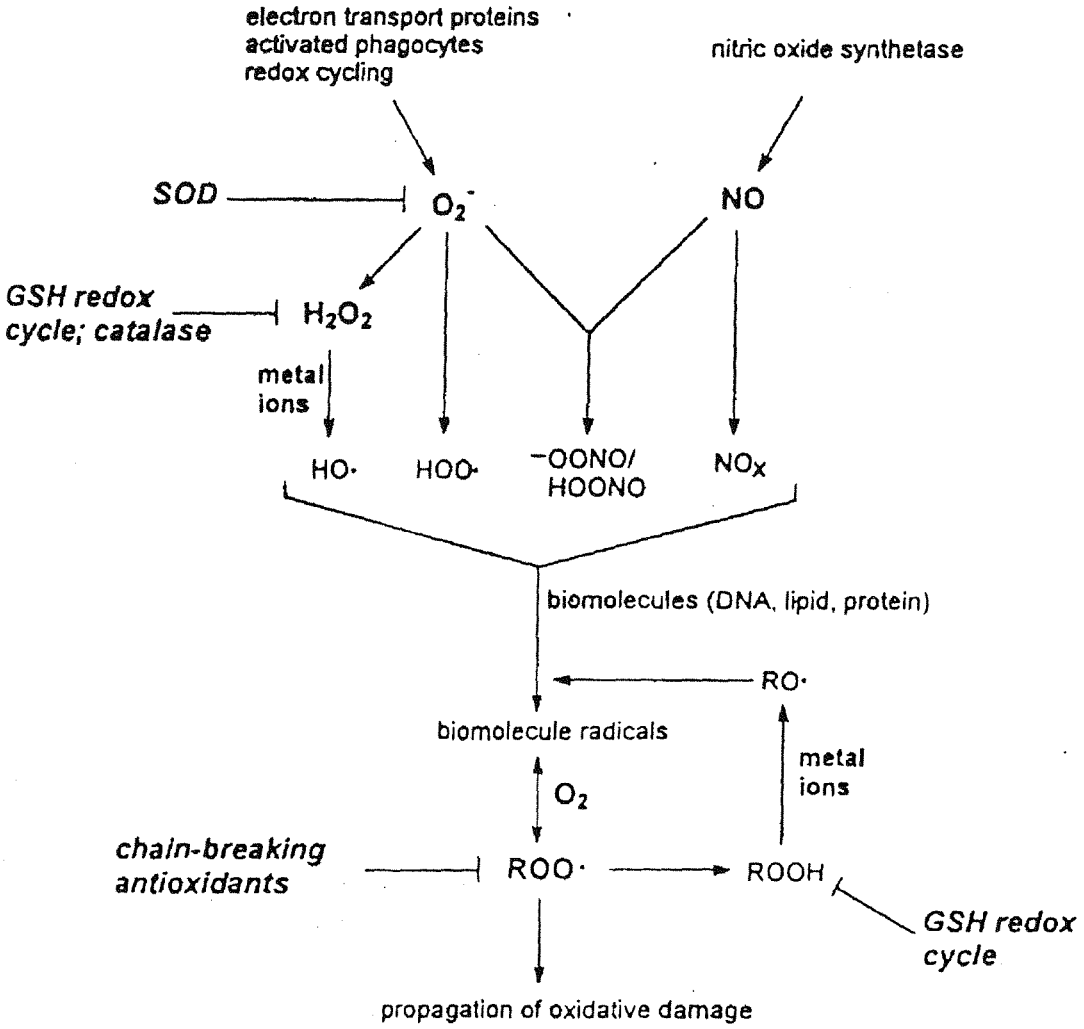

Fig. 1 體內抗氧化防禦系統扮演的位置

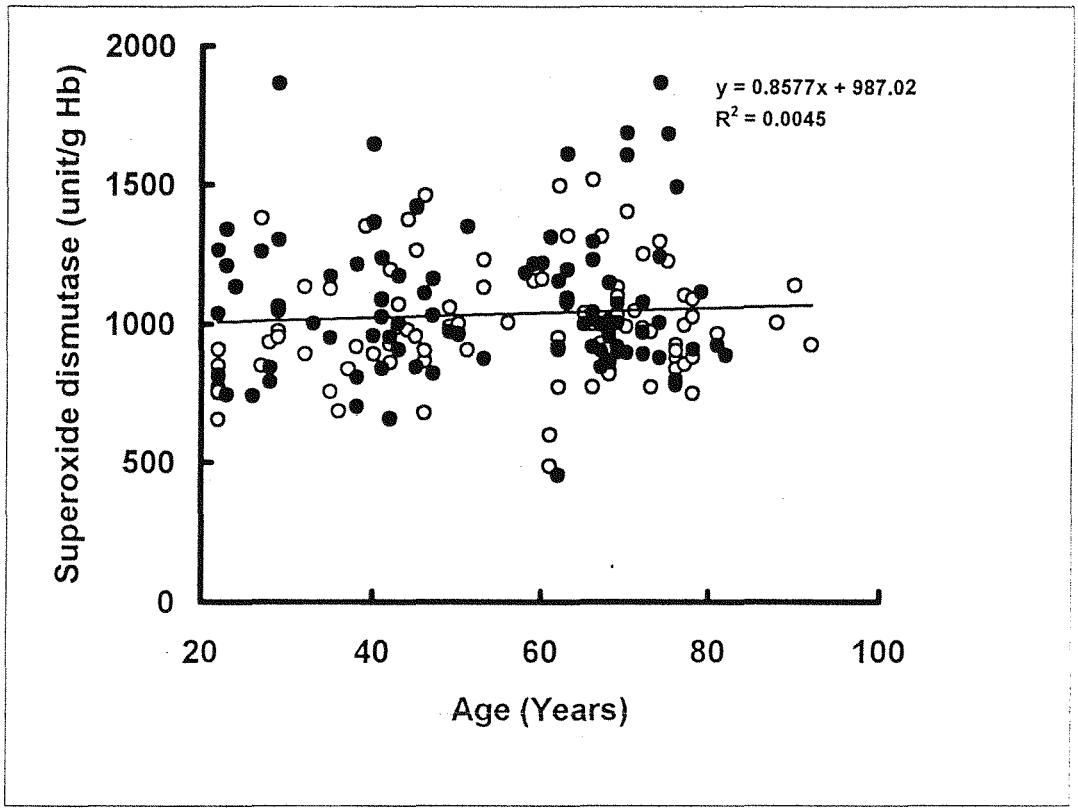

Fig. 2 紅血球內 superoxide dismutase activity 與年齡之關係

●: 男性　○: 女性

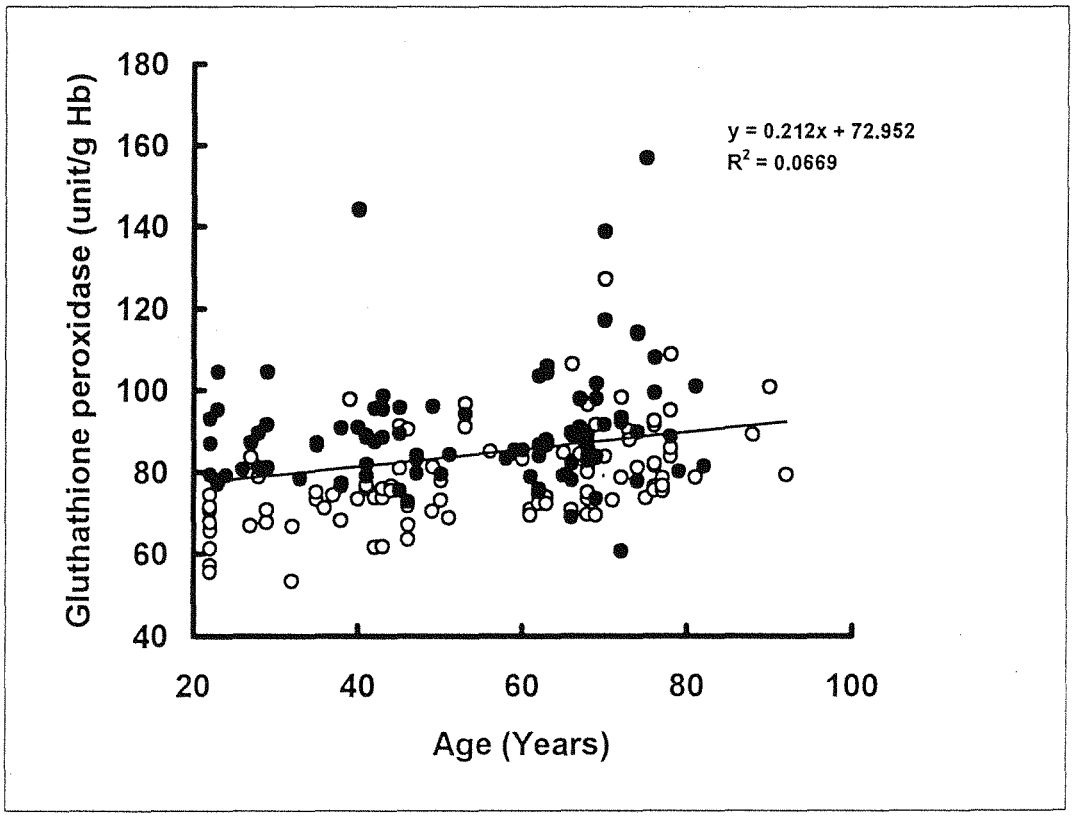

Fig. 3 紅血球內 glutathione Peroxidase activity 與年齡之關係

●：男性　○：女性

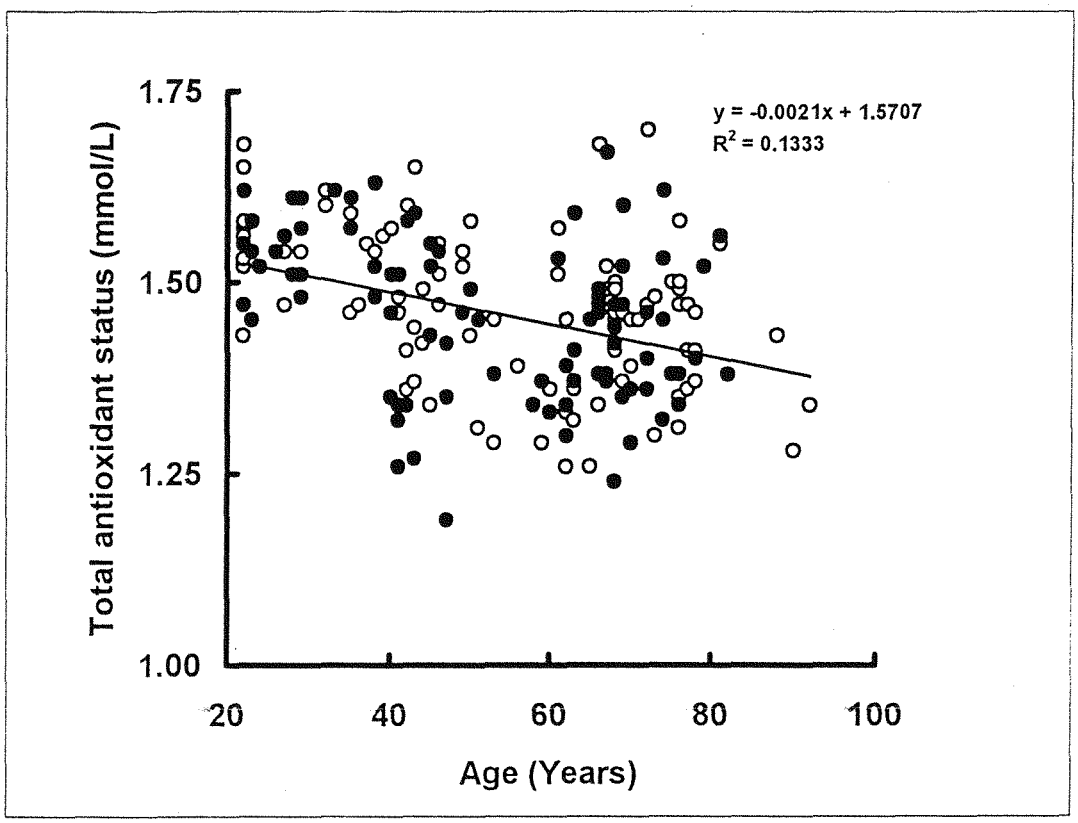

Fig. 4 人體內 total antioxidant status 與年齡之關係

●: 男性　○: 女性

附　　錄

Quantification of Superoxide Dismutase, Glutathione Peroxidase and Total Antioxidant capacity in Blood of Healthy Taiwanese by A Modified Automated Procedure

Whei-Lin Chiang[1,2,3], Jyh-Chang Lai[4], Yie-Tin Tasy[3], Jer-Yuh Liu[2], Shu-Chen Chu[3] and Yih-Shou Hsieh[2]

[1]*School of Medical Technology,*
[2]*Institute of Biochemistry, Chung Shan Medical and Dental College, Taichung, Taiwan;*
[3]*Department of Clinical Biochemistry, Chung Shan Medical and Dental College Hospital, Taichung, Taiwan;*
[4]*Department of Clinical Biochemistry, Veterans General Hospital, Taichung, Taiwan*

A rapid automated system for assessing the activities of superoxide dismutase (SOD), total antioxidant status (TAS), and glutathione peroxidase (GSH-Px) in blood was developed using a set of commercial kits and the Beckman Synchrom CX-5 automatic analyzer. Reproducibility data for assaying SOD, TAS and GSH-Px by our proposed procedure were excellent (CVs < 6.0 %). We then recruited a total of 188 apparently healthy individuals and their antioxidative status were assessed by our proposed method. We found that female (n=90) had a significant higher SOD (1082 ± 261 unit/g Hb) and GSH-Px (91 ± 16 unit/g Hb) than its male counterparts (n=98) [SOD: 989 ± 196 unit/g Hb; GSH-Px: 79 ± 11 unit/g Hb] ($p < 0.01$). Interestingly, we also found that alcoholics and motor cyclists seemed to have lower GHS-Px activities as compared to their respective counterparts ($p < 0.05$). In contrast, we did not find any significant discrepancy in SOD, GSH-Px and TAS between smokers and non-smokers.

Key words: Antioxidant enzyme activities, total antioxidative status, superoxide dismutase, glutathione peroxidase

Introduction

The discovery of superoxide dismutase (SOD) in erythrocytes [1], the key component of the antioxidant system, shed light on the understanding of mechanisms in the enzymatic clearance of oxygen free radicals. When the capacity of the antioxidant system reduces, the highly reactive cytotoxic oxygen species such as superoxide anions, hydrogen peroxide, and hydroxyl radicals can cause a wide spectrum of cell damage including lipid peroxidation, inactiva-

Received: June 24, 1999 Accepted: June 25, 1999
Address for correspondence: Dr. Yih-Shou Hsieh, Institute of Biochemistry, Chung Shan Medical and Dental College, No. 110, Sec. 1, Chien Kuo N. Road, Taichung 40203, Taiwan

tion of enzymes, alteration of intracellular oxidation-reduction state as well as damage to DNA [2]. The oxygen free radicals have been reported to be involved in the etiology of atherosclerosis and cancer [3] as well as neurodegenerative diseases [4]. Moreover, they have been attributed to be implicated in the aging process [5].

