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DISSERTATION

IMMUNE RESPONSE MODULATION IN THE INTERNAL PUDENDAL ARTERY IN WISTAR RATS WITH STRESS CONDITION

A Psychoneuroimmunology study towards the pathogenesis of psychogenic Erectile Dysfunction

Rudi Yuwana

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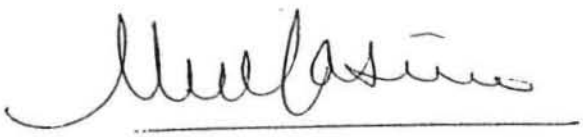
Promotor



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RINGKASAN

Sudah lama diyakini, bahwa stresor psikogenik seperti kecemasan, depresi, ketakutan dapat mengakibatkan disfungsi ereksi (DE). Banyak penelitian klinik telah membuktikan adanya hubungan antara keadaan psikogenik dan timbulnya DE, namun sejauh ini mekanisme timbulnya DE pada penderita yang mengalami stresor psikogenik belum diketahuui dengan jelas. Sampai sekarang diagnosa DE psikogenik hanya ditentukan setelah semua sebab-sebab organik disingkirkan.

Untuk mengungkap patogenesis DE psikogenik, digunakan penelitian dengan paradigma psikoneuroimunologi (PNI). PNI adalah ilmu pengetahuan dengan model berfikir, bahwa suatu stresor, apapun macamnya, fisik atau psikis menyebabkan suatu perubahan biologik pada suatu makhluk. Perubahan biologik ini dapat pada sistem, organ, sel maupun gen dan perubahan ini merupakan suatu modulasi respon imun (MRI) (Ader, 1991; Putra, 1999).

Pada penelitian ini digunakan desain penelitian *Randomized Control Group Posttest only Design,* yang dipakai untuk percobaan adalah tikus putih Rattus Nervigicus strain Wistar. Empat puluh tikus dibagi secara acak (random assingment) dalam 4 kelompok. Kelompok I (kelompok kontrol 5 hari), kelompok II (kelompok eksperimen 5 hari), kelompok III (kelompok kontrol 10 hari) dan kelompok IV (kelompok eksperimen 10 hari). Tikus berumur 3-4 bulan dan mempunyai berat badan 150 – 200 gram.

Suatu renjatan listrik (*electric Footshock* = EF) dipakai untuk menimbulkan stresor psikogenik, sebagai variabel indedenden. Setelah tikus mendapat paparan EF selama 5 dan 10 hari, dibunuh, lalu dideseksi a pundendus internusnya dan diproses untuk diperiksa secara imunohistokimia dengan menggunakan antibodi monoklonal. Lalu diamati adanya MRI, yang tercermin dalam perubahan sel endotel penghasil VCAM-1 dan limfosit penghasil sitokin IL-2, IFNγ, IL-4 dan IL-10 pada dinding arteria pundendus internus, pembuluh darah utama untuk ereksi. Kemudian diperiksa juga keadaan kortisol dan katekolamin didalam darah sebelum dan sesudah eksperimen.

Untuk menganalisa data digunakan analisa multivariat, oleh karena pada suatu perubahan biologik merupakan interaksi antara beberapa variabel. Hasil yang didapat setelah 5 hari EF menunjukkan peningkatan kadar kortisol, adrenalin, noradrenalin

dalam darah; peningkatan jumlah sel endotel penghasil VCAM-1 dan penurunan jumlah limfosit penghasil IL-2, IFNγ, IL-4 dan IL-10 menurun banyak. Pada renjatan listrik 10 hari didapatkan peningkatan kadar kortisol. Kadar adrenalin juga meningkat tetapi lebih sedikit. Endotel penghasil VCAM-1 berkurang dan limfosit penghasil IL-2, IFNγ, IL-4 dan IL-10 lebih berkurang.

Untuk menjelaskan mekanisme diperlukan kontribusi fungsi setiap variabel terhadap kejadian DE. Berdasarkan analissis diskriminan hanya 5 variabel (yaitu kortisol, adrenalin, VCAM-1, IL-4, IL-10), yang mempunyai kontribusi berperan pada MRI. Setelah 5 hari EF didapatkan peningkatan peran adrenalin, tetapi penurunan peran kortisol. Peran VCAM-1 meningkat dan peran IL-4, IL-10 juga meningkat. Setelah 10 hari EF didapatkan peningkatan peran kortisol, tetapi penurunan peran adrenalin. Peran IL-4, IL-10 menjadi lebih mwningkat dan peran VCAM-1 menurun.

Dapat disimpulkan bahwa setelah 5 hari EF, peran adrenalin dan peran VCAM-1 meningkat berdasarkan kerangka konseptual menyebabkan disfungsi endotil, yang secara potential dapat menyebabkan kerusakan pembuluh darah arteri pudendus internus, dan selanjutnya menyebabkan DE psikogenik.

Setelah 10 hari EF peran kortisol yang lebih meningkat menyebabkan peran IL-4, IL-10 lebih meningkat. Keadaan yang menunjukkan sel Th2 lebih dominan berperan dapat potensial merusak pembuluh darah arteria pudendus internus lewat proses ADCC (*antibody dependent cell cytotoxicity*) dan selanjutnya menyebabkan DE psikogenik.

Ini adalah suatu penemuan baru yang dapat menerangkanmekanisme timbulnya kerusakan endotel pada a pudendus internus dan potensial menimbulkan DE.

ABSTRACT

It has long been stated that psychogenic stressors like anxiety, depression, and stress may cause Erectile Dysfunction (ED). Many clinical studies have shown the relationship between psychogenic conditions and the development of ED (psychogenic ED). However, until recently, the exact mechanism of ED in patients with psychogenic stressors had not been understood.

To clarify the pathogenesis of psychogenic ED, an animal experimental research using psychoneuroimmunology (PNI) paradigm was conducted. The design chosen was a Randomized Control Group Posttest only Design.

Forty male rats, Rattus Nervigicus of Wistar strain were used and at random divided into 4 groups of ten rats. Group I (control group for 5 days experiment), group II (5 days experiment group), group III (control group for 10 days experiment), group IV (10 days experiment group). The rats were 3-4 months old and had a body weight of 150-200 gr

An electric footshock (EF) was used as a psychogenic stressor (as independent variable) exposed to the rats.

The dependent variables observed were Immune Response Modulations (IRM) of the neurohormonal changes expressed in cortisol, cathecholamines (adrenalin, noradrenalin) blood level, amount of VCAM-1 expressing endothelial cells (ECs) and amount of IL-2, IFNγ, IL-4, IL-10 producing lymphocytes in the internal pudendal artery wall, the main artery for erection. A multivariate analysis was used to evaluate the data.

The results after 5 days of EF exposure showed cortisol, adrenalin and noradrenalin blood levels increased; amount of VCAM-1 expressing ECs increased and amount of IL-2, IFNg, IL-4, IL-10 producing lymphocytes decreased.

After 10 days of EF exposure cortisol, adrenalin and noradrenalin blood levels also increased; amount of VCAM-1 expressing ECs decreased and amount of IL-1, IFNg, IL-4, IL10 producing lymphocytes were more decreased.

To explain the mechanism, the contribution function of each variables to the development of ED is needed. Based on discriminant analysis, only 5 variables (cortisol, adrenalin, VCAM-1, IL-4, IL-10) have contribution in IRM pattern.

It showed that after 5 days of EF exposure, IRM caused an increased adrenalin function, but a decreased cortisol function. VCAM-1 expressing ECs and IL-4, IL-10 producing lymphocytes functions were increased.

After 10 days of EF exposure there was a prominent increased cortisol function, but a decreased adrenalin function. IL-4 and IL-10 producing lymphocytes functions were more increased whereas VCAM-1 expressing ECs function was decreased.

In conclusion: the increased adrenalin function after 5 days of EF exposure and increased VCAM-1 expressing ECs function (according to the theoritical framework) may cause endothelial dysfunction and potentially may cause endothelial damage in the internal pudendal artery wall, and hence causes psychogenic ED.

After 10 days of EF exposure cortisol function increased, which consequently caused increased IL-4 and IL-10 function. This condition, where Th2 cells are dominant may cause endothelial damage of the internal pudendal artery wall through ADCC (antibody-dependent cell-cytotoxicity) process, and hence may cause psychogenic ED.

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ABREVIATIONS

A	: Adrenalin
ACTH	: Adrenocorticotropic hormone
ADCC	: Antibody-Dependent Cell-mediated Cytotoxicity
AGE	: Advanced Glycosylation End-product
APC	: Antigen Presentation Cell
Apo (a)	: Apolipoprotein (a)
AVP	: Arginin Vaso Presin
CC	: Corpus Cavernosum
cGMP	: ciclic Guanosine Mono Phospate
CMV	: Cytomegalo Virus
CRF	: Corticotropic Releasing Factor
CRH	: Corticotropic Releasing Hormone
CRP	: C-Reactive Protein
CS	: Corpus Spongiosum
DM	: Diabetes Mellitus
DTH	: Delayed Type Hypersensitivity
EC	: Endothelial Cell
ED	: Erectile Dysfunction
EDRF	: Endothelium Derived Relaxing Factor
Egr-1	: Early Growth Response-1
eNO	: endothelial Nitric Oxide
eNOS	: endothelial Nitric Oxide Synthase
GAS	: General Adaptation Syndrom
GC	: Guanulate Cyclase

HPA-axis	: Hypothalamic Pituitary Adrenal-axis
HSV	: Herpes Virus
ICAM-1	: Intercellular Adhesion Molecule-1
IFN-γ	: Interferon-γ
IHC	: Immunohistochemistry
IL-1	: Interleukin-1
iNOS	: Inducible NOS
IRM	: Immune Response Modulation
LAG-3	: Lymphocyte Activation Gen-3
LCAT	: Lecetin Cholesterol Acyltransferase
Lp (a)	: Lipoprotein (a)
MCP-1	: Monocyte Chemotactic Protein-1
MCSF	: Macrophages Colony Stimulating Factor
m AB	: Monoclonal Antibody
mp PVN	: medial parvocellular division of the Para Verticular Nucleus
MPOA	: Medial Preoptic Area
NA	: Nor Adrenalin
NANC	: Non Adrenergic – Non Cholinergic
NE	: Nor Epinefrin
NF-κB	: Nuclear Factor – Kappa Beta
NIH	: National Institute of Health
nNOS	: Neural NOS
NO	: Nitric Oxide
NOS	: Nitric Oxide Synthase
NPT	: Nocturnal Penile Tumescence test

PA-1	: Protein Activator-1
PAC	: Pituitary-adreno-corticol system
PBS	: Phosphate Buffer saline
PDGF	: Platelet Derived Growth Factor
PNI	: Psychoneuroimunology
POMC	: Pro-Opio-Melano-Cortrin
SAM	: Sympathatic Adrenal Medullary System
SMC	: Smooth Muscle Cell
STATs	: Signal Tranducers and Activators of Transcription
T dth	: T delayed type hypersensitivity
TA	: Tunica Albuginea
TGF-α	: Transforming Growth Factor Alpha
TGF-β	: Transforming Growth Factor-β
TH cell	: T Helper cell
TNF	: Tumor Necrosis Factor
Ts	: T Supressor
VCAM-1	: Vascular Cell Adhesion Molecule-1
VIP	: Vasoactive Intestinal Peptide
VO	: Veno Oclusi

CHAPTER 1

INTRODUCTION

1.1 Research Background

Psychogenic condition has long been stated as one of the causes of Erectile Dysfunction (ED). Many clinical studies have shown the relationship between psychogenic conditions (like stress, depression, anxiety) and the development of ED. However, the exact mechanism on how psychogenic factors may cause ED has not yet been understood.

If the pathogenesis of psychogenic ED is known, then treatment and prevention may be managed better. There must be a more basic pathobiological research that might explain the mechanism.

In 1970, Masters and Johnson stated that 95% of patients with ED had psychogenic causes. The development of more sophisticated diagnostic tools have made better diagnosis possible. So more and more organic causes were identified. Karacan found in 1970 after the development of Nocturnal Penile Tumescene test (NPT) organic causes in more than 50% of the patients. More sophisticated investigations like pharmacodynamic studies are able to elucidate that more than 80% of ED patients have a specific organic background, such as a vascular, neurological and/or endocrinological diseases. In 1988, Melman, Tiefer, and Pederson concluded from epidemiological studies that causes of ED were 40% purely psychogenic, 25% organic and psychological, and in 29% the cause was purely of organic origin. With the present diagnostic procedures, it is possible indeed to differentiate more organic causes, but sometimes it is still difficult to know whether only organic factors do play

a role in the development of ED or do also psychogenic factors play a role. According to Watkins (1993), psychological factors may aggravate ED caused by organic factors. That is why it is important to know how psychological factors may influence the pathogenesis of ED.

Until recently, psychological factors are assumed to be the cause of ED if all organic factors have been excluded. Organic causes of ED are classified as vascular, neurological and/or hormonal impairment (Yuwana, 1994). Damage to the main artery, the internal pudendal artery, which supply blood to the penis for erection can cause ED. Also trauma or diseases that involve the nerve system to the penis will impair erection. The androgen hormone testosterone has not a direct influence on the physiology of erection, but may improved libido and the quality of erection (Thomas et al, 1992).

The pathway of how psychological factors can cause ED is not clear yet. Psychological factor can not be diagnosed by an interview or a psychometric assessment alone. There must be more objective method to diagnose psychological causes.

At the present time, the therapy for ED is symptomatic, it implies that the condition is treated only, when symptoms are already evident. Several treatment options such as vacuum assisted device, intracavernous pharmaco injection (Yuwana, 1993), as well as reconstructive vascular and penile prosthesis operations (Yuwana, 1998) are performed when ED is already there. Also oral, topical and even intraurethral drugs convey a symptomatic therapy. To be able to reduce the prevalence of ED, it is necessary to find changes before symptoms are apparent. Some

counts for 50% of DM complications (Tjokroprawiro A, 1996 and 1997), only by regulation of the blood sugar level is not successful. Also, prevention of the growth of atherosclerosis, which may cause ED, by the treatment of risk factors such as DM, dyslipidemia, smoking and alcohol abuse, presents no good results either. It might be possible that psychological factors like stress have a role in the development of ED and therefore, the prevention is not sufficiently managed by treating the organic causes alone.

As previously stated, the mechanism on how stress (psychogenic condition) can cause ED is not yet known. It is, however, already known that stress condition may relate to the development of several disease conditions, when the normal immune responses of the human body decrease. (Riley, 1981; Jacobs et al, 1970). The science that tries to explain the relationship between stress and the immune system is nowadays called psychoneuroimmunology (PNI), which developed very fast in the last few years (Putra, 1998; 1999a). Many investigations have already shown that the relationship between stress and the immune system involve the neurological and the endocrine system.

Stress may trigger the Central Nervous System (CNS) and which influence the endocrine and autonomic nervous system, effecting the immune system. This channel is called the Hypothalamic-Pituitary-Adrenal-axis (HPA-axis) (Dunn, 1995). The adrenal gland secretes cortisol from the cortex after a stimulation by adrenocorticotropic hormone (ACTH) from the pituitary gland and cathecholamines are secreted by the medulla under the influence of adrenergic autonomic nervous pathways.

These enzymes may change the immune system. If the immune system is disturbed, the homeostatic mechanism of the body is impaired also; and consequently it may damage several organs. From the above concept, we can assume that stress may also damage the vascular and neurological system in general and if it involves the main artery for erection it will cause ED.

Sergio Romagnani in 1997 explained through his Th1/Th2 paradigm how the immune system may contribute to the damage of several organs and causes diseases. This theory is based on studies that immuno-competent T-cells are able to polarize into Th1 and Th2 subsets depending on the environment and genetic factors. The damaging mechanism depends on which type of lymphocytes will dominate. When Th1 cells dominate, then the process is through Delayed Type Hypersensitivity (DTH) and macrophages, whereas when Th2 cells dominate, the damaging process can be explained through an autoimmune or Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) process. There is no study about the polarization of Th cells in the vascular wall after stress. If this assumption is happening then the damaging process which mostly the development of atherosclerosis in the arterial wall can be explained by the Th1 / Th2 paradigm. Atherosclerosis starts mostly in the middle size muscular vessels, with predelection in arteries with a "Fork Shape", or places where the artery makes branches. In this study, the internal pudendal artery will be investigated. The internal pudendal artery is a branch of the internal iliac artery and the main supplier of blood for the penis.

Stress stimulates the adrenal medulla via the sympathetic nerve to secrete cathecholamines (Dunn, 1995). Cathecholamines (adrenalin and noradrenalin) cause vasoconstriction and increase intravascular tension. Then cause "Shear stress" and shear stress results in endothelial dysfunction (Khder et al, 1998). Endothelial

dysfunction can be recognized by decreasing NO-level, endothelial Nitric Oxide (eNO) produced by endothelial cells. Before endothelial dysfunction occurs, adhesion molecule as Vascular Cell Adhesion Molecule-1 (VCAM-1) is expressed on the surface of the endothelial cells (Khder et al, 1998). Endothelial dysfunction causes higher permeability of the endothelium and intravascular cells among others lymphocytes and monocyte will migrate from the intravascular wall compartment into the sub-endothelial tissue. In the sub-endothelial tissue immuno competent lymphocyte will differentiate into Th1 and Th2 cells.

Therefore, it is important to prove that there will be a polarization of Th cells in the arterial wall after expression of VCAM-1 on the endothelial cells before vascular damage occurs, which will rise to atherosclerosis. The expression of VCAM-1 on the endothelium and the polarization of Th1 and Th2 cells will be studied before and after giving a stressor. Rats are chosen for these experiments because this allows easy dissection of the internal pudendal artery, which can be processed to study the endothelial changes (Carter, Bazim, 1979). The presence of VCAM-1 can be demonstrated by immunohistochemistry (IHC) using monoclonal antibody (m AB) staining.

The polarization of Th1 and Th2 cells is shown by the production of cytokines. Interleukin-1 (IL-1), IL-2 and Interferon-γ (= IFNγ) are products of Th1 and Il-4, IL-5, IL-10 an IL-12 are made by Th2 (Staines, Broscoft, James, 1993a; Romagnani, 1997). These cytokines can also be demonstrated by immunohistochemistry staining.

1.2 Identification of the Problem

The exact pathogenesis how stress (psychological factor) may cause ED is not yet known. Psychoneuroimunology, the science that studies the relationship between stress and the immune system, might explain and solve the problem.

Stress via the HPA-axis, the autonomic nerve and the endocrine system causes immune response modulation (= IRM) (dynamic changes) in the immune system, before the pathologic condition with atherosclerosis and hence luminal obstruction appears.

Stress causes decreased production of eNO and consequently expression of VCAM-1 on the ECs of the blood vessels. This causes endothelial dysfunction and when endothelial dysfunction occurs then Th lymphocyte migrates from the intravascular into the subendothelial compartment. In this subendothelial compartment the process of polarization of lymphocytes into Th1 and Th2 cells starts. If this continues, destruction of the vascular wall will occur and atherosclerosis develops.

Development of atherosclerosis in the internal pudendal artery may cause diminished vascularization of the cavernous tissues and causes some degree of ED.

In our study, we used rats, Rattus Norvigicus strain Wistar, with "Electric Footshock" as psychological stressor. "Electric Shock" causes anxiety and a noxious condition, which serves as a psychologic stressor for the rats. We used an exposure of 5 days and 10 days based on previous experiments that after five days IRM already take place.

From the above statements we raise the following questions:

1. Would IRM occur (presented as changes in : cortisol and cathecholamine blood level; ECs expressing VCAM-1, and Th1 cell secreting IL-2, IFNγ & Th2 cell

secreting IL-4, IL-10 polarization) in internal pudendal artery wall of rats in stress condition after exposure to an "electric footshock" stressor of 5 days?

2. Would IRM occur (presented as changes in cortisol and cathecholamine blood level; ECs expressing VCAM-1, and Th1 cell secreting IL-2, IFNγ & Th2 cell secreting IL-4, IL-10 polarization) in internal pudendal artery wall of rats in stress condition after exposure to an "electric footshock" stressor of 10 days?
3. Are there any differences in IRM in the internal pudendal artery of rats in stress condition after electric footshock exposure of 5 days or 10 days?

1.3 Objective of the Research

1.3.1 General Objective

To elucidate the mechanism of vascular damage in the internal pudendal artery in rats through IRM produced by "electric footshock" stressor for 5 and 10 days, and to explain the possible mechanism of psychogenic ED.

1.3.2 Specific Objectives

1. To prove that there is an IRM (presented as changes in cortisol and cathecholamine blood level; ECs expressing VCAM-1 and Th1 cell secreting IL-2, IFNγ, and Th2 cell secreting IL-4, IL-10 polarization) in the internal pudendal artery wall of rats with stress condition caused by exposure to "electric footshock" stressor for 5 days.
2. To prove that there is an IRM (presented as changes in cortisol and cathecholamine blood level; ECs expressing VCAM-1 and Th1 cell secreting IL-2, IFNγ, and Th2 cell secreting IL-4, IL-10 polarization) in the internal pudendal artery wall of

rats with a stress condition caused by exposure "electric footshock" stressor for 10 days.

3. To prove that there are differences in IRM in the internal pudendal artery wall in rats with stress condition dependent on the length of the exposure to "electric footshock" stressor for 5 or 10 days.
4. To explain the mechanism of vascular damage in the internal pudendal artery wall through IRM in rats with stress conditions caused by "electric footshock" stressor.

1.4 Significance of the Research

When it is proven that psychogenic stressor causes an IRM in the internal pudendal artery wall, further destructive process of the vessel can be explained through psychoneuroimmunology paradigm.

The IRM might be used as an indicator for the initiation of the damaging process, because changes in the immune system already occur before the damaging process of the internal pudendal artery and symptoms of ED become manifested.

If it is proven that stress modulates the immune system, managing stress might prevent further destruction of the vessel wall and hence reduce the psychological factors for ED.

If localized cytokines play a role in the pathogenesis of ED, probably pharmacotherapy against these cytokines may stop this phenomenon.

CHAPTER 2
LITTERATURE STUDY

2.1 Sexual Dysfunction, Impotence and Erectile Dysfunction

Male Sexual dysfunction may involve the following disorders:

1. Erectile dysfunction or impotence
2. Ejaculation changes
3. Lack of orgasm
4. Diminished libido
5. Dysfunction of Emission

In general the term "Impotence" is used to indicate Erectile Dysfunction (ED). Impotence means actually only "not potent" or "not capable".

We distinguish 2 (two) kinds of impotence

1. Impotentia Coeundi means not capable to have an erection
2. Impotentia Generandi indicates not capable to have descendents

To avoid misunderstanding, since 1992 the "National Institute of Health (NIH) Consensus Development Conference on Impotence" in USA, suggested to use the term Erectile Dysfunction when the incapability of having an erection is indicated, without the presence of ejaculatory, orgasm or libido changes.

Erectile dysfunction is thus defined as the consistent inability to attain and maintain a penile erection to permit a satisfactory sexual intercourse (Lue, 1992). This definition will be used in this research.

Ejaculatory, orgasm and libido disorders will not be discussed in this text.

2.2 Functional Anatomy of the Penis

2.2.1 Corpus Cavernosum, Corpus Spongiosum, and Glans Penis

The erectile part of penis is called Corpus Cavernosum (CC). It consists of two spongy, paired cylinders envelopped by a thick layer called tunica albuginea. Their proximal ends, the crura, originate at the undersurface of the puboischial rami as two separate structures which merge at the distal end of the pubic arch and remain attached distally up to the Glans Penis (GP). The septum between the two CC is incomplete in humans but complete in someother species, such as the dog.

The tunica albuginea consists of layers of wavy collagen and elastic fibers, which can accommodate to the elongation and expansion during erection of the penis. The tunical covering is complete except for those areas penetrated by the arteries and nerves. These places are surrounded by a sheath of loose areolar tissue that protects the arteries and nerves from compression during erection. The emissary veins, however, are in direct contact with the tunica albuginea and thus can be occluded easily by the stretching of the tunica during erection.

Within the tunica are numerous sinusoids among the interwoven trabeculae of smooth muscles supporting connective tissue that contain the terminal cavernous nerves and helicine arterioles. The entire CC is a collection of sinusoids, larger in the center and smaller in the periphery. In the flaccid state, the blood slowly diffuses from the central to the peripheral sinusoids and the blood gas levels are similar to those of venous blood. During erection the rapid entry of arterial blood to both the central and peripheral sinusoids alters the intracavernous blood gas values to those of arterial blood.

In the corpus spongiosum (CS) and the GP, the structure is similar, except that the sinusoids are larger in diameter and the covering tunica is much thinner in the CS and

even absent in the GP.

2.2.2 The Penile Arterial System

The paired internal pudendal artery is usually the main source of blood supply to the penis. The internal pudendal artery becomes the common penile artery after giving off branches to the perineum. The three branches of the penile artery are the dorsal artery, the bulbo-urethral artery and the cavernous artery (Fig. 2-1a).

The cavernous artery enters the corpus cavernosum at the hilum of the penis, where the two crura merge. At the penile base, the cavernous artery is close to the septum, in the mid and distal penis it becomes centrally located. Along its course it gives off many helicine arteries that supply the trabecular erectile tissue and the sinusoids. This helicine arteries are contracted and tortuous in the flacid state and become dilated and straight during erection (Lue TF, 1994).

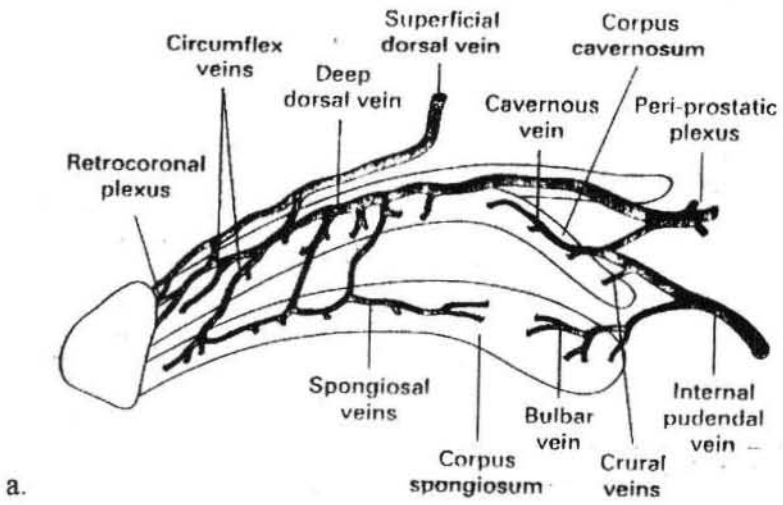
2.2.3 The Penile Venous System

The venous drainage from the three corpora originates as small venules leading from the peripheral sinusinoidal spaces immediately beneath the tunica albuginea. The venules travel in the trabeculae between the tunica and the peripheral sinusinoids for some distances to form the subtunical venular pexus before they exit as the emissary veins (Lue and Tanagho, 1988).

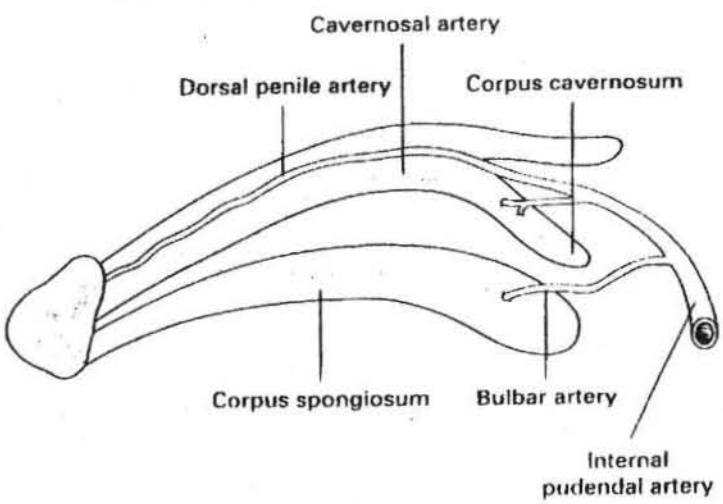
The majority of these veins exit dorsally to join the deep dorsal vein or laterally to the circumplex veins, some exit ventrally to join the periurethral veins. In the proximal CC and the crura, these emissary veins empty in the cavernous veins and the crural veins. These in turn join the urethral veins to form the internal pudendal vein (Fig. 2-1b). Because the GP has no tunical covering, the glandular sinusoids empty directly

large and small veins that form a retrocoronal plexus, the origin of the deep dorsal vein. Along its course, the deep dorsal vein receives several circumplex veins; together these ascend behind the pubic bone to become the periprostatic plexus.

The superficial dorsal veins are small venous channels in the subcutaneous layer, which drain the skin and subcutaneous penile tissue and usually empty the saphenous vein.



a.



b.

Figure 2-1 a. Arterial supply b. Venous drainage (Lue TF, 1994)

2.2.4 Neuroanatomy and Neurophysiology of Penile Erection

a. Peripheral Pathway

The penis is innervated by two sets of nerves. The autonomic nerve system (Sympathetic and Parasympathetic) and somatic nerve systems (Sensory and Motor). From the neurons in the spinal cord and peripheral ganglia, the sympathetic and parasympathetic nerves merge to form the corpus cavernosum nerves, which enter the corpus cavernosum and corpus spongiosum to regulate the neurovascular events during erection and detumescence. The somatic nerves are primarily responsible for sensation of the penis and contraction of the bulbocavernosus and ischiocavernosus muscles.

a) Autonomic Pathways

The parasympathetic nerve fibers to the penis arise from neurons in the intermediolateral cell columns of the second, third and fourth sacral spinal cord segments in man. The preganglionic nerves enter the pelvic plexus, where they are joined by sympathetic nerves from the hypogastric plexus.

Branches of this plexus innervate the rectum, bladder, prostate and splincters. The cavernosus nerves are branches of the pelvic plexus to innervate the penis. Their relationship to the urinary tract is as follows: Posterolateral to the apex of the prostate (5 and 7 o'clock positions); lateral to the membranous urethra (3 and 9 o'clock) and anterior to the bulbous urethra (1 and 11 o'clock), where they enter the hilum of the penis (Lue et al, 1984). An understanding of the course of these nerves is essential in the prevention of iatrogenic impotence during prostate and urethral surgery (Walsh and Donker, 1982).

Sympathetic nerves originate from the eleventh thoracic to the second lumbar

spinal segments and descend through the preaortic plexus and abdominal chains to the superior and inferior hypogastic plexuses. Branches from these plexus communicate with parasympathetic nerve fibers (Burnstock, 1986) to form the cavernous nerves.

Stimulation of the pelvic plexus and the cavernous nerves in animals induce erection, where as stimulation of the hypogastric nerve or the sympathetic trunk causes detumescence. This clearly implies that the sacral parasympathetic nerve is responsible for tumescence and the thoracolumbar sympathetic pathway for detumescence.

