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嬰幼兒心室中隔缺損
血清中獨特的預後因子分析

Novel Serum Prognostic Factors for
Infants with Ventricular Septal Defect

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致謝

(Acknowledgement)

努力不一定有收穫,

但是要有收穫一定要努力。

謹以此書獻給

關心我及鼓勵我的

師長、朋友及小兒科同仁。

目　錄

嬰幼兒心室中隔缺損血清中獨特的預後因子分析

摘要

心室中隔缺損為最常見的先天性心臟病，發生率約為每千名活產嬰中 2 至 4 位，主要表徵為充血性心臟衰竭，呈現肺臟及全身鬱血，心肌收縮功能，心臟負擔，供血狀況及身體內各種調適機制運作下，不同程度失調的結果。

心臟超音波可作為診斷心臟疾病及畸形，預估心臟功能及心臟負擔情形的技術，而體內神經內分泌的調節機制在心臟衰竭過程中亦扮演著重要角色，足以影響心臟功能的預後。類胰島素生長因子-I 及 II(IGF-I，II)在胚胎的發育及心肌細胞的分化過程中扮有重要角色，但有關心肌生長因子對心室中隔缺損的影響研究訊息則甚少。目前雖然心臟超音波是診斷及評估心室中隔缺損及心臟功能的主要工具，但仍無有效方法可作為心室中隔缺損合併心臟衰竭預後的預測方法，我們的研究主要是提出一個特別的診斷標準：「心室中隔缺損合併輕度心臟衰竭者，血清中類胰島素因子－Ⅰ(IGF-I)及類胰島素因子結合蛋白 3 (IGF-BP3）呈輕度減少，而心室中隔缺損合併嚴重性心臟衰竭者，類胰島素因子－I，II (IGF-I ,IGF-II)及類胰島素因子– I 與類胰島素結合蛋白 3 (IGF1/IGFBP3)比率明顯過低且血中生長激素同時呈現代償性遽增」可作為預測心室中隔缺損合併心臟衰竭的預後，以早期規劃手術治療。

本研究中收錄嬰兒年齡從 3 個月到一歲，出生體重及妊娠週數，檢查時體重並無明顯差異下，分成四組為控制組,心室中隔缺損合併輕度心臟衰竭（Mild-VSD)、心室中隔缺損合併嚴重性心臟衰竭（Severe-VSD）及經外科手術後（post-surgery)。我們也利用 ELISA 方法，首次訂出台灣嬰幼兒血

中類胰島素因子－Ⅰ(IGF-I) 的標準值 112 ± 23 (微克/毫升)及類胰島素因子－II（IGF-II）的標準值(549 ± 45 微克/毫升)，相較於正常嬰幼兒，心室中隔缺損合併輕度心臟衰竭組(Mild-VSD) 血中類胰島素因子－Ⅰ(IGF-I)及攜帶蛋白(IGF-BP3)較低，分別下降約 43%及 32%。更特別的是，心室中隔缺損合併嚴重性心臟衰竭嬰幼兒中，不僅僅血中類胰島素因子－Ⅰ(IGF-I) 下降 79%，結合蛋白(IGF-BP3)下降 57%，IGF-I / IGF-BP3 比值下降 39%，且生長激素呈代償性遽增 3.1 倍，但類胰島素因子-II (IGF-II)則下降 24%，IGF-BP3 攜帶蛋白分解脢及 Pro-MMP- 9 下降 40%。在嚴重組中，所有的血清變化，有可能導因於心室中隔缺損導致血液溶積負荷過重而發生過度代謝，產生血中乳酸堆積增加。但是，一當手術後六個月，大部分的變化均可復原。結論：血清中類胰島素因子 I、II、結合蛋白(IGF-BP3)及生長激素的改變，可提供成為一特別的方法來訂出心臟衰竭的程度，而做為預後的指標及做為外科手術的依據，在釐清這些生長因子的作用機制後,將可成為新的治療方法。

Novel Serum Prognostic Factors for Infants with Ventrcicular Septal Defect

ABSTRACT:

Ventricular septal defect, the most common congenital heart disease with incidence around 2-4/1000 livebirth, represents the major manifestions of congestive heart failure of the admixture of components that may reflex pulmonary and systemic congestion, the contractile status of the myocardium, loading conditons, perfusion status, and the operation of various adaptive mechanisms. Cardiac ultrasonography provides the key data to define the specific disorder or malformation and the functional status of the myopericardium, particularly the nature of the loading conditon. The major neurohormonal mechanisms play a key role in the pathophysiologic process of congestive heart failure and influence the natural history of certain volume- and pressure-loading. Insulin-like growth factors (IGF-I,II) play a major role for embryonic development and cardiomyocytes differentiation. However, very little information is available regarding the cardiac growth factors involved in ventricular septal defect (VSD). Although cardiac echocardiography plays the major tool for diagnosis and evaluation of heart functions., but there are no efficient methods to predict the outcome of VSD with congestive heart failure of different severity and to determine the optional time for surgical treatment. Our study was to provide a novel diagnostic criteria, a slight reduction of IGF-I and IGFBP3 for VSD with mild symptoms of congestive heart failure and a high reduction of IGF-I, IGF-II, and IGF-I/IGFBP3 ratio resulting in a compensative elevation of growth hormone for VSD with severe heart failure, to predict the outcome of infants with VSD of variable severity of congestive heart failure. In present study, infants aged from 3 months to one year were divided into four groups as control, mild-VSD (Qp/Qs $\leqq$ 1.5), severe-VSD (Qp/Qs $\geqq$ 2.0, symptoms of intractable heart failure), and post surgery. For the first report, we set up the standard value of IGF-I (112±23ng/ml) and IGF-II (549±45ng/ml) of normal infants in Taiwan by ELISA. Compared to normal infants, IGF-I and its

carry protein IGFBP3 are lower in mild-VSD infants by 43% and 32%, respectively. Moreover, it was found that not only more significant reduction of IGF-I (79%), IGFBP3 (57%), and IGF-I/IGFBP3 ratio(39%), which resulted in the compensative elevation of growth hormone by 3.1-fold, but also IGF-II reduced 24% and the protease of IGFBP3, pro-MMP-9, decreased 40% were observed in severe-VSD infants. All these changes of severe-VSD group might result from the increase of serum lactate concentration leading to the reduction of pH due to hyperdynamics of flow overloading. Following surgery reversed most of the changes. In conclusion, these results supply a novel approach to define mild and severe VSD to prevent intactable heart failure and help to make the decision for surgery. Furthermore, the elucidation of the mechanism of the results could supply a new strategy for treatment of infants with VSD.

序論

心室中隔缺損為最常見的先天性心臟病，發生率約為每千名新生中佔二至四位，主要表徵為身體內多種因素混合運作的結果，包括肺部及全身充血情形、心臟肌肉收縮的功能、心臟負擔血液灌注組織及體內各種調適機制的運作，最後造成充血性心臟衰竭。然而，對於控制心臟間隔形成的分子層次資訊大都未知。類胰島素生長因子(IGFs)在哺乳動物的生長及細胞分化的過程中，包括調節胎兒心臟發育的功能，扮有重要角色。IGFs 是與胰島素相關的胜類家族成員之一，包括胰島素、類胰島素生長因子-Ⅰ，Ⅱ(IGF-Ⅰ，-Ⅱ)及放鬆素(relaxin)，均源自一共同基因。其中 IGFs 是在研究生長激素對出生後胎兒生長影響的調控中發現其是依賴生長激素生成。IGF 經由細胞表面的接受器 IGF-Ⅰreceptor 結合後，發揮如同內分泌腺的功能。另外，許多組織，包括心臟內的心肌均有能力表現 IGF 基因並且合成蛋白質。

IGF-Ⅰ的數量在胎兒血清、肝臟、腎臟及心臟內均低於 IGF-Ⅱ，而出生後則隨年齡逐年增加。IGF-Ⅰ能夠調節體內多種類型細胞的增生及分化，並能發揮如胰島素般的代謝功能，且存在血液循環中；與胰島素不同的是 IGF-Ⅰ由體內多種不同組織所產生，因此 IGF 可發揮內分泌、自體分泌及旁分泌的功能。而 IGF-Ⅰ在出生後生物體內扮演的角色已被充分瞭解，在許多研究中發現，從成年早期到老年均可於心臟內測得 IGF-Ⅰ的轉殖體。

