國立臺灣大學臨床醫學研究所

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細胞間隙接合於心肌細胞自噬之角色

Cardiomyocyte Gap Junction Intercellular Communication and Autophagy

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口試委員會審定書

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Cardiomyocyte gap junction intercellular Communication and autophagy

本論文係 陳盈憲 君（P97421017）在國立臺灣大學臨床醫學研究所完成之碩士學位論文，於民國 99 年 6 月 23 日承下列考試委員審查通過及口試及格，特此證明

口試委員：陳明豐（簽名）

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（是否須簽章依各院系所規定）

誌謝

寫這篇序言時，代表已進入撰寫臨床醫學研究所論文的尾聲，吾所投入之研究題材「細胞間隙接合於心肌細胞自噬之角色」從研究之研擬、討論乃至實驗進行，一直到論文撰寫，整個過程，一路走來之體驗，雖未必是篳路藍縷或披荊斬棘，但也可稱得上血汗交織、百感交集。憶兩年前，受到陳明豐教授的鼓勵，懵懂地踏上了醫學研究之路，選擇了自己不熟悉的道途-基礎研究；然而，在臨床、家庭與課業三頭燒的情況下，僅能在有限時間的空隙中擠進研究的時程表，幸得陳教授與何奕倫教授適時給予提綱挈領地指導下，這個實驗有了最重要的骨幹，並繼續向前，乃至完竣。實驗過程仰賴的是臨床醫學研究所裡研習而來的實驗方法，以及不斷重複試驗的研究精神填補而成，在此特別感謝實驗室許老師、承先、如慶、寶珠、佩玟、第三共研同仁以及臨床醫學研究內師長之經驗分享，讓整個實驗化為可能。回想多少的夜裡，獨自手持實驗手札，一人挑燈夜戰，培養細胞、萃取核糖核酸、電泳、做聚合酵素鏈鎖反應等，一次又一次失敗的實驗幾乎耗盡了殘存的氣力，澆熄了僅有的熱忱。然而，當實驗有了一點點結果時，彷彿又亮起一絲曙光。箇中滋味，實不足為外人道也。實驗終於告個段落，雖未稱完美，但也唯有自身體驗整個過程，乃將它的不完美視為美麗。我知道，今日只是醫學研究之路的開端，我將有機會更進一步見識到這殿堂的廣大。

這段期間內，還要感謝李啟明教授在許多方面的提攜以及心臟科同仁的協助。感謝距我 253 公里遠的至親，我與你們內心零距離；感恩岳父母的關懷與體諒，謝謝你們視我如子；感謝吾兒泓旭開心地成長，大寶你真的很乖；感謝佳鈴奔波斗六與台北兩地，默默地、持續地支持與幫忙。

最後僅將此論文獻給曾經協助過我的師長、朋友與家人，謝謝你們。

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研究背景

細胞之生死存亡的命運，可能與細胞所接受之外在環境刺激以及相鄰細胞所傳遞的訊號有關。而當中，細胞間隙接合可能是傳遞細胞間存活或死亡訊號的重要媒介。細胞自噬長久來被認為是一種細胞保護機制，透過細胞內部調控以達到清除壞損胞器或蛋白質的功能，進而促成延續細胞生存之目的。細胞自噬之研究者，莫不期望有朝一日能將調控細胞自噬化身為治療與預防疾病的利器。而目前有諸多研究顯示，細胞內部具同時調控細胞自噬與細胞凋亡的機制，細胞依據存在的環境狀況、所承受環境刺激之程度以及細胞壞損嚴重度，來決定細胞開啟何種生理機制；當中細胞內部的細胞自噬調控已被廣泛研究，但細胞間之調控研究卻付之闕如。反觀細胞凋亡之細胞間調控之研究，已證實細胞間隙接合可能是細胞間傳遞與溝通細胞凋亡訊號的重要窗口；為了讓細胞自噬之調控更為深入，進而運用這些調控方法於醫療使用，細胞間調控細胞自噬之研究的進行有其必要性。

研究目的

本實驗主要探討心肌細胞間隙接合對於心肌細胞於承受氧化壓力刺激引發細胞自噬中所扮演的角色。我們假設心肌細胞利用細胞間隙接合傳遞細胞之間的細胞自噬之訊號。

研究方法

我們使用 H9c2 作為實驗之模型，使用正庚醇(1-Heptanol)作為抑制細胞間隙接合之藥劑，並使用過氧化氫(Hydrogen peroxide, H_2O_2)作為氧化壓力源以誘發細胞自噬表現。細胞自噬偵測方式包括分析 Light chain 3 (LC3)與 Beclin-1 之蛋白質與 mRNA 表現，同時利用流式細胞儀分析 Acridine orange 染色陽性比例。

本實驗進行，共分成三大部分:

實驗第一部分，計畫建立自身實驗室常模，探討 H9c2 細胞接受正庚醇以及過氧化氫之作用後之細胞反應，並尋求最適當的正庚醇與過氧化氫作用條件。

實驗第二部分，計畫合成 pCMV-Lc3-Gluc 質體，並將此質體以短暫轉染方式植入 H9c2 細胞內。同時檢驗經 pCMV-Lc3-Gluc 轉染之 H9c2 細胞能否作為偵測 Light Chain 3 (LC3)表現之 Luciferase Report System。

實驗第三部分，計畫在標準的細胞培養條件下我們將 wild type H9c2 細胞以及含 Luciferase Report System 之 H9c2 (轉染 pCMV-Lc3-Gluc 之 H9c2)各分成四大組包括: 第一組(Control)、第二組(Heptanol)、第三組(H_2O_2)以及第四組(H_2O_2+Heptanol)。透過比較第三組(H_2O_2)與第四組(H_2O_2+Heptanol)，我們進而判斷使用細胞間隙接合抑制劑，對於細胞表現細胞自噬與細胞凋亡之影響。

實驗結果

第一部分實驗: 我們發現使用 100 至 400μM 過氧化氫處理超過六小時可誘發細胞自噬表現，而當過氧化氫作用濃度超過 200 μM 時，可能會同時誘發細胞凋亡，減少 Connexin 43 表現，以及降低細胞之存活率。使用正庚醇將不影響 Connexin 43 之表現，同時長時間使用正庚醇將不影響細胞之存活。

第二部分實驗，我們成功的合成 pCMV-Lc3-Gluc 質體，並將該質體成功轉染到 H9c2 細胞內以做為 Gaussia Luciferase Report System，透過施予氧化壓力 400μM 過氧化氫之刺激驗證發現，大約於作用 8 小時後，LC3 表現量已明顯增加，作用至 24 小時後，LC3 表現量增加 38% (n=4, P<0.001)。

第三部分實驗，我們發現使用正庚醇抑制細胞間隙接合將使承受過氧化氫刺激之 H9c2 細胞減少 24.2%之 LC3 mRNA 表現(P>0.05, n=3)；透過 Gaussia Luciferase Report Assay，我們發現正庚醇於作用 16 小時後將使承受過氧化氫刺激之 H9c2 細胞減少 20.6% LC3 表現量 (P<0.001, n=4)。

結論與討論

我們研究發現抑制心肌細胞之細胞間隙接合，將使細胞自噬之表現減少。顯示細胞自噬，在可能需要透過細胞間隙接合達到傳遞、延續或增強細胞自噬表現。至於細胞間隙所傳遞的訊息為何? 細胞接受到細胞自噬訊號後究竟如何決定細胞之生或死的命運? 以及是否還有其他細胞間的調控機制，則需要進一步實驗來印證與回答。

關鍵字: 細胞自噬、氧化壓力、間隙接合、過氧化氫、正庚醇

英文摘要 (abstract)

Study background: The gap junction intercellular communication may involve in signal exchange for both cell death and cell survival. The cell destiny depends on the cell status and environmental context of the cell that receives the signals. Autophagy was once considered as a protective mechanism in cells. Through removing the diseased organelles or protein, autophagy functioned in maintaining cellular homeostasis, nutrient or energy preservation and providing cell survival. Scientists who devoted in autophagy study are trying to transform autophagy concept into therapeutic tool enthusiastically. However, the best studied intracellular regulation of autophagy was known to be complex, and in the contrast the interaction with apoptosis was well-documented. In fact, the intercellular communication of autophagy signal between cells was never investigated and the role of gap junction in autophagy was unknown.

Study purpose: We investigate the contribution of gap junction to cardiomyocyte autophagy which was produced by oxidative stress. We hypothesized that autophagy signal between individual cardiomyocyte was conducted through gap junction.

Material and methods: We use H9c2 cell line as study model, 1-Heptanol as gap junction uncoupler, and hydrogen peroxide as oxidative stresser for autophagy induction. Autophagy activity was investigated by reverse-transcription polymerase chain reaction (RT-PCR) and Western blot for light chain 3 and Beclin-1 detection. Quantity analysis

of autophagy was determined by densitometry by Alpha Innotech and Gaussia luciferase report assay after transfecting pCMV-Lc3-Gluc to H9c2 cell. Our study composed of 3 main parts. The first part of the study was designed to establish the laboratory model. We test the effects of 1-Heptanol and hydrogen peroxide on H9c2 and measure the change of autophagy expression, connexin 43 expressions and survival. The second part of the study included constructing pCMV-Lc3-Gluc plasmid and transfecting the plasmid into H9c2. The whole system was taken as Gaussia luciferase report system. We determined the effect of transfection on autophagy and testify the feasibility of Gaussia luciferase report system for the role of reporting LC3. During the third part of study, we divided wild type H9c2 and Gaussia luciferase report system transfected H9c2 into 4 groups, including the Control group, the Heptanol group, the H_2O_2 group and the H_2O_2+Heptanol group. In the H_2O_2+Heptanol group, 1-Heptanol was administered for gap junction blockade along with hydrogen peroxide for generating oxidative stress-induced autophagy. In the Heptanol group, we use 1-Heptanol only. We also setup negative control (the Control group) using DMEM culture medium and positive control with hydrogen peroxide only (the H_2O_2 group).

Results: We found that 100 to 400μM hydrogen peroxide could induce autophagy in H9c2 when treatment duration is longer than 6 hours. However, higher concentration of hydrogen peroxide (>200μM) might decrease H9c2 survival measured by MTT assay. When hydrogen peroxide treatment was longer than 14 hours, H9c2 Cx43 expression

might reduce. Heptanol when used at 0.5mM will not decrease Cx43 expression. Blocking gap junction by 1-heptanol (H_2O_2+Heptanol group), we found there is about 24.2% reduction of LC3 mRNA expression when compare to H_2O_2 group which use hydrogen peroxide ($P>0.05$, $n=3$). Using Gaussia luciferase report assay, the LC3 expression was attenuated by 20.6 % when compare to H9c2 cell that receive 1-Heptanol pre-treatment before hydrogen peroxide stressing ($P<0.001$, $n=4$).

Conclusion: Blocking the gap junction inter-cellular communication showed trends in decreasing oxidative stress induced autophagy activity. It's possible that autophagy regulation required complex machinery which involved autocrine, paracrine and probable gap junction intercellular communication. Further studies are required to figure out the exact signal molecules, the true effect of autophagy flux and the existence other intercellular regulation.

Key words: autophagy, oxidative stress, H9c2, gap junction, Heptanol, hydrogen peroxide, connexin

第一章 緒論

1.1 細胞自噬

細胞自噬(Autophagy)首次於文獻中被的描述，大約要朔及四十到五十年前(Clark, 1957; De Duve and Wattiaux, 1966)，但真正對於此**溶小體**(Lysosome)相關的細胞機制之研究發展，則是最近十年才快速累積。當中包括對細胞自噬之誘發、調控與生物角色與功能定位的研究等(Kroemer and Levine, 2008; Mizushima et al., 2008)。細胞自噬為生物體於演化過程中持續被保存下來的細胞反應，整體而言，細胞自噬是一種非選擇性分解機制，目前已觀察有三大類之細胞自噬，包括(1) Macroautophagy (Yorimitsu and Klionsky, 2005)、(2) Microautophagy (Kunz et al., 2004)與(3) Chaperon-mediated Autophagy (Dice, 2007)。

以研究最為透徹之 Macroautophagy 為例，細胞受傷害性刺激後，細胞內逐漸形成雙層膜之細胞自噬體(Autophagosome)，成熟之細胞自噬體隨後透過與溶小體的結合形成 Autolysosome; 於 Autolysosome 中細胞可以將壞損之蛋白質與粒線體或其它胞器進行分解、回收、再利用，以調控與維持細胞生態之穩定與和諧(Mizushima et al., 2008)。除了上述的功用外，細胞自噬，可能同時參與了細胞死亡調控，命名為細胞自噬式死亡(Autophagic Cell Death)或稱為第二型細胞死亡 (Scarlatti et al., 2009)。

細胞自噬式死亡之特色乃於細胞內，產生空泡化(vacuolization)，而相對於細胞凋亡(Apoptosis)，細胞自噬僅有少量的核染質濃縮(chromatin condensation)或細胞減縮表現。總論而言，目前認為細胞自噬，為細胞面對外界刺激改變如缺氧、養分來源缺乏、荷爾蒙失調與氧化壓力或是面對細胞內在需求改變，所因應的細胞反應，而它的存在對於細胞之存活、分化與發展皆有密切之關聯性，學者甚至認為細胞自噬可以對抗氧化壓力與幫助生物體對抗疾病(Hamacher-Brady et al., 2006b; Mizushima et al., 2008; Nakai et al., 2007)。

1.2 細胞自噬之分子調控

在外界環境刺激下，透過**誘發性**的 Class III PI3-K 之作用(phosphoinositide 3-kinase, 或稱為 Vps34)，細胞自噬相關的早期蛋白質 Beclin-1 (Atg 6)可以與其他細胞自噬相關蛋白結合，包括 Atg 12 合併 Atg 5 或 LC3 (Light chain 3)合併 PE (phosphatidylethanolamine)等。經後續相互作用，形成成熟之細胞自噬體 (Hsieh et al., 2009)。整個過程當中 Atg4 會將 LC3 切割成 LC3-I 與 LC3-II，其中 LC3-II 會參與細胞自噬體之外膜結構建立，因此 LC3-II 之表現量或是 LC3-II 與 LC3-I 之比值，則被運用於定量細胞自噬之表現量。

除了誘發性之 Class III PI3-K 外，另有 mammalian target of rapamycin (mTOR) 調節之 Class I PI3-K 於調控上扮演了**抑制性**的調控角色。其它牽涉細胞自噬之上游訊息傳遞包括 Growth Factor Signaling、Phosphatidylinositol (PI)3-kinase/Akt、Mitogen-Activated Protein Kinases、AMP-Dependent Protein Kinase (AMPK)、small GTPases、Trimeric G Proteins、Inositol Triphosphates 與 Calcium Signaling 等(Nishida et al., 2008)。

1.3 細胞自噬與細胞凋亡之交互關係

研究顯示，形成細胞自噬體之前，細胞自噬早期蛋白質 Beclin-1 若與 Bcl-2/BclXL 結合，則相關之細胞自噬體形成將受到抑制，代表 Bcl-2/BclXL 具有抑制細胞自噬之功能(Saeki et al., 2000)。學者認為與細胞凋亡相關之 Bcl-2/BclXL，於細胞內可能同時扮演調控細胞自噬與細胞凋亡的角色(Levine et al., 2008)。Bcl-2 (B-cell lymphoma 2)的存在可抑制因為養分缺乏所誘發出之 Beclin-1 (Pattingre et al., 2005)。雖然 Bcl-2 與細胞自噬之相關性未完全通盤研究清楚，但可以知道 Bcl-2 在許多細胞種類中皆與細胞自噬之調控相關，而 Bcl-2 與 Beclin-1 之作用很可能就是細胞自噬與細胞凋亡間調控的重要訊息傳遞機制。而細胞之存與亡，也很有可能透過這一層關係達到調控。

研究同時細胞凋亡相關蛋白 BNIP3，可以活化心肌細胞之細胞自噬表現，特別是當心肌細胞處於缺氧/再灌流刺激時(Dorn and Kirshenbaum, 2008; Hamacher-Brady et al., 2007)。

而細胞自噬蛋白如 Atg 5 本身也具有 pro-apoptotic 之影響力，過量 Atg 5 表現會透過抑制 Bcl-XL，增加細胞對於細胞凋亡反應的感受性與促發細胞凋亡(Yousefi et al., 2006)，甚至於透過 FADD (Fas associated death domain protein) 誘發外途徑細胞凋亡(Pyo et al., 2005)。因此 Atg 5 可能是決定細胞自噬或細胞凋亡重要的物質。

整體而言，包括雖然細胞自噬與細胞凋亡於細胞表現上是截然不同的細胞現象，目前認為細胞透過 Bcl-2 /$BclX_L$ ， Beclin-1，Atg5，BNIP3，Bid 與 Bax/Bak 之作用，細胞內確實存在著調控細胞自噬與細胞凋亡之機制，細胞自噬與細胞凋亡兩種細胞反應可以互存、互補或互相調控與影響(Nishida et al., 2008)。

1.4 細胞自噬與心臟疾病

目前認為細胞自噬與許多人類疾病相關包括退化性神經病變、老化、腫瘤、肝臟疾病以及心臟疾病等(Mizushima et al., 2008)。細胞自噬於心臟之表現最早之記錄始於 1976 年(Sybers et al., 1976)，目前認為，正常情況，心肌細胞表現穩定之基礎值細胞自噬活性，用以維持細胞恆定(Kim et al., 2007)。當心臟之細胞自噬活性降低時，如缺少 Lysosome-associated membrane protein 2 (LAMP2)之老鼠模式，其細胞自噬體無法與溶小體結合，乃導致細胞自噬功能不全，如此將讓心臟進展至擴張性心肌病(Tanaka et al., 2000)。

其他心臟疾病狀態如缺氧、切乏養分、心肌梗塞、心臟衰竭或壓力過負荷狀態時，心肌細胞內可觀察到增量之細胞自噬活性與細胞自噬體累積。鑒於疾病狀態之心肌細胞內，可見到大量之細胞自噬體，迄今仍有大量爭論細胞自噬體究竟是造成心肌細胞死亡之主因，抑是群起抵抗細胞死亡、細胞壞死或細胞凋亡之努力，目前仍有待驗證。

1.4.1 心肌面對缺氧狀態

心肌缺氧時維持心肌細胞存活之養分與氧氣供應皆匱乏，心肌細胞將誘發 AMP-activated protein kinase (AMPK)，同時抑制 mammalian target of rapamycin (mTOR)，進而誘發細胞自噬以幫助細胞存活。心肌細胞長期缺氧狀態時，心肌細胞整體代謝活性下降呈現類似冬眠狀態(Hibernation)，而產生 Ischemic cardiomyopahty，而此時 BNIP3 (BCL2/adenovirus E1B 19 kd-interacting protein) 之表現將被誘發，此與粒線體功能異常或細胞死亡相關(Regula et al., 2002)，然而又有證據顯示，BNIP3 透過 Bcl-2、Bcl-XL 或是抑制 mTOR 進而造成細胞自噬之活化 (Li et al., 2007; Tracy et al., 2007)。

1.4.2 心肌面對再灌流狀態

於再灌流階段，AMPK 不再被活化，而在 Beclin-1 大量表現下，其結果導致細胞表現大量細胞自噬體，此時誘發細胞凋亡之 Bax 蛋白表現量亦下降(Hamacher-Brady et al., 2006a)。相對於缺氧狀態時細胞自噬的細胞保護角色，於再灌流階段，細胞自噬表現過量將引發細胞自噬式死亡，而於再灌流階段抑制細胞自噬似乎可以減少降低心肌梗塞大小(Matsui et al., 2007)，同時抑制缺氧誘發之細胞自噬可以增加細胞存活(Valentim et al., 2006)。顯示細胞自噬於再灌流階段，不見得是用於保護細胞；細胞自噬於再灌流階段之角色未定，而過度活化之細胞自噬將導致細胞死亡的證據也越來越明顯 (Pattingre et al., 2005)。

1.4.3 心肌肥大與心衰竭

研究心肌梗塞後兔子模式發現，代表細胞自噬活性之 LC3-II/LC3-I 比值，與左心室射出分率(LVEF)成反比(Chen et al., 2010)，顯示細胞自噬狀態與心臟功能相關。研究發現 Rapamycin 可以預防心肌肥厚(Kuzman et al., 2007)，同時 Rapamycin 作用於壓力負荷誘發之心肌厚，可以讓已產生之心肌肥厚回復(McMullen et al., 2004)，而 Rapamycin 抑制的正是減緩細胞自噬表現的 mTOR。陸續研究也證實細胞自噬

又與壓力負荷誘發之心肌肥厚、心臟衰竭(Zhu et al., 2007)與擴張心肌病變相關(Shimomura et al., 2001)。

1.4.4 細胞自噬與粥狀動脈硬化

目前認為細胞自噬與動脈壁上阻塞斑塊穩定度相關，血管壁受刺激產生大量之細胞自噬將誘發平滑肌細胞死亡導致動脈壁上阻塞斑塊不穩定；容易造成斑塊破裂導致血栓(Levine and Yuan, 2005)。

1.5 細胞間隙接合

既然細胞自噬與諸多心臟血管疾病相關，因此研究細胞自噬之學者莫不相信細胞自噬可以成為一種調控與治療心臟疾病的方法(Bao et al., 2010)。然而運用細胞自噬於心臟疾病治療之前，更重要的是要先知道細胞自噬是如何受到調控的，包括細胞自噬之產生、誘發、細胞內部調控與細胞間相互調控等。現今對於細胞自噬之內部抑制性調控及誘發性調控已有相當程度之研究，但細胞之間之調控機制研究卻付之闕如。

而討論細胞間之訊息傳遞，莫不需要提及細胞間隙接合(Gap junction)。動物細胞透過間隙接合讓細胞之細胞體直接連接，並同時選擇性的讓特定傳遞物質進出細胞間 (Lampe and Lau, 2000)。包括 small second messengers、inositol triphosphate (IP3)與鈣離子 (Ca2+)或其他小於 1000 Da 大小之物質進出。間隙接合主要功能包括(1)可選擇性交換細胞間的離子與代謝物；(2)可形成電位性耦合(electrical coupling)及代謝性耦合(metabolic coupling)細胞；(3)可直接在結構上連結細胞；(4) 可直接在功能上參與細胞的訊息傳遞。

反觀細胞凋亡細胞間之調控研究，已證實細胞間隙接合可能是細胞間傳遞與溝通細胞凋亡訊號的重要窗口；在細胞自噬之細胞間調控機制研究未明之今日，研究細胞自噬之細胞間調控的進行有其必要性。

1.6 細胞自噬與細胞間訊息傳遞

對於探討細胞生死調控最為透徹的莫過於細胞凋亡之研究。對於細胞凋亡，目前認為細胞或組織間可透過間隙接合(Gap junction)傳遞細胞凋亡之訊號，而造成細胞死亡(Contreras et al., 2004; Kalvelyte et al., 2003)。若使用細胞間隙接合之抑制劑如 Carbenoxolone (CBX)，可發現腦細胞接受缺氧刺激產生的細胞凋亡量將大幅減少(de Pina-Benabou et al., 2005)。暫時性轉染 Cx43 至 BHK 細胞 (baby hamster kidney)，讓 BHK 細胞表現正常功能之間隙接合，結果顯示轉染後之細胞比起未轉染之細胞，於誘發細胞凋亡物質 CyC (cytochrome C)作用後更容易產生細胞凋亡(Udawatte and Ripps, 2005)。

雖然如此，細胞間隙接合究竟是扮演加工殺害抑是扮演幫助細胞存活之角色，近年來之爭議逐漸浮現(Decrock et al., 2009; Taimor, 2000)，特別是在心臟之研究，以心肌細胞培養之研究顯示，如果心肌細胞間有正常的間隙接合，則心肌細胞較不易產生細胞凋亡(Yasui et al., 2000)。同時心肌細胞內之粒線體內有正常之 Connexin 43 表現，可以預防心肌細胞死亡(Rottlaender et al., 2010)。對於支持細胞受益於細胞間隙接合而免於細胞死亡之學者，他們認為可能的機轉是因為細胞間隙接合限制了毒害物質的傳遞如過氧化物等，同時可提供幫助細胞存活之訊號如維他命 C、ATP 與葡萄糖或是抗細胞凋亡之訊號(Decrock et al., 2009; Nakase et al., 2003)。但是否細胞自噬的訊號也是透過細胞間隙接合達成訊息傳遞，此問題一直未被證實。

1.7 研究目的

有鑑於細胞自噬之調控未被徹底研究，特別是細胞間隙接合於細胞自噬所扮演之角色未定。同時而若能操控細胞自噬，進而利用細胞自噬於治療心臟相關疾病包括心肌缺氧、心肌梗塞、心肌肥厚、心臟衰竭或血管粥狀動脈硬化與阻塞等，其對醫界貢獻不言可喻。因此我們著手細胞自噬之研究，探討心肌細胞間隙接合對於心肌細胞於承受氧化壓力刺激引發細胞自噬中所扮演的角色，同時研究假設: **心**

肌細胞乃透過細胞間隙接合傳遞訊細胞自噬之訊息，進而影響整體細胞群表現細胞自噬活性。

第二章 研究材料與方法

2.1 使用之化學藥劑

正庚醇 (1-Heptanol): 正庚醇購自 Sigma, St. Louis, MO, USA。為脂溶性物質於諸多實驗中運用於抑制細胞間隙連接(Keevil et al., 2000; Kimura et al., 1995; Lin et al., 2009)。當正庚醇濃度<1 mM 時，正庚醇被視為相對選擇性的間隙接合抑劑(Christ et al., 1999)。文獻上，實驗運用之正庚醇藥劑濃度為 0.16mM~2.0mM，而使用正庚醇並不影響細胞表現 Connexin 43 (Kimura et al., 1995)，僅在功能上抑制隙細胞間隙接合，將正庚醇清洗掉後，細胞間隙接合將恢復正常功能。

過氧化氫(Hydrogen Peroxide, H_2O_2): 過氧化氫購自 Sigma, St Louis, MO, USA，依據文獻紀載使用過氧化氫 (1.0 mM)可使 HEK293 與 U87 細胞誘發出細胞自噬與細胞凋亡(Chen et al., 2008)。而過氧化氫於 H9c2 之影響未定，我們將先將檢測不同作用濃度與作用時間之過氧化氫對細胞之影響。

pCMV-Lc3: pCMV-Lc3 購自 OriGene, Rockville, MD, USA。

pCMV-Gluc: pCMV-Gluc 購自 New England Biolabs。

Dulbecco's modified Eagle's medium (DMEM), F12 medium、 phosphate-buffered saline (PBS)、 0.25% trypsin-EDTA, streptomycin-penicillin 以及 amphotericin B solutions 購自 Gibco, Gaithersburg, MD, USA。

Fetal bovine serum 購自 US Bio-Technologies, Parkerford, PA, USA

TurboFect ™ in vitro Transfection Reagent (Fermentas)

MTT (3-(4,5-dimethyl-2- thiazolyl)-2,5- diphenyltetrazolium bromide)。

2.2 H9c2 (2-1)細胞培養

本實驗採用細胞模式進行實驗。細胞之取得來自台灣食品工業發展研究所(FIRDI: Food Industry Research and Development Institute)的之生物資源保存及研究中心(Bioresource Collection and Research Center)。生物資源保存及研究中心所提供之 H9c2 細胞株，來源為 ATCC® (American Type Culture Collection)，原 ATCC 產品編號為 CRL-1446™。

H9c2(2-1)乃分離自 *Rattus norvegicus* 大鼠心肌組織的胚胎 BD1X 細胞，具有一般橫膈肌肉細胞特性，需附著生長但本身不具肌束收縮特性(Kimes and Brandt, 1976)。培養心肌細胞株 H9c2(2-1)，採用 90% DMEM 培養液(Dulbecco's modified Eagle's medium)包括 4 mM L-glutamine、1.5 g/L sodium bicarbonate、4.5 g/L glucose 以及 10% 胎牛血清 (FBS: fetal bovine serum)。培養時控制環境溫度為 37℃，並且維持適當之濕度，以及 5% CO_2 與 95%空氣混和狀態(Koudssi et al., 1998; Long et al., 1991)。當細胞生長到 80%的生長空間時，先置換新鮮的培養液於培養容器內。翌日，利用 0.25% (w/v) Trypsin 將細胞從培養皿上分離取下，使用 Trypan blue 染色法計算細胞的濃度與實驗所需的細胞總數，取足夠量的細胞後，用滅菌後以 PBS (phosphate buffered salin)清洗與離心 2 次後，再用培養溶液調整到需要的實驗細胞數量 $5x10^4$~$4x10^5$/Well，將細胞植入新的細胞培養槽(6 well-plate、12 well-plate 或 96 well-plate)，並置入細胞培養箱內培養靜置 24 小時後開始著手進行細胞實驗。

