行政院國家科學委員會專題研究計畫成果報告
計畫編號：NSC 87-2314-B-038-143
執行期限：87 年 8 月 1 日至 88 年 7 月 31 日
主持人：曾啟瑞
執行機構：台北醫學院醫學系婦產學科

對培養在子宮內膜異位症病患腹膜液內小白鼠胚胎的影響

Effects of superoxide dismutase, catalase and thioedoxin on mouse embryo cultured in peritoneal fluid of patients with endometriosis.

分別來自子宮內膜異位症的不孕症患者及不具子宮內膜異位症患者腹腔液中的抗氧化酵素活性於本實驗中測定，抗氧化酵素如 catalase 及 glutathione reductase 等酵素活性分別加以偵測。10 位正常的不孕症患者為第一組；17 位具有子宮內膜異位症第一、二期者為第二組；9 位具有子宮內膜異位症第三、四期者為第三組。經由光譜分析儀，於腹腔液中的 catalase 活性藉由 H_2O_2 的衰解來偵測。glutathione reductase 活性則由 NADPH 減少的量來測得。Catalase 在腹腔液中的活性於三組中分別為 7.58 ± 0.55；8.49 ± 0.57 及 12.61 ± 1.85 (units/mg protein)。glutathione reductase 於腹腔液中活性在三組中分別為 0.948 ± 0.048；0.883 ± 0.054 及 1.338 ± 0.153 (units/mg protein x 10^{-3})。鐵離子濃度於三組中分別為 0.41, 0.36 及 1.10 (ppm)。鐵離子濃度與 Catalase 活性之比值會影響 hydroxyl radical 的產生並與子宮內膜異位症的嚴重程度有統計上的相關(0.091 of group 3 vs 0.042 of group 2)。我們的實驗數據顯示 catalase 及 glutathione reductase 活性於子宮內膜異位症患者腹腔液中上升並隨著子宮內膜異位症嚴重度而增加。子宮內膜異位症中的氧化壓力隨著鐵離子濃度的增加而上升。抗氧化酵素活性增加可能由於代償性的增加以清除自由基，同時也說明 reactive oxygen species 與子宮內膜異位症病理成因間的關係。

The activities of the antioxidant enzymes, catalase (CAT) and glutathione reductase (GRx) in the peritoneal fluid (PF) from infertile patients with or without endometriosis were studied. There were 10 normal infertile cases in group 1 (control), 17 cases with stage I-II endometriosis in group 2, and 9 cases with stage III-IV endometriosis in group 3. By spectrophotometry, the activity of CAT by measuring the breakdown of H_2O_2 in the PF was determined. The activity of GRx was measured by decreased amount of NADPH (Bergmeyer's method). The activity of CAT (units/mg protein) in the PF (mean ± SE) was 7.58 ± 0.55; 8.49 ± 0.57; 12.61 ± 1.85 ($P < 0.05$) in group 1,2 and 3 respectively. The activity of GRx (units/mg protein x 10^{-3}) in the PF was 0.948 ± 0.048; 0.883 ± 0.054; 1.338 ± 0.153 ($P < 0.05$) in group 1,2 and 3 respectively. Iron (Fe) concentration (ppm) was 0.41, 0.36 and 1.10 ($P < 0.05$) in group 1,2 and 3, respectively. The ratio of Fe/CAT reflecting the generation activity of hydroxyl radical and therefore oxidative stress was relevant to severity of endometriosis (0.091 of group 3, vs 0.042 of group 2) ($p < 0.05$). We conclude the activities of both CAT and

GRx increase in PF of endometriosis and the increase is stage dependent. The increased oxidative stress in endometriosis is related to iron concentrations by the generation of hydroxyl radicals. The increase of antioxidant enzyme which may attenuate and scavenge the excessive oxygen-derived free radicals is elucidated part of the pathogenesis between endometriosis and reactive oxygen species.

