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睪固酮對於心臟細胞凋亡的探討(第 2 年)
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計畫主持人：李信達
共同主持人：黃志揚
計畫參與人員：教授-主持人(含共同主持人)：黃志揚
副教授-主持人(含共同主持人)：李信達
碩士-兼任助理人員：蔡靜怡

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行政院國家科學委員會補助專題研究計畫期中進度報告

睪固酮對於心臟細胞凋亡的探討(第二年)

計畫類別：■ 個別型計畫　　□ 整合型計畫
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共同主持人：黃志揚
計畫參與人員：　蔡靜怡

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執行單位：中國醫藥大學物理治療學系

中　華　民　國　　97　年　05　　月　　11　　　日

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計畫主持人：李信達　中國醫藥大學　　物理治療學系

CARDIAC FAS RECEPTOR AND TNF RECEPTOR-1 DEPENDENT APOPTOTIC PATHWAY IN ORCHIECTOMY RATS

Abstract

Background. Very limited information regarding the cardiac apoptosis or fibrosis after andropause or bilateral orchiectomy in men was available. The purpose of this study was to evaluate whether orchiectomized rats will increase cardiac Fas and TNFR-1 dependent apoptotic pathways. ***Methods.*** In this study, fifteen male Wistar rats at 7-8 weeks of age randomly were divided into sham-operated group (Sham) and bilateral orchiectomized group (ORX). After two month of bilateral orchiectomy, the cardiac characteristics, myocardial architecture, Fas and TNFR-1 dependent apoptotic pathways, and fibrotic pathway in the excised left ventricle from rats were measured by heart weight index, histopathological analysis, western blotting and positive TUNEL assays. ***Results.*** The body weight and the whole heart weight were significantly decreased in ORX relative to Sham. Whereas the ratios of whole heart weight to body weight were no significantly differences in ORX relative to Sham. Abnormal myocardial architecture, enlarged interstitial spaces, no cardiac fibrosis, and more cardiac TUNEL-positive apoptotic cells were observed in ORX. The key components of Fas and TNFR-1 dependent apoptosis (Fas ligand, Fas death receptors, TNF-alpha, TNF receptor 1, FADD, activated caspase 8, and activated caspase 3) were significantly increased and fibrosis associated proteins (uPA, PAI-1, TGF-beta 1, Sp1, CTGF, MMP9, MMP2) were no changed in ORX hearts after two month orchiectomy. ***Conclusions.*** The absence of male tests will activate the cardiac Fas and TNFR-1 dependent apoptotic pathways, which may provide one of possible mechanism for developing heart failure in aging and orchiectomized patient.

Key words: heart, Fas and TNFR-1 dependent apoptosis, andropause, orchiectomy, caspase

Introduction

A progressive decrease in androgen production is common in aging men or in orchiectomized patients.(1) The increased risks of cardiovascular disease are associated with andropausal aging. Testosterone therapy improves cardiovascular risk in aging males and orchiectomized animal models.(2, 3) Previous studies demonstrate that testosterone can ameliorate chronic stable angina and cardiac ischemia in men, possibly because testosterone has effects of coronary vasodilation. (4, 5) However, very limited information regarding the development of cardiac apoptosis in andropausal or orchiectomized men was available.

Apoptosis, a physiological program of cellular death, may contribute to many cardiac disorders (6-8). The occurrence of apoptosis has been reported to contribute to the loss of cardiomyocytes in cardiomyopathy, and is recognized as a predictor of adverse outcomes in subjects with cardiac

diseases or heart failure (8). The 'extrinsic' Fas receptor and Tumor necrosis factor receptor 1 (TNFR1) apoptotic pathway were believed to be one of the major pathways directly to trigger cardiac apoptosis (6, 9, 10). This Fas receptor and TNF receptor 1 apoptotic pathway was initiated by binding of Fas ligand to the Fas receptor and TNFα to the TNF-receptor 1, which results in clustering of receptors and initiates the extrinsic pathway (9). Fas ligand and TNFα binding followed by Fas-receptor and TNF-receptor 1 oligomerisation led to formation of a death-inducing signal complex starting with recruitment of the the Fas-associated death domain (FADD) of the adaptor protein (9). Fas receptor and TNF-receptor 1 oligomerization recruits FADD and pro-caspase 8 to the complex and results in the activation of caspase 8. The activated caspase 8 cleaves pro-caspase 3, which then undergoes autocatalysis to form active caspase 3, a principle effector caspase of apoptosis (11, 12).