2.3 製作質體 pCMV-Lc3-Gluc

限制酶反應: 第一部分，將 pCMV-Lc3 (1.5μg/λ, OriGene, Rockville, MD, USA),以限制酶 *Eco*R V(1U/λ)切開，再以 Shrimp Alkaline Phosphatase(1.5μg/λ)將 DNA 兩端去磷酸化，最後以 Gel Extraction Kit 純化之以取得 Linear pCMV-Lc3。第二部分，pCMV-Gluc (2.0 μg/λ, New England Biolabs) 以限制酶 *Hind* III (1U/λ) 與 *Xba* I(1U/λ)切開，再以 T4 DNA polymerase 補平 5'端和移除 3'端，最後以 Gel

Extraction Kit 純化以取得 608 bp 之 DNA 片段 Gluc (Tannous et al., 2005; Verhaegent and Christopoulos, 2002)。

接合反應: 將 Linear pCMV-Lc3 與 608 bp 之 Gluc DNA 片段以分子數比 5 比 1 混合，以 T4 DNA ligase (5U/λ)於 4℃進行隔夜之接合反應。

轉形反應: 將 10μL 結合反應液與 100 μL E. coli DH5α 受容細胞混合，冰浴 5 分鐘，再於 42℃熱水浴 90 秒，加入 300 μL Lysogeny broth (LB) 培養基，於 37℃震盪培養 1 小時，各取 200μL 以 L 棒均勻塗抹於含有 25 μg/mL 之洋菜培養基，於 37℃培養隔夜，利用 Kanamycin selection，隔日檢查是否有菌落形成。

篩選轉形株: 挑選部分菌落接種於 10 mL Lysogeny broth (LB)，於 37℃培養隔夜，進行質體之抽取，將抽取之質體以限制酶 Not I (1U/λ)切開，若有 5872 bp 與 637 bp 之 DNA 片段，即可能為正確接合的質體，委託 DNA 定序服務，進行 DNA 序列比對以確認之，確定所建立之 pCMV-Lc3-Gluc 質體序列是否正確。

2.4 暫時性轉染作用與 Luciferase 活性測量

我們待細胞生長達 80%生長空間飽和度後進行 H9c2 細胞轉染，轉染過程使用 TurboFect ™ in vitro Transfection Reagent (Fermentas)，轉染方式依據製造商建議指引進行實驗。我們將取 10 μg plasmid pCMV-Lc3-Gluc，轉染到生長於 T75 Flask 內之 H9c2，經 24 小時與 TurboFect ™ in vitro Transfection Reagent (Fermentas) 完成轉染。轉染後細胞經由 0.25% trypsin-EDTA 作用分離後，將轉染後之 H9c2 平均接種至數個 12-wells plate 中，每個 well 之細胞數為 $5x10^4$，每個 well 含有定體積 1.5 ml 之 10%FBS/DMEM，置於 37℃培養箱培養 24 小時後，即可以開始作各種比較實驗。經化學藥劑實驗後，立刻添加 BioLux™ GLuc Assay Buffer 進行冷光酵素反應測量，利用微盤冷光光譜儀計算各個細胞培養槽內一秒鐘之冷光酵素反應值(CPS, count per second)。利用此冷光酵素反應值代表 LC3 之表現量，作為偵測細胞自噬活性之方式(Ju et al., 2009; Verhaegent and Christopoulos, 2002; Wu et al., 2007)。

2.5 細胞存活率測量

H9c2 細胞存活率測定以 MTT 法進行(3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyltetrazolium bromide)。將 H9c2 細胞依照 $5x10^4$/well 之數量，植入 12-well culture plates 細胞培養器內，經藥劑作用包括正庚醇與過氧化氫處理，包括不同作用濃度的與時間之條件。細胞存活率之計算，乃於正庚醇與過氧化氫處理後，將 DMEM 培養液去除，於各個培養槽內加入溶於 PBS 中最終濃度為(0.2g/dL)之 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma)。 每一培養器內加入 1ml MTT 放置 37℃ 培養約 1 小時，之後會產生的紫色的 formazan 產物，再加入 1ml DMSO 回溶，並測定此產物在 550nm 吸光度；測定此產物在 550nm 吸光度可決定細胞的存活率，細胞存活率之計算為: $\frac{\text{處理後細胞之吸光值}}{\text{對照組細胞之吸光值}} \times 100\%$ (Monastyrskaya et al., 2002; Mosmann, 1983)。

2.6 細胞自噬之偵測

2.6.1 Reverse-transcription polymerase chain reaction (RT-PCR)

細胞表現 LC3 與 Beclin-1 mRNA 可以視為細胞自噬之表現，因此實驗過程利用反轉錄聚合酵素鏈鎖反應(RT-PCR)偵測 LC3 與 Beclin-1 mRNA，可作為偵測細胞自噬之方式。先將 H9c2 細胞內的總量 RNA 物質利用 Trizol reagent (Invitrogen)，依據製造商建議之處理方式處理。利用 ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA)以分析所萃取出之總量 RNA 濃度(Chen et al., 2007)。運用所萃取之 RNA 隨即進行，備製第一股的互補 DNA (complementary DNA): 取樣 1μg 總量 RNA，利用逆轉錄酶 RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania)，依照製造商建議進行實驗。反轉錄(Reverse transcription)操作條件為 42 ℃ 作用 60 分鐘，隨後調溫為 70 ℃ 維持 5 分鐘。製造出第一股 cDNA 後，利用聚合酵素鏈鎖反應(Polymerase Chain Reaction, PCR)方式複製放大 cDNA 的總量，聚合酵素鏈鎖反應作用條件為，95 ℃ 作用 10 分鐘，接著進行 35 循環的三種溫度條件作用包括: 94 ℃ 持續 30 秒鐘; 50 ℃ 作用 1 分鐘以

及 72 ℃作用 1 分鐘，循環結束後，最終延長作用溫度 72 ℃之時間到 10 分鐘。最終聚合酵素鏈鎖反應產物取其中 5 μl ，利用 2% agarose gel 進行電泳分析。電泳結果經由 EtBr 染色後，利用膠體影像分析儀 Alpha Innotech 擷取影像，影像結果利用 Alpha.Imager® Mini 進行資料分析。所使用的引子包括:

Rat Cx43 sense: 5'-AAAGGCGTTAAGGATCGCGTG -3';

Rat Cx43 antisense: 5'- GTCATCAGGCCGAGGCCT -3';

Rat LC3 sense: 5'-GCCTGTCCTGGATAAGACC-3';

Rat LC3 antisense: 5'-TTGGGAGGCATAGACCATGT-3';

Rat GAPDH sense: 5'-GCACCACCAACTGCTTAGC-3';

Rat GAPDH antisense: 5'-TGAGTGGCAGTGATGGCAT-3'

2.6.2 西方墨點法(Western blotting)

將分離出的心肌細胞蛋白質溶解於 RIPA lysis buffer(Santa Cruz, Santa Cruz, CA)，並測量其蛋白質濃度(Chen et al., 2007)。取 20μg 的蛋白質以 SDS-PAGE 系統進行電泳分析，隨後將結果轉漬到 PVDF membrane (Amersham Bioscience)上。主要加入下列各項物質的特異性抗體偵測 LC3-I (18 kDa, 細胞質 LC3)、 LC3-II (16 kDa, 細胞自噬體膜上 LC3) 以及 actin (Santa Cruz)，並以 ECL(enhanced chemiluminescence) 將其顯色(Liu et al., 2007)。最後以 QUANTITY ONE (Bio-Rad) 定量之，資料分析經 Actin 校正後呈現。

2.6.3 流式細胞儀

Acridine Orange (AO)為弱鹼物質，可以堆積在酸性環境的細胞空間內。測量時先將細胞收集起來以 1 ml PBS 回溶，加入 AO (100 mg/ml)染色 15-20 分鐘，染色後

以 PCS 清洗兩次後以 0.3ml 之 PBS 回溶，並利用流式細胞儀(BECTON DICKINSON, FACS Vantage)進行測量。

利用流式細胞儀檢驗 acidic vesicular organelles (AVOs)可以推算細胞自噬比例。細胞自噬的特色就是形成 acidic vesicular organelles (AVOs)，AVOs 代表的就細胞自噬過程當中形成的細胞自噬體與 autolysosomes。我們透過 Acridine Orange (AO)進行細胞染色後，進而利用流式細胞儀，偵測 AVOs 的情況，而 AVOs 陽性比例即代表細胞自噬比例(Kanzawa et al., 2005; Levine and Yuan, 2005; Mujumdar et al., 2010)。

2.7 實驗設計

實驗設計分三大部分(圖表 1)，依序說明如下:

2.7.1 第一部分

主要建立實驗室常模；包括檢驗 H9c2 細胞單獨接受過氧化氫以及正庚醇作用後，所產生之細胞凋亡反應、細胞自噬反應、細胞間隙接合表現變化以及細胞存活率。

2.7.2 第二部分

企圖合成 pCMV-Lc3-Gluc 質體，並將此質體以短暫轉染方式殖入 H9c2 細胞內，並將轉染後之 H9c2 細胞作為偵測 Light Chain 3 (LC3)表現之 Luciferase Report System。進行但三部分實驗前，同時先評估轉染 pCMV-Lc3-Gluc 後 H9c2 細胞是否產生細胞自噬表現改變，同時評估利用 Luciferase Report Assay 以偵測細胞自噬之可行性，利用轉染 pCMV-Lc3-Gluc 質體後 wild type H9c2，比較過氧化氫刺激之有或無，對冷光酵素反應值表現之差異。

2.7.3 第三部分

此研究進行分兩大主軸，第一主軸，在標準的細胞培養條件下，利用 wild type H9c2 做實驗；分成四組包括第一組(Control)、第二組(Heptanol)、第三組(H_2O_2)、第四組(H_2O_2+Heptanol)，利用第一部分實驗所得到之結論，選取適當之正庚醇與過氧化氫濃度作用，並比較正庚醇對於細胞自噬之影響力，以印證實驗假說。第二主軸利用轉染 pCMV-Lc3-Gluc 之 H9c2 進行藥物測試，同時分成四組包括第一組(Control)、第二組(Heptanol)、第三組(H_2O_2)、第四組(H_2O_2+Heptanol)，利用 Luciferase Report Assay 檢定冷光酵素反應值，以推估 LC3 表現之特性，相互比較以印證實驗假說之成立。

2.8 統計分析與圖表繪製

所以檢驗數值利用 PC version of SPSS statistical software, 15th version，比較平均值差異將運用無母數分析 Mann Whitney U 運算；對於多組數之差異比較，將採用 analysis of variance (ANOVA)檢測。P 值以<0.05 視為統計上顯著差異；圖表繪製利用 GraphPad Prism 5 軟體製作。

第三章 實驗結果

3.1 第一部分結果-建立實驗常模

3.1.1 過氧化氫刺激 H9c2 後之細胞反應

由於過氧化氫提供的氧化壓力，是否可誘發 H9c2 產生細胞自噬仍屬未知，因此在採用過氧化氫做為細胞自噬誘發藥劑之前，我們進行初步過氧化氫藥物試驗，以觀察 H9c2 接受 400 μM 過氧化氫處理後的細胞反應。

3.1.1.1 測試過氧化氫是否能誘發細胞自噬

我們將 1.36 x10 ^{5} 的 H9c2 植入 12-well plate，以過氧化氫 400 μM 作用 0-180 分鐘後，使用反轉錄聚合酵素鏈鎖反應(RT-PCR)檢驗 LC3、Beclin-1 與 Bcl-2 之 mRNA

表現情形，可發現，在作用時間超過 90 分鐘之後，代表細胞自噬表現的 LC-3 與 Beclin-1 的表現逐漸增加 (n=2)，而調控細胞凋亡的 Bcl-2 mRNA 的表現則保持穩定狀態(圖表 2)。

利用膠體影像分析儀(Alpha.Imager® Mini)，將各個時間點所測量到之 mRNA 表現量量化，經由 gapdh 校正後，更可以發現較長的作用時間，對於後續誘發細胞自噬表現之影響越大；經 180 分鐘之後，LC3 之表現量大約增加 29.3%，而 Beclin-1 之表現量大約增加 36.7% (圖表 3)，而 Bcl-2 之表現則無明顯變化。顯示氧化氫能誘發細胞自噬相關基因表現。

3.1.1.2 過氧化氫作用時間對誘發細胞自噬影響

除了細胞自噬相關的基因(mRNA)表現可能受到過氧化氫 400 μM 誘發外，延長過氧化氫 400 μM 作用時間是否會造成細胞自噬或細胞凋亡之蛋白質表現改變，需要進一步釐清。

我們將 2.0 x10 ^{5} 個細胞的 H9c2 植入 10cm^{2} 的細胞培養槽中，利用 400 μM 過氧化氫藥物刺激 1.5 hr、 3 hr、6 hr、12 hr、16 hr 與 24 hr，觀察當中 H9c2 相關細胞自噬與細胞凋亡的蛋白質表現。400 μM 過氧化氫作用後，Western Blot 顯示整個 LC3 之表現量，隨時間延長而持續增加；而大約到作用 6 小時以後，包括 LC3-I 與代表細胞自噬體之膜上 LC3-II 之表現，皆明顯增加(圖表 4)。

量化所測得之 LC3-I 與 LC3-II，並將測量值與 Actin 作校正，發現 400 μM 過氧化氫作用六小時後 LC3-I 之表現量約增加 45.2%，而 LC3-II 之表現量則為起始值之 4.54 倍。當作用 24 小時後 LC3-I 表現量升高至起始值之 2.5 倍，而 LC3-II 表現量升高至起始值之 7.06 倍(圖表 5)。而 LC3-II/LC3-I 比值，於 400 μM 過氧化氫刺激 6 小時後，比值持續升高並延續至 24 小時以後(圖表 6 與表格 1)。

利用 Western Blot 偵測代表細胞凋亡的 Bax，顯示過氧化氫 400 μM作用時間越長，所誘發的 Bax 蛋白質表現量越高，且開始誘發時間也與產生細胞自噬時間點相同，約發生於過氧化氫 400 μM 處理 6 小時以後(圖表 8)。

利用流式細胞儀檢測 Acidic vacuoles 之細胞比例。結果顯示過氧化氫 400 μM 刺激後 Acridine orange 染色陽性的 Acidic vacuoles 比例增加，而 AVOs 可以間接代表細胞自噬體。因此，意味著細胞自噬體於過氧化氫 400 μM 作用後也明顯增加(圖表 7)。進一步，利用流式細胞儀測量過氧化氫 400 μM 刺激後 Annexin V stain 陽性比例，顯示過氧化氫 400 μM 作用 24hr 後，Annexin V stain 陽性比例增加超過 20%，代表細胞凋亡之比例也大幅增加(圖表 9）。

3.1.1.3 過氧化氫作用時間對細胞存活率影響

除了細胞蛋白質表現上外，我們同時運用 MTT assay 來評估不同作用時間的過氧化氫作用，對細胞存活的影響。

我們以過氧化氫 200 μM 刺激 H9c2 細胞 0-24 小時，比較不同時間點造成細胞存活比例改變情形。結果顯示，相較於作用之前，過氧化氫作用 12 小時後細胞存活率佔 86.5% (n=5, P =0.13)，而過氧化氫作用 24 小時以後細胞存活率佔 85.2% (n=6, P=0.009)（圖表 10 與表格 2）。

3.1.1.4 過氧化氫作用濃度對誘發細胞自噬影響

探究高濃度過氧化氫與低濃度過氧化氫對於細胞自噬或細胞凋亡表現上的差異，我們進行固定作用時間，但不同濃度的過氧化氫氧化壓力進行測試。

我們將 1.2 x10 ^{5} 的 H9c2 植入 12-well plate，利用 0-1000μM 過氧化氫作用 24 小時施予氧化壓力，使用反轉錄聚合酵素鏈鎖反應(RT-PCR)檢驗 LC3 mRNA 表現情形，可以發現面對較高濃度的過氧化氫，LC3 之 mRNA 表現無明顯變化(圖表 11)。顯示過氧化氫作用時間超過 24 小時時，作用濃度對於 H9c2 誘發細胞自噬可能不具鑑別力。

乃將作用時間修定為 12 小時，利用 0-400μM 過氧化氫作用，比較不同濃度對於 H9c2 表現細胞自噬 LC3 蛋白質之影響。使用 Western Blot 測定發現，細胞自噬蛋白質表現相對於過氧化氫氧化壓力具有 dose-response 關係(圖表 12)，使用 100μM 過氧化氫作用 12hr 可使 LC3-II 增加 2.48 倍(圖表 13)，而 LC3-II/LC3-I 由基礎值的 0.45，升高至 0.84，持續升高過氧化氫濃度至 800μM，LC3-II 之表現量為基礎值的 2.56 倍，而 LC3-II/LC3-I 增加為 1.29 倍，顯示當濃度越高越能刺激產生細胞自噬相關蛋白質產生，而且增加部分以細胞自噬體之膜上蛋白 LC3-II 為主（圖表 14、圖表 15、表格 3）。

3.1.1.5 過氧化氫作用濃度對細胞存活率影響

對於高濃度過氧化氫作用 12hr 對於細胞存活之關係，我們利用 MTT 方式，計算不同濃度之過氧化氫對於細胞存活的影響，結果顯示當作用濃度過氧化氫大於 200μM，其細胞之存活率將小於 50% (圖表 16)。

3.1.1.6 過氧化氫作用對細胞表現細胞間隙接合影響

探究過氧化氫是否造成細胞間隙接合表現改變，我們測驗不同濃度之過氧化氫刺激並比較細胞表現 connexin 43 的差異。

我們將 $2.0x10^5$ H9c2 細胞植入 6 well-plate 細胞培養槽，施予高濃度的 400μM 過氧化氫作用 0-24 小時，利用反轉錄聚合酵素鏈鎖反應(RT-PCR)方式檢定 Cx43 mRNA 表現量，結果發現當作用時間超過 14 小時後，Connexin43 之 mRNA 表現量可能會減少為基礎值之 77%，一直到作用 24 小時以後，Connexin43 之 mRNA 表現量可能僅為基礎值之 26%（圖表 17、圖表 18），顯示高濃度過氧化氫作用時間越長，將直接影響細胞間隙接合之基因表現。

同時比較細胞間隙接合之蛋白質的表現，可發現若使用過氧化氫 400μM 處理 24 小時候，將使 Membrane fraction 的 Cx43 蛋白質表現減少(圖表 19)，顯示高濃度過氧化氫作用同時減少了細胞間隙接合之蛋白質的表現。

3.1.2 正庚醇處理 H9c2 後之細胞表現

3.1.2.1 正庚醇作用濃度對細胞表現細胞間隙接合影響

我們將 $2.0x10^5$ H9c2 植入 6 well-plate 細胞培養槽，利用不同濃度的正庚醇作用於 H9c2 細胞，並利用反轉錄聚合酵素鏈鎖反應(RT-PCR)檢定 Cx43 mRNA 表現，結果顯示正庚醇濃度對於 Cx43 表現並沒有顯著的變化。即便使用更高濃度之正庚醇處理，細胞之 Connexin 43 mRNA 表現依舊維持基礎值 72%以上(圖表 20、圖表 21)。

3.1.2.2 正庚醇作用濃度對細胞存活率影響

運用 MTT assay 方式，計算正庚醇藥物是否影響 H9c2 細胞的細胞存活率，我們將 $2.0x10^5$ H9c2 細胞植入 6 well-plate 細胞培養槽中，利用 0.5mM 正庚醇作用細胞中，依據作用時間點採樣。結果顯示即使經過持續長達 24 小時之 0.5mM 正庚醇作用，細胞存活率依舊可以維持高於 85%。顯示 0.5mM 正庚醇並不會大量毒害細胞造成細胞之直接死亡(圖表 22)。

3.1.3 第一部分結論

過氧化氫研究顯示，過氧化氫作用可誘發 H9c2 表現細胞自噬。而隨著過氧化氫作用時間的延長，H9c2 的細胞自噬以及細胞凋亡的蛋白質表現量也增加。所染出 Acidic vacuole 陽性之細胞以及 Annexin V 呈現陽性之細胞比例也增加。而最明顯的表現變化出現在 6-12 小時以後，但隨著作用時間繼續延長，並沒有看見明顯 dose-response curve 趨勢，顯示過氧化氫的氧化壓力提供可能於 6-12 小時即達到最高峰。但使用長時間與高濃度過氧化氫作用，可能直接誘發細胞凋亡、減少細胞存活率以及降低細胞表現 Connexin 43。因此依據實驗結果，最理想做為誘發細胞自噬之條件為過氧化氫濃度介於 100~200μM 而作用時間則為 6~12 小時。

正庚醇研究顯示，正庚醇之使用不會明顯直接降低 H9c2 表現 Connexin 43，同時長時間使用 0.5 mM 正庚醇作用於 H9c2，其細胞存活率亦可維持超過 85%。

3.2 第二部分結果-合成 Luciferase Report System

3.2.1 建立 Gaussia Luciferase report assay 以偵測細胞自噬

運用「實驗方法」中描述之基因重組方式(recombinant DNA)，以含 pCMV 質體(Plasmid)作為載體，將 LC-3 與 Gaussia Luciferase 基因做連結，合成含 pCMV-Lc3-Gluc 片段之質體。將 pCMV-Lc3-Gluc 轉染入 H9c2 細胞，形成一套具有可快速反應細胞自噬表現(LC3 表現)，之 Gaussia Luciferase Report System。成功合成後，進行進一步測試。

3.2.1.1 測試轉染 pCMV-Lc3-Gluc 對 H9c2 之影響

比較正常培養的 H9c2 與轉染入 pCMV-Lc3-Gluc 之 H9c2 可發現 pCMV-Lc3-Gluc 轉染後之 H9c2 有較高的 LC-3 mRNA 表現，大約增加 42.9% (P=0.10, n=3) (圖表 23、圖表 24)。利用流式細胞儀偵測 Acridine orange stain 陽性比例之細胞，發現經 pCMV-Lc3-Gluc 轉染之 H9c2 有較高的比例(圖表 25)，也就是表示轉染 pCMV-Lc3-Gluc 後，細胞表現細胞自噬之基因表現量將提高。

3.2.1.2 測試轉染 pCMV-Lc3-Gluc 作為細胞自噬偵測系統

依據第一部分實驗，已知過氧化氫將誘發細胞自噬表現，乃利用過氧化氫刺激檢定，所製作出之 Gaussia Luciferase Report System (pCMV-Lc3-Gluc 轉染之 H9c2)能否做為快速偵測細胞自噬之工具。

利用過氧化氫 400μM 分別刺激對照組 H9c2 以及含有 Gaussia Luciferase Report System 之 H9c2，記錄刺激 0-24 小時期間之冷光酵素反應值。結果發現細胞於作用 8 小時後，含有 Gaussia Luciferase Report System 之 H9c2 其產生之 Luciferin 冷光酵素值增加 12%(P=0.04, n=4)。作用 12 小時後，其產生之 Luciferin 冷光酵素值增加 31% (P<0.001, n=4)。整體增加，約於 14 小時後增加之幅度達到高原期(plateau)， 大約 40-49%，並於統計分析上達到顯著意義(P<0.001, n=4) (圖表 26

圖表 27 與表格 6)，顯示 Gaussia Luciferase Report System (pCMV-Lc3-Gluc 轉染之 H9c2)可做為快速偵測細胞自噬之工具。

3.2.2 第二部分結論

我們成功的建立了 Gaussia Luciferase Report System，利用轉染此系統之 H9c2，我們可以快速有效比較 H9c2 細胞之細胞自噬表現，經氧化壓力刺激後，細胞自噬表現大約在氧化壓力刺激 8 小時後即達顯著差異，超過 14 小時其差異將達高原期。最終利用過氧化氫 400μM 刺激 24 小時以後，利用 Gaussia Luciferase Report System 檢定，大約可以產生超過 40%之細胞自噬反應。

3.3 第三部分結果-檢定細胞間隙之細胞自噬角色

3.3.1 利用 wild type H9c2 測試間隙接合對細胞自噬之影響

實驗進行分成四組，分別為第一組(Control)、第二組(Heptanol)、第三組(H_2O_2)、第四組(H_2O_2+Heptanol)，將 wild type H9c2 分別經由 0.5mM 正庚醇與 200μM 過氧化氫藥物作用後，我們同時進行四組之相關 LC3、 Beclin-1、Bax mRNA 表現情形，同時量化 mRNA 表現量，數值資料皆經與 gapdh 表現校正，結果顯示於(圖表 28&圖表 29)。

3.3.1.1 比較第一組(Control)與第二組(Heptanol)

經過 12 小時的正庚醇作用，LC3 mRNA 表現量降為原本基礎值的 90.8 % (P=0.2, n=3)。相對而言 Beclin-1 與 Bax 之 mRNA 表現量無明顯改變。顯示正庚醇於基礎狀態，對於細胞表現細胞自噬無明顯影響力。

3.3.1.2 比較第一組(Control)與第三組(H_2O_2)

結果發現大約於作用 12 小時以後，LC3 mRNA 相較於第一組(Control)基礎狀態的 H9c2 大約增加了 10.8% (P=1.0, n=3)。

3.3.1.3　比較第一組(Control)與第四組(H_2O_2+Heptanol)

比較第一組(Control)與第四組(H_2O_2+Heptanol)之間之 LC3 mRNA 發現，作用 12 小時後第四組(H_2O_2+Heptanol)之 mRNA 表現量為第一組(Control) 基礎狀態的 H9c2 之 84.0%(P=0.1, n=3)。顯示正庚醇，似乎皆有趨勢降低 LC3 mRNA 之表現。

3.3.1.4　比較第三組(H_2O_2)與第四組(H_2O_2+Heptanol)

比較第三組(H_2O_2)與第四組(H_2O_2+Heptanol)間之 LC3 mRNA 之表現發現，有正庚醇的共同作用情形下，H9c2 細胞於作用時間 12 小時以後，其 LC3 mRNA 之表現大約降低了 24.2% (P=0.4, n=3)，顯示抑制細胞間隙接合，有趨勢的減少細胞自噬基因表現。

3.3.2 利用轉染 pCMV-Lc3-Gluc 之 H9c2 測試間隙接合對細胞自噬之影響

實驗進行分成四組，分別為第一組(Control)、第二組(Heptanol)、第三組(H_2O_2)與第四組(H_2O_2+Heptanol)，將轉染 pCMV-Lc3-Gluc 之 H9c2 經藥物測試後進行比較分析。利用 Luciferin 冷光酵素值代表 H9c2 表現 LC3 量的特性進行實驗。將 H9c2 植入 12 well-Plate 培養槽，每一個細胞培養槽，植入 $5x10^4$ H9c2 細胞，每組各進行四重覆試驗，結果顯示如下(圖表 30、圖表 31、圖表 32、圖表 33 與表格 7)：

3.3.2.1　比較第一組(Control)與第二組(Heptanol)

比較第一組(Control)與第二組(Heptanol)發現，無論作用的時間長短，正庚醇似乎不影響 H9c2 Luciferin 的冷光酵素值，意味著於基礎狀態代表細胞自噬表現之 LC3 不受正庚醇影響，即使作用延長至 16 小時，第二組(Heptanol)所累積的 Luciferin 冷光測驗值為第一組(Control)的 96.5% (P >0.05, n=4)。

3.3.2.2　比較第一組(Control)與第三組(H_2O_2)

