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

（計畫名稱）

**自體骨髓間質幹細胞移植於急性心肌梗塞的迷你豬：** 分析心臟功能恢復的細胞/分子生物學機轉, 比較震波與非震波治療冠狀動脈攝影的結果, 及這些治療對心臟功能長期影響

計畫類別：■ 個別型計畫　　□ 整合型計畫

計畫編號：NSC　　96—2314—B—182A—132

執行期間：　　96 年 08 月 01 日 至 97 年 07 月 31 日

計畫主持人：**葉 漢 根**

共同主持人：王 清 貞, 許 俊 傑

計畫參與人員：吳 炯 仁, 孫灼均, 裴 松 南, 江 強 華, 梁馨仁

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：高雄長庚紀念醫院心臟血管內科

中　華　民　國　97 年 07 月 21 日

附件二

# 可供推廣之研發成果資料表

□ 可申請專利　■ 可技術移轉　日期：97 年 07 月 21 日

| | |
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| **國科會補助計畫** | 計畫名稱：**自體骨髓間質幹細胞移植於急性心肌梗塞的迷你豬：** 分析心臟功能恢復的細胞/分子生物學機轉,比較震波與非震波治療冠狀動脈攝影的結果,及這些治療對心臟功能長期影響<br>計畫主持人：**葉 漢 根**<br>計畫編號：96-2314-B-182A-132 學門領域：心臟血管內科 |
| **技術/創作名稱** | |
| **發明人/創作人** | |
| **技術說明** | 中文：中文：1）利用開胸的辦法將蘭嶼迷你豬的冠狀動脈左前降枝(left anterior descending artery)自中段後綁死作為心肌梗塞的模型.<br>2)骨髓間質幹細胞治療（以幹細胞治療**: 3.0 x $10^7$ cells**)梗塞心肌區(infarcted area)組 vs. 實驗 control 組(AMI treated by saline only)治療; 3）2-D echo evaluate 心臟功能的恢復; 4）評估骨髓間質幹細胞治療對於心臟功能恢復及阻止左心室 remodeling 的影響力(包括評估:梗塞區的厚度,減低左心室的擴張及改善左心室心縮功能）<br>英文：1) The mini-pig (Taitung Animal Propagation Station, Livestock Research Institute, Taiwan) was anesthetized by intramuscular injections of ketamine (15 mg/kg) and maintained with an inhalation of 1.5% isoflurane during the procedure.<br>2) After being shaved on the chest, the mini-pig was placed in supine position on a warming pad at 37 $^0$C and then endotracheally intubated with positive-pressure ventilatory support (180 mL/min.) with room air using a ventilator (Sn: Q422ZO, SIMS PneuPAC, Ltd.) during the procedure.<br>3) EKG monitor and defibrillator were connected to the chest wall of each mini-pig. One ample of amiodarone (150 mg) was intravenously given to each min-pig before the procedure.<br>4) Under sterile conditions, the heart was exposed through mid-thoracotomy. The pericardium was gently opened and the mid-LAD was ligated just after the first diagonal branch at two separated sites with 5-0 prolene suture. |

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| | 5) Regional myocardial ischemia was confirmed by EKG tracings and rapid color change from reddish to whitish-dark and then to reddish-black color of anterior surface of the LV, and the rapid development of akinesia as well as dilatation in the area at risk. AAWMI was confirmed by EKG finding following the procedure. LAD ligation was performed in 18 mini-pigs which were equally divided into group 1 [AMI plus saline (1000 μl) injection in IA, n = 6], group 2 [AMI plus BMDMNC transplantation into non-IA (i.e. the remote viable myocardium of left ventricle)], group 3 (AMI plus BMDMNC implantation into IA), and group 4 (sham control: Thoracotomy only without coronary artery ligation).<br>6) One-week cultured BMDMNCs ($3.0 \times 10^7$) in 1000 μL culture medium DMEM were immediately implanted into non-IA of group 2 and IA of group 3 following AMI induction.<br>7) The LV ejection fraction (LVEF) was calculated as follows: LVEF (%) = $[(LVEDD^3-LVEDS^3)/LVEDD^3] \times 100$ on day 90 following AMI induction<br>8) By 6 months after BMDMNC implantation, cardiac catheterization was performed using right common carotid artery approach. |
| | |
| **可利用之產業<br>及<br>可開發之產品** | |
| **技術特點** | To utilize an open heart surgery method to ligation of anterior descending artery. Therefore, acute anterior wall MI in mini-pig model was induced, followed by bone marrow stem cell implantation |
| **推廣及運用的價值** | 此方法有如下值得推廣及運用的價值：<br>1. creating an animal model of AMI for cellular therapy。<br>2. using mini-pig model of AMI that could be easily performed coronary angiographic study。<br>3. The anatomical structure of mini-pig is similar to human beings. Such a animal model, therefore, can more really reflect stem cell therapy in setting of AMI in human beings.<br>4. 從本研究結果顯示**自體骨髓間質幹細胞移植於急性心肌梗塞的迷你豬**對心臟功能, 心肌受損功能恢復重生, 有很不錯的效果。 |

※ 1. 每項研發成果請填寫一式二份，一份隨成果報告送繳本會，一份送貴單位研發成果推廣單位（如技術移轉中心）。

※ **2. 本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。**

**Six-Month Angiographic Study of Immediate Autologous Bone Marrow-Derived Mononuclear Cell Implantation on Acute Anterior Wall Myocardial Infarction using a mini-pig model**

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**Running title:** angiographic outcomes of bone marrow cell transplantation following myocardial infarction utilizing mini-pig model

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**ABSTRACT**

**Background** This study investigated six-month angiographic results of autologous bone marrow-derived mononuclear cell (BMDMNC) transplantation immediately following acute anterior wall myocardial infarction (AWMI) in a mini-pig model.

