行政院國家科學委員會專題研究計畫　期中進度報告

以微量體液化學發光法定量早產兒血液中活性氧及抗氧化狀態來評估與視網膜病變嚴重度之相關性(1/2)

計畫類別： 個別型計畫
計畫編號： NSC92-2314-B-002-214-
執行期間： 92 年 08 月 01 日至 93 年 07 月 31 日
執行單位： 國立臺灣大學醫學院小兒科

計畫主持人： 謝武勳
共同主持人： 鄭劍廷

報告類型： 精簡報告

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中　華　民　國 93 年 5 月 31 日

附件一

行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告 ■ 期中進度報告

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共同主持人：鄭劍廷
計畫參與人員：楊中美、曹伯年、周弘傑

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：台灣大學醫學院附設醫院小兒部

中　華　民　國　93　年　5　月　31　日

中英文摘要

（一）計畫中文摘要

關鍵詞：早產兒、視網膜病變、自由基、抗氧化狀態、化學發光法（chemiluminescence）

新生嬰兒的生產過程中，常會合併有氧化自由基的侵襲；因為胎兒在母體的子宮內是處於相對性的缺氧狀況，血氧分壓為 20 到 50 毫米汞柱，但出生後立即暴露於相對高氧狀態，血氧分壓為 100 毫米汞柱，肺上皮細胞更直接接觸於氧分壓為 140 毫米汞柱的空氣中，因此新生兒一誕生，需馬上面對氧化侵襲的嚴苛考驗。

近年來的研究認為氧氣、一氧化氮等自由基的過量產生可能會造成新生兒，尤其是早產兒的各種疾病，包括新生兒慢性肺疾，早產兒視網膜病變，壞死性腸炎，溶血性黃疸等。在懷孕週數低於 28 週的早產兒，視網膜病變發生率甚至可高達 80%，其中的百分之二十會有進行性的變化，嚴重者甚至造成視網膜剝離或失明。針對視網膜病變的致病機轉，近年來的研究傾向於和早產兒體內的活性氧及抗氧狀態有相關性，但是仍缺乏一致性的結論。無法下定論的其中原因，也許是新生兒單單早產本身就會出現視網膜病變，另一方面也有可能和研究方法有關，大部分檢查以測試蛋白質或脂類的氧化產物作為間接證據，僅有少數研究直接測試 glutathione 的氧化還原狀態來評估與視網膜病變之相關性，此外，檢驗項目的特異性值得商榷。台大醫院醫學研究部近年來發展出新的化學發光測定法，僅需採用微量體液即可分析血中的活性氧化自由基，而且不易造成早產兒的醫源性貧血。針對住在加護病房的早產兒，在接受氧氣的治療後，可能引起氧化自由基的上昇，造成進一步早產兒的各種病變，值得作相關的研究。我們希望採用這種化學發光測定法研究活性氧自由基與早產兒視網膜病變之嚴重度的相關性。

第一年我們已針對足月兒與早產兒各收集?例及?例，分別抽取臍帶血及出生後第三天例行篩檢時的微量血液作檢驗，比較兩組間之差異；我們發現出生三天後血中的活性氧自由基比起臍帶血明顯的上昇，確認出生後新生兒對於新環境中相對高氧濃度的反應。此外針對懷孕週數未滿 30 週或出生體重低於 2000 公克的早產兒共?例，經由收集 5 c.c.臍帶血及出生後第 1, 7, 14, 21, 28 天分別留取淚液來測定淚液中的自由基；並且由視網膜專家定期追蹤與評估早產兒視網膜病變的發生率與嚴重度，探討氧化自由基及抗氧化狀態與早產兒視網膜病變之嚴重度的相關性。

（二）計畫英文摘要

關鍵詞（Key words）: prematurity, neonate, reactive oxygen species, reactive nitrogen species, free radical, antioxidant status, retinopathy of prematurity, chemiluminescence

Neonates undergo a dramatic change during the process of childbirth by an increase in oxidative aggression. The fetus exchange a low oxygen intrauterine environment, with PO_2 of 20-25 mmHg and a low presence of free radicals, for another with a relative high oxygen extrauterine environment, with PO_2 of 100 mmHg. This change results in considerable oxidative stress.