Introduction:

Antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase are the major antioxidant scavenging enzymes in the body. The catalase activity of a mammalian varies greatly, it is highest in liver and kidney and lowest in connective tissues. In these cells it is mainly particle-bound (in mitochondria and peroxisomes), whereas in erythrocytes it exists in a soluble state. Glutathione reductase is found in the soluble fraction of cells. The primary role of the enzyme is apparently the maintenance of the intracellular-reduced glutathione (GSH) concentration, this being available for the direct reduction of oxidized protein thiol groups (e.g., in enzymes and hemoglobin). Some authors have suggested that the antioxidants may protect against adhesion formation in endometriosis (Portz et al., 1991), while others have demonstrated that total antioxidant status in the peritoneal fluids from women with endometriosis do not increase (Ho et al., 1997).

At first, Zeller et al. (1987) showed that in the peritoneal fluid of endometriosis, there is increased chemiluminescence from macrophages which they attributed to the increased generation of ROS such as superoxide anions ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and singlet oxygen ($\bullet O_2$). On the other hand, Portz et al. (1991) used antioxidant enzymes such as SOD and catalase to block oxygen free radical toxicity to prevent adhesion formation in an endometriosis model. Ishikawa et al (1993) found that Cu/Zn SOD is decreased in the peritoneal fluid of patients with endometriosis. Arumugam (1994) also found a significant increase of iron concentration in the peritoneal fluid from patients with moderate to severe endometriosis. All these studies support that haemoproteins and iron are abundant in the pelvic peritoneal cavity of endometriosis. Because endometriosis may have local inflammatory reaction during the inflammatory reaction of endometriosis,and hence ROS released from macrophages are increased. Iron, promoters of the Fenton reaction in presence of hydrogen peroxide (H_2O_2 + $Fe^{2+} \rightarrow \bullet OH + -OH + Fe^{3+}$), can be released from the breakdown of hemoglobin (Gutteridge, 1986). Finally the most toxic hydroxyl radical (•OH) can accelerate lipid peroxidation (Arumugam, 1994). But Arumugam reported that the level of Malondialdehyde, a stable breakdown product of lipid hydroperoxides in the peritoneal fluid, was not correlated with the severity of endometriosis (Arumugam et al.,1995b). There must be some mechanism that can remove free radicals to prevent the Fenton reaction and lipid peroxidation. Antioxidant enzymes such as catalase and glutathion reductase from tissue may play an important role in scavenging the excess oxygen-derived free radicals and prevent tissue toxicity and cell damage in patients with endometriosis.

Our study evaluated the activities of the antioxidant enzymes, catalase and glutathione reductase, in the peritoneal fluid from infertile patients with or without endometriosis.

Results:

The activities of catalase and glutathione reductase in the peritoneal fluid were analyzed in 10 patients with

normal pelvis, 17 patients with minimal to mild endometriosis, and 9 patients with moderate to severe endometriosis. Mean values (±SE) for the activity of catalase in the PF for the three groups are shown in table I. There was a significant difference between normal patients and those with moderate to severe endometriosis.

Mean values (±SE) for the activity of glutathione reductase in the PF for the three groups are shown in table I. There was a significant difference between normal patients and those with moderate to severe endometriosis, and the increase of activity of glutathione reductase was stage dependent.

The activity of catalase of the moderate to severe endometriosis with well-encapsulized leions only shows no significant difference in comparison with the normal group (Table II). On the contrary, the activity is significantly higher in the group with red lesions.

The increase of iron content (Table III) is stage dependent, but only stage III-IV has significant increase. The ratio of Fe/CAT reflecting the generation activity of hydroxyl radical and therefore oxidative stress was relevant to severity of endometriosis (0.091 of group 3, vs 0.042 of group 2) ($p < 0.05$).