Some patients with sacral spinal cord injury however, retain psychogenic erectile ability even if reflexogenic erection is abolished. The psychogenic erection is mediated via the hypogastric nerves and thoracolumbar pathways. A study of paraplegic patients by Chapelle and associates (1980) demonstrated that psychogenic stimuli evoke minimal rigidity whereas tactile stimulation produced an erection of better quality in the corpus cavernosum and spongiosum. This observation suggests that rigid erection requires interaction between separate erectile pathways and that the sacral parasympathetic pathway is the primary erectile center.

b) Somatic Pathways

The Somatosensory pathway begins at the sensory receptors in the penile skin, glans, and urethra and within the corpus cavernosum. The nerve fibers from these receptors converge to form bundles of the dorsal nerve of the penis, which join other nerves to become the internal pudendal nerve. This then ascends via the dorsal roots of the second to fourth nerves to the spinal cord.

Activation of these sensory receptors sends messages of pain, temperature and touch via the dorsal and pudendal nerves, spinal cord and spinothalamic tract to the

thalamus and sensory cortex for sensory perceptions.

The motor pathway. Onuf's nucleus is the center of somatomotor penile innervation (Onuf, 1900). These nerves travel in the sacral nerve to the pudendal nerve, to innervate the bulbocavernosus and ischiocavernosus muscles. Contraction of the ischiocavernosus muscles produces the rigid erection by compressing the engorged corpora cavernosa and increasing the intracavernous pressure to several hundred millimeter of mercury (mm Hg) Rithmic contraction of the bulbocavernosus muscles expels the semen down the narrowed urethral lumen and results in external ejaculation from the meatus.

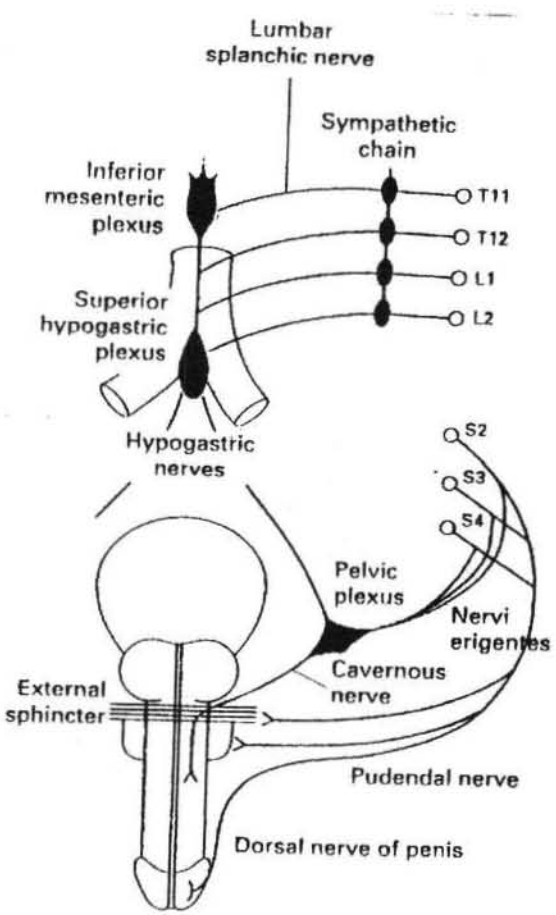


Figure 2-2 Nerve supply of the penis (Lue TF, 1984)

c) Neurotransmitters for Erection and Detumescence

The neurotransmitters responsible for erection and detumescence are still under investigation. Achetylcholine is recognized for ganglionic transmission (by nicotinic receptors) and vascular smooth muscle relaxation (by muscarinic receptors). Cholinergic nerves have been demonstrated within the human cavernous, smooth muscle and surrounding penile arteries. (Mc Connell et al, 1979) and ultrastructural examination has also identified terminals containing cholinergic vesicles in the same area (Steers et al, 1984).

Bilateral sectioning of the cavernous nerves abolishes erectile function and produces within the erectile tissue a marked reduction in choline acetyltransferase, a biochemical marker for cholinergic neurons (Dall and Hamill 1989). Direct injection of acetylcholine into the corpus cavernosum induces a transient increase of intracavernous pressure (Stief et al, 1989). All the above supports the role of acetylcholine in penile erection. However, intravenous or intracavernous injection of atropine fails to abolish erection induced in animals by electrical neurostimulation (Carati et al., 1987: Stief et al., 1989) and in men by erotic stimuli (Wagner and Uhrenholdt, 1980).

Therefore the role of acetylcholine in affecting smooth muscle relaxation during erection, is important, but probably not exclusive (Steers, 1990).

Saenz de Tejada and associate (1989a) showed that acetylcholine-induced relaxation of human cavernous smooth muscle in vitro is also mediated in part by endothelium-derived relaxing factor (EDRF). This factor has been identified as Nitric Oxide (Vanhoutte, 1982), which causes muscle relaxation by stimulating guanalate cyclase (= GC), resulting in increased levels of cyclic guanosine monophosphate (= cGMP) in smooth muscle cells (Hoffman, 1985). Endothelial cells can also synthesize

other potent vasodilators, such as prostacycline and a potent vasoconstrictor, endothelin (Saenz de Tejada et al., 1989b). In addition, researcher, has also shown that human cavernous tissue is capable of synthesizing prostaglandins, which may also be involved in the erectile process (Roy et al., 1984).

Other investigators believe that vasoactive intestinal polypeptide (VIP) may be one of the transmitters responsible for erection. VIP-immunreactive nerve fibers have been identified within the cavernous trabeculae and surrounding penile arteries. Ultra structural studies have shown that VIP immunoactive vesicles are located within varicocities containing presumably cholinergic vesicles (Steers et al, 1984). Achetylcholine and VIP appear to be localized in parasympathetic pathway, they may act synergistically to initiate and maintain erection. Other possible neurotransmitters are calcitonin-gene-related peptide (Stief et al, 1990) and prostaglandins.

Penile detumescence may also involve several neurotransmitters. Adrenergic nerve fibers and receptors have been demonstrated in the cavernous trabeculae and surrounding the cavernous arteries (Benson et al., 1980). Neuropeptide-γ, which has a vasocontrictive property, has also been demonstrated in penile erectile tissue (Carrillo et el., 1989). Endothelin, a potent vasoconstrictor produced by the endothelial cells, has also been suggested to be a transmitter for detumescence (Saenz de Tejada et al., 1989b).

b. Spinal and Supra Spinal Pathways

a) Spinal Pathways

Tectile stimulation of the external genitalia produces penile erection in normal men and patients with supra sacral spinal cord lesions, but not patients with sacral injury. The pudendal afferent fibers from the penis terminate in the medial dorsal horn

and dorsal commissure of the sacral spinal cord in the cat and rat. Second order neurons, the anterolateral spinothalamic tract, relay the message to the brain or activate parasympathetic neurons to initiate erection. Axon studies in several species have shown the spinal erection centers to be located in the inter mediolateral column of the sacral cord.

b) Supra Spinal Pathways

Studies in primates and rodents have identified the medial preoptic area (MPOA) as an important integration center for sexual drive and penile erection (Perachio et al, 1979, Sachs and Meisel, 1988). Electrical stimulation of this area in animals produces erection, lesions at this site limit the ability for copulation. Efferent pathways from MPOA enter the medial forebrain bundle and the mid-brain tegmental region. Pathologic processes in these regions such as Parkinson's disease or Stroke are associated with impotence.

A variety of neurotransmitters, including dopamine, norepinephrine, and serotonin, have been identified in the MPOA (Simerly and Swanson, 1988). Recent research suggests that dopaminergic adrenergic receptors may promote and that serotonin receptors inhibit sexual drive (Foreman and Wernicke, 1990).

Axonal tracing in monkeys, cats and rats has shown direct projection from hypothalamic nuclei to the lumbosacral autonomic erction centers. The neurons in these hypothalamic nuclei contain peptidergic neurotransmitters, including oxytoxin and vasopressin, which may be involved in penile erection. (Sachs and Meisel, 1988).

2.3 Physiology of Erection

2.3.1 Mechanism of Erection

In order to have a better understanding of the pathogenesis in penile erection problems, a knowledge of the current concept about the mechanism of penile erection is needed.

Local sensory stimulation of the genital organs initiates penile erection (reflexogenic erection). Erection can also be a consequence of central psychogenic stimuli received by or generated within the brain (psychogenic erection). The mechanism acts synergistically (De Groat and Steers, 1988). In the detumescent state, penile smooth muscle is kept contracted, probably by release of Noradrenalin (NA) acting on postjunctional α-adrenoreceptors in the cavernous and helicine arteries and trabecular smooth muscle. A contribution of myogenic activity and additional contractant factors can not be excluded (Anderson et al., 1991)

According to the Current Concept (Lue and Tanagho, 1987), erection follows when the sinusoids and the cavernosal and helicinal arteries dilate with subsequent increase in bloodflow of the lacunar spaces of the corpora cavernosa. It is believed that this is achieved partly by a decrease in NA mediated tone, but also through the release of relaxing non-adrenergic, non-cholinergic transmitters from nerves and from the endothelium.

As the trabecular smooth muscle relaxes, the sinusoids will be filled with blood and compress the plexus of the subtunical venules against the tunica albuginea. The "veno-oclusive méchanism" reduces venous outflow from the lacunar spaces, increases the pressure within the corpora and makes the penis rigid (fig. 2-3).

When the penile contractile tissue resumes its contractile state, detumescence is produced. Thus as tone of the helicine arteries and the trabeculae is increased, arterial

inflow is reduced, the pressure within the lacunar spaces decreases, venous outflow increases, and the penis becomes flaccid. Detumescence is achieved by activation of the sympathetic nerve.

2.3.2 Neurogenic and Endothelium Mediated Control of Penis Smooth Muscle

Many investigations have been performed to elucidate the mechanism of neurogenic and endothelial control of the tone of penile blood vessels.

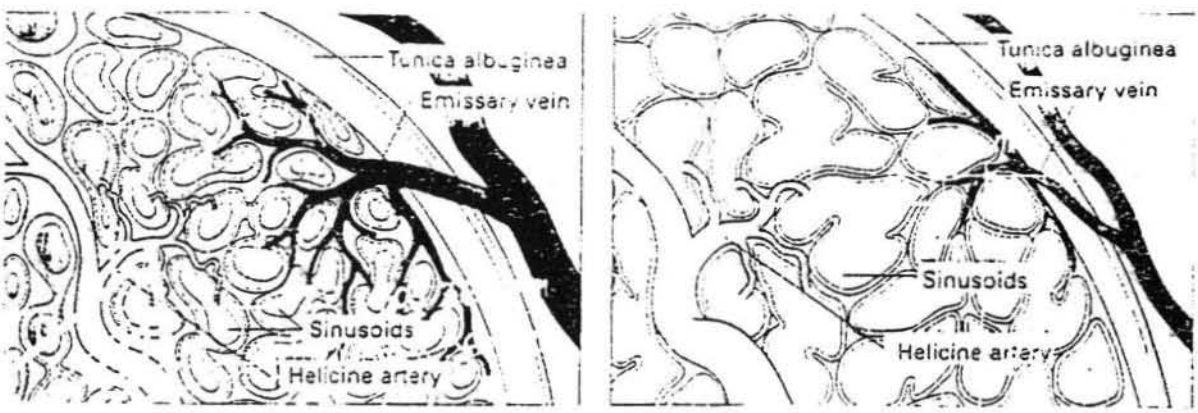
Histochemical studies of the cavernosal and helicine arteries have demonstrated the presence of adrenergic nerves, acetilcholinesterase-containing (probably cholinergic) nerves, as well as vasoactive intestinal polypeptide and neuropeptide γ immunereactive nerves (Kimoto, Ito, 1987).

Adrenergic nerves constrict corporal smooth muscle via norepinephrine acting on alpha-adreno receptors. Relaxation is controlled by cholinergic and non-adrenergic, non-cholinergic neurotransmitter (NANC).

Cholinergic nerves seem to have a modulatory role over the other two neuro effector systems and not a direct effect on the smooth muscle. Vasoactive intestinal polipeptide (VIP), a 28-amino acid peptide, has been proposed as the NANC neurotransmitter in penile smooth muscle by several investigators. This is supported by the observation that VIP-immuno reactive fibers densely innervate the trabecular smooth muscle and that VIP elicits relaxation of the trabecular smooth muscle (Benson, 1993).

In a separate study, however, NANC-mediated relaxation in rabbit corpus cavernosum was shown to release nitric oxide and simultaneously causes the accumulation of cGMP in this tissue. In addition several studies have proposed that nitric oxide is a neurotransmitter in the peripheral nervous system in various organs, accounting for

NANC neurogenic inhibitory responses. Furthermore, nitric oxide synthase has been localized in the peripheral autonomic nerves of several organs which contain smooth muscle (Bredt, Huang, Snyder, 1990). In preparations of human corpus cavernosum in which the endothelium has been removed, the NANC neurogenic relaxation is inhibited by substances that interfere with the synthesis or the effects of nitric oxide



a b

Figure 2-3 a. Cavernosal sinusoids during flaccidity b. Cavernosal sinusoid during-penile erection (Lue TF, 1989)

Such evidents strongly suggest that a Nitric Oxide-like substance mediate NANC-madiated relaxation in the trabecular smooth muscle (Kim et al, 1991).

The relaxation induced by various vasodilators (i.e. acetylcholine, bradykinin) and physical stimulus (i.e. shear stress) requires the presence of functional endothelium. This phenomenon, first described by Furchgott in the rabbit aorta, also has been demonstrated in the corpus cavernosum vascular bed (Furchgott and Zawadski, 1980).

In the human corpus cavernosum, the endothelium-derived relaxing factors (EDRF) released by acetylcholine have the chemical properties as Nitric Oxide, which is proposed to be the EDRF in various vascular beds (Palmer, Ferrige and Moncada, 1987).

Nitric Oxide stimulates Guanylate cyclase with accumulation of cyclic Guanosin Monophosphate (cGMP), which then leads to smooth muscle relaxation. This second messenger pathway is thought to be of key importance for trabecular smooth muscle relaxation, because Nitric Oxide mediates both endothelium and nerve-mediated relaxation.

2.4 Pathogenesis of ED

2.4.1 Psychogenic ED

Impulses from the brain send both facilitatory and inhibitory messages to the spinal erection centers, which in turn induce and inhibit the erectile process via the spinal nerves. Therefore, a psychologic stimulus (e.g., visual images, fantasies) itself is a very strong inducer of erection and can also enhance the erection induced by genital stimulation (reflexogenic erection).

Other stimuli such as anxiety or depression, religious inhibition, sexual phobias or deviation, obsessive compulsive personality (Lo Piccolo, 1986) or a traumatic past experience, can also send strong messages from the brain to the spinal erection center to inhibit or terminate erection.

The exact mechanism of psychogenic inhibition is still speculative. It can be a result of direct inhibition from the brain to the spinal centers or of increased peripheral catecholamine levels that render the cavernous smooth muscle less responsive to neurotransmitters.

2.4.2 Neurogenic ED

Because erection is a neurovascular event, erectile dysfunction can be caused by diseases or dysfunction of the brain, spinal cord, cavernous and pudendal nerves, and receptors in the terminal arterioles and cavernous smooth muscles. Among these, spinal disorders are probably the most common cause. Bors and Comarr (1971) reported that about 95 percent of patients with complete upper cord lesions are capable of erection (reflexogenic erection), whereas only 25 percent of those with complete lower cord lesions can attain (psychogenic) erection. It is believed that the thoracolumbar sympathetic pathway may carry impulses for psychogenic erection. However, because only 25 percent of patients are able to achieve erection through the sympathetic pathway, sacral parasympathetic neurons are obviously the most important erectile pathways.

Lesions affecting the brain (e.g. Cerebrovascular accidents, Parkinson's or Alzheimer's disease, tumor, injury) cause erectile dysfunction through a derangement of the hypothalamic center or overinhibition of the spinal center. Dysfunction at the spinal level (e.g. spina bifida, disc herniation, syringomyelia, tumor and multiple sclerosis) may affect either the afferent or efferent nerve pathways. Neuropathy, such as seen in alcoholism, vitamin deficiency, or diabetes may affect the cavernous nerve terminals and may result in deficiency of neurotransmitters. Injury to the cavernous or pudendal nerve from pelvic injury or surgery may disrupt the neural pathway (Hansen et al, 1989).

Sensory ED (from dysfunction of somatosensory nerves) is characterized by the inability to maintain a rigid erection despite normal nocturnal erections and an initially normal response to sexual stimulation. In autonomic ED (from disruption or dysfunction of the parasympathetic pathways) all types of erections will be impaired.

When the nerves are transected from radical surgery or injury, most neurologic dysfunction is incomplete.

As no direct test for the autonomic innervation of the penis exists at the present time, clinicians should use caution in making the diagnosis of neurogenic ED.

2.4.3 Arteriogenic ED

A manifold increase in arterial flow via the cavernous arteries is required to initiate erection. Therefore, diseases of the terminal aorta or the hypogastric, pudendal, or penilé arteries can result in erectile failure. The cavernous artery, the helicine arterioles, and the intercellular and intracellular architecture and function of the penile erectile tissue can also be affected by diminished inflow. Michal and Ruzbarsky (1980) found that the incidence and age at onset of coronary disease and ED are parallel.

Although trauma or a congenital anomaly may cause arterial insufficiency, in the majority of the cases arteriogenic ED is a component of a generalized atherosclerotic process. Associated risk factors include, hypercholesterolemia, cigarette smoking, diabetes, radiation, hypertension, and perineal trauma. A study of penile segmental pulsatile blood flow during nocturnal erection showed that the amplitudo was lower in potent hypertensive patients than in normal controls and lowest in impotent hypertensive patients (Karacan et al, 1989). A histologic study of diabetic men and men over 38 years old revealed a high incidence of fibrotic lesions of the cavernous artery, with initial proliferation, calcification and luminal stenosis (Michal and Ruzbarsky, 1980).

The initial symptoms of arteriogenic impotence are delayed onset of erection, increase stimulation before response, and early detumescence. With the increasing reduction of

luminal diameter, partial to complete erectile failure can occur. The degree can vary from patient to patient, because of the great variation of penile size and adequacy of the venous occlusion mechanism. Other factors, such as obesity, cigarette smoking, and psychologic overlay, can also aggravate erectile failure.

2.4.4 Venogenic ED

The many causes of venogenic ED are still under investigations. Some of the known causes are:

a. Abnormal venous channels or communications, such as ectopic drainage of the corpora cavernosa via large superficial dorsal veins or abnormally large cavernous and crural veins, an abnormally large communication between the corpus spongiosum and cavernosum or between the corpus cavernosum and the glans after a shunting operation for priapism.
b. Tunical abnormality, as in Peyronie' s disease, with rupture from injury or thinning from primary or secondary causes.
c. Functional impairment of the cavernous erectile tissue, resulting from a lack of neurotransmitters, psychologic inhibition, cigarette smoking, or ultrastructural changes.

2.4.5 Hormonal ED

Diabetes mellitus is the most common endocrinological cause of ED, but the impairment is not a result from the hormone deficiency alone, but vascular, neurologic and psychologic factors play also a role (Forsberg et al, 1989). Duplex ultrasonographic studies clearly indicate a high incidence of vascular disease in patients with both hypertension and diabetes (Lue et al, 1989). Diabetic patients have

also been reported to have a lower heart rate response to deep breathing and abnormal vesicourethro-bulbocavernosus reflex (Sarica and Karacan, 1987) indicating autonomic dysfunction.

Androgens are essential for male sexual maturity. In the adult, androgen deficiency results in a loss of sexual interest, impaired seminal emission and decreased frequency and magnitude of nocturnal erections. In a study of hypogonadal men undergoing testosterone replacement therapy, the number of episodes of nocturnal penile tumescences (NPT), maximal increase in penile circumference, penile rigidity, and total tumescence line were found to rise after testosterone therapy and to decline with a low serum testosterone level (Cunningham et al, 1990). A progressive decline in testosterone occurs after the seventh decade, and testicular or hypothalamic-pituitary dysfunction is suggested (Deslypere and Vermeulen, 1984). Studies have shown that erection in response to visual sexual stimulation is not affected by androgen withdrawal in hypogonadal men, suggesting that androgen enhances but is not essential for erection (Brancroft and Wu, 1983). In a controlled study in patients with normal testosterone levels, more than half of the men reported marked improvement in sexual potency regardless of whether they recieved androgen or placebo (Benkert et al, 1979).

Therefore, androgen replacement therapy is indicated only in hypogonadal patients.

Any dysfunction of the hypothalamic-pitutary-gonadal axis can result in hypogonadism. Hypo gonadotropic hypogonadism can be caused by malignancy, injury, or aging; hypergonadotropic hypogonadism (a disease of the testes) by malignancy, injury, surgery and mumps.

Hyperprolactinemia, caused by a pituitary adenoma, chronic renal failure or medication, inhibits the hypothalamic center and results in low testosterone and

decreased libido. Hyperthyroidism or hypothyroidism can also affect sexual function through inhibition of sexual drive.

2.5 Atherosclerosis

Atherosclerosis is the most common vascular disease, which occurs especially in old ages, but also in patients with risk factors like DM, hyperlipidemia and smoking. The disease belongs to a group of vascular disorders which is called Arteriosclerosis. Arteriosclerosis means "hardening of the arteries, or it has in common a thickening and loss of elasticity of the arterial walls".

Three distinctive morphologic variants are included within the term arteriosclerosis:

1. Atherosclerosis, characterized by intimal thickening and lipid deposition.
2. Monckeberg's medial calcific sclerosis, characterized by calcification of the media of muscular arteries.
3. Arteriolosclerosis, marked by proliferative or hyalin thickening of the walls of small arteries and arterioles.

Atherosclerosis accounts for more death and serious morbidity in the western world than any other disorder. It has a global distribution which is significantly high in economically developed societies. Although any artery may be affected, the aorta and the coronary and cerebral systems are the prime targets. So myocardial infarction, cerebral infarction and aortic aneurysms are the major consequences of this disease. Atherosclerosis causes also disease as a consequence of bad perfusion to the target organs, so like gangrene of the legs, mesenteric occlusion, sudden cardiac death, chronic ischemic heart disease and ischemic encephalopathy and ED may occur. Despite the reduction in morbidity from myocard infarction and other form of ischeamic hearth disease, nearly 50% of all deaths in United States continue to be

attributed to atherosclerosis-related diseases.

Atherosclerosis is a disease primarily of the elastic arteries (e.g. aorta, carotid; and iliac arteries and large and medium-sized muscular arteries (e.g. coronary and popliteal arteries). The basic lesion, the atheroma, or fibrofatty plaque consists of a raised focal plaque within the intima, having a core of lipid (mainly cholesterol and cholesterol esters) and a covering fibrous cap (Schoen, 1994).

Atheroma are at first sparsely distributed, but as the disease advances, they become more and more numerous, sometimes covering the entire circumferences of severely affected arteries. As the plaques increase in size, they progessivcly encroach on the lumen of the artery as well as on the subjacent media. Consequently in small arteries, plaques are occlusive, inhibiting blood flow to distal organs and causing ischemic injury, but in large arteries they are destructive, weakening the affected vessel wall causing an aneurysm or rupture of the thrombosis. Moreover, extensive atheromas are friable, often causing emboli into the distal circulation (atheroemboli).

Epidemiological studies have identified several risk factors leading to the development of atherosclerosis. Of the various risk factors like age and sex, four are most significant:

1. Hyperlipidemia,, 2. Hypertension, 3. Cigarette smoking and 4. Diabetes Mellitus (Neaton and Wenwoth, 1992, Tjokroprawiro, 1997).

2.5.1 Pathogenesis of Atheroschlerosis

There are historically two hypotheses for atherogenesis, which are dominant. One enphasized cellular proliferation in the intima as reaction to insudation of plasma proteins and lipids from the blood, whereas the other postulated that organization and repetitive growth of thrombi resulted in the plaque formation. The temporary view of

the pathogenesis of atherosclerosis incorporates elements of both older theories and is called the "response to injury hypothesis". It is formulated in 1973 and modified in 1986 and 1993.(Ross, 1986 and Ross, 1993) It states that the lesions of atherosclerosis are initiated as a response to some form of injury to arterial endothelium. The injury postulated is a form of endothelial dysfunction without necessary denudation, which increases permeability to plasma constituents, including lipids, and permits blood monocytes and eventually platelets to adhere to the endothelium. Monocytes adhere and subsequently enter the intima, transform into macrophages, and accumulate lipid to become foam cells, contributing to the evolution of the lesions. Factors released from the activated platelets at the surface or monocytes then cause migration of smooth muscle cells (SMC's) from media into intima, followed by proliferation and synthesis of extracellular matrix components by SMC's, leading to the accumulation of collagen and proteoglycans. Single or short-lived injurious events can be followed by restoration of endothelial function and regression of the lesion. But repeated or chronic injury, however, results in the development of an atheromatous plaque, by permitting continuing increased permeability, migration of monocytes and platelets subendothelially for possible interaction.

To understand the role of the various factors contributing in the process of atherogenesis, the following factors will be discussed.

a. Role of Endothelial Injury

There is considerable experimental support for endothelial damage as a major factor in atherogenesis. Endothelial injury induced in experimental animals by mechanical denudation, hemodynamic forces (AV Fistula), immune complex deposition, irradiation and chemicals cause intimal smooth muscle proliferation and in

the presence of high lipid diets, typical atheromas. It should be stressed, however, that early lesion, develop at sites of morphologically intact endothelium. Thus some form of endothelial dysfunction is thought to initiate the process.

Three manifestations of such dysfunction appear to be most important:

Increased endothelial permeability, increase monocyte adhesion, and increased EC replication. All of these are early events in experimental hypercholesterolemia.

Blood monocyte adherence to ECs is probably mediated by induction of specific receptor molecules on the surface of activated ECs. Indeed, the expression of vascular cell adhesion molecule (VCAM-1), the EC-leucocyte adhesion molecule was noted as an early molecular marker of lesion prone areas a response to experimental hypercholesterolemia in rats. In humans, intercellular adhesion molecule (ICAM-1) and VCAM-1 expression is increased in the endothelium of atherosclerosis plaques.

Risk factors such as hypertension and cigarette smoking may also cause endothelial damage or increased endothelial permeability. Endothelial alteration induced by hemodynamic forces (Shear stress and turbulent flow) can possibly explain the distribution of plaques at branches and fork points of arteries and in portion of the abdominal aorta. The molecular mechanism by which the various risk factors cause induction of endothelial leucocyte adhesion molecules and other changes in the endothelium are currently being explored. One postulated common pathway is related to injury-induced activation of a specific family of transcription factors (NFκB) that regulate expression of inducable EC genes.

b. Role of Macrophages and other Inflammatory / Immunologic Mechanism

Following adherence to endothelium early in the course of experimental hypercholesterolemia, monocytes emigrate the intima and subsequently accumulate

LDL. The migration of monocytes into the subendothelial space is presumably in response to chemotactic factors produced in the intima including oxidized lipids, fragments of tissue matrix proteins or inflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1) and macrophage colony stimulating factor (MCSF). Macrophages also proliferate in the intima.

Most importantly, however, macrophages ingest lipid to produce foam cells. Although monocytes / macrophages express the LDL receptor, the rate at which they take up native LDL is too low, to generate foam cells. They can, however, take up a modified form of LDL, not recognized by the LDL receptor. The specific receptor on macrophages for oxidized LDL is called the scavenger receptor. Foam cells may therefore be considered to be specialized macrophages. They are present in variable numbers in all stages of human atheromatous plaques. Fatty streaks, probably the most elementary lesions of atherosclerosis are collections of macrophage-derived foam cells.

The precise stimuli for recruitement and the role of the T-lympohcytes (both CD4+ and CD8+) found within progressing atheromas are uncertain.

In view of the large number of secretory products and biologic activities of macrophages, it is likely that they play a role in the progression of atherosclerotic lesions, for example, macrophages produce IL-1 and TNF (which increase adhesion of leukocyte) as well as chemotactic factors for leukocytes (e.g. MCP-1 and MCSF) that may further recruit leukocytes into the plaque. They produce toxic oxygen species that also cause oxydation of the LDL in the lesions and oxidized LDL is recognized by the scavenger receptor. Finally, growth stimulators and growth inhibitors elaborated by macrophages (PDGF, FCF and TGF-β) may modulate the proliferation of smooth muscle cells and the deposition of extracellular matrix.

c. Role of Smooth Muscle Cell Proliferation

In addition to lipid accumulation, SMC proliferation and extra cellular matrix deposition in the intima are the major processes that account for the progressive growth of atherosclerotic lesions. The proliferating SMCs originate from the media and possibly also in some cases from pre-existing miointima cells. A number of SMC mitogens and chemotactic agents derived from cells and found in atherosclerotic lesions or serum have been implicated in such proliferation. Principal among these are platelet derived growth factor (PDGF), present in the platelet alpha granules and released after platelet adhesion to foci of injury, but also produced by macrophages, ECs, and SMCs; FGF and other cytokines that may be produced by ECs, monocytes, macrophages, T-lymphocytes and the SMCs themselves, especially IL-1, and transforming growth factors alpha (TGFα). In addition, growth stimulation can also be theoritically accomplished by loss of growth inhibitors, such as hiparin like molecules elaborated by both ECs and SMCs, or TGF-β produced by macrophages or ECs, SMCs also elaborate and remodel the extracellular components of the atheromatous plaque and growth factors are also involved in such processes. Furhermore, SMCs can accumulate large amounts of cholesterol and cholesterol esters and together with infiltrating macrophages, also give rise to the foam cells in the plaque.

d. Role of Hyperlipidemia

All lipids in plasma circulate in combination with protein. The plasma lipoproteins are complexes of lipid and protein assembled as globular particles, each consisting of a core of neutral lipid (primarily trigliceride and cholesterol esters), surrounded by a coat composed of polar lipids (phospholipid and free cholesterol) and

apolipoproteins (also called apoproteins). Lipoprotein transport is also controlled by the lipoprotein processing proteins (Lecithin-cholesterol acyltransferase [LCAT], lipoprotein lipase, hepatic lipase and cholesterol ester transfer protein) and the lipoprotein receptors that recognize lipoproteins and mediate their cellular uptake and catabolism (LDL receptor, chylomicron remnant receptor and the scavenger receptor). In the past decade, nearly all the genes that code for these proteins have been isolated, sequenced, and mapped in the human genome; abnormalities in them may explain premature atherosclerosis in some cases.

The abnormality common to most syndromes of premature atherosclerosis is hyperlipoproteinemia. A number of genetic and acquired derangements influence both exogenous and endogenous pathways of cholesterol metabolism and can result in hyperlipoproteinemia. Hyperlipoproteinemia can be due to either primary genetic defects in lipid metabolism or secondary to some other underlying disorder, such as the nephrotic syndrome, alcoholism, hypothyroidism or diabetes mellitus. Four types of lipoproteins abnormality are frequently found in the population: (1) increased LDL cholesterol levels, (2) decreased HDL cholesterol levels, (3) increased chylomicron remnants and IDL and (4) increased levels of an abnormal lipoprotein L(a).