**Methods and Results** AWMI was induced by left anterior descending artery ligation. Twenty-four mini-pigs were equally divided into group 1 [AWMI plus saline injection in infarcted area (IA)], group 2 (AWMI plus BMDMNC transplantation into non-IA), group 3 (AMI plus BMDMNC implantation into IA), and group 4 (sham control). One-week cultured BMDMNCs ($3.0 \times 10^7$) were immediately transplanted following AWMI induction. Angiographic studies over six months demonstrated that mitral regurgitation (MR) was lower in groups 3 and 4 than in groups 1 and 2 (all $p<0.01$). Wall motion scores and left ventricular ejection fraction (LVEF) were higher in groups 3 and 4 than in groups 1 and 2 (all $p<0.05$). Collateral circulation was higher in group 3 than in groups 1 and 2 ($p<0.01$). The wall thickness of IA was higher, whereas the heart weight was lower in group 3 than in groups 1 and 2 (all $p<0.01$).

**Conclusion** Immediate autologous BMDMNC transplantation into IA is superior to saline-treated only or BMDMNC transplantation into non-IA following AWMI for reducing MR and improving LVEF.

**Key words:** acute myocardial infarction, bone-marrow cell transplantation, mini-pig, angiographic studies

# 迷你豬模型探討急性前壁心肌梗塞後移植自體骨髓間質幹細胞衍生單核細胞之六個月後心導管研究報告

**背景**: 本計劃以迷你豬模型探討急性前壁心肌梗塞後移植自體骨髓間質幹細胞衍生單核細胞(BMDMNC)之六個月後心導管研究。

方法: 急性前壁心肌梗塞引導乃經由冠狀動脈左前降枝綁死. 二十四隻迷你豬均分為四組,組 1 為急性前壁心肌梗塞引導後注射生理食鹽水心肌梗塞區域,組 2 為急性前壁心肌梗塞引導後注射自體骨髓間質幹細胞衍生單核細胞於非心肌梗塞區域,組 3 為急性前壁心肌梗塞引導後注射自體骨髓間質幹細胞衍生單核細胞於心肌梗塞區域,組 4 為對照組。急性前壁心肌梗塞引導後立即注射體外培養七天之骨髓間質幹細胞衍生單核細胞($3.0 \times 10^7$)至梗塞區域。六個月後血管攝影研究顯示二尖瓣閉鎖不全回流現象在組 3 及組 4 比組 1 與組 2 低(all $p<0.01$)。心室壁運動指數及左心室射出比例在組 3 及組 4 比組 1 與組 2 高($p<0.05$)。側支循環在組 3 比組 1 與組 2 高($p<0.01$)同時心室壁厚度在心肌梗塞區域也有相同情況,但是心臟重量在組 3 比組 1 與組 2 輕($p<0.01$)。

**結論**: 急性前壁心肌梗塞引導後立即注射骨髓間質幹細胞衍生單核細胞至心肌梗塞區域遠優於單純生理食鹽水或注射自體骨髓間質幹細胞衍生單核細胞於非心肌梗塞區域 此方法能降低二尖瓣閉鎖不全回流現象及改善左心室射出比例。

**鍵詞**:急性心肌梗塞,骨髓細胞移植, 迷你豬, 心導管研究

**A**cute myocardial infarction (AMI) is the leading cause of death of patients hospitalized for cardiovascular disease[1-3] mainly due to the fact that dead cardiomyocytes following AMI cannot be regenerated. Growing data, including those from animal models and limited clinical trials, have shown that autologous transplantation of bone marrow-derived mesenchymal stem cells (BMDMSCs) improves left ventricular (LV) function in the settings of ischemic cardiomyopathy and acute myocardial infarction (AMI).[4-8]

Although the majority of the investigators prefer utilizing small animals, e.g. either rats or mice, for their experimental models.[4,6,7,9], anesthesia can significantly suppress the heart rate, blood pressure, and especially LV contractility, resulting in inaccurate measurements using pressure transducers, electrocardiogram (EKG), and transthoracic echocardiography.[10,11] In addition, the anatomical distribution of coronary arteries in small animals is different from that in human beings that distinctively consists of the left main trunk, left anterior descending artery, and left circumflex artery. Furthermore, the LV end diastolic pressure, an index of left ventricular compliance, is difficult to be accurately measured in small animals due to their rapid heart rate. Hence, the findings from the use of small animal AMI model may not truly reflect the clinical picture of AMI. Moreover, the size of small animals precludes the application of coronary angiographic studies in assessing the impact of BMDMSC therapy on LV performance. Similarly, the effectiveness of BMDMSC implantation into the non-infarcted area (IA) in improving LV function cannot be easily assessed in small animals. To investigate the impact of BMDMNCs implantation in distinctive anatomical locations of the heart (i.e. IA vs. non-IA) and to study the subsequent changes in cardiac functions, a large animal model of AMI comparable to clinical setting is needed. Compared with human beings, the mini-pigs have many similar baseline characteristics, including the heart rate, anatomical distribution of coronary arteries, ratio of heart to body weight, the arterial blood pressure, LV pressure and LV ejection fraction. Furthermore, coronary angiographic studies and LV angiogram are easily evaluated without difficulty in a mini-pig model. Therefore, this study utilized a mini-pig AMI model by ligation of the middle left anterior descending artery (LAD) to investigate the impact of immediate autologous bone marrow-derived mononuclear cell (BMDMNC) implantation following acute anterior wall myocardial infarction (AAWMI) on cardiac function using angiography and echocardiography over a six-month period..

## Methods

*Ethics*

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at our hospital and performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, National Academy Press, Washington, DC, USA, revised 1996).

*Animals, Protocol and Procedure*

The mini-pig (Taitung Animal Propagation Station, Livestock Research Institute, Taiwan) was anesthetized by intramuscular injections of ketamine (15 mg/kg) and maintained with an inhalation of 1.5% isoflurane during the procedure. After being shaved on the chest, the mini-pig was placed in supine position on a warming pad at 37 $^{0}$C and then endotracheally

intubated with positive-pressure ventilatory support (180 mL/min.) with room air using a ventilator (Sn: Q422ZO, SIMS PneuPAC, Ltd.) during the procedure. EKG monitor and defibrillator were connected to the chest wall of each mini-pig. One ample of amiodarone (150 mg) was intravenously given to each min-pig before the procedure.