IGF-II在胎兒生長的角色也已清楚知道，若阻止 IGF-II與其接受器結合將導致胎兒生長遲緩。在老鼠胎兒許多組織中，都有 IGF-II的表現，但出生後，除在腦及心臟外，則大幅減少，而 IGF-IImRNA 的減少也導致出生後血液循環中 IGF-II蛋白量的減少。

IGF 主要由六種攜帶蛋白以蛋白質分解作用的方式調節，而基質金屬蛋白分解酵素(Matrix-MetalloProteinase，MMP)-1，-3，-9 已被証實為其蛋白分解脢。此外，MMP 也與組織重建及致病過程有關。MMP-1 活性可使已與 IGF 結合的類胰島素生長因子結合蛋白(IGFBP)複合物彼此的親和性降低，即增加了 IGF 對其接受器的利用效率，促進平滑肌細胞的再生。當 IGFBP-3 與 IGF 結合時，則會使 IGF 與其接受器結合的數量減少，造成前列腺瘤細胞的增生。此外，IGFBP-3 則由 MMP-9 分解蛋白作用而調控。若血中 IGFBP-3 愈多，則 IGF 的活性降低，而 IGF-I與 IGFBP-3 的比值增加，則可表示 IGF-I在血液中游離的數量增加。除此之外，血清中 IGF-I、IGFBP-3，也反應出正常小孩血中內生性生長激素的量，然而調控 IGF 的內分泌及旁分泌功能及局部產生生長因子的功能尚未瞭解。

目前已有相當多的臨床運用及証據指出，循環中的生長激素及類胰島素生長因子主要作用在心臟上，因此許多的研究都針對在心血管系統。近年研究發現甲狀腺過低症會出現心臟病致死，而生長激素缺乏，則發生在左心室及右心室功能失常。因此，我們認為類胰島素生長因子可能與心臟間

隔缺損有關，我們針對其中的心室中隔缺損與類胰島素生長因子的關係，以期提出獨特的標準作為預測嬰幼兒心室中隔缺損的指標。

方法：

Ⅰ. 病人與檢體之收集

我們研究心室中隔缺損嬰幼兒血清中 IGFs、IGFBP3、hGH、tPA、total protein 及 MMPs 的值。這些嬰幼兒又以 QP/QS 值大於 2 或小於 2；有無臨床症狀及心臟充血徵候分為 severe-VSD 跟 mild-VSD 兩組。並且以正常嬰幼兒的血清當控制組(normal control)來比較上述因子對心臟生長的影響。我們收集了手術之前(severe-VSD、mild-VSD)跟手術之後(post surgery-VSD)兩個不同時期的血清，每次採集 6ml 的全血在血清管中，於 4°C、3000g 離心 5 分鐘，收集血清保存在-20°C待用。研究期間之病人都有做記錄。心臟負擔過重的量是由心臟超音波掃描及導管分析測得的。

病嬰基本資料

Infant Characteristics	normal	Mild-CHF	Severe-CHF	Post surgery-CHF
Male:female	6 : 4	5 : 5	14 : 10	12 : 8
Birth weight (kg)	3.4±0.8	3.3±0.7	3.3±0.6	3.4±0.6
Gestation (wk)	37.4±2.6	37.0±2.4	37.2±1.8	37.8±1.6
Age at study (mo)	6.6±2.8	7.0±3.6	6.8±3.7	8.4±2.8
Weight at study (kg)	6.4±2.8	6.2±3.2	6.0±1.8	8.2±1.6
Heart rate (min^{-1})	136±18	140±12	142±18	137±18
Respiratory rate(min^{-1})	46.8±8.6	48.4±16.2	52.6±18.4	50.4±16.4

(沒有顯著差異)

II. 用 ELISA 方法測 IGFs、 hGH、IGFBP-3 及 tPA：

1.IGF-I 由不用萃取的 IGF-I ELISA KIT(DSL-10-2800)測得：

在分析之前用 sample buffer 將 unknown samples 稀釋 100 倍。在 microplate 的 well 中加入 20μl 的 standard, control, and diluted unknown samples，然後加入 100μl 之 assay buffer，在室溫下以 microplate shaker (500-600rpm)搖 2 個小時。倒掉 wells 中之溶液，用 wash solution 洗 5 次之後加入 100μl 之 antibody-enzyme conjugate solution，在室溫(25℃)下以 microplate shaker (500-600rpm)搖 30 分鐘。用 wash solution 洗 5 次之後加入 100μl 之 TMB 呈色劑，在室溫(25℃)下以 microplate shaker (500-600rpm)避光搖 10 分鐘，然後加 100μl 之 stopping solution 使呈色穩定, 於 30 分鐘內測 OD.值(450nm)。

2.IGF-II 由不用萃取的 IGF-II ELISA KIT(DSL-10-2600) 測得:

在分析之前用 sample buffer 將 unknown samples 稀釋 100 倍。在 microplate 的 well 中加入 20μl 的 standard, control, and diluted unknown samples，然後加入 200μl 之 assay buffer，在室溫(25℃)下以 microplate shaker (500-600rpm)搖 2 個小時。倒掉 wells 中之溶液，用 wash solution 洗 5 次之後加入 100μl 之 antibody-enzyme conjugate solution，在室溫下以 microplate shaker (500-600rpm)搖 30 分鐘。

用 wash solution 洗 5 次之後加入 100μl 之 TMB 呈色劑，在室溫(25℃)下以 microplate shaker (500-600rpm)避光搖 10 分鐘，然後加 100μl 之 stopping solution 使呈色穩定，於 30 分鐘內測 OD.值(450nm)。

3.hGH(human growth hormone)由 hGH ELISA KIT (IBL-MG-59121) 測得:

Standards、Controls、以及 unknown samples 各吸 50μl 至 microplate 的 wells 中, 操作時間必須在 30mins 內完成 加入 50μl 之 anti-hGH HRP conjugate 後在室溫(25℃)下於 microplate shaker(700 ±100 rpm)搖 1 個小時。倒掉 wells 中之溶液，用 wash solution 洗 5 次之後在 15mins 內加入 200μl 新鮮配製之 revelation solution (chromogen TMB)， 在室溫(25℃)下以 microplate shaker(700 ±100 rpm)避光搖 15 分鐘，然後加 50μl 之 stopping solution 使呈色穩定，於 1 個小時內測 OD.值(450nm)。

4.IGFBP-3 由 IGFBP-3 ELISA KIT(DSL-10-6600) 測得:
在分析之前用 zero standard 將 unknown samples 稀釋 100 倍。在 microplate 的 well 中加入 25μl 的 standard, control, and diluted unknown samples，然後加入 50μl 之 assay buffer，在室溫(25℃)下以 microplate shaker (500-700rpm)搖 2 個小時。倒掉 wells 中之溶液，用 wash solution 洗 5 次之後加入 100μl 之 antibody-enzyme conjugate solution，在室溫下以 microplate shaker (500-700rpm)搖 1 個小時。用 wash solution 洗 5 次之後加入 100μl 之 TMB 呈色劑，在室溫(25℃)下以 microplate shaker (500-700rpm)避光搖 10 分鐘，然後加 100μl 之 stopping solution 使呈色穩定，於 30 分鐘內測 OD.值(450nm)。

5.tPA(human tissue-type plasminogen activator antigen) was measured by IMUBIND®tPA ELISA KIT(Product NO. 860) :
於每個 well 中各加入 50μl 之 PET Buffer, 將不同濃度之 tPA Standards 及 unknown samples 各吸 20μl 至適當的 wells 中， wells 蓋起來後在室溫(25℃)下以 microplate shaker 搖 1 個小時，600rpm。加入 50μl 之 antibody conjugate，將 wells 蓋起來後在室溫(25℃)下以 microplate shaker 搖 15mins，600rpm。倒掉 wells 中之溶液，用 wash solution 洗 5 次之後加入 100μl 之 OPD/H2O2 Substrate，將 wells 蓋

起來後在室溫(25℃) 以 microplate shaker 搖 15mins，600rpm。然後加 100μl 之 stopping solution 避光反應 10mins，使呈色穩定，於 30 分鐘內測 OD.值(490nm)。

III. Gelatin Zymography 蛋白酵素分析

Gelatin Zymography 蛋白酵素分析是在 0.1% glatin - 8% SDS-PAGE 稀釋 40 倍的 serum samples，150V 電泳 2.5 個小時後以 washing buffer (2.5%Triton X-100 in dH2O) washing gel 2 次，每次 30mins。然後加 reaction buffer(40 mM Tris-HCl, pH 8.0; 10 mM CaCl2, 0.01% NaN3) 在 37°C 反應 16 個小時以後, 用 0.25% Coomassie brilliant blue R-250 染色 30 分鐘，再用退染液(875 ml dH2O, 50 ml methanol, and 75 ml acetic acid)退染, 縮膠 1hr 之後，用玻璃紙固定在玻璃片上乾膠 Marker 之來源為人類乳癌細胞。

