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外傷出血性休克所導致發炎反應與基因體表現之性別差異—與外傷後多重器官衰竭機轉之可能關連

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The Gender Dimorphism of Inflammatory Response Following Traumatic Hemorrhagic Shock: A study based on its functional genomics and validation of a novel therapeutic approach for post-traumatic multiple organ failure by hormonal modulation

大白鼠外傷性出血性休克後炎性反應在性別差異上之研究：基於功能性基因體學並應用賀爾蒙調節來治療因創傷導致多重器官衰竭之嶄新療法的研究

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中英文摘要：

關鍵詞：性別差異，雌激素，細胞激素，外傷出血性休克，多重器官衰竭，功能性基因體，DNA 微陣列。

背景：在台灣外傷一直位居國人第四大死亡原因。外傷早期死亡主要原因包括頭部外傷與出血性休克，晚期則為器官衰竭。如果於嚴重的外傷病人受傷後二十四小時尚能存活，將會出現更多的多重器官衰竭，成為外傷後期死亡之主要原因。研究顯示，外傷好發於年輕男性。許多研究指出性別差異源自於性賀爾蒙效用，外傷後性賀爾蒙導致免疫不同反應。同時性賀爾蒙已經被證明造成男女性間心臟血管反應，免疫反應，血中細胞激素，及 T 細胞活性之明顯地不同；並可藉由其合成的賀爾蒙製劑與拮抗劑來調節上述之反應。肝臟是一個代謝功能複雜且重要的器官，各類休克常導致肝臟衰竭，同時合併極高的死亡率。DNA 微陣列提供一個樞紐性工具，讓生物學界能夠了解複雜疾病之基因體變化，但是目前為止，甚少被應用於嚴重外傷病患。但是外傷提供一個獨特機會得以研究功能性蛋白質體與基因體變化。因為這種對生物體急速與巨大衝擊，反而是癌症等慢性疾病及遺傳疾病所無法呈現的。因此應用基因科技研究生物於外傷後，信使核糖核蛋白(mRNA)如何受轉錄，並轉譯成蛋白質造成失調性發炎反應之路徑，以期待能解開感染(Infection)或發炎(imflamation)與器官衰竭間之微控關係。

目的：藉由微陣列技術，全面瞭解出血性休克導致基因體表現之變化在性別上的差異，並觀察基因體變化與細胞層級變化及臨床指標，如：細胞激素與雌激素，以期找出其間之關連性。

方法：本實驗之對象為大白鼠，共分三組，每組 12 隻，雌雄各半。第一組：控制組，第二組：出血性休克組，第三組：出血性休克及急救組。出血性休克採用抽血 2.5 毫升血液/公斤體重，並維持平均動脈壓為 45-55 毫米汞柱達一小時。實驗結束後四小時給予安樂死，同時將大白鼠肝臟組織取下並且由心臟抽血。取出之肝臟組織立即放入-196°C液態氮急凍，再保存於-70°C之冰箱，直到接受微陣列之分析。各種血中細胞激素 TNF-α,IL-1β，IL-6 及 IL-10，同時雌激素(17β-estradiol)也予以檢測。

結果：研究發現三組動物除了第二組外，第一組及第三組 4 小時的存活率均為 100%。第二組雄性 4 小時存活率為 33.3%；而雌鼠則為 50%。在雌激素方面，雌性明顯高於雄性，兩者有統計學上的意義 (<0.001)，但雌鼠彼此間雌激素的差異極大。無論雌雄，第二組血清中四種細胞激素均有最高的表現，而第一組血清中濃度最低（p<0.001 第二組 vs.第一組，第二組 vs.第三組）。第二組較第一組血清細胞激素升高倍數分別為：TNF-α：雄性（5.7 倍）、雌性（2.3

倍）；IL-1β：雄性（18.1 倍）、雌性（2.8 倍）；IL-6：雄性（15.9 倍）、雌性（2.6 倍）；IL-10：雄性（1.9 倍）、雌性（4.0 倍）。第三組較第二組血清細胞激素降低比例分別為：TNF-α：雄性（85.8%）、雌性（60.2%）；IL-1β：雄性（92.5%）、雌性（61.8%）；IL-6：雄性（91.8%）、雌性（57.8%）；IL-10：雄性（46.8%）、雌性（72.2%）。微陣列的實驗目前仍進行中，詳細實驗結果將於補充報告中提出。

討論： 根據本實驗的結果，三組動物中只有休克組(第二組)有死亡個案，死亡率分別為雄性 66.7%， 雌性 50%。在死亡個案當中，雄鼠平均存活時間亦較短 (81.3 分鐘 vs.123 分鐘)。在刺激性細胞激素方面（TNF-α，IL-1β，IL-6），雄鼠因出血性休克導致血清濃度升高之比例較雌鼠明顯，但在抑制性細胞激素方面（IL-10），雌鼠則較雌鼠明顯。這樣的結果顯示雌鼠對外傷性出血性休克有較高的忍受性，其中導致外傷性出血性休克對死亡率及血清細胞激素變化上在性別差異上最主要的因素為性荷爾蒙。在本實驗中雌性荷爾蒙（17β-estradiol）在兩性是有明顯差異的（$p<0.001$），而 17β-estradiol 在外傷性出血性休克中對細胞激素的調節及對身體的保護作用在許多其他相關的論文中已被廣泛的討論也已被證實，而在本實驗中則是再次證實此一論點。至於休克組(第三組)無論雌雄，血清中的細胞激素均較控制組(第一組)稍高，但並無統計學上差異($p>0.05$)，這樣的結果是否顯示經由急救(輸血及輸液治療)即能克服性別差異使血清中細胞激素從高濃度(如第二組所示)回復至正常的水準(如第一組所示)？我們將於討論中做詳細說明。微陣列的實驗目前仍進行中，詳細實驗結果將於補充報告中提出。

Key words: gender dimorphism, cytokines, estrogen, traumatic hemorrhagic shock, multiple organ failure (MOF), functional genomics, cDNA Microarray.

Background：Injury remains the fourth leading cause of death among persons of all ages in Taiwan. The majority of trauma patients in the acute and early categories died as a result of neurologic dysfunction, and hemorrhage. If they survived the first 24 h, most of the severely injured patients are at high risk of multiple organ failure (MOF). Moreover, multiple organ failure was found to be the most common contributor to late death. As all are known, the mortality rate of male following trauma is higher compared tothat of females. A number of studies indicated that this sex difference was primarily the effect of sex hormones. The liver is a complex metabolically active organ, particularly susceptible to shock, and liver failure carries a high mortality rate. Microarray technology is a pivotal tool in understanding the functional genomics of complex diseases. The application of genomic technology to the study of injury will yield important new insights into the regulatory networks of inflammation-induced organ dysfunction.

Purpose：Under the application of microarray technology, we can have a global understanding of the pattern and difference of immune restoration and gene expression following traumatic hemorrhagic shock in the male and female rats. We also want to correlate these observed gene expressions with cellular change and clinical parameters such as hormone levels and serum cytokine concentrations.

Methods：In our study, mature Sprague-Dawley rats with half for each sex, will be divided into three groups, each group having 12 rats. Group I: control group (sham operation). Group II:hemorrhagic group (sham operation + shock). Group III:resuscitative group (sham operation + shock+ resuscitation). Hemorrhagic shock will be induced by withdrawing 2.5 mL blood/kg body weight for 10 minutes. After shock, animals are resuscitated with the shed blood and lactated Ringer's (RL) solution (2 times the shed blood volume, 5 mL/kg/h). The animals will be sacrificed by euthanasia at 4 hrs after the finish of experiment to obtain the liver and whole blood. The liver sample will be harvest and frozen rapidly in liquid nitrogen(-196℃) and storage at refreezer(-70℃)until microarray and pathological analysis. The cytokines including TNF-α,IL-1β, IL-6 及 IL-10, as well as 17β-estradiol will be measured through the blood samples.

Results: The 4-hour survival rates of group I and group III were 100%. However, in group II (hemorrhagic group), the 4-hour survival rate was 33% in male sub-group, and 50% in female sub-group. In regard to 17-βestradiol, plasma levels in females were significantly higher than those in males (p<0.001). However, the variation of plasma levels of 17-βestradiol among

females were significant. Regardless of sexes, group II had the highest and group I had the lowest plasma levels of TNF-α,IL-1β,IL-6 and IL-10. As compared with sham operation group (group I), the plasma levels cytokines following traumatic hemorrhagic shock (group II) increased 5.7-fold (male) and 2.3 fold (female) for TNF-α, 18.1-fold (male) and 2.8-fold (female) for IL-1β, 15.9-fold (male) and 2.6-fold (female) for IL-6, and 1.9-fold (male) and 4.0-fold (female) for IL-10 respectively. On the other hand, as compared with shock group, levels of cytokine in plasma following resuscitation decreased 85.8% (male) and 60.2% (female) for TNF-α, 92.5% (male) and 61.8% (female) for IL-1β, 91.8% (male) and 57.8% (female) for IL-6, and 46.% (male) and 72.2% (female) for IL-10 respectively. The microarray study is still in progress. Thorough results will be presented in the supplementary report.