Prior to opening the chest wall, local anesthesia with 8 ml of 2% xylocain was injected into the third, fourth, and fifth intercostal spaces. Under sterile conditions, the heart was exposed through mid-thoracotomy. The pericardium was gently opened and the mid-LAD was ligated just after the first diagonal branch at two separated sites with 5-0 prolene suture (Fig 1). Regional myocardial ischemia was confirmed by EKG tracings (Fig 1) and rapid color change from reddish to whitish-dark and then to reddish-black color of anterior surface of the LV, and the rapid development of akinesia as well as dilatation in the area at risk (Fig 1). AAWMI was confirmed by EKG finding following the procedure.

LAD ligation was performed in 23 mini-pigs. Five mini-pigs were dead due to ventricular tachycardia/ventricular fibrillation (n = 3), injury of trachea during intubation (n = 1), and perforation of lung (n = 1) during the procedure. The remaining 18 mini-pigs were equally divided into group 1 [AMI plus saline (1000 μl) injection in IA, n = 6], group 2 [AMI plus BMDMNC transplantation into non-IA (i.e. the remote viable myocardium of left ventricle)], group 3 (AMI plus BMDMNC implantation into IA), and group 4 (sham control: Thoracotomy only without coronary artery ligation). One-week cultured BMDMNCs ($3.0 \times 10^7$) in 1000 μL culture medium DMEM were immediately implanted into non-IA of group 2 and IA of group 3 following AMI induction (Fig 1). Muscle and skin were closed in layers. Mini-pig remained on the warm pad and was allowed to recover under care.

*Preparation of BMDMNCs for Autologous Transplantation*

Under general anesthesia, BMDMNCs were aspirated from the iliac crest of group 2 and group 3 animals 1 week before AMI induction (Fig 1). The detailed procedures of separating BMDMNCs after aspiration and cell culture were performed according to our recently described method.[9] Briefly, the BMDMNCs were cultured in DMEM high glucose medium (supplemented with 10% bovine serum and antibiotics). Non-adherent hematopoietic cells were removed, and the medium was replaced. The adherent, BMDMNC population about $3.0 \times 10^7$ cells was obtained one week after they were first plated.

For 24 h cellular stimulation, 5-azacytidine (Sigma) [300 μL in 3mL DMEM-high glucose (10% FBS)] was added to the culture medium on day 3 following BMDMNC culture. On day 7, 30 min before implanting BMDMNCs, CM-Dil (Vybrant™ Dil cell-labeling solution, Molecular Probes, Inc.) [3 μL in 3 mL DMEM high glucose (serum free)] was added to the culture medium. This highly lipophilic carbocyanine dye, which has properties of low cytotoxicity and high resistance to intercellular transfer, can be added directly to normal culture media to uniformly label suspended or attached culture cells for their visibility in an implanted area due to the distinctive fluorescent colors.

*Functional Assessment by Echocardiography (Figure 2)*

With the animals in supine position, transthoracic echocardiography was performed preoperatively and on day 90 after AMI induction under general anesthesia as previously

described[12, 9] using a commercially available echocardiographic system (UF-750XT) equipped with a 8-MHz linear-array transducer for animals (FUKUDA Denshi Co. Hongo, Bunkyo-Ku, Tokyo, Japan). Left ventricular internal dimensions, including end-systolic diameter (ESD) and end-diastolic diameter (EDD), were measured according to the American Society of Echocardiography leading-edge method using at least 3 consecutives cardiac cycles.[13] The LV ejection fraction (LVEF) was calculated as follows:

$LVEF\ (\%) = [(LVEDD^3 - LVEDS^3)/LVEDD^3] \times 100$

All measurements were performed by an animal cardiologist blind to the treatment and non-treatment groups.

*Cardiac Catheterization and Definition (Figure 3)*

By 6 months after BMDMNC implantation, cardiac catheterization was performed using right common carotid artery approach. A 6-French pigtail was used for measuring the arterial blood pressure in ascending aorta, LV systolic and end diastolic pressure as well as performing left ventriculogram. Coronary angiographic study was performed using a 6-French Kimny guiding catheter (Boston Scientific, Scimed, Inc. Maple Grove, MN).

Left ventriculogram, which was immediately performed after the arterial sheath was inserted into right common carotid artery, was recorded for $30^0$ right anterior oblique and $60^0$ left anterior oblique views. The LVEF, LV contractility and the presence or absence of mitral regurgitation (MR) were determined by left ventriculographic study. Severity of MR was categorized into grades 1 (mild), 2 (moderate), 3 (moderate-severe) and 4 (severe) in accordance with traditional method. Coronary collateral flow was determined according to method previously published.[14]

The mini-pigs were sacrificed by intra-coronary injection of potassium chloride after the procedure. The heart was carefully removed and weighted with the infarct area excised for measuring wall thickness at papillary muscle level and for immunohistochemical study.

*Immunohistochemical Study*

Engraftment of troponin I-positive and CD31-positive BMDMNCs was assessed by examining the previously implanted areas after immunohistochemical labeling with respective primary antibodies, including anti-troponin I (Abcam) and anti-CD31 (Serotec) as well as secondary anti-mouse conjugate FITC antibody (Molecular Probe), followed by incubation for 30 minutes at room temperature. Irrelevant antibodies were used as negative controls.

*Measurement of Infarct Wall Thickness at Papillary Muscle Level*

To determine the wall thickness of the IA, cross sections at papillary muscle level of left ventricle were observed with three thickest regions chosen and the thickness measured for each mini-pig. The variables were further summated and divided by 3 for statistical analysis for each animal. All measurements were performed by a technician blinded to the treatment and non-treatment groups.