Cardiac fibrosis, the disproportionate accumulation of extracellular matrix (ECM) in the heart that occurs after myocyte death, inflammation, hypertrophy, is a common feature of end-stage heart disease independent of etiology.(13, 14) The proliferation of interstitial fibroblasts and increased deposition of ECM components results in myocardial stiffness and diastolic dysfunction, which ultimately leads to heart failure.(13)

Several factors have been implicated in the development of cardiac fibrosis. These include matrix metalloproteinases (MMPs) which are proteinases that participate in extracellular matrix remodelling and degradation.(15) Another various cytokines and growth factors such as transforming growth factor (TGF) β 1, urokinase plasminogen activator(uPA), connective tissue growth factor (CTGF), Sp1 may activated MMPs lead to fibrosis.(15-17) PAI-1 inhibits plasminogen activator activity, decreasing the production of fibrosis.(18)

The role of cardiac Fas-TNFR1 apoptosis and cardiac fibrosis in orchiectomized rats was still unknown. In the current study, to understand whether cardiac abnormality in orchiectomized rats is associated with more activated Fas receptor and TNFR-1 dependent apoptotic pathway and cardiac fibrosis, the myocardial morphology and key components of Fas receptor and TNFR-1 dependent apoptotic pathway and cardiac fibrosis were determined by histopathological analysis and Western blotting from the exercised cardiac tissue in bilateral orchiectomized wistar rats. We hypothesized that cardiac abnormality in orchiectomized rats may predispose to more activated Fas receptor and TNFR-1 mediated cardiac apoptosis.

Materials and Methods

Animals

Fifteen 7-8 weeks male Wister rats were purchased from National Laboratory Animal Center, Taiwan. Ambient temperature was maintained at 21°C and the animals were kept on an artificial 12-h light-dark cycle. The light period began at 6:00 A.M. The rats were allowed free access to water and chow (Purina chow, Ralston Purina, St. Louis, MO, USA). All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taiwan.

Orchiectomy and Sham operation

All fifteen rats were randomly divided into either sham-operated group or orchiectomied group (ORX). All animals were conducted by survival

surgical procedures with aseptic technique at age of 8-9 weeks. After anesthetized with intramuscular injection of ketamine (100 mg/kg), animals were bilaterally orchiectomized through vertical incisions over the left and right hemiscrotum. Incisions were closed in two layers using 4-0 chromic on the tunica dartos and 4-0 silk on the skin and each rat was injected with Penicillin-G procaine (0.2 ml, 20,000 IU, IM). The sham-operated group underwent the same surgical procedure except for the removal of the testes. After ORX or Sham operation, the rats were kept individually in plastic cages (25 × 41 × 19 cm) for recovery for about 7 days, and then grouped back to their home cages.

Cardiac characteristics.

After one months of orchiectomied or Sham operation, all 15 rats were weighed and decapitated at age of 12-13 weeks. The hearts of 7 Sham and 8 ORX animals were soaked in formalin and further analyzed by Hematoxylin-eosin, Masson trichrome staining, DAPI staining and TUNEL assay as well as the other 7 Sham and 8 ORX were analyzed by heart weight index and Western Blotting. The hearts of animals were excised, cleaned with phosphate-buffered saline (PBS). The left atrium and ventricle were separated and body weight (BW), whole heart weight (WHW), left ventricular weight (LVW) were measured by scale. The right tibias were also separated and tibia length was measured by the electronic digital vernier caliper for correcting the WHW and LVW. The ratios of the WHW to BW, the ratios of the LVW to BW, the LVW to the WHW, the WHW to tibia length, and the LVW to to tibia length were calculated.

Hematoxylin-eosin and Masson trichrome staining and Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL)

The hearts from animals were excised and were soaked in 10% formalin and embedded in paraffin. In heart tissues, the 3-μm thick paraffin sections were cut from formalin- fixed, paraffin-embedded tissue blocks. The sections were heated overnight in 60 °C incubator, and then deparaffinized by immersing in xylene and rehydrated through a series of graded alcohols (100%, 95%, 85% and 75%) and distilled H_2O, 5 minutes for each. The slides were then dyed with hematoxylin and eosin or Masson trichrome. A slice chosen from each group was analyzed under 400× microscope.