A well-integrated antioxidant system is determined by the a dynamic interaction between individual components. These components include vitamins A, E and C, β-carotene, reduced glutathione (GSH) and the antioxidative enzymes [3]. Among the enzymatic antioxidants, copper-zinc superoxide dismutase (CuZn-SOD; E. C. 1.15.1.1.), catalyzes dismutation of the superoxide anion into hydrogen peroxide and oxygen. The hydrogen peroxide is further reduced to water and oxygen by catalase (CAT; E. C. 1.11.1.6.) and glutathione peroxidase (GSH-Px; E. C. 1.11.1.9.) [6]. Therefore, measuring the levels superoxide dismutase, glutathione peroxidase and catalase in blood may provide valuable information on the antioxdiant status of an individual.

Since the antioxidant system is relatively complex, analyzing individual components is a painstaking task. Therefore, determination of the total antioxidant status (TAS) has been reported to be a favorable alternative to give an overall indication of the antioxidant status of an individual [7-15]. The total peroxyl radical trapping assay [7], ABTS (2, 2'-azion-di-(3-ethylbenz thiazoline sulphonate)) method [12], enhanced chemiluminescent method [9], and oxygen radical absorbance capacity assay [10, 15] were developed for TAS analysis. The values of TAS have been reported to be elevated in patients with cardiovascular diseases [16-17].

