EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT FOR THE DEGREEE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY (FACULTY OF SCIENCE)

BY

Mrs. BINDU. M. P.

AMALA CANCER HOSPITAL AND RESEARCH CENTRE,
AMALA NAGAR
THRISSUR-680553
Kerala, India.

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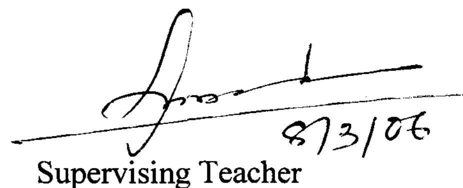
CERTIFICATE

Certified that this thesis entitled **"The effect of different types of stress on lipid metabolism and antioxidant status in albino rats"** is a bonafied record of research work carried out by Bindu. M.P under my guidance and supervision in Amala Cancer Hospital and Research Centre, Thrissur and same has been not submitted for any other degree, title or associateship.



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Professor of Biochemistry

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DECLARATION

I hereby declare that this thesis entitled **"The effect of different types of stress on lipid metabolism and antioxidant status in albino rats"** is a bonafied record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any other degree, diploma, associateship or other similar title of any other university or society.

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DEDICATED TO MY BELOVED FATHER

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

INTRODUCTION

Stress is defined as the total response of an organism to environmental demands or pressure. It is expressed as an external response that can be measured by changes in glandular secretions, skin reactions and other physical functions or it is an internal interpretation of a reaction or reaction to a stressor or it is both. During the stress response, somewhere in the neighborhood, several biochemical reactions can occur in the body. Neurotransmitters are activated, hormones are released, and nutrients are metabolized. Some body systems (e.g., the cardiovascular system) accelerate their functions and others (e.g., the gastrointestinal system) slow down their operations in response to stress. This is commonly referred to as the fight or flight response. The body is being prepared to expend physical energy, which in prehistoric times was necessary for survival. In modern times most human stress is psychosocial in nature, so the need to respond physically in most cases is unnecessary. Unfortunately the by products of the stress response continue to circulate in the body and have the potential to create physical illness (e.g., cortisol secretion's impact on the immune system). Regular exercise is useful in removing the byproducts of the stress response by providing the opportunity to simulate the fighting or running away dictated by the fight or flight phenomenon. As such, regular exercise allows the body to return to homeostasis faster and reduce the physical impact of psychosocial stress. Some key factors about the brain are that adrenaline aids in awareness and memory in the short term. Prolonged exposure of the brain to corticosteroid from continued stress can be very damaging, both decreasing hippocampus function and decreasing cell proliferation rates.

Generally, the body acclimatizes in a variety of ways depending upon the environmental stresses to which it is exposed. Passive stresses are those, which are persistent and relatively invariant (e.g., altitude, climate) and active stresses are those, which are occasionally variable (e.g., exercise, emotions, diet). Reactions to excessive stresses are

modified by the individual attributes of each person. Stress in humans results from interaction between persons and their environment that are perceived as straining or exceeding their adaptive capabilities and threatening their well - being. All systems: the heart and blood vessels, the immune system, the lungs, the digestive system, the sensory organs and brain are modified to meet the perceived danger. The causes of stress can include any event or occurrence that a person considers a threat to his or her coping strategies or resources.

Researchers generally agree that a certain degree of stress is a normal part of living organism's response to the inevitable changes in its physical or social environment, and that positive, as well as negative events can generate stress as well as negative occurrences. The symptoms of stress can be either physical or psychological. Stress related physical illness, such as irritable bowel syndrome, heart attack, and chronic headache result from long term over stimulation of a part of the nervous system that regulates the heart rate, blood pressure and digestive system.

External and internal stressors

People can experience either external or internal stressors. External stressors include adverse physical conditions (such as pain or hot or cold temperatures) or stressful psychological environments (such as poor working conditions or abuse relationships). Humans, like animals can sometimes experience external stressors. Internal stressors can also be physical (infection, inflammation) or psychological. An example of an internal psychological stressor is intense worry about a harmful event that may or may not occur.

Acute and chronic stress

Stressors can be short term (acute) or long term (chronic). Acute stress is the reaction to an immediate threat, commonly known as the fight or flight response.

Common acute stressors include

Noise

Crowding

Isolation

Hunger

Infection

Imagining a threat or remembering a dangerous event.

Modern life poses on-going stressful situations that are not short lived and the urge to act (to fight or to flee) must be suppressed.

Common chronic stressors include

Ongoing highly pressured work

Long-term relationship problems

Loneliness

Persistent financial worries

Effect of acute and chronic stress

The part of the hypothalamic-pituitary- adrenal (HPA) system is activated by stress. HPA system triggers the production and release of steroid hormones (glucocorticoids : cortisol). Cortisol is very important in marshaling systems throughout the body (including the heart, lungs, circulation, metabolism, immune systems and skin)

Hypothalamic pituitary- adrenal (HPA) system also releases certain neurotransmitters such as dopamine, norepinephrine and epinephrine. Neurotransmitters signal the hippocampus to store emotionally loaded experience in long-term memory. During the stressful event catecholamines also suppress activity in the frontal areas of brain concerned with short-term memory, concentration, inhibition and rational thought. Heart rate and blood pressure increase immediately. Breathing becomes rapid and lungs take in more oxygen.

Blood flow may actually increase 300% to 400%, priming the muscles, lungs and brain for added demands. Spleen discharges red and white blood cells, allowing the blood to transport more oxygen. The immune boosting troops are sent to the body's front lines where injury or infection is most likely, such as the skin, the bone marrow, and the lymph node. Stress can cause the spasms of the throat muscles, making it difficult to swallow. Stress shuts down digestive activity. Some evidence suggests that repeated release of stress hormone produces hyperactivity in the hypothalamus- pituitary -adrenal axis and disrupts normal level of serotonin. Stress diminishes the quality of life by reducing feelings of pleasure and accomplishment and relationships are often threatened. Mental stress is a trigger for angina as physical stress. Incidents of acute stress have been associated with a higher risk of cardiac events, such as heart rhythm abnormalities and heart attacks, and death from such events in people with heart disease. Stress activates the sympathetic nervous system and affects heart.

1. Sudden stress increases the pumping action and rate of heart beat and causes the arteries to constrict thereby posing a risk for blocking blood flow to the heart.
2. Emotional effects of stress alter the heart rhythms and pose a risk for various arrhythmias in people with existing heart rhythm disturbances.
3. Stress can increase the likelihood of an artery – clogging blood clot.
4. Stress may signal the body to release fat into the bloodstream, raising cholesterol levels in blood, at least temporarily.
5. In woman, chronic stress may reduce estrogen levels, which are important for cardiac health.
6. Stressful events may cause men and women who have relatively low levels of serotonin to produce more of certain immune system proteins - cytokines, which in high amounts cause inflammation and damage to cells including heart cells. People who regularly experience sudden increases in blood pressure caused by mental stress, develop injuries in

the inner lining of their blood vessels. In a 20- year study, men who periodically measured highest on the stress scale were twice as likely to have high blood pressure as those with normal stress.

The stress implies any condition that harms the body, damages or causes the death of a few or many cells. The body immediately tries to repair the damaged cells, but it can do so only if the diet is adequate, providing a generous supply of all the essential nutrients. If, however, rebuilding of cells is not able to keep pace with their destruction, the condition will result in disease. The most common diseases associated with stress are heart disease, diabetes, head-ache, peptic ulcer, ulcerative colitis, chronic dyspepsia, asthma, psoriasis and sexual disorders. Stress induced homeostatic changes and immune reduction tends to affect the balance between oxidants and antioxidants in the body. Any alteration in this balance in favor of antioxidants may result in pathological responses causing functional disorders and diseases such as cancer and Alzheimers disease. It can also accelerate aging process.

Stress and health are closely linked. It is well known that stress; either quick or constant can induce risky body and mind disorders. Immediate disorders such as dizzy spells, anxiety, tension, sleeplessness and muscle cramps can all result in chronic health problems and it may also affect our immune, cardiovascular and nervous system.

It is observed that the deleterious effects of stress in the body are brought about by alteration taking place in the concentration of various body parameters. Some of the ways by which stress can be induced experimentally include: extreme temperatures, constriction, handling (of the rats), physical exertion (forced swimming), vibration, and isolation. The changes taking place in the tissue parameters can best be studied in animal models. It is considered important to study the alteration in the metabolism of lipids and also of free radicals in variously stressed animals.

1.0. REVIEW OF LITERATURE

1.1. STRESS

Stress is an inherent part of daily living. Stress in the modern world has become a part of our life style. Prolonged stress can overwork many organ systems, especially the heart, blood vessels, adrenals and immune system. When stress is over-whelming, the response may be general depression, low blood pressure and low heart rate, increased cortisol or decreased sex hormone secretions. Failure to cope with stress can lead to stress related disorders such as head ache, hypertension, heart disease, stroke and ulcers. Stress is a threat or challenge to the integrity and survival of the organism (Herbert Weiner et al, 1991). Stress is an adaptive response that prepares the organism for a threatening situation. It induces strain upon both emotional and physical endurances, which has been considered a basic factor in the aetiology of number of diseases --- cardiovascular diseases, cancer, diabetes mellitus, etc (Halliwell et al 1984). Stressful condition leads to the formation of excessive free radicals which is a major internal threat to cellular homeostasis of aerobic organisms (Yu,1994). Free radicals are formed in the human body both in physiological and pathological conditions in cytosol, mitochondria, lysosomes, peroxisomes and plasma membranes (Hemnani et al 1988). Stress causes biochemical changes in the body that can raise blood glucose levels. A hormone released from the adrenals causes the liver to convert amino acids into glucose for the raised energy levels needed to deal with the demands of stress. "Stress response" describes the condition caused by a person's reaction to physical, chemical, emotional or environmental factors. Stress can refer to physical effort and mental tension. It's hard to define a high level of emotional or psychological stress to measure in a precise way. All people feel stress, but they feel it in different amounts and react to it in different ways.

More and more evidence suggests a relationship between the risk of cardiovascular disease and environmental and psychosocial factors. These factors include job strain, social isolation and personality traits. But, more research is needed to understand how stress contributes to heart disease risk. Clinical observations continue to suggest that hypertension may be an apparent cause for heart failure in both men and women (Kannel et al, 1996). Acute and chronic stress may affect other risk factors and behaviors, such as high blood pressure and cholesterol levels, smoking, physical inactivity and overeating. The detrimental effects of stress on the cardiovascular system have been documented through research in animal models and humans. Primarily two systems mediate the stress response, by exerting an acute influence on cardiovascular function: the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympatho-adrenomedullary system (SAS). Individuals with confirmed cardiovascular disease or its risk factors respond differently to these two systems. Although humans are physiologically equipped to respond to acute stressors, chronic stress disrupts the HPA axis and the SAS, resulting in harmful effects on human health. An exercise-induced increase in myocardial heat shock proteins is a potential mechanism to explain the cardioprotection associated with exercise. A recent study indicates that exercise training in a cold environment provides cardioprotection during I-R (ischemia reperfusion) injury but does not elevate myocardial levels of heat shock proteins (Hamilton et al., 2001).

Briefly, in response to a stress, the hypothalamus releases corticotropin-releasing hormone (CRH). In turn, CRH acts on the pituitary gland triggering the release of another hormone, adrenocorticotropin (ACTH) into the bloodstream. Next, ACTH signals the adrenal glands to release a number of hormonal compounds. These compounds include epinephrine, norepinephrine and cortisol. All three hormones enable the body to respond to a threat. Epinephrine increases blood pressure and heart rate, diverts blood to the

muscles, and speeds reaction time. Cortisol, also known as glucocorticoid, releases sugar (in the form of glucose) from the body reserves so that this essential fuel can be used to power the muscles and the brain. Normally, cortisol also exerts a feedback effect to shut down the stress response after the threat has passed, acting upon the hypothalamus and causing it to stop producing CRH. This stress circuit affects systems throughout the body. The hormones of the HPA axis exert their effect on the autonomic nervous system, which controls such vital functions as heart rate, blood pressure, and digestion.

1.2. Hormonal changes during stress

1.2.1. Release of Steroid Hormones.

The HPA systems trigger the production and release of steroid hormones (glucocorticoids), including the primary stress hormone cortisol. Cortisol is very important in marshaling systems throughout the body (including the heart, lungs, circulation, metabolism, immune systems, and skin)

1.2.2. Release of Catecholamines.

The HPA system also releases the neurotransmitters catecholamines, particularly dopamine, norepinephrine, and epinephrine Catecholamines activate the amygdala, which apparently triggers an emotional response to a stressful event. Neurotransmitters then signal the hippocampus to store the emotionally loaded experience in long-term memory. In primitive times, this combination of responses would have been essential for survival, when long-lasting memories of dangerous stimuli would be critical for avoiding such threats in the future. In post-traumatic stress disorder (PTSD), which is triggered by uncontrollable stress, medial prefrontal cortex activity is reduced. Proposing an analogous mechanism, Maier (Maier et al, 2005) speculated that loss of inhibition from the medial prefrontal cortex might explain increased activity of the amygdala in PTSD.

1.3. Effects of stress on reproductive system

Stress suppresses the reproductive system at various levels. For example, stress hormones inhibit the testes and ovaries directly, hindering production of the male and female sex hormones - testosterone, estrogen, and progesterone. Psychosocial stress influences fertility in females (Sanders,1987). First, CRH prevents the release of gonadotropin releasing hormone (GnRH), the hormone that signals a cascade of hormones that direct reproduction and sexual behavior. Similarly, cortisol and related glucocorticoid hormones not only inhibit the release of GnRH, but also the release of luteinizing hormone, which promots ovulation and sperm release.

1.4. The gastro- intestinal tract and stress

Stress can result in digestive problems (Mark G. Swain, 2000). Stress hormones directly hinder the release of stomach acid and emptying of the stomach and can directly stimulate the colon, speeding up the emptying of its contents. Stress is an ever-present part of modern life. The "stress response" constitutes an organism's mechanism for coping with a given stress and is mediated via the release of glucocorticoids and catecholamines. The influence of stress on the clinical course of a number of intestinal diseases is increasingly being recognized (Johan et al, 1998). This article focuses on recent findings related to the effects of stress on mucosal barrier function in the small intestine and colon. Experiments using animal models demonstrate that various types of psychological and physical stress induce dysfunction of the intestinal barrier, resulting in enhanced uptake of potentially noxious material (e.g., antigens, toxins, and other proinflammatory molecules) from the gut lumen. Prolonged stress can disrupt the digestive system, irritating the large intestine and causing diarrhoea, constipation, cramping, and bloating. Excessive

production of digestive acids in the stomach may cause a painful burning. Stress has become an etiological factor in the development of gastric ulcers (Das et al, 1997)

1.4.1. Peptic Ulcers.

Stress-related mucosal disease is common in critically ill patients and can result in significant morbidity (Duerksen et al, 2003). Studies still suggest that stress may predispose someone to ulcers or sustain existing ulcers (Brodie et al, 1960).