Determination of apoptosis using DNA fragmentation technique

Apoptosis detection was performed using the TUNEL Assay Kit (Roche Applied Science, Indianapolis, IN, USA).Tissue slides were deparaffinized and rehydrated, then treated with proteinase K (20 μg/ml) for 25 min at room temperature. After rinsing in phosphate-buffered saline (PBS), pH 7.4, specimens were incubated in 1× equilibration buffer for 8mins and washed in PBS. The slides were then incubated with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 60 min at 37 °C. Next, after washing PBS twice, the sections were also stained with 4', 6-diamidine-2-phenylindole dihydrochloride (DAPI) (Sigma Chemicals, St. Louis, MO, USA) for 5 min to detect cell nucleus by UV light microscopic observations (blue). TUNEL-positive nuclei (fragmented DNA) fluoresced bright green at 450-500 nm, whereas DAPI-positive nuclei (intact DNA) fluoresced blue at 360 nm. The mean number of TUNEL-positive and DAPI-labeled cells were counted for at least 5-6 separate fields x 2 slides x 3 LV regions (upper, middle, lower) excised from six rat hearts in each group. All counts were performed by at least 2 independent individuals in a blinded manner.

Tissue Extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a lysis buffer (20mM Tris, 2mM EDTA, 50mM 2-mercaptoethanol, 10% glycerol, PH 7.4, proteinase inhibitor (Roche), phosphatase inhibitor cocktail (sigma)) at a ratio of 100 mg tissue/1ml buffer for 1 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 g for 40 min twice. The supernatant was collected and stored at -70°C for further experiments.

Electrophoresis and Western Blot

The tissue extract samples were prepared as described by homogenizing with buffer. Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis was done with 12% polyacrylamide gels. The samples were electrophoresed at 80 V for 3 hours and equilibrated for 5 min in 25 mM Tris-HCl, pH 8.3, containing 192 mM glycine and 20% (V/V) methanol. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) with a Bio-rad Scientific Instruments Transphor Unit at 100 V for 2 hr. PVDF membranes were incubated at room temperature for 1 hours in blocking buffer containing 100 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl. Antibodies used in the current study include Fas ligand, Fas receptor, FADD, uPA, PAI-1, Sp1, MMP2, α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cleaved caspase-3, TNF-α, TGFβ1 (Cell Signaling Technologies, Danvers, MA, USA), caspase-8, MMP9 (CHEMICON international, Inc., CA, USA), TNFR-1, CTGF (abcam Inc., Cambridge, MA, USA) were diluted to 1:500 in TBS buffer containing 100 mM Tris-HCL, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 and incubations were performed at 4°C overnight. The immunoblots were washed three times in TBS buffer (Tris-Base, Nacl, Tween-20, PH7.4) for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti goat IgG-HRP (Santa Cruz) for 1 hour and diluted 500-fold in TBS buffer. The immunoblots were then washed in blotting buffer for 10 min three times. The immunoblotted proteins were visualized by using an enhanced chemiluminescence ECL western Blotting luminal Reagent (Santa Cruz) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (FUJIFILM, Tokyo, Japan).

Statistical Analysis

The data of body weight and heart weight index, protein levels, and the percentage of TUNEL positive cells relative to total cells assay were compared between Sham and ORX groups using student t-test. Sham group serves as negative control group for ORX group. In all cases, a difference at $P<0.05$ was considered statistically significant.

Results

Cardiac characteristics

Body weight (BW), whole heart weight (WHW) and left ventricular weight (LVW) were significantly lower in orchiectomied group(ORX) than those in Sham group, whereas the ratio of WHW to BW, the ratio of LVW to BW, the ratio of LVW to WHW and the ratio of WHW to tibia length were not changed. Beside the ratio of LVW to tibia length was significant decreased, compared with sham group. (Table 1)

Myocardial architecture, fibrosis, and apoptosis

To understand the myocardial architecture, cardiac fibrosis and apoptosis in ORX group, we did a histopathological analysis of left ventricular tissue with Hematoxylin-eosin staining, Masson trichrome staining and TUNEL assay in Sham and ORX groups. Hearts stained

with Hematoxylin-eosin showed that the ventricular myocardium in the ORX group showed abnormal architecture and increased interstitial space compared with sham group (Fig 1A). Hearts stained with Masson trichrome showed that no fibrosis was observed in ORX group (Fig 1B). Hearts stained with TUNEL assay showed that increased TUNEL-postive cardiac cells in the ORX group. (Fig 1C)

Upstream components of cardiac Fas receptor and TNF receptor -1 dependent apoptotic pathways

To investigate the upstream components of cardiac death receptor apoptotic signaling pathways ORX group. , the protein levels of TNF-α, TNFR-1, Fas ligand, Fas receptor, and FADD in the excised hearts of Sham and ORX groups were examined by Western blotting. Compared with the Sham group, the levels of TNF-α, TNFR-1, (Fig 2) Fas ligand, Fas receptor (Fig 3) and FADD (Fig 4) were significantly higher in the ORX group.