Although there is evidence for the automation in the analyses of the antioxidative enzyme activities [18], the determination of these enzymes is mainly based on manual procedures. Recently, commercial kits for assessing the CuZn-SOD, GSH-Px and TAS in blood have been commercially available. These kits can detect the antioxidative enzyme activities from whole blood sample. We report herein the development of an automated procedure for assaying SOD, GSH-Px and TAS for clinical laboratory usage based on these commercially-prepared reagents. By using these proposed methods, we also assessed the levels of antioxidant enzymes and antioxidative capacities among the general population in Taichung area.

Materials and Methods

Subjects

Blood specimens were collected from 188 subjects (98 males and 90 females; aged 22 to 92 years) who participated in the general health examination at the Chung Shan Medical and Dental college Hospital. These subjects passed all examination items and no medical or surgical problems were found. In addition to recording the sex and age of each subject, daily activities (cigarette smoking, alcohol consumption, and motor cycle riding, etc.) were also inquired.

Blood Collection

The subjects were fast overnight. Whole blood sampples (2 ml) were collected by venipuncture into Venoject tubes with EDTA. These blood

samples were kept at 4 °C and analyzed within 8 hours.

Analytical Methods

Assay of CuZn-SOD activity. The activity of CuZn-SOD was determined using a kit (Ransod; Randox Labs. Cat. No. SD125) which is based on the method of McCord and Fridovich [1]. The reaction between xanthine and xanthine oxidase is used to generate superoxide radicals. These radicals react with p-iodonitrotetrazolium salts (INT) to produce a red formazan dye. SOD in the sample competes with the INT for superoxide radicals and so inhibits the production of the formazan dye. SOD is measured by the degree of inhibition of formazan dye formation. 0.5 ml heparinized whole blood for were centrifuged 10 minutes at 3000 rpm. The RBC was washed four times with 3 ml of 0.9% NaCl solution and centrifuged for 10 minutes at 3000 rpm after each wash. The washed RBC was then made up to 2.0 ml with cold distilled water, mixed and left to stand at 4 °C for 15 minutes. The lysate was then diluted with 0.01 M phosphate buffer pH 7.0 to reduce the inhibition reaction to the range of 30 to 60%. A sample of 6 μL with the primary inject reagent (200 μ L) was added to the cuvette of an autoanalyzer(Beckman CX-5). After adding the secondary inject reagent (30 μ L), absorbance was monitored at 520 nm for 180s. The final volume reaction volume was 236 μ L and the unit of activity was calculated based upon as the amount of enzyme that inhibits the rate of the formazan dye formation by 50%.

Assay of GSH-Px activity. A kit (Ransel; Randox Labs. Cat. No. SD125) was used to determine the activity of GSH-Px. This kit is based on the method of Paglia and Valentine [19]. GSH-Px catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured. Whole blood (0.05 ml) was added to 1 ml diluting agent and the mixture was incubated for 5 min. After adding 1 ml double strength Drabkin's reagent to the mixture, 5 μL of the sample was transfer to the curvette of the autoanalyzer (Beckman CX-5). Absorbance was monitor at 340 nm for 128s.

Assay of TAS activity. The Randox Total Antioxidant Status Kit (Randox Labs. Cat. No. 2332) was employed to determine the TAS activity. This kit is based on the fact that the ompound ABTS is capable of producing a stable blue-green colored cation radical ($ABTS^+$) in the presence of peroxidase and hydrogen peroxide. Antioxidants in the sample can suppress the production of this color to a degree which is proportional to their concentration. Plasma (4 μ L) was transferred into the curvette of the autoanalyzer (Beckman CX-5). A chromgen (1 ml) was added and the solution was mixed thoroughly before taking an initial reading of absorbance at 600 nm for 32s. After adding a substrate (hydrogen peroxide, 200μL), another reading was taken 3 minutes later exactly. The concentration was calculated as the difference of these two readings.

Statistical Analysis

The coefficients of variation (CV) were calcu-

lated to determine the accuracy and precision of the assays. Means and standard deviations of the results obtained from the normal subjects were calculated. Student's test was used to determine whether they exist any differences in the mean values between groups. The relationships between the antioxidative enzyme activities and the respective variables were assessed by correlation and stepwise regression analysis.

Results

Precision studies

Reproducibilities as reflected by the within-run and between-run precision data for SOD, GSH-Px and TAS determined by the proposed automated procedure were excellent (Table 1). Twenty repetitive determinations on the same control material had mean values for 219 unit/g Hb (SOD), 484 unit/g Hb (GSH-Px) and 1.34 mmol/L (TAS), respectively, with CV's of < 6.0%. The same control material assayed in several consecutive runs showed mean values of 223 unit/g Hb (SOD), 482 unit/g Hb (GSH-Px) and 1.32 mmol/L (TAS), with CV's of < 6.0%. Again, the between-run reproducibility was also excellent.

Evaluation of SOD, GSH-Px and TAS levels in a group of apparently healthy subjects

One hundred and eighty-eight subjects were recruited for this study. Among these, they were also subgrouped according to age and gender. Also, their daily activities, namely: cigarette smoking, alcohol consumption and motor cycle rider, were recorded. As indicated in Table 2, the overall SOD and GSH-Px levels in female subjects had a significant higher value than its male counterparts ($p < 0.01$). However, TAS levels was shown to be insignificantly different from each other between the two groups ($p > 0.05$).