1.4.2. Inflammatory Bowel Disease.

Chronic stress seems to impair the immune system's capacity to respond to glucocorticoid hormones that normally are responsible for terminating an inflammatory response following infection or injury (Gregory E. Miller, 2002)

1.5. The Immune System's Response to Acute Stress

The steroid hormones dampen parts of the immune system, so that infection fighters (including important white blood cells) or other immune molecules can be redistributed. These immune-boosting troops are sent to the body's frontlines where injury or infection is most likely, such as the skin, the bone marrow, and the lymph nodes. Stress interacts with the immune system (Gregory E Miller, 2002), making more vulnerable to colds and flu, fatigue and infections. In addition, the high cortisol levels resulting from prolonged stress could serve to make the body more susceptible to disease by switching off disease-fighting white blood cells. Conversely, too little corticosteroid, can lead to a hyperactive immune system and increased risk of developing autoimmune diseases.. Breast cancer patients who feel high levels of stress concerning their diagnosis and treatment show evidence of a weakened immune system compared to patients experiencing less stress. A preliminary study (Felicia et al, 1995) found that the highly stressed women had lower levels of natural killer cells than women who reported less stress. However, the immune system is a highly specialized network whose activity is

affected not only by stress but also by a number of other factors. It has not been shown that stress-induced changes in the immune system directly cause cancer. Chronic stress not only makes people more vulnerable to catching illnesses but can also impair their immune system's ability to respond to its own anti-inflammatory signals that are triggered by certain hormones, say researchers, possibly altering the course of an inflammatory disease (*Health Psychology*, published by the American Psychological Association - APA, 1998). It's no surprise that being diagnosed with cancer is stressful. A more interesting problem for many researchers (Beth Azar, 1998) is whether heightened stress can increase a person's susceptibility to cancer or worsen the prognosis of a person with cancer.

1.6. The Brain's Response to Acute Stress

The stress system orchestrates body and brain responses to the environment. Corticosteroid hormones secreted by the adrenal cortex are implicated in both modes through their high affinity type 1 (mineralocorticoid receptors - MR) and lower affinity type 2 (glucocorticoid receptors - GR) receptors that are co-localised in limbic neural circuitry (De Kloet. et al 2003). MR controls in specific afferents the threshold or sensitivity of the fast CRH-1 driven stress system mode and thus prevents disturbance of homeostasis, while GR facilitates its recovery by restraining in these very same circuits stress responses and by mobilizing energy resources. In preparation for future events GR facilitates behavioural adaptation and promotes storage of energy. The balance in the two stress system modes is thought to be essential for cell homeostasis, mental performance and health. Imbalance induced by genetic modification or chronic stressors changes specific neural signalling pathways underlying psychic domains of cognition and emotion, anxiety and aggression. This Yin-Yang stress concept is fundamental for

genomic strategies to understand the mechanistic underpinning of cortisol-induced stress-related disorders such as severe forms of depression and co-morbid diseases.

Rats exposed to uncontrollable stress develop learned helplessness, a syndrome similar to depression and post traumatic stress disorder (PTSD) (Amat et al, 2005). They lose the ability to learn how to escape stressors. Activation of a brain stem area (dorsal raphe nucleus) has been implicated in such reactions. But this area is too small and lacks the proper sensory inputs to judge whether a stressor is controllable. Many of its inputs come conspicuously from the mid-prefrontal cortex area (medial prefrontal cortex), seat of higher order functions, such as problem solving and learning from experience. These signals are sent via the chemical messenger, serotonin, which is involved in mood regulation and in mediating the effects of the most widely prescribed antidepressants. In response to seeing the stress, a part of the brain hypothalamic-pituitary-adrenal (HPA) system is activated. An increased activity of serotonergic neurons in the brain is an established consequence of stress. An increase in brain tryptophan levels on the order of that produced by eating a carbohydrate-rich/protein-poor meal causes parallel increases in the amounts of serotonin released into synapses (Takeda et al, 2004). Eating is suppressed during stress, due to anorectic effects of corticotrophin releasing hormone, and considered to be increased during recovery from stress, due to appetite stimulating effects of residual cortisol.

1.7. Response by the Heart, Lungs, and Circulation to Acute Stress

Breathing becomes rapid and the lungs take in more oxygen and blood flow may actually increase 300% to 400%, priming the muscles, lungs, and brain for added demands. The spleen discharges red and white blood cells, allowing the blood to transport more oxygen.

1.8. The Acute Response in the Mouth and Throat

As the threat increases, fluids are diverted from nonessential locations, including the mouth. This causes dryness and difficulty in talking. In addition, stress can cause spasms of the throat muscles, making it difficult to swallow.

1.9. The Skin's Response to Acute Stress

The stress effect diverts blood flow away from the skin to support the heart and muscle tissues. The physical effect is a cool, clammy, sweaty skin. The scalp also tightens so that the hair seems to stand up.

1.10. Metabolic Response to Acute Stress

Stress shuts down digestive activity, a nonessential body function during short-term periods of physical exertion or crisis.

1.11. The Relaxation Response: the Resolution of Acute Stress

Once the stress has passed and the effect has not been harmful the stress hormones return to normal. This is known as the relaxation response. In turn, the body's systems also normalize.

Stress-related conditions that are most likely to produce negative physical effects include: an accumulation of persistent stressful situations, particularly those that a person cannot easily control (for example, high-pressured work plus an unhappy relationship), persistent stress following a severe acute response to a traumatic event (such as an automobile accident), an inefficient or insufficient relaxation response, acute stress in people with serious illness, such as heart disease.

1.12. Stress and cardiovascular system

The detrimental effects of stress on the cardiovascular system have been documented through research in animal models and humans. Primarily two systems

mediate the stress response, by exerting an acute influence on cardiovascular function: the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympatho-adrenomedullary system (SAS). Individuals with confirmed cardiovascular disease or its risk factors respond differently to these two systems. Although humans are physiologically equipped to respond to acute stressors, chronic (longtime) stress disrupts the HPA axis and the SAS, resulting in harmful effects on human health. Moreover, cross-sectional (and, to a lesser extent, longitudinal) epidemiological data show that chronic job stress and cardiovascular reactivity in response to stress are associated with hypertension, coronary heart disease, and stroke (Kenneth et al, 1976). However, the specific physiological and behavioral mechanisms, as well as the degree of cardiovascular risk attributable to chronic stress, remain poorly understood. Besides the mobilizing of our body for flight, the chronic stress increased flow of adrenaline produces a number of other less helpful and more serious consequences.

1.12.1. Biochemical changes:

- An increase in the production of blood cholesterol
- A narrowing of the capillaries and other blood vessels that can shut down the blood supply to the heart muscle.
- A decrease in the body's ability to remove cholesterol
- An increase in the blood's tendency to clot
- An increase in the depositing of plaque on the walls of the arteries

1.12.2. Stress related cardiovascular system disorders

1. High blood pressure
2. Atherosclerosis

3. Heart attacks
4. Strokes
5. Angina pectoris
6. Myocardial infarction

Acute or chronic psychological stress could induce an acute phase response and subsequently a chronic inflammatory process such as atherosclerosis (Black, 2003). Stress can induce an acute phase response and inflammation, has been extended to include a chronic inflammatory process, characterized by the presence of certain cytokines and acute phase reactants (APR), which is associated with certain metabolic diseases. The loci of origin of these cytokines, particularly interleukin 6 (IL-6), and their induction, have been considered. Evidence is presented that the liver, the endothelium, and fat cell depots are the primary sources of cytokines, particularly IL-6, and that IL-6 and the acute phase protein, C-reactive protein (CRP), are strongly associated with, and likely play a dominant role in the development of this inflammatory process. This process leads to insulin resistance, non-insulin dependent diabetes mellitus type II, and metabolic syndrome X. The possible role of psychological stress and the major stress-related hormones as etiologic factors in the pathogenesis of these metabolic diseases, as well as atherosclerosis, is discussed. The fact that stress can activate an acute phase response, which is part of the innate immune inflammatory response, is evidence that the inflammatory response is contained within the stress response or that stress can induce an inflammatory response. The evidence that the stress, inflammatory, and immune systems all evolved from a single cell, the phagocyte, is further evidence for their intimate relationship which almost certainly was maintained throughout evolution. Mental stress is as major a trigger for angina as physical stress. Incidents of acute stress have been

associated with a higher risk for serious cardiac events, such as heart rhythm abnormalities and heart attacks, and even death from such events in people with heart disease.

Stress activates the sympathetic nervous system (the autonomic part of the nervous system that affects many organs, including the heart). Such actions and others may negatively affect the heart in several ways. Sudden stress increases the pumping action and rate of the heart and causes the arteries to constrict, thereby posing a risk for blocking blood flow to the heart. Emotional effects of stress alter the heart rhythms and pose a risk for serious arrythmias in people with existing heart rhythm disturbances. Stress causes blood to become stickier (possibly in preparation of potential injury), increasing the likelihood of an artery-clogging blood clot. Stress may signal the body to release fat into the bloodstream, raising blood-cholesterol levels, at least temporarily. In women, chronic stress may reduce estrogen levels, which are important for cardiac health.

Stressful events may cause men and women who have relatively low levels of the neurotransmitter serotonin (and therefore a higher risk for depression or anger) to produce more of certain immune system proteins (called *cytokines*), which in high amounts cause inflammation and damage to cells, including possibly heart cells (Rabin, 1999).

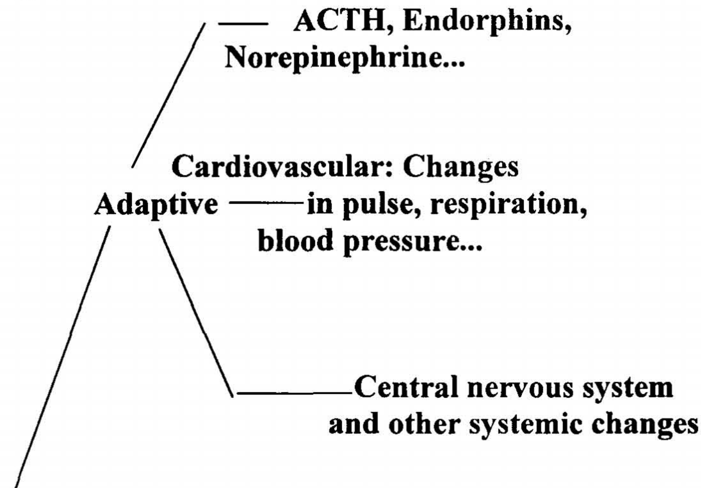
Recent evidence confirms the association between stress and hypertension. People who regularly experience sudden increases in blood pressure caused by mental stress may, over time, develop injuries in the inner lining of their blood vessels.

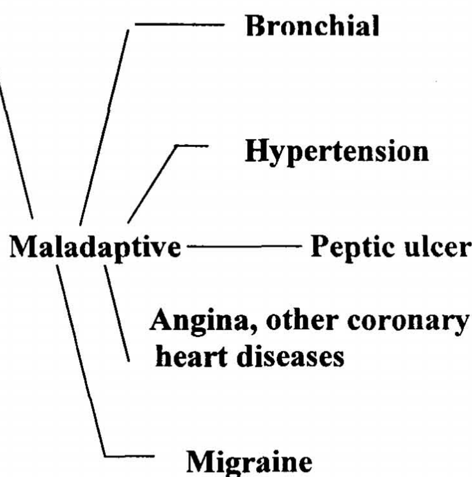
1.3. Responses to Stressful Life Events

A person is not only in constant interaction with the world around him, but also in continuous contact with his inner reality. Human responses to life stress of diverse nature

can, therefore, be divided into four groups: psychological, physiological, social, and spiritual.

TABLE I- Physiological response- Adaptive and maladaptive responses





Stress can be considered as a state of disharmony or threatened homeostasis. Stress condition varies and can range from physical to psychological, from mild to severe and from acute to chronic (Vogel, 1993 and Selye, 1950). An animal or organism is said to be in a state of stress, if it is required to make some abnormal or extreme adjustments in its

physiology or behaviour in order to cope with adverse aspects of its environment as quoted (Frazer et al, 1990). Adrenocorticotropic hormone (ACTH) is released by anterior pituitary during stress bringing about the release of corticosteroids as cortisol and cortisone resulting in a relative decrease in carbohydrate metabolism, an increase in protein metabolism and mobilization of fat deposits. Stress research in the laboratory animals has assumed an important role in the biological and psychological sciences over the past decade due to the view that stressful stimulus may influence the onset and progression of a number of diseases in human beings leading to hypertension, stroke, depression, etc. Cardiovascular response during and after psychological stressful situation has been frequently investigated with an objective to correlate behavioral and physiological aspects of stress (Koolhas, 1991). Stress, age and behavioral characteristics are considered to be risk factors for disturbances of the cardiovascular system in animal and man. It has been suggested that chronic stress can contribute to the development or exacerbation of cardiovascular dysfunction. Previous studies have revealed significant interaction between individual responsiveness to changing environment and susceptibility for high blood pressure leading to hypertension in chronically stressed rats [Henry et al, 1981 and De Quattro et al, 1985, and Muldoon et al,1995]. Psychological stress affected cardiovascular and adrenal physiology in five different types of rat strains (Henry et al, 1993). Stress is known to induce more secretion of epinephrine and norepinephrine from adrenal medulla (Axelrod et al, 1984). The body and mind react to any stress factor. A large number of physical changes take place at the time of stress, stress induced nervous system become intensively active, the pupils of the eye dilate, digestion slows down muscles become tense, the heart start pumping blood harder and faster, hormones such as adrenaline are released into the system along with glucose from the liver and sweating starts. Stress implies an ability to withstand a defined amount of strain. Dr. Hands Selye

(Hands Selye, 1956) described stress as a state manifested by specific syndrome, which consists of all the non-specifically induced changes within a biological system. The term implies any condition that harms the body and damages or causes the death of a few or many cells. The body immediately tries to repair the damaged cells but it can do so only if the diet is adequate, providing a generous supply of all the essential nutrients. If however, rebuilding of cells is not able to keep pace with destruction, the condition will result in diseases, such as diabetics, headache, peptic ulcer and ulcerative colitis, chronic dyspepsia, asthma, psoriasis and sexual disorders. Reaction to stress is manifold. No one situation is stressful to all the people all the time. Stress is a pervasive factor in everyday life that critically affects development and functioning. Severe and prolonged stress exposure impairs homeostatic mechanisms, particularly associated with the onset of depressive illness. An increased activity of serotonergic neurons in the brain is an established consequence of stress. An increase in brain tryptophan levels on the order of that produced by eating a carbohydrate-rich/protein-poor meal causes parallel increases in the amounts of serotonin released into synapses (Takeda, 2004). Eating is thought to be suppressed during stress, due to anorectic effects of corticotrophin releasing hormone, and increased during recovery from stress, due to appetite stimulating effects of residual cortisol. A short single experience of stress can have long-term consequences for the animal's stress responsivness and behaviour (Sutantowet et al, 1994). One predominant feature of chronic stress is the finding that repeated stress leads to adaptation or habituation. Chronic or repeated stress can cause a wide range of physiological and neuroendocrine changes (Naleson et al, 1988). Swimming in small laboratory animals has been widely used for studying the physiological changes and capacity of the organism in response to stress (Tan et al, 1988 and Greenen et. al., 1988). The amount of work done during swimming exercise is far greater than that during excercise of identical time

duration. Forced swimming is not always a simple exercise stress, because emotional factors are difficult to be eliminated (Kramer et. al., 1993). The forced swimming stress developed by Porsolt (Porsolt et. al., 1977) has now become a widely accepted model for studying the physical stress in animals. Water temperature is another factor in the forced swimming test. By varying the temperature Richter (Richter et. al., 1957) found that rats could survive as long as 80 hours in lukewarm water (36^0C). Increase or decreasing the temperature above/ below this point influence the overall behaviour of the animal and changes the involvement of glucocorticoids (Abel et al , 1991). Enzyme activities after swimming were studied (Di Simplicio et al, 1997) and the enzyme activities found to be modified in a complex way. A decrease in the enzyme activity observed in the adductor muscle seem to confirm the sensitivity of this organ to overproduction of reactive oxygen species, oxidized glutathione (GSSG) decrease observed in blood was a new and unexpected findings, one that indicates a very prompt adaptation of red cells to increased oxidant stress. Effect of immobilization stress was also studied (Hemlata et al, 2004). The forced immobilization stress enhances lipid profiles (Sighal et al,1997). Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameter in broilers were studied (Altan et al, 2003). Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities and MDA concentration were increased. Cows exposed to moderate heat stress (39.52^0c) due to summer temperature, show high erythrocyte SOD, GSH, GPx and TABRS, indicating a condition of oxidative stress in summer transition cows (Bernabuci et al 2002). Ronchi and Trout (Ronchi et al1999 and Trout et al, 1999) reported no effects of heat stress on plasma concentration of vitamin E and ß-carotene or on muscle content of thiobarbituric acid reactive substances. Calamari [Calamari et al, (1999)] observed weak negative effects of heat stress on some plasma markers of oxidative status in midlactating cows.