The most well-studied example of genetically induced hyperlipoproteinemia causing severe atherosclerosis in young individuals is familial hypercholesterolemia caused by defects in the LDL receptor; this leads to inadequate hepatic uptake of LDL and markedly increasing circulating LDL. Genetic defects in apoproteins may also be associated with hyperlipoproteinemia and accelerated atherosclerosis.

A number of mechanisms have been postulated to account for the role of lipids in lesion formation:

- Increased in plasma levels of LDL or some component of hyperlipidemic serum

may increase the role of lipid penetration into the artery wall.

- Local modification of LDL may render it more atherogenic. In addition to being readily ingested by macrophages through the scavenger receptor, oxidized LDL could accelerate atherogenesis by other mechanisms: (1) It is chemotactic for circulating monocytes. (2) It increases monocyte adhesion. (3) It inhibits the mortility of macrophages already in lesions, thus favoring the recruitment and retention of macrophages in the lesion. (4) It stimulates the release and growth of growth factors and cytokines. (5) It is cytotoxic to ECs and SMCs. (6) It is immunogenic. This suggests that antioxidants (e.g. Vitamin E) might be efficacious in preventing atherosclerosis by reducing LDL oxidative modification.
- Hyperlipoproteinemia may directly alter EC function, without leading to denudation, through focal EC death, increased permeability, increased replication or increased monocyte adhesion.

e. Role of Thrombosis

Thrombosis is a complication of late-stage atherosclerosis, and organization of thrombi may contribute to plaque formation and their encroachment on the lumen. Although platelets generally do not adhere to the arterial wall without prior severe injury or endothelial denudation, more biochemical disruption of the normal EC could render it thrombogenic.

Lipoprotein (a) (L[a]) is an altered form of LDL, that contains the apolipoprotein B-100 portion of LDL linked to apolipoprotein (a) (apo[a]) itself a large glycoprotein molecule sharing a high degree of structural homology to plasminogen (a key protein in fibrinolysis). L(a) could be atherogenetic by various mechanism, including interference with both LDL and plasminogen metabolism or

promotion of SMC proliferation. Epidemiological studies have shown a significant correlation between increased blood levels of L(a) and coronary and cerebral vascular disease, independent of the level of total cholesterol or LDL.

f. Other Theories of Atherogenesis

The development of atheromatous plaque could also be explained if SMC proliferation were in fact the primary event. For example, the monoclonal hypothesis of atherogenesis was based on the observation that some human plaques are monoclonal or at the most oligoclonal. This was interpreted by Benditt (1977) as evidence that plaques may be equivalent to benign monoclonal neoplastic growth (such as leiomyomas), perhaps induced by exogenous chemical (e.g. cholesterol or some of its oxidized products) or an oncogenic virus. Although the universal monoclonal nature of plaques has been questioned, there is evidence in animals that certain viruses (e.g. the agent of Marek's disease in chicken) cause plaques in the aorta, and both herpes virus and cytomegalovirus have been detected in human atheromatous plaques.

The summarized major proposed mechanism of atherogenesis is as follows:

The current trend is to consider that atherosclerosis is a chronic inflammatory response of the vascular wall to a variety of initiating events that can occur early in life. Multiple pathogenic mechanism contributes to the plaque formation and progression, including endothelial dysfunction, monocyte adhesion and infiltration, smooth muscle proliferation, extracellular matrix deposition, lipid accumulation and thrombosis.

2.6 Psychoneuroimmunological approach for ED

The mechanism how stress (psychogenic factor) may cause ED is until recently not yet clear. The diagnosis of psychogenic ED is still subjective and is made after all organic factors have been excluded.

Many investigations explain only the role of vascular, neurological, hormonal disorders and the contractile tissue of the penis as the main factors causing organic ED. Studies to prove that stress may also cause changes in the main artery (internal pudendal artery) of the penis and develop ED is so far not yet been performed.

It is already proven that stress may alter the immune system in human through the endocrine and the autonomic nerve system and consequently damage several organs (Ader, Felter and Cohen, 1991).

The questions are: Does stress also cause changes in the main vascular system to the penis? And does the damaging process also involve the immune system? All these questions may probably be clarified through a psychoneuroimmunological study.

2.6.1 Stress axis and the Immune System

All organisms from bacteria to human have evolved mechanisms to deal with significant changes in their external or internal environments called stressors.

In mammals this function is carried out in part by the hypothalamus-pituitary-adrenal (HPA)-axis (Akil, Morano, 1995).

This system integrates various inputs indicative of stress, converging on a final common path in the brain, the neurons of the medial parvocellular division of paraventricular neucleus of the hypothalamus (mp PVN). These neurons synthesize corticotropin-releasing factor (CRF) and arginin vasopressin (AVP), and project to the external layer of the median eminence. Activation by stressors leads to release of

peptides into the periferal blood, carrying these secretory products to the anterior pituitary.

In turn CRF and AVP receptors on the anterior pituitary are responsible for the release into the total circulation, of adrenocorticotropic hormone (ACTH) and related peptides derived from the common precursor pro-opio-melano-cortin (POMC).

ACTH activates the biosynthesis and release of glycocorticoids, corticosterone in rodents and cortisol in primates by the cells of the adrenal cortex.

These steroids possess extremely broad actions mediated by specialized receptors, affecting expression and regulation of genes throughout the body and preparing the organism for the changes in energy and metabolism required for coping.

It is now well known that stress may impair the immune system. As already mentioned the defence mechanisms against stress (internal as well as external) is conducted by the nervous system.

The relationship between the nervous system and the immune system has also been proven by several investigations. A number of specific mechanism by which the nervous system might affect immune functions are postulated (Dunn, 1995). These include, cathecholamines secreted from sympathetic nerve terminals and the adrenal medulla. Other hormones secreted by the pituitary and other endocrine organs, and peptides (including endorphine) secreted by the adrenal medulla and autonomic nerve terminals are also involved. The network includes not only the autonomic nervous system and classical neuroendocrine mechanism, but also involves an endocrine function of the immune system.

A variety of immune system produces e.g cytokines, peptide, other factors that function to coordinate the immune response, may also provide important signals for the nervous system. This chemical messengers can account for a variety of

interactions between the nervous system and the immune system. (Fig. 2-4)

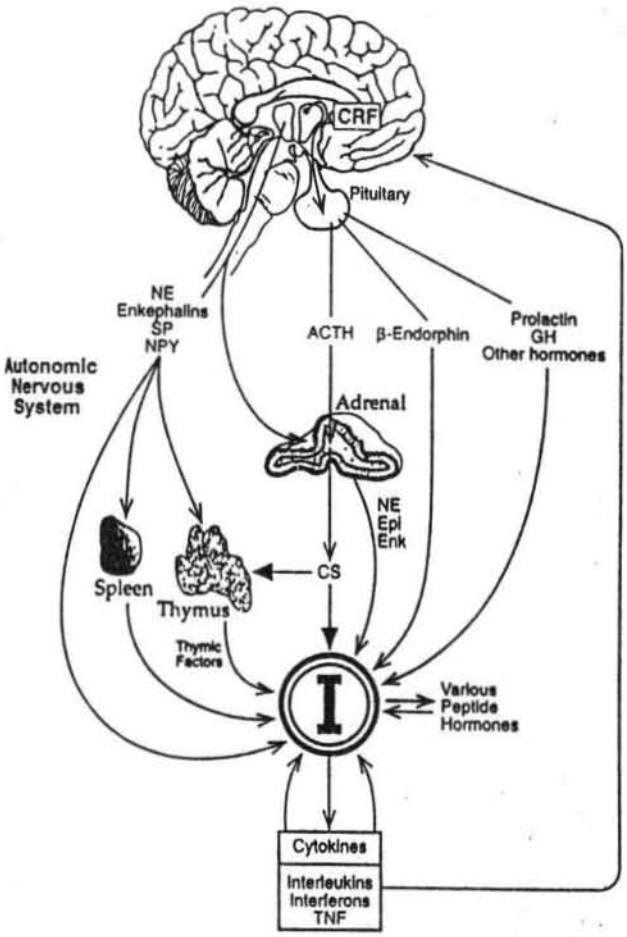


Figure 2-4 Interaction between the nervous system, the endocrine and immune system (Dunn, 1995)

The dogma that stress suppresses immunity is to some extent based on the "well established" immuno suppressive effects of glucocorticoids. In fact, endogenous glucocorticoids at physiological doses are not universally immunosuppressive and

actually may enhance immune function (fig. 2-5).

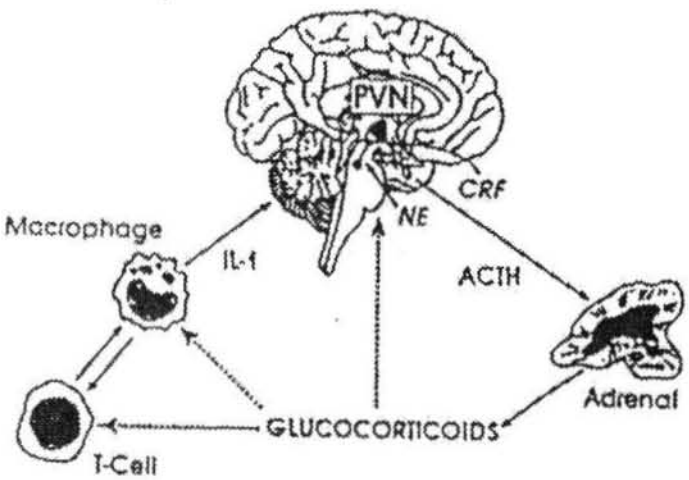


Figure 2-5 Relationship between brain, HPA-axis and immune cells (Dunn, 1995)

Furthermore, glucocorticoids may not even be the major mechanism by which stress suppresses immune function. More recent studies have suggested an important role for the circulating cathecholamines derived from the sympathetic nervous system and the adrenal medulla, in the chronic studies.

2.6.2 Stress concept and Psychoneuroimmunology

There is a general opinion that stress is difficult to be defined and to be measured. But it is important that there is an agreement on the nature of the concept. Lack of such agreement would make research on stress and the subsequence of effective stress management strategies difficult.

Cox in 1995 concluded from several reviews of the literature that there are essentially three different but overlapping approaches to the definition and study of stress:

1. Engineering Approach

The engineering approach has treated stress as a stimulus characteristic of the person's environment, usually shown in terms of the load or level of demand on the individual or as some aversive or noxious element of that environment (Cox, 1990). Occupational stress, for example, is treated as a property of the work environment, and usually as an objectively measurable aspect of that environment. Stress is a set of causes not a set of symptoms. According to this approach, stress was said to produce a strain reaction which, although often reversible, could on occasions prove to be irreversible and damaging (Sutherland and Cooper, 1990). The concept of a stress threshold grew up from this way of thinking and individual differences in this threshold have been used to account for differences in stress resistance and vulnerability.

In this approach stress is treated as an independent variable, the environmental cause of ill health.

2. Medicophysiological Approach

The medicophysiological approach for the definition and study of stress started with the work of Selye (1956). He defined stress as "a state manifested by a specific syndrome which consists of non specific changes within the biologic system" that occurs when challenged by aversive or noxious stimuli. Stress is treated as a generalized and non-specific physiological response syndrome. For many years, the stress response was considered as the activation of two neuroendocrine systems, the anterior pituitary-adreno cortical system (PAC) and the sympathetic-adrenal medullary system (SAM) Cox and Thirlaway (1983). Selye (1956) said that this

response has three phases in nature.

1. Involving an initial alarm stage (sympathetic-adrenal medullary activation),
2. Followed by a stage of resistance (adreno-cortical activation)
3. Giving way under some circumstances to a final stage of exhaustion (terminal reactivation of the sympathetic-adrenal medullary system).

Selye (1956) stated that stress response can significantly contribute to physical pathology.

This was true when the response was frequent, of long duration or severe. The disease that had such a stress-related aetiology is termed "disease of adaptation" by Selye. A paradoxal situation arises when the stress response had evolved as adaptive in the short time, increasing the animal's (or person's) ability to respond actively to an aversive or noxious environment. However, in the long term it could contribute to the disease process.

Putra (1999a) stated that a stressor causes the "triad" of "general adaptation syndrom" (GAS) e. g: 1. The alarm stage, 2. Adaptation stage or, 3. The stage of exhaustion

In this approach stress is treated as a dependent variable, as a particular physiological response to a threatening or damaging environment.

3. Physiological approach

The third approach to the definition and study of stress generally conceptualizes it in terms of the dynamic interaction between the person and their environment. When studied stress is either inferred from the existence of difficult person-environment interaction or measured in terms of the cognitive and emotional processes that underpin those interactions. This approach has been termed as the "psychological model". Variants of this psychological model dominate contemporary stress theory, and among them two distinct types can be identified: the interactional

and the transactional.

The former focuses on the structural features of the person's interaction with their environment, whereas the latter is more concerned with the psychological processes underpinning that interaction. Transactional models are primarily concerned with cognitive appraisal and coping. In a sense, they represent a development of interactional models, and offer little which is not consistent with such models. Transactional theories owe much to the work of Lazarus (1966) and his notion of "cognitive appraisal".

The "medicophysiological approach" of stress is the basic paradigm for psychoneuroimmunology. Whatever the sort of stressor, it causes a non specific alteration in the biological system (eg the immune system in psychoneuroimmunology) in response to a wide range of aversive or noxious stimuli. Hans Selye in 1996 stated that response against stressor might be manifected as a biological stress but not a psychological condition.

Psychoneuroimmunological basic concept, is a science with distinct paradigm, that is a model of thinking which is focussed on the observation of stress immune modulation. The stressor does not depends on wether it is psychogenic or physical. The modulation may be derived from effect of stress individuals, system, organs, cells, molecules or genes (Putra, 1999b).

2.6.3 Stress Response

It is already known that stress via the stress axis causes changes in response of the neuroendocrine and immune system, which disturb the homeostasis and might cause several diseases.

Some metabolic changes include the metabolism of cathecholamine, cortisol,

and other hormones like: endorphine, growth hormone, prolactin and testosteron (Selby J, Mc Cance KL, 1998).

2.6.4 The Role of Lymphocytes in Immune Response

The important role of lymphocytes in the immune response of the body is well known. We distinguish two sets of lymphocytes; B lymphocytes and T lymphocytes, both originating from Stem cells in the bone marrow.

B cells maturate in the bone marrow itself before they enter the blood circulation. When a B cell is exposed to an antigen it differentiates from an antigen reactive B cell either into an antibody secreting cell which becomes a plasma cell, or a memory cell. The combination of antigen with the antibody receptor on the cell surface of the B cell generate signals inside the cell that switches on its antibody-producing machinery. In the first stages the cell becomes activated, it then multiplies and differentiates to form a clone of daughter cells which start secreting antibody of the same type as that expressed originally on the surface of the parent cell. This antibody combines with the antigen, initiating a chain of events leading to the destruction or neutralization of the antigen. Most of the daughter cells die after a few weeks. A proportion of them, however, do not make antibody to any great extent, but recirculate in the body and may persist for many years as memory cells. If they are subsequently exposed again to the same antigen they rapidly become activated and make more antibody. In this way secondary immune responses occur. The same antibody is produced again but at a faster rate then in the primary response which follows the first exposure to the antigen. This happens because more antigen reactive cells exist as a result of clonal expression and because they go through fewer devisions before they secrete antibody. These enhances specific secondary responses

are said to demonstrate immunological memory.

Clonal expression provides a highly efficient way to produce large amounts of antibody without the necessity of initially processing large number of each specific lymphocyte. The importance of this can be appreciated when it is realized that after immunization there may be thousands of cells making the same antibody and that the immune system has the potential to make millions of different antibodies.

The maturation process of T-cells occur in the thymus before they enter the blood circulation. T-cells do not make antibodies or secrete special forms of their antigen receptor molecules.

Instead, after reacting with antigen they go through similar stages of activation, growth and differentiation and secrete a variety of cytokines. These mediators are especially important in regulating the function of other lymphocytes. It is recognized that there are different types of subsetts of T-cells that serve different function by virtue of the different cytokines they produce. Furthermore T-cells express particular accessory molecules on their cell surface that are important to their function, but which incidentally are very useful to immunologists as an aid to identify and classify them.

Thus T-helper cells (also known as Th2. cells) express structures known as CD-4 molecules on their surface. T-helper cells are important in helping B-cells make antibody responses by synthesizing and secreting cytokines that promote the activation, growth and differentiation of B-cells. Other cells also known as T-suppressor cells express CD8 molecules on their surface, and counterbalance the effects of T-helper cells and suppress immune responses. Therefore T-cells control the activity of each other and other cells of the immune system through making cytokines. The measurement of T-cells expressing either CD4 or CD8 has become clinically

important as changes in the natural balance between helper and suppressor cells which may be an indication of changes in the healthy functioning of the immune system. In AIDS, for example the CD8 population dominates in the peripheral blood, indicating that immune function is compromised.

However not all T-cells are regulatory. Some of the $CD8^+$ cells kill other cells and are known as T-cytotoxic cells. They are primarily responsible for destroying cells of the body that have been infected with viruses. Some $CD4^-$ T-cells are important in certain types of hypersensitivity reactions.

They are known as T dth cells or Th1 cells, (DTH means: delayed-type hypersensitivity) and they are involved in the cell-mediated immune response. Such as the destruction of intracellular bacteria, some types of delayed hypersensitivities, auto-immune diseases and the rejection of organ transplant. Although the T-cells that help B-cells make antibodies (Th2) and the T-cells that are involved in delayed-type hypersensitivity (DTH) all have CD4 molecules, they differ from each other in the types of cytokines that they make. It is this that enables them to different effects (Stainess, Brostoff, James, 1993a).

2.6.5 The Th1/Th2 Paradigm

The Th1/Th2 paradigm provides a useful model for understanding the pathogenesis of several diseases, as well as for developing new immunotherapeutic strategies. Sergio Romagnani (1997) examines Th1/Th2 polarization in the context of associated pathophysiological conditions.

In the past few years considerable evidence has shown the existence of functionally polarized responses by the CD4 T helper (Th) and the CD8 T cytotoxic (Tc) cell subsets, that depend on the cytokines they produce.

Th1 cells produce interferon γ (IFN-γ) and tumor necrosis factor β (TNF-β), which activate macrophages and are involved in delayed-type-hypersensitivity (DTH) reactions. By contrast Th2 cells produce interleukin 4 (IL-4), IL-5, IL-10 and IL-13, which are responsible for strong antibody responses, including IgE production, and inhibits several macrophage functions.

In general, activated CD8-T cells exhibit a Tc1 cytokine profile but, in some pathological condition, CD8 –T cells with Tc2 profile can also arise:

Furthermore, Th1 and Th2 are not the only cytokine pattern possible. T cells expressing cytokines of both patterns have been designated as Th0 and additional patterns have been described among long term clones. Th0 cells represent a heterogenous population of partially differentiated effector cells comprising multiple discrete subsets that can secrete Th1 on Th2 cytokines.

Polarized human Th1 on Th2 cells not only produce different sets of cytokines, which result in distinct functional properties, but also exhibit the preferential expression of some activation markers. For example CD30 (a member of the TNF receptor family) is mainly expressed in Th2 or Th c2 like cells, whereas lymphocyte activation gene 3 (LAG-3) a member of the immunoglobulin super family, is preferentially associates with Th1-like cells.

The functional meaning of Th1-LAG-3 and Th2-CD30 association is not being clarified.

The factors responsible for the polarization of the specific immune responses into a predominant Th1 and Th2 profile have been extensively investigated in mice and humans. Strong evidents suggets that Th1 and Th2 do not derive from different lineage, but rather develop from the same Th-cell precursor under the influence of environmental and genetic factors acting at the level of antigen presentation. Among

the environmental factors, a role has been demonstrated for the route of the antigen entry, the phisical form of antigen, the type of adjuvant and the dose of antigen. The genetic mechanisms that occur in controlling the type of Th-cells differentiation remain elusive.

The environmental and genetic factors influence Th1/Th2 differentiation mainly by determining the predominance of a given cytokines in the microenvironment of the responding Th cell. The early presence of IL-6 is the most potent stimulus for Th2 differentiation, whereas IL-12 and IFN favour Th1 development. The mechanism responsible for the early IL-4 production involved in the differentiation of naive Th cells into Th2 effectors as well as the source of IL-4 remain unclear.

Under certain circumstances, one such source may be a small subset of $CD4^+$ NK 1, 1^- cells capable of recognizing antigens presented in association with the non-polymorphic 2-microglobulin-associated molecule CD1. However, naive Th cells themselves are able to produce small amount of IL-4 from their initial activation and the concentration of Il-4 that accumulates at the level of the Th cell response, increases with increasing lymphocytes activation.

The inducing effect of IL-4 dominates over other cytokines so that, if IL-4 levels reach a necessary threshold, differentiation of the Th cell into the Th2 phenotype occurs. Recently it has been shown that IL-6 derived from antigen-presenting cells is able to polarize naive Th cells to effector Th2 cells by inducing the initial production of Il-4 in CD-4 T cells. Prostaglandin E, has also been suggested to favor the development of Th2 responses by inhibiting both the production of IL12 by dendritic cells and the production of IFN-γ by T cells.

The molecular mechanisms by which IL-4 and IL-12 promote the development of

naive Th cells into Th2 or Th1 effectors are also being clarified. The binding of cytokines to their receptors typically results in rapid tyrosine phosphorylation of signal transducers and activators of transcription (STATs). Of these, STAT-4 appears to be sensitively activated by IL-12 and targeting of the STAT4 gene results in the inhibition of Th1 responses. On the other hand, signaling by IL-4 occurs through activation of STAT6 and knockout of the STAT6 gene results in deficient Th2 responses.

In autoimmune disease like Insulin Dependent Diabetes Mellitus (IDDM), it is obvious that Th1 plays an important role in the development of the disease.

Depending on which immune responses are dominant, Th1 or Th2, the following diseases (like in the diagram) may be developed.

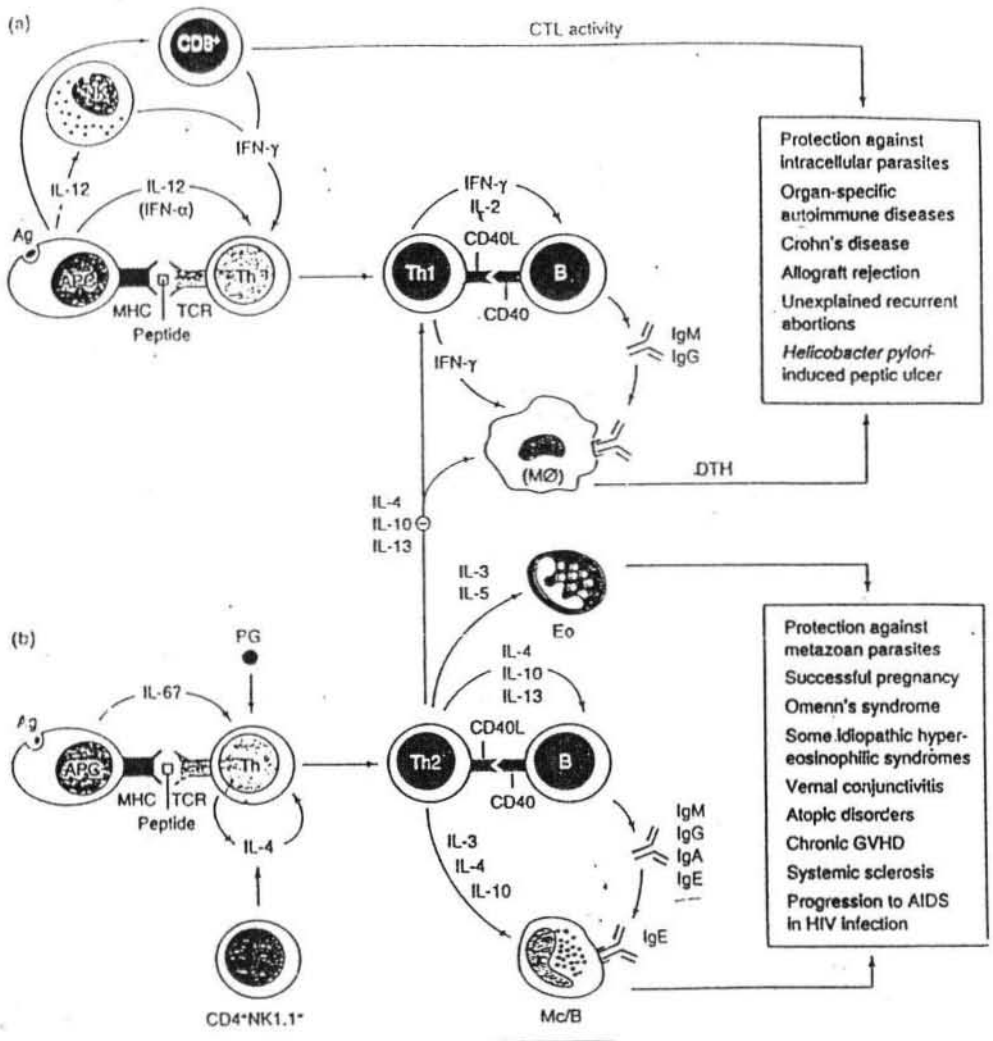


Figure 2-6 Diagram of Th1/Th2 paradigm in disease (Romagnani, 1994)

a. Early production of IL-12 and IFNs by cells of the innate immune system promotes the development of Th1 cells, which induces the production of opzonizing antibodies and the activation of Mϕ (i. e. a DTH reaction). Th1

responses, together with frequently CTL activity are highly protective against infections caused by intra cellular parasites and are responsible for anti allograft-rejection, but may result in organ specific autoimmune disease and other immuno pathological disorders.

b. Autonomous IL-4 production by naive Th cells, possibly stimulated by APC-derived IL-6 or early production of IL-4 provided by the CD4 NK1.1 subset, promotes the development of Th2 cells, which stimulate high antibody production (including Ig E), eosinophil and mast cells activity and inhibit (indicated by the minus sign) Th1 cells and macrophages. Th2 responses are protective against metazoon parasites and hamper the rejection of the fetal allograft, but can be responsible for reduced resistance against several infectious agents, including HIV, as well as development of atopy and other immunopathological disorders.

2.6.6 Delayed Type Hypersensitivity (Type IV)

Hypersensitivity reactions in immune responses arise from the usual adaptive immune responses and the way they work depends upon immunological memory for a particular antigen or allergen.

The problem with these reactions is that they are exagerated or inapproriate forms of adaptive responses coincidentally damage one's own tissues in the process of producing the immune reaction, which is actually directed to the destruction of the foreign antigen. This damage occurs because the body is either exposed to an excessive amount of antigen or because antibody to it is made in too high a quantity. In special cases hypersensitivity reactions happen when antibody or T cells are directed against antigens of one's own body as in the so-called autoimmune diseases.

Hypersensitivity reactions are classified into four types according to their

mechanisms:

1. Atopy and Anaphylaxis type I
2. Cytotoxic Hypersensitivity type II
3. Immune Complex Hypersensitivity type III
4. Delayed Type hypersensitivity Type IV

The first three types are mediated by antibody, the fourth being antibody-independent and mediated by T cells and macrophages.

It is called delayed because the reactions are slow to express themselves after antigen challenged. T cells necessary for producing the delayed response are cells which have become specifically sensitized to the particular antigen by previous encounter, and they act by recruiting other cell types to the side of reaction. Mostly it appears to be CD4 Tdth cells (also known as Th1 cells) that secrete selected cytokines such as IL2 and IFNγ, that cause the typical lesions of delayed hypersensitivity. The action of these cells is probably controlled by other CD4 T cells (known as Th2 cells) that secrete IL-4 and IL-10. So the balance between protective and damaging immunity partly reflects the way in which different lymphocyte populations interact with each other (Staness, Brostoff, James, 1993b).

2.6.7 Antibody-Dependent Cell-Mediated Cytotoxicity

A number of cells that have cytotoxic potential express membrane receptors for the Fc region of the antibody molecule. When antibody is specially bound to a target cell, these receptor-bearing cells can bind to the antibody Fc region, and thus to the target cells and subsequently cause lysis of the target cell. Although these cytotoxic cells are nonspecific for antigen, the specificity of the antibody directs them

to specific target cells. This type of cytotoxicicity is referred to as antibody-dependent cell-mediated cytoxicity (ADCC).

Among the cells that can mediate ADCC are NK cells, macrophages, monocytes, neutrophils and eosinophils.

Target-cell killing by ADCC does not involve complement-mediated lysis, but appears to involve a number of different cytotoxic mechanism. When macrophages, neutrophils, or eosinophils bind to a target cell by way of the Fc receptor, they become more active metabolically; as a result, the lytic enzymes in their cytoplasmic lysosomes or granules increase. Release of these lytic enzymes at the site of the Fc-mediated contact may result in damage to the target cell. In addition, activated monocytes, macrophages, and NK cells have been shown to secrete tumor necrosis factor (TNF), which way have a cytotoxic effect on the bound target cell. Since both NK cells and eosinophils contain perforin in cytoplasmic granules, their target-cell killing also may involve perforin-mediated membrane damage (Goldsby, Kindt, 2000).

2.7 Endothelial Dysfunction

Endothelial dysfunction is generally defined as a decrease in the synthesis, release and / or activity of Nitric Oxide (NO), which originates from the endothelium. NO is a soluble gas and is continuously synthesized by the Endothelial Cells (EC). This molecule has various biological functions in regulating vascular homeostasis, including vascular rhythm, local cell growth, and the protection of vascular damage as a consequence of thrombotic aggregation and of circulating cells or molecules adhesions.

Conditions which are generally associated with risk factors in atherogenesis, like

smoking, hypertention, hypercholesterolaemia, hyperhomocysteinemia, bacterial / viral infection, result in a diminishing release of NO in the arterial wall.

The decrease in the amount and activity of NO is a result of the synthesis impairment and excessive oxidative degradiation. Shortness in NO bioactivity may develop vasoconstriction during heavy physical activity or mental stress and may cause myocard ischaemia in patient with coronary arterial disease.

Further decline in NO bioactivity may activate ECs. Activated ECs then express adhesion molecules, like vascular cell adhesion molecule (VCAM-1), intra cellular adhesion molecule (ICAM-1) and E-Selection, which cause adhesion of leucocytes from the circulation to the endothelium. Monocyte adhesion to the vascular wall and their differentiation into macrophages are important factor for the development of foam cell in atherosclerotic plaques (Peter et al, 1997). Cytokines, oxidized-LDL, and bacterial infection like chlamydia pneumonia increase the inflammatory process, which causes further activation of ECs. Endothelial dysfunction and their activation by e.g risk factors for coronary heart diseases and together with vascular inflammation are thus the basic in atherogenesis and acute coronary syndrome. Therefore, restoration of endothelial function is the main focus in the prevention of coronary heart disease.

2.7.1 Endothelial Dysfunction and Atherosclerosis

Atherosclerosis in the vascular system is related to vasomotor dysfunction, because the absence or the decrease in NO bioactivity. Until recently it is not yet clear, whether endothelial dysfunction is a marker for atherosclerosis or is an important contributor for the process of atherogenesis.

