行政院國家科學委員會補助專題研究計畫成果報告

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※全靜脈營養引起之黏膜免疫功能不良的機制－　　※
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※ 腸道相關淋巴組織細胞凋亡的角色　　　　　　　※
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※Mechanisms of mucosal immune dysfunction following ※
※total parenteral nutrition - the role of apoptosis of ※
※gut-associated lymphoid tissues ※
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計畫主持人：許 文 明

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一、中文摘要

全靜脈營養(Total parenteral nutrition, TPN)對於有腸胃道疾病而無法攝取足夠營養的病患來說是一項非常重要的治療方式。然而已有許多研究顯示使用全靜脈營養會造成黏膜免疫力受損，以致造成腹膜炎、肺炎及敗血症。全靜脈營養造成黏膜免疫力受損的機制並不清楚，目前僅知使用全靜脈營養會造成分泌性免疫球蛋白 A 及腸道相關淋巴組織內的淋巴細胞數量減少。細胞凋亡和細胞增生在生物體內相互平衡以維持正常的組織型態及細胞數量，如果細胞凋亡增加將會造成細胞數量減少。因此我們假設使用全靜脈營養後腸道相關淋巴組織內的淋巴細胞可能發生細胞凋亡以致數量減少。本研究將 20 隻大白鼠隨機分為 10 隻控制組及 10 隻 TPN 組，在使用 TPN 三天之後將老鼠犧牲，取出全部小腸並萃取腸黏膜及淋巴結內之淋巴細胞，再以流式細胞儀分析 T 細胞及 B 細胞之數量，以及其發生凋亡之比例。實驗結果顯示在使用 TPN 三天之後，腸黏膜及淋巴結內之淋巴細胞總數均大量減少($p<0.001$)，而其中 T 細胞的比例下降 B 細胞則上升($p<0.05$)，而且不論 T 細胞或 B 細胞其發生凋亡的比例均增加($p<0.05$)。TPN 之使用確實會使得腸道內的淋巴細胞減少，而且細胞凋亡的比例也增加，顯示細胞凋亡在 TPN 引起的免疫不全應扮有相當角色。而 TPN 如何引起淋巴細胞凋亡，則需進一步探討。

關鍵詞：全靜脈營養，腸到相關淋巴組織，細胞凋亡

Abstract:

Total parenteral nutrition (TPN) is an effective alternative to gastrointestinal feeding in patients with gastrointestinal disorders. However, several studies have shown impaired mucosal immunity (increased bacterial translocation, and decreased secretory IgA and lymphocytes in gut-associated lymphoid tissue [GALT]) after TPN. The mechanisms of TPN induced mucosal immunity impairment are not clear. Apoptosis is a controlled type of cell death that counter-balances cellular proliferation to maintain normal tissue morphology and cellular population. Increased apoptosis may result in morphologic atrophy and decreased cellular numbers. We then hypothesize that impaired mucosal immunity is caused by increased apoptosis of lymphocytes in GALT. To testify this hypothesis, Twenty rats are randomly assigned to the CHOW group (n=10) and TPN treated group (n=10). After 3 days of TPN, the rats in two groups are sacrificed and the whole intestine is removed. Then the total viable cell yield and extent of apoptosis of GALT (including Peyer's patches, and lymphocytes in the intraepithelial spaces) of the whole intestine of both CHOW and TPN treated groups are determined by way of trypan blue exclusion and three-color flow cytometric analysis. The phenotypes of the lymphocyte populations are also examined. The results show that there is significant decrease of the total viable cell yield ($p<0.001$) associated with increased apoptosis of B and T cells ($p<0.05$) from the GALT in the TPN treated group. The percentage of B cells increases and T cells decreases after TPN, which correlates with greater extent of apoptosis of T cells. The results of this study confirms that TPN will induce atrophy of GALT and that there is increased apoptosis of lymphocytes in GALT after TPN, suggesting that apoptosis should play an important role in the TPN induced mucosal immunity dysfunction. The mechanism of TPN induced Lymphocyte apoptosis deserves further investigation.