The body and mind react to stress factor. A large number of physical changes take place at the time of stress-induced arousal. The brain and nervous system become intensively active, the pupils of the eye dilate, the digestion slows down, muscle become tense the heart starts pumping harder and faster, hormones such as adrenaline are released into the system along with glucose from the liver and sweating starts. All theses changes take place in a split second under the direction of nervous system. If the stress factors are immediately removed no harm occurs and all the changes are reversed. Stress in earlier stage leads to poor sleep, bad temper, continual grumbling, longer hours of work with lesser achievement, domestic conflict with spouse and children, repeated minor sickness, absenteeism and prolonged absence of each spell of sickness, accident proneness, feeling of frustration and persecution by colleagues and complaints of lack of cooperation and increase in alcohol intake. Harmon (Harmon et al, 1997) reported a reduction of antioxidant activity of plasma in midlactating heat-stressed Holstein cows.

1.4. Stress response

Stress may be caused by a variety of factors both outside the body and within. External factors include loud noises, blinding lights, extreme heat or cold, X rays and other forms of radiation, drugs, chemicals, bacteria and other toxic substances, and pain and inadequate nutrition. Stress is known to worsen many immune related medical conditions, including diabetes. Cortisol produced during stress situations may suppress the body's immune response and increasing susceptibility to infectious diseases.

Stress has become an etiological factor in the development of gastic ulcer during experimental procedures (Piere et al, 1998, Brodie et al, 1960) found rats highly susceptible to restraint stress and noted ulceration of gastric mucosa. Bharihoke, (Bharihoke et al, 2000) subjected their experimental animals to stress of immobilization and cold in a plexi glass container for 2 hours for five days. Sanchez (Sanchez et al,1996)

studied the restrained stress in rats and noted a rise in cortisol level. Francis (Francis et al, 1979) established the serum cholesterol level as an index of stress. Use of temporary immobilization in small plastic devices as stress induced in rats has been commonly employed in their study (Coscum et al,1995 and Brodie et al, 1960 and Brennan et al 1996 and Bharihoke et al 2000). This stress was sufficient as evidenced by production of gastric ulcer (Brodie et al 1960). Varley (Varley et al 1992) reported higher values of serum cholesterol in men as compared to that of women. Vyas (Vyas et al ,1992) noted a rise of 20% cholesterol level in pregnant women as compared to that of nonpregnant women. Various theories have been propounded to explain this rise of serum cholesterol. Patterson (Patterson et al,1993) are of the view that increased cholesterol synthesis in immobilization stress is due to hypovolemia, while Koob (Koob 1985) associated stress response with hyperinsulinemia and increased cholesterol synthesis, (Alveraz et al, 1989 and Mayer et al, 1988). The forced immobilization stress enhances significant increase in heart weight was observed after the rats were exposed to stress (Horie et al 1991 and Gelsema et al, 1992). Stress is known to induce more secretion of epinephrine and norepinephrine from adrenal gland (Axelord et al 1984). These hormones acting on heart exert positive influence on the force of contraction (inotropism) which gradually might have caused the cardiac hypertrophy. [During stress there is uniform arousal of both the fight-flight sympatho-adrenal and pituitary-adrenal cortical systems. These two systems acting together participate in the stress response of an organism. Increased heart rate and force of cardiac contraction is considered as the immediate response of the organism to stress (Herd et al, 1991). Several workers reported tachycardia as a response to both acute and chronic stress (Gomez et al, 1989 and Vike et al.1968). Vasopressin (VP) stimulates pituitary ACTH secretion through interaction with receptors of the V1b subtype (V1bR, V3R), located in the plasma membrane of the pituitary corticotroph, mainly by

potentiating the stimulatory effects of corticotropin releasing hormone (CRH). Chronic stress paradigms associated with corticotroph hyper-responsiveness lead to preferential expression of hypothalamic VP over CRH and upregulation of pituitary V1bR, suggesting an important role for VP during adaptation of the hypothalamic-pituitary-adrenal (HPA) axis to stress.(Volpi et al, 2004)

"Stress response" describes the condition caused by a person's reaction to physical, chemical, emotional or environmental factors. Stress can refer to physical effort and mental tension. It's hard to define a high level of emotional or psychological stress to measure in a precise way. All people feel stress, but they feel it in different amounts and react to it in different ways. More and more evidence suggests a relationship between the risk of cardiovascular disease and environmental and psychosocial factors. These factors include job strain, social isolation and personality traits. But more research is needed on how stress contributes to heart disease risk. It is not known whether stress acts as an "independent" risk factor for cardiovascular disease. Acute and chronic stress may affect other risk factors and behaviors, such as high blood pressure and cholesterol levels, smoking, physical inactivity and overeating. The experience of stress affects cellular immunity, an important aspect of many medical problems, including controlling/curing cancer and the immunobiology of autism. Treating disease with immunological components means also treating and managing psychological stress. Free radicals play an important role as mediators of skeletal muscle damage and inflammation after strenuous exercise. Simmons (Simmon et al, 1990) reported a decrease in glutathione levels in mice on exposure to cold.

. Human immune function is mediated by the release of cytokines and nonantibody messenger molecules from a variety of cells of the immune system and from other cells such as endothelial cells. There are Th1 and Th2 cytokines. Autoimmune and

allergic diseases involve a shift in the balance of cytokines toward Th2. The autoimmune aspect of autism has been related to excessive Th2 cytokines resulting, in part, from vaccination. Gulf War syndrome and asthma have been similarly linked to excess immunization in the presence of increased environmental toxins and pollutants (high antigenic load). Cytokines stimulate cellular release of specific compounds involved in the inflammatory response. Stress-induced activation of the sympathetic nervous system and the sympathetic-adrenal medullary and hypothalamic-pituitary adrenal axes lead to the release of cytokines (Rabin et al, 1999). Blocking the response of the sympathetic nervous system by pre-treating subjects in stressful experiments with adrenergic antagonists can reduce this release of cytokines and decrease the resulting inflammatory response (Bachen et al, 1994 and Benschop et al, 1994). Discrete areas of the brain (for example, the hypothalamus and the locus coeruleus) regulate the sympathetic nervous system and therefore the levels of circulating adrenergic stress hormones, thereby influencing the activity of the immune system (Wetmore et al, 1991 and Rassnick et al, 1994). Adrenergic stress hormones alter the synthesis and release of cytokines by white blood cells .The effects of stress on immunity has been experimentally studied in animals. The stress of crowding prior to and following tuberculosis infection affects the outcome of the infection in mice (Tobach et al, 1956). Social disruption in mice causes reactivation of latent herpes simplex virus (Padgett et al, 1998). Stress enhances the reactivation of latent herpes viruses including the Epstein-Barr virus in humans (Glaser et al, 994). Psychological stress inhibits many aspects of the immune response including innate immunity (eg, natural killer cell lysis), T-cell responses, and antibody production (Rabin et al, 1999). Outside of proven clinical interventions, there is reason to think that certain changes in lifestyle might increase host resistance to infectious diseases.

1.5. Oxidative stress and free radicals

Oxidative stress resulting from increased production of free radicals and reactive oxygen species, and a decrease in antioxidant defense, leads to damage of biological macromolecules and disruption of normal metabolism and physiology (Trivisan et al, 2001). When reactive forms of oxygen are produced faster than they can be safely neutralized by antioxidant mechanisms, oxidative stress results (Sies, 1991).

Free radical or reactive oxygen species (ROS) were formed by various biochemical reactions. It was towards the end of the 18th century that oxygen emerged as the paragon among the elements that sustained life, promoted physical health and stimulated mental vigor. But too much of even the best is bad and it is known that oxygen in high concentration can damage the brain, lungs and other organs. The phenomenon of oxygen toxicity in early days referred as the toxic effects of oxygen at high pressure. Free radicals are an unstable and extremely reactive chemical species, which have an unpaired electron in their structure. The most important free radicals are the by products of energy generation and are formed during oxidation, such as that occurs in the electron transport chain.

The term reactive oxygen species (ROS) collectively describes free radicals such as superoxide radicals ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), and hypochlorous acid (HOCl). These reactive oxygen intermediates form in reactions, which give rise to free radicals species. Unstable free radical species attack cellular components, causing damage to lipids, proteins and DNA, which can initiate a chain of events which results in the onset of diseases. Oxidative stress results when the balance between the reactive oxygen species overrides the antioxidant capability of the target cells. ROS may interact with and modify cellular proteins, lipids and DNA, which results in altered target cell function. Free radicals are extremely reactive. Their half-life is only a few milliseconds. When a

radical reacts with a new compound more free radicals are generated. This chain reaction leads to thousands of events. Peroxidation of polyunsaturated fatty acids (PUFA) in the plasma membrane results in the inhibition and loss of membrane functions such as absorbtion, secretion, inhibition of protein and enzyme synthesis and indirectly cause cell death.

1.6. Free radicals can originate in various ways

Biochemical redox reactions involving oxygen, which occur as a part of normal metabolism. Eg.O_2, NO, H_2O_2.

By phagocytosis as a controlled inflammatory reaction. Eg. HOCl.

Occasionally in response to exposure to ionizing radiations, UV light, environmental pollution, cigarette smoke,stress, hyperoxia, excessive exercise and ischemia eg $O_2^{\cdot}$,$OH^{\cdot}$, and $ROO^{\cdot}$

Many free radicals and ROS have been implicated in disease development. $OH^{\cdot}$ is a highly reactive radical, which can travel in the blood and attack a number of biological targets. O_2 can also act as a vasodilator and may have a role in intracellular signaling and growth regulation. $NO^{\cdot}$ act on smooth muscle cells in vessel wall causing relaxation. H_2O_2 crosses cellular membrane easily and may cause alteration in the expression of virus gene in infected cells. Eg HIV. This ROS has only cellular targets but can result in the production of hydroxyl radicals. It is now widely believed that stress play important role in the development of free radicals and diseases.

1.7. Biologically relevant ROS

Superoxide radical

Hypochlorous acid

Hydrogen peroxide

Hydroxy radical

Peroxyl radical

Peroxynitrile

Heme proteins

Singlet oxygen

Nitric oxide

Though all classes of biomolecules may be attacked by free radicals, lipids are the most susceptible. Human cells are rich in polyunsaturated fatty acids (PUFA) and hence are readily attacked by oxidizing radicals by a process known as lipid peroxidation, which is a highly damaging self-perpetuating chain reaction.

1.7. The antioxidant system

Body possesses a number of mechanisms both to control the production of ROS and to limit or repair the damaged tissues. The integrated antioxidant system consists of (i) preventive antioxidants, which prevent the formation of new ROS [ceruloplasmin(cu), tranferrin (Fe), ferritin (Fe) and myoglobin (Fe). (ii) Scavenging antioxidants which remove ROS once formed, thus prevent free radical chain reaction [eg- superoxide dismutase (SOD), catalase (CAT) glutathione peroxidase (GPx) and glutathione reductase. (iii) Repair enzymes, which repair or remove ROS-damaged biomolecules (eg DNA repair enzymes and methionine sulphoxide reductase). The first defense against ROS is mainly by the antioxidant enzymes. By the combined action of these enzymes, the free radicals are removed very effectively.

1.7.1. Superoxide dismutase

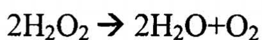
Superoxide dismutase (SOD) is a metalloenzyme that convert superoxide radical to hydrogen peroxide.

$O_2^{\cdot} + O_2^{\cdot} + 2H \rightarrow H_2O_2 + O_2$

SOD is most important enzyme because it is found virtually in all aerobic organisms. SOD is found in four different isoforms : copper dependent (Cu-SOD), copper- zinc dependent (Cu-Zn-SOD), Manganese dependent (Mn-SOD) and iron dependent (Fe-SOD). Human SOD is the Cu-Zn-SOD. The transition metals of the enzyme react with oxygen radical by abstracting its electron (Oberely and Oberley, 1984). The only known substrate for SOD is superoxide radical, which is converted to hydrogen peroxide by the action of the enzyme.

1.7.2. Catalase

Catalase also serves as a free radical scavenging enzyme. It is present in almost all the cells especially in erythrocytes. Catalase catalyses the decomposition of hydrogen peroxide to water and oxygen.



$2H_2O_2 \rightarrow 2H_2O+O_2$

The toxic hydrogen peroxide formed by the action of SOD on superoxide radical is converted to water by catalase. Catalase is a haeme containing protein and is found to act 104 times faster than peroxidases. It is localized mainly in mitochondria and in sub-cellular respiratory organelle (Pryor et al, 1986). Catalase is also present in peroxisomes and cytosol.

1.7.3. Glutathione peroxidase

Glutathione peroxidase (GPx) is another well known enzyme defense against oxidative stress, which in turn requires glutathione as cofactor. Among the many functions of glutathione it is involved in the generation of nucleotide precursors of DNA via the reduction of ribonucleotides to deoxyribonucleotides (Meister,1994). Gpx catalyses the oxidation of GSH to GSSG at the expense of hydrogen peroxides.

$$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG.$$

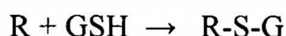
The reverse reaction is catalysed by the glutathione reductase (GR) to retain the reduced glutathione.

$$NADPH + H^{+} + GSSG \rightarrow NADP^{+} + 2GSH$$

The combined action of these enzymes the oxidative stress insduced by free radicals has been eliminated. Other antioxidant molecule such as glutathione, albumin, bilurubin and uric acid also found to defense against the oxidative stress induced by free radicals or ROS.

