2015

高雄長庚紀念醫院

外傷科

醫學研究論文集

目錄

臨床

Bicycle-related hospitalizations at a Taiwanese level I Trauma Centers

BMC Public Health. 2015 Jul 29;15:722.

Hang-Tsung Liu, Cheng-Shyuan Rau, Chi-Cheng Liang, Shao-Chun Wu, Shiun-Yuan Hsu, Hsiao-Yun Hsieh, Ching-Hua Hsieh

本研究在於了解南台灣騎腳踏車時受傷住院病患之情況。我們發現騎腳踏車受傷的人，和機車騎士相比，其戴安全帽之比例較低、於夜間急診就診情況較少、且酒駕比例較低。但是其根據年齡及受傷嚴重程度調整後之死亡率卻為機車騎士的 4.4 倍

Motorcycle-related hospitalization of adolescents in a Level I trauma center in southern Taiwan: a cross-sectional study

BMC Pediatr. 2015 Aug 28;15:105.

Chi-Cheng Liang, Hang-Tsung Liu, Cheng-Shyuan Rau, Shiun-Yuan Hsu, Hsiao-Yun Hsieh, Ching-Hua Hsieh

使用外傷登錄的資料比較 13-19 歲青少年機車住院患者，和同期成年人機車住院患者相比，其創傷嚴重程度、住院天數、加護病房住院人數比例、以及死亡率並無明顯之差別。但是青少年機車騎士戴安全帽的比例則明顯較低，而這些未戴安全帽的騎士，和有戴安全帽的騎士相比，其到院時昏迷的比例較高、頭頸部受傷比例較高、顱骨骨折的人也較多

Obese patients who fall have less injury severity but a longer hospital stay than normal-weight patients

World J Emerg Surg. 2016 Jan 4;11:3.

Jung-Fang Chuang, Cheng-Shyuan Rau, Hang-Tsung Liu, Shao-Chun Wu, Yi-Chun Chen, Shiun-Yuan Hsu, Hsiao-Yun Hsieh, Ching-Hua Hsieh

統計發現身體質量指數大於或等於30以上之肥胖患者於跌倒中所受之傷害比一般正常體重患者來的輕，但是其病房之住院天數卻較長，即便考慮了原有之各項慢性疾病及創傷嚴重程度之不同，跌倒之肥胖患者比一般正常體重患者會明顯地多了一天半的住院時間

Use of the reverse shock index for identifying high-risk patients in a five-level triage system

Scand J Trauma Resusc Emerg Med. 2016 Feb 9;24(1):12.

Jung-Fang Chuang, Cheng-Shyuan Rau, Shao-Chun Wu, Hang-Tsung Liu, Shiun-Yuan Hsu, Hsiao-Yun Hsieh, Yi-Chun Chen, Ching-Hua Hsieh

正常人之心跳數值是不會高於動脈收縮血壓。對於受傷之病人，尤其是出血而近休克之患者，心跳會加快，血壓會降低，當血壓小於心跳時，我們定義其為逆轉休克指數 reverse shock index(RSI) <1，代表病人正處於可能休克的情況，而需要更積極之治療。我們發現使用逆轉休克指數 <1 為篩檢條件時，可以幫助於急診檢傷分類 Level II 及 III 的病患中，找出有著高危險性之患者，將有利於提高急診檢傷分類之準確性

A regime of two intravenous injections of tranexamic acid reduces blood loss in minimally invasive total hip arthroplasty

Bone Joint J. 2015 Jul;97-B(7):905-10.

Chih-Hsiang Hsu, Po-Chun Lin, Feng-Chih Kuo, Jung-Wen Wang

對於60位接受微創全髖關節置換術手術之前瞻性隨機雙盲的研究顯示，傷口切開前十分鐘及三個小時後，分別於靜脈注射一劑傳明酸可以減少術中及術後之出血量及總出血量，並可以減少整體輸血之需求

Mini-midvastus total knee arthroplasty in patients with severe varus deformity

Orthopedics. 2015 Feb;38(2):e112-7.

Hao-Chen Liu, Feng-Chih Kuo, Chung-Chen Huang, Jun-Wen Wang, Jung-Wen Wang

雖然膝關節嚴重內翻畸形患者一般不是使用微創全膝關節置換術之好的手術候選人，但此研究發現，如果經股四頭肌微創全膝關節置換術手術治療膝關節嚴重內翻畸形，並和那些接受手術之膝關節內翻畸形較不嚴重的患者相比較，其臨床預後、回復之膝功能分數、以及X光之檢查結果，兩者之間差異性不大，而且病患也都很滿意手術後之結果

基礎

Altered exosomal protein expression in the serum of NF-κB knockout mice following skeletal muscle ischemia-reperfusion injury.

J Biomed Sci. 2015 Jun 10;22:40.

Johnson Chia-Shen Yang, Ming-Wei Lin, Cheng-Shyuan Rau, Seng-Feng Jeng, Tsu-Hsiang Lu, Yi-Chan Wu, Yi-Chun Chen, Siou-Ling Tzeng, Chia-Jung Wu, Ching-Hua Hsieh

此對於小鼠大腿肌肉缺血再灌流後血液中外排小體的蛋白質體分析研

究顯示，NF-κB 基因剔除之小鼠其外排小體中對於代謝過程及生物功能調控之蛋白質較正常 C57BL/6 小鼠少，顯示肌肉缺血再灌流可能藉由血循中外排小體來對遠處標的進行進一步之調控

Identification of Circulating miRNAs in a Mouse Model of Nerve Allograft Transplantation under FK506 Immunosuppression by Illumina Small RNA Deep Sequencing.

Dis Markers. 2015;2015:863192.

Shao-ChunWu, Cheng-Shyuan Rau, Johnson Chia-Shen Yang, Tsu-Hsiang Lu, Yi-ChanWu, Yi-Chun Chen, Siou-Ling Tzeng, Chia-JungWu, Chia-Wei Lin, Ching-Hua Hsieh

使用次世代基因定序方法來分析小鼠異體神經移植後其血液中微型核醣核酸之表現，我們發現有九種微型核醣核酸於異體神經移植三天後會有明顯之表現，但在第七及第十四天時卻沒有明顯之差異。此只有短暫出現微型核醣核酸異常之情況，代表說如果使用血液中微型核醣核酸的表現來作為監測小鼠異體神經移植後有無排斥之生物指標可能不太合適

Regulatory and Effector Helper T-Cell Profile after Nerve Xenografting in the Toll-Like Receptor-Deficient Mice.

Int J Med Sci. 2015 Aug 1;12(8):650-4..

Cheng-Shyuan Rau, Ming-Wei Lin, Shao-Chun Wu, Yi-Chan Wu, Tsu-Hsiang Lu, Siou-Ling Tzeng, Yi-Chun Chen, Chia-Jung Wu, Ching-Hua Hsieh

研究 C57BL/6、NF-κB 基因剔除、TLR2 基因剔除小鼠異體神經移植後血液中 T 細胞之表現，我們發現於 TLR2 及 NF-κB 基因剔除情況下，小鼠異體神經移植後之 Treg 減少情況會減緩，並會抑制 Th1-及 Th17 驅動之免疫反應。此實驗亦顯示使用 TLR 抑制來調控移植後 T 細胞相關免疫反應之潛力

Weight-reduction through a low-fat diet causes differential expression of circulating microRNAs in obese C57BL/6 mice

BMC Genomics. 2015 Sep 16;16(1):699.

Ching-Hua Hsieh, Cheng-Shyuan Rau, Shao-Chun Wu, Johnson Chia-Shen Yang, Yi-Chan Wu, Tsu-Hsiang Lu, Siou-Ling Tzeng, Chia-Jung Wu, Chia-Wei Lin

肥胖常被視為是一種身體慢性發炎之疾病，我們的研究顯示，肥胖鼠食用低脂飲食可以導致其血液中微型核醣核酸表現的改變，而這些改變的微型核醣核酸調控基因牽涉到和代謝、胰島素、還有脂肪細胞素的訊息傳遞途徑的調控有關

Effect of Weight-Reduction in Obese Mice Lacking Toll-Like Receptor 5 and C57BL/6 Mice Fed a Low-Fat Diet

Mediators Inflamm. 2015;2015:852126.

Shao-Chun Wu, Cheng-Shyuan Rau, Tsu-Hsiang Lu, Siou-Ling Tzeng, Yi-ChanWu, Chia-Jung Wu, Chia-Wei Lin, Ching-Hua Hsieh

類鐸樣受體第五型基因剔除小鼠比正常 C57BL/6 小鼠更容易導致肥胖的型態及症狀。食用低脂飲食可以改善正常小鼠肥胖程度、血糖不耐症、及體內脂肪細胞素不當增加的情況，但是食用低脂飲食對於類鐸樣受體第五型基因剔除之肥胖鼠效果卻不佳，尤其是血液中的飢餓素(ghrelin)及脂肪中的抵抗素(resistin)的降低變得更不明顯，代表說類鐸樣受體第五型基因的剔除導致了減肥效果受到了明顯的影響。

編言

高雄長庚醫院外傷科一直以來除了服務南台灣之大量外傷病患，不斷提升臨床之照護水準外，尤兢兢業業於各項臨床與基礎方面之研究工作。每年於農曆過年前，彙整我們過去一年來由科內醫師擔任第一或指導作者所發表之學術研究工作內容，不僅是在於對過去一路走來之足跡做一個回顧及檢討，也在於對未來的各項研究方向括劃出一個方向。並希望能藉由於增加外傷醫學領域之學術交流外，促進對外傷病患之全方面照顧。

謝青華/劉約維

Liu et al. BMC Public Health (2015) 15:722
DOI 10.1186/s12889-015-2075-9

BMC Public Health

RESEARCH ARTICLE Open Access

Bicycle-related hospitalizations at a Taiwanese level I Trauma Center

Hang-Tsung Liu[1†], Cheng-Shyuan Rau[2†], Chi-Cheng Liang[1], Shao-Chun Wu[3], Shiun-Yuan Hsu[1], Hsiao-Yun Hsieh[1] and Ching-Hua Hsieh[1*]

Abstract

Background: This study aimed to investigate differences in injury severity and mortality between patients who met with bicycle or motorcycle accidents and were hospitalized at a Level I trauma center in Taiwan.

Methods: We performed a retrospective analysis of bicycle-related injuries that have been reported in the Trauma Registry System in order to identify and compare 699 bicyclists to 7,300 motorcyclists who were hospitalized for treatment between January 1, 2009 and December 31, 2013. Statistical analyses of the injury severity, associated complications, and length of stay in the hospital and intensive care unit (ICU) were performed to compare the risk of injury of bicyclists to that of motorcyclists with the corresponding unadjusted odds ratios and 95 % confidence intervals (CIs). Adjusted odds ratios (AORs) and 95 % CIs for mortality were calculated by controlling for confounding variables that included age, and an Injury Severity Score (ISS) was calculated.

Results: More of the cyclists were under 19 years of age or over 70 than were the motorcyclists. In contrast, fewer bicyclists than motorcyclists wore helmets, arrived at the emergency department between 11 p.m. and 7 a.m., and had a positive blood alcohol concentration test. The bicyclists sustained significantly higher rates of injuries to the extremities, while motorcyclists sustained significantly higher rates of injuries to the head and neck, face, and thorax. Compared to motorcyclists, the bicyclists had significantly lower ISSs and New Injury Severity Scores, shorter length hospital stays, and a smaller proportion of admittance into the ICU. However, the bicyclists had higher AORs for in-hospital mortality (AOR: 1.2, 95 % CI: 1.16–1.20). In terms of critical injury severity (ISS ≥ 25), the bicyclists had 4.4 times (95 % CI: 1.95–9.82) the odds of mortality than motorcyclists with the same ISSs.

Conclusions: Data analysis indicated that the bicyclists had unique injury characteristics including bodily injury patterns and lower ISSs, but had higher in-hospital mortality compared to motorcycle riders. In this study, given that only 9 % of bicyclists reported wearing helmets and considering the high mortality associated with head injury, it is possible that some bicycle riders underestimated the gravity of cycling accidents.

Keywords: Bicyclist, Motorcyclist, Helmet, Head injury, Injury severity score, Mortality, Trauma registry system

Background

Bicycles are a popular form of transport and recreation worldwide. However, cyclists are likely to suffer more severe consequences of road accidents than users of motorized vehicles. Road accidents involving cyclists have widely varying consequences, ranging from minor abrasions to fractures and death. One nationally representative study found that bicyclists had 2.3 times as many fatalities and 1.8 times as many nonfatal injuries as motor vehicle occupants per 100 million person-trips [1]. It is estimated that for every two million trips, 600 injuries will occur and one bicyclist will die in a crash [2]. Approximately a third of the injured cyclists required hospitalization [3, 4]. In the United States, there were more than 25,000 bicycling-related hospitalizations annually between 2002 and 2009 [5]. These hospitalizations accounted for a national estimate of one billion dollars in total hospital charges per year [5].

* Correspondence: m93chinghua@gmail.com
†Equal contributors
[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan
Full list of author information is available at the end of the article

To improve the safety of cyclists, multiple governing bodies have been involved in planning bicycle-friendly urban spaces, designing traffic management solutions at intersections and bicycle roundabouts, and maintaining cycle paths [6]. The investment in infrastructure development significantly improved the safety of cyclists and lead to a steady decrease in the number of bicycle fatalities and road fatalities in general [6]. However, because of the growing popularity of bicycle transportation and the rising number of cyclists, a high incidence of bicycle-related injuries is still observed [2]. The identification of high-risk injury patterns and a greater understanding of major trauma epidemiology are vital in order to maximize the provision of services and the quality of care delivered [7, 8]. In addition, compared to the many studies that have focused on accident and injury rates, relatively few studies have focused on cyclist injury severity. It was previously reported that the median Injury Severity Score (ISS) was four (range 1–41) for individuals with bicycle-related injuries who required hospitalization in the United Arab Emirates [9]. At one university hospital in Japan, the average ISS was 23.9 for 115 bicyclists who died as a consequence of traffic accidents [10]. When speeding was involved in bicycle-motor vehicle collisions, the probability of a fatal injury increased by 300 % [11]. Given that most bicycle accidents occur on relatively crowded streets in Asian cities, bicycle-related injuries occur at relatively low velocities, similar to most motorcycle-related injuries, which represent a major proportion of traffic accident-related hospital admissions in South Taiwan [12]. Therefore, the aim of this study was to investigate differences in injury severity and mortality between patients who sustained bicycle or motorcycle accidents and were hospitalized at a Level I trauma center in Taiwan using data from a population-based trauma registry.

Methods

Ethics statement

This study was pre-approved by the Institutional Review Board (IRB) of the hospital (approval number 103-4599B). Informed consent was waived according to IRB regulations.

Study design

Data

In this retrospective study, all data added to the Trauma Registry System of a 2,400-bed Level I regional trauma center, which provided care to trauma patients who were primarily from South Taiwan, were reviewed. Cases involving hospitalization for trauma sustained in motorcycle accidents between January 1, 2009 and December 31, 2013 were selected. Among the 16,548 hospitalized and registered patients who were entered into the database, 669 (4.0 %) were bicyclists (which included 657 bicyclists and 12 moped riders and accounted for the legal speed limit of < 25 km/h in Taiwan) and 7,300 (44.1 %) were motorcyclists (which included motorcycle, motorized tricycle, and all-terrain vehicle riders).

Study variables

Detailed patient information was obtained from our institutional Trauma Registry System, which included patient age, sex, arrival time, mode of transportation, vital signs upon admission, collision manner, and helmet use. A blood alcohol concentration (BAC) of 50 mg/dL, the legal limit for drivers in Taiwan, was defined as the threshold. Other data collected included the first emergency department Glasgow Coma Scale (GCS) assessment, details of the emergency procedures performed (i.e. cardiopulmonary resuscitation, intubation, chest tube insertion, and blood transfusion), an Abbreviated Injury Scale (AIS) for each body region, the Injury Severity Score (ISS), New Injury Severity Score (NISS), Trauma Injury Severity Score (TRISS), length of hospital stay (LOS), LOS in the intensive care unit (ICU), in-hospital mortality, and associated complications. Stratified ISS data were compared to identify differences in injury severity using clinically relevant ISS cut-offs: ≥ 16 for severe and ≥ 25 for critical injuries. In our study, the primary outcome was injury severity as measured by various scoring systems (GCS, AIS, ISS, NISS, and TRISS) and in-hospital mortality. The secondary outcomes were the associated complications, and hospital and ICU LOS.

Exploratory analysis

The data collected were analyzed using the SPSS v.20 statistical software (IBM, Armonk, NY). Chi-square tests were used to determine the significance of associations between the predictor and outcome variables among the categorical variables. Student t-tests were used evaluate the significance of associations between the predictor and outcome variables among the continuous variables. Univariate logistic regression analyses were initially performed to identify the significant predictor variables of the injury or mortality risk of bicyclists. The corresponding unadjusted odds ratios (ORs) with 95 % confidence intervals (CIs) for each variable were obtained.

Regression analysis

The adjusted odds ratios (AORs) and 95 % CIs for mortality were estimated through stepwise model selection of a multiple regression model that was adjusted by controlling the cofounding variables of age and ISS. All of the results are presented as the mean ± standard error. A p-value < 0.05 was considered statistically significant.

Results

Patient characteristics

As shown in Table 1, the mean patient ages were 50.7 ± 24.8 and 42.3 ± 19.0 years in the bicyclist and motorcyclist groups, respectively. Among the bicyclists, a greater number of patients were aged 0–9, 10–19, 70–79, 80–89, and ≥90 years, and fewer patients were aged 20–29, 30–39, and 40–49 years. More of the bicyclists than motorcyclists were children, teenagers, or elderly. No statistically significant difference regarding sex was identified between bicyclists and motorcyclists. The helmet-wearing status was recorded as 99.6 % and 97.7 % for bicyclists and motorcyclists, respectively; however, at the time of injury, significantly fewer bicyclists were wearing helmets than motorcyclists (9.0 % vs. 84.7 %, respectively; $p < 0.001$). Most of the bicyclists and motorcyclists arrived at the emergency department between 7 a.m. and 5 p.m., while more of the motorcyclists arrived between 11 p.m. and 7 a.m. ($p < 0.001$). In comparison to motorcyclists, more injured bicyclists were transported to the hospital in a private vehicle (OR: 2.2, 95 % CI: 1.85–2.64; $p < 0.001$) and less injured bicyclists were transported by emergency medical services (OR: 0.6, 95 % CI: 0.48–0.67; $p < 0.001$). More bicycle accidents occurred as a result of riders losing control (including sliding and turn-over), while more motorcycle accidents involved collisions with cars, buses, trucks, or obstacles (such as a wall, tree, pillar, or pedestrian). A positive BAC test result was less frequent among bicyclists than among motorcyclists (4.2 % vs. 9.6 %, respectively; $p < 0.001$).

Table 1 Demographics of bicyclists hospitalized due to trauma

Variables	Bicycle *n* = 669	Motor *n* = 7300	*Odds ratio (95 % CI)*	*p*
Age	50.7±24.8	42.3±19.0	-	<0.001
Age category				
0-9years	40(6.0)	75(1.0)	6.1(4.14-9.07)	<0.001
10-19years	106(15.8)	823(11.3)	1.5(1.19-1.85)	<0.001
20-29 years	16(2.4)	1575(21.6)	0.1(0.05-0.15)	<0.001
30-39 years	32(4.8)	947(13.0)	0.3(0.24-0.48)	<0.001
40-49 years	65(9.7)	1015(13.9)	0.7(0.51-0.87)	0.002
50-59 years	108(16.1)	1268(17.4)	0.9(0.74-1.14)	0.422
60-69 years	107(16.0)	980(13.4)	1.2(0.99-1.53)	0.064
70-79yeas	135(20.2)	503(6.9)	3.4(2.77-4.21)	<0.001
80-89years	56(8.4)	106(1.5)	6.2(4.44-8.66)	<0.001
≧90 years	4(0.6)	8(0.1)	5.5(1.65-18.26)	0.002
Gender				0.141
Male	410(61.3)	4260(58.4)	1.1(0.96-1.33)	–
Female	259(38.7)	3040(41.6)	0.9(0.75-1.04)	–
Helmet				
Yes	60(9.0)	6185(84.7)	0.02(0.01-0.02)	<0.001
No	606(90.6)	946(13.0)	64.6(49.41-84.49)	<0.001
Unknown	3(0.4)	169(2.3)	0.2(0.06-0.60)	0.001
Time				
7:00–17:00	194(29.0)	2145(29.4)	1.0(0.82-1.17)	0.834
17:00–23:00	383(57.2)	3909(53.5)	1.2(0.99-1.36)	0.066
23:00–7:00	92(13.8)	1239(17.0)	0.8(0.62-0.98)	0.033
Unknown	0(0.0)	7(0.1)	-	0.423
Transportation				
Private vehicle	202(30.2)	1195(16.4)	2.2(1.85-2.64)	<0.001
EMS	235(35.1)	3575(49.0)	0.6(0.48-0.67)	<0.001
Transferred	232(34.7)	2530(34.7)	1.0(0.85-1.18)	0.991
Mechanism, hit with				
Bicycle	11(1.6)	75(1.0)	1.6(0.85-3.05)	0.139
Motorcycle	144(21.5)	1663(22.8)	0.9(0.77-1.13)	0.458
Car	89(13.3)	2346(32.1)	0.3(0.26-0.41)	<0.001
Bus or Truck	11(1.6)	372(5.1)	0.3(0.17-0.57)	<0.001
Out of control	387(57.8)	2105(28.8)	3.4(2.88-3.98)	<0.001
Obstacle	27(4.0)	739(10.1)	0.4(0.25-0.55)	<0.001
BAC > 50 mg/dL, n(%)	28(4.2)	700(9.6)	0.4(0.28-0.61)	<0.001

We found a significant difference in the GCS between bicyclists and motorcyclists (14.3 ± 2.2 vs. 14.2 ± 2.5, respectively; $p = 0.045$) (Table 2), as well as in the distribution of scores among patients with a GCS ≥ 13. However, the difference in GCS between bicyclists and motorcyclists was less than one point. In contrast, there was no difference in the proportion of patients with a GCS of either ≤ 8 or 9–12 between bicyclists and motorcyclists. Our analysis of the AIS scores revealed that bicyclists sustained significantly higher rates of injury to the extremities, while motorcyclists sustained significantly higher rates of injury to the head or neck, face, and thorax. The comparison of injury scores between the bicyclists and motorcyclists indicated significant differences in the ISS (8.7 ± 7.1 vs. 9.6 ± 7.7, respectively; $p < 0.001$). When stratified by injury severity (ISS < 16, 16–24, or ≥ 25), more bicyclists had an ISS of less than 16 compared to motorcyclists (86.7 % vs. 82.0 %, respectively; $p = 0.002$), while more motorcyclists had an ISS of 16–24, compared to bicyclists (12.5 % vs. 9.4 %, respectively; $p = 0.021$). There was no significant difference in these two groups of patients with an ISS of ≥ 25. We also found significant differences between bicyclists and motorcyclists regarding the NISS (10.1 ± 9.1 vs. 11.2 ± 9.3, respectively; $p < 0.001$), TRISS (0.949 ± 0.137 vs. 0.960 ± 0.112, respectively; $p = 0.033$), and in-hospital mortality rates (2.8 % vs. 1.7 %, respectively; $p = 0.030$). Among patients with critical injuries (ISS ≥ 25), bicyclists had a higher OR (OR: 4.4, 95 % CI: 1.95–9.82; $p < 0.001$) of mortality than motorcyclists. However, no difference was found between the injured cyclists and motorcyclists

Table 2 Injury severity and mortality of bicyclists hospitalized due to trauma

Variables	Bicycle	Motor	*Odds ratio*	*p*
	n = 669	*n* = 7300	*(95 % CI)*	
GCS	14.3±2.2	14.2±2.5	-	0.045
n (%)				
≤ 8	30(4.5)	435(6.0)	0.7(0.51-1.08)	0.119
9-12	23(3.4)	324(4.4)	0.8(0.50-1.18)	0.225
≥ 13	616(92.1)	6541(89.6)	1.3(1.01-1.80)	0.043
AIS, n (%)				
Head/Neck	189(28.3)	2411(33.0)	0.8(0.67-0.95)	0.012
Face	96(14.3)	1834(25.1)	0.5(0.40-0.62)	<0.001
Thorax	60(9.0)	1212(16.6)	0.5(0.38-0.65)	<0.001
Abdomen	31(4.6)	577(7.9)	0.6(0.39-0.82)	0.002
Extremity	492(73.5)	5274(72.2)	1.1(0.89-1.28)	0.473
ISS	8.7±7.1	9.6±7.7	-	<0.001
>16	580(86.7)	5986(82.0)	1.4(1.14-1.80)	0.002
16-24	63(9.4)	910(12.5)	0.7(0.56-0.96)	0.021
≧25	26(3.9)	404(5.5)	0.7(0.46-1.03)	0.071
NISS	10.1±9.1	11.2±9.3	-	<0.001
TRISS	0.949±0.137	0.960±0.112	-	0.033
Mortality, n (%)	19(2.8)	123(1.7)	1.7(1.05-2.78)	0.031
>16, n	2	9	2.3(0.50-10.67)	0.274
16-24, n	3	29	1.5(0.45-5.13)	0.498
≧25, n	14	85	4.4(1.95-9.82)	<0.001
AOR			1.2(1.16-1.20)	<0.001

with ISSs of < 16 and from 16 to 24. After adjusting for age and ISS, we found that bicyclists had a significantly higher AOR for patient mortality than did motorcyclists (AOR: 1.2, 95 % CI: 1.16–1.20; p < 0.001), indicating that the differences in injury severity between bicyclists and motorcyclists were not entirely responsible for their distinct mortality rates.

The findings regarding the types of injuries associated with bicycle accidents are shown in Table 3. Bicyclists had a higher OR for sustained humeral fracture (OR: 2.2, 95 % CI: 1.65–2.80; p < 0.001) as well as radial (OR: 1.4, 95 % CI: 1.15–1.83; $p = 0.002$), ulnar (OR: 1.7, 95 % CI: 1.25–2.25; $p = 0.001$), and femoral fractures (OR: 1.6, 95 % CI: 1.32–2.02; $p < 0.001$). However, compared to motorcyclists, bicyclists had a significantly lower percentage of cranial, orbital, maxillary, mandibular, rib, clavicle, metacarpal, pelvic, tibial, and metatarsal fractures, as well as epidural hemorrhage, subarachnoid hemorrhage (SAH), hemothorax, hepatic injury, and splenic injury. A significantly shorter hospital LOS was found for bicyclists compared to motorcyclists (7.8 days vs. 9.8 days, respectively; $p = 0.001$) (Table 4). Moreover, a significantly smaller proportion of bicyclists than motorcyclists was admitted to the ICU (15.7 % vs. 19.1 %, respectively; $p = 0.033$). No differences were noted in the proportion of bicyclists and motorcyclists who were admitted into the ICU, or in the LOS in the ICU after the stratification into either group based on injury severity (ISS of < 16, 16–24, or ≥ 25).

The major injuries associated with mortality are listed in Table 5, and the data revealed that the bicyclists were significantly older (50.7 ± 24.8 and 42.3 ± 19.0 years, respectively; $p = 0.001$) and were more likely to ride without a helmet (89.5 % vs. 26.8 %, respectively; $p = 0.001$). There was no significant difference between the bicyclists and motorcyclists in terms of the collision mechanisms of accidents responsible for mortality. Of the 19 bicyclists and 123 motorcyclists who died, the bicyclists did not have higher odds for major injuries than the motorcyclists; however, they did have greater odds for sustaining SAH (OR: 2.8, 95 % CI: 1.00–7.76; $p = 0.046$).

Discussion

Age distribution and injury region of the bicyclists

It was previously reported that older bicyclists were at a significantly higher risk for sustaining fractures to the upper extremities [13]. In addition, thoracic and abdominal injuries were rare among younger cyclists, except for in cases of isolated ruptures of the spleen or liver by bicycle handlebars [14]. In a study of 12,429 hospital admissions that resulted from bicycle-related injuries involving motor vehicles, three out of every 100 patients presented with a splenic injury, and two out of every 100 patients sustained a liver injury [13]. The analysis of AIS scores in this study revealed that bicyclists sustained significantly higher rates of injuries to the extremities, while motorcyclists sustained significantly higher rates of injuries to the head or neck, face, and thorax. Bicyclists had a higher OR for sustained humeral fracture (OR: 2.2, 95 % CI: 1.65–2.80), radial fracture (OR: 1.4, 95 % CI: 1.15–1.83), ulnar fracture (OR: 1.7, 95 % CI: 1.25–2.25), and femoral fracture (OR: 1.6, 95 % CI: 1.32–2.02) than motorcyclists. In contrast, the ORs of hepatic injury (OR: 0.4, 95 % CI: 0.23–0.88) and splenic injury (OR: 0.3, 95 % CI: 0.09–0.86) were significantly lower for bicyclists than for motorcyclists. In the present study, more cyclists were under 19 years of age or over 70 compared to motorcyclists. Cyclists who were greater than 60 years of age accounted for 45.2 % of all injured cyclists. In contrast, 21.9 % of all injured motorcyclists were > 60 years of age. In Taiwan, the rate of hip fracture is among the highest in the world, and the age-specific incidence rate of hip fracture has been found to increase exponentially with age for both sexes, after the age of 65 [15]. The larger number of elderly patients may, in part, explain the higher odds of extremity fractures for injured cyclists than that for motorcyclists.

Table 3 Associated injuries of bicyclists hospitalized due to trauma

Variables	Bicycle	Motor	*Odds ratio*	*p*
	n = 669	*n* = 7300	*(95 % CI)*	
Head/Neck trauma, n(%)				
Neurologic deficit	3(0.4)	66(0.9)	0.5(0.16-1.58)	0.223
Cranial fracture	36(5.4)	616(8.4)	0.6(0.44-0.87)	0.006
Epidural hematoma (EDH)	22(3.3)	382(5.2)	0.6(0.40-0.95)	0.028
Subdural hematoma (SDH)	65(9.7)	763(10.5)	0.9(0.71-1.20)	0.550
Subarachnoid hemorrhage (SAH)	63(9.4)	885(12.1)	0.8(0.58-0.99)	0.039
Intracerebral hematoma (ICH)	16(2.4)	182(2.5)	1.0(0.57-1.61)	0.872
Cerebral contusion	42(6.3)	441(6.0)	1.0(0.75-1.45)	0.806
Cervical vertebral fracture	9(1.3)	65(0.9)	1.5(0.75-3.06)	0.240
Maxillofacial trauma, n(%)				
Orbital fracture	6(0.9)	206(2.8)	0.3(0.14-0.70)	0.003
Maxillary fracture	29(4.3)	715(9.8)	0.4(0.29-0.61)	<0.001
Mandibular fracture	12(1.8)	269(3.7)	0.5(0.27-0.86)	0.011
Nasal fracture	5(0.7)	110(1.5)	0.5(0.20-1.21)	0.115
Rib fracture	41(6.1)	863(11.8)	0.5(0.35-0.67)	<0.001
Sternal fracture	0(0.0)	15(0.2)	-	0.241
Hemothorax	7(1.0)	159(2.2)	0.5(0.22-1.01)	0.050
Pneumothorax	8(1.2)	166(2.3)	0.5(0.26-1.06)	0.068
Hemopneumothorax	9(1.3)	129(1.8)	0.8(0.38-1.50)	0.423
Lung contusion	6(0.9)	119(1.6)	0.5(0.24-1.25)	0.144
Thoracic vertebral fracture	2(0.3)	54(0.7)	0.4(0.10-1.65)	0.191
Abdominal trauma, n(%)				
Intra-abdominal injury	7(1.0)	134(1.8)	0.6(0.26-1.21)	0.138
Hepatic injury	9(1.3)	216(3.0)	0.4(0.23-0.88)	0.016
Splenic injury	3(0.4)	119(1.6)	0.3(0.09-0.86)	0.017
Retroperitoneal injury	1(0.1)	15(0.2)	0.7(0.10-5.51)	0.757
Renal injury	4(0.6)	52(0.7)	0.8(0.30-2.33)	0.735
Urinary bladder injury	0(0.0)	18(0.2)	-	0.199
Lumbar vertebral fracture	5(0.7)	86(1.2)	0.6(0.26-1.56)	0.316
Sacral vertebral fracture	1(0.1)	45(0.6)	0.2(0.03-1.75)	0.127
Scrotum injury	0(0.0)	15(0.2)	-	0.241
Extremity trauma, n(%)				
Scapular fracture	12(1.8)	173(2.4)	0.8(0.42-1.36)	0.344
Clavicle fracture	50(7.5)	945(12.9)	0.5(0.40-0.73)	<0.001
Humeral fracture	73(10.9)	393(5.4)	2.2(1.65-2.80)	<0.001
Radial fracture	94(14.1)	740(10.1)	1.4(1.15-1.83)	0.002
Ulnar fracture	55(8.2)	370(5.1)	1.7(1.25-2.25)	0.001
Metacarpal fracture	7(1.0)	266(3.6)	0.3(0.13-0.60)	<0.001
Pelvic fracture	10(1.5)	275(3.8)	0.4(0.21-0.73)	0.002
Femoral fracture	113(16.9)	809(11.1)	1.6(1.32-2.02)	<0.001

Table 3 Associated injuries of bicyclists hospitalized due to trauma *(Continued)*

Patella fracture	14(2.1)	207(2.8)	0.7(0.42-1.27)	0.263
Tibia fracture	44(6.6)	778(10.7)	0.6(0.43-0.81)	0.001
Fibular fracture	27(4.0)	420(5.8)	0.7(0.46-1.03)	0.065
Calcaneal fracture	29(4.3)	396(5.4)	0.8(0.54-1.16)	0.230
Metatarsal fracture	7(1.0)	201(2.8)	0.4(0.18-0.80)	0.008

The incidence pattern of bicycle accidents

Prior studies have shown that motor vehicle collisions with cyclists have resulted in an increase in the overall severity of injury [16] and a 4.6-fold increase in the odds of serious injury [17] compared to non-collisions. Heavy vehicles such as trucks (to a large extent) and buses (to a lesser extent) have been associated with higher cyclist injury severity [17]. A recent study of trauma hospitalizations revealed that bicycle crashes involving motor vehicles resulted in a 10-fold greater risk of death in hospital for adults (95 % CI: 1.8–34.3) and a eight-fold greater risk for children under 17 years of age (95 % CI: 1.2–85.3) [18]. In this study, loss of control was the main cause of bicycle injuries and accounted for 57.8 % of all patients with bicycle-related injuries. Collision with a motorcycle (21.5 %) was the second most common cause of injury. While 1.6 % of accidents involved buses or trucks, an additional 13.3 % of accidents involved cars. The incidence of bicycle accidents involving motor vehicle collisions was markedly lower than in prior reports, potentially reflecting a distinct epidemiology of bicycle accidents in a relatively crowded city and resulting in bicyclists who presented with differences in injury severity.

Head injury of the bicyclists

The main cause of death and moderate disability after bicycle-related incidents was head injury [19, 20]. The overall incidence of head injury was 28.3 %, starting at 29.9 % in the pediatric group and increasing to 38.6 % in the elderly population [13]. Furthermore, the nature of the intracranial injuries differed significantly between the various age groups. Although the incidence of epidural hematomas was similar across age strata, the incidence of other intracranial injuries such as subdural hematoma and SAH was found to increase proportionally with age [13]. In this study, head or neck injury was noted in 189 of 669 (28.3 %) bicyclists who were admitted to the hospital, a result that is comparable to a study in which approximately a third of the 1,859 patients who were hospitalized with bicycle-related injured had one or more head injuries [3]. In this study, the bicyclists who suffered fatal injuries were significantly older and neglected to wear a helmet than those motorcyclists. Notably, among the fatal cases, although bicyclists had a significantly lower percentage of SAH than motorcyclists, they had greater odds for sustaining SAH.

Table 4 Length of stay (LOS) in the hospital and intensive care unit (ICU) of bicyclists hospitalized due to trauma

Variables	ISS	Bicycle	Motor	*Odds ratio*	*p*
		n = 669	*n* = 7300	*(95 % CI)*	
Hospital LOS		7.8±9.3	9.8±10.4	-	<0.001
ICU LOS		105(15.7)	1391(19.1)	0.8(0.64-0.98)	0.033
n (%)	<16	40(6.9)	472(7.9)	0.9(0.62-1.21)	0.397
	16-24	42(66.7)	574(63.1)	1.2(0.68-2.01)	0.568
	≥25	23(88.5)	345(85.4)	1.3(0.38-4.51)	0.666
days		7.2±8.8	7.3±8.8	-	0.907
	<16	4.9±5.0	5.2±8.8	-	0.406
	16-24	7.3±7.7	6.8±5.9	-	0.244
	≥25	11.2±13.2	10.9±6..8	-	0.942

Helmet use and the mortality of the bicyclists

Among the various preventive measures, wearing a helmet in particular has been shown to protect against head injuries in both groups of riders [21, 22]. The odds of sustaining a head injury increased 1.98–3.89 times for cyclists who did not wear a helmet [3]. In addition, compared to cyclists who did not wear helmets, helmeted cyclists were less likely to sustain serious bodily injuries other than to the head, less likely to disobey a traffic light, less likely to have a BAC over 0.05 mg/dL, and more likely to be riding during the day [3]. A case-controlled study demonstrated that wearing a helmet reduced the risk of head injury by 63 % and the risk of loss of consciousness by 86 % among children [23]. Moreover, the average number of deaths per year decreased by 52 % after the institution of a mandatory helmet law [24]. The present study revealed a very low rate (9 %) of helmet use among South Taiwanese cyclists in comparison to cyclists in Germany (12 %–15 %) [25, 26], Finland (13 %) [4], Canada (50 %) [27], the United States (54 %) [28], and the state of Victoria in Australia (75.4 %) [3], which in 1990 became one of the first regions worldwide to introduce mandatory helmet legislation for cyclists on public roadways. In Taiwan, helmet use is not mandatory and is only required for competitive cyclists. Although the Taiwanese Government has invested in infrastructure and has conducted health programs to promote bicycle safety, there are no

Table 5 Demographics and associated injuries of bicycle-related fatalities

Variables	Bicycle	Motor	*Odds ratio*	*p*
	n = 19	*n* = 123	*(95 % CI)*	
Age	67.4±13.7	51.2±19.0	-	0.001
Gender				0.294
Male	11(57.9)	86(69.9)	0.6(0.22-1.59)	
Female	8(42.1)	37(30.1)	1.7(0.63-4.54)	
Helmet				
Yes	2(10.5)	62(50.4)	0.1(0.03-0.52)	0.001
No	17(89.5)	33(26.8)	23.2(5.08-105.83)	<0.001
Unknown	0(0.0)	28(22.8)	-	0.020
Mechanism				
Bicycle	0(0.0)	1(0.8)	-	0.693
Motorcycle	4(21.1)	17(13.8)	1.7(0.49-5.61)	0.409
Car	6(31.6)	42(34.1)	0.9(0.32-2.51)	0.826
Bus or Truck	2(10.5)	12(9.8)	1.1(0.22-5.29)	0.917
Out of control	3(15.8)	29(23.6)	0.6(0.17-2.23)	0.450
Others	4(21.1)	22(17.9)	1.2(0.37-4.05)	0.740
Head/Neck trauma, n(%)				
Cranial fracture	9(47.4)	40(32.5)	1.9(0.70-4.96)	0.205
Epidural hematoma (EDH)	7(36.8)	33(26.8)	1.6(0.58-4.39)	0.367
Subdural hematoma (SDH)	12(63.2)	67(54.5)	1.4(0.53-3.89)	0.478
Subarachnoid hemorrhage (SAH)	13(68.4)	54(43.9)	2.8(1.00-7.76)	0.046
Intracerebral hematoma (ICH)	4(21.1)	15(12.2)	1.9(0.56-6.56)	0.291
Cerebral contusion	8(42.1)	29(23.6)	2.4(0.87-6.42)	0.087
Cervical vertebral fracture	0(0.0)	5(4.1)	-	0.371
Thoracic trauma, n(%)				
Rib fracture	0(0.0)	14(11.4)	-	0.121
Sternal fracture	0(0.0)	1(0.8)	-	0.693
Hemothorax	0(0.0)	11(8.9)	-	0.175
Pneumothorax	0(0.0)	8(6.5)	-	0.252
Hemopneumothorax	0(0.0)	9(7.3)	-	0.223
Lung contusion	0(0.0)	16(13.0)	-	0.095
Thoracic vertebral fracture	0(0.0)	1(0.8)	-	0.693
Abdominal trauma, n(%)				
Intra-abdominal injury	2(10.5)	8(6.5)	1.7(0.33-8.64)	0.524
Hepatic injury	1(5.3)	15(12.2)	0.4(0.05-3.22)	0.374
Splenic injury	0(0.0)	8(6.5)	-	0.252
Retroperitoneal injury	0(0.0)	3(2.4)	-	0.491
Renal injury	0(0.0)	3(2.4)	-	0.491
Extremity trauma, n(%)				
Pelvic fracture	2(10.5)	8(6.5)	1.7(0.33-8.64)	0.524
Femoral fracture	2(10.5)	13(10.6)	1.0(0.21-4.80)	0.995

compulsory helmet laws for cyclists, contrary to the laws for motorcyclists. Indeed, it is possible that some bicycle riders underestimated the seriousness of cycling accidents. In this study, although bicyclists had significantly lower ISSs than motorcyclists, their mortality rate was higher. In addition, bicyclists with critical ISSs (ISS ≥ 25) had approximately four times the odds of mortality than motorcyclists with ISSs. Without adequate protection, riding a bicycle is more dangerous than riding a motorcycle if you are severely injured.

Limitations of the study

The limitations of this study include the retrospective design and the lack of available data regarding injury mechanisms and circumstances, including speed, helmet material, exposure data (e.g., number of trips, hours of riding, and/or miles traveled), and details regarding the accident location (e.g., infrastructure characteristics and land use, light and weather conditions, as well cyclist behavior and maneuvers). Additionally, the number of patients in the study was not adequate to analyze the association of age with different accident characteristics other than mortality. The relatively small number of hospitalized bicyclists precluded an in-depth examination of risk factors such as age, type of head injury, and injury severity. Finally, the injured patients who were pronounced dead at the scene of the accident or those who were discharged from the emergency department were not included in the sample, which may have introduced a survival bias.

Conclusions

This study indicates that bicyclists have unique injury characteristics including bodily injury patterns, as well as lower ISSs but higher in-hospital mortality when compared to motorcycle riders. In the study, because only 9 % of bicyclists reported wearing a helmet, and considering the high mortality associated with head injury, it is possible that some bicycle riders underestimated the gravity of cycling accidents.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HTL and CSR wrote and revised the manuscript, CCL and SCW contributed to the collection of data, SYH carried out the analysis and edited the tables, HYH prepared the English-editing, and CHH designed the study, contributed to the analysis and interpretation of data, and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was supported by a grant from CDRPG8C0031.

Author details

[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan. [2]Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung City, Taiwan. [3]Department of Anesthesiology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung City, Taiwan.

Received: 17 February 2015 Accepted: 20 July 2015
Published online: 29 July 2015

References

1. Beck LF, Dellinger AM, O'Neil ME. Motor vehicle crash injury rates by mode of travel, United States: using exposure-based methods to quantify differences. Am J Epidemiol. 2007;166(2):212–8.
2. Administration NHTS. Traffic Safety Facts: Bicyclists and Other Cyclists. Washington, DC: National Highway Traffic Safety Administration; 2007.
3. Bambach MR, Mitchell RJ, Grzebieta RH, Olivier J. The effectiveness of helmets in bicycle collisions with motor vehicles: a case–control study. Accid Anal Prev. 2013;53:78–88.
4. Airaksinen N, Luthje P, Nurmi-Luthje I. Cyclist Injuries Treated in Emergency Department (ED): Consequences and Costs in South-eastern Finland in an Area of 100 000 Inhabitants. Ann Adv Automot Med. 2010;54:267–74.
5. Hamann C, Peek-Asa C, Lynch CF, Ramirez M, Torner J. Burden of hospitalizations for bicycling injuries by motor vehicle involvement: United States, 2002 to 2009. The J Trauma and Acute Care Surgery. 2013;75(5):870–6.
6. Kaplan S, Vavatsoulas K, Prato CG. Aggravating and mitigating factors associated with cyclist injury severity in Denmark. J Safety Res. 2014;50:75–82.
7. Densmore JC, Lim HJ, Oldham KT, Guice KS. Outcomes and delivery of care in pediatric injury. J Pediatr Surg. 2006;41(1):92–8. discussion 92–98.
8. Rogers SC, Campbell BT, Saleheen H, Borrup K, Lapidus G. Using trauma registry data to guide injury prevention program activities. J Trauma. 2010;69 (4 Suppl):S209–13.
9. Hefny AF, Eid HO, Grivna M, Abu-Zidan FM. Bicycle-related injuries requiring hospitalization in the United Arab Emirates. Injury. 2012;43(9):1547–50.
10. Gomei S, Hitosugi M, Ikegami K, Tokudome S. Assessing injury severity in bicyclists involved in traffic accidents to more effectively prevent fatal bicycle injuries in Japan. Med Sci Law. 2013;53(4):194–8.
11. Kim JK, Kim S, Ulfarsson GF, Porrello LA. Bicyclist injury severities in bicycle-motor vehicle accidents. Accid Anal Prev. 2007;39(2):238–51.
12. Rau CS, Liu HT, Hsu SY, Cho TY, Hsieh CH. Alcohol-related hospitalisations of trauma patients in Southern Taiwan: a cross-sectional study based on a trauma registry system. BMJ Open. 2014;4(10), e005947.
13. Lustenberger T, Inaba K, Talving P, Barmparas G, Schnuriger B, Green D, et al. Bicyclists injured by automobiles: relationship of age to injury type and severity–a national trauma databank analysis. J Trauma. 2010;69(5):1120–5.
14. Erez I, Lazar L, Gutermacher M, Katz S. Abdominal injuries caused by bicycle handlebars. Eur J Surg. 2001;167(5):331–3.
15. Chie WC, Yang RS, Liu JP, Tsai KS. High incidence rate of hip fracture in Taiwan: estimated from a nationwide health insurance database. Osteoporos Int. 2004;15(12):998–1002.
16. Heng KW, Lee AH, Zhu S, Tham KY, Seow E. Helmet use and bicycle-related trauma in patients presenting to an acute hospital in Singapore. Singapore Med J. 2006;47(5):367–72.
17. Rivara FP, Thompson DC, Thompson RS. Epidemiology of bicycle injuries and risk factors for serious injury. Inj Prev. 1997;3(2):110–4.
18. Siman-Tov M, Jaffe DH, Peleg K. Bicycle injuries: a matter of mechanism and age. Accid Anal Prev. 2012;44(1):135–9.
19. Yeung JH, Leung CS, Poon WS, Cheung NK, Graham CA, Rainer TH. Bicycle related injuries presenting to a trauma centre in Hong Kong. Injury. 2009;40(5):555–9.
20. Sikic M, Mikocka-Walus AA, Gabbe BJ, McDermott FT, Cameron PA. Bicycling injuries and mortality in Victoria, 2001–2006. Med J Aust. 2009;190(7):353–6.
21. Elvik R. Publication bias and time-trend bias in meta-analysis of bicycle helmet efficacy: a re-analysis of Attewell, Glase and McFadden, 2001. Accid Anal Prev. 2011;43(3):1245–51.
22. Amoros E, Chiron M, Martin JL, Thelot B, Laumon B. Bicycle helmet wearing and the risk of head, face, and neck injury: a French case–control study based on a road trauma registry. Inj Prev. 2012;18(1):27–32.
23. Priest P. Effectiveness of cycle helmets. Remains debatable. BMJ (Clinical research ed). 1994;308(6937):1164–5.

24. Wesson DE, Stephens D, Lam K, Parsons D, Spence L, Parkin PC. Trends in pediatric and adult bicycling deaths before and after passage of a bicycle helmet law. Pediatrics. 2008;122(3):605–10.
25. Ritter N, Vance C. The determinants of bicycle helmet use: evidence from Germany. Accid Anal Prev. 2011;43(1):95–100.
26. Orsi C, Ferraro OE, Montomoli C, Otte D, Morandi A. Alcohol consumption, helmet use and head trauma in cycling collisions in Germany. Accid Anal Prev. 2014;65:97–104.
27. Page JL, Macpherson AK, Middaugh-Bonney T, Tator CH. Prevalence of helmet use by users of bicycles, push scooters, inline skates and skateboards in Toronto and the surrounding area in the absence of comprehensive legislation: an observational study. Inj Prev. 2012;18(2):94–7.
28. Fischer CM, Sanchez CE, Pittman M, Milzman D, Volz KA, Huang H, et al. Prevalence of bicycle helmet use by users of public bikeshare programs. Ann Emerg Med. 2012;60(2):228–31.

Liang et al. BMC Pediatrics (2015) 15:105
DOI 10.1186/s12887-015-0419-3

BMC Pediatrics

RESEARCH ARTICLE Open Access

Motorcycle-related hospitalization of adolescents in a Level I trauma center in southern Taiwan: a cross-sectional study

Chi-Cheng Liang[1†], Hang-Tsung Liu[1†], Cheng-Shyuan Rau[2], Shiun-Yuan Hsu[1], Hsiao-Yun Hsieh[1] and Ching-Hua Hsieh[1*]

Abstract

Background: The aim of this study was to investigate and compare the injury pattern, mechanisms, severity, and mortality of adolescents and adults hospitalized for treatment of trauma following motorcycle accidents in a Level I trauma center.

Methods: Detailed data regarding patients aged 13–19 years (adolescents) and aged 30–50 years (adults) who had sustained trauma due to a motorcycle accident were retrieved from the Trauma Registry System between January 1, 2009 and December 31, 2012. The Pearson's chi-squared test, Fisher's exact test, or the independent Student's t-test were performed to compare the adolescent and adult motorcyclists and to compare the motorcycle drivers and motorcycle pillion.

Results: Analysis of Abbreviated Injury Scale (AIS) scores revealed that the adolescent patients had sustained higher rates of facial, abdominal, and hepatic injury and of cranial, mandibular, and femoral fracture but lower rates of thorax and extremity injury; hemothorax; and rib, scapular, clavicle, and humeral fracture compared to the adults. No significant differences were found between the adolescents and adults regarding Injury Severity Score (ISS), New Injury Severity Score (NISS), Trauma-Injury Severity Score (TRISS), mortality, length of hospital stay, or intensive care unit (ICU) admission rate. A significantly greater percentage of adolescents compared to adults were found not to have worn a helmet. Motorcycle riders who had not worn a helmet were found to have a significantly lower first Glasgow Coma Scale (GCS) score, and a significantly higher percentage was found to present with unconscious status, head and neck injury, and cranial fracture compared to those who had worn a helmet.

Conclusion: Adolescent motorcycle riders comprise a major population of patients hospitalized for treatment of trauma. This population tends to present with a higher injury severity compared to other hospitalized trauma patients and a bodily injury pattern differing from that of adult motorcycle riders, indicating the need to emphasize use of protective equipment, especially helmets, to reduce their rate and severity of injury.

Keywords: Abbreviated injury scale, Adolescent, Glasgow coma scale, Injury severity score, New injury severity score, Motorcycle, Trauma, Trauma injury severity score

* Correspondence: m93chinghua@gmail.com
†Equal contributors
[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Sung District, Kaohsiung City 833, Taiwan
Full list of author information is available at the end of the article

Background

Road traffic accidents have been reported as the most common causes of blunt pediatric injuries [1, 2]. Among the various means of transportation, motorcycle use is becoming popular in many cities as a cheaper, easier, and more fuel-efficient means. However, the increased use of motorcycles for recreation, the availability of more powerful motorcycles, and a greater number of older riders has led to increased incidence of motorcycle fatalities and injuries [3]. Motorcycle drivers are 35 times more likely than pillion-car occupants (i.e., motorcycle riders) to die in a motor vehicle traffic crash, 8 times more likely to be injured per vehicle mile [4], and 58 times more likely to be killed on a per-trip basis [5]. Among motorcycle drivers, young motorcyclists have the highest fatality rates of any age group, perhaps owing to their inexperience, skill level, and risky riding behavior [6]. In the United States, the national population estimate for all motorcycle-related hospital discharges for patients aged 12–20 years in 2006 was 5,662, a figure that represented 3.0 % of all hospitalized injuries for this age group [7]. The Centers for Disease Control and Prevention (CDC) reported that the motorcyclist fatality rate for individuals aged 12–20 had increased from 0.52 deaths per 100,000 population in 1999 to 0.98 deaths per 100,000 population in 2006, an increase of 88 % [7].

Pediatric patients sustain distinct patterns of injuries from causes that differ from those of adults because of their unique anatomical, physiologic, and behavioral characteristics. Young motorcyclists are considered a high-risk traffic group [8, 9] because they are more likely to be at fault in the event of a collision due to being under the influence of alcohol, riding without insurance, or not wearing a helmet [9]. While a significant link has been found between risk perception and traffic condition awareness for experienced drivers (ages 25–28), it has not been found for younger drivers (ages 18–24) [10].

The figures regarding the number of major trauma patients and the subsequent volume of surgery performed for those aged 10–17 years have been reported to differ from those reported for younger patients [3]. The identification of high-risk injury patterns may lead to improved care and ultimately further improvements in outcome in children and adolescents admitted to the hospital with trauma [11, 12]. In addition, gaining greater understanding of the epidemiology of pediatric major trauma is vital to integrate the knowledge of pediatric trauma into the trauma system to maximize the provision of services and quality of care delivered. To assist in achieving these aims, this study investigated the injury pattern, mechanisms, severity, and mortality of adolescents treated for injuries sustained in motorcycle accidents in a Level I trauma center in southern Taiwan using data from a population-based trauma registry.