Discussion: According to the results of our reperiment,， the mortality rates were zero in all except the shock group. In shock group (group II), the mortality was 66.7% in male sub-group and 50% in female sub-group. Amongthe mortality rats, male rats had shorter mean survival time than females did (81.3 min. vs. 123 min.). In regard to pro-inflammatory cytokines,TNF-α，IL-1β and IL-6，the plasma levels following traumatic hemorrhagic shock increased more predominantly in male subgroups as compared with those in female sub-groups. On the other hand, levels of anti-inflammatory cytokine, IL-10 in plasma following traumatic hemorrhagic shock increased more significantly in females as compared with those in males. The current data of mortality and plasma levels of cytokines in group II suggesting that females have more protective from traumatic hemorrhagic shock than males do. The protective effect may be due to the modulation of sex hormone, 17β-estradiol in particularly. The regulation of 17β-estradiol for the release and function of cytokines and the protection of the body in traumatic hemorrhagic shock have been widely discussed and proven. The contention is further supported in our experiment. In resuscitation groups, all of the studied cytokines were decreased significantly as compared with those in shock groups (p<0.001), but they showed no significant differences between sham operation group and resuscitation group. Whether or not the above finding reveals that the protective effects of sex hormone may be neglected when resuscitation was incorporated？ We will make a detailed discussion in this report. The microarray study is still in progress. Thorough discussions will be presented in the supplementary report.

報告內容

- **Introduction**

[Trauma Epidemiology and Death]

Injury continues to be the leading cause of death in the first four decades of life, and remains the third or fourth leading cause of death among persons of all ages in most developed countries including Taiwan.[1-4] Because it is a “disease” of the young, trauma is responsible for loss of more years of productive life than any other illness.[4-9] In Taiwan, the Department of Health (DOH) reported total 9,513 trauma deaths on 2001. In fact, we have more than ten thousand annular trauma deaths in the last twenty years.[3]

Trunkey[10-11] reported the famous trimodal distribution of trauma death in 1983. In their study, the immediate and early deaths mostly resulted from neurologic dysfunction or major hemorrhage. Late deaths, those more than 1 week after injury, most commonly resulted from infection or multiple organ failure. Patients who survive the immediate consequences of their injuries and the accompanying loss of blood are susceptible to subsequent sepsis and multiple organ dysfunctions.[12] The mechanisms responsible for the development of multiple organ dysfunctions have not yet been established; nonetheless, it has been shown that macrocirculatory and microcirculatory disturbances are important factors in the pathogenesis of impaired organ and immune functions.[13] It appears that after an initial insult such as hemorrhagic shock, the capacity of the cardiovascular and immune system to respond to a septic challenge is reduced, contributing to increased susceptibility to sepsis in trauma victims.

[Hemorrhagic shock]

Several studies in the past decade have investigated the effects of hemorrhagic shock alone or in conjunction with soft-tissue trauma (ie, laparotomy) on cell-mediated and humoral immunity.[14-21] In this respect, a marked depression in specific and nonspecific immunity has been reported following hemorrhagic shock.[14-15] These alterations in various organ (such as the cardiovascular, liver, gut, and adrenals) and immune functions are apparent immediately after hemorrhage (starting as early as 30 minutes after induction of hypotension), and they remains depressed for 24 hours in experimental rats.[14, 16-18] Major blood loss has been also reported to induce endocrine alterations, including increased release of adrenocorticotropic hormone, corticosterone, and beta-endorphin.[19]

The response of the hormonal and inflammatory mediator systems in patients with hemorrhagic shock appears to represent a distinct set of responses different from those of other forms of shock.[20,21] Activation of the systemic inflammatory response by hemorrhage and tissue injury is an important component of the pathophysiologic condition of post-injury

hemorrhagic shock. Activators of this systemic inflammatory response include ischemia/reperfusion injury and neutrophil activation. Capillary "no-flow" with prolonged ischemia and "no-reflow" with reperfusion may initiate neutrophil activation in patients in hemorrhagic shock. The mechanisms that lead to decompensated and irreversible hemorrhagic shock include (1) "arteriolar hyposensitivity" as manifested by progressive arteriolar vasodilation and decreased responsiveness of the microcirculation to alpha-agonists, and (2) cellular injury and activation of both proinflammatory and counterinflammatory mechanisms. These changes represent a failure of the microcirculation. Redistribution of cardiac output and persistent gut ischemia after adequate resuscitation may also contribute to the development of irreversible hemorrhagic shock.

Previous studies have shown that vascular endothelial cell dysfunction occurs during hemorrhagic shock and persists despite fluid resuscitation. Vascular endothelial cells play a critical role in the maintenance of tissue perfusion.[55-57] Therefore, it is also important to investigate potential therapeutic approaches for maintaining endothelial cell function after hemorrhagic shock. One of the cornerstones in the management of trauma and hemorrhagic shock is the rapid replacement of the lost blood with fluids to maintain blood pressure and organ perfusion. However, despite seemingly successful resuscitation, a significant number of trauma patients who survive the initial insult later develop sepsis and multiple organ failure. [22-25]

[Post-injury Multiple Organ Failure]

Most studies reported an incidence of multiple organ failure (MOF) in multiple trauma patients between 8 and 42%, depending upon the scoring system used and the study population.[22-25] Sauaia et al. [24] described in a prospective study with 411 multiple trauma patients an incidence of MOF of 19% and a MOF-related mortality of 37%. Regel et al.[23] in 1995 observed an incidence of MOF 8 to 28% with a mortality of 26 to 35% in 3,406 multiple trauma patients during an observation period of 20 years. Despite this intensive research interest, the incidence of post-injury MOF appears to be increasing as a result of optimized prehospital care and regional trauma system development, fewer critically injured patients are dying at the scene of their accident, and more are surviving the first 24 h of their trauma center care. Consequently, there are more ICU patients who are at high risk of MOF. Moreover, despite tremendous clinical efforts and basic applied research, there have been no new proven effective interventions for MOF in the past decade, and the mortality remains high (40-50%).[22-25]

[Pathogenesis of Post-injury MOF]

Over the past 25 years, significant research efforts have focused on the pathogenesis of post-injury MOF.[26] In a 1975 editorial, Arthur Baue[27] made the seminal observation that ICU patients were no longer dying of isolated organ failure, but rather of a new syndrome characterized by progressive sequential organ failure. Two years later, Ben Eiseman[28] coined the term "multiple organ failure"(MOF) and identified the important risk factors to be preexisting disease, shock, and sepsis. Soon thereafter, Hiram Polk and Don Fry observed a consistent association between infection and MOF and concluded that MOF was the "fatal expression of uncontrolled infection".[29] As a result, research efforts in the early 1980s were focused on determining how the initial traumatic insult promotes infections and how these infections cause MOF. In the mid 1980s, observations from Europe by Eugene Faist [30] and Jan Goris [31] changed this research focus. They observed that a significant portion of blunt trauma patients who developed MOF did not have an identifiable infection. They concluded that MOF can occur as a result of an "autodestructive inflammatory response," and research efforts subsequently refocused on explaining how a variety of noninfectious insults could cause a systemic inflammatory response (SIRS) that would result in MOF independent of a bacterial infection.

The global hypothesis is that post-injury MOF occurs as a result of a dysfunctional inflammatory response. This occurs in a bimodal fashion; early MOF occurs as a result of an exuberant proinflammatory response, and late MOF occurs as a result of delayed immunosuppression and subsequent infections. After trauma, patients are resuscitated into a state of systemic hyperinflammation (i.e., SIRS). Mild to moderate SIRS is most likely beneficial (i.e., it is the normal injury stress response), but severer SIRS can precipitate early tissue injury, the severity of shock, and a variety of host factors. As time proceeds, negative feedback mechanisms down-regulate certain aspects of SIRS to restore homeostasis and potential autodestructive inflammation. This compensatory anti-inflammatory response results in delayed immunosuppression. Again, mild to moderate immunosuppression is presumed to be beneficial, but when severe, it is associated with late infections (principally pneumonia) that can either worsen early MOF or precipitate late MOF.