Keywords: Total parenteral nutrition(TPN), Gut-associated lymphoid tissue(GALT), Apoptosis

Background and Purpose:

In pediatric surgery, it becomes necessary to maintain the nutritional condition of the patients by intravenous alimentation or even by complete parenteral nutrition. The majority of patients who require such intravenous nutrition have serious complications also in the digestive tract, such as short

bowel syndrome or pseudo-obstruction. In these patients, it is important to maintain the general condition solely by intensive intravenous nutrition for a prolonged period. However, several animal and human studies have shown the adverse effects of prolonged use of total parenteral nutrition (TPN) on the intestinal mucosal structure and functions [1-12].

Although the body weight and nitrogen balance can be maintained by TPN under fasting, the intestinal mucosal weight, height, protein and DNA all decrease significantly without enteral feeding, that is inappropriate with the changes of other organs [1-6]; suggesting that enteral feeding has a special role on the intestine. The intestinal mucosal enzymes and mucous gel decrease and mucosal permeability increases after TPN [1,4,5,7,9,10,12], representing the mucosal functional deterioration. Some studies correlate the increased permeability to increased bacterial translocation after TPN [9,12], however, this finding is not consistent [6]. In a rat model, the most striking alterations in intestinal mucosa may occur only after 3 days of TPN [4]. Within 3 days of starting TPN, mean jejunal mucosal thickness decreased by 16% and after 15 days it had fallen by 28% [4]. In human studies, there seems to be similar intestinal mucosal changes as in animal models, however, the findings occur in humans are substantially less significant than observed in animal models [8,11], and much longer periods are required to realize the changes [7].

One major complication after TPN is impaired mucosal immunity. Several major septic complications including generalized sepsis, pneumonia, catheter- associated infections have been noted with the use of TPN [13,14]. It is strongly suggested that the source of the sepsis is the gastrointestinal tract [15]. The mechanisms of impaired mucosal immunity are not clear. Secretory IgA (S-IgA) is an important component of the mucosal defense system, which can be found in tears, saliva, breast milk, bile, and intestinal mucus. The S-IgA has been shown to decrease in bile and intestinal and respiratory tract after TPN [16,17], suggesting that gut-associated lymphoid tissue (GALT) plays an important role in the pathogenesis of TPN associated immune depression. The GALT, as an independent immune organ, not only provides indispensible immunologic protection against resident microbial flora and infectious pathogens, but also provides significant immunologic protection for distant mucosal sites, such as the nasopharynx, breast, salivary glands and lung [18]. Morphologically, the GALT consists of both affector and effector limbs [19]. The affector components consist of organized lymphoid follicles (i.e. Peyer's patches [PP], mesenteric lymph nodes, tonsils and the appendix) acting as antigen detectors and processors. Lamina propria (LP) and intraepithelial (IE) lymphocytes compose the nonaggregated lyphoid tissue acting as the effector cells that control and produce S-IgA. Several studies have demonstrated both B and T lymphocytes decrease significantly in GALT after TPN and also there is decreased ratio of helper T to suppressor T lymphocytes [17,20-23]. Why does the lymphocytes decrease after TPN is not known. We specculate that increased mucosal permeability might enhance translocation of enteric antigens, endotoxin, etc. from bowel lumen which may serve as a stimulant for macrophages and lymphocytes in the intestinal mucosa and submucosa. This in turn may lead to the release of mediators, such as the proinflammatory cytokines, i.e. TNF, IL-1, etc., which may act to suppress normal immune responses in intestinal mucosa [24]. Apoptosis is a deliberate and genetically controlled cellular response to specific developmental and environmental stimuli and is regulated by both survival (Bcl-2, etc) and death (TNF, Fas ligand, Fas, etc) factors [25]. We hypothesize that prolonged use of TPN may induce inappropriate apoptosis of lymphocytes in GALT, which in turn results in decrease of lymphocytes in GALT then impairs the mucosal immunity. The purpose of our study is to examine whether there is inappropriate apoptosis in GALT after TPN.