Methods

Study design

The study was conducted at Kaohsiung Chang Gung Memorial Hospital, a 2,400-bed facility and a Level I regional trauma center that provides care to trauma patients primarily from South Taiwan. Approval for this study was obtained by the hospital institutional review board (approval number 103-2186B) before its initiation. An informed consent was waived according to the regulation of IRB. This retrospective study was designed to review all the data added to the Trauma Registry System from January 1, 2009 to December 31, 2012 for selection of cases that met the inclusion criteria of (1) age 13–19 years (adolescents) or age 30–50 years (adults) and (2) hospitalization for treatment of trauma following a motorcycle accident. The lower age limit for adolescents was chosen because of the recent sharp increase in incidence observed for cases as young as age 13. Exclusion criteria included those patients with incomplete data. The aim of selection of this age group (age 13–19) rather than a younger or older group was to narrow the selected range of ages to avoid comparison with those just older than 20 years and to avoid the introduction of the possibly confounding factor of inability to control a motorcycle due to advanced age, a factor generally affecting those over 60 years. To compare the injury pattern, mechanisms, severity, and mortality of adolescents from those of adults hospitalized for treatment of trauma following motorcycle accidents, the data of patients who had sustained injuries in a motorcycle accident, including road and off-road motorcyclist accidents, were collected for further analysis.

Among the 13,233 hospitalized registered patients entered in the database, 1,033 (7.8 %) were adolescents ages from 13 to 19 years and 3,470 (26.2 %) adults between 30 and 50 years. Among them, 635 (61.5 %) adolescents and 1,566 (45.1 %) adults had been admitted due to a motorcycle accident. Detailed patient information was retrieved from the Trauma Registry System of our institution and included data regarding age, sex, admission vital signs, injury mechanism, helmet use, the first Glasgow Coma Scale (GCS) in the emergency department, Abbreviated Injury Scale (AIS) severity score of each body region, Injury Severity Score (ISS), New Injury Severity Score (NISS), Trauma-Injury Severity Score (TRISS), length of hospital stay (LOS), length of intensive care unit stay (LICUS), in-hospital mortality, and rates of associated complications. Odd ratios (ORs) of the associated injuries of adolescents and adults in the motorcycle accidents were calculated with 95 % confidence intervals (CIs). The data collected regarding the combined population of drivers and pillions (hereafter referred to as riders) were compared using SPSS v.20 statistical software (IBM, Armonk, NY, USA) for

performance of Pearson's chi-squared test, Fisher's exact test, or the independent Student's t-test, as applicable. All results are presented as the mean ± standard error. A p-value less than 0.05 was considered statistically significant.

Results

Characteristics of all trauma patients

The mean age was 16.9 ± 1.9 and 40.5 ± 6.1 years, respectively, in the adolescent and adult patient groups (Table 1). Of the 1,033 adolescents, 737 (71.3 %) were male and 296 (28.7 %), female. Of the 3,470 adult patients, 2,438 (70.3 %) were male and 1,032 (29.7 %) female. No statistically significant difference was found between the groups regarding sex. Among the injured patients, 2053 (547 [53.0 %] of the adolescents and 1506 [43.4 %] of the adults) were the drivers of motorcycles and only 148 (88 [8.5 %] of the adolescents and 60 [1.7 %] of the adults) were the riders.

Injury severity of all trauma patients

Comparison of trauma injury scores for the adolescent and adult groups did not indicate any significant difference regarding ISS (7.8 ± 7.0 vs. 7.7 ± 6.7, respectively, $p = 0.571$) for any subgroup of injury severity (ISS <16, 16–24, and ≥25) or regarding NISS (8.9 ± 7.8 vs. 8.7 ± 7.7, respectively, $p = 0.460$), TRISS (0.976 ± 0.099 vs. 0.979 ± 0.093, respectively, $p = 0.451$), or in-hospital mortality (0.39 % vs. 0.95 %, respectively, $p = 0.078$). In contrast, a significant difference in ISS was found between the 635 adolescent riders (ISS = 9.2 ± 7.6) and the other 398 adolescent non-motorcycle riders ($p < 0.001$).

Table 1 Demographics of hospitalized trauma patients aged 13–19 years (adolescents) and 30–50 years (adults)

Variable	Adolescent	Adult	*p*
	N = 1033	*N* = 3470	
Age	16.9 ± 1.9	40.5 ± 6.1	
Gender, n(%)			0.502
Male	737(71.3)	2438(70.3)	
Female	296(28.7)	1032(29.7)	
Mechanism, n(%)			
Drivers of MV	2(0.2)	96(2.8)	
Pillions of MV	14(1.4)	35(1.0)	
Drivers of Motorcycle	547(53.0)	1506(43.4)	
Pillions of Motorcycle	88(8.5)	60(1.7)	
Bicyclists	67(6.5)	89(2.6)	
Pedestrians	14(1.4)	44(1.3)	
Fall	140(13.6)	570(16.4)	
Unspecific	161(15.6)	1070(30.8)	
ISS	7.8 ± 7.0	7.7 ± 6.7	0.571
< 16	903(87.4)	3041(87.6)	0.850
16-24	89(8.6)	318(9.2)	0.590
≥ 25	41(4.0)	111(3.2)	0.229
NISS	8.9 ± 7.8	8.7 ± 7.7	0.460
TRISS	0.976 ± 0.099	0.979 ± 0.093	0.451
Mortality, n(%)	4(0.39)	33(0.95)	0.078

Characteristics of the motorcycle-related trauma patients

The data regarding the 635 (61.5 %) adolescent and 1566 (45.1 %) adult patients who had been motorcycle riders were further compared for identification of differences regarding motorcycle-related major trauma injury. As shown in Fig. 1, of the 75, 80, 97, 139, 154, 233, and 255 hospitalized patients aged 13, 14, 15, 16, 17, 18, and 19 years, respectively, 7 (9.3 %), 21 (26.3 %), 38 (39.2 %), 76 (54.7 %), 111 (72.1 %), 182 (78.1 %), and 200 (78.4 %) patients, respectively, had been admitted for treatment subsequent to a motorcycle accident. Among these adolescent motorcycle riders, 77.6 % ($n = 493$) were aged from 17 to 19 years. As shown in Table 2, of the 635 adolescent and 1566 adult motorcycle riders, the mean age was 17.5 ± 1.4 and 40.1 ± 6.2 years, respectively. No statistically significant difference was found regarding sex between the adolescent motorcycle riders, of whom 416 (65.5 %) were male and 219 (34.5 %) female, and the adult motorcycle riders, of whom 988 (63.1 %) were male and 578 (36.9 %) female. Analysis of the data regarding helmet-wearing status, which were recorded for 97.5 % of the adolescent and 97.3 % of the adult patients, revealed that significantly more adolescent motorcycle drivers had not been wearing a helmet compared to the adult motorcycle drivers (12.4 % vs. 10.0 %, respectively, $p = 0.012$). In contrast, no significant difference regarding helmet-wearing status was found between the adolescent and adult motorcycle pillions. In addition, 455 of the 534 adolescent drivers and 67 of the 85 adolescent pillions had worn a helmet ($p = 0.133$) and 1307 of the 1464 adult drivers and 53 of the 59 adult pillions had

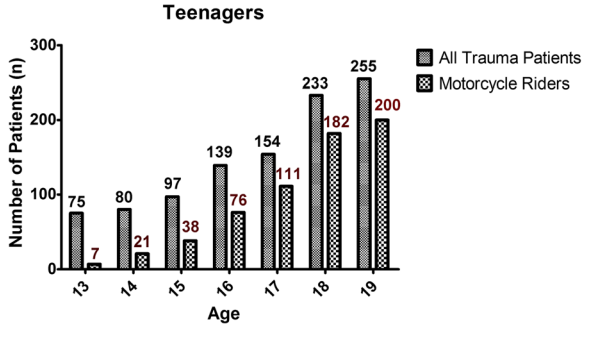

Fig. 1 Number of adolescent patients admitted for treatment of all trauma injury and number admitted for treatment of motorcycle-related trauma injury

Table 2 Injury characteristics of adolescent and adult motorcycle riders

Motorcycle Accident			
	Adolescent	Adult	*p*
	N = 635	*N* = 1566	
Age	17.5 ± 1.4	40.1 ± 6.2	
Gender, n(%)			0.284
Male	416(65.5)	988(63.1)	
Female	219(34.5)	578(36.9)	
Helmet wearing, n(%)			
Yes			
Drivers	455(71.7)	1307(83.5)	0.038
Pillions	67(10.6)	53(3.4)	0.063
No			
Drivers	79(12.4)	157(10.0)	0.012
Pillions	18(2.8)	6(0.4)	0.090
Unknown	16(2.5)	43(2.7)	0.766
GCS	14.2 ± 2.3	14.1 ± 2.6	0.457
≤ 8	30(4.7)	85(5.4)	0.502
9–12	37(5.8)	98(6.3)	0.702
≥ 13	568(89.4)	1383(88.3)	0.447
AIS n(%)			
Head/Neck	224(35.3)	521(33.3)	0.368
Face	194(30.6)	403(25.7)	0.021
Thorax	54(8.5)	250(16.0)	0.000
Abdomen	65(10.2)	111(7.1)	0.014
Extremity	442(69.6)	1183(75.5)	0.004
ISS	9.2 ± 7.6	9.2 ± 7.2	0.914
< 16	525(82.7)	1301(83.1)	0.821
16–24	79(12.4)	195(12.4)	0.994
≥ 25	31(4.9)	70(4.5)	0.6766
NISS	10.5 ± 8.4	10.5 ± 8.2	0.945
TRISS	0.971 ± 0.110	0.975 ± 0.095	0.386
Mortality, n(%)	3(0.5 %)	20(1.3 %)	0.093
LOS (days)	9.5 ± 9.5	9.1 ± 9.3	0.460
ICU			
Patients, n(%)	125(19.7)	262(16.7)	0.099
< 16	45(8.6)	91(7.0)	0.245
16–24	55(69.6)	114(58.5)	0.085
≥ 25	25(80.6)	57(81.4)	0.926
LOS in ICU (days)	6.4 ± 6.2	6.4 ± 6.3.	0.791
< 16	5.67.1	4.8 ± 5.2	0.207
16–24	6.6 ± 5.7	5.6 ± 4.7	0.061
≥ 25	7.3 ± 5.6	10.5 ± 8.7	0.068

worn a helmet ($p = 0.$ 0.893). No significant differences regarding helmet wearing was found between drivers and pillions in either group.

Injury severity of the motorcycle-related trauma patients

No significant difference was found between the adolescent and adult patients regarding GCS score (14.2 ± 2.3 vs.14.1 ± 2.6, respectively, $p = 0.457$) or distribution of patients at different levels of consciousness (GCS ≤8, 9–12, or ≥13; Table 3). Moreover, no significant differences in GCS score was found between the adolescent drivers ($n = 547$, 14.2 ± 2.4) and the adolescent pillions ($n = 88$, 14.1 ± 2.6; $p = 0.737$) or between the adult drivers ($n = 1506$, 14.1 ± 2.6) and the adult pillions ($n = 60$, 14.0 ± 3.0; $p = 0.788$). Likewise, no significant differences were found between the adolescent and adult motorcycle riders regarding ISS (9.2 ± 7.6 vs. 9.2 ± 7.2, respectively, $p = 0.914$) regardless of subgroup of injury severity; NISS (10.5 ± 8.4 vs. 10.5 ± 8.2, respectively, $p = 0.945$); TRISS (0.971 ± 0.110 vs. 0.975 ± 0.095, respectively, $p = 0.386$); or in-hospital mortality (0.5 % vs. 1.3 %, respectively, $p = 0.093$). Moreover, no significant differences regarding hospital LOS (9.5 days vs. 9.1 days, respectively, $p = 0.460$), proportion of patients admitted to the intensive care unit (ICU; 19.7 % vs. 16.7 %, respectively, $p = 0.099$), or LICUS (6.4 days vs. 6.4 days, respectively, $p = 0.791$), regardless of injury severity, were found between the adolescent and adult motorcycle riders.

Injury pattern of the motorcycle-related trauma patients

Analysis of AIS revealed that the adolescent patients had sustained significantly higher rates of facial injury (30.6 % vs. 25.7 %, respectively, $p = 0.021$) and abdominal injury (10.2 % vs. 7.1 %, respectively, $p = 0.014$) compared to the adult patients, while the adult patients had sustained significantly higher rates of thorax injury (16.0 % vs. 8.5 %, respectively, $p = 0.000$) and extremity injury (75.5 % vs. 69.6 %, respectively, $p = 0.004$). On the other hand, no significant differences regarding injury to the head and neck region were found between the adolescent and adult patients. Table 3 shows the findings regarding injury associated with motorcycle accidents. As can be observed, a significantly higher percentage of adolescent riders had sustained cranial fracture (OR = 1.6, 95 % CI = 1.20–2.10), mandibular fracture (OR = 2.3, 95 % CI = 1.55–3.49), hepatic injury (OR = 2.5, 95 % CI = 1.55–4.19), or femoral fracture (OR = 2.3, 95 % CI: 1.76–3.05) compared to adult riders. In contrast, a significantly lower percentage of adolescent motorcycle riders had sustained rib fracture (OR = 0.1, 95 % CI = 0.06–0.21), hemothorax (OR = 0.3, 95 % CI = 0.12–0.79), scapular fracture (OR = 0.4, 95 % CI = 0.16–0.80), clavicle fracture (OR = 0.3, 95 % CI = 0.23–0.46), and humeral fracture (OR = 0.5, 95 % CI = 0.32–0.88).

Table 3 Associated injuries of adolescent and adult motorcycle riders

Motorcycle accident				
	Adolescent $N = 635$	Adult $N = 1566$	*Odds Ratio (95%CI)*	*p*
Head trauma, n(%)				
Neurologic deficit	6(0.9)	20(1.3)	0.7(0.30–1.85)	0.513
Cranial fracture[a]	89(14.0)	146(9.3)	1.6(1.20–2.10)	0.001
Epidural hematoma (EDH)	13(2.0)	24(1.5)	1.3(0.68–2.65)	0.395
Subdural hematoma (SDH)	34(5.4)	79(5.0)	1.1(0.71–1.61)	0.766
Subarachnoid hemorrhage (SAH)	40(6.3)	134(8.6)	0.7(0.50–1.04)	0.075
Intracerebral hematoma (ICH)	12(1.9)	25(1.6)	1.2(0.59–2.38)	0.628
Cerebral contusion	29(4.6)	72(4.6)	1.0(0.64–1.54)	0.975
Cervical vertebral fracture	2(0.3)	14(0.9)	0.4(0.08–1.55)	0.147
Maxillofacial trauma, n(%)				
Maxillary fracture	65(10.2)	182(11.6)	0.9(0.64–1.17)	0.351
Mandibular fracture[a]	47(7.4)	52(3.3)	2.3(1.55–3.49)	0.000
Orbital fracture	28(4.4)	54(3.4)	1.3(0.81–2.06)	0.281
Nasal fracture	13(2.0)	29(1.9)	1.1(0.57–2.15)	0.761
Thoracic trauma, n(%)				
Rib fracture[+]	9(1.4)	183(11.7)	0.1(0.06–0.21)	0.000
Hemothorax[+]	5(0.8)	39(2.5)	0.3(0.12–0.79)	0.010
Pneumothorax	17(2.7)	30(1.9)	1.4(0.77–2.57)	0.263
Lung contusion	7(1.1)	24(1.5)	0.7(0.31–1.67)	0.438
Hemopneumothorax	9(1.4)	26(1.7)	0.9(0.40–1.83)	0.680
Thoracic vertebral fracture	1(0.2)	15(1.0)	0.2(0.02–1.24)	0.053
Abdominal trauma, n(%)				
Intra-abdominal injury	21(3.3)	33(2.1)	1.6(0.91–2.77)	0.099
Hepatic injury[a]	32(5.0)	32(2.0)	2.5(1.55–4.19)	0.000
Splenic injury	13(2.0)	29(1.9)	1.1(0.57–2.15)	0.761
Retroperitoneal injury	1(0.2)	4(0.3)	0.6(0.07–5.52)	0.662
Renal injury	6(0.9)	7(0.4)	2.1(0.71–6.35)	0.167
Urinary bladder injury	2(0.3)	4(0.3)	1.2(0.23–6.75)	0.808
Lumbar vertebral fracture	4(0.6)	24(1.5)	0.4(0.14–1.18)	0.087
Sacral vertebral fracture	4(0.6)	12(0.8)	0.8(0.26–2.56)	0.733
Extremity trauma, n(%)				
Scapular fracture[+]	7(1.1)	47(3.0)	0.4(0.16–0.80)	0.009
Clavicle fracture[+]	41(6.5)	273(17.4)	0.3(0.23–0.46)	0.000
Humeral fracture[+]	19(3.0)	86(5.5)	0.5(0.32–0.88)	0.013
Radial fracture	59(9.3)	150(9.6)	1.0(0.71–1.33)	0.835
Ulnar fracture	26(4.1)	68(4.3)	0.9(0.59–1.49)	0.794
Femoral fracture[a]	107(16.9)	126(8.0)	2.3(1.76–3.05)	0.000
Patella fracture	21(3.3)	45(2.9)	1.2(0.68–1.96)	0.589
Tibia fracture	60(9.4)	117(7.5)	1.3(0.93–1.79)	0.122
Fibular fracture	43(6.8)	94(6.0)	1.1(0.78–1.65)	0.499
Metacarpal fracture	24(3.8)	50(3.2)	1.2(0.73–1.96)	0.489

Table 3 Associated injuries of adolescent and adult motorcycle riders *(Continued)*

Metatarsal fracture	23(3.6)	38(2.4)	1.5(0.89–2.56)	0.122
Calcaneal fracture	27(4.3)	99(6.3)	0.7(0.43–1.02)	0.058
Pelvic fracture	20(3.1)	49(3.1)	1.0(0.59–1.71)	0.980

[+] and [a] indicated significant lower and higher incidences of the associated injury, respectively, in the adolescents than those adult patients ($p<0.05$).

Helmet-wearing status of the motorcycle-related trauma patients

Table 4 shows the results of the analysis of helmet-wearing status among adolescent riders. As can be observed, adolescent riders who had not worn a helmet presented with a significantly lower first GCS score compared to those who had worn a helmet (13.1 ± 2.9 vs. 14.4 ± 2.1, respectively, $p = 0.000$). A significantly greater percentage of adolescent riders who had not worn a helmet presented with unconscious status based on GCS score ≤8 (12.4 % vs. 4.4 %, respectively, $p = 0.002$), head and neck injury based on AIS (52.6 % vs. 30.8 %, respectively, $p = 0.000$), and cranial fracture (26.8 % vs. 10.9 %, respectively, $p = 0.000$), while a significantly lower percentage presented with extremity injury based on AIS (59.8 % vs. 72.2 %, respectively, $p = 0.014$). In contrast, no significant differences were found between adolescent riders who had and had not worn a helmet regarding incidence of maxillofacial trauma, regardless of the type of maxillofacial trauma. While significantly more patients who had not worn a helmet had sustained severe injury (ISS 16–24; 18.6 % vs. 10.5 %, respectively, $p = 0.025$), significantly fewer patients who had not worn a helmet had an ISS less than 16 (76.3 % vs. 85.2 %, respectively, $p = 0.028$). Although a significantly higher percentage of adolescents who had not worn a helmet required admission to the ICU (32.0 % vs. 16.7 %, respectively, $p = 0.000$), no significant differences were found between adolescents who had and had not worn a helmet regarding incidence of very severe injury (ISS ≥ 25), NISS, TRISS, mortality, LOS, or LICUS.

Discussion

This study analyzed the demographics and characteristics of injuries observed in a population of adolescents with motorcycle-related injuries presenting at a Level I trauma center. Analysis of the data indicates that adolescent motorcycle riders comprise a major population of hospitalized trauma patients, have a higher severe injury score compared to adolescents hospitalized for all trauma injury, and present with a bodily injury pattern that differs from that of adult motorcycle riders. It also revealed that a significant percentage of adolescent motorcycle riders do not wear a helmet, which, as motorcyclists have little other protection from injury, puts them at high risk of injury.

A previous study found that the youngest motorcyclists, defined as those aged 16–19 years, were 1.30 (95 % CI = 1.10–1.54), 3.09 (95 % CI = 2.61–3.66), and 4.79 (95 % CI = 4.04–5.67) times more likely to be killed and 3.67 (95 % CI = 3.34–4.03), 10.68 (95 % CI = 9.73–11.71), and 18.03 (95 % CI = 16.43–19.78) times more likely to be nonfatally injured compared to motorcyclists aged 20–29, 30–39, and 40–49 years, respectively [9]. In the current study, no significant differences were found between adolescent and adult motorcycle riders regarding ISS, regardless of the subgroup of injury severity; NISS, TRISS; mortality; hospital LOS; proportion admitted to the ICU; or LICUS. Similar studies in Singapore also reported that most motorcyclist riders hospitalized for treatment of trauma had a low ISS [13]. Considering that almost all of motorcycles are forbidden on highways in Asian cities and that most traffic accidents occur in relatively crowded streets in these cities, we hypothesize that the reason for the discrepancy between our findings and those of previous Western studies is that most motorcycle injuries in the Asian region occur at relatively low velocity.

In contrast, the adolescent motorcycle riders were found to have presented with a different bodily injury pattern compared to the adult motorcycle riders. Based on analysis of AIS, the adolescent riders presented with a higher rate of injury to the face and abdomen, but a lower rate to the thorax and extremities, and a higher rate of cranial, mandibular, hepatic, and femoral fracture but a lower rate of hemothorax and rib, thoracic vertebral, scapular, clavicle, and humeral fracture. Notably, the adolescent motorcycle riders sustained a more than 2-fold greater incidence of mandibular fracture, hepatic injury, and femoral fracture compared to the adult motorcycle riders, whereas the latter sustained a significantly higher rate of injury around the thorax region; rib fracture, hemothorax, and scapular, clavicle, and humeral fracture, all of which are considered within the category of extremity injury.

A previous study by Jou et al. in Taiwan revealed that motorcyclist fatality accounted for nearly 60 % of all driving fatalities in the country between 2006 and 2008 [14]. They also found an association between higher fatality rates and the factors of male sex, advanced age, unlicensed status, not wearing a helmet, riding after alcohol consumption, and alcohol consumption of more than 550 cc [14]. In the current study, 3 of 4 (75 %) fatalities among adolescents aged 13–19 years and 20 of 33 (61 %) among adults aged 30–50 years were found to have involved motorcycle use. However, the number

Table 4 Injury characteristics of adolescent motorcycle riders according to helmet-wearing status

Motorcycle accident			
	Helmet + *N* = 522	Helmet- *N* = 97	*p*
Gender, n(%)			0.972
Male	340(65.1)	63(64.9)	
Female	182(34.9)	34(35.1)	
GCS	14.4 ± 2.1	13.1 ± 2.9	0.000
≤ 8	23(4.4)	12(12.4)	0.002
9–12	22(4.2)	2(2.1)	0.313
≥ 13	477(91.4)	83(85.6)	0.073
AIS n(%)			
Head/Neck	161 (30.8)	51 (52.6)	0.000
Face	156 (29.9)	33 (34.0)	0.417
Thorax	46 (8.8)	6 (6.2)	0.392
Abdomen	54 (10.3)	9 (9.3)	0.750
Extremity	377 (72.2)	58 (59.8)	0.014
Head trauma, n(%)			
Neurologic deficit	4(0.8)	1(1.0)	0.789
Cranial fracture[a]	57(10.9)	26(26.8)	0.000
Epidural hematoma (EDH)	9(1.7)	3(3.1)	0.369
Subdural hematoma (SDH)	20(3.8)	8(8.2)	0.055
Subarachnoid hemorrhage (SAH)	30(5.7)	7(7.2)	0.575
Intracerebral hematoma (ICH)	9(1.7)	1(1.0)	0.619
Cerebral contusion	21(4.0)	3(3.1)	0.663
Cervical vertebral fracture	2(0.4)	0(0.0)	0.541
Maxillofacial trauma, n(%)			
Maxillary fracture	52(10.0)	10(10.3)	0.917
Mandibular fracture	38(7.3)	7(7.2)	0.982
Orbital fracture	25(4.8)	3(3.1)	0.460
Nasal fracture	10(1.9)	3(3.1)	0.458
ISS	8.9 ± 7.5	9.8 ± 7.2	0.383
< 16	445(85.2)	74(76.3)	0.028
16–24	55(10.5)	18(18.6)	0.025
≥ 25	22(4.2)	5(5.2)	0.677
NISS	10.2 ± 8.3	11.0 ± 8.0	0.455
TRISS	0.973 ± 0.110	0.971 ± 0.106	0.997
Mortality, n(%)	2(0.4 %)	0(0.0 %)	0.541
LOS (days)	9.1 ± 8.7	8.7 ± 7.0	0.077
ICU			
Patients, n(%)	87(16.7)	31(32.0)	0.000
LICUS (days)	6.3 ± 6.4	5.9 ± 5.5	0.612

of fatalities among the adolescent motorcycle riders examined was too small to analyze and draw any conclusions from.

Among several preventive measures, helmet wearing in particular has been shown to protect against head and other serious injuries and to be cost effective [9, 15, 16]. One study found a 37 % increased risk of serious/severe traumatic brain injury that required hospitalization for young motorcycle riders in states with limited-age helmet laws compared with youth in states with universal helmet laws, with the greatest increase in risk observed for the most severe type of head injury in the largest group of injured young motorcycle riders: those aged 18–20 [4]. The study also revealed that the decrease in helmet-usage rates for youth when universal helmet laws are repealed leads to increases youth motorcycle fatality rates and overall morbidity [4]. In the current study, adolescent motorcycle drivers, but not pillions, were found to be less likely to wear a helmet than adult motorcycle drivers. Compared to patients who had worn a helmet, a greater number of patients who had not worn a helmet presented with unconscious status (GCS score ≤8); had sustained head and neck injury, cranial fracture, and severe injury (ISS 16–24); and had required admission to the ICU. These findings indicate that wearing a helmet may prevent head injury and reduce injury severity among adolescent motorcycle riders. However, among adolescent motorcycle riders who had sustained very severe injury (ISS ≥25), no significant difference was found regarding the percentage who had and had not worn a helmet. Moreover, no significant differences regarding NISS, TRISS, mortality, LOS, or LICUS were found between those adolescent motorcycle riders who had and had not worn a helmet.

The limitations of this study include the use of a retrospective design and the lack of availability of data regarding the circumstances of the mechanism of injury. Although a study regarding the factors influencing motorcycle crash victim outcomes found that traveling in excess of 50 kph increased the risk of intracranial injury (OR = 4.8) [17], lack of data regarding the motorcycle speed during accidents prevented analysis of the effect of speed in the current study. Lack of data also prevented the ability to analyze the impact of the type of motorcycle; type of helmet material; or the use of any other protective materials, such as knee braces. Lastly, lack of exposure data prevented analysis of motorcycle-related hospitalization based on exposure-based risk (e.g., number of trips, hours of riding, and/or miles traveled). As younger motorcycle riders generally do not own motorcycles or travel as much as their older counterparts in terms of distance and time, inability to analyze exposure data may have led to underestimation of the true risk for younger age groups.

Conclusion

Adolescent motorcycle riders comprise a major population of patients hospitalized for treatment of trauma. This population tends to present with a higher injury severity compared to other trauma patients and a bodily injury pattern differing from that of adult motorcycle riders, indicating the need to emphasize the use of protective equipment, especially helmets, to reduce their rate and severity of injury.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HTL and CCL wrote and revised the manuscript; CSR drafted the manuscript; SYH carried out the analysis and edited the tables; HYH revised the English and conducted the proofreading; and CHH designed the study, contributed to the analysis and interpretation of data, and drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was supported by a grant from CDRPG8C0031.

Author details

[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Sung District, Kaohsiung City 833, Taiwan. [2]Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung City, Taiwan.

Received: 18 June 2014 Accepted: 14 August 2015
Published online: 28 August 2015

References

1. Naci H, Chisholm D, Baker TD. Distribution of road traffic deaths by road user group: a global comparison. Inj Prev. 2009;15(1):55–9.
2. Odero W, Garner P, Zwi A. Road traffic injuries in developing countries: a comprehensive review of epidemiological studies. Trop Med Int Health. 1997;2(5):445–60.
3. Deasy C, Gabbe B, Palmer C, Babl FE, Bevan C, Crameri J, et al. Paediatric and adolescent trauma care within an integrated trauma system. Injury. 2012;43(12):2006–11.
4. Weiss H, Agimi Y, Steiner C. Youth motorcycle-related brain injury by state helmet law type: United States, 2005-2007. Pediatrics. 2010;126(6):1149–55.
5. Beck LF, Dellinger AM, O'Neil ME. Motor vehicle crash injury rates by mode of travel, United States: using exposure-based methods to quantify differences. Am J Epidemiol. 2007;166(2):212–8.
6. Wong JT, Chung YS, Huang SH. Determinants behind young motorcyclists' risky riding behavior. Accid Anal Prev. 2010;42(1):275–81.
7. Weiss H, Agimi Y, Steiner C. Youth motorcycle-related hospitalizations and traumatic brain injuries in the United States in 2006. Pediatrics. 2010;126(6):1141–8.
8. Lin MR, Chang SH, Pai L, Keyl PM. A longitudinal study of risk factors for motorcycle crashes among junior college students in Taiwan. Accid Anal Prev. 2003;35(2):243–52.
9. Schneider WH, Savolainen PT, Van Boxel D, Beverley R. Examination of factors determining fault in two-vehicle motorcycle crashes. Accid Anal Prev. 2012;45:669–76.
10. Chung YS, Wong JT. Beyond general behavioral theories: structural discrepancy in young motorcyclist's risky driving behavior and its policy implications. Accid Anal Prev. 2012;49:165–76.
11. Densmore JC, Lim HJ, Oldham KT, Guice KS. Outcomes and delivery of care in pediatric injury. J Pediatr Surg. 2006;41(1):92–8. discussion 92-98.
12. Rogers SC, Campbell BT, Saleheen H, Borrup K, Lapidus G. Using trauma registry data to guide injury prevention program activities. J Trauma. 2010;69(4 Suppl):S209–213.
13. Wong TW, Phoon WO, Lee J, Yiu IP, Fung KP, Smith G. Motorcyclist traffic accidents and risk factors: a Singapore study. Asia Pac J Public Health. 1990;4(1):34–8.
14. Jou RC, Yeh TH, Chen RS. Risk factors in motorcyclist fatalities in Taiwan. Traffic Inj Prev. 2012;13(2):155–62.
15. Hundley JC, Kilgo PD, Miller PR, Chang MC, Hensberry RA, Meredith JW, et al. Non-helmeted motorcyclists: a burden to society? A study using the National Trauma Data Bank. J Trauma. 2004;57(5):944–9.
16. MacLeod JB, Digiacomo JC, Tinkoff G. An evidence-based review: helmet efficacy to reduce head injury and mortality in motorcycle crashes: EAST practice management guidelines. J Trauma. 2010;69(5):1101–11.
17. Cunningham G, Chenik D, Zellweger R. Factors influencing motorcycle crash victim outcomes: a prospective study. ANZ J Surg. 2012;82(7-8):551–4.

Chuang et al. World Journal of Emergency Surgery (2016) 11:3
DOI 10.1186/s13017-015-0059-9

World Journal of Emergency Surgery

RESEARCH ARTICLE Open Access

Obese patients who fall have less injury severity but a longer hospital stay than normal-weight patients

Jung-Fang Chuang[1†], Cheng-Shyuan Rau[2†], Hang-Tsung Liu[1], Shao-Chun Wu[3], Yi-Chun Chen[1], Shiun-Yuan Hsu[1], Hsiao-Yun Hsieh[1] and Ching-Hua Hsieh[1*]

Abstract

Background: The effects of obesity on injury severity and outcome have been studied in trauma patients but not in those who have experienced a fall. The aim of this study was to compare injury patterns, injury severities, mortality rates, and in-hospital or intensive care unit (ICU) length of stay (LOS) between obese and normal-weight patients following a fall.

Methods: Detailed data were retrieved for 273 fall-related hospitalized obese adult patients with a body mass index (BMI) ≥30 kg/m^2 and 2357 normal-weight patients with a BMI <25 kg/m^2 but ≥18.5 kg/m^2 from the Trauma Registry System of a Level I trauma center between January 1, 2009, and December 31, 2013. We used the Pearson's chi-squared test, Fisher's exact test, the Mann Whitney *U* test, and independent Student's *t*-test to analyze differences between the two groups.

Results: Analysis of AIS scores and AIS severity scaling from 1 to 5 revealed no significant differences in trauma regions between obese and normal-weight patients. When stratified by injury severity (Injury Severity Score [ISS] of <16, 16–24, or ≥25), more obese patients had an ISS of <16 compared to normal-weight patients (90.5 % vs. 86.0 %, respectively; $p = 0.041$), while more normal-weight patients had an ISS between 16 and 24 (11.0 % vs. 6.6 %, respectively; $p = 0.025$). Obese patients who had experienced a fall had a significantly lower ISS (median (range): 9 (1–45) *vs.* 9 (1–50), respectively; $p = 0.015$) but longer in-hospital LOS than did normal-weight patients (10.1 days *vs.* 8.9 days, respectively; $p = 0.049$). Even after taking account of possible differences in comorbidity and ISS, the obese patients have an average 1.54 day longer LOS than that of normal-weight patients. However, no significant differences were found between obese and normal-weight patients in terms of the New Injury Severity Score (NISS), Trauma-Injury Severity Score (TRISS), mortality, percentage of patients admitted to the ICU, or LOS in the ICU.

Conclusion: Obese patients who had experienced a fall did not have different injured body regions than did normal-weight patients. However, they had a lower ISS but a longer in-hospital LOS than did normal-weight patients.

Keywords: Fall, Obesity, Injury severity score, Mortality, In-hospital length of stay

* Correspondence: m93chinghua@gmail.com
†Equal contributors
[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Sung District, Kaohsiung City 833, Taiwan
Full list of author information is available at the end of the article

Background

Falls are a leading cause of injury and a significant public health issue [1–3]. The incidence of falls that lead to emergency unit admission is growing with the increased size and rapid growth of the geriatric population [4, 5]. In addition, obesity is a worldwide health problem leading to a range of health consequences [6, 7]. While obesity is known to increase the risk for a variety of medical conditions including hypertension, diabetes mellitus, cardiac disease, and pulmonary thromboembolism [8], the effect of obesity on the injury pattern and outcome of trauma patients after a fall remains unclear. Evidence was found that the effect of weight on the risk of falling appeared to be linear; greater obesity was related to greater risk of falling [9–11]. Compared with normal-weight respondents, the odds ratios (OR) for risk of falling were 1.12 (95 % confidence interval [CI] = 1.01–1.24) for obesity Class 1 (BMI 30.0–34.9 kg/m^2), 1.26 (95 % CI = 1.05–1.51) for obesity Class 2 (BMI 35.0–39.9 kg/m^2), and 1.50 (95 % CI = 1.21–1.86) for obesity Class 3 (BMI ≥ 40.0 kg/m^2) [9]. In addition, obesity was associated with a 25 % higher risk (95 % CI = 1.11–1.41; $p < 0.0003$) of having fallen in the previous 12 months compared to non-obese individuals [12].

Identification of the high-risk injury patterns and better understanding of the epidemiology and outcome of fall injury in obese patients are important in order to cope with a rising number of obese patients. Therefore, this study was designed to investigate the injury characteristics, injury patterns, injury severities, and mortality rates of adult obese patients admitted and treated for fall-related injury in southern Taiwan over a five-year period at a level I trauma center.

Methods

Ethics statement

Approval for this study was obtained from the hospital's institutional review board (IRB) before its initiation (approval number 103-7110B). Given its observational nature, the requirement for written informed consent from each patient was waived by the IRB.

Study design

This retrospective study was designed to review all 16,548 hospitalized and registered patients added to the Trauma Registry System from January 1, 2009, to December 31, 2013, and select cases that met the following inclusion criteria: (1) age ≥ 18 years, (2) BMI ≥ 30 kg/m^2 for obese patients and BMI < 25 but ≥ 18.5 kg/m^2 for normal-weight patients according to the World Health Organization definition [13, 14], and (3) admittance due to a fall accident. The patients who had sustained fall injuries from all fall heights (<1 m, 1–6 m, >6 m) were included, but those who had attempted suicide in the fall or who had non-validated BMI values or incomplete data were excluded.

To compare the injury patterns, mechanisms, severity, and mortality of obese patients with those of normal-weight patients, detailed data were retrieved on age, sex, vital signs in the emergency department (ED), injury mechanism, fall height (<1 m, 1–6 m, >6 m), transportation, injury time, Glasgow Coma Scale (GCS) upon arrival at the ED, Abbreviated Injury Scale (AIS) severity score for each body region, Injury Severity Score (ISS), New Injury Severity Score (NISS), Trauma-Injury Severity Score (TRISS), in-hospital length of stay (LOS), LOS in the ICU, and in-hospital mortality. In addition, the pre-existed comorbidities and chronic diesases including diabetes mellitus (DM), hypertension (HTN), coronary artery diseases (CAD), congestive heart failure (CHF), cerebrovascular accident (CVA), and end-stage renal disease (ESRD) were identified. A blood alcohol concentration (BAC) of 50 mg/dL at the time of arrival at the hospital was defined as the cut-off value according to the legal limit for drivers in Taiwan. The primary outcomes were injury severity scores (i.e., GCS, AIS, ISS, NISS, and TRISS), and the secondary outcomes were LOS, ICU LOS, and in-hospital mortality.

The ORs of the injured regions and associated conditions sustained by obese and normal-weight patients were calculated with 95 % CIs. Data collected regarding the obese and normal-weight population of patients who had experienced a fall were compared using SPSS v.20 statistical software (IBM, Armonk, NY, USA). Pearson's chi-squared tests, Fisher's exact tests, and independent Student's t-tests were used to analyze data as applicable. The Mann Whitney *U* test was used to compare the AIS severity scaling from 1 to 5 in each injury region. Ordinal data, like ISS and NISS, is presented as median (range). Data were further analyzed by a multiple linear regression adjusted for the effect of confounding variables (ie, comorbidity and ISS) to show the main effects of obesity on LOS in hospital. All other results are presented as the mean ± standard error.

Results

Injury characteristics

Among the 2630 adult patients with fall accidents, 273 (10.4 %) were obese (BMI ≥ 30 kg/m^2), and 2357 (89.6 %) were of normal weight (25 > BMI ≥ 18.5 kg/m^2) (Table 1). No statistically significant difference regarding sex was found between the obese and normal-weight patients. The mean ages of the obese and normal-weight patients were 60.6 ± 16.8 and 65.7 ± 17.1 years, respectively (p <0.001). There were significant higher incidence rates of the pre-existed comorbidities and chronic diseases including DM, HTN, and CAD in the obese patients. The majority of patients in both groups fell from a height < 1 m, implying that the majority of the patients

Table 1 Demographics of the obese and normal-weight patients with a fall injury

Variables	Obese BMI ≥ 30 *n* = 273	Normal 25 > BMI ≥ 18.5 *n* = 2357	*Odds ratio (95 %)*	*P*
Gender				
Male	114(41.8)	1080(45.8)	0.8(0.66–1.09)	0.202
Female	159(58.2)	1277(54.2)	1.2(0.92–1.52)	0.202
Age	60.6 ± 16.8	65.7 ± 17.1	—	<0.001
Comorbidity				
DM	98(35.9)	497(21.1)	2.1(1.61–2.74)	<0.001
HTN	153(56.0)	995(42.2)	1.7(1.36–2.25)	<0.001
CAD	26(9.5)	137(5.8)	1.7(1.10–2.65)	0.016
CHF	8(2.9)	51(2.2)	1.4(0.64–2.91)	0.418
CVA	25(9.2)	216(9.2)	1.0(0.65–1.54)	0.997
ESRD	0(0.0)	7(0.3)	—	1.000
Height of fall				
< 1 m	213(78.0)	1859(78.9)	1.0(0.70–1.29)	0.745
1–6 m	59(21.6)	471(20.0)	1.1(0.81–1.50)	0.525
> 6 m	1(0.4)	27(1.1)	0.3(0.04–2.34)	0.354
Alcohol > 50, n(%)	4(1.5)	51(2.2)	0.7(0.24–1.88)	0.445
GCS	14.6 ± 1.7	14.4 ± 2.0	—	0.104
≤ 8	8(2.9)	80(3.4)	0.9(0.41–1.80)	0.687
9–12	6(2.2)	88(3.7)	0.6(0.25–1.34)	0.196
≥ 13	259(94.9)	2189(92.9)	1.4(0.81–2.49)	0.218
AIS, n(%)				
Head/Neck	48(17.6)	537(22.8)	0.7(0.52–1.00)	0.050
Face	12(4.4)	154(6.5)	0.7(0.36–1.20)	0.169
Thorax	20(7.3)	174(7.4)	1.0(0.61–1.60)	0.973
Abdomen	16(5.9)	131(5.6)	1.1(0.62–1.81)	0.837
Extremity	217(79.5)	1792(76.0)	1.2(0.90–1.66)	0.203
ISS, median(range)	9(1–45)	9(1–50)	—	0.015
< 16	247(90.5)	2027(86.0)	1.5(1.02–2.36)	0.041
16–24	18(6.6)	259(11.0)	0.6(0.35–0.94)	0.025
≥ 25	8(2.9)	71(3.0)	1.0(0.46–2.04)	0.940
NISS, median(range)	9(1–66)	9(1–75)	—	0.070
TRISS	0.960 ± 0.112	0.958 ± 0.085	—	0.645
Mortality, n(%)	7(2.6)	55(2.3)	1.1(0.50–2.44)	0.812
Height of fall				
< 1 m	4(1.5)	44(1.9)	0.8(0.28–2.19)	0.639
1–6 m	3(1.1)	11(0.5)	2.4(0.66–8.55)	0.171
LOS (days)	10.1 ± 10.3	8.9 ± 8.9	—	0.049
Controlled by Comorbidity & ISS	—	—	1.01(1.01–1.03)	0.004
ICU				
Patients, n(%)	33(12.1)	375(15.9)	0.7(0.50–1.06)	0.099
< 16	15(5.5)	145(6.2)	0.9(0.51–1.53)	0.667
16–24	11(4.0)	167(7.1)	0.6(0.30–1.03)	0.057

Table 1 Demographics of the obese and normal-weight patients with a fall injury *(Continued)*

≥ 25	7(2.6)	63(2.7)	1.0(0.43–2.11)	0.916
LOS in ICU (days)	8.2 ± 8.8	7.8 ± 9.6	—	0.833
< 16	6.6 ± 6.7	6.3 ± 8.7	—	0.908
16-24	7.5 ± 6.6	7.7 ± 10.0	—	0.963
≥ 25	11.9 ± 12.5	13.3 ± 13.4	—	0.782
AIS ≥3 sites, n(%)	6(0.2)	78(3.3)	0.7(0.28–1.52)	0.323
Mortality	0(0.0)	0(0.0)	—	—
LOS (days)	16.2 ± 10.2	14.8 ± 12.2	—	0.784
ICU Patients, n(%)	3(50.0)	36(46.2)	1.2(0.22–6.14)	1.000
LOS in ICU (days)	11.3 ± 9.1	7.1 ± 6.4	—	0.293

sustained a ground-level fall occurring upon walking or with movement; however, this difference in patient number stratified by fall height (<1 m, 1–6 m, >6 m) was not statistically significant.

Injury severity

No significant differences were found between obese and normal-weight patients in GCS scores (14.6 ± 1.7 *vs.* 14.4 ± 2.0, respectively; $p = 0.104$) and the distribution of the proportion of patients at different levels of consciousness (GCS ≤8, 9–12, or ≥13) (Table 1). Analysis of AIS scores revealed no significant differences in trauma regions between obese and normal-weight patients. Comparison of the composition of AIS severity scaling from 1 to 5 in each region between obese and normal-weight patients also did not show a significant difference (Table 2). A significant difference in ISS was found between obese and normal-weight patients (median (range): 9 (1–45) *vs.* 9 (1–50), respectively; $p = 0.015$). When stratified by injury severity (ISS of <16, 16–24, or ≥25), more obese than normal-weight patients had an ISS < 16 (90.5 % vs. 86.0 %, respectively; $p = 0.041$), while more normal-weight than obese patients had an ISS between 16 and 24 (11.0 % vs. 6.6 %, respectively; $p = 0.025$). However, no significant difference were found for NISS (median (range): 9 (1–66) *vs.* 9 (1–75), respectively; $p = 0.070$), TRISS (0.960 ± 0.112 *vs.* 0.958 ± 0.085, respectively; $p = 0.645$), or in-hospital mortality (2.6 % *vs.* 2.3 %, respectively; $p = 0.645$). We found that obese patients had a significantly longer average in-hospital LOS than did normal-weight patients (10.1 *vs.* 8.9 days, respectively; $p = 0.049$). Because the detected significant higher incidence rates of DM, HTN, and CAD in the obese patients or a higher ISS may be positively correlated to a longer hospital stay, therefore, we performed a multiple linear regression analysis to investigate the effect of obesity, DM, HTN, CAD, and ISS on LOS (days) of these patients. The analysis of variance table (Table 3) indicates that the relationship between obesity and LOS is significant ($p = 0.005$), LOS in obesity tends to be 1.54 day

Table 2 Explanatory variables by a multiple regression analysis to investigate the effect of obesity, comorbidity, and ISS on LOS of these patients

Variables	Obese BMI ≥ 30 n(%)	Normal 25 > BMI ≥ 18.5 n(%)	Mann–Whitney *U* Test *P*
Head/Neck	48	537	0.210
AIS 1	2(29.2)	112(20.9)	
AIS 2	4(8.3)	44(8.2)	
AIS 3	10(20.8)	104(19.4)	
AIS 4	15(31.3)	225(41.9)	
AIS 5	5(10.4)	52(9.7)	
Face	12	154	0.550
AIS 1	4(33.3)	71(46.1)	
AIS 2	8(66.7)	83(53.9)	
Thorax	20	176	0.804
AIS 1	4(20.0)	19(10.8)	
AIS 2	5(25.0)	56(31.8)	
AIS 3	5(25.0)	77(43.8)	
AIS 4	6(30.0)	22(12.5)	
AIS 5	0(0.0)	2(1.1)	
Abdomen	17	131	0.413
AIS 1	4(23.5)	6(4.6)	
AIS 2	7(41.2)	83(63.4)	
AIS 3	6(35.3)	40(30.5)	
AIS 4	0(0.0)	2(1.5)	
Extremity	217	1792	0.073
AIS 1	9(4.2)	43(2.4)	
AIS 2	86(39.6)	633(35.3)	
AIS 3	121(55.8)	1114(62.2)	
AIS 4	0(0.0)	2(0.1)	
AIS 5	1(0.5)	0(0.0)	

Table 3 Comparison of the composition of AIS severity scaling from 1 to 5 in each region between obese and normal-weight patients

Variable	Parameter estimate	Standard error	95 % CI for parameter	*P*-value
Intercept	2.600	0.362	(1.891–3.310)	0.000
Obesity	1.543	0.544	(0.477–2.610)	0.005
DM	0.990	0.418	(0.170–1.809)	0.018
HTN	0.485	0.354	(−0.209–1.178)	0.171
CAD	0.962	0.690	(−0.392–2.315)	0.164
ISS	0.642	0.032	(0.579–0.705)	0.000

The multiple linear regression equation is given by: LOS (days) =2.60 + 1.54*(obesity) + 0.99*(DM) + 0.49*(HTN) + 0.96*(CAD) + ISS*0.64, depending on obesity (o = normal weight, 1 = obesity), DM (0 = no DM, 1 = DM), HTN (0 = no HTN, 1 = HTN), CAD (0 = no CAD, 1 = CAD), and ISS (scores) of these patients

longer, on average, than that of normal-weight patients, even after taking account of possible differences in comorbidity and ISS. In addition, the relationship between ISS and LOS is highly significant ($p < 0.0005$), with a one score increase in ISS being associated with an average increase of 0.64 day LOS, after adjusting for obesity and comorbidity. However, no differences were noted in the proportion of obese and normal-weight patients admitted to the ICU as well as LOS in the ICU after stratification into either group of injury severity (ISS of <16, 16–24, or ≥25). In addition, among those who had sustained 3 or more body area injury (AIS ≥3 sites), there were no difference in obese and normal-weight patients admitted to the ICU, LOS in the ICU and in the hospital, and the mortality.

Physiological response & procedures performed at the ED

Upon arrival at the ED, no significant differences were found for GCS of <13, systolic blood pressure (SBP) of <90 mmHg, heart rate of >100 beats/min, or respiratory rates of <10 or >29. Furthermore, no significant differences were found in the odds for requiring procedures, including cardiopulmonary resuscitation, intubation, chest tube insertion, and blood transfusion, at the ED (Table 4).

Discussion

In a retrospective review of all blunt trauma patients admitted to the ICU at a Level I trauma center, prior study demonstrated that there was no difference between obese and lean patients in the type of traumatic brain injury [15]. Another study demonstrated similar injury patterns of fewer head but more chest and lower extremity injuries [16]. It has also been reported that obese trauma patients sustained more pelvic, rib, and lower extremity fractures but fewer liver injuries, mandibular fractures, and cerebral injuries than those non-obese trauma patients [17]. Based on our analysis of the AIS scores, obese patients presented no significant difference of injuries to body region from normal-weight patients. Comparison of the composition of AIS severity scaling from 1 to 5 in each region also did not show a significant difference between obese and normal-weight patients.

Longer hospitalizations of obese patients result in increased morbidity and are associated with impaired mobility, higher incidence of respiratory complications, more venous thromboembolic events, and higher nosocomial infection rates [18]. For example, obesity resulted in nearly twofold-increased odds for developing cardiac and pulmonary complications after a hip fracture as well

Table 4 Physiological response and procedures performed upon arrival at the ED

Variables	Obese BMI ≥ 30 *n* = 273	Normal 25 > BMI ≥ 18.5 *n* = 2357	*Odds ratio* (95 %)	*P*
Physiology at ER, n(%)				
GCS < 13	14(5.1)	168(7.1)	0.7(0.40–1.23)	0.218
SBP < 90 mmHg	3(1.1)	26(1.1)	1.0(0.30–3.31)	1.000
Heart rate > 100 beats/min	52(19.0)	357(15.1)	1.3(0.96–1.82)	0.092
Respiratory rate < 10 or >29	0(0.0)	6(0.3)	—	1.000
Procedures at ER, n(%)				
Cardiopulmonary resuscitation	0(0.0)	1(0.0)	—	1.000
Intubation	3(1.1)	34(1.4)	0.8(0.23–2.49)	1.000
Chest tube insertion	2(0.7)	23(1.0)	0.7(0.18–3.19)	1.000
Blood transfusion	8(2.9)	56(2.4)	1.2(0.59–2.63)	0.574

as significantly increasing the odds of developing infectious complications (OR 3.8; 95 % CI 1.9–7.6, $p < 0.001$) [19]. It has been reported that the mean duration of orthopedic surgery in morbidly obese patients was 30 % longer than in non-obese patients [20]. Moreover, medically stable obese patients were found to be almost twice as likely to experience delayed fracture fixation due to preference of the surgeon [20]. In addition, obesity in critically ill patients is significantly related to a prolonged duration of mechanical ventilation and intensive care unit length of stay [21, 22]. In this study, although obese patients had a significant lower ISS than normal-weight patients who had experienced a fall, the obese patients had a significantly longer in-hospital LOS than did normal-weight patients. Even after taking account of possible differences in comorbidity and ISS, the obese patients have an average 1.54 day longer LOS than that of normal-weight patients.

Limitation of this study involves the use of a retrospective design with its inherent selection bias and the lack of available data on the circumstances of the mechanisms of injury. In addition, the patients dead on hospital arrival or accident scene are not included into the Trauma Registry Database, thus creating a selection bias. Moreover, some well-established risk factors, including prior falls, inappropriate use of medications, gait or balance problems, and functional limitations, were not documented and analyzed in this study. Finally, this study is only descriptive and, therefore, unable to assess the effects of any particular treatment intervention; it could only rely on the assumption that assessment and management are uniform between obese and normal-weight populations.

Conclusion

The obese adult patients presented with similar injury to the body region following a fall in comparison with normal-weight patients. The obese patients had significantly lower ISS but significantly longer in-hospital LOS than did normal-weight patients. However, mortality, the percentage of patients admitted to the ICU, and LOS in the ICU exhibited no statistically significant differences between obese and normal-weight patients.

Level of evidence

Epidemiological study, level III.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JFC revised the manuscript; CSR drafted the manuscript; HTL wrote the manuscript; SCW, YCC, and SYH performed the analysis and edited the tables; HYH revised and proofread the manuscript; CHH designed the study, contributed to the data analysis and interpretation, and drafted the manuscript. All authors read and approved the final manuscript.

Author details

[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Sung District, Kaohsiung City 833, Taiwan. [2]Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan. [3]Department of Anesthesiology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan.