比較第一組(Control)與第三組(H_2O_2)結果發現大約於作用 12 小時以後，細胞自噬之活性相較於基礎狀態的 H9c2 大約增加了 60.6% ($P<0.001$, n=4)。一直到作用 16 小時後，細胞自噬之活性相較於基礎狀態的 H9c2 大約增加了 78.0% ($P<0.001$, n=4)，顯示 200 μM 過氧化氫處理確實增加了代表 LC3 數量的冷光酵素值。

3.3.2.3 比較第一組(Control)與第四組(H_2O_2+Heptanol)

比較第一組(Control)與第四組(H_2O_2+Heptanol)之間之 Luciferin 冷光酵素值發現，一直作用到 8 小時，第四組(H_2O_2+Heptanol)之檢測值與第一組(Control)相似，待延長到 12 小時，Luciferin 冷光酵素值乃增加 31.3% ($P>0.05$, n=4)，而作用延長至 24 小時 Luciferin 冷光酵素值乃增加 41.3% ($P<0.001$, n=4)。

3.3.2.4 比較第三組(H_2O_2)與第四組(H_2O_2+Heptanol)

比較第三組(H_2O_2)與第四組(H_2O_2+Heptanol)間之 Luciferin 冷光測量值發現，有正庚醇的共同作用情形下，H9c2 細胞於作用時間超過 4 小時以後，可觀察到一致性較低的 Luciferin 冷光酵素值，而當作用時間超過 12 小時時，這樣的一致性 Luciferin 冷光測量值降低，達到統計分析上之差異($P<0.001$, n=4)。而經 16 小時共同作用後，正庚醇可以減少大約 20.6%的 Luciferin 冷光酵素值。顯示抑制細胞間隙接合，有意義的減少細胞自噬蛋白質表現。

3.3.3 第三部分結論

綜合考量，過氧化氫誘發之細胞自噬之表現似乎在正常細胞間隙接合時，表現量較高；但當細胞間隙接合受到抑制，則細胞自噬之基因表現量有趨勢顯示下降，降幅約為 24%，但統計上未達顯著差異，顯示抑制細胞間隙接合，有趨勢的減少細胞自噬基因表現。

若利用 Gaussia Luciferase Report System 檢測，則 LC3 之表現量將有意義的下降約 20%。顯示抑制細胞間隙接合，有意義的減少細胞自噬蛋白質表現。而使用細胞間隙接合抑制劑阻斷了細胞間之訊息傳遞，對於基礎狀態之細胞較不受影響，但當

細胞承受氧化壓力傷害時細胞，細胞間之交互作溝通失去了；細胞自噬之能力也下降了。

第四章 研究討論

本實驗首次印證心肌之細胞自噬的細胞間調控，需仰賴細胞間隙接合進行。我們發現若心肌細胞之細胞間隙接合受到抑制，基礎值的細胞自噬表現不會受到細胞間隙接合抑制影響；但當細胞受到氧化壓力時，則細胞間隙接合抑制將減少細胞自噬之表現。顯示細胞自噬在某種程度上需要透過細胞間隙接合達到傳遞、延續或增強細胞自噬表現，特別是當細胞面臨傷害性刺激時。對於未來醫療界企圖利用調控細胞自噬達到治療疾病的同時，本實驗提供了另一個可能的調控機制，或許下一代的藥物可以瞄準調控細胞間隙接合。

4.1 本實驗的臨床意涵

4.1.1 細胞間隙接合與心臟疾病

細胞間隙接合於心肌細胞主要之功能為電氣訊號之傳遞(Electrical coupling)，但在諸多分子研究進行的年代之前，學界就發現細胞間隙可能影響心臟細胞之存活，特別是在心肌梗塞之心臟組織面對再灌流階段。急性心肌梗塞時隨著阻塞的時間延長，心肌細胞所受的損傷也越大(Reimer et al., 1977)，因此快速打通阻塞血管可以降低心肌梗塞之區域成為學界深信的道理(Maroko et al., 1972)。這樣的理念從動物實驗出發，乃迄今日心臟介入性治療的蓬勃發展，制定治療指引運用於人類心肌梗塞治療(Antman et al., 2004)。盡速打通阻塞血管建立血流供應固然重要，但再灌流之次發性傷害卻逐漸受到重視，甚至認為再灌流之次發性傷害中，細胞間隙接合能扮演依定之角色(Garcia-Dorado et al., 1989; Schroeder, 2007)。於再灌流階段，若施予正庚醇抑制心肌細胞間隙接合，可發現降低 Lactate Dehydrogenase 釋放、產生較少的 Contraction Band Necrosis(Garcia-Dorado et al., 1997)，更顯示細胞間隙接

是促成再灌流之心臟損傷兇手之一，而其可能原因乃間隙接合之電氣訊號之傳遞(Electrical coupling)可能導致過度心肌收縮，造成心肌壞死。降低心肌再灌流損傷之現象，亦可在缺氧狀態時施與細胞間隙接合之抑制劑中觀察到(Rodriguez-Sinovas et al., 2006)。如果總結上述之研究並以此下結論，可能會認為細胞間隙接合的存在於心肌缺氧與梗塞是有害的，而抑制細胞間隙接合將成為治療心肌梗塞的治療工具，但事實上卻不然。

隨著 Ischemia Pre-condition (IPC)的研究陸續加入，我們卻發現事情上細胞間隙接合的影響並非如此單純。Ischemic Pre-conditioning (IPC) 指的是在心肌承受持續性缺氧導致心肌梗塞前，接受數次短暫的缺氧事件；經 Ischemia precondition (IPC) 處理後之心臟，將更能抵抗心肌梗塞造成的心肌壞死、心律不整或再灌注心肌損傷(Yellon and Downey, 2003)。目前認為 Ischemic Pre-conditioning (IPC)的調控可能牽涉了 Src、 protein kinase C (PKC)以及 p38 mitogen-activated protein kinase (Steenbergen, 2002; Thijssen et al., 2002)；甚至包括了細胞間隙接合(Miura et al., 2010)與細胞自噬等(Yan et al., 2009)。近期研究顯示，利用可模擬 Ischemic Pre-conditioning (IPC) 之化學藥劑(D-Ala2,D-Leu5)-enkephaline acetate (DADLE)處理老鼠心臟，可降低心肌梗塞的區域。但如果同時使用抑制細胞間隙接合之藥劑: 正庚醇，則 DADLE 引發 IPC 的心臟保護能力將下降 65% (Li et al., 2002)。同時 IPC 對心臟的保護力，於 Connexin 43 基因剔除的老鼠也將不復見(Maejima et al., 2005; Schwanke et al., 2002)。總結來說，若要維持 IPC 時對心臟的保護力，心臟間隙接合必須需要持續維持正常功能。雖然，Connexin 43 對於 Ischemic Post-conditioning 的重要性，不若對於 Ischemic Pre-conditioning (Heusch et al., 2006)。部分學者認為細胞間隙接合是有保護細胞的功能，但心臟保護效果究竟是如何透過細胞間隙接合來達成仍屬未知，但合理懷疑其中必定與傳遞了重要的生存訊號或細胞自噬訊號相關，而此推測與本實驗之結果相互輝映。

4.1.2 細胞自噬與缺血/再灌流傷害

缺血/再灌流傷害(ischemia/reperfusion injury)意思指的是供應組織之血流的中止或再分布進而導致的細胞傷害。缺血狀態時，組織將呈現缺氧狀態時；長時間之缺血將導致能量缺乏與細胞壞死。但於再灌流時，可能會快速累積過氧化物(reactive oxygen species)，反而導致細胞損傷。再者，再灌流階段時許多炎症反應細胞也會隨著血液循環到達心肌受損區域，進而產生局部炎症反應，產生再灌流時心肌損傷。許多研究顯示，於心肌細胞面對缺血/再灌流狀態或長期缺氧狀態時將會產生大量的細胞自噬表現產生 AVOs (Takagi et al., 2007)；同時前述的 Ischemia preconditioning (IPC)也認為與細胞自噬相關，利用豬隻進行動物實驗發現，經 Ischemia preconditioning (IPC)處理，除了可降低大約 60%心肌梗塞大小外，同時也增加了細胞自噬基因之表現包括 Beclin-1 與 LC3(Gurusamy et al., 2009)。而細胞自噬可能扮演之角色包括(1)移除受氧化壓力導致壞損之細胞胞器與蛋白質(2)移除內質網內異常組裝之蛋白質 (3)維持能量恆定 (4)抑制細胞凋亡。

本實驗利用過氧化氫作為氧化壓力源，使其成為促發細胞自噬的物質，其產生之效果就如同心肌細胞面對 reactive oxygen species 或缺血/再灌流傷害狀況一般，因此維持細胞間隙接合穩定或許可以做為心肌梗塞與缺氧之心臟保護方式。

4.1.3 調控細胞自噬與心臟疾病治療

調控 Mammalian target of rapamycin (mTOR)目前被視為一個重要的疾病治療機制，抑制 mTOR 可以誘發細胞自噬表現。研究顯示，使用可抑制 mTOR 之 Everolimus (3.0 mg/kg/day)治療心肌梗塞後之老鼠可發現，處理 28 天後，心肌梗塞區域將顯著減少，同時心臟將有較小之 LV end-diastolic diameters、較低 end-diastolic volumes 與較小的心肌細胞大小。顯示抑制 mTOR 誘發細胞自噬將避免心臟梗塞後之心肌重塑(Buss et al., 2009)。

而目前藥物塗層支架所運用之 Sirolimus, Everolimus, Zotarolimus 皆為抑制 mTOR 機制相關，而這些藥物塗層支架產品之成功也被認為可能與內皮細胞之細胞自噬

誘發相關(Hayashi et al., 2009)。因此，可誘發細胞間隙接合的物質例如 4-oxoretinoic acid (Hanusch et al., 1995)，或許可以考慮做為下一個治療性藥物研發的方向。

4.2 研究之限制

4.2.1 藥劑作用方式使間隙接合角色重要性降低

利用過氧化氫作為氧化壓力源，使其成為促發細胞自噬的物質，可以模擬心肌細胞面對 Reactive Oxygen Species 或缺血/再灌流傷害狀況。但因為實驗過程中，過氧化氫配合 DMEM+PBS 細胞培養液的操作，將整體作用濃度調整為 100-400μM。如此設計雖可以避免過氧化氫氧化壓力作用不平均的狀況。但也因為此讓整個培養心肌細胞暴露到等量之過氧化氫氧化壓力，因而讓細胞間隙接合的訊息傳遞與細胞間交互影響的重要性降低。對於此可以考慮參考使用 Scrape Loading Methods 局部施予氧化壓力促發細胞自噬表現 (Udawatte and Ripps, 2005)，其後再評估鄰近之細胞是否接受到細胞自噬訊號，影響鄰近細胞表現細胞自噬。而使用 Scrape Loading Methods，可以運用較高濃度之過氧化氫或其他可以促發細胞自噬之物質包括 Rapamycin 或其他作用於 mTOR 之藥物包括 Everolimus。

4.2.2 正庚醇抑制細胞間隙接合不具專一性

本實驗運用細胞間隙抑制劑為正庚醇，在心臟組織實驗與細胞培養實驗皆證實可以有效抑制細胞間隙接合(Kimura et al., 1995; Lin et al., 2009)，但正庚醇屬於非專一性、可逆性的細胞間隙抑制劑，作用機轉未明，很有可能與降低細胞間隙接合之打開機率下降有關；而其他可能作用與機轉包括降低其他離子通道之通透性，改變細胞膜之流動性與結構皆曾被提出 (Bastiaanse et al., 1993; Takens-Kwak et al., 1992)。也因為正庚醇的影響可能經由細胞膜作用，並且特異性與專一性較低，因此正庚醇是否透過其他機制而達到抑制細胞自噬則仍有討論空間，而干擾我們實驗之判讀。對此可考慮運用更專一性抑制方式，包括使用 Connexin 43 mimetic peptides (Evans and Leybaert, 2007)、Connexin 43 抗體 (Meyer et al., 1992)或使用

Connexin 43 SiRNA(Ai et al., 2010; Yasui et al., 2000)。目前也可以考慮使用 Connexin 43 細胞外之第一生肽鏈片段，Gap26 最為抑制 Cx 43 的實驗方式。除了單方面抑制 Connexin 43 之外，同時可以運用另一方向思考，利用基因合成轉染細胞增加細胞間隙接合表現量 (Tolmachov et al., 2006; Udawatte and Ripps, 2005)，或是利用藥物如 4-oxoretinoic acid (Hanusch et al., 1995)以及 Antiarrhythmic peptide 10 (Quan et al., 2009)來增加 Connexin 43 的活性，反向印證是否會因為細胞間隙接合表現量增加而導致細胞自噬之活性較容易受到激發或感染周邊之心肌細胞。

4.2.3 H9c2 細胞株繼代培養代數不同

本實驗使用細胞，為 H9c2 細胞株，原為 cardiomyoblast 於 1976 年自 *Rattus norvegicus* Embryonic BD1X 分化出來 (Kimes and Brandt, 1976)。根據實驗觀察，於實驗當中隨著繼代培養(passage)的過程，細胞可能持續進行細胞分化，而進一步分化後，細胞的表現型與細胞特性可能進而改變導致後續細胞對於藥物之作用的反應改變 (Hescheler et al., 1991)。本實驗進行約維持兩年時間，著繼代培養之差異可達 10-20 繼代培養以上，因實驗非同一時間完成，無法將細胞控制在同一個繼代培養代數，因此部分實驗結果可能因此受到影響，導致實驗誤差、影響判讀。

4.2.4 正庚醇抑制細胞間隙接合效果未定

本實驗使用之細胞為不具收縮力的H9c2，相對於其他運用於心臟或心肌細胞之正庚醇實驗，我們無法利用觀察細胞是否停止收縮來判斷正庚醇能否有效抑制細胞間隙接合。因此需要考慮使用 Microinjection dye (LY) transfer method (Garcia-Dorado et al., 1997; Kimura et al., 1995)，Detect intercellular Ca^{2+} fluctuation (Garcia-Dorado et al., 1997; Kimura et al., 1995)，Tracking the movement of fluorescent dye calcein (Garcia-Dorado et al., 1997)。同時需要進一步證實，正庚醇僅於功能上抑制間隙接合之作用，而並不影響心肌細胞上 Cx43 之分布，因為從文獻可知，心肌細胞之細胞間隙接合之表現可能受疾病狀態影響導致表現量下降(Ai

and Pogwizd, 2005; Kostin et al., 2003)，或是導致表現分布位置改變情形(Hesketh et al., 2010)。因此可以進一步透過免疫螢光染色來確定細胞間隙接合之表正常。

4.2.5 忽略其他可能的細胞間交通方式

本研究重心為細胞自噬的細胞間交通與調控，乃採討論細胞間隙接合做完研究主軸，但事實上細胞間的交通與調控除了間隙接合外，可能還包括了 Tight Junction (Schneeberger and Lynch, 2004)、Desmosome (Lewis et al., 1997)、Paracrine Signal (Noseda and Schneider, 2009)、Juxtacrine Signal (Walker et al., 2008)等，在忽略其他細胞間交通方式的前提下，進行研究結果判讀可能過於武斷與偏頗。

4.2.6 Luciferase Report System 無法代表 LC3-II 的表現量

測量細胞自噬表現可以透過測量 Beclin-1、LC3、利用電顯偵測細胞自噬體、利用流噬細胞儀偵測 Acridine orange 陽性染色比例等(Klionsky et al., 2008; Mizushima, 2004)。其中 LC3-II 的表現量或是 LC3 II/LC3-I 比值更能反應細胞自噬體的表現量(Kabeya et al., 2000)。本實驗利用可以快速檢測並且將訊號強度放大之 Luciferase Reporter Assay 作為偵測細胞自噬體的方法，但該方法無法直接反應 LC3-II 的表現量與 LC3 II/LC3-I 比值。雖然許多實驗使用其他方式如 GFP-LC3 (Mizushima and Kuma, 2008)、mCherry-LC3 (Terada et al., 2010)或是 Gaussia luciferase (Ju et al., 2009)作為 Report Assay，但也僅止於顯示 LC3 的表現量而已。未能直接表現 LC3-II 的表現量，為利用 pCMV-LC3-Gluc 作為 Reporter Assay 最大的缺點。

但 Luciferase reporter assay 之方式，可以間接反應整體細胞自噬之功能與活性，因其表現量同時也反應著 ATG4b 活性，而 ATG4b 則負責將 LC3 切割成 LC3-II 與 LC3-I(Ketteler and Seed, 2008)。

4.2.7 過氧化氫同時造成細胞凋亡與細胞自噬

本實驗企圖利用模擬組織承受心肌缺氧/再灌注傷害之模式，因此利用過氧化氫提供氧化壓力，進而誘發細胞表現細胞自噬。但過氧化氫在此同時除了誘發細胞自噬的表現外，還有可能產生細胞壞死(Necrosis)以及細胞凋亡，進而干擾實驗的結果判讀。雖然我們有其他方式誘發細胞自噬如 Starvation (Munafo and Colombo, 2001)、 Insulin Deprivation (Baek et al., 2009)、 Heat Stress (Schwartz et al., 1992) 或是 Hypoxia (Jiang et al., 2010)等方式，但依舊無法避免作用後引發不同程度的細胞自噬、細胞凋亡與細胞壞死，畢竟這些細胞的現象存在可能的調控關係(Fimia and Piacentini, 2010)。但若使用直接使用 Rapamycin、Valproate、Tamoxifen 來誘發細胞自噬表現 (Rubinsztein et al., 2007)，雖可單純化實驗並減少干擾，但這樣的研究卻也無法貼切細胞的生理病理反應。

第五章 未來展望

研究細胞自噬之學者莫不抱持希望，有朝一日將細胞自噬運用於臨床治療。調控細胞間隙似乎可以左右細胞自噬之活性，並對於心臟之缺氧/再灌流傷害或 ischemia preconditioning 造成影響。要達成理想，仍有許多問題需解決包括: 細胞間隙所傳遞的是何種細胞自噬調控訊號? 細胞接受到細胞自噬訊號後究竟如何決定細胞之生或死? 以及是否還有其他細胞間的調控機制? 解決這些問題，仍有待其他實驗來印證。

未來我們將著手規劃更完善的實驗模組，包括細胞培養模或是動物模組、運用更專一性的細胞間隙抑制方式、並企圖找出細胞間隙間傳遞的細胞訊號為何、以及研究是否有其他的細胞間溝通方式影響細胞自噬。

第六章 論文英文簡述

1. Introduction

1.1 Autophagy

Autophagy was first observed about 50 years ago in the kidneys of newborn mice (Clark, 1957) and the term autophagy was first coined by de Duve (De Duve and Wattiaux, 1966) which open the field of this lysosome associated cellular process. Autophagy was described as an intracellular degradation process for the turnover of long-lived cytosolic proteins and organelles such as mitochondria and endoplasmic reticulum. Despite the early start 50 years ago, it was until recent 10 years where knowledge toward the induction, regulation and function of autophagy were accumulated rapidly (Kroemer and Levine, 2008; Mizushima et al., 2008). Autophagy was considered as an evolutionally conserved cellular mechanism which is essential for cell homeostasis under normal conditions and for the cell defense and adaptation against environmental stress.

There are three main autophagic pathways: macroautophagy (Yorimitsu and Klionsky, 2005), microautophagy (Kunz et al., 2004) and chaperon-mediated autophagy(Dice, 2007). Macroautophagy is the most prevalent form. The hallmark of macroautophagy is the de novo formation of the double-membrane vacuoles which were called autophagosomes. Mature autophagosome will fuse with lysosome, thereby generating

an autophagolysosome or autolysosome. The incorporation of the outer autophagosomal membrane with the lysosomal membrane eventually allows the degradation of the remaining inner single-membrane and the cytoplasmic content of the autophagosome by lysosomal hydrolases (Mizushima et al., 2008). By removing or recycling of long-lived proteins and organelles, autophagy became an essential mechanism for maintaining or lengthening cell survival. Moreover, Autophagy may also involved in cell death regulation, which called autophagic cell death or type 2 cell death (Scarlatti et al., 2009). Autophagic cell death was characterized by massive vacuolization of the cytoplasm and accumulation of double-membraned vacuoles. In contrast to apoptosis, there is little (or no) cell shrinkage and chromatin condensation.

To date, autophagy had been implicated in adaptive mechanism for confronting cellular stress such as starvation, hypoxia, oxidative stress or increased cell demands. The existence of autophagy provides cell survival, differentiation, development, and homeostasis as well as for fighting against oxidative stress and biological disease (Hamacher-Brady et al., 2006b; Mizushima et al., 2008; Nakai et al., 2007).

1.2 Regulation of autophagy

Induction of autophagy requires activity of Beclin-1 and its interacting partner, class III phosphoinositide 3 kinase (PI3K), also known as Vps34. Through binding of Atg 12, Atg 5, light chain 3 (LC3), phosphatidylethanolamine (PE) and other autophagy related

protein, autophagosomes are formed (Hsieh et al., 2009). LC3 is cleaved by the protease ATG4 to generate LC3-I and LC3-II. LC3-II is recruited into the forming of autophagosomal membrane. Therefore the expression of LC3-II or LC3-II/LC3-I ratio could be used for semi-quantification of autophagy expression. In addition to inducible class III phosphoinositide 3 kinase (PI3K) pathway, autophagy is negatively regulated by class I PI3K through mammalian target of rapamycin (mTOR). Other upstream regulatory signal includes growth factor signaling, phosphatidylinositol (PI)3-kinase/Akt, mitogen-activated protein kinases, AMP-dependent protein kinase (AMPK), small GTPases, trimeric G Proteins, inositol triphosphates and calcium Signaling (Nishida et al., 2008).

1.3 Autophagy meets apoptosis

Beclin-1as an essential activator of autophagy, when binds with Bcl-2/BclXL, the formation of autophagsome will be inhibited (Saeki et al., 2000). It's possible that Bcl-2/BclXL involved the regulation of both apoptosis and autopahy (Levine et al., 2008). The anti-apoptotic protein, Bcl-2, interacts with the evolutionarily conserved autophagy protein, Beclin-1. Thus, Bcl-2 not only functions as an anti-apoptotic protein, but also as an anti-autophagic protein via its inhibitory interaction with Beclin-1 (Pattingre et al., 2005). Although the detail interaction and regulation of Bcl-2 in autophagy was not completly prevailed. The regulatory role of Bcl-2 was indeed

observed in many cell types. The interaction of Bcl-2 and Beclin-1 was the key to the cross talk between autophagy and apoptosis.

Moreover, the autophagy related protein, Atg5 also possessed pro-apoptotic effect. Autophagy-related gene (Atg) 5 is a gene product required for the formation of autophagosomes. Over-expression of Atg5 provokes apoptotic cell death, therefore, represents a molecular link between autophagy and apoptosis (Yousefi et al., 2006). Although autophagy and apoptosis had differed biological behavior, the two mechanisms existed in a complementary and interactive way (Nishida et al., 2008).

1.4 Autophagy and heart disease

Autophagy was implicated in many human disease including aging, neurodegenerative disease, immunity, liver disease, development, tumorigenesis, differentiation and cardiovascular disease (Mizushima et al., 2008). The earliest description of autophagy in heart was documented in 1976(Sybers et al., 1976). Under basal or mildly stressed conditions, autophagy degrades and recycles diseased organelles, mis-folded protein or damaged mitochondria for maintaining homeostasis (Kim et al., 2007). In lysosome-associated membrane protein-2 (LAMP-2) deficiency mice, large accumulation of autophagic vacuoles and cardiomyopathy were observed (Tanaka et al., 2000). Increased autophagy activity was also observed during ischemia/ reperfusion injury, myocardial infarction, heart failure, hypertrophy and pressure overload state

(Nishida et al., 2009). Though autophagy had been observed in many disease conditions, it remained unclear whether autophagy is protective or detrimental in response to cardiovascular disease.

1.4.1 Autophagy versus myocardial ischemia:

During myocardial ischemia with hypoxia and nutrition deprivation, autophagy was activated through an AMP activated protein kinase (AMPK)-dependent mechanism (Matsui et al., 2007). AMPK inhibits mTOR, a negative regulator of autophagy, as well in turn to activate autophagy. BNIP3 is dramatically induced by prolonged hypoxia in neonatal cardiac myocytes, thereby mediating mitochondrial dysfunction and cell death (Regula et al., 2002). It is also evident that BNIP3 will induce autophagy via titrating Bcl-2 and/or Bcl-XL away from Beclin-1 (Tracy et al., 2007) or possibly through inhibiting mTOR (Li et al., 2007). In summary, autophagy seems to be a key pro-survival mechanism in hibernating cardiomyocytes during ischemia (May et al., 2008).

1.4.2 Autophagy versus reperfusion

During reperfusion period, AMPK was no longer been activated, whereas reperfusion further stimulates autophagy through Beclin-1 dependent mechanisms. Therefore, cell accumulated massive autophagosome and reduced activation of pro-apoptotic Bax (Hamacher-Brady et al., 2006a). However, in contrast to the generally protective effects

of autophagy during ischemia, autophagy during the reperfusion phase may not necessarily be protective. Some study even showed increased cardiomyocyte survival when hypoxia and reoxygenation-induced autophagy was inhibited (Valentim et al., 2006). Inhibition but not activation of autophagy during the reperfusion phase is accompanied by significant reduction in the size of myocardial infarction and cardiac myocyte apoptosis (Matsui et al., 2007). In summary, autophagy role during reperfusion period remained detrimental. Since excessive activation of autophagy could cause cell death; the combination of up-regulation of Beclin-1 and down regulation of Bcl-2 during the reperfusion phase may stimulating cell death; and autophagy and apoptosis are interconnected by common mediators (Pattingre et al., 2005).

1.4.3 Autophagy versus hypertrophy and heart failure

In study of ligation induced myocardial infarction rabbit model, the LC3-II/LC3-I ratio, which represent autophagy activity, was inversely correlated with post infarction left ventricular ejection fraction (Chen et al., 2010). This shade the light that autophagy correlates with heart systolic function. Rapamycin, which inhibits mTOR and in turn activates autophagy, prevents thyroid hormone-induced cardiac hypertrophy (Kuzman et al., 2007). Rapamycin was shown to regress the established cardiac hypertrophy which was (McMullen et al., 2004). Serial evidence also support that autophagy was

correlated with some other form of heart failure such as pressure overload heart failure (Zhu et al., 2007) and dilated cardiomyopathy (Shimomura et al., 2001).

1.4.4 Autophagy and atherosclerosis

The role of autophagy in atherosclerosis is poorly understood. Autophagy of smooth muscle cells (SMCs) in the fibrous cap may be the important mechanism underlying plaque stability. Autophagy may safeguards plaque cells against oxidative stress, by degradation of the damaged mitochondria (Kiffin et al., 2006). However, the postulated role is not always positive. Excessively stimulated autophagy may cause autophagic SMC death, which in turn results in plaque instability, owing to the reduced synthesis of collagen and thinning of the fibrous cap. Autophagic death of endothelial cells may be a primary mechanism for acute lesional thrombosis (Levine and Yuan, 2005).

1.5 Gap junction intercellular communication

With broad understanding of autophagy, scientist was about to formulate therapeutic strategies based on autophagy intervention (Bao et al., 2010). However, before utilizing autophagy as therapeutic tool, the design should involve understanding the detailed processes, including induction, formation and degradation, to intra-cellular and inter-cellular regulation. The intra-cellular control and molecular mechanism of autophagy had been investigated vigorously. However, the intercellular regulation of autophagy signal between cells was lacking. When talking about intercellular signal

exchange, gap junction intercellular communication always plays a pivotal role. Gap junction directly connects the cytoplasm between two cells, and allows selective various molecules and ions to pass freely between cells (Lampe and Lau, 2000). The signal for chemical communication between cells includes small second messengers, inositol triphosphate (IP3) and calcium (Ca2+) and molecules smaller than 1,000 Daltons. The main functions include electrical and metabolic coupling, electrical and metabolic exchange, cytoplasmic connection and direct cytoplasmic signal transduction. The intercellular regulation of apoptosis had been well-documented. In contrast, the intercellular communication of autophagy signal between cells was never investigated and the role of gap junction in autophagy was unknown.