IV. Zymography 之定量分析：

血清中 92 kd (proMMP-9) 及 72 kd (proMMP-2) gelatinase 是由 AlphaImager 2000 densitimetor 測得的。所測的結果由 ANOVA test 統計分析之後。若 $P < 0.05$ 表示有顯著差異。

V. Lowry 蛋白質分析：

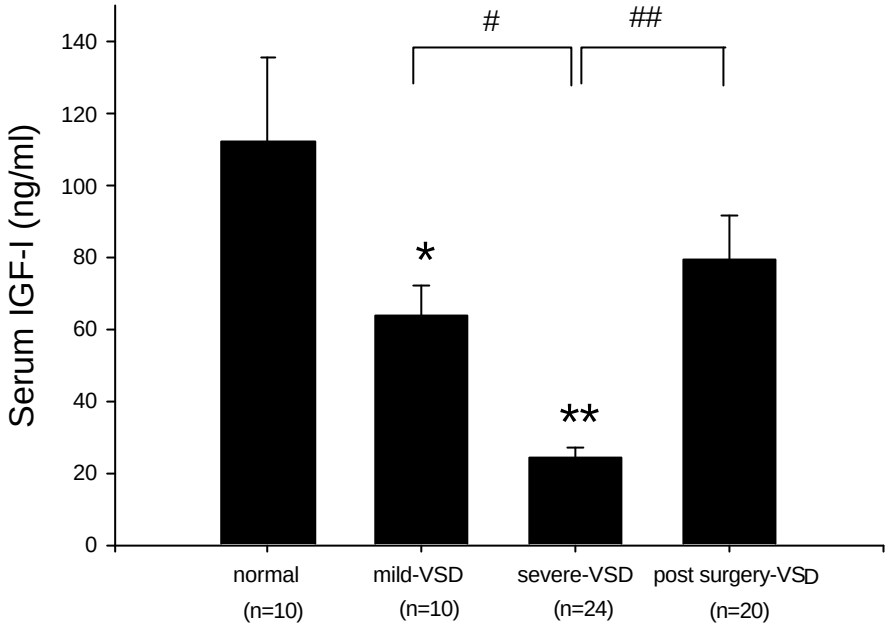
Serum samples 用 ddH2O 稀釋 20 倍，在 15ml 之乾淨試管中加入 1ml 之 standard 或 sample(每個稀釋倍數都做二重複), 然後加入 5.0ml 之鹼性銅試劑，用震盪器混合後在室溫下反應 10 分鐘，於 750nm 測 OD.值。

結果

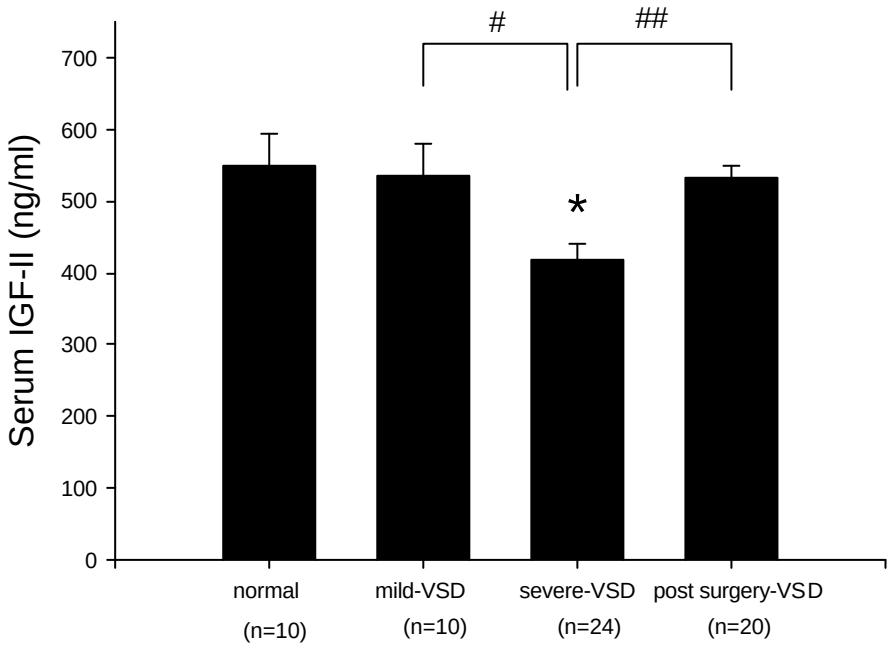
在正常、輕微及嚴重心室中隔缺損嬰兒術前與術後之血清中 IGF-I，IGFBP-3，IGF-I/IGFBP-3 比率及 IGF-II 的數值比較。

嬰兒年齡從三個月至十二個月大。分為正常、輕微(Qp/Qs≦1.5)及嚴重(Qp/Qs≧2.0)心室中隔缺損及手術後嬰兒，共四組。我們發現在輕微心室中隔缺損嬰兒中 IGF-I (正常 111.99±23.40ng/ml，n=10，輕微心室中隔缺損 63.80±8.22ng/ml，n=10，手術後 79.45±12.15ng/ml，n=20)及其結合蛋白 IGFBP-3(正常 22.06±2.37ng/ml，n=10，輕微心室中隔缺損 17.13±1.63ng/ml，n=10，手術後 20.31±1.37ng/ml，n=20)分別下降了 43%與 32%。然而，在嚴重心室中隔缺損的嬰兒中，IGF-I (正常 111.99±23.40ng/ml，n=10，嚴重 24.07±2.69ng/ml，n=24)、IGFBP-3(正常 22.06±2.37ng/ml，n=10，嚴重 9.41±0.77ng/ml，n=24)，甚至 IGF-I 與 IGFBP-3 的比值(正常 4.17±0.78ng/ml，n=10，嚴重 2.55±0.16ng/ml，n=24)下降的更明顯，大約下降了 79%、57%與 39%。同時，IGF-II(正常 549.30±44.95ng/ml，n=10，嚴重 417.60±23.93ng/ml，n=24)下降了 24%。然而，所有的下降情況在手術後六個月都恢復了。

(a)


Serum IGF-I (ng/ml)
140
120
100
80
60
40
20
0
#
##
*
**
normal
(n=10)
mild-VSD
(n=10)
severe-VSD
(n=24)
post surgery-VSD
(n=20)

(b)


Serum IGF-II (ng/ml)
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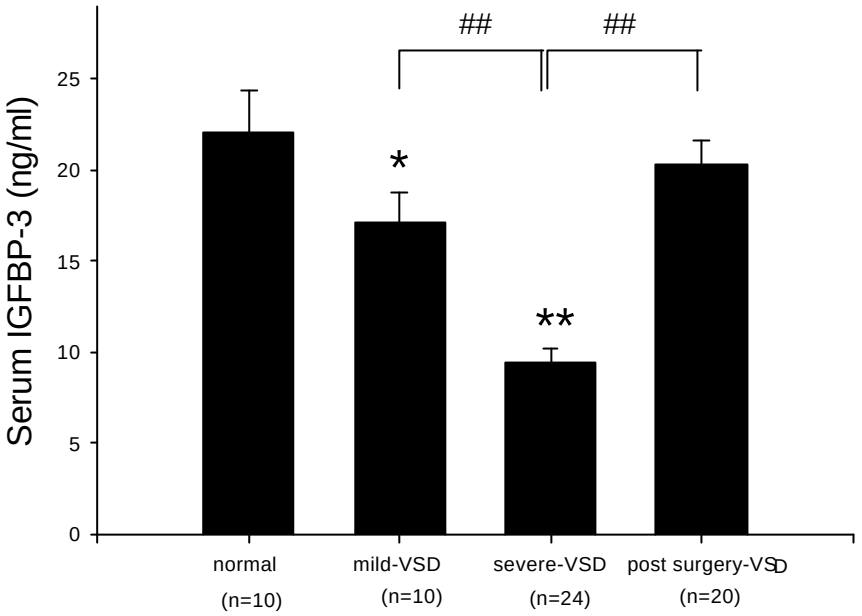
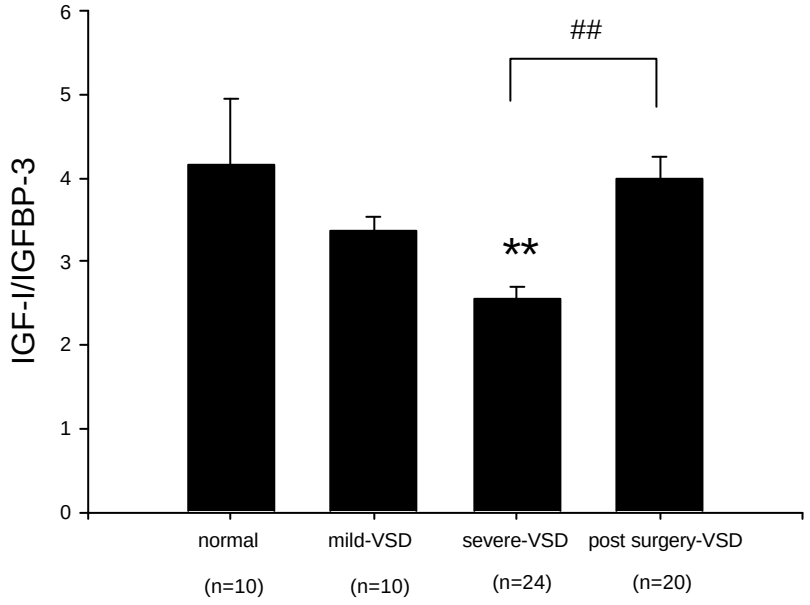
600
500
400
300
200
100
0
#
##
*
normal
(n=10)
mild-VSD
(n=10)
severe-VSD
(n=24)
post surgery-VSD
(n=20)