Received: 28 August 2015 Accepted: 31 December 2015
Published online: 04 January 2016

References

1. Ambrose AF, Paul G, Hausdorff JM. Risk factors for falls among older adults: a review of the literature. Maturitas. 2013;75(1):51–61.
2. Rosen T, Mack KA, Noonan RK. Slipping and tripping: fall injuries in adults associated with rugs and carpets. J Inj Violence Res. 2013;5(1):61–9.
3. Hester AL, Wei F. Falls in the community: state of the science. Clin Interv Aging. 2013;8:675–9.
4. Meschial WC, Soares DF, Oliveira NL, Nespollo AM, Silva WA, Santil FL. Elderly victims of falls seen by prehospital care: gender differences. Rev Bras Epidemiol. 2014;17(1):3–16.
5. Wendelboe AM, Landen MG. Increased fall-related mortality rates in New Mexico, 1999–2005. Public Health Rep. 2011;126(6):861–7.
6. Pi-Sunyer FX. The obesity epidemic: pathophysiology and consequences of obesity. Obes Res. 2002;10 Suppl 2:97s–104.
7. Wang Y, Lobstein T. Worldwide trends in childhood overweight and obesity. Int J Pediatr Obes. 2006;1(1):11–25.
8. Rosenfeld HE, Tsokos M, Byard RW. The association between body mass index and pulmonary thromboembolism in an autopsy population. J Forensic Sci. 2012;57(5):1336–8.
9. Himes CL, Reynolds SL. Effect of obesity on falls, injury, and disability. J Am Geriatr Soc. 2012;60(1):124–9.
10. Mitchell RJ, Lord SR, Harvey LA, Close JC. Associations between obesity and overweight and fall risk, health status and quality of life in older people. Aust N Z J Public Health. 2014;38(1):13–8.
11. Finkelstein EA, Chen H, Prabhu M, Trogdon JG, Corso PS. The relationship between obesity and injuries among U.S. adults. Am J Health Promot. 2007;21(5):460–8.
12. Mitchell RJ, Lord SR, Harvey LA, Close JC. Obesity and falls in older people: mediating effects of disease, sedentary behavior, mood, pain and medication use. Arch Gerontol Geriatr. 2015;60(1):52–8.
13. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser. 1995;854:1–452.
14. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser. 2000;894:i-xii, 1–253.
15. Brown CV, Rhee P, Neville AL, Sangthong B, Salim A, Demetriades D. Obesity and traumatic brain injury. J Trauma. 2006;61(3):572–6.
16. Brown CV, Neville AL, Rhee P, Salim A, Velmahos GC, Demetriades D. The impact of obesity on the outcomes of 1,153 critically injured blunt trauma patients. J Trauma. 2005;59(5):1048–51. discussion 1051.
17. Boulanger BR, Milzman D, Mitchell K, Rodriguez A. Body habitus as a predictor of injury pattern after blunt trauma. J Trauma. 1992;33(2):228–32.
18. Goulenok C, Monchi M, Chiche JD, Mira JP, Dhainaut JF, Cariou A. Influence of overweight on ICU mortality: a prospective study. Chest. 2004;125(4):1441–5.
19. Belmont Jr PJ, Garcia EJ, Romano D, Bader JO, Nelson KJ, Schoenfeld AJ. Risk factors for complications and in-hospital mortality following hip fractures: a study using the National Trauma Data Bank. Arch Orthop Trauma Surg. 2014;134(5):597–604.
20. Childs BR, Nahm NJ, Dolenc AJ, Vallier HA. Obesity is associated with more complications and longer hospital stays after orthopaedic trauma. J Orthop Trauma. 2015;29(11):504–9.
21. Akinnusi ME, Pineda LA, El Solh AA. Effect of obesity on intensive care morbidity and mortality: a meta-analysis. Crit Care Med. 2008;36(1):151–8.
22. Mica L, Keel M, Trentz O. The impact of body mass index on the physiology of patients with polytrauma. J Crit Care. 2012;27(6):722–6.

Chuang et al. Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine (2016) 24:12
DOI 10.1186/s13049-016-0208-5

Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine

ORIGINAL RESEARCH Open Access

Use of the reverse shock index for identifying high-risk patients in a five-level triage system

Jung-Fang Chuang[1†], Cheng-Shyuan Rau[2†], Shao-Chun Wu[3], Hang-Tsung Liu[1], Shiun-Yuan Hsu[1], Hsiao-Yun Hsieh[1], Yi-Chun Chen[1] and Ching-Hua Hsieh[1*]

Abstract

Background: The ratio of systolic blood pressure (SBP) to heart rate (HR), called the reverse shock index (RSI), is used to evaluate the hemodynamic stability of trauma patients. To minimize undertriage in emergency departments (EDs), we evaluated whether RSI < 1 (i.e., SBP lower than HR) could be used as an additional variable to identify patients at high risk for more severe injury within a level category of the five-level Taiwan Triage and Acuity Scales (TTAS) system.

Methods: Data obtained from the Trauma Registry System, including triage level according to the TTAS system, were retrospectively reviewed for trauma admissions from January 2009 through December 2013 in a Level I trauma center. In our study, the primary outcomes were injury severity as measured using different scoring systems, including the Glasgow coma scale (GCS), abbreviated injury scale scores, and the injury severity score (ISS), and in-hospital mortality. The secondary outcomes were hospital and intensive care unit (ICU) length of stay (LOS).

Results: Of 10,814 trauma patients, 348 patients (3.2 %) had RSI < 1, whereas 10,466 (96.8 %) had RSI ≥ 1. Those with RSI < 1 had greater injury severity, a higher incidence of commonly associated injuries, lower GCS scores, greater deterioration of vital signs, and a higher incidence of procedures those with RSI ≥ 1. Patients with RSI < 1 also worse outcomes including hospital and ICU LOS, a greater frequency of ICU admission, and higher in-hospital mortality. Although the five-level TTAS system provides good prioritization of patients with major trauma, using the additional criterion of RSI < 1 could identify the patients at higher risk within the same triage level (I–III).

Discussion: The alert of a trauma patient's SBP being lower than his/her HR (RSI < 1) without the requirement of any additional equipment makes the concept of RSI particularly valuable in crowded EDs for identifying high-risk patients. RSI < 1 may serve as a principle trigger for action in the ED to alert trauma surgeons to the need for early intervention and timely preparation upon patient arrival particularly for those patients triaged in levels II and III of the TTAS system.

Conclusions: RSI < 1 upon arrival at an ED is an alarming sign of an associated worse outcome. Within the same triage level from level I to level III, patients with RSI < 1 had worse outcomes than those with RSI ≥ 1. The inclusion of RSI in the TTAS system may help to identify patients with more serious injuries who need an upgraded management level.

Keywords: Reverse shock index (RSI), Shock, Injury severity score (ISS), Mortality, Five-level triage system

* Correspondence: m93chinghua@gmail.com
†Equal contributors
[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan
Full list of author information is available at the end of the article

Background

In Taiwan, the five-level Taiwan Triage and Acuity Scales (TTAS) system was adapted from the Canadian Triage and Acuity Scales (CTAS) and was found to be a reliable triage system that accurately prioritizes the treatment of patients in the emergency department (ED) [1, 2]. The TTAS guidelines recommend a time to physician assessment based on the triage acuity level according to a classification of patients in descending order as follows: level I, resuscitation; level II, emergency; level III, urgent; level IV, less urgent; and level V, non-urgent [3]. It has been estimated that nearly one in three patients who experienced major trauma were undertriaged according to an analysis of 36,395 major trauma patients from the Nationwide Emergency Department Sample of the United States in 2010 [4]. Accurate triage of trauma patients is essential for trauma centers to avoid undertriage and poorer care or conversely overtriage and wasting of valuable resources and funding. To minimize undertriage, it is of the utmost importance to identify patients who are at high risk for severe injury; therefore, continuous evaluation of the triage system to provide greater acuity for use in emergency care is valuable for ensuring greater patient safety and more timely utilization of appropriate ED resources.

Hypovolemic shock is the most common type of shock in patients who experience traumatic injury. To identify hypovolemic shock, isolated vital signs such as heart rate (HR) or systolic blood pressure (SBP) have been revealed to be unreliable [5, 6]. Tachycardia failed to be useful as an isolated vital sign to predict the need for interventions commonly associated with hemorrhage control [6]. In addition, because there are compensatory mechanisms to increase cardiac output and maintain tissue perfusion despite a relatively low blood pressure, the reliance on SBP alone may delay recognition of the shock state [7]; for example, young patients who present with tachycardia and mild hypotension are at risk of losing their compensatory mechanisms, and therefore, they may slip into profound shock [7]. Furthermore, hemorrhagic shock occurs more rapidly in patients with penetrating injuries than in those who experience blunt trauma, and similar patterns regarding the cardiac index, mean arterial pressure, pulse oximetry, and transcutaneous oxygen tension indexed to FiO_2 were found [8]. Moreover, different parameters other than vital signs, such as the Glasgow coma scale (GCS), are useful for triaging patients with head injuries [9]. By contrast, the shock index (SI), or the ratio of HR to SBP, has also been studied as a marker of significant injury in trauma patients with hypovolemic shock [10]. The SI upon ED arrival may be considered a clinical indicator of hypovolemic shock with respect to transfusion requirements and hemostatic resuscitation [11]. The SI has been previously emphasized to serve as a capable measure for assessing hemodynamic instability [11–15] and identifying patients requiring hospital admission and/or intensive care therapy despite vital signs that may not appear strikingly abnormal [16, 17]. When healthy blood donors were subjected to a defined blood loss of 450 mL, the SI substantially increased, whereas HR and SBP, taken as separate values, remained within their normal ranges [18]. It has been demonstrated that an abnormal SI portends a worse outcome in critically ill patients [15]. In addition, patients with SI ≥ 1.0 despite

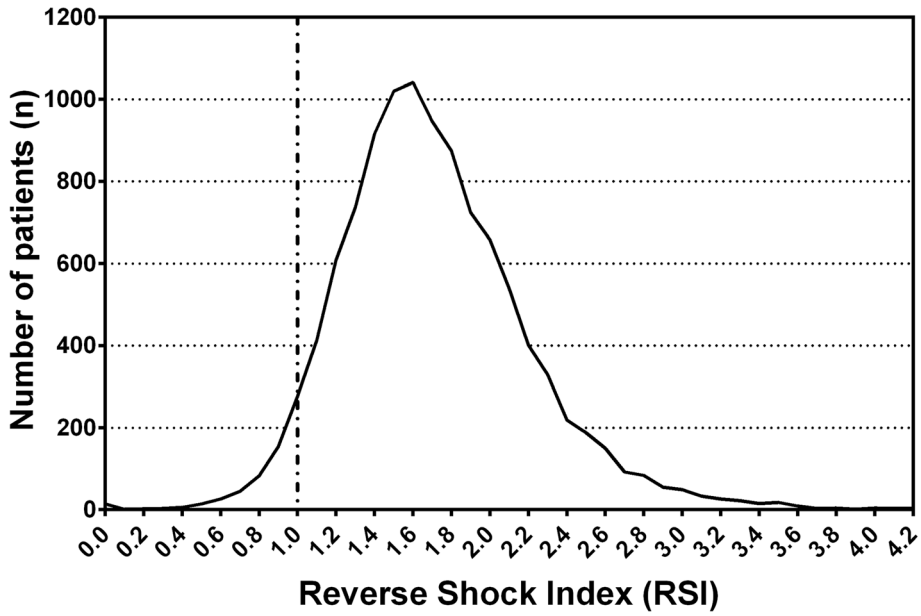

Fig. 1 Distribution of trauma patients according to the reverse shock index (RSI)

prehospital crystalloid resuscitation had significantly higher transfusion requirements and higher mortality than general major trauma patients [12].

Although the SI is an extremely practical and valuable predictor of outcome in trauma patients, the calculation of SI as the ratio of HR to SBP is odd and appears contradictory to the basic concept of shock. Generally, the idea of an unstable hemodynamic status is impressed upon practitioners as the patient's SBP being lower than the HR but not the HR being higher than the SBP, as indicated by the SI. Therefore, we prefer to calculate the ratio of SBP to HR as the reverse shock index (RSI) to evaluate hemodynamic stability in trauma patients. RSI < 1 indicates that the SBP is lower than the HR, implying that the patient is probably in shock. Moreover, the RSI could be used upon the arrival of first responders at the site of injury without any additional calculation or equipment.

To minimize the chance of undertriage, many systems have adopted extensive lists of variables related to the mechanism of injury and patient demographics, but they often have little or no scientific validation. The objective of this study was to minimize the risk of undertriage in the ED by evaluating whether RSI < 1 as an additional variable can identify patients who are at high risk for more severe injury within a level category of the TTAS system.

Methods

Study design

This retrospective study was conducted at the Kaohsiung Chang Gung Memorial Hospital, a 2400-bed facility and Level I regional trauma center that provides care to trauma patients primarily from South Taiwan. This study reviewed all 16,548 hospitalized and registered patients added to the Trauma Registry System from January 1, 2009 to December 31, 2013. During this time, patients who were transferred from other hospitals were not included in the study population because their condition was generally stable after management or procedures

Table 1 Demographics and injury characteristics of the hospitalized trauma patients

Variables	RSI < 1	RSI ≧ 1	*Odds ratio*	*p*
	N = 348	*N* = 10466	*(95 % CI)*	
SBP Mean (mmHg)	90.9±29.0	146.0±29.7	-	-
HR Mean (beats/min)	123.4±61.3	86.4±16.0	-	-
RSI Mean	0.8±0.2	1.7±0.5	-	-
Range	0.0 0.9	1.0 8.1	-	-
Gender				
Male	255 (73.3)	6631 (63.4)	1.6 (1.25–2.02)	<0.001
Female	93 (26.7)	3835 (36.6)	0.6 (0.50–0.80)	<0.001
GCS	12.1±4.0	14.4±2.0	–	<0.001
≤8	69 (19.8)	411 (3.9)	6.1 (4.57–8.02)	<0.001
9–12	36 (10.3)	338 (3.2)	3.5 (2.41–4.96)	<0.001
≥13	243 (69.8)	9717 (92.8)	0.2 (0.14–0.23)	<0.001
AIS, n (%)				
Head/Neck	139 (39.9)	2513 (24.0)	2.1 (1.69–2.62)	<0.001
Face	83 (23.9)	1946 (18.6)	1.4 (1.07–1.76)	0.013
Thorax	114 (32.8)	1197 (11.4)	3.8 (3.00–4.76)	<0.001
Abdomen	100 (28.7)	652 (6.2)	6.1 (4.75–7.76)	<0.001
Extremity	226 (64.9)	7590 (72.5)	0.7 (0.56–0.88)	0.002
ISS, median (IQR)	13 (5–22)	5 (4–9)	-	<0.001
<16	192 (55.2)	9152 (87.4)	0.2 (0.14–0.22)	<0.001
16–24	81 (23.3)	947 (9.0)	3.0 (2.36–3.95)	<0.001
≥25	75 (21.6)	367 (3.5)	7.6 (5.74–9.96)	<0.001
Mortality	34 (9.8)	103 (1.0)	10.9 (7.28–16.31)	<0.001
Ward LOS (days)	17.0±17.8	8.9±9.7	-	<0.001
ICU				
n (%)	168 (48.3)	1606 (15.3)	5.1 (4.15–6.40)	<0.001
LOS (days)	12.3±19.5	8.6±10.2	-	<0.001

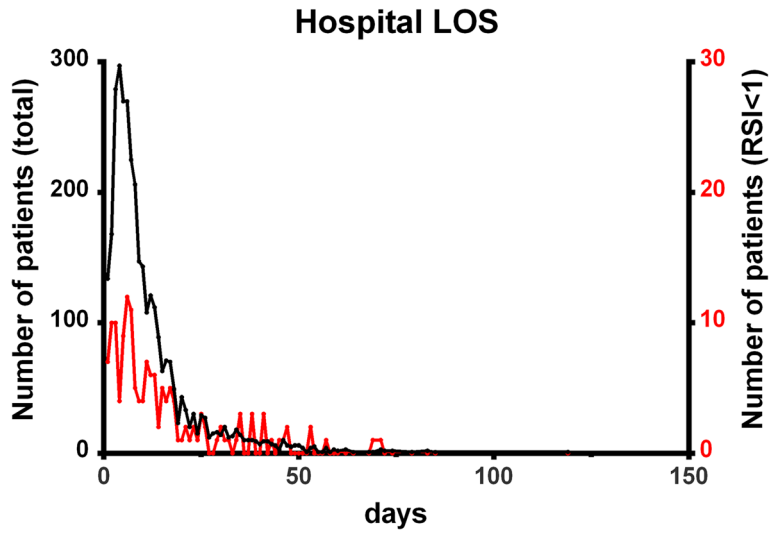

Fig. 2 The hospital length of stay (LOS) of the trauma patients with reverse shock index (RSI) < 1 (red line) and RSI ≥ 1 (black line)

had been performed in the previous hospital. In addition, patients who had incomplete data regarding vital signs, GCS, or triage level were excluded. In total, 10,814 trauma patients were enrolled in the study. Approval for this study was obtained from the hospital's institutional review board (IRB) with approval numbers 103-4599B and 103-5679B before its initiation. The informed consent requirement was waived according to the IRB regulations.

Detailed patient information was retrieved from the Trauma Registry System of our institution, and it included data regarding triage level according to the TTAS system, age, sex, vital signs on arrival, GCS score assessed on arrival at the ED, details of procedures performed at the ED (intubation, chest tube insertion, and blood transfusion), abbreviated injury scale (AIS) scores for each body region, injury severity score (ISS), length of stay (LOS) in the hospital, LOS in the intensive care unit (ICU), in-hospital mortality, and complications associated with the injuries. The ISS is expressed as the median and interquartile range (IQR, Q_1-Q_3). The RSI was calculated as the ratio of SBP to HR (RSI = SBP/HR). In our study, the primary outcomes were injury severity as measured using different scoring systems (GCS, AIS, and ISS) and in-hospital mortality. The secondary outcomes were hospital LOS and ICU LOS. Odds ratios (ORs) were calculated with 95 % confidence intervals (CIs).

Data were compared using SPSS version 20 statistical software (IBM Corporation, Armonk, NY, USA). We used Pearson's χ^2 test, Fisher's exact test, or the independent Student's *t*-test, as applicable. All results are presented as means ± standard errors. A p-value of <0.05 was considered as statistically significant.

Results

Worse outcomes of patients with RSI < 1

From January 1, 2009 to December 31, 2013, the Trauma Registry System included 16,548 hospitalized and registered patients. After excluding 5734 patients who were transferred from other hospitals or who had incomplete

Table 2 Physiological responses of and procedures underwent by the hospitalized trauma patients

Variables	RSI < 1	RSI ≧ 1	*Odds ratio*	*p*
	N = 348	*N* = 10466	*(95 % CI)*	
Physiological response, *n* (%)				
GCS < 13	105 (30.2)	749 (7.2)	5.6 (4.41–7.13)	<0.001
Respiratory rate <10 or >29	15 (4.3)	33 (0.3)	14.2 (7.66–26.47)	<0.001
Procedures performed, *n* (%)				
Cardiopulmonary resuscitation	12 (3.4)	11 (0.1)	33.9 (14.87–77.48)	<0.001
Intubation	82 (23.6)	369 (3.5)	8.4 (6.45–11.03)	<0.001
Chest tube insertion	36 (10.3)	172 (1.6)	6.9 (4.74–10.06)	<0.001
Blood transfusion	44 (12.6)	264 (2.5)	5.6 (3.99–7.85)	<0.001

data, the study group included 10,814 patients. Of these patients, 348 patients (3.2 %) had RSI < 1, and 10,466 (96.8 %) had RSI ≥ 1 (Fig. 1). The mean RSIs of the patients with RSI < 1 and RSI ≥ 1 were 0.8 ± 0.2 and 1.7 ± 0.5, respectively (Table 1). A statistically significant difference regarding sex was found between patients with RSI < 1 (255 men [73.3 %] and 93 women [26.7 %]) and those with RSI ≥ 1 (6631 men [63.4 %] and 3835

Table 3 Associated injuries of the hospitalized trauma patients

Variables	RSI < 1	RSI ≧ 1	*Odds ratio*	*p*
	N = 348	*N* = 10466	*(95 % CI)*	
Head trauma, *n* (%)				
Cranial fracture	37 (10.6)	623 (6.0)	1.9 (1.32–2.67)	<0.001
Epidural hematoma (EDH)	22 (6.3)	401 (3.8)	1.7 (1.09–2.64)	0.018
Subdural hematoma (SDH)	38 (10.9)	770 (7.4)	1.5 (1.09–2.18)	0.013
Subarachnoid hemorrhage (SAH)	51 (14.7)	869 (8.3)	1.9 (1.40–2.57)	<0.001
Intracerebral hematoma (ICH)	14 (4.0)	169 (1.6)	2.6 (1.47–4.45)	0.001
Cervical vertebral fracture	11 (3.2)	91 (0.9)	3.7 (1.97–7.02)	<0.001
Maxillofacial trauma, *n* (%)				
Orbital fracture	3 (0.9)	226 (2.2)	0.4 (0.13–1.24)	0.098
Nasal fracture	6 (1.7)	120 (1.1)	1.5 (0.66–3.46)	0.323
Maxillary fracture	31 (8.9)	680 (6.5)	1.4 (0.97–2.05)	0.074
Mandibular fracture	14 (4.0)	265 (2.5)	1.6 (0.93–2.79)	0.084
Thoracic trauma, *n* (%)				
Rib fracture	56 (16.1)	833 (8.0)	2.2 (1.65–2.98)	<0.001
Sternal fracture	3 (0.9)	14 (0.1)	6.5 (1.86–22.69)	0.001
Hemothorax	20 (5.7)	150 (1.4)	4.2 (2.60–6.77)	<0.001
Pneumothorax	16 (4.6)	163 (1.6)	3.0 (1.80–5.15)	<0.001
Hemopneumothorax	30 (8.6)	125 (1.2)	7.8 (5.16–11.81)	<0.001
Lung contusion	15 (4.3)	111 (1.1)	4.2 (2.42–7.28)	<0.001
Thoracic vertebral fracture	14 (4.0)	93 (0.9)	4.7 (2.64–8.29)	<0.001
Abdominal trauma, n (%)				
Intra-abdominal injury	18 (5.2)	126 (1.2)	4.5 (2.70–7.42)	<0.001
Hepatic injury	42 (12.1)	182 (1.7)	7.8 (5.44–11.05)	<0.001
Splenic injury	26 (7.5)	87 (0.8)	9.6 (6.13–15.14)	<0.001
Retroperitoneal injury	10 (2.9)	12 (0.1)	25.8 (11.06–60.07)	<0.001
Renal injury	12 (3.4)	45 (0.4)	8.3 (4.34–15.78)	<0.001
Urinary bladder injury	3 (0.9)	17 (0.2)	5.3 (1.56–18.32)	0.003
Lumbar vertebral fracture	14 (4.0)	179 (1.7)	2.4 (1.38–4.20)	0.001
Sacral vertebral fracture	7 (2.0)	62 (0.6)	3.4 (1.57–7.58)	0.001
Extremity trauma, n (%)				
Scapular fracture	13 (3.7)	160 (1.5)	2.5 (1.41–4.45)	0.001
Humeral fracture	15 (4.3)	468 (4.5)	1.0 (0.57–1.63)	0.886
Radial fracture	28 (8.0)	1069 (10.2)	0.8 (0.52–1.14)	0.188
Ulnar fracture	28 (8.0)	514 (4.9)	1.7 (1.14–2.52)	0.008
Pelvic fracture	44 (12.6)	269 (2.6)	5.5 (3.91–7.70)	<0.001
Femoral fracture	57 (16.4)	842 (8.0)	2.2 (1.67–3.00)	<0.001
Tibial fracture	37 (10.6)	768 (7.3)	1.5 (1.06–2.13)	0.021
Fibular fracture	26 (7.5)	425 (4.1)	1.9 (1.26–2.88)	0.002

women [36.6 %]). There were also significant differences in GCS scores and the distribution of scores (≤8, 9–12, or ≥13) between the RSI < 1 and RSI ≥ 1 groups. Analysis of AIS scores revealed that patients with RSI < 1 had significantly higher rates of injuries to the head/neck, face, thorax, and abdomen, whereas patients with RSI ≥ 1 displayed significantly higher rates of injuries to the extremities. Comparisons of trauma injury scores of the patients with RSI < 1 and those with RSI ≥ 1 revealed significant differences in the ISS between the groups (13 [5–22] vs. 5 [4–9]; p < 0.001) regardless of the stratification by injury severity (ISS of <16, 16–24, or ≥25). Likewise, we also found significant differences in in-hospital mortality rates between the two patient groups. The OR of mortality of the patients with RSI < 1 was 10.9-fold greater than that of patients with RSI ≥ 1 (95 % CI = 7.28–16.31). A significantly longer hospital LOS was found among patients with RSI < 1 than among those with RSI ≥ 1 (Fig. 2). Likewise, a significantly larger proportion of patients with RSI < 1 were admitted to the ICU, and the ICU LOS was significantly longer in this group. Furthermore, the patients with RSI < 1 exhibited a higher OR for presenting with worse hemodynamic measures than those with RSI ≥ 1 (Table 2). These measures included a GCS score < 13, SBP < 90 mmHg, HR > 100 beats/min, and a respiratory rate of <10 or >29 times/min. In addition, patients with RSI < 1 had higher odds for requiring procedures at the ED, including cardiopulmonary resuscitation, intubation, chest tube insertion, and blood transfusion. Patients with RSI < 1 had a statistically significantly higher OR for sustaining multiple types of trauma in the head, thorax, abdomen, and extremities (Table 3), although there were no significant differences regarding the rates of maxillofacial trauma and certain types of extremity trauma (humeral fracture and radial fractures).

Good prioritization of patients using the TTAS system

The overall study population of 10,814 trauma patients was triaged into levels I (623 patients), II (3333 patients), III (6522 patients), IV (314 patients), and V (22 patients). The comparative injury characteristics between levels I and II as well as between levels II and III are shown in Table 4. Differences between sexes were found between levels II and III (p < 0.001) but not between levels I and II ($p = 0.173$). Good prioritization using the five-level TTAS system was found with significant differences among patients with major trauma (levels I–III) regarding ISS, hospital LOS, the proportion of patients admitted to the ICU, ICU LOS, and mortality.

Patients with RSI < 1 within levels I–III had worse outcomes

We compared patients with RSI < 1 and those with RSI ≥ 1 in levels I–III (Table 5). In level I, there were 130 patients with RSI < 1 and 493 patients with RSI ≥ 1. No sex-related differences were found in this group. The patients with RSI < 1 had worse outcomes than those with RSI ≥ 1 regarding ISS and ICU LOS but not hospital LOS, the proportion of patients admitted to the ICU, or mortality. In level II, there were 153 patients with RSI < 1 and 3180 patients with RSI ≥ 1. A higher proportion of male patients had RSI < 1 in this level. The patients with RSI < 1 had worse outcome than those with RSI ≥ 1 regarding ISS, hospital LOS, the proportion of patients admitted to the ICU, and mortality but not ICU LOS. In level III, there were 59 patients with RSI < 1 and 6463 patients with RSI ≥ 1. No sex-related differences were found in this group. The patients with RSI < 1 had worse outcomes than those with RSI ≥ 1 regarding ISS, hospital LOS, the proportion of patients admitted to the ICU, and mortality but not ICU LOS.

Table 4 Comparison of the trauma patients regarding sex and outcomes in levels I–III in the Taiwan Triage and Acuity Scales system

Variables	Triage								
	I	II			III			IV	V
	N = 623	*N* = 3333	I vs. II		*N* = 6522	II vs. III		*N* = 314	*N* = 22
			Odds ratio (95 % CI)	*p*		*Odds ratio (95 % CI)*	*p*		
Gender				0.173			<0.001		
Male (n, %)	455 (73.0)	2344 (70.3)	1.1 (0.94–1.38)		3899 (59.8)	1.6 (1.46–1.74)		175 (55.7)	13 (59.1)
Female (n, %)	168 (27.0)	989 (29.7)	0.9 (0.72–1.06)		2623 (40.2)	0.6 (0.57–0.69)		139 (44.3)	9 (40.9)
ISS									
Median (IQR)	17 (10–25)	8 (4–13)		<0.001	4 (4–9)		<0.001	4 (4–9)	4 (4–9)
LOS (days)	19.3±19.8	11.3±11.1		<0.001	7.3±7.3		<0.001	7.2±7.1	9.0±8.5
ICU									
n (%)	416 (66.8)	809 (24.3)	6.3 (5.21–7.54)	<0.001	499 (7.7)	3.9 (3.43–4.37)	<0.001	47 (15.0)	3 (13.6)
Days	12.5±14.9	8.3±11.3		<0.001	7.2±7.1		0.040	8.1±8.7	4±2.6
Mortality	96 (15.4)	27 (0.8)	22.3 (14.4–34.5)	<0.001	14 (0.2)	3.8 (1.99–7.25)	<0.001	0 (0.0)	0 (0.0)

Table 5 Sex and outcomes of the trauma patients with reverse shock index (RSI) < 1 or RSI ≥ 1 in levels I–III of the Taiwan Triage and Acuity Scales system

Triage: I	RSI < 1	RSI ≧ 1	*Odds ratio*	*p*
Variables	*N* = 130	*N* = 493	*(95 % CI)*	
Gender				0.990
Male (*n*, %)	95 (73.1)	360 (73.0)	1.0 (0.65–1.55)	
Female (*n*, %)	35 (26.9)	133 (27.0)	1.0 (0.65–1.54)	
ISS				
Median (IQR)	20 (12–29)	17 (9–25)	-	0.007
LOS days	21.8±22.9	18.7±18.8	-	0.102
ICU				
n (%)	92 (70.8)	324 (65.7)	1.3 (0.83–1.92)	0.277
Days	15.5±22.9	11.7±11.5	-	0.030
Mortality	26 (20.0)	70 (14.2)	1.5 (0.92–2.49)	0.105
Triage: II	RSI < 1	RSI ≧ 1	*Odds ratio*	*p*
Variables	*N* = 153	*N* = 3180	*(95 % CI)*	
Gender				0.025
Male (n, %)	120 (78.4)	2224 (69.9)	1.6 (1.06–2.32)	
Female (n, %)	33 (21.6)	956 (30.1)	0.6 (0.43–0.95)	
ISS				
Median (IQR)	10 (4–17)	6 (4–13)	-	<0.001
LOS days	15.0±14.3	11.1±10.9	-	<0.001
ICU				
n (%)	61 (39.9)	748 (23.5)	2.2 (1.54–3.01)	<0.001
days	8.8±14.8	8.2±11.0	-	0.698
Mortality	7 (4.6)	20 (0.6)	7.2 (2.98–17.31)	<0.001
Triage III	RSI < 1	RSI ≧ 1	*Odds ratio*	*p*
Variables	*N* = 59	*N* = 6463	*(95 % CI)*	
Gender				0.846
Male (*n*, %)	36 (61.0)	3863 (59.8)	1.1 (0.62–1.78)	
Female (*n*, %)	23 (39.0)	2600 (40.2)	0.9 (0.56–1.61)	
ISS				
Median (IQR)	5 (4–16)	4 (4–9)	-	<0.001
LOS days	12.2±10.4	7.2±7.2	-	<0.001
ICU				
n (%)	15 (25.4)	484 (7.5)	4.2 (2.33–7.62)	<0.001
Days	7.0±6.9	7.2±7.2	-	0.932
Mortality	1 (1.7)	13 (0.2)	8.5 (1.09–66.15)	0.041

Inaccurate prioritization of patients with RSI < 1 within levels II and III in the TTAS system

As shown in Table 6, the 348 trauma patients with RSI < 1 were triaged into levels I (130 patients), II (153 patients), III (59 patients), IV (5 patients), and V (1 patient). The comparative injury characteristics between levels I and II as well as between levels II and III are shown. Sex-related differences were found between levels II and III ($p = 0.010$) but not between levels I and II ($p = 0.293$). Good prioritization of trauma patients with RSI < 1 using the TTAS system was achieved between levels I and II but not between levels II and III. The difference between levels I and II was significant concerning ISS, hospital LOS, the proportion of patients admitted to the ICU, ICU LOS, and mortality. However, none of these outcome measurements was significantly different between levels II and III, implying there was an inaccurate prioritization between levels II and III for patients with RSI < 1.

Inaccurate prioritization of patients with RSI < 1 within levels II and III in the TTAS system

To investigate the outcomes of patients with SBP < 90 who were inappropriately triaged to a level other than level I in the TTAS system, further stratification of 153 patients with RSI < 1 in level II or III using the criterion SBP < 90 was performed. We found that in level III, no patient had SBP < 90. In triage level II, there were 67 patients with RSI < 1 and SBP < 90 and 86 patients with RSI < 1 and SBP ≥ 90 (Table 7). Notably, these nonhypotensive patients with RSI < 1 who were triaged in level II displayed significant differences from those with RSI ≥ 1 concerning ISS, hospital LOS, the proportion of patients admitted to the ICU, and mortality, indicating the RSI < 1 is useful for identifying high-risk patients triaged in level II who displayed a nonhypotensive status.

Discussion

The assessment and treatment of trauma patients upon arrival to the ED is essential in the presence of life-threatening injuries. Prospectively identifying patients who would benefit from trauma care is essential to the success of trauma systems. However, this remains an ongoing challenge for prehospital providers and the physicians in attendance in the emergency room, who have limited data to make the decision. Moreover, this decision has been clearly illustrated to have implications for patient outcomes [19]. In this study, we analyzed the injury characteristics of all trauma patients hospitalized in a Level I trauma center and found that patients who presented with RSI < 1 had a higher injury severity, a higher incidence of commonly associated injuries, a worse physiological response, and a greater frequency of undergoing procedures than those with RSI ≥ 1. Furthermore, patients with RSI < 1 also had worse outcomes including hospital and ICU LOS, the proportion of patients admitted to the ICU, and in-hospital mortality. Moreover, although we found that the five-level TTAS system could provide good prioritization of patients with major trauma, within the same triage level from level I to level

Table 6 (RSI < 1). Comparison of the trauma patients with reverse shock index < 1 in levels I–III in the Taiwan Triage and Acuity Scales system

Variables	Triage								
	I	II			III			IV	V
	N = 130	*N* = 153	I vs. II		*N* = 59	II vs. III		*N* = 5	*N* = 1
			Odds ratio (95 % CI)	*p*		*Odds ratio (95 % CI)*	*p*		
Gender				0.293			0.010		
Male (n, %)	95 (73.1)	120 (78.4)	0.7 (0.43–1.29)		36 (61.0)	2.3 (1.21–4.45)		3 (60.0)	1 (100)
Female (n, %)	35 (26.9)	33 (21.6)	1.3 (0.78–2.31)		23 (39.0)	0.4 (0.23–0.82)		2 (40.0)	0 (0)
ISS									
Median (IQR)	20 (12–29)	10 (4–17)		<0.001	5 (4–16)		0.098	5 (3–14)	10 (10–10)
LOS (days)	21.8±22.9	15.0±14.3		0.002	12.2±10.4		0.169	4.3±9.6	11-
ICU									
n (%)	92 (70.8)	61 (39.9)	3.7 (2.22–6.01)	<0.001	15 (25.4)	1.9 (1.00–3.80)	0.051	0 (0)	0 (0)
Days	15.5±22.9	8.8±14.8		0.046	7.0±6.9		0.648	–	–
Mortality	26 (20.0)	7 (4.6)	5.2 (2.18–12.47)	<0.001	1 (1.7)	2.8 (0.34–23.10)	0.324	0 (0)	0 (0)

III, patients with RSI < 1 had worse outcomes than those with RSI ≥ 1.

Under the circumstance of many patients waiting in crowded EDs for hours before being evaluated [20], triage tools are expected to have high sensitivity in discriminating emergency conditions in terms of risk management for the care of trauma patients. However, it is also important for triage systems to balance both patient safety and system efficiency. In a study of the reproducibility of the five-level CTAS, the overall interrater agreement was moderate among five experienced nurses, with a global κ of 0.44 (95 % *CI* = 0.40–0.48) [21]. It has been reported that through years of practice, nurses might interpret and integrate the CTAS differently, developing an individualized usage of the tool, which also could lead to lower interrater reliability [21]. The diversity among several aspects of nursing triage may point to a safety risk for patients. One of the major benefits of using the RSI for evaluation at the ED is that it can be used quickly when first responders arrive without the requirement of any additional equipment or cost. Following the advanced trauma life support paradigm of "keep algorithms simple," RSI < 1 may serve as a principle trigger for action in the ED. RSI < 1 also can alert trauma surgeons to the need for early intervention and timely preparation upon patient arrival. In this study, the OR for mortality of the entire trauma patient population with RSI < 1 was more than 10-fold greater than that of patients with RSI ≥ 1. In addition, the ORs for mortality of the patients with RSI < 1 were approximately 8.5- and 7.2-fold greater than those for patients with RSI ≥ 1 in triage levels III and II, respectively. The inclusion of the RSI in the TTAS triage system may help to identify patients with serious injuries who need an upgraded management level and avert morbidity or mortality after a severe injury. Even if we are unable to identify reductions of morbidity and mortality after addition of the RSI in the TTAS triage system, we may conservatively assume they exist. However, a prospective study is warranted to validate the aforementioned hypothesis.

Table 7 Comparison of trauma patients with reverse shock index (RSI) < 1 and systolic blood pressure ≧ 90 with those with RSI ≧ 1 in level II

Triage II	RSI < 1	RSI > 1	*Odds ratio*	*p*
Variables	SBP ≧ 90	*n* = 3180	*(95 % CI)*	
	n = 86			
Gender				
Male (n, %)	72 (83.7)	2224 (69.9)	2.2 (1.24–3.94)	0.006
Female (n, %)	14 (16.3)	956 (30.1)	0.5 (0.25–0.81)	0.006
ISS				
Median (IQR)	10 (4–18)	6 (4–13)	-	<0.001
LOS				
Days	15.8±15.3	11.1±10.9	-	0.006
LOS in ICU				
n (%)	36 (41.9)	748 (23.5)	2.3 (1.51–3.62)	<0.001
Days	10.3±18.4	8.2±11.0	-	0.499
Mortality	10 (11.6)	20 (0.6)	20.8 (9.41–45.92)	<0.001

In this study, good prioritization of the five-level TTAS system is found with significant differences among patients with major trauma (levels I–III). However, inaccurate prioritization in levels II and III of the TTAS system was found when the patients had RSI < 1. The middle group (level III) is most problematic because it includes the greatest number of patients, but this group had a relatively low mortality rate. These findings may result in physicians not paying close attention to these patients to avoid using limited resources that could be reserved for other potentially sicker patients, which

that may make the physician less alert and make the limited resources being taken away from other potentially sicker patients [22]. The original recommendation of those endorsing the TTAS was that level III patients should be evaluated by a physician within 30–60 min. This recommendation is likely to be safe for these patients. Unfortunately, there are instances in which level III patients may wait for hours in a crowded ED before they are actually placed in a treatment area and evaluated by a physician. In this study, level III comprised 60 % of all patients, but this group had a low mortality rate (0.2 %). If these higher-risk level III patients are reassigned to level II by delineating the high-risk group of patients using RSI < 1 as an additional criterion, one would expect that placing these patients in a higher triage category may reduce delays in their evaluation and treatment and subsequently reduce the morbidity associated with such delays. Although different parameters such as age [23, 24], GCS [9], injury mechanisms [25], and injury regions [26] appear to be outcome predictors of trauma patients irrespective of vital signs, the alert of a trauma patient's SBP being lower than his/her HR (RSI < 1) without the requirement of any additional equipment makes the concept of RSI particularly valuable in crowded EDs for identifying high-risk patients.

Our analysis has several limitations. First, our data were collected prospectively as part of the required trauma registry process, but our questionnaires and analyses were performed retrospectively, making them subject to the limitations of all retrospective studies. Second, injured patients who died prior to arrival at the hospital or who were discharged from the ED were not included in the sample, which could result in bias. Third, the impact of pre-existing comorbidities on the course of hospitalization and mortality remains unclear. In addition, the lack of available data regarding the circumstances of the injury and the factors influencing the decision-making regarding patient management may have biased the outcome, particularly because the study population was limited to a single urban trauma center. Last, some important data other than LOS and mortality, including costs, delays in treatment, and complications, were not evaluated, and these data may provide limited information concerning the outcome evaluation.

Conclusion

Our analysis of data on trauma admissions at a Level I trauma center spanning 5 years indicated that patients with RSI < 1 had worse outcomes including hospital and ICU LOS, the proportion of patients admitted to the ICU, and in-hospital mortality. Although the five-level TTAS system could provide good prioritization of patients with major trauma, within the same triage level (I–III), the patients with RSI < 1 had worse outcomes than those with RSI ≥ 1. The inclusion of the RSI in the TTAS triage system may help to identify the patients with serious injuries who need to be shifted to a higher triage category.

Abbreviations

ORs: Odd ratios; AIS: Abbreviated injury scale; CI: Confidence intervals; GCS: Glasgow coma scale; HR: Heart rate; ICU: Intensive care unit; ISS: Injury severity score; LOS: Length of stay; RSI: Reverse shock index; SI: Shock index; SBP: Systolic blood pressure; TTAS: Taiwan Triage and Acuity Scales.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JFC draft the manuscript, CSR and SCW wrote the manuscript, HTL analyzed the collected data, SYH conducted the collection and analysis of the data, HYH revised the manuscript, YCC edited the tables, and CHH designed the study and contributed to the analysis and interpretation of data. All authors read and approved the final manuscript.

Acknowledgments

The research reported in this publication was supported by a grant from Chang Gung Memorial Hospital (CDRPG8C0031).

Author details

[1]Department of Trauma Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan. [2]Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung City, Taiwan. [3]Department of Anesthesiology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung City, Taiwan.

Received: 17 August 2015 Accepted: 5 February 2016
Published online: 09 February 2016

References

1. Ng CJ, Yen ZS, Tsai JC, Chen LC, Lin SJ, Sang YY, et al. Validation of the Taiwan triage and acuity scale: a new computerised five-level triage system. Emerg Med J. 2011;28(12):1026–31.
2. Chang YC, Ng CJ, Wu CT, Chen LC, Chen JC, Hsu KH. Effectiveness of a five-level Paediatric Triage System: an analysis of resource utilisation in the emergency department in Taiwan. Emerg Med J. 2013;30(9):735–9.
3. Ng CJ, Hsu KH, Kuan JT, Chiu TF, Chen WK, Lin HJ, et al. Comparison between Canadian Triage and Acuity Scale and Taiwan Triage System in emergency departments. J Formos Med Assoc. 2010;109(11):828–37.
4. Xiang H, Wheeler KK, Groner JI, Shi J, Haley KJ. Undertriage of major trauma patients in the US emergency departments. Am J Emerg Med. 2014;32(9):997–1004.
5. Victorino GP, Battistella FD, Wisner DH. Does tachycardia correlate with hypotension after trauma? J Am Coll Surg. 2003;196(5):679–84.
6. Brasel KJ, Guse C, Gentilello LM, Nirula R. Heart rate: is it truly a vital sign? J Trauma. 2007;62(4):812–7.
7. Gutierrez G, Reines HD, Wulf-Gutierrez ME. Clinical review: hemorrhagic shock. Crit Care. 2004;8(5):373–81.
8. Lu KJ, Chien LC, Wo CC, Demetriades D, Shoemaker WC. Hemodynamic patterns of blunt and penetrating injuries. J Am Coll Surg. 2006;203(6):899–907.
9. Matsuyama T, Okuchi K, Seki T, Murao Y. Prognostic factors in hanging injuries. Am J Emerg Med. 2004;22(3):207–10.
10. King RW, Plewa MC, Buderer NM, Knotts FB. Shock index as a marker for significant injury in trauma patients. Acad Emerg Med. 1996;3(11):1041–5.
11. Mutschler M, Nienaber U, Munzberg M, Wolfl C, Schoechl H, Paffrath T, et al. The Shock Index revisited - a fast guide to transfusion requirement? A retrospective analysis on 21,853 patients derived from the TraumaRegister DGU. Crit Care. 2013;17(4):R172.
12. Mitra B, Fitzgerald M, Chan J. The utility of a shock index >/= 1 as an indication for pre-hospital oxygen carrier administration in major trauma. Injury. 2014;45(1):61–5.

13. McNab A, Burns B, Bhullar I, Chesire D, Kerwin A. An analysis of shock index as a correlate for outcomes in trauma by age group. Surgery. 2013;154(2):384–7.
14. DeMuro JP, Simmons S, Jax J, Gianelli SM. Application of the Shock Index to the prediction of need for hemostasis intervention. Am J Emerg Med. 2013;31(8):1260–3.
15. Cannon CM, Braxton CC, Kling-Smith M, Mahnken JD, Carlton E, Moncure M. Utility of the shock index in predicting mortality in traumatically injured patients. J Trauma. 2009;67(6):1426–30.
16. Yealy DM, Delbridge TR. The shock index: all that glitters. Ann Emerg Med. 1994;24(4):714–5.
17. Rady MY, Smithline HA, Blake H, Nowak R, Rivers E. A comparison of the shock index and conventional vital signs to identify acute, critical illness in the emergency department. Ann Emerg Med. 1994;24(4):685–90.
18. Birkhahn RH, Gaeta TJ, Terry D, Bove JJ, Tloczkowski J. Shock index in diagnosing early acute hypovolemia. Am J Emerg Med. 2005;23(3):323–6.
19. MacKenzie EJ, Rivara FP, Jurkovich GJ, Nathens AB, Frey KP, Egleston BL, et al. A national evaluation of the effect of trauma-center care on mortality. N Engl J Med. 2006;354(4):366–78.
20. Travers DA, Waller AE, Bowling JM, Flowers D, Tintinalli J. Five-level triage system more effective than three-level in tertiary emergency department. J Emerg Nurs. 2002;28(5):395–400.
21. Dallaire C, Poitras J, Aubin K, Lavoie A, Moore L. Emergency department triage: do experienced nurses agree on triage scores? J Emerg Med. 2012;42(6):736–40.
22. Ruger JP, Lewis LM, Richter CJ. Identifying high-risk patients for triage and resource allocation in the ED. Am J Emerg Med. 2007;25(7):794–8.
23. Bruijns SR, Guly HR, Bouamra O, Lecky F, Lee WA. The value of traditional vital signs, shock index, and age-based markers in predicting trauma mortality. J Trauma Acute Care Surg. 2013;74(6):1432–7.
24. Zarzaur BL, Croce MA, Magnotti LJ, Fabian TC. Identifying life-threatening shock in the older injured patient: an analysis of the National Trauma Data Bank. J Trauma. 2010;68(5):1134–8.
25. Haider AH, Chang DC, Haut ER, Cornwell 3rd EE, Efron DT. Mechanism of injury predicts patient mortality and impairment after blunt trauma. J Surg Res. 2009;153(1):138–42.
26. Van Belleghem G, Devos S, De Wit L, Hubloue I, Lauwaert D, Pien K, et al. Predicting in-hospital mortality of traffic victims: A comparison between AIS-and ICD-9-CM-related injury severity scales when only ICD-9-CM is reported. Injury. 2016;47(1):141–6.

■ HIP

A regime of two intravenous injections of tranexamic acid reduces blood loss in minimally invasive total hip arthroplasty

A PROSPECTIVE RANDOMISED DOUBLE-BLIND STUDY

C-H. Hsu,
P-C. Lin,
F-C. Kuo,
J-W. Wang

From Kaohsiung Chang Gung Memorial Hospital and Chang Gung University, College of Medicine, Taiwan

Tranexamic acid (TXA), an inhibitor of fibrinolysis, reduces blood loss after total knee arthroplasty. However, its effect on minimally invasive total hip arthroplasty (THA) is not clear. We performed a prospective, randomised double-blind study to evaluate the effect of two intravenous injections of TXA on blood loss in patients undergoing minimally invasive THA.

In total, 60 patients (35 women and 25 men with a mean age of 58.1 years; 17 to 84) who underwent unilateral minimally invasive uncemented THA were randomly divided into the study group (30 patients, 20 women and ten men with a mean age of 56.5 years; 17 to 79) that received two intravenous injections 1 g of TXA pre- and post-operatively (TXA group), and a placebo group (30 patients, 15 women and 15 men with a mean age of 59.5 years; 23 to 84). We compared the peri-operative blood loss of the two groups. Actual blood loss was calculated from the maximum reduction in the level of haemoglobin. All patients were followed clinically for the presence of venous thromboembolism.

The TXA group had a lower mean intra-operative blood loss of 441 ml (150 to 800) *versus* 615 ml (50 to 1580) in the placebo (p = 0.044), lower mean post-operative blood loss (285 ml (120 to 570) *versus* 392 ml (126 to 660) (p = 0.002), lower mean total blood loss (1070 ml (688 to 1478) *versus* 1337 ml (495 to 2238) (p = 0.004) and lower requirement for transfusion (p = 0.021). No patients in either group had symptoms of venous thromboembolism or wound complications.

This prospective, randomised controlled study showed that a regimen of two intravenous injections of 1 g TXA is effective for blood conservation after minimally invasive THA.

Cite this article: *Bone Joint J* 2015;97-B:905–10.

Anaemia is a recognised complication of total hip arthroplasty (THA).[1] The blood loss after primary THA has been reported to range from 646 ml to 1770 ml.[2-4] In a retrospective study of 370 hip and knee arthroplasties, a rate of transfusion of 69% was reported when the patients' pre-operative level of haemoglobin was < 13 g/dl.[5] Allogeneic transfusion is associated with a risk of infection, viral transmission, immunological reaction and fluid overload.[6-9] Therefore, some form of blood-saving strategy is mandatory for patients undergoing THA.

Tranexamic acid (TXA), an inhibitor of fibrinolysis, reportedly reduces peri-operative blood loss in cardiac surgery as well as in total knee arthroplasty (TKA).[10-12] However, the timing and dosage of TXA in THA remains controversial. Administration of TXA after cementing the femoral component failed to reduce bleeding.[13] Other uncertainties regarding the efficacy of TXA in THA relate to variation in dose and whether to use TXA with or without a subsequent bolus or infusion.[14-18] Low doses of TXA (10 mg/kg) administered pre-operatively to patients undergoing THA have yielded contrasting results, with some authors reporting an increase in the need for transfusion, others a reduction in blood loss and use of transfused blood and some that the peri-operative blood loss remained unchanged.[13-15,19,20] It has been reported that a single pre-operative 1 g intravenous bolus of TXA reduced the early post-operative blood loss but not the intra-operative and late blood loss in THA.[21] One report examining pharmokinetic data in healthy volunteers has suggested that a larger dose of TXA (20 mg/kg), which can be maintained for eight hours, may cause thromboembolism.[22] There is also some evidence of benefit when it is applied topically.[23]

Minimally invasive THA has become popular in recent years. The potential benefits of this technique are believed to be a reduction in soft-tissue trauma, less post-operative pain, more rapid rehabilitation, and shorter hospital stay.[24-26] However, there has been little

■ C-H. Hsu, MD, Orthopaedic Surgeon, Lecturer
■ P-C. Lin, MD, Orthopaedic Surgeon, Assistant Professor
■ F-C. Kuo, MD, Orthopaedic Surgeon, Lecturer
■ J-W. Wang, MD, Orthopaedic Surgeon, Professor
Kaohsiung Chang Gung Memorial Hospital, 123, Ta Pei Road, Niao Sung Dist, Kaohsiung City, Taiwan.

Correspondence should be sent to Prof Dr J. W. Wang; e-mail: wangjw@adm.cgmh.org.tw

doi:10.1302/0301-620X.97B7.35029 $2.00

Bone Joint J 2015;97-B:905–10.
Received 26 August 2014; Accepted after revision 10 February 2015

research documenting the blood-saving effect of TXA in minimally invasive THA. Hourlier et al[27] reported a 20% reduction of blood loss using erythropoietin, if the pre-operative level of haemoglobin was < 11.5 g/dl, and TXA (30 mg/kg) before operation in minimally invasive primary THA (133 patients) compared with a matched historical control group (82 patients) which did not receive TXA. However, the use of an historical control group may have introduced a confounding variable as surgical technique may have altered. Therefore, we undertook a prospective randomised, double-blind study to evaluate the effect of TXA on peri-operative blood loss and transfusion requirements in minimally invasive THA.

Patients and Methods

A power study was based on the results of a pharmacokinetic study on TXA in THA by Benoni et al.[22] In that study, which compared ten patients with ten matched controls, TXA reduced post-operative blood loss by a mean of 255 ml. A sample size calculation showed that 30 patients in each group were required to confirm this difference at an 85% statistical power and alpha error of 0.05.

The inclusion criteria for this study were patients aged ≥ 18 years with osteoarthritis of the hip secondary to degeneration, inflammatory arthritis, gouty arthritis, acetabular dysplasia or osteonecrosis of the femoral head, and undergoing primary unilateral minimally invasive THA. Patients were excluded if the pre-operative level of haemoglobin was < 10 g/dl, or there was a history of ischaemic heart disease, myocardial infarction, cerebrovascular disease, thromboembolic disease or ipsilateral infection of the hip.

Between July 2011 and June 2013, a total of 96 patients were assessed for eligibility; 26 were excluded because they did not meet the inclusion criteria (n = 5) or they refused to participate (n = 21). In all 70 patients were randomly allocated into two groups. Randomisation was undertaken using a computer-generated method. TXA (50 mg/ml, total 1 g/20 ml) or placebo (saline, 20 ml) was packed in numbered envelopes by an assistant (Y-J Yang) not involved in this study. A total of ten of the 70 patients dropped out of the study because of incomplete data, or refusal, after allocation. An intention-to-treat analysis was performed for the remaining 60 patients (35 women and 25 men, mean age 58.1 years; 17 to 84). Complete data were available for 30 patients in each group (Fig. 1). Those in the TXA group received two doses of TXA (1 g) intravenously, one ten minutes before the incision and one three hours later. Those in the placebo group received two equivalent volumes of normal saline, with the same timing as the TXA group. All the drugs were handled by the anaesthetist during the operation and the nurse in charge at the operating theatre recovery suite, both of whom were not involved in the study. The study had ethical approval and all patients provided informed consent.

Pre-operative laboratory data including the level of haemoglobin (Hb), haematocrit (Hct), platelet count, activated partial thromboplastin time (APTT), and prothrombin time (PT) were collected. The demographic details, including age, gender, body mass index, diagnosis, American Society of Anesthesiologists (ASA)[28] grade and pre-operative laboratory data, were similar in the two groups (Table I).

All THAs were unilateral and uncemented using the same prosthesis (Versys system, Zimmer, Warsaw, Indiana). They were performed or supervised by the same surgeon (JWW), under general anaesthesia, using a posterior approach and a minimally invasive method according to Dorr et al.[24] The patients received 1 g cefazolin which was given intravenously as a prophylactic antibiotic. The incision was a mean of 10 cm long (standard deviation (SD) 2 cm), splitting the gluteus maximus for 6 cm and using a 3 cm to 4 cm posterior capsular flap with preservation of quadratus femoris muscle and the gluteus maximums tendon.