Downstream components of cardiac Fas receptor and TNF receptor -1 dependent apoptotic pathways

To identify the downstream components of cardiac Fas receptor and TNF receptor-1 dependent apoptotic pathways, the protein levels of activated caspase 8 and 3 were measured in the excised hearts of Sham and ORX groups by Western Blotting. The activated forms of caspase 8 and 3 protein products were increased in the ORX group compared with Sham group (Fig 5).

Cardiac fibrosis related protein

To test the fibrosis related proteins including uPA, PAI-1, TGFβ1, Sp1, CTGF, MMP9 and MMP2 in ORX group by Western Blotting analysis. Changes in protein levels were not found in ORX group compared with sham group in 12-13 weeks rats (Fig 6).

Discussion

Major findings

Our main findings can be summarized as follows: (1) body weight and heart weight both decrease in orchiectomized animal whereas the ratio of heart weight to body weight were not changed. (2) Abnormal myocardial architecture, enlarged interstitial space and TUNEL-positive apoptotic cells increase in orchiectomized animal but no changes cardiac fibrosis stained masson trichrome were found. (3) The cardiac TNF receptor and Fas-dependent apoptotic pathways were significantly more activated in orchiectomized animal, the evidence for which is based on increases in TNF-alpha, TNF receptor 1, Fas ligand, Fas death receptors, FADD, activated caspase-8, and activated caspase-3. (4) Changes in fibrosis related proteins were not found. After integrating our current findings into previously proposed apoptotic theories, our hypothesis proposed that cardiac TNF receptor and Fas-dependent apoptotic pathways might be more activated after the absence male testis (Fig 7).

Table 1. Cardiac characteristics of Sham and ORX group

	Sham	ORX
Body weight (BW), g	387±12	328±29**
Whole heart weight (WHW), g	1.00±0.05	0.87±0.03***
Left ventricle weight(LVW), g	0.71±0.03	0.61±0.02***
WHW / BW *10^3	2.59±0.17	2.67±0.20
LVW / BW *10^3	1.83±0.09	1.88±0.16
LVW / WHW	0.71±0.01	0.71±0.02
WHW / Tibia length (mm)*10^3	27.56±0.99	25.12±2.27
LVW / Tibia length (mm)*10^3	19.53±0.44	17.71±1.46*

Values are means ± SD (n=7, 8). Sham, sham-operated rats; ORX, Orchiectomized rats. * P<0.05, ** P<0.01, *** P<0.001 Significant differences between Sham group and ORX group.

Fig 1

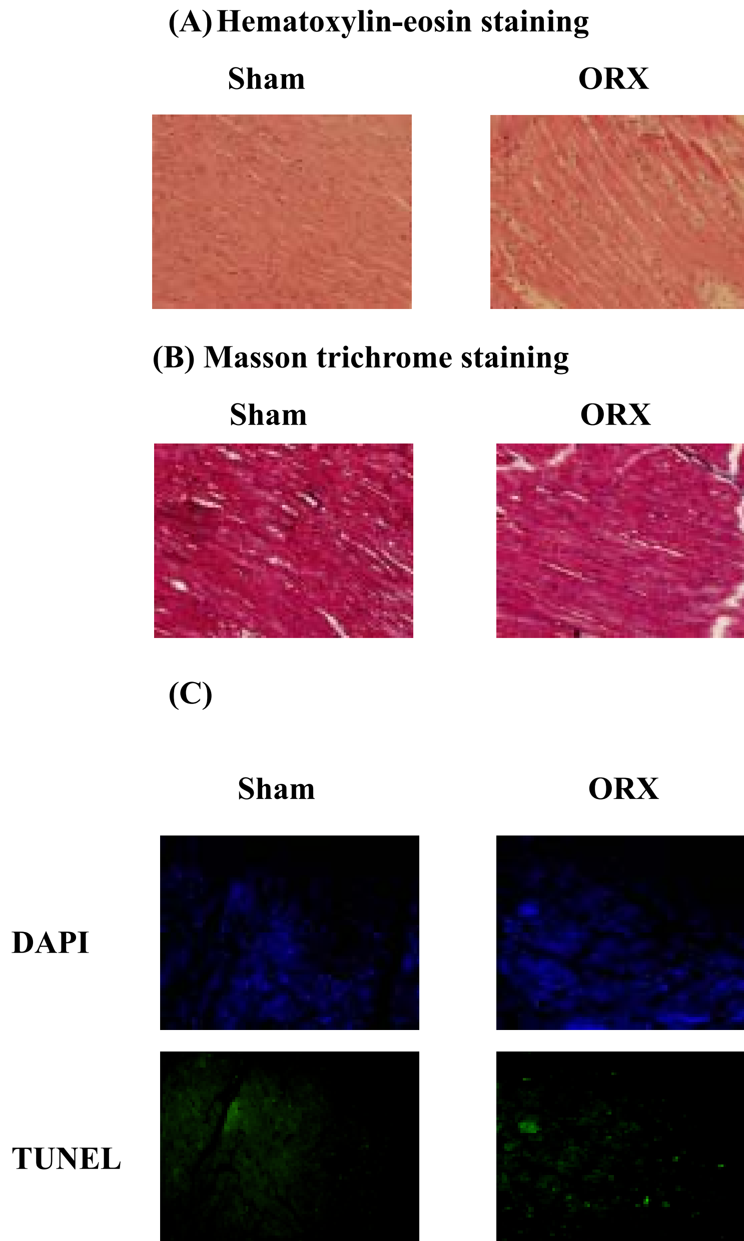
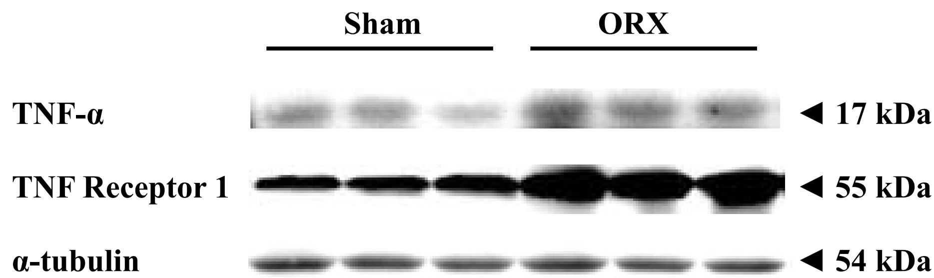