Among these subjects, 27 of 188 (14.4%) were cigarette smokers, 25 of 188 (13.3%) were alcoholics and 57 of 188 (30.3%) were motor cyclist. Comparatively, we found that there was no significant difference in antioxidant enzyme levels between smokers and non-smokers (Table 3). In addition, among all variables compared, only alcoholics and motor cyclists had had suppressed levels of GSH-Px ($p < 0.05$) (Table 3).

Discussion

Since disorders in the antioxidant system may

Table 1. Precision data for the proposed automated procedures for analyzing SOD, GSH-Px and TAS levels in erythrocytes

Item	Within-run precision			Between-run precision		
	Mean	SD	CV (%)	Mean	SD	CV (%)
Superoxide dismutase (unit/g Hb)	219	12.3	5.6	222.6	12.2	5.4
Glutathione Peroxidase (unit/g Hb)	484	5.8	1.2	482	10.9	2.3
Total Antioxidant Status (mmol/L)	1.34	0.016	1.2	1.32	0.05	3.8

Table 2. SOD, GSH-Px and TAS levels in erythrocytes of a group of apparently healthy subjects in Taichung area

Age group (years)	Superoxide dismutase (unit/g Hb)		Glutathione peroxidase (unit/g Hb)		Total antioxidant status (mmol/L)	
	Male	Female	Male	Female	Male	Femal
20 - 29	876.98 ± 175.75[a] *N* = 13	1095.23 ± 299.96* *N* = 15	68.59 ± 7.89 *N* = 13	87.48 ± 8.88*** *N* = 15	1.55 ± 0.07 *N* = 13	1.54 ± 0.05 *N* = 15
30 - 39	961.07 ± 222.59 *N* = 8	973.52 ± 198.61 *N* = 6	72.50 ± 12.34 *N* = 8	82.77 ± 6.08 *N* = 6	1.55 ± 0.06 *N* = 8	1.57 ± 0.06 *N* = 6
40 - 49	1028.85 ± 186.18 *N* = 19	1080.36 ± 241.77 *N* = 20	74.54 ± 8.07 *N* = 19	92.98 ± 18.89** *N* = 20	1.49 ± 0.08 *N* = 19	1.41 ± 0.12 *N* = 20
50 - 59	1054.69 ± 116.80 *N* = 7	1115.37 ± 194.23 *N* = 5	82.45 ± 9.83 *N* = 7	85.32 ± 5.42 *N* = 5	1.39 ± 0.11 *N* = 7	1.41 ± 0.06 *N* = 5
60 - 69	1006.35 ± 239.46 *N* = 25	1038.04 ± 206.72 *N* = 27	84.1 ± 9.88 *N* = 25	87.46 ± 9.50* *N* = 27	1.43 ± 0.10 *N* = 25	1.43 ± 0.10 *N* = 27
70 - 79	991.02 ± 167.51 *N* = 26	1170.72 ± 352.05* *N* = 17	86.38 ± 12.27 *N* = 26	100.30 ± 23.27* *N* = 17	1.45 ± 0.10 *N* = 26	1.42 ± 0.09 *N* = 17
Overall	989.24 ± 196.31 *N* = 98	1082.03 ± 261.32** *N* = 90	78.79 ± 11.73 *N* = 98	90.68 ± 15.66** *N* = 90	1.46 ± 0.10 *N* = 98	1.47 ± 0.10 *N* = 90

Student's test: *p<0.05, **p<0.01, ***p<0.001, [a]Mean ± Standard deviation.

Table 3. Comparison of SOD, GSH-Px and TAS levels in erythrocytes in grouped individuals according to their daily activities

	Cigarette Smoking		Alcohol consumption		Riding motor cycle	
	Yes (*N*=27)	No (*N*=161)	Yes (*N*=25)	No (*N*=163)	Yes (*N*=57)	No (*N*=131)
SOD (unit/g Hb)	984.76 ± 138.29	1041.86 ± 245.55	989.18 ± 191.61	1040.48 ± 239.34	1039.01 ± 239.35	1031.34 ± 232.20
GSH-Px (unit/g Hb)	79.54 ± 11.43	85.31 ± 15.34	77.91 ± 9.21	85.49 ± 15.42*	80.63 ± 10.73	86.16 ± 16.21*
TAS (mmol/L)	1.44 ± 0.12	1.46 ± 0.10	1.47 ± 0.09	1.46 ± 0.11	1.47 ± 0.11	1.46 ± 0.10

*Student's t-test: *p<0.05. aMean ± Standard deviation.

cause cell damage and damage to DNA [2], antioxidative enzymes may be employed as an indicator for the health status as well as the risk of certain diseases. However, in the analysis of these enzymes, manual weighing and pipetting may cause significant variations in the results. Thus, it is imperative to devise methods of analyses with high degree of reproducibility in quantifying SOD, GSH-Px and TAS levels in human erythrocytes (RBCs). For this reason, we have developed a rapid and simple automated procedure for analyzing SOD, GSH-Px and TAS levels in RBCs using commercially-prepared reagents. These procedures have been evaluated and excellent reproducibilities have been obtained (Table 1). Thus, they are suitable for