But it is accepted that NO has an anti-atherogenetic and anti-inflammatory effect. As

is already mentioned the absence of NO, because the decrease in its synthesis as well as the inactivation by superoxide anion, results in the disturbance of vascular relaxation, in increased thrombocyte aggregation, smooth muscle cell proliferation and endothelial leukocyte interaction. It is therefore clear that NO has an important role in the suppression of atherogenesis.

Some studies have shown that endothelial dysfunction is an early manifestation of the presence of atherosclerosis, even if it is not yet evident on angiographic examination.

That is why early restoration of endothelial function is an important step in the prevention of atherosclerosis through the modification of risk factors.

It is already known that there is a close relationship between risk factors of coronary heart disease and endothelial dysfunction. For example LDL, especially oxidized LDL is a strong inhibitor for endothelial function. The mechanism how LDL inhibits NO activity, involves the declining expression of "endothelial Nitric Oxide Synthase" (eNOS), the decreasing release of NO, which is influenced by the receptors and inactivation of NO by the production of superoxide anion. This means that endothelial dysfunction is an important marker in hypercholesterolaemia and will be quickly restored by aggressively decreasing the cholesterol level. And many investigations have been done that have confirmed this mechanism. Oxidative stress will increase oxidized LDL and inhibit NO bioactivity, which implies endothelial dysfunction. One of the mechanism how oxidative stress may contribute to the process of atherogenesis is through the induction of pro inflammatory mediators.

Activation of pro inflammatory transcription factors like "nuclear factor-kappa B" (NF-kB), "Protein Activator" (PA)-1, and "Early Growth Factor" (egr-1) occur through "oxidant sensitive" mechanism, which involves hydrogen peroxide (H_2O_2).

Adhessions molecules like VCAM-1, ICAM-1, E Selection, as well as some cytokines and Growth Factors like "Monocyte Chemoattractant Protein (MCP)", "Macrophage Colony Stimulating Factor (MCSF)" are stimulated by activation of this transcription factor NF-kB for their expression which occurs in ECs and SMCs in the arterial wall.

It is attractive that NF-kB activation may be blocked by antioxidants and anti inflammatory drugs like salicylate and glucocorticoid.

These findings showed that atherosclerosis is an inflammatory process, initiated by an oxidative stress. The presence of cytokines and inflammatory mediators in the atherosclerotic vascular wall suggest the speculative theory that atherogenesis is a consequence of chronic infection, like duodenal ulcer which is caused by the Helicobacter pylori microorganism.

This is also shown by the presence of a chronic inflammatory marker like C-reactive protein (CRP) and amyloid-A serum protein, which are increased in the atherosclerotic patients. Some viral and bacterial infection, which is proven to have their role in the process of atherogenesis is Herpes Virus (HSV), cytomegalo virus (CMV), Helicobacter pylori and Chlamydia pneumoniae. Tjokroprawiro in 1998 summarized that oxidative stress in "HDL syndrome", improves the NFkB expression, which in turn causes VCAM-1 expression and also of MCP-1. Both are serum makers for atherosclerosis (Fig. 2-7)

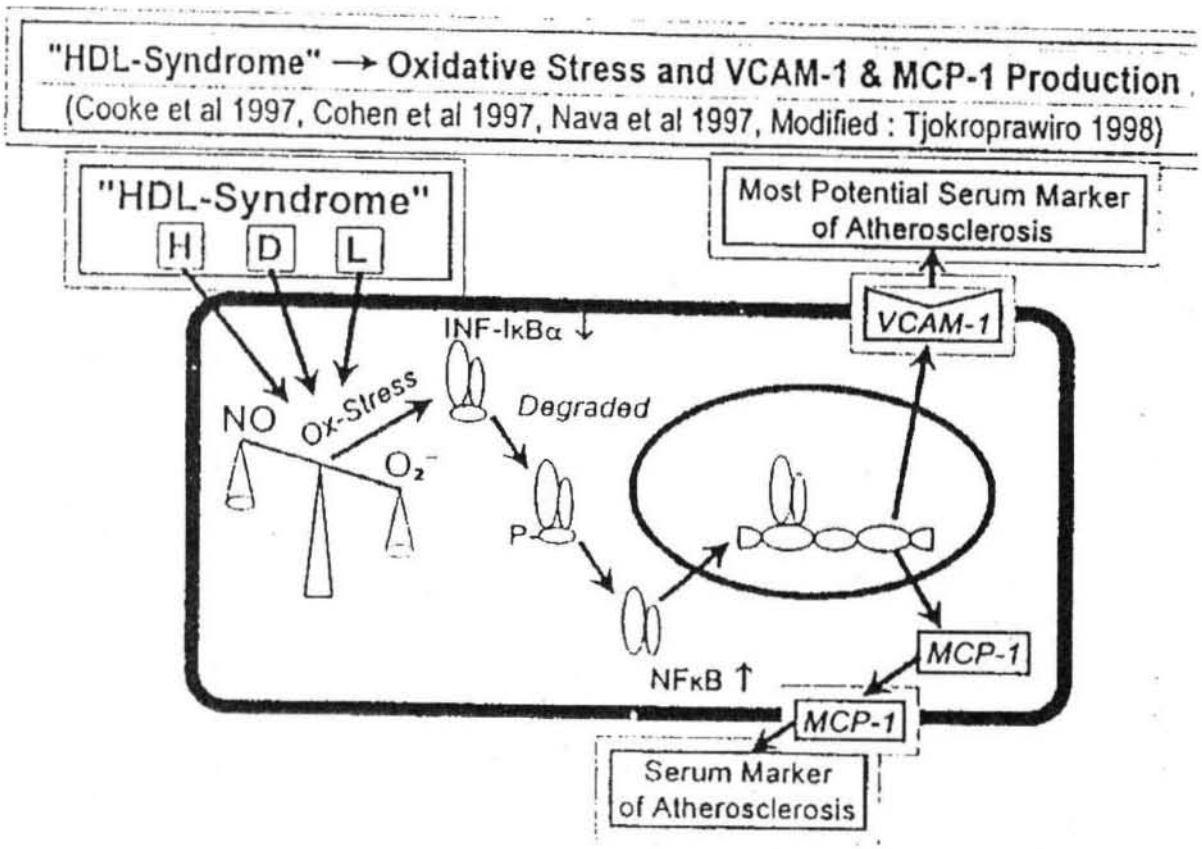


Figure 2-7 Scheme of Oxidative Stress and VCAM-1 & MCP-1 Production (Tjokroprawiro, 1998)

2.7.2 The role of NO in Endothelial dysfunction and Atherosclerosis

Nitric oxide (NO) has an important biological role as an "endothelium derived relaxing factor = EDRF", a substance to maintain the normal vascular tone. It also has an important role to suppress lipoprotein oxidation, a potential anti atherogenic property.

In the last few years NO is known as a molecule present in many organs. It has the role in giving signals to the nervous system and cardiovascular system and as a cytotoxic compound in the body defence mechanism. It is a simple inorganic molecule with unpaired electron (a free radical), that can freely move through the cell membrane to interact directly with its target. This molecule differs from other

signaling molecules that its specificity is not controlled by the distribution of membrane receptors. The activity of NO is controlled by its rates of production and the reaction with the target molecules.

NO is synthesized from L-Arginin by a group of enzymes, known as "Nitric Oxyde Synthase" (NOS), which are expressed in a wide range of tissues.

The enzymes responsible for the production of EDRF is called "Endothelial Nitric Oxyde synthase" (eNOS or NOS_3) a protein regulated by intracellular calcium.

In the cardiovascular system NO is the source of EDRF, a vasodilator, which has the role in maintaining the vascular tone. In atherosclerotic artery, EDRF (NO) activity declines, which is easily detected in the early stage of the disease, even in hypercholesterolaemia patient, without arterial changes (Jessep, 1996).

Three kinds of NOS isoforms are identified, NOS originally detected in the brain is called "Neural NOS" or nNOS, in macrophage is called inducible NOS or iNOS and in endothelial cell is endothelial NOS or eNOS. They share 50-60 % homology of their amino acid sequence and are encoded by three different genes, respectively NOS 1, NOS 2 and NOS 3.

Various cells in the cardiac muscle may express one or more of the three kinds of NOS isoforms. nNOS is expressed in sympathetic autonomic nerve terminals and regulates the release of catecholamine in cardiac muscles.

eNOS, expressed in the endothelium cells inhibits the contractile tone, vascular SMCs proliferation, inhibits platelet aggregation and monocyte adhesion, promotes diastolic relaxation and decreases O_2 consumption in the cardiac muscle through paracrinally produced NO. eNOS is also expressed in cardiac myocytes from rodent and human, where it autocrinally counteracts the inotropic action of catecholamines after muscarinic cholinergic and beta-adrenergic receptor stimulation.

iNOS-gene transcription and protein expression happens in all cell types, after exposure to a variety of inflammatory cytokines. Besides its role participating in the immune defence against intracellular microorganisms and viruses, most NO produces autocrinally or paracrinally, mediate the vasoplegia and myocardial depression properties of systemic immune stimulation and promote cell death through apoptosis. NO is able to regulate type L calcium channel and contraction, by activation of cGMP dependent protein kinase and cGMP modulated phophodiesterase. Other mechanisms not depending on the cGMP elevation may work through the interaction between NO and heme proteins, non-heme iron or free thiol residues on target signaling proteins, enzymes, or ion channels. .

When considering the various expression of NOS isoforms in the cardial muscles and the potential molecular targets for the produced NO, a tight molecular regulation of NOS expression and activity at the transcription and posttranscriptional level appears necessary to coordinate the many roles of NO.

Tjokroprawiro in 1998 further summarized from the publication of Drexler (1998), "NO as vasodilator and SMC-growth inhibitor", the eleven roles of NO as presented in figure 2-8:

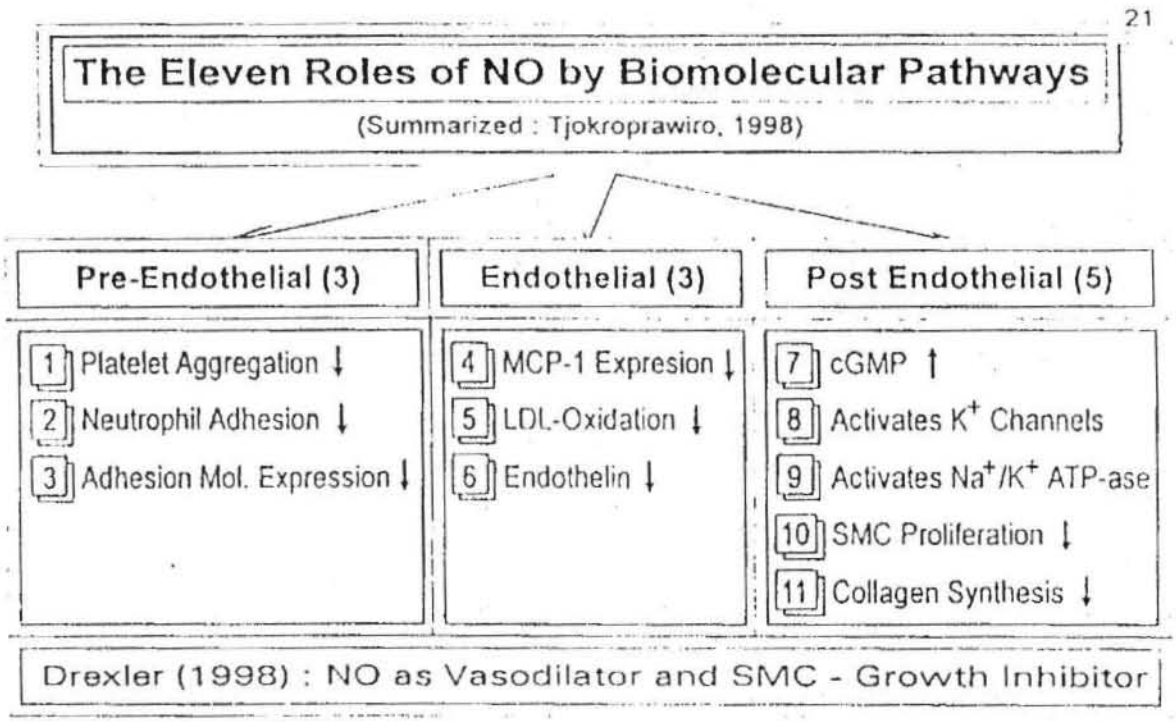


Figure 2-8 NO Biomolecular Pathways (Tjokroprawiro, 1998)

2.7.3 VCAM-1 and the role in atherosclerosis

At the beginning of atherogenesis, aggregation of lipid-rich macrophage and T lymphocytes are found in the intima.

Adhesion of leucocyte at the EC membrane and leucocyte transendothelial migration are mediated by adhesion molecules at the EC membrane. They belong to two protein families: Selectin and immunoglobulin superfamily adhesion molecules.

To the first group belongs E-Selectin and P-selectin and the second group includes Intercellular Ashesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1). Their expression is found on various cells, constituting atherosclerotic plaques, e.g. ECs, vascular SMCs and macrophages.

VCAM-1 is a kind of protein expressed on activated EC's and is found in an

early stage of atherosclerosis. Some of these proteins are released in the circulation and can be detected also in the peripheral blood plasma. VCAM-1 level has a strong correlation with the severity of atherosclerosis.

It is not yet completely clarified about the pathophysiology whose stimulus may cause expression of adhesion molecules. Lisophosphatidilcholine (a component of oxidized – LDL) and Advanced Glycosylation End-product (AGE) has been mentioned to be able to increase the VCAM-1 level. Meanwile it is also proven that antioxidants, several fatty acids n-3 or Nitric Oxide may selectively decrease VCAM-1 expression. Based on the above evidence it may be proposed,that the regulation of VCAM-1 can become a selective prevention for atherogenesis, in comparison with other adhesion molecules, which are not selective.

2.7.4 Evaluation of Endothelial Dysfunction

Endothelial cells produce vasoactive materials in response to environmental factors, like changes in blood flow, hemodynamic pressure ("Shear Stress") and receptor stimulation.

Because Endothelial dysfunction is an early sign of atherosclerosis and is a consequence of several risk factors, like hypercholesterolaemia, hypertention, smoking and hyperhomocysteinaemia, free radicals and others, therefore, prevention through modification of those factors is important Decreasing cholesterol levels, administration of antioxidants and anti-inflammatory drugs are alternatives to be concidered.

Endothelial dysfunction assessment through measurement of the vascular diametre is less reliable in indicating the presence of endothelial dysfunction.

The present of inflammation, the increase of endothelial permeability, LDL oxidation,

platelet aggregation, increasing coagulation activity, fibrinolysis disturbances are better assessed by biochemical and immunological parameters.

Biochemical parameters which are commonly used for Endothelial Dysfunction assessment are:

1. For the presence of inflammation: VCAM-1, ICAM-1, E-Selection.
2. Parameter for platelet aggregations and procoagulen activation: von Wildebrand factor (vWF), thrombomodulin (sTM).
3. Parameter for fibrinolysis disturbances: Plasminogen Activator Inhibitor-1 (PAI-1), tissue –type Plasminogen Activator (tAP).
4. Parameter for endothelial permeability increasement: mikroalbuminuria.
5. Parameter for oxydized LDL: F2-isoprostan

The presence of VCAM-1 expression on the ECs at the early stage of atherogenesis can be detected by immunohistochemistry, even before morphologic changes appear.

CHAPTER 3

THEORETICAL FRAMEWORK AND HYPOTHESIS

From the above literature study it is evident that stress stimulates the hypothalamus in the brain and through the sympathatic nerve system causes release of cathecholamine (norepinephrin =NE) by the adrenal medulla.

Cathecholamine in the circulation causes vasoconstriction and haemodynamic changes on the vascular wall. If this happens continuously may results in endothelial dysfunction. When endothelial dysfunction occurs, monocytes and platelets adhere to the EC, and there are increasement in permeability and repliction of EC's.

The adherence of monocytes and thrombocytes is mediated by the expression of VCAM-1 on

EC's. Lymphocytes T (CD4 and CD8) then migrate from the intravascular to the subendothelial compartment. This is followed by the polarization of lymphocyte T into Th1 and Th2 cells. If Th1 is dominant, then the disease process is via the DTH and macrophage mechanism, whereas on the otherhand if Th2 is dominant, then the disease process is via the ADCC mechanism.

Furthermore T lymphocytes together with macrophages and monocytes proceed the atherogenesis process in the internal pudendal artery as according to the "respons to injury hypothesis".

If atherosclerosis occurs in the internal pudendal artery, erectile dyfunction may develop.

Internal pudendal artery may be one of the predelection places for develloping atherosclerosis, because its "fork sharpe" condition.

The theoretical frame work is outlined in a scheme (see Fig. 3-1).

3.1 Theoretical frame work

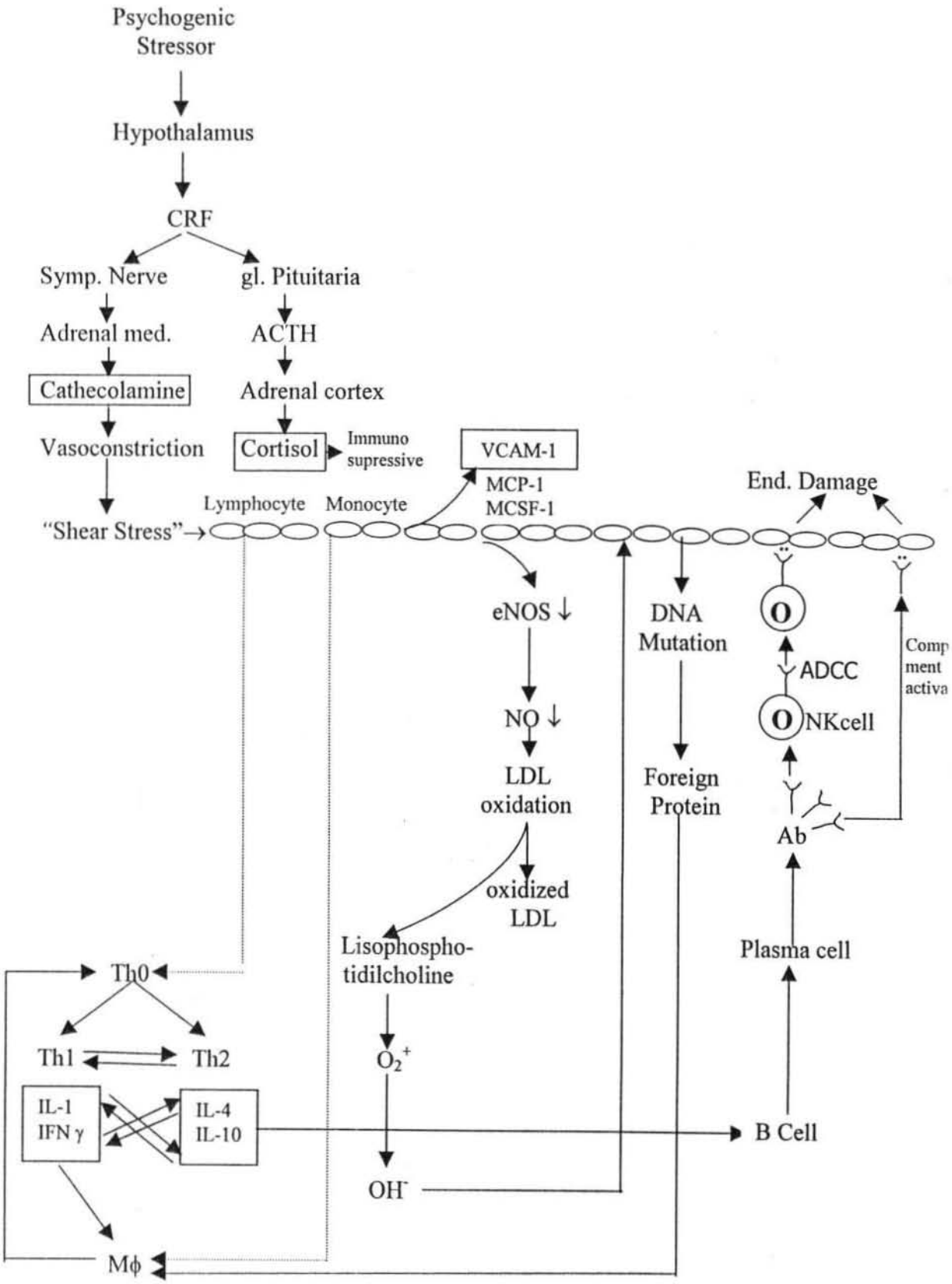


Figure 3-1 Theoretical frame work

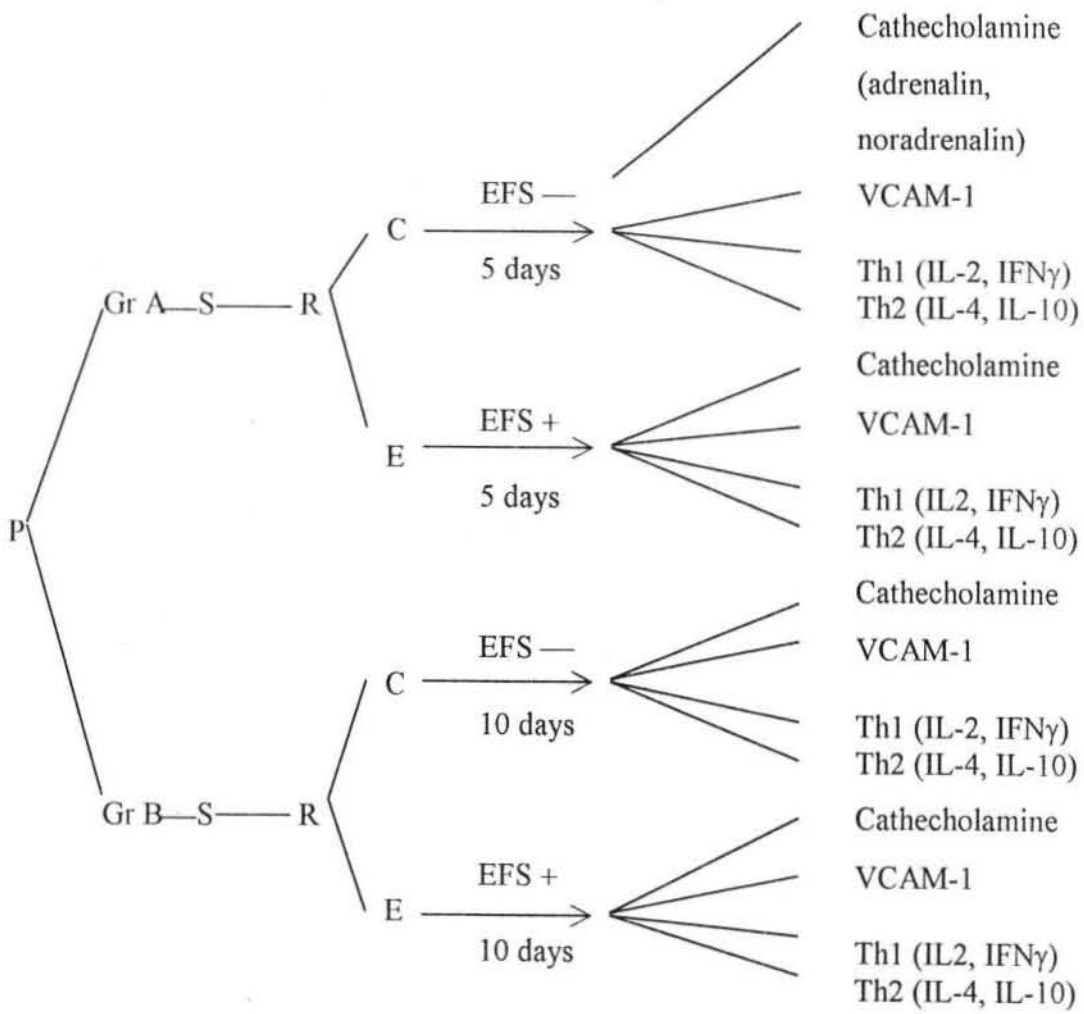
3.2 Hypothesis

1. Immune response modulation occurs in the internal pudendal artery wall (presented as changes in cortisol, cathecholamine blood level; ECs expressing VCAM-1 and Th1 cell secreting IL-2, IFNγ & Th2 cell secreting IL-4, IL-10 polarization) in rats with stress condition caused by electric footshock stressor exposure during 5 days.
2. Immune response modulation occur in the internal pudendal artery wall (presented as changes in cortisol, cathecholamine blood level; ECs expressing VCAM-1 and Th1 cell secreting IL-2, IFNγ & Th2 cell secreting IL-4, IL-10 polarization) in rats with stress condition caused by electric footshock stressor exposure during 10 days.
3. There are differences in immune response modulation in the internal pudendal artery wall in rats with stress condition caused by electric footshock stressor exposure during 5 or 10 days.

CHAPTER 4
RESEARCH METHODS

4.1 Reseach Design and Experimental Chart

1. This reseach is an experimental animal reseach using a "Randomized Posttest only Control Group Design".
2. Experimental chart.



P = Population
S = Sample
R = Random

C = Control
E = Experiment
EFS = Electric Foot Shock

Figure 4-1 Experimental chart

After termination of the animal the following are examined:

- VCAM-1
- IL-2, IFNγ
- IL-4, IL-10
- Cathecholamine

The Stressor used, is an "Electrical foot shock" with the following application schedule:

Day	Amount of shock	Session
1	4	2
2	8	2
3	10	3
4	12	3
5	14	4
6	16	5
7	18	5
8	20	6
9	22	6
10	24	6

The time between two shocks = 1-2 minutes

The time between two session = 4 minutes

4.2 Population, sample and sample size

4.2.1 Population:

The animals for experiment are rats Rattus Norvigicus strain Wistar, bought from the breeding laboratorium in Malang, Bandung, Yogyakarta.

4.2.2 Sample:

The samples are taken by random assignment, for the experimental group as well as the control group.

Inclution Criteria :

Healthy male wistar rats, weight between 150-200 gr, age 3-4 month.

Exclusion Criteria:

Sick and disabled rats

4.2.3 Sample Size:

The sample size according to Daniel formula (1987) is

$$n = \frac{(Z\alpha + Z\ 1\text{-}\beta)^2 \text{ X } d^2}{d^2}$$

$Z\alpha$	= 1,96	d	= 0,05
Z 1-β	= 0,842	α	= 0,05
δ	= 0,05	1-β	= 0,80

$$n = \frac{(1,96 + 0,842)^2 \text{ X } (0,05)^2}{(0,05)^2} = 7,85$$

n = 8

For this experiments are used 10 rats in each group.

4.3 Variables, Classification of variables and Operational definition

Classification

Independent variable :

Stressor Electric footshock

It is measured as the dosis of shock, one dose is amount of shock x amount of session.

Scale = Ratio.

Dependent variable:

1. Cathecholamines

   Are the hormones adrenalin and nor-adrenalin secreted by the adrenal medulla in the blood serum, expressed in nmol/L.

   Scale = Ratio.

2. Cortisol

   Is a hormone secreted by the adrenal cortex, expressed in nmol/L.

   Scale = Ratio

3. VCAM-1

   Is adhesion molecule, expressed in amount on endothelial cells.

   Scale = Ratio.

4. IL-2, IFN-γ, IL-4, IL-10

   Are cytokines secreted by Th1 and Th2 lymphocytes, expressed in amount.

   Scale = Ratio.

Operational definition of variables.

Stressor

Stressor , is given as an artificial shock, using a electric footshock apparatus, with a current power of 0,3 mA.

The shocks are given with an increasing frequency and session.

VCAM-1 is an adhesion molecule expressed on the endothelium of the internal pudendal artery after stress, can be detected by immunohistochemistry examination using monoclonal antibody (m AB). The amount of ECs is concordant to the amount of their VCAM-1.

IL-2, IFN-γ, IL-4 and IL-10 are cytokines secreted by Th lymphocytes (Th1 and Th2). They can be detected by immunohistochemistry using mAB also. The amount of secreting lympocytes is concordant to the amount of IL-2, IFNγ, IL-4, and IL-10.

Changes of the amount before and after given stress can be examined.

Cathecholamine and cortisol concentration in the arterial blood serum can be detected by the Elisa method before and after stress.

4.4 Materials

4.4.1 Experimental Animal

Adult male rats Rattus Norvigicus strain Wistar are used, age 3-4 month, weight 150 – 200 gr.

4.4.2 Rat Boxes

The rats lived in plastic boxes, 30 x 20 x 15 cm, covered by woven metal wire and the bottom covered with straw.

4.4.3 Electric Foot Shock Apparatus

This instrument is a basin made from transparant glass, measured 30 x 20 x 15 cm. The bottom is laminated by metal plates, which conduct electrical current. The conducted electrical current can be regulated by an ampere meter. The instrument is made in cooperation with the "The Institute of Technology Surabaya".

4.4.4 Rats Foods

The rats are fed with standarized pellets made by Characan Pokhpound Indonesia Factory, Mojokerto East Java (Indonesia)

4.4.5 A Set of Chemicals, reagents for Immunohistochemistry examination consist of

Acetone

Dacopen

H_2O_2

PBS

Primary Antibody

4.5 Instruments

Light microscope to count the VCAM-1 and cytokines expression.

4.6 Location for experiments

All experiments were done at the School of Medicine Airlangga University Surabaya.

1. Department of Pathological Anatomy for making the "Freezed Coupe" Slides.
2. "Gramik" Pathobiology Laboratory for the Immunohistochemistry examination.
3. Laboratory of Biochemistry Department for caring the experimental rats (animals) and dissecting, harvesting the blood and tissues.
4. "Makmal endokrinologi" Laboratory for examining the cathecholamine and cortisol.

4.7 Procedures of Data Collection

The rats are taken by random assignment from the boxes and divided into two groups, group A and group B. Group A get a sequence of shocks during 5 days, and group B during 10 days. Each group has its controle group (fig. 4-1).

Harvesting the specimen.

After finishing the procedure,

1. Rats are dissected according to the common surgical procedure (Wagenforth, 1988).

   Arterial blood samples are taken from the heart, with a 3 cc syringe and put in a sterile tube. This tube is contrifuged, the serum is separated and temporary stored in deep freeze for further cathecholamines and cortisol examination.
2. The left and right internal pudendal artery are dissected, using a loupe, a segment are taken and put in a sterile small specimen bottle. These specimens are deep freezed (-40^0) for further immunohistochemistry examination (Green, 1959).

Processing the specimen

The blood serum samples are further processed for cathacholamines examination using the following method.

1. Cathecholamine is examined by RIA method, using Kit: kat-combi RIA (Cat No RE 29291) 1 BL-Immuno-Biological-Laboratories, Hamburg.
2. Cortisol is examined by IRMA method, using Kit: Cool-A-Count and DPC USA (Cat No TK CO1).

The internal pudendal arteries specimens are further processed for immunohistochemistry examination of VCAM-1, IL-2, IFNγ and IL-4, IL-10 using purified mouse anti rat monoclonal antibody staining.

Three Freezed coupe slide are made from three places of each desected artery for staining.