The intra-operative blood loss was recorded from the contents of the suction bottle and the estimated blood loss through weighing the swabs. The post-operative blood loss was recorded from the contents of two intra-articular drains every two hours in the first eight hours, and then every eight hours in the next 32 hours. Routine thromboprophylaxis involved enoxaparin 40 mg subcutaneously each day between the first post-operative day and discharge, after which, patients were given 25 mg of indomethacin three times a day (orally or by suppository) for four weeks.[29] No other techniques such as anti-embolic stockings or pneumatic compressive devices were used. The Hb and Hct levels were measured on the first, second and fourth days after operation. The blood volume was assumed to normalise on the fourth day. The total Hb loss was calculated by subtracting the Hb level on the fourth post-operative day from the pre-operative value. Actual blood loss was calculated as described by Nadler et al[30] using the maximum post-operative decrease in the Hb level adjusted for the weight and height of the patient. The formula is summarised as actual blood loss = (total blood volume × change in Hb level/mean Hb) + volume transfused.

The indication for intra-operative red blood cell transfusion was blood loss exceeding 25% of total blood volume or 1000 ml. The trigger for the transfusion of allogeneic red blood cells post-operatively was a Hb level < 8 g/dl (or 8 g/dl to 9 g/dl in older patients with cardiopulmonary disease).

The patients were reviewed at two weeks, and one and three months post-operatively. The status of the wound including haematoma formation, ecchymosis or infection, was recorded. If deep vein thrombosis was suspected, for example, by swelling of the leg by > 3 cm compared with the contralateral leg,[31] bilateral antegrade venography was performed, and CT angiography if pulmonary embolism was suspected clinically.

Statistical analysis. The Mann–Whitney U test was used to determine differences in the distribution of demographics

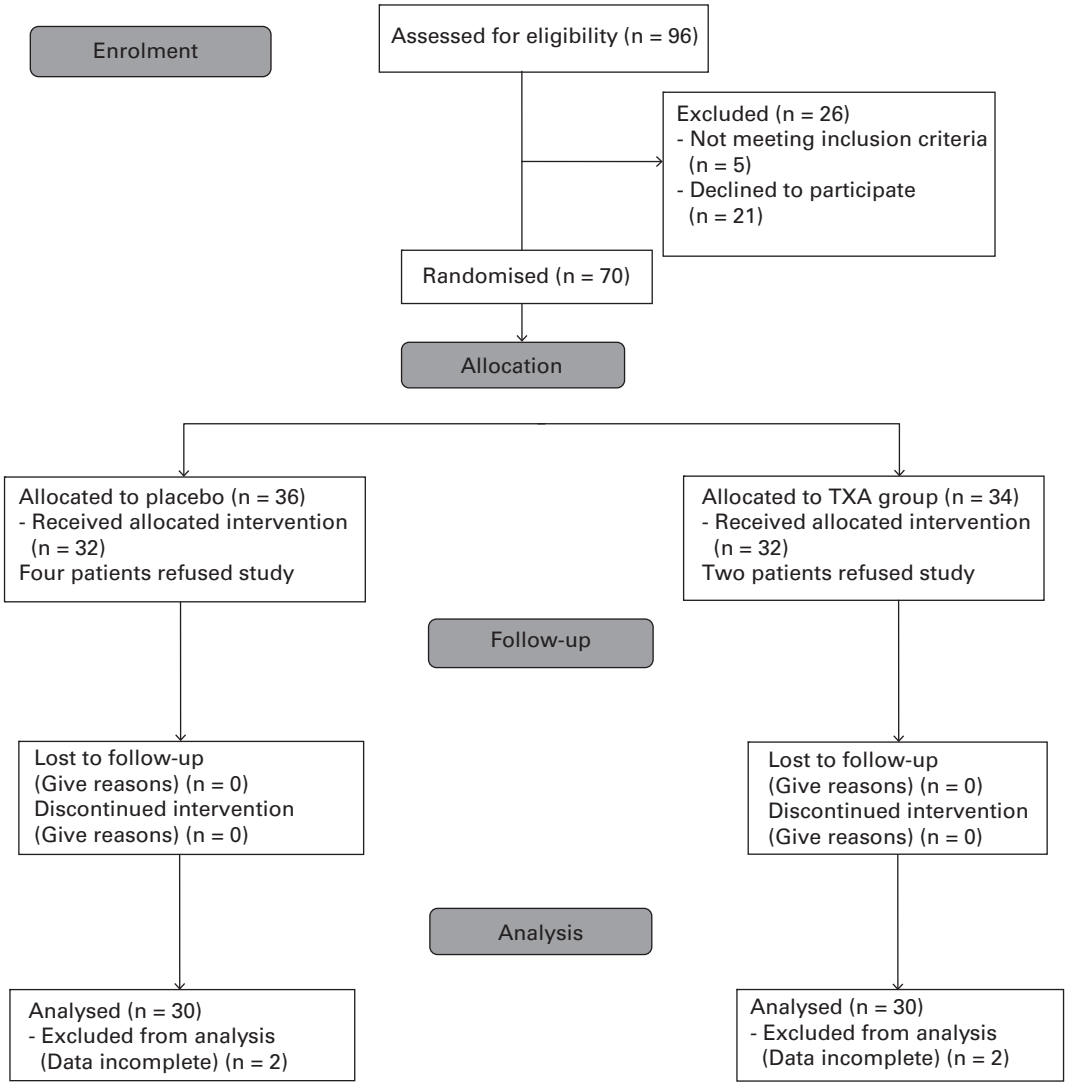

Fig. 1

Flow chart of the patients included in the trial. TXA, tranexamic acid.

and in pre-operative clinical data and also the differences in the length of the wound, hospital stay, actual blood loss, intra-operative blood loss, post-operative Hb level, and post-operative drainage between the two groups. Descriptive data, including the rate of transfusion and mean units of blood transfused, were compared using the chi-squared test. The Statistical Package for Social Sciences version 13 (SPSS Inc., Chicago, Illinois) was used for all statistical comparisons. The level of statistical significance was set at $p < 0.05$.

Results

The mean follow-up was six months (four to 20). The mean length of the wound was similar in the two groups (TXA, 9.16 cm *vs* placebo 9.23 cm), as was the mean length of stay (5.66 *vs* 5.86 days) (Table II). The mean operating time was 118 minutes (98 to 163) in the TXA group and 113 minutes (83 to 166) in the placebo group; this difference was not statistically significant ($p = 0.214$, Table II).

The mean number of red cell transfusions was significantly less in the TXA group than in the placebo group (2; 6.7% *vs* 9; 30%, $p = 0.02$, Table II). The mean number of units of red cell transfusion was less in the TXA group than the placebo group (0.13 U *vs* 0.32 U, $p = 0.02$, Table II). Despite differences in the rate and amount of transfusion between the groups, the post-operative levels of Hb on the first, second and fourth days were similar (Table II).The mean intra-operative blood loss, the total drainage and the volume of drainage in the first four, six and eight hours were all significantly less in the TXA group than in the placebo group (Table III). The mean actual blood loss was also less in the TXA group than in the placebo group (Table III) representing a total reduction of blood loss of 20% in the TXA group.

Table I. Details of the patients

	TXA (n = 30)	Placebo (n = 30)	p-value*
Mean age (yrs) (range)	56.5 (17 to 79)	59.5 (23 to 84)	0.637
Mean BMI (kg/m^2) (range)	25.2 (18.9 to 48.6)	25.6 (17.4 to 33.0)	0.281
Mean female gender (n, %)	20 *(62.5)*	15 *(50)*	
Diagnosis			
Osteoarthritis, Osteonecrosis, and others	16/14/2	14/11/5	0.064
Haemoglobin (g/dl) (range)	13.4 (10 to 16.1)	13.3 (10.8 to 16)	0.894
Mean haematocrit (n, %; range)	*40.4* (30.9 to 48.9)	*40.4* (33.9 to 47)	0.905
Mean PT INR (range)	0.98 (0.9 to 1.09)	0.99 (0.9 to 1.06)	0.453
Mean APTT INR (range)	0.99 (0.78 to 1.36)	0.99 (0.89 to 1.27)	0.840
Mean platelet count (1000/mL) (range)	255.2 (155 to 350)	240.7 (124 to 384)	0.248
ASA risk (n, %)			0.118
ASA I	4 *(1.25)*	0	
ASA II	23 *(71.8)*	23 *(76.7)*	
ASA III	5 *(15.6)*	7 *(23.3)*	

* The Mann–Whitney U test was used for statistical analysis
TXA, tranexamic acid; BMI, body mass index, SD, standard deviation; ASA, American Society of Anesthesiologists; PT-INR, prothrombin time-international normalised ratio; APTT-INR, activated partial thromboplastin time-international normalised ratio

Table II. Post-operative data for all patients

	TXA (n = 30)	Placebo (n = 30)	p-value*
Mean operating time (mins) (range)	118 (98 to 163)	113 (83 to 166)	0.214
Mean wound length (cm) (range)	9.16 (6.5 to 15)	9.23 (7 to 12)	0.783
Mean hospital stay (days) (range)	5.66 (4 to 9)	5.86 (4 to 8)	0.603
Patients requiring a blood transfusion (n, %)	2 *(6.7)*	9 *(30)*	0.021
Mean units of transfusion (SD)	0.13 *(0.50)*	0.32 *(0.54)*	0.02
Mean post-operative haemoglobin level (g/dl) (range)			
Day 1	10.8 (9.2 to 13.9)	10.4 (8 to 13.9)	0.251
Day 2	10.1 (8.3 to 13.4)	9.7 (7 to 13.4)	0.088
Day 4	9.8 (8.1 to 12.5)	9.3 (7.1 to 11.8)	0.290

* The Mann–Whitney U test was used for statistical analysis except for transfusion rate and volume of transfusion, in which, the chi-squared test was used
TXA, tranexamic acid; SD, standard deviation

Table III. Peri-operative blood loss of all patients

	TXA (n = 30)	Placebo (n = 30)	p-value*
Mean intra-operative (ml) (range)	441 (150 to 800)	615 (50 to 1580)	0.044
Mean drainage amount (ml) (range)			
0 to 4 hrs	55 (10 to 210)	99 (40 to 390)	0.003
0 to 6 hrs	75.4 (10 to 240)	140 (40 to 470)	0.004
0 to 8 hrs	92.5 (5 to 260)	155 (10 to 530)	0.011
> 8 hrs	195 (60 to 450)	237 (25 to 450)	0.051
Mean total drainage (ml)	285 (120 to 570)	392 (126 to 660)	0.002
Mean actual blood loss (ml)	1070 (688 to 1478)	1337 (495 to 2238)	0.004

* The Mann–Whitney U test was used for statistical analysis
TXA, tranexamic acid; SD, standard deviation

Throughout the study, there were no significant complications, including deep infection, deep-vein thrombosis, pulmonary embolism, dislocation of the hip or periprosthetic fracture.

Discussion

The mean reduction in the total blood loss of 20% in the TXA group compared with the placebo group was statistically significant (p = 0.004). Both intra- and post-operative blood loss were less in the TXA group. These findings are consistent with other studies using TXA in patients undergoing THA with a dose of 10 mg/kg, 15 mg/kg with or without continuous infusion or one 1 g bolus before surgery.[14-16,21,32-35]

The reported efficacy of TXA in studies on THA has shown a greater discrepancy than its use in TKA. TXA administered before deflation of the tourniquet after TKA saved 45% and 48% of blood in two studies.[11,12] Previous studies reporting promising results using TXA in THA,[14,15,32-35] have recorded blood loss intra- and post-operatively; this only partially reflects actual blood loss, which should be measured from the maximum Hb loss after surgery adjusted for the weight and height of the patients and the amount of allogenic blood transfusion.[21]

Table IV. Studies on transfusion reduction after THA with tranexamic acid prophylaxis

Authors	Regime of intravenous tranexamic acid	Number of patients	VTE prophylaxis	Transfusion protocol	Reduction of transfusion
Ido et al[33]	1 g pre-op. and 3 hrs after operation	42	None	None	No change
Benoni et al[14]	10 mg/kg pre-op only	40	LWMH	Hb 8 to 10 g/dl	No change
Husted et al[32]	10 mg/1kg pre-op and then 1 mg/kg/h x 10 hrs	40	LWMH	None	Effective
Johansson et al[16]	15 mg/kg only	100	LWMH	Hb < 9 g/dl	Effective
Niskanen and Korlala[35]	10 mg/kg pre-op and 8 hrs, 16 hrs later	39	LWMH	Hct 0.28 to 0.30	No change
Ekbäck et al[15]	10 mg/kg pre-op. and 10 mg/kg 3 hrs later with 1 kg/kg/h infusion x 10 h	40	LMMH	Hb < 27%	No change
Lemay et al[19]	10 mg/kg pre-op and 1 mg/kg/h infusion before skin closure	40	LMWH + mechanical	Hb < 7 g/dl or Hb < 9 g/dl (elderly patient)	Effective
Claeys et al[34]	15 mg/kg pre-op only	40	LMWH	Hb < 8.5 g/dl Hct < 27%	Effective
Rajesparan et al[21]	1 g pre-op only	73	LMWH	None	Effective
Garneti and Field[20]	10 mg/kg pre-op only	50	Mechanical	None	Effective
Yamasaki et al[37]	1 g pre-op only	42	None	None	Not mentioned

THA, total hip arthroplasty; Pre-op, pre-operative; VTE, venous thromboembolism; LWMH, low molecular weight heparin; Hb, haemoglobin; Hct, haematocrit

In a previous study, we reported that one intra-operative bolus of TXA in minimally invasive TKA gave a reduction of 43% in mean actual blood loss, compared with a placebo group.[36] In this study, the blood-saving effect of two intravenous doses of TXA for patients undergoing minimally invasive THA was 20%, which is similar to the 18.4% in the recent report by Rajesparan et al,[21] who measured actual blood loss using maximum Hb reduction. One bolus of 10 mg/kg TXA before the incision was thought to produce a therapeutic blood concentration of 5 mg to 10 mg/L for three hours,[33] thus, theoretically reducing bleeding during surgery. Husted et al[32] reported that a pre-operative bolus of 10 mg/kg TXA followed by continuous infusion of 1 mg/kg/hour for ten hours reduced total and post-operative blood loss but not the intra-operative blood loss in primary THA. Other factors that affect intra-operative bleeding during THA may be related to the patient's comorbidities, the surgeon's commitment to haemostasis, the operating time and the method of anaesthesia.[32] The pharmacokinetic effect of TXA is to delay fibrinolysis by blocking the lysine binding sites on the plasminogen molecule, thereby inhibiting the activation of plasminogen to plasmin.[15] Theoretically, it has no influence on primary haemostasis and coagulation.[31] Our findings were similar to those of Ekbäck et al[15] who used 10 mg/kg TXA pre-operatively in routine THA.

The reported doses of TXA are variable. Some studies have reported a blood-saving effect of TXA at 10 mg/kg with or without continuous infusion before THA.[13-15] However, Claeys et al[34] considered that a dose of 10 mg/kg would not be sufficient to prevent post-operative bleeding. Lemay et al[19] reported no change in total blood loss in patients undergoing THA using a 10 mg/kg bolus before surgery and then a 1 mg/kg/hour infusion until skin closure. Others have reported that a pre-operative bolus of 15 mg/kg is cost effective in patients undergoing THA.[5,8] The administration of 20 mg/kg before THA would reduce the fall in Hb from a mean of 3.8 g/dl to 2.8 g/dl ($p < 0.05$).[17] However, in a study on healthy volunteers, a larger dose of 20 mg/kg maintained for eight hours was predicted to influence the risk of thromboembolism.[22] In TKA, one bolus of 1 g TXA was thought to produce a plasma concentration above minimum therapeutic level for four hours.[33] Previous studies have shown a promising reduction of post-operative blood loss in primary THA.[33,37] However, operative blood loss may increase if the operating time is extended. Yamasaki et al[37] suggested that the regime of TXA should be changed if the operation is prolonged. DiGioia et al[38] reported a mean operating time of two hours in a group of patients undergoing minimally invasive THA patients which is longer than a mean of 76 minutes reported by Husted et al in patients undergoing routine THA.[32] Our regime was 1 g TXA before incision, followed by a bolus three hours later and our mean operating time for minimally invasive THA was 118 minutes in the TXA group and 113 minutes in the placebo group, which is similar to that reported by DiGioia et al.[38] This regime reduced the mean intra-operative blood loss from 615 ml to 441 ml ($p = 0.044$) and mean actual blood loss from 1337 ml to 1070 ml ($p = 0.004$) in patients undergoing minimally invasive THA.

The requirement for transfusion has varied in different studies investigating the blood-conservation effect of TXA in THA. In 11 prospective randomised trials, only six studies reported fewer transfusions in the TXA group than in a placebo group (Table IV). This might be because of the absence of strict criteria for transfusion, which we applied in our study. In the current study, the number of patients receiving red cell transfusion was decreased to 2/30 (6.7%) from 9/30 (30%) when TXA was used ($p = 0.021$). One patient in the TXA group and four in the placebo required red cell transfusions intra-operatively. The decision to transfuse was made by the anaesthetist. A similar situation has been

mentioned in a meta-analysis of the use of TXA in THA.[39] Our data showed that TXA is effective in decreasing intra- and post-operative bleeding and, therefore, decreasing the requirement for transfusion in patients undergoing minimally invasive THA.

A limitation of this study is that the design used only two arms involving two doses of TXA and placebo. The efficacy and safety of one dose TXA was therefore not investigated.

In conclusion, our prospective randomised, double-blind study showed that two intravenous injections of 1 g of TXA, one before operation and one three hours later, reduced the peri-operative and actual blood loss and the need for transfusion in patients undergoing primary minimally invasive cementless THA.

Author contributions:
C-H. Hsu: Data collection, Data analysis, Writing the paper.
P-C. Lin: Data collection, Data analysis, Project design and patients selection
F-C. Kuo: Performed surgeries, Data collection, Project design.
J-W. Wang: Performed surgeries, Writing the paper, Data collection, Project design and patients selection.

We thank Professor L. Sheng-Nan and Miss Y. Ya-Ju for assistance with the statistical analysis, Miss Y. Ya-Ju for preparation of the study medications and all the anesthetists who helped us during the surgery.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

This article was primary edited by G. Scott and first proof edited by J. Scott.

References

1. **McSwiney MM, O'Farrell D, Joshi GP, McCarroll SM.** Blood transfusion in total hip arthroplasty: guidelines to eliminate overtransfusion. *Can J Anaesth* 1993;40:222–226.
2. **Callaghan JJ, O'Rourke MR, Liu SS.** Blood management: issues and options. *J Arthroplasty* 2005;20 (suppl 2):51–54.
3. **Kubota R, Nozawa M, Matsuda K, et al.** Combined preoperative autologous blood donation and intra-operative cell salvage for hip surgery. *J Orthop Surg (Hong Kong)* 2009;17:288–290.
4. **Smith LK, Williams DH, Langkamer VG.** Post-operative blood salvage with autologous retransfusion in primary total hip replacement. *J Bone Joint Surg [Br]* 2007;89-B:1092–1097.
5. **Salido JA, Marín LA, Gómez LA, Zorrilla P, Martínez C.** Preoperative hemoglobin levels and the need for transfusion after prosthetic hip and knee surgery: analysis of predictive factors. *J Bone Joint Surg [Am]* 2002;84-A:216–220.
6. **Lemaire R.** Strategies for blood management in orthopaedic and trauma surgery. *J Bone Joint Surg [Br]* 2008;90-B:1128–1136.
7. **Salo M.** Immunosuppressive effects of blood transfusion in anesthesia and surgery. *Acta Anaesthesiol Scand* 1988;32 (suppl 89):26–34.
8. **Murphy P, Heal JM, Blumberg N.** Infection or suspected infection after hip replacement surgery with autologous or homologous blood transfusions. *Transfusion* 1991;31:212–217.
9. **Kendall SJH, Weir J, Aspinall R, Henderson D, Rosson J.** Erythrocyte transfusion causes immunosuppression after total hip replacement. *Clin Orthop Relat Res* 2000;381:145–155.
10. **Shore-Lesserson L, Reich DL, Vela-Cantos F, Ammar T, Ergin MA.** Tranexamic acid reduces transfusions and mediastinal drainage in repeat cardiac surgery. *Anesth Analg* 1996;83:18–26.
11. **Hiippala S, Strid L, Wennerstrand M, et al.** Tranexamic acid (Cyklokapron) reduces perioperative blood loss associated with total knee arthroplasty. *Br J Anaesth* 1995;74:534–537.
12. **Benoni G, Fredin H.** Fibrinolytic inhibition with tranexamic acid reduces blood loss and blood transfusion after knee arthroplasty: a prospective, randomised, double-blind study of 86 patients. *J Bone Joint Surg [Br]* 1996;78-B:434–440.
13. **Benoni G, Lethagen S, Nilsson P, Fredin H.** Tranexamic acid, given at the end of the operation, does not reduce postoperative blood loss in hip arthroplasty. *Acta Orthop Scand* 2000;71:250–254.
14. **Benoni G, Fredin H, Knebel R, Nilsson P.** Blood conservation with tranexamic acid in total hip arthroplasty: a randomized, double-blind study in 40 primary operations. *Acta Orthop Scand* 2001;72:442–448.
15. **Ekbäck G, Axelsson K, Ryttberg L, et al.** Tranexamic acid reduces blood loss in total hip replacement surgery. *Anesth Analg* 2000;91:1124–1130.
16. **Johansson T, Pettersson LG, Lisander B.** Tranexamic acid in total hip arthroplasty saves blood and money: a randomized, double-blind study in 100 patients. *Acta Orthop* 2005;76:314–319.
17. **Hynes MC, Calder P, Rosenfeld P, Scott G.** The use of tranexamic acid to reduce blood loss during total hip arthroplasty: an observational study. *Ann R Coll Surg Engl* 2005;87:99–101.
18. **Irwin A, Khan SK, Jameson SS, et al.** Oral versus intravenous tranexamic acid in enhanced-recovery primary total hip and knee replacement: results of 3000 procedures. *Bone Joint J* 2013;95-B:1556–1561.
19. **Lemay E, Guay J, Côté C, Roy A.** Tranexamic acid reduces the need for allogenic red blood cell transfusions in patients undergoing total hip replacement. *Can J Anaesth* 2004;51:31–37.
20. **Garneti N, Field J.** Bone bleeding during total hip arthroplasty after administration of tranexamic acid. *J Arthroplasty* 2004;19:488–492.
21. **Rajesparan K, Biant LC, Ahmad M, Field RE.** The effect of an intravenous bolus of tranexamic acid on blood loss in total hip replacement. *J Bone Joint Surg [Br]* 2009;91-B:776–783.
22. **Benoni G, Bjorkman S, Fredin H.** Application of pharmacokinetic data from healthy volunteers for the prediction of plasma concentrations of tranexamic acid in surgical patients. *Clin Drug Investig* 1995;10:280–287.
23. **Alshryda S, Sukeik M, Sarda P, et al.** A systematic review and meta-analysis of the topical administration of tranexamic acid in total hip and knee replacement. *Bone Joint J* 2014;96-B:1005–1015.
24. **Dorr LD, Maheshwari AV, Long WT, Wan Z, Sirianni LE.** Early pain relief and function after posterior minimally invasive and conventional total hip arthroplasty. A prospective, randomized, blinded study. *J Bone Joint Surg [Am]* 2007;89-A:1153–1160.
25. **Chimento GF, Pavone V, Sharrock N, et al.** Minimally invasive total hip arthroplasty: a prospective randomized study. *J Arthroplasty* 2005;20:139–144.
26. **Nakamura S, Matsuda K, Arai N, Wakimoto N, Matsushita T.** Mini-incision posterior approach for total hip arthroplasty. *Int Orthop* 2004;28:214–217.
27. **Hourlier H, Fennema P, Liné B.** A novel blood-saving plan for less-invasive primary total hip replacement. *Orthopedics* 2008;31 (suppl 2).
28. **Owens WD, Felts JA, Spitznagel EL Jr.** ASA physical status classification: a study of consistency of ratings. *Anesthesiology* 1978;49:239–243.
29. **Wang CJ, Wang JW, Weng LH, et al.** Prevention of deep-vein thrombosis after total knee arthroplasty in Asian patients. Comparison of low-molecular-weight heparin and indomethacin. *J Bone Joint Surg [Am]* 2004;86-A:136–140.
30. **Nadler SB, Hidalgo JH, Bloch T.** Prediction of blood volume in normal human adults. *Surgery* 1962;51:224–232.
31. **Wells PS, Anderson DR, Bormanis J, et al.** Value of assessment of pretest probability of deep-vein thrombosis in clinical management. *Lancet* 1997;350:1795–1798.
32. **Husted H, Blønd L, Sonne-Holm S, et al.** Tranexamic acid reduces blood loss and blood transfusions in primary total hip arthroplasty: a prospective randomized double-blind study in 40 patients. *Acta Orthop Scand* 2003;74:665–669.
33. **Ido K, Neo M, Asada Y, et al.** Reduction of blood loss using tranexamic acid in total knee and hip arthroplasties. *Arch Orthop Trauma Surg* 2000;120:518–520.
34. **Claeys MA, Vermeersch N, Haentjens P.** Reduction of blood loss with tranexamic acid in primary total hip replacement surgery. *Acta Chir Belg* 2007;107:397–401.
35. **Niskanen RO, Korkala OL.** Tranexamic acid reduces blood loss in cemented hip arthroplasty: a randomized, double-blind study of 39 patients with osteoarthritis. *Acta Orthop* 2005;76:829–832.
36. **Lin PC, Hsu CH, Chen WS, Wang JW.** Does tranexamic acid save blood in minimally invasive total knee arthroplasty? *Clin Orthop Relat Res* 2011;469:1995–2002.
37. **Yamasaki S, Masuhara K, Fuji T.** Tranexamic acid reduces blood loss after cementless total hip arthroplasty-prospective randomized study in 40 cases. *Int Orthop* 2004;28:69–73.
38. **DiGioia AM III, Plakseychuk AY, Levison TJ, Jaramaz B.** Mini-incision technique for total hip arthroplasty with navigation. *J Arthroplasty* 2003;18:123–128.
39. **Sukeik M, Alshryda S, Haddad FS, Mason JM.** Systematic review and meta-analysis of the use of tranexamic acid in total hip replacement. *J Bone Joint Surg [Br]* 2011;93-B:39–46.

Mini-Midvastus Total Knee Arthroplasty in Patients With Severe Varus Deformity

HAO-CHEN LIU, MD; FENG-CHIH KUO, MD; CHUNG-CHEN HUANG, MD; JUN-WEN WANG, MD

abstract

Patients with severe varus deformity of the knee (≥15° varus) usually are not considered good candidates for minimally invasive total knee arthroplasty (TKA). The goal of this study was to retrospectively investigate outcomes in patients with severe varus deformity after minimally invasive TKA. A study group of 52 patients with a tibiofemoral mechanical axis of 195° or greater was compared with a matched control group of 55 patients with a tibiofemoral mechanical axis of less than 195°. Clinical and radiographic evaluations according to the American Knee Society rating system were obtained preoperatively and postoperatively, and postoperative patient satisfaction in the 2 groups was compared. All patients were followed at a mean of 3 years (range, 2-5 years). Preoperatively, clinical knee and function scores and range of motion were inferior in the study group compared with the control group ($P<.001$). However, at the latest follow-up, both groups of patients were satisfied with the clinical results, and no significant differences were found in the knee and function scores ($P>.05$). Radiographic evaluation showed no differences in the mechanical axis, femoral component valgus angle, and tibial component valgus angle, and all outliers of the radiographic parameters between the 2 groups postoperatively ($P > .05$). The study results showed that mini-midvastus TKA did not result in more inaccurate implant positioning in patients with severe varus deformity of the knee. The clinical outcome in the group with severe varus was comparable to that in the group with less severe varus [*Orthopedics*. 2015; 38(2):e112-e117.]

The authors are from the Department of Orthopaedic Surgery (H-CL, F-CK, J-WW) and the Department of Radiology (C-CH), Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Kaohsiung City, Taiwan.

The authors have no relevant financial relationships to disclose.

Correspondence should be addressed to: Jun-Wen Wang, MD, Department of Orthopaedic Surgery, Kaohsiung Chang Gung Memorial Hospital, Chang Gung University College of Medicine, 123 Ta Pei Rd, Niao Sung District, Kaohsiung City, Taiwan (wangjw@adm.cgmh.org.tw).

Received: December 14, 2013; Accepted: April 28, 2014.

doi: 10.3928/01477447-20150204-58

Severe varus deformity of the knee is a challenge for the orthopedic surgeon during total knee arthroplasty (TKA). Successful TKA requires returning the knee alignment to 5° to 7° of valgus, with good soft tissue balancing in flexion and extension. This is sometimes difficult in knees with severe varus deformity.[1,2] Teeny et al[2] reported significantly higher varus mechanical axis of the knee and longer operative times in patients with preoperative severe varus deformity than in patients without deformity undergoing TKA. However, other authors reported no difference in knee scores and postoperative alignment between severely deformed and slightly deformed knees undergoing TKA.[3,4] Using the technique of posteromedial release and osteotomy of the tibial flare, severe varus deformity can be corrected and result in pain-free motion and stability in a high-volume joint center.[5]

Minimally invasive TKA has been associated with a shorter hospital stay, less postoperative pain, and more rapid return of function than standard TKA.[6-8] However, because of increased malalignment of the tibial component in some reports,[9,10] this procedure is not suggested for use in knees with severe varus deformity (>15° varus), more than 10° flexion contracture, or range of motion (ROM) of less than 110°.[8,11] Furthermore, because of a preoperatively larger bowing deformity of the femur in knees with severe varus, worsened postoperative mechanical axis was noted after minimally invasive TKA in these knees compared with knees with less varus deformity.[12] The goal of this study was to retrospectively investigate whether severe varus deformity would increase malalignment of the components and therefore affect clinical outcome in patients undergoing midvastus minimally invasive TKA and followed up for 2 to 5 years.

Materials and Methods

Records of a consecutive series of 467 patients who underwent unilateral primary TKA between January 2006 and December 2007 were reviewed. Patients who underwent minimally invasive TKA because of varus osteoarthritis during that period were selected for this study. Exclusion criteria were inflammatory arthritis, posttraumatic arthritis, postosteotomy knee arthritis not amenable to minimally invasive TKA, valgus knee deformity, and previous knee surgery. The study group included 52 patients (52 knees) with a preoperative tibiofemoral mechanical axis of 195° or greater (≥15° varus). A selected matched group of 55 patients (55 knees) with less severe varus deformity and a tibiofemoral mechanical axis of less than 195° (<15° varus) was included as the control group. The control group and the patient group were matched in terms of sex, age, body mass index, and disease, which was osteoarthritis in all patients. The authors' institutional review board approved this study.

All operations were performed or supervised by the senior author (J.-W.W.), who had performed more than 100 minimally invasive TKA procedures before the study. Minimally invasive TKA was performed using the mini-midvastus approach of Haas et al,[13,14] who wrote that "the skin incision was made along the medial aspect of the patella to the medial border of the mid to distal tibial tubercle. The vastus medialis oblique muscle was split approximately 2 cm in line with its fibers from the super medial pole of the patella." All patients received general anesthesia. All TKA procedures were unilateral and were cemented with the same prosthesis (NexGen, Legacy Posterior-Stabilized Prosthesis; Zimmer, Warsaw, Indiana). A tourniquet was inflated to a pressure of 300 mmHg before the incision and deflated after wound closure. The tourniquet time was recorded. Specific instruments were used to accommodate the small incisional wound. The patella was subluxed laterally but was not everted. The distal femur was resected from the medial to the lateral side with an intramedullary guide. The goal was to place the femoral component in 6° valgus. In the case of severe femoral bowing (>5°), a 7° valgus cut was selected. An extramedullary guide system was used to resect the proximal tibia from the medial to the lateral side. The goal was to place the tibial component at 90° to its anatomic axis in the coronal plane and at a 3° downslope in the lateral plane.[7] Soft tissue balancing after bone resection in patients with severe deformity was achieved with the technique described by Clayton et al.[15] The technique includes step-by-step subperiosteal release of the medial collateral ligament and pes anserinus tendons from their tibial insertions until medial and lateral soft tissue balance is achieved. Posterior capsular release and posteromedial release are necessary for additional severe flexion contracture of the knee. The entire procedure was performed through the mini-incision without lengthening the wound. Two drains connected to a vacuum bag were placed postoperatively for hematoma drainage. The drains were removed on the second postoperative day. The total amount of blood loss, which included blood drainage accumulated in the drain 36 to 48 hours postoperatively, was measured.

All patients received enoxaparin 20 mg subcutaneously every 12 hours until discharge; after that, they received indomethacin orally or by suppository for at least 4 weeks.[16] No other modalities of thromboembolic prophylaxis, such as foot pumps or antiembolic stockings, were used. The postoperative rehabilitation program included routine mobilization and continuous passive motion exercise on the first postoperative day. The criteria for discharge were ROM of the knee of greater than 90°, a clean wound without discharge, and the ability to walk with walker support.

All patients were evaluated clinically preoperatively and postoperatively at 3 months, 1 year, and annually thereafter using the Knee Society knee scoring systems.[17] The Western Ontario and Mc-

Table 1

Demographic Data

Characteristic	Study Group (n=52)	Control Group (n=55)	*P*
Age, mean, y	72.4	73.7	.152
SD (range)	4.2 (66-80)	5.3 (53-84)	
Female, No. (%)	43 (83)	45 (82)	.906
Body mass index, mean, kg/m^2	27.6	26.9	.160
SD (range)	2.51 (22.7-33)	3.2 (20.5-38.47)	

Table 2

Intraoperative Data

Parameter	Study Group	Control Group	*P*
Wound length in knee extension, mean, cm	9.7	9.3	.522
SD (range)	1.2 (8-13)	0.9 (8-12)	
Tourniquet time, mean, min	96.3	92.6	.373
SD (range)	7.3 (83-117)	5.6 (80-101)	
Blood loss, mean, mL	553	460	.041
SD (range)	186 (300-1050)	155 (201-850)	

Master Universities Osteoarthritis Index (WOMAC) scoring system[18] was used only at the latest follow-up for evaluation of patient satisfaction. Radiographic evaluation was performed with standard anteroposterior and lateral weight-bearing views of the knee and skyline patellar and long-leg weight-bearing radiographs of the leg, including the hip, knee, and ankle, both preoperatively and postoperatively at follow-up. The coronal tibiofemoral mechanical axis was defined by the angle between the femoral and tibial mechanical axes as measured on the weight-bearing anteroposterior long-leg radiographs.[12] Femoral bowing angle was defined as the angulation between the proximal and distal quarters of the femoral diaphysis in the coronal plane.[19] A femoral bowing angle of greater than than 5° was considered substantial.[20] The postoperative component alignment angles in the coronal plane, including the femoral valgus angle and tibial valgus angle, were measured with standing anteroposterior radiographs of the knee as well as the radiolucent lines at the bone-cement interface, as recommended by the Knee Society.[21] All measurements were performed by an independent blinded observer (C.-C.H.). The ideal postoperative coronal tibiofemoral mechanical axis was defined as a neutral mechanical axis (180°), the ideal femoral valgus angle was defined as 96°, and the ideal tibial valgus angle was defined as 90° in the coronal plane. Deviation of greater than 3° from the ideal was considered an outlier.[12]

Statistical Analysis

Collection and data analysis were performed with SPSS version 15 software (SPSS, Inc, Chicago, Illinois). The authors identified differences in demographic features and preoperative clinical and radiographic data, including age, body mass index, wound length, tourniquet time, operative blood loss, preoperative ROM, tibiofemoral mechanical axis, knee score, function score, WOMAC score, and postoperative clinical and radiographic data between the study group and the control group using the Mann-Whitney *U* test. Categorical data were compared with the chi-square test. A power of 83% was achieved with group sample sizes of 52 and 55 to detect a difference of -0.7 between the 2 groups. The test statistic used was the 2-sided Mann-Whitney *U* test, with a significance level of $P<.05$.

Results

Mean follow-up was 3 years (range, 2-5 years). The demographic features of the patients, including sex, age, and body mass index, were similar between groups (**Table 1**), and there were no statistically significant differences between the study group and the control group in wound length in knee extension, tourniquet time, or blood loss (**Table 2**).

Because of the more severe varus deformity in the study group than in the control group, the patients in the study group tended to have worse preoperative knee ROM (98°±9.3° vs 118°±9.2°, $P<.001$), Knee Society knee score (39±4.8 vs 51±7.8, $P<.001$), and function score (42±5.9 vs 52±6.7, $P<.001$) (**Table 3**). At the latest follow-up, knee ROM was consistently less in the study group (124°±6°) than in the control group (128°±8°) ($P=.024$). Nevertheless, postoperative knee scores, function scores, and WOMAC scores were similar between the 2 groups (**Table 4**).

Several complications occurred in both groups of patients, including 1 superficial wound infection in each group, 1 deep venous thrombosis in the study group, 2 deep venous thromboses in the control group, and 1 pulmonary embolism in the control group, but no pulmonary embolisms occurred in the study group. No patients in either group had deep infection, periprosthetic fracture, instability, or patellar dislocation.

Other than the more severe tibiofemoral mechanical axis in the varus angle of the study group preoperatively (≥195°), the severity of femoral bowing preoperatively was also greater in the study group than in the control group (patients with >5° bowing, 56% vs 13%, *P*<.001) (**Table 3**). At the latest follow-up, no difference was noted in the postoperative tibiofemoral mechanical axis between the 2 groups (180.6° vs 181.2°, *P*=.097). The percentage of outliers with a postoperative tibiofemoral mechanical axis greater than 3° was 8% in the study group and 4% in the control group (*P*=.364). Femoral valgus angle was 94.7° (range, 92°-97°) in the study group, which did not differ from the finding of 95.5° (range, 92.5°-98.2°) in the control group (*P*=.21). The percentage of postoperative femoral valgus angle outliers (>3°) did not differ between the 2 groups (11% vs 8%, *P*=.451), nor did postoperative tibial component coronal alignment tibial valgus angle (90.7° vs 91.4°, *P*=.41). The percentage of postoperative tibial valgus angle outliers (>3°) was 4% in the study group and 2% in the control group, showing no significant difference (*P*=.527) (**Table 4**). No radiolucent lines, loosening of the components at the cement-bone interface, or wear of polyethylene or osteolysis around the prosthesis occurred in any patient in either group.

Table 3

Preoperative Clinical and Radiographic Data

Parameter	Study Group	Control Group	*P*
Range of motion, mean	98°	118°	<.001
SD (range)	9.3° (75°-115°)	9.2° (100°-135°)	
Knee Society knee score, mean, points	39	51	<.001
SD (range)	4.8 (30-50)	7.8 (40-63)	
Knee Society function score, mean, points	42	52	<.001
SD (range)	5.9 (30-54)	6.7 (40-61)	
Tibiofemoral mechanical axis, mean	160.4°	171.8°	<.001
SD (range)	4.7° (145.6°-165°)	1.7° (167°-174°)	
Femoral bowing >5°, No. (%)	29 (56)	7 (13)	<.001

Table 4

Postoperative Clinical and Radiographic Data

Parameter	Study Group	Control Group	*P*
Range of motion, mean	124°	128°	.024
SD (range)	6° (105°-130°)	8° (110°-135°)	
Knee Society knee score, mean, points	90	92	.052
SD (range)	3.9 (84-98)	4.6 (84-99)	
Knee Society function score, mean, points	92	93	.065
SD (range)	3.1 (86-98)	3.8 (85-98)	
WOMAC score, mean, points	33.2	32.8	.112
SD (range)	10.1 (14-55)	5.8 (26-37)	
Tibiofemoral mechanical axis, mean	180.6°	181.2°	.097
SD (range)	1.8° (176.2°-183.6°)	1.3° (177.4°-184.3°)	
Outliers ±3°, No. of knees (%)	4 (8)	2 (4)	.364
Femoral valgus angle, mean	94.7°	95.5°	.21
SD (range)	1.3° (92°-97°)	1.5° (92.5°-98.2°)	
Outliers ±3°, No. of knees (%)	6 (11)	4 (8)	.451
Tibial valgus angle, mean	90.7°	91.4°	.41
SD (range)	1.1° (88°-94°)	1.3° (87.5°-93.4°)	
Outliers ±3°, No. of knees (%)	2 (4)	1 (2)	.527

Abbreviation: WOMAC, Western Ontario and McMaster Universities Osteoarthritis Index.

Discussion

A limited number of studies have investigated clinical outcome and radiographic accuracy of the implant position in minimally invasive TKA procedures in patients with severe varus deformity because patients with severe varus deformity (≥15° varus) of the knee have not been considered good candidates for minimally invasive TKA.[11] However, with the increasing experience of some surgeons in knee arthroplasty in high-volume centers, minimally invasive TKA is now being gradually accepted in patients with severe varus deformity.[12] In the current study, preoperative characteristics, including age, sex, primary diagnosis, and body mass index, were similar between the study group (≥15° varus) and the control group (<15° varus) (**Table 1**). Intraoperative data, including wound length and tourniquet time, were also similar, except that the study group had higher drainage blood loss than the control group, possibly because of more extensive soft tissue

release in the study group (**Table 2**). Nevertheless, at the latest follow-up after minimally invasive TKA, clinical outcomes (Knee Society knee score, function score, and WOMAC score) and radiographic findings were similar in the study group (≥15° varus) and the control group, even though the patients in the study group had more severe deformities and worse clinical scores preoperatively. However, these findings were not consistent with the study by Niki et al,[12] who reported inferior radiographic accuracy of coronal alignment of the mechanical axis postoperatively because of the greater bowing angle of the femoral diaphysis after minimally invasive TKA in the group with severe varus compared with the group with less varus. In that report, minimally invasive TKA was performed with the compact cutting guide developed for quadriceps-sparing approaches in which the instrument landmark is used only for resection of the medial side of the distal femur. Cutting of the lateral side of the distal femur was performed free-hand, and this technique is more likely to be inaccurate and to cause outliers of the femoral valgus angle of the component. The mini-midvastus approach of minimally invasive TKA offers better visualization of the lateral femoral condyle, and the specific instrument is used to cut the medial and lateral femoral condyle at the same time, allowing more accurate distal femoral cutting.[7] Furthermore, the authors tend to cut the distal femur at 7° valgus in patients with a femoral bowing angle greater than 5° and at 6° valgus in patients with normal femoral angulation. Therefore, the current study results showed no difference in the postoperative tibiofemoral mechanical axis and femoral valgus angle between knees with severe varus deformity and those with less varus deformity, even though 56% of the patients with severe varus deformity had a femoral bowing angle greater than 5°, which was similar to the 53% of patients with femoral bowing of greater than 4° in the report by Niki et al.[12]

Dalury and Dennis[9] reported that minimally invasive TKA was more likely to result in varus tibial alignment (<87° in 4 of 30 patients) than was the standard incision (no malalignment). Early failure caused by malpositioning of the components and instability is considered a risk factor for minimally invasive TKA if the procedure is performed by low-volume surgeons.[10] In view of the learning curve and the surgical volume of an arthroplasty surgeon, minimally invasive TKA showed significantly better clinical outcomes in length of hospital stay, need for inpatient rehabilitation after discharge, narcotic use, and need for assistive devices postoperatively in the hands of a high-volume arthroplasty surgeon after a substantial learning curve of 50 procedures.[22] The annual number of TKA procedures performed at the authors' institution is approximately 900, and the senior author (J.-W.W.) had experience with more than 100 minimally invasive TKA procedures before this study. The authors' finding of low numbers of outliers in the postoperative tibiofemoral mechanical axis, femoral valgus angle, and tibial valgus angle (>3° deviation from the ideal) after minimally invasive TKA in knees with severe varus was comparable with the results of King et al[23] after a substantial learning curve. In a study of 600 mini-subvastus TKA procedures, Schroer et al[24] reported no increase in complications during an initial learning curve of 100 procedures.

Limitations

This study had several limitations. First, the authors' radiographic evaluation was based on 2-dimensional analysis of standing radiographs, which may show projection-related errors in varus-valgus alignment.[25] Three-dimensional computed tomographic evaluation is more accurate in coronal and rotational alignment of the components.[26] Second, no patients in this study underwent minimally invasive TKA with computer-assisted navigation. Because of the high surgical volume in the operating room, computer-assisted navigation is not the authors' first choice in minimally invasive TKA. Third, this was not a prospective randomized study. However, the authors retrospectively analyzed 2 groups of patients undergoing the same type of surgery in the same period performed by the same surgeons using the same technique, the same implant, and the same protocol. However, the patients differed in severity of knee deformity. Finally, the postoperative outliers of the mechanical axis were 4% in the control group and 8% in the study group, which favored the control group. Even though this difference was not significant, a greater sample size with a longer-term follow-up may show some differences.

Conclusion

This study investigated the effect of minimally invasive TKA without computer-assisted navigation in patients with severe varus deformity and clinical follow-up of more than 3 years. The findings showed no adverse clinical outcomes as a result of poor implant position after minimally invasive TKA in patients with severe varus deformity. Further follow-up is necessary to validate the long-term clinical effectiveness of mini-midvastus TKA in patients with severe varus deformity.

References

1. Insall JN, Binazzi R, Soudry M, Mestriner LA. Total knee arthroplasty. *Clin Orthop Relat Res*. 1985; (192):13-22.
2. Teeny SM, Krackow KA, Hungerford DS, Jones M. Primary total knee arthroplasty in patients with severe varus deformity: a comparative study. *Clin Orthop Relat Res*. 1991; (273):19-31.
3. Karachalios T, Sarangi PP, Newman JH. Severe varus and valgus deformities treated by total knee arthroplasty. *J Bone Joint Surg Br*. 1994; 76(6):938-942.
4. Ritter MA, Faris GW, Faris PM, Davis KE. Total knee arthroplasty in patients with angular varus or valgus deformities of ≥20°. *J Arthroplasty*. 2004; 19(7):862-866.
5. Mullaji AB, Padmanabhan V, Jindal G. Total

knee arthroplasty for profound varus deformity. *J Arthroplasty*. 2005; 20(5):550-561.

6. Bounutti PM, Mont MA, McMahon M, Ragland PS, Kester M. Minimally invasive total knee arthroplasty. *J Bone Joint Surg Am*. 2004; 86(suppl 2):26-32.
7. Laskin RS, Beksac B, Phongjunakorn A, et al. Minimally invasive total knee replacement through a mini-midvastus incision: an outcome study. *Clin Orthop Relat Res*. 2004; (428):74-81.
8. Tria AJ Jr, Coon TM. Minimal incision total knee arthroplasty: early experience. *Clin Orthop Relat Res*. 2003; (416):185-190.
9. Dalury DF, Dennis DA. Mini-incision total knee arthroplasty can increase risk of component malalignment. *Clin Orthop Relat Res*. 2005; 440:77-81.
10. Barrack RL, Barnes CL, Burnett RS, Miller D, Clohisy JC, Maloney WJ. Minimal incision surgery as a risk factor for early failure of total knee arthroplasty. *J Arthroplasty*. 2009; 24(4):489-498.
11. Chen AF, Alan RK, Redziniak DE, Tria AJ Jr. Quadriceps sparing total knee replacement: the initial experience with results at two to four years. *J Bone Joint Surg Br*. 2006; 88(11):1448-1453.
12. Niki Y, Matsumoto H, Otani T, Enomoto H, Toyama Y, Suda Y. Accuracy of implant positioning for minimally invasive total knee arthroplasty in patients with severe varus deformity. *J Arthroplasty*. 2010; 25(3):381-386.
13. Haas SB, Manitta MA, Burdick P. Minimally invasive total knee arthroplasty: the mini-midvastus approach. *Clin Orthop Relat Res*. 2006; 452:112-116.
14. Haas SB, Cook S, Beksac B. Minimally invasive total knee replacement through a mini midvastus approach: a comparative study. *Clin Orthop Relat Res*. 2004; (428):68-73.
15. Clayton ML, Thompson TR, Mack RP. Correction of alignment deformities during total knee arthroplasties: staged soft-tissue releases. *Clin Orthop Relat Res*. 1986; (202):117-124.
16. Wang CJ, Wang JW, Weng LH, Hsu CC, Huang CC, Yu PC. Prevention of deep-vein thrombosis after total knee arthroplasty in Asia patients: comparison of low-molecular-weight heparin and indomethacin. *J Bone Joint Surg Am*. 2004; 86(1):136-140.
17. Insall JN, Dorr LD, Scott RD, Scott WN. Rationale of the Knee Society clinical rating system. *Clin Orthop Relat Res*. 1989; (248):13-14.
18. Bellamy N, Buchanan WW, Goldsmith CH, Campbell J, Stitt LW. Validation study of WOMAC: a health status instrument for measuring clinically important patient relevant outcomes to antirheumatic drug therapy in patients with osteoarthritis of the hip or knee. *J Rheumatol*. 1988; 15(12):1833-1840.
19. Yau WP, Chiu KY, Tang WM, Ng TP. Coronal bowing of the femur and tibia in Chinese: its incidence and effects on total knee arthroplasty planning. *J Orthop Surg (Hong Kong)*. 2007; 15(1):32-36.
20. Mullaji A, Shetty GM. Computer-assisted total knee arthroplasty for arthritis with extra-articular deformity. *J Arthroplasty*. 2009; 24(8):1164-1169.
21. Ewald FC. The Knee Society total knee arthroplasty roentgenographic evaluation and scoring system. *Clin Orthop Relat Res*. 1989; (248):9-12.
22. Wülker N, Lambermont JP, Saccetti L, Lazaro JG, Nardi J. A prospective randomized study of minimally invasive total knee arthroplasty compared with conventional surgery. *J Bone Joint Surg Am*. 2010; 92(7):1584-1590.
23. King J, Stamper DL, Schaad DC, Leopold SS. Minimally invasive total knee arthroplasty compared with traditional total knee arthroplasty: assessment of the learning curve and the postoperative recuperative period. *J Bone Joint Surg Am*. 2007; 89(7):1497-1503.
24. Schroer WC, Diesfeld PJ, Reedy ME, LeMarr AR. Evaluation of complications associated with six hundred mini-subvastus total knee arthroplasties. *J Bone Joint Surg Am*. 2007; 89(suppl 3):76-81.
25. Krackow KA, Pepe CL, Galloway EJ. A mathematical analysis of the effect of flexion and rotation on apparent varus/valgus alignment at the knee. *Orthopedics*. 1990; 13(8):861-868.
26. Matziolis G, Krocker, Weiss U, Tohtz S, Perka C. A prospective, randomized study of computer-assisted and conventional total knee arthroplasty: three-dimensional evaluation of implant alignment and rotation. *J Bone Joint Surg Am*. 2007; 89(2):236-243.

Yang et al. Journal of Biomedical Science (2015) 22:40
DOI 10.1186/s12929-015-0147-x

科 技 部 Ministry of Science and Technology
The cost of publication in Journal of Biomedical Science is borne by the Ministry of Science and Technology, Taiwan.

JOURNAL OF BIOMEDICAL SCIENCE

RESEARCH Open Access

Altered exosomal protein expression in the serum of NF-κB knockout mice following skeletal muscle ischemia-reperfusion injury

Johnson Chia-Shen Yang[1†], Ming-Wei Lin[1†], Cheng-Shyuan Rau[2†], Seng-Feng Jeng[3], Tsu-Hsiang Lu[1], Yi-Chan Wu[1], Yi-Chun Chen[1], Siou-Ling Tzeng[1], Chia-Jung Wu[1] and Ching-Hua Hsieh[1*]

Abstract

Background: The NF-κB signaling pathway plays a role in local and remote tissue damage following ischemia-reperfusion (I/R) injury to skeletal muscles. Evidence suggests that exosomes can act as intercellular communicators by transporting active proteins to remote cells and may play a role in regulating inflammatory processes. This study aimed to profile the exosomal protein expression in the serum of NF-κB knockout mice following skeletal muscle ischemia-reperfusion injury.

Results: To investigate the potential changes in protein expression mediated by NF-κB in secreted exosomes in the serum following I/R injury, the levels of circulating exosomal proteomes in C57BL/6 and NF-κB$^{-/-}$ mice were compared using two dimensional differential in-gel electrophoresis (2-DE), liquid chromatography tandem mass spectrometry (LC-MS/MS), and proteomic analysis. In C57BL/6 mice, the levels of circulating exosomal proteins, including complement component C3 prepropeptide, PK-120 precursor, alpha-amylase one precursor, beta-enolase isoform 1, and adenylosuccinate synthetase isozyme 1, increased following I/R injury. However, in the NF-κB$^{-/-}$ mice, the expression of the following was upregulated in the exosomes: protease, serine 1; glyceraldehyde-3-phosphate dehydrogenase-like isoform 1; glyceraldehyde-3-phosphate dehydrogenase; and pregnancy zone protein. In contrast, the expression of apolipoprotein B, complement component C3 prepropeptide, and immunoglobulin kappa light chain variable region was downregulated in NF-κB$^{-/-}$ mice. Bioinformatic annotation using the Protein Analysis Through Evolutionary Relationships (PANTHER) database revealed that the expression of the exosomal proteins that participate in metabolic processes and in biological regulation was lower in NF-κB$^{-/-}$ mice than in C57BL/6 mice, whereas the expression of proteins that participate in the response to stimuli, in cellular processes, and in the immune system was higher.

Conclusions: The data presented in this study suggest that NF-κB might regulate exosomal protein expression at a remote site via circulation following I/R injury.