1.7.4. Glutathione-S- transferase

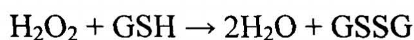
Glutathione -S- transferase (GST) utilizes glutathione in reaction contributing to the transformation of a wide range of compounds and products of oxidative stress. GST act by catalyzing the reaction of glutathione with an acceptor molecule to form an S-substituted glutathione. R is an electrophilic xenobiotic.



$$R + GSH \rightarrow R\text{-}S\text{-}G$$

1.7.5. Reduced glutathione

Glutathione a cofactor for the enzyme GPx and GST. GSH is known as as a cofactor in both conjugation and reduction reactions, catalysed by glutathione –S – transferase enzymes in cytosol, microsomes and mitochondria. GSH antioxidant system participate directly in the destruction of reactive oxygen compounds. Glutathione destroys free radicals involved in detoxification. The reaction is catalysed by glutathione peroxidase.



$$H_2O_2 + GSH \rightarrow 2H_2O + GSSG$$

The ratio of GSSG/ GSH in the cell is an important marker of oxidative stress. Glutathione reduces the toxic substances before they can damage other molecule or important part of cell

1.8. Alcohol

Alcohol abuse is the commonest cause of liver damage (Doll et al, 1994). Light or moderate ingestion of alcohol may reduce the risk of coronary artery disease (Fuchs, 1995). Intake of more than 30gm alcohol per day has been associated with cirrhosis in both sexes (Becker et al, 1996). The mechanisms involved in alcohol induced liver disease are poorly understood (Friedman, 1993). Only 8 to 30% of long term alcohol abusers develop alcoholic cirrhosis and a minority of individuals will not progress beyond stage of fatty liver despite persistent drinking. The risk of severe liver damage is dose dependent (Bellantani , 1997). The technique of feeding ethanol as part of a totally liquid diet was invented two decades ago. This technique results in much higher ethanol intake than the conventional procedures. As a consequence, various complications observed in alcoholics were reproduced in animal models. The amount of ethanol metabolized by the individual differs by sex (Frezza et al, 1990) and that chronic ethanol consumption induces the microsomal ethanol oxidizing system (Liber et al , 1967). Chronic liver damage caused by alcohol consumption diminishes livers capacity to synthesize and export lipids (Baraona et al). The involvement of free radicals in the pathogenesis of liver injury has been investigated for many years in a few well defined system (Poly,1993). There is now good evidence that moderate consumption of alcohol is associated with a lower risk of coronary artey disease than either teetotalism or heavy drinking (Steinberg et al, 1991). Increased alcohol consumption is associated with an increased risk of cardiovascular and other diseases due to hypertension and haemorrhagic stroke or sudden arrhythmic death (Kauhnen et al, 1997)

1.8.1. Ethanol metabolism

Alcohol reaching the liver in the portal blood is oxidized by alcohol dehydrogenase (ADH) in the hepatocyte cytosol to acetaldehyde, with simultaneous

reduction of the cofactor NAD to NADH. Acetaldehyde is highly reactive substance whose toxicity greatly exceeds that of ethanol, and is metabolized to acetate by the mitochondrial isoenzyme of aldehyde dehydrogenase (ALDH). The cytochrome P450 system present in endoplasmic recticulam provides an alternative pathway for alcohol metabolism that may account for up to 10% of ethanol oxidation in chronic abusers due to enzyme induction. Factors that increase ethanol oxidation or reduce acetaldehyde clearance will result in increased acetaldehyde levels in the liver and greater injury.

1.8.2. Ethanol administration and alteration in cell biochemistry

During ethanol oxidation, acetaldehyde forms both stable and unstable adducts with proteins, glycoproteins and membrane phopholipids (Sorrel et al,1985). Evidence from rat model suggests that this process is dependent upon ADH activity (Lin et al, 1990). Free-radical formation results in increased lipid peroxidation, which exacerbates membrane injury (Lauterburg et al, 1988). Many enzyme systems are effected by the redox shift that occurs due to increased NADH production relative to NAD (Lieber et al ,1998). In addition profound changes occur in lipid, carbohydrate and protein metabolism, leading to ketosis, the accumulation of triglycerides and lactate and very occasionally hypoglycemia occurs in acute alcoholic states. Chronic ethanol ingestion induces oxidative stress in the liver as result of the generation of superoxide radicals by ethanol metabolism via cytochrome P_{450} system. In alcoholic hepatitis infiltration of neutrophils may contribute to this oxidative stress (Williams et al, 1987). Free radical mediated damage is aggravated by a reduction in cytoprotective enzymes and other antioxidants. Glutathione synthesis is reduced due to the interaction of acetaldehyde with precursor essential amino acids. In addition, nutritional deficiencies and malabsorbtion contribute to the reduced levels of natural antioxidants (Bjorneboe et al ,1993).

The interaction of ethanol with lipid metabolism is complex. When ethanol is present, it becomes a preferred fuel for the liver and displaces fat as a source of energy. This favours fat accumulation. In addition, the altered redox state secondary to the oxidation of ethanol promotes lipogenesis, through enhanced formation of acylglycerols. The high-density lipoprotein (HDL) have been suggested to be responsible for the lower incidence of coronary complications of moderate drinkers compared to teetotalers (Lieber, 1984). Schlorff (Schlorff et al, 1999) found that ethanol ingestion perturbs the plasma antioxidant system in a dose and time dependent manner. The significant changes in the ratios of catalase/superoxide dismutase, glutathione peroxidase/superoxide dismutase, glutathione reductase/glutathione peroxidase and reduced glutathione/ oxidised glutathione in the plasma may be used as an index of alcohol induced oxidative stress. Krikun (Krikun et al,1986) reported that microsomes isolated from chronic ethanol fed rats displayed elevated rates of malondialdehyde production when compared to pair fed controls. Husain (Husain etal ,1998) studied the effect of exercise training and chronic ethanol ingestion and combination selectively inhibited hypothalamic cholinesterase activity and the inhibition was correlated with increased lipid peroxidation which may perturb hypothalamic function. Leptin, the hormone that regulates appetite discovered in 1994, has implications beyond its originally designated role In rats, one month of alcohol use stimulates leptin (Nicolas et al. 2001), presenting another potential and intriguing mechanism for alcohol-induced hypogonadism.

1.9. Cigarette smoke

Tobacco smoking leads to increased leukocytosis and elevation of acute phase reactants Cigarette smoke is produced by incomplete combustion of tobacco. It is a heterogenous aerosol containing more than 4000 substances (Anthony et al, 1998). Cigarette smoke contains various cytotoxic, mutagenic and carcinogenic agents like

biphenyl and polycyclic aromatic hydrocarbons and oxidants like oxygen, nitrous oxide and free radicals. Free radicals are capable of independent existence and can cause oxidative tissue damage (Ansari, 1997). Free radicals are capable of initiating and promoting oxidative damage (Chow et al, 1993 and Church et al, 1985). Smoking can significantly increase CAD mortality and morbidity related to the amount of tobacco smoked daily and the duration of smoking (Manson et al, 1992 and Wilhelmsen, 1998). Nicotine stimulates release of adrenaline leading to increased serum concentrations of fatty acids (Shepherd et al, 1978). Free fatty acid is a stimulant of hepatic secretion of LDL and triglycerides (TG). The free fatty acids can also stimulate hepatic synthesis and release of cholesterol (Banonome et al, 1992). In addition to this, cigarette smoking can alter coagulation system, produce various free radicals, all of which may contribute to atherosclerosis. The benefit of smoking cessation is seen regardless of how long and how much the person previously smoked (Kawachi, 1993). Tobacco is the most commonly known carcinogen for human society. Two most important etiological factors, implicated in the development of oral cancer are tobacco smoking and alcohol consumption.

1.9.1. Toxicity of cigarette smoke

Cigarette smoke predisposes to emphysema, lung and several other cancers, atherosclerosis and many other diseases.

1.9.2. Chemistry of cigarette smoke

Cigarette smoke is a complex mixture of toxic agents, some of which are free radicals themselves, others are redox cycling agents, cytotoxic aldehydes and carcinogens such nitrosamines and benzpyrene. Other constituents of cigarette smoke are ammonia, carbon monoxide, hydrogen cyanide, ethanol, formaldehyde, benzene vapour, vinyl chloride, tar, nicotine, phenols, lead, iron and carcinogenic hydrocarbons etc. The tar in smoke contains about 10^{17} radicals per gram; most of them are highly stable, persisting

for hours. The tar contains 3000 aromatic compounds and at least four different radical species. Aqueous extracts of cigarette tar generates O_2 free radicals and hydrogen peroxide and have been shown to damage isolated DNA. It has been estimated that more than one microgram of iron is inhaled per pack of cigarettes. Lung macrophages and respiratory tract lining fluids in smokers have elevated iron contents. Fresh cigarette smoke contains high concentrations of NO and NO_2.

1.9.3. Mechanisms of damage by cigarette smoke

i. RO_2 and oxides of nitrogen can cause direct damage, stimulating lipid peroxidation and oxidizing DNA bases. Plasma and urine from cigarette smokers show elevated levels of isoprostanes. Cigarette smokers exhale more pentane immediately after smoking

ii. The aldehyde present, especially acrolein, other unsaturated acetaldehyde and formaldehyde, can cause GSH depletion and modify protein - SH groups and amino groups.

iii. The hydroquinones/quinines in the tar phase may leach out into lung lining fluids, diffuse across the cell membranes and undergo both extracellular and intracellular redox cycling to generate semiquinones.

iv. The carcinogens are partially absorbed and usually after the metabolism can initiate and promote carcinogenesis.

v. Cigarette smoke acts as an irritant to lung macrophages and may activate them to produce hydrogen peroxide. It also promotes recruitment and retention of neutrophils in the lung.

vi. Both surfactant and alpha-1-antiproteinase can be inactivated by species within cigarette smoke or generated by activated phagocytes. Smoking predisposes to the development of emphysema and bronchitis. The term chronic obstructive

pulmonary diseases (COPD) is often used to encompass both chronic bronchitis and emphysema, since the two conditions often co-exist to some extent in smokers. Cigarette smoking is the primary risk factor for COPD.

vii. Cigarette smoke not only contains but also be capable of releasing iron from ferritin in lining fluids, possibly leading to hydroxyl formation from hydrogen

1.10. Aim of the present study

From the above review it is evident that stress both physical, and psychological as well as oxidative stress have a major role to play in the pathogenesis of number of diseases. Our present study is aimed to study the effect of different types of stress on lipid metabolism and antioxidant status.

1.11. Relevance of the present study

The assay of lipid profiles- total cholesterol, triglycerides, HDL- cholesterol and LDL-cholesterol and scavenger enzymes of reactive oxygen species- superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione content are important to understand the role of stress in the incidence of various diseases.

Hence the objectives are

1) To study the effect of various types of stress (Fresh water swimming, cold water swimming, isolation, overcrowding, alcohol administration and cigarette smoke exposure) on the lipid parameters (serum and tissue cholesterol, LDL-cholesterol, HDL-cholesterol,triglycerides) and lipogenic enzymes (glucose-6- phosphate dehydrogenase, malic enzyme and HMG Co A reductase) in the liver tissue of experimental animals.

2) To study the effect of above mentioned stress conditions on antioxidant enzymes (Superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase) and reduced glutatione content in tissue of

experimental animals.

3) The study also envisages to evaluate the combined effect of alcohol and cigarette smoke on lipid parameters and antioxidant enzymes in serum and tissues of experimental animals.

4) The assay of the above parameters (lipids and antioxidant enzymes) are done with a view to understand the effects of various stress conditions on the incidence of coronary heart disease.

5. The study is also extended to understand the effect of S-allyl cysteine sulphoxide and diallyl disulphide (SACS and DADS) on animals treated with alcohol.

EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT FOR THE DEGREEE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY
(FACULTY OF SCIENCE)

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March 2006

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Nicotinamide adenine dinucleotide phosphate-reduced (NADPH), nitroblue tetrazolium (NBT),1-chloro 2,4 dinitrobenzene (CDNB), 5, 5' dithio-bis 2 nitrobenzoic acid (DTNB) and reduced- glutathione (GSH) were purchased from Sisco Research Laboratories, Mumbai. Chloroform, thiobarbituric acid (TBA) was purchased from E-Merck (India), Riboflavin from Loba Chemicals Mumbai, India. All other chemicals and reagents used for the study were of analytical reagent grade.

2.2. Digonostic kits

Cholesterol	Ecoline, E-Merck Ltd, Mumbai.
Cholesterol HDL	Ecoline, E-Merck Ltd, Mumbai
Triglycerides	Ecoline, E-Merck Ltd, Mumbai.
Glutamate pyruvate Transaminase (ALT)	Ecoline, E-Merck Ltd, Mumbai.
Glutamate oxaloacetate Transaminase (AST)	Ecoline, E Merck Ltd, Mumbai.
Glutathione peroxidase (GPx)	Randox laboratories Ltd, United Kingdom.
Glutathione reductase	Randox laboratories Ltd, United Kingdom. (G R)

2.3. Instruments

Spectrophotometer	ELICO (SL 159and SL 177)
Cooling centrifuge	Remi
Deep freezer (-70^{0}C)	Remi
Automatic analyzer	Hitachi
Microlab 2000	Merck
pH meter	ELICO
Cold lab	LKB

2.4. Animals

Sprague Dawley albino rats were obtained from the Small Animal Breeding Station, Veterinary College, Mannuthy, Thrissur, Kerala

2.5. Materials and methods

Adult male albino rats of Sprague Dawley strain weighing (150-200g) were maintained under environmentally controlled conditions with free access to standard food (Lipton, India) and water. Rats were divided into seven groups.

Group I - Normal control rats: These groups of rats were kept in the ideal laboratory conditions without any kind of stress.

Group II - Fresh water swimming stress: In this type of stress, rats were forced to swim in a small plastic tub for 45 minutes (height; 60cm, diameter; 40cm containing water at room temperature, 28°C). Water depth was always maintained at 30cm. The forced swimming stress was studied for a period of 30 days.

Group III - Cold -water swimming stress: In this type of stress, rats were forced to swim in the cold water maintained at 10°C. Water depth was always maintained at 30 cm. Cold water swimming stress was studied for a period of 30 days.

Group IV- Overcrowding stress: In this type of stress rats were kept in a small cage (mice cage 290 x 220 x 140 mm) in such a way that minimum movement was possible for the rats inside the cages. The overcrowding stress was studied for a period of 30 days.

Group V – Isolation stress: Rats were individually kept in a specially designed isolation cage and isolated totally. Isolation stress was studied for a period of 30 days.

Group VI - Cigarette smoke exposure: Rats were kept in a polypropylene cage of size 43.5x29.0x16.0 cm and fed with standard diet. The animals were exposed to cigarette smoke keeping a bottomless rectangular metal container on the top of the polypropylene cage containing rats. The metallic container contains 2 holes of about 3 cm diameter. One in front

and other in the back of the container. A burning cigarette was introduced through one hole. Animals were exposed to cigarette smoke for 45 minutes daily for a period of 30 days.

Group VII- Alcohol treatment: Male albino rats were given 18% alcohol orally (4gm alcohol/kg body weight) for a period of 30 days.

At the end of the experimental period of 30 days the animals were sacrificed and blood and tissues were collected for various biochemical analysis.

2.6. Laboratory investigations

The following investigations were carried out:

2.6.1. Cholesterol

Cholesterol and its esters are released from lipoproteins by detergents. Cholesterol esterase hydrolyses the esters. In the subsequent enzyme oxidation by cholesterol oxidase, H_2O_2 is formed. This is converted into coloured quinonimine in a reaction with 4-aminoantipyrine and phenol catalysed by peroxidase (Duncan et al, 1982)

The reaction mixture contained 10 μl of serum or plasma or standard (200 mg/dl), 1ml reaction solution, which contained PIPES buffer pH 7.5 (99mmol/L), salicylic alcohol (3.96mmol/L), 4-amino antipyrine (0.5mmol/L), peroxidase (≥100U/L), cholesterol oxidase (≥100U/L), cholesterol esterase (≥100U/L). Mixed well and incubated for 5 minutes at 37°C. Then the absorbance was measured at 565 nm.

2.6.2. HDL-cholesterol

HDL-cholesterol was estimated from the supernatant after precipitation by phosphotungstic acid and 8.6mmol/L magnesium chloride (Harris *et al* 1996). 1ml of the supernatant was mixed with 1ml of reaction solution (same as that for cholesterol). Mixed well and incubated for 5 minutes at 37°C. The absorbance was measured against reagent blank at 546 nm.

2.6.3. Triglycerides

Triglycerides in presence of lipase are converted to glycerol and fatty acids. This glycerol by the action of glycerokinase is converted to glycerol-3-phosphate, which is oxidised to form dihydroxyacetone phosphate and H_2O_2. H_2O_2 generated in this step will react with aminoantipyrine and 4-chlorophenol in presence of peroxidase (POD) to form chinonimine, which is read at 546 nm. (National Cholesterol Education Programme, 1995).