Evaluating the data results

The results are examined and evaluated by the reseacher himself and confirmed and reevaluated by a second observer (expert).

A light microscope is used to detect and count the ECs which expressed VCAM-1 and the lymphocyte with secreted IL-1, IFN-γ, Il-4 and Il-10. The total amount of cells counted from 3 microscopic fields for each slide is recorded.

4.8 Data Analysis:

1. For analysis testing normality of the data a Kolmogorov-Smirnof test is used.
2. To test the reliability a "paired T-test" analysis used.

3. To compare the control group and experiment group during 5 days as well as 10 days a manova analysis is used.
4. To know which variable has the largest contribution to the immune respons modulation a discriminant analysis is used. The size of the contribution as calculated by Fisher discriminant function. Further a pattern of the discriminant is made, to help explain the mechanism of the immune response modulation caused by electric footshock.

CHAPTER 5

Results and Statistical Analysis

5.1 Results of the data:

Some examples of data, obtained by IHC staining of internal pudendal artery of rats.
P = Positive N = Negative

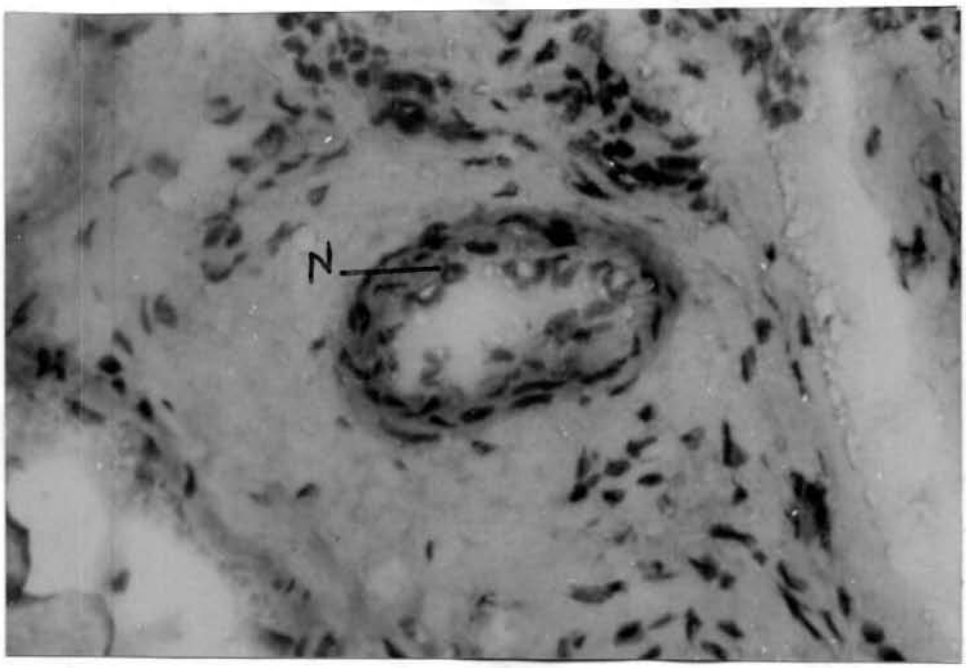


Figure 5-1 Rat internal pudendal artery IHC staining with VCAM-1 m AB. Control group (LM photograph : x 100)

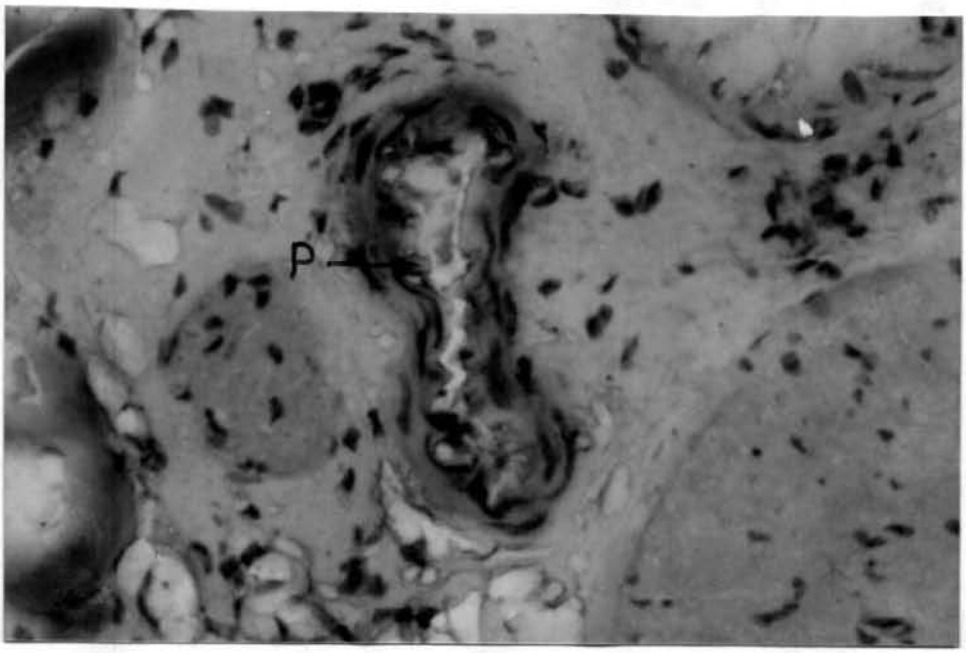


Figure 5-2 Rat internal pudendal artery IHC staining with VCAM-1 m AB. Experiment group. (LM Photograph : x 100)

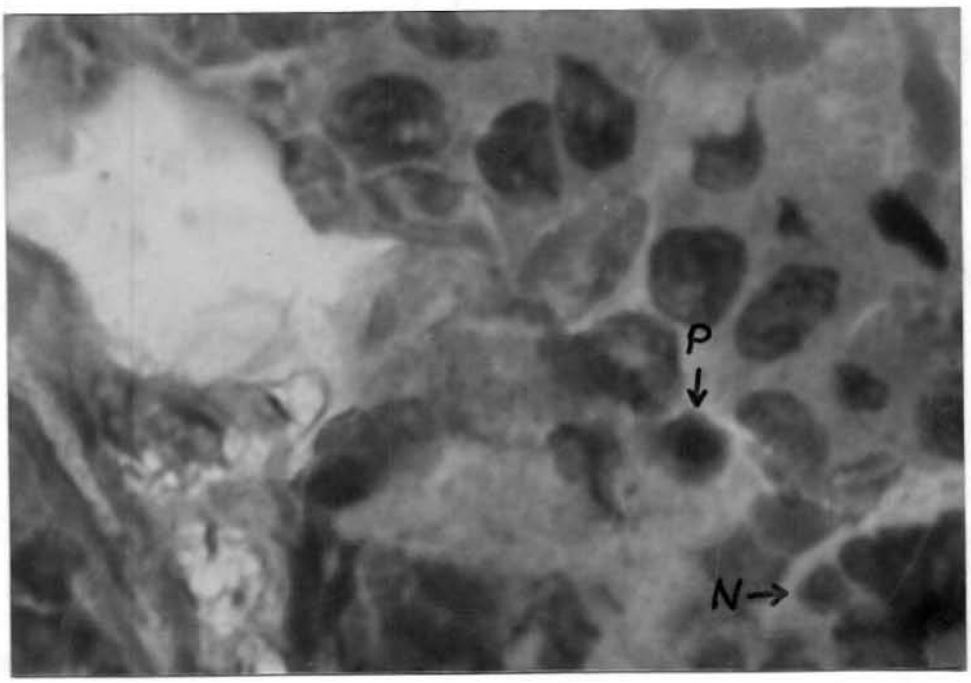


Figure 5-3 Rat internal pudendal artery. IHC staining with IL-2 m AB
(LM photograph : x 450)

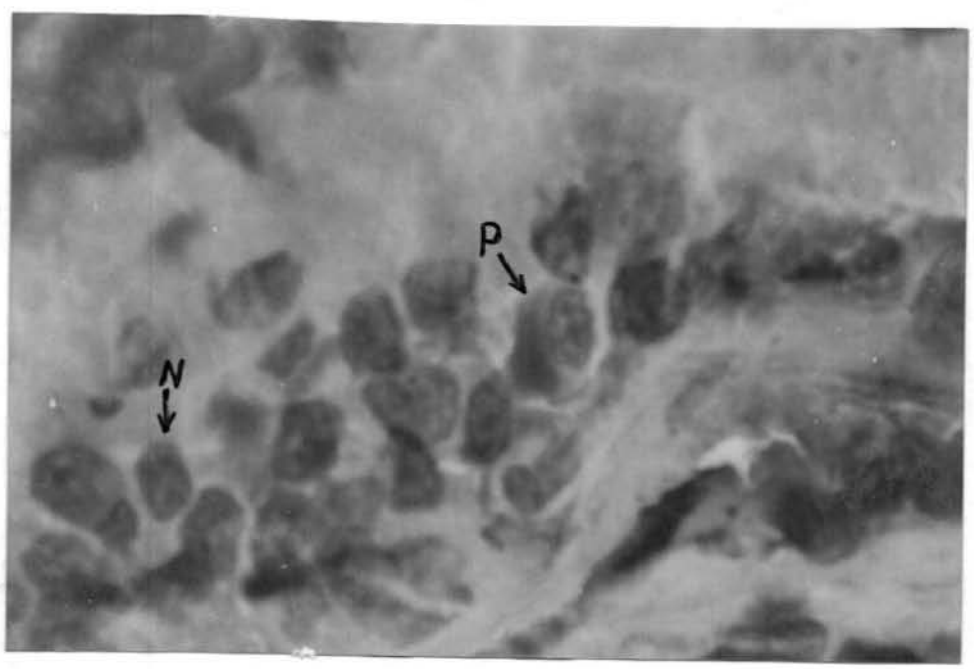


Figure 5-4 Rat internal pudendal artery. IHC staining with IFN-γ m AB
(LM photograph : x 450)

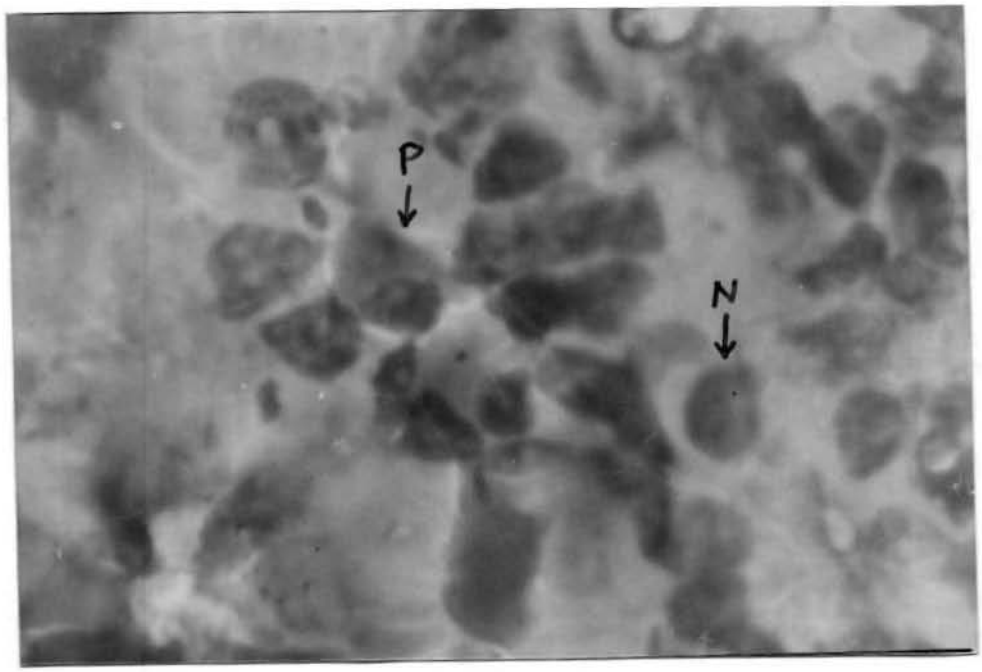


Figure 5-5 Rat internal pudendal artery. IHC staining with IL-10 m AB
(LM photograph : x 450)

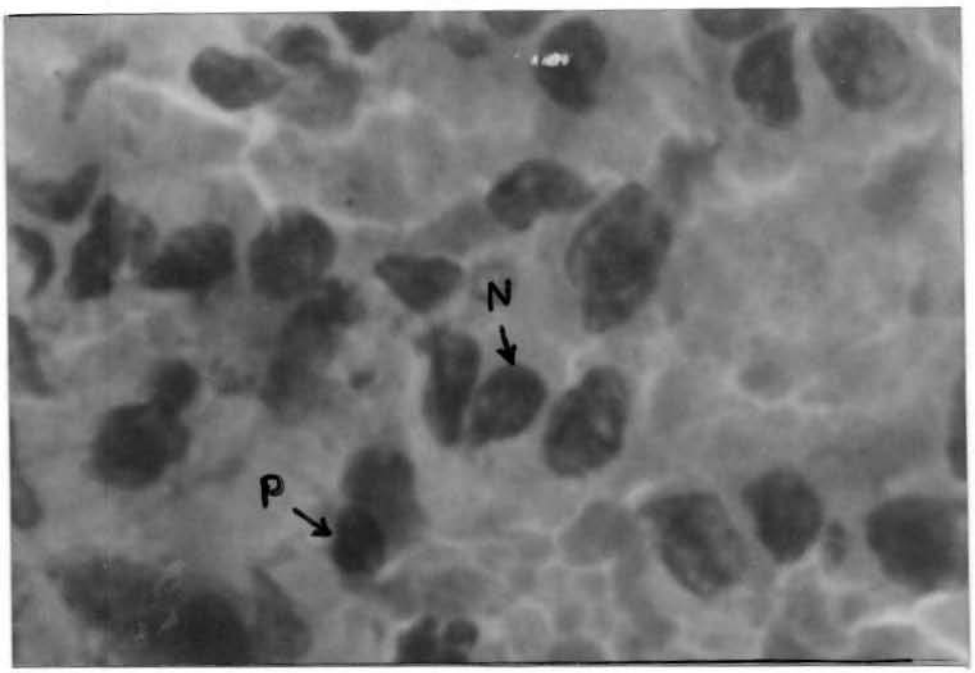


Figure 5-6 Rat internal pudendal artery IHC staining with IL-4 m AB
(LM photograph : x 450)

5.2 Statistical Analysis

The following statistical analysis are used:

1. Analysis for reliability:

To test the consistency of the researcher's findings a reliability. Paired –T test is used. The findings of the researchers are compaired to the findings of a second observer (expert) obtained from an at random taken 20 samples.

Table 5-1 Results of Data obtained by researcher and expert (second observer)

No	Variables	Researcher	Expert
1	VCAM	9	9
2	VCAM	0	1
3	VCAM	2	2
4	VCAM	1	2
5	IL2	1	1
6	IL2	6	6
7	IL2	0	0
8	IL2	4	4
9	IFNg	4	4
10	IFNg	3	4
11	IFNg	2	2
12	IFNg	2	2
13	IL4	2	2
14	IL4	4	5
15	IL4	2	2
16	IL4	6	5
17	IL10	1	1
18	IL10	4	5
19	IL10	2	3
20	IL10	3	2

$t_{sig} = 0.163$ ($P>0.05$)

There is no different between the data compiled by the researcher or the expert (second observer) (see appendix 11).

2. All data have been tested for IIDN (Identically, InDependent, and Normality) before further statistical analysis may be performed. It shows from the statistical

analysis, that all data fulfill those criteria (see appendix 4).

a. Identically is tested by making a Time Sequence Plot Graphic, to know the variation of each group during time. The result is that there is nearly no variation in fluctuation of the data. This means that although the mean is different but it has relatively the same SD.

b. InDependency is tested by making an Estimated Autocorrelation Graphic, to prove the independency observation of the researcher. Observation of the second sample data is not influenced by the observation of the first data sample. This is done because previous observation of the same factor may influence the next observation for instance the color, shape, and size of the cell. The tolerance limit influenced by observation is shown by an estimated autcorrellation line. Our data show within the independency of correlation.

c. Normality is tested by making a Normal Probability Plot Graphic. If the distribution shows a linear line then the distribution is normal. Our result show a linear line so that the distribution is normal for all variables.

3. Comparison analysis test between groups.

a. To compare that there is an immune response modulation in the blood circulation and internal pudendal artery wall between the control group (5days) and the experimental group multivariate Wilks Lambda analysis is used.

The result is Wilk's Lambda $= 0{,}000$ ($P < 0{,}05$). This means that there are differences in immune response modulation between the control group (5 days) and experimental group (5 days).

Table 5-2 Mean and SD variables of experimental groups and control groups during 5 days EF

Variables	Group			
	Contr 5 D		Exp 5 D	
	Mean	SD	Mean	SD
Cortisol	240,700	43,151	329,100	63,898
Adrenalin	0,508	0,081	0,911	0,068
Nor-Adrenalin	1,358	0,175	2,388	0,326
VCAM-1	10,200	5,116	20,500	10,190
IL-2	30,800	6,477	11,900	5,840
IFNγ	27,300	4,473	17,500	3,979
IL-4	23,300	3,401	8,400	5,948
IL-10	21,000	5,457	10,000	4,422

Wilks Lambda = 0,000 ($P < 0,05$)

Table 5-2 shows that there are differences between the control group 5 days and experimental group 5 days. This means that there are differences in IRM between the control group (5 days) and experimental group (5 days).

b. To prove that there is an immune response modulation in the blood circulation and internal pudendal artery wall, between the control group (10 days) and the experimental group .

Multivariate Wilks Lambda analysis is used.

While for the 10 days group the result is a significant rate of Wilk's Lambda = 0,000 ($P < 0,05$).

This means that there are differences in immune response modulation between the control group 10 days and experimental group 10 days.

Table 5-3 Mean and SD variables of experimental groups and control groups during 10 days EF

Variables	Group			
	Contr 10 D		Exp 10 D	
	Mean	SD	Mean	SD
Cortisol	179,700	49,751	478,200	66,101
Adrenalin	0,464	0,077	0,803	0,053
Nor-Adrenalin	1,152	0,182	2,286	0,225
VCAM-1	8,200	2,394	4,100	3,635
IL-2	31,700	5,658	2,600	2,459
IFNγ	37,400	7,677	8,700	5,293
IL-4	26,600	4,742	2,500	1,841
IL-10	24,300	3,917	3,900	2,514

Wilks Lambda = 0,000 ($P < 0,05$).

Table 5-3 shows that there are differences between the control group 10 days and experimental group 10 days. This means that there are differences in immune response modulation between the control group 10 days and experimental group 10 days.

c. To prove the maturation process, between the control group 5 days or 10 days, multivariate Wilks Lambda analysis is used.

The result is Wilk's Lambda = 0, 222 ($P > 0, 05$). This means that between the control group 5 days and 10 days are no differences in response immune modulation (see table 5-2 and table 5-3).

d. To prove the differences in immune response modulation, between the experiment group 5 days and 10 days a multivariate Wilks Lambda test is used.

The result is Wilk's Lambda = 0, 003. This means that there are differences in immune response modulation in the experiment group 5 days and 10 days (see table 5-2 and table 5-3).

e. Although between the experiment group 5 and 10 days are significant differences in immune response modulation, nearly and between control group 5 and 10 days is statistically significant, homogen, it is impossible that the means of both control group are the same. That is way it is important to calculate the delta (δ) between the experimental group 5 days substracted by the control group 5 days and the delta (δ) between the experiment group 10 days substracted by control group 10 days to prove that the different of immune response modulation is purely caused by the electric footshock (see table 5-4).

Table 5-4 Mean and SD of differences (δ) of variables between the experimental group and control group, both at 5 and 10 days exposures

Variable	Group			
	5 D		10 D	
	Mean	SD	Mean	SD
Cortisol	+88,400	77,027	+298,500	62,443
Adrenalin	+0,403	0,106	+0,339	0,085
Nor-Adrenalin	+1,030	0,226	+1,134	0,348
VCAM-1	+10,300	10,563	-4,100	4,909
IL-2	-18,000	6,839	-29,100	5,607
IFNγ	-9,800	6,359	-28,700	10,709
IL-4	-14,900	4,458	-24,100	3,872
Il-10	-11,000	5,617	-20,400	5,211

Wilks Lambda = 0, 002 (P < 0, 05).

To know the difference of the immune response modulation between the experiment group 5 days and 10 days, which is purely caused by electric footshock a multivariate Wilks Lambda analysis is used for δ (E-K) 5 days and δ (E-K) 10 days. The result is Wilks Lambda = 0, 002 (P < 0, 05).

This means there is a significant difference in immune response modulation purely caused by electric shock exposure between the experiment group 5 and 10 days.

4. Dicriminant Analysis

A discriminant analysis is used to know the differential variables (discriminats) that has the role in immune response modulation during 5 days and 10 days electric footshock.

From the δ variables examined in this research it shows out that there are 5 differential variables (discriminants) those are cortisol, VCAM-1, IL-10, adrenalin, and IL-4. IL-2, IFNγ were not discriminants (see table 5-5).

Table 5-5 Summary Table

Step	Action Entered remove	Vars In	Wilks' Lambda	Sig
1	CORT	1	0,28619	0,0000
2	VCAM	2	0,20521	0,0000
3	IL-10	3	0,17687	0,0000
4	ADRF	4	0,16840	0,0000
5	IL-4	5	0,16233	0,0000

Five variables included in the discriminant analysis possess a discriminant power of 100%; for the Fisher linear discriminant model analysis the 5 variables are used and gives the following results (see table 5-5).

Table 5-6 Fisher Linear Discriminant Function

KEL =	1	2
	5 D	10 D
CORT	-0,7534718E-03	+0,4504974E-01
ADRE	+35,20852	+22,04504
IL-4	-0,5639694	-0,8094136
IL-10	-0,2734687	-0,5505554
VCAM	+0,1543054	-0,7129820E-01
(constant)	+14,25468	+26,66872

To know the power of the role of the various variables in each group (5 days and 10 days electric footshock) in immune respons modulation a calculation of the Fisher linear model times the original data (δ) is needed.

The results gives the following pattern of variables (Fig. 5-7).

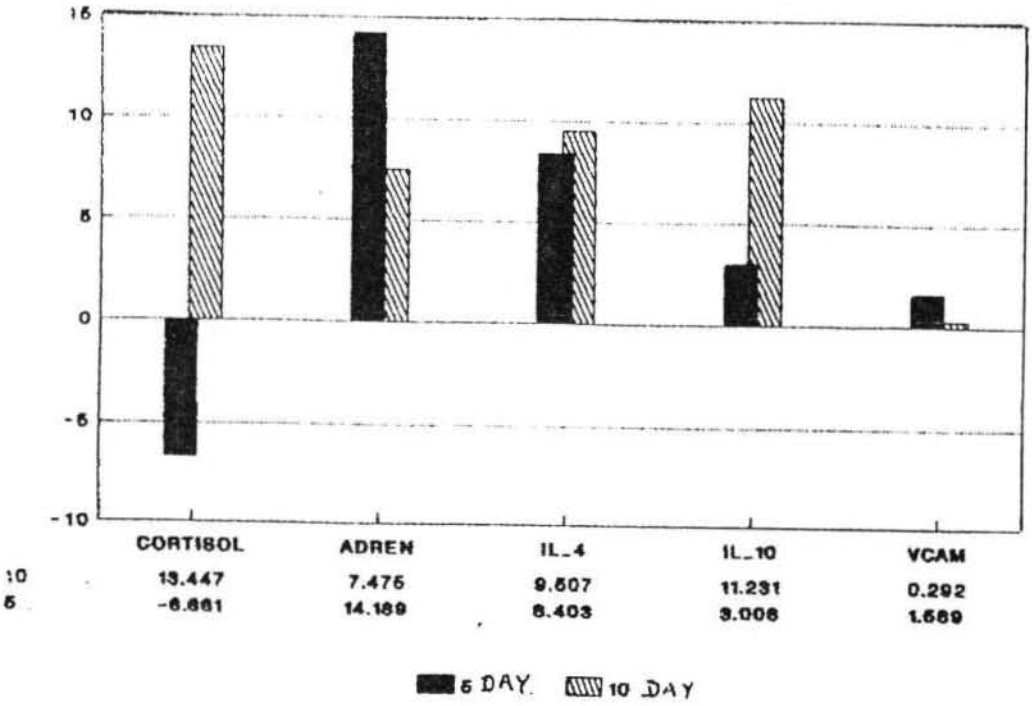


Figure 5-7 Relative power of variables after 5 days and 10 days exposure

The grafic of pattern may help to explain the mechanism of immune response modulation towards the pathogenesis of endothelial damage and hence psychogenic ED.

CHAPTER 6
DISSCUSSION

As stated before, the objective of this research is to elucidate the pathobiogenesis of ED in stress condition (psychogenic ED).

There are until recently no studies that explain the mechanism on how psychogenic stressor may cause ED, so that treatment and prevention of psychogenic ED is still unsatisfactory. In this study, the psychoneuroimmunology paradigm is introduced. This relatively new study endeavors to explain the relationship between stress condition and changes in the immune system via the autonomic nerve system and endocrine system. Through this research it is proven that electric footshock (physical stressor), which acts as a psychogenic stressor for rats, can cause immune response modulation of the immune competent cells like Th1 and Th2 cells in the internal pudendal artery of those rats.

If the immune system is disturbed, damage of the internal pudendal arterial wall can occur, which may further cause ED. Until 1993, the presence of lymphocyte-T in atherosclerotic plaque is not yet clearly understood (Ross et al, 1993). This study may explain the damaging role of those cells.

In this study wistar rats, Rattus Norvigicus are used as experimental animals, because after the electric footshock, the animals must be terminated. The internal pudendal artery is dissected for imunohistochemistry examination.

The stressor used in this experiment is "electric footshock". There are actually other physical stressors that can be used to elucidate psychogenic stressor, e.g.

"Electric tailshock", "Housing isolation", and "Sound stress" (Keller, Schleifer, 1991). However, in this experiment, the "electric footshock" is used mainly because it is easy to use and because it is developed at the Institute for Technology (Institut

Tehnologi Surabaya) here in Surabaya. The dosage of shock (amount of stressor X amount of shock) has been established in previous experiments, so that it will not cause direct injury to the rats, except for causing stress.

An EF, a physical stressor, which acts as a psychogenic stressor may give various effects. Depending on the intensity and duration of the electric current, EF causes different pathologic conditions. It may causes just fear, pain, or injury (burn wound) with pain.

When the rats are only frightened by the EF, then this psychogenic stressor will stimulate the brain and via the HPA-axis will cause a rise in cathecholamine and cortisol and cause IRM.

However if the EF causes pain to the rats, then this pain-stimulus will lead via the spino-thalamicus channel to the brain, before causing the release of cortisol and cathecholamine via the HPA-axis and also IRM (Ludwig – Beymer, Heuther, Schoessler, 1994)

It is said, that when animals feel pain, they will cry or beep. In a restless condition we can observe that some of them will urinate or deficate. But it is indeed difficult to know wether the effect of EF is only frightening or causes also pain. In this research it seems that the predominant effect of psychogenic stressor is only a restless condition, because none of the rats beep, but some did urinate during the experiments and no lesion did appear.

In this research we used an EF exposure during 5 and 10 days. It is based on previous investigations, which showed that when rats got an electric stressor, after 5 days there were already changes in immune response of the immunocompetent cells. However when the stressor was continued during 20 days the function of the immunocompetent cells recovered, because of adaptation (Keller, Schleifer, 1991).

That is way it was decided to use an exposure during 5 days and 10 days period, which was also proven in a prelimanary study that during this period an IRM already occurs.

Hypothesis that must be proven in this study are:

1. There is immune response modulation in the internal pudendal artery wall after electric footshock exposure during the 5 day period.
2. There is immune response modulation in the internal pudendal artery wall after electric footshock exposure during the 10 day period.
3. There are differences in immune response modulation after electric footshock exposure during the 5 day and 10 day period.

The only research design used in this experiment is the "Randomized Post Test only Control Group Design", because in this animal experiment it is impossible to use the same animal in the pre test to be followed up after the electric footshock.

For analysis of the data, a multivariate Wilks-Lambda analysis is used, because an IRM is a biologic event, where many variables interact with each other. It is proven in this research that after a multivariate analysis of the data an immune response modulation did occur after electric footshock exposure during the 5 as well as 10 day period (table 5-2; 5-3). The cathecholamine and cortisol levels increase after 5 and 10 days, followed by a decrease in IL-2, IFNγ, IL-4, and IL-10 production. It is well known already that stress (psychologic stressor) via the HPA-axis causes an increase in cathecholamine and cortisol. Cortisol as well as cathecholamine then suppress the activity of T-cells in their production of cytokines (Mc Cance, Shelby, 1994).

It is also proven from the multivariate analysis that there is a significant difference in the immune response modulation after the electric footshock exposure

during the 5 day and 10 day period. IL-2, IFNγ, IL-4, and IL-10 all get more depressed after being exposed to electric footshock during a 10 day period (table 5-4).

Adrenalin level declines, whereas cortisol level is still increasing. It is indeed proven that adrenalin will rise suddenly in acute stress and then decline quickly. In chronic stress cortisol will usually rise, which will depress more the production of cytokines.

The increase in adrenalin which is first seen at day 5 can be explained in that short time stress or acute stress, through the sympathetic nerve system causes adrenalin to be excreted instantaneously. But adrenalin activates macrophage to produce IL-1, which consequently activates the hypothalamus and via the HPA-axis stimulates the adrenal cortex to secrete cortisol (Dunn, 1995). VCAM-1 which increases after 5 days, declines after electric shock of 10 days. Adrenalin which is first secreted is also followed by an increase in VCAM expression at day 5 and adrenalin will disintegrate quickly.

The increase in adrenalin at day 5 causes vaso constriction and the secretion of eNOS by the ECs diminishes. When eNOS diminishes, NO production will decrease. Lowering of NO causes endothelial dysfunction, and expression of VCAM-1. Expression of VCAM-1 causes monocyte, lymphocyte and thrombocyte agregation to the EC and migration to the subendothelial compartment.

The declining VCAM-1 expression at day 10 is probably caused by the decreasing EC function, which is already exhausted and the decrease of adrenalin.

In order to clarify the pathogenesis of psychogenic ED through the damaging process in the internal pudendal artery which is actually the main objective of this study, it is important to find the differential variables (discriminating variables) that play the main role in the pathogenesis.

All variables influence each other but not all variables have the same power to conduct this interaction. For this purpose a multivariate discriminant analysis is applied.

The result of this discriminant analysis shows that 5 of the 8 variables do act as a discriminant of the process. They are cortisol, adrenalin, VCAM-1, IL-4 and IL-10. (See table 5-5)

It can be seen in table 5-6 and figure 5-1 that after 5 days of stress, the role of adrenalin is more prominent than that of cortisol. After 10 days of stress cortisol shows to have more impact than adrenalin in immune response modulation. The condition is followed by the increasing role of IL-4 and IL-10 after 10 days of EF exposure. It can then be concluded that after 10 days of exposure, Th2 is more dominant and the hypothesis is proven.