(c)


Serum IGFBP-3 (ng/ml)
25
20
15
10
5
0
##
##
*
**
normal
(n=10)
mild-VSD
(n=10)
severe-VSD
(n=24)
post surgery-VSD
(n=20)

(d)

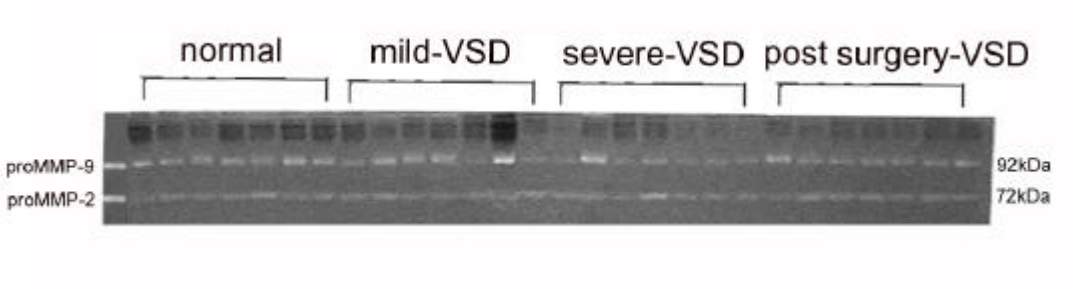

IGF-I/IGFBP-3
6
5
4
3
2
1
0
##
**
normal
(n=10)
mild-VSD
(n=10)
severe-VSD
(n=24)
post surgery-VSD
(n=20)

圖一：酵素連結免疫吸附法分析：(a)類胰島素生長因子 I (b) 類胰島素生長因子 II (c) 類胰島素生長因子結合蛋白 3 (d) 類胰島素生長因子 I 與類胰島素生長因子結合蛋白 3 在正常、輕微及嚴重心室中隔缺損嬰兒術前與術後的比值。嬰兒年齡從三個月至十二個月大。數值顯示為平均值±標準差值，* p<0.05 表示與正常嬰兒比較有 95%的顯著差異； p<0.01 表示與正常嬰兒比較有 99%的極顯著差異。# p<0.05 表示與嚴重心室中隔缺損嬰兒有 95%的顯著差異，## p<0.01 表示與嚴重心室中隔缺損嬰兒有 99%的極顯著差異。**

Figure 1. The ELISA analysis of (a) insulin-like growth factor I (IGF-I) (b) insulin-like growth factor II (IGF-II) (c) insulin-like growth factor binding protein 3 (IGFBP-3)(d) IGF-I/IGFBP3 ratio in serum of normal, mild-, and severe-ventricular septal defect infants before and after surgery. All of the infants were from three to twelve month old. Data are expressed as mean ± SEM. * given represents levels of significant differences compared with normal control group. * $p<0.05$, ** $p<0.01$. #given represents levels of significant differences compared with severe VSD. # $p<0.05$, ## $p<0.01$.

前基質金屬蛋白酵素-2， -9 及組織血纖維溶解酵素促進因子之變化

在四組嬰兒血清中，pro-MMP-2(正常，1.00±0.09,n=10；輕微心室中隔缺損，1.01±0.07,n=10；嚴重心室中隔缺損，0.85±0.11，n=24；手術後，1.01±0.10，n=20)的光學密度與血清纖維破壞因子 tPA (正常，6.01±0.47ng/ml,n=10；輕微心室中隔缺損，6.22±0.67ng/ml,n=10；嚴重心室中隔缺損，6.13±0.69ng/ml，n=24；手術後，7.26±1.14ng/ml，n=20) 沒有顯著差異。但是 pro-MMP-9(正常，1.00±0.23,n=10；輕微心室中隔缺損，0.71±0.19,n=10；嚴重心室中隔缺損，0.39±0.10，n=24；手術後，0.60±0.05，n=20)(IGFBP-3 的蛋白酵素)在嚴重心室中隔缺損嬰兒血清中，下降了 40%，而在心術後六個月亦恢復了。



圖二．以膠原蛋白酵素電泳分析正常、輕微及嚴重心室中隔缺損嬰兒術前與術後基質金屬蛋白酵素-2，-9。

Figure 2. The zymography analysis of matrix-metallo-proteinase-2, -9(MMP-2,9) in serum of normal, mild-, and severe-ventricular septal defect infants before and after surgery.

表一.血清中基質金屬蛋白酵素活性及組織型血纖維溶解酵素促進因子濃

Table 1. Serum MMP-2, -9 activities and tPA concentration of the subject groups

	Normal (n=10)	mild-VSD (n=10)	severe-VSD (n=24)	post surgery-VSD (n=20)
ProMMP 9 (O.D.)	1.00±0.23	0.71±0.19	0.39±0.10 *	0.60±0.05
proMMP 2 (O.D.)	1.00±0.09	1.01±0.07	0.85±0.11	1.01±0.10
tPA (ng/ml)	6.01±0.47	6.62±0.67	6.13±0.69	7.26±1.14

正常、輕微及嚴重心室中隔缺損嬰兒術前與術後血清中基質金屬蛋白酵素活性及組織型血纖維溶解酵素促進因子濃度。嬰兒年齡從三個月至十二個月大。光學密度以密度儀來偵測，而組織型血纖維溶解酵素促進因子濃度則以酵素連結免疫吸附法分析。數值顯示為平均值±標準差值，* p<0.05 表示與正常嬰兒比較有 95%的顯著差異。

Serum metallo-matrix protease- 2, -9 (MMP-2,9) activities and tissue plasminogen activator (tPA) concentration of normal, mild-, and severe-ventricular septal defect infants before and after surgery. All of the infants were from three- to twelve-month old. The optical density (O.D.) of MMP2 and MMP9 were analyzed by desnitometer, and the tPA was analyzed by ELISA. Data are expressed as mean ± SEM. * given represents significant differences compared with normal control group. * p<0.05.

血清中總蛋白與白蛋白之濃度

在四組嬰兒血清中，總蛋白(正常，128.44±3.35mg/ml,n=10；輕微心室中隔缺損，121.66±5.59mg/ml,n=10；嚴重心室中隔缺損，125.80±4.23mg/ml，n=24；手術後，126.99±3.51mg/ml，n=20)、白蛋白(正常，37.00±0.05mg/ml,n=10；輕微心室中隔缺損，38.88±0.64mg/ml,n=10；嚴重心室中隔缺損，39.04±0.55mg/ml，n=24；手術後，39.21±0.48mg/ml，n=20)的濃度都沒有改變。

表二．血清總蛋白、白蛋白及肌酸激酵素活性

Table 2. Serum total protein, albumin, and creatine kinase (CK) activity of the subject groups.

