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中文摘要

（一）計畫中文摘要

關鍵詞：早產兒、視網膜病變、呼吸窘迫症候群，新生兒慢性肺疾，開放性動脈導管，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。

　　在兩年的研究期間，我們針對早產兒共收集 45 例，其中 4 例最後出現嚴重的視網膜病變，需接受開刀治療。出生體重分別為 2119 ± 997 公克及 1195 ± 598 公克 ($p = 0.042$)，懷孕週數分別為 33.71 ± 4.77 週及 28.50 ± 4.04 週 ($p = 0.041$)。針對這些早產兒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。但是兩組間之差異僅於出生後第三天的 Luminol-dependent ROS 有統計上的意義。此外針對這些早產兒分別於出生後第 7、14、21、28、35、42、49、56 天分別留左右眼收集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我們發現絕大部份兩組間之差異並不明顯僅在第八週發生視網膜病變的早產兒淚液中的活性氧自由基比未發生視網膜病變的早產兒具統計上有意義較高的現象，但是由於眼淚收集的不容易導致收集量少亦可能影響研究的結果。

英文摘要

（二）計畫英文摘要

關鍵詞（Key words）: prematurity, neonate, respiratory distress syndrome, patent ductus arteriosus, chronic lung disease 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.

During the first year of our study, 10 preterm and 14 term infants had been enrolled. We found that the plasma ROS level was significantly higher then those of the cord blood. However, the levels were not affected by the gestational age, the gender and the mode of delivery. The elevation was supposed due to the oxidative stress after the birth.

During the second year of our study, we continually collected the tears from the preterm infants. Totally 45 infants were enrolled including 41 infants without retinopathy of prematurity and 4 infants with retinopathy of prematurity. We found that the plasma ROS levels at the age of three says were also significantly elevated after birth when compared with those of the cord blood both in the preterm infants with ROP and those without ROP. This result was compatible with our first year study. Again, we believed that the neonates suffered from oxidative stress after birth.

In the analysis of ROS in tears of the preterm infants, we found that both of the Luminol-dependent ROS and Lucigen-dependent ROS were not significantly different at the 7, 14, 21, 28, 35, 42 and 49 days of age. The difference was significant at the eighth week of age. We speculated that the difference was associated with the development of ROP. However, that collection of the tears from the preterm infants was not satisfactory and the amount of the tears was usually limited. The limitation of the samples collection may substantially affect the results of our study. Further improved methods for the analysis of tears ROS is necessary for the interpretation of the pathophysiology of ROP.

<center>報告內容</center>

(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.

<center>1</center>

(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, we 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, 28,35,42,49,56 after birth in these preterm infants. In the second year of the project, further case collection of preterm infants will be enrolled for study.

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.

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 August 2003 and July 2005 in the Neonatology Unit of this institution. Written informed parental consent was obtained before enrollment in the study. During the first year of our study, 10 preterm and 14 term infants had been enrolled. We found that the plasma ROS level was significantly higher then those of the cord blood. However, the levels were not affected by the gestational age, the gender and the mode of delivery. The elevation was supposed due to the oxidative stress after the birth.

During the second year of our study, we continually collected the tears from the preterm infants. Totally 45 infants were enrolled including 41 infants without retinopathy of prematurity and 4 infants with retinopathy of prematurity. We found that the plasma ROS levels at the age of three says were also significantly elevated after birth when compared with those of the cord blood both in the preterm infants with ROP and those without ROP. This result was compatible with our first year study. Again, we believed that the neonates suffered from oxidative stress after birth.

In the analysis of ROS in tears of the preterm infants, we found that both of the Luminol-dependent ROS and Lucigen-dependent ROS were not significantly different at the 7, 14,21,28,35,42 and 49 days of age. The difference was significant at the eighth week of age. We speculated that the difference was associated with the development of ROP. However, that collection of the tears from the preterm infants was not satisfactory and the amount of the tears was usually limited. The limitation of the samples collection may substantially affect the results of our study. Further improved methods for the analysis of tears ROS is necessary for the interpretation of the pathophsiology of ROP.

第一年我們已針對足月兒與早產兒各收集 14 例及 10 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 **(Fig)**。但是依性別、生產方式、或懷孕週數分析並未造成活性氧自由基的差異。

　　在兩年的研究期間，我們針對早產兒共收集 45 例，其中 4 例最後出現嚴重的視網膜病變，需接受開刀治療。出生體重分別為 2119 ± 997 公克及 1195 ± 598 公克 (p = 0.042)，懷孕週數分別為 33.71 ± 4.77 週及 28.50 ± 4.04 週 (p = 0.041) 。針對這些早產兒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。但是兩組間之差異僅於出生後第三天的 Luminol-dependent ROS 有統計上的意義。此外針對這些早產兒分別於出生後第 7、14、21、28、35、42、49、56 天分別留左右眼收集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。我們發現絕大部份兩組間之差異並不明顯僅在第八週發生視網膜病變的早產兒淚液中的活性氧自由基比未發生視網膜病變的早產兒具統計上有意義較高的現象，但是由於眼淚收集的不容易導致收集量少亦可能影響研究的結果。

Table 1. Demographic data between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Gestational age	33.71 ± 4.77	28.50 ± 4.04	0.041
Birth body weight	2119 ± 997	1195 ± 598	0.042
Maternal age	32.8 ± 4.9	30 ± 1.2	0.275
1-minute Apgar score	7.2 ± 2.1	6 ± 1.4	0.293
5-minute Apgar score	8.2 ± 1.6	7.8 ± 1.9	0.581