1.6 Intercellular mechanism of autophagy

For cell death and survival regulation, apoptosis was implicated in an important role. Evidence showed that, there are apoptotic signal exchanges through gap junction (Contreras et al., 2004; Kalvelyte et al., 2003). Administration of carbenoxolone, a gap junction uncoupler, to ischemic brain cell prevented caspase-3 activation and dramatically reduced neuron death (de Pina-Benabou et al., 2005). When stably transfected with the gap-junctional protein connexin32 to baby hamster kidney (BHK) cells, apoptosis had spread from dying cells to healthy neighbor cells. Despite the fact that cyC is too large to traverse the gap junctional (Udawatte and Ripps, 2005). It seems

that gap junction assists in cell death, but recent evident question the point of view (Decrock et al., 2009; Taimor, 2000). In neonatal rat ventricular myocytes treated with antisense oligonucleotide for connexin43, which down regulate of Cx43 expression, the progress of apoptosis was accelerated (Yasui et al., 2000). Mitochondrial connexin 43 also acts as a cytoprotective mediator of signal transduction in mouse cardiomyocytes (Rottlaender et al., 2010). The proponents for pro-survival gap junction, considered that the potential mechanism rely on limiting the large molecule toxic substance transfer, offer pro-survival signal transfer such as vitamin C, ATP, glucose or anti-apoptotic signal transduction (Decrock et al., 2009; Nakase et al., 2003). Will autophagy signal be part of the mechanism?

1.7 Study purpose

For autophagy inter-cellular regulation remain to be elucidated, especially the role of gap junction. Autophagy control possessed therapeutic potential in heart failure, left ventricular hypertrophy, myocardial ischemia, infarction, reperfusion injury, and atherosclerosis. We investigate the contribution of gap junction to cardiomyocyte autophagy which was produced by oxidative stress. We hypothesized that autophagy signal between individual cardiomyocyte was conducted through gap junction.

2. Material and methods

2.1 Chemicals

1-Heptanol (Sigma, St. Louis, MO, USA). Hydrogen Peroxide (H_2O_2, Sigma, St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), F12 medium, phosphate-buffered saline (PBS), 0.25% trypsin-EDTA, streptomycin-penicillin, amphotericin B solutions (Gibco, Gaithersburg, MD, USA). Fetal bovine serum (US Bio-Technologies, Parkerford, PA, USA), pCMV-Lc3 (OriGene, Rockville, MD, USA)、TurboFect ™ in vitro Transfection Reagent (Fermentas), MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyltetrazolium bromide) (Sigma, St. Louis, MO, USA). All chemicals were used according to the manufacturer's instruction.

2.2 Cell culture

We use H9c2 (2-1) cell culture as study model. The source of H9c2 comes from the Bioresource Collection and Research Center of Food Industry Research and Development Institute, Taiwan (FIRDI) which was derived from American Type Culture Collection (ATCC ®). H9c2 were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and supplemented with 10% fetal bovine serum (FBS) and 0.6% penicillin/streptomycin (Invitrogen) at 37 ℃ with 5% CO2 in a humidified atmosphere (Koudssi et al., 1998; Long et al., 1991). H9c2 at the concentration of $5x10^4$~$4x10^5$/well were seeded in 6 well-plate, 12 well-plate and 96 well-plate overnight before beginning any chemical treatment.

2.3 Construction of plasmid pCMV-Lc3-Gluc

To generate a plasmid coding for a fusion protein containing Lc3 and luciferase (plasmid pCMV-Lc3-Gluc), the DNA fragment coding for luciferase insert was excised from plasmid pCMV-Gluc with Hind III and Xba I and blunted with T4 DNA polymerase. The pCMV-Lc3 vector was linearized by digestion with EcoR V and dephosphorylated by shrimp alkaline phosphatase. The linearized pCMV-Lc3 vector and the Lc3 insert were ligated using T4 DNA ligase. E. coli transformants were selected based on kanamycin resistance. Plasmids from transformed E. coli were screened by restriction enzyme digestion and confirmed by sequence analysis. The plasmid pCMV-Lc3 and pCMV-Gluc were purchased from Origene and New England BioLabs respectively (Tannous et al., 2005; Verhaegent and Christopoulos, 2002).

2.4 Transient Transfection and Luciferase Activity Measurement

All transfections were performed at 80% cell confluence using TurboFect ™ in vitro Transfection Reagent (Fermentas) according to the manufacturer's instructions. For experiments involving luciferase assays, 10 μg reporter plasmid pCMV-Lc3-Gluc was transfected into H9c2 cultured on T75 Flask. Transfected H9c2 cells were seeded to 12-well plate at concentration of $5x10^4$ while each well contained 1.5 ml of 10%FBS/DMEM. Cells were incubated at 37 ℃ with 5% CO2 in a humidified atmosphere for 24 hr and all medium were refreshed before study. We added BioLux™

GLuc Assay Buffer for luciferase release assay. Chemiluminescence analyzer were used for luminometric (CPS, count per second) and the luminometric results stand for LC3 expression and autophagy activity (Ju et al., 2009; Verhaegent and Christopoulos, 2002; Wu et al., 2007).

2.5 Cell viability analysis

Cell viability was measured using MTT bioreduction assay(Mosmann, 1983). MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, was purchased from Sigma. Cell culture plates were washed with PBS, exposed to 0.2g/dL of MTT solution, and kept at 37 ℃ for 60 min. The blue crystals that appeared were dissolved in DMSO at the end of incubation. We examine the optical density of each plate at 550 nm on a scanning multi-well spectrophotometer. Percentage of cell survival was expressed as $\frac{\text{absorbance of treated cells}}{\text{absorbance of control cells}} \times 100\%$ (Monastyrskaya et al., 2002).

2.6 Autophagy detection

2.6.1 Reverse-transcription polymerase chain reaction (RT-PCR)

Measuring LC3 and Beclin-1 mRNA were used for detecting autophagy expression. Total RNA was isolated from H9c2 using Trizol reagent (Invitrogen) according to the manufacturer's protocol. ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) was use for quantifying gathered total RNA (Chen et al., 2007). The first stranded complementary DNA was synthesized from 2 μg of total

RNA using a RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol.

Reverse transcription (RT) was carried out at 42 ℃ for 60 min following incubation at 70 ℃ for 5 min. Complementary DNA amplification was carried out according to the following temperature profile: 94 ℃, 30 s; 50 ℃, 1 min; and 72 ℃, 1 min. At the end of 35 cycles, the reaction was prolonged for 10 min at 72 ℃, and 5 μl of product was analyzed on a 2.0 % agarose gel. The intensity of the bands was measured using Alpha Innotech, Alpha.Imager® Mini software.

The following are the sequences of the primers:

Rat Cx43 sense: 5'-AAAGGCGTTAAGGATCGCGTG -3';

Rat Cx43 antisense: 5'- GTCATCAGGCCGAGGCCT -3';

Rat LC3 sense: 5'-GCCTGTCCTGGATAAGACC-3';

Rat LC3 antisense: 5'-TTGGGAGGCATAGACCATGT-3';

Rat GAPDH sense: 5'-GCACCACCAACTGCTTAGC-3';

Rat GAPDH antisense: 5'-TGAGTGGCAGTGATGGCAT-3'

2.6.2 Western blotting

H9c2 were washed twice in cold phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer(Santa Cruz, Santa Cruz, CA)(Chen et al., 2007). A total of 20μg protein of cellular lysates was then electrophoresed on a 10% SDS-polyacrylamide gel and transferred to polyvinylidine difluoride membranes (Amersham Bioscience) by electroblotting. The membranes were incubated in 5% nonfat milk overnight, then incubated with mouse anti-LC3-I, anti-LC3-II, anti-Bax antibody for 2 hours, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG for 1 hour. Immunoreactive proteins were visualized using a commercially available enhanced chemiluminescence kit with exposure of the transfer membrane to X-ray film. As a loading control, blots were stripped and reprobed with an antibody against β-actin (Liu et al., 2007). The density of the bands on the membranes was scanned and results were quantified using QUANTITY ONE (Bio-Rad).

2.6.3 Flow cytometry

The acidic autophagic vesicles were visualized by supravital staining with a pH-sensitive dye acridine orange. The alkaline acridine orange would enter acidic compartments such as lysosomes and autolysosome for staining. Briefly, cells were washed with 1ml PBS and stained with acridine orange (100 mg/ml) for 15-20 min. Acridine orange-stained cells were trypsinized, washed with 0.3 ml PBS, and analyzed by flow cytometry on BECTON DICKINSON, FACS Vantage. We quantify autophagy

intensity by measuring the red/green fluorescence ratio (FL3/FL1), which increases during enhanced autophagy (Kanzawa et al., 2005; Levine and Yuan, 2005; Mujumdar et al., 2010).

2.7 Study design

Our study composed of 3 parts, which was described in detail below. The first part of the study composed of examinations for establishing the laboratory models. We test the H9c2 cell effect after treating with hydrogen peroxide and 1-Heptanol. The second part of the study composed of reporter plasmid pCMV-Lc3-Gluc construction. The constructed luciferase report plasmid when transfected into H9c2 was taken as reporting system for autophagy expression. During the third part of study, we test our hypothesis by dividing wild type H9c2 and reporter plasmid pCMV-Lc3-Gluc transfected H9c2 into 4 groups, including the control group, the Heptanol group, the H_2O_2 group and the H_2O_2+Heptanol group. We selected the ideal drug concentration from our first part study and apply to the groups. 1-Heptanol was administered for gap junction blockade and hydrogen peroxide was taken as source of oxidative stresser. By comparing the H_2O_2 group which was oxidatively stressed and the H_2O_2+Heptanol group which had gap junction blocked during oxidative stress, we are able to tell the role of gap junction in autophagy regulation (**圖表 1**).

2.8 Statistic analysis

All data were process by PC version of SPSS statistical soft ware, 15th version. We use Mann Whitney U for non-parametric analysis and analysis of variance (ANOVA) were used for multiple groups. P value less than 0.05 was considered statistically significant. The graph was depicted by GraphPad Prism 5 software.

3 Results

3.1 Part I- hydrogen peroxide and 1-heptnol effect

3.1.1 Hydrogen peroxide Oxidative stress on H9c2

Hydrogen peroxide had been use as a source of oxidative stresser in many other studies, but whether the drug will induce autophagy in H9c2 or not was still unknown. We tested the H9c2 response after exposure to hydrogen peroxide.

3.1.1.1 To test the autophagy induction ability of hydrogen peroxide

We seeded 1.36 $x10^5$ H9c2 to 12-well plates. After overnight culture in the incubator, we applied H_2O_2 400 μM to each well for different duration, 0 min, 30 min, 60 min, 90 min, 120 min and 180 min. RT-PCR was use to analyze the expression of LC3, Beclin-1and Bcl-2 mRNA (**圖表 2**). The expression of LC-3 and Beclin-1 which represents for autophagy activity was dramatically increased after 90 min of hydrogen peroxide treatment. While Bcl-2 as the pro-apototic protein and also possible regulator in autophagy, the expression remained constant. Band analysis by Alpha.Imager® Mini

after adjusting to gapdh level, revealed that at the end of 180 min treatment, LC3 expression increased 29.3% while Beclin-1 increased about 36.7% (圖表 3).

3.1.1.2 To testify the effect of treatment duration

We also tested the level of protein expression by Western Blot after hydrogen peroxide stimulation. After seeding 2.0 x105 of H9c2 to 10cm2 culture dish overnight in the incubator, hydrogen peroxide 400 μM was administered to the culture dish for 1.5 hr, 3 hr, 6 hr, 12 hr, 16 hr and 24 hr. We tested the autophagic and apototic protein expression level a different time point. With the hydrogen peroxide treatment, the expression of LC3 increased gradually atfer 6 hour later (圖表 4).

. Band analysis results after adjusting to actin was expressed in (圖表 5). At the end of 6 hours, LC3-I expression increased 45.2% while LC3-II increased dramatically up to 4.54 folds. At the end of 24 hrs, LC3-I expression increased 2.5 folds while LC3-II increased dramatically increased 7.06 folds. We also calculated LC3-II/LC3-I which also showed the trends of increment after treatment (圖表 6 & 表格 1).

At the mean time, the expression of pro-apoptotic protein Bax was also induced 6 hours after hydrogen peroxide treatment (圖表 8). The timing of enhanced Bax expression coincided with LC3 expresssion.

Alternatively, flow cytometry study for acidic vacuole detection after hydrogen peroxide stimulation showed that hydrogen peroxide 400 μM increased acridine orange

stain positive acidic vacuoles which means that autophagy expression was induced by hydrogen peroxide (圖表 7). Meanwhile, for detecting positive Annexin V stain percentage, flow cytometry showed exceeding 20% of expression after 24 hours of hydrogen peroxide 400 μM treatment (圖表 9) which may explain the concomitant induction of autopahy and apoptosis by hydrogen peroxide.

3.1.1.3 To test the viability after long period of hydrogen peroxide treatment

H9c2 cell viability after hydrogen peroxide stimulation was investigated by MTT assay. We studied the time factor on H9c2 by using constant hydrogen peroxide concentration at 200 μM and different stimulation duration up to 24 hours. It showed that after 12 hours of hydrogen peroxide treatment, the cell viability became 86.5%(n=5, P =0.13) while after 24 hours of treatment, the viability remained 85.2% (n=6, P=0.009)(**圖表 10** &表格 2).

3.1.1.4 To test the concentration effect

To determine the ideal concentration for autophagy induction by hydrogen peroxide, we designed the study using different concentration of hydrogen peroxide. We seeded 1.2 x105 of H9c2 to 12-well plate, and 0 μM, 50μM, 100μM, 500μM and 1000μM of hydrogen peroxide was administered to H9c2. After 24 hours of incubation, we determined the level of LC3 and Bcl-2 mRNA expression by RT-PCR. There was no

significant difference in LC3 expression at any concentration level when treatment duration exceeded 24 hours (圖表 11).

We also studied the concentration factor on H9c2 protein expression when hydrogen peroxide was administered for 12 hours. From the Western blot study, we found that the higher the hydrogen peroxide concentration, the higher the expression of LC3 protein (圖表 12). Using 100μM H_2O_2 for 12 hours, the LC3-II expression increased 2.48 folds (圖表 13), while LC3-II/LC3-I ratio elevated from 0.45 at basal state to 0.84. Further increasing the concentration to 800 μM, the LC3-II expression increased 2.56 folds and LC3-II / LC3-I ratio increased 1.29 folds in contrast to the basal state (圖表 14, 圖表 15 &表格 3).

3.1.1.5 To test the viability after high concentration treatment

Cell viability under high concentration of hydrogen peroxide was studied by MTT assay. When 0, 50, 100, 200, 400 and 800 μM of hydrogen peroxide were applied for 12 hours, we found that a dramatic decrement of cell viability below 50% when hydrogen peroxide exceeded 200 μM (圖表 16).

3.1.1.6 Hydrogen peroxide and Cx 43 expression

In addition to testify the ability of hydrogen peroxide for autophagy induction, we also evaluate the influence on gap junction, Connexin 43 expression. We seeded $2.0x10^5$ H9c2 cell to 6 well-plates. After overnight incubation, we added 400μM hydrogen

peroxide to each wells. By using RT-PCR for RNA expression analysis, we found that Connexin 43 mRNA expression dropped 23 % when treatment exceeded 14 hours. When treatment beyond 24 hours, the remaining Connexin 43 mRNA level was 26% of the basal level (圖表 17 & 圖表 18). Protein expression of membrane fraction Connexin 43 was also decreased after 24 hours of hydrogen peroxide 400μM treatment (圖表 19).

3.1.2 1-Heptanol on H9c2

We also investigated the influence of 1-Heptanol on H9c2 before utilizing 1-heptanol as gap junction uncoupler.

3.1.2.1 Heptanol and Cx 43 expression

We seeded $2.0x10^5$ H9c2 to 6 well-plates with overnight incubation. After 5 minutes of 0, 0.01, 0.1, 0.5, 1.0 and 2.0 mM of heptanol of treatment, Cx43 expression was analyzed by RT-PCR. The Connexin 43 mRNA expression remained constant, and exceeded 72% even under 2.0 mM heptanol (圖表 20 & 圖表 21).

3.1.2.2 Heptanol and cell viability

Cell viability after 1-Heptanol treatment was evaluated by MTT assay. After seeding $2.0x10^5$ H9c2 to 6 well-plates with overnight incubation, we investigated the time factor

of 1-heptanol treatment, which at constant concentration of 0.5 mM. Cell viability remained higher than 85% after 24 hours of incubation (圖表 22).

3.1.3 Summary to Part I study

We found that hydrogen peroxide will induce apoptosis as well as autophagy after long duration of stimulation. Lengthening the reaction time may not exactly disclose a dose-responsive curve, and the largest reactive change happened probably at 6 to 12 hours. High concentration, especially exceeded 200μM, and long duration, especially exceeded 12 hours of hydrogen peroxide treatment may results in significant reduction of cell viability, apoptosis activation and decrease Cx 43 expression. The ideal autophagy induction by hydrogen peroxide should be 200μM concentration for 12 hours. Meanwhile, 1-heptanol as a gap junction uncoupler will not interfere the expression of Cx 43 expression. Despite long duration of 1-heptanol stimulation, the cell viability remained higher than 85%.

3.2 Part-II: Utilizing Luciferase Report System

3.2.1 Constructing Gaussia Luciferase report assay

We had constructed pCMV-Lc3-Gluc by inserting DNA fragment coding for luciferase into pCMV-Lc3 vector. The plasmid was screened by restriction enzyme digestion and confirmed by sequence analysis. The pCMV-Lc3-Gluc was taken as reporter plasmid for Lc3 expression which also stands for autophagy expression. By transfecting H9c2,

the reporter plasmid was delivered into H9c2 and the whole system was taken as Gaussia Luciferase report system.

3.2.1.1 Effect of transfection

We compare the expression of LC3 among wild type H9c2 and pCMV-Lc3-Gluc transfected H9c2. We found that pCMV-Lc3-Gluc transfected H9c2 had slight increment of LC3 mRNA expression up to 42.9% (P=0.10, n=3) (圖表 23 & 圖表 24). From flow cytometry, we also found that pCMV-Lc3-Gluc transfected H9c2 had higher percentage acridine orange stain positive acidic vacuoles (圖表 25).

3.2.1.2 Confirm the role for reporting autophagy

We applied hydrogen peroxide to pCMV-Lc3-Gluc transfected H9c2 in order to confirm the use of Gaussia Luciferase report system for autophagy report. After 400μM hydrogen peroxide stimulation for 0-24 hours, we found that at the beginning of 8 hours stimulation, there is a significant increment in the luciferin optical density level which is about 12% (P=0.04, n=4). At the end of 12 hours hydrogen peroxide stimulation, the luciferin optical density level increased 31% (P<0.001, n=4). The luciferin optical density level reached plateau since 14 hours of stimulation, and the increment was about 40-49% (P<0.001, n=4) (圖表 26, 圖表 27 &表格 6).

3.2.2 Summary to part II

We had successfully established the Gaussia Luciferase Report System by contracting pCMV-Lc3-Gluc and transfecting into H9c2. Though, trasnfecting H9c2 with pCMV-Lc3-Gluc resulted in higher level of LC3 expression. The whole system could be used to detect autophagy in a fast, convenient and sensitive way. The report assay showed significant increment of LC3 expression at 8 hours after hydrogen peroxide stimulation and the largest autophagy expression may developed about 14 hours of treatment.

3.3 Part III: determine the role of Gap junction in autophagy

3.3.1 Wild type H9c2 model

With wild type H9c2 during the third part of the study, we use four-group model with the Control group, the Heptanol group, the H_2O_2 group and the H_2O_2+Heptanol group. In the control group, we use DMEM culture medium throughout the study. In the Heptanol group, we use 0.5mM 1-heptanol for gap junction blocking. In the H_2O_2 group, and we used 200 μM hydrogen peroxide for autophagy induction. Finally in the H_2O_2+Heptanol group, we use 1-heptanol at working concentration of 0.5mM and hydrogen peroxide at working concentration of 200μM. After seeding $2.0x10^5$ H9c2 into 6-well plates with overnight incubation, we administered 1-heptanol and/or hydrogen peroxide into culture plate according to the grouping condition. After another

12 hours of incubation, we analyzed mRNA expression in each groups (圖表 28 & 圖表 29). Comparing the Control group and the Heptanol group, the expression of LC3 mRNA in the Heptanol group was about 90.8% of the Control group (P=0.20, n=3) while Beclin-1 and Bax expression remained no significant change. Comparing the Control group and the H_2O_2 group, we found that the LC3 mRNA expression increased 10.8% under hydrogen peroxide stress after 12 hours (P=1.00, n=3). While in the H_2O_2+Heptanol group, LC3 mRNA expression had 24.2% decrement when comparing to the H_2O_2 group (P=0.4, n=3).

3.3.2 pCMV-Lc3-Gluc transfected H9c2 model

We utilized pCMV-Lc3-Gluc transfected H9c2 and group into the control group, the Heptanol group, the H_2O_2 group and H_2O_2+Heptanol group. After seeding $5x10^4$ H9c2 into 12-well plates with overnight incubation, we administered 1-heptanol and/or hydrogen peroxide into culture plates according to the grouping condition. The optical density value of luciferin in each well, which represent LC3 expression, therefore the autophagy expression, were measured (圖表 30, 圖表 31, 圖表 32, 圖表 33 & 表格 7).

In comparing the control group and the Heptanol group, we found that 1-heptanol did not itself induce further autophagy expression nor making significant reduction of basal autophagy activity. At the end of 16 hours incubation, the luciferin optical density value

was 96.5% to the control group (P >0.05, n=4). In comparing the control group and the H_2O_2 group, we found that hydrogen peroxide induced 60.6% increment of autophagy expression after 12 hours of stimuli(P<0.001, n=4), and 78.0% of increment after 16 hours of stimuli (P<0.001, n=4). Finally, comparing the H_2O_2 group and the H_2O_2+Heptanol, we found 1-heptanol result in 20.6% reduction of autophagy expression (P<0.001, n=4).

3.3.3 Summary of Part-III

In summary, we found that gap junction uncoupler, 1-heptanol, will reduce 24% of LC3 mRNA expression when H9c2 cell was under the stress of hydrogen peroxide. Although the results were statistically insignificant, the trends were constant.

In H9c2 with Gaussia Luciferase Report System, we had found 20% reduction of autophagy under the use of 1-heptanol. In both study, reduction of autophagy gene and protein expression was obsereved after blockade of gap junction.

4. Conclusion and discussion

Our study had shown the evidence that gap junction plays a role in intercellular regulation of autophagy for the first time. We found that the basal expression of autophagy was not interferred by gap junction uncoupler, but in oxidative stress, autophagy expression was lessened. This finding showed that gap junction may involve in intercellular communication of autophagy signal. In the era where scientist tried to

achieve the disease treatment by using autophagy control, our study opened another window for approaching it.

4.1 Gap junction and heart disease

Traditionally, gap junctions were known to mediate electrical coupling between cardiomyocyte, which enables physiological synchronized contraction of the atria and ventricles However, gap junctions were more than that. Long before the molecular era, gap junctions were noted to have an additional role in mediating cardiomyocyte survival. During myocardial infarction, irreversible ischemic myocardial cell injury develops in an increasing number of cells as the duration of coronary occlusion is prolonged (Reimer et al., 1977). While early coronary artery revascularization helped in salvaging of myocardial tissue (Maroko et al., 1972). From this point of view, interventional field became the main stay in cardiology and guidelines were edited in order to lead the treatment of myocardial infarction (Antman et al., 2004). In the heart, gap junction contributes to necrosis of cardiomyocytes after ischemia-reperfusion and the physical cell-to-cell interaction as a cell death mechanism is necessary to reproduce “confluent” myocardial infarct in a computer simulation(Garcia-Dorado et al., 1989). The concept of reperfusion injury were gaining notice daily (Schroeder, 2007), while most of the intervention cardiologist tried to open the occluded vessel.

During reperfusion in swine hearts, a significant limitation of infarct size, lower lactate dehydrogenase release, and fewer contraction band necrosis were achieved by administration of a gap junction blocker (Garcia-Dorado et al., 1997). These results demonstrated that hypercontracture may be transmitted to adjacent myocytes through gap junctions and that heptanol may interfere with this transmission and reduce the final extent of myocardial necrosis during reoxygenation or reperfusion. Moreover, gap junction uncouplers, when given during hypoxia, also attenuated lactate dehydrogenase release during subsequent reoxygenation (Rodriguez-Sinovas et al., 2006). Some may conclude that gap junction assisted cardiomyocyte death during ischemia or reperfusion, and blocking gap junction can be used to treat acute myocardial infarction. However, this is not where we are today. Since the declaration of ischemia precondition (IPC) study, we had exactly the opposite concept now. Ischemic preconditioning (IPC), denotes a brief episode or episodes of ischemia before long sustained ischemia, affords protection against myocardial necrosis and arrhythmias during the sustained ischemia-reperfusion (Yellon and Downey, 2003). The regulation of IPC may involve Src, protein kinase C (PKC), p38 mitogen-activated protein kinase which are also known to participate in GJC regulation (Steenbergen, 2002; Thijssen et al., 2002). The effects of IPC on the gap junction and its functional significance have been investigated (Miura et al., 2010). Moreover, autophagy may also involve in IPC (Yan et al., 2009). Using (D-Ala2,D-Leu5)-enkephaline acetate (DADLE), an IPC mimetic, show infarct

size-limiting effect. But the pro-survival effect of DADLE was decreased for about 65% under gap junction uncoupler 1-Heptqanol use (Li et al., 2002). Furthermore, IPC failed to protect the myocardium of Cx43 heterozygous knockout mouse from infarction (Maejima et al., 2005; Schwanke et al., 2002). To sum up, these results suggest that gap junctions need to be opened for protective signal transduction during the trigger phase of IPC. Although, the role of Cx43 in ischemic post-conditioning is less documented (Heusch et al., 2006). Scientists' point of view toward gap junction in cardiomyocyte ischemia is now evolving. It's reasonable to say that there must be some kind of signal exchange through gap junction during the physiological or pathological state.

4.1.1 Autophagy and ischemia/reperfusion injury

Ischemia-reperfusion injury refers to the arrest and restitution of blood flow that may occur during circulation arrest and myocardial infarction, organ hypoperfusion and hypoxia, or by mechanical intervention. Prolonged ischemia can result in loss of energy source (ATP depletion) and necrotic cell death. Additionally, during the reoxygenation of tissue after ischemic/hypoxic episodes which may result in the increased production of reactive oxygen species and further promotes tissue injury. Moreover during reperfusion, inflammatory cells and cytokines will be recruited to the site of injury which makes the thing worse. During ischemia-reperfusion phase, accumulated formation of acidic vacuole had be observed (Takagi et al., 2007). Besides decreasing

infarction size up to 60% during IPC, autophagic expression such as Beclin-1 and LC3 were also enhanced in swine model (Gurusamy et al., 2009). Our study using hydrogen peroxide as oxidative stress mimics the state of ischemia-reperfusion injury further showed the evidence that autophagic signal through gap junction intercellular communication may be an important adaptive mechanism.

4.1.2 Therapeutic potential of autophagy in cardiovascular disease

Regulation of mammalian target of rapamycin (mTOR) had been utilized in clinical post-transplantation use. Meanwhile, there were studies considered mTOR having additional therapeutic potential base on the concept of autophagy regulation. The concept was built up from the belief that inhibiting mTOR can induce autophagy activity. For example, mammalian target of rapamycin inhibition was believed to be beneficial on left ventricular remodeling after myocardial infarction. In mouse infarction model, after 28 days, everolimus treatment (3.0 mg/kg/day) had reduced post-MI remodeling, with improved LV function and smaller LV end-diastolic diameters (Buss et al., 2009). While currently we had coronary stents eluted with sirolimus, everolimus and zoztarolimus in clinical use. These drugs targeted toward mTOR, and the product success may also have something to say with autophagy induction (Hayashi et al., 2009). Therefore, gap junction regulation agents such as 4-oxoretinoic acid maybe the next generation drugs (Hanusch et al., 1995).