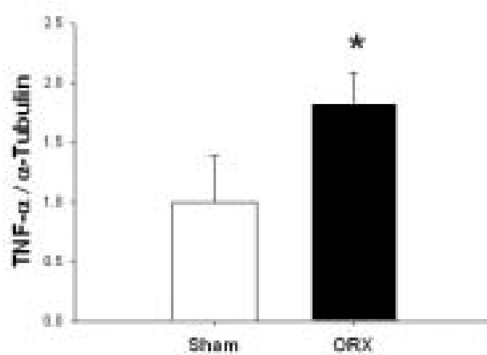
Fig 1. Representive histopathological analysis of cardiac tissue sections with (A) Hematoxylin and eosin staining and with (B) Masson trichrome staining (fibrosis: blue color) in sham-operated rats (Sham) and orchiectomized rats (ORX). (C) Apoptotic cells of cardiac sections from left ventricles in sham-operated rats and ORX rats were measured by staining with 4',6-diamidino-2-phenylindole (DAPI) (upper panels, blue spots) and Terminal deoxynucleotidyltransferase UTP Nick End Labeling (TUNEL) assay with dark background (lower panels, green spots). (D) Bars present the percentage of TUNEL positive cells relative to total cells (6 rats x 30 scope field count in each group). The images of myocardial architecture were magnified by 400 times (n=6 in each group).

Fig 2

(A)



(B)



(C)

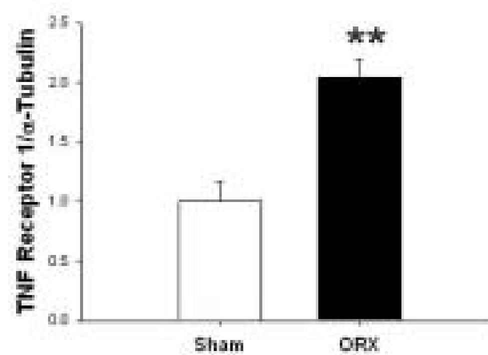


Fig 2. (A) The representive protein products of tumor necrosis factor-alpha (TNF-α) (17kDa) and tumor necrosis factor receptor 1 (TNFR1) (55kDa) extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and orchiectomized rats (ORX) were measured by Western Blotting analysis. (B)(C) Bars represent the relative protein quantification of TNF-α and TNFR1 on the basis of α-tubulin, and indicate mean values±SD (n=6 in each group). $^{*}P<0.05$, $^{**}P<0.01$, significant differences between Sham and ORX group.