Discussion:

Repeated inflammatory reactions in endometriosis will cause macrophages to release large amounts of oxygen-derived free radicals into the peritoneal fluid. Then through the reaction of superoxide dismutase or other oxidative-reductive reactions, these oxygen-derived free radicals will become hydrogen peroxide (H_2O_2). The main clearance pathways of H_2O_2 in the body are: (1) catalase by reduction of H_2O_2 to H_2O; and (2) the glutathione oxidative-reductive cycle. Through these pathways, less H_2O_2 will become hydroxyl radicals (·OH) through the Fenton reaction in the presence of free iron derived from the breakdown of red blood cells (Fig.1).

The increased activities of catalase and glutathione reductase in the patients with moderate to severe endometriosis may represent increased oxidative stress, and hence antioxidant enzymes being induced or activated to scavenging excessive ROS. It has been confirmed that elevated cytokine (Keenan et al., 1995; Overton et al., 1996; Skrzypczak et al., 1995; Oosterlynck et al., 1994) and ROS levels (Zeller et al., 1987) in the peritoneal fluid indicate that the oxidative stress of moderate to severe endometriosis increased. During the inflammatory reaction of endometriosis, macrophages induced by endometriosis may release ROS, iron promoters of the Fenton reaction following breakdown of hemoglobin also were elevated (Gutteridge et al., 1986). Therefore, increasing free iron concentration in the peritoneal fluid may induce the activity of both catalase and glutathione reductase enzymes in order to scavenge excessive hydrogen peroxide (H_2O_2) to avoid hydroxyl radical formation and further lipid peroxidation. Elevated iron concentrations of the peritoneal fluid from patients with moderate to severe endometriosis have been demonstrated in a previous study (Arumugam, 1994).

This study showed that the activity of catalase in the peritoneal fluid from infertile patients with moderate to severe endometriosis increased to 1.7 fold compared with the control group. That was especially true for an advanced disease (stages III-IV) combined with fresh activity (red lesions) in cases with moderate to severe endometriosis but not in the patients with well-encapsulized lesions (black lesions) (Table 2). A red lesion indicates neovasculization, inflammation, and a tissue reaction that may attract macrophage accumulation and by which abundant ROS are released, as well as IL-1, IL-6 (Punnonen et al., 1996; Tseng et al., 1996) and vascular endothelial growth factor (VEGF)

(McLaren et al., 1996a & b; Donnez et al., 1998). Previous studies proved that the content of VEGF, an important regulator of normal angiogenesis and pathological neovascularization, was higher in the stromal cells of red lesions than black lesions during all phases of the menstrual cycle (Donnez et al., 1998). The increase of ROS is a plausible explanation for the increased activities of catalase in PF of stage III-IV endometriosis. Under these conditions, the activity of catalase may be higher than that of the well-encapsulized lesions (black lesions) of the same stage. This means that, according to the revised American Fertility Society Classification, even though patients can be classified to the same stage, biochemical activities of the advanced stage can be very different (Vernon et al., 1986). Whether the prognosis after treatment for infertility may be affected by the activity of catalase remains to be evaluated. Another possible reason for the higher activity of catalase in red lesions is that catalase can be released from red blood cells after their breakdown in the peritoneal fluid. In endometriosis, there is cyclical hemorrhage within the pelvic cavity (Sampson et al., 1927). As a result, hemoproteins and iron are released into the pelvic peritoneal fluid, at the same time the intracellular catalase and glutathione reductase are released. Furthermore, we also found the activity of glutathione reductase to show a 1.4-fold increase in the advanced disease, and there was a stage-dependent increase in activity reflecting the association of severity of disease and levels of antioxidant enzyme activity. We conclude that endometriosis may increase catalase and glutathione reductase activities by scavenging the excessive oxygen-derived free radicals.

References:

Arumugam, K. (1994) *Hum. Reprod.*, **9,** 1153-1157.

Arumugam, K. and Yip, Y.C. (1995a) *Fertil. Steril.*, **64**, 62-64.

Beers, R.F. and Sizer, I.W. (1952) *J. Biol. Chem.*, **195**, 133-140.

Bergmeyer, H.U., (1983) *Methods of Enzymatic Analysis.* Volume III, Verlag Chemie, Florida, 258-265pp.