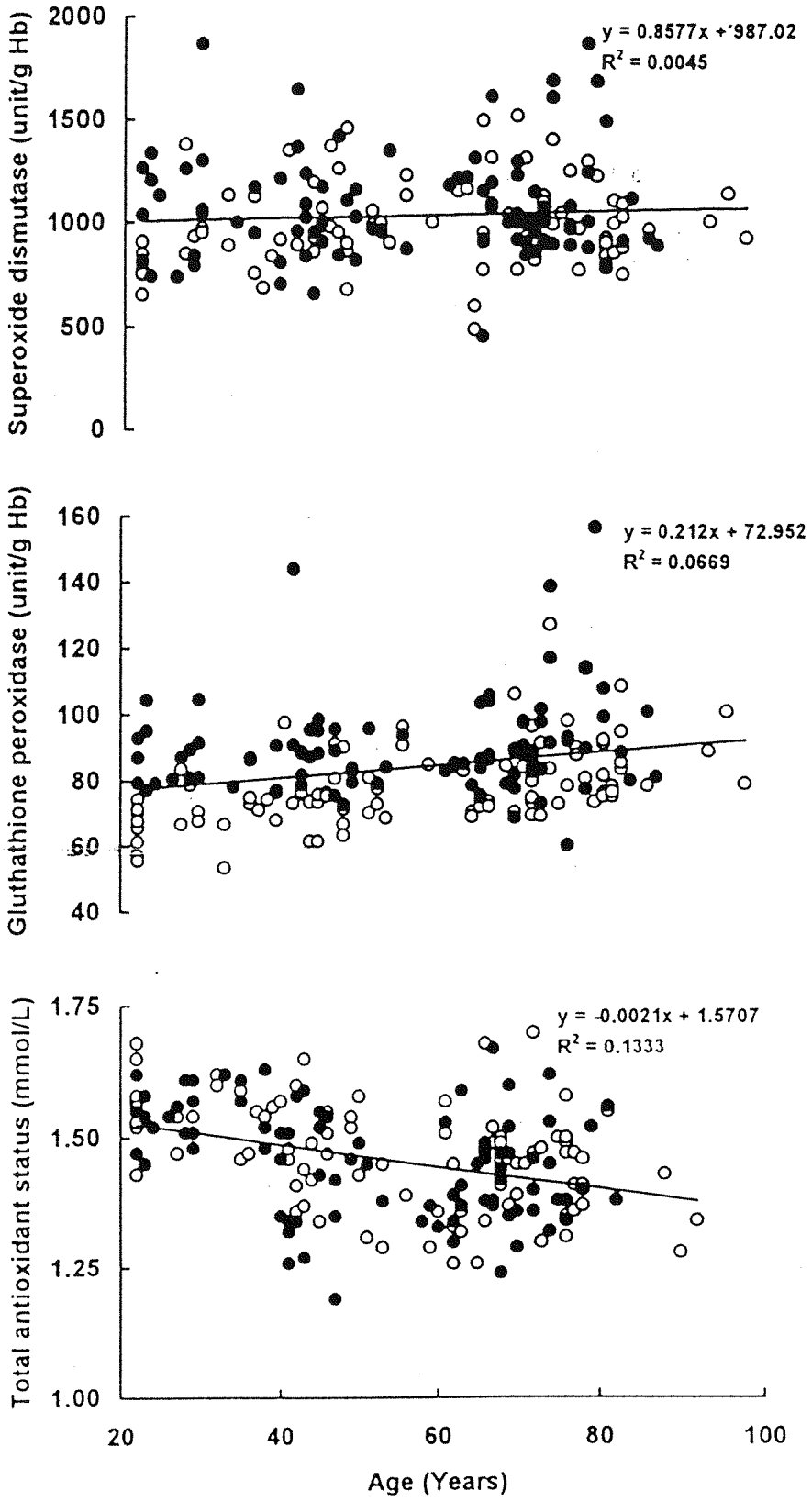

Fig. 1. Correlation studies between SOD, GSH-Px and TAS levels in erythrocytres and age of subjects.

both clinical applications as well as for basic studies.

Biological variability in the antioxidative enzymes activities has been reported in several populations [18-23]. In this reports, we provide some reference ranges of SOD, GSH-Px and TAS levels among local residents in Taichung area (Table 2). Additionally, it is interesting to note that female subjects have had mean values for SOD and GSH-Px higher than their respective counterparts. These data are consistent with those reported elsewhere [19-20]. Conversely, Ne've et al [21] as well as Ceballos-Picot et al [22] reported that gender was not differed in both SOD and GSH-Px levels in French and Belgian.

Cigarette smoking have been reported to be negatively correlated with activities of antioxidative enzymes [18, 24]. However, we did not find any significant difference in SOD, GSH-Px and TAS between smokers and non-smokers. This finding agrees with the reports of Guemouri et al. [20] and Leonard et al. [25]. Conflicting results have been obtained in the relationship between alcoholism and levels of antioxidative enzymes [20]. We have found that alcoholics had a lower GSH-Px value. This finding is inconsistent with that reported by Guemouri et al. [20] and may be due to the difference in the population studies. Lower values in GSH-Px have also been observed in the persons riding motor cycle everyday. It is possible that prolong exposure to the pollutants on the road may cause disturbance in the antioxidant system.

Finally, we noted that SOD, GSH-Px and TAS levels in RBCs of our population evaluated were considerably higher than those reported in the Westerns [18-22] and Japanese. Whether genetic factor will play a role in causing this discrepancy or not is warranted for further investigation.

Acknowledgement

This study was supported by the Chung Shan Medical and Dental College (Grant CSMC 85-OM-B-023).