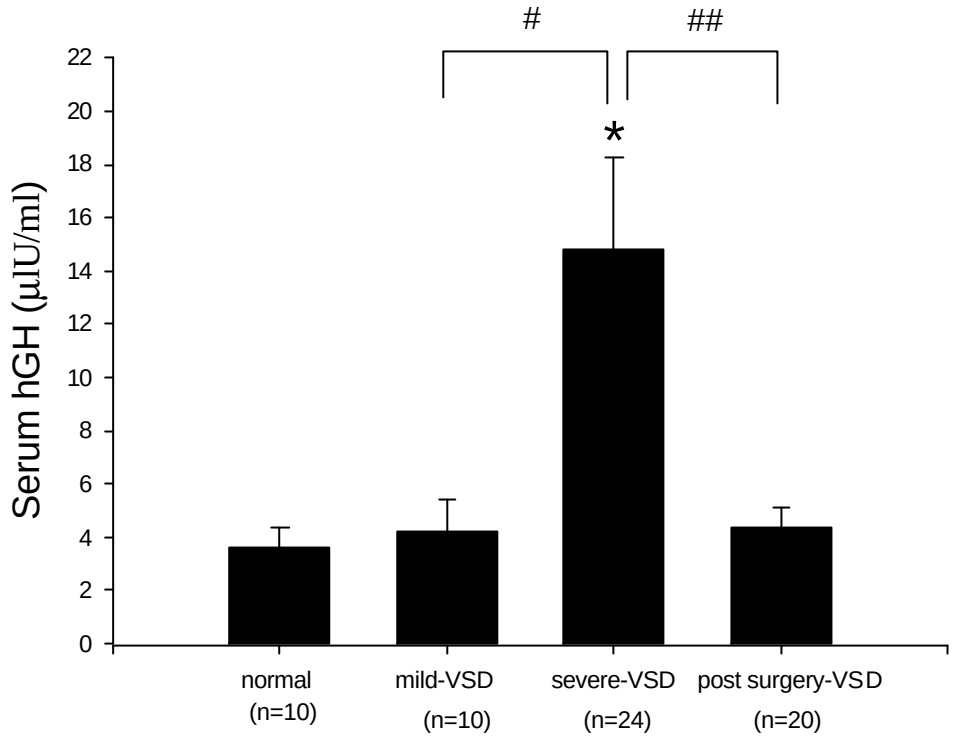
	Normal (n=10)	mild-VSD (n=10)	severe-VSD (n=24)	post surgery-VSD (n=20)
Toltal protein (mg/ml)	128.44±3.35	121.66±5.59	125.80±4.23	126.99±3.51
Albumin(mg/ml)	37.00±0.05	38.88±0.64	39.04±0.55	39.21±0.48
CK(IU/L)	112.82±15.98	110.22±11.93	142.00±20.75	99.78±14.15

正常、輕微及嚴重心室中隔缺損嬰兒術前與術後血清總蛋白、白蛋白及肌酸激酵素活性。嬰兒年齡從三個月至十二個月大。數值顯示為平均值±標準差值，* p<0.05 表示與正常嬰兒比較有 95%的顯著差異

Serum total protein, albumin, and (CK) activity of normal, mild-, and severe- ventricular septal defect infants before and after surgery. All of the infants were from three- to twelve- month old. Data are expressed as mean ± SEM.

血清中人類生長荷爾蒙(hGH)的不同

在嚴重心室中隔缺損嬰兒血清中，GH(正常,3.6±0.78 μIU/ml,n=10; 輕微心室中隔缺損,4.17±1.26μIU/ml,n=10; 嚴重心室中隔缺損，，14.79±3.51μIU /ml,n=24; 手術後，4.36±0.75μIU /ml,n=20)上升超過三倍。然而，手術後六個月完全恢復。



圖三．正常、輕微及嚴重心室中隔兒術前與術後血清中人類生長荷爾蒙酵素連結免疫吸附法分析。嬰兒年齡從三個月至十二個月大。數值顯示為平均值±標準差值，* p<0.05 表示與正常嬰兒比較有 95%的顯著差異； p<0.01 表示與正常嬰兒比較有 99%的極顯著差異。# p<0.05 表示與嚴重心室中隔缺損嬰兒有 95%的顯著差異，## p<0.01 表示與嚴重心室中隔缺損嬰兒有 99%的極顯著差異。**

Figure 3. The ELISA analysis of serum human growth hormone (hGH in serum of normal, mild-, and severe- ventricular septal defect infants before and after surgery. All of the infants were from three to twelve month old. Data are expressed as mean ± SEM. * given represents levels of significant differences compared with normal control group. * p<0.05, ** p<0.01. #given represents levels of significant differences compared with severe VSD. # p<0.05, ## p<0.01.

討論：

心室中隔缺損為嬰幼兒最常見的先天性心臟病，常導致明顯的血流量負擔過大而引發心臟衰竭，需早期治療。心臟超音波檢查為目前診斷的主要工具，並可評估心肌的功能狀態。神經荷爾蒙的調節機制，在心臟衰竭的病態生理上扮演著重要角色，並且可影響心臟對血流量及壓力負擔的後續反應。

類胰島素生長因子為胚胎期心臟發育的重要影響因子。然而，有關類胰島素生長因子與心室中隔缺損嚴重程度的關係，則未被研究過。尤其特別的，目前除心臟超音波外，在血液中尚無有效的"預測標的"能有效界定心室中隔缺損的狀況。先前的研究報告曾顯示大多數的先天性心臟病(34)血液中類胰島素生長因子-I 較低，我們的研究更指出，在輕度心室中隔缺損中，類胰島素因子-I 輕微減少 43%，但嚴重心室中隔缺損組則明顯大幅減少 79%(Figure)，類胰島素生長因子不僅扮演局部生長因子功能，並可如自體分泌或旁分泌一般，以內分泌的方式促進生長，並作用至心臟肌肉。

類胰島素生長因子的作用及生物利用率主要由 6 種攜帶蛋白以蛋白質分解作用的方式調節，其中主要的類胰島素因子攜帶蛋白 3(IGFBP-3)及與酸性活性次體(ALS)可與類胰島素生長因子 I 及 II 結合而形成一複合體。此複合體可決定類胰島素生長因子與其細胞表面接受器之親和性，故可作為一標的物。類胰島素因子攜帶蛋白(IGFBP-3)及酸性活性次體(ALS)在人

體中依賴著生長激素類胰島素因子攜帶蛋白(IGFBP-3)由體內許多組織局部產生時，可以自體分泌及旁分泌功能調節細胞的生長。除此之外，在過去研究中發現，類胰島素因子攜帶蛋白(IGFBP-3)可直接轉錄到細胞核而誘發前列腺瘤細胞及乳瘤細胞的致死(apoptosis)。

在心室中隔缺損的嬰幼兒中，血清中類胰島素生長因子攜帶蛋白(IGFBP-3)與類胰島素生長因子的量會減少，於輕度心室中隔組達 32%，嚴重心室中隔缺損組達 57%，但游離態的類胰島素生長因子-I(IGF-I)及類胰島素生長因子-I 與類胰島素生長因子結合蛋白3(IGF-I/IGFBP-3)的比值亦同樣有下降趨勢，在輕度心室中隔缺損組為 19%，嚴重心室中隔缺損組則高達 39%。

雖然類胰島素生長因子結合蛋白(IGFBP)複合體如何激化類胰島素生長因子(IGF)的方式尚未完全清楚，但目前已知類胰島素生長因子(IGF)的複合體有較長的循環半衰期，且其安定度則由酸性活性次體判定。類胰島素生長因子結合蛋白 3(IGFBP-3)的濃度與生長因子的活性呈正相關，舉例而言，臍帶血中類胰島素生長因子結合蛋白 3(IGFBP-3)量與出生體重呈正比，因此血液中類胰島素生長因子結合蛋白 3(IGFBP-3)在整個成長過程中逐年增加。由以上可知，類胰島素生長因子結合蛋白 3(IGFBP-3)及其與類胰島素生長因子的複合體是生長發育所必需。研究得知，促使類胰島素生長因子釋放的可能機轉是因為母親懷孕時體內蛋白分解脢導致類胰島素生

長因子結合蛋白 3(IGFBP-3)量減少。

膠原纖維蛋白分解胺及蛋白分解酵素-2,-9 (MMP-2,-9)在組織發育過程中能促進膠原纖維更新，並幫助受傷組織的修補。另外，MMP 蛋白分解酵素也存在心臟組織。在原發性高血壓病人中，因合併左心室肥厚，故血中 MMP 蛋白分解酵素受到抑制，因此當膠原纖維更新受阻時，則會加速器官的纖維化。