*Statistical Analysis*

Data were expressed as mean values (mean ± SD) or (%) of mini-pigs. The significance of differences between groups was evaluated with one-way analysis of variance. Continuous variables among 4 groups were compared using the Duncan's multiple comparison procedure. The data on MR which were not normally distributive were analyzed by Kruskal-Wallis test,

followed by multiple comparison procedure with the Wilcoxon's rank sum test and Bonferroni's correction. Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC). A probability value $< 0.05$ was considered statistically significant.

## Results

*Group Morbidity and Mortality*

No malignant ventricular tachyarrhythmia or mortality was noted in the AMI group with saline injection (group 1), AMI with BMDMNC implanted into non-IA (group 2), and the control group (group 4) within the study period. However, by two months after AMI induction, right forelimb infection was observed in 1 mini-pig of AMI with BMDMNC implantation into IA (group 3). To avoid transmission of infection to other mini-pigs, this animal was prematurely sacrificed. A replacement mini-pig was utilized following the same procedure and treatment as in group 2.

*Initial and Final Body Weight, Heart Weight and Serial Echocardiographic Findings (Table 1)*

The initial body weight did not differ among the four groups. Additionally, the final body weight did not differ between group 1 and group 2 and between group 3 and group 4. However, the final body weight was significantly lower in groups 1 and 2 than in group 3 and 4. The final heart weight was not different between group 3 and group 4. However, the final heart weight was significantly higher in group 1 than in group 2, 3 and 4, and significantly higher in group 2 than in group 3 and 4. Furthermore, the ratio of heart to body weight was significantly higher in group 1 and 2 than in group 3 and 4. We, therefore, speculated that the above findings, including body weight and heart weight, implicate that the congestive heart failure should develop in group 1 and 2.

There was no significant difference in initial LVEF, LVEDD, or LVESD among the four groups. On day 90 following AMI induction, the LVEF and LVESD did not differ between group 1 and 2. Additionally, the LVEDD was also similar among group 1, 2 and 3. However, the LVEF was remarkably higher, whereas the LVESD was notably lower in group 3 and 4 than in group 1 and 2. Furthermore, the LVEF was significantly lower, whereas the LVESD was significantly higher in group 3 than in group 4.

*Six-Month Angiographic Follow-Up Results (Table 1 and Figure 3)*

By six months after AMI induction, cardiac catheterization was performed via right common carotid artery for each group of mini-pigs. There were no significant differences in terms of heart rate, systolic and diastolic blood pressure in ascending aorta, and LV systolic blood pressure among the four groups. The LV end diastolic blood pressure tended to be higher in group 1 and 2 than in group 3 and 4, although there was no statistical significance. These findings indicate that the compliance of left ventricle was poorer in group 1 and 2 than in group 3 and 4 following AMI induction.

Left ventriculogram demonstrated that LVEF was significantly higher in group 2 than in group 1. Additionally, LVEF was remarkably higher in group 3 and 4 than in group 1 and 2, and significantly higher in group 4 than in group 3. These findings highlight the positive impact of BMDMNC transplantation on LV function after AMI and further indicate that the effect of BMDMNC implantation in IA is better than the BMDMNC implantation in non-IA for improving

heart function.

The wall motion of the individual segment, including anterobasal, anterolateral, apical diaphragm, and posterobasal, was also evaluated by left ventriculographic study. The results demonstrated that the wall motion of anterobasal, anterolateral, and posterobasal was significantly higher in group 3 and 4 than in group 1 and 2. These findings implicate an improvement in contractility of left ventricle after BMDMNC implantation into the IA following AMI.

The mean grade of coronary collateral circulation was significantly higher in group 3 than in group 1 and 2. Interestingly, more than 80% of collaterals were intra-coronary rather than inter-coronary in the mini-pigs. These findings implicate that the collateral circulation was elicited by BMDMNC implantation rather than by spontaneous development.

To investigate whether ischemic-related MR is present following AMI induction and whether BMDMNC implantation can amenriorate this complication, the degree of MR was evaluated by biplane left ventriculogram. As expected, MR was significantly higher in group 1 and 2 than in group 3 and 4. This finding indicates that BMDMNC implantation in IA markedly inhibits the development of ischemic-related MR after AMI.

*Identification of Implanted BMDMNCs in Infarct and Non-Infarct Areas (Figure 4)*

By day 90, numerous Dil-stained undifferentiated BMDMNCs were identified in IA (Fig 4-A). Additionally, some Dil-stained engrafted cells present as troponin I-positive myogenic-like cells were also identified in IA (Fig 4-A). By six months following transplantation, numerous Dil-stained undifferentiated BMDMNCs were engrafted into the IA and non-IA (Fig 4-B). On the other hand, only some Dil-stained engrafted cells present as troponin I-positive myogenic-like cells in the IA and non-IA (Fig -B). Moreover, some Dil-stained engrafted cells positive for CD31 (an endothelial cell surface marker) were also clearly identified in IA and non-IA (Fig 4-B).

*Measurement of the Wall Thickness in Infarct Area (Table 1)*

The wall thickness in IA at the level of papillary muscle was significantly thinner in group 1 and 2 than in group 3 and 4, and significantly thinner in group 3 than in group 4. This finding indicates that BMDMNC implantation into the IA may be able to repair the infarct myocardium.

## Discussion

*Evidences of angiogenesis/Vasculogenesis and Long-term Survival of BMDMNCs at the Implantation Sites*

Although there is evidence showing the survival of BMDMSCs in ischemic LV myocardium for up to three months after implantation,[4,6,7,9] the long-term survival of implanted BMDMSCs in an ischemic-related organ is currently unclear. One of distinctive findings in the present study was that engrafted autologous BMDMNCs in IA and non-IA of the LV myocardium can survive for more than six months. Therefore, our findings further support and extend the findings of the recent studies.[4,6,7,9]

Angiogenesis and vasculogenesis in the infarcted LV myocardium were identified in the IA three months after autologous BMDMNC implantation in a rat AMI model in our recent study.[9] Interestingly, in the present study, we also found that implanted BMDMNCs participated in

angiogenesis/vasculogenesis (indirectly proved by the presence of CD31-positive cells) in IA. Therefore, the findings of the present study corroborated with those from our recent study.[9] Also of importance in the current study was that angiographic investigation after six months demonstrated that the majority of collateral circulation was intra-coronary rather than inter-coronary. Furthermore, the incidence of collateral circulation was significantly higher in group 3 than in group 1 and 2. Taken together, our basic and angiographic findings support that BMDMNC therapy elicits angiogenesis/vasculogenesis which in turn enhances intra-coronary collateral circulation in the infarcted myocardium.