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Analysis of Atherosclerosis Risk Factor Among Pre-school Children in Taichung Area

Whei-Ling Chiang[1,2], Jia-Yuh Chen[3], Ung-Paw Ling[4], Jyh-Chang Lai[4] and Yih-Shou Hsieh[2]

[1]*School of Medical Technology and*
[2]*Institute of Biochemistry, Chung Shan Medical and Dental College, Taichung, Taiwan;*
[3]*Department of Pediatrics, Chung Shan Medical and Dental College Hospital, Taichung, Taiwan;*
[4]*Department of Clinical Biochemistry, Veterans General Hospital, Taichung, Taiwan*

A group of 128 pre-school children were randomly selected from Taichung area to participate in this evaluation program. Anthropometric measurements and biochemical determinations of serum glucose, total cholesterol, triglyceride, apolipoprotein A, apolipoprotein B, lipoprotein (a), fructosamine and total proteins were performed on this group of subjects in order to establish if there is a possible correlation between obesity and the metabolic status of lipids and carbohydrates. Firstly, we found that regardless of sex, there was no significant differences in these measurements. Secondly, we found a relatively low prevalence of obesity (4.7%) among the children participated in this study. No significant correlation was found between obesity and the anthropometric as well as biochemical measurements. However, it is of interest to point out that body mass index (BMI) was found to be significantly correlated with the levels of glucose, triglyceride and apolipoprotein B. Conversely, BMI was also found to be inversely correlated with the ratio of apolipoprotein A to apolipoprotein B and fructosamine. Taken together, these findings indicate that changes of BMI with these factors in children may be a progressive process rather than an immediate reflective response at the pre-school stage.

Key words: Pre-school children, lipoprotein (a), fructosamine, body mass index, correlation

Introduction

Obesity in childhood or adolescence has been reported to have significant influence on the health status in adulthood [1, 2]. It poses a serious risk for the development of diabetes mellitus, hypertension, heart disease, gall bladder disease, and certain forms of cancer [3, 4]. Currently, obesity is considered as a consequence of the interaction between environmen-

Received: June 24, 1999 Accepted: June 25, 1999
Address for correspondence: Dr. Yih Shou Hsieh Institute of Biochemistry, Chung Shan Medical and Dental College, No. 110, Sec 1, Chien Kuo N. Road, Taichung 40203, Taiwan

tal factors and the individual genetic substrate, in particular with susceptibility genes [5]. After the obesity (ob) gene in ob/ob mouse was isolated [6], its product, leptin, has been determined to play an important role in controlling food intake and energy metabolism [7-10].

Although the basis for genetic susceptibility to obesity has been documented, researches on the relationships between metabolism and obesity in childhood deserves extensive studies. In obese children, the metabolism of apolipoprotein B has been reported to be impaired [11] and the levels of serum lipoproteins and apolipoproteins elevate significantly [12]. These changes of the lipid metabolism in childhood have also been considered to be a risk factors for coronary artery disease in adulthood [13, 14]. In addition, significant associations between child obesity and abnormalities in glucose tolerance as well as insulin secretion have been reported [15-19]. Therefore, obesity affects not only the lipid, but also the carbohydrate metabolisms in obese children. Thus, these changes may be employed as predicators of obesity in childhood.

Information on the relationships between carbohydrate and lipid metabolisms and obesity is quite abundant. However, this information is obtained mainly based on the children of school age. Thus far, information pertaining preschool children is scanty. The present study was designed to obtain these information for preschool children and to find risk factors that may be used as indicators for obesity.

Materials and Methods

Subjects

The subjects were children selected randomly among the kindergartens located at Taichung area. Informed consents were obtained from the parents prior to participation in this study.

Anthropometric Measurements

All measurements were taken by pediatricians. The children were weighed on a calibrated weighing scale (Nagata, Tokyo) with a precision of 0.5 kg. Standing height was measured to the nearest 0.1cm. At least two measurements were taken on each child and the average of these measurements were recorded. Body mass index (BMI) calculated from height and weight (kg/m^2) was used to estimate the degree of obesity. Children with BMI above the 95th percentile were considered to be obese [20]. Ages of the children were determined from their dates of birth.

Biochemical measurements

Blood samples of the participants were also taken by pediatricians between 8 a.m. and 9 a.m. after an overnight fast. Serum was then separated when clotting was complete and stored in at -70 °C for further analyses.

The concentrations of serum glucose (GLU), total cholesterol (TCh), triglyceride (TG), and total protein (TP) were determined by an automatic biochemical analyzer (CX-5, Beckman, Los Angles) according to established procedures of the manufacturer. Fructosamine (FRC) was assayed using the nitroblue tetrazolium method adopted from the Beckman CX5 autoanalyzer. The levels of apolipoprotein A (Apo A), apolipoprotein B (Apo B), and lipoprotein (a) [Lp (a)] were determined by Array analyzer (Beckman, Los Angles). All laboratory measurements were obtained in duplicate and averaged.

The concentrations of serum FRC was corrected according to Peheim et al. [21]:

$$[\text{Fructosamine}]_{\text{related to protein}} = [\text{Fructosamine}] / [\text{Protein}] \times 70\ \text{g/L}.$$

Statistical Analyses

Values were expressed as mean ± SD. Differences in the anthropometric and biochemical measurements were determined by the Student's t test. The correlation between these measurements and obesity was determined by Spearman's rank correlation. Correlation coefficients among the anthropometric and biochemical variables were also calculated.

Results

A total of 128 pre-school children (75 boys, 53 girls, aged 5.03 ± 0.71 years) participated in this study. The means of anthropometric and biochemical measurements categorized based on gender are shown in Table 1. There were no significant differences in these measurements with respect to gender status. Regardless of sex, six children were found to have BMI above the 95th percentile and, thus, the prevalence of obesity were 4.7%.

The results of the anthropometric and biochemical measurements of obese and normal children are tabulated in Table 2. Regardless of the obese children had higher values in height, weight, GLU, TCh, TG, Apo B, and TP and lower values in Apo A, Apo A/Apo B, Lp (a) and FRC, there is actually no significant correlation between these measurements and obesity.

As shown in Table 3, BMI was not only significantly correlated with glucose, triglyceride, and apolipoprotein B, but also inversely correlated with the ratio of apolipoprotein A to apolipoprotein B and fructosamine.