Keywords: Muscle ischemia-reperfusion (I/R) injury, Exosome, NF-κB, Two-dimensional-gel electrophoresis, Proteomics

* Correspondence: m93chinghua@gmail.com
†Equal contributors
[1]Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No. 123, Ta-Pei Road, Niao-Sung District, Kaohsiung City 833, Taiwan
Full list of author information is available at the end of the article

Background

Ischemia-reperfusion (I/R) injury to the skeletal muscle leads to the production of oxygen free radicals, resulting in the occurrence of tissue lipid peroxidation upon re-oxygenation, and release of pro-inflammatory cytokines such as IL-6, IL-1, and TNF-α through the NF-κB signaling pathway. In addition, I/R injury to the skeletal muscle not only affects the muscle but also causes injury to remote organs, which can lead to multiple organ failure and death [1–3]. NF-κB plays an important role in the pathogenesis of I/R injury to the skeletal muscle. Regulation of the initial phase of NF-κB activation provides physiological protection against severe ischemic stress [4, 5]. Selective inhibition of NF-κB has been suggested as a potential therapeutic intervention to treat I/R injury [6, 7]. Moreover, it has been reported that inhibition of NF-κB prevents local and remote organ injury following I/R injury [8].

Exosomes are small, spherical vesicles that are secreted upon fusion of the limiting membrane of multivesicular bodies with the plasma membrane [9]. It has been proposed that exosomes act as intercellular communicators with the carried active cytosolic proteins, mRNAs, and miRNAs [10]. The exosome content can also be transferred into the target cells, either through the direct fusion of exosomes with the cell membrane or through active uptake, which is mediated by endocytosis [11, 12]. The secretion of functional proteins by several cell types, including those of the skeletal muscle [13, 14], has been described as being associated with exosomes [15–17], and is involved in cellular stress responses [18–20] as well as in the modulation of immunological responses [19, 21, 22]. Importantly, the presence of oxidative or hypoxic stress can modulate the expression of biologically active proteins in cell-derived exosomes [9, 20, 22–24], suggesting that stress-related signaling via the exosomes could occur through the transfer of the exosomal protein content.

However, the active circulating exosomal proteins that may potentially participate in the regulation of biological responses following I/R injury to the skeletal muscle remain poorly understood. Moreover, although the inhibition of the NF-κB signaling pathway prevents injury to remote organs following I/R injury [8], it is not known whether there is an NF-κB-mediated change in the expression of circulating exosomal proteins during I/R injury. Therefore, the aim of this study was to profile the change in the expression of exosomal proteins in the serum of C57BL/6 and NF-κB$^{-/-}$ mice following I/R injury to the skeletal muscle, using the complementary techniques of two-dimensional electrophoresis (2-DE) and liquid chromatography tandem mass spectrometry (LC-MS/MS).

Methods

Animal handling

Twelve NF-κB$^{-/-}$ (B6.Cg-Nfkb1tm1Bal/J) mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and twelve C57BL/6 mice purchased from BioLasco were used in this study (Taipei, Taiwan). The animals were housed in a specific-pathogen-free (SPF) facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All surgical procedures, including analgesia, were performed according to national and institutional guidelines. Animal protocols were approved by the Institutional Animal Care and Usage Committee of the Chang Gung Memorial Hospital (permission number No. 2012091304). Briefly, mice were anesthetized using intraperitoneal injection of an anesthetic cocktail, which consisted of 0.1 mg/g ketamine and 0.01 mg/g xylazine (0.01 mL/g body weight). The anesthetized mice were restrained in the supine position on a heated pad to maintain their body temperature at 37 °C. The model of skeletal muscle I/R injury was performed according to our previous report [25]. The quadriceps muscle was perfused at the femoral artery and was then carefully separated from the femoral bone and from the underlying adductor muscle group. In the ischemic experimental group, ischemia was induced by carefully placing a microvascular clamp across the proximal site of the vascular pedicle for 4 h, after which the clamp was removed. The presence of good vascular flow through the pedicle was verified with direct magnified vision. In the sham-operated group, the muscle was isolated without inducing ischemia with a microvascular clamp. The incision wound was closed with interrupted sutures (4–0 nylon sutures), and the animals were allowed to awaken during the remaining reperfusion time.

Muscle histochemistry

To evaluate basic muscle morphology after I/R injury, the muscles of sham-operated mice and of experimental mice subjected to ischemia for 4 h and reperfusion for 4 h, 1 day, or 7 days were harvested. The muscles were subsequently covered in Tissue Tek O.C.T. compound (Sakura Finetek Inc., Torrance, CA, USA), frozen in liquid nitrogen–cooled isopentane, and stored at −80 °C until cryosectioning (7 μm). Sections were stained with hematoxylin and eosin.

Western blotting of nuclear p65

Nuclear protein extracts of the experimental muscles of C57BL/6 and NF-κB$^{-/-}$ mice at 4, 16, and 48 h after I/R injury were used for Western blot analysis using NE-PER extraction reagents according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL). Protein extracts (30 μg) were separated on 10 % SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked using nonfat

milk in Tween-20/Tris-buffered saline (TBST), and incubated with monoclonal rabbit anti-p65 antibody (Cell Signalling) and anti-lamin B1 (Santa Cruz, CA, USA), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies. Nuclear extract of human umbilical vein endothelial cells (HUVECs) against 10 ug/mL LPS (L3755; Sigma, St Louis, MO) treatment for 24 h was used for positive control of p65 expression according to our previous report [26]. The expression of nuclear p65 protein was assessed against that of lamin B1 using a FluorChem 8900 imaging system (Alpha Innotech, San Leandro, CA) ($n = 4$), and the intensity of each band was quantified using auto-background subtraction during spot density analysis using the AlphaEaseFC software (Alpha Innotech).

Exosome isolation

For proteomic analysis of the exosome contents, six C57BL/6 mice and six NF-κB$^{-/-}$ mice were used; in both cases, three mice were from the sham-operated group and three from the I/R injury experimental group. Whole blood was drawn from the sham-operated mice and mice subjected to ischemia for 4 h and reperfusion for 1 day, respectively, and collected in RNAprotect Animal Blood Tubes (Qiagen, Valencia, CA, USA) without anticoagulant. The whole blood samples were incubated at room temperature for 15 min and centrifuged at $3000 \times g$ for 15 min. Subsequently, white blood cells were carefully removed from the corresponding layers, and the serum (250 μL) was extracted and thawed on ice. The supernatants were transferred to sterile tubes containing 63 μL ExoQuick Precipitation Solution (System Biosciences, Mountain View, CA, USA) and were then mixed. The mixtures were incubated for a minimum of 12 h at 4 °C and were subsequently centrifuged at $1500 \times g$ for 30 min at 4 °C. The resuspended exosome pellets were then lysed in a protein lysis buffer.

Scanning electron microscopy

The exosome isolates were attached to double-sided adhesive tape, fixed to a stage, and then coated with nanogold particles. The exosomes were photographed using a JEOL JSM-5300 scanning electron microscope (Tokyo, Japan) for analysis of size and morphology.

Proteomic analysis

The exosomes were lysed in lysis buffer containing 2 % sodium dodecyl sulfate (SDS), 1 % Triton-X100, 0.1 M Tris (pH 7.4), and one tablet of Complete EDTA-free protease inhibitors (Roche, Indianapolis, IN, USA). Protein concentrations in the exosome lysates were determined using a BCA protein assay (Pierce, Rockford, IL, USA). The lysis mixture was incubated at room temperature for 60 min and was then centrifuged at $15{,}000 \times g$ for 60 min at 4 °C. Following centrifugation, the resulting supernatant was collected and then quantified with a 2D QUANT Protein Assay Kit (GE Healthcare, Piscataway, NJ, USA). The supernatant containing 300 μg of total cellular protein (20 μL) was mixed with a sample buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 65 mM DTT, 0.2 % ampholytes, and a small amount of bromophenol blue) to obtain a final volume of 450 μL. 2-DE analysis was performed using a 24 cm Immobiline DryStrip (GE Healthcare). Subsequent rehydration followed by isoelectric focusing (set at the highest current, 50 μA/gel, 20 °C) and then SDS-polyacrylamide gel electrophoresis were performed. Following electrophoresis, silver staining, which is compatible with mass spectrometry, was conducted. The gel was scanned using a UMAX Power Look 1100 transmission scanner to obtain images, which were subsequently analyzed with the PDQuest software, version 7.1.0. The protein spots (protein expression with changes greater than twofold, following I/R injury) were excised from the gels and were then subjected to in-gel digestion, after which LC-MS/MS was performed. The resulting data were analyzed using the Mascot database.

Enzyme-linked immunosorbent assay (ELISA)

To investigate whether the exosomal proteins identified are also present in the serum or the experimental skeletal muscle after I/R injury, expression of a representative exosomal protein, the complement component C3 prepropeptide, which was upregulated in C57BL/6 mice but downregulated in the exosomes of NF-κB$^{-/-}$ mice, was measured. The analysis was performed using exosomes from the sham-operated mice and from mice subjected to 4-h ischemia and reperfusion for 1 d ($n = 4$ for each group). C3 prepropeptide expression was measured by ELISA using a commercially supplied kit (Genway Biotech, San Diego, CA, USA). Briefly, each sample was diluted 1/100,000 in blocking buffer (50 mM Tris, 0.14 M NaCl, 1 % BSA, pH 8.0) and added to the wells of a 96-well plate coated with 100 μl of 2 μg/ml rabbit anti-human C3 prepropeptide. After incubation with 1:10,000 diluted horseradish peroxidase conjugate, 100 μl enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added for 30 min, then 100 μl of 2 M H_2SO_4 was applied to each well to stop the TMB reaction. The absorbance at 450 nm was measured using an ELISA plate reader. Each dilution was measured alongside a set of standards and the results averaged. Results are expressed as micrograms per milliliter (μg/mL) of serum and exosomes or per milligram (μg/mg) of tissue protein.

Functional analysis of the exosomal proteome

Proteins that had twofold or greater expression and were identified by LC-MS/MS were analyzed using the Protein Analysis Through Evolutionary Relationships (PANTHER;

http://www.pantherdb.org) classification system. The PANTHER classification system is a comprehensive method that combines gene function, gene ontology (GO), pathway, and statistical analysis tools to facilitate the analysis of large-scale, genome-wide data from sequencing, proteomics, or gene expression experiments [27]. The PANTHER system allows the prediction of protein classifications based on the proteins' GO, biological processes, and molecular functions.

Results

Histological analysis involving hematoxylin and eosin staining of the muscles from the experimental group confirmed the presence of I/R injury (Fig. 1). In comparison to the findings in the sham-operated group, increased swelling of muscle fibers with loss of contact integrity among muscle fibers and increased cellular infiltration were found after I/R injury. This morphological change in muscle fibers could still be observed 7 days after the I/R injury. Western blot analysis of the nuclear protein extracts revealed a significant p65 nuclear translocation in the experimental muscle at 4 and 16 h but not 48 h after I/R injury in the C57BL/6 mice. In contrast, no p65 nuclear translocation was found in the NF-κB$^{-/-}$ mice (Fig. 2). The electron microscopy experiments performed using the purified exosome samples obtained from the serum revealed the presence of vesicles that were derived from a C57BL/6 mouse, with an approximate diameter of 50–200 nm (Fig. 3). A proteomics approach involving 2-DE was used to characterize the changes occurring in the serum exosomes following I/R injury and to compare such changes seen in C57BL/6 and NF-κB$^{-/-}$ mice. The resulting protein pattern was observed in a silver-stained 2-DE gel, and a representative image is shown in Fig. 4. The imaging software facilitated the analysis of protein spots on the gel. Protein spots that exhibited a twofold or greater change in expression levels were excised from the silver-stained gels and identified using LC-MS/MS analysis (Table 1). Following I/R injury to the skeletal muscle in C57BL/6 mice, an increase was found in the level of circulating exosomal proteins, including complement component C3 prepropeptide, PK-120 precursor, alpha-amylase 1 precursor, beta-enolase isoform 1, and adenylosuccinate synthetase isozyme 1. However, in the NF-κB$^{-/-}$ mice, the expression of the following were upregulated in the exosomes: protease, serine 1; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-like isoform 1; GAPDH; and pregnancy zone protein. The expression of apolipoprotein B (ApoB), complement component C3 prepropeptide, and immunoglobulin kappa light chain variable region was downregulated in NF-κB$^{-/-}$ mice. In C57BL/6 mice, the expression levels of C3 prepropeptide in the exosomes and serum after I/R injury were significantly higher than those in the sham group (Fig. 5). However, the fold increase in expression was much higher in the exosomes than in the serum, indicating that the majority of the C3 prepropeptide was located in exosomes. In contrast, the expression level of C3 prepropeptide was significantly lower in both exosomes and serum of the NF-κB$^{-/-}$ mice after I/R injury. There was no significant change in C3 prepropeptide expression in the I/R skeletal muscle in both C57BL/6 and NF-κB$^{-/-}$ mice, indicating that the I/R muscle is not the source of C3 prepropeptide in the exosomes. The data were subsequently subjected to ontology and pathway analyses using the PANTHER software and were then classified based on their respective protein classes, molecular functions, and biological processes. Exosomal proteins that were found to have a greater than twofold change

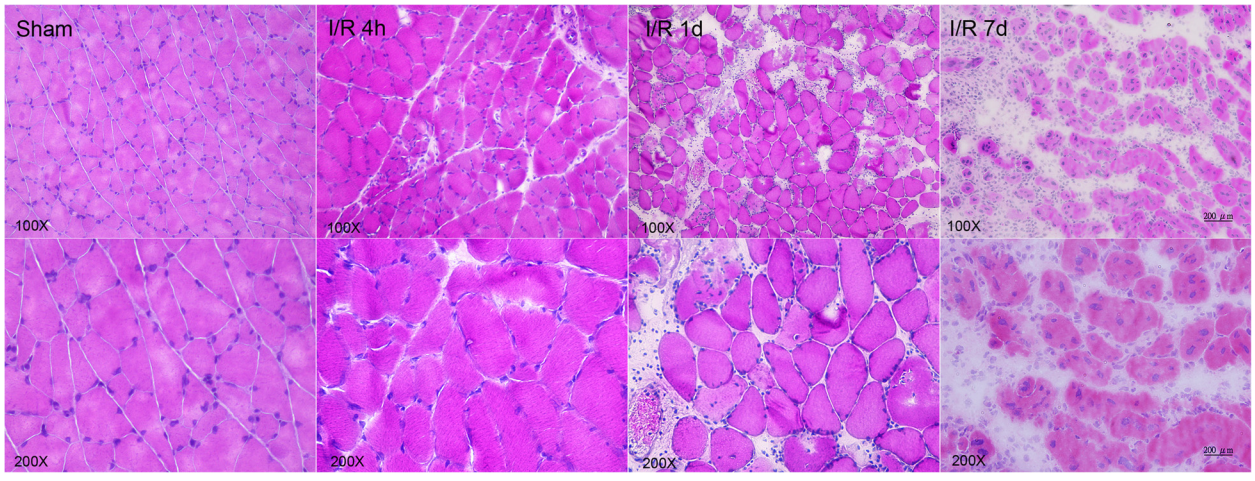

Fig. 1 Hematoxylin and eosin staining of the cryosectioned muscles of sham-operated mice and the muscles of experimental mice subjected to ischemia for 4 h and reperfusion for 4 h, 1 day, or 7 days

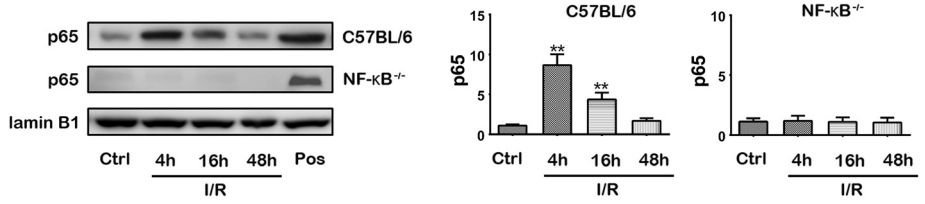

Fig. 2 Expression fold of p65 in Western blot analysis of the nuclear protein extracts of the experimental muscles at 4, 16, and 48 h after I/R injury in C57BL/6 and NF-κB$^{-/-}$ mice. (*$p < 0.01$, compared to sham-operated mice in quadruplicate) Ctrl, Control sham-operated mice; Pos, Positive control of p65 expression

in expression following I/R injury in the NF-κB$^{-/-}$ mice were classified into six GO categories, that is, immunity proteins (22.2 %), enzyme modulators (22.2 %), hydrolases (11.1 %), oxidoreductases (11.1 %), proteases (11.1 %), and signaling molecules (11.1 %). However, in the C57BL/6 group, the functions of the differentially expressed genes consisted of enzyme modulators (28.6 %), hydrolases (14.3 %), ligases (14.3 %), lyases (14.3 %), signaling molecules (14.3 %), and immunity proteins (14.3 %) (Fig. 6). In terms of the molecular function categories, the proteins in both mouse groups that belonged to the functional categories of regulation of enzyme activity, binding, and catalytic activity were highly overrepresented (Fig. 6). In terms of the biological processes, most of the clusters identified were associated with the categories of metabolic processes, responses to stimuli, biological regulation, cellular processes, and immune system processes (Fig. 7). The expression of proteins that participate in metabolic processes and biological regulation was lower in NF-κB$^{-/-}$ mice than in C57BL/6 mice, whereas the expression of proteins that participate in the response to stimuli, cellular processes, and immune system processes was higher.

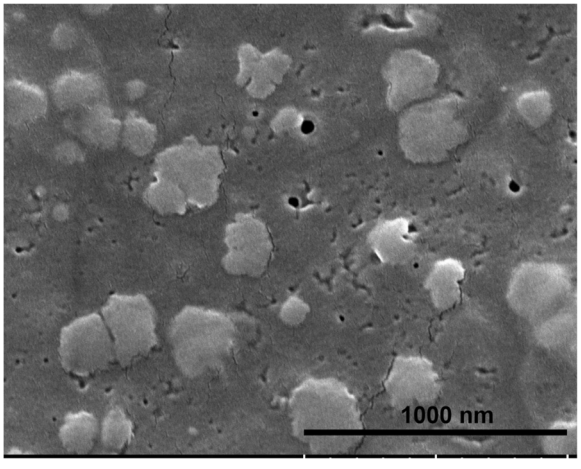

Fig. 3 Electron microscopic image of purified serum exosomes derived from a normal C57BL/6 mouse

Discussion

Our analysis revealed that, during I/R injury, the NF-κB-mediated alterations in the expression of circulating exosomal proteins are related to biological processes that are involved in complement activation, proteolysis, cellular processes, response to stimuli, regulation of catalytic activities, and glycolysis. The putative influences of these proteins on the downstream pathways that are related to I/R include cellular processes, cell communication, and signaling pathways. Based on the classes of exosomal proteins, our data also revealed that the expression of oxidoreductases is upregulated in NF-κB groups, suggesting that the inhibition of NF-κB may modify the expression of the redox modulation enzymes in the exosomes secreted. Oxidoreductases are enzymes that catalyze numerous redox reactions. Their actions include the catalysis of the transformation of free, neutral oxygen gas into oxygen free radicals, superoxide, hydroperoxide, single oxygen molecules, and hydrogen peroxide. They also make up the most important free radical

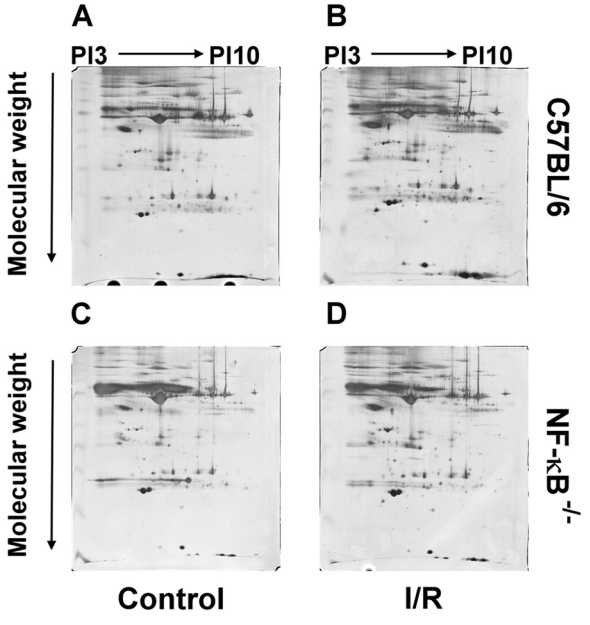

Fig. 4 2-DE protein patterns in serum exosomes from C57BL/6 and NF-κB$^{-/-}$ mice after I/R injury. (a) C57BL/6 sham control; (b) C57BL/6 I/R injury; (c) NF-κB-/- sham control; (d) NF-κB-/- I/R injury.

Table 1 Proteins exhibiting a twofold or greater change in expression levels in serum exosomes from C57BL/6 and NF-κB$^{-/-}$ mice in response to I/R injury ($p < 0.05$)

	ID	Name	Score	Expression
C57BL/6	gi|387114	complement component C3 prepropeptide	1963	up
	gi|2739028	PK-120 precursor	2421	up
	gi|160358819	alpha-amylase 1 precursor	2557	up
	gi|6679651	beta-enolase isoform 1	850	up
	gi|6671519	adenylosuccinate synthetase isozyme 1	1577	up
NF-κB$^{-/-}$	gi|16716569	protease, serine, 1	1330	up
	gi|309266468	glyceraldehyde-3-phosphate dehydrogenase-like isoform 1	1346	up
	gi|6679937	glyceraldehyde-3-phosphate dehydrogenase	1075	up
	gi|34785996	pregnancy zone protein	1192	up
	gi|27371137	apolipoprotein B	2617	down
	gi|387114	complement component C3 prepropeptide	2372	down
	gi|158346648	immunoglobulin kappa light chain variable region	3238	down

scavenger systems exemplified by catalase, superoxide dismutase, and glutathione peroxidase [28]. Oxidoreductases represent one of the most important free radical scavenger systems and play a cytoprotective role beyond their antioxidant function [29–31]. The possibility of exosomal oxidoreductases in the NF-κB$^{-/-}$ mouse serum contributing to a protective role for the distal organs after I/R muscle injury is interesting and requires further investigation.

The complement system is a host defense system that identifies injured cells, recruits inflammatory cells, and induces cell lysis [32]. The complement component C3 is a pro-inflammatory protein and an important component of the complement cascade. Expression of complement component one is elevated through oxidative stress following ischemic reperfusion [33]. Moreover, many reports have attributed the protective effects of antioxidants to their capacity to suppress the expression of complement component C3 [34–36] and the subsequent blocking of NF-κB activation which inhibits complement component 3 [37]. Importantly, the complement cascade has been implicated in the process of I/R injury. In this study, the expression of the complement component C3 prepropeptide in the serum exosomes of C57BL/6 mice was upregulated in response to I/R muscle injury; however, the expression of this protein was downregulated in NF-κB$^{-/-}$ mice. In addition, although the expression levels of C3 prepropeptide were significantly higher in the exosomes and serum after I/R injury, the fold increase in expression was much higher in the exosomes than in the serum, indicating that most of the C3 prepropeptide was located in the exosomes. Thus, exosomes may participate in inflammatory processes in remote organs during I/R muscle injury; however, such inflammatory effects transmitted by exosomes may decrease during NF-κB inhibition. However, this hypothesis requires further experiments to be validated.

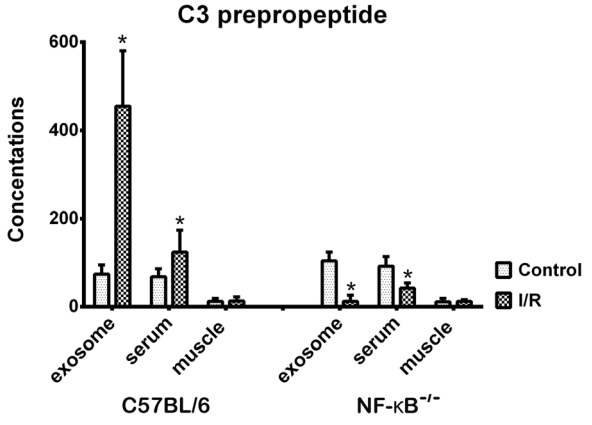

Fig. 5 Expression levels of C3 prepropeptide measured by enzyme-linked immunosorbent assay (ELISA) in the exosomes, serum, and experimental skeletal muscle of C57BL/6 and NF-κB$^{-/-}$ mice after I/R injury (*$p < 0.01$, compared to sham-operated mice in quadruplicate)

The upregulation of protease, serine one and pregnancy zone protein, an inhibitor of proteinases, was observed in the NF-κB$^{-/-}$ groups, suggesting that the modulation of the function of T lymphocytes and fibrinolysis by these proteins was negatively regulated through the NF-κB pathway following I/R injury [38, 39]. GAPDH, a glycolytic enzyme, has been recently identified as being involved in the initiation of apoptosis [40]. The expression of a phosphorylation-defective GAPDH mutant during I/R injury reduces cell apoptosis [41], suggesting that GAPDH may play a critical role in the progression and spread of ischemic damage [42]. In this study, the expression of exosomal GAPDH was upregulated in the NF-κB$^{-/-}$ groups, suggesting that the expression of exosomal GAPDH is NF-κB-independent and that there may be a negative

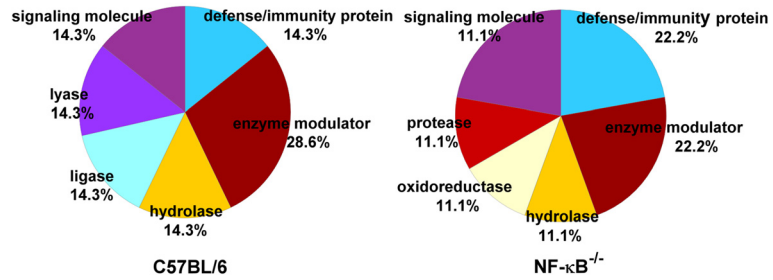

Fig. 6 Alteration in serum exosome protein expression after I/R injury. Protein classes were identified and analyzed by mass spectrometry and PANTHER software

feedback loop of regulation following I/R injury through NF-κB-independent pathways.

In our study, the expression of ApoB was downregulated in NF-κB$^{-/-}$ mice but not in C57BL/6 mice. ApoB is the primary apolipoprotein of low-density lipoproteins and is responsible for transporting cholesterol to the tissues. The retention and modification of ApoB in the extracellular matrix, followed by proliferation and inflammation, can lead to the chronic progression of atherosclerotic lesions [43]. Alpha-amylase is an enzyme that hydrolyses the bonds of large, α-linked polysaccharides, such as those in starch and glycogen, yielding glucose and maltose. Our results indicated that the expression of alpha-amylase precursor was elevated in C57BL/6 mice. In previous studies,

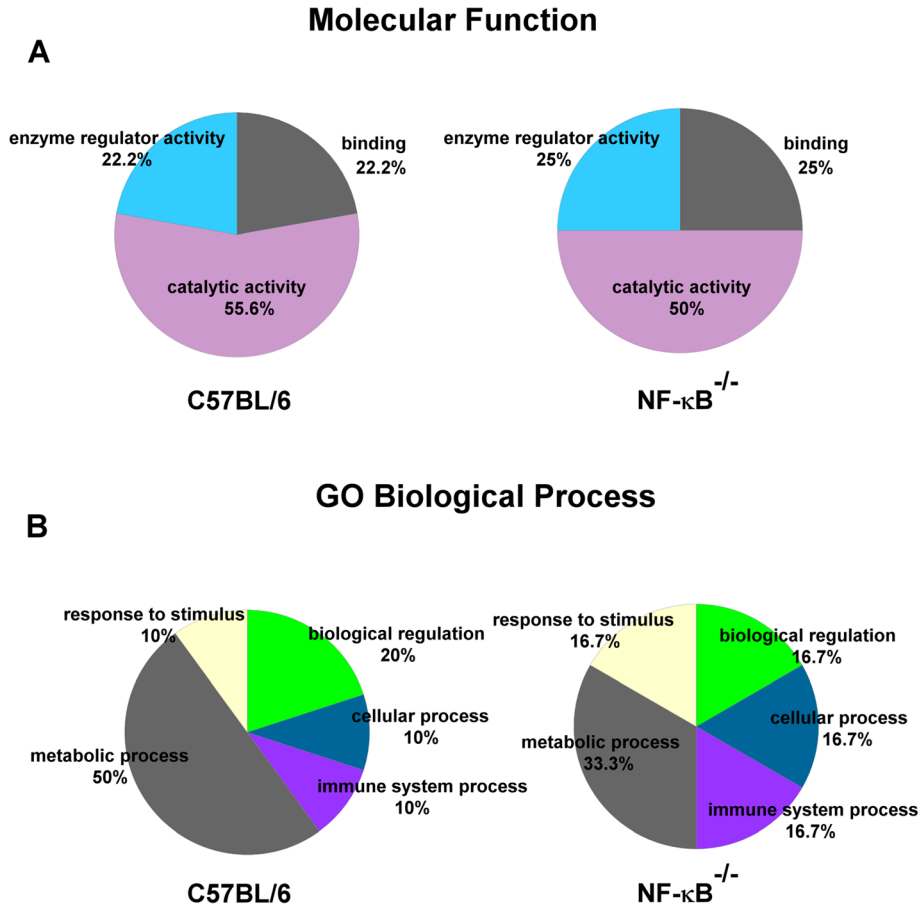

Fig. 7 Analysis of exosomal proteins identified by mass spectrometry using PANTHER software. Exosomal proteins isolated from C57BL/6 or NF-κB$^{-/-}$ mice were classified using PANTHER based on (a) their molecular functions and (b) biological processes

this enzyme has been described as a biomarker of injury to peripheral organs in the serum [44, 45]. Ischemia caused a moderate release of enzymes and an increase in the activity of alpha-amylase [46]. The damage due to ischemia and reperfusion in the pancreas of rats was associated with increased levels of serum alpha-amylase [47]. The elevated plasma levels of alpha-amylase were also investigated in advanced chronic heart failure secondary to ischemic cardiomyopathy [48]. In response to I/R injury, exosomes may serve as carriers of alpha-amylase in the serum and elevation of levels of alpha-amylase in circulation is inhibited by the NF-κB signaling pathway.

Although our data do not provide definitive and exact evidence of the function of the serum exosomes in remote organs following I/R injury, our findings do supplement the current knowledge base regarding their potential function in the regulation of inflammation. The data presented in this study suggest that NF-κB might regulate exosomal protein expression at remote sites via the circulation, following I/R injury.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JCY and CHH were responsible for the writing of the manuscript. MWL and SFJ were responsible for the design and coordination of the data acquisition and analysis. CSR and THL participated in the animal experiments and acquisition of the study specimens. YCW and YCC were involved in the proteomic experiment. SLT and CJW contributed to the histochemistry, Western blotting, and ELISA. All authors read and approved the final manuscript.

Acknowledgements
The work was supported by the Genomic & Proteomic Core Laboratory, Department of Medical Research, Kaohsiung Chang Gung Memorial Hospital (CMRPG8D0131 to CS Rau & CMRPG8D0311 to CH Hsieh) and E-Da Hospital (EDPJ102035)

Author details
[1]Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No. 123, Ta-Pei Road, Niao-Sung District, Kaohsiung City 833, Taiwan. [2]Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung City, Taiwan. [3]Department of Plastic Surgery, E-Da Hospital, I-Shou University, Kaohsiung City, Taiwan.

Received: 16 December 2014 Accepted: 15 May 2015
Published online: 10 June 2015

References
1. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg. 2002;10(6):620–30.
2. Walker PM. Ischemia/reperfusion injury in skeletal muscle. Ann Vasc Surg. 1991;5(4):399–402.
3. Lentsch AB, Yoshidome H, Warner RL, Ward PA, Edwards MJ. Secretory leukocyte protease inhibitor in mice regulates local and remote organ inflammatory injury induced by hepatic ischemia/reperfusion. Gastroenterology. 1999;117(4):953–61.
4. Lille ST, Lefler SR, Mowlavi A, Suchy H, Boyle Jr EM, Farr AL, et al. Inhibition of the initial wave of NF-kappaB activity in rat muscle reduces ischemia/reperfusion injury. Muscle Nerve. 2001;24(4):534–41.
5. Park JW, Qi WN, Cai Y, Urbaniak JR, Chen LE. Proteasome inhibitor attenuates skeletal muscle reperfusion injury by blocking the pathway of nuclear factor-kappaB activation. Plast Reconstr Surg. 2007;120(7):1808–18.
6. Kim YS1, Kim JS, Kwon JS, Jeong MH, Cho JG, Park JC, Kang JC, Ahn Y: BAY 11-7082, a nuclear factor-κB inhibitor, reduces inflammation and apoptosis in a rat cardiac ischemia-reperfusion injury model. International heart journal 2010;5(5):348-353.
7. Andrade-Silva AR, Ramalho FS, Ramalho LN, Saavedra-Lopes M, Jordao Jr AA, Vanucchi H, et al. Effect of NFkappaB inhibition by CAPE on skeletal muscle ischemia-reperfusion injury. J Surg Res. 2009;153(2):254–62.
8. Suzuki T, Yamashita K, Jomen W, Ueki S, Aoyagi T, Fukai M, et al. The novel NF-kappaB inhibitor, dehydroxymethylepoxyquinomicin, prevents local and remote organ injury following intestinal ischemia/reperfusion in rats. J Surg Res. 2008;149(1):69–75.
9. Burke M, Choksawangkarn W, Edwards N, Ostrand-Rosenberg S, Fenselau C. Exosomes from myeloid-derived suppressor cells carry biologically active proteins. J Proteome Res. 2014;13(2):836–43.
10. Yoon YJ, Kim OY, Gho YS. Extracellular vesicles as emerging intercellular communicasomes. BMB Reports 2014;47(10):531-539
11. Svensson KJ, Christianson HC, Wittrup A, Bourseau-Guilmain E, Lindqvist E, Svensson LM, et al. Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. J Biol Chem. 2013;288(24):17713–24.
12. Nanbo A, Kawanishi E, Yoshida R, Yoshiyama H. Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells. J Virol. 2013;87(18):10334–47.
13. Hudson MB, Woodworth-Hobbs ME, Zheng B, Rahnert JA, Blount MA, Gooch JL, et al. miR-23a is decreased during muscle atrophy by a mechanism that includes calcineurin signaling and exosome-mediated export. Am J Physiol Cell Physiol. 2014;306(6):C551–8.
14. Forterre A, Jalabert A, Chikh K, Pesenti S, Euthine V, Granjon A, et al. Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. Cell Cycle. 2014;13(1):78–89.
15. Ung TH, Madsen HJ, Hellwinkel JE, Lencioni AM, Graner MW. Exosome proteomics reveals transcriptional regulator proteins with potential to mediate downstream pathways. Cancer Sci. 2014;105(11):1384-1392
16. Jaworski E, Narayanan A, Van Duyne R, Shabbeer-Meyering S, Iordanskiy S, Saifuddin M, et al. Human T-lymphotropic Virus Type 1-infected Cells Secrete Exosomes That Contain Tax Protein. J Biol Chem. 2014;289(32):22284–305.
17. Beckham CJ, Olsen J, Yin PN, Wu CH, Ting HJ, Hagen FK, et al. Bladder cancer exosomes contain EDIL-3/Del1 and facilitate cancer progression. J Urol. 2014;192(2):583–92.
18. Fruhbeis C, Frohlich D, Kuo WP, Amphornrat J, Thilemann S, Saab AS, et al. Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. PLoS Biol. 2013;11(7), e1001604.
19. Beninson LA, Fleshner M. Exosomes: An emerging factor in stress-induced immunomodulation. Seminars Immunol. 2014;26(5):394-401.
20. de Jong OG, Verhaar MC, Chen Y, Vader P, Gremmels H, Posthuma G, et al. Cellular stress conditions are reflected in the protein and RNA content of endothelial cell-derived exosomes. J Extracellular Vesicles. 2012;1.
21. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol. 2014;14(3):195–208.
22. Sonoda H, Yokota-Ikeda N, Oshikawa S, Kanno Y, Yoshinaga K, Uchida K, et al. Decreased abundance of urinary exosomal aquaporin-1 in renal ischemia-reperfusion injury. Am J Physiol Renal Physiol. 2009;297(4):F1006–16.
23. Salomon C, Kobayashi M, Ashman K, Sobrevia L, Mitchell MD, Rice GE. Hypoxia-induced changes in the bioactivity of cytotrophoblast-derived exosomes. PLoS One. 2013;8(11), e79636.
24. Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, et al. Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int. 2006;70(10):1847–57.
25. Yang JC, Wu SC, Rau CS, Chen YC, Lu TH, Wu YC, et al. TLR4/NF- kappa B-Responsive MicroRNAs and Their Potential Target Genes: A Mouse Model of Skeletal Muscle Ischemia-Reperfusion. Injury. 2015;2015:410721.
26. Rau CS, Yang JC, Chen YC, Wu CJ, Lu TH, Tzeng SL, et al. Lipopolysaccharide-induced microRNA-146a targets CARD10 and regulates angiogenesis in human umbilical vein endothelial cells. Toxicol Sci. 2014;140(2):315–26.
27. Mi H, Muruganujan A, Casagrande JT, Thomas PD. Large-scale gene function analysis with the PANTHER classification system. Nat Protoc. 2013;8(8):1551–66.
28. Bellisola G, Casaril M, Gabrielli GB, Caraffi M, Corrocher R. Catalase activity in human hepatocellular carcinoma (HCC). Clin Biochem. 1987;20(6):415–7.

29. Dick RA, Kwak MK, Sutter TR, Kensler TW. Antioxidative function and substrate specificity of NAD(P)H-dependent alkenal/one oxidoreductase. A new role for leukotriene B4 12-hydroxydehydrogenase/15-oxoprostaglandin 13-reductase. J Biol Chem. 2001;276(44):40803–10.
30. Dinkova-Kostova AT, Talalay P. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector. Arch Biochem Biophys. 2010;501(1):116–23.
31. Brass EP, Hiatt WR, Gardner AW, Hoppel CL. Decreased NADH dehydrogenase and ubiquinol-cytochrome c oxidoreductase in peripheral arterial disease. Am J Physiol Heart Circ Physiol. 2001;280(2):H603–9.
32. del Zoppo GJ. In stroke, complement will get you nowhere. Nat Med. 1999;5(9):995–6.
33. Eisenhardt SU, Schmidt Y, Karaxha G, Iblher N, Penna V, Torio-Padron N, et al. Monitoring molecular changes induced by ischemia/reperfusion in human free muscle flap tissue samples. Ann Plast Surg. 2012;68(2):202–8.
34. Yang J, Ahn HN, Chang M, Narasimhan P, Chan PH, Song YS. Complement component 3 inhibition by an antioxidant is neuroprotective after cerebral ischemia and reperfusion in mice. J Neurochem. 2013;124(4):523–35.
35. Olsson S, Stokowska A, Holmegaard L, Jood K, Blomstrand C, Pekna M, et al. Genetic variation in complement component C3 shows association with ischaemic stroke. Eur J Neurol. 2011;18(10):1272–4.
36. Mocco J, Mack WJ, Ducruet AF, Sosunov SA, Sughrue ME, Hassid BG, et al. Complement component C3 mediates inflammatory injury following focal cerebral ischemia. Circ Res. 2006;99(2):209–17.
37. Moon MR, Parikh AA, Pritts TA, Fischer JE, Cottongim S, Szabo C, et al. Complement component C3 production in IL-1beta-stimulated human intestinal epithelial cells is blocked by NF-kappaB inhibitors and by transfection with ser 32/36 mutant IkappaBalpha. J Surg Res. 1999;82(1):48–55.
38. Skornicka EL, Kiyatkina N, Weber MC, Tykocinski ML, Koo PH. Pregnancy zone protein is a carrier and modulator of placental protein-14 in T-cell growth and cytokine production. Cell Immunol. 2004;232(1–2):144–56.
39. Chaitanya GV, Eeka P, Munker R, Alexander JS, Babu PP. Role of cytotoxic protease granzyme-b in neuronal degeneration during human stroke. Brain Pathol. 2011;21(1):16–30.
40. Sirover MA. Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and in cell pathology. J Cell Biochem. 1997;66(2):133–40.
41. Yogalingam G, Hwang S, Ferreira JC, Mochly-Rosen D. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) phosphorylation by protein kinase Cdelta (PKCdelta) inhibits mitochondria elimination by lysosomal-like structures following ischemia and reoxygenation-induced injury. J Biol Chemist. 2013;288(26):18947–60.
42. Tanaka R, Mochizuki H, Suzuki A, Katsube N, Ishitani R, Mizuno Y, et al. Induction of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in rat brain after focal ischemia/reperfusion. J Cereb Blood Flow Metab. 2002;22(3):280–8.
43. Hurt-Camejo E, Camejo G. Why are plasmatic apoB lipoproteins atherogenic? The hypothesis of response to retention. Investig Clin. 2001;42 Suppl 1:43–73.
44. Michiue T, Ishikawa T, Kawamoto O, Chen JH, Wang Q, Zhu BL, et al. Postmortem serum levels of amylase and gamma glutamyl transferase (GGT) as markers of systemic tissue damage in forensic autopsy. Legal Med. 2013;15(2):79–84.
45. Malinoski DJ, Hadjizacharia P, Salim A, Kim H, Dolich MO, Cinat M, et al. Elevated serum pancreatic enzyme levels after hemorrhagic shock predict organ failure and death. J Trauma. 2009;67(3):445–9.
46. Sokolowski A, Spormann H, Urbahn H, Letko G. Contribution of pancreatic edema and short-term ischemia to experimental acute pancreatitis in the rat. II. Behaviour of serum parameters. Zeitschrift fur experimentelle Chirurgie, Transplantation, und kunstliche Organe : Organ der Sektion Experimentelle Chirurgie der Gesellschaft fur Chirurgie der DDR. 1986;19(6):331–9.
47. Viola G, Al-Mufti RA, Sohail M, Williamson RC, Mathie RT. Nitric oxide induction in a rat model of selective pancreatic ischemia and reperfusion. Hepato-Gastroenterology. 2000;47(35):1250–5.
48. Parissis JT, Adamopoulos SN, Venetsanou KF, Karas SM, Kremastinos DT. Elevated plasma amylase levels in advanced chronic heart failure secondary to ischemic or idiopathic dilated cardiomyopathy: correlation with circulating interleukin-6 activity. J Interferon Cytokine Res. 2003;23(6):329–33.

IVYSPRING INTERNATIONAL PUBLISHER

International Journal of Medical Sciences

2015; 12(8): 650-654. doi: 10.7150/ijms.12304

Research Paper

Regulatory and Effector Helper T-Cell Profile after Nerve Xenografting in the Toll-Like Receptor-Deficient Mice

Cheng-Shyuan Rau[1*]; Ming-Wei Lin[2*]; Shao-Chun Wu[3*]; Yi-Chan Wu[2], Tsu-Hsiang Lu[2], Siou-Ling Tzeng[2], Yi-Chun Chen[2], Chia-Jung Wu[2], Ching-Hua Hsieh[2]✉

1. Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taiwan
2. Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taiwan
3. Department of Anesthesiology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taiwan

*These authors contributed equally to this work

✉ Corresponding author: Ching-Hua Hsieh, M.D., PhD., FACS, Department of Plastic and Reconstructive Surgery Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan. Tel: 886-7-7327476; E-mail: m93chinghua@gmail.com

Received: 2015.04.01; Accepted: 2015.07.18; Published: 2015.08.01

Abstract

Introduction: The balance between regulatory T cells (Tregs) and effector T help cells (Th cells) is critical for the control of adaptive immune response during nerve transplantation. However, whether the homeostasis of immune regulation between Tregs and Th cells requires toll-like receptor (TLR) signaling is unclear. The aim of this study is to profile the distribution of spleen Tregs and Th cells in a mouse model of nerve xenografting in the TLR2 and NF-κB gene knockout mice.

Methods: The sciatic nerve was taken from a SD rat or an allogeneic mouse and transplanted to a right back leg of recipient C57BL/6, TLR2$^{-/-}$, or NF-κB$^{-/-}$ mice by subcutaneous transplantation. After 7 days, the T lymphocytes were then isolated from spleen, stained with phenotyping kits, and analyzed by flow cytometry.

Results: The results showed that Tregs were decreased after nerve xenografting in the recipient C57BL/6 mouse. In addition, nerve xenografting also increased the Th1 and Th17 but not the Th2 cell populations. In contrast, amelioration of the Tregs elimination was found in TLR2$^{-/-}$ and NF-κB$^{-/-}$ mice after transplantation of the nerve xenograft. Moreover, the mice lacking TLR2 or NF-κB showed attenuation of the increase in Th1 and Th17 cells after nerve xenografting.

Conclusions: TLR signaling is involved in T cell population regulation during tissue transplantation. Knock-out of TLR2 and NF-κB prevented Tregs elimination and inhibited Th1- and Th17-driven immune response after nerve xenografting. This study highlighted the potential of inhibiting TLR signaling to modulate T cell-mediated immune regulation to facilitate tolerance to nerve transplantation.

Key words: Nerve xenografting, Toll-like receptor (TLR), Regulatory T cells (Tregs), T help cells (Th cells)

Introduction

Nerve allograft had been reported to successfully treat the gap of the injured peripheral nerve. However, neurologic recovery with nerve allotransplantation is still limited by immune response over-activation and graft rejection and requires a short-term use of immunosuppressive agents. Nerve allo- or xeno-transplantation results in activation of both innate and adaptive immunity. TLRs are sensors

of both innate and adaptive immunity, and they play critical roles in nerve graft rejection and dysfunction after transplantation [1, 2]. Moreover, pathogens, surgical trauma and ischemic injury in the graft may also result in TLR stimulation [3]. Activation of TLRs may initiate intracellular signal transduction and lead to activation of the transcription factor, NF-κB, to release proinflammatory cytokines, such as IL-1β, TNF-α, and IL17 [4, 5].

$CD4^+$ regulatory T cells (Tregs) expressing FOXP3 play a critical role in the maintenance of immune tolerance during tissue transplantation [6]. The presence of Tregs is associated with allograft survival [7-9]. However, other phenotypes of T lymphocytes such effector T helper cells including $CD4^+INF\gamma^+$ Th1, $CD4^+IL4^+$ Th2, and $CD4^+IL17^+$ Th17 cells exert deleterious effects on tissue by activating of dendritic cells macrophages, and antigen-presenting cells. They secrete cytokines to activate inflammatory pathways, mainly through macrophage activation. However, overactivation of either pathway may cause tissue damage and result in graft rejection [10-12]. Tregs can regulate and suppress Th cells function mainly by cell-to-cell contact-dependent and antigen-independent mechanisms [13, 14]. Therefore, prolonged allograft acceptance requires Tregs, and the balance between Tregs and Th cells is critical for the inhibition of autoimmunity and the magnitude of the adaptive immune response [10, 15].

A previous study demonstrated that TLRs play a role in the maintenance of Tregs, are involved in T cell development [16]. Activation of TLRs can reprogram naïve T cells or Tregs to become effector Th cells [2, 17]. Therefore, TLR2 and its downstream target, NF-κB, may play a role in the homeostasis of immune regulation between Tregs and Th cells. Moreover, nerve xenografting had been reported to induce more severe immunoresponse of the recipient than the nerve allografting. Therefore, in this study, the aim is to investigate the role of TLR2/NF-κB on the homeostasis of immune regulation between Tregs and Th cells by profiling their distribution in the spleen in a mouse model of nerve xenografting using the TLR2 and NF-κB gene knockout mice.

Methods

Animal experiments

C57BL/6 mice and SD rats were purchased from BioLasco (Taipei, Taiwan). $Tlr2^{-/-}$ (B6.129-Tlr2tm1Kir/J), and $NF\text{-}\kappa B^{-/-}$ (B6.Cg-Nfkb1tm1Bal/J) mice were purchased from Jackson Laboratory (Bar Harbor, USA). All housing conditions were maintained, and surgical procedures, including analgesia, were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited SPF facility according to national and institutional guidelines. Animal protocols (permission number No. 2012091304) were approved by Chang Gung Memorial Hospital. Briefly, mice or rats were anesthetized with a combination of ketamine and xylazine, and the right back leg incision was made. The sciatic nerve (1 cm) was taken from a SD rat and transplanted to a right back leg of recipient mice by subcutaneous transplantation. This type of transplantation is defined as xenograft. The donor sciatic nerve taken from the same species of mouse is defined as allograft. The mice were sacrificed after 7 days after the surgery, and the spleen was removed for the T lymphocytes isolation.

T lymphocyte isolation and flow cytometry analysis

The spleen was removed from the mice and dissected. Splenic cell suspensions were gently pressed through a sterile 100 μm nylon mesh, and lymphocytes were isolated by ficoll gradient centrifugation (GE Healthcare, Sweden). The isolated lymphocytes were stained with a mouse Th1/Th2/Th17 phenotyping kit (the fluorescent antibodies: CD4, IFN-γ, IL-4 or IL-17A for detecting Th1, Th2 or Th17) and a mouse Th17/Treg phenotyping kit (the fluorescent antibodies: Foxp3 and CD4 for detecting Tregs) (BD Pharmingen, USA), and were acquired in a BD LSR II flow cytometer (BD Biosceinces, USA).

Statistical analysis

The data collected were analyzed using SPSS v.20 statistical software (IBM, Armonk, NY) for the independent Student's t-tests. All results are presented as the mean ± standard error. A p-value less than 0.05 was considered statistically significant.

Results

Nerve xenografting decreased Tregs population in wild type mice but not in $TRL2^{-/-}$ or $NF\text{-}\kappa B^{-/-}$ mice

To determine whether TLR2 or NF-κB plays a role in Tregs population modulation in nerve transplantation, we isolated spleen T lymphocytes from wild type or $TLR2^{-/-}$ or $NF\text{-}\kappa B^{-/-}$ mice to analyze $CD4^+Foxp3^+$ Tregs population in nerve allografts or xenografts. By flow cytometry analysis, we found that the $CD4^+Foxp3^+$ Tregs population was decreased in the spleen of control wild-type mice after nerve xenografting compared to that after allografting. However, elimination of the Tregs population was ameliorated in $NF\text{-}\kappa B^{-/-}$ mice (Fig 1). The results indicate that NF-κB may participate in Treg-mediated immune suppression after nerve xenografting.

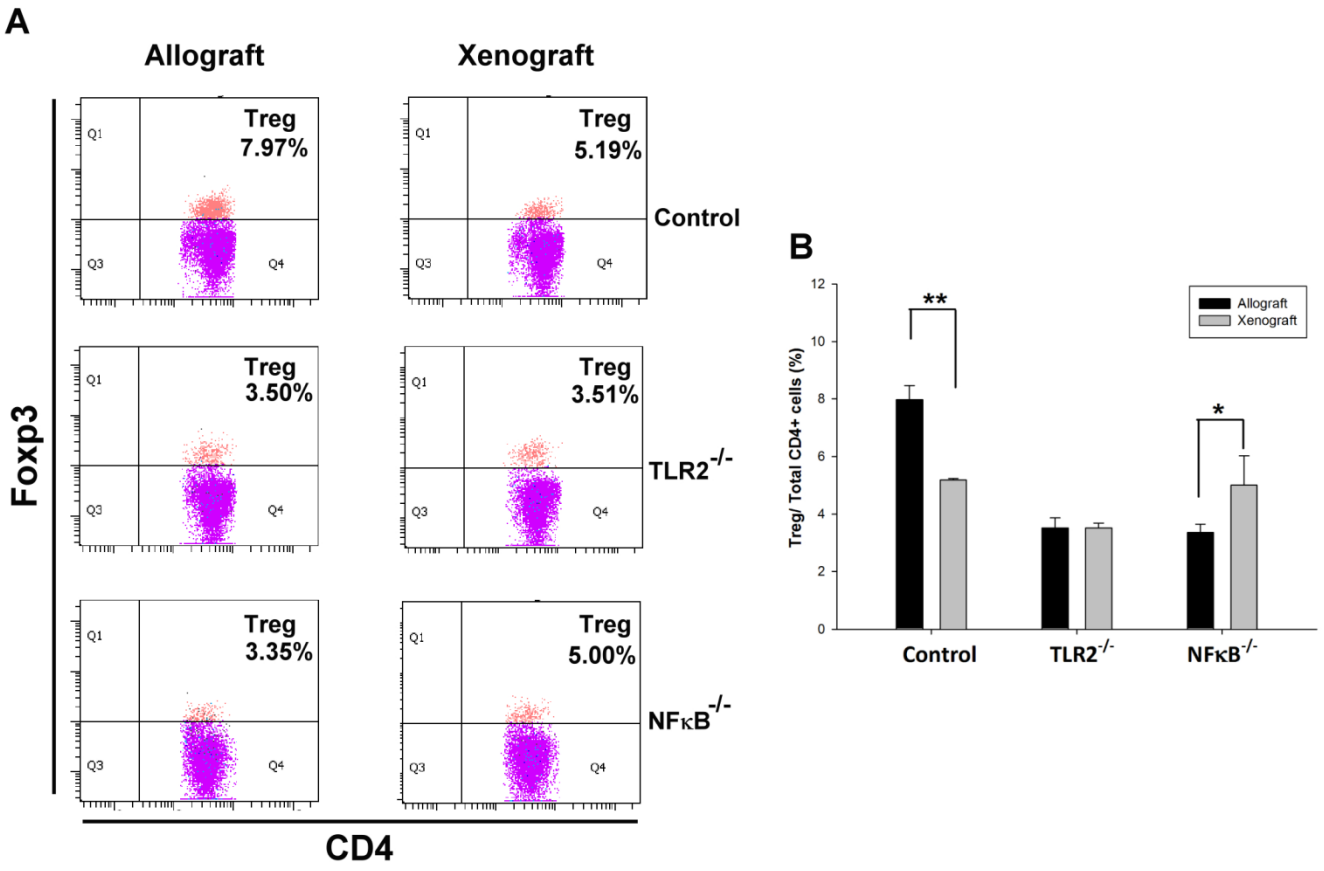

Figure 1. Knock-out of TLR2 and NF-κB prevented Tregs elimination after nerve xenografting. (A) Flow cytometry analysis of $CD4^+Foxp3^+$ Tregs population in nerve allografts or xenografts. (B) Quantification of the $CD4^+Foxp3^+$ Tregs population from flow cytometry analysis. The $CD4^+Foxp3^+$ Tregs population was decreased after nerve xenografting compared to allografting. Data are representative of 3 experiments with similar results. (n=3-5, *$p < 0.05$ and **$p < 0.01$ vs corresponding allograft).