*Prevention of LV remodeling following Autologous BMDMNC Therapy*

LV remodeling following AMI, as a consequence of LV dilatation and pump failure, largely accounts for poor clinical outcomes.[15-17] Severity of pump failure typically depends on infarct size and ischemic area.[15-18] In the present study, the principal finding was the significantly elevated LVEDD and LVESD in group 1 and 2 than in group 4 (i.e. control group) on 90-day transthoracic echocardiographic study. Conversely, LVEF was significantly lower in group 1 and 2 than in group 4. However, LVEDD and LVESD were lower in group 3 than in group 1 and 2, whereas LVEF was significantly higher in group 3 than in group 1 and 2. Additionally, the wall thickness of the IA was significantly increased in group 3 than in group 1 and 2. These findings suggest that immediate autologous BMDMNC transplantation into IA, in addition to improving LV function, also effectively prevented LV remodeling early after AMI.

*Implications of Six-Month Angiographic Findings*

The exact mechanisms underlying LV functional improvement after BMDMSC therapy remain uncertain.[5,7,9,19] In fact, proposed mechanisms, including angiogenesis/vasculogenesis,[4,5,9] myogenesis,[4,20] chemokine effects,[5,9] effect of paracrine mediators,[7,20,21] or a myocardial homing by bone marrow-derived mesenchymal stem cells to the myocardium for angiogenesis and repair,[23,23] have been extensively debated. The most important finding in the current study was that LV wall motion was notably improved and LVEF was significantly preserved following BMDMNC implantation into the IA of LV myocardium. Surprisingly, immunohistochemical investigation demonstrated that only a small proportion of implanted BMDMNCs differentiated into myogenic-like cells in the IA. Conclusively, our findings, based on both bench works and angiographic studies, suggest that the improvement in heart function following BMDMNCs implantation to the infarcted myocardium could be due to a broad-spectrum of mechanisms rather than a single one.

Ischemic-related MR following AMI has been well recognized in many clinical observational studies.[24-26] An association between ischemic-related MR and poor prognostic outcome has been extensively debated in patients following AMI with or without undergoing primary coronary angioplasty.[24-27] Another distinctive finding in the present study was the high incidence of MR observed in group 1 and 2 of mini-pigs following AMI induction. Interestingly, the incidence of MR was substantially reduced in group 3. Our findings, in addition to strengthening the findings of previous clinical observational studies,[24-27] further suggest that immediate implantation of BMDMNCs to the IA following AMI can abrogate the development of ischemic-related MR. The result may raise a need for prospective clinical trials for further

elucidation of the safety and effectiveness of BMDMNC therapy for ischemic-related MR in human subjects.

*Study limitations*

This study has several limitations. Firstly, the sample size was relatively small. Secondly, six-month follow-up may be still not long enough to extrapolate the favorable results to the conclusion of improved long-term outcomes. Finally, the design protocol of this study did not provide a satisfactory parameter for evaluating the incidence and severity of AMI-induced congestive heart failure in the studied animals.

## Conclusion

The present study demonstrated that autologous BMDMNC transplantation into the IA in acute phase of AWMI provides persistent benefit of improving heart function and abrogating ischemia-induced MR. These findings encourage us to prospectively investigate the application of BMDMNC therapy in the clinical setting of AMI.

## Acknowledgement

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**Table 1 Summarized Data on Body Weight, Final Heart Weight, LV Dimension, LV Function and 6-Month Angiographic Results in Four Groups of Mini-Pigs**