If Th2 cells have a more prominent role, then the damaging process of the vessels can be explained via the ADCC pathway. But the investigation of Law (2000) shows that IL-10 may stimulate secretion of IFNγ by the Th1 cells, which may also explain that the endothelial damage occurs via DTH and macrophage pathway. However, in this research IFNγ did not show up as a discriminant variable, implying that the damaging process of the vessel is more probable via the ADCC pathway.

At the stage of 5 days, it can be explained that the damaging process may directly be caused by the effect of an increasing adrenalin level. Adrenalin lower eNOS secretion and NO production and the endothelial dysfunction that occurs causes leakage of intravascular materials, so that it may cause further atherogenesis.

The damaging process of the endothelium after a psychogenic stressor can be explained by different pathways. Some of the possibilities are :

1. The high contribution of adrenalin function in the IRM after 5 days EF exposure in this research causes vasoconstriction, which in turn causes oxidative stress on the epithelium. IFN-$_k$Bα decreases and as a consequence NK$_k$B increases, which causes the expression of VCAM-1 and MCP-1 (fig. 2-7). VCAM-1 causes inhibition of NO production, so that endothelial dysfunction occurs.
2. Adrenalin binds to α_1 receptor on the surface of SMC. It increases the activity of membrane bound enzyme phospholipase C (PLC), which converts phosphotidil inositol biphosphate (PIP) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 liborates Ca^{++} from sarcoplasmic reticulum and DAG stimulates the enzyme protein kinase C (PKC). PKC in turn open the L-type channel. The open calcium channel, leading to Ca^{++} influx into the cell. PKC also closes the potassium channel. There will be a resultant rise in cytoplasmic Ca^{++} concentration, with consequent SMC contraction (Eardly, Sethia, 1998) and causes ED.

   Adrenalin also binds to α_2 receptor on the cell membrane, causes opening of calcium channel and influx of Ca^{++} from extracellular compartment in to intracellular compartment. Adrenergic β-receptor has in general not an important role in the regulation of smooth muscle tone, becausee the present is only 1 : 10, in comparison with the amount of adrenergic α-receptors (Eardly, Sethia, 1998).
3. The predominant contribution function of IL-4 and IL-10 in this research may cause endothelial damage through the ADCC pathway.

   The decreased eNOS production causes decreased NO, which caused LDL oxidation to become oxydized LDL. During this process lisophosphotidilcholin molecule is liberalized (Wijaya, 1998). Lisophosphotidilcholin stimulates the

production of superoxide radicals (O_2^-), which in turn stimulates the production of hydroxil radicals (OH^-), according to Haber - Weis - Fenton reaction. Hydroxil radicals cause DNA mutation of the epithelial cell and consequently causes foreign protein expression (Tjokroprawiro, Sutjahyo, 1995; Tjokroprawiro, 1999).

The foreign protein stimulates, immune competent cells, in this research Th2 cells, which elaborates IL-4 and IL-10, with the help of macrophage to activate plasma cells to produce antibodies.

Antibody binds with its Fc region to NK cell, which then specifically binds with the foreign protein and destroys the epithelial cell (ADCC).

The antibody that is produced may also directly binds to the foreign protein on the cell membrane and stimulates complements activation for the distruction of the cell. It may be assumed that after 5 days EF exposure, the epithelial damage follows the pathway by direct binding of adrenalin with the α-receptors, while in the chronic stress after 10 days EF exposure, the damaging process is via ADCC.

From this research is proven that immune response modulation occurs after psychogenic stressor and from the pattern of the discriminating variables it is possible to explain the pathogenesis of endothelial damaging in the internal pudendal artery, which explains the cause of psychogenic ED. The main objective of this research is thus confirmed.

The damaging process of the EC of the internal pudendal artery through the the immune system in individual during stress condition has been explained in this research. This is a new finding that explain the mechanism how an psychogenic stressor may cause endothelial damage and potentially causes ED.

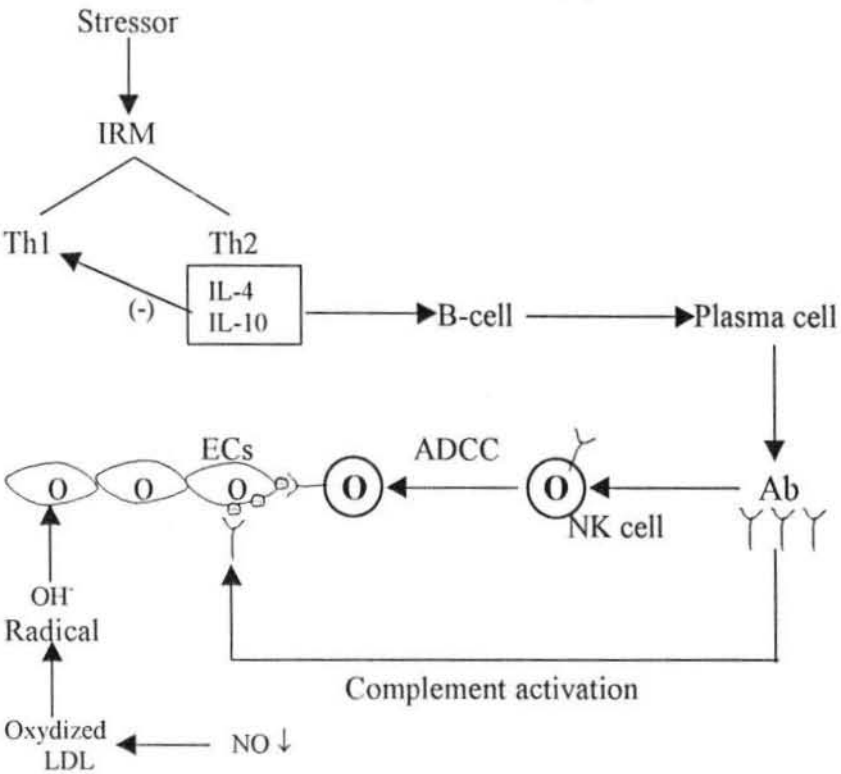


Figure 6-1 New concept for the development of psychogenic ED

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 Some conclusions can be made from this research:

1. It is proven by empirical study that immune response modulation indeed occurs in the internal pudendal artery wall after a psychogenic stressor.
2. From this research it is possible to conclude that the mechanism towards psychogenic ED takes the ADCC pathway through a damaging process of the internal pudendal artery. This is a new finding provided via the psychoneuroimmunology paradigm. Previously, the diagnosis of psychogenic ED was assumed only when organic factors were excluded.
3. With short term stressor exposure (5 days) the damaging process of the vessels may be explained by the direct influence of the sudden rise of cathecholamine, which causes depression of NO production and endothelial dysfunction. Endothelial dysfunction causes leakage of intravascular material, which promoted atheroschlerosis and may cause ED.

7.2 Recommendation

1. A further investigation about the neural damage caused by stress condition with the same paradigm is recommended, because the neural system has also an important role in the pathogenesis of ED, besides it is not yet clear which is involved first the vascular or the neural system.
2. Part of the research with animals can be continued in human subjects. The immune response modulation that occurs in the vascular tissue has

probably correlation with the changes in the blood serum. By examination of the blood, an immune response modulation may be detected before ED becomes apparent. However the application of this study in humans need to take the behavioral and coping abilities of humans in consideration

3. This research may be continued for a longer period of stress exposure. It is possible that longer stress condition might take another pathway to develop damage to the vessels.
4. For the treatment of psychogenic ED, the management of stress may be important to prevent vascular exchange.
5. Also pharmacotherapy to prevent immune response modulation might be developed to stop the destructive process.

REFERENCES

Aakvaag A, Bentdal O, Quigstad K, Walstad P, Rohnningen H and Fonnum F, 1978. Testosterone and testosterone-binding globulin (TeBG) in young men during prolonged stress. International Journal of Andrology, 1, 22-31.

Ader R, Felter DC, Cohen N 1991. Psychoneuroimmunology 2nd ed. San Diego : Academic Press.

Akil HA, Morano MI 1995. Stress. In (Bloom FE, Kupper DJ eds) Psychopharmacology 2nd ed. New York : Raven Press pp 773 -783.

Anderson KE, Hedlund H, Holmquist F, 1991. Current concept of erection. In (Rogers S Kirby, Culey Carson, George D Webster eds). Impotence diagnosis and management 1st ed. Oxford : Butterworth–Heinmann Ltd, pp 32.

Apasou SG, Sitkorsky MV, 1999. T-Cell-mediated Immunity, In Principles of Immuno Pharmacology (Nijkamp FP, Painhane MJ eds). Basel Birkhansen Verlag.

Balligand JL, Cannon PJ, 1997. Nitric Oxide and Synthases and Cardiac muscle. Aterosklerosis, Thrombosis and Vascular Biology 17 : 10.

Bancroft J and Wu FCW. 1983. Changes in erectile responsiveness during androgen therapy. Arch. Sex. Behav 12 : 59 -66.

Benditt EP, 1977. Implication of the monoclonal characters of human atherosclerotic plaques. Am J Path 86 : 693.

Benkert O, Witt W, Adam W and Leits A. 1979. Effects of testosterone undecanoate on sexual potency and the hypothalamic pituitary-gonadal axis of impotent males. Arch Sex Behav 8 : 471 - 479.

Benson GS, 1993. Penile Erection. In search of a neurotransmitter. World. J Urol 1 : 209.

Benson GS, Mc Connell J, Lipschutz LI, Corrier JH jr and Wood J, 1980. Neuromorphology and neuropharmacology of the human penis. J. Clin Invest 65 : 506 – 513.

Berne RM, Levy MN, (Eds.), 1990. Principles of Physiology. St. Louis: Mosby-Year Book.

Bors E, Comarr AE. 1971. Neurological Urology; Physiology of micturition, its neurological disorders and sequelae. Baltimore, University Press.

Bredt DS, Huang PM, Snyder SH, 1990. Localization of NO synthase indicating a neural role for NO. Nature 347 : 495.

Burguera B, Muruais C, Penalva A, Dieguez C, Casanueva F, 1990. Dual and selective actions of glucocorticoid upon basal and stimulated growth hormone release in man. Neuroenddocrinology, 51, 51-58.

Burnstock G, 1986. The changing face of autonomic neurotransmission. Acta Physiol. Scand 126 : 67 - 91.

Calogero A et al, 1988. Multiple regulatory feedback loops in hypothalamic corticotropin-releasing hormone secrtion, J Clint Invest 82 : 767.

Cannon RO, 1998. Role of Nitric Oxide in Cordio Vascular disease : Focus on the endothelium. Clin. Chem 44 : 1809 - 1819.

Carati CK, Creed LE, Keogh EJ, 1987. Autonomic control of penile erection in the dog. J. Physiol. (Lond) 384 : 525 – 538.

Carrillo Y, Fernandez E, Dail WG et al, 1989. Neuropeptide Y innervation of penile erectile tissue. Society for Neuroscience (Abstracts) 15 : 631.

Carter DB, Bazim, 1979. Imunology in The Laboratory Rat (Baker HJ, Lindsey JR, Weisbroth S.H, eds). San Diego Academis press, Inc. pp 181 – 212.

Chapelle PA, Durant J, Lacert P, 1980. Penile erection following complete spinal cord injury in man. Br. J. Urol 52 : 216 - 219.

Chrousos GP, Gold PS, 1992. The Concepts of Stress and Stress System disorders: Overview of physical and behavioral homeostasis. Journal of the American Medical Association, 267 (9), 1244-1252.

Cohen MR, Pickar D, Dubois M, Bunney WE, Jr. 1982. Stress-induced plasma beta endorphin immunoreactivity may predict post-operative morphine usage. Psychiatry Reseach, 6, 7-12.

Collins T, 1993. Endothelial nuclear factor-kB and the initiation of the atherosclerotic lesion. Lab Invest 68 : 499.

Colt EWD, Wardlaw SL, and Franz AG, 1981. The effect of running on plasma β-endorphin. Life Science, 28, 1637-1640.

Cox T, 1990. The recognition and measurement of stress: conceptual and methodological issues in methods in ergonomics (eds Wilson J, Corlett N). Taylor and Francis, London.

Cox T, 1995. Stress, coping and physical health in (Broome A, Liewelyn S eds) Health Psychology 2nd ed. London : Chapman and Hall : pp 21 - 33.

Cox T, Cox S, Thirlaway M, 1983. The psychological and physiological response to stress, in Physiological Correlates of Human Behaviour (eds Gale A, Edward JA) . Academic Press, London.

Cunningham GR, Hirskowitz M, Korenman SG and Karacan I. 1990. Testosterone replacement therapy and sleep related erections in hypogonadal men. J . Clin. Endocrin. Metab 70 : 792 - 797.

Cybulsky MI, Gimbrone MA 1991. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 251 : 788.

Dall WG, Hamill RW, 1989. Parasympathetic nerve in penile erectile tissue of the rat containing choline acetyltransferase. Brain. Res 487 : 165 - 170.

Daniel, Wayne W, 1987. Biootatistic : A Faundation for analysis in the Health Siences, Fourth Ed. John Wiley and Sons

De Groat WC and Steers WD, 1988. Neuroanatomy and neurophysiology of penile erection. In (EA Tanagho, TF Lue and RD Mc Clure eds). Contemporary management of impotence and infertility. Baltimore: Williams and Wilkins, pp 3 – 27.

Deslypere JP and Vermeulen A. 1984. Leydig cell functions in normal men : Effect of age, life style, residence, diet and activity. J. Clin. Endocrinol. Metab, 59 : 955 - 962.

Dunn AJ, 1995. Interaction between the nervous system and immune system. In (Bloom FE, Kupper DJ eds) Psychopharmacology 2nd ed. New York : Raven Press pp 719 -731.

Eardly I, Sethia K, 1998. Erectile dysfunction. Current investigation and management. 1st ed, London, Mosby – Wolfe pp 18.

Fauci AS, 1975. Corticosteroids and Circulating Lymphocytes. Transplant Proceeding, 7, 37-48.

Foreman MM, Wernicke JF, 1990. Aproaches for the development of oral drug therapy for erectile dysfunction. Semin. Urol 8 : 107 – 112.

Forsberg L, Hojerback T, Olsson AM, Rosen I. 1989. Eteologic aspects of impotence in diabetics. Scand. J Urol Nephrol 23 : 173 - 175.

Furchgott RF, Zawadski JV 1980. The obligation role of endothelial Cells in relaxation of arterial Smooth muscle to acetylcholin. Nature 288 : 373.

Gimbrone MA et al, 2000. Endothelial dysfunction, hemodynamic forces and atherogenesis. Ann NY Acad Sci 902: 230-239.

Goldsby RA, Kindt TJ, Antibody-Dependent Cell-mediated Cytotoxicity. In KUBY Immunology 2000, Osborne BA eds, New York, WH Freeman and Coy, p 362.

Graigners DJ et al, 1993. Proleferation of human smooth muscle cells, promoted by lipoprotein (a). Science 260 : 693.

Granner DK, 1988. Hormones of the adrenal medulla. In RK Murray et al (Eds.). Harper's biochemistry (21st ed.). New York: Lange medical Books.

Green EC, 1959. Anatomy of the Rat. New York : Hafner Publishing Co.

Guillemin R, Vargo T, Rosier J, Minick S, Ling N, Rivier C, Valle W, Bloom F, 1977. Beta endorphin and adrenocorticotropin are secreted concomitantly by the pituitary gland. Science, 197, 1367-1369.

Hajjar DP, 1991. Viral pathogenesis of atherosclerosis. Impact of molecular mimicry and viralgenes. Am J Pathol 139 : 1195.

Hansen MV, Ertekin C, Larson LE and Pedersen K. 1989. A neurophysiological study of patients undergoing radical prostatectomy. Scand J Urol. Nephrol 23 : 267 - 273.

Herd JA, 1991. Cardiovascular response to stress. Physiological Review, 71 (1), 305-330.

Hoffman F, 1985. The molecular basis of the second messenger systems for regulation of smooth muscle contractility: State of the art lecture. J. Hypertention 3 : 53 – 58.

Houldin AD, Lev E, Prytowsky MB, Redei E and Lowery BJ, 1991. Psychoneuroimmunology: A review of literature. Hollistic Nursing Practice, 5 (4), 10-21.

Jacobs MA, spilken A, Norman MM and Andersson LS, 1970. Life stress and respiratory illness. Psychosomatic Medicine, 32, 233-242.

Jessep W, 1996. Oxidized lipoprotein and Nitric Oxide Current Opinion in Lipodology. 7 : 274 - 280.

Jungkunz G, Engel RR, King UG, Kuss HJ, 1983. Endogenous opiates increase pain tolerance after stress in humans. Psychiatry Reseach, 8, 13-18.

Kaplan JH, 1983. Impotence introduction. Postrad med. 74 : 191.

Karacan I, 1970. Clinical value of nocturnal erection in the prognosis and the diagnoses of impotence. med aspects human sex 4: 27.

Karacan I, Salis PJ, Hirshkowitz M, Borreson RE, Narter E and William RL. 1989. Erectile dysfunction in hypertensive men : sleep related erections, penile blood flow and musculovasculer events. J Urol. 142 : 56 - 61.

Keller SE, Schleifer SJ, Demetrikopoulos MK, 1991. Stress – Induced changes in immune function in animal : HPA influences. In (Ader R, Felten DL, Cohen N) Psychoneuroimmunology, 2nd ed, Academic Press. Pp 771-777.

Khder Y, Briancon S, Petermann R et al. 1998. Shear stress abnormalities contribute to endothelial Dysfunction in Hipertension, but not in Type II DM. Journal of hypertension 16 : 1619 - 1625.

Kim N, Azadzoi KM, Goldstein et al, 1991. A Nitric Oxyde like factor mediates non adrenergic non cholinergic neurogenic relaxation of penile cavernosum smooth muscle. J Clin Invest 88 : 1991.

Kimoto Y, Ito Y, 1987. Autonomic innervation of the canin penile artery and vein in relation to neural mechanism involved in erection. BJ Urol 59 : 463.

Kitayama J et al, 2000. Shear stress affects migration behavior of poly morpho nuclear cells arrested endothelium. Cell Immunol 2000. 203 (1) : 39-46.

Law FH, 2000. Pro-inflammatory effect of IL-10 during human endotoxemia. J Immunol, 165(5) : 2783-9.

Lazarus RS and Folkman S, 1984. Stress, Appraisal and Coping New York : Spinger Publications.

Lazarus RS, 1966. Psychological Stress and the Coping Process, Mc Graw-Hill, New York.

Lo Piccolo J, 1986. Diagnosis and treatment of male sexual dysfunction. J sex marit ther. 11 : 215 - 232.

Ludwig – Beymer, Huetter SE, Schoessler M, 1994. Pain, Temperature Regulation, Sleep and Sensory function. In Pathophysiology, The Biologic Basis for Disease in Adults and Children (Mc Cance KL, Huetter SE ed) 2nd ed. London, Mosby, pp 437 – 447.

Lue TF, 1992. Male Sexual Dysfunction. In Smith's General Urology 13 th ed. Connecticut : Lange Med Book, pp 699 – 700.

Lue TF, 1994. Functional Anatomy of The Penis, The Arterial System. In Campbell's Urology 6 th ed. Philadelphia : WB Saunders Coy, pp 709.

Lue TF, Mueller SC, Jow YR and Hwang TI. 1989. Functional evaluation of penile arteries with duplex ultrasound in vasodilator induced erection. Urol Clin. North AM, 16 : 799 - 807.

Lue TF, Tanagho EA, 1988. Functional Anatomy and Mechanism of penile erection. In (Tanagho EA, Lue TF, Meclure RD). Contemporary management of impotence and infertility 1st ed. Baltimore : Williams and Wilkins, pp 30 – 50.

Lue TF, Tanagho, 1987. Physiology of erection and pharmacological management of impotence. J Urol 137 : 829 – 836.

Lue TF, Zeineh SJ, Schmidt RA, Tanagho EA, 1984. Neuro Anatomy of penile erection : Its relevance to iatrogenic impotence. J Urol 131 : 273 – 280.

Masters WH, Johnson VE. 1970. Human Sexual inadequacy. Boston : Little Brown 157.

Matsumoto K, Takeyasu K, Mizutani S, Hamamaka Y and Uozumi T, 1970. Plasma testosterone levels following surgical stress in male patients. Acta Endocrinology, 65, 11-17.

Mc Cance, Shelby, 1994. Stress and disease in (Mc Cance KL, Huether SE, eds) Pathophysiology. The Biologic Basis for Disease Stress and Disease in Adult and Children, 2nd ed. Baltimore : Mosby pp 2919-317.

Mc Connell J, Benson GS, Wood J, 1979. Autonomic innervation of the mammalian penis: A histochemical and physiological study. J. Neural Trans 45 : 227 - 238.

Melman A, Tiefer L and Pederson R. 1988. Evaluation of the first 406 patients in urology department based center for male sexual deviation. Urology, 32 : 6 - 10.

Meuleman EJH, 1994. Diagnosis of erectile dysfunction. In clinical urology (eds Krane RJ, Siroky MB, Fitzpatric JM). Philadelphia: JB Lippencott coy, p:1217.

Michal V and Rubarsky V. 1980. Histological change in the penile arterial bed with aging and diabetes. In Vasculogenic Impotence. Proceedings of the first International Conference on Corpus Cavernosum Revascularization. Springfield III, Charles C Thomas pp 113 - 119.

Mohan S et al, 1999. Regulation of law shear flow-induced HAEC VCAM-1 expression and monocyte adhesion. Am. J. Physiol 276.

National Institutes of Health Consensus Developments Conference on Impotence, Dec 7-9, 1992. Wellness Web. The Patients Network.

Neaton JD, Wentworth D, 1992. Serum cholesterol, blood pressure, cigarette smoking, and death from coronary hearth disease. Overal findings and differences by age for 316.099 white men. Ach Intern Med 152 : 56.

Noel GL, Suh HK, Stone JG and Franz AG, 1972. Human Prolactin and growth hormone release during surgery and other conditions of stress. Journal of Clinical Endocrinology and Metabolism, 35, 840 - 851.

Onuf (Onufrowicz) B, 1900. On the arrangement and function of the cell groups of the sacral region of the spinal cord in man. Arch. Neurol. Psychopathol 3 : 387 - 411.

Palmer RMJ, Ferrige AG, Moncada S, 1987. Nitric Oxide release accounts for biological activity of EDRF. Nature 333 : 664.

Pellerier KR, Herzing DL, 1988. Psychoneuroimmunology: Review. Advances, 5 (1), 27-56.

Perachio AA, Marr LD, Alexander M, 1979. Sexual behavior in male rhesus mongkeys elicited by electrical stimulation of preoptic and hypotholanic areas. Brain Res 177 : 127 – 144.

Peter K, Nawroth P, Conradt C, et al., 1997. Circulating Vascular Cell Adhesion Molecule-1 Correlates with the Extent of Human Atherosclerosis in Contrast to Circulating Intercellular Adhesion Molecule-1, E-Selectin, P-Selectin, and thrombomodulin. *Arterioscler Thromb Vasc Biol* 1997 ; 17 : 505 - 512

Pierce GN, Beamish RE, Dhalla NS. Sosiety , Ny HAPNER. Publishing 60, 188. Reseach model of diabetis mellitus in Heart dysfunction in Diabetis. Florida. CRC Press, inc. pp 23 – 25.

Poston Rol et al, 1992. Expression of intercellular adhesion molecule-I. in atherosclerotic plaques. Am J Pathol 140 : 665.

Putra ST, 1998. Perkembangan konsep Psikoneuroimunologi. Naskah, Kajian Ilmiah Psikoneuroimunologi Surabaya 12 November 1998.

Putra ST, 1999a. Stress Concept in Psychoneuroimmunology : Makalah kelompok Studi Psikoneuroimunologi FK Unair.

Putra ST, 1999b. History and concept of psychoneuroimmunology. Folia Medica Indonesia, XXXV, pp 1-4.

Riley, 1981. Psychoneuroendocrinology influences on immunocompetence and neoplasma, Science, 212.

Romagnani S, 1997. The Th 1 / Th 2 Paradigm. Imun. Today 18 : 263 – 266.

Rose RM, 1985. Psychoendocriinology. In JD Wilson and DW Foster (Eds.), William text book of endocrinology (7th ed.). Philadelphia: Saunders.

Ross R et al, 1993. The pathogenesis of athereosclerosis. A perspective for the 1990's. Nature 362 : 801.

Ross R, 1986. The pathogenesis of atherosclerosis an update. N. Engl. J. Med 314 : 488.

Roy AC, Tan SM, Kattegoda SR, and Ratnam SS, 1984. Ability of human corpora cavernosa muscle to generate prostaglandin and thromboxanes in vitro. Res. Comm. Syst. Med. Sci 12 : 610.

Sachs BD, Meisel RL, 1988. The physiology of male sexual behavior. In (Knobil E, Neill JD, Ewing L, Greenwarld GS, Markert CL, Pfaff DW eds). The physiology of reproduction Vol 2. New York : Karger, pp 1393 – 1423.

Saenz de Tejada 1, Carson MP, Tarish A, Eastman EH, Goldstein 1, 1989 b. Role of endothelin in the local control of penile smooth muscle tone. J Vasc. Med. Biol. 1 : 112.

Saenz de Tejada 1, Goldstein 1, Azadzoi K, Krane RJ and Cohen RA, 1989 a. Impaired neurogenic and endothelium mediated relaxation of penile smooth muscle from diabetic men with impotence. N Engl. J Med 320 : 1025 – 1030.

Sarica Y, Karacan I. 1987. Bulbocavernosus reflex to somatic and visceral nerve stimulation in normal subjects and in diabetics with erectile impotence. J Urol 130 : 55 - 58.

Schalach DS, 1967. The influence of physical stress and exercise on growth hormone and insulin secretion in man. Journal of Laboratory and Clinical Medicine, 69, 256-269.

Schoen FJ, 1994. Atherosclerosis and other forms of arteriosclerosis. In (Cortran, Kumar, Robbins eds) Pathologic basic of diseases 5th ed. Philadelphia : WB Saunders, pp 473 – 484.

Selby J, Mc Cance KL, 1998. Stress and Disease. In (Mc Cance KL, Huether SE eds) Pathophysiology. The Biologic Basis for Disease in Adult and Children 3rd ed. Baltimore pp 291-295.

Selye H, 1956. Stress of life, Mc Graw-Hill, New York.

Selye, 1950. Stress Acta in corporates markeal.

Shiu RPC, Friesen HG, 1980. Mechanismc of action of pro;actin in the control of mammary gland function. Annual Review of Physiology, 42, 83-96.

Simerly RB, Swanson LW, 1988. Projections of the medical preoptic nucleus: A phaseolus vulgaris leucoagglutinin anterograde tract-tracing in the rat. J. Comp. Neurol 270 : 209 –242.

Staines NA, Brostoff J, James K 1993 a. T Cell activation. In Introducing Immunology 2nd ed. London : M Mosby pp 23 - 27.

Stainess NA, Brostoff J, James K, 1993 b. Delayed type Hipersensitivity (type IV). In Introducing Immunology, Ed 2. London : M Mosby pp 109 - 110.

Steers WD, 1990. Neural Controle of Penile Erection Semi Urology. Urol : 66 –79.

Steers WD, Mc Connell J, Benson G, 1984. Anatomical localization and some pharmacological effects of VIP in human and monkey corpus covermosum. J Urol 132 : 1048 – 1053.

Stein N, 1985. Bereavement, depression, stress, and immunity. In R. Guillemin and M Cohen (Eds.). Neural modulation of immunity. New York: Raven Press, pp 29-44.

Stief C, Bernard F, Bosch R, Aboseif S, Nunes L, Lue TF, Tangho EA, 1990. Acetylcholine as a possible neurotransmitter in penile erction. J. Urol 141 : 1444 – 1448.

Stief CG, Bernard F, Bosch RJLH, Lue TF, Tanagho EA, 1989. Possible role of calcitonin-gene-related peptide in the regulations of Smooth muscle tone in the bladder and penis. J Urol. 143 : 392 - 397.

Sutherland VJ, Cooper CL, 1990. Understanding stress: Psychological Perspective for Health Professionals. Psychology and Health 5. Chapman and Hall, London

Thomas M, Mills, Vernon T. Wiedmeier, and Vivienne S. Stopper, 1992. Androgen Maintenance of Erectile Function in the Rat Penis. Biol. of Reprod. 46, 342 – 348.

Tjokroprawiro A, 1996. Penyulit Menahun Diabetes. Naskah lengkap Surabaya diabetes update – I, 16-17 Nop : pp 9.

Tjokroprawiro A, 1997. Aterosklerosis : Peran sindroma 29. Naskah Seminar Nasional XIII dan Konggres VIII. Perhimpunan Biokimia dan Biologi Molekuler Indonesia. Surabaya : 7 – 9 Juli 1997.

Tjokroprawiro A, 1999. Radikal bebas dan Diabetes melitus. Makalah pada Pertemuan Ilmiah Tahunan Nasional PERKEMI, Yogyakarta 8 - 9 Oktober 1999.

Tjokroprawiro A, Sutjahyo A, 1995. Radikal bebas dan DM. Simposium dampak negative radikal bebas pada organ tubuh dan manfaat antioksidan. Dies Natalis Unair XII FK Unair; Nov.

Vanhoutte PM, 1982. The end of the quest? Nature 327 : 459 – 460.

Wagner G, Uhrenholdt A, 1980. Blood flow measurement by the clearance method in the human corpus cavernosum in the flaccid and errect states. In (Zorgniotti AW, Rossi G Eds). Vasculogenic Impotence. Proceeding of the First International Conference on Corpus Cavernosum Revascularitation. Springfield, III, Charles C Thomas pp 41 – 46.

Walsh PC, Donker PJ. 1982. Impotence following radical postatectomy : Insight into etiology and prevention. J Urol 128 : 492 – 497.

Watkins SE, 1993. Psychometric asessment of diabetes impotence. BJ Psychiatry, June 1993.

Wagenforth HB, 1988. Experimental and surgical technic in rats. 1st ed London, Academic Press.

Wijaya A, 1998. Forum Diagnosticum. Prodia Diagnostic Educational Services : no 1, pp 11-20.

Witztum JL, Steinberg D, 1991. Role of Oxidized low density lipoprotein in atherogenesis. J Clin Invest 88 : 1785.

Yuwana R, 1993. The acceptance of intracavernous papaverin injection for ED. (Clinical based study). Proceeding APSIR meeting Bali.

Yuwana R, 1994. Prevalence, diagnostic and therapeutic options for ED in Semarang. The Second National Symposium on Impotence.

Yuwana R, 1998. Penatalaksanaan terapi pembedahan untuk DE. (pengalaman operasi prothesis penis) Muktamar IDI, Purwokerto.