Knock-out of TLR2 or NF-κB decreased Th1 but not Th2 cells after nerve xenografting

To determine whether TLR2 or NF-κB plays a role in Th1 or Th2 population regulation after nerve xenografting, we analyzed the Th1 or Th2 population after nerve xenografting compared to allografts in wild-type, TLR2$^{-/-}$ and NF-κB$^{-/-}$ mice. The flow cytometry analysis results indicate that knock-out of TLR2 or NF-κB decreased of Th1 but not Th2 population relative to the populations in control wild-type mice (Fig 2).

Knock-out of TLR2 or NF-κB inhibited the Th17 population increase after nerve xenografting

To determine whether the Th17 population is involved in TLR2- or NF-κB-dependent pathways, we analyzed Th17 cell population in wild-type, TLR2$^{-/-}$, or NF-κB$^{-/-}$ mice after nerve xenografting compared to allografting. We found that the Th17 population elevation was inhibited after nerve xenografting in TLR2$^{-/-}$ and NF-κB$^{-/-}$ mice (Fig 3). TLR2 and NF-κB may play a role in Th17-medated immune response after nerve xenografting.

Treg/(Th1+Th17) balance is TLR2 or NF-κB dependent after nerve xenografting

The balance between Tregs and Th cells is critical for the inhibition of autoimmunity. The results of the flow cytometry analysis showed that balance of the Tregs population over the Th1 and Th17 population was disturbed in wild-type mice after nerve xenografting. The value of Treg/(Th1+Th17) was decreased in control wild type but not in TLR2$^{-/-}$ or NF-κB$^{-/-}$ mice (Fig 4).

Discussion

Successful transplantation depends on the modulation of adaptive immunity in graft transplantation, as previous studies demonstrated that T cells are necessary and sufficient to reject almost all allogeneic tissues [18]. Tregs function as immune suppressors in regulating effector Th cells [13]. Therefore, the balance between Tregs and Th cells is critical for inhibition of the autoimmune response and graft rejection.

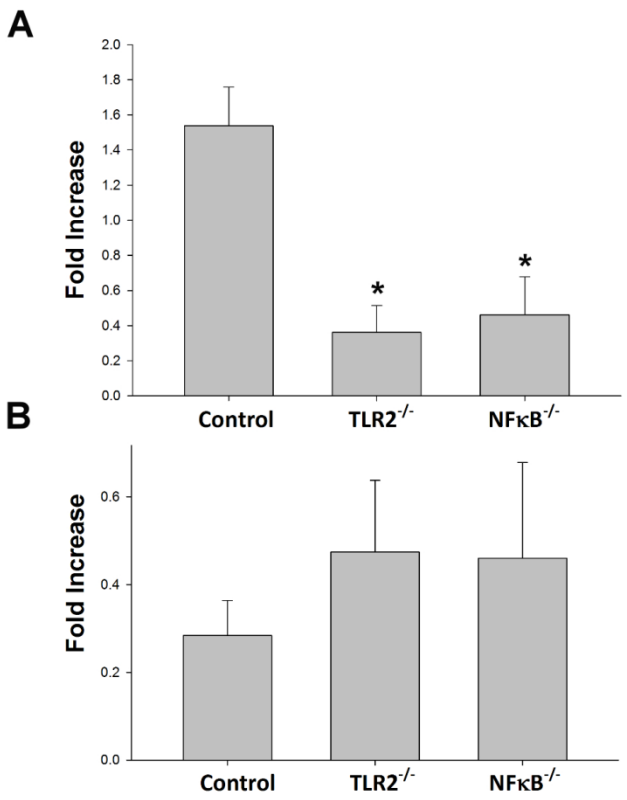

Figure 2. Knock-out of TLR2 and NF-κB prevented Th1 but not Th2 elevation after nerve xenografting. (A) Th1 and (B) Th2 cell expression in wild type or TLR2$^{-/-}$ or NF-κB$^{-/-}$ mice in xenografts compared to allografts by flow cytometry analysis. Data are expressed as fold increases (mean±SD) and are representative of 3 experiments with similar results. (n=3-5, *p < 0.05 vs control wild-type mice).

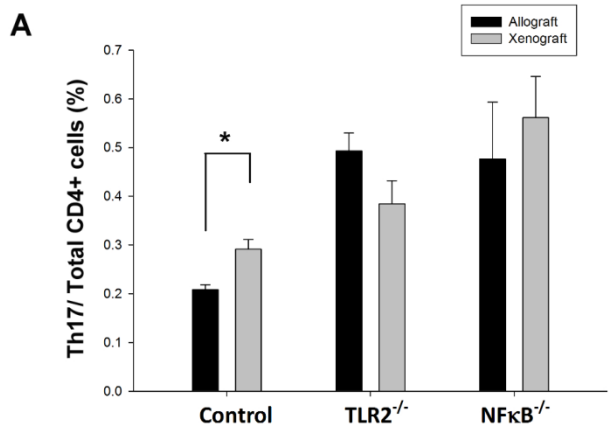

Figure 3. Knock-out of TLR2 and NF-κB inhibited Th17 increase after nerve xenografting. The Th17 cell population was increased after nerve xenografting compared to allografts in wild-type but not in TLR2$^{-/-}$ or NF-kB$^{-/-}$ mice, as observed by flow cytometry analysis. Data are presented as the mean ± SD and are representative of 3 experiments with similar results. (n=3-5, *p < 0.05 vs control wild type mice).

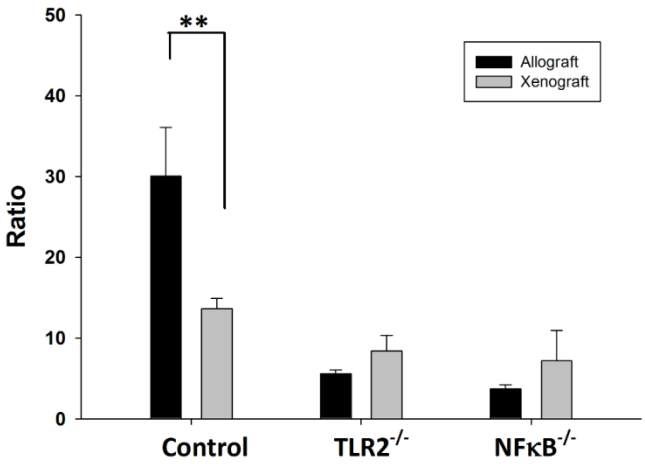

Figure 4. Ratio of Treg/(Th1+Th17) in wild type, TLR2$^{-/-}$, NF-κB$^{-/-}$ mice after nerve xenografting. The value of Treg/(Th1+Th17) was decreased in control wild type but not in TLR2$^{-/-}$ or NF-kB$^{-/-}$ mice. Data are presented as the mean ± SD. (n=3, *p<0.05 vs control wild-type mice).

TLRs are the first-line sensor for innate immunity, and mounting evidence suggests that stimulation of TLR activated serious inflammatory responses which resulted in tissue injury and graft rejection after transplantation [1, 18, 19]. Although TLRs and T cells are known to play major roles in innate immunity, whether knock-out of TLRs can regulate the T cell population after tissue transplantation remains unknown. To determine whether TLR2 is important in the modulation of Tregs and Th cells in tissue transplantation, we used a model of nerve transplantation in TLR2 or NF-κB knock-out mice. Our study demonstrated that knock-out of TLR2 or NF-κB prevented Tregs elimination and increased the population of Th1 and Th17 cells after nerve xenografting. Recent studies suggested that TLR ligands regulate T cell activation, as well as T cell differentiation [17, 19]. Therefore, TLR2 ligands may modulate T cell population through NF-κB by direct action because TLR2 are present on Tregs and Th cells. TLR2 was reported play a role in Tregs proliferation, and activation of TLR2 can cause increased activity of Tregs [20, 21]. In addition, reduction of Tregs was observed in TLR2$^{-/-}$ mice [22]. However, TLR2 also promotes Th1 and Th17 cell polarization [23]. Activation of TLRs also activates effector Th cells to enhance Th cell-mediated cytotoxicity and result in transplantation failure [8, 20]. Therefore, the graft immune tolerance and transplantation success may correlate with the balance of Treg/Th cells [8, 24].

The TLR2-coupled protein, MyD88, is reported to be important for Th17 immunity against allogeneic grafts [25]. A similar study also suggested that TLR2 signaling leads to Th1 immunity [26]. Although a previous study suggested that TLR2 activation promotes the Th2 immune response [27], knock-out of

TLR2 altered Th1 but not Th2 population after nerve xenografting in this study. According to the Th1/Th2 balance hypothesis [10], the TLR2-NF-κB signaling pathway might have influenced the Th1-driven immune response in our nerve xenograft animal model.

Th17 cells, a newly recognized distinct subset of T helper cells, have been shown to play an important role in murine autoimmune diseases. They express a variety of potent proinflammatory cytokines in several autoimmune states [28]. Stimulation of TLR-induced NF-κB activation promotes differentiation of activated T cells into Th1 and Th17 cells [29]. Th1 and Th17 cells both activate in response to tissue transplantation to produce cytotoxicity [2]. Our results demonstrated that decrease of the Th1 and Th17 population in nerve xenografting in the TLR2$^{-/-}$ or NF-κB$^{-/-}$ mice, implying that reduction of Th1 and Th17 population may ameliorate tissue damage after nerve transplantation.

Conclusions

In conclusion, our results suggest that TLR2 and its downstream target NF-κB are important in immune homeostasis. Knock-out of TLR2 or NF-κB prevented Tregs elimination and inhibited Th1 and Th17 populations increase, and thus may impair Th1- and Th17-driven immune response after nerve xenografting. TLR2 inhibitors may further provide a potential prevention strategy for innate immunity-mediated graft rejection.

Acknowledgements

The work was supported by Chang Gung Memorial Hospital (CMRPG8A0022 & CMRPG8A0023) to Ching-Hua Hsieh.

Competing Interests

The authors have declared that no competing interest exists.

References

[1] Andrade CF, Waddell TK, Keshavjee S, Liu M. Innate immunity and organ transplantation: the potential role of toll-like receptors. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2005;5:969-75.
[2] Porrett PM, Yuan X, LaRosa DF, Walsh PT, Yang J, Gao W, et al. Mechanisms underlying blockade of allograft acceptance by TLR ligands. Journal of immunology. 2008;181:1692-9.
[3] Ivashkiv LB. A signal-switch hypothesis for cross-regulation of cytokine and TLR signalling pathways. Nature reviews Immunology. 2008;8:816-22.
[4] Kwok SK, Cho ML, Her YM, Oh HJ, Park MK, Lee SY, et al. TLR2 ligation induces the production of IL-23/IL-17 via IL-6, STAT3 and NF-kB pathway in patients with primary Sjogren's syndrome. Arthritis research & therapy. 2012;14:R64.
[5] Zhang P, Liu X, Li Y, Zhu X, Zhan Z, Meng J, et al. Protein tyrosine phosphatase with proline-glutamine-serine-threonine-rich motifs negatively regulates TLR-triggered innate responses by selectively inhibiting IkappaB kinase beta/NF-kappaB activation. Journal of immunology. 2013;190:1685-94.
[6] Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nature reviews Immunology. 2008;8:523-32.
[7] Wolf D, Schreiber TH, Tryphonopoulos P, Li S, Tzakis AG, Ruiz P, et al. Tregs expanded in vivo by TNFRSF25 agonists promote cardiac allograft survival. Transplantation. 2012;94:569-74.
[8] Sagoo P, Lombardi G, Lechler RI. Relevance of regulatory T cell promotion of donor-specific tolerance in solid organ transplantation. Frontiers in immunology. 2012;3:184.
[9] Shalev I, Selzner N, Shyu W, Grant D, Levy G. Role of regulatory T cells in the promotion of transplant tolerance. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2012;18:761-70.
[10] Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Alternative medicine review : a journal of clinical therapeutic. 2003;8:223-46.
[11] Chen Y, Wood KJ. Interleukin-23 and TH17 cells in transplantation immunity: does 23+17 equal rejection? Transplantation. 2007;84:1071-4.
[12] Zhang X, Beduhn M, Zheng X, Lian D, Chen D, Li R, et al. Induction of alloimmune tolerance in heart transplantation through gene silencing of TLR adaptors. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012;12:2675-88.
[13] Mandapathil M, Hilldorfer B, Szczepanski MJ, Czystowska M, Szajnik M, Ren J, et al. Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. The Journal of biological chemistry. 2010;285:7176-86.
[14] Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of treg-mediated T cell suppression. Frontiers in immunology. 2012;3:51.
[15] Hanidziar D, Koulmanda M. Inflammation and the balance of Treg and Th17 cells in transplant rejection and tolerance. Current opinion in organ transplantation. 2010;15:411-5.
[16] Zhang N, Kruger B, Lal G, Luan Y, Yadav A, Zang W, et al. Inhibition of TLR4 signaling prolongs Treg-dependent murine islet allograft survival. Immunology letters. 2010;127:119-25.
[17] Jin B, Sun T, Yu XH, Yang YX, Yeo AE. The effects of TLR activation on T-cell development and differentiation. Clinical & developmental immunology. 2012;2012:836485.
[18] Land WG. Innate immunity-mediated allograft rejection and strategies to prevent it. Transplantation proceedings. 2007;39:667-72.
[19] Goldstein DR, Palmer SM. Role of Toll-like receptor-driven innate immunity in thoracic organ transplantation. The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation. 2005;24:1721-9.
[20] Oberg HH, Juricke M, Kabelitz D, Wesch D. Regulation of T cell activation by TLR ligands. European journal of cell biology. 2011;90:582-92.
[21] Dai J, Liu B, Li Z. Regulatory T cells and Toll-like receptors: what is the missing link? International immunopharmacology. 2009;9:528-33.
[22] Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. The Journal of clinical investigation. 2006;116:485-94.
[23] Aliahmadi E, Gramlich R, Grutzkau A, Hitzler M, Kruger M, Baumgrass R, et al. TLR2-activated human langerhans cells promote Th17 polarization via IL-1beta, TGF-beta and IL-23. European journal of immunology. 2009;39:1221-30.
[24] Yang J, Fan H, Hao J, Ren Y, Chen L, Li G, et al. Amelioration of acute graft-versus-host disease by adoptive transfer of ex vivo expanded human cord blood CD4+CD25+ forkhead box protein 3+ regulatory T cells is associated with the polarization of Treg/Th17 balance in a mouse model. Transfusion. 2012;52:1333-47.
[25] Wu H, Noordmans GA, O'Brien MR, Ma J, Zhao CY, Zhang GY, et al. Absence of MyD88 signaling induces donor-specific kidney allograft tolerance. Journal of the American Society of Nephrology : JASN. 2012;23:1701-16.
[26] Byun EH, Kim WS, Kim JS, Jung ID, Park YM, Kim HJ, et al. Mycobacterium tuberculosis Rv0577, a novel TLR2 agonist, induces maturation of dendritic cells and drives Th1 immune response. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2012;26:2695-711.
[27] Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, et al. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. Journal of immunology. 2004;172:2739-43.
[28] Korn T, Oukka M, Kuchroo V, Bettelli E. Th17 cells: effector T cells with inflammatory properties. Seminars in immunology. 2007;19:362-71.
[29] Lobo PI, Bajwa A, Schlegel KH, Vengal J, Lee SJ, Huang L, et al. Natural IgM anti-leukocyte autoantibodies attenuate excess inflammation mediated by innate and adaptive immune mechanisms involving Th-17. Journal of immunology. 2012;188:1675-85.

IVYSPRING INTERNATIONAL PUBLISHER

International Journal of Medical Sciences

2015; 12(8): 650-654. doi: 10.7150/ijms.12304

Research Paper

Regulatory and Effector Helper T-Cell Profile after Nerve Xenografting in the Toll-Like Receptor-Deficient Mice

Cheng-Shyuan Rau[1*]; Ming-Wei Lin[2*]; Shao-Chun Wu[3*]; Yi-Chan Wu[2], Tsu-Hsiang Lu[2], Siou-Ling Tzeng[2], Yi-Chun Chen[2], Chia-Jung Wu[2], Ching-Hua Hsieh[2]✉

1. Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taiwan
2. Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taiwan
3. Department of Anesthesiology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taiwan

*These authors contributed equally to this work

✉ Corresponding author: Ching-Hua Hsieh, M.D., PhD., FACS, Department of Plastic and Reconstructive Surgery Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No.123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan. Tel: 886-7-7327476; E-mail: m93chinghua@gmail.com

Received: 2015.04.01; Accepted: 2015.07.18; Published: 2015.08.01

Abstract

Introduction: The balance between regulatory T cells (Tregs) and effector T help cells (Th cells) is critical for the control of adaptive immune response during nerve transplantation. However, whether the homeostasis of immune regulation between Tregs and Th cells requires toll-like receptor (TLR) signaling is unclear. The aim of this study is to profile the distribution of spleen Tregs and Th cells in a mouse model of nerve xenografting in the TLR2 and NF-κB gene knockout mice.

Methods: The sciatic nerve was taken from a SD rat or an allogeneic mouse and transplanted to a right back leg of recipient C57BL/6, TLR2$^{-/-}$, or NF-κB$^{-/-}$ mice by subcutaneous transplantation. After 7 days, the T lymphocytes were then isolated from spleen, stained with phenotyping kits, and analyzed by flow cytometry.

Results: The results showed that Tregs were decreased after nerve xenografting in the recipient C57BL/6 mouse. In addition, nerve xenografting also increased the Th1 and Th17 but not the Th2 cell populations. In contrast, amelioration of the Tregs elimination was found in TLR2$^{-/-}$ and NF-κB$^{-/-}$ mice after transplantation of the nerve xenograft. Moreover, the mice lacking TLR2 or NF-κB showed attenuation of the increase in Th1 and Th17 cells after nerve xenografting.

Conclusions: TLR signaling is involved in T cell population regulation during tissue transplantation. Knock-out of TLR2 and NF-κB prevented Tregs elimination and inhibited Th1- and Th17-driven immune response after nerve xenografting. This study highlighted the potential of inhibiting TLR signaling to modulate T cell-mediated immune regulation to facilitate tolerance to nerve transplantation.

Key words: Nerve xenografting, Toll-like receptor (TLR), Regulatory T cells (Tregs), T help cells (Th cells)

Introduction

Nerve allograft had been reported to successfully treat the gap of the injured peripheral nerve. However, neurologic recovery with nerve allotransplantation is still limited by immune response over-activation and graft rejection and requires a short-term use of immunosuppressive agents. Nerve allo- or xeno-transplantation results in activation of both innate and adaptive immunity. TLRs are sensors

of both innate and adaptive immunity, and they play critical roles in nerve graft rejection and dysfunction after transplantation [1, 2]. Moreover, pathogens, surgical trauma and ischemic injury in the graft may also result in TLR stimulation [3]. Activation of TLRs may initiate intracellular signal transduction and lead to activation of the transcription factor, NF-κB, to release proinflammatory cytokines, such as IL-1β, TNF-α, and IL17 [4, 5].

$CD4^+$ regulatory T cells (Tregs) expressing FOXP3 play a critical role in the maintenance of immune tolerance during tissue transplantation [6]. The presence of Tregs is associated with allograft survival [7-9]. However, other phenotypes of T lymphocytes such effector T helper cells including $CD4^+INF\gamma^+$ Th1, $CD4^+IL4^+$ Th2, and $CD4^+IL17^+$ Th17 cells exert deleterious effects on tissue by activating of dendritic cells macrophages, and antigen-presenting cells. They secrete cytokines to activate inflammatory pathways, mainly through macrophage activation. However, overactivation of either pathway may cause tissue damage and result in graft rejection [10-12]. Tregs can regulate and suppress Th cells function mainly by cell-to-cell contact-dependent and antigen-independent mechanisms [13, 14]. Therefore, prolonged allograft acceptance requires Tregs, and the balance between Tregs and Th cells is critical for the inhibition of autoimmunity and the magnitude of the adaptive immune response [10, 15].

A previous study demonstrated that TLRs play a role in the maintenance of Tregs, are involved in T cell development [16]. Activation of TLRs can reprogram naïve T cells or Tregs to become effector Th cells [2, 17]. Therefore, TLR2 and its downstream target, NF-κB, may play a role in the homeostasis of immune regulation between Tregs and Th cells. Moreover, nerve xenografting had been reported to induce more severe immunoresponse of the recipient than the nerve allografting. Therefore, in this study, the aim is to investigate the role of TLR2/NF-κB on the homeostasis of immune regulation between Tregs and Th cells by profiling their distribution in the spleen in a mouse model of nerve xenografting using the TLR2 and NF-κB gene knockout mice.

Methods

Animal experiments

C57BL/6 mice and SD rats were purchased from BioLasco (Taipei, Taiwan). $Tlr2^{-/-}$ (B6.129-Tlr2tm1Kir/J), and $NF\text{-}\kappa B^{-/-}$ (B6.Cg-Nfkb1tm1Bal/J) mice were purchased from Jackson Laboratory (Bar Harbor, USA). All housing conditions were maintained, and surgical procedures, including analgesia, were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited SPF facility according to national and institutional guidelines. Animal protocols (permission number No. 2012091304) were approved by Chang Gung Memorial Hospital. Briefly, mice or rats were anesthetized with a combination of ketamine and xylazine, and the right back leg incision was made. The sciatic nerve (1 cm) was taken from a SD rat and transplanted to a right back leg of recipient mice by subcutaneous transplantation. This type of transplantation is defined as xenograft. The donor sciatic nerve taken from the same species of mouse is defined as allograft. The mice were sacrificed after 7 days after the surgery, and the spleen was removed for the T lymphocytes isolation.

T lymphocyte isolation and flow cytometry analysis

The spleen was removed from the mice and dissected. Splenic cell suspensions were gently pressed through a sterile 100 μm nylon mesh, and lymphocytes were isolated by ficoll gradient centrifugation (GE Healthcare, Sweden). The isolated lymphocytes were stained with a mouse Th1/Th2/Th17 phenotyping kit (the fluorescent antibodies: CD4, IFN-γ, IL-4 or IL-17A for detecting Th1, Th2 or Th17) and a mouse Th17/Treg phenotyping kit (the fluorescent antibodies: Foxp3 and CD4 for detecting Tregs) (BD Pharmingen, USA), and were acquired in a BD LSR II flow cytometer (BD Biosceinces, USA).

Statistical analysis

The data collected were analyzed using SPSS v.20 statistical software (IBM, Armonk, NY) for the independent Student's t-tests. All results are presented as the mean ± standard error. A p-value less than 0.05 was considered statistically significant.

Results

Nerve xenografting decreased Tregs population in wild type mice but not in $TRL2^{-/-}$ or $NF\text{-}\kappa B^{-/-}$ mice

To determine whether TLR2 or NF-κB plays a role in Tregs population modulation in nerve transplantation, we isolated spleen T lymphocytes from wild type or $TLR2^{-/-}$ or $NF\text{-}\kappa B^{-/-}$ mice to analyze $CD4^+Foxp3^+$ Tregs population in nerve allografts or xenografts. By flow cytometry analysis, we found that the $CD4^+Foxp3^+$ Tregs population was decreased in the spleen of control wild-type mice after nerve xenografting compared to that after allografting. However, elimination of the Tregs population was ameliorated in $NF\text{-}\kappa B^{-/-}$ mice (Fig 1). The results indicate that NF-κB may participate in Treg-mediated immune suppression after nerve xenografting.

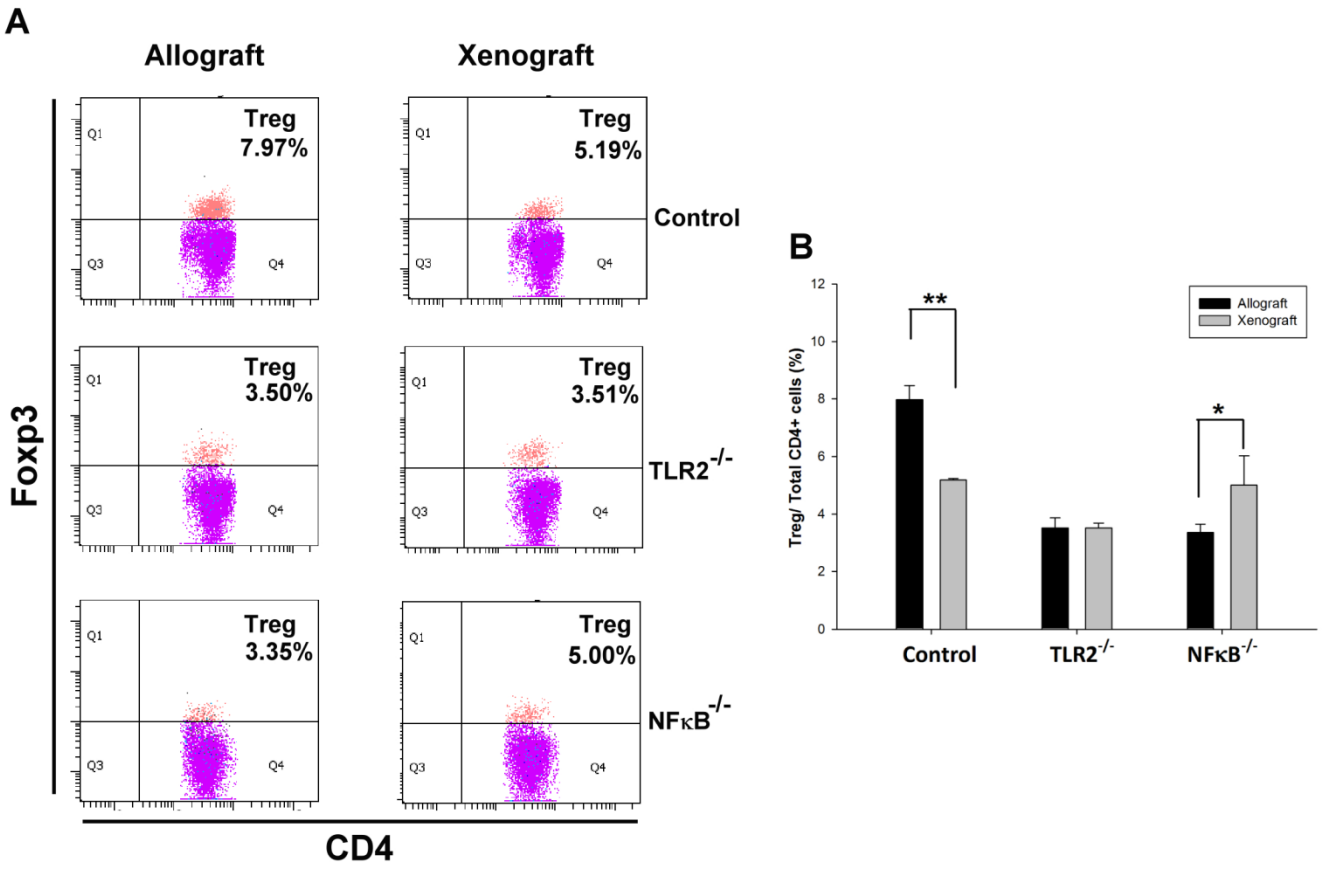

Figure 1. Knock-out of TLR2 and NF-κB prevented Tregs elimination after nerve xenografting. (A) Flow cytometry analysis of $CD4^+Foxp3^+$ Tregs population in nerve allografts or xenografts. (B) Quantification of the $CD4^+Foxp3^+$ Tregs population from flow cytometry analysis. The $CD4^+Foxp3^+$ Tregs population was decreased after nerve xenografting compared to allografting. Data are representative of 3 experiments with similar results. (n=3-5, *$p < 0.05$ and **$p < 0.01$ vs corresponding allograft).

Knock-out of TLR2 or NF-κB decreased Th1 but not Th2 cells after nerve xenografting

To determine whether TLR2 or NF-κB plays a role in Th1 or Th2 population regulation after nerve xenografting, we analyzed the Th1 or Th2 population after nerve xenografting compared to allografts in wild-type, TLR2$^{-/-}$ and NF-κB$^{-/-}$ mice. The flow cytometry analysis results indicate that knock-out of TLR2 or NF-κB decreased of Th1 but not Th2 population relative to the populations in control wild-type mice (Fig 2).

Knock-out of TLR2 or NF-κB inhibited the Th17 population increase after nerve xenografting

To determine whether the Th17 population is involved in TLR2- or NF-κB-dependent pathways, we analyzed Th17 cell population in wild-type, TLR2$^{-/-}$, or NF-κB$^{-/-}$ mice after nerve xenografting compared to allografting. We found that the Th17 population elevation was inhibited after nerve xenografting in TLR2$^{-/-}$ and NF-κB$^{-/-}$ mice (Fig 3). TLR2 and NF-κB may play a role in Th17-medated immune response after nerve xenografting.

Treg/(Th1+Th17) balance is TLR2 or NF-κB dependent after nerve xenografting

The balance between Tregs and Th cells is critical for the inhibition of autoimmunity. The results of the flow cytometry analysis showed that balance of the Tregs population over the Th1 and Th17 population was disturbed in wild-type mice after nerve xenografting. The value of Treg/(Th1+Th17) was decreased in control wild type but not in TLR2$^{-/-}$ or NF-κB$^{-/-}$ mice (Fig 4).

Discussion

Successful transplantation depends on the modulation of adaptive immunity in graft transplantation, as previous studies demonstrated that T cells are necessary and sufficient to reject almost all allogeneic tissues [18]. Tregs function as immune suppressors in regulating effector Th cells [13]. Therefore, the balance between Tregs and Th cells is critical for inhibition of the autoimmune response and graft rejection.

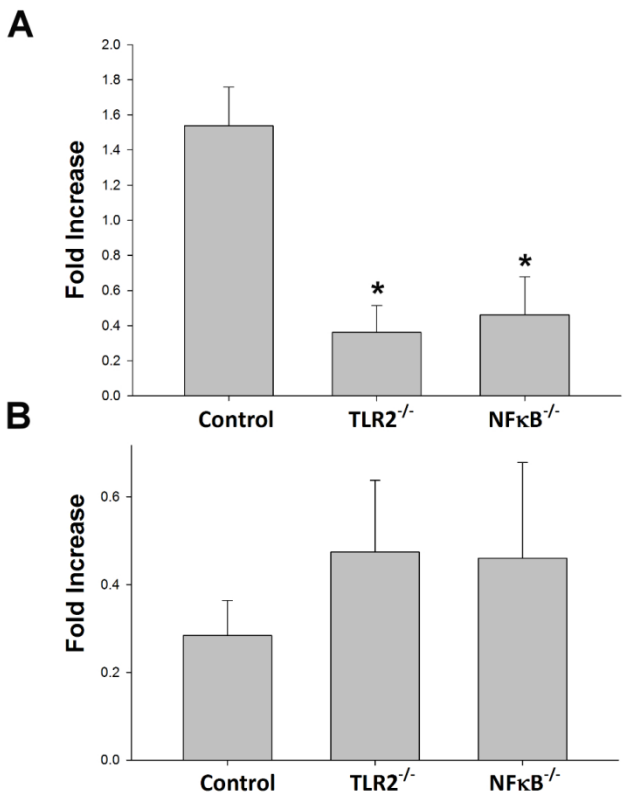

Figure 2. Knock-out of TLR2 and NF-κB prevented Th1 but not Th2 elevation after nerve xenografting. (A) Th1 and (B) Th2 cell expression in wild type or TLR2$^{-/-}$ or NF-κB$^{-/-}$ mice in xenografts compared to allografts by flow cytometry analysis. Data are expressed as fold increases (mean±SD) and are representative of 3 experiments with similar results. (n=3-5, *p < 0.05 vs control wild-type mice).

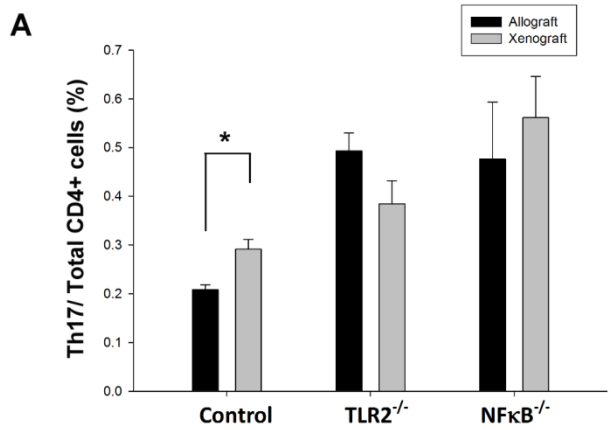

Figure 3. Knock-out of TLR2 and NF-κB inhibited Th17 increase after nerve xenografting. The Th17 cell population was increased after nerve xenografting compared to allografts in wild-type but not in TLR2$^{-/-}$ or NF-kB$^{-/-}$ mice, as observed by flow cytometry analysis. Data are presented as the mean ± SD and are representative of 3 experiments with similar results. (n=3-5, *p < 0.05 vs control wild type mice).

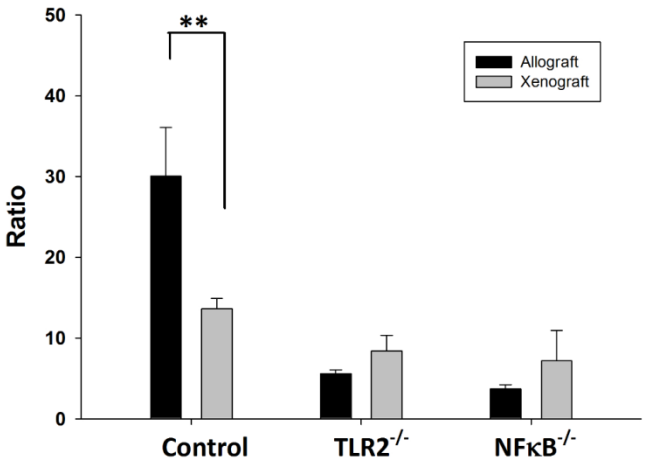

Figure 4. Ratio of Treg/(Th1+Th17) in wild type, TLR2$^{-/-}$, NF-κB$^{-/-}$ mice after nerve xenografting. The value of Treg/(Th1+Th17) was decreased in control wild type but not in TLR2$^{-/-}$ or NF-kB$^{-/-}$ mice. Data are presented as the mean ± SD. (n=3, *p<0.05 vs control wild-type mice).

TLRs are the first-line sensor for innate immunity, and mounting evidence suggests that stimulation of TLR activated serious inflammatory responses which resulted in tissue injury and graft rejection after transplantation [1, 18, 19]. Although TLRs and T cells are known to play major roles in innate immunity, whether knock-out of TLRs can regulate the T cell population after tissue transplantation remains unknown. To determine whether TLR2 is important in the modulation of Tregs and Th cells in tissue transplantation, we used a model of nerve transplantation in TLR2 or NF-κB knock-out mice. Our study demonstrated that knock-out of TLR2 or NF-κB prevented Tregs elimination and increased the population of Th1 and Th17 cells after nerve xenografting. Recent studies suggested that TLR ligands regulate T cell activation, as well as T cell differentiation [17, 19]. Therefore, TLR2 ligands may modulate T cell population through NF-κB by direct action because TLR2 are present on Tregs and Th cells. TLR2 was reported play a role in Tregs proliferation, and activation of TLR2 can cause increased activity of Tregs [20, 21]. In addition, reduction of Tregs was observed in TLR2$^{-/-}$ mice [22]. However, TLR2 also promotes Th1 and Th17 cell polarization [23]. Activation of TLRs also activates effector Th cells to enhance Th cell-mediated cytotoxicity and result in transplantation failure [8, 20]. Therefore, the graft immune tolerance and transplantation success may correlate with the balance of Treg/Th cells [8, 24].

The TLR2-coupled protein, MyD88, is reported to be important for Th17 immunity against allogeneic grafts [25]. A similar study also suggested that TLR2 signaling leads to Th1 immunity [26]. Although a previous study suggested that TLR2 activation promotes the Th2 immune response [27], knock-out of

TLR2 altered Th1 but not Th2 population after nerve xenografting in this study. According to the Th1/Th2 balance hypothesis [10], the TLR2-NF-κB signaling pathway might have influenced the Th1-driven immune response in our nerve xenograft animal model.

Th17 cells, a newly recognized distinct subset of T helper cells, have been shown to play an important role in murine autoimmune diseases. They express a variety of potent proinflammatory cytokines in several autoimmune states [28]. Stimulation of TLR-induced NF-κB activation promotes differentiation of activated T cells into Th1 and Th17 cells [29]. Th1 and Th17 cells both activate in response to tissue transplantation to produce cytotoxicity [2]. Our results demonstrated that decrease of the Th1 and Th17 population in nerve xenografting in the TLR2$^{-/-}$ or NF-κB$^{-/-}$ mice, implying that reduction of Th1 and Th17 population may ameliorate tissue damage after nerve transplantation.

Conclusions

In conclusion, our results suggest that TLR2 and its downstream target NF-κB are important in immune homeostasis. Knock-out of TLR2 or NF-κB prevented Tregs elimination and inhibited Th1 and Th17 populations increase, and thus may impair Th1- and Th17-driven immune response after nerve xenografting. TLR2 inhibitors may further provide a potential prevention strategy for innate immunity-mediated graft rejection.

Acknowledgements

The work was supported by Chang Gung Memorial Hospital (CMRPG8A0022 & CMRPG8A0023) to Ching-Hua Hsieh.

Competing Interests

The authors have declared that no competing interest exists.

References

[1] Andrade CF, Waddell TK, Keshavjee S, Liu M. Innate immunity and organ transplantation: the potential role of toll-like receptors. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2005;5:969-75.
[2] Porrett PM, Yuan X, LaRosa DF, Walsh PT, Yang J, Gao W, et al. Mechanisms underlying blockade of allograft acceptance by TLR ligands. Journal of immunology. 2008;181:1692-9.
[3] Ivashkiv LB. A signal-switch hypothesis for cross-regulation of cytokine and TLR signalling pathways. Nature reviews Immunology. 2008;8:816-22.
[4] Kwok SK, Cho ML, Her YM, Oh HJ, Park MK, Lee SY, et al. TLR2 ligation induces the production of IL-23/IL-17 via IL-6, STAT3 and NF-kB pathway in patients with primary Sjogren's syndrome. Arthritis research & therapy. 2012;14:R64.
[5] Zhang P, Liu X, Li Y, Zhu X, Zhan Z, Meng J, et al. Protein tyrosine phosphatase with proline-glutamine-serine-threonine-rich motifs negatively regulates TLR-triggered innate responses by selectively inhibiting IkappaB kinase beta/NF-kappaB activation. Journal of immunology. 2013;190:1685-94.
[6] Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. Nature reviews Immunology. 2008;8:523-32.
[7] Wolf D, Schreiber TH, Tryphonopoulos P, Li S, Tzakis AG, Ruiz P, et al. Tregs expanded in vivo by TNFRSF25 agonists promote cardiac allograft survival. Transplantation. 2012;94:569-74.
[8] Sagoo P, Lombardi G, Lechler RI. Relevance of regulatory T cell promotion of donor-specific tolerance in solid organ transplantation. Frontiers in immunology. 2012;3:184.
[9] Shalev I, Selzner N, Shyu W, Grant D, Levy G. Role of regulatory T cells in the promotion of transplant tolerance. Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society. 2012;18:761-70.
[10] Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Alternative medicine review : a journal of clinical therapeutic. 2003;8:223-46.
[11] Chen Y, Wood KJ. Interleukin-23 and TH17 cells in transplantation immunity: does 23+17 equal rejection? Transplantation. 2007;84:1071-4.
[12] Zhang X, Beduhn M, Zheng X, Lian D, Chen D, Li R, et al. Induction of alloimmune tolerance in heart transplantation through gene silencing of TLR adaptors. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012;12:2675-88.
[13] Mandapathil M, Hilldorfer B, Szczepanski MJ, Czystowska M, Szajnik M, Ren J, et al. Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. The Journal of biological chemistry. 2010;285:7176-86.
[14] Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of treg-mediated T cell suppression. Frontiers in immunology. 2012;3:51.
[15] Hanidziar D, Koulmanda M. Inflammation and the balance of Treg and Th17 cells in transplant rejection and tolerance. Current opinion in organ transplantation. 2010;15:411-5.
[16] Zhang N, Kruger B, Lal G, Luan Y, Yadav A, Zang W, et al. Inhibition of TLR4 signaling prolongs Treg-dependent murine islet allograft survival. Immunology letters. 2010;127:119-25.
[17] Jin B, Sun T, Yu XH, Yang YX, Yeo AE. The effects of TLR activation on T-cell development and differentiation. Clinical & developmental immunology. 2012;2012:836485.
[18] Land WG. Innate immunity-mediated allograft rejection and strategies to prevent it. Transplantation proceedings. 2007;39:667-72.
[19] Goldstein DR, Palmer SM. Role of Toll-like receptor-driven innate immunity in thoracic organ transplantation. The Journal of heart and lung transplantation : the official publication of the International Society for Heart Transplantation. 2005;24:1721-9.
[20] Oberg HH, Juricke M, Kabelitz D, Wesch D. Regulation of T cell activation by TLR ligands. European journal of cell biology. 2011;90:582-92.
[21] Dai J, Liu B, Li Z. Regulatory T cells and Toll-like receptors: what is the missing link? International immunopharmacology. 2009;9:528-33.
[22] Sutmuller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like receptor 2 controls expansion and function of regulatory T cells. The Journal of clinical investigation. 2006;116:485-94.
[23] Aliahmadi E, Gramlich R, Grutzkau A, Hitzler M, Kruger M, Baumgrass R, et al. TLR2-activated human langerhans cells promote Th17 polarization via IL-1beta, TGF-beta and IL-23. European journal of immunology. 2009;39:1221-30.
[24] Yang J, Fan H, Hao J, Ren Y, Chen L, Li G, et al. Amelioration of acute graft-versus-host disease by adoptive transfer of ex vivo expanded human cord blood CD4+CD25+ forkhead box protein 3+ regulatory T cells is associated with the polarization of Treg/Th17 balance in a mouse model. Transfusion. 2012;52:1333-47.
[25] Wu H, Noordmans GA, O'Brien MR, Ma J, Zhao CY, Zhang GY, et al. Absence of MyD88 signaling induces donor-specific kidney allograft tolerance. Journal of the American Society of Nephrology : JASN. 2012;23:1701-16.
[26] Byun EH, Kim WS, Kim JS, Jung ID, Park YM, Kim HJ, et al. Mycobacterium tuberculosis Rv0577, a novel TLR2 agonist, induces maturation of dendritic cells and drives Th1 immune response. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2012;26:2695-711.
[27] Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, et al. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. Journal of immunology. 2004;172:2739-43.
[28] Korn T, Oukka M, Kuchroo V, Bettelli E. Th17 cells: effector T cells with inflammatory properties. Seminars in immunology. 2007;19:362-71.
[29] Lobo PI, Bajwa A, Schlegel KH, Vengal J, Lee SJ, Huang L, et al. Natural IgM anti-leukocyte autoantibodies attenuate excess inflammation mediated by innate and adaptive immune mechanisms involving Th-17. Journal of immunology. 2012;188:1675-85.

Hsieh et al. BMC Genomics (2015) 16:699
DOI 10.1186/s12864-015-1896-3

BMC Genomics

RESEARCH ARTICLE Open Access

Weight-reduction through a low-fat diet causes differential expression of circulating microRNAs in obese C57BL/6 mice

Ching-Hua Hsieh[1*†], Cheng-Shyuan Rau[2†], Shao-Chun Wu[3†], Johnson Chia-Shen Yang[1], Yi-Chan Wu[1], Tsu-Hsiang Lu[1], Siou-Ling Tzeng[1], Chia-Jung Wu[1] and Chia-Wei Lin[1]

Abstract

Background: To examine the circulating microRNA (miRNA) expression profile in a mouse model of diet-induced obesity (DIO) with subsequent weight reduction achieved via low-fat diet (LFD) feeding.

Results: Eighteen C57BL/6NCrl male mice were divided into three subgroups: (1) control, mice were fed a standard AIN-76A (fat: 11.5 kcal %) diet for 12 weeks; (2) DIO, mice were fed a 58 kcal % high-fat diet (HFD) for 12 weeks; and (3) DIO + LFD, mice were fed a HFD for 8 weeks to induce obesity and then switched to a 10.5 kcal % LFD for 4 weeks. A switch to LFD feeding led to decreases in body weight, adiposity, and blood glucose levels in DIO mice. Microarray analysis of miRNA using The Mouse & Rat miRNA OneArray® v4 system revealed significant alterations in the expression of miRNAs in DIO and DIO + LFD mice. Notably, 23 circulating miRNAs (mmu-miR-16, mmu-let-7i, mmu-miR-26a, mmu-miR-17, mmu-miR-107, mmu-miR-195, mmu-miR-20a, mmu-miR-25, mmu-miR-15b, mmu-miR-15a, mmu-let-7b, mmu-let-7a, mmu-let-7c, mmu-miR-103, mmu-let-7f, mmu-miR-106a, mmu-miR-106b, mmu-miR-93, mmu-miR-23b, mmu-miR-21, mmu-miR-30b, mmu-miR-221, and mmu-miR-19b) were significantly downregulated in DIO mice but upregulated in DIO + LFD mice. Target prediction and function annotation of associated genes revealed that these genes were predominantly involved in metabolic, insulin signaling, and adipocytokine signaling pathways that directly link the pathophysiological changes associated with obesity and weight reduction.

Conclusions: These results imply that obesity-related reductions in the expression of circulating miRNAs could be reversed through changes in metabolism associated with weight reduction achieved through LFD feeding.

Keywords: Diet-induced Obesity, MicroRNAs, High-fat Diet, Low-fat Diet

Background

Obesity is associated with insulin resistance and an abnormal inflammatory response [1], and the strong associations suggest that adipose tissue plays a prominent role in the onset and progression of these comorbidities [2]. White adipose tissue (WAT) has been characterized as an endocrine organ [3], as it produces endocrine-acting peptides such as leptin, and it is metabolically important, with excess levels being associated with metabolic syndrome [4, 5]. High fat uptake leads to metabolic alterations in adipose tissue that increase the levels of circulating free fatty acids in the blood [6]. This leads to macrophage activation and the production of proinflammatory cytokines via Toll-like receptors, resulting in inflammation in adipose tissue [6]. When allowed *ad libitum* access to a high-fat diet (HFD), C57BL/6J mice develop insulin resistance and obesity in a manner that resembles disease progression in humans [7]. Increased energy expenditure and decreased energy intake are the two most commonly recommended lifestyle changes to reduce adiposity and restore insulin sensitivity in the treatment of diet-induced obesity (DIO) and associated comorbidities [8]. Calorie restriction is effective in improving insulin sensitivity and decreasing both body weight and percent body fat [9]. In addition, reductions in

* Correspondence: m93chinghua@gmail.com
†Equal contributors
[1]Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No. 123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan
Full list of author information is available at the end of the article

body weight and improvements in insulin sensitivity can also be achieved by reducing the percentage fat in a diet, i.e., by switching from a HFD to a low-fat diet (LFD) [10].

MicroRNAs (miRNAs) are endogenous small RNAs that post-transcriptionally regulate gene expression, and they have been demonstrated to have important roles in numerous disease processes. There is growing evidence that miRNAs play an important role in regulating adipose tissue pathways that control a range of processes, including adipogenesis, insulin resistance, and inflammation [11–13]. Many miRNAs are dysregulated in the metabolic tissues of obese animals and humans, potentially contributing to the pathogenesis of obesity-associated complications [11–13]. In addition, recent studies identified several miRNAs expressed in metabolic organs that could be used as feasible therapeutic targets for obesity and its consequent pathologies [11, 13]. Recently, circulating serum miRNAs were found to display specific expression patterns, suggesting that miRNA profiles may represent fingerprints for various diseases [14, 15]. In addition, despite the ubiquitous presence of ribonucleases, serum miRNAs levels are remarkably stable and reproducible [16, 17], and they function in cell-to-cell communication [18]. Currently, how changes in miRNA profiles might affect adipose tissue at the functional and molecular level and to what extent they differ in response to weight-reduction strategies are not well understood. This information is important in the development of dietary anti-obesity interventions [19]. As circulating miRNAs potentially play an important role in regulating the pathophysiology of obesity and they are potential therapeutic targets, we hypothesized the weight reduction may change the circulating miRNAs expression. Our study aim was to profile the expression of circulating miRNAs in a mouse model of DIO with subsequent weight reduction achieved through LFD feeding.

Methods

Ethics statement

This study was conducted in strict accordance with guidelines on the use of laboratory animals, and every effort was made to minimize the suffering of affected animals. Animal protocols were approved by the IACUC of Chang Gung Memorial Hospital, Taiwan (permission number No. 2012091002).

Animal experiments

C57BL/6NCrl mice were purchased from BioLasco (Taipei, Taiwan). Animals were housed, and surgical procedures, including analgesia, were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited SPF facility according to national and institutional guidelines. In this experiment, 18 male, wild-type C57BL/6NCrl mice were randomly assigned to three subgroups ($n = 6$ in each group) as follows: (1) control, mice were fed a standard AIN-76A (fat: 11.5 kcal %) diet *ad libitum* for 12 weeks; (2) DIO, mice were fed a 58 kcal % HFD (D12331; Research Diets Inc., New Brunswick, NJ) *ad libitum* for 12 weeks to induce obesity; and (3) DIO + LFD, mice were fed a 58 kcal% HFD (D12331) *ad libitum* for 8 weeks to induce obesity and then fed a 10.5 kcal% LFD (D 12329; Research Diets Inc.) for 4 weeks. Weight measurements were performed weekly, and a glucose tolerance test was performed at the beginning and end of the experiment to confirm that HFD-fed mice developed an obese and glucose intolerance phenotype. Briefly, mice were fasted for 5 h, and baseline blood glucose levels were measured with an Accu-Check Advantage blood glucose meter (Roche, New Jersey, USA) using blood collected from the tail vein. Mice ($n = 6$ in each group) were injected intraperitoneally with 2 g of glucose per kilogram body weight in sterile PBS. The glucose level was measured via tail vein blood (~10 μL) at t = -30 and 0 (pre) and t = 15, 30, 60, 90, and 120 min after the glucose infusion. Data were averaged and graphed as blood glucose level as a function of time. To reflect the circulating levels of glucose during the glucose tolerance test (GTT), we calculated the total area under the curve (AUC) of the glucose concentration versus time by the linear trapezoidal rule for the period of 0 - 120 min after glucose infusion. To avoid the effect of loss of blood in GTT experiment or uncertain effect or repeat tail punctures on subsequent miRNAs expression and cytokine assay, additional groups of mice under the same model were used for further experiments. After the end of the experiment, all mice were euthanized, and the abdominal mesenteric WAT of each mouse was removed and weighed. The adipose tissue block embedded in paraffin was sectioned at 5 μm to measure the adipocyte area. Three 5 μm-thickness sections of the same fat specimen at 50 μm distance was mounted on glass plate and stained with hematoxylin and eosin. Two different microscopic fields (magnification × 100) per plate were photographed and 100 adipose cells were arbitrarily selected in the center of field and their cell diameters were assessed by tracing the outline of each adipocyte. The mean adipocyte area was measured from the WAT of control and experimental mice ($n = 4$ in each group) using Image-Pro Plus image analysis software (Carl Zeiss, Oberkochen, Germany) and expressed in terms of square micrometers. The cells were randomly chosen, and the person analyzing the images was blinded to the group assignments. At the indicated time of the experiment, 1 mL of whole blood was collected via cardiac puncture into a plain tube and

allowed to clot for 1 h. Samples were centrifuged at 3000 × *g* for 10 min, and sera were aliquoted and stored at −80°C until further analysis.

Cytokine assays

Serum cytokine concentrations were analyzed using two complementary Bio-Plex suspension arrays (M60-00003J7 and M60-00007NY) covering all cytokine biomarkers potentially involved in inflammation ($n = 6$ in each group). Eleven biomarkers (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-17, GM-CSF, IFN-γ, and TNF-α) were assessed simultaneously using the Bio-Plex system (BioRad, Hercules, CA). Assays were performed on four biological replicates per the manufacturer's instructions. Results are expressed in picograms per milliliter of serum.

RNA isolation and preparation

Total RNA was extracted from serum using the mirVana™ miRNA Isolation Kit (Life Technologies, NY). Purified RNA was quantitatively evaluated by measuring its absorbance at 260 nm using an SSP-3000 Nanodrop spectrophotometer (Infinigen Biotechnology, Inc., City of Industry, CA), and RNA quality was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA (10 ng) was reverse-transcribed into cDNA using a TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Target miRNAs were reverse-transcribed using sequence-specific stem-loop primers, and cDNA was used for quantitative real-time polymerase chain reaction (qPCR).

miRNA microarray analysis

The Mouse & Rat miRNA OneArray® v4 (Phalanx Biotech Group, Hsinchu, Taiwan) array used in this experiment contains 144 experimental control probes, 1157 unique mouse miRNA probes, and 680 rat miRNA probes, based on the miRBase 18 database. Three biological replicates of each group of mice were used in miRNA microarray experiments. Mouse genome-wide miRNA microarray experimental and statistical analyses were performed by Phalanx Biotech Group. Briefly, fluorescent targets were prepared from 2.5 μg of total RNA using the miRNA ULS™ Labeling Kit (Kreatech Diagnostics, Amsterdam, Netherlands). Labeled miRNA targets enriched using NanoSep 100K (Pall Corporation, Port Washington, NY) were hybridized to The Mouse & Rat miRNA OneArray® v4 in Phalanx hybridization buffer in the OneArray® Hybridization Chamber. After overnight hybridization at 37 °C, non-specifically bound targets were removed by three washing steps (Wash I, 37 °C, 5 min; Wash II, 37 °C, 5 min and 25 °C, 5 min; and Wash III, rinse 20 times). Slides were dried by centrifugation and scanned using an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA). The signal intensities of Cy5 fluorescence in each spot were analyzed using GenePix 4.1 software (Molecular Devices, Sunnyvale, CA) and processed using R language (http://www.r-project.org/) with two packages: limma (http://www.bioconductor.org/packages/release/bioc/html/limma.html) and genefilter (http://www.bioconductor.org/packages/release/bioc/html/genefilter.html). Spots with flag < 0 were filtered out, and the remaining spots were log 2 transformed and normalized using the 75 % media scaling normalization method. Normalized spot intensities were converted into gene expression ratios between the control and treatment groups. Spots with expression ratios ≤0.5 or ≥2, as well as with $p < 0.05$, were selected for further analysis. Differentially expressed miRNAs were subjected to hierarchical cluster analysis using average linkage and Pearson's correlation as the measure of similarity. The miRNA array data have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE61005. Five miRNAs detected by array analysis were selected and quantified by qPCR using the Applied Biosystems 7500 Real-Time PCR System (Life Technologies) to confirm the upregulation of miRNA expression in the DIO + LFD group. Twenty-five femtomoles of single-stranded cel-miR-39 synthesized by Invitrogen (Carlsbad, CA) was spiked into 400 μL of serum as an internal control for the expression of each miRNA.