10μl of serum or plasma was mixed with 1ml of reaction solution which contained Goods buffer pH 7.2 (50mmol/L), 4-chlorophenol (4mmol/L), ATP (2mmol/L), Mg^{2+} (15mmol/L), glycerokinase (≥.4KU/L), peroxidase (≥KU/L), lipoprotein lipase (≥KU/L), 4-aminoantipyrine (0.5mmol/L), glycerol-3-phosphate oxidase (≥1.5KU/L). 10 μl of triglycerides (200mg/dl) was used as the standard. Mixed and incubate at 37°C for 10 minutes. The absorbance was measured at 546 nm.

2.6.4. LDL cholesterol

LDL-cholesterol was calculated using Friedewald's formula (Friedewald et al, 1972)

LDL-cholesterol (mg%)= total cholesterol- (HDL-cholesterol+TG/5)

2.6.5. Extraction of tissue for lipid estimation

The tissues were homogenized with washed powdered glass and extracted with chloroform: methanol (2:1) and 0.5 g of tissue corresponds to 25 ml of the extract in the case of tissues other than aorta. For aorta, aortas from 2 rats (60 mg) were pooled and extract was made up to 10 ml.

2.6.5.1. Tissue cholesterol

Total cholesterol was estimated by the method of Abell (Abell et al, 1952). An aliquot from the lipid extract was pipette in to a glass stoppered centrifuge tube and evaporated to dryness. 5ml of ethanolic KOH was added and stoppered and shaken well. It was then warmed in a water bath at 37-40°C for 55 minutes. After cooling to room temperature, 10 ml of

petroleum ether (60-80°C) was added and mixed. 5 ml of water was added to this and shaken vigorously for one minute. It was then centrifuged at a low speed for 5 minutes. 4 ml of petroleum ether layer was pipetted out in to a test tube and evaporated to dryness at 60°C. A standard was treated in the same manner. 6 ml of color reagent (20 ml of acetic anhydride+1 ml of con H_2SO_4) was added to acetic acid taken as the blank. After 30-35 minutes, the optical density was read at 620 nm.

2.6.5.2. Tissue triglycerides

Triglycerides were estimated by the method of Van Handel and Zilversmit (Van Handel et al, 1957) with the modification that florisil was used to remove phospholipids. 2 g of florisil were taken in a glass-stoppered tube and 3 ml of chloroform was added. An aliquot of the extract was layered on the top of the florisil and mixed. More chloroform was than added to a total volume of 10ml. It was then stoppered, shaken intermittently for about 10 minutes and filtered through a filter paper. 1ml of filtrate was pipetted out into each of three tubes. The solvent was evaporated at 60-70^{0}C. Then 0.5 ml ethanolic KOH (0.4%) was added to 2 out of 3 tubes (saponified sample). The solvent was evaporated at 60-70^{0}C. Then 0.5ml of ethanol was added to the unsaponified sample. The tubes were closed and kept at 60-70^{0}C for 5 minutes. 0.5ml of 0.2 N H_2SO_4 was added to each tube and tubes were then placed in a gently boiling water bath for 15 minutes to remove alcohol. They were then cooled to room temperature; 0.1 ml of sodium arsenite solution (0.5 M) was then added. A yellow colour of iodine appeared and vanished within a few minutes. 5 ml of chromotropic acid was added to each tube and mixed. The tubes were closed and then heated in a boiling water bath for 30 minutes. They were cooled and the absorbance was read at 570 nm. Glycerol was used as the standard.

2.6.6. Lipid peroxidation

Lipid peroxidation is a chain reaction initiated by the attack of membrane lipids by free radicals that has sufficient reactivity to abstract a hydrogen atom from the methylene group. This leaves behind an unpaired electron on the carbon atom. The carbon radical is stabilized by molecular rearrangement to produce conjugated diene, which then react with an oxygen molecule to form a peroxy radical. Peroxy radical can form cyclic peroxide and cyclic endoperoxide. Fragmentation of these peroxides leads to the formation of malondialdehyde (MDA). This reacts with thiobarbituric acid to form coloured complex, which is measured at 532 nm. (Yoshioka et al, 1979).

0.2 ml of the serum was mixed with 1ml of 20% trichloroaceticacid (TCA). To the mixture 0.4 ml of 0.67% thiobarbituric acid (TBA) was added, shaken and kept for 30 minutes in a boiling water bath. After cooling to room temperature, 1.6ml of butanol was added and the mixture was shaken. The organic mixture was separated by centrifugation and its absorbance was measured at 532 nm. The breakdown product of 1,1,3,3 tetramethoxy propane was used as the standard

2.6.7. Tissue lipid peroxidation

The lipid peroxide formation was measured by the method of Okhawa (Okhawa et al, 1979). 0.1 ml of tissue homogenate (25%) in tris HCl buffer (pH 7.4) was incubated in a reaction mixture containing KCl (0.1ml), ascorbic acid 0.1 ml and ferrous ammonium sulphate, 0.1ml for one hour at 37°C. The reaction mixture was allowed to cool and centrifuged at 2000 rpm. The incubated reaction mixture 0.4 ml was taken and treated with SDS (0.2 ml, 8%) and acetic acid 1.5 ml (20%, pH 3.5). The total volume was made up to 4 ml by adding distilled water and 5 ml of a mixture of N- butanol- pyridine (15:1) were added and shaken vigorously. The absorbance of the organic layer was measured at 560 nm after centrifugation.

2.6.8. Conjugated dienes

Conjugated dienes estimated by the method of Brein (Brein et al, 1966). 1 ml of aqueous tissue homogenate was mixed thoroughly with 5 ml chloroform:methanol (2:1) followed by centrifugation at 1000 rpm for 5 minutes to separate the phases. The lower layer of chloroform was evaporated to dryness under a stream of nitrogen at 45°C. The lipid residue was dissolved in 1.5 ml of cyclohexane. Optical density at 233 nm was determined against a cyclohexane blank. The amount of conjugated dienes produced can be calculated using the molar extinction coefficient of $2.52x\ 10^4$/ M/cm

2.6.8. Hydroperoxides

Hydroperoxides estimated by the method of Nair R.D (Nair et al, 1971). 1ml of aqueous tissue homogenate was mixed thoroughly with 5 ml chloroform:methanol (2:1) followed by centrifugation at 1000 rpm for 5 minutes to separate the phases. The lower layer of chloroform was evaporated to dryness under a stream of nitrogen at 45°C, while still under a stream of nitrogen. 1 ml of acetic acid:chloroform followed 0.05 ml potassium iodide (1.2g / mlwater) were quickly added and mixed. The samples were placed in the dark at room temperature for 5 minutes followed by addition of 3 ml of cadmium acetate (0.5g /100mlwater). The solution was mixed and centrifuged at 1000 rpm for 10 minutes. The optical density of upper phase was determined at 353 nm against a blank containing the complete assay mixture except the lipid. Standardization of the reaction was done by cumene hydroperoxides as peroxide standard. The molar extinction coefficient of cumene hydroperoxide is $1.73x10^4$/M/ cm.

2.6.9. Superoxide dismutase (SOD)

SOD is measured by the degree of inhibition of formazan dye, which is formed by the reduction of nitroblue tetrazolium (NBT) in presence of riboflavin (Mc Cord et al, 1969). The assay system consists of 0.6 M phosphate buffer pH 7.4, 0.12 M riboflavin (50 µl), 0.1M

EDTA containing 0.0015% sodium cyanide (200μl), 1.5mM nitroblue tetrazolium (100 μl) and various concentrations of test materials, in a total volume of 3 ml. The tubes were illuminated under an incandescent lamp for 15 minutes. Optical density at 530 nm was measured before and after illuminations and the percentage inhibition was calculated using the formula.

$$\frac{\text{Optical density of control tube - optical density of treated tubes}}{\text{Optical density of control tube}} \times 100$$

One unit of enzyme activity is defined as the enzyme concentration required to inhibiting optical density measurements at 560 nm of chromogen production by 50% in one minute under assay condition.

2.6.10. Catalase

In the UV range, hydrogen peroxide shows a continued increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in extinction at 240nm (E_{240}). The difference in extinction (ΔE_{240}) per unit time is a measure of the catalase activity (Aebi, 1983). The estimation was done spectrophotometrically following the decrease in optical density measurements at 230 nm. The assay system consisted of phosphate buffer (50 mM, pH 7) 1 ml, 0.1 ml enzyme solution, 1 ml H_2O_2 solution (30 mM) in test. Control tube contained 2 ml of enzyme solution, the reaction occurred by the addition of H_2O_2. The decrease in extinction was recorded at 240 nm at 15 seconds intervals for 3 minutes.

2.6.11. Reduced glutathione (GSH)

The glutathione was estimated by the reaction of GSH with DTNB to give a yellow colored complex with absorption maximum at 412 nm (Moron et al, 1979).

The reaction mixture consisted of 0.5 ml oftissue homogenate. 125 μl of 25% TCA was added to precipitate proteins. The tubes were cooled on ice for 5 minutes and the mixture was further diluted with 0.6 ml of 5% TCA, Centrifuged for 10 minutes and 0.3 ml of the resulting

supernatant was taken for GSH estimation. The volume of aliquot was made up to 1 ml with 0.2 M phosphate buffer pH 8). 2 ml of freshly prepared 0.6 mM DTNB was added to the tubes and the intensity of yellow colour was read at 412 nm. Values were expressed as nmol/mg protein.

2.6.12. Glutathione peroxidase (GPx)

Glutathione peroxidase was estimated by the method of Hafemann (Hafemann et al, 1974). The reaction mixture consisted of 50mM phosphate buffer (pH 7.0), 1 mM sodium azide and 0.2 mM H_2O_2 in a total volume of 2.5 ml was incubated at 37°C for 6 minutes. After addition of 2 ml of 1.67% phosphoric acid, the mixture was centrifuged at 8000g for 15 minutes. The supernatant (2 ml) was added to a mixture of 2 ml of 0.4 M Na_2HPO_4 and 1 ml of 1 mM DTNB. After 10 minutes incubation at 37°C the absorbance of the reaction mixture was measured at 412 nm. One unit of enzyme activity was defined as the decrease in log GSH by 0.001/minute, after subtraction of the decrease in log GSH / minute for the non-enzymatic reaction and expressed as units / mg protein.

2.6.13. Glutathione-S- transferase (GST)

Glutathione –S- tranferase was estimated by the method of Habig (Habig et al, 1974). The reaction mixture contained 2.79 ml phophate buffer (0.1M, pH 6.5), 100 µl of GSH (30 mM), 10 µl of homogenate and 100 µl of CDNB (30 mM). The absorbance was noted for 3 minutes at one-minute interval at 340 nm at 37°C.

2.6.14. Glutathione reductase (GR)

Glutathione reductase activity was determined by the method of Racker (Racker et al, 1995). The amount of NADPH consumed in the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH). The reaction is catalysed by glutathione reductase.

The reaction mixture contained 100 µl of GSSG (2.2mmol/I), 100 µl of EDTA and

100 μl of NADPH and 680 μl of phosphate buffer (pH 6.6). The reaction commences with the addition of 20 μl sample and a decrease in absorbance/ minute was noted and followed at every 1minute interval for 5 minutes at 340 nm.

2.6.15. Serum Glutamate Oxaloacetate Transaminase (SGOT orAST)

AST was estimated by the meyhod of IFCC (IFCC).

L- aspartate + α ketoglutarate $\xrightarrow{GOT}$ Oxaloacetate + L-Glutamate

Oxaloacetate + NADH + H^+ $\xrightarrow{MDH}$ L- Malate + NAD^+.

AST - Aspartate aminotransferase

MDH - Malate dehydrogenase

The reaction mixture contained I ml of the reagent 100 μl of the sample, mix well and read the OD at 340 nm. Reagents: MDH (≥ 600 U/ L), LDH (≥ 900 U/L), NADH (0.20 mmol / L), α keto glutarate (12 mmol/L), Tris buffer [(pH 7.8) 88mmol / L], L- Aspartate (260 mmol/L)

2.6.16. Glutamate Pyruvate Transaminase (ALT)

ALT was estimated by the method of IFCC (IFCC).

L- Alanine + α ketoglutarate $\xrightarrow{GPT}$ Pyruvate + L-Glutamate

Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ L- Lactate + NAD^+.

ALT - Alanine aminotransferase

MDH - Malate dehydrogenase

The reaction mixture contained I ml of the reagent 100 μl of the sample, mix well and read the OD at 340 nm. (Reagent LDH (≥1200 U/L), NADH (0.20 mmol / L), α keto glutarate (16mmol/L), Tris buffr [(pH 7.5) 110mmol / L], L- Alanine (550 mmol/L)

2.6.17. HMG CoA reductase

HMG CoA reductase activity of the liver tissue was estimated as described by Rao (Rao et al, 1975) by determining the ratio of HMG CoA to mevalonate.

Equal volumes of freshly prepared 10% tissue homogenate and dilute perchloric acid (50 ml made up to 1 litre) were mixed, kept for 5 minutes and centrifuged at 2000 rpm for 10 minutes. To 1 ml supernatent 0.5 ml of freshly prepared hydroxylamine hydrochloride reagent (alkaline hydroxylamine reagent in case of HMG Co A) was added,mixed and after 5 minutes, 1.5ml of ferric chloride reagent was added (5.2 g TCA+10 g $FeCl_3$ in 50 ml of 0.65 N HCl and made up to 100 ml with latter). After shaking well, readings were taken after 10 minutes at 540 nm against a similarly treated saline arsenate blank. The ratio of HMG Co A to mevalonate is taken as index of activity of the enzyme which catalyses the conversion of β hydroxy β methyl glutaryl CoA to mevalonate, lower the ratio, higher the enzyme activity.

2.6.18. Glucose-6- phosphate dehydrogenase

The enzyme was assayed by the method of Kornberg (Kornberg et al, 1955). The chilled tissue was homogenized with 3 volumes of glycyl glycine buffer pH 7.5. The homogenate was centrifuged at 0°C for 10 minutes. The supernatent was used as the enzyme source. To 1 ml of substrate, (0.02 ml glucose-6-phosphate, 0.1 ml of $NADP^+$ (0.0015 M), 0.25 ml of buffer (glycyl-glycine 0.04 M pH 7.5) and 0.2 ml of $MgCl_2$ (0.1 M) were added and the absorbance was read immediately at 340 nm at one minute intervals for 5 minutes. One unit of enzyme activity is defined as the amount of enzyme which cause an increase of 1.00 in optical density / minute per g protein.

2.6.19. Malic enzyme

The enzyme was assayed by the method of Ochoa (Ochoa etal,1955). The chilled tissue was homogenized with 3 volumes of glycyl-glycine buffer pH 7.4. The homogenate was centrifuged at 2000 rpm at 0°C for 10 minutes. The supernatant was used as the enzyme source. The reaction mixture consisted of 0.3 ml of 0.25 M glycyl-glycine buffer pH 7.4, 0.06 ml of 0.05 M $MnCl_2$ (3 mM), 0.1 ml of $NADP^+$ (0.135 μmoles), enzyme and water to a final volume of 3 ml. The enzyme assay was carried out at room temperature (23-25°C). The reaction was

started by the addition of either malate or enzyme and optical measurements at 340 nm were taken against a blank cuvette containing all components except $NADP^+$ at 15 seconds intervals for two minutes. One unit of enzyme activity is defined as the amount of enzyme, which causes an increase of 0.01 in optical density/ minute/g protein.

2.6.20. Isocitrate dehydrogenase

The enzyme was assayed by the method of Ochoa (Ochoa et al, 1955). The reaction mixture consisted of 0.3 ml of glycyl glycine buffer (pH 7.4), 0.1 ml of Mn Cl_2 (1.8 μm), 0.1 ml of $NADP^+$ (0.135m), 0.1ml of disodium isocitrate (0.006 M), enzyme and water to a final volume of 3 ml. The enzyme assay was carried out at room temperature (23-25°C), the reaction was started by the addition of either isocitrate or enzyme and optical density measurements at 340 nm were taken against a blank containing all components except $NADP^+$ at 15 seconds intervals for two minutes.