Damage due to free radicals, including reactive oxygen species and reactive nitrogen species, is thought to be one of the common mechanisms for several neonatal diseases especially in preterm infants. Chronic lung disease of neonate, retinopathy of prematurity, necrotizing enterocolitis, and hemolytic diseases of neonate are accepted as caused by the excessive production of oxygen or nitrogen free radicals. The oxidative aggression suffered by the neonate is counteracted by the maturation of complex antioxidant defense systems, including both enzymatic systems (superoxide dismutase, catalase, glutathione peroxidase, etc.), and non-enzymatic systems (vitamins E, A, C). Despite the knowledge that has been advanced in recent years regarding the oxidative stress and antioxidant mechanism, there have remained questions about the maternal-fetal transfer of antioxidant defense mechanism and the free radical and antioxidant status in neonates during perinatal period.

Retinopathy of prematurity (ROP) is a disease of the incompletely vascularized immature reteina characterized by retinovitreal neovascularization. It develops in more than 80% of premature survivors born at <28 weeks gestation. Some evidences, by estimating the associations between protein oxidation and lipid peroxidation products and retinopathy of prematurity, indirectly suggest that reactive oxygen species and reactive nitrogen species may play important roles in the pathogenesis of ROP. However, direct evidence that oxidation plays a causative role in ROP is limited. It is not always clear whether the association between oxygen and ROP just reflect a higher disease incidence in the very-low- birth-weight infants who receive more oxygen. Besides, the assay procedures of protein oxidation and lipid peroxidation products may be different and nonspecific, newer and more sensitive tests is warranted.

We have developed to use a characteristic emission spectrum analysis of the chemiluminescence (CL-spectrum) with small amount of body fluids for the first time to evaluate the specific reactive oxygen species (ROS) activity including H_2O_2 and HOCl in the plasma and to adapt a chemiluminescence-high performance liquid chromatography (CL-HPLC) for measurement of PCOOH before and after the dialysis session, in the absence and presence of antioxidant treatment.

We hypothesize that the contents of free radicals and the antioxidant defense system are different between the preterm infants with ROP and those without ROP. We propose a two-year research project. In the first year of the project, 20 preterm and 20 term infants will be enrolled. We will detect the oxygen free radicals of cord blood and blood/ urine samples collected 3 days

after birth from these neonates. In addition, we will prospectively enroll 20 preterm infants with gestational age less than 30 weeks or birth weight less than 2000 grams. The clinical data including birth weight, sex, gestation age, days on oxygen and/or ventilator will be collected. The ROP check-up will be performed by an ophthalmologist regularly after postnatal age of 4 weeks. We will also detect the oxygen free radicals of the tears samples collected on Days 7, 14, 21, 28 after birth in these preterm infants. In the second year of the project, further cases of preterm infants will be enrolled for study.

報告內容

(一)、前言:

Oxidative Stress in the Neonate

Neonates undergo a dramatic change during the process of childbirth by an increase in oxidative aggression. The fetus exchange a low oxygen intrauterine environment, with PO_2 of 20-25 mmHg and a low presence of free radicals, for another with a relative high oxygen extrauterine environment, with PO_2 of 100mmHg. This change results in considerable oxidative stress in the neonates[1,2].

Oxidative Radical Disease in Neonatology

Damage due to free radicals, including reactive oxygen species and reactive nitrogen species, is thought to be one of the common mechanisms for several neonatal diseases especially in preterm infants. Chronic lung disease of neonate, retinopathy of prematurity, necrotizing enterocolitis, and hemolytic diseases of neonate are accepted as caused by the excessive production of oxygen or nitrogen free radicals[3-5].