Cross, E.C. (1987) *Ann Int. Med.*, **107**, 526-545.

Donnez, J., Smoes, P., Gillerot, S., Casanas-Roux, F. and Nisolle, M. (1998) *Hum. Reprod.*, **13**, 1686-1690.

Gutteridge, J.M.C. (1986) *FEBS Lett*, **201**, 291- 295.

Ho, H.N., Wu, M.Y., Chen, S.U., Chao, K.H., Chen, C.D. and Yang, Y.S. (1997) *Hum. Reprod.*, **12**, 2810-2815.

Ishikawa, M., Nakata, T., Yaginuma, Y., Nishiwaki, K., Goishi, K. and Saitoh, S. (1993) *Am. J. Obstet. Gynecol.*, **169**, 730-734.

McLaren, J., Prentice, A., Charnock-Jones, D.S., Millican, S.A., Muller, K.H., Sharkey, A.M. and Smith, S.K. (1996a) *J. Clin. Invest.*, **98**, 482-489.

Oosterlynck, D.J., Meuleman, C. and Waer, M. (1994) *Obstet. Gynecol.*, **83**, 287-292.

Portz, D.M., Elkins, T.E, White, R., Warren, J., Adadevoh, S. and Randolph, J. (1991) *Int. J. Fertil.*, **36**, 39-42.

Punnonen, J., Teisala, K., Ranta, H., Bennett, B. and Punnonen, R. (1996) *Am. J. of Obstet. Gynecol.*, **174**, 1522-1526.

Sampson, J.A. (1927) *Am. J. Obstet. Gynecol.*, **14**, 422-429.

Skrzypczak, J., Jedrzejczak, P. and Kasprzak, M. (1995) *Ann. N.Y. Acad. Sci.*, **762**, 496- 498.

The American Fertility Society. (1985) Revised American Fertility Society classification of endometriosis. *Fertil. Steril.*, **43**, 351-352.

Tseng, J.F., Ryan, I.P., Milam, T.D., Murai, J.T., Schriock, E.D., Landers, D.V. and Taylor, R.N. (1996) *J. Clin. Endo. Metab.*, **81**, 1118-1122.

Vernon, M.W., Beard, J.S., Graves, K. and Wilson, E.A. (1986) *Fertil. Steril.*, **46**,

801-806.

Table I. Activities of catalase and glutathione reductase in peritoneal fluid

Antioxidant enzyme	Control N = 10	Endometriosis stage I-II N = 17	Endometriosis stage III-IV N = 9
Catalase (unit/mg protein)	7.58 ±0.55	8.49 ±0.57	12.61 ±1.85 [a]
Glutathione reductase (x 10^{-3} unit/mg protein)	0.948 ±0.048	0.883 ±0.054[c]	1.338 ±0.153[b]

Values are means ±SE.

[a] Unpaired t-test: endometriosis stage III-IV against normal, P = 0.028.

[b] Unpaired t-test: endometriosis stage III-IV against normal, P = 0.036.

[c] Unpaired t-test: endometriosis stage I-II against stage III-IV, P = 0.019.

Table II. Activity of catalase in peritoneal fluid from normal and endometriosis stage III-IV

Antioxidant enzyme	Control N = 10	stage III-IV with red lesions N = 6	stage III-IV with well-encapsulized lesions only N = 3
Catalase (unit/mg protein)	7.58 ± 0.55[a]	14.17 ± 2.58[a]	9.49 ± 0.83

Values are means ± SE.

[a] $P < 0.05$

Table III. The concentrations of Selenium and Iron in peritoneal fluid of patients with endometriosis

	Control	Endometriosis stageI-II	Endometriosis stageIII-IV
Selenium (ppb)	19.62	22.53	25.13
Iron (ppm)	0.41*	0.36	1.10*
Fe/CAT	0.051 ± 0.03	0.042 ± 0.019*	0.091 ± 0.054*

* $P < 0.05$