| Variables | Group 1* (n = 6) | Group 2* (n = 6) | Group 3* (n = 6) | Group 4* (n = 6) | p value |
|---|---|---|---|---|---|
| Initial BW (kgm) | 17.1 ± 0.9 | 17.1 ± 0.7 | 16.9 ± 1.0 | 16.8 ±1.0 | 0.910 |
| Final BW (kgm)† | 20.0 ± 1.7[a] | 20.8 ± 1.3[a] | 25.1 ± 1.2[b] | 26.6 ± 1.5[b] | <0.0001 |
| Final HW (gm)† | 133.8 ± 4.5[a] | 127.6 ± 6.5[b] | 105.8 ± 4.3[c] | 102.0 ± 3.6[c] | <0.0001 |
| Ratio of HW to BW (x $10^{-3}$)† | 6.6 4 ± 0.86[a] | 6.01 ± 0.61[a] | 4.23 ± 0.29[b] | 3.84 ± 0.28[b] | <0.0001 |
| Initial LVEF (%) | 70.5 ± 7.3 | 68.7 ± 5.8 | 71.3 ± 5.6 | 70.3 ± 5.9 | 0.904 |
| Initial LVEDD (mm) | 3.06 ± 0.43 | 3.32 ± 0.27 | 2.99 ± 0.23 | 3.07 ± 0.28 | 0.300 |
| Initial LVESD (mm) | 1.87 ± 0.36 | 2.05 ± 0.11 | 1.93 ± 0.33 | 1.86 ± 0.19 | 0.610 |
| 90-day LVEF (%)† | 46.8 ± 4.0[a] | 50.0 ± 3.7[a] | 58.1 ± 3.6[b] | 71.4 ± 5.1[c] | <0.0001 |
| 90-day LVEDD (mm)† | 3.68 ± 0.23[a] | 3.78 ± 0.19[a] | 3.44 ± 0.29[a] | 2.93 ± 0.38[b] | 0.0002 |
| 90-day LVESD (mm)† | 2.94 ± 0.23[a] | 2.95 ± 0.15[a] | 2.43 ± 0.27[b] | 1.74 ± 0.24[c] | <0.0001 |
| 6-month angiographic results | | | | | |
| Heart rate (beat/minute) | 99.8 ± 13.9 | 98.0 ± 22.7 | 98.2 ± 14.5 | 103.0 ± 8.2 | 0.941 |
| AsAo SBP (mmHg) | 127.3 ± 25.2 | 120.8 ± 34.4 | 130.5 ± 34.4 | 117.2 ± 12.8 | 0.841 |
| AsAo DBP (mmHg) | 74.3 ± 31.5 | 77.3 ± 35.2 | 94.8 ± 34.7 | 76.3 ± 11.8 | 0.619 |
| LV-SBP (mmHg) | 141.2 ± 44.9 | 151.7 ± 29.5 | 142.8 ± 29.6 | 126.0 ± 19.6 | 0.588 |
| LV-EDBP (mmHg) | 19.2 ± 5.9 | 19.8 ± 9.6 | 13.7 ± 4.8 | 10.7 ± 5.6 | 0.083 |
| LVEF (%) by ventriculogram† | 33.3 ± 9.5[a] | 43.8 ± 10.0[b] | 58.3 ± 11.0[c] | 64.0 ± 6.4[c] | <0.0001 |
| Wall motion (%) | | | | | |
| anterobasal† | 14.3[a] ± 5.1 | 16.0[a] ± 13.3 | 27.0[b] ± 5.7 | 33.0[b] ± 9.9 | 0.005 |
| anterolateral† | 12.0[a] ± 8.4 | 19.5[b] ± 13.0 | 30.2[c] ± 9.3 | 33.7[c] ± 10.2 | 0.006 |
| apical | 12.8 ± 9.1 | 11.5 ±1.8 | 15.0 ± 8.3 | 18.8 ± 7.4 | 0.343 |
| diaphragmatic | 19.0 ± 6.3 | 16.5 ± 7.8 | 28.7 ± 12.1 | 29.5 ± 11.2 | 0.064 |
| posterobasal† | 18.0[a] ± 8.4 | 17.8[a] ± 1.7 | 27.8[b] ± 6.5 | 23.8[a, b] ± 6.6 | 0.034 |
| Ischemia-related MR‡ | 2.17[a] ± 1.0¶ | 2.17[a] ± 1.33¶ | 0.33[b] ± 0.82¶ | 0[b] ± 0¶ | 0.002 |
| Collateral circulations‡ | 0.50 ± 0.55¶ | 0.83 ± 0.41¶ | 2.83 ± 0.41¶ | 0 ± 0¶ | 0.0008 |
| Wall thickness of IA (mm) | 0.69 ± 0.10[a] | 0.80 ± 0.10[a] | 1.14 ± 0.13[b] | 1.40 ± 0.05[c] | < 0.0001 |

Data are expressed as mean value ± SD or (%) of mini-pigs.

AsAo SBP = ascending aorta systolic blood pressure; BW = body weight; DBP = diastolic blood pressure; EDBP = end diastolic blood pressure; HW= heart weight; LVEF = left ventricular ejection fraction; LVEDD = left ventricular end diastolic dimension; LVESD = left ventricular systolic dimension; MR = mitral regurgitation.

* Group 1 = acute myocardial infarction (AMI) by saline treated in infarcted area (IA); Group 2 = AMI plus bone marrow-derived mononuclear cells (BMDMNC) implantation into non-IA; Group 3 = AMI plus BMDMNC implantation into the IA; Group 4 (sham control).

**Figure Legends**

**Figure 1.** **A)** Echocardiographic examination. **B)** Aspiration of bone marrow-derived mononuclear cells (BMDMNCs) from iliac crest of min-pig. **C)** BMDMNC aspiration. **D)** Ligation of anterior descending artery (LAD). **E)** Reddish-black discoloration of left ventricular (LV) anterior wall (yellow arrows) just after successful LAD ligation. **F)** BMDMNCs implantation into infarct area one week after culture. **G)** Electrocardiogram (EKG) monitor showed acute ST-segment elevation after LAD ligation (Note: Time interval between two strips was five minutes).

**Figure 2.** Echocardiographic examinations before and on day 90 after AMI induction. Good LV performance was observed before acute myocardial infarction (AMI) induction (**A and C**). Improved left ventricular ejection fraction (LVEF) in AMI treated by BMDMNC implantation (**B**) than by saline (**D**) (56% vs. 45%, respectively). Better septal motion (yellow arrows) with less dilated LV chamber in BMDMNC therapy group than in saline-treated group.

**Figure 3-A.** AMI treated with saline only (Upper panel), with BMDMNCs implanted into remote viable LV myocardium [defined as non-infarct area (non-IA)] (Middle panel), and with BMDMNCs implanted into infarct area (IA). **A, D and G)** Mid-LAD ligation just after first diagonal branch (yellow arrows). Better antegade flow in **(G)** than in **(A)** and **(D)**. **B, G and H)** No collateral circulation from right coronary artery to LAD. Notably higher LVEF in **(I)** than in **(C)** and **(F)** (57% vs. 34% and 44%, respectively).

**Figure 3-B.** Sham control plus BMDMNC therapy (Upper panel). Sham control group (Middle panel). **A and D)** Normal LAD. **B and E)** Normal right coronary artery. Note similar LVEF between (**C**) and (**F**).

**Figure 4-A.** Confocal imaging studies on day 90 after AMI induction in a mini-pig. **A)** Implanted BMDMNCs (yellow arrows) identified in IA on day 90 after implantation with CM-Dil staining. **B)** Positive troponin I staining in IA, indicating viable myocardium in IA. **C)** Merged image after double staining with CM-Dil and Troponin I **(A)** and **(B).** Double staining with CM-Dil and troponin I indicating differentiation of only a minority of implanted BMDMNCs into myogenic-like phenotype (yellow arrows) with the majority of IA-engrafted BMDMNCs staying undifferentiated (red arrows). Nuclei counter-stained by 4',6-diamidino-2-phenylindole (DAPI) (blue color). Scale bars in right lower corner represent 200 μm.