Fig 3

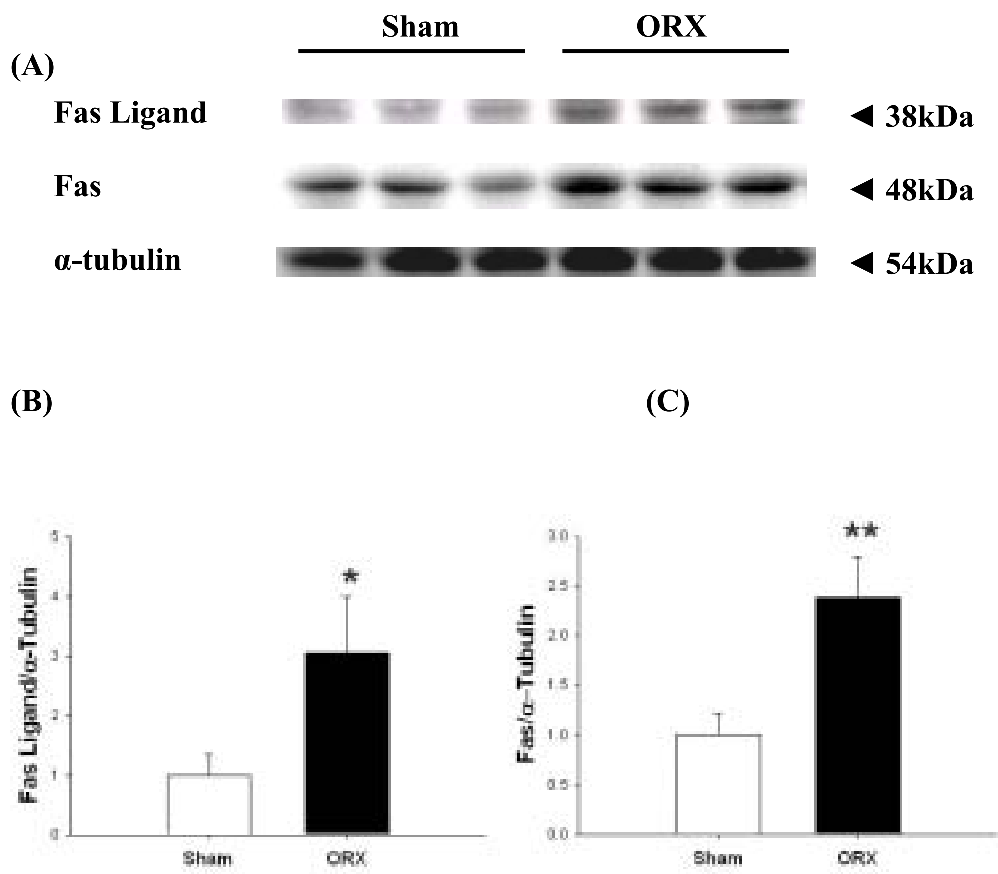
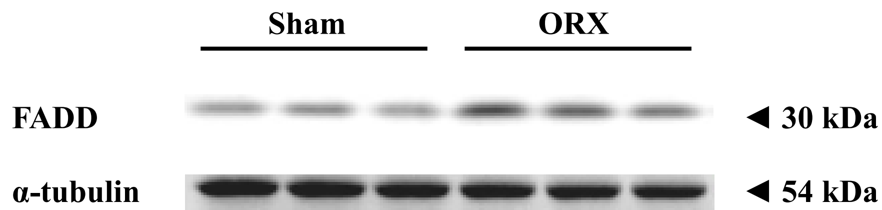


Fig 3. (A) The representive protein products of Fas ligand (38kDa) and Fas receptor (48kDa) extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and orchiectomized rats (ORX) were measured by Western Blotting analysis. (B)(C) Bars represent the relative protein quantification of Fas ligand and Fas receptor on the basis of α-tubulin, and indicate mean values±SD (n=6 in each group). $^{*}P<0.05$, $^{**}P<0.01$, significant differences between Sham and ORX group.

Fig 4

(A)



(B)

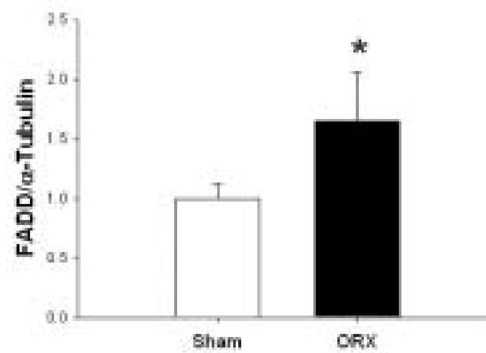
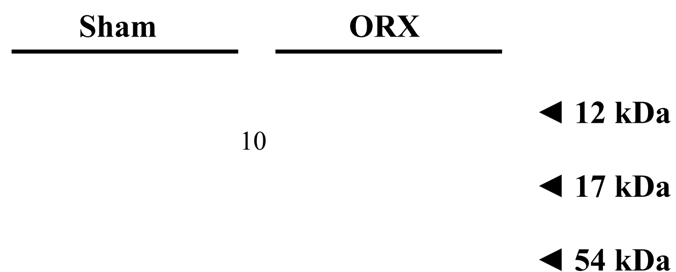


Fig 4. (A)The representive protein products of Fas-associated death domain (FADD) (30kDa) extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and orchiectomized rats (ORX) were measured by Western Blotting analysis. (B) Bars represent the relative protein quantification of FADD on the basis of α-tubulin, and indicate mean values±SD (n=6 in each group). $^{*}P<0.05$, significant differences between Sham and ORX group.