Materials and Methods:

Animals and study design

Twenty male Sprague-Dawley rats are randomly assigned into control group (n=10) and TPN group (n=10). All rats receive catheter implantation through internal jugular vein for sham operation or administration of TPN. The rats in the control group receive normal saline infusion and take food and water ad libitum, while rats in the TPN group receive intravenous nutrition only. The TPN solution contains a final concentration of 4.25% amino acids, and 28% glucose, in addition to electrolytes and vitamines. The rats will receive 307 Kcal/Kg/day of sterile TPN solution. The body weight of each rat will be recorded before the study and at the end of the study. Rats of the TPN group will be sacrificed after starting TPN for 3 days. The whole intestine of each rat will be harvested for further studies.

Cell isolations and total viable cell yields

Peyer's patch(PP). The PP are excised from the serosal side of the inestine and teased apart with 18-gauge needles. Fragments are treated with type I collagenase in Minimal Essential Medium for 60 min at 37℃ with constant rocking. After collagenase digestion, cell suspensions are passed through nylon filters [26].

IE cells. IE and LP lymphocytes are isolated as follows. First the small intestine is flushed with phosphate-buffered Solution (PBS) to remove intestinal contents. After excision of PP, the intestine is opened lengthwise and cut into 5 mm pieces. The pieces are incubated three times, 30 min each time, with prewarmed (37℃) calcium and magnesium-free HBSS containing 5 mM EDTA in a flask on a

magnetic stirrer at 20 rpm at 37℃. Supernatant containing released sloughed epethelial cells and IE lymphocytes from each incubation period arepooled and stored on ice for further purification later. To block remaining EDTA activity, the remaining tissue pieces are incubated for 30 min at 37℃ with RPMI 1640 containing 5% heat-inactivated fetal bovine serum (FBS). The RPMI/5% FBS is decanted and 30 ml RPMI containing 40 U/ml collagenase (type I: 30 U/ml; type III: 10 U/ml), and 5% inactivated FBS is added to the flask that is then incubated on a magnetic stirrer (100rpm). Released cells are decanted from the tissue fragments. Fresh enzyme-containing media is added, and the process is repeated twice, 30 min each tme, for a total 90 min. After the third extraction, pooled cells are gently mixed and placed on ice for 10-15 min to let larger debris sediment. Supernatants containing lymphocytes, debris, and dead cells are filtered through a glass wool column. Suspensions are centrifuged, the pellets resuspended in 40% Percoll, and the cell suspensions overlaid on 70 % Percoll. After centrifugation for 20 min at 600 g at 4℃, viable lymphocytes are recovered from the 40/70% interface and washed twice in RPMI 1640.

Total viable cell yields. Total viable cell yields are determined by trypan blue exclusion. Cell counts are adjusted to $5x10^5$ using PBS and 1 ml of this cell suspenion is placed into 1.5 ml microcentrifuge tubes for cell staining.

Cell staining and flow cytometry

For the detection of apoptosis and cell phenotype, cells are stained with monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or DNA binding agent Anexin-V. In a typical staining protocol, cells ($5 x10^5$) are centrifuged at 400g for 5 min, the pellets is resuspended in 100 μ l buffer and 10 μ g/ml Fc blocker and incubated on ice for 15 min. After addition of 0.1 μ g/ml of anti-mouse CD3-FITC or B cell-PE mAbs, the cells are left for another 45 min on ice, followed by wash with the same buffer. The cells are resuspended in 1 ml of 1% paraforaldehyde in PBS overnight at 4℃.The fixed cells are centrifuged, resuspended in 0.5 ml of Anexin-V staining solution and stored in the dark room at room temperature until flow cytometric analysis. Flow cytometric analysis is performed on FACSORT based on Telford et al [27]. Stained cells are excited at the 488-nm line with a 15 MW argon laser for FITC, PE, PI, and cytochrome, respectively. The detection of FITC fluorescence emission is at 525 nm, PE at 575 nm, Anexin-V at 620 nm, and cytochrome at 670 nm. FITC or PE single-positive as well as double-positive and double-negative Anexin-V cell cycle analysis is determined following gating of cell debris and doublet for no less than 5000 cells/sample. Histograms of the regionalized cells are then produced of cell number *vs* Anexin-V stain intensity from which the cell cycle can be determined.