血液中類胰島素生長因子結合蛋白 3(IGFBP-3)主要由 MMP-2 及 MMP-9 蛋白分解胺來調節，我們的研究顯示，MMP-9 在嚴重心室中隔缺損嬰兒中大幅減少 61%(Figure 2)，而所有的類胰島素生長因子，包括自由態類胰島素生長因子-I(IGF-I), 類胰島素生長因子結合蛋白 3(IGFBP-3)及 MMP-9 蛋白分解酵素在心室中隔缺損病嬰中均減少，特別值得注意的是，其減少幅度與心室中隔缺損心臟衰竭嚴重度有關，但所有病徵在手術後均可恢復。此外，缺血性心肌病變中，組織血漿蛋白胺激活因子(tPA)可將血漿蛋白胺轉化成血漿蛋白，再激活 MMP 蛋白分解胺，而類胰島素生長因子-I(IGF-1)阻斷 MMP 蛋白分解胺活性的方法，主要是藉由抑制 tPA，因此 tPA 的量在心肌梗塞的病人中，可能是心臟缺血及病人猝死的危險因子。然而，在心室中隔缺損嬰幼兒中血清 tPA 並無變化，表示 tPA 並非為 MMP-9 改變的變因，可能是由類胰島素生長因子(IGF)及類胰島素生長因子結合蛋白 3(IGFBP-3)的改變所造成。

為了証明血液中類胰島素生長因子(IGF)減少並非因為營養不良及病人體質差異所造成，我們也測量了血清中總蛋白及白蛋白的濃度，發現並無明顯差異(Table 1) ；除此，在正常嬰兒血中類胰島素生長因子-I(IGF-I)及類胰島素生長因子結合蛋白 3(IGFBP-3)也反應出生長激素分泌情形，類胰島素生長因子-I(IGF-I)較類胰島素生長因子結合蛋白 3(IGFBP-3)易受生長激素調節。但某些慢性心臟衰竭、續發性生長激素停止分泌合併類胰島素生長因子-I(IGF-I)缺乏者，給予生長激素補充治療可改善擴張性心肌病變及預防心臟衰竭，且可治療先天性心臟病及生長發育遲緩的嬰兒。因代償效應，在嚴重心室中隔缺損組，生長激素明顯提高 3.1 倍，但輕度心室中隔缺損者，則僅小幅增加 16%，更令人驚訝的是，這些改變在手術後 6 個月，全部恢復至正常範圍。因此，心室中隔缺損嬰幼兒血中類胰島素生長因子(IGF)、 類胰島素生長因子結合蛋白 3(IGFBP-3)及 MMP9 減少可能不是因為 GH / IGF-I 比例的異常。

心室中隔缺損將導致心臟過度代謝，而誘發慢性代謝酸中毒，這可能是嚴重心室中隔缺損病例中，血乳酸過高及 PH 值降低的原因，而導致 GH /IGF 比值的異常，進而引發類胰島素生長因子(IGF)、類胰島素生長因子結合蛋白 3(IGFBP-3)及 MMP9 降低。過去報導指出，酸中毒的老鼠其 GH receptor 和類胰島素生長因子-I(IGF-I)接受器會顯著減少，以生長激素及類胰島素生長因子-I(IGF-I)治療對其生長速率並無改善，因此，生長激素的治療可

能向上調節乳酸、alanine 的量，而減低 PH 值，導致病況惡化。這也可以解釋為什麼在嚴重心室中隔缺損嬰幼兒血中 PH 值低、生長激素過高，但卻導致病情惡化的負回饋機制。因此，外科手術可去除酸中毒而改善病況，若釐清這些生理機轉，將可提供成為治療心室中隔缺損的方法，即以藥物去抑制乳酸、調節 PH 值或利用類胰島素生長因子(IGF)強化的配方，使用基因治療來過度表現類胰島素生長因子(IGF)以恢復 GH/IGF 比例，以關閉心室中隔缺損。

最近有報告指出某些轉錄因子如活化 T-細胞轉錄因子(NF-ATc)是心臟間隔及瓣膜發育的主要因子，在 NF-ATc exon 3 的基因剔除鼠中，發現心室中隔及瓣膜發育異常，並死於心室肥厚及心臟衰竭。然而，GSK-3 的磷酸化可促進 NF-ATC 的轉錄，且主要發生在白血球及心膜內，因此 NF-ATC 基因上任何突變都足以抑制 IGF promoter region 或抑制 VSD 病嬰。

未來解釋心室中隔缺損的發生將上溯至基因層次的探討，進一步利用基因突變的篩檢及 Electro-mobility shift assay 分析類胰島素生長因子-I(IGF-I)及類胰島素生長因子-II(IGF-II)Promoter region 是否含有 NF-ATC 的 binding Site。同時利用基因雜合來偵測人類心臟 cDNA 序列中交互作用的蛋白質，以找出突變的轉殖因子。

總結：基於輕度心室中隔缺損併發輕微減少類胰島素生長因子-I(IGF-I)，類胰島素生長因子結合蛋白 3(IGFBP-3)及嚴重心室中隔缺損併發類胰島素

生長因子-I(IGF-I)、類胰島素生長因子-II(IGF-II)、類胰島素生長因子 I 與類胰島素生長因子結合蛋白 3(IGF-I/IGFBP3)比值極度過低，導致血中生長因子代償性遽增的發現，我們提出此方法以預測心室中隔缺損的發生，將有助於確定及篩檢輕度及重度心臟衰竭者，決定手術的必要性，且檢視手術結果的好壞。未來利用基因治療或添加類胰島素生長因子-I (IGF-I)強化牛奶配方，將為改變心室中隔缺損狀況及預防心臟衰竭不可或缺的良方。

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INTRODUCTION:

Ventricular septal defect, the most common congenital heart disease with the incidence around 2-4/1000 of all newborn (1)(2), represents the major manifestions of congestive heart failure of the admixture of multiple components that may reflex pulmonary and systemic congestion, the contractile status of the myocardium, loading conditons, perfusion status, and the operation of various adaptive mechanisms. However, the molecular signals that regulate cardiac septation formation are largely unknown(3). The insulin-like growth factors (IGFs) play a key role in mammalian growth and differentiation processes(4,5,6), including a regulatory function in fetal cardiac development(7). IGFs are the members of insulin-related peptide family, insulin, IGF-I, IGF-II, and relaxin, which are diverged from a common ancestral gene(8). The IGFs were discovered in a search for factors that could mediate the effects of growth hormone on postnatal growth(9), and serum levels of IGFs are growth hormone-dependent in postnatal animals. The IGFs carries out an endocrine function mediated through the IGF-I receptor located on the surface of responsive cells(5,6,10). Further, a wide variety of tissues, including the myocardium, are capable of expressing the IGFs gene and synthesizing the peptides (6,9,11).

IGF-I transcripts in fetal serum, liver, kidney, and heart are lower in abundance compared with IGF-II and rise progressively with increasing postnatal age(12). IGF-I regulates proliferation and differentiation of multiple cell types in bodies and is capable of exerting insulin-like metabolic effects. Like insulin, IGF-I is abundant in the circulation. On the other hand, unlike insulin, it is produced by most tissues of the body. Thus, IGF-I has the potential to act via endocrine as well as autocrine and/or paracrine mechanisms(13). The biological role of IGF-I

in postnatal growth has been well established(14), and its transcripts detected in the heart from early adulthood through senescence were investigated in many studies(15).

The role for IGF-II in fetal growth has been demonstrated(16). Disruption of the interaction between IGF-II and its receptor results in embryonic growth deficiency(17). In fetal rats, IGF-II gene expression is abundant in most tissues, but decreases dramatically at birth in all tissues except in brain and heart. Also, the decrease in IGF-II mRNA levels corresponds to the decrease in circulating levels of IGF-II postnatally(16).