4.2 Study limitation

There were several limitations in our study. We use hydrogen peroxide as oxidative stress for autophagy induction. Ideally this model mimics ischemia-reperfusion injury model in cardiomyocyte. However, during the study, we administered hydrogen peroxide evenly to the culture plate which means that every cell in the culture plate exposed to the drug at the same time. This method avoided uneven drug stimulation. However, the design may make intercellular gap junction communication less important. For each individual cell had direct autophagy induction from surrounding oxidative stress, but not through the gap junction intercellular communication. To solve the problem, we may use scrape loading method, which delivered oxidative stress locally and investigate the neighborhood cells for any change of autophagy expression(Udawatte and Ripps, 2005). Under such design, we need a sensitive method for autophagy induction and may need to use higher intensity of oxidative stress. Besides direct hydrogen peroxide stimulation, we may deliver rapamycin or everolimus focally by scrape loading method alternatively

In our study, we use 1-heptanol as gap junction uncoupler which had been used in both cardiomyocyte and heart tissue model (Kimura et al., 1995; Lin et al., 2009). However, the gap junction uncoupler mechanism for 1-heptanol was unknown. It's blocking is not specific enough either. Proposed mechanism includes interfering nonjunctional

membrane ionic currents, disrupting structure of the lipid membrane or decreasing decrease in the fluidity of membranous cholesterol-rich domains (Bastiaanse et al., 1993; Takens-Kwak et al., 1992). Therefore, we are not confident enough to say that, lessened autophagy expression after 1-heptanol use 100% based on the gap junction blocking. We still need and specific blocking methodology in the future, such as using Connexin 43 mimetic peptides (Evans and Leybaert, 2007), Connexin 43antibody (Meyer et al., 1992) or Connexin 43 SiRNA (Ai et al., 2010; Yasui et al., 2000). Alternatively, by increasing gap junction expression, we can also investigate the physiological effect(Udawatte and Ripps, 2005).

5. Future direction

We proved that autophagy may be regulated by gap junction intercellular communication. However, we have not figure the exact autophagy signal yet, nor other potential intercellular regulatory mechanism such as paracrine or autocrine signals. We have not figure the key determinants for cell destiny under autophagic flux. These will be the important issues before we put autophagy into clinical practice.

第七章 參考文獻

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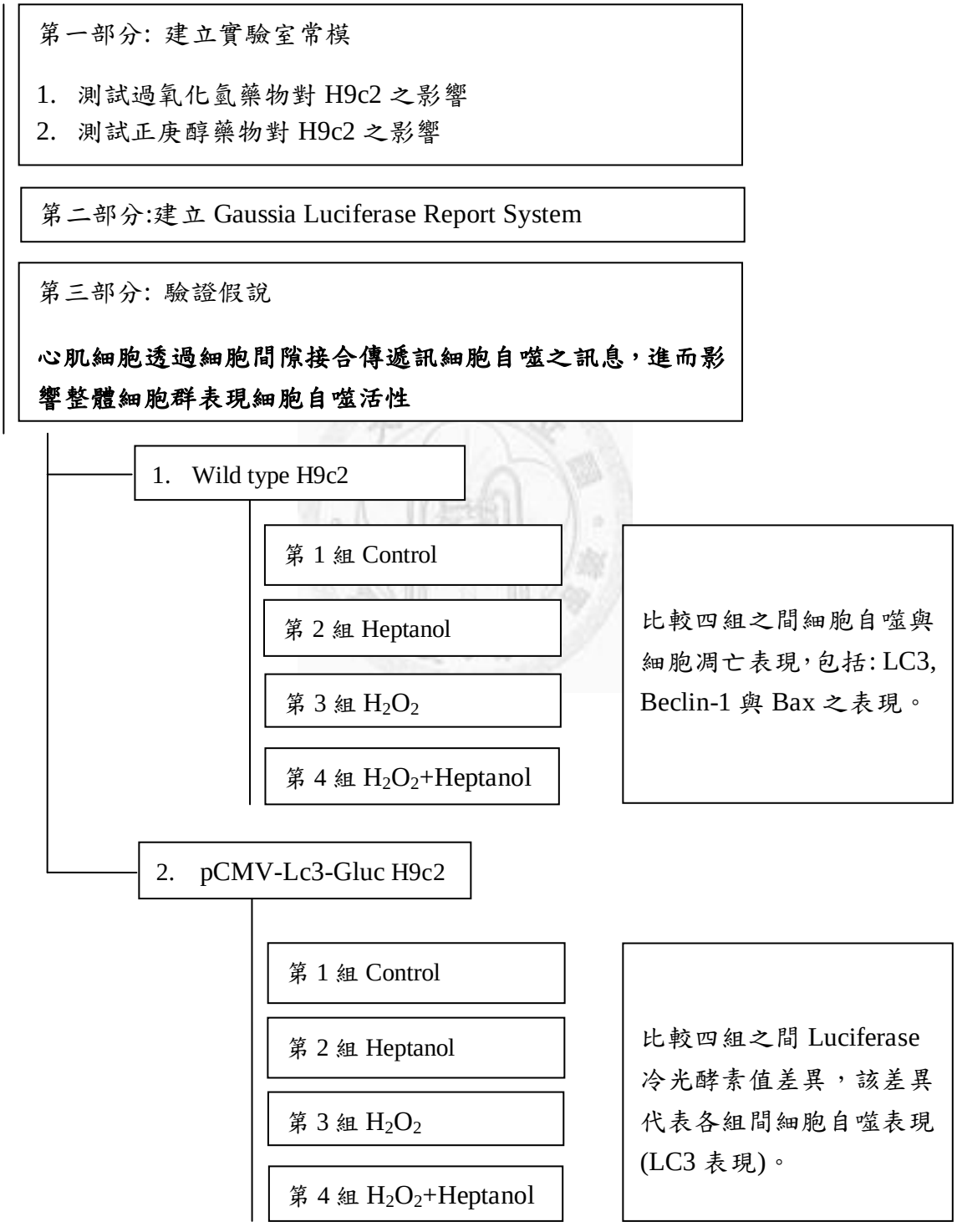
圖表附錄

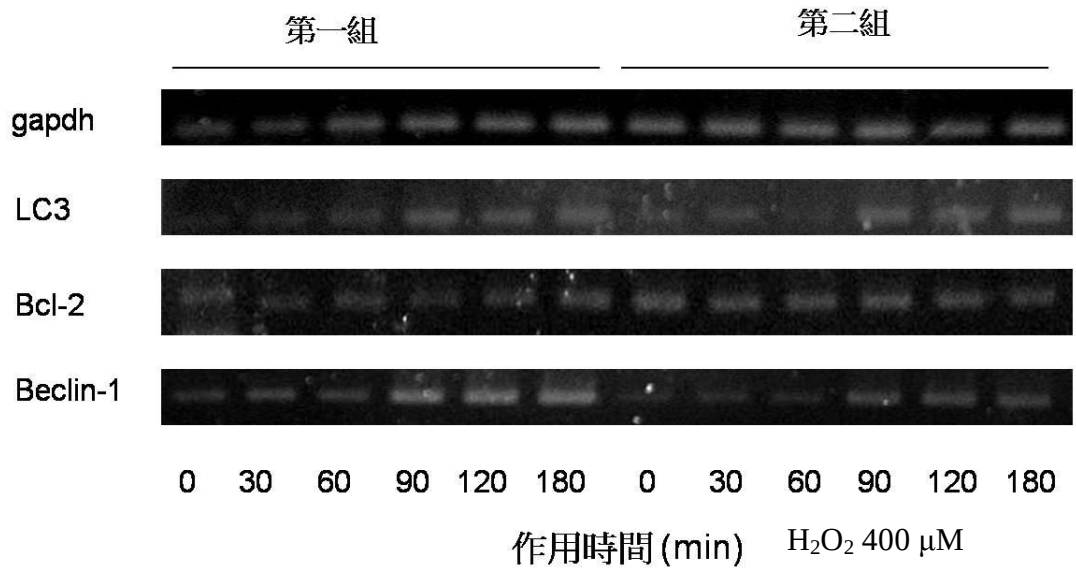
圖表 1: 實驗流程圖



圖表 2: 過氧化氫可誘發 H9c2 細胞自噬基因

實驗進行二重覆(第一組與第二組)，將 1.36 x10 ^{5} /well 之 H9c2，培養於 6-well plate 24 小時後，利用過氧化氫(H_2O_2 400μM)處理 0-3 小時。三小時後，可以發現代表細胞自噬活性之 LC3 與 Beclin-1 表現量明顯上升。

Gapdh: glyceraldehyde 3-phosphate dehydrogenase, LC3: light chain 3, Beclin-1: Atg 6, Bcl2: B-cell leukemia/lymphoma 2, H_2O_2: hydrogen peroxide.

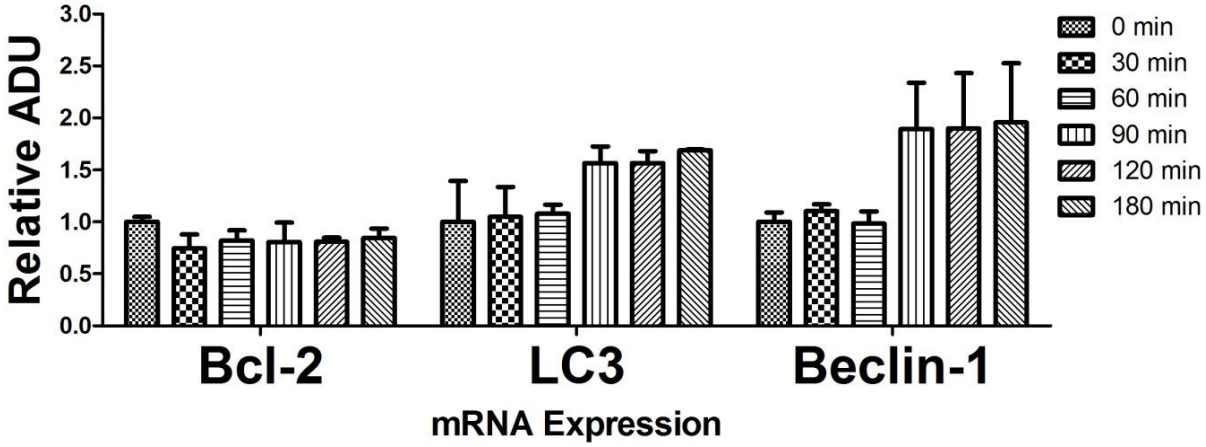


圖表 3: 過氧化氫可增加 LC3 mRNA 表現

將圖表 2 之各種 mRNA 表現資料，量化處理並經 gapdh 校正後，再以相對於過氧化氫作用前之基礎值的比值表示。

整體而言，LC3 經 3 小時過氧化氫作用，其表現增加 29.3%，Beclin-1 增加 36.7%，Bcl-2 無明顯變化。

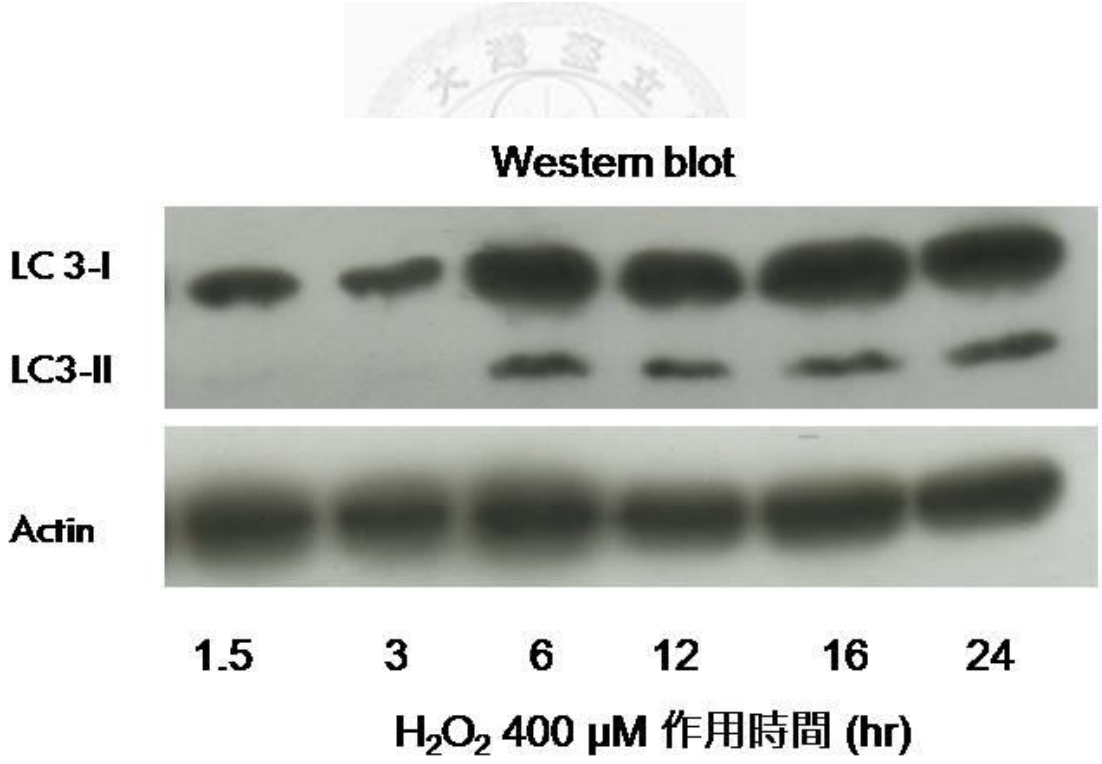
ADU: Arbitrary densitometry units, Gapdh: glyceraldehyde 3-phosphate dehydrogenase, LC3: light chain 3, Beclin-1: Atg 6, Bcl2: B-cell leukemia/lymphoma 2



圖表 4: 過氧化氫增加細胞自噬蛋白表現

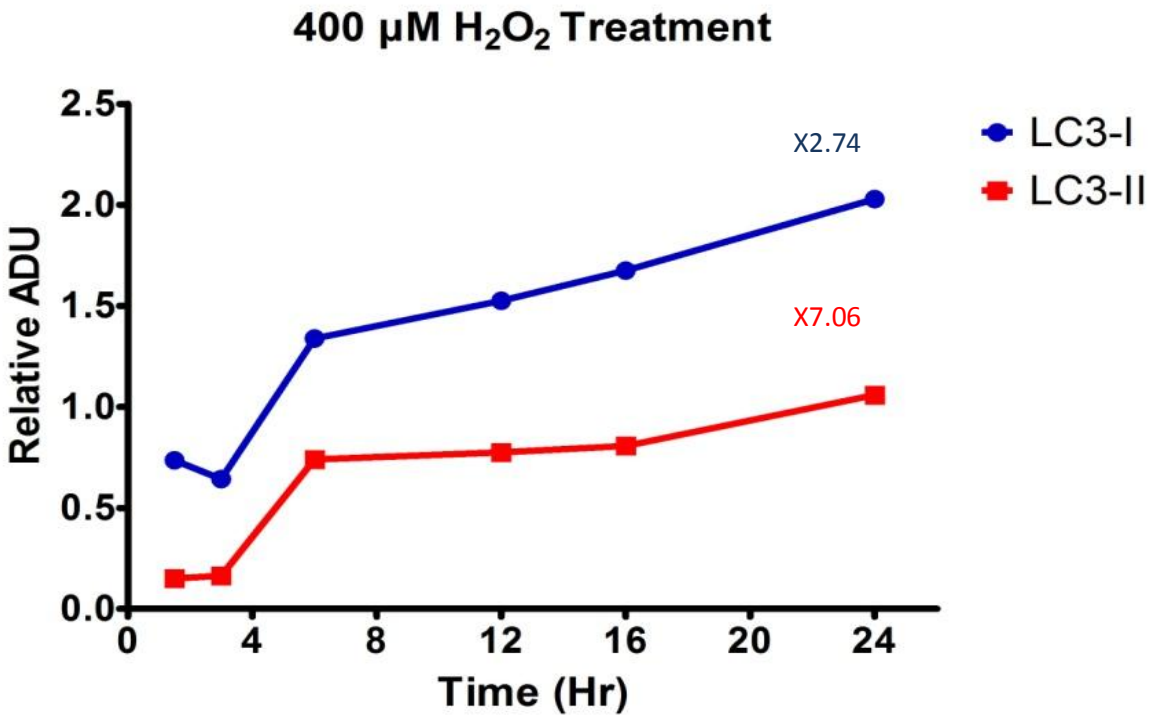
將 2.0 x10^5 /well 之 H9c2，培養於 10cm dish 24 小時後，利用過氧化氫(H_2O_2 400μM)處理 0-24 小時，可發現細胞自噬蛋白包括 LC3-I 與 LC3-II 隨作用時間越長，表現量越高。

LC3: Light Chain 3 protein，LC3-II: Membrane form Light chain 3, LC3-I: cytosol form light chain 3, H_2O_2: hydrogen peroxide.



圖表 5: 過氧化氫增加 7 倍 LC3-II 蛋白質表現

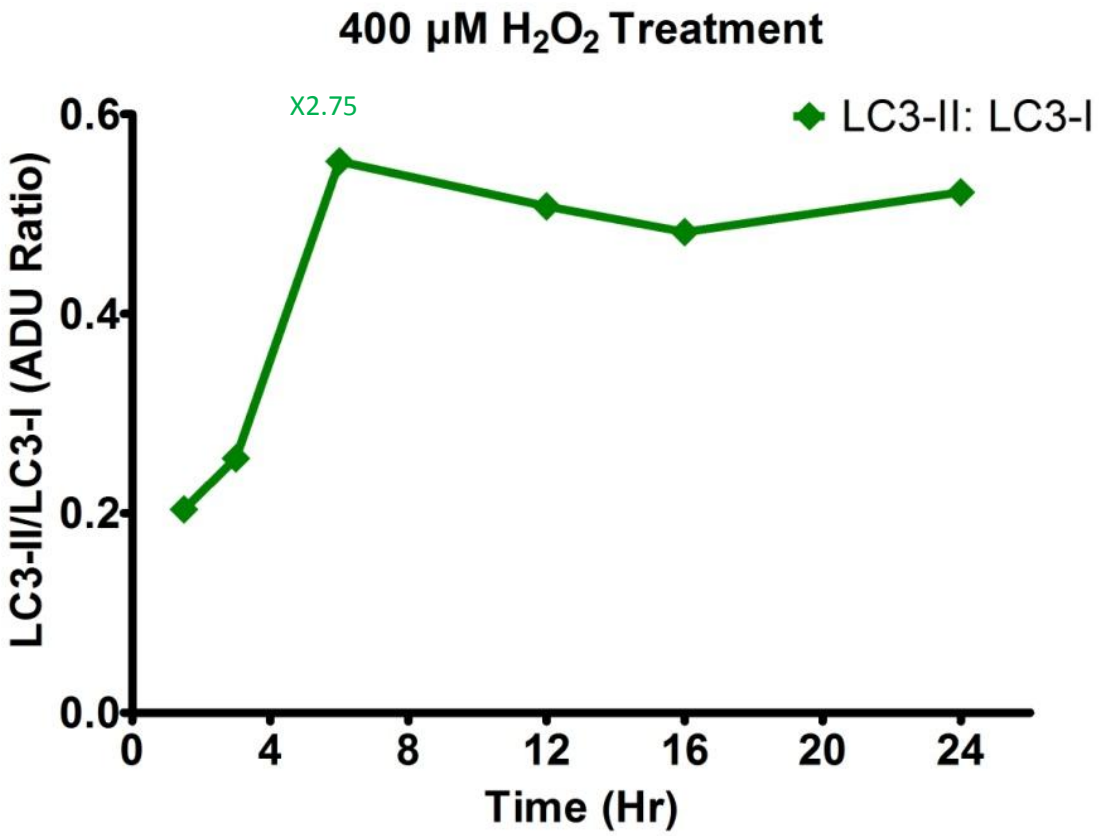
將圖表 4 之蛋白質表現資料量化處理，經 Actin 校正後呈現，可見過化氫作用 24 小時後，LC3-II 增加 7.06 倍，而 LC3-I 增加 2.74 倍。Relative ADU: relative arbitrary densitometry unit, LC3-II: Membrane form Light chain 3, LC3-I: cytosol form light chain 3, H_2O_2: hydrogen peroxide.



圖表 6: 過氧化氫作用增加 LC3-II/LC3-I 比值

將圖表 4 之蛋白質表現資料，量化處理並經 Actin 校正後，計算 LC3-II/LC3-I 比值，發現作用 6 小時後 LC3-II/LC3-I 比值達 2.75 倍上升。

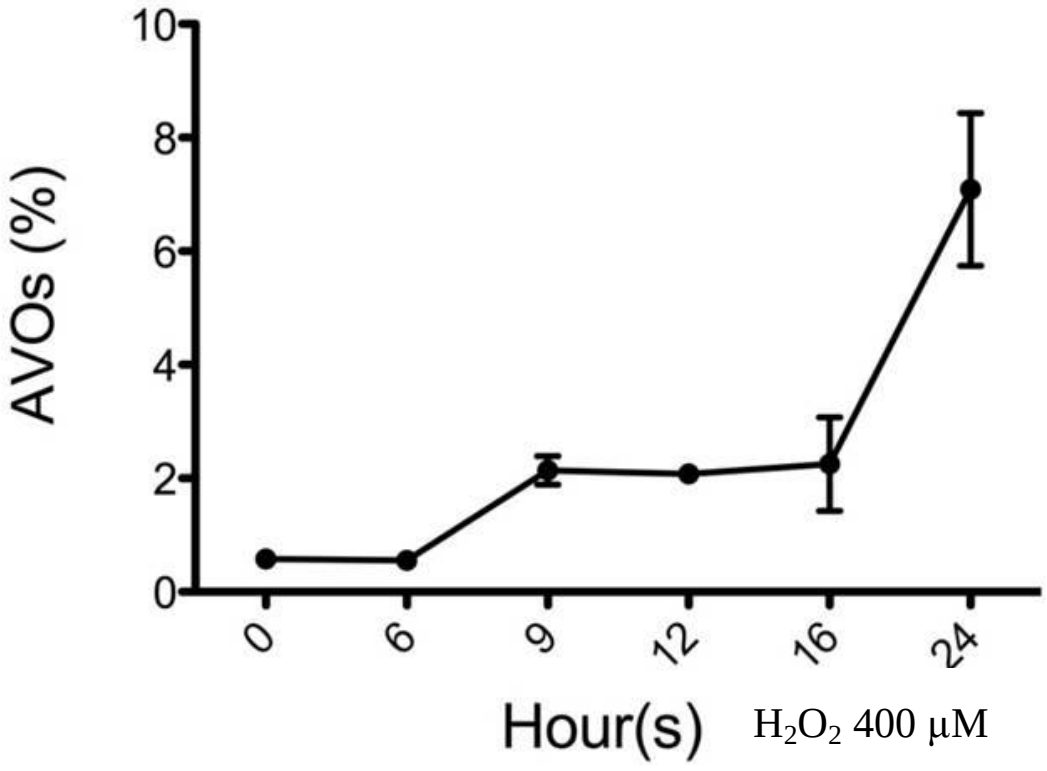
Relative ADU: relative arbitary densitometry unit, LC3-II: Membrane form Light chain 3, LC3-I: cytosol form light chain 3, H_2O_2: hydrogen peroxide.



圖表 7: 過氧化氫作用增加 Acidic vacuole 表現

將 2.0 x10 ^{5} /well 之 H9c2，培養於 10cm dish 24 小時後，利用過氧化氫(H_2O_2 400μM) 處理 0-24 小時，利用流噬細胞儀，測量含 Acridine orange 染色陽性的細胞比例 (AVOs)。可見過氧化氫作用後，細胞含有可偵測之 AVOs 比例升高。

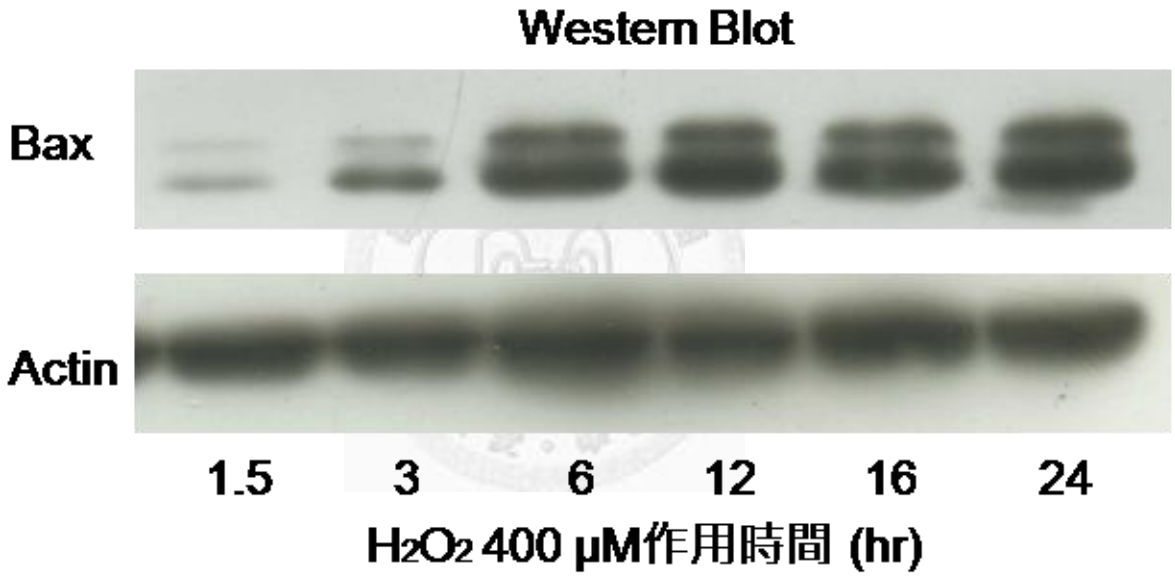
AVOs : Acidic vacuole organelles, H_2O_2: hydrogen peroxide.