[Gut Theory]

In the late 1980s and early 1990s, the experimental work at a number of trauma research centers persuasively focused attention on bacterial translocation (BT) as a unifying mechanism for MOF.[32- 35] Alexander[36] has defined bacterial translocation as "the passage of both viable and nonviable microbes and microbial products such as endotoxin across an anatomically intact mucosal barrier". Although bacterial translocation was not a new concept in the late 1980s, it provided an enticing explanation of how traumatic shock could induce the "sepsis syndrome" (i.e., SIRS without an infection) that characterized early post-injury MOF.

More recently, laboratory studies have implicated that the gut is a cytokine-generating organ after shock-induced gut hypoperfusion[37-39] and that gut-associated bacteria may play an important role in this inflammatory response[40-41]. For instance, Deitch and Chaudry[37] found greater increases in IL-6 and TNF levels in portal vs. systemic circulation in rats subjected to hemorrhagic shock and resuscitation. The exact pathogenesis of post-injury MOF remains elusive and is undoubtedly multifactorial.

[Risk Factors of Post-injury MOF & Mortality]

Based on previous studies of Sauaia et al.[24], age greater than 55 years, ISS greater than or equal to 25, and greater than 6 U RBC/12 hours are found as the early independent predictors of postinjury MOF. Another multiple logistic regression analysis performed by Tran et al.[42] on 206 consecutive trauma patients, advancing age, chronic disease, ISS, and MOF score were selected as major predictors of death. In a statewide population-based study of 22,332 trauma patients after multiple logistic regression analysis, the predictors of in-hospital mortality were found as age, injury severity, admission physiological parameters, preexisting diseases.[43] Therefore, the risk factors of the post-injury MOF and trauma death were similar and determined under three headings, as: host factors, severity of tissue injuries, and clinical status of shock.

[Gender Difference in Trauma Outcome]

Oberholzer and colleagues[44] demonstrated in 1,276 trauma patients with ISS≧9 that the incidence of posttraumatic sepsis and multiple organ dysfunction syndrome was significantly increased in severely injured males with ISS≧25 when compared with an equivalent female group(sepsis, 30.7% versus17.0%; multiple organ dysfunction syndrome, 29.6% versus 16.0%). They concluded that sex influences posttraumatic morbidity in severely injured patients and supports the concept that females are immunologically better positioned toward a septic challenge. Majetschak and colleagues[45] also examined 84 patients with blunt trauma and ISS≧16 and discovered in severely injured males development of severe posttraumatic sepsis is associated with an increased cytokine producing capacity of whole blood in the early posttraumatic period. These findings may correspond to overwhelming inflammatory responses, which increase susceptibility for sepsis in male trauma patients. The data provide evidence for a gender specific regulation of the initial inflammatory response following severe blunt trauma.

[Gender Difference of Immune Response]

These alterations in endocrine and immune functions have been investigated using male laboratory animal, although gender differences in the susceptibility to and morbidity from

sepsis have been observed in several clinical and epidemiological studies.[46-48] Recent studies have suggested that male sex steroid hormones play a significant role in producing immunodepression following trauma and hemorrhagic shock.[49-51] Moreover, these depressed immune responses have been associated with an increased susceptibility to and mortality due to subsequent sepsis.[49,52] Furthermore, a number of investigators have shown that sexual dimorphism exists during circulatory stress, and those androgens and estrogens play a pivotal role in regulating the stress responses. [49-52]

In this regard, studies by Wichmann et al.[53] and Zellweger et al.[54] have shown that female mice have enhanced immune responses as opposed to decreased responses in male mice after trauma-hemorrhage. Moreover, castration of male animals 14 days before hemorrhagic shock prevented the depression in myocardial functions and immune responses usually observed under those conditions.[50,55,56] Furthermore, administration of the testosterone receptor antagonist flutamide improved the depressed immune responses and cardiac and hepatic functions in male animals after trauma and severe hemorrhage.[57] Thus, male sex hormones appear to play a deleterious role in the development of cell and organ dysfunction after trauma and hemorrhage.[58] In contrast to male mice, the elevated levels of female sex hormones, ie, prolactin and estrogen, in the proestrus state contribute to sexual dimorphism in the immune response following trauma-hemorrhage.[59]

[Liver Dysfunction in Post-injury MOF]

The liver is a complex metabolically active organ, particularly susceptible to shock. Liver failure carries a high mortality rate because no real treatment or supportive care is known.[60] Causes of these hepatic cellular changes are probably mutifactorial. A reduction in hepatic oxygen supply and subsequent hypoxia has been postulated. Neutrophil-mediated hepatic changes and oxygen radical-mediated injury are also thought to play a role in shock-induced hepatic injury.[21] The hepatocyte's ability to generate ATP is grossly reduced in an anaerobic metabolic state, which leads to cellular membrane and organelle damage followed by loss of liver function. Hepatocyte ATP level is drastically reduced during ischemia and is noted to rise again during reperfusion.[61] Cellular swelling is accompanied by increased total cellular and mitochondrial calcium content, contributing to the further dysfunction of the energy-producing mechanism. The Kupffer cells and coagulations factors synthesis are also severely reduced in the shocked liver.

In the review by Jarrar & Chaudry[62], they focus first on factors and mediators responsible for producing cell and organ dysfunctions, especially hepatocellular dysfunction, following trauma, hemorrhagic shock, and sepsis. The changes in signaling transduction pathways will also be discussed, specifically the role of mitogen-activated protein kinases, transcription factors, nitric oxide, heat shock proteins, and inflammatory cytokines in the

development of cell and organ dysfunctions following trauma-hemorrhage and sepsis. Moreover, potential therapeutic approaches for improving cell and organ functions under adverse circulatory conditions are included.

[Microarray]

The completion of the Human Genome Project (sequencing of all ~30,000 human genes) will provide an unprecedented opportunity to study the molecular basis of injury-induced disease. Harnessing the power of rapidly surveying global variations in gene sequence and expression, microarrays afford the first tool permitting a truly integrated view of life at the molecular level. Arrays are capable currently of profiling patterns of expression for tens of thousands of genes (mouse or human) in a single experiment. The technique requires isolation of mRNA from the cells or tissue of interest, creation of complementary nucleotide "target", and hybridization of the labeled target to gene-specific "probe" nucleotides affixed to a glass or nylon matrix (the array).[63] With the aid of a template describing the unique location of each probe oligonucleotide (gene), computer-driven differential analysis can be used to determine changes in gene expression (either up or down) relative to a standard (control). Far from being "fishing expeditions", arrays in recent months have determined unanticipated relationships between patterns of gene expression, indicating "cytoplasmic signaling proteins form networks of interactions rather than simple linear pathways as they are usually depicted", consistent with our overarching hypothesis.[64]

[Why Trauma Need Micrarray Study?]

cDNA, microarray technology, promises to become a pivotal tool in understanding the functional genomics of complex diseases, and is rarely applied on critically ill or injured patients.[65] However, trauma and burns offer a unique population that is quite unlike NIH's current clinical study populations (e.g. AIDS, cancer, heart disease, arthritis, diabetes, and so forth). Most of trauma patients were entirely normal, immediately prior to the insult. This offers an unparalleled opportunity to study the physiological, proteomic, and genomic changes beginning with normal, deviating to grossly abnormal, and in most cases, return to the normal state. This scenario does not exist in any of the populations mentioned above.

Therefore, there is a strong likelihood that causal relationships between injury and changes in gene expression will be discovered in the field of inflammation and the host response in injury. First, those who suffer from injury begin as normal healthy individuals. Most survive the injury and return to a normal physiological and genomic state. However, the most attractive feature of this phenomenon is that the exact moment of the perturbation for these normal individuals is known, and that very dramatic physiological changes in the host response occur in a temporally related sequence. The phenotypic expression of these

changes can be profound and tissue specific. For example, cardiac index, oxygen consumption, and resting energy expenditure may increase twofold. Furthermore, this response is tissue-specific. The energy requirement of the liver, heart, and wound may be increased threefold or more whereas skeletal muscle and brain remain at or below their normal requirements for energy. There are similarly dramatic changes in the acute phase and immuno- inflammatory response after injury.

[Injury and Functional Genomics]

Functional genomics is to describe the regulatory relationships between genes and their programs of expression, as reflected by changes in mRNA abundance and protein levels. [66,67] Critically ill or injured patients frequently die of incompletely understood conditions such as septic shock, acute respiratory distress syndrome, and ultimately multiple system organ dysfunction or failure. Activation of host inflammatory pathways causes tissue injury and thereby acts as a major pathogenic mechanism in these syndromes.