Presentation of data and statistical analysis

The results are presented as means±SE for each group. Student's *t* test are used for comparison of means

Results:

***Body weight and Intestinal weight:*(Table 1)**

Table 1. Animal body weight, weight gain and intestinal Weight

	CHOW (n=10)	TPN (n=10)
Body Weight (g)	265.22±31.75	240.85±12.05
Weight Gain (g)	10.64±3.78	7.64±3.29
Intestine Weight (g/10cm)	0.82±0.07*	0.38±0.05*

Values are expressed as mean±SE

* $p< 0.001$

The pre-experiment weights of both groups were similar, and the weight gains of both groups were also similar. However, there was severe intestinal atrophy in the TPN group ($p<0.001$).

***Total cell yield and percentage of B and T cells from GALT:* (Table 2)**

Total cell yields from GALT, including PP and IE, decreased significantly after TPN ($p<0.001$). The percentage of B cells increased and T cells decreased after TPN ($p<0.05$)

Table 2. Total cell yield and percentage of B and T cells from GALT

	CHOW (n=10)	TPN (n=10)
PP (x106)	7.44±2.41	1.34±0.42*
B (%)	62.98±6.04	73.86±7.24#
T (%)	26.74±4.08	20.76±5.44#
IE (x106)	0.82±0.19	0.28±0.08*
B (%)	5.68±1.06	35.585.73*
T (%)	79.66±6.70	54.14±6.19*

Values are expressed as mean±SE

* $p<0.001$, # $p<0.05$, vs. CHOW

***Percentage of apoptosis of B cells and T cells from GALT:* (Table 3)**

The percentage of apoptosis of B and T cells, both increase after TPN. There was a greater extent of increased apoptosis of T cells than that of B cells.

Table 3. Percentage of apoptosis of B and T cells from GALT

		CHOW (n=10)	TPN (n=10)
PP	B (%)	3.71±0.92	5.34±0.73#
	T (%)	1.66±0.68	12.45±2.04*
IE	B (%)	4.11±1.06	5.76±1.26&
	T (%)	2.08±0.53	13.62±3.74*

Values are expressed as mean±SE

# $p<0.01$, * $p<0.001$, & $p<0.05$, vs. CHOW

Discussion:

It has been shown that TPN may induce GALT atrophy with decreased IgA production, which results in impaired mucosal immunity [17,20,22,28,29]. King et al [17] showed that B cells and T cells from GALT began to decrease after TPN for just one day and reached statistical significance and stabilized by day 3. However, the mechanisms of GALT atrophy still remain obscured.

Our studies confirmed that lymphocytes from GALT decreased significantly after TPN for 3 days and that there was significantly increased apoptosis of both B and T lymohocytes in GALT. Also there was a greater extent of increased apoptosis of T cells than that of B cells, which resulted in increased percentage of B cells and decreased percentage of T cells in GALT.

The cause of increased apoptosis of lymphocytes in GALT after TPN was not clear. Glutamine, which is routinely added in the TPN, serves a primary oxidative fuel for rapidly growing cells such as intestinal mucosal cells and lymphocytes [30,31]. Glutamine has been shown to restore the GALT and mucosal immunity after TPN [32,33]. It has also been shown that glutamine deprivation might induce apoptosis in lymphoma cell lines [34]. So lacking of glutamine or other elemental factors in TPN might be a possible cause of increased apoptosis of GALT cells after TPN.

It was also possible that the increased bacteria or endotoxin translocation due to impaired mucosa integrity after TPN [9,12] might be another cause of increased apoptosis of GALT cells after TPN. As the T cells were antigen processors, they might have greater challenge than the B cells. Our results confirmed that the T cells had a greater extent of apoptosis than the B cells had after TPN.

In conclusion, our results showed that apoptosis might play an important role in TPN induced GALT atrophy. The causes of TPN induced GALT cells apoptosis need further investigation. Apoptosis is a genetically controlled type of cell death, that responses to specific environmental and developmental factors and depends on both survival (Bcl-2, etc.) and death (TNF, Fas ligand, etc.), suggesting that apoptosis can be modified by genetical and environmental factors. In the future the TPN associated immunal dysfunction could be prevented by antiapoptotic treatment [35].

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