圖表 8: 過氧化氫同時誘發細胞凋亡蛋白質表現

將 2.0 x10 ^{5} /well 之 H9c2，培養於 10cm dish 24 小時後，再利用 H_2O_2 400μM 處理 0-24 小時，可見 Bax 表現量逐漸增加。

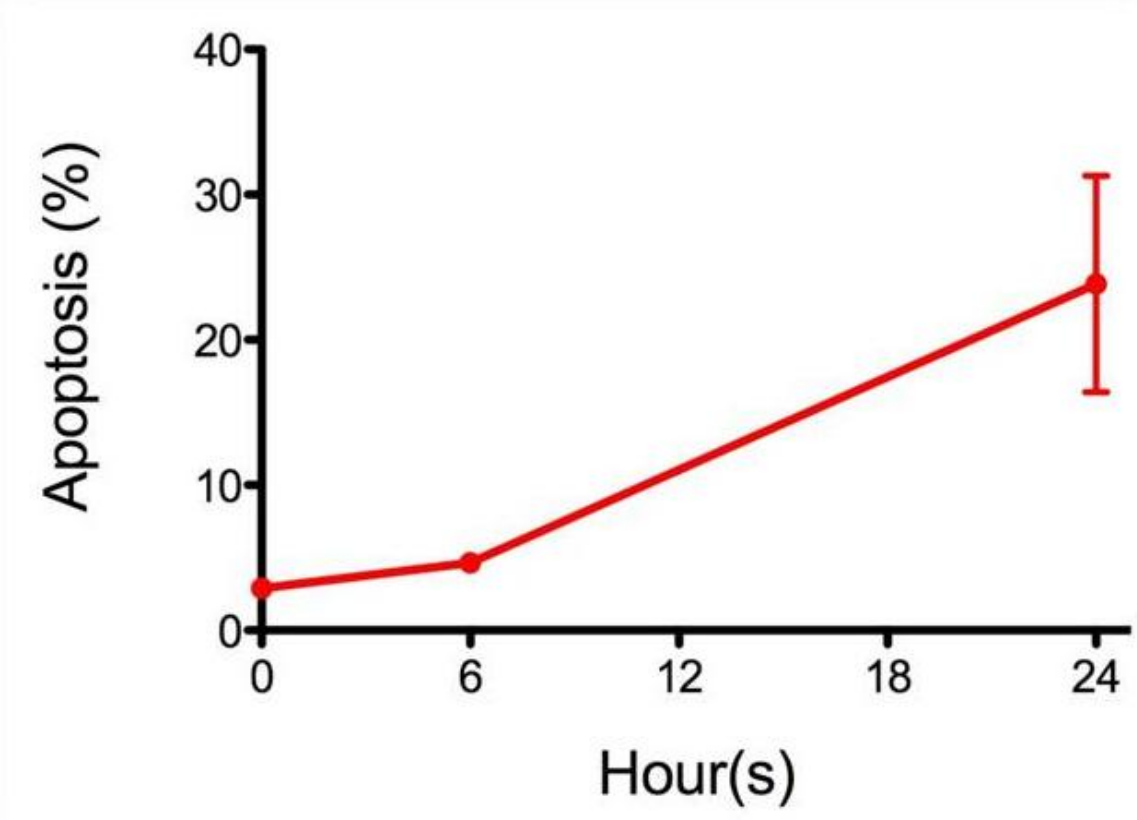
H_2O_2: hydrogen peroxide, Bax: pro-apoptotic protein，可促進細胞凋亡



圖表 9: 過氧化氫作用時間與細胞凋亡比例

將 2.0 x10 ^{5} /well 之 H9c2，培養於 10cm dish 24 小時後，利用過氧化氫(H_2O_2 400μM)處理 0-24 小時，利用流式細胞儀，測量含 Annexin V 陽性之的細胞比例，Annexin V 陽性比例視為細胞凋亡比例。實驗結果顯示，作用後細胞 Annexin V 陽性比例升高。

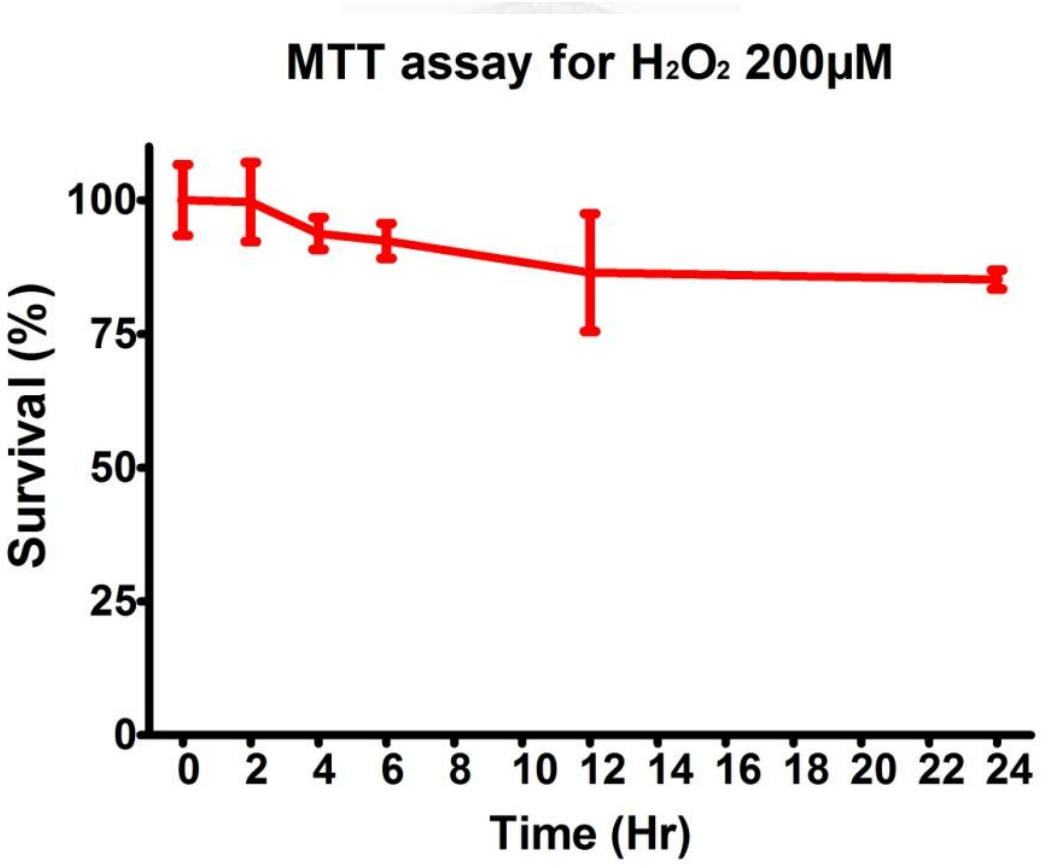
H_2O_2: hydrogen peroxide



圖表 10: 過氧化氫作用後的細胞存活率

將 $5x10^4$/well 之 H9c2，培養於 12-well culture plates 細胞培養器內 24 小時後，添加過氧化氫(200μM H_2O_2) 作用 0-24 小時，利用 MTT assay 計算細胞存活率，發現 24 小時候細胞約有 85%存活率。

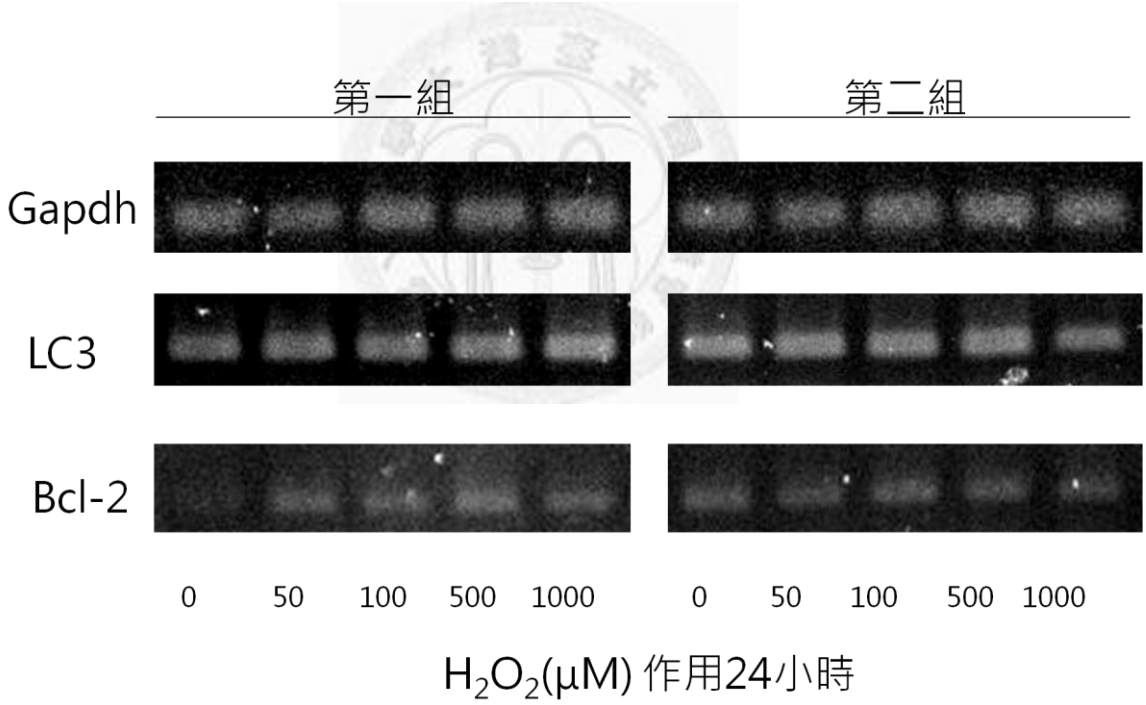
H_2O_2: hydrogen peroxide, MTT: 3-(4,5-dimethyl-2- thiazolyl)-2,5- diphenyltetrazolium bromide, 細胞存活率之計算公式: $\frac{\text{處理後細胞之吸光值}}{\text{處理前對照組細胞之吸光值}}$ x 100%



圖表 11: 過氧化氫輕微影響細胞自噬基因表現

實驗進行二重覆(第一組與第二組)，將 1.2x10^5/well 之 H9c2，培養於 12-well culture plates 細胞培養器內 24 小時後，添加不同濃度過氧化氫(0-1000μM)作用 24 小時，顯示細胞自噬基因表現僅受輕微影響。

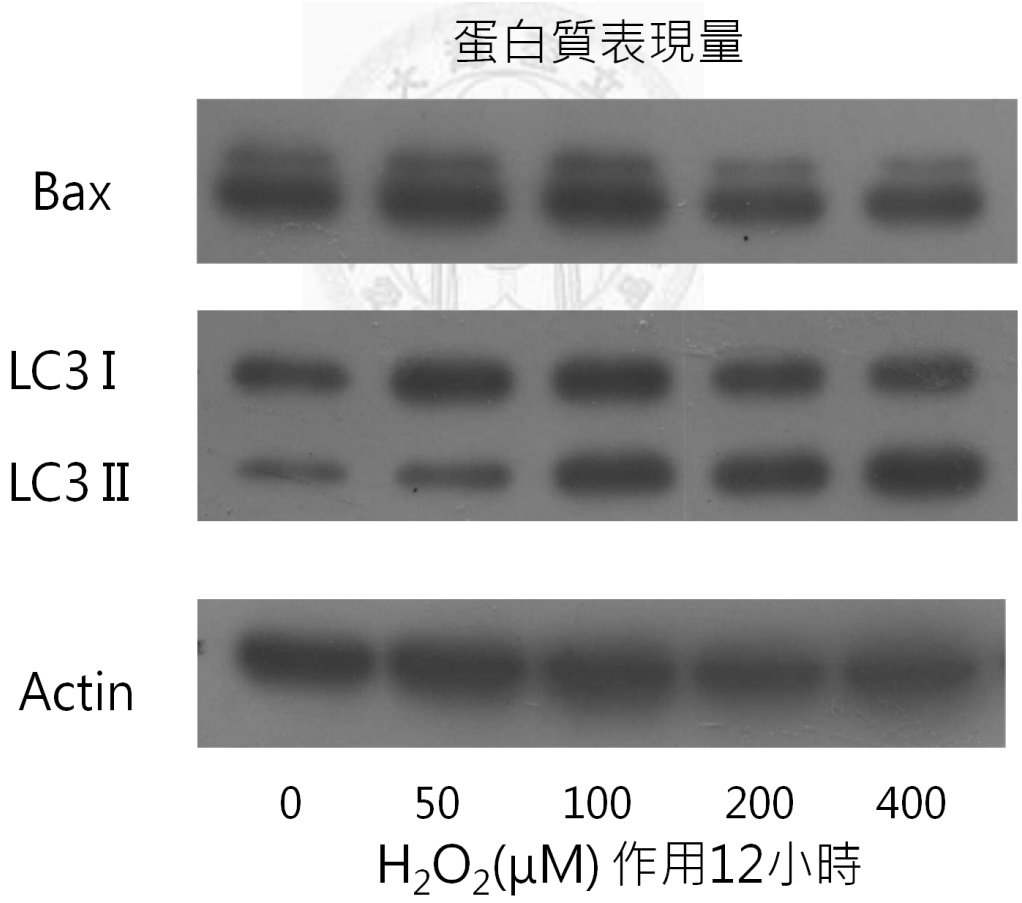
Gapdh: Glyceraldehyde 3-phosphate dehydrogenase, LC3: light chain 3, Bcl-2: B-cell leukemia/lymphoma 2, H_2O_2: hydrogen peroxide



圖表 12: 過氧化氫誘發細胞自噬蛋白表現

將 $4x10^4$ 之 H9c2，培養於 12-well culture plates 細胞培養器內 24 小時後，添加不同濃度過氧化氫(0-400μM)作用 12 小時，顯示隨作用濃度增加，細胞自噬蛋白表現也增加。

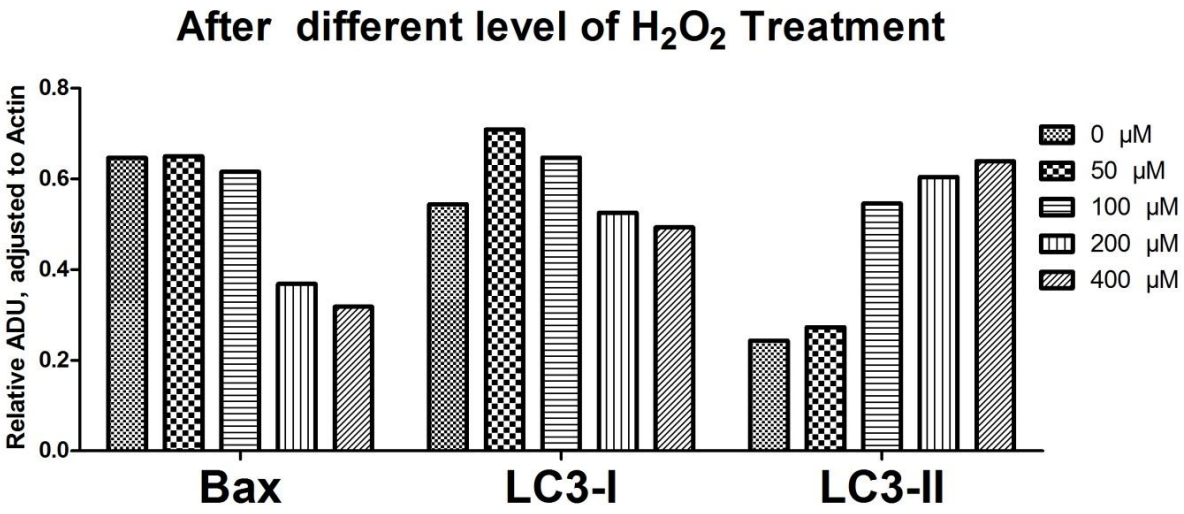
LC3-II: Membrane form Light chain 3, LC3-I: cytosol form light chain 3, H_2O_2: hydrogen peroxide, Bax: pro-apoptotic protein



圖表 13: 高濃度過氧化氫更能誘發細胞自噬

將圖表 12 資料量化，並經 actin 校正後呈現，顯示確實高濃度過氧化氫更能誘發細胞自噬。

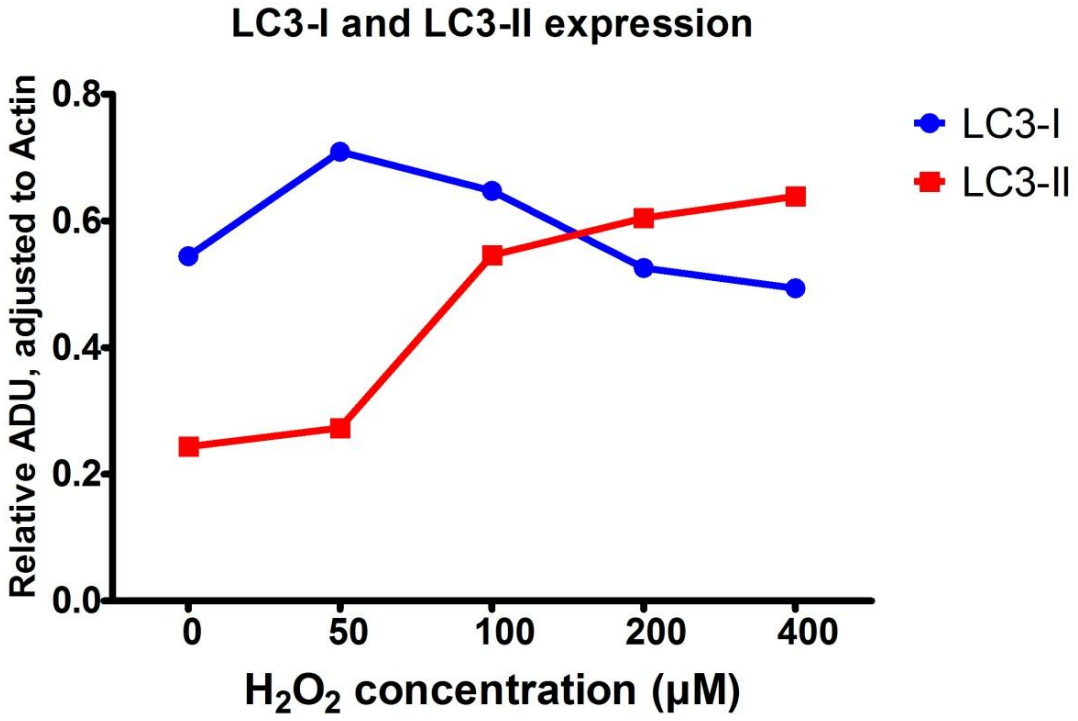
Bax: pro-apoptotic protein; LC3-II: Membrane form Light chain 3, LC3-I: cytosol form light chain 3, ADU: arbitrary densitometry units, H_2O_2: hydrogen peroxide



圖表 14: 增加過氧化氫濃度主要增加 LC3-II 表現

將圖表 12 資料量化，並經 actin 校正後呈現，顯示確實高濃度過氧化氫主要增加 LC3-II 表現，大約增加 2.67 倍。

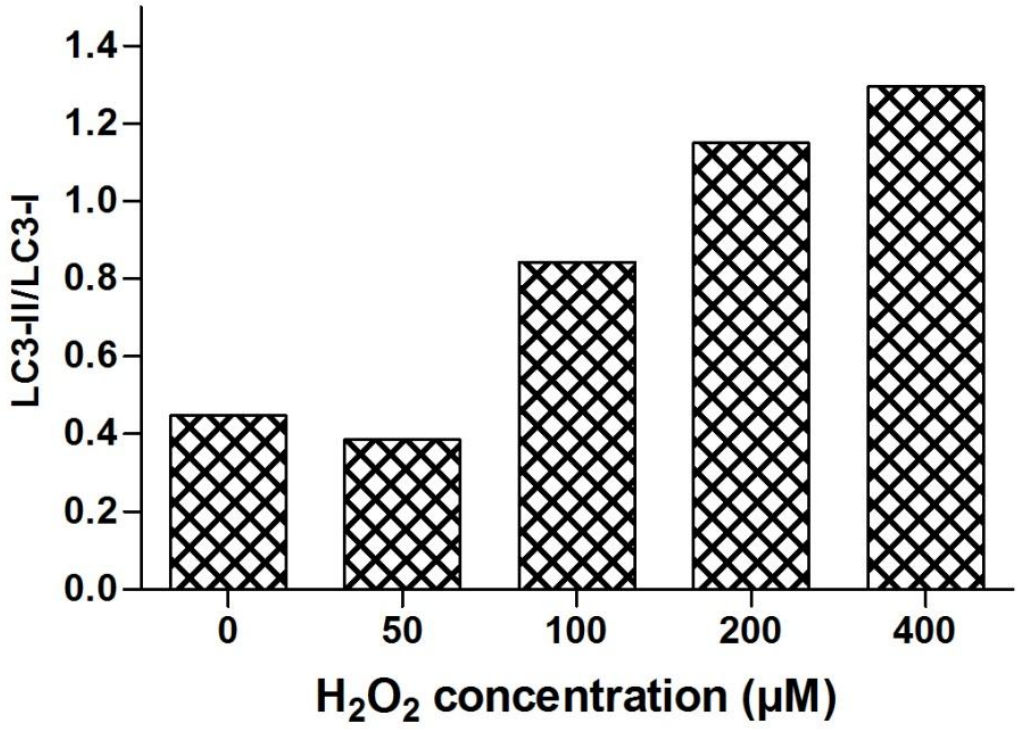
Bax: pro-apoptotic protein, LC3-II: Membrane form Light chain 3, LC3-I: cytosol form light chain 3, ADU: arbitrary densitometry units, H_2O_2: hydrogen peroxide



圖表 15: 過氧化氫濃度對 LC3-II/LC3-I 比值影響

將圖表 12 之蛋白質表現資料量化處理，經 Actin 校正後，計算 LC3-II/LC3-I 比值，發現最大比值出現於作用 24 小時後，約為 1.29，為基礎值的 2.87 倍。

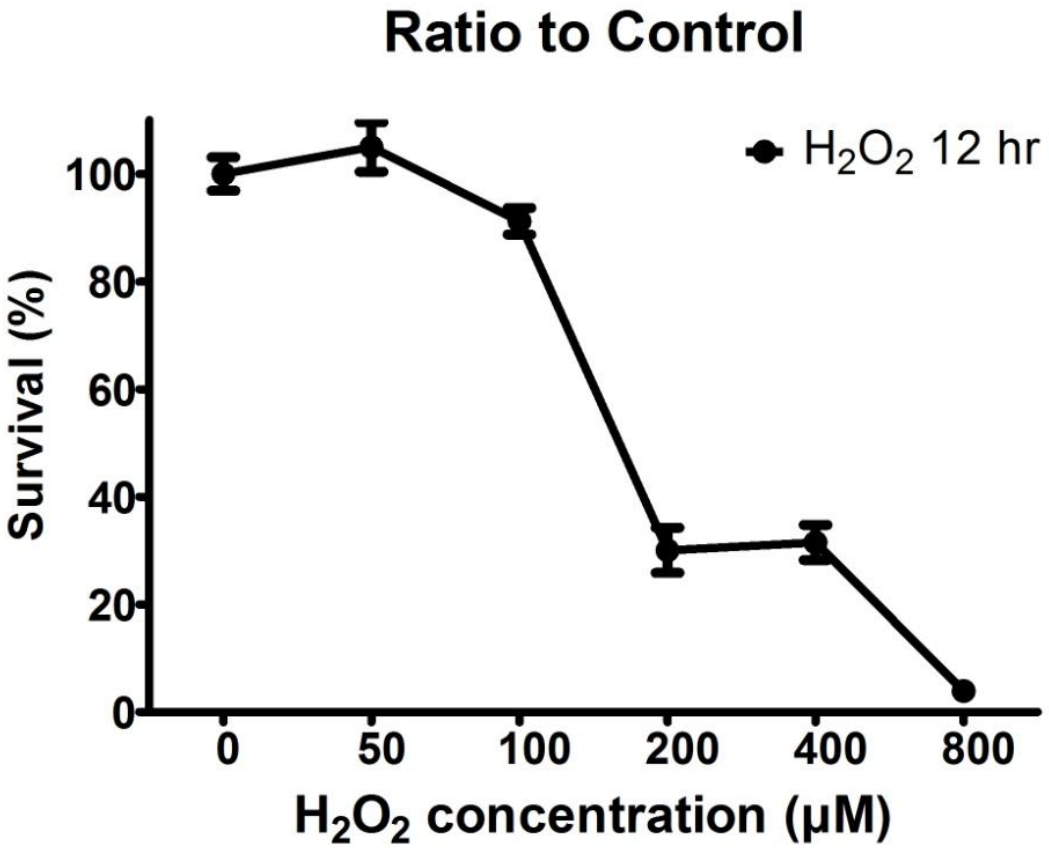
LC3-II: Membrane form Light chain 3, LC3-I: cytosol form light chain 3, H_2O_2: hydrogen peroxide



圖表 16: 過氧化氫濃度對細胞存活率影響

將 $4x10^4$/well 之 H9c2，培養於 12-well culture plates 細胞培養器內 24 小時後，添加過氧化氫(0-800μM H_2O_2) 作用 12 小時，利用 MTT assay 計算細胞存活率，發現高濃度過氧化氫將減少細胞存活率。

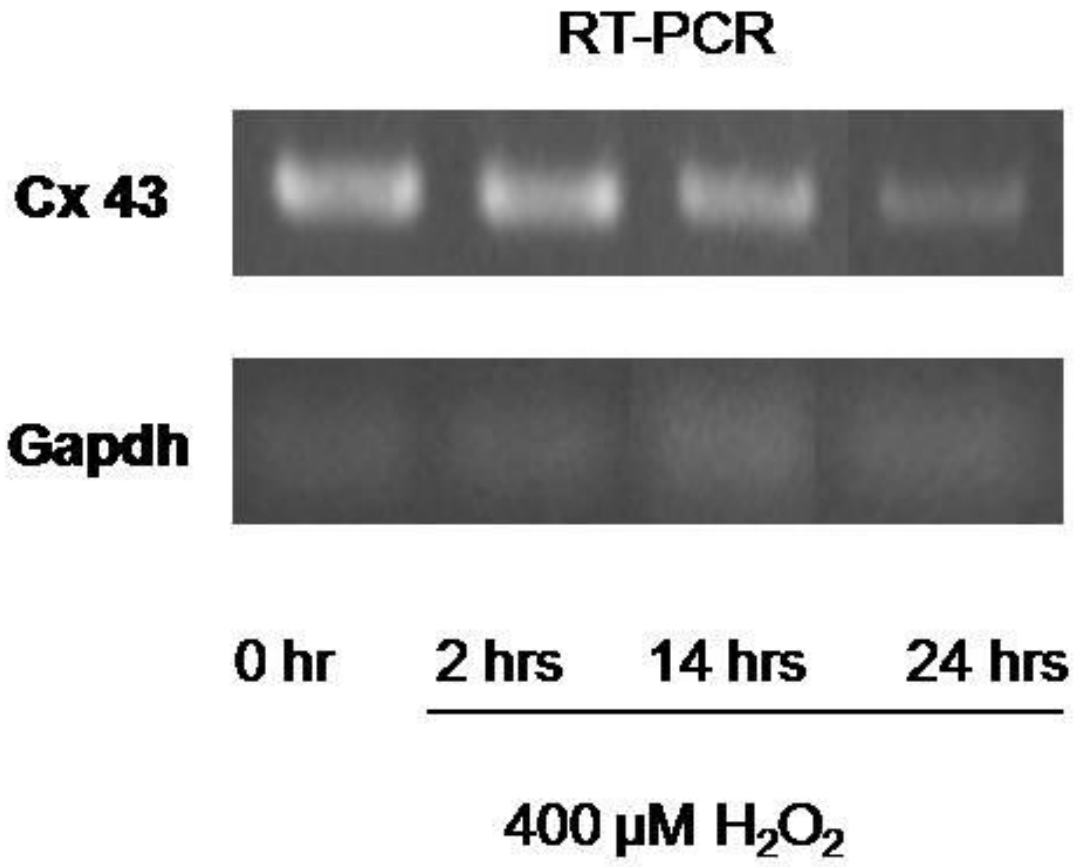
H_2O_2: hydrogen peroxide, MTT: 3-(4,5-dimethyl-2- thiazolyl)-2,5- diphenyltetrazolium bromide，細胞存活率之計算公式: $\frac{\text{處理後細胞之吸光值}}{\text{處理前對照組細胞之吸光值}}$ x 100%



圖表 17: 過氧化氫作用將減少 Connexin 43 mRNA 表現

將 $2x10^5$/well 之 H9c2，培養於 6-well culture plates 細胞培養器內 24 小時後，利用過氧化氫(H_2O_2 400 μM)作用，可發現作用時間越長，Cx43 之基因表現將下降。