The application of genomic technology to the study of sepsis will yield important new insights into the regulatory networks underlying pathogenic mechanisms relevant to treatment of infection- and inflammation-induced organ dysfunction. At a basic level, the clinical and biological manifestations of host responses are determined by quantitative and qualitative changes in gene expression. Therefore, organ injury syndromes might be defined by their associated patterns of altered gene expression. From paired samples of cells or tissues, cDNA microarrays can measure relative changes in mRNA levels for thousands of genes simultaneously.[68]

Further, cDNA microarrays can be used to detect genetic polymorphisms that affect outcome and to identify new gene targets for drug development. Because cDNA microarrays generate huge data sets even from relatively simple experiments, difficulties with validating and conceptually handling this quantity of information need to be resolved. Clustering data from genes with shared characteristics, developing software to aid in the interpretation of results, and rapidly making results widely available are some potential approaches to these problems. Data derived from microarrays will allow for the first time a truly global, integrated view of the adaptive response to injury.

[Gene Expression Profiles of Septic Mice]

Cobb et al. [69] use the cecal ligation and puncture (CLP) in a murine septic model, and examined two organs of interest, spleen (sensitive to stress-induced apoptosis) and liver (resistant to stress-induced apoptosis). By using Clontech Atlas (Clontech, Palo Alto, CA) microarray technology, they found that the sepsis-induced changes in expression of the 588 murine genes studied were organ specific.[69] The changes in liver gene expression at 24 hrs

of sepsis in this model were largely at the level of membrane proteins and receptors. In contrast, changes in the expression of a much larger number of spleen genes were observed, most of which are associated with signaling cascades, cell death pathways, and cytokine production. Recently Chung et al.[70] also reported that they can determine not only changes in the murine sepsis transcriptome, but also which of these changes are associated with antibiotic treatment and, thereby, survival vs. death after cecal ligation and puncture.

[Significance and Aim of this Study]

We plan to use the more detrimental hemorrhagic shock in the rat model and followed by resuscitation or no resuscitation. And based on the above hypothesis, we intended to investigate the sex dimorphism of the hormone, immunology, and functional genomics in the hemorrhagic shock of rat. Through the studies, we anticipate the miscellaneous hormonal, immunological, and genomic responses of rats can yield important new insights into the clinical resuscitation on severely injured patients with hemorrhagic shock and achieving an improved outcome in the future trauma resuscitation.

- **Materials and Methods**

[Animal preparation]

Sprague-Dawley rats (body weight, male:300-350 g; female:200-300 g), with half for each sex, average age 8-12 weeks old, were housed in the animal facility at constant room temperature in a 12-hour light-dark cycle. They were allowed to acclimatize to laboratory conditions for at least 3 days before use. The experiments described conform to the current guidelines for care and use of laboratory animals as established by the Animal Care Committee of Chinese Medical University. All animals received rat chow and water ad libitum throughout the acclimatization period and until the time of operation.

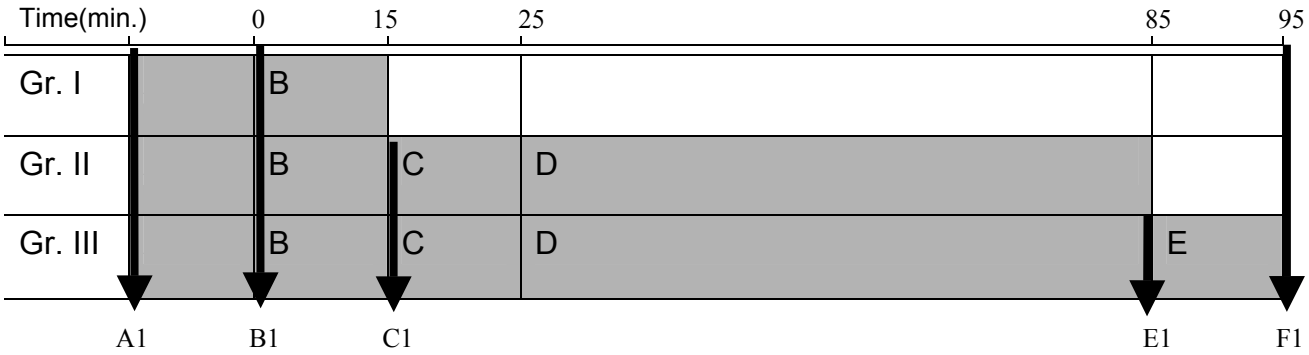
Thirty-six rats were randomized into three groups with each groups of twelve rats, Group I: control group; Group II: hemorrhagic shock without resuscitation;Group III: hemorrhagic shock with resuscitation. Each group was further divided into two sub-groups: male and female (each sub-group contains 6 rats). Sprague-Dawley rats will be anesthetized with intraperitoneal injections of citosol (25-40 mg/kg) till adequate anesthesia. Polyethylene tubes (PE-50) were placed into the right jugular vein to administer drugs or fluids and continuously monitor central venous pressure (CVP) by a Cardiomax -II model 85 (Columbus Instruments International Co., Ohio, USA). The right femoral artery was cannulated (PE-50) to monitor mean arterial blood pressure (MABP) by a Cardiomax -II model 85 (Columbus Instruments International Co., Ohio, USA). The left femoral artery was cannulated (PE-50) for blood withdraw and replacement. All rats received a basic infusion of lactated Ringer's solution (RL) (10 mL/kg/h) to replace evaporative losses, and are allowed to stabilize for 15 minutes after preparation. Body temperature is kept at 37°C ± 0.5 by a heating device.

[Hemorrhagic Shock and Resuscitation Model]

The hemorrhagic shock model was used in the current study is a well-established model of fixed-pressure hemorrhage. In Group II and III, hemorrhagic shock was induced by withdrawing 2.5 mL blood/kg body weight for 10 minutes and maintaining MABP at 50±5 mm Hg for 60 minutes by withdrawing or replacing blood.

After this shock interval, animals were followed the following resuscitation as previous assigned groups: (II) without resuscitation; (III) with resuscitation: the shed blood will be returned and lactated Ringer solution (2 times the shed blood volume, 5 mL/kg/h) will be infused over 10 minutes to provide adequate fluid resuscitation. Sham-operated animals (Group I) are instrumented as described, but not subjected to hemorrhagic shock and serve as the control group. After resuscitation, the animals will be allowed awake after the catheters removed and vessels ligated. All the animals were sacrificed 4 hours later.

[Procedure]



A1: Start celiotomy: ventral incision from sub-xiphoid to pubic symphysis， bowel manipulation and then close the wound by silk.

A: Prepared period (90 minutes): Includes celiotomy、cannulation(right jugular vein and bilateral femoral arteries), monitor setting and tubes connection. The experiment will be start if all the above procedures are completed.

B1: Start experiment

B: Stabilization period (15 minutes)：

C1:Start blood withdraw

C: Shock inducing period (10 minutes): withdraw 2.5 mL blood/kg body weight for 10 minutes to maintain MABP at 50±5 mmHg

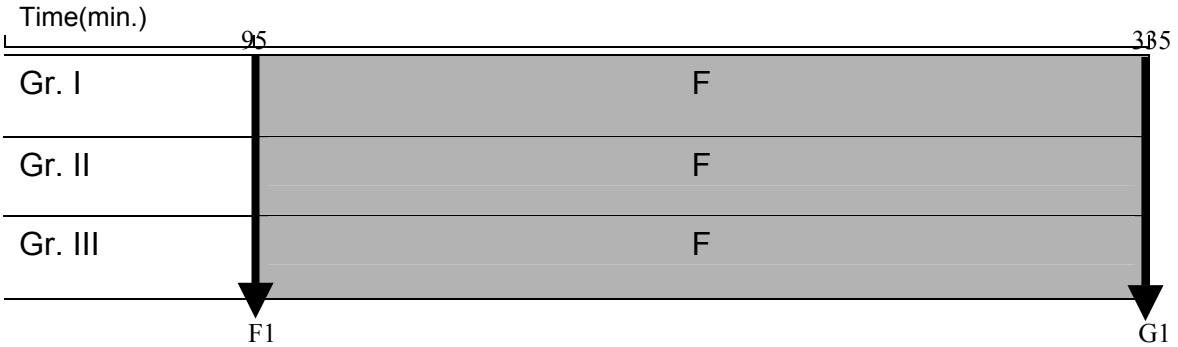
D: Shock maintaining period: maintaining MABP at 50±5 mmHg for 60 minutes by withdrawing or replacing blood.