One unit of enzyme is defined as the amount of enzyme, which causes an initial change in OD of 0.01/ minute under the above conditions. Specific activity is expressed as units /mg protein.

2.6.21. Total proteins

Protein was estimated in all enzyme extracts by the method of Lowry (Lowry et al, 1951).

1. Reagent A: 2 g Na_2CO_3 in 100 ml 0.1 N NaOH
2. Reagent B: 5 g $CuSO_4$ in 100 ml, 2% Rochelle salt (Sodium potassium tartarate) solution.
3. Reagent C: Mix 50 ml of A with 1 ml of B
4. Phenol reagent (Folin Ciocalteu reagent) :(1:1dilution). It is a mixture of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid.

0.1 ml of enzyme source is taken and made up to 1ml with distilled water. To this 5 ml of reagent C is added and kept for ten minutes. Then 0.5 ml of Folin Ciocalteu reagent is added. Optical density is read after 30 minutes at 670 nm.

2.7. Statistical evaluation

Statistical significance were determined using students 't ' test (Lutz N, 1967) according to the formula. To find out the statistical significance between group X and group Y. Value of 't' was found out from the equation

$$t = \frac{x - y}{\sqrt{S^2(1/nx + 1/ny)}}$$

x is the mean value of group (X). y is the mean value of groupY.

nx—number of sample X

ny---- number of sample Y.

S was found out from the equation.

$$S = \sqrt{\frac{(Sx^2\,(nx-1) + Sy^2\,(ny-1)}{(nx+ny-2)}}$$

Sx--- Standard deviation of sample x.

Sy--- Standard deviation of sample y

nx+ny—2 is the degree of freedom, statistical significance (p values) are found out from't' distribution table.

EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT FOR THE DEGREEE OF

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CHAPTER 3

EFFECT OF STRESS ON LIPID PROFILES, LIPOGENIC ENZYMES AND TRANSAMINASES

Stress is considered as state of disharmony or threatened homeostasis. Stressful stimulus influences the onset and progression of a number of disorders in human beings leading to hypertension, stroke or depression. Stress induces the production of free radicals, which leads to lipid peroxidation. Stress induced psychiatric problems, psychosomatic disorders, hypertension and heart diseases are on the rise across the globe. Stress has become an important etiological factor in the development of gastric ulcers (Das et al, 1997). Adenocorticotropic hormone is released by anterior pituitary during stress, bringing about the release of corticosteroids such as cortisol and cortisone, resulting in a relative change in carbohydrate metabolism, an increase in protein metabolism and mobilization of fat depots.

In the present chapter our aim is to study the effect of physical stress (fresh water swimming, cold water swimming), psychological stress (isolation and overcrowding) and stress by external agents (exposure to cigarette smoke and alcohol administration) on lipid parameters, (Blood and tissue cholesterol, triglycerides, HDL- cholesterol, LDL-cholesterol), transaminases [glutamate oxaloacetate transaminase (AST), glutamate pyruvate transaminase (ALT)] and the lipogenic enzymes, HMG CoA reductase, the key enzyme involved in cholesterol synthesis and enzymes glucose-6-phosphate dehydrogenase, and malic enzyme.

3.1. Materials and methods

3.1.1. Animal model

Male albino rats (Sprague-Dawley strain) weighing (150-200g), were divided into seven groups of 6 rats each.

Group I - Normal control rats

Group II - Fresh water swimming (F W S)

Group III - Cold water swimming (C W S)

Group IV - Overcrowding

Group V - Isolation

Group VI - Exposure to cigarette smoke

Group VII - Alcohol administered

Animals were subjected to the above-mentioned stress for a period of one month. Detailed procedure adopted for the animal experiments are given in chapter 2. At the end of the experimental period the animals were sacrificed and their blood, liver, heart, kidney and aorta were collected for various biochemical estimations.

3.1.2. Methods

Total cholesterol was estimated by CHOD-PAP method, HDL –cholesterol was done by the precipitation using phosphotungstic acid and $MgCl_2$. Triglycerides was done by the GPO-PAP method and LDL-cholesterol was estimated by using Friedewald's formula, glutamate oxaloacetate transaminase (AST) and glutamate pyruvate transaminase (ALT) assayed using enzymatic kit supplied by Bayer diagnostics. HMG CoA reductase activity of the liver tissue was estimated by the method of Rao and Ramakrishnan (Venugopala Rao et al, 1975), glucose-6-phosphate dehydrogenase by the method of Kornberg , (Kornberg et al,1955), malic enzyme by Ochoa (Ochoa et al,1955). The detailed procedure for various estimations is given in chapter 2.

3.2. Results

3.2.1. Body weight

Table 3a depicts the body weight of normal animals and animals exposed to different types of stress. The body weight significantly increased in the isolated group

(P<0.001), cigarette smoke exposed (P<0.01) group, when compared with that of control rats. A significant decrease (P<0.05) was observed in the fresh water-swimming group when compared with that of control rats.

3.2.2. Serum cholesterol

Table 3b represents the concentration of serum cholesterol, LDL-cholesterol, HDL-cholesterol, atherogenic index (Total cholesterol/HDL cholesterol) of normal control rats and animal exposed to different types of stress. Serum cholesterol and LDL-cholesterol were significantly increased (P<0.001) in isolation, overcrowding, alcohol administered and smoke exposed when compared with that of control rats. A significant decrease was observed in fresh water swimming (P<0.05), and cold-water swimming (P<0.01) group. HDL-cholesterol was significantly (P<0.001) decreased in the smoke exposed, alcohol administered and isolated group when compared with normal control rats. Values of HDL-cholesterol were also significantly increased in fresh water swimming (P<0.01) and cold-water swimming (P<0.05) group when compared with normal control rats. A significant decrease was observed in the atherogenic index (total cholesterol / HDL chol) of animals exposed to fresh water swimming and cold-water swimming stress when compared with that of normal control rats. Values of atherogenic index were significantly increased (P<0.001) in the smoke exposed, alcohol administered and isolated group when compared with the normal control rats.

3.2.3. Tissue cholesterol

Table 3c represents values of cholesterol in the liver, heart, kidney and aorta of animals exposed to different types of stress. The values of liver and heart cholesterol were significantly increased (P<0.001) in the isolated group, alcohol administered group and smoke exposed group when compared with control rats. A significant decrease was observed in the liver (P<0.05) and heart (P<0.01) tissue of the fresh water swimming and

cold-water swimming group compared with control group. No significant change was observed in the overcrowding group compared with control group. A significant increase ($P<0.001$) in cholesterol was observed in the kidney and aorta of rats exposed to cigarette smoke, alcohol administration, and isolation stress compared with control group. A significant decrease ($P<0.01$) was observed in the kidney of fresh water swimming group. No change was observed in the aorta of animals exposed to swimming stress.

3.2.3. Serum and tissue tryglycerides

Table 3d represents the concentration of triglycerides in the serum, liver, heart, kidney and aorta of animals exposed to different types of stress. The value of triglycerides increased significantly ($P<0.001$) in the serum of rats exposed to all types of stress except fresh water swimming and cold-water swimming stress compared with normal control rats. A significant decrease was observed in the serum of rats exposed to fresh water swimming stress ($P<0.05$), cold-water swimming stress ($P<0.01$). Values of triglycerides increased significantly ($P<0.001$) in the liver, heart, kidney and aorta of rats exposed to cigarette smoke, isolation stress and alcohol administration when compared with the control rats. No significant change was observed in the kidney and aorta of rats exposed to overcrowding and swimming stress when compared with the control rats.

3.2.4. Transaminases

Table 3e represents the activities of serum glutamate pyruvate transaminase and glutamate oxaloacetate transaminase in the control group and rats subjected to all types of stress. The activities of serum glutamate oxaloacetate transaminase (AST) and pyruvate transaminase (ALT) were found to be significantly increased in the serum of rats subjected to alcohol administration and cigarette smoke exposure when compared with that of control rats. A significant increase in AST activity ($P<0.01$) and ALT activity ($P<0.001$) was observed in the fresh water swimming and cold water swimming group

when compared with normal control rats. A significant increase ($P<0.05$) in AST activity was observed in the overcrowding and isolated group when compared with normal control rats. A significant increase ($P<0.01$) in ALT was observed in the isolated group when compared with normal control rats.

3.2.5. Lipogenic enzymes

Table 3f represents the activities of HMG CoA reductase , glucose-6-phosphate dehydrogenase and malic enzyme in the liver of rats exposed to different types of stress conditions. The HMG CoA reductase is the key enzyme involved in the synthesis of cholesterol. HMG CoA reductase activity was significantly increased ($P<0.05$) in the fresh water swimming ($P<0.001$), cold-water swimming ($P<0.05$) group respectively. The values of HMG CoA reductase is significantly increased ($P<0.001$) in the isolation, overcrowding and cigarette smoke exposed group compared with normal control rats. No significant change was observed in the alcohol treated group. The glucose-6-phosphate dehydrogenase activity was significantly increased ($P<0.05$) in the liver of rats exposed to fresh water swimming, cold-water swimming, isolation and. A significant increase ($P<0.001$) was observed in the liver of rats exposed to cigarette smoke and alcohol administration. A significant increase ($P<0.001$) was observed in the activity of malic enzyme of rats exposed to fresh-water swimming, cold-water swimming stress rats administered with alcohol and cigarette smoke exposure compared with normal control rats. A significant increase in malic enzyme ($P<0.01$) was observed in the liver of rats exposed to overcrowding stress compared with normal control rats.

3.3. Results and Discussion

In the present study body weights were increased in isolated and smoke exposed group when compared to normal control rats. Rats subjected to alcohol administration,

overcrowding, fresh water swimming and cold-water swimming showed a decrease in body weight compared with normal control rats.

The values of the lipid parameters such as cholesterol, triglycerides (TG) and LDL- cholesterol were found to be significantly increased, and the values of HDL-cholesterol was found to be significantly decreased in the serum, liver, heart, kidney and aorta of the rats exposed to isolation, alcohol administration and smoke exposure. Swimming stress and overcrowding stress showed a decrease in cholesterol, triglycerides and LDL-cholesterol. This observation indicates that all the above-mentioned stress except swimming stress can lead to significant alteration in lipid metabolism.

Swimming is a strenuous exercise. During swimming large amount of energy is spend. Lipids are utilized for this purpose. Isolation and overcrowding causes the release of corticosteroids and which induces hyperinsulinemia resulting in increased lipogenesis. Though there was no change observed in the food intake initially, a significant increase in the food intake was observed in the later part of the experimental period. There was a significant reduction in the water intake in the stressed animals. Ethanol feeding has been found to enhance the endogenous synthesis of triglycerides resulting in their accumulation in the liver. The elevated levels of cholesterol and triglycerides by smoke exposure may be due to trace amount of cadmium, a metallic element present in cigarette smoke. In general, the increased cholesterol level may be due to the increased activity of HMG CoA reductase, which is the key enzyme involved in cholesterol synthesis.

AST and ALT are markers for hepatic function as their elevation indicate parenchymal liver damage. AST and ALT were increased significantly in animals exposed to all types of stress. A significant increase ($P<0.001$) was observed in the group of rats subjected to alcohol, and cigarette smoke. A significant increase ($P<0.05$) was observed in the enzyme activities in fresh water- swimming group, cold-water swimming

group, isolated group and overcrowded group. The observed increase in serum AST and ALT in swimming group, isolation group was in accordance with various reports published earlier (Prabakaran et al, 1988, and Lalitha et al, 1988). The observed increase in these transaminases in stressful situation might be as a result of cortisol-induced gluconeogenesis in the liver (Rosen et al, 1959). Of the two transaminases AST level is particularly interesting to correlate with myocardial damage, also because the duration and extent of increase in this transaminase can be related to the size of infarct, if any. The HMG CoA reductase activity increased in all stress condition accepts alcohol administered and overcrowding group. The activity of HMG CoA reductase increased in different stress condition may be due to the release of adrenaline and cortisol. The HMG CoA reductase activity decreased in alcohol fed group due to the fatty liver. The glucose-6- phosphate dehydrogenase activity helps to increase HMP shunt and hence the supply of NADPH. The activities of HMG Co A reductase, glucose-6-phosphate dehyrogenase and malic enzyme is also found to be increased, which may be due to the increased release of stress hormones cortisol and adrenaline. Hypercholesterolemia and hypertriglyceridemia observed in the present study in ethanol-administered rats is in agreement with the results reported earlier (Augusti , 2002).

From the result it is evident that in the types of stress studied: isolation, overcrowding, alcohol intake and cigarette smoke exposure produces significant changes in the lipid metabolism.

Table 3a

Values of the body weight of control animals and animals subjected to different types of stress

Group	Body weight (g)
Normal	178 ± 3.86
F W S	172 ± 3.98***
C W S	174 ± 4.62
Over crowding	176 ± 3.86
Isolation	193 ± 3.42*
Alcohol administration	177 ± 3.08
Cigarette smoke exposure	186 ± 4.01**

Values represent mean ±SD n=6
*P<0.001 **P<0.01 ***P<0.05

FWS : Fresh water swimming stress
CWS : Cold water swimming stress

Normal group is compared with FWS similarly with CWS, Overcrowding, isolation, alcohol administration and smoke exposure respectively

Table: 3b

Concentration of total cholesterol, LDL-cholesterol, HDL-cholesterol (mg%) and atherogenic index in the serum of control and rats subjected to different types of stress

Group	Totalcholesterol	HDL-cholesterol	LDL-cholesterol	Atherogenic index Total chol/HDL chol
Normal	62.39 ±1.89	32.72 ± 0.72	27.27 ± 0.78	1.90 ± 0.09
F W S	59.13 ±1.32***	34.87 ± 0.92**	22.14 ± 0.53*	1.69 ± 0.07**
C W S	57.09 ±1.53**	34.18 ± 0.87***	21.07 ± 0.69*	1.67 ± 0.03**
Overcrowding	68.12 ±1.32*	30.97 ± 0.87**	33.84 ± 0.35*	2.40 ± 0.05*
Isolation	70.22 ±1.12*	29.17 ± 0.78*	37.48 ± 0.49*	2.43 ± 0.10*
Cigarette smoke	93.87 ±1.82*	24.28 ± 0.68*	63.95 ± 0.39*	3.86 ±0.06*
Alcohol	89.06 ±1.47*	30.74 ± 0.92**	53.22 ± 0.92*	2.89 ± 0.07*

Values represent mean ±SD n = 6
*P<0.001 **P<0.01 ***P<0.05

FWS : Fresh water swimming stress
CWS : Cold water swimming stress

Normal group is compared with FWS similarly with CWS, Overcrowding, isolation, alcohol administered and smoke exposed group respectively

Table: 3c

Concentration of tissue cholesterol in the control and rats subjected to different types of stress (mg /100g wet tissue).