The insulin like growth factors are regulated by a family of six IGF binding proteins(IGFBPs). IGFBP regulation of IGFs may involve proteolysis. MMP-1(18,19),MMP-3(19),MMP-9(20,21) have been identified as IGFBP proteinase. In addition, matrix metalloproteinases(MMPs) are involved in tissue remodeling and diseases(22,23). MMP-1 activity can produce IGFBP fragments with a low affinity to IGFs, thus increasing the bioavailability of IGFs for IGF receptors and consequently inducing smooth muscle cells proliferation(18). IGFBP-3 binding to IGFs also modulates the proliferation of prostate adenocarcinoma cell line by reducing signaling through IGF-I receptor. Moreover, IGF-BP3, in turn, is regulated by MMP-9 proteolysis(20). IGFBP-3 is critical for circulating IGFs bioactivity and the increase in the molar ratio between IGF-I and IGFBP-3 reflects an increase in free, biologically active IGF-I (24). Additionally, the serum levels of IGF-I and IGF-BP3 reflect the endogenous GH secretion in normal children (25, 26). So far, the regulation of the autocrine / paracrine IGFs as well as the functions of the locally-produced growth factor are poorly understood(27). There have been increasing clinical use

and abundant evidences indicating that circulating growth hormone(GH) and insulin-like growth factors target the heart , and more and more systematic investigation of their effects are focusing on cardiovascular system(28). Recent studies suggest that excess of cardiac mortality appear in hypopituitarism(29,30), LV and RV dysfunction in GH deficiency(31,32). Therefore, it is postulated that IGFs axis could be linked to cardiac septal defects. Our results provide a new insight into the relationship of IGFs axis and cardiac septal diseases, and **suggest a novel criteria to predict the outcome of infants with VSD of variable severity of congestive heart failure.**

METHODS

I. Sample collection :

All the infants of VSD were diagnosed by echocardiography as isolated VSD with variable size and shunt in different severity of congestive heart failure. All the infants (table 1), aged from 3 months to one year, were divided into four groups which are control, mild-VSD (without symptoms of congestive heart failure; Qp/Qs≦1.5), severe-VSD (with intractable heart failure, Qp/Qs≧2.0), and postoperation for 6 months recovery. 4 ml blood was punctured, followed by centrifugation at 3000g for 5 minutes. Then the serum was aspirated and stored at -20℃ until used in the determination of zymography assay, total protein and ELISA for IGFs, hGH,IGFBP-3 and tPA. The samples were collected after six months of surgery in postoperative group. The quantitation of overload volume was measured by echocardiograhic measurements and catheterization analyse.

II. ELISA for IGFs, hGH, IGFBP-3 and tPA

Serum IGF-I, IGF-II, IGFBP-3 were determined by a commercially available non-extraction enzyme-linked immunosorbent assay (ELISA) kit (Diagnostic Systems Laboratories, Inc USA) using a monoclonal antibody. All of the procedures were following the manufacturer' s instructions *(DSL-10-2800, Texas, USA. IBL-MG-59121, DSL-10-6600 , IMUBIND® Product NO. 860).* All of the sample target proteins were immunosorbented by enzyme-conjugated antibody, followed by adding substrate solutions for color development. The contents of the proteins were determined by comparing relative absorbance of

samples to that of known amounts of standard, using a microplate reader (Model:RS01, Kansin instruments. CO, LTD).

Serum hGH and tPA were also determined by ELISA (Biosource Europe, S.A. and American Diagnostica, Inc.) All of the procedures were following the manufacturer's instructions, and similar to that of IGF-I, IGF-II and IGFBP-3.

III. Gelatin Zymography Protease Assay and quantitative Analysis

Gelatin zymography analysis was carried out by loading 10-µl sample of serum (1/40) on 0.1% glatin - 8% SDS-PAGE. Electrophoresis was run at 150 V for 2.5 h. Enzymes on the gels were renatured by washing twice in a 2.5% Triton X-100 solution with shaking for 30 mins. The gels were then incubated with 50 ml of a reaction buffer (40 mM Tris-HCl, pH 8.0; 10 mM CaCl2, 0.01% NaN_3) at 37°C for 16 h and then stained with 0.25% Coomassie brilliant blue R-250 for 30 min. Quantitative analysis was carried out after discoloring the stain in a discoloring solution (875 ml dH_2O, 50 ml methanol, and 75 ml acetic acid). The extract of a random-chosen human breast cancer biopsy was used as a marker. Expression of 92 kd (proMMP-9) and 72 kd (proMMP-2) gelatinase in the serum were determined using the AlphaImager 2000 densitimetor.

IV. Lowry Protein Assay

Sample protein was measured by the method of Lowry et al using bovine serum albumin as standard.

V. The Measurement of Serum pH value, Lactate and Creatine Kinase(CK)

The lactate reagent, in conjunction with SYNCHRON CX System CX MULTI™ Calibrator(kit #445875), was intended for the quantitative determination of lactate concentration in serum. The creatine kinase reagent was intended for the quantitative determination of CK activity in serum on the SYNCHRON CX clinical systems(kit #442635). The change in absorbance measured by sepectrophotometer (Beckman, CS-7) is directly proportional to the concentration of lactic acid and creatine kinase in the serum sample and is used by the SYNCHRON CX System to calculate the concentration.

VI. Statistical analysis:

The data was compared between groups of animals and treatments of samples, using one-way analysis of variance (ANOVA). Fisher's Least Significant Difference test was used to determine differences. $P<0.05$ was taken as significant.

RESULTS

The serum levels of IGF-I, IGFBP-3, IGF-I/IGFBP-3 ratio and IGF-II

The results of IGFs and its binding protein are described in Fig (1). We found serum IGF-I (normal 111.99±23.40ng/ml, mild-VSD 63.80±8.22ng/ml) and its carry protein IGFBP3(normal 22.06±2.37ng/ml, mild-VSD 17.13±1.63ng/ml) reduced 43% and 32%, respectively, in mild-VSD children (n=10, fig. 1a,b). However, more significant reduction of IGF-I(normal 111.99±23.40ng/ml, severe-VSD 24.07±2.69 ng/ml), IGFBP3(normal 22.06±2.37ng/ml, severe-VSD 9.41±0.77ng/ml), and IGF-I/IGFBP3 ratio(normal 4.17±0.78ng/ml, severe-VSD 2.55±0.16ng/ml) occurred in severe-VSD children, which are about 79%,57% and 39%, respectively, (n=24 fig. 1a,b,c). At the same time, IGF-II(normal 549.30±44.59ng/ml, severe-VSD 417.60±23.93ng/ml) reducing 24% was observed(fig. 1d). However, all the reductions were reversed at the sixth month after surgery, n=20.

The variation of serum pro-metallo-matrix-2,-9(pro-MMP-2,9) and tissue plasminogen activator(tPA)

There are no significant differences were observed in the optical densities of serum pro-MMP-2 (normal, 1.00±0.09,n=10; mild-VSD, 1.01±0.07,n=10; severe-VSD, 0.85 ± 0.11, n=24; post-surgery, 1.01 ± 0.10, n=20), the concentration of serum fibrolysis factor, and tissue-type plasminogen activator(tPA) (normal, 6.01±0.47ng/ml,n=10; mild-VSD, 6.62±0.67ng/ml,n=10; severe-VSD,6.13±0.69ng/ml, n=24; post-surgery, 7.26±1.14 ng/ml, n=20) among four groups (fig. 2a,b). The optical density of pro-MMP-9, the protease of IGFBP3, decreased 40% in severe-VSD children, and then restored by surgery(normal 1.00±0.23 optical density, mild-VSD 0.71±0.67 optical density;

severe-VSD 0.39±0.10 optical density, post surgery-VSD 0.60±0.05 optical density) (fig. 2a,b).

The concentrations of serum total protein and albumin

There are no obvious alterations observed on serum total proteins (normal,128.44±3.35mg/ml,n=10; mild-VSD,121.66±5.59mg/ml,n=10; severe-VSD, 125.80±4.23mg/ml,n=24; post-surgery,126.99±3.51mg/ml,n=20) and albumin (normal, 37.00±0.05mg/ml,n=10; mild-VSD,38.88±0.64mg/ml,n=10; severe-VSD,39.04±0.55 mg/ml,n=24; post-surgery,39.21±0.48mg/ml,n=20) in all groups (table 1).

The differentiation of serum human growth hormone(hGH)

The 311% elevation of serum growth hormone occurred in severe-VSD. However, the changes were totally reversed after six months of surgery. The exact values are: normal,3.6±0.78 μIU/ml,n=10; mild-VSD,4.17±1.26μIU/ml,n=10; severe-VSD, 14.79±3.51μIU /ml,n=24; post-surgery, 4.36±0.75μIU /ml,n=20)(fig. 3).