E1: Start resuscitation：

E: Resuscitation period (10 minutes):

a、 replace the shed blood that was previously withdrawn.

b、 IV fluid challenge(Lactated Ringer solution :5ml/kg body weight)



F1: Remove catheters、vessels ligation and animal awake

F: Recovery period (4 hours):

G1: The experiment was stopped at 335th min.(4 hours after the end of resuscitation period) or any time if the animals were dead during recovery period. The animals were

sacrificed immediately after the experiment was stopped. The blood was withdrawn by cardiac puncture and the liver tissues were harvested thereafter.

[Animal Plasma Collection and Storage]

Whole blood will be sampled by cardiac puncture after rats were sacrifice. Plasma will be separated by centrifugation in pyrogen-free microcentrifuge tubes and samples are immediately frozen and stored (-70°C) until assayed.

1. Assessment of 17β-Estradiol and Cytokine Concentrations

17β-estradiol plasma concentrations were determined using a commercially available enzyme-linked immunosorbent assay as described by the manufacturer (Cayman Chemical Company, USA). Activity of IL-1β, IL-6, IL-10 and TNF-α were determined by enzyme-linked immunosorbent assay according to the manufacturer's recommendations (R&D Systems, Inc, USA).

[Animal Liver Tissue Harvest]

The harvest of rat liver was performed through a midline ventral incision from pubis to xiphoid process. One segment (weight > 1 g) of middle lobe of liver is excised and hemostasis is done by ligation with 3-0 silk at the proximal part of middle lobe. The liver sample will be frozen rapidly in liquid nitrogen. The tissue will be kept in a -70^{o}C freezer until microarray analysis.

[Microarray & Technique[71,72]]

1. Introduction

Expression of liver RNA was analysed by microarray technique using GeneChip®Probe Arrays (Affymetrix, Inc.). GeneChip probe arrays are manufactured using technology that combines photolithography and combinatorial chemistry. Up to 1.3 million different oligonucleotide probes are synthesized on each array. Each oligonucleotide is located in a specific area on the array called a probe cell. Each probe cell contains hundreds of thousands to millions of copies of a given oligonucleotide.

During the laboratory procedure described in this manual, biotin-labeled RNA or DNA fragments referred to as the "target" are hybridized to the probe array. The hybridized probe array is stained with streptavidin phycoerythrin conjugate and scanned by the GeneChip® Scanner 3000, or the GeneArray® Scanner. The amount of light emitted at 570 nm is proportional to the bound target at each location on the probe array[74].

2. GeneChip® Expression Analysis

A. RNA Preparation

Total RNA was isolated from rat liver tissue samples and electrophoresed on a 1% agarose-formaldehyde gel to determine the integrity of the RNA preparation. All RNA samples should meet assay quality standards to ensure the highest quality RNA is hybridized to the gene expression arrays. A 260/280 absorbance reading should be obtained for both total RNA and biotinylated cRNA. Acceptable $A_{260/280}$ ratios fall in the range of 1.8 to 2.1. Ratios below 1.8 indicate possible protein contamination. Ratios above 2.1 indicate presence of degraded RNA, runcated cRNA transcripts, and/or excess free nucleotides. Then double-stranded cDNA is synthesized from total RNA isolated from rats' liver tissue. An in vitro transcription (IVT) reaction is then done to produce biotin-labeled cRNA from the cDNA. The cRNA is fragmented before hybridization. The use of gel electrophoresis will aid in quality control following the sample from step to step in the assay and hybridization protocol. Gel electrophoresis can be performed after cDNA synthesis (if using poly-A mRNA as starting material), after cRNA synthesis, and after fragmentation. This will be helpful in estimating quantity and size distribution of the labeled sample. During this phase of technical evaluation, cRNA yield from a standard total RNA sample is another simple and effective method to assess consistency.

B. Gene microarray hybridization

A hybridization cocktail is prepared, including the fragmented target, probe array controls, BSA, and herring sperm DNA. It is then hybridized to the probe array during a 16-hour incubation. The hybridization process is described in the respective sections for the different probe array types.

C. Fluidics Station Setup

Specific experimental information is defined using Affymetrix® Microarray Suite or GeneChip Operating Software (GCOS) on a PC-compatible workstation. The probe array type, sample description, and comments are entered and saved with a unique experiment name. The fluidics station is then prepared for use by priming with the appropriate buffers.

D. Probe Array Washing and Staining

Immediately following hybridization, the probe array undergoes an automated washing and staining protocol on the fluidics station.

E. Probe Array Scan

Once the probe array has been hybridized, washed, and stained, it is scanned. Each workstation running Affymetrix Microarray Suite or GCOS can control one scanner. The software defines the probe cells and computes an intensity for each cell. Each complete probe array image is stored in a separate data file identified by the experiment name and is saved with a data image file (.dat) extension.

F. Data analysis

The .dat image is analyzed for probe intensities; results are reported in tabular and graphical formats. Information on data analysis is provided in the enclosed GeneChip® Expression Analysis: Data Analysis Fundamentals booklet (P/N 701190).

[Statistical analysis]

Group analyzed using one-way analysis of variance (ANOVA). Statistical analysis of differences between groups was determined by post hoc Student Newman Keuls test. Comparisons between different periods of hemodynamic data in the animal were analyzed using the paired Student's *t* test. Data are presented as means $\pm$ standard error of means (SEM). Significance was achieved when $p<0.05$. Statistical analysis was performed using commercially available software (SPSS13.0 for window).

- **Results**

[Age versus Body Weight]

There were no significant differences in age among three groups, but the body weights in male rats were significantly greater than those in female rats (p<0.05, Table 1).

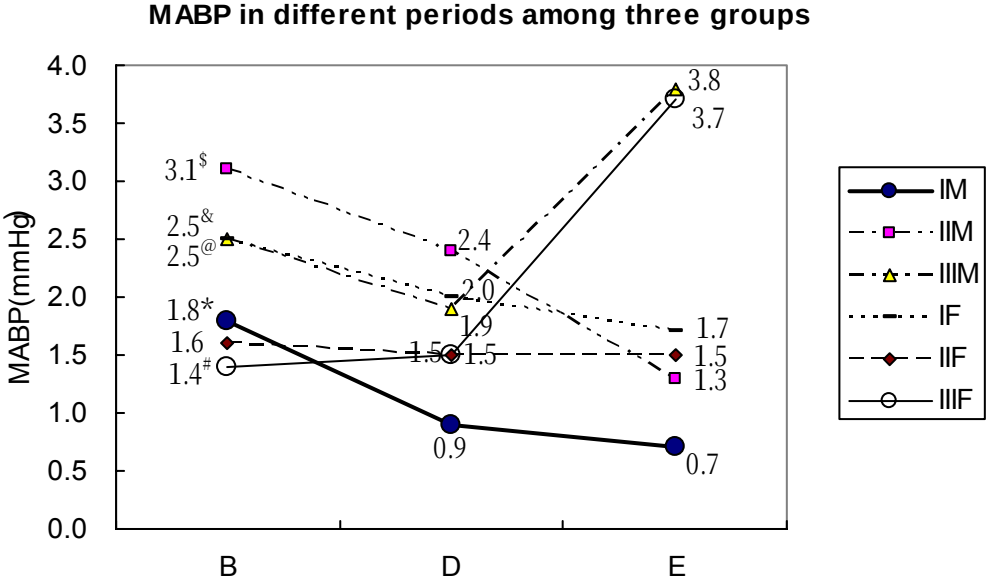
Table1:

	Group I		Group II		Group III	
	I_M (n=6)	I_F(n=6)	II_M(n=6)	II_F(n=6)	III_M(n=6)	III_F(n=6)
Age(wks)	9-11	9-11	9-11	9-11	9-11	9-11
Wt(kg)	338.2±29.0	274.7±24.4	351.2+13.0	286.7+34.5	338.2+19.8	285.2+22.3
(range)	(314-384)	(250-316)	(328-366)	(233-336)	(306-360)	(253-305)
p value	<0.05		<0.05		<0.05	

[Hemodynamic Change]

In regard to mean central venous pressure (MCVP) levels, female sub-group had significant greater levels than that of male sub-group during shock period in group I (p<0.05, Table 2). In group I and group II, there were significant differences in MCVP among three periods in males (p<0.001), but there were no significant differences in females. In group III, there were significant differences in MCVP among three periods in males (p<0.001), but there only showed significant differences in MCVP between group III vs. group II and group III vs. group I.(p<0.001). Regardless of sex, group III had significant greater MCVP levels than those of group I and group II in resuscitation period (p<0.05, Fig.1).