Target prediction, GO enrichment, and KEGG pathway analyses

Target prediction was performed to identify the target genes of the identified dysregulated miRNAs by integrating all three public databases (TargetScan, PicTar, and miRanda). This method firstly mapped all target gene candidates to GO terms in the database (http://www.geneontology.org/), calculated gene numbers for each term, and then used a hypergeometric test to find significantly enriched GO terms in target gene candidates compared to the reference gene background. Bonferroni's correction for the *p*-value was used to obtain a corrected *p*-value. GO terms with corrected *p*-values ≤ 0.05 were defined as significantly enriched in target gene candidates. To reveal the main pathways in which the target gene candidates are involved, pathway analysis using a major public pathway-related database, KEGG, was performed to identify significantly enriched metabolic pathways or signal transduction pathways in target gene candidates compared with the whole reference gene background. Genes with FDR ≤ 0.05 were considered significantly enriched among the target gene candidates.

Statistical analysis

The body weight of mice are expressed as the mean with a confidence interval. All other experimental data are

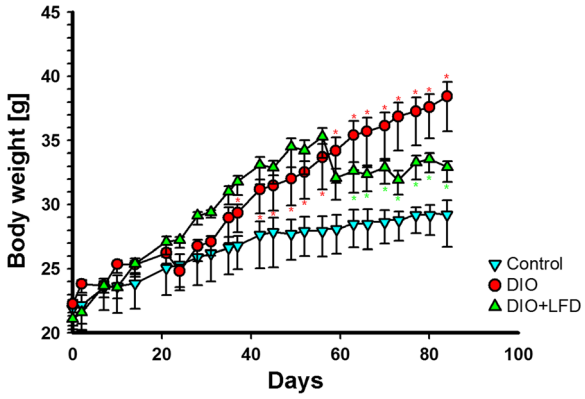

Fig. 1 The body weight of C57BL/6NCrl mice on a weekly basis till 12 weeks ($n = 6$ in each group). Control: the mice fed with the standard diet; DIO: the mice fed with high-fat diet; DIO + LFD: the mice fed with high-fat diet for 8 weeks to induce obesity, then change to low-fat diet for subsequent 4 weeks. *(red color), $P < 0.05$ vs. control. *(green color), $P < 0.05$ vs. DIO. The body weight of mice are expressed as the mean a ± 80 % confidence interval

expressed as the mean ± standard error of the mean. Analysis of variance combined with a Bonferroni post hoc correction was performed to identify significant differences in body weight, weight of fat, adipocyte area, glucose levels, and serum cytokine levels. A p value of 0.05 was regarded as the level of statistical significance.

Results

LFD decreased body weight and adiposity

In comparison with the C57BL/6NCrl mice fed the standard diet, feeding with the HFD significantly increased body weight (Fig. 1). By contrast, after the change from HFD feeding to LFD feeding, the body weight of DIO mice decreased quickly, with the mean body weight stabilizing after 4 weeks on the LFD. Abdominal mesenteric WAT was also significantly larger in the DIO mice than in control mice, and the mice that were switched to the LFD had significantly less abdominal mesenteric WAT than those that remained on the HFD (Fig. 2a). However, the lower amount of abdominal mesenteric WAT was not accompanied by a significantly smaller adipocyte area (Figs. 2b and 3). Notably, because the fat specimen chosen at 50 μm distance was less than the mean diameter of the adipocytes, there may be exist a selection bias that some measured adipocytes were repeatedly calculated. However, we expect this selection bias is not significant because the tissue section was chosen in a fixed distance and the counting of high number of adipocytes could decrease the deviation of calculated mean adipocyte area. After injection of glucose to the control mice, blood glucose levels increased to a peak of 350 mg/dL after 15 min, and then gradually returned to baseline after 120 min. In the DIO mice, blood glucose concentrations at 30 to 120 min during the GTT were significantly higher than those in the control mice (Fig. 4a). HFD-fed animals displayed significant impairment in glucose tolerance, as evidenced by a 90 % higher incremental glucose AUC (Fig. 4b). In addition, significantly lower glucose level was observed at 30 min after glucose injection for the LFD-fed mice relative to the HFD-fed mice (Fig. 4a), resulting in an around 15 % lower glucose AUC (Fig. 4b).

LFD did not induce the differential expression of inflammatory cytokines

Of the 11 biomarkers measured (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-17, GM-CSF, IFN-γ, and

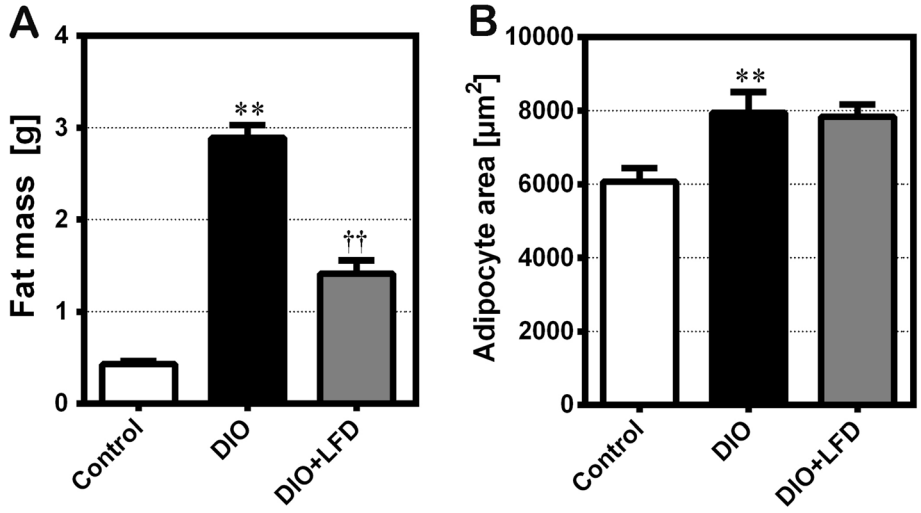

Fig. 2 a The weight of abdominal mesenteric white adipose tissue and **b** the adipocyte area of C57BL/6NCrl mice in groups of control, DIO, DIO + LFD at 12w. **,$P < 0.01$ vs. control. ††, $P < 0.01$ vs. DIO ($n = 4$ in each group)

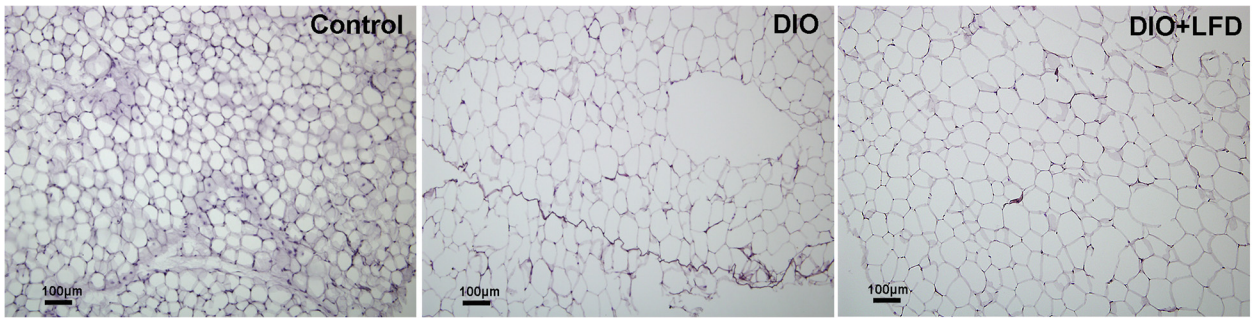

Fig. 3 Hematoxylin and eosin stain of the paraffin-embedded white adipose tissue at 5 μm section of C57BL/6NCrl mice in groups of control, DIO, DIO + LFD at 12w

TNF-α), IL-1β had the highest expression in serum, followed by TNF-α, IL-10, and IL-12 (p70) (Fig. 5). However, there were no significant differences in expression among the groups.

Upregulated miRNA targets in microarray analysis

Greater than 2-fold differences in serum miRNA expression between DIO mice and controls as well as between DIO + LFD and DIO mice ($p < 0.05$) were identified for further analysis. The microarray and qPCR results were in agreement, with a Pearson correlation value of 0.891 (Additional file 1), under the limitation of only five miRNAs being selected for analysis. Unsupervised hierarchical clustering of all differentially expressed serum miRNAs was conducted to separate samples from experimental or control subjects into different groups (Fig. 6). In microarray experiments of DIO mouse sera, eight miRNAs were upregulated, and 34 were downregulated (Table 1). In addition, in the sera of DIO + LFD mice, 28 miRNAs were upregulated, and 10 were downregulated (Table 2). As shown in the Venn diagram in Fig. 7, notably, 23 of the 28 upregulated miRNAs in DIO + LFD mice (mmu-miR-16, mmu-let-7i, mmu-miR-26a, mmu-miR-17, mmu-miR-107, mmu-miR-195, mmu-miR-20a, mmu-miR-25, mmu-miR-15b, mmu-miR-15a, mmu-let-7b, mmu-let-7a, mmu-let-7c, mmu-miR-103, mmu-let-7f, mmu-miR-106a, mmu-miR-106b, mmu-miR-93, mmu-miR-23b, mmu-miR-21, mmu-miR-30b, mmu-miR-221, and mmu-miR-19b) were downregulated in the DIO mice. Only five miRNAs (mmu-miR-451, mmu-miR-223, mmu-miR-92a, mmu-miR-200c, and mmu-miR-873) were differentially expressed, implying that the majority of miRNA downregulation associated with obesity could be reversed by LFD treatment. By contrast, of the eight upregulated miRNAs in DIO mice, only one (mmu-miR-711) was significantly downregulated in DIO + LFD mice.

Target prediction and function annotation

To further understand the physiological functions and biological processes associated with the 23 miRNAs, target prediction was performed by integrating three public databases (TargetScan, PicTar, and miRanda), and 1082 target genes were identified. GO annotation and KEGG pathway analysis was also performed to identify functional modules regulated by these 23 miRNAs. In GO annotation analysis, cellular processes, biological regulation, metabolic processes, primary

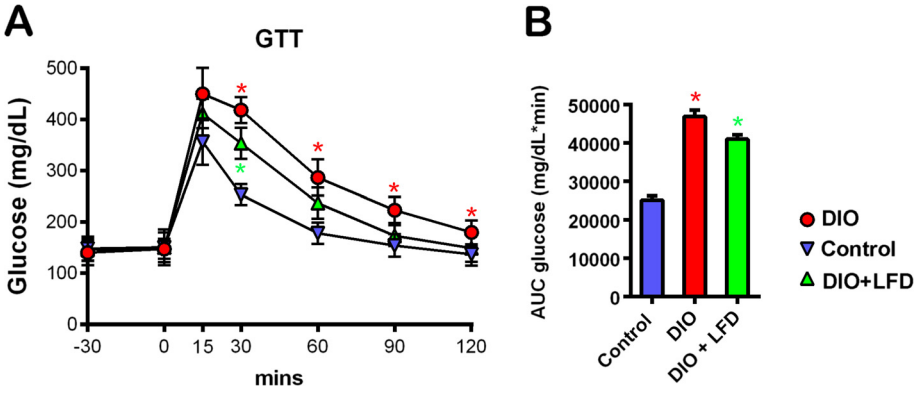

Fig. 4 Blood glucose concentrations (**a**) and area under the curve (AUC) quantification (**b**) during a 120-min glucose tolerance test in groups of control, DIO, DIO + LFD at 12w. *(red color), $P < 0.05$ vs. control. *(green color), $P < 0.05$ vs. DIO

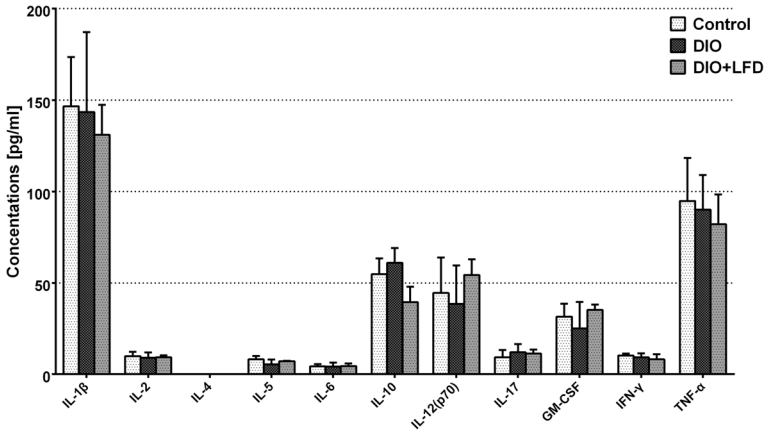

Fig. 5 Concentrations of serum cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-17, GM-CSF, IFN-γ, TNF-α) analyzed by the Bio-Plex Multiplex cytokine assay at 12w in the mice in groups of control, DIO, DIO + LFD ($n = 6$ in each group)

metabolic processes, and cellular metabolic processes were the most significantly enriched GO terms (Fig. 8). KEGG pathway analysis revealed 142 pathways associated with these miRNA targets. Among these, metabolic pathways were the most enriched, with 1024 associated genes, followed by MAPK signaling, actin cytoskeleton regulation, secondary metabolite biosynthesis, focal adhesion, insulin signaling, calcium signaling, cytokine-cytokine receptor interaction, tight junctions, phagosomes, and adipocytokine signaling pathways (Table 3). These results suggest that these targets have a high possibility of being regulated by miRNAs during obesity and weight reduction through LFD feeding; however, the possibility of false-positive results from the prediction algorithm always exists.

Discussion

Switching to a LFD is an effective intervention to promote weight loss and improve metabolic health parameters in obesity [19]. Although morbid obesity is considered a systemic inflammatory state, the serum inflammatory profile of C57BL/6 mice, as measured by an antibody array, revealed that DIO mice had higher leptin, IL-6, and LPS-induced chemokine concentrations and lower concentrations of all other chemokines/cytokines than control mice [20]. In this study, we demonstrated that LFD feeding reduced the body weight and adiposity of DIO mice; however, there was no significant difference in the expression of the 11 measured cytokines between DIO and DIO + LFD mice, suggesting that DIO mice may be in an early state of obesity. By contrast, significantly dysregulated miRNAs were identified in both groups. Notably, most (23 of 28) of these circulating miRNAs were upregulated in DIO + LFD and downregulated in DIO mice, implying that the downregulation of these miRNAs by obesity could be reversed by LFD treatment. In addition, target prediction and function annotation revealed that the target genes associated with these 23 differentially expressed miRNAs are involved in metabolic, insulin, and adipocytokine signaling pathways that directly link the pathophysiological changes that occur during obesity and weight reduction. Therefore, whether miRNA supplementation represents a potential therapeutic strategy to treat obesity is an interesting topic requiring further robust investigations to clarify.

As many miRNAs are extensive regulators of adipocyte development and function, their differential expression in the adipose tissue of mice in response to HFD-induced obesity has been explored and discussed in a number of articles [11–13, 21]. For example, in a microarray experiment, 26 miRNAs were upregulated in WAT in response to HFD feeding, including mmu-miR-342-3p, mmu-miR-222, mmu-miR-221, mmu-miR-142-3p, mmu-miR-142-5p, mmu-miR-21, mmu-miR-335-5p, mmu-miR-146a, mmumiR-146b, mmu-miR-647*, and mmu-miR-379, whereas the following miRNAs were downregulated: mmu-miR-141, mmu-miR-200a, mmu-miR-200b, mmumiR-200c, mmu-miR-122, mmu-miR-204, mmu-miR-133b, mmu-miR-1, mmu-miR-30a*, mmu-miR-130a, mmu-miR-192, mmu-miR-193a-3p, mmu-miR-203, mmu-miR-378, and mmumiR-30e* [21]. Some of the circulating miRNAs identified in this study have also been reported in the adipose tissue of DIO mice or implicated in adipogenic processes [11–13], including *Let-7*, miR-103, miR-15, the miR-17-92 cluster (miR-17, miR-20a, and miR-92a), miR-21, miR-221, and miR-30b. However, these miRNA that were previously reported to be upregulated in adipose tissue were downregulated in DIO mice but upregulated in DIO + LFD mice in this study, casting doubt on the suggestion that these circulating miRNAs originated

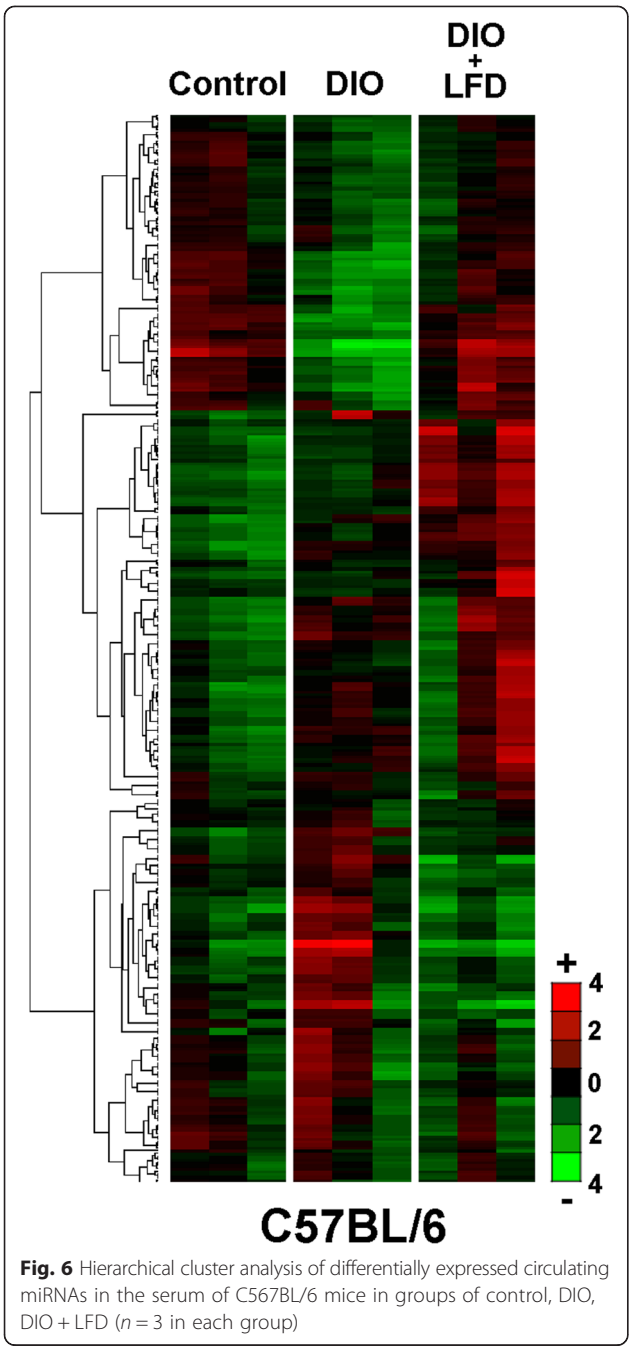

Fig. 6 Hierarchical cluster analysis of differentially expressed circulating miRNAs in the serum of C567BL/6 mice in groups of control, DIO, DIO + LFD ($n = 3$ in each group)

Table 1 miRNA targets dys-regulated more than 2-fold in serum of C57BL/6NCrl mice at 12w high-fat diet-induced obesity vs. control (P-value < 0.05)

Up-regulated			Down-regulated		
DIO	Fold (log_2)	p-value	DIO	Fold (log_2)	p-value
mmu-miR-711	2.15	0.00	mmu-let-7i	−3.98	0.01
mmu-miR-712	1.58	0.04	mmu-miR-16	−3.83	0.01
mmu-miR-713	1.41	0.03	mmu-miR-15a	−3.02	0.01
mmu-miR-714	1.12	0.05	mmu-miR-26a	−2.61	0.00
mmu-miR-715	1.07	0.01	mmu-miR-107	−2.53	0.00
mmu-miR-716	1.05	0.04	mmu-miR-106b	−2.51	0.01
mmu-miR-717	1.05	0.05	mmu-miR-17	−2.49	0.00
mmu-miR-574	1.03	0.03	mmu-miR-93	−2.36	0.00
			mmu-let-7b	−2.33	0.00
			mmu-miR-15b	−2.31	0.02
			mmu-miR-25	−2.26	0.04
			mmu-let-7c	−2.20	0.00
			mmu-miR-20a	−2.17	0.00
			mmu-miR-103	−2.16	0.00
			mmu-miR-221	−1.97	0.00
			mmu-miR-195	−1.86	0.03
			mmu-miR-21	−1.85	0.00
			mmu-miR-106a	−1.82	0.00
			mmu-let-7a	−1.70	0.01
			mmu-let-7f	−1.67	0.04
			mmu-miR-30c	−1.55	0.05
			mmu-let-7d	−1.52	0.02
			mmu-miR-19b	−1.41	0.01
			mmu-miR-30e	−1.35	0.02
			mmu-miR-30a	−1.34	0.02
			mmu-miR-23b	−1.32	0.03
			mmu-miR-486	−1.28	0.03
			mmu-miR-30b	−1.25	0.01
			mmu-miR-130b	−1.25	0.05
			mmu-miR-106b*	−1.14	0.02
			mmu-miR-185	−1.10	0.03
			mmu-miR-18a	−1.06	0.01
			mmu-miR-17*	−1.04	0.04
			mmu-miR-148b	−1.01	0.04

from the adipose tissue of obese mice. Notably, although the origin of circulating miRNAs is debatable, the expression profile of circulating miRNAs is obviously different from those in pathological tissues [22–24]. Whether these circulating miRNAs originated from adipose tissue, blood cells, or other cells of the circulating system requires further experimentation to clarify.

In this study, some of the identified dysregulated miRNAs have been linked to obesity or adipogenesis in the literature. Among them, five members of the Let-7 family (mmu-let-7a, mmu-let-7b, mmu-let-7c, mmu-let-7f, and mmu-let-7i) were dysregulated in response to obesity and weight reduction following LFD feeding. Mice with global overexpression of *Let-7* are viable, but they have reduced body size and weight [25]. In mice, 12 genes encode members of the *Let*-7 family, which includes nine slightly different miRNAs (*Let*-7a, *Let*-c, and *Let*-7f [all encoded by two genes], and *Let*-7b, *Let*-7d,

Table 2 miRNA targets dys-regulated more than 2-fold in serum of C57BL/6NCrl mice following 4 w low-fat diet after 8 w high-fat diet-induced obesity vs. 12 w high-fat diet-induced obesity (*P*-value < 0.05)

Up-regulated			Down-regulated		
DIO + LFD	Fold (log_2)	*p*-value	DIO + LFD	Fold (log_2)	*p*-value
mmu-miR-16	3.48	0.00	mmu-miR-1983	−3.29	0.05
mmu-let-7i	3.23	0.01	mmu-miR-5112	−2.39	0.02
mmu-miR-26a	2.57	0.02	mmu-miR-1894	−2.18	0.02
mmu-miR-17	2.38	0.02	mmu-miR-5109	−1.88	0.01
mmu-miR-107	2.35	0.02	mmu-miR-711	−1.80	0.00
mmu-miR-451	2.34	0.00	mmu-miR-351*	−1.37	0.04
mmu-miR-195	2.28	0.01	mmu-miR-700	−1.19	0.01
mmu-miR-20a	2.06	0.01	mmu-miR-1940	−1.15	0.03
mmu-miR-25	1.88	0.03	mmu-miR-204*	−1.14	0.02
mmu-miR-15b	1.85	0.01	mmu-miR-125b	−1.09	0.03
mmu-miR-15a	1.83	0.01			
mmu-let-7b	1.73	0.00			
mmu-let-7a	1.69	0.00			
mmu-let-7c	1.64	0.02			
mmu-miR-103	1.61	0.01			
mmu-let-7f	1.57	0.01			
mmu-miR-106a	1.56	0.01			
mmu-miR-106b	1.48	0.04			
mmu-miR-93	1.37	0.01			
mmu-miR-23b	1.34	0.02			
mmu-miR-21	1.23	0.01			
mmu-miR-223	1.18	0.04			
mmu-miR-30b	1.16	0.02			
mmu-miR-221	1.11	0.01			
mmu-miR-19b	1.06	0.02			
mmu-miR-92a	1.05	0.02			
mmu-miR-200c	1.03	0.02			
mmu-miR-873	1.00	0.04			

Let-7e, *Let*-7g, *Let*-7i, and miR-98 [all encoded by one gene]). All *Let*-7 family members are believed to have similar functions because they share a common seed region (nucleotides 2–8), which mediates interactions between miRNA and target mRNAs [25]. Furthermore, *Let-7* transgenic mice exhibit impaired glucose tolerance because of diminished glucose-induced insulin secretion, and anti-miR–induced silencing of *Let-7* has been proven to improve blood glucose levels and insulin resistance in obese mice [25].

In vivo, miR-103 is downregulated in the mature adipocytes of obese mice [26] and upregulated during the differentiation of human and murine pre-adipocytes [27, 28]. A 9-fold upregulation of miR-103 was noted during early adipogenesis in 3T3-L1 pre-adipocyte cells, and lipid droplet formation was accelerated when it was ectopically expressed [26]. miR-103 is also upregulated during porcine adipogenesis, and its inhibition suppresses adipogenesis [26].

miR-15a overexpression leads to a decrease in the number, but an increase in the size, of murine adipocytes by inhibiting Delta-like 1 homolog expression [29]. In a study of miRNA libraries reconstructed from pre- and post-differentiated 3T3-L1 cells, it was noted that miR-15a may not be related to the actual differentiation process, but it may induce growth arrest and/or hormonal stimulation [30]. In addition, the miR-17-19 cluster, which comprises seven miRNAs (miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20, miR-19b, and miR-92-1) and promotes cell proliferation in various cancers, has been demonstrated to be significantly upregulated at the clonal expansion stage of adipocyte differentiation. MiR-17-92 has been revealed to target Rb2/p130, an important early regulator of pre-adipocyte clonal expansion [31], and the stable transfection of 3T3L1 cells with miR-17-92 resulted in accelerated differentiation and increased triglyceride accumulation after hormonal stimulation [32]. The adipogenic miR-21 has also been demonstrated to be upregulated in human obesity [33] and to enhance adipogenesis in human adipose tissue-derived mesenchymal stem cells (hASCs) by mediating TGF-β signaling [34].

The miR-30 family has been found to be important for adipogenesis [12]. In this study, miR-30a, miR-30b, and miR-30c were significantly downregulated in obese mice, and miR-30b was significantly upregulated after LFD feeding. MiR-30 family members are strongly upregulated during adipogenesis in human cells, and inhibition of miR-30 inhibits adipogenesis [12]. miR-30 family members have also been demonstrated to act as positive regulators of adipocyte differentiation in a human adipose tissue-derived stem cell model [35]. Overexpression of miR-30a and miR-30d stimulates adipogenesis, and it has been demonstrated that miR-30a and miR-30d target RUNX2, a major regulator of osteogenesis and a potent inhibitor of PPARγ, the master gene in adipogenesis [36]. MiR-30c has been found to be upregulated in adipogenesis and to enhance adipogenesis in hASCs, and it appears to target two genes (PAI-1 and ALK2) in distinct pathways [37]. Moreover, miR-30d has been identified as a positive regulator of insulin transcription [38].

Furthermore, in this study, eight miRNAs (mmu-miR-711, mmu-miR-712, mmu-miR-713, mmu-miR-714, mmu-miR-715, mmu-miR-716, mmu-miR-717, and mmu-miR-574) were upregulated in DIO mice. Of these, miR-712 is a mechanosensitive miRNA that is upregulated in endothelial cells by disturbed flow, which regulates

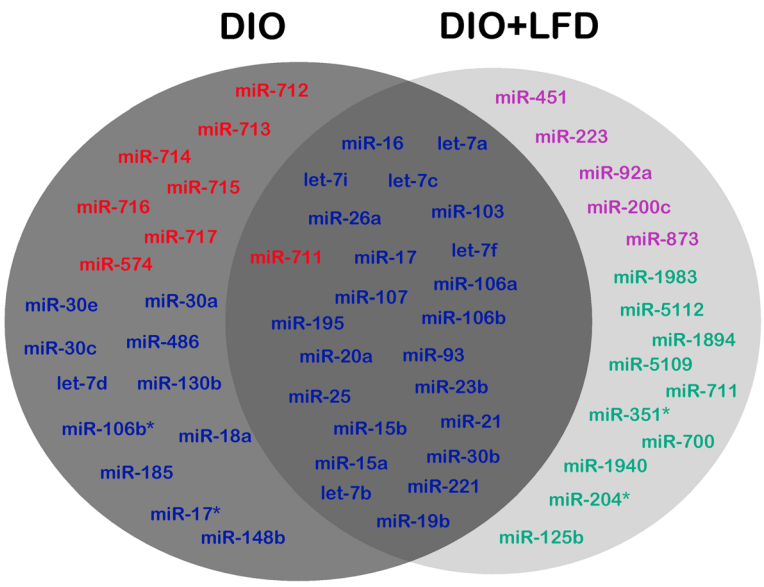

Fig. 7 Venn diagram of the changed miRNAs in the serum of C567BL/6 mice in groups of DIO and DIO + LFD

endothelial dysfunction and atherosclerosis [39, 40]. MiR-717, which was first reported in mice, is encoded by intron 3 of the body mass-associated glypican-3 (Gpc3) gene, and it plays an important regulatory role in renal osmoregulation. Meanwhile, Gpc3 knockout mice display increased body mass, renal dysplasia, and perinatal mortality [41]. Bioinformatics analysis enables functional annotation of MiR-717 orthologs to determine the effect of its target genes on fat-related traits [42]. However, the effects and mechanisms of these eight upregulated miRNAs in the obese mice in this study are poorly understood.

Conclusion

This study identified the expression profile of circulating miRNAs in a mouse model of DIO and DIO with

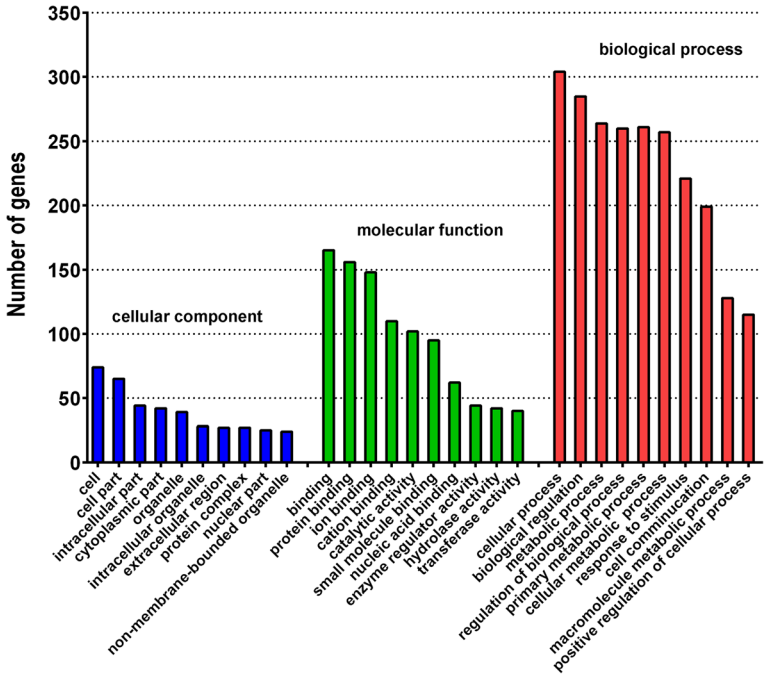

Fig. 8 Partial gene ontology (GO) classification annotated for the predicted target genes of 23 interesting up-regulated miRNAs. The figure shows partial GO enrichment for the predicted target genes in cellular component, molecular function, and biological process. All the $P < 0.001$

Table 3 The most enriched KEGG pathways of target genes for 23 differentially expressed miRNAs between the sera of C57BL/6NCrl mice during obesity and weight reduction by low-fat diet

Pathway	Count of genes	*P*-value	Pathway ID
Metabolic pathways	1024	2.11E06	ko01100
MAPK signaling pathway	514	1.82E05	ko04010
Regulation of actin cytoskeleton	471	3.09E05	ko04810
Biosynthesis of secondary metabolites	392	4.72E05	ko05200
Focal adhesion	342	6.21E10	ko04510
Insulin signaling pathway	318	5.13E05	ko04910
Calcium signaling pathway	295	5.34E05	ko04020
Cytokine-cytokine receptor interaction	228	2.16E04	ko04060
Tight junction	172	4.22E06	ko04530
Phagosome	144	2.14E05	ko04145
Adipocytokine signaling pathway	117	1.27E04	ko04920

subsequent weight reduction through LFD feeding. The results demonstrated that the majority of miRNA down-regulation in association with obesity could be reversed by LFD feeding. Target prediction and function annotation revealed that the target genes associated with these 23 differentially expressed miRNAs are involved in metabolic, insulin, and adipocytokine signaling pathways that directly link the pathophysiological changes that occur during obesity and weight reduction.

Additional file

Additional file 1: Correlation of the miRNA expression in the microarray and qPCR. (TIFF 83 kb).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
CHH was responsible for the design and coordination of the data acquisition and analysis, as well as writing and revising the manuscript. CSR contributed to the drafting of the manuscript. SCW was responsible for the animal study and revision of the manuscript. JCY participated by providing and coordinating the resources. CJW and CWL were involved in the acquisition of microRNA array data. THL, YCW, and SLT contributed to the acquisition of the study specimens. All authors read and approved the final manuscript.

Acknowledgements
The work was supported by a grant from Chang Gung Memorial Hospital (CMRPG8B1421 awarded to Ching-Hua Hsieh and CMRPG8C0351 awarded to Cheng-Shyuan Rau) and a grant from E-Da Hospital (EDPJ104021 awarded to Seng-Feng Jeng).

Author details
[1]Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No. 123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan. [2]Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No. 123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan. [3]Department of Anesthesiology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, No. 123, Ta-Pei Road, Niao-Song District, Kaohsiung City 833, Taiwan.

Received: 20 October 2014 Accepted: 7 September 2015
Published online: 16 September 2015

References
1. Tsukumo DM, Carvalho-Filho MA, Carvalheira JB, Prada PO, Hirabara SM, Schenka AA, et al. Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. Diabetes. 2007;56(8):1986–98.
2. Davis JE, Braucher DR, Walker-Daniels J, Spurlock ME. Absence of Tlr2 protects against high-fat diet-induced inflammation and results in greater insulin-stimulated glucose transport in cultured adipocytes. J Nutr Biochem. 2011;22(2):136–41.
3. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab. 2004;89(6):2548–56.
4. Despres JP, Lemieux I. Abdominal obesity and metabolic syndrome. Nature. 2006;444(7121):881–7.
5. Wajchenberg BL. Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome. Endocr Rev. 2000;21(6):697–738.
6. Betanzos-Cabrera G, Estrada-Luna D, Belefant-Miller H, Cancino-Diaz JC. Mice fed with a high fat diet show a decrease in the expression of "toll like receptor (TLR)2 and TLR6 mRNAs in adipose and hepatic tissues. Nutr Hosp. 2012;27(4):1196–203.
7. Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN. Diet-induced type II diabetes in C57BL/6J mice. Diabetes. 1988;37(9):1163–7.
8. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc Natl Acad Sci U S A. 2003;100(12):7265–70.
9. Weiss EP, Racette SB, Villareal DT, Fontana L, Steger-May K, Schechtman KB, et al. Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial. Am J Clin Nutr. 2006;84(5):1033–42.
10. Muurling M, Jong MC, Mensink RP, Hornstra G, Dahlmans VE, Pijl H, et al. A low-fat diet has a higher potential than energy restriction to improve high-fat diet-induced insulin resistance in mice. Metabolism. 2002;51(6):695–701.
11. Peng Y, Yu S, Li H, Xiang H, Peng J, Jiang S. MicroRNAs: emerging roles in adipogenesis and obesity. Cell Signal. 2014;26(9):1888–96.
12. Hilton C, Neville MJ, Karpe F. MicroRNAs in adipose tissue: their role in adipogenesis and obesity. Int J Obes (2005). 2013;37(3):325–32.
13. Alexander R, Lodish H, Sun L. MicroRNAs in adipogenesis and as therapeutic targets for obesity. Expert Opin Ther Targets. 2011;15(5):623–36.
14. Hsieh CH, Rau CS, Jeng JC, Chen YC, Lu TH, Wu CJ, et al. Whole blood-derived microRNA signatures in mice exposed to lipopolysaccharides. J Biomed Sci. 2012;19(69):1423–0127.
15. Hsieh CH, Yang JC, Jeng JC, Chen YC, Lu TH, Tzeng SL, et al. Circulating microRNA signatures in mice exposed to lipoteichoic acid. J Biomed Sci. 2013;20(2):1423–0127.
16. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513–8.
17. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem. 2010;285(23):17442–52.
18. Chen X, Liang H, Zhang J, Zen K, Zhang CY. Secreted microRNAs: a new form of intercellular communication. Trends Cell Biol. 2012;22(3):125–32.
19. Hoevenaars FP, Keijer J, Herreman L, Palm I, Hegeman MA, Swarts HJ, et al. Adipose tissue metabolism and inflammation are differently affected by weight loss in obese mice due to either a high-fat diet restriction or change to a low-fat diet. Genes & nutrition. 2014;9(3):391.
20. Fenton JI, Nunez NP, Yakar S, Perkins SN, Hord NG, Hursting SD. Diet-induced adiposity alters the serum profile of inflammation in C57BL/6N mice as measured by antibody array. Diabetes Obes Metab. 2009;11(4):343–54.
21. Chartoumpekis DV, Zaravinos A, Ziros PG, Iskrenova RP, Psyrogiannis AI, Kyriazopoulou VE, et al. Differential expression of microRNAs in adipose tissue after long-term high-fat diet-induced obesity in mice. PLoS One. 2012;7(4):e34872.
22. Hsieh CH, Rau CS, Jeng JC, Chen YC, Lu TH, Wu CJ, et al. Whole blood-derived microRNA signatures in mice exposed to lipopolysaccharides. J Biomed Sci. 2012;19:69.

23. Hsieh CH, Yang JC, Jeng JC, Chen YC, Lu TH, Tzeng SL, et al. Circulating microRNA signatures in mice exposed to lipoteichoic acid. J Biomed Sci. 2013;20:2.
24. Rau CS, Yang JC, Wu SC, Chen YC, Lu TH, Lin MW, et al. Profiling circulating microRNA expression in a mouse model of nerve allotransplantation. J Biomed Sci. 2013;20:64.
25. Frost RJ, Olson EN. Control of glucose homeostasis and insulin sensitivity by the Let-7 family of microRNAs. Proc Natl Acad Sci U S A. 2011;108(52):21075–80.
26. Xie H, Lim B, Lodish HF. MicroRNAs Induced During Adipogenesis that Accelerate Fat Cell Development Are Downregulated in Obesity. Diabetes. 2009;58(5):1050–7.
27. Esau C, Kang X, Peralta E, Hanson E, Marcusson EG, Ravichandran LV, et al. MicroRNA-143 regulates adipocyte differentiation. J Biol Chem. 2004;279(50):52361–5.
28. Sun T, Fu M, Bookout AL, Kliewer SA, Mangelsdorf DJ. MicroRNA let-7 regulates 3T3-L1 adipogenesis. Mol Endocrinol (Baltimore, Md). 2009;23(6):925–31.
29. Andersen DC, Jensen CH, Schneider M, Nossent AY, Eskildsen T, Hansen JL, et al. MicroRNA-15a fine-tunes the level of Delta-like 1 homolog (DLK1) in proliferating 3T3-L1 preadipocytes. Exp Cell Res. 2010;316(10):1681–91.
30. Kajimoto K, Naraba H, Iwai N. MicroRNA and 3T3-L1 pre-adipocyte differentiation. RNA (New York, NY). 2006;12(9):1626–32.
31. Prince AM, May JS, Burton GR, Lyle RE, McGehee Jr RE. Proteasomal degradation of retinoblastoma-related p130 during adipocyte differentiation. Biochem Biophys Res Commun. 2002;290(3):1066–71.
32. Wang Q, Li YC, Wang J, Kong J, Qi Y, Quigg RJ, et al. miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor-suppressor Rb2/p130. Proc Natl Acad Sci U S A. 2008;105(8):2889–94.
33. Keller P, Gburcik V, Petrovic N, Gallagher IJ, Nedergaard J, Cannon B, et al. Gene-chip studies of adipogenesis-regulated microRNAs in mouse primary adipocytes and human obesity. BMC Endocr Disord. 2011;11:7.
34. Kim YJ, Hwang SJ, Bae YC, Jung JS. MiR-21 regulates adipogenic differentiation through the modulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue. Stem Cells (Dayton, Ohio). 2009;27(12):3093–102.
35. Zaragosi LE, Wdziekonski B, Brigand KL, Villageois P, Mari B, Waldmann R, et al. Small RNA sequencing reveals miR-642a-3p as a novel adipocyte-specific microRNA and miR-30 as a key regulator of human adipogenesis. Genome Biol. 2011;12(7):R64.
36. Enomoto H, Furuichi T, Zanma A, Yamana K, Yoshida C, Sumitani S, et al. Runx2 deficiency in chondrocytes causes adipogenic changes in vitro. J Cell Sci. 2004;117(Pt 3):417–25.
37. Karbiener M, Neuhold C, Opriessnig P, Prokesch A, Bogner-Strauss JG, Scheideler M. MicroRNA-30c promotes human adipocyte differentiation and co-represses PAI-1 and ALK2. RNA Biol. 2011;8(5):850–60.
38. Tang X, Muniappan L, Tang G, Ozcan S. Identification of glucose-regulated miRNAs from pancreatic {beta} cells reveals a role for miR-30d in insulin transcription. RNA (New York, NY). 2009;15(2):287–93.
39. Kumar S, Kim CW, Simmons RD, Jo H. Role of Flow-Sensitive microRNAs in Endothelial Dysfunction and Atherosclerosis: Mechanosensitive Athero-miRs. Arterioscler Thromb Vasc Biol. 2014;34(10):2206–16.
40. Son DJ, Kumar S, Takabe W, Kim CW, Ni CW, Alberts-Grill N, et al. The atypical mechanosensitive microRNA-712 derived from pre-ribosomal RNA induces endothelial inflammation and atherosclerosis. Nat Commun. 2013;4:3000.
41. Cano-Gauci DF, Song HH, Yang H, McKerlie C, Choo B, Shi W, et al. Glypican-3-deficient mice exhibit developmental overgrowth and some of the abnormalities typical of Simpson-Golabi-Behmel syndrome. J Cell Biol. 1999;146(1):255–64.
42. Kunej T, Skok DJ, Horvat S, Dovc P, Jiang Z. The glypican 3-hosted murine mir717 gene: sequence conservation, seed region polymorphisms and putative targets. Int J Biol Sci. 2010;6(7):769–72.

Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2015, Article ID 852126, 12 pages
http://dx.doi.org/10.1155/2015/852126

Research Article

Effect of Weight-Reduction in Obese Mice Lacking Toll-Like Receptor 5 and C57BL/6 Mice Fed a Low-Fat Diet

Shao-Chun Wu,[1] Cheng-Shyuan Rau,[2] Tsu-Hsiang Lu,[3] Siou-Ling Tzeng,[3] Yi-Chan Wu,[3] Chia-Jung Wu,[3] Chia-Wei Lin,[3] and Ching-Hua Hsieh[3]

[1]*Department of Anesthesiology, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taoyuan City, Taiwan*
[2]*Department of Neurosurgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taoyuan City, Taiwan*
[3]*Department of Plastic and Reconstructive Surgery, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Taoyuan City, Taiwan*

Correspondence should be addressed to Ching-Hua Hsieh; m93chinghua@gmail.com

Received 27 July 2015; Revised 20 October 2015; Accepted 25 October 2015

Academic Editor: Luca Cantarini

Background. This study aims to investigate the effect of feeding low-fat diet (LFD) to diet-induced obesity (DIO) mice lacking TLR5 ($TLR5^{-/-}$), which have a tendency to develop glucose intolerance with increased adiposity, compared to that in C57BL/6 mice. *Results*. $TLR5^{-/-}$ and C57BL/6 male mice were divided into three subgroups: (1) control, mice were fed a standard AIN-76A (fat: 11.5 kcal%) diet for 12 weeks; (2) DIO, mice were fed a 58 kcal% high-fat diet (HFD) for 12 weeks; and (3) diet, mice were fed a HFD for 8 weeks to induce obesity and then switched to a 10.5 kcal% LFD for 4 weeks. The glucose intolerance in DIO $TLR5^{-/-}$ mice was more significant than that in DIO C57BL/6 mice and was not attenuated by a switch to the LFD. Weight-reduction with LFD had significantly decreased the epididymal fat mass in C57BL/6 mice but not in $TLR5^{-/-}$ mice. In addition, the LFD-fed $TLR5^{-/-}$ mice showed significantly higher expression of ghrelin in the serum and resistin in the epididymal fat than that in C57BL/6 mice. *Conclusions*. This study demonstrated that *TLR5* gene knockout impairs some effects of weight-reduction in DIO.

1. Introduction

Obesity is associated with insulin resistance and an abnormal inflammatory response [1, 2]. High-fat uptake leads to metabolic alterations in the adipose tissue that is associated with the synthesis and release of a huge amount of proinflammatory adipokines and cytokines such as leptin, resistin, PAI-1, IL-6, IL-10, and TNF-α [2]. Increased level of the circulating free fatty acids also leads to macrophage activation and the production of proinflammatory cytokines via Toll-like receptors (TLRs) [3]. The adipocytes and preadipocytes isolated from the adipose tissues of the *ob/ob* and *db/db* mice, which are leptin and leptin-receptor-deficient, respectively, were characterized by significant upregulation of *TLR1* to *TLR9* expression than that with wild type cells [4–6]. Upregulated expressions of *TLR1* to *TLR9* and *TLR11* to *TLR13* are also observed in adipose tissues of high-fat diet- (HFD-) induced obese mice or leptin-deficient obese mice [7]. The magnitudes of the obesity-induced upregulation of the *TLR1*, *TLR4*, *TLR5*, *TLR8*, *TLR9,* and *TLR12* genes in the visceral adipose tissue were even greater in the diet-induced obesity (DIO) mice than in the *ob/ob* mice [7]. These upregulated expressions of TLRs in the expanded adipose tissues of obese mice are linked with downstream NF-κB, IRFs, and STAT-1 activation and upregulated expressions of cytokines and chemokines via MyD88-dependent and MyD88-independent cascades [7].

Evidence collected from these inbred mouse strains suggests that the detrimental effects in metabolism due to HFD are strain dependent, and some strains such as C57BL/6J and C57BL/6N are genetically predisposed to metabolic defects resulting from HFD feeding [8, 9]. When C57BL/6J mice were fed a high-fat diet (HFD) with *ad libitum*, they developed

insulin resistance and obesity in a manner that resembles disease progression in humans [10]. Furthermore, mice lacking Toll-like receptor 5 (TLR5), on a mixed C57BL/6J and C57BL/6N genetic background, develop insulin resistance and increased adiposity [11–13]. These mice exhibit hyperphagia and develop hallmark features of metabolic syndrome including hyperlipidemia, hypertension, insulin resistance, and increased adiposity [13]. The TLR5$^{-/-}$ mice consumed about 10% more food, had greater stool output, and did not significantly impact the efficiency of dietary energy harvest than wild type littermates [13]. The TLR5$^{-/-}$ mice also exhibited a reduced response to exogenous insulin relative to wild type mice. In addition, the insulin resistance of TLR5$^{-/-}$ mice is not entirely dependent on increased food consumption or adiposity seeing that the lean TLR5$^{-/-}$ mice after 12 weeks of food restriction regimen still exhibited a decreased response to exogenous insulin [13]. TLR5 is a transmembrane protein that is highly expressed in the intestinal mucosa and recognizes bacterial flagellin. HFD and bacteria interact to promote early inflammatory changes in the small intestine that contribute to the development of susceptibility to obesity and insulin resistance [12]. The low-grade proinflammatory signaling in TLR5-deficient mice may attenuate insulin signaling, resulting in increased food consumption that drives other manifestations of metabolic syndrome [13]. Moreover, the humans with the segregation of a dominant nonsense polymorphism (R392X, rs5744168) lack TLR5 function and become susceptible to type 2 diabetes [14].

Decreased energy intake and increased energy expenditure, which reduces adiposity and restore insulin sensitivity, are the two most commonly recommended lifestyle changes to treat DIO and its related disorders [15]. Calorie restriction is effective in improving the insulin sensitivity and decreasing both body weight and percent body fat [16]. Moreover, switching from a HFD to a low-fat diet (LFD) can reduce body weight and improve insulin sensitivity by reducing the percentage of fat in a diet [17]. Although food restriction prevents obesity, but not insulin resistance, in TLR5-deficient mice [13], limited information is known regarding the LFD effect on TLR5-deficient obese mice. In this study, we aim to investigate the diet effect of LFD feeding on the DIO mice lacking TLR5 against C57BL/6 mice.

2. Materials and Methods

2.1. Animal Experiments. C57BL/6NCrl mice were purchased from BioLasco (Taipei, Taiwan). TLR5$^{-/-}$ (B6.129S1-Tlr5tm1Flv/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed, and surgical procedures, including analgesia, were performed in Association for Assessment and Accreditation of Laboratory Animal Care International-accredited SPF facility according to national and institutional guidelines to minimize the suffering of affected animals. Animal protocols were approved by the IACUC of Chang Gung Memorial Hospital, Taiwan (permission number 2012091002). In this experiment, 24 male TLR5$^{-/-}$ and 24 male C57BL/6NCrl mice were randomly assigned to three subgroups ($n = 8$ in each group) as follows: (1) control, where mice were fed a standard AIN-76A (fat: 11.5 kcal%) diet with *ad libitum* for 12 weeks; (2) DIO, where mice were fed a 58 kcal% HFD (D12331; Research Diets Inc., New Brunswick, NJ) with *ad libitum* for 12 weeks to induce obesity; and (3) diet, where mice were fed a 58 kcal% HFD (D12331) *ad libitum* for 8 weeks to induce obesity and then fed a 10.5 kcal% LFD (D 12329; Research Diets Inc.) for 4 weeks. Weight measurements were recorded weekly, and an intraperitoneal glucose tolerance test (IPGTT) was performed at the beginning and end of the experiment to confirm that HFD-fed mice developed an obese and insulin-resistant phenotype. Briefly, mice were allowed to fast for 5 h, and baseline blood glucose levels were measured with an Accu-Check Advantage blood glucose meter (Roche, NJ) using blood samples collected from the tail vein. Mice were injected intraperitoneally with 2 g of glucose (in sterile PBS) per kilogram body weight. The glucose level was measured via tail vein blood (~10 μL) at $t = -30$ and 0 (pre) and $t = 15$, 30, 60, 90, and 120 min after the glucose infusion. Data were averaged and graphed as blood glucose level as a function of time. To reflect the circulating levels of glucose during the glucose tolerance test, we calculated the total area under the curve (AUC) of the glucose concentration versus time by the linear trapezoidal rule for the period of 0–120 min after glucose infusion. In the end of the experiment, all mice were euthanized, and the epididymal white adipose tissue (WAT) of each mouse was collected and weighed. The adipose tissue block embedded in paraffin was sectioned at 5 μm to measure the adipocyte area. Three 5 μm-thick sections of the same fat specimen at 50 μm distance were mounted on glass plate and stained with hematoxylin and eosin. Two different microscopic fields (magnification ×100) per plate were photographed and 100 adipose cells were arbitrarily selected in the center of field and their cell diameters were assessed by tracing the outline of each adipocyte. The mean adipocyte area was measured from the epididymal WAT of control and experimental mice ($n = 8$ in each group) using Image-Pro Plus image analysis software (Carl Zeiss, Oberkochen, Germany) and expressed in terms of square micrometers. The liver embedded in paraffin was sectioned at 5 μm and stained with hematoxylin and eosin. At the end of the experiment, 1 mL of whole blood was collected via cardiac puncture into a plain tube and allowed to clot for 1 h. Samples were centrifuged at 3000 ×g for 10 min, and sera were aliquoted and stored at −80°C until further analysis.

2.2. Cytokine Assays. Cytokine concentrations in the serum and epididymal WAT were analyzed using Bio-Plex Cytokine Assay (Mouse Diabetes 8-Plex, cat. number 171-F7001M, Bio-Rad, Hercules, CA) including ghrelin, gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1), insulin, leptin, plasminogen activator inhibitor type 1 (PAI-1), glucagon, and resistin, which are potentially involved in obesity-associated diabetes. Expressions of IL-6, IL-10, and TNF-α in the adipose tissue were also assessed using the Bio-Plex system (BioRad). Assays were performed on four biological replicates as per the manufacturer's instructions. Results were expressed in picograms per milliliter of serum or per milligram of adipose tissue.