Reactive Oxygen Species and Reactive Nitrogen Species in the Developing Ocular Vasculature

Retinopathy of prematurity (ROP) is a disease of the incompletely vasularized immature retina characterized by retinovitreal neovasularization[6].It develops in more than 80% of premature survivors born at < 28 weeks gestation[7]. Several evidences indirectly suggest that free radicals including reactive oxygen species and reactive nitrogen species have associations with the pathogenesis of retinopathy of prematurity. However, direct evidence that oxidation plays a causative role in ROP is limited[10]. Evidence for free radical injury comes mainly from measurements of biochemical markers of protein oxidation and lipid peroxidation. Besides, the associations of oxidant markers with ROP were not consistent [7-10]. The inconsistency of these associations may just reflect a higher disease incidence in the lowest birth weight infants who receive more oxygen, and uncertainty of assay specificity is an another concern[10]. Protein carbonyls, lipid peroxidation products such as malondialdehyde, and glutathione status has usually been used for evaluating the associations[8-10]. However, the concentrations of oxygen free radicals including hydrogen peroxide, superoxide, nitric oxide products have not been measured for evaluation of the associations with ROP. There is a great similarity between the retina and red blood cells in human beings concerning the membrane structure, the metabolism, and the antioxidant mechanism[11-12]. The direct measurement of free radicals and antioxidantstatus in preterm infants for evaluating the association with ROP is warranted.

(二)研究目的：

Using our newly developed technique of chemiluminescence spectrum, we can measure the oxygen free radicals with small amount of body fluids in preterm infants. With coordination among Departments of Pediatrics, Ophthalmology, and Medical Research in National Taiwan University Hospital and National Taiwan University College of Medicine, we will perform a collaborative study to explore the possible relationships between the reactive oxygen species, the reactive nitrogen species, the antioxidant status, and the pathogenesis and the severity of ROP.

(三)研究方法、進行步驟及執行進度。

We propose a two-year research project. In the first year of the project, w expect that 20 preterm and 20 term infants will be enrolled. We will detect the oxygen free radicals, nitric oxide products, and antioxidant status of cord blood and blood/ urine samples collected 3 days after birth from these neonates. In addition, we will prospectively enroll preterm infants with gestational age less than 30 weeks or birth weight less than 2000 grams. The clinical data including birth weight, sex, gestation age, days on oxygen and/or ventilator will be collected. The ROP check-up will be performed by an ophthalmologist regularly after postnatal age of 4 weeks. We will also detect the oxygen free radicals, nitric oxide products, and antioxidant status of the blood samples and/or urine samples collected from cord blood and on Days 1, 7, 14, 21, 28 after birth in these preterm infants. In the second year of the project, further case collection of preterm infants will be enrolled for study.

Measurement of oxidized parameters

In the presence of H_2O_2, the enzyme MPO can generate tyrosyl radicals by abstraction of $H^{\bullet}$ from the –OH group on tyrosine, and the tyrosyl radicals may participate in the oxidation of lipid and proteins. When a tyrosyl radical is generated in biological systems, it often cross-links to give a fluorescent adduct, dityrosine, which can be determined with a fluorometer (Hitachi F-2500, Tokyo, Japan), as described previously[11]. The sample (5 μl of serum in 1 ml of distilled water) will be measured at an excitation wavelength of 315 nm and an emission wavelength of 410 nm.

The hydroxyl radical, an important product of ROS, is known to play a role in the biosynthesis of MG, which contributes to toxicity in uremic patients. The MG with fluorescent activity, as an indirect measure of hydroxyl radical activity, will be determined as described previously[12]. The sample will be assayed at an excitation wavelength of 395 nm and an emission wavelength of 500 nm. The concentrations of dityrosine or MG will be displayed as fluorescence intensity/mL (FI/mL).

(四) 結果與討論:

The study was approved by the institutional review board of National Taiwan University hospital, Taipei, Taiwan. It was performed between January and December 2003 in the Neonatology Unit of this institution. Written informed parental consent was obtained before enrollment in the study. In our study, 15 preterm and 15 term infants would be enrolled. The gender, gestational age, Apgar score, and modes of delivery were also collected for analysis.