<Fig.1>

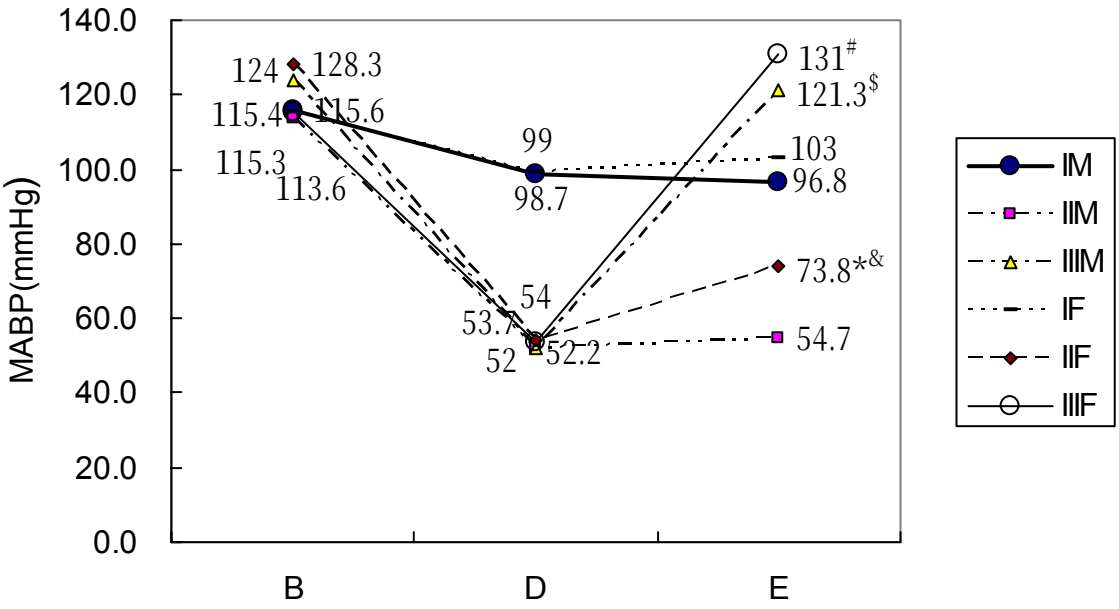


※ : B: stabilization period; D: shock period; E: resuscitation period

※ *$p<0.05 vs. shock and resuscitation period(II_M); &p<0.05 vs. resuscitation period(I_F); @p<0.05 vs. resuscitation period(III_M); *p<0.05 vs. shock and resuscitation period(I_M); #p<0.05 vs. resuscitation period(III_F).*

With regard to mean systolic blood pressure (MSBP) levels, there were no significant differences as compared with the corresponding group and period in both sexes, except in the resuscitation period of group II: the females had significant greater MSBP levels than those of males (p=0.048). Regardless of sexes, there were no significant differences among three periods in group I. In group II, there were no significant differences in MSBP between shock and resuscitation period in males, but there showed significant higher MSBP in resuscitation period those in shock period in females. In group III, both sexes had significant higher MSBP in resuscitation period than those in shock period (p<0.001, Table 3).

<Fig.1> **MABP in different periods among three groups**



※ B: *stabilization period*; D: *shock period*; E: *resuscitation period.*

※ **p<0.05 vs. IIM in E; #p<0.05 vs. shock period (III_F); $p<0.05 vs. shock period (III_M); &p<0.05 vs. shock period (II_F)*

[Mortality of Model]

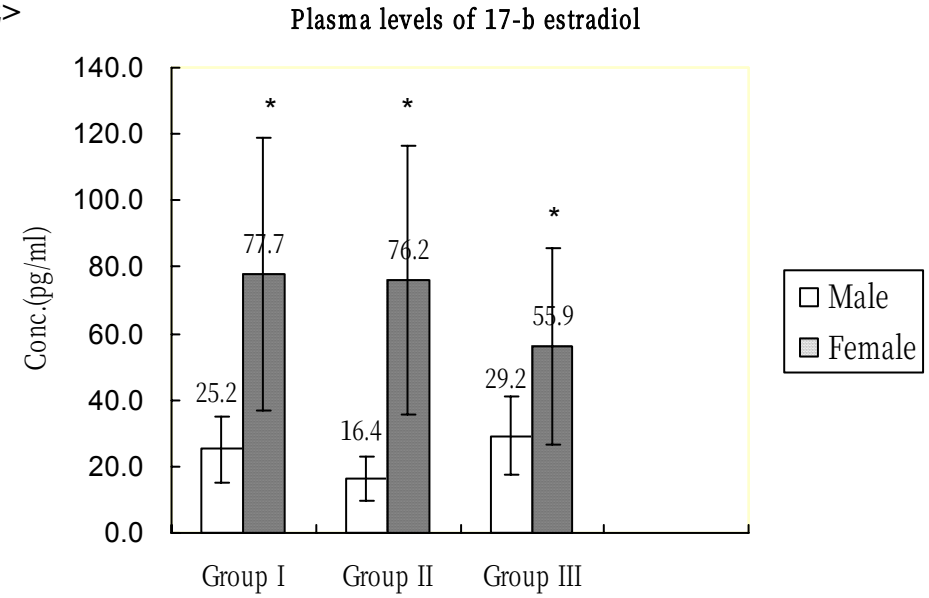
All rats in group I and group III were alive 4 hours after trauma-hemorrhage and resuscitation in the present study. Before 4 hours after trauma-hemorrhage and

resuscitation, 4 (66.7%) were dead in group II_M (at 14^{th}, 52^{th}, 72^{th}, and 187^{th} respectively) and 3 (50%) were dead in group II_F (at 91^{th}, 93^{th}, and 185^{th} respectively).

[Alteration in Plasma Levels of 17β-estradiol]

In each group, the male sub-group has the significant lower 17β-estradiol levels in plasma ($p<0.05$,Table 4). However, there were no significant differences in plasma levels of estradiol among three groups whether males or females.

<Fig.2>



※**p=0.035 vs male in group I;. * p=0.004 vs male in group II; * p=0.012 vs male in group III*

[Cytokine Levels]

1. Gender difference

In regard to TNF-α, there were no significant plasma levels (p=0.221 and 0.523 respectively) between males and females in group I and group III. In group II, female sub-group had significant lower plasma levels (p<0.001) than those of male sub-group.

In regard to IL-1β and IL-6, females had significant lower plasma levels (p<0.001) than those of males in group I and group III. However, females had significantgreater plasma levels (p<0.001) than those of males in group II.

In regard to IL-10, females had greater plasma levels than those of males in group I, but they showed no significant difference (p=0.117). In group II and group III, females had significant greater plasma levels (p<0.001) than those of males.

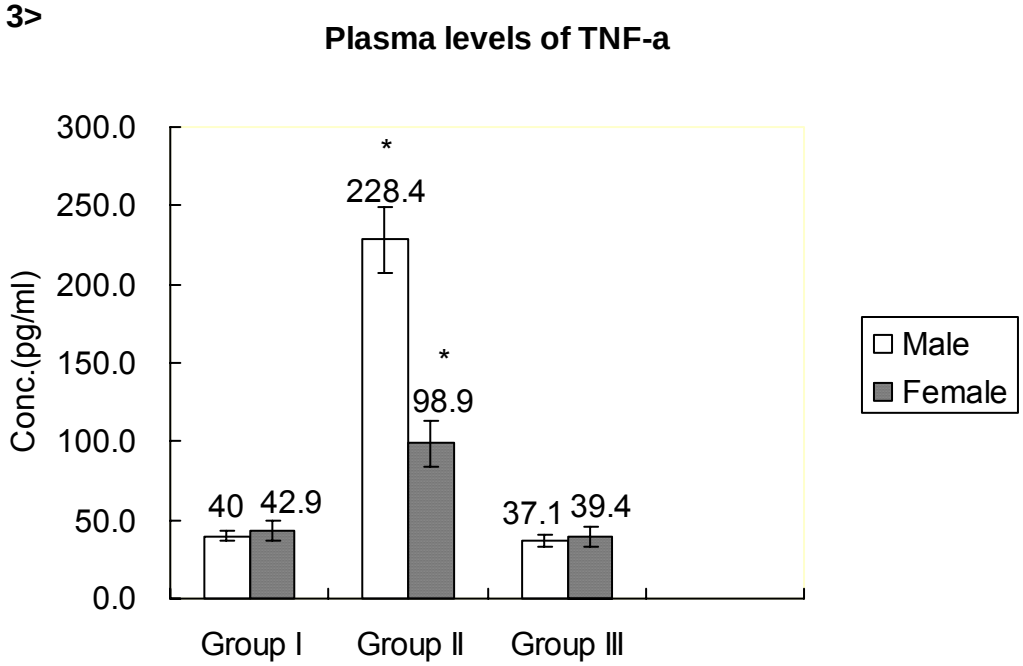
2. Differences among three groups

Regardless of sexes, group II had the highest and group I had the lowest plasma levels of TNF-α, IL-1β, IL-6, and IL-10.