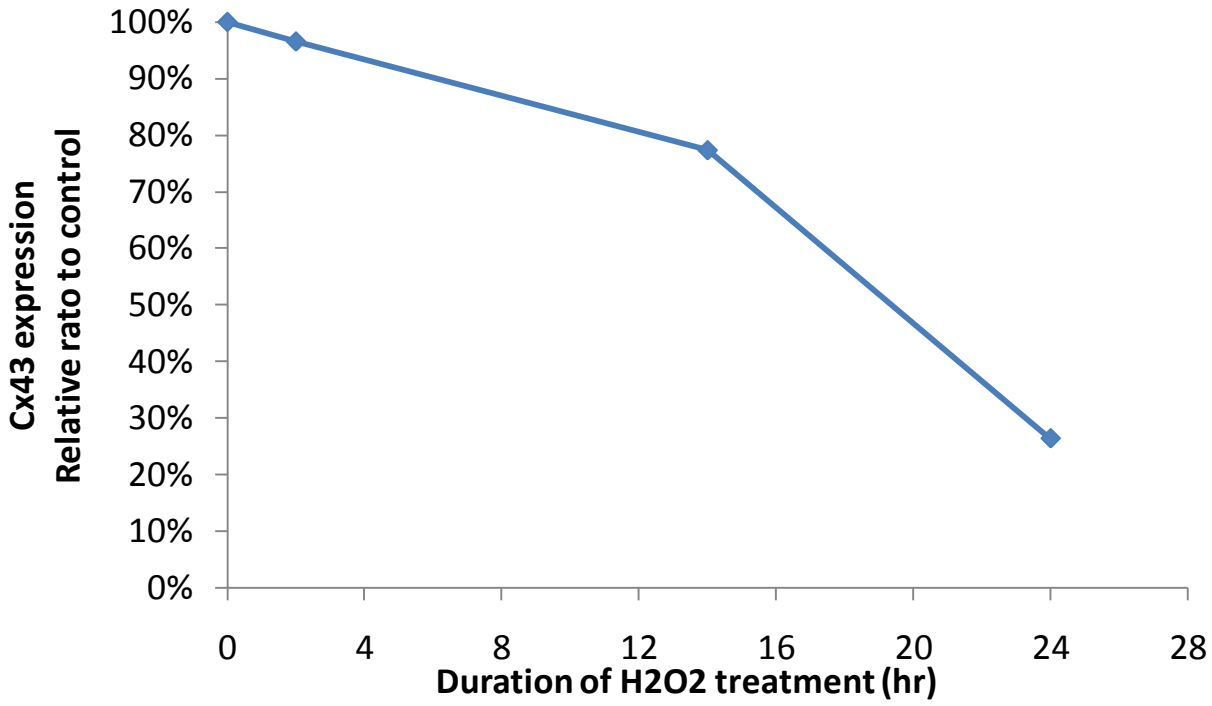
Cx43: Connexin 43, RT-PCR: reverse transcriptase polymerase chain reaction, Gapdh: glyceraldehyde 3-phosphate dehydrogenase, H_2O_2: hydrogen peroxide



圖表 18: 過氧化氫作用時間延長將影響 Cx43 基因表現

量化圖表 17 的資料，並利用 gapdh 校正後，以相對於 0 hr 基礎值之比例表示，發現 Cx43 之表現於作用 14 小時候，將維持 77%，但於 24 小時後減為基礎值之 26%。

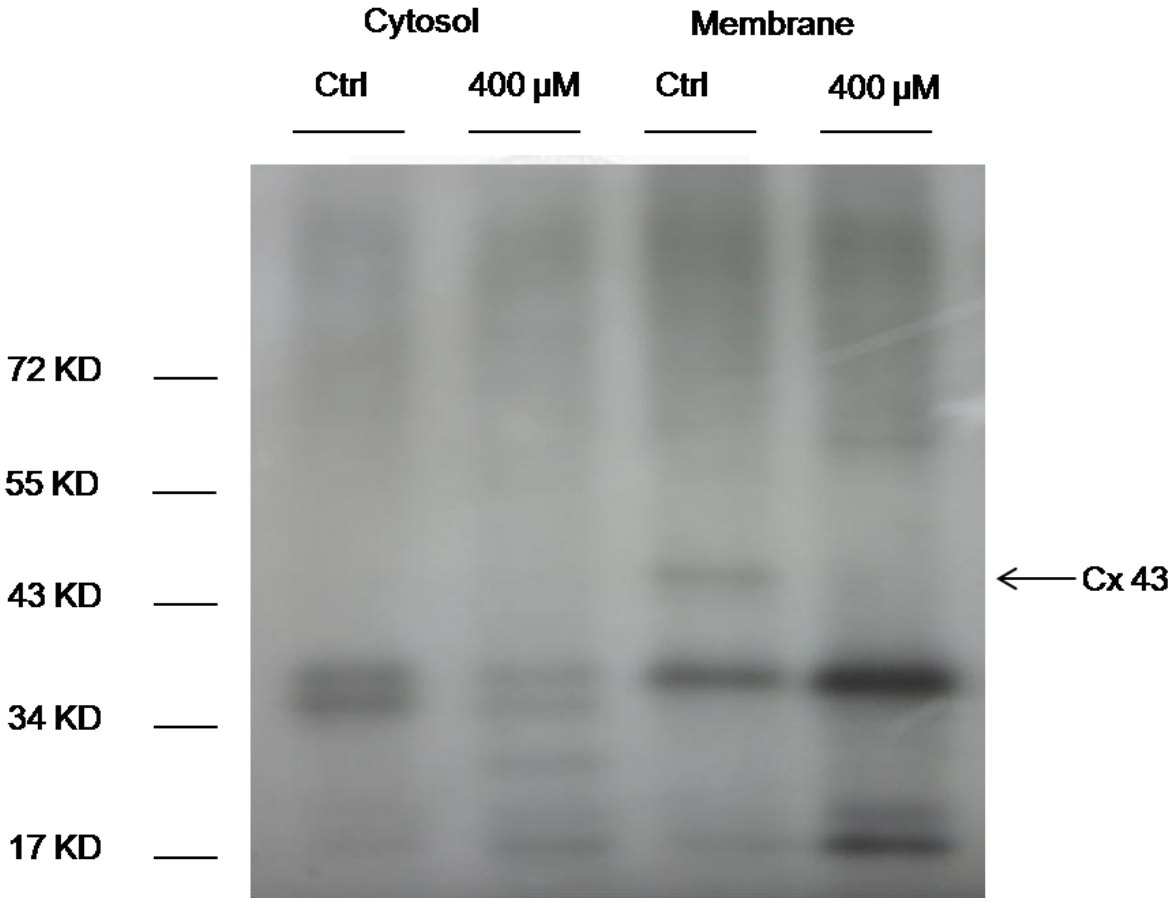
ADU: arbitrary densitometry unit; Cx 43: Connexin 43, H_2O_2: hydrogen peroxide



圖表 19: 過氧化氫作用減少細胞膜上 Cx43 蛋白表現

將 2.0 x10^5 /well 之 H9c2，培養於 10cm dish 24 小時後，利用過氧化氫(H_2O_2 400μM) 處理 24 小時，發現細胞膜上 Cx43 將減少表現。

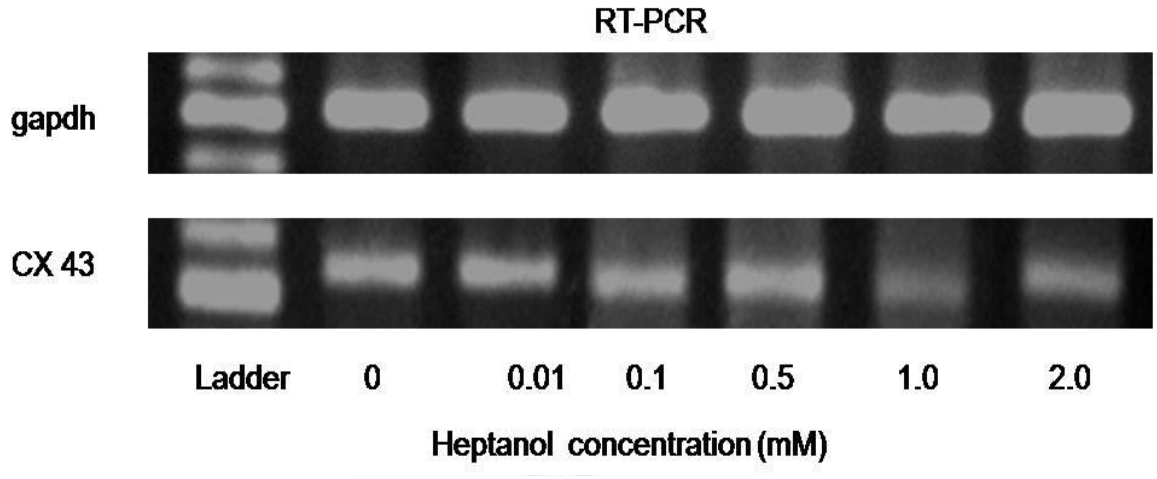
Cx43: Connexin 43, H_2O_2: hydrogen peroxide



圖表 20: 正庚醇不影響 Cx43 基因表現

將 1x10^5 之 H9c2，培養於 6 well culture plates 細胞培養器內 24 小時候，以正庚醇作用 5 分鐘後，發現 Cx43 基因表現不受影響。

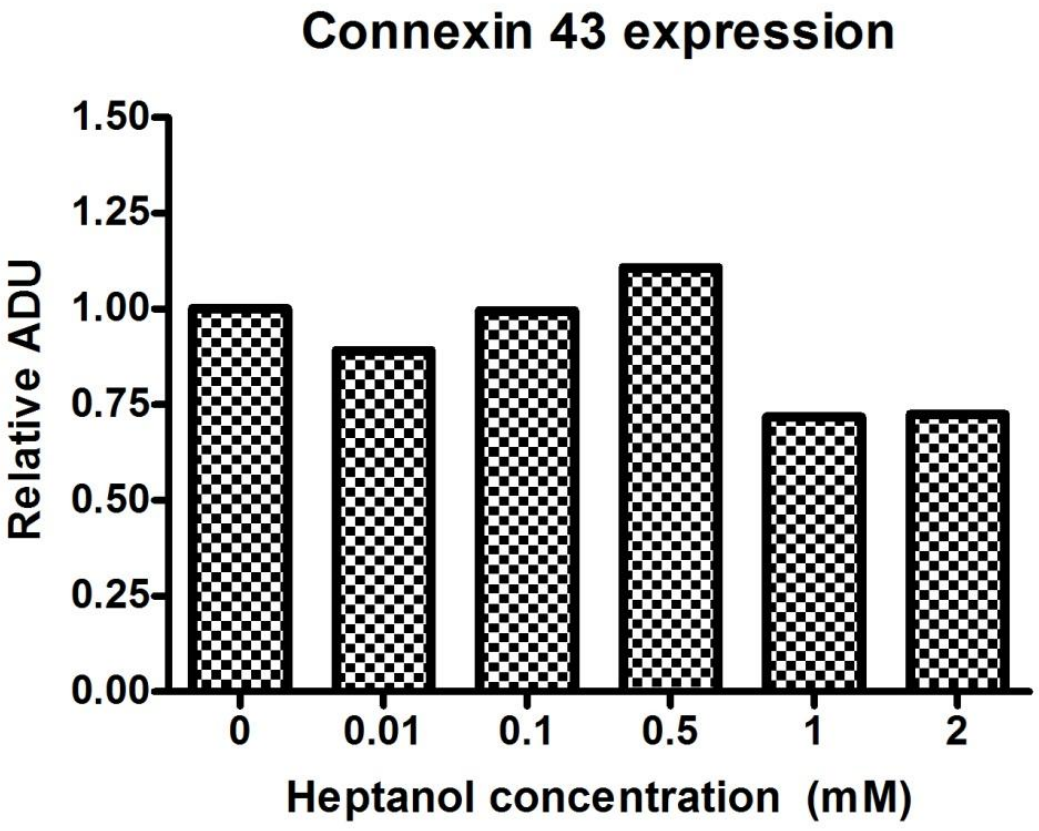
Gapdh: glyceraldehyde 3-phosphate dehydrogenase , Cx43: Connexin 43, RT-PCR: reverse transcriptase polymerase chain reaction, Heptanol : 正庚醇



圖表 21: 量化不同濃度正庚醇處理後 H9c2 Cx43 mRNA 相對表現量

將圖表 20 資料量化，並經 Gapdh 校正後，以相對於 0mM 正庚醇之比值表示。結果顯示以正庚醇作用 5 分鐘後，發現 Cx43 基因表現不受影響。

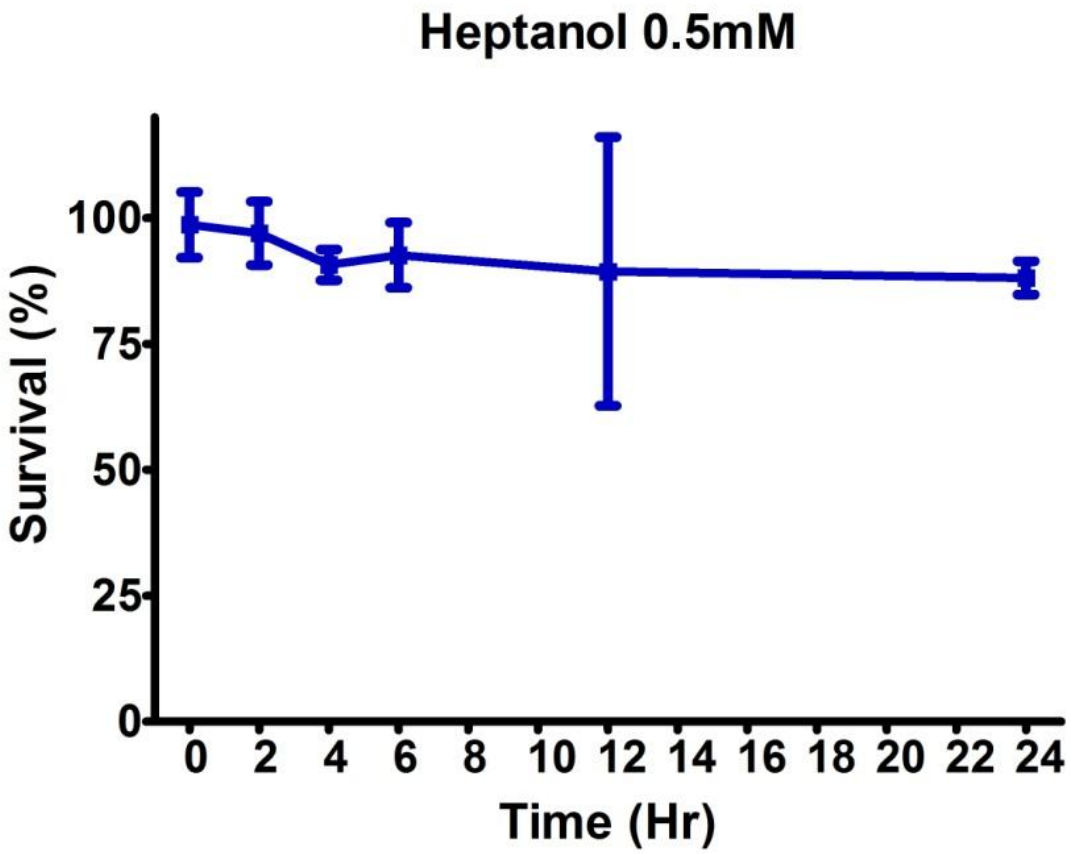
ADU: arbitrary densitometry units, Heptanol: 正庚醇



圖表 22: 正庚醇 0.5mM 對細胞存活率之影響

將 $5x10^4$/well 之 H9c2，培養於 12-well culture plates 細胞培養器內 24 小時後，添加正庚醇(0.5 mM Heptanol)作用 0-24 小時，利用 MTT assay 計算細胞存活率，發現 24 小時候細胞約有 88%存活率。

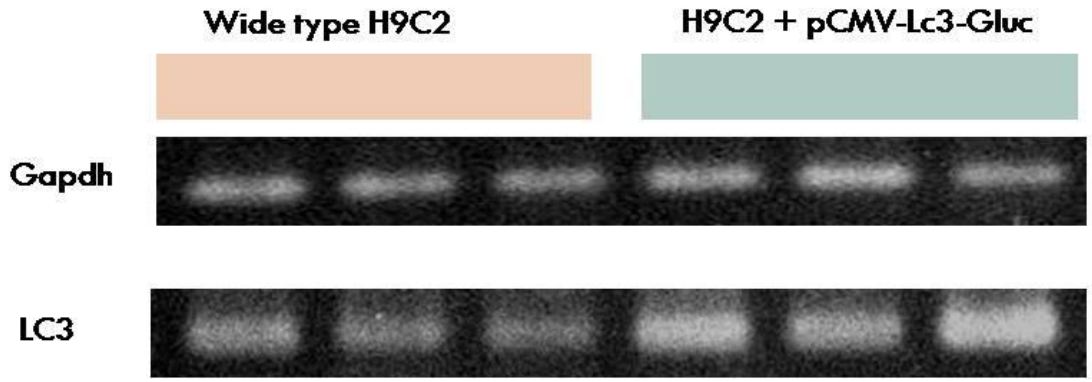
Heptanol: 正庚醇, MTT: 3-(4,5-dimethyl-2- thiazolyl)-2,5- diphenyltetrazolium bromide，細胞存活率之計算公式: $\frac{\text{處理後細胞之吸光值}}{\text{處理前對照組細胞之吸光值}}$ x 100%



圖表 23: 轉染後之 H9c2 表現較高 LC3 基因

將 2x10^5/well 之 H9c2，培養於 6-well culture plates 細胞培養器內 24 小時後，進行 pCMV-LC3-Gluc 轉染，顯示轉染後之 H9c2 其冷光酵素反應值較高。

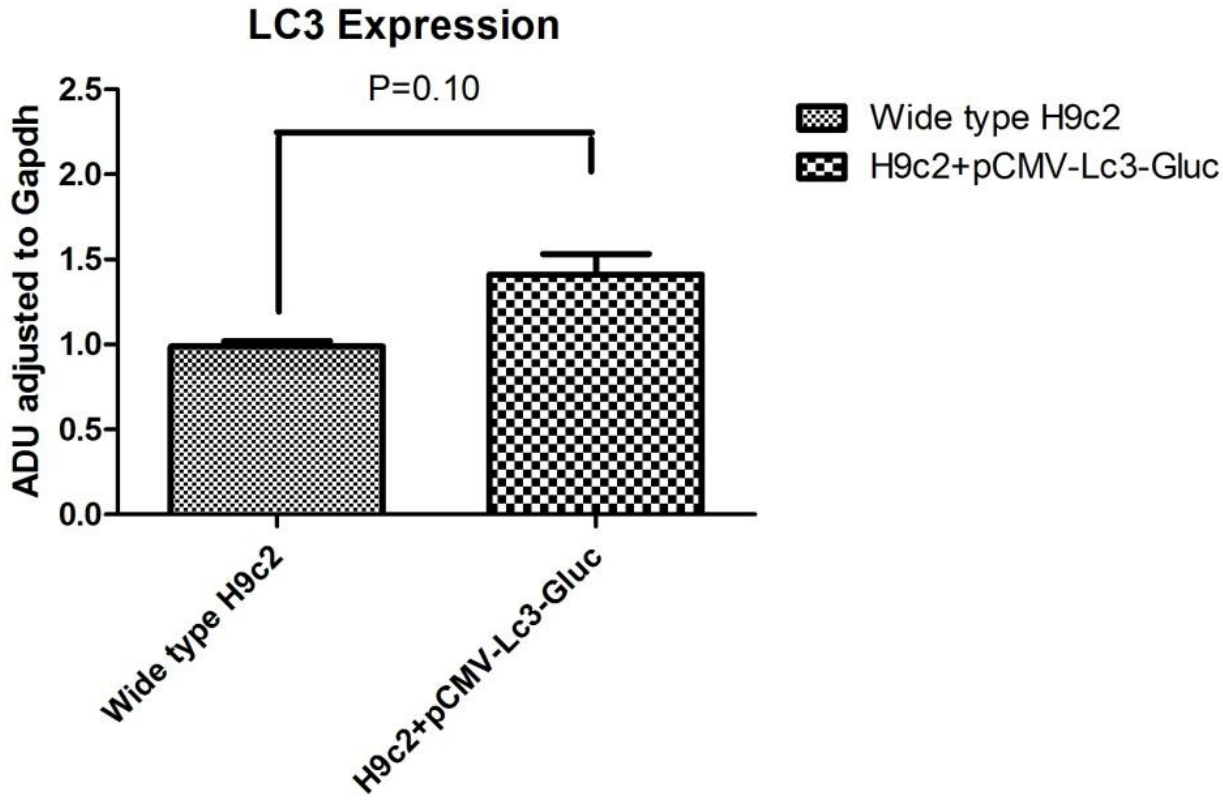
Wide type H9c2: 為未轉染 pMCV-Lc3-Gluc 之 H9c2 細胞株，H9c2+pMCV-Lc3-Gluc: 為轉染後 H9c2 細胞株，Gapdh: glyceraldehyde 3-phosphate dehydrogenase, LC3: light chain 3, Gluc: Gaussia luciferase



圖表 24: 轉染前後細胞 LC3 基因表現量

將圖表 23 之資料量化後，經 gapdh 校正後，以相對於 wild type H9c2 之表現值表示，可發現轉染後細胞表現細胞自噬量將增加。

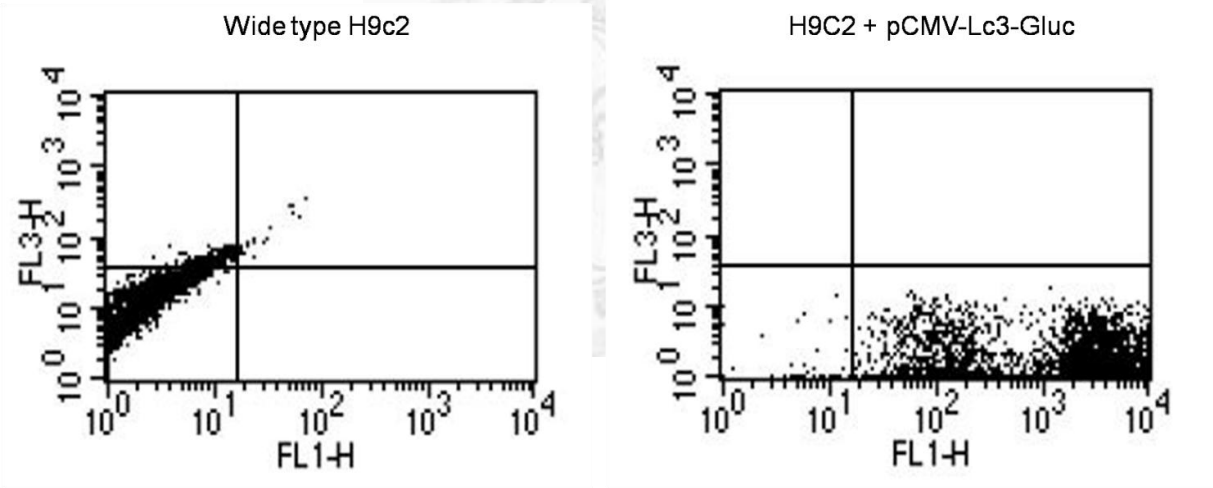
Wide type H9c2 為未轉染 pMCV-Lc3-Gluc 之 H9c2 細胞株，H9c2 + pMCV-Lc3-Gluc 為轉染後 H9c2 細胞株，ADU: arbitrary densitometry units, LC3: light chain 3, Gluc: Gaussia luciferase



圖表 25: 流式細胞儀偵測轉染後細胞表現細胞自噬

流式細胞儀顯示細胞轉染後，Acridine orange 陽性比例將增加。

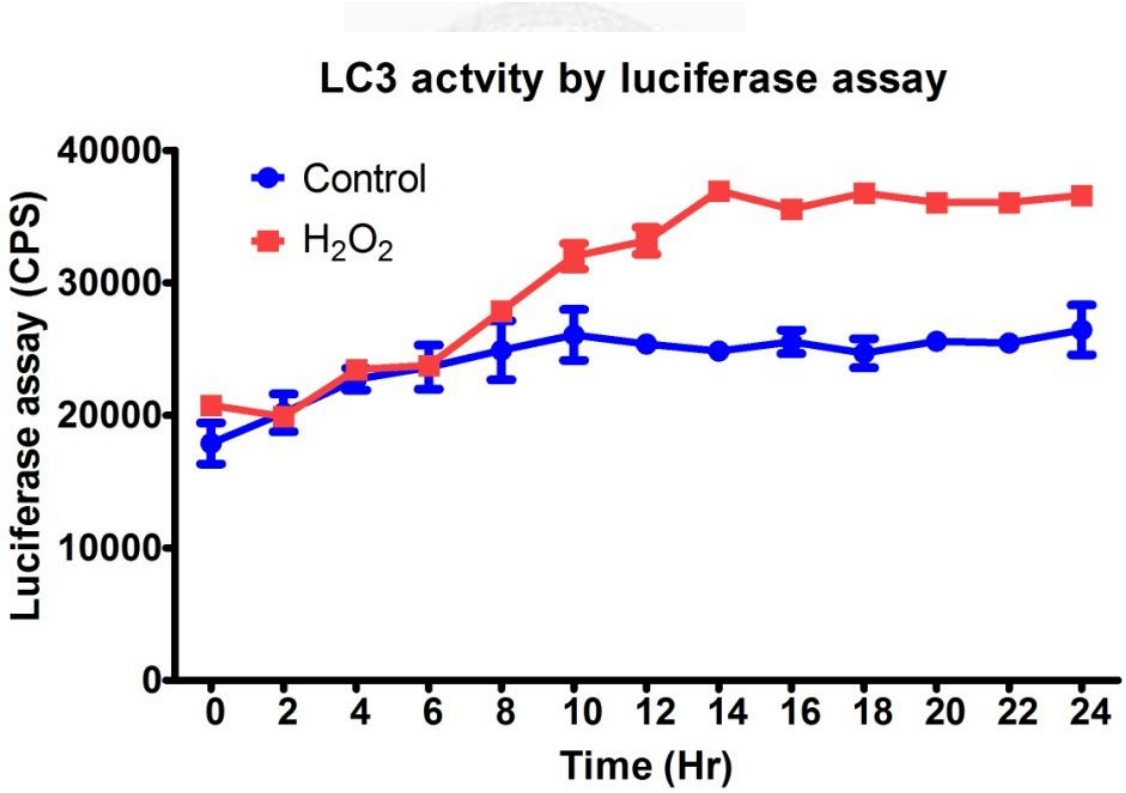
Wide type H9c2 為未轉染 pMCV-Lc3-Gluc 之 H9c2 細胞株，H9c2 + pMCV-Lc3-Gluc 為轉染後 H9c2 細胞株，FL1: Acridine orange (autophagosome staining)，FL3: 7-AAD (Dead staining), Gluc: Gaussia luciferase



圖表 26: 鑑定 H9c2+pMCV-Lc3-Gluc 為 Luciferase Report Assay

將 $5x10^4$ 之 H9c2+pMCV-Lc3-Gluc，培養於 12-well culture plates 細胞培養器內 24 小時後，進行過氧化氫(400 μM H_2O_2) 作用 0-24 小時。可發現於 8 小時後冷光酵素值逐漸增加，而且與對照組之差異隨時間延長，差距持續擴大。

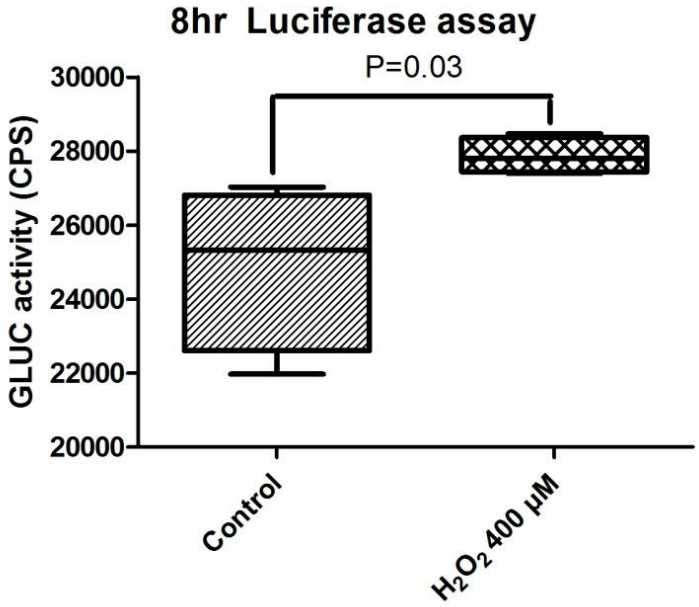
Control group: 單純轉染 pMCV-Lc3-Gluc 之 H9c2，H_2O_2 group: H9c2+pMCV-Lc3-Gluc 接受 400 μM H_2O_2，LC3: light chain 3, CPS: count per seconds, H_2O_2: hydrogen peroxide, Gluc: Gaussia luciferase