Discussion

Serum lipoprotein (a) concentrations have been investigated in a group of five-year-old Japanese children [22]. The frequency distribution of the concentrations was found to be

Table 1. Anthropometic and biochemical measurements of a group of pre-school children in Taichung area

Item	Boys (n=75)	Girls (n=53)
Height (cm)	112.9 ± 8.1	112.5 ± 7.0
Weight (kg)	23.9 ± 5.9	22.5 ± 5.3
Body mass index (kg/m^2)	18.5 ± 3.3	17.6 ± 3.2
Glucose (mg/dL)	79.1 ± 10.4	78.5 ± 10.7
Cholesterol (mg/dL)	167.2 ± 27.2	173.2 ± 34 5
Triglycerides (mg/dL)	61.4 ± 18.6	65.3 ± 26 1
Apolipoprotein A (Apo A) (mg/dL)	135.0 ± 21.0	136.7 ± 20 7
Apolipoprotein B (Apo B) (mg/dL)	102.9 ± 24 5	108.6 ± 28 5
Apo A/Apo B	1.4 ± 0.3	1.3 ± 0.3
Lipoprotein (a) (mg/dL)	16.9 ± 31.5	14.7 ± 19.5
Fructosamine (g/L)	220.8 ± 30.1	221.0 ± 18 1
Total Protein (g/dL)	7 8 ± 3 1	7.5 ± 0 5

Table 2. Anthropometic and biochemical measurements obese and non-obese pre-school children in Taichung area

Item	Obese children* (n=6)	Non-obese children (n=122)
Height (cm)	113.7 ± 7.2	112.7 ± 7.7
Weight (kg)	33.8 ± 4.2	22.8 ± 5.2
Body mass index (kg/m^2)	26.1 ± 0.8	17.8 ± 2.8
Glucose (mg/dL)	86.3 ± 4.3	78.5 ± 10.6
Total Cholesterol (mg/dL)	164.8 ± 33.5	169.9 ± 30.4
Triglycerides (mg/dL)	65.8 ± 28.3	62.9 ± 21.8
Apolipoprotein A (Apo A) (mg/dL)	129.0 ± 19.5	136.0 ± 20.9
Apolipoprotein B (Apo B) (mg/dL)	109.0 ± 31.0	105.1 ± 26.1
Apo A/Apo B	1.3 ± 0.3	1.4 ± 0.3
Lipoprotein (a) (mg/dL)	15.4 ± 10.7	16.0 ± 27.7
Fructosamine (g/L)	205.7 ± 15.1	221.7 ± 25.9
Total Protein (g/dL)	8.0 ± 0.5	7.7 ± 2.4

*Children with BMI above the 95th percentile

Table 3. Correlations among anthropometric and biochemical measurements of pre-school children in Taichung area

	HT	WT	BMI	GLU	TCh	TG	Apo A	Apo B	Apo A/Apo B	Lp (a)	FRC
WT	0.69**										
BMI	0.24	0.86**									
GLU	0.23	0.49**	0.50								
TCh	-0.09	-0.00	0.04	0.13							
TG	0.12	0.23	0.23*	0.30**	0.11						
Apo A	0.26*	-0.21	-0.09	-0.00	0.34**	-0.11					
Apo B	-0.00	0.18	0.23*	0.26*	0.77**	0.27*	0.19				
Apo A/Apo B	-0.14	-0.29**	-0.28*	-0.29**	-0.46**	-0.39**	0.40**	-0.75**			
Lp (a)	-0.06	-0.05	-0.04	0.02	0.13	-0.03	-0.01	0.10	-0.05		
FRC	-0.16	-0.32**	-0.33**	-0.14	-0.03	-0.21	0.04	-0.09	0.14	0.72**	
TP	0.13	0.08	0.02	0.05	-0.06	0.05	0.02	-0.03	0.04	-0.53**	-0.81**

*p < 0.01, **p < 0.001.
HT: Height; WT: Weight; BMI=Body mass index; GLU=Glucose; TCh=Total cholesterol; TG=Triglyceride Apo A=Apolipoprotein A; Apo B=Apolipoprotein B, Apo A/Apo B=Ratio of apolipoprotein A to apolipoprotein B; Lp (a)=Lipoprotein (a); FRC=Fructosamine; TP=Total protein.

highly skewed. The concentration was also inversely correlated with weight and BMI. We obtained similar findings in frequency distribution of the concentrations. The concentration of serum lipoprotein (a) among the pre-school children in Taichung area ranged from 2 to 242 mg/dL. However, no significant correlation was found between this analyte and total choles-

terol, or weight, or BMI in the present study.

Low prevalence of obesity among pre-school children in Taichung area makes the analysis of the relationships between the measurements and obesity difficult. By the Spearman's rank correlation, we did not found significant correlation between obesity and the anthropometic as well as biochemical measurements. However, BMI was significant correlated with TG and Apo B and inversely correlated with Apo A/Apo B and FRC. However, recent investigations have established that apolipoprotein-A1 and apolipoprotein B as well as their ratio (Apo B/Apo-A1) are strongly correlated with coronary heart disease [23]. Our findings indicate that changes of BMI with these factors in children may be a progressive process. Significant influence on the metabolism of lipid and carbohydrate by obesity did not reflect immediately at the pre-school stage.

Carbohydrate intolerance is known to occur in obese adult and can be determined by serum fructosamine assay [24]. We found that there is a significant inverse correlation between the level of fructosamine and BMI. Thus, an impairment in the metabolism of carbohydrate may occur in children with lower fructosamine levels. Therefore, serum fructosamine assay may also be used as an indicator for obesity in children.

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