With regard to TNF-α and IL-10, group II had significant greater plasma levels than those of group I and group III in both sexes (p<0.001).

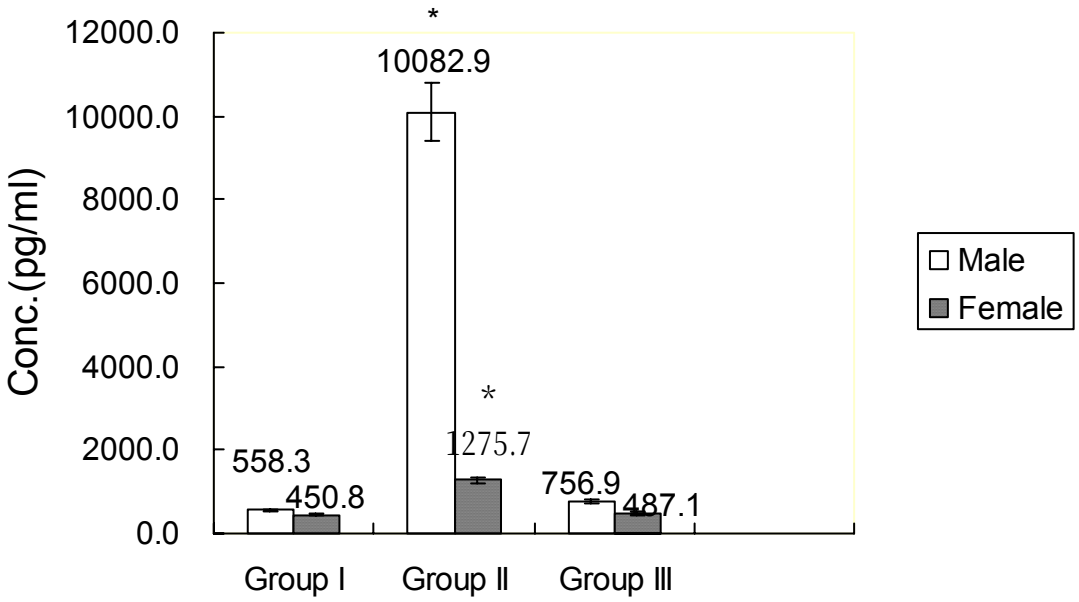
With regard to IL-1β and IL-6, there were significant differences among three groups in males (p<0.001), but there only showed significant differences between group I and II, group II and group III in females (p<0.001).

<Fig. 3>



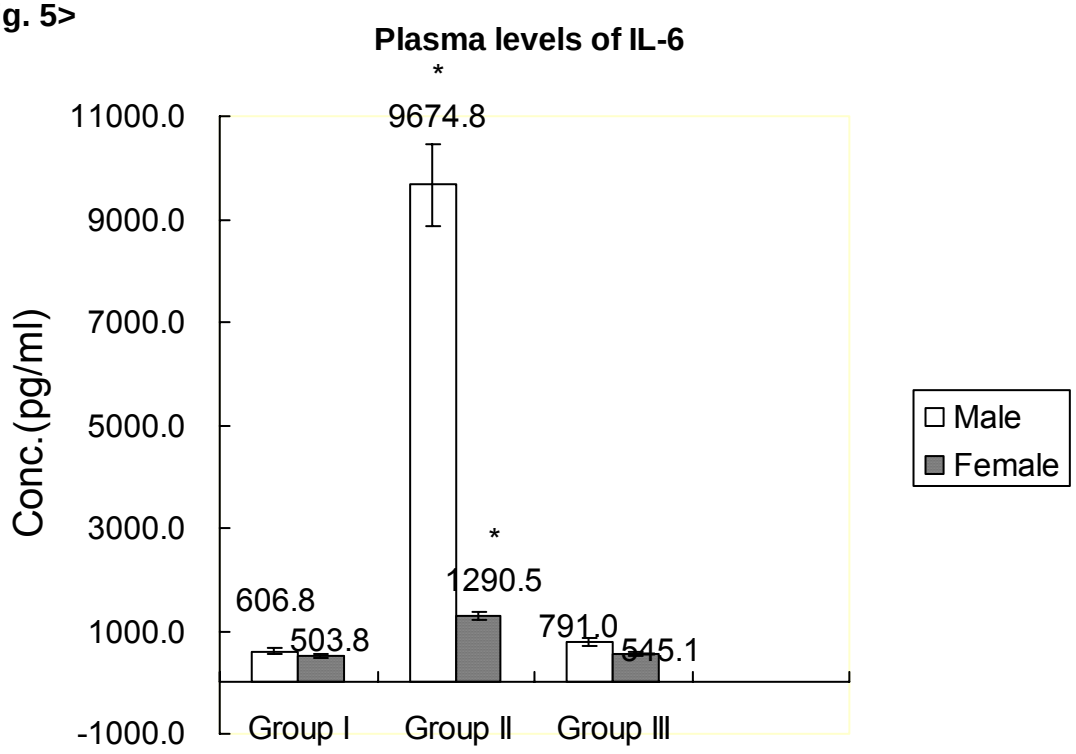
※*p<0.001 vs. other groups*

<Fig. 4>



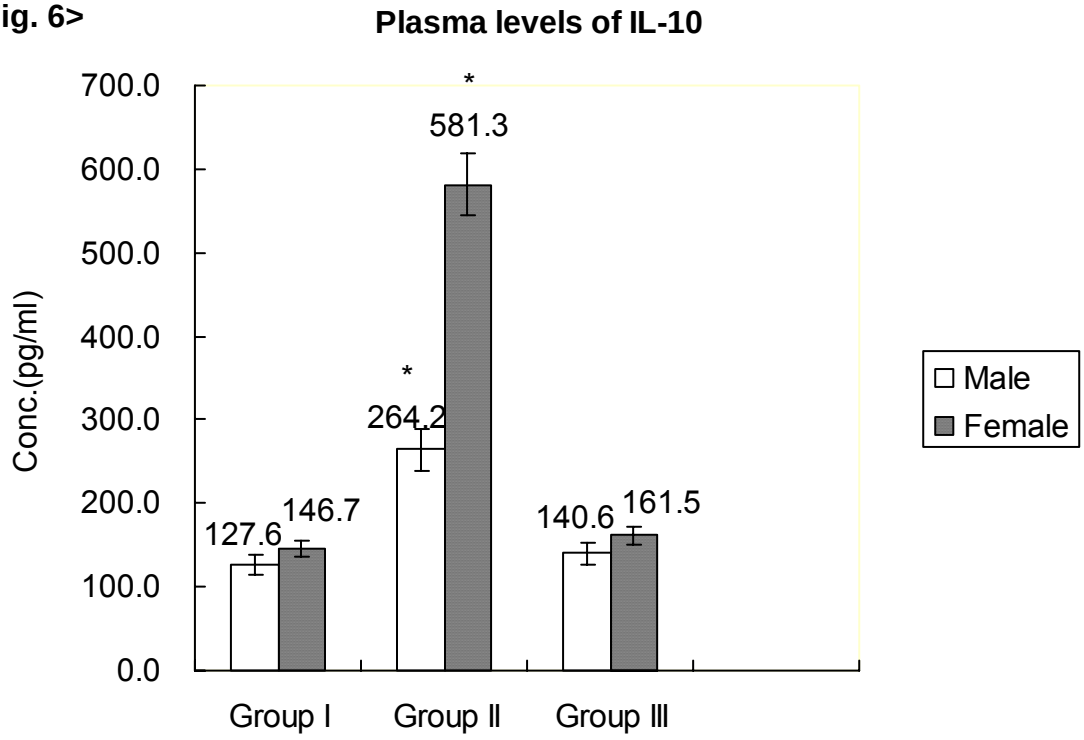
※*p<0.001 vs. other groups

<Fig. 5>



※*p<0.001 vs. other groups

<Fig. 6>



※*p<0.001 vs. other groups

- **Discussion**

[Age versus Body Weight]

If the body weight may influence the hemodynamic change, hormonal change, inflammatory response and genomics？ The question is negative. According to De Maio A. et al[73], the clinical outcome from injury is a combination of several factors including the initiating insult, the environment, the genetic makeup of the subject, sex and age. Body weight play little role on the response to injury. Therefore, the significant difference in body weight between males and females in our experiment (Table 1) may not influence the result of this study.