Fig 5

(A)



(B) **(C)**

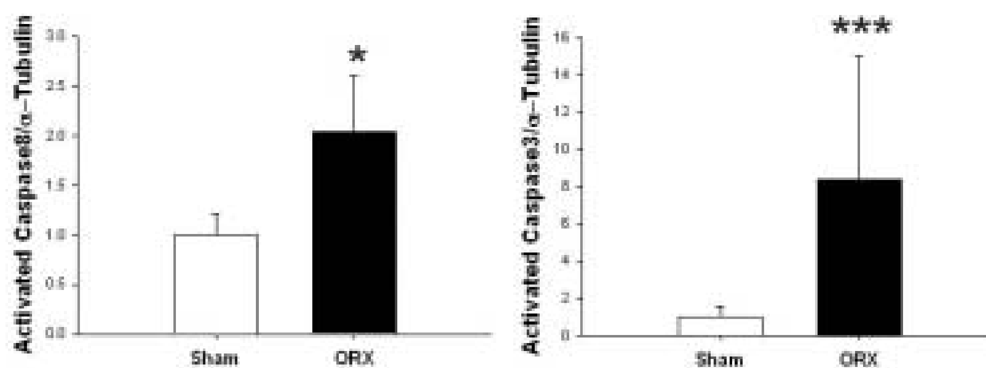


Fig 5. (A)The representive protein products of activated caspase 8 (12kDa) and activated caspase 3 (17kDa) extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and orchiectomized rats (ORX) were measured by Western Blotting analysis. (B)(C) Bars represent the relative protein quantification of activated caspase 8 and caspase 3 on the basis of α-tubulin, and indicate mean values±SD (n=6 in each group). $^{*}P<0.05$, $^{***}P<0.001$, significant differences between Sham and ORX group.

Fig 6

(A)

Sham **ORX**

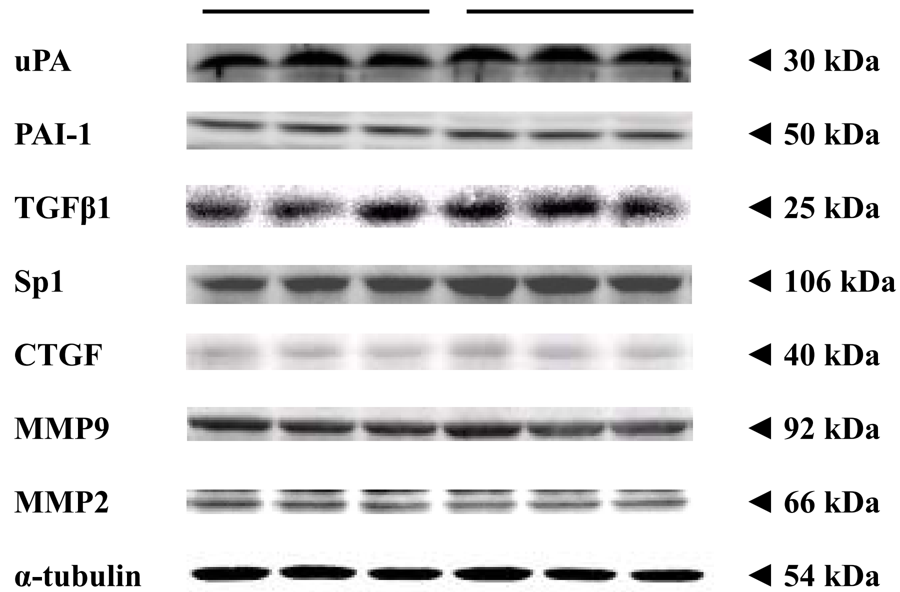


Fig 6. (A) The representive protein products of uPA (30kDa), PAI 1 (50kDa), TGFβ1 (25kDa), Sp1 (106kDa), CTGF (40kDa), MMP9 (92kDa) and MMP2 (66kDa) extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and orchiectomized rats (ORX) were measured by Western Blotting analysis. (B) Bars represent the relative protein quantification of uPA, PAI-1, TGFβ1, Sp1, CTGF, MMP9 and MMP2 on the basis of α-tubulin, and indicate mean values±SD (n=6 in each group). No significant differences between Sham and ORX group.