**Figure 4-B.** Confocal imaging studies six months after AMI induction.

CD31 (surface maker of endothelial cell) staining (Upper panel). **A)** AMI treated by saline only. CD31-positively stain cells (red arrows) observed in both longitudinal and cross sections, indicating some viable small vessels in IA. **B)** AMI treated by BMDMNCs implantation into IA, showing numerous Dil-positive and undifferentiated BMDMNCs (yellow arrows). Doubly stained (CM-Dil and CD31) cells also frequently observed in IA (red arrows), indicating differentiation of implanted BMDMNCs into endothelial phenotype. **C)** AMI treated by BMDMNCs implantation into non-IA, showing CD31positive staining (red arrows). **D)** Sham control. CD31-positively stained cells identified in an intact vessel (cross section), implicating development of angiogenesis following BMDMNC implantation [**(C)** and **(D)**].

Troponin I staining (Lower panel). **E)** AMI treated by saline only. **F)** AMI treated by BMDMNCs implantation into IA. Identification of only a minority of BMDMNCs having differentiated into myogenic-like cells (double stains of CM-Dil and Troponin I) in IA (greenish arrows). **G)** AMI treated by BMDMNCs implantation into non-IA, showing CM-Dil positively stained BMDMNCs. **H)** Positive troponin I staining in sham control indicating intact myocardium.

Nuclei counter-stained with 4',6-diamidino-2-phenylindole (DAPI) (blue color). Scale bars in right lower corner represent 200 μm.