Group	Liver	Heart	Kidney	Aorta
Normal	145.89 ± 4.01	87.67 ± 1.71	2.56 ± 0.02	120.6 ± 2.73
F W S	140.02 ± 3.02***	83.70 ± 1.62**	2.49 ± 0.01 **	120.7 ± 2.95
C W S	138.01 ± 2.99***	83.31 ± 2.01**	2.54 ± 0.02	121.1 ± 2.34
Over crowd	147.20 ± 3.01	88.00 ± 2.14	2.60 ± 0.01***	119.9 ± 2.15
Isolation	160.91 ± 3.98*	93.90 ± 1.93*	3.80 ± 0.03*	130.7 ± 1.62*
Cigarette	188.01 ± 3.90*	100.5 ± 2.15*	3.90 ± 0.03*	138.6 ± 3.28*
Alcohol	208.31 ± 5.01*	116.5 ± 2.41*	3.59 ± 0.04*	140.6 ± 2.73*

Values represent mean ±SD. n= 6
*P<0.001 **P<0.01 ***P<0.05

FWS : Fresh water swimming stress
CWS : Cold water swimming stress
Normal group is compared with FWS similarly with CWS, Overcrowding, isolation, alcohol administered and smoke exposed group respectively

Table: 3d

Concentration of triglycerides (mg%) in the serum, and tissues (mg/100g wet tissues) liver heart, kidney and aorta of control and rats subjected to different types of stress

Group	Serum	Liver	Heart	Kidney	Aorta
Normal	12.05 ± 0.98	480.1 ± 8.38	545.3 ± 16.8	182.1 ± 4.01	1025 ± 22.55
F W S	10.65 ± 0.83***	476.9 ± 8.99	540.8 ± 19.3	179.8 ± 3.97	1024 ± 28.75
C W S	09.91 ± 0.72**	475.9 ± 9.38	541.2 ± 20.3	180.1 ± 5.84	1025 ± 19.99
Over crowding	16.55 ± 0.88*	485.5 ± 10.2	568.3 ± 15.1***	185.1 ± 5.90	1028 ± 29.13
Isolation	16.25 ± 0.83*	513.2 ± 12.0*	600.9 ± 19.4*	200.3 ± 3.87*	1048 ± 31.03
Cigarette smoke	24.38 ± 0.37*	562.1 ± 9.23*	610.0 ± 20.4*	281.2 ± 3.97*	1323 ± 38.23*
Alcohol	25.38 ± 0.29*	648.9 ± 10.7*	700.2 ± 18.3*	310.2 ± 4.81*	1454 ± 33.45*

Values represent mean ±SD n = 6
*P<0.001 **P<0.01 ***P<0.05

FWS : Fresh water swimming stress
CWS : Cold water swimming stress

Normal group is compared with FWS similarly with CWS, Overcrowding, isolation, alcohol administered and smoke exposed group respectively

Table: 3e

Activities of serum glutamate oxaloacetate transaminase(AST) and pyruvate transaminase (ALT) in the control and rats subjected to different types of stress

Group	AST (U/L)	ALT (U/L)
Normal	118.49 ± 2.70	61.18 ± 1.70
F W S	126.32 ± 2.91**	68.99 ± 2.01*
C W S	128.23 ± 3.10**	70.97 ± 2.33*
Over crowding	124.99 ± 3.41***	67.18 ± 2.19**
Isolation	124.01 ± 3.60***	64.01 ± 2.08
Cigarette smoke	180.38 ± 2.87*	92.32 ± 3.23*
Alcohol	161.32 ± 2.93*	76.28 ± 2.38*

Values represent mean ±SD of six rats in each group
*P<0.001 **P<0.01

FWS : Fresh water swimming stress
CWS : Cold water swimming stress

Normal group is compared with FWS similarly with CWS, Overcrowding, isolation, alcohol administered and smoke exposed group respectively

Table: 3f

Activity of HMG CoA reductase, glucose-6-phosphate dehydrogenase and malic enzyme in the liver of rats exposed to different types of stress.

Group	Stress	Ratio of HMG A CoA to mevalonate	Glucose-6-p dehydrogenase	Malic enzyme
1	Control	1.910 ± 0.036	110.8 ± 5.29	820.3 ± 20.1
2	F W S	1.780 ± 0.039*	120.3 ± 1.98*	831.2 ± 18.2*
3	C W S	1.832 ± 0.038***	125.2 ± 2.01*	830.6 ± 22.3*
4	Overcrowding	1.718 ± 0.030*	113.8 ± 2.65	822.7 ± 29.3**
5	Isolation	1.608 ± 0.029*	120.3 ± 2.93*	821.2 ± 30.0
6	Alcohol	2.010 ± 0.044	138.7 ± 4.20*	1025 ± 23.2*
7	Cigarette smoke	1.500 ± 0.039*	134.3 ± 3.18*	1003 ± 28.1*

Value represents mean ±SD of 6 rats in each group
*P<0.001 **P<0.05

FWS - Fresh water swimming group
CWS - Cold water swimming group

Normal group is compared with FWS, similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposure respectively

EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT FOR THE DEGREEE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY (FACULTY OF SCIENCE)

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CHAPTER 4

EFFECT OF STRESS ON LIPID PEROXIDATION

Assessing the stress may be difficult because of the fact that an activity that may be stressful for one person may be regarded as a positive challenge by another. The possible mechanisms by which stress exerts its negative effects on coronary heart disease has been cited as increase in blood pressure, heart rate, increased plasma cholesterol levels and adverse effects on coagulation and fibrinolysis (Jhonston et al, 1993). With the urbanization and westernization of our society stress is increasing day-by-day, the result of which is an increased incidence of psychosomatic, psychiatric and cardiovascular diseases.

In the present chapter we are aiming at understanding the effect of physical stress, (fresh water and cold water swimming) psychological stress (isolation and overcrowding) and oxidative stress (exposure to cigarette smoke, alcohol intake and heat) on lipid peroxidation. Lipid peroxidation is known to play an important role in atherogenesis (Coresh et al, 1996). Increase in serum lipid peroxide level has been reported in diabetics, stress conditions, in patients with cardiovascular disorders, (Gallou et al, 1993) and in aging subjects (Vysal et al, 1986).

4.1 Materials and method

4.1.1 Animal model:

Male albino rats (Sprague-Dawley strain) weighing 150-200g were divided into seven groups of six rats each.

Group I - Normal control rats

Group II - Fresh water swimming

Group III - Cold water swimming

Group IV - Over crowding

Group V - Isolation

Group VI - Cigarette smoke exposure

Group VII - Alcohol administration

Animals were subjected to the above-mentioned stress for a period of one month. Detailed experimental procedure is given in the chapter 2. At the end of the experimental period animals were sacrificed and their blood, liver, lungs, kidney and aorta were removed to ice cold containers for various estimations.

4.1.2. Methods

The lipid peroxidation product - serum malondialdehyde (MDA) was estimated by the method of Yoshioka et al (Yoshioka, 1979). Tissue malondialdehyde was estimated by the method of Ohkawa et al (Ohkawa et al, 1979). Conjugated dienes was estimated by the method of Brien (Brien, 1969) and hydroperoxides by the method of R.D Nair (R D Nair, 1963). The detailed procedures for various estimations are discussed in chapter 2.

4.2. Result and discussion

4.2.1. Malondialdehyde in blood

Table 4a represents the concentration of malondialdehyde in blood of control and rats subjected to different types of stress. Serum malondialdehyde was significantly increased ($P<0.001$) in fresh water swimming, cold-water swimming, alcohol administered and cigarette smoke exposed groups when compared with normal control rats. A significant increase ($P<0.05$) was observed in the isolation group compared with normal control rats.

4.2.2. Malondialdehyde in the tissues

Table 4b represent the concentration of malondialdehyde in tissues of rats exposed to different types of stress. Concentration of malondialdehyde was found to be increased significantly ($P<0.001$) in the liver tissue of rats exposed to fresh water swimming, cold water swimming, overcrowding, alcohol administered and cigarette smoke exposed group when compared with normal control rats. A significant increase ($P<0.05$) was observed in the liver tissue of rats exposed to isolation stress. A significant increase was observed ($P<0.001$) in the lung tissue of rats exposed to cigarette smoke when compared with normal control rats. No

significant increase was observed in the lung tissue of rats exposed to fresh water swimming, cold- water swimming, isolation, overcrowding and alcohol administered groups when compared with control rats. A significant increase ($P<0.001$) was observed in the heart tissue of rats exposed to fresh water swimming, cold-water swimming and alcohol administration when compared with normal control rats. A significant increase ($P<0.05$) was also observed in the heart tissue of rats exposed to overcrowding stress when compared with normal control rats. No significant change was observed in the heart tissue of rats exposed to cigarette smoke and isolation stress. A significant increase ($P<0.001$) was observed in the kidney of rats exposed to alcohol and cigarette smoke exposure when compared with normal controlled rats. A significant increase was observed in the kidney of rats exposed to fresh water swimming ($P< 0.05$) and cold- water swimming stress ($P<0.01$) when compared with normal control rats. No significant change was observed in the kidney of rats exposed to isolation and overcrowding stress.

4.2.3. Lipid peroxides

Table 4c represents concentration of lipid peroxides in the liver, lungs, heart and kidney of rats exposed to all types of stress. Concentration of hydroperoxides increased significantly ($P<0.001$) in the liver tissue of rats exposed to fresh water swimming, cold-water swimming, alcohol administration and cigarette smoke exposure. A significant increase ($P<0.05$) was observed in the liver tissue of rats exposed to isolation stress. No significant change was observed in the liver tissue of rats exposed to overcrowding stress. No significant increase was observed in the concentration of hydroperoxides in the lung tissue of rats exposed to all types of stress except cigarette smoke. A significant increase ($P<0.001$) was observed in the lung tissue of rats exposed to cigarette smoke when compared with normal control rats. Concentration of hydroperoxides in the heart tissue was significantly increased ($P<0.001$) in the fresh water swimming group, cold- water swimming group and alcohol-

administered group when compared with that of control rats. No significant increase was observed in the overcrowding, cigarette smoke exposed and isolated group compared with normal control rats. Concentration of hydroperoxides in the kidney was significantly ($P<0.001$) increased in the group of rats subjected to fresh water swimming stress, cold-water swimming, alcohol administration and smoke exposure compared with normal control rats. No significant increase was observed in the kidney of rats exposed to isolation and overcrowding stress.

4.2.4. Conjugated Dienes

Table 4d represents concentration of conjugated dienes in the liver, heart, lungs and kidney of rats exposed to different types of stress. Concentration of conjugated dienes significantly increased ($P<0.001$) in the liver, heart and kidney of rats exposed to cigarette smoke and alcohol administration, when compared with normal control rats. A significant increase ($P<0.001$) was observed in the heart tissue of rats exposed to fresh water swimming and cold- water swimming stress. A significant increase was observed in the liver tissue of rats exposed to fresh water swimming ($P<0.05$), and cold-water swimming stress ($P<0.001$). A significant increase ($P<0.01$) was observed in the kidney of rats exposed to fresh water swimming and cold-water swimming stress. No significant change was observed in the liver, heart and kidney of rats exposed to isolation and overcrowding stress. A significant increase ($P<0.001$) was observed in the lung tissue of rats exposed to cigarette smoke when compared with normal control rats. No significant change was observed in the lung tissue of rats exposed to fresh water swimming, cold-water swimming, overcrowding, isolation and alcohol administration when compared with normal control rats.

Results of the present study clearly shows that the concentration of lipid peroxides, hydroperoxides and conjugated dienes in the serum, liver and heart of fresh water swimming, cold-water swimming, alcohol administered and cigarette smoke exposed group were

increased when compared to that of normal control rats. No significant change was observed in the heart and kidney of rats exposed to isolation and overcrowding stress.

Stress induces the production of free radicals in the body. Swimming is a strenuous exercise. During swimming large amount of energy is utilized, which increases oxygen supply to active tissues. The rate of oxygen uptake by the body, during swimming, may increase by 10-15 fold, which increases lipid peroxidation. Most of the oxygen is reduced to water and a few are reduced to various intermediates such as oxygen free radicals and hydroxyl radicals and it induces lipid peroxidation. Strenous physical exercise induces oxygen free radicals and induces lipid peroxidation and oxidative damage to all the tissues. Similarly isolation and overcrowding stress induces the formation of free radicals and enhances lipid peroxidation, though the reason behind this is not clearly understood. Ethanol is said to enhance the generation of oxygen free radicals during its oxidation in the liver. Ethanol induced toxic effects are mediated through enhanced generation of free radicals. Oxygen free radicals enhance lipid peroxidation in the serum and tissues of rats fed with ethanol. Free radicals and oxidants in cigarette smoke are responsible for lipid peroxidation in serum and different tissues of rats. The massive surface area makes lungs a target organ of oxidative stress due to cigarette smoke. The increased value of lipid peroxidation products in different stress conditions indicates the involvement of free radicals leading to various diseases. It has been proposed that increased lipid peroxidation products in blood indicate the occurrence of membrane damage provoked by free radical induced diseases (Stringer et al, 1989)

From the present study it is clear that stress - physical, and psychological induces lipid peroxidation in the serum and tissues of rats when compared with normal control rats. The alcohol administration and smoke exposure induces more lipid peroxidation compared with control rats. The fresh water swimming and cold-water swimming, stress induces less

lipid peroxidation compared with stress induced by the external agents. On the other hand isolation and overcrowding stress induces less elevation in the lipid peroxidation products.

Table: 4a

Values of lipid peroxidation products in the serum of rats subjected to different types of stress

Stress	Lipid peroxidation products (n mol/ml of serum)
Control	1.25 ± 0.027
F W S	2.93 ± 0.063*
C W S	3.01 ± 0.071*
Overcrowding	2.39 ± 0.029*
Isolation	1.31 ± 0.026***
Alcohol administration	4.50 ± 0.079*
Cigarette smoke	4.90 ± 0.067*

Values are mean ± SD (n=6)
*P<0.001
Control group is compared with FWS similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposure respectively.

FWS: Fresh water swimming stress.
CWS: Cold water swimming stress.

Table: 4b

Values of malondialdehyde (nmoles/mg protein) in the liver, lungs, heart and kidney of rats subjected to different types of stress

Stress	Liver	Lungs	Heart	Kidney
Control	0.59 ± 0.012	0.53 ± 0.018	0.30 ± 0.101	1.27±0.120
F W S	2.37 ± 0.052*	0.56 ± 0.027	0.90 ± 0.082*	1.63±0.201***
C W S	2.90 ± 0.071*	0.54 ± 0.051	0.88 ± 0.031*	1.78±0.192**
Overcrowding	0.69 ± 0.067*	0.50 ± 0.030	0.50± 0.091**	1.30±0.131
Isolation	0.72 ± 0.060**	0.53 ± 0.023	0.31 ± 0.050	1.30±0.180
Alcohol	3.78 ± 0.091*	0.58 ± 0.023	4.08 ± 0.101*	3.99±0.165*
Cigarette smoke	4.98 ± 0.121*	4.97 ± 0.091*	0.32 ± 0.072	4.01±0.101*

Values represent mean ±SD of six rats (n=6)
*P<0.001 **P<0.01 ***P< 0.05
Control group is compared with FWS, CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposure

FWS: Fresh water swimming stress.
CWS: Cold water swimming stress.

Table 4c

Values of hydroperoxide (μmoles/100g wet tissue) in the liver, heart, lungs and kidney of rats subjected to different types of stress.

Stress	Liver	Heart	Lungs	Kidney
Control	14.85 ± 1.29	09.92 ± 0.59	10.36 ± 0.32	17.01 ± 0.89
F W S	19.56 ± 0.93*	14.28 ±1.94*	11.08 ±0.37***	21.01 ± 0.93*
C W S	21.01 ± 1.52*	14.81 ± 0.97*	10.38 ± 0.29	20.13 ± 0.78*
Overcrowding	15.01 ± 0.87	10.19 ± 0.68	10.05 ± 0.33	17.81 ± 0.61
Isolation	16.81 ± 0.99***	12.23 ± 0.73	10.98 ± 0.36	16.99 ± 0.53
Alcohol	26.33 ±1.38*	22.08 ±1.09*	12.68 ± 0.30*	26.08 ±1.12*
Smoke	24.36 ±1.27*	10.01 ± 0.69	30.06 ±1.01*	28.01 ± 0.99*

Values represent mean ±SD of 6 rats
*P<0.001 **P<0.01, *** P< 0.05.
Control group is compared with FWS, CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposure respectively.