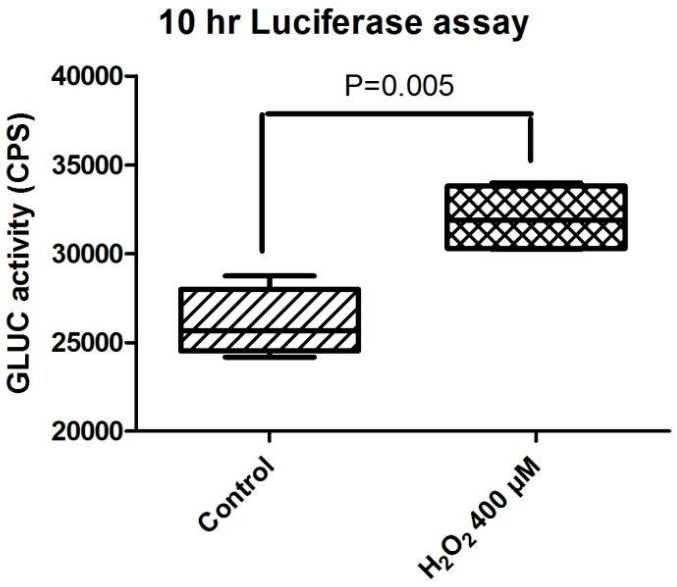


圖表 27: H9c2+pMCV-Lc3-Gluc 之冷光酵素值於 8 小時後即達顯著差異

統計分析 400 μM H_2O_2 作用後之冷光酵素值顯示，8 小時後 control group 與 H_2O_2 之差異(P=0.03, n=4)，10 小時後 control group 與 H_2O_2 之差異(P=0.005, n=4)。

CPS: Count per second, H_2O_2: hydrogen peroxide, Gluc: Gaussia luciferase



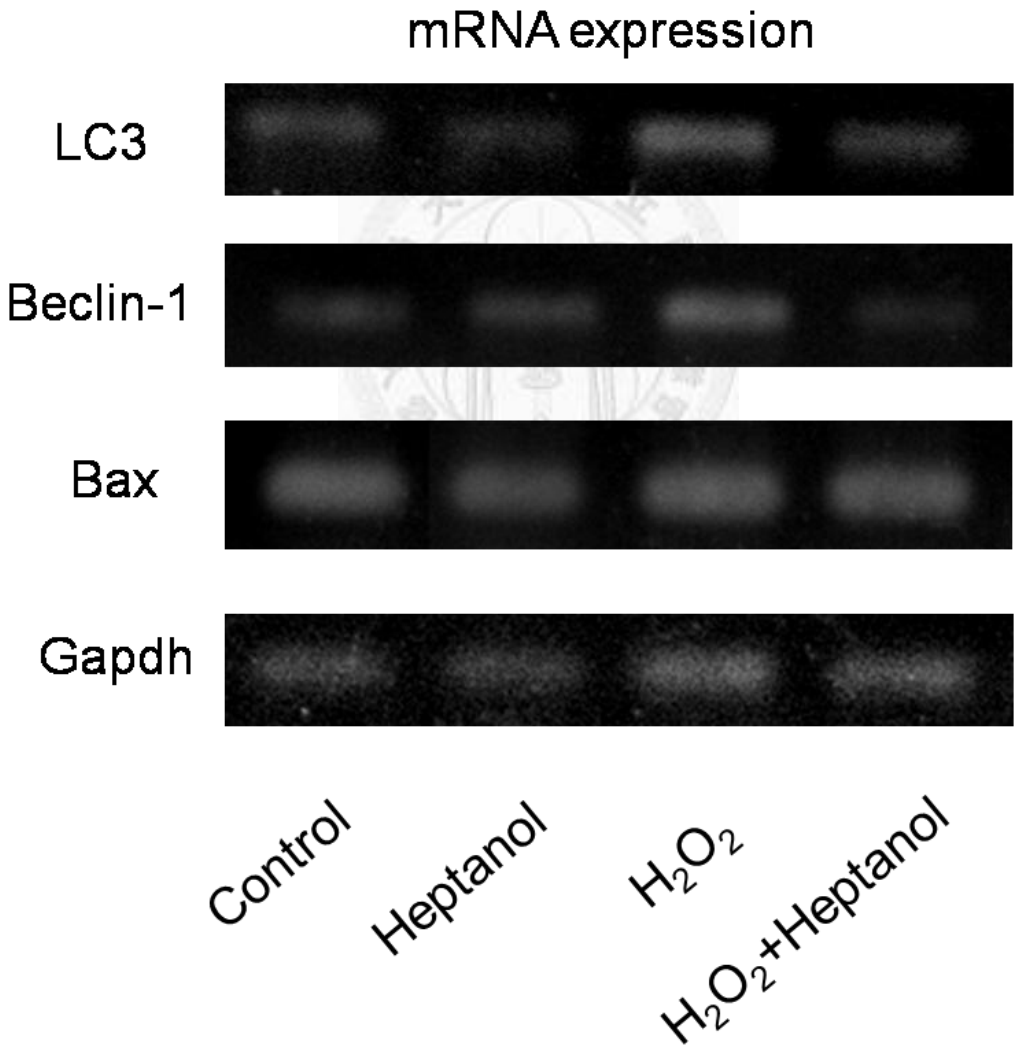


圖表 28: 比較抑制細胞間隙接合是否會減少 LC3 mRNA 表現。

抑制細胞間隙接合將減少細胞經過氧化氫刺激後激發之 LC3 基因表現。

Control group: 只添加培養液，Heptanol group: 使用正庚醇 0.5mM 作用 12 小時，H_2O_2 group: H_2O_2 200μM 作用 12 小時，H_2O_2 +Heptanol:同時添加正庚醇與 H_2O_2。

Gapdh: glyceraldehyde 3-phosphate dehydrogenase, LC3: light chain 3, Beclin-1: Atg 6, Bax: pro-apoptotic protein, H_2O_2: hydrogen peroxide

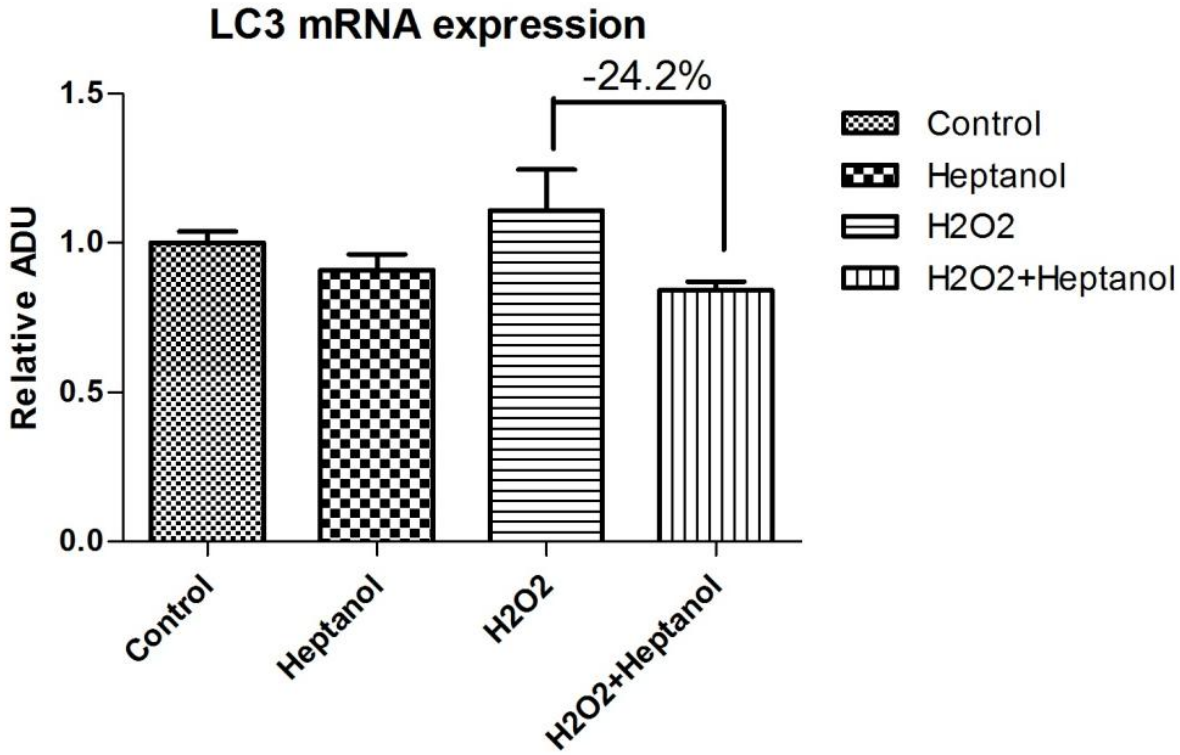


圖表 29: 比較抑制細胞間隙接合是否會減少 LC3 mRNA 表現(相對表現量)

量化圖表 28 之資料，經 gapdh 校正，並與相對於 control 表現量表示，結果顯示抑制細胞間隙接合可降低細胞自噬基因表現約 24.2% (P=0.10, n=3)。

Control group: 只添加培養液，Heptanol group: 使用正庚醇 0.5mM 作用 12 小時，H_2O_2 group: H_2O_2 200μM 作用 12 小時，H_2O_2 +Heptanol:同時添加正庚醇與 H_2O_2,

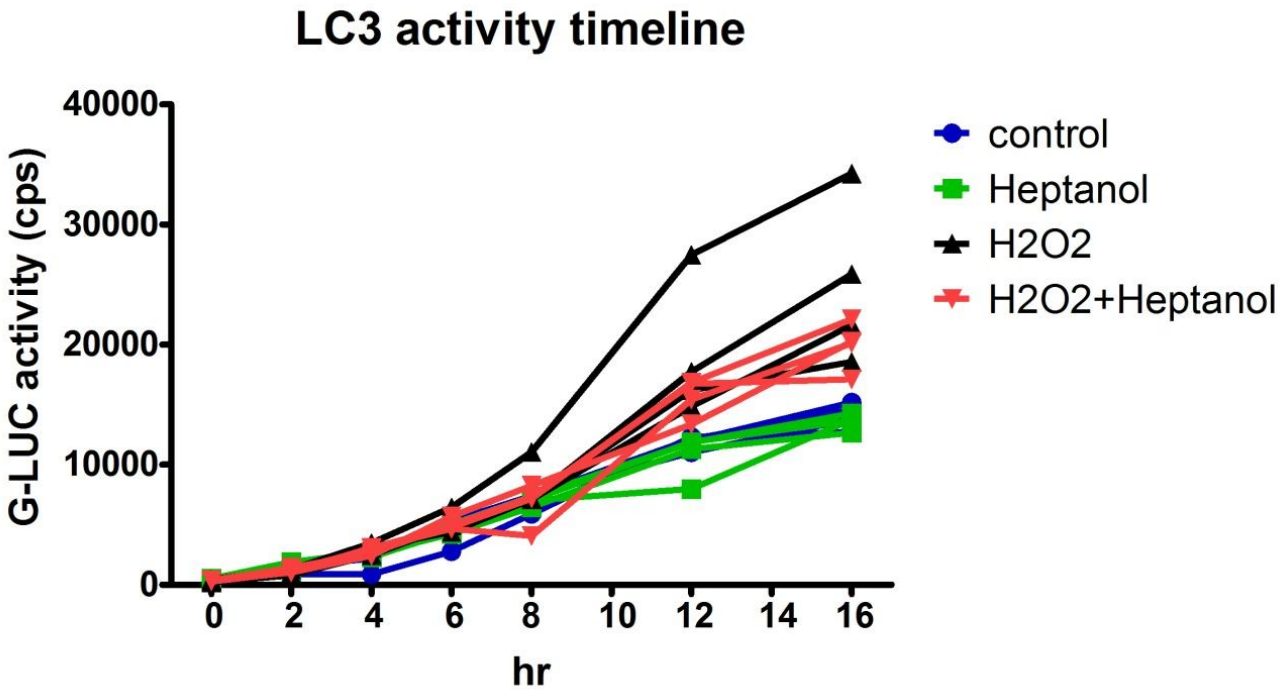
ADU: arbitrary densitometry units,



圖表 30: 抑制細胞間隙接合減少細胞自噬表現(個別冷光酵素反應)

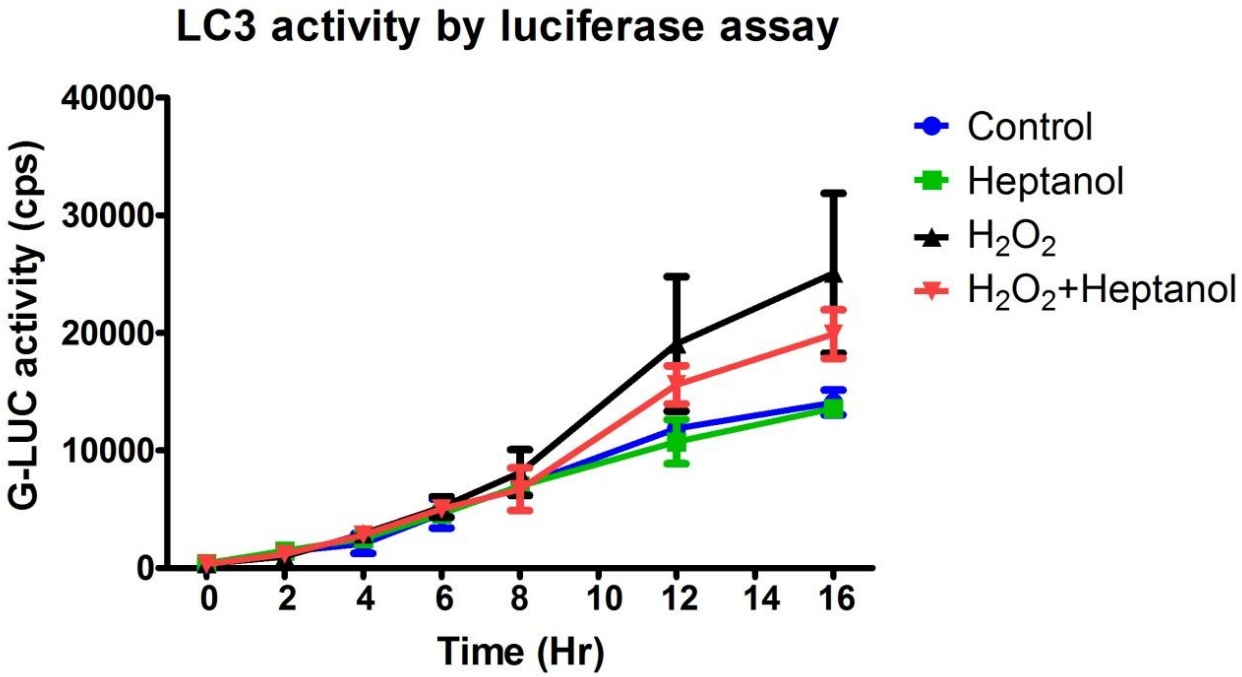
進行 4 重覆實驗，顯示抑制細胞間隙接合將減少冷光酵素反應。

Control group: 只添加培養液，Heptanol group: 使用正庚醇 0.5mM 作用，H_2O_2 group: H_2O_2 200μM 作用，H_2O_2 +Heptanol:同時添加正庚醇與 H_2O_2，Gluc: Gaussia luciferase，CPS: count per second。



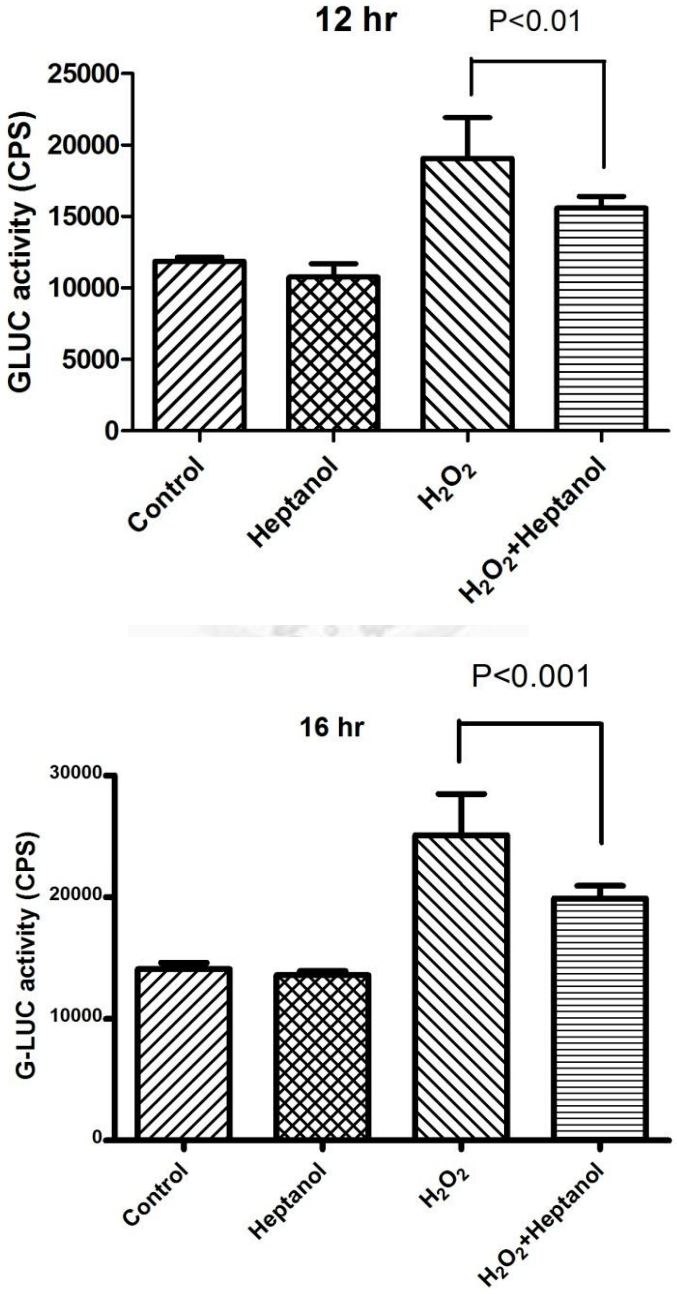
圖表 31: 抑制細胞間隙接減少細胞自噬表現(平均冷光酵素反應)

抑制細胞間隙接合將減少冷光酵素反應。Control group: 只添加培養液，Heptanol group: 使用正庚醇 0.5mM 作用，H_2O_2 group: H_2O_2 200μM 作用，H_2O_2+Heptanol: 同時添加正庚醇與 H_2O_2，Gluc: Gaussia luciferase，CPS: count per second。



圖表 32: 抑制間隙接合減少細胞自噬表現(12 小時、16 小時結果)

抑制細胞間隙接合將減少冷光酵素反應。Control group: 只添加培養液，Heptanol group: 使用正庚醇 0.5mM 作用，H_2O_2 group: H_2O_2 200μM 作用，H_2O_2+Heptanol: 同時添加正庚醇與 H_2O_2，Gluc: Gaussia luciferase，CPS: count per second。

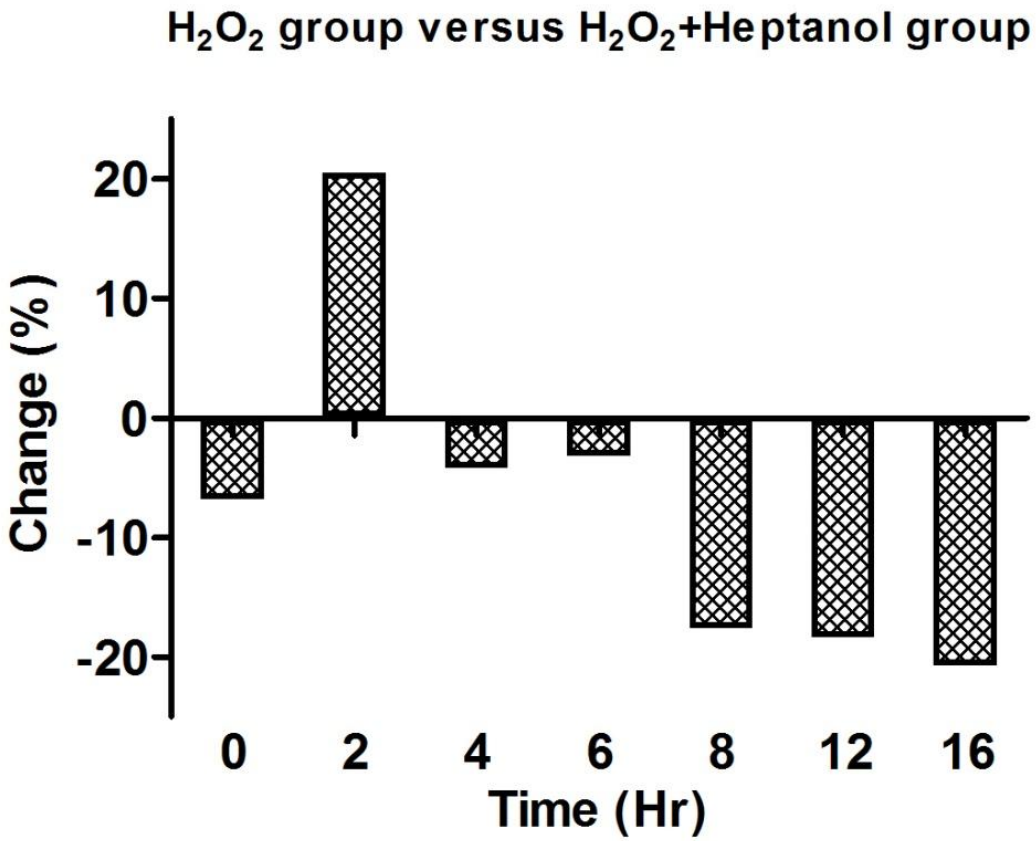


圖表 33: 抑制細胞間隙接合將降低細胞自噬

計算各時間點之細胞自噬下降率，顯示於 16 小時後，細胞自噬下降約 20%。

H_2O_2: hydrogen peroxide,

下降率計算公式 $\text{Change}(\%)=\left(1-\frac{\text{H2O2+Heptanol group Luciferase assay}}{\text{H2O2 group luciferase assay}}\right)\times 100\%$



表格附錄

表格 1: 過氧化氫增加 LC3-I, LC3-II 與 Bax 表現

H_2O_2 400 μM 作用於 H9c2

Time (hr)	ADU after Adjusting to Actin			LC3-II/LC3-I
	LC3-I	LC3-II	Bax	
1.5	0.74	0.15	0.08	0.20
3	0.64	0.16	0.33	0.26
6	1.34	0.74	0.83	0.55
12	1.53	0.77	0.90	0.51
16	1.67	0.81	0.91	0.48
24	2.03	1.06	1.14	0.52

ADU= Arbitrary Densitometry Units

All Data were expressed as relative level to Actin

LC3-I: Cytosol form light chain 3

LC3-II: membrane form light chain 3

Bax: pro-apoptotic protein

表格 2：過氧化氫對細胞存活率之影響

200 μM H_2O_2 作用於 H9c2 細胞之存活率

作用時間	細胞存活率 (%)	N	P-value
0 hr	100.0± 6.7	6	N.S.
2 hr	99.7± 7.4	4	N.S
4 hr	93.8± 3.0	5	N.S
6 hr	92.4± 3.3	6	N.S
12 hr	86.5± 11.0	5	N.S
24 hr	85.2± 1.8	4	<0.01

細胞存活率之計算為($\frac{\text{處理後細胞之吸光值}}{\text{200μM H2O2 對照組細胞之吸光值}}$ x 100%)

H_2O_2: Hydrogen peroxide

表格 3: 不同濃度過氧化氫對表現 LC3-I, LC3-II 與 Bax 之影響

不同濃度過氧化氫作用 12 小時

H_2O_2 濃度	ADU after Adjusting to Actin			ADU ratio of
	LC3-I	LC3-II	Bax	LC3-II/LC3-I
0 μM	0.54	0.24	0.65	0.45
50 μM	0.71	0.27	0.65	0.39
100 μM	0.65	0.55	0.62	0.84
200 μM	0.53	0.60	0.37	1.15
400 μM	0.49	0.64	0.32	1.29

H_2O_2: Hydrogen peroxide

LC3-I: Cytosol light chain 3

LC3-II: membrane form light chain 3

Bax: pro-apoptotic protein

ADU= Arbitrary Densitometry Units

表格 4: 不同濃度的過氧化氫對細胞存活的影響

不同濃度 H_2O_2 作用 12 小時後之細胞存活率

H_2O_2 濃度 (μM)	細胞存活率 (%)	N	P-value
0	100.0± 6.9	5	N.S.
50	105.0±10.2	5	N.S
100	91.2± 5.5	5	N.S
200	30.1± 9.4	5	0.03
400	31.5± 7.2	5	0.03
800	3.9± 0.1	5	0.03

細胞存活率之計算為($\frac{\text{處理後細胞之吸光值}}{\text{0μM H2O2 對照組細胞之吸光值}}$ x 100%)

H_2O_2: Hydrogen peroxide

表格 5: 正庚醇 0.5mM 對 H9c2 細胞存活率影響

正庚醇 0.5mM 處理

作用時間 (hr)	Survival (%)	N	P-value
0	100.0±6.7	6	N.S.
2	97.0±6.3	4	N.S
4	90.7±3.1	3	N.S
6	92.7±6.5	6	N.S
12	89.4±26.7	5	N.S
24	88.1±3.3	6	0.004

細胞存活率之計算為($\frac{\text{處理後細胞之吸光值}}{\text{處理前對照組細胞之吸光值}}$ x 100%)

Heptanol:正庚醇， gap junction uncoupler

表格 6: 比較過氧化氫對誘發細胞自噬的影響(冷光酵素反應)

過氧化氫 400μM 作用後冷光酵素反應(Guassia Luciferase Assay, count per second)

Time (Hr)	Control (N=4)	H_2O_2 (N=4)	H_2O_2/Control
0 hr	17885.3±1547.7	20754.0±673.7	1.16
2 hr	20196.0±1424.1	19911.5±841.9	0.99
4 hr	22728.5±825.0	23453.5±1210.4	1.03
6 hr	23636.8±1660.8	23768.8±563.4	1.01
8 hr	24911.0±2222.5	27867.8±494.6 *	1.12
10 hr	26065.3±1920.8	31995.3±1943.1 ¥	1.23
12 hr	25391.8±570.9	33174.3±2028.8 §	1.31
14 hr	24847.8±643.1	36956.3±890.0 §	1.49
16 hr	25552.5±883.8	35555.8±992.5 §	1.39
18 hr	24690.8±1095.9	36769.5±595.0 §	1.49
20 hr	25591.0±723.4	36070.3±844.8 §	1.41
22 hr	25466.0±622.5	36070.3±637.8 §	1.42
24 hr	26452.0±1879.2	36608.0±603.4 §	1.38

Control group: H9c2 轉染 pCMV-Lc3-Gluc 後，未添加過氧化氫，H_2O_2 group: H9c2 轉染 pCMV-Lc3-Gluc 後，加入過氧化氫 400μM，*: P value<0.05, ¥: P value <0.01, §: P value <0.001

表格 7: 正庚醇對氧化壓力誘發細胞自噬的影響（冷光酵素反應）

冷光酵素反應(Guassia Luciferase Assay, count per second)

Time	Control N=4	Heptanol N=4	H_2O_2 N=4	H_2O_2+Heptanol N=4
0 hr	308.0±76.2	413.8±56.5	363.3±53.3	338.8±26.3
2 hr	1384.8±169.1	1468.5±167.7	983.8±115.4	1184.5±93.3
4 hr	2082.8±415.3	2520.5±87.2	2919.8±231.8	2800.8±153.4
6 hr	4644.5±618.2	4636.3±174.6	5190.8±438.4	5030.5±229.6
8 hr	6990.3±368.0	6986.3±179.7	8131.3±971.7	6708.3±908.9
12 hr	11870.3±285.1	10755.5±934.7	19069.0±2863.1 £	15590.5±807.9 ¥
16 hr	14084.8±529.3	13599.3±331.9	25081.0±3402.7 £	19904.3±1034.5 §

Control group: 只添加培養液，Heptanol group: 使用正庚醇 0.5mM 作用，H_2O_2 group: H_2O_2 200μM 作用，H_2O_2+Heptanol:同時添加正庚醇與 H_2O_2 作用

¥: P value <0.01; §: P value <0.001; Data compare to H_2O_2 group

£: P value <0.001; data was compare to control group