Appendix 1. Body Weight Data

Body Weight rats during 5 days exposure

Sample	Before exp. 5 days	After exp. 5 days
A1	140	125
A2	141	127
A3	168	159
A4	139	135
A5	140	130
A6	165	246
A7	145	128
A8	130	122
A9	135	121
A10	165	151

Body Weight rats during 5 days without exposure

Sample	Before 5 days	After 5 days
CA1	160	162
CA2	165	167
CA3	165	167
CA4	145	145
CA5	160	165
CA6	140	155
CA7	140	145
CA8	160	165
CA9	140	150
CA10	140	142

Body Weight rats during 10 days exposure

Sample	Before exp. 10 days	After exp. 10 days
B1	153	165
B2	140	150
B3	151	109
B4	170	140
B5	140	149
B6	115	131
B7	118	146
B8	129	156
B9	120	137
B10	105	117

Body Weight rats during 10 days without exposure

Sample	Before 10 days	After 10 days
CB1	210	205
CB2	195	195
CB3	190	190
CB4	223	229
CB5	220	225
CB6	197	200
CB7	220	225
CB8	203	200
CB9	207	205
CB10	180	160

Appendix 2. Statistic Body Weight

Title 'Differencies between 5 days and 10 days (EF)'

Cell Means and Standard Deviations

Variable .. BW1

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	146.800	13.839	10	136.900	156.700
GROUP	10 Days	134.100	20.267	10	119.602	148.598
For entire sample		140.450	18.103	20	131.977	148.923

Variable .. BW2

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	144.400	37.819	10	117.346	171.454
GROUP	10 Days	140.000	17.250	10	127.660	152.340
For entire sample		142.200	28.697	20	128.769	155.631

Variable .. WEIGHT

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	-2.400	29.564	10	-23.549	18.749
GROUP	10 Days	5.900	23.197	10	-10.694	22.494
For entire sample		1.750	26.211	20	-10.517	14.017

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
BW1	806.45000	5420.50000	806.45000	301.13889	2.67800	.119
BW2	96.80000	15550.4000	96.80000	863.91111	.11205	.742
BERAT	344.45000	12709.3000	344.45000	706.07222	.48784	.494

Title 'Differencies between controls 5 days and 10 days (EF)'

Cell Means and Standard Deviations
Variable .. BW1

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	151.500	11.316	10	143.405	159.595
GROUP	10 Days	204.500	14.215	10	194.331	214.669
For entire sample		178.000	29.926	20	163.994	192.006

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Variable .. BW2

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	156.300	10.078	10	149.091	163.509
GROUP	10 Days	203.400	20.414	10	188.797	218.003
For entire sample		179.850	28.797	20	166.372	193.328

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Variable .. WEIGHT

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	4.800	4.541	10	1.551	8.049
GROUP	10 Days	-1.100	7.608	10	-6.542	4.342
For entire sample		1.850	6.808	20	-1.336	5.036

- - - - - - - - - - -
Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
BW1	14045.0000	2971.00000	14045.0000	165.05556	85.09256	.000
BW2	11092.0500	4664.50000	11092.0500	259.13889	42.80349	.000
BERAT	174.05000	706.50000	174.05000	39.25000	4.43439	.050

Title 'Differencies between EF exposure 5 days and controls 5 days

Cell Means and Standard Deviations
Variable .. BW1

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	146.800	13.839	10	136.900	156.700
GROUP	Control	151.500	11.316	10	143.405	159.595
For entire sample		149.150	12.537	20	143.282	155.018

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Variable .. BW2

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	144.400	37.819	10	117.346	171.454
GROUP	Control	156.300	10.078	10	149.091	163.509
For entire sample		150.350	27.620	20	137.423	163.277

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Variable .. WEIGHT

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	-2.400	29.564	10	-23.549	18.749
GROUP	Control	4.800	4.541	10	1.551	8.049
For entire sample		1.200	20.915	20	-8.588	10.988

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Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
BW1	110.45000	2876.10000	110.45000	159.78333	.69125	.417
BW2	708.05000	13786.5000	708.05000	765.91667	.92445	.349
BERAT	259.20000	8052.00000	259.20000	447.33333	.57943	.456

Title 'Differencies between EF exposure 10 days and controls 10 days

Cell Means and Standard Deviations
Variable .. BW1

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	134.100	20.267	10	119.602	148.598
GROUP	Control	204.500	14.215	10	194.331	214.669
For entire sample		169.300	39.932	20	150.611	187.989

- - - - - - - - - - -
Variable .. BW2

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	140.000	17.250	10	127.660	152.340
GROUP	Control	203.400	20.414	10	188.797	218.003
For entire sample		171.700	37.365	20	154.213	189.187

- - - - - - - - - - -
Variable .. WEIGHT

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	5.900	23.197	10	-10.694	22.494
GROUP	Control	-1.100	7.608	10	-6.542	4.342
For entire sample		2.400	17.181	20	-5.641	10.441

- - - - - - - - - - -
Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
BW1	24780.8000	5515.40000	24780.8000	306.41111	80.87435	.000
BW2	20097.8000	6428.40000	20097.8000	357.13333	56.27534	.000
BERAT	245.00000	5363.80000	245.00000	297.98889	.82218	.377

Appendix 3. Results Variables

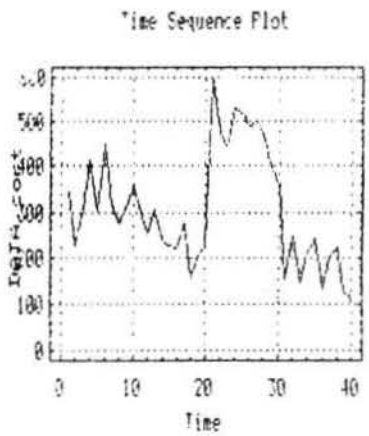
TABLE DATA

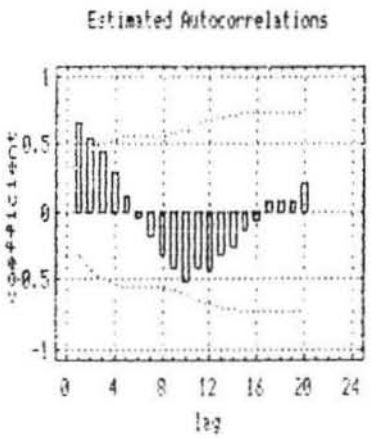
group	kel	cort	adre	noadre	bb1	bb2	il_2	ifn_g	il_4	il_10	vcam
1	1	342	.88	2.75	140	125	7	13	3	5	5
1	1	229	.84	1.99	141	127	11	22	6	4	21
1	1	301	.99	2.63	168	159	9	13	7	11	18
1	1	411	.93	2.32	139	135	6	12	4	7	18
1	1	303	.97	1.98	140	130	13	20	6	12	22
1	1	447	.95	2.67	165	246	9	19	6	8	12
1	1	312	.97	2.42	145	128	6	18	14	10	12
1	1	274	.82	1.89	130	122	21	24	23	19	27
1	1	313	.95	2.56	135	121	22	17	6	14	41
1	1	359	.81	2.67	165	151	15	17	9	10	29
2	1	298	.64	1.61	160	162	34	25	23	27	14
2	1	251	.46	1.32	165	167	27	21	16	16	2
2	1	301	.58	1.41	165	167	26	27	22	19	4
2	1	241	.39	1.29	145	145	34	28	21	10	8
2	1	229	.51	1.31	160	165	34	28	24	23	10
2	1	223	.48	1.57	140	155	27	28	25	19	7
2	1	276	.47	1.43	140	145	29	23	26	24	17
2	1	160	.61	.98	160	165	36	28	29	22	13
2	1	207	.52	1.28	140	150	42	38	24	29	17
2	1	221	.42	1.38	140	142	19	27	23	21	10
1	2	593	.87	1.97	153	165	2	6	5	3	4
1	2	472	.75	2.21	140	150	0	15	1	10	0
1	2	441	.81	2.35	151	109	0	4	2	1	5
1	2	532	.82	2.36	170	140	6	3	1	6	4
1	2	519	.75	2.05	140	149	5	20	4	2	3
1	2	488	.81	2.61	115	131	5	11	6	3	8
1	2	502	.72	2.52	118	146	0	6	1	4	0
1	2	473	.79	1.98	129	156	4	9	2	4	11
1	2	398	.82	2.43	120	137	0	6	1	3	0
1	2	364	.89	2.38	105	117	4	7	2	3	6
2	2	152	.59	1.28	210	205	41	44	36	21	4
2	2	247	.51	.99	195	195	29	29	24	19	12
2	2	146	.38	1.37	190	190	35	41	30	24	10
2	2	212	.55	1.27	223	229	28	37	22	25	7
2	2	240	.47	1.43	220	225	32	29	25	23	8
2	2	135	.39	.89	197	200	32	54	29	31	6
2	2	199	.41	1.17	220	225	28	37	19	28	11
2	2	223	.52	1.09	203	200	38	37	29	29	9
2	2	130	.37	.96	207	205	21	36	27	22	7
2	2	113	.45	1.07	180	160	33	30	25	21	8

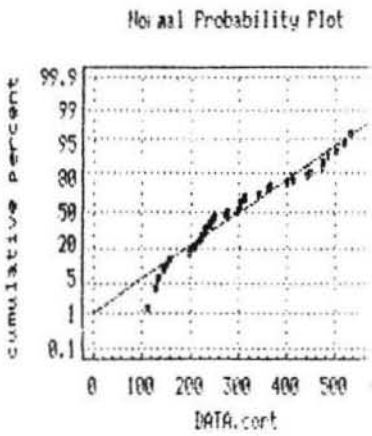
Result Cortisol and Cathecholamine

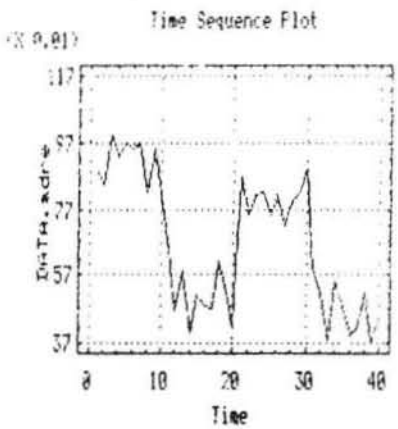
No.	Sampel	Kortisol (nmol/L)	Adrenalin (nmol/L)	Nor-adrenalin (nmol/L)
1	aA1	342	0.88	2.75
2	aA2	229	0.84	1.99
3	aA3	301	0.99	2.63
4	aA4	411	0.93	2.32
5	aA5	303	0.79	1.98
6	aA6	447	0.85	2.67
7	aA7	312	0.97	2.42
8	aA8	274	0.82	1.89
9	aA9	313	0.95	2.56
10	aA10	359	0.81	2.67
11	Ca1	298	0.64	1.61
12	Ca2	251	0.46	1.32
13	Ca3	301	0.58	1.41
14	Ca4	241	0.39	1.29
15	Ca5	229	0.51	1.31
16	Ca6	223	0.48	1.57
17	Ca7	276	0.47	1.43
18	Ca8	160	0.61	0.98
19	Ca9	207	0.52	1.28
20	Ca10	221	0.42	1.38
21	B1	593	0.87	1.97
22	B2	472	0.75	2.21
23	B3	441	0.81	2.35
24	B4	532	0.82	2.36
25	B5	519	0.75	2.05
26	B6	488	0.81	2.61
27	B7	502	0.72	2.52
28	B8	473	0.79	1.98
29	B9	398	0.82	2.43
30	B10	364	0.89	2.38
31	CB1	152	0.59	1.28
32	CB2	247	0.51	0.99
33	CB3	146	0.38	1.37
34	CB4	212	0.55	1.27
35	CB5	240	0.47	1.43
36	CB6	135	0.39	0.89
37	CB7	199	0.41	1.17
38	CB8	223	0.52	1.09
39	CB9	130	0.37	0.96
40	CB10	113	0.45	1.07

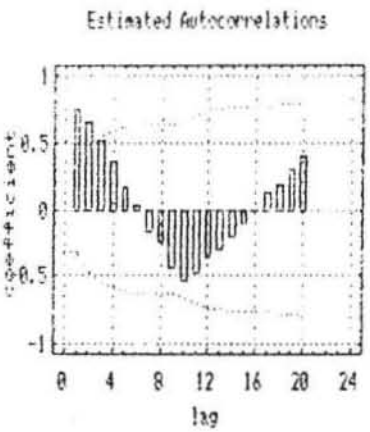
Appendix 4. IIDN test

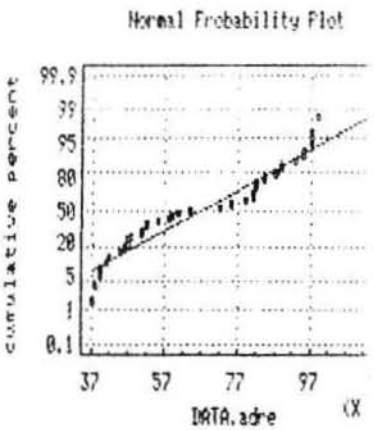


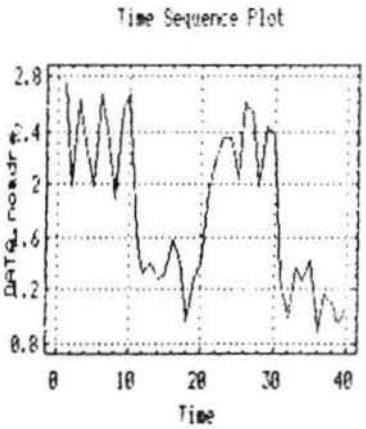


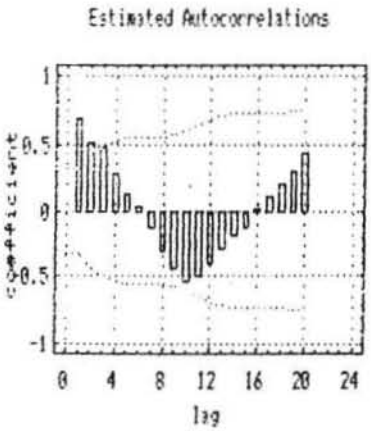


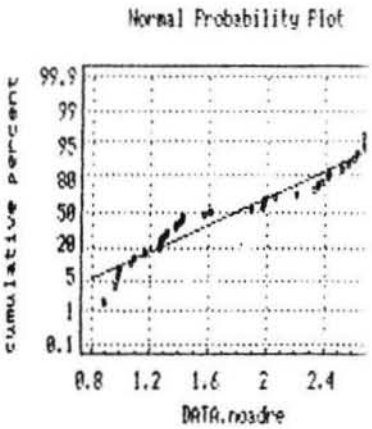




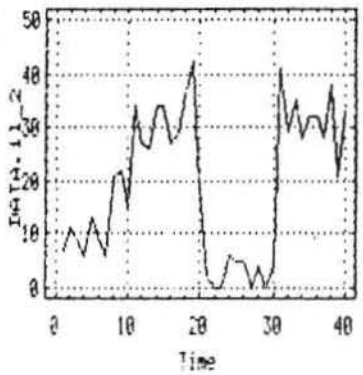




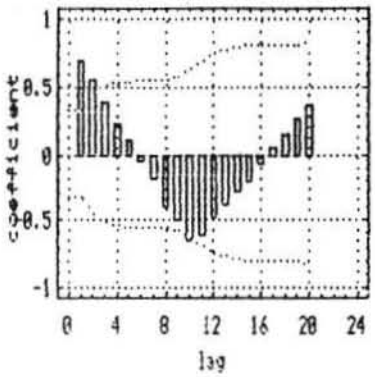




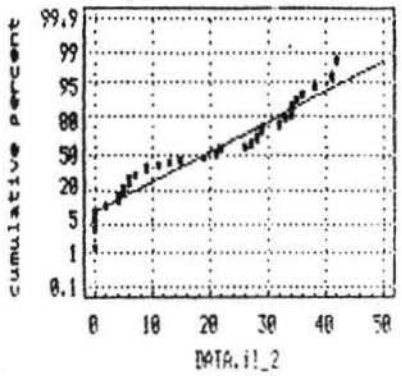
Time Sequence Plot



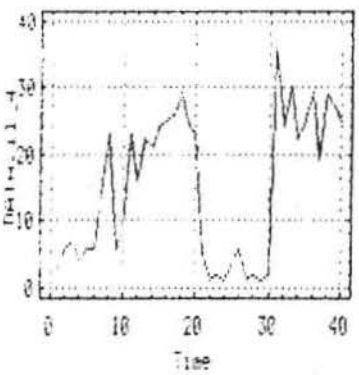
Estimated Autocorrelations



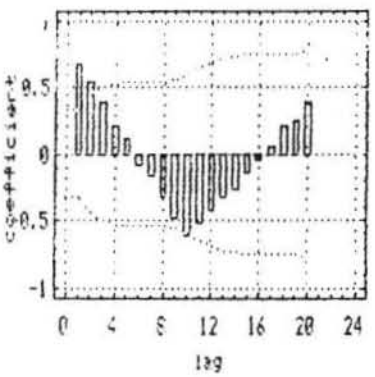
Normal Probability Plot



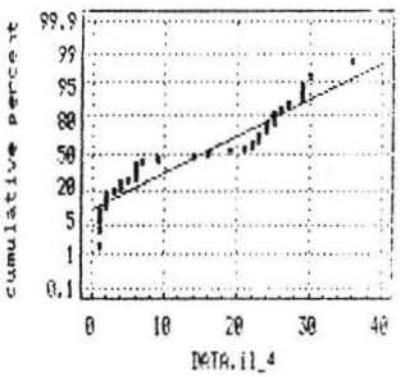
Time Sequence Plot



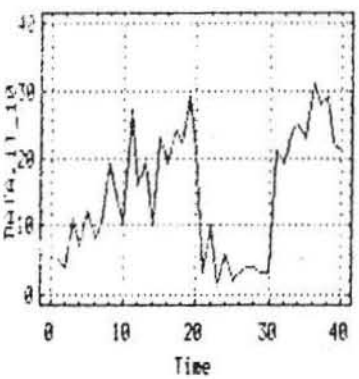
Estimated Autocorrelations



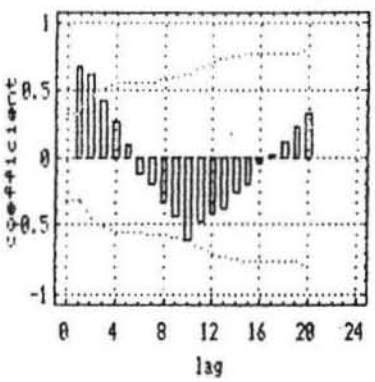
Normal Probability Plot



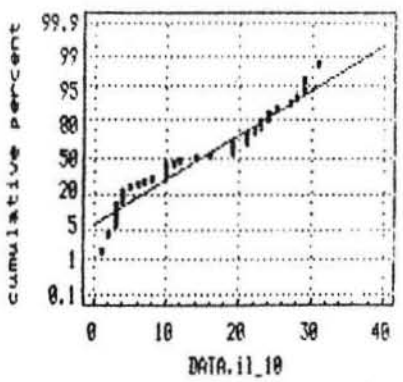
Time Sequence Plot

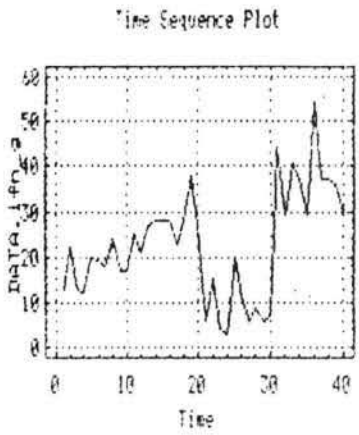


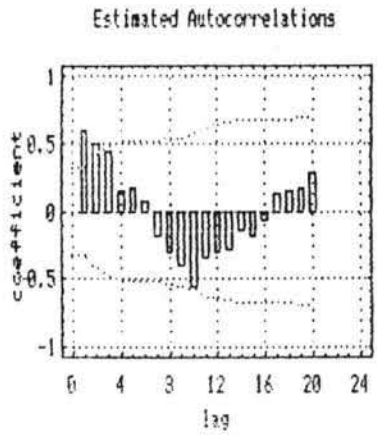
Estimated Autocorrelations

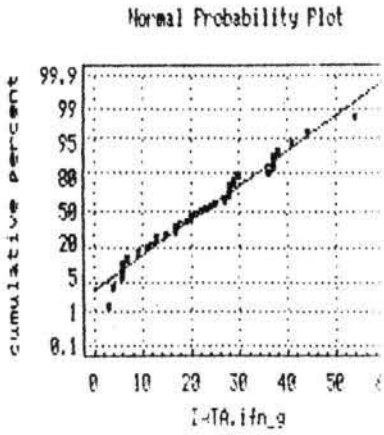


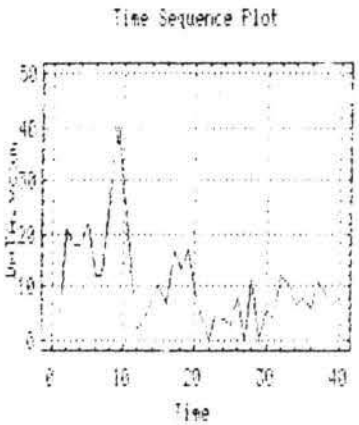
Normal Probability Plot

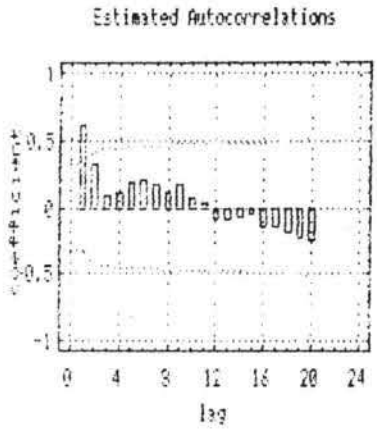


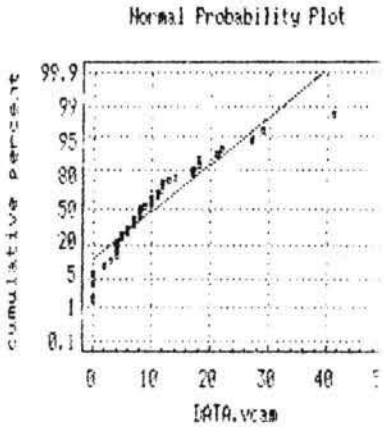

Time Sequence Plot
DATA.ifn_g
Time


Estimated Autocorrelations
coefficient
lag


Normal Probability Plot
cumulative percent
DATA.ifn_g


Time Sequence Plot
Time


Estimated Autocorrelations
lag


Normal Probability Plot
cumulative percent
DATA.vcam

Appendix 5. Statistic Exposure 5 days and Controls 5 days

Title 'Differencies between EF exposure 5 days and controls 5 days'.

```
PRO IF (kel=1).
MAN cort adre noadre il_2 ifn_g il_4 il_10 vcam BY group(1,2)/pri cell (all)/pri homo (all)/pri
signif (all)/disc/desig.

        20 cases accepted.
         0 cases rejected because of out-of-range factor values.
         0 cases rejected because of missing data.
         2 non-empty cells.
         1 design will be processed.

- - - - - - - - - -
              CELL NUMBER
                1    2
Variable
  GROUP         1    2

Cell Means and Standard Deviations
Variable .. CORT
     FACTOR           CODE                Mean  Std. Dev.         N   95 percent Conf. Interval

 GROUP            Experimen             329.100     63.898        10    283.390    374.810
 GROUP            Control               240.700     43.151        10    209.832    271.568
For entire sample                       284.900     69.803        20    252.231    317.569

- - - - - - - - - -
Variable .. ADRE
     FACTOR           CODE                Mean  Std. Dev.         N   95 percent Conf. Interval

 GROUP            Experimen               .911       .068        10       .863       .959
 GROUP            Control                 .508       .081        10       .450       .566
For entire sample                         .709       .219        20       .607       .812

- - - - - - - - - -
Variable .. NOADRE
     FACTOR           CODE                Mean  Std. Dev.         N   95 percent Conf. Interval

 GROUP            Experimen              2.388       .326        10      2.155      2.621
 GROUP            Control                1.358       .175        10      1.233      1.483
For entire sample                         1.873       .586        20      1.599      2.147

- - - - - - - - - -
Variable .. IL_2
     FACTOR           CODE                Mean  Std. Dev.         N   95 percent Conf. Interval

 GROUP            Experimen             11.900      5.840        10      7.723     16.077
 GROUP            Control               30.800      6.477        10     26.166     35.434
For entire sample                       21.350     11.403        20     16.013     26.687

- - - - - - - - - -
Variable .. IFN_G
     FACTOR           CODE                Mean  Std. Dev.         N   95 percent Conf. Interval

 GROUP            Experimen             17.500      3.979        10     14.654     20.346
 GROUP            Control               27.300      4.473        10     24.100     30.500
For entire sample                       22.400      6.500        20     19.358     25.442

- - - - - - - - - -
Variable .. IL_4
     FACTOR           CODE                Mean  Std. Dev.         N   95 percent Conf. Interval

 GROUP            Experimen              8.400      5.948        10      4.145     12.655
 GROUP            Control               23.300      3.401        10     20.867     25.733
For entire sample                       15.850      8.981        20     11.647     20.053
```

Variable .. IL_10

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	10.000	4.422	10	6.837	13.163
GROUP	Control	21.000	5.457	10	17.096	24.904
For entire sample		15.500	7.430	20	12.022	18.978

Variable .. VCAM

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	20.500	10.190	10	13.211	27.789
GROUP	Control	10.200	5.116	10	6.540	13.860
For entire sample		15.350	9.461	20	10.922	19.778

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. GROUP

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.97326	50.03843	8.00	11.00	.000
Hotellings	36.39159	50.03843	8.00	11.00	.000
Wilks	.02674	50.03843	8.00	11.00	.000
Roys	.97326				

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	36.39159	100.00000	100.00000	.98654

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	39072.8000	53505.0000	39072.8000	2972.50000	13.14476	.002
ADRE	.81205	.10045	.81205	.00558	145.51328	.000
NOADRE	5.30450	1.23092	5.30450	.06838	77.56881	.000
IL_2	1786.05000	684.50000	1786.05000	38.02778	46.96698	.000
IFN_G	480.20000	322.60000	480.20000	17.92222	26.79355	.000
IL_4	1110.05000	422.50000	1110.05000	23.47222	47.29207	.000
IL_10	605.00000	444.00000	605.00000	24.66667	24.52703	.000
VCAM	530.45000	1170.10000	530.45000	65.00556	8.16007	.010

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	43590.66655	56550.03137	5448.83332	392.70855	13.87501	.000

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. CONSTANT

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.99745	538.29621	8.00	11.00	.000
Hotellings	391.48815	538.29621	8.00	11.00	.000
Wilks	.00255	538.29621	8.00	11.00	.000
Roys	.99745				

- - - - - - - - - - -

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	391.48815	100.00000	100.00000	.99873

- - - - - - - - - - -

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	1623360.20	53505.0000	1623360.20	2972.50000	546.12622	.000
ADRE	10.06780	.10045	10.06780	.00558	1804.08626	.000
NOADRE	70.16258	1.23092	70.16258	.06838	1026.00194	.000
IL_2	9116.45000	684.50000	9116.45000	38.02778	239.73134	.000
IFN_G	10035.2000	322.60000	10035.2000	17.92222	559.93056	.000
IL_4	5024.45000	422.50000	5024.45000	23.47222	214.05941	.000
IL_10	4805.00000	444.00000	4805.00000	24.66667	194.79730	.000
VCAM	4712.45000	1170.10000	4712.45000	65.00556	72.49303	.000

- - - - - - - - - - -

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	1657133.98039	56550.03137	207141.74755	392.70855	527.46941	.000

Appendix 6. Statistic Exposure 10 days and Controls 10 days

Title 'Differencies between EF Exposure 10 days and Control 10 days'

```
PRO IF (kel=2).
MAN cort adre noadre il_2 ifn_g il_4 il_10 vcam BY group(1,2)/pri cell (all)/pri homo (all)/pri
signif (all)/disc/desig.

        20 cases accepted.
         0 cases rejected because of out-of-range factor values.
         0 cases rejected because of missing data.
         2 non-empty cells.
         1 design will be processed.

- - - - - - - - - -
              CELL NUMBER
                1    2
Variable
  GROUP         1    2

Cell Means and Standard Deviations
Variable .. CORT
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           Experimen             478.200     66.101        10     430.915     525.485
 GROUP           Control               179.700     49.751        10     144.111     215.289
For entire sample                      328.950    163.371        20     252.490     405.410

- - - - - - - - - -
Variable .. ADRE
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           Experimen               .803       .053        10        .765        .841
 GROUP           Control                 .464       .077        10        .409        .519
For entire sample                        .633       .185        20        .547        .720

- - - - - - - - - -
Variable .. NOADRE
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           Experimen              2.286       .225        10       2.125       2.447
 GROUP           Control                1.152       .182        10       1.022       1.282
For entire sample                       1.719       .615        20       1.431       2.007

- - - - - - - - - -
Variable .. IL_2
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           Experimen              2.600      2.459        10        .841       4.359
 GROUP           Control               31.700      5.658        10      27.653      35.747
For entire sample                      17.150     15.520        20       9.886      24.414

- - - - - - - - - -
Variable .. IFN_G
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           Experimen              8.700      5.293        10       4.914      12.486
 GROUP           Control               37.400      7.677        10      31.908      42.892
For entire sample                      23.050     16.061        20      15.533      30.567

- - - - - - - - - -
Variable .. IL_4
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           Experimen              2.500      1.841        10       1.183       3.817
 GROUP           Control               26.600      4.742        10      23.208      29.992
For entire sample                      14.550     12.849        20       8.536      20.564
```

Variable .. IL_10

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	3.900	2.514	10	2.101	5.699
GROUP	Control	24.300	3.917	10	21.498	27.102
For entire sample		14.100	10.944	20	8.978	19.222

Variable .. VCAM

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	Experimen	4.100	3.635	10	1.500	6.700
GROUP	Control	8.200	2.394	10	6.487	9.913
For entire sample		6.150	3.660	20	4.437	7.863

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. GROUP

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.98780	111.30866	8.00	11.00	.000
Hotellings	80.95175	111.30866	8.00	11.00	.000
Wilks	.01220	111.30866	8.00	11.00	.000
Roys	.98780				

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	80.95175	100.00000	100.00000	.99388

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	445511.250	61599.7000	445511.250	3422.20556	130.18249	.000
ADRE	.57461	.07845	.57461	.00436	131.84056	.000
NOADRE	6.42978	.75160	6.42978	.04176	153.98624	.000
IL_2	4234.05000	342.50000	4234.05000	19.02778	222.51942	.000
IFN_G	4118.45000	782.50000	4118.45000	43.47222	94.73751	.000
IL_4	2904.05000	232.90000	2904.05000	12.93889	224.44354	.000
IL_10	2080.80000	195.00000	2080.80000	10.83333	192.07385	.000
VCAM	84.05000	170.50000	84.05000	9.47222	8.87331	.008

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	458939.65439	63323.93005	57367.45680	439.74951	130.45485	.000