Cord blood and blood samples at age of three days were collected for the detection of Luminol-dependent Reactive Oxygen Species (mainly H2O2) and Lucigen-dependent Reactive Oxygen Species (mainly superoxide). (Five milliliters of cord blood and one milliliter of neonatal blood will be placed in a heparin-rinsed tube on ice. Plasma will be separated from blood cells by centrifugation at 3,000 ×g for 5 min at 4°C.) The plasma were immediately stored at -70°C and analyzed within 2 weeks. Chemiluminescence (CL) signals emitted from the "test mixture" of plasma, H2O2 (or HOCl), and CL-emitting substance [i.e.,luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and lucigen, Sigma, St. Louis, MO, USA] will be measured with a multi-wavelength chemiluminescence spectrum analyzer (CLA-SP2, Tohoku Electronic Ind. Co., Sendai, Japan). All values will be expressed as mean±SEM, and $p < 0.05$ will be considered to indicate statistical significance. The values will be analyzed by t-test and ANOVA for repeated measures where appropriate.

Table 1. Comparison of plasma reactive oxygen species (ROS) levels between preterm and term infants.

	Preterm infants (N = 3)	Term infants (N = 14)	P value
Cord Luminol-dependent ROS	847 ± 892	1277 ± 1302	0.598
Day 3 Luminol-dependent ROS	10685 ± 14726	20101 ± 26667	0.571
Cord Lucigen-dependent ROS	1493 ± 524	716 ± 953	0.198
Day 3 Lucigen-dependent ROS	6434 ± 5095	13007 ± 18588	0.303

Table 2. Comparison of plasma reactive oxygen species (ROS) levels between infants born of normal spontaneous delivery and cesarean section.

	NSD (N = 11)	C-Section (N = 6)	P value
Cord Luminol-dependent ROS	1483 ± 1383	685 ± 707	0.21
Day 3 Luminol-dependent ROS	23798 ± 296323	9232 ± 9896	0.268
Cord Lucigen-dependent ROS	886 ± 1014	793 ± 838	0.85
Day 3 Lucigen-dependent ROS	14287 ± 21423	7799 ± 5161	0.405

Table 3. Comparison of plasma reactive oxygen species (ROS) levels between male and female infants.

	Male (N = 8)	Female (N = 9)	P value
Cord Luminol-dependent ROS	1669 ± 1431	786 ± 899	0.143
Day 3 Luminol-dependent ROS	19825 ± 32128	17177 ± 19243	0.84
Cord Lucigen-dependent ROS	1009 ± 1141	718 ± 739	0.532

Day 3 Lucigen-dependent ROS	12820 ± 22493	10940 ± 13294	0.841

Fig Dramatic changes of plasma ROS levels at age of three days compared to cord bloods in the neonates

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計劃成果自評

1.第一年的計劃目前進展順利，我們已依照計劃進度於第一年成功收集新生足月兒與早產兒各約近 20 例，並分析其臍帶血及出生後第三天的微量血液的活性氧自由基。我們證實臍帶血的活性氧自由基包括 Luminal－dependent ROS 及 Lucigen－dependent ROS 含量在初生時並不高，但是出生後第三天則明顯的上昇，顯示出生後的環境中比起在懷孕期的環境會受到相對的高氧分壓而呈現體內血液中活性氧自由基的驟變。

2.此外，有關於早產兒視網膜病變和氧自由基的相關性研究，我們直接以淚液收集片依出生後每週收集兩眼之淚水，分別測定 Luminal－dependent ROS 及 Lucigen－dependent ROS 的含量，目前已收集八例早產兒，但其中僅二例有第一度視網膜病變，一例已死亡，需在第二年的計劃繼續收集個案，以進一步分析。

3.整體而言，我們已依規劃中的進度順利執行本研究，期待在未來的一年四個月內，完成整個研究計劃的進度，並且發表完整的研究成果。