**Figure 1**

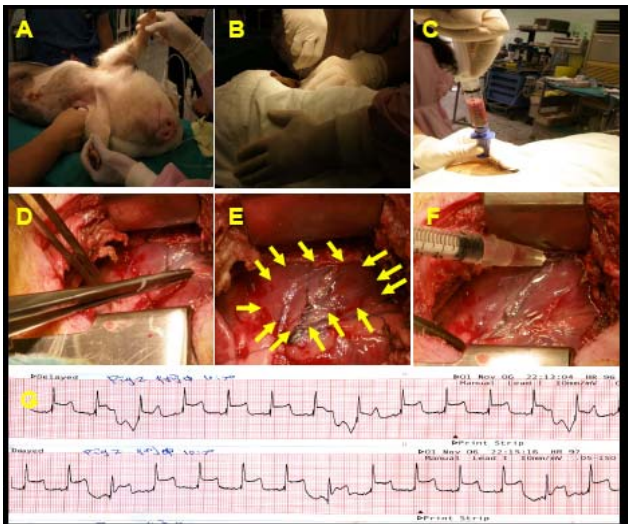



**Figure 2**

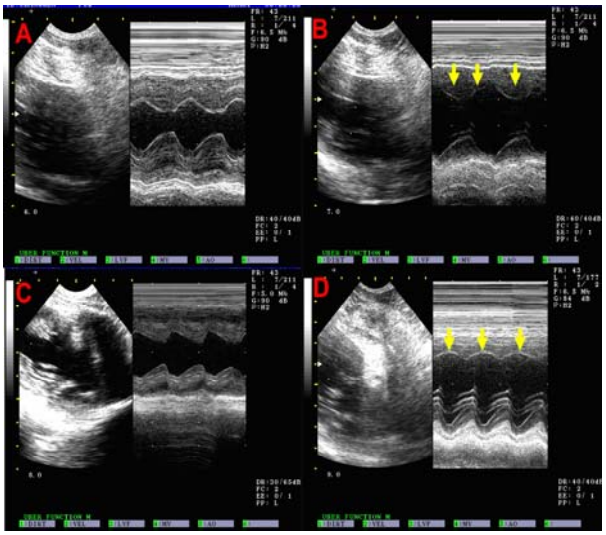



**Figure 3-A**

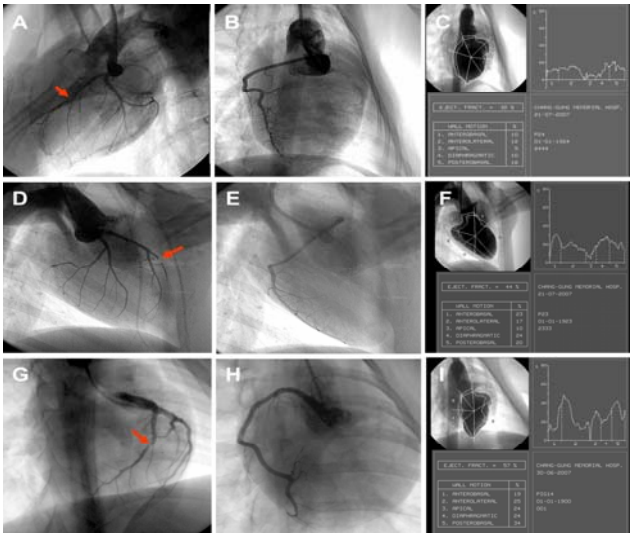



**Figure 3-B**

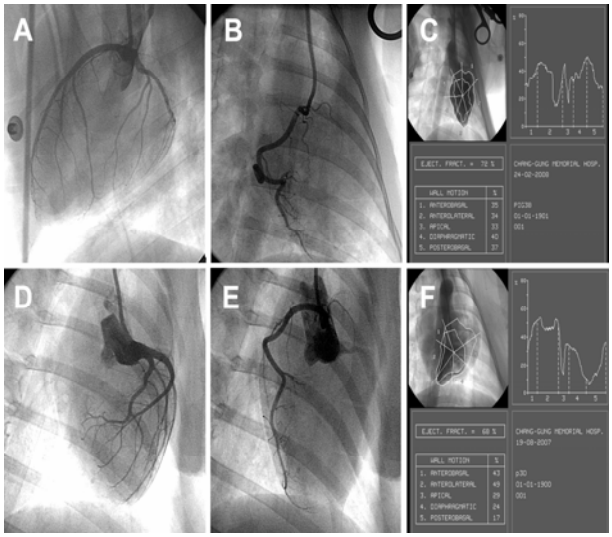



**Figure 4-A**

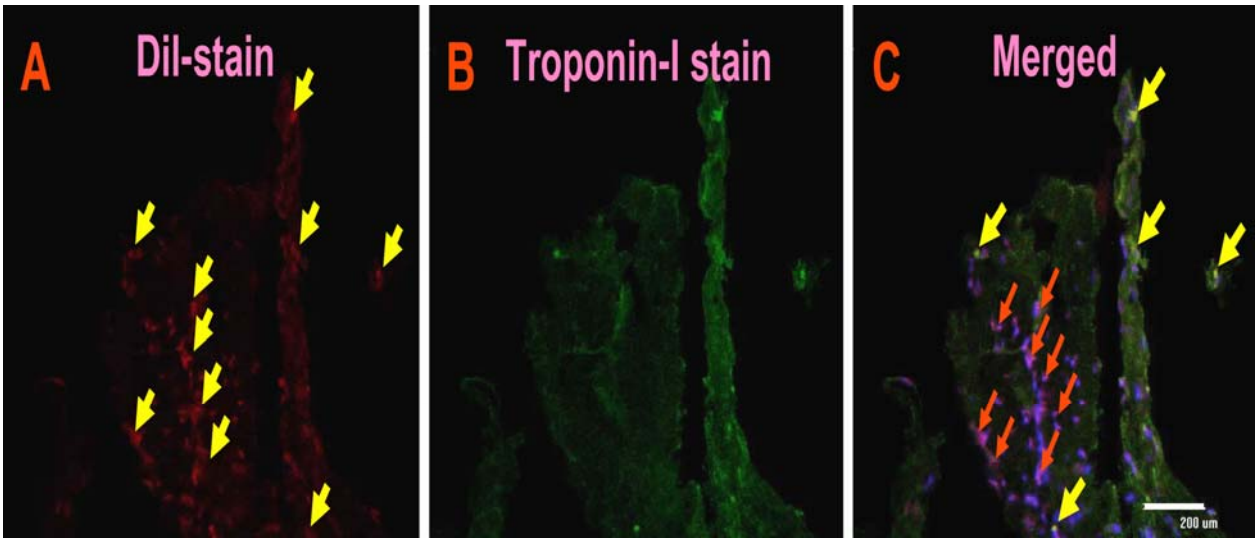
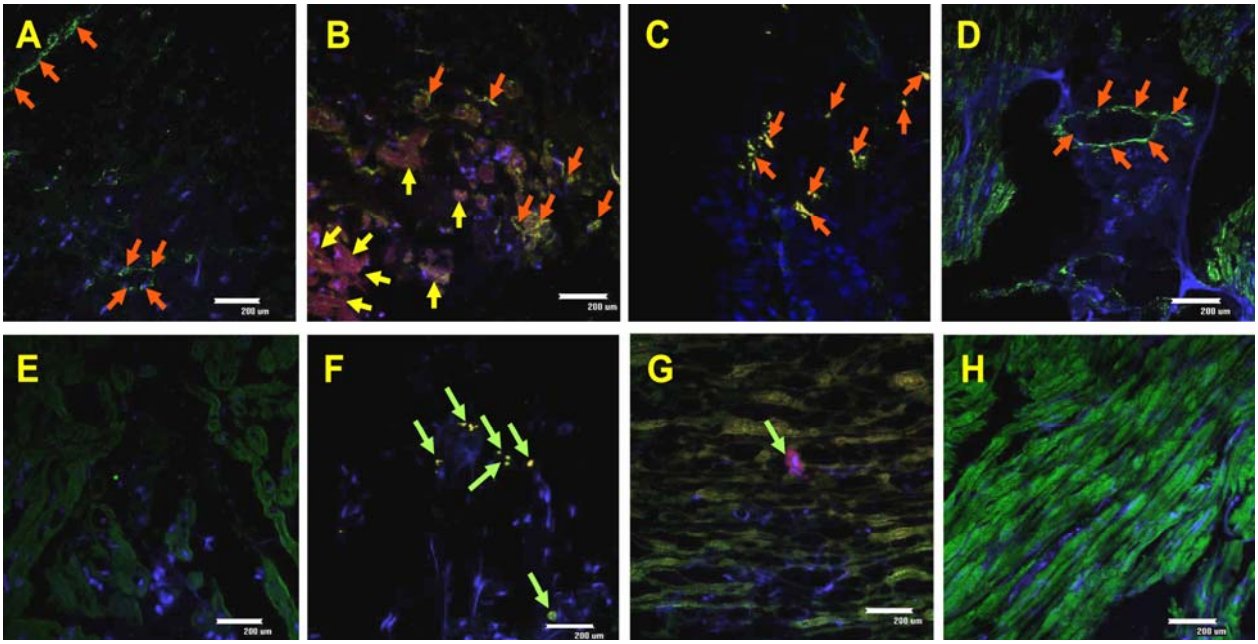


**Figure 4-B**




**自我評比(SELF ESTIMATION)**

我們很高興我們能完成國科會計劃,到目前為止,我們已投出去 1 份稿件。

本研究結果發現幾項重要訊息:

1. 利用開胸的辦法將蘭嶼迷你豬作為心肌梗塞的模型for**幹細胞治療**study能提供更多好的效果。
2. **利用骨髓間質幹細胞治療心肌梗塞壞死的心肌的長期預後及心臟功能的持續性改善程度**有很好的幫助, 且結果令人鼓舞。
3. 這個研究將會使我們進一步了解在心肌梗塞後骨髓間質幹細胞治療的分子生物機轉(molecular mechanisms)。同時，提供以後心血管疾病治療的新方向。
4. 希望將來在國科會的經費支持下做更大型的這方面的研究。