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. CONSTANT

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.99784	634.62802	8.00	11.00	.000
Hotellings	461.54765	634.62802	8.00	11.00	.000
Wilks	.00216	634.62802	8.00	11.00	.000
Roys	.99784				

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	461.54765	100.00000	100.00000	.99892

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
COPT	2164162.05	61599.7000	2164162.05	3422.20556	632.38809	.000
ADRE	8.02644	.07845	8.02644	.00436	1841.63202	.000
NOADRE	59.09922	.75160	59.09922	.04176	1415.36205	.000
IL_2	5882.45000	342.50000	5882.45000	19.02778	309.15066	.000
IFN_G	10626.0500	782.50000	10626.0500	43.47222	244.43310	.000
IL_4	4234.05000	232.90000	4234.05000	12.93889	327.23444	.000
IL_10	3976.20000	195.00000	3976.20000	10.83333	367.03385	.000
VCAM	756.45000	170.50000	756.45000	9.47222	79.85982	.000

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	2189704.37566	63323.93005	273713.04696	439.74951	622.42945	.000

Appendix 7. Statistic Different Control 5 days and Control 10 days

```
Title 'Different between control 5 days and control 10 days'

PRO IF (group=2).
MAN cort adre noadre il_2 ifn_g il_4 il_10 vcam BY kel(1,2)/pri cell (all)/pri homo (all)/pri signif
(all)/disc/desig.

        20 cases accepted.
         0 cases rejected because of out-of-range factor values.
         0 cases rejected because of missing data.
         2 non-empty cells.
         1 design will be processed.

- - - - - - - - - - -
              CELL NUMBER
                1    2
Variable
  GROUP         1    2

Cell Means and Standard Deviations
Variable .. CORT
     FACTOR           CODE                  Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 240.700     43.151        10    209.832    271.568
 GROUP           10 Days                179.700     49.751        10    144.111    215.289
For entire sample                       210.200     55.079        20    184.422    235.978

- - - - - - - - - - -
Variable .. ADRE
     FACTOR           CODE                  Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                   .508        .081        10       .450       .566
 GROUP           10 Days                  .464        .077        10       .409       .519
For entire sample                         .486        .080        20       .448       .524

- - - - - - - - - - -
Variable .. NOADRE
     FACTOR           CODE                  Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                  1.358        .175        10      1.233      1.483
 GROUP           10 Days                 1.152        .182        10      1.022      1.282
For entire sample                        1.255        .203        20      1.160      1.350

- - - - - - - - - - -
Variable .. IL_2
     FACTOR           CODE                  Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 30.800       6.477        10     26.166     35.434
 GROUP           10 Days                31.700       5.658        10     27.653     35.747
For entire sample                       31.250       5.937        20     28.471     34.029

- - - - - - - - - - -
Variable .. IFN_G
     FACTOR           CODE                  Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 27.300       4.473        10     24.100     30.500
 GROUP           10 Days                37.400       7.677        10     31.908     42.892
For entire sample                       32.350       8.015        20     28.599     36.101

- - - - - - - - - - -
Variable .. IL_4
     FACTOR           CODE                  Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 23.300       3.401        10     20.867     25.733
 GROUP           10 Days                26.600       4.742        10     23.208     29.992
For entire sample                       24.950       4.359        20     22.910     26.990

- - - - - - - - - - -
```

Variable .. IL_10

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	21.000	5.457	10	17.096	24.904
GROUP	10 Days	24.300	3.917	10	21.498	27.102
For entire sample		22.650	4.923	20	20.346	24.954

- - - - - - - - - - -

Variable .. VCAM

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	10.200	5.116	10	6.540	13.860
GROUP	10 Days	8.200	2.394	10	6.487	9.913
For entire sample		9.200	4.021	20	7.318	11.082

* * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. GROUP

- - - - - - - - - - -

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.54262	1.63125	8.00	11.00	.222
Hotellings	1.18636	1.63125	8.00	11.00	.222
Wilks	.45738	1.63125	8.00	11.00	.222
Roys	.54262				

- - - - - - - - - - -

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	1.18636	100.00000	100.00000	.73663

- - - - - - - - - - -

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	18605.0000	39034.2000	18605.0000	2168.56667	8.57940	.009
ADRE	.00968	.11240	.00968	.00624	1.55018	.229
NOADRE	.21218	.57192	.21218	.03177	6.67793	.019
IL_2	4.05000	665.70000	4.05000	36.98333	.10951	.745
IFN_G	510.05000	710.50000	510.05000	39.47222	12.92175	.002
IL_4	54.45000	306.50000	54.45000	17.02778	3.19772	.091
IL_10	54.45000	406.10000	54.45000	22.56111	2.41344	.138
VCAM	20.00000	287.20000	20.00000	15.95556	1.25348	.278

- - - - - - - - - - -

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	19248.22186	41410.88432	2406.02773	287.57559	8.36659	.000

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. CONSTANT

- - - - - - - - - - -

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.99430	239.95281	8.00	11.00	.000
Hotellings	174.51113	239.95281	8.00	11.00	.000
Wilks	.00570	239.95281	8.00	11.00	.000
Roys	.99430				

- - - - - - - - - - -

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	174.51113	100.00000	100.00000	.99715

- - - - - - - - - - -

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	883680.800	39034.2000	883680.800	2168.56667	407.49533	.000
ADRE	4.72392	.11240	4.72392	.00624	756.49964	.000
NOADRE	31.50050	.57192	31.50050	.03177	991.41317	.000
IL_2	19531.2500	665.70000	19531.2500	36.98333	528.10951	.000
IFN_G	20930.4500	710.50000	20930.4500	39.47222	530.25771	.000
IL_4	12450.0500	306.50000	12450.0500	17.02778	731.16117	.000
IL_10	10260.4500	406.10000	10260.4500	22.56111	454.78478	.000
VCAM	1692.80000	287.20000	1692.80000	15.95556	106.09471	.000

- - - - - - - - - - -

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	948582.02442	41410.88432	118572.75305	287.57559	412.31857	.000

Appendix 8. Statistic different Exposure 5 days and Exposure 10 days

Title 'Different between EF exposure 5 days and EF exposure 10 days

```
PRO IF (group=1).
MAN cort adre noadre il_2 ifn_g il_4 il_10 vcam BY kel(1,2)/pri cell (all)/pri homo (all)/pri signif
(all)/disc/desig.

        20 cases accepted.
         0 cases rejected because of out-of-range factor values.
         0 cases rejected because of missing data.
         2 non-empty cells.
         1 design will be processed.

- - - - - - - - - -
              CELL NUMBER
                1    2
Variable
  GROUP         1    2

Cell Means and Standard Deviations
Variable .. CORT
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                329.100     63.898        10    283.390    374.810
 GROUP           10 Days               478.200     66.101        10    430.915    525.485
For entire sample                      403.650     99.267        20    357.192    450.108

- - - - - - - - - -
Variable .. ADRE
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                  .911       .068        10       .863       .959
 GROUP           10 Days                 .803       .053        10       .765       .841
For entire sample                        .857       .081        20       .819       .895

- - - - - - - - - -
Variable .. NOADRE
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 2.388       .326        10      2.155      2.621
 GROUP           10 Days                2.286       .225        10      2.125      2.447
For entire sample                       2.337       .277        20      2.207      2.467

- - - - - - - - - -
Variable .. IL_2
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                11.900      5.840        10      7.723     16.077
 GROUP           10 Days                2.600      2.459        10       .841      4.359
For entire sample                       7.250      6.463        20      4.225     10.275

- - - - - - - - - -
Variable .. IFN_G
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                17.500      3.979        10     14.654     20.346
 GROUP           10 Days                8.700      5.293        10      4.914     12.486
For entire sample                      13.100      6.415        20     10.098     16.102

- - - - - - - - - -
Variable .. IL_4
     FACTOR           CODE                Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 8.400      5.948        10      4.145     12.655
 GROUP           10 Days                2.500      1.841        10      1.183      3.817
For entire sample                       5.450      5.246        20      2.995      7.905
```

- - - - - - - - - - -
Variable .. IL_10

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	10.000	4.422	10	6.837	13.163
GROUP	10 Days	3.900	2.514	10	2.101	5.699
For entire sample		6.950	4.696	20	4.752	9.148

- - - - - - - - - - -
Variable .. VCAM

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	20.500	10.190	10	13.211	27.789
GROUP	10 Days	4.100	3.635	10	1.500	6.700
For entire sample		12.300	11.235	20	7.042	17.558

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. GROUP
- - - - - - - - - - -
Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.86782	9.02785	8.00	11.00	.001
Hotellings	6.56571	9.02785	8.00	11.00	.001
Wilks	.13218	9.02785	8.00	11.00	.001
Roys	.86782				

- - - - - - - - - - -
Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	6.56571	100.00000	100.00000	.93157

- - - - - - - - - - -
Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	111154.050	76070.5000	111154.050	4226.13889	26.30156	.000
ADRE	.05832	.06650	.05832	.00369	15.78586	.001
NOADRE	.05202	1.41060	.05202	.07837	.66380	.426
IL_2	432.45000	361.30000	432.45000	20.07222	21.54470	.000
IFN_G	387.20000	394.60000	387.20000	21.92222	17.66244	.001
IL_4	174.05000	348.90000	174.05000	19.38333	8.97936	.008
IL_10	186.05000	232.90000	186.05000	12.93889	14.37913	.001
VCAM	1344.80000	1053.40000	1344.80000	58.52222	22.97931	.000

- - - - - - - - - - -
Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	113670.71034	78463.07710	14209.83879	544.88248	26.07872	.000

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. CONSTANT
- - - - - - - - - - -
Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.99828	797.56170	8.00	11.00	.000
Hotellings	580.04487	797.56170	8.00	11.00	.000
Wilks	.00172	797.56170	8.00	11.00	.000

Roys .99828

- - - - - - - - - - -

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	580.04487	100.00000	100.00000	.99914

- - - - - - - - - - -

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	3258666.45	76070.5000	3258666.45	4226.13889	771.07415	.000
ADRE	14.68898	.06650	14.68898	.00369	3975.96430	.000
NOADRE	109.23138	1.41060	109.23138	.07837	1393.84998	.000
IL_2	1051.25000	361.30000	1051.25000	20.07222	52.37337	.000
IFN_G	3432.20000	394.60000	3432.20000	21.92222	156.56260	.000
IL_4	594.05000	348.90000	594.05000	19.38333	30.64746	.000
IL_10	966.05000	232.90000	966.05000	12.93889	74.66252	.000
VCAM	3025.80000	1053.40000	3025.80000	58.52222	51.70344	.000

- - - - - - - - - - -

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	3267859.72036	78463.07710	408482.46505	544.88248	749.67076	.000

Appendix 9 Statistics Changes Exposure 5 days and 10 days

Title 'Differencies changes EF exposure 5 days and 10 days'

```
PRO IF (group=1).
MAN cort adre noadre il_2 ifn_g il_4 il_10 vcam BY kel(1,2)/pri cell (all)/pri homo (all)/pri signif
(all)/disc/desig.

        20 cases accepted.
         0 cases rejected because of out-of-range factor values.
         0 cases rejected because of missing data.
         2 non-empty cells.
         1 design will be processed.

- - - - - - - - - - -
              CELL NUMBER
                1    2
Variable
  GROUP         1    2

Cell Means and Standard Deviations
Variable .. CORT
     FACTOR           CODE                 Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                -88.400     77.027        10   -143.502    -33.298
 GROUP           10 Days              -298.500     62.443        10   -343.169   -253.831
For entire sample                     -193.450    127.569        20   -253.154   -133.746

- - - - - - - - - - -
Variable .. ADRE
     FACTOR           CODE                 Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                  -.403       .106        10      -.479      -.327
 GROUP           10 Days                 -.339       .085        10      -.400      -.278
For entire sample                        -.371       .099        20      -.417      -.325

- - - - - - - - - - -
Variable .. NOADRE
     FACTOR           CODE                 Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 -1.030       .226        10     -1.191      -.869
 GROUP           10 Days                -1.134       .348        10     -1.383      -.885
For entire sample                       -1.082       .290        20     -1.218      -.946

- - - - - - - - - - -
Variable .. IL_2
     FACTOR           CODE                 Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 18.900      6.839        10     14.008     23.792
 GROUP           10 Days                29.100      5.607        10     25.089     33.111
For entire sample                       24.000      8.026        20     20.244     27.756

- - - - - - - - - - -
Variable .. IFN_G
     FACTOR           CODE                 Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                  9.800      6.356        10      5.253     14.347
 GROUP           10 Days                28.700     10.709        10     21.039     36.361
For entire sample                       19.250     12.941        20     13.194     25.306

- - - - - - - - - - -
Variable .. IL_4
     FACTOR           CODE                 Mean  Std. Dev.         N  95 percent Conf. Interval

 GROUP           5 Days                 14.900      4.458        10     11.711     18.089
 GROUP           10 Days                24.100      3.872        10     21.330     26.870
For entire sample                       19.500      6.228        20     16.585     22.415

- - - - - - - - - - -
```

Variable .. IL_10

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	11.000	5.617	10	6.982	15.018
GROUP	10 Days	20.400	5.211	10	16.672	24.128
For entire sample		15.700	7.146	20	12.356	19.044

- - - - - - - - - - -

Variable .. VCAM

FACTOR	CODE	Mean	Std. Dev.	N	95 percent Conf. Interval	
GROUP	5 Days	-10.300	10.563	10	-17.856	-2.744
GROUP	10 Days	4.100	4.909	10	.588	7.612
For entire sample		-3.100	10.901	20	-8.202	2.002

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. GROUP

- - - - - - - - - - -

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.84033	7.23657	8.00	11.00	.002
Hotellings	5.26296	7.23657	8.00	11.00	.002
Wilks	.15967	7.23657	8.00	11.00	.002
Roys	.84033				

- - - - - - - - - - -

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	5.26296	100.00000	100.00000	.91670

- - - - - - - - - - -

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	220710.050	88490.9000	220710.050	4916.16111	44.89480	.000
ADRE	.02048	.16570	.02048	.00921	2.22474	.153
NOADRE	.05408	1.54624	.05408	.08590	.62955	.438
IL_2	520.20000	703.80000	520.20000	39.10000	13.30435	.002
IFN_G	1786.05000	1395.70000	1786.05000	77.53889	23.03425	.000
IL_4	423.20000	313.80000	423.20000	17.43333	24.27533	.000
IL_10	441.80000	528.40000	441.80000	29.35556	15.04996	.001
VCAM	1036.80000	1221.00000	1036.80000	67.83333	15.28452	.001

- - - - - - - - - - -

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	224918.17456	92655.31194	28114.77182	643.43967	43.69450	.000

* * * * * ANALYSIS OF VARIANCE -- DESIGN 1 * * * * *

EFFECT .. CONSTANT

- - - - - - - - - - -

Multivariate Tests of Significance (S = 1, M = 3 , N = 4 1/2)

Test Name	Value	Approx. F	Hypoth. DF	Error DF	Sig. of F
Pillais	.99188	168.02635	8.00	11.00	.000
Hotellings	122.20098	168.02635	8.00	11.00	.000
Wilks	.00812	168.02635	8.00	11.00	.000
Roys	.99188				

- - - - - - - - - - -

Eigenvalues and Canonical Correlations

Root No.	Eigenvalue	Pct.	Cum. Pct.	Canon Cor.
1	122.20098	100.00000	100.00000	.99593

- - - - - - - - - - -

Univariate F-tests with (1,18) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
CORT	748458.050	88490.9000	748458.050	4916.16111	152.24441	.000
ADRE	2.75282	.16570	2.75282	.00921	299.03898	.000
NOADRE	23.41448	1.54624	23.41448	.08590	272.57130	.000
IL_2	11520.0000	703.80000	11520.0000	39.10000	294.62916	.000
IFN_G	7411.25000	1395.70000	7411.25000	77.53889	95.58107	.000
IL_4	7605.00000	313.80000	7605.00000	17.43333	436.23327	.000
IL_10	4929.80000	528.40000	4929.80000	29.35556	167.93414	.000
VCAM	192.20000	1221.00000	192.20000	67.83333	2.83342	.110

- - - - - - - - - - -

Averaged F-test with (8,144) D. F.

VARIABLES	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
1 to 8	780142.46730	92655.31194	97517.80841	643.43967	151.55704	.000

Appendix 10. Discriminant Analysis

Title 'Differencies changes EF exposure 5 days and 10 days'

```
PRO IF (group=1).
dsc group kel(1,2)/VAR cort adre noadre il_2 ifn_g il_4 il_10 vcam /met rao/PIN=0.5/POUT=0.5/ana
all/stat all.

- - - - - - - - - - - - - - -  D I S C R I M I N A N T   A N A L Y S I S  - - - - - - - - - - - - - - -

On groups defined by GROUP

           20 (unweighted) cases were processed.
            0 of these were excluded from the analysis.
           20 (unweighted) cases will be used in the analysis.

Number of Cases by Group

              Number of Cases
  GROUP     Unweighted     Weighted  Label
        1           10         10.0  5 Hari
        2           10         10.0  10 Hari
    Total           20         20.0

Group Means

  GROUP       CORT       ADRE      NOADRE      IL_2       IFN_G      IL_4       IL_10      VCAM
     1   -88.40000     -.40300   -1.03000   18.90000    9.80000   14.90000   11.00000  -10.30000
     2  -298.50000     -.33900   -1.13400   29.10000   28.70000   24.10000   20.40000    4.10000
 Total  -193.45000     -.37100   -1.08200   24.00000   19.25000   19.50000   15.70000   -3.10000

Group Standard Deviations

  GROUP       CORT       ADRE      NOADRE      IL_2       IFN_G      IL_4       IL_10      VCAM
     1     77.02698     .10605     .22568    6.83862    6.35610    4.45845    5.61743   10.56251
     2     62.44331     .08465     .34767    5.60654   10.70877    3.87155    5.21110    4.90918
 Total    127.56855     .09899     .29022    8.02627   12.94065    6.22812    7.14585   10.90099


Wilks' Lambda (U-statistic) and univariate F-ratio
with    1 and         18 degrees of freedom

 Variable    Wilks' Lambda          F          Significance
 --------    -------------    -------------    ------------
 CORT             .28619           44.89            .0000
 ADRE             .89000           2.225            .1531
 NOADRE           .96621           .6296            .4379
 IL_2             .57500           13.30            .0018
 IFN_G            .43866           23.03            .0001
 IL_4             .42578           24.28            .0001
 IL_10            .54463           15.05            .0011
 VCAM             .54079           15.28            .0010


- - - - - - - - - - - - - - - -  D I S C R I M I N A N T   A N A L Y S I S  - - - - - - - - - - - -

On groups defined by GROUP

Analysis number       1

Stepwise variable selection

     Selection rule:  Maximize Rao's V
     Maximum number of steps.....................      16
     Minimum Tolerance Level.....................  .00100
     Maximum significance of F to enter.....  .50000
```

```
     Minimum significance of F to remove.....  .50000
     Minimum increase in Rao's V.............  .00000

Canonical Discriminant Functions

     Maximum number of functions.............       1
     Minimum cumulative percent of variance...  100.00
     Maximum significance of Wilks' Lambda....  1.0000

Prior probability for each group is  .50000

---------------- Variables not in the analysis after step   0 ----------------

                    Minimum   Signif. of
Variable  Tolerance Tolerance F to enter        Rao's V
CORT      1.0000000 1.0000000     .0000        44.89480
ADRE      1.0000000 1.0000000     .1531        2.224744
NOADRE    1.0000000 1.0000000     .4379        .6295530
IL_2      1.0000000 1.0000000     .0018        13.30435
IFN_G     1.0000000 1.0000000     .0001        23.03425
IL_4      1.0000000 1.0000000     .0001        24.27533
IL_10     1.0000000 1.0000000     .0011        15.04996
VCAM      1.0000000 1.0000000     .0010        15.28452

* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
At step   1, CORT     was included in the analysis.

                                 Degrees of Freedom  Signif.   Between Groups
Wilks' Lambda          .28619        1     1        18.0
Equivalent F         44.8948               1        18.0   .0000
RAO'S V             44.89480               1                .0000 (APPROX.)

---------------- Variables in the analysis after step   1 ----------------
                    Signif. of
Variable  Tolerance F to remove       Rao's V
CORT      1.0000000     .0000

---------------- Variables not in the analysis after step   1 ----------------

                    Minimum   Signif. of
Variable  Tolerance Tolerance F to enter        Rao's V
ADRE       .9823813  .9823813     .2286        50.66530
NOADRE     .9983070  .9983070     .7909
IL_2       .9879889  .9879889     .1460        53.48461
IFN_G      .7650960  .7650960     .3692        48.04281
IL_4       .8918577  .8918577     .1521        53.21219
IL_10      .9952958  .9952958     .0372        63.81058
VCAM       .9770617  .9770617     .0191        69.71319

F statistics and significances between pairs of groups after step   1
Each F statistic has   1 and       18.0 degrees of freedom.

               Group          1
                        5 Days
   Group
       2   10 Days         44.895
                            .0000

* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
At step   2, VCAM     was included in the analysis.

                                 Degrees of Freedom  Signif.   Between Groups
Wilks' Lambda          .20521        2     1        18.0
Equivalent F         32.9201               2        17.0   .0000
RAO'S V             69.71319               2                .0000 (APPROX.)

---------------- Variables in the analysis after step   2 ----------------
                    Signif. of
Variable  Tolerance F to remove       Rao's V
CORT       .9770617     .0001
```

```
VCAM        .9770617     .0191

---------------- Variables not in the analysis after step   2 ----------------

                       Minimum    Signif. of
Variable   Tolerance  Tolerance  F to enter         Rao's V
ADRE       .9768671   .9628780      .3977          73.85305
NOADRE     .9963360   .9748638      .9006
IL_2       .8720615   .8624165      .5855
IFN_G      .7382371   .7382371      .2563          77.31143
IL_4       .8807692   .8631090      .3318          75.20335
IL_10      .9793509   .9614089      .1289          83.76687

F statistics and significances between pairs of groups after step   2
Each F statistic has   2 and       17.0 degrees of freedom.

              Group        1
                       5 Days
   Group
      2  10 Days          32.920
                            .0000

* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
At step   3, IL_10    was included in the analysis.

                                  Degrees of Freedom  Signif.   Between Groups
Wilks' Lambda          .17687       3     1        18.0
Equivalent F          24.8198             3        16.0    .0000
RAO'S V               83.76687            3                .0000 (APPROX.)

---------------- Variables in the analysis after step   3 ----------------
                      Signif. of
Variable   Tolerance  F to remove        Rao's V
CORT        .9747041     .0004
IL_10       .9793509     .1289
VCAM        .9614089     .0657

---------------- Variables not in the analysis after step   3 ----------------

                       Minimum    Signif. of
Variable   Tolerance  Tolerance  F to enter         Rao's V
ADRE       .9733787   .9549504      .3987          88.88683
NOADRE     .9687946   .9522790      .9006
IL_2       .8618564   .8564719      .7337
IFN_G      .5710264   .5710264      .6773
IL_4       .8136887   .8136887      .6088

F statistics and significances between pairs of groups after step   3
Each F statistic has   3 and       16.0 degrees of freedom.

              Group        1
                       5 Days
   Group
      2  10 Days          24.820
                            .0000

* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
At step   4, ADRE     was included in the analysis.

                                  Degrees of Freedom  Signif.   Between Groups
Wilks' Lambda          .16840       4     1        18.0
Equivalent F          18.5181             4        15.0    .0000
RAO'S V               88.88683            4                .0000 (APPROX.)

---------------- Variables in the analysis after step   4 ----------------
                      Signif. of
Variable   Tolerance  F to remove        Rao's V
CORT        .9598546     .0005
ADRE        .9733787     .3987
IL_10       .9758536     .1380
VCAM        .9549504     .0997
```

-------------- Variables not in the analysis after step 4 ---------------

Variable	Tolerance	Minimum Tolerance	Signif. of F to enter	Rao's V
NOADRE	.8872767	.8872767	.9038	
IL_2	.8580711	.8480364	.7083	
IFN_G	.5254722	.5254722	.5211	
IL_4	.7614945	.7614945	.4812	92.88556

F statistics and significances between pairs of groups after step 4
Each F statistic has 4 and 15.0 degrees of freedom.

	Group 1 5 Days
Group 2 10 Days	18.518 .0000

* *

At step 5, IL_4 was included in the analysis.

		Degrees of Freedom			Signif.	Between Groups
Wilks' Lambda	.16233	5	1	18.0		
Equivalent F	14.4489		5	14.0	.0000	
RAO'S V	92.88556		5		.0000 (APPROX.)	

---------------- Variables in the analysis after step 5 ----------------

Variable	Tolerance	Signif. of F to remove	Rao's V
CORT	.8445632	.0094	
ADRE	.9109410	.3386	
IL_4	.7614945	.4812	
IL_10	.9046148	.2536	
VCAM	.9448648	.1425	

---------------- Variables not in the analysis after step 5 ----------------

Variable	Tolerance	Minimum Tolerance	Signif. of F to enter	Rao's V
NOADRE	.8482231	.7279772	.7944	
IL_2	.6386186	.5667415	.9863	
IFN_G	.4913469	.4913469	.6506	

F statistics and significances between pairs of groups after step 5
Each F statistic has 5 and 14.0 degrees of freedom.

	Group 1 5 Days
Group 2 10 Days	14.449 .0000

F level or tolerance or VIN insufficient for further computation.□ □

Summary Table

Step	Action Entered	Removed	Vars In	Wilks' Lambda	Sig.	Rao's V	Sig.	Change in V	Sig.	Label
1	CORT		1	.28619	.0000	44.89480	.0000	44.89480	.0000	
2	VCAM		2	.20521	.0000	69.71319	.0000	24.81839	.0000	
3	IL_10		3	.17687	.0000	83.76687	.0000	14.05368	.0002	
4	ADRE		4	.16840	.0000	88.88683	.0000	5.11996	.0237	
5	IL_4		5	.16233	.0000	92.88556	.0000	3.99873	.0455	

Classification Function Coefficients
(Fisher's Linear Discriminant Functions)

GROUP =	1 5 Days	2 10 Days
CORT	-.7534718E-03	+.4504974E-01
ADRE	+35.20852	+22.04504
IL_4	.5639694	-.8094136
IL_10	-.2734687	.5505554
VCAM	+.1543054	-.7129820E-01
(constant)	-14.25468	-26.66872

Canonical Discriminant Functions

Function	Eigenvalue	Percent of Variance	Cumulative Percent	Canonical Correlation	After Function	Wilks' Lambda	Chi-squared	D.F.	Signif
					0	.1623295	28.181	5	.0000
1*	5.16031	100.00	100.00	.9152434					

* marks the 1 canonical discriminant functions remaining in the analysis.

Standardized Canonical Discriminant Function Coefficients

	FUNC 1
CORT	-.74511
ADRE	.29303
IL_4	.23777
IL_10	.34831
VCAM	.43110

Structure Matrix:
Pooled within-groups correlations between discriminating variables
and canonical discriminant functions
(Variables ordered by size of correlation within function)

	FUNC 1
CORT	-.69522
IL_4	.51122
VCAM	.40565
IL_10	.40253
IFN_G	.39951
IL_2	.37424
ADRE	.15476
NOADRE	-.00807

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On groups defined by GROUP

Analysis number 2
Direct method: All variables passing the tolerance test are entered.
Minimum Tolerance Level..... .00100

Canonical Discriminant Functions

Maximum number of functions..... 1
Minimum cumulative percent of variance... 100.00
Maximum significance of Wilks' Lambda.... 1.0000

Prior probability for each group is .50000

Classification Function Coefficients
(Fisher's Linear Discriminant Functions)

GROUP =	1 5 Hari	2 10 Hari

CORT	-.6269965E-01	-.1036748
ADRE	-21.48398	-6.773362
NOADRE	-43.69553	43.65737
IL_2	1.737529	1.725575
IFN_G	-1.620777	-1.520570
IL_4	.5852805	.7949907
IL_10	.7710228	.9914122
VCAM	-1.020818	-.7689234
(constant)	-52.63271	-63.47112

Canonical Discriminant Functions

Function	Eigenvalue	Percent of Variance	Cumulative Percent	Canonical Correlation	After Function	Wilks' Lambda	Chi-squared	D.F.	Signif
					0	.1596689	25.685	8	.0012
1*	5.26296	100.00	100.00	.9166958					

* marks the 1 canonical discriminant functions remaining in the analysis.

Standardized Canonical Discriminant Function Coefficients

	FUNC 1
CORT	-.66004
ADRE	.32426
NOADRE	.00257
IL_2	-.01717
IFN_G	.20272
IL_4	.20116
IL_10	.27433
VCAM	.47662

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On groups defined by GROUP

Analysis number.. 1

Number of Canonical Discriminant Functions.. 1

List of the 5 Variables used..

Variable	Label
CORT	
ADRE	
IL_4	
IL_10	
VCAM	

Classification Results -

Actual Group	No. of Cases	Predicted Group Membership 1	2
Group 1	10	10	0
5 Days		100.0%	.0%
Group 2	10	0	10
10 Days		.0%	100.0%

Percent of "grouped" cases correctly classified: 100.00%

Classification Processing Summary
20 Cases were processed.
0 Cases were excluded for missing or out-of-range group codes.
0 Cases had at least one missing discriminating variable.
20 Cases were used for printed output.

- - - - - - - - - - - - - - - D I S C R I M I N A N T A N A L Y S I S - - - - - - - - - - - - - - - - - -

On groups defined by GROUP

Analysis number.. 2

Number of Canonical Discriminant Functions.. 1

List of the 8 Variables used..

Variable	Label
CORT	
ADRE	
NOADRE	
IL_2	
IFN_G	
IL_4	
IL_10	
VCAM	

Classification Results -

Actual Group		No. of Cases	Predicted Group Membership 1	2
Group	1	10	10	0
5 Days			100.0%	.0%
Group	2	10	0	10
10 Days			.0%	100.0%

Percent of "grouped" cases correctly classified: 100.00%

Classification Processing Summary
        20 Cases were processed.
         0 Cases were excluded for missing or out-of-range group codes.
         0 Cases had at least one missing discriminating variable.
        20 Cases were used for printed output.

Appendix 11 Statistic Reliability Researcher & observer

T-Test

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Researcher	2,90	20	2,22	,50
	Observer	3,10	20	2,15	,48

Paired Samples Correlations

	N	Correlation	Sig.
Pair 1 Researcher & Observer	20	,961	,000

Paired Samples Test

	Paired Differences			t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean			
Pair 1 Researcher Observer	-,20	,62	,14	-1,453	19	,163

Correlations

Correlations

		Researcher	Observer
Researcher	Pearson Correlation	1,000	,961**
	Sig. (2-tailed)	.	,000
	N	20	20
Observer	Pearson Correlation	,961**	1,000
	Sig. (2-tailed)	,000	.
	N	20	20

**. Correlation is significant at the 0.01 level (2-tailed).

Curve Fit

```
MODEL:  MOD_1.

Dependent variable.. PEN                Method.. LINEAR

Listwise Deletion of Missing Data

Multiple R           ,96088
R Square             ,92328
Adjusted R Square    ,91902
Standard Error       ,63228
```