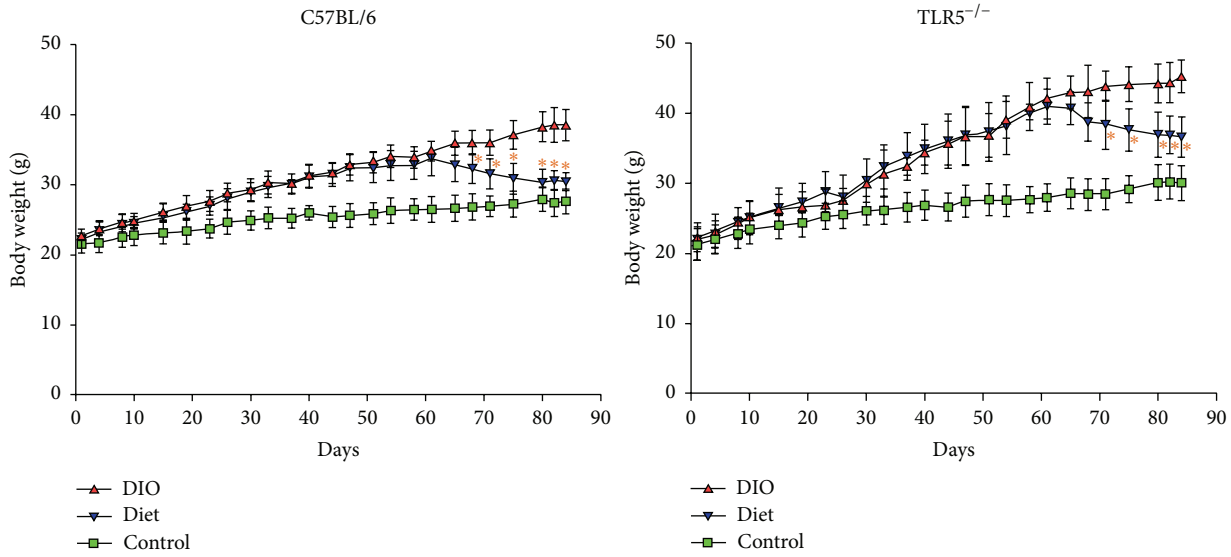

Figure 1: The body weight of C57BL/6 and TLR5$^{-/-}$ mice on a weekly basis till week 12 ($n = 8$). Control: the mice fed with the standard diet; DIO: the mice fed with high-fat diet; diet: the mice fed with high-fat diet for 8 weeks to induce obesity and then we change to low-fat diet for subsequent 4 weeks. * (red color), $p < 0.05$ versus DIO.

2.3. Statistical Analysis. All experimental data are expressed as the mean ± standard error of the mean. Analysis of variance combined with a Bonferroni post hoc correction was performed to identify significant differences in body weight, weight of fat, adipocyte area, glucose levels, and serum cytokine levels. $p \geq 0.05$ was regarded as the level of statistical significance.

3. Results

3.1. LFD Reduces the Body Weight Gain by HFD Feeding. HFD-fed C57BL/6 and TLR5$^{-/-}$ mice gained more body weight compared to regular chow, as shown in Figure 1. At the end of the experiment, the difference in body weight gained between the DIO and control groups reached 10.8 g in C57BL/6 mice (38.5 ± 1.2 versus 27.7 ± 1.8 g) and 15.3 g in TLR5$^{-/-}$ mice (45.3 ± 2.3 versus 30.0 ± 2.5 g). The TLR5$^{-/-}$ mice gained more weight by HFD feeding than C57BL/6 mice at week 12. The LFD-induced weight-reduction in DIO mice became significant at week 2 and week 3 for C57BL/6 and TLR5$^{-/-}$ mice, respectively, and the body weight decreased by 8.1 g in C57BL/6 mice (38.5 ± 1.2 versus 30.4 ± 1.3 g) and by 8.7 g in TLR5$^{-/-}$ mice (45.3 ± 2.3 versus 36.6 ± 2.9 g) 4 weeks later. After 4 weeks of LFD feeding, 21.0% and 19.2% reduction in body weight of the DIO C57BL/6 and TLR5$^{-/-}$ mice, respectively, were recorded.

3.2. TLR5$^{-/-}$ Present Severe Glucose Intolerance Than C57BL/6 Mice. In the control C57BL/6 mice, glucose infusion increased the blood glucose levels to a peak of 300 mg/dL after 15 min and then gradually returned to baseline after 120 min (Figure 2). HFD-fed animals displayed significantly higher blood glucose concentrations at 30 to 120 min during the IPGTT compared to control mice, which is evident by a 36% higher incremental glucose AUC. In addition, significantly lower glucose level was observed in LFD-fed mice at 30 and 60 min after the glucose infusion against the HFD-fed mice, resulting in around 15% lower glucose AUC. In control TLR5$^{-/-}$ mice, the response to glucose infusion was quite similar to that in C57BL/6 mice. In DIO TLR5$^{-/-}$ mice, glucose infusion increased blood glucose levels to a peak of 420 mg/dL after 30 min and then gradually decreased but did not return to the baseline after 120 min. With a 60% higher incremental glucose AUC, the glucose intolerance in DIO TLR5$^{-/-}$ mice was more significant compared to DIO C57BL/6 mice. Furthermore, unlike C57BL/6 mice, no significantly lower glucose level or glucose AUC was observed in LFD-fed TLR5$^{-/-}$ mice during IPGTT after glucose infusion against DIO TLR5$^{-/-}$ mice.

3.3. TLR5$^{-/-}$ Present Different Adiposity Response to Weight-Reduction Than C57BL/6 Mice. As revealed in Figure 3, the histologic examination of epididymal fat demonstrates that HFD increased the size of adipocytes and induced a significant level of adipocyte hypertrophy at the end of the experiment in both C57BL/6 and TLR5$^{-/-}$ mice. HFD for 8 weeks increased the average epididymal fat mass by ~1.9 g and ~ 1.7 g in C57BL/6 and TLR5$^{-/-}$ mice, respectively, compared to those fed on regular chow. Notably, histologic examination also revealed that the size of adipocytes was significantly larger in the control TLR5$^{-/-}$ mice compared to C57BL/6 mice. In addition, although a switch to the LFD had significantly decreased the epididymal WAT in C57BL/6 mice, the decrease of epididymal fat mass was not significant in TLR5$^{-/-}$ mice. Moreover, there was no reduction in the diameter of fat lobules after a switch to LFD feeding in both C57BL/6 and TLR5$^{-/-}$ mice.

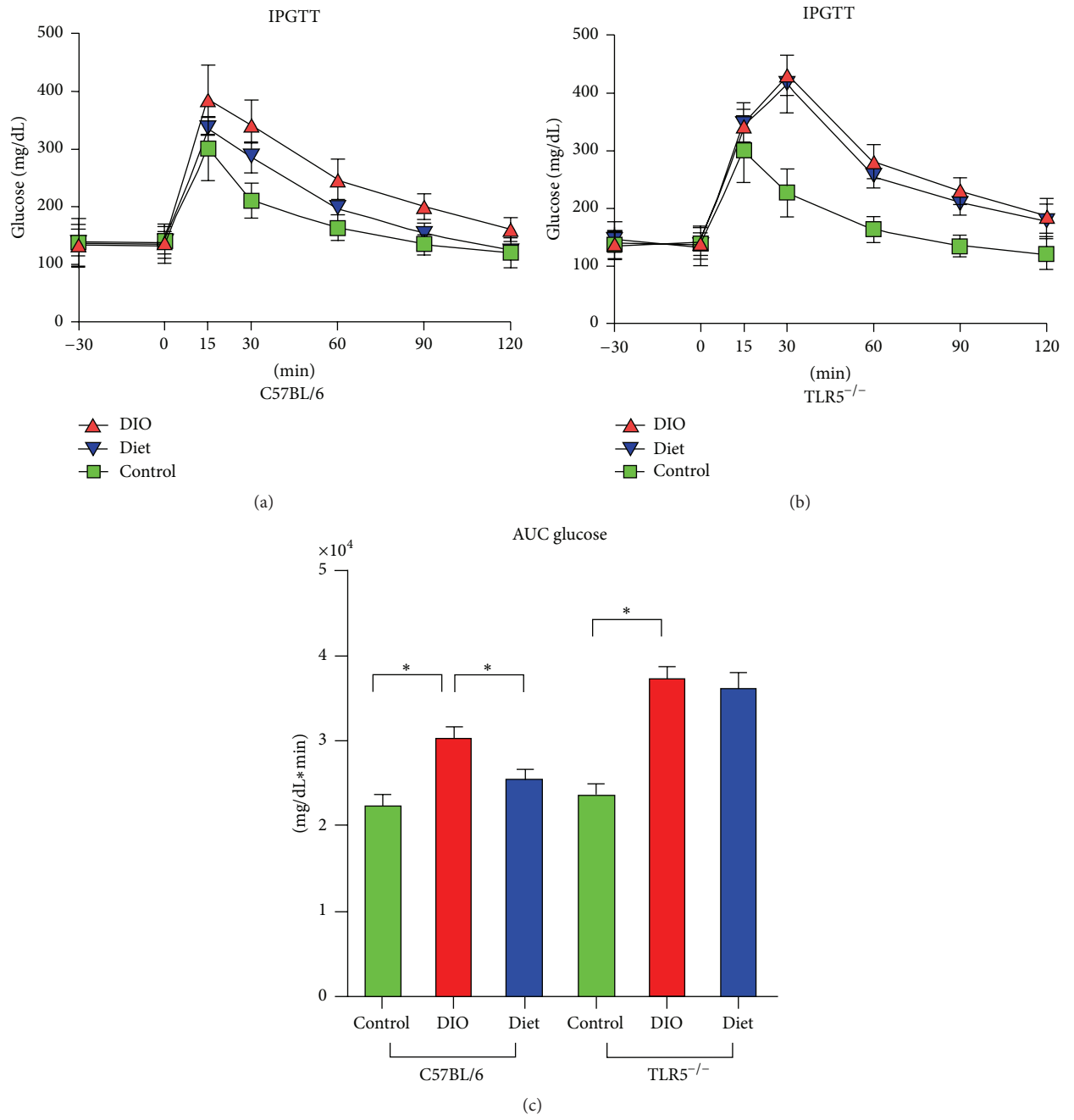

Figure 2: Intraperitoneal blood glucose concentrations of (a) C57BL/6 and (b) TLR5$^{-/-}$ mice and (c) the quantification of area under the curve (AUC) during a 120 min intraperitoneal glucose tolerance test (IPGTT) in groups of control, DIO, and diet at week 12. $^{*}p < 0.05$.

3.4. HFD Induces Severer Hepatic Steatosis in TLR5$^{-/-}$ Than C57BL/6 Mice. Hepatic steatosis is a common metabolic complication associated with obesity. In this study, the histological examination revealed that HFD induced progressively enlarged vacuoles, suggesting hepatic fat deposition in the liver of C57BL/6 and TLR5$^{-/-}$ mice. Moreover, the liver of DIO TLR5$^{-/-}$ mice had more hepatic fat deposition with various size of fat cells compared to DIO C57BL/6 mice (Figure 4). However, no fat deposition was found in the liver of C57BL/6 and TLR5$^{-/-}$ mice after LFD feeding for 4 weeks.

3.5. TLR5$^{-/-}$ Present Different Cytokine Response to Weight-Reduction Than C57BL/6 Mice in Serum. DIO increased the expression of ghrelin, GIP, GLP-1, insulin, leptin, PAI-1, glucagon, and resistin (Figure 5) in the serum of C57BL/6 mice, and weight-reduction with LFD feeding significantly reduced their expressions. However, in TLR5$^{-/-}$ mice, GIP, GLP-1, insulin, leptin, PAI-1, glucagon, and resistin were already higher in the control mice compared to C57BL/6 control mice. DIO in the TLR5$^{-/-}$ mice was not associated with the upregulation of the cytokines ghrelin, GIP, GLP-1, insulin, leptin, PAI-1,

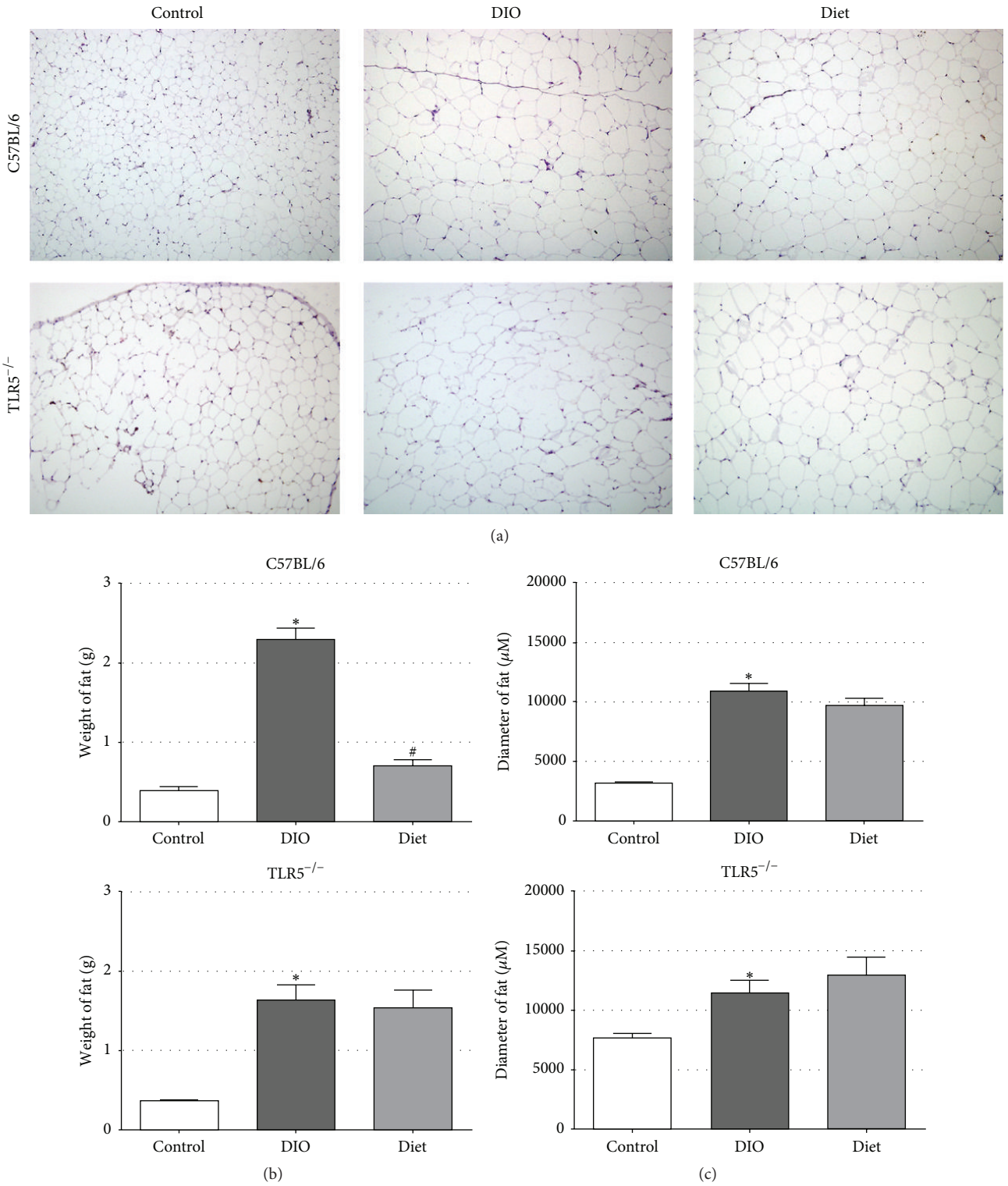

FIGURE 3: (a) Hematoxylin and eosin stain of the paraffin-embedded epididymal white adipose tissue at 5 μm section of C57BL/6 and TLR5$^{-/-}$ mice in groups of control, DIO, and diet at week 12. (b) The weight of epididymal white adipose tissue and (c) adipocyte area of C57BL/6 and TLR5$^{-/-}$ mice in groups of control, DIO, and diet at week 12. $^{*}p < 0.05$ versus control. $^{\#}p < 0.05$ versus DIO.

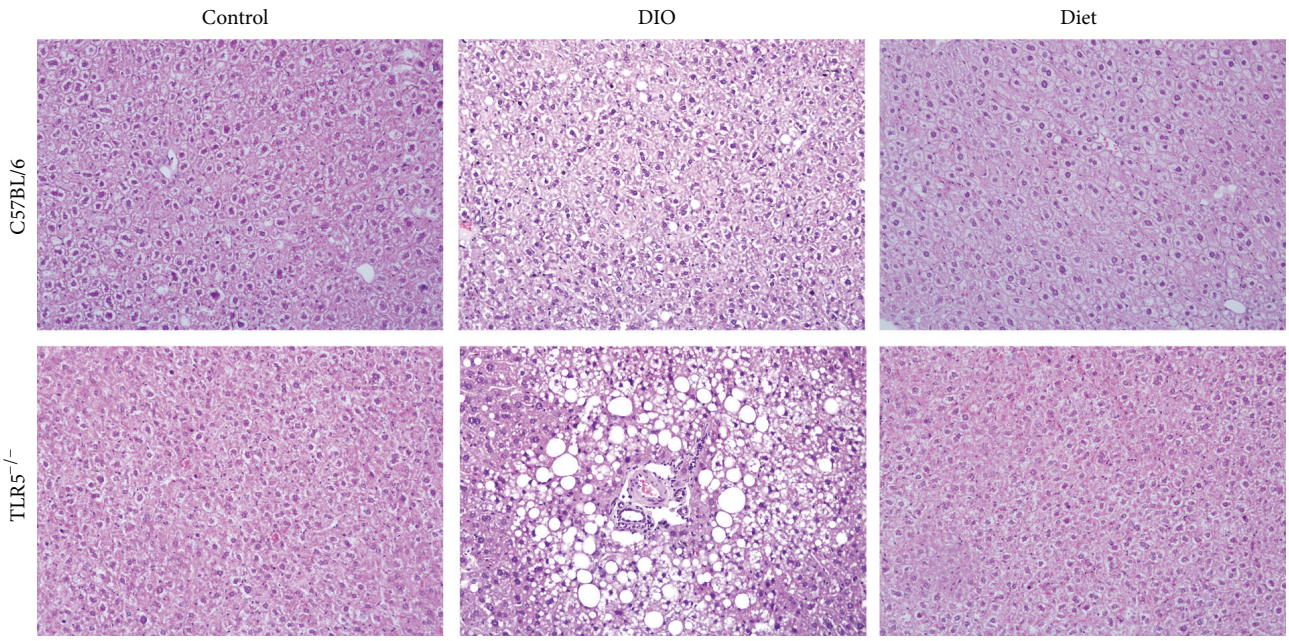

Figure 4: Hepatic steatosis in the hematoxylin and eosin stain of the paraffin-embedded liver at 5 μm section of C57BL/6 and TLR5$^{-/-}$ mice in groups of control, DIO, diet at week 12.

glucagon, and resistin. Weight-reduction via LFD feeding significantly reduced the expression of circulating GIP, GLP-1, insulin, leptin, glucagon, and resistin in the TLR5$^{-/-}$ mice but not ghrelin and PAI-1. The LFD-fed TLR5$^{-/-}$ mice showed significantly higher ghrelin expression in the serum compared to DIO TLR5$^{-/-}$ mice.

3.6. TLR5$^{-/-}$ Present Different Cytokine Response to Weight-Reduction Than C57BL/6 Mice in Fat. In C57BL/6 mice, DIO increased the expression of leptin, PAI-1, and resistin (Figure 6) as well as IL-6, IL-10, and TNF-α (Figure 7) in the epididymal WAT. Weight-reduction via LFD feeding significantly reduced their expression. In TLR5$^{-/-}$ mice, leptin, PAI-1, glucagon, and resistin as well as IL-6, IL-10, and TNF-α were already higher in the control mice than those in the C57BL/6 control mice. In contrast, DIO in the TLR5$^{-/-}$ mice results in significant downregulation of leptin, PAI-1, glucagon, resistin, IL-6, IL-10, and TNF-α. In addition, weight-reduction via LFD feeding resulted in an increased expression of resistin in the epididymal WAT of the TLR5$^{-/-}$ mice, but no significant change in the expression of leptin, PAI-1, glucagon, IL-6, IL-10, and TNF-α was found.

4. Discussion

In this study, HFD feeding significantly increased the body weight and adipocyte size in both TLR5$^{-/-}$ and C57BL/6 mice. After 12 weeks of HFD feeding, the TLR5$^{-/-}$ mice gained more weight and showed significantly higher glucose intolerance and hepatic steatosis than C57BL/6 mice. Although, switching to a LFD is effective in weight-reduction and improves metabolic health parameters in obesity [18], weight-reduction with LFD in the TLR5$^{-/-}$ mice resulted in a different response regarding the change of weight of epididymal WAT, glucose tolerance, and cytokines response in the serum and adipose tissue compared to the C57BL/6 mice.

Visceral fat is a highly active tissue from the metabolic point of view [19, 20]. Nowadays it is assumed that unfavorable changes in the secretion of adipose tissue hormones and inflammatory cytokines caused by obesity influence the development of metabolic syndrome [21]. Excess of visceral adipose tissue and increased production of adipokines are mostly responsible for metabolic complications [21]. As the big adipocytes are more prone to rupture and therefore obviously constitute a focus of inflammation, a positive correlation between adipocyte size and TNF-α, IL-6, and C-reactive protein was reported [22]. Adipose inflammation and ectopic fat deposition in extra-adipose tissues collectively resulted in impaired glucose homeostasis [23] and several lines of evidence prove that chronic inflammation causatively contributes to insulin resistance development in obesity [24, 25]. Additionally, because approximately 75% of weight lost by dieting is composed of adipose tissue [26], it is believed that the health benefits that result from weight loss are due to the reductions in proinflammatory secretions by adipocytes and the influence those secretions have on cell types in other tissues [27–29]. In this study, although a switch to the LFD had significantly decreased the epididymal fat mass in C57BL/6 mice, this effect was not found in TLR5$^{-/-}$ mice. Moreover, glucose intolerance was still significant in LFD-fed TLR5$^{-/-}$ mice than C57BL/6 mice. However, there was no reduction in the diameter of fat lobules after a switch to LFD feeding both in C57BL/6 and in TLR5$^{-/-}$ mice.

In this study, the LFD-induced weight-reduction resulted in a different cytokine response in serum and adipose tissues

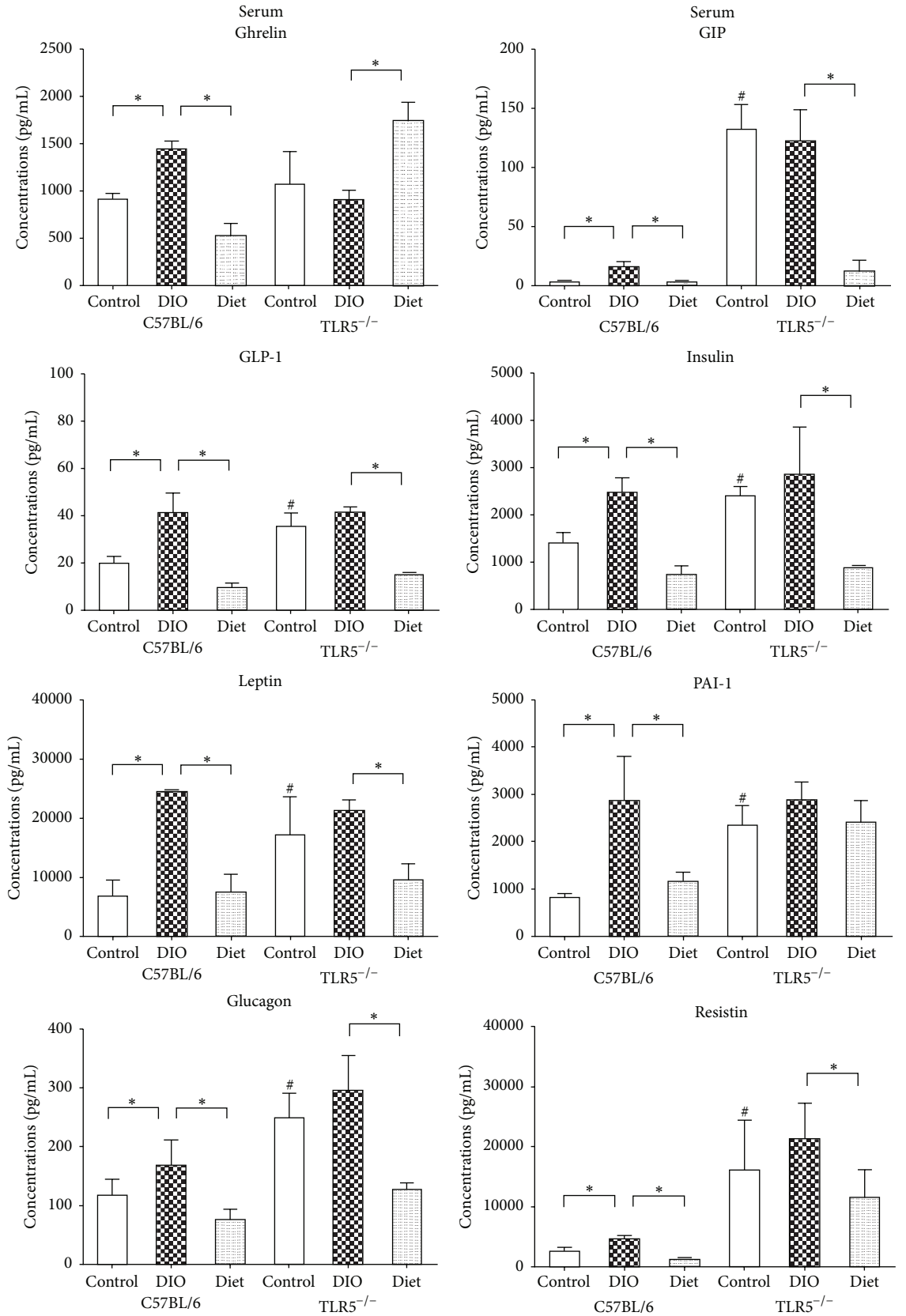

Figure 5: Concentrations of serum cytokines including ghrelin, gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1), insulin, leptin, plasminogen activator inhibitor type 1 (PAI-1), glucagon, and resistin analyzed by the Bio-Plex Multiplex cytokine assay at week 12 in the C57BL/6 and TLR5$^{-/-}$ mice in groups of control, DIO, and diet. $^{*}p < 0.05$ versus indicated group. $^{\#}p < 0.05$ versus control C57BL/6 mice.

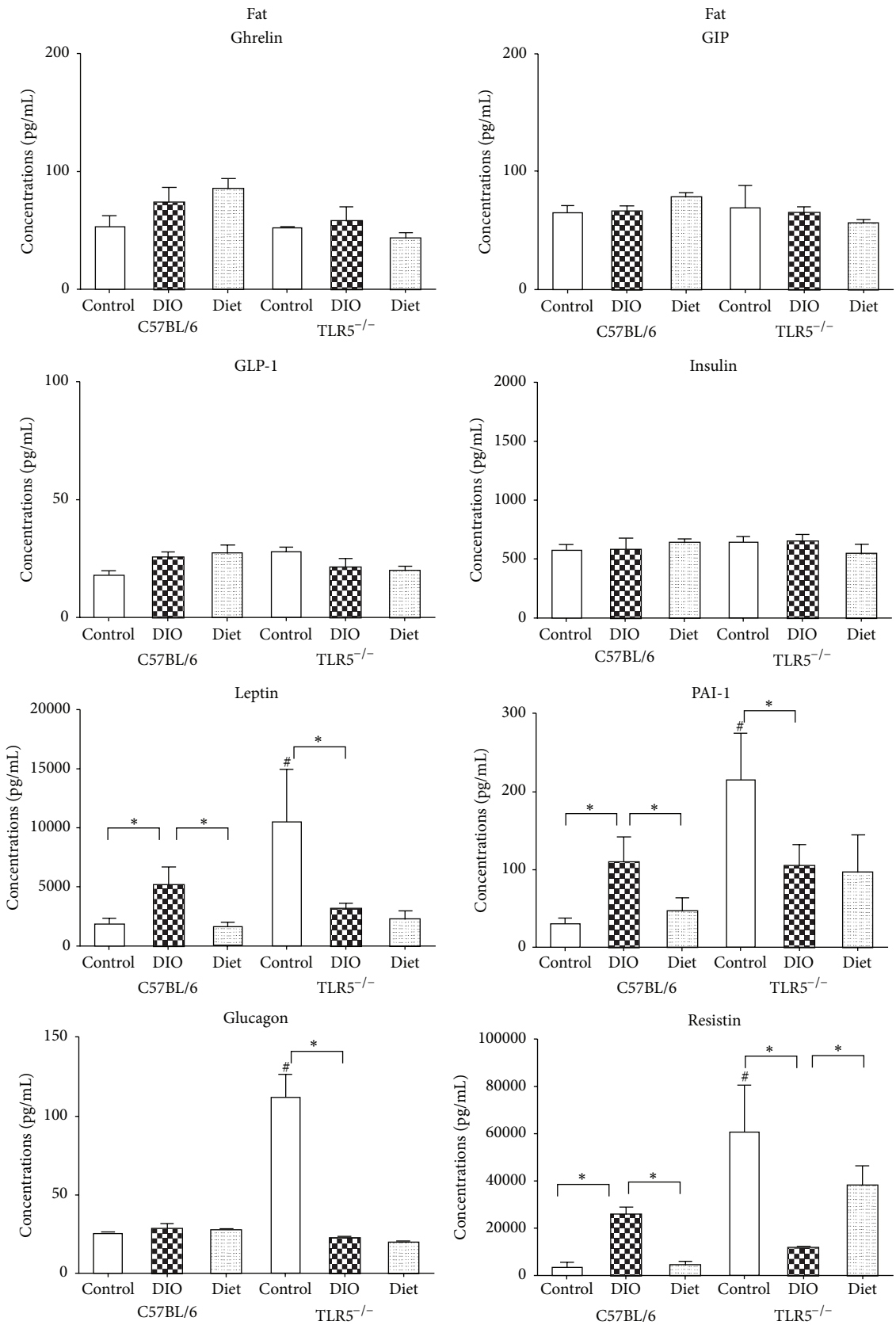

FIGURE 6: Concentrations of adipose tissue cytokines analyzed by the Bio-Plex Multiplex cytokine assay at week 12 in C57BL/6 and TLR5$^{-/-}$ mice in groups of control, DIO, and diet. $^{*}p < 0.05$ versus indicated group. $^{\#}p < 0.05$ versus control C57BL/6 mice.

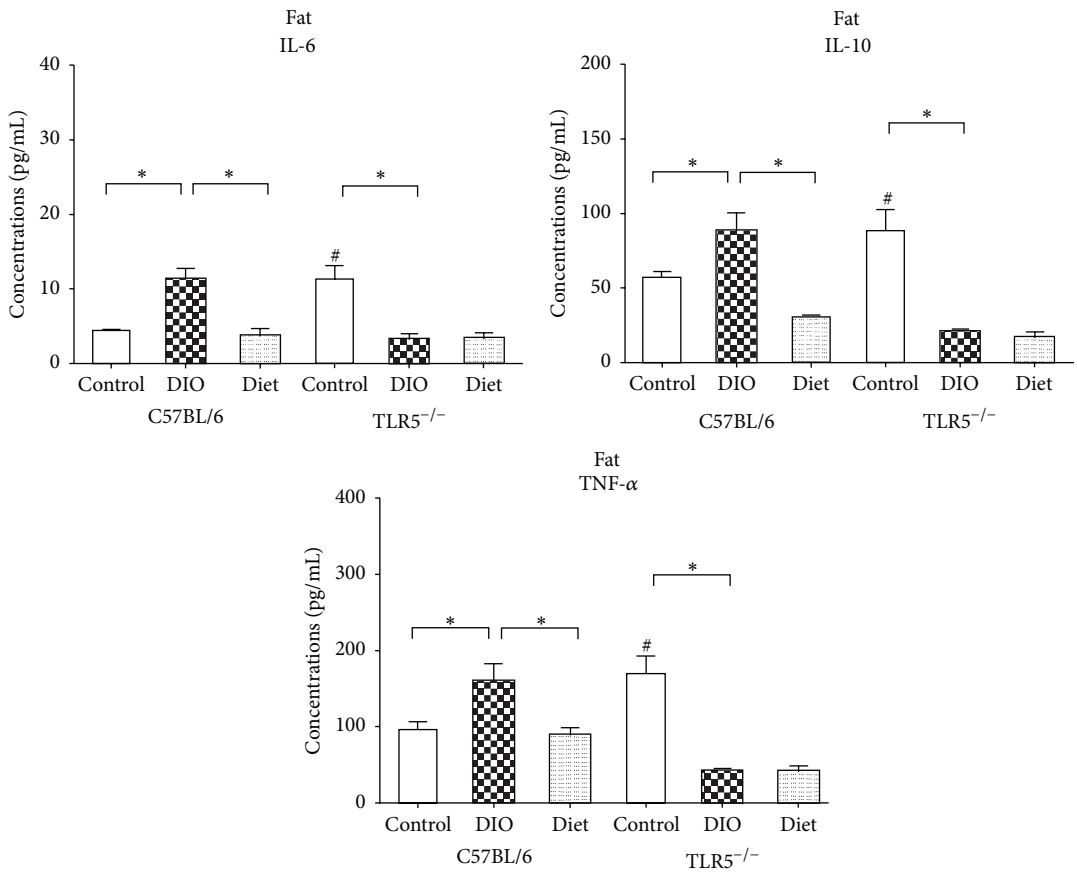

Figure 7: Concentrations of adipose tissue cytokines including IL-6, IL-10, and TNF-α at week 12 in C57BL/6 and TLR5$^{-/-}$ mice in groups of control, DIO, and diet. $^{*}p < 0.05$ versus indicated group. $^{\#}p < 0.05$ versus control C57BL/6 mice.

of TLR5$^{-/-}$ and C57BL/6 mice. In C57BL/6 mice, weight-reduction via LFD feeding could significantly reduce the DIO-related expression of ghrelin, GIP, GLP-1, insulin, leptin, PAI-1, glucagon, and resistin in the serum and PAI-1, resistin, IL-6, IL-10, and TNF-α in the epididymal WAT. In the TLR5$^{-/-}$ mice, although LFD feeding significantly reduced the expression of circulating GIP, GLP-1, insulin, leptin, glucagon, and resistin, no such effect was found in the ghrelin and PAI-1. The LFD-fed TLR5$^{-/-}$ mice showed significantly higher ghrelin expression in the serum compared to the DIO mice. In addition, weight-reduction via LFD feeding resulted in an increased expression of resistin in the epididymal WAT of the TLR5$^{-/-}$ mice but there were no significant changes in the expression of leptin, PAI-1, glucagon, IL-6, IL-10, and TNF-α.

Among these investigated cytokines, the difference in response of ghrelin, PAI-1, and resistin between TLR5$^{-/-}$ and C57BL/6 mice during the weight-reduction gained much attention. Ghrelin and the ghrelin receptor are expressed by lymphocytes, monocytes, and dendritic cells; therefore, there was no significant change in ghrelin expression in the fat deposits of TLR5$^{-/-}$ or C57BL/6 mice during DIO with HFD and weight-reduction with LFD. Potent anti-inflammatory effects of ghrelin were reported on the expression of IL-1β, IL-6, and TNFα in the liver, spleen, lungs, and mesenteric lymph nodes of LPS-treated mice associated with an attenuation of the LPS-induced anorexia [30]. Activation of the ghrelin receptor also results in an inhibition of proinflammatory cytokine expression and an increase in survival in various inflammatory disease models [31, 32]. The strong correlation of plasma level of PAI-1 with body mass index (BMI) and visceral accumulation of body fat suggests that PAI-1 is an adipose tissue-derived circulating peptide [33]. A direct correlation between the expression of PAI-1 in adipocytes and its serum concentration has been observed [34–36]. In addition, increased concentration of PAI-1 was found in the blood of obese patients, with some exhibiting insulin resistance [37]. Concentration of PAI-1 in blood decreases with weight-reduction via increased physical activity and caloric restriction [38]. The reduction in PAI-1 levels after weight loss is more associated with the degree of weight loss than with triglyceride or insulin changes [39]. Resistin is predominantly expressed in adipocyte and immunocompetent cells as a proinflammatory cytokine and participate in obesity-associated inflammation [40–42]. Increased resistin concentration has been observed in mice with genetically and diet-induced obesity [43]. In obese individuals, the amount of resistin in

adipose tissue, especially visceral adipose tissue, is significantly higher compared to individuals with normal weight [43, 44]. It has also been reported that increased serum resistin levels in obese patients with insulin resistance [45] and resistin impair glucose homeostasis and insulin action in mice [44, 46].

The variation in the expression of cytokines during weight-reduction with LFD feeding is yet to be explored. It may be attributed to the significantly higher expression of cytokines in the serum (GIP, GLP-1, insulin, leptin, PAI-1, glucagon, and resistin) and in the fat (leptin, PAI-1, glucagon, resistin, IL-6, IL-10, and TNF-α) deposits of the control TLR5$^{-/-}$ mice. Because the proteins measured in this study might originate from myriad cell types, including adipocytes, immune and epithelial cells, it is hard to ascertain the source of production. However, there was no evidence that TLR5 plays the role of a direct upstream mediator of the differently expressed ghrelin, PAI-1, and resistin. In this study, whether there are different expressions of IL-1β, IL-18, and IL-22, which had been reported to be involved in metabolic disorder and insulin sensitivity [47–49], is interesting but yet investigated. Notably, it had been reported that the mice deficient in IL-22 receptor are prone to developing metabolic disorders after the feeding with HFD [49]. In addition, the administration of exogenous IL-22 in genetically obese leptin-receptor-deficient (*db/db*) mice and mice fed with HFD reverses many of the metabolic symptoms, including hyperglycemia and insulin resistance [49]. However, whether there is similar effect of IL-22 administration in the TLR5$^{-/-}$ mice, which had already a higher expression of leptin that differs from the *db/db* mice, warrants further investigation.

Gastrointestinal tract plays an important role in DIO and other nutrition-related disorders, as it represents the route by which all nutrients and other sources of energy are ingested, processed, and absorbed [50]. Microbiota play an important role in the complex network of molecular and cellular interactions that link genotype to phenotype and have potential implications for obesity and diabetes. Evidence of the connection between overall gut microbial composition and obesity had been provided [51, 52]. In addition, emerging literature has implicated HFD-induced alterations in gut microbiota in the obesity epidemic [53]. Notably, development of obesity in genetically or diet-induced obese mice is associated with dramatic changes in the composition and metabolic function of the microbiota. This trait is transmissible as colonization of germ-free mice with an "obese-gut-derived" microflora results in a much greater increase in total body fat and leads to obesity [11, 12]. In addition, gut microbiota of TLR5$^{-/-}$ and wild type littermate mice were significantly different in their species composition [13]. Transplantation of TLR5$^{-/-}$ microbiota into wild type germ-free mice conferred many aspects of the TLR5$^{-/-}$ phenotype to the wild type germ-free hosts, including hyperphagia, obesity, hyperglycemia, insulin resistance, and elevated levels of proinflammatory cytokines [13]. Lean TLR5$^{-/-}$ mice exhibited a decreased response to exogenous insulin, which suggests that their insulin resistance is not entirely dependent on increased food consumption or adiposity [13]. Therefore, the study of the change and its impact on the gut microbiota of TLR5$^{-/-}$ mice during weight-reduction with LFD feeding might provide more important information regarding the distinct effects of weight-reduction in obese TLR5$^{-/-}$ and C57BL/6 mice.

5. Conclusion

In conclusion, this study demonstrates that weight-reduction with LFD resulted in a difference in response in TLR5$^{-/-}$ and C57BL/6 mice regarding the change of epididymal fat weight, glucose tolerance, and cytokines response in the serum and adipose tissue. These results also indicate that the knockout of *TLR5* gene impaired some effect of weight-reduction in DIO.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

Shao-Chun Wu contributed to the drafting of the paper. Cheng-Shyuan Rau provided and coordinated the resources. Chia-Jung Wu and Chia-Wei Lin were involved in the acquisition of histological morphology and protein expression. Tsu-Hsiang Lu, Yi-Chan Wu, and Siou-Ling Tzeng contributed to the acquisition of the study specimens. Ching-Hua Hsieh was responsible for the design and coordination of the data acquisition and analysis, as well as writing and revising the paper. All authors read and approved the final paper. Shao-Chun Wu and Cheng-Shyuan Rau indicate equal contribution to the authorship.

Acknowledgment

The work was supported by the grant from Chang Gung Memorial Hospital CMRPG8C0351 awarded to Cheng-Shyuan Rau with TLR-related experiments and CMRPG8B1421 and CMRPG8D1351 awarded to Ching-Hua Hsieh.

References

[1] D. M. L. Tsukumo, M. A. Carvalho-Filho, J. B. C. Carvalheira et al., "Loss-of-function mutation in toll-like receptor 4 prevents diet-induced obesity and insulin resistance," *Diabetes*, vol. 56, no. 8, pp. 1986–1998, 2007.

[2] R. Monteiro and I. Azevedo, "Chronic inflammation in obesity and the metabolic syndrome," *Mediators of Inflammation*, vol. 2010, Article ID 289645, 10 pages, 2010.

[3] G. Betanzos-Cabrera, D. Estrada-Luna, H. Belefant-Miller, and J. C. Cancino-Diaz, "Mice fed with a high fat diet show a decrease in the expression of 'toll like receptor (TLR)2 and TLR6 mRNAs in adipose and hepatic tissues," *Nutricion Hospitalaria*, vol. 27, no. 4, pp. 1196–1203, 2012.

[4] A. Schäffler, J. Schölmerich, and B. Salzberger, "Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs," *Trends in Immunology*, vol. 28, no. 9, pp. 393–399, 2007.

[5] J. Pietsch, A. Batra, T. Stroh et al., "Toll-like receptor expression and response to specific stimulation in adipocytes and preadipocytes: on the role of fat in inflammation," *Annals of the New York Academy of Sciences*, vol. 1072, pp. 407–409, 2006.

[6] A. Batra, J. Pietsch, I. Fedke et al., "Leptin-dependent toll-like receptor expression and responsiveness in preadipocytes and adipocytes," *The American Journal of Pathology*, vol. 170, no. 6, pp. 1931–1941, 2007.

[7] S.-J. Kim, Y. Choi, Y.-H. Choi, and T. Park, "Obesity activates toll-like receptor-mediated proinflammatory signaling cascades in the adipose tissue of mice," *Journal of Nutritional Biochemistry*, vol. 23, no. 2, pp. 113–122, 2012.

[8] C. Podrini, E. L. Cambridge, C. J. Lelliott et al., "High-fat feeding rapidly induces obesity and lipid derangements in C57BL/6N mice," *Mammalian Genome*, vol. 24, no. 5-6, pp. 240–251, 2013.

[9] S. Collins, T. L. Martin, R. S. Surwit, and J. Robidoux, "Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics," *Physiology and Behavior*, vol. 81, no. 2, pp. 243–248, 2004.

[10] R. S. Surwit, C. M. Kuhn, C. Cochrane, J. A. McCubbin, and M. N. Feinglos, "Diet-induced type II diabetes in C57BL/6J mice," *Diabetes*, vol. 37, no. 9, pp. 1163–1167, 1988.

[11] H. Tilg, "Obesity, metabolic syndrome and microbiota: multiple interactions," *Journal of Clinical Gastroenterology*, vol. 44, supplement 1, pp. S16–S18, 2010.

[12] S. Ding and P. K. Lund, "Role of intestinal inflammation as an early event in obesity and insulin resistance," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 14, no. 4, pp. 328–333, 2011.

[13] M. Vijay-Kumar, J. D. Aitken, F. A. Carvalho et al., "Metabolic syndrome and altered gut microbiota in mice lacking toll-like receptor 5," *Science*, vol. 328, no. 5975, pp. 228–231, 2010.

[14] N. M. Al-Daghri, M. Clerici, O. Al-Attas et al., "A nonsense polymorphism (R392X) in TLR5 protects from obesity but predisposes to diabetes," *Journal of Immunology*, vol. 190, no. 7, pp. 3716–3720, 2013.

[15] P. Sartipy and D. J. Loskutoff, "Monocyte chemoattractant protein 1 in obesity and insulin resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 12, pp. 7265–7270, 2003.

[16] E. P. Weiss, S. B. Racette, D. T. Villareal et al., "Improvements in glucose tolerance and insulin action induced by increasing energy expenditure or decreasing energy intake: a randomized controlled trial," *The American Journal of Clinical Nutrition*, vol. 84, no. 5, pp. 1033–1042, 2006.

[17] M. Muurling, M. C. Jong, R. P. Mensink et al., "A low-fat diet has a higher potential than energy restriction to improve high-fat diet-induced insulin resistance in mice," *Metabolism: Clinical and Experimental*, vol. 51, no. 6, pp. 695–701, 2002.

[18] F. P. M. Hoevenaars, J. Keijer, L. Herreman et al., "Adipose tissue metabolism and inflammation are differently affected by weight loss in obese mice due to either a high-fat diet restriction or change to a low-fat diet," *Genes & Nutrition*, vol. 9, no. 3, article 391, 2014.

[19] E. Bertin, P. Nguyen, M. Guenounou, V. Durlach, G. Potron, and M. Leutenegger, "Plasma levels of tumor necrosis factor-alpha (TNF-α) are essentially dependent on visceral fat amount in type 2 diabetic patients," *Diabetes & Metabolism*, vol. 26, no. 3, pp. 178–182, 2000.

[20] T. You, B. J. Nicklas, J. Ding et al., "The metabolic syndrome is associated with circulating adipokines in older adults across a wide range of adiposity," *Journals of Gerontology A Biological Sciences and Medical Sciences*, vol. 63, no. 4, pp. 414–419, 2008.

[21] M. Gnacińska, S. Małgorzewicz, M. Stojek, W. Łysiak-Szydłowska, and K. Sworczak, "Role of adipokines in complications related to obesity: a review," *Advances in Medical Sciences*, vol. 54, no. 2, pp. 150–157, 2009.

[22] R. Monteiro, P. M. S. T. de Castro, C. Calhau, and I. Azevedo, "Adipocyte size and liability to cell death," *Obesity Surgery*, vol. 16, no. 6, pp. 804–806, 2006.

[23] M. Gao, Y. Ma, D. Liu, and K. Stadler, "High-fat diet-induced adiposity, adipose inflammation, hepatic steatosis and hyperinsulinemia in outbred CD-1 mice," *PLoS ONE*, vol. 10, no. 3, Article ID e0119784, 2015.

[24] Y. Wu, T. Wu, J. Wu et al., "Chronic inflammation exacerbates glucose metabolism disorders in C57BL/6J mice fed with high-fat diet," *Journal of Endocrinology*, vol. 219, no. 3, pp. 195–204, 2013.

[25] J. M. Olefsky and C. K. Glass, "Macrophages, inflammation, and insulin resistance," *Annual Review of Physiology*, vol. 72, pp. 219–246, 2009.

[26] D. L. Ballor and E. T. Poehlman, "Exercise-training enhances fat-free mass preservation during diet-induced weight loss: a meta-analytical finding," *International Journal of Obesity*, vol. 18, no. 1, pp. 35–40, 1994.

[27] O. González, C. Tobia, J. L. Ebersole, and M. J. Novak, "Caloric restriction and chronic inflammatory diseases," *Oral Diseases*, vol. 18, no. 1, pp. 16–31, 2012.

[28] L. M. Redman and E. Ravussin, "Caloric restriction in humans: impact on physiological, psychological, and behavioral outcomes," *Antioxidants & Redox Signaling*, vol. 14, no. 2, pp. 275–287, 2011.

[29] J. Ye and J. N. Keller, "Regulation of energy metabolism by inflammation: a feedback response in obesity and calorie restriction," *Aging*, vol. 2, no. 6, pp. 361–368, 2010.

[30] V. D. Dixit, E. M. Schaffer, R. S. Pyle et al., "Ghrelin inhibits leptin- and activation-induced proinflammatory cytokine expression by human monocytes and T cells," *The Journal of Clinical Investigation*, vol. 114, no. 1, pp. 57–66, 2004.

[31] D. D. Taub, "Neuroendocrine interactions in the immune system," *Cellular Immunology*, vol. 252, no. 1-2, pp. 1–6, 2008.

[32] D. D. Taub, "Novel connections between the neuroendocrine and immune systems: the ghrelin immunoregulatory network," *Vitamins and Hormones*, vol. 77, pp. 325–346, 2007.

[33] M. C. Alessi, F. Peiretti, P. Morange, M. Henry, G. Nalbone, and I. Juhan-Vague, "Production of plasminogen activator inhibitor 1 by human adipose tissue: possible link between visceral fat accumulation and vascular disease," *Diabetes*, vol. 46, no. 5, pp. 860–867, 1997.

[34] I. Mertens and L. F. Van Gaal, "Obesity, haemostasis and the fibrinolytic system," *Obesity Reviews*, vol. 3, no. 2, pp. 85–101, 2002.

[35] M. Michalska, I. Iwan-Zietek, W. Gnilka et al., "PAI-1 and α2-AP in patients with morbid obesity," *Advances in Clinical and Experimental Medicine*, vol. 22, no. 6, pp. 801–807, 2013.

[36] D. J. Schneider and B. E. Sobel, "PAI-1 and diabetes: a journey from the bench to the bedside," *Diabetes Care*, vol. 35, no. 10, pp. 1961–1967, 2012.

[37] P. Vague, I. Juhan-Vague, M. F. Aillaud et al., "Correlation between blood fibrinolytic activity, plasminogen activator inhibitor level, plasma insulin level, and relative body weight in normal and obese subjects," *Metabolism*, vol. 35, no. 3, pp. 250–253, 1986.

[38] L. M. Belalcazar, C. M. Ballantyne, W. Lang et al., "Metabolic factors, adipose tissue, and plasminogen activator inhibitor-1 levels in type 2 diabetes: findings from the look AHEAD study," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 7, pp. 1689–1695, 2011.

[39] A. R. Folsom, H. T. Qamhieh, R. R. Wing et al., "Impact of weight loss on plasminogen activator inhibitor (PAI-1), factor VII, and other hemostatic factors in moderately overweight adults," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 13, no. 2, pp. 162–169, 1993.

[40] S. Bo, R. Gambino, A. Pagani et al., "Relationships between human serum resistin, inflammatory markers and insulin resistance," *International Journal of Obesity*, vol. 29, no. 11, pp. 1315–1320, 2005.

[41] M. P. Reilly, M. Lehrke, M. L. Wolfe, A. Rohatgi, M. A. Lazar, and D. J. Rader, "Resistin is an inflammatory marker of atherosclerosis in humans," *Circulation*, vol. 111, no. 7, pp. 932–939, 2005.

[42] M. Bokarewa, I. Nagaev, L. Dahlberg, U. Smith, and A. Tarkowski, "Resistin, an adipokine with potent proinflammatory properties," *The Journal of Immunology*, vol. 174, no. 9, pp. 5789–5795, 2005.

[43] P. G. McTernan, C. M. Kusminski, and S. Kumar, "Resistin," *Current Opinion in Lipidology*, vol. 17, no. 2, pp. 170–175, 2006.

[44] C. M. Steppan and M. A. Lazar, "The current biology of resistin," *Journal of Internal Medicine*, vol. 255, no. 4, pp. 439–447, 2004.

[45] A. Baranova, S. J. Gowder, K. Schlauch et al., "Gene expression of leptin, resistin, and adiponectin in the white adipose tissue of obese patients with non-alcoholic fatty liver disease and insulin resistance," *Obesity Surgery*, vol. 16, no. 9, pp. 1118–1125, 2006.

[46] M. Nakata, T. Okada, K. Ozawa, and T. Yada, "Resistin induces insulin resistance in pancreatic islets to impair glucose-induced insulin release," *Biochemical and Biophysical Research Communications*, vol. 353, no. 4, pp. 1046–1051, 2007.

[47] M. G. Netea, L. A. B. Joosten, E. Lewis et al., "Deficiency of interleukin-18 in mice leads to hyperphagia, obesity and insulin resistance," *Nature Medicine*, vol. 12, no. 6, pp. 650–656, 2006.

[48] R. Stienstra, L. A. B. Joosten, T. Koenen et al., "The inflammasome-mediated caspase-1 activation controls adipocyte differentiation and insulin sensitivity," *Cell Metabolism*, vol. 12, no. 6, pp. 593–605, 2010.

[49] X. Wang, N. Ota, P. Manzanillo et al., "Interleukin-22 alleviates metabolic disorders and restores mucosal immunity in diabetes," *Nature*, vol. 514, no. 7521, pp. 237–241, 2014.

[50] M. M. Wolfe and M. O. Boylan, "Obesity and the gastrointestinal tract: you are what you eat," *Journal of Clinical Gastroenterology*, vol. 48, no. 10, pp. 817–822, 2014.

[51] R. E. Ley, P. J. Turnbaugh, S. Klein, and J. I. Gordon, "Microbial ecology: human gut microbes associated with obesity," *Nature*, vol. 444, no. 7122, pp. 1022–1023, 2006.

[52] P. J. Turnbaugh, R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon, "An obesity-associated gut microbiome with increased capacity for energy harvest," *Nature*, vol. 444, no. 7122, pp. 1027–1031, 2006.

[53] E. A. Murphy, K. T. Velazquez, and K. M. Herbert, "Influence of high-fat diet on gut microbiota: a driving force for chronic disease risk," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 18, no. 5, pp. 515–520, 2015.