Fig 7

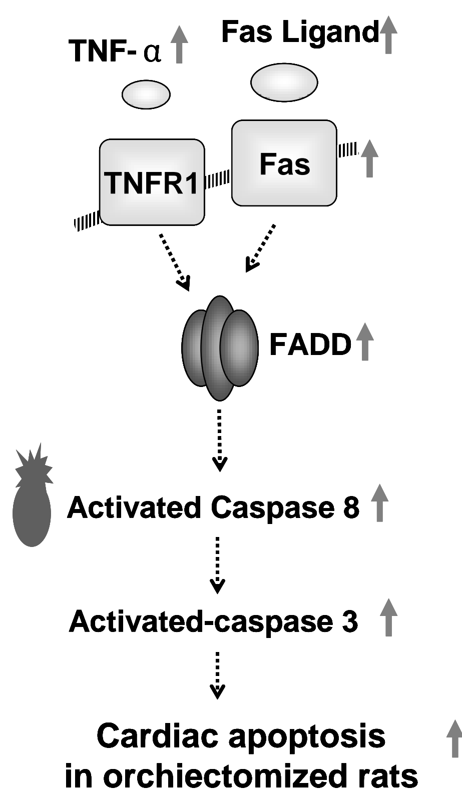


Fig 7. In our proposed hypothesis, cardiac Fas-dependent apoptotic pathways will be activated in orchiectomized rats (ORX) compared with Sham. Cardiac Fas-dependent apoptotic pathway appears to be increased due to increases in TNF-α, TNFR1, Fas ligand, Fas receptor, FADD, activated caspase-8, and activated caspase-3. Up arrows and down arrows on the right side represent increases and decreases, respectively.

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出席國際學術會議心得報告

計畫編號	NSC 95-2314-B-039-043-MY3（2/3）
計畫名稱	睪固酮對於心臟細胞凋亡的探討
出國人員姓名 服務機關及職稱	李信達 中國醫藥大學　物理治療學系 副教授兼系主任
會議時間地點	民國 97 年 5 月 16 日至 5 月 21 日舉辦在 Toranto, Canada
會議名稱	2008 年美國胸腔協會國際會議年會 (2008 ATS International Conference)
發表論文題目	長期間歇性低氧和肥胖對大鼠心臟凋亡途徑的加乘性的影響 The Coexistence of Mimic Nocturnal Sustained Hypoxia and Obesity Additively Increases Cardiac Apoptosis

一、參加會議經過

2008 年美國胸腔協會國際會議年會 (2008 ATS International Conference)是在呼吸胸腔學界最大且最重要科學研討會議, 全球呼吸胸腔學界菁英在此交流和提出最新的研究，今年舉行的地點在 Toranto, Canada，已超過 16,000 人從 90 國家的呼吸胸腔學界學者專家去參加此盛會。

本人於此次會議中發表的兩篇論文為”長期間歇性低氧和肥胖對大鼠心臟凋亡途徑的加乘性的影響 (The Coexistence of Mimic Nocturnal Sustained Hypoxia and Obesity Additively Increases Cardiac Apoptosis) Poster presentation at a Poster Discussion session, A107 - ANIMAL MODELS FOR CONSEQUENCES OF HYPOXIA AND SLEEP APNEA, scheduled for Sunday, 5/18/2008 from 1:30 pm to 4:15 pm.。於此次會議中也出席參與” Sleep and Respiratory Neurology Assembly”興趣小組的會議。多種缺氧或睡眠呼吸終止症的相關主題研討報告會例如: “Cardiovascular Consequences of Sleep-Disordered Breathing”、“Sleep Medicine Grand Rounds”、“Sleep Apnea as a mechanism of Atherosclerosis from bench to beside”、“Sleep and Heart failure”、“Sleep and Type II DM”“Upper Airway”、….等主題研討報告會。

二、與會心得

全球菁英在 Toranto, Canada 交流和提出最新的研究，呼吸胸腔學界學者更新最新醫療科學資訊最重要的必要課程。今年參加完美國胸腔協會國際會議年會收穫很多，由其是參與”

Sleep and Respiratory Neurology Assembly” 興趣小組的會議參與興趣小組的選舉，不僅面對面的深度討論，也相對得慢慢融入興趣小組的團體，在美國胸腔協會國際會議年會與多位興趣小組的學者溝通討論之後，除了更了解目前興趣小組的最新的研究成果與國際發展方向，深深的覺得整個學術歷程中興趣小組的整合國際研究扮演了一個非常重要的一個環節。也深深覺得研究不再持續提升、不再更加油，很容易被進步神速的學術潮流給洮汰。