The concentrations of serum creatinine kinase, lactate and pH value

There are no significant differences observed in the serum creatinine kinase among four groups (normal, 112.82±15.98 IU/L,n=10; mild-VSD, 110.22±11.93 IU/L,n=10; severe-VSD, 142.00±20.75 IU/L,n=24; post-surgery, 99.78±14.15 IU/L, n=20) (table 1). Similarly, no significantly changes were found on serum lactate (normal 5.28±0.85 mmol/L, mild-VSD 5.21±0.99 mmol/L) and pH value(normal 8.09±0.05, mild-VSD 7.99±0.04) in mild-VSD

children,n=10(table 2), However, significantly reduction of lactate(normal 5.28±0.85 mmol/L, severe-VSD 8.66±1.11 mmol/L), pH(normal 8.09±0.05, severe-VSD 8.09±0.04), appeared in severe-VSD group, by 64% and 2%, respectively, n=24(table 2).

DISCUSSION

Ventricular septal defect, the most common congenital heart disease in infant, will cause significantly hemodynamic volume overloading and induce congestive heart failure requiring earlier treatment. Cardiac ultrasonography provides the key tool to define the specific disorder and assess the functional status of the myocadium. The major neurohormonal mechanisms play a key role in the pathophysiologic process of congestive heart failure and influence the natural history of certain volume- and pressure-loading

The IGFs are very important for the embryonic heart development (33), but the correlation between IGFs and the severity of VSD in patients has never been investigated. Particularly, so far, except echocardiography, there are no efficient predictive markers in blood to define the VSD situation. One previous report showed that serum IGF-I lowered in most congenital heart disease(34). Our data further indicated that serum IGF-I slightly declined 43% in mild-VSD and highly declined 79% in severe-VSD infants(fig. 1a). IGFs not only play important roles as local(autocrine or paracrine) growth factor, but also exert growth-promoting actions in an endocrine manner(35,36) and target to myocardium. The actions and bioavilability of IGFs are regulated by the family of six IGF binding proteins. Among them, IGFBP-3, the major circulating IGFBPs, combines with a glycoprotein, the acid-labile subunit(ALS), to form a ternary complex with IGF-I or IGF-II(37,38). This carries and/or determines the release of IGFs to target organ. Both IGFBP-3 and ALS show growth hormone dependence in human(39,40). IGFBP-3 is also produced locally in many tissues(41), where it serves autocrine and paracrine roles in modulating cellular growth(42). Additionally, IGFBP-3 has been even shown to directly translocate into nuclei and induce apotosis in prostate cancer cells (43) and breast cancer cells(44).

In VSD infants, we found serum IGFBP-3 correlate with IGFs reduction by 32% and 57% in mild- and severe VSD children, respectively, and even the free type-IGF-I and IGF-I/IGFBP-3 ratio which represents IGF decrease is not resulting from IGFBP-3 reduction have the same declining tendency, mild-VSD with 19% and severe-VSD with 39% (fig. 1b,c). Although how the IGFBP complex exactly allows the activation of IGF activity is not totally clear, IGFs in the complex have a greatly extended circulating half-life, and their stability is conferred by the ALS(37). Mostly, the concentration of IGFBP-3 also shows a positive association with growth activity. For example, cord serum IGFBP-3 correlates postively with birth weight(45), and serum IGF-BP3 increase throughtout the growth peroid of childhood and puberty(39,40). The both phenomena imply that IGFBP-3 and the complex, far from blocking IGF-mediating growth promotion, are necessary for growth(37). The possible mechanism to facilitate the release of IGFs is the reduction of IGFBP-3 by proteolysis which occurs in pregnancy(46). The collagen proteases, MMP-2,-9 mainly produced by connective tissue, are thought to contribute to collagen turnover for tissue remodeling in development, and as part of repairing processes following tissue damage. The target organ, heart, is one of the highly turnover tissues by MMPs(47). Due to the depression of serum MMPs in the essential hypertension patients with left ventricular hypertrophy, a depressed degradation of collagen then facilitates organ fibrosis(48). Furthermore, the serum IGF-BP3, in turn, is regulated by MMP-9(20) and MMP-2(49) proteolysis. Our data showed that MMP-9 significantly reduced 61% in severe-VSD infants(fig. 2). The above data indicated that total IGFs, including free-type IGF-I, carrying protein-IGFBP-3, and the proteolysis factor-MMP-9, all declined in VSD patients. Interestingly, the declination level is correlate with the severity of this

disease. However, all the suppressions are mostly reversed by surgery. At the same time, tissue plasminogen activator(tPA) converts plasminogen to plasmin which, in turn, activates MMPs following ischemic cardiomyopathy(50). Moreover, IGF-I blocked MMPs activation also mediated through the suppression of tPA(51). Consequently, tPA could be a risk factor of myocardial infarction or sudden death in patients with angina pectoris(52). However, the alterations on serum tPA level in VSD infants was not found, indicating that the change of MMP-9 was not resulting from tPA variation, and probably led by the IGFs and IGFBP-3 alteration.

In order to exclude the declination is not resulting from malnutrition effects which was reported in some congenital heart disease cases(34), we measured serum total proteins and the level of albumin as well. The result showed no differences among the infants (Table 1). Additionally, the levels of IGF-I and IGFBP-3 in serum reflect spontaneous GH secretion in normal individuals, and IGF-I level are more sensitive to GH regulation than IGFBP-3 (53). IGF-I protects heart from heart failure by maintaining on physiological hypertrophy (54). But some chronic heart failure patients acquired growth hormone resistance with deficient IGF-I (55) and GH treatment which activated IGF-I did improve the dilated cardiomyopathy patients and prevent heart failure(56), and GH treatment did recovery the congenital heart disease and growth retardation in infants(57). However, probably due to the compensative effect(fig. 3), the GH level significantly elevated about 3.1-folds in severe-VSD infants. Minor increase (16%) of serum hGH appeared only in mild-VSD infants. Surprisingly, all the changes were restored again by the sixth month after surgery. Therefore, the lower level of IGFs ,IGFBP-3 and MMP-9 in VSD infants is

probably not due to the abnormality of GH/IGF-I axis.

In VSD infants, hyperdynamic cardiac metabolism will induce chronic metabolic acidosis which is probably the reason why higher lactate and lower pH value were observed in severe-VSD infants then causing abnormal GH/IGFs axis and resulting in low serum level of IGFs, IGFBP3 and MMP9. However, the previous papers reported that acidotic young rats had significant decrease of GH receptor and IGF-I, and the GH and IGF-I therapy produced no improvement in growth rate(58). Besides, GH treatment might even upregulated the lactate, alanine level and lower pH value, which lead to a worse condition(59). This might explain why severe-VSD infants, with low pH, low IGF-I and high GH, and still lead to a worst positive feedback situation. Therefore, the surgery removed the acidosis stress and released the situation. The elucidation of these mechanism will provide a novel strategy for curing the VSD problem by applying some medicine to suppress lactate and adjust the pH value, fortifying milk formula with IGFs, or applying gene therapy to overexpress IGFs to recovery the GH/IGFs axis and shunt the hole off.

Recent reports also indicated that some transcription factors, such as nuclear factor of activated T cells (NF-ATc) is essential for cardiac septum and value formation. The NF-ATc exon 3 knock out mice displayed abnormalities of valve and septum structure and died in ventricular hypertrophy and heart failure(60,61). However, the nuclear export of NF-ATc enhanced by phosphorylation of glycogen synthase kinase 3 (GSK-3), the nuclear import activated by de-phosphorylation of calcineurin (phosphatase)(62), then bind to Tcf-4 response element and turn on gene expression. It happened only in leukocytes and endocardium (63). Therefore, either any mutation appears in NF-ATc gene of VSD patients or this mutant occurs to suppress IGFs promoter

region and inhibit their gene expression could provide another possible mechanism to explain the occurrence of VSD. Further gene mutations screening and electro-mobility shift assay should be performed to analyze if the binding sites exist in IGF-I & II promoter for NF-ATc mutuant. At the same time, two-hybrid system could be used to find out the interactive proteins in the human heart cDNA library for these mutated transcriptional factors.

In conclusion, based on the results of a slight reduction of IGF-I and IGF-binding protein-3(IGFBP3) in mild-VSD infants, and a high reduction of IGF-I, IGF-II, and IGF-I/IGFBP3 ratio which resulted in compensative elevation of growth hormone in severe-VSD infants, we proposed a novel approach to predict VSD condition in infants. The finding of these novel prognostic factors will help to define and screen the mild- and severe symptoms of patients, determine the necessity of surgery, and examine the efficiency of surgery. For the future, either overexpressimg by gene therapy or fortifying milk added IGF-I could be a good way to improve the VSD problem. All these finding should be helpful and essential for the VSD prediction and heart failure prevention.