[Hemodynamic Change]

CVP is not a reliable indicator for hemodynamic monitor because wide variety of MCVP among experiment rats in each group were found in our experiment. Although, monitor of central venous pressure is a routine practice in critical care. However, it continues to be a question whether it is necessary to evaluate the hemodynamic condition during acute injury or not. As Dr. Graham Hocking stated[74], the main reasons for inserting a central venous catheter are: 1.measurement of central venous pressure (CVP), 2.venous access when no peripheral veins are available, 3.administration of vasoactive/inotropic drugs which cannot be given peripherally, 4.administration of hypertonic solutions including total parenteral nutrition, and 5.haemodialysis/plasmapheresis. The factors influencing the wide variety of MCVP in each group in our experiment include right heart function, venous return, right heart compliance, intrathoracic pressure and positioning of rats. Therefore, It should always be interpreted alongside other measures of cardiac function and fluid state (pulse, BP, urine output etc.). The absolute value is not as important as serial measurements and the change in response to therapy.

With regard to mean systolic blood pressure (MSBP) levels, the female sub-group had significant greater MSBP levels than those of male sub-group during resuscitation period in group II ($p=0.048$), suggesting that females had greater reserve for hemodynamic change than males.

[Mortality of Model]

Several animal studies have revealed that female rats show an enhanced immunological response in compared with males, resulting in better survival after injury[75]. The difference in response between female and male rats has been predominantly attributed to the different hormonal circumference in each sex. Although, there were no significant difference between males and females in group II, the 4-hour mortality rates were lower in females than in males (50% vs. 66.7%). The reasons that it could not reach the significant

difference in mortality between males and females may be: first, too small animals in each sub-group and secondly, too short period was observed. Therefore, we may expect that the differences may become significant if more rats are studied and longer time was observed.

[Alteration in Plasma Levels of 17β-estradiol]

As previous mentioned, animal studies have revealed that female rats have protective effect of 17β-estradiol on immune function and physiological responses following trauma-hemorrhage. However, whether women experience better outcomes from injury as compared with men is still controversial in clinical studies[76-79]. In clinical studies, the female sex hormone could difficultly been controlled due to varying plasma levels during menstrual cycle. On the other hand, the female sex hormone could be controlled easier in rat studies because they have short length of the estrus cycle., making them ideal for investigation of changes occurring during the reproductive cycle. The estrous cycle lasts four days and is divided as: proestrus, estrus, metestrus and diestrus, which can be determined according to the cell types observed in the vaginal smear by light microscopy[80] as in our experiment. During estrous cycle in rats, the proestrus state shows the highest plasma concentration of estradiol and prolactin [81]. The plasma levels of both hormones are low on the morning of estrus and then gradually increase over diestrus to achieve their peak levels on the morning of proestrus [82]. Wichmann et al.[53] have shown that female mice subjected to hemorrhage during the proestrus state have enhanced immune responses as opposed to decreased responses in males. In summary, to achieve satisfactory results of female sex hormone (17 β-estradiol) on immune function and physiological responses following trauma-hemorrhage, adequate controlling of female rats in proestrus stage to keep high plasma levels of 17 β-estradiol plays an important role on this study.

However, poorly controlling of 17 β-estradiol was encountered in our experiment. The female sub-groups had significant greater plasma levels of 17 β-estradiol, however, the diversity of plasma hormonal levels among female rats were large, representing that there was poorly controlling of the hormone in plasma, which may distorted the results in our study. The reason for poorly controlling of 17 β-estradiol is that we cannot decide the initial time of proestrus stage even though we can decide the proestrus under light microscopy. Therefore, the plasma levels of 17 β-estradiol may be reduced when the rats were sacrificed because they were not in proestrus stage at that time.

[Cytokine Levels in Rats]

1. Introduction:

A. TNF-α:

Tumor necrosis factor alpha (TNF-α), also known as cachectin[83], is a 17.5 kD, 157 amino acid protein that is potently lymphoid factor, which exerts cytotoxic effects on a wide range of tumor cells and other target cells.[84] TNF-α has been suggested to play a pro-inflammatory role and it is the primary mediator of immune regulation. According to Deitch[73], TNF-α, rather than IL-I or IL-6 appears to be the messenger that initiates and orchestrates the septic response. The biosynthesis of TNF-α is tightly controlled, being produced in extremely small quantities in quiescent cells, but is a major screted factor in activated cells.[85]

B. IL-1 β

Interleukin-1 β (IL-1 β) is translated as a 31 kD precursor that is glycosylated and cleaved into a cytosolic pro-IL-1 β molecule. IL-1 β with the aid of inducible transcription factors initiates the expression of a pro-inflammatory cascade of proteins and cytokines by selective regulation of other molecules.[86] The half-life of IL-1 β mRNA depends upon the cell type and the conditions of stimulation. In human blood monocytes and macrophage cell lines, the half-life of IL-1 β mRNA is about 4 hours.[83]

C. IL-6:

Interleukin-6 (IL-6) is a cytokine critical to the regulation of immune and hematopoietic systems. A 211-amino acid protein, rat IL-6 is expressed by cell type such as T-cells, mast cells, monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, and many tumor cell lines. It appears to take part in acute phase reactions and response to injury and inflammation. Elevation of serum IL-6 levels have been observed in a number of pathological conditions, including bacterial and viral infections, trauma, autoimmune diseases, inflammations and malignancies.[83]

D. IL-10

Interleukin-10 (IL-10), originally known as cytokine synthesis inhibitory factor (CSIF), is a pleiotropic cytokine with a myriad of immunomodulatory effects on a variety of cell types. On macrophages, IL-10 is known to downregulate TNF-α, IL-1, and IL-6 production.[87]

2. Cytokines of the model

Regardless of sexes, all of the studied cytokines were increased significantly in traumatic hemorrhagic shock groups as compared with those in sham operation group (p<0.001). The plasma levels of pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 following traumatic hemorrhagic shock increased more predominantly in male subgroups (5.7-fold, 18.1-fold and 15.9-fold respectively) as compared with those in female sub-groups (2.3-fold, 2.8-fold and 2.6-fold respectively). On the other hand, levels of anti-inflammatory

cytokine, IL-10 in plasma following traumatic hemorrhagic shock increased more significantly in females (4.0-fold) as compared with those in males (1.9-fold). In resuscitation groups, all of the studied cytokines were decreased significantly as compared with those in shock groups (p<0.001). The plasma levels of TNF-α, IL-1, and IL-6 following resuscitation decreased more predominantly in male subgroups (85.8%, 92.5% and 91.8% respectively) as compared with those in female sub-groups (60.2%, 61.8% and 57.8% respectively). On the other hand, levels of anti-inflammatory cytokine, IL-10 in plasma following traumatic hemorrhagic shock decreased more significantly in females (72.2%) as compared with those in males (46.8%). The current data of plasma levels of cytokines in our experiment suggesting that females have more protective from traumatic hemorrhagic shock than males. According to Chaudry et.at,[88] the protective effect may be due to the modulation of sex hormone, 17β-estradiol in particularly. However, when resuscitation was incorporated, the protective effects of sex hormone may be neglected as in our experiment.

[Microarray Analysis]

To be presented in the supplementary report.

[Study Limitations and Future Directions]

There are two major limitations in our experiment. First, poor control of female sex hormone was encountered. Although, the female sub-groups had significant greater plasma levels of 17β-estradiol. However, the diversity of plasma 17β-estradiol levels among female rats were large, representing that there was poor control of 17β-estradiol levels in plasma, which may distorted the results in our study. The reason for poor control of female sex hormone is that we cannot decide the initial time of proestrus stage even though we can decide the proestrus under light microscopy. Therefore, the plasma levels of 17β-estradiol may be reduced when the rats were sacrificed because they were not in proestrus stage at that time. How to keep high stable plasma levels of 17β-estradiol should be concerned in the following related study.

Secondly, long term survival rate and the correlation with plasma levels of cytokines and liver mRNA expression were not studied in this experiment. Although we planned to evaluate the survivals, plasma levels of cytokines and liver tissue mRNA expression at the acute stage following traumatic-hemorrhagic shock using traumatic-hemorrhage and resuscitation model in rat, 4-hour survival evaluation may reflect the systemic inflammatory response at the acute stage. However, we cannot find out the thorough change of plasma levels of cytokines, including TNF-α, IL-1β, IL-6, and IL-10 in the short period of time. Longer evaluation, including 12-hour and 24-hour survival, should be considered in the following study.

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