Table 2. Comparison of plasma reactive oxygen species (ROS) levels at birth and on Day-3 between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Cord Luminol-dependent ROS	705 ± 1131	1157 ± 1006	0.589
Day 3 Luminol-dependent ROS	21185 ±42812	4189 ± 5413	0.025
Cord Lucigen-dependent ROS	427 ± 777	1766 ± 320	0.441
Day 3 Lucigen-dependent ROS	8718 ± 13482	1808 ± 2044	0.322

Table 3. Comparison of tear reactive oxygen species (ROS) levels at age of 1-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	24.5± 44.5	NA	NA
Right eye Luminol-dependent ROS	1.94± 5.5	NA	NA
Left eye Lucigen-dependent ROS	73.3± 136.7	140.7 ± 128.3	0.517
Left eye Lucigen-dependent ROS	0.1 ± 0.3	NA	NA

Table 4. Comparison of tear reactive oxygen species (ROS) levels at age of 2-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	70.2 ± 146.1	1.75 ± 3.0	0.056
Right eye Luminol-dependent ROS	76.8 ± 171.9	13.1 ± 22.7	0.151
Left eye Lucigen-dependent ROS	10.2 ± 19.4	87.0 ± 99.1	0.002
Left eye Lucigen-dependent ROS	77.4 ± 181.1	5.3 ± 7.5	0.112

Table 5. Comparison of tear reactive oxygen species (ROS) levels at age of 3-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	68.5 ± 226.2	1.97 ± 3.41	0.216
Right eye Luminol-dependent ROS	10.0 ± 36.22	0 ± 0	0.272
Left eye Lucigen-dependent ROS	32.6 ± 63.4	0 ± 0	0.043
Left eye Lucigen-dependent ROS	2.71 ± 8.40	3.47± 6.01	0.860

Table 6. Comparison of tear reactive oxygen species (ROS) levels at age of 4-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	20.0 ± 39.1	79.1 ± 135.5	0.125
Right eye Luminol-dependent ROS	0.19 ± 0.46	1.88 ± 3.75	0.096
Left eye Lucigen-dependent ROS	14.8 ± 18.7	6.9 ± 13.8	0.444
Left eye Lucigen-dependent ROS	2.13 ± 7.38	15.8 ± 31.5	0.111

Table 7. Comparison of tear reactive oxygen species (ROS) levels at age of 5-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	45.6 ± 86.2	4.67 ± 6.60	0.104
Right eye Luminol-dependent ROS	0.16 ± 0.41	0.0 ± 0.0	0.165
Left eye Lucigen-dependent ROS	9.46 ± 15.91	1.77 ± 2.50	0.131
Left eye Lucigen-dependent ROS	12.64 ± 45.74	0.0 ± 0.0	0.320

Table 8. Comparison of tear reactive oxygen species (ROS) levels at age of 6-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	37.42 ± 76.23	0.94 ± 1.326	0.523
Right eye Luminol-dependent ROS	1.40 ± 2.98	0.0 ± 0.0	0.663
Left eye Lucigen-dependent ROS	102.87 ± 284.9	42.58 ± 57.45	0.512
Left eye Lucigen-dependent ROS	3.52 ± 9.68	0.0 ± 0.0	0.627

Table 9. Comparison of tear reactive oxygen species (ROS) levels at age of 7-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	34.61 ± 63.31	27.04 ± 15.08	0.737
Right eye Luminol-dependent ROS	2.59 ± 7.88	4.63 ± 6.54	0.742
Left eye Lucigen-dependent ROS	23.06 ± 53.248	58.44 ± 74.629	0.425
Left eye Lucigen-dependent ROS	6.24 ± 12.359	10.54 ± 12.963	0.661

Table 10. Comparison of tear reactive oxygen species (ROS) levels at age of 8-weeek between Non-ROP and ROP preterm infants.

	Non-ROP(N = 41)	ROP (N=4)	P value
Right eye Luminol-dependent ROS	1.52 ± 2.781	58.51 ± 43.272	0.001
Right eye Luminol-dependent ROS	0.0 ± 0.0	5.83 ± 8.25	0.024
Left eye Lucigen-dependent ROS	12.56 ± 24.048	109.56 ± 42.221	0.01
Left eye Lucigen-dependent ROS	2.51 ± 4.94	0.0 ± 0.0	0.165

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計劃成果自評

1. 在兩年的研究期間進展順利，在第一年的計劃中，我們成功收集新生足月兒與早產兒各 14 例及 10 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. 有關於早產兒視網膜病變和氧自由基的相關性研究，我們首先繼續分析早產兒其臍帶血及出生後第三天的微量血液的活性氧自由基，發現臍帶血的活性氧自由基包括 Luminal－dependent ROS 及 Lucigen－dependent ROS 含量在出生後第三天比初生時明顯的上昇，再次證實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。

3. 持續第一年的計劃，在第二年我們繼續共收集四十五例早產兒，其中四例有嚴重的視網膜病變；我們以淚液收集片依出生後每週收集兩眼之淚水，共連續收集八週以上分別測定 Luminal－dependent ROS 及 Lucigen－dependent ROS 的含量，我們發現絕大部份兩組間之差異並不明顯僅在第八週發生視網膜病變的早產兒淚液中的活性氧自由基比未發生視網膜病變的早產兒具統計上有意義較高的現象。

4. 整體而言，我們已依規劃中的進度順利執行本研究，但是由於眼淚收集的不容易導致收集量少亦可能影響研究的結果，因此未來的研究需考慮眼淚收集的困難度或其它體液的收集來源。