FWS: Fresh water swimming stress.
CWS: Cold water swimming stress.

Table 4d

Values of conjugated dienes (µmoles/100g wet tissue) in the liver, heart, lungs and kidney of rats subjected to different types of stress

Stress	Liver	Heart	Lungs	Kidney
Control	46.99 ± 3.02	08.99 ± 0.36	10.96 ± 0.29	17.01 ± 0.09
F W S	49.03 ± 2.07***	12.19 ±0.41*	09.99 ± 0.23	21.91 ± 0.13**
C W S	50.81 ± 1.97*	13.33 ± 0.39*	10.99 ± 0.21	22.09 ± 0.20**
Overcrowding	47.01 ± 2.99	09.08 ± 0.33	10.91 ± 0.32	18.02 ± 0.12
Isolation	46.01 ± 3.10	09.12 ± 0.29	09.93 ± 0.20	18.99 ± 0.20
Alcohol	76.50 ± 1.59*	18.18 ± 0.58*	11.01 ± 0.27	28.01 ± 0.09*
Smoke	66.98 ± 3.70*	09.98 ± 0.29**	30.26 ± 0.53*	30.01 ± 0.60*

Values represent mean ±SD of six rats
*P<0.001 **P<0.01, ***P<0.05
Control group is compared with FWS similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposure and respectively

FWS: Fresh water swimming stress.
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EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

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CHAPTER 5

EFFECT OF STRESS ON ANTIOXIDANT STATUS

The involvement of reactive oxygen species (ROS) in the pathogenesis of various diseases has been under intense investigations during the past decade. ROS are continuously generated in cells exposed to an aerobic environment during the course of normal metabolism. ROS damage proteins, DNA and other biomolecules (Davies, 1993 and Hussain et al, 1994). It has been postulated that age dependent diseases such as atherosclerosis, arthritis, and also cancer involve ROS at least at some stage of their development. The term ROS refers to forms of oxygen exhibiting high reactivity and having at least one unpaired electron. However, other reactive forms of oxygen are also known which are non-free radicals. Both of these forms are collectively referred to as reactive oxygen species (ROS) and include singlet oxygen, superoxide anion, hydrogen peroxide, hydroxyl radical, etc. ROS are short-lived species that are regenerated in normal cells. In addition, the metabolism of xenobiotics, exposure to ionizing radiation and stress also generate these species. An important feature of free radical reaction with non-radical species is the formation of new free radical species. Free radical driven reactions are usually chain reactions (Halliwell et al, 1984). Electron acceptors such as molecular oxygen react easily with free radicals and become radicals themselves, the ROS. This explains why in aerobic life, where molecular oxygen is ubiquitous, ROS become the primary mediators of cellular free radical mediated cellular injury.

The first line of cellular defense against oxidative stress by free radicals consists of enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase(GR). These enzymes react directly with the oxidizing radicals to yield non-radical products. Selenium dependent glutathione peroxidase removes both H_2O_2 and lipid peroxides. This protects the cell membrane from damage by these highly reactive species.

Catalase catalyses the dismutation of hydrogen peroxides. H_2O_2 formed by the dismutation of superoxide anion by SOD is decomposed to water by catalase, thus ameliorating the deleterious effects of H_2O_2 in the body. Glutathione plays a major role in cellular protection

against oxidative damage (Mallika et al, 2000). Conditions that perturb intracellular levels of glutathione have been shown to result in significant alteration in the cellular metabolism. The levels of SOD, CAT, GPx, GR and glutathione-S- transferase (GST) are indicators of oxidative damage and these parameters may be altered in different stress conditions. The present chapter deals with the effect of different types of stress on these antioxidant enzymes.

5.1. Materials and methods

5.1.1. Animal model

Male albino rats (Sprague-Dawley strain) weighing 150-200g were divided into seven groups of six rats each.

Group I - Normal control rats

Group II - Fresh water swimming

Group III - Cold water swimming

Group IV - Over crowding

Group V - Isolation

Group VI - Cigarette smoke exposure

Group VII - Alcohol administration

Animals were subjected to the above-mentioned stress for a period of one month. Detailed experimental procedure is given in the chapter 2. At the end of the experimental period animals were sacrificed and their blood, liver, lungs and kidney were removed to ice cold containers for various estimations.

5.1.2. Methods

Superoxide dismutase (SOD) activity was measured by NBT reduction method of Mc Cord and Fridovich (Mc Cord et al,1969), catalase by the method of Aebi (Aebi et al, 1983), reduced glutathione by the method of Moron (Moron et al ,1979), glutathione peroxide by the method of Hafemann, (Hafemann et al, 1972) and glutathione reductase by the method of

Racker (Racker et al,1955), glutathione –S- transferase by the method of Habig (Habig et al,1974) and proteins by the method of Lowry (Lowry et al,1951). The detailed procedure for various estimations is discussed in chapter 2.

5.2. Results and discussion

5.2.1. Superoxide dismutase

Table 5a represents the values of superoxide dismutase activity in the liver, lungs, heart and kidney of normal rats and rats subjected to different types of stress. The value of SOD was significantly decreased ($P<0.001$) in the liver tissue of rats exposed to fresh water swimming, cold- water swimming and alcohol administration when compared with that of normal control rats. A significant decrease was observed in the liver tissue of rats exposed to overcrowding ($P<0.01$) and isolation stress ($P<0.05$) when compared to normal control rats. A significant increase was observed in the liver tissue of rats exposed to cigarette smoke ($P<0.001$). No significant change was observed in the lung tissue of rats exposed to fresh water swimming, cold-water swimming, over crowding, isolation and alcohol administration. Only the lung tissue of rats exposed to cigarette smoke showed a significant increase ($P<0.001$) when compared with normal control rats. No significant change in the lung tissue was observed in groups of rats exposed to all other types of stress. A significant decrease was observed in the heart tissue of rats subjected to alcohol administration ($P<0.001$) compared with normal control rats. No significant decrease was observed in the heart tissue of rats, exposed to overcrowding, isolation and cigarette smoke exposure. A significant decrease ($P<0.001$)was observed in the heart and kidney of rats exposed to fresh water swimming, cold-water swimming and alcohol administration. A significant decrease was observed in the kidney of rats exposed to overcrowding stress ($P<0.05$). A significant increase ($P<0.001$) was observed in the kidney of rats exposed to cigarette smoke when compared with normal control rats.

5.2.2. Catalase

Table 5b depicts the values of catalase activity in the liver, heart, lungs and kidney of normal rats and rats subjected to different types of stress. The value of catalase activity was significantly decreased ($P<0.001$) in liver and heart tissue of rats exposed to cold-water swimming and alcohol administration when compared with control rats. A significant decrease was observed in the heart tissue of rats exposed to fresh water swimming stress ($P<0.01$) when compared with control rats. A significant decrease was observed in the heart tissue of rats exposed to overcrowding stress ($P<0.05$). A significant increase was also observed in the liver and lung tissue of rats exposed to cigarette smoke ($P<0.001$) when compared with normal control rats. No significant change was observed in the lung tissue of rats exposed to fresh water swimming, cold-water swimming, isolation, overcrowding and alcohol administration. No significant change was observed in the heart tissue of rats, exposed to cigarette smoke. A significant decrease was observed in the kidney of rats exposed to fresh water swimming ($P<0.05$) and cold-water swimming stress ($P<0.05$) when compared with normal control rats. A significant decrease ($P<0.001$) was observed in the kidney of rats administered with alcohol. A significant decrease was observed in the kidney of rats exposed to fresh water swimming ($P<0.05$) and cold-water swimming ($P<0.05$) stress. No significant change was observed in the kidney of rats exposed to overcrowding and isolation stress. A significant increase ($P<0.001$) was observed in the kidney of rats exposed to cigarette smoke ($P<0.001$).

5.2.3. Reduced glutathione

Table 5c represents the values of reduced glutathione content in the liver, heart, lungs and kidney of normal control rats and rats subjected to different types of stress. Concentration of reduced glutathione content was significantly decreased ($P<0.001$) in the liver tissue of rats exposed to alcohol administration and cigarette smoke exposure when compared with normal control rats. A significant decrease ($P<0.001$) was observed in the heart tissue of rats

administered with alcohol. A significant decrease (P<0.001) was observed in the heart, liver and kidney of rats exposed to fresh water swimming and cold-water swimming stress when compared with normal control rats. No significant change was observed in the liver tissue of rats exposed to overcrowding and isolation stress when compared with normal control rats. A significant decrease (P<0.001) was observed in the heart tissue of rats exposed to cigarette smoke and alcohol when compared with normal control rats. No significant change was observed in the lungs and kidney of rats exposed to overcrowding and isolation stress. A significant decrease (P<0.001) was observed in the kidney of rats exposed to cigarette smoke and alcohol administration when compared with normal control rats. A significant decrease in reduced glutathione content was observed in the lung tissue of rats exposed to cigarette smoke (P<0.001) when compared with normal control rats. No significant change was observed in the lung tissue of rats subjected to fresh water swimming, cold-water swimming, isolation and overcrowding.

5.2.4 Glutathione peroxidase

Table 5d represents the value of glutathione peroxidase in the liver, heart and kidney of normal rats and rats subjected to different types of stress The value of glutathione peroxidase was significantly increased (P<0.001) in the liver heart and kidney of rats, exposed to cigarette smoke and alcohol administration when compared with normal control rats. A significant decrease (P<0.05) was observed in the liver and heart tissue of rats exposed to fresh water swimming and cold -water swimming stress. No significant change was observed in the liver, heart and kidney of rats subjected to isolation and overcrowding. No significant change was observed in the kidney of rats subjected to fresh water swimming and cold-water swimming stress.

Glutathione-S- transferase

Table 5e represents the values of glutathione-S- transferase in the liver, heart and kidney of rats subjected to different types of stress. The values of glutathione-S- transferase were significantly increased (P<0.001) in the liver, heart and kidney of rats exposed to cigarette smoke and alcohol administration. A significant decrease was observed in the liver tissue of rats exposed to cold- water swimming stress (P<0.001). No significant change was observed in the liver, heart and kidney of rats, those subjected to fresh water swimming, overcrowding and isolation stress. No significant change was observed in the heart and kidney of rats, those subjected to cold-water swimming stress.

Results of the present study clearly show the decreased values of antioxidant enzymes such as SOD, CAT, GPx and GST in the tissues of rats subjected to all types of stress except cigarette smoke. The values of reduced glutathione (GSH) were also reduced in different stress conditions. The antioxidant enzymes such as SOD, CAT, GPx and GST were increased in all the tissues of rats exposed to cigarette smoke. No change was observed in the lung tissue of rats subjected to different types of stress except exposure to cigarette smoke. Fresh water swimming and cold-water swimming are strenuous exercises. During swimming large amount of energy is utilized, which increases oxygen supply to the active tissues. The rate of oxygen uptake by the body during swimming may increase 10-15 fold, which increases free radical formation and thereby increase lipid peroxidation. Most of the oxygen is reduced to water and a few are reduced to various intermediates such as oxygen free radical, hydroxyl radical and it induce lipid peroxidation. All the above-mentioned stress induces lipid peroxidation. Activities of antioxidant enzymes are reduced due to the attack of free radicals on DNA, which damage the DNA and may impair gene expression resulting in impaired functions of proteins. But in the cigarette smoke exposed group, a significant increase in the activities of SOD and CAT and GPx and GST were observed. Increased antioxidant enzyme activity due to smoke is

suggestive of an attempt made by the antioxidant defense to minimize the oxidative stress caused by increased free radical burden. In the alcohol treated groups the lipid peroxidation products are increased due to the microsomal membrane free radical generation. Ethanol induced hepatotoxic effects are mediated through enhanced generation of free radicals. The antioxidant enzyme activity was found to be decreased due to the attack of free radicals on DNA and proteins. From the present study it was observed that all the above-mentioned stress except cigarette smoke leads to the formation of free radicals that increases lipid peroxidation and there by reduces the activities of antioxidant enzymes. Values of reduced glutathione content were also reduced in all the tissues of rats exposed to different types of stress.

SOD protects the tissues from free radical damage; hence it can be used as a marker of tissue damage. From the present study it is evident that the level of SOD is reduced in liver, heart and kidney of rats exposed to all types of stress except the cigarette smoke exposed groups when compared with normal control rats. The value of catalase was also found to be low in stress condition when compared to that of the normal control rats. The decreased activity of catalase in the different stress group could be due to increase in MDA which can cross link with amino group of protein to form intra- and inter-molecular cross links thereby inactivating several membrane bound enzymes such as catalase (Kikugawa et al, 1984). The decreased activity of catalase may result in the accumulation of H_2O_2 in the body. Thus, the inadequacy of these enzymes leads to the accumulation of free radicals and increased oxidative stress.

Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defense processes. Perturbation of glutathione status of a biological system has been reported to lead to serious consequences. Administration of thiol compounds such as glutathione, cysteine and methionine are shown to protect against oxidative stress and also acts as a substrate for dehydroascorbate reductase. We found low activity of

GPx in different stress conditions except exposure to cigarette smoke and alcohol administration, which may be attributed to the unavailability of GSH, the substrate for GPx. The capacity of the glutathione system to cope with free radicals mainly depends upon the activity of peroxidase and glutathione reductase. GST plays an essential role in the liver by eliminating toxic compounds by conjugating them with glutathione. The substrate variability of GST accounts for the reactivity of these enzymes with a wide range of endogenous, environmental and therapeutic substrates (Halliwell et al, 1992). We observed a low concentration of GST in the heart and liver tissue of rats exposed to cigarette smoke and alcohol administration.

From the present study we understand that stress induces the production of free radicals, which reduces the activities of antioxidant enzymes. Alcohol administration and exposure to cigarette smoke produces more oxidative damage to the tissues followed by fresh water swimming and cold-water swimming stress. Overcrowding and isolation stress cause least damage to the tissues compared with other stress conditions.

Table: 5a

Value of superoxide dismutase(SOD : U/mg protein)) in the liver, lungs, heart and kidney of rats subjected to different types of stress

Stress	Liver	Lungs	Heart	Kidney
Control	12.30 ± 0.57	8.09 ± 0.101	5.99 ± 0.31	16.01 ± 0.13
F W S	08.38 ± 0.78*	8.12 ± 0.132	3.01 ± 0.23*	13.98 ± 0.18*
C W S	07.99 ± 0.88*	8.01 ± 0.210	3.25 ± 0.24*	13.81 ± 0.20*
Overcrowding	11.01 ± 0.47**	8.18 ± 0.187	5.59 ± 0.13	15.41 ± 0.13**
Isolation	11.38 ± 0.39***	8.13 ± 0.139	5.61 ± 0.21	15.99 ± 0.09
Alcohol	05.09 ± 0.23*	7.93 ± 0.231***	2.01 ± 0.01*	10.03 ± 0.19*
Cigarette smoke	16.28 ± 0.31*	12.8 ± 0.290*	5.91 ± 0.18	20.01 ± 0.31*

Values represent mean ±SD (n=6)
*P<0.001 **P<0.01 ***P <0.05
Control group is compared with FWS, similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposure respectively.

FWS: Fresh water swimming stress.
CWS: Cold water swimming stress.

Table: 5b

Value of catalase (CAT) U/mg protein) in the liver, lungs, heart and kidney of rats subjected to different types of stress.

Stress	Liver	Lungs	Heart	Kidney
Control	66.61 ± 3.57	21.99 ± 1.10	50.20 ± 1.90	20.20 ± 1.30
F W S	53.38 ± 2.98*	19.03 ± 0.99**	45.38 ± 2.01**	18.32 ± 0.92***
C W S	49.39 ± 3.02*	20.99 ± 0.98	40.18 ± 3.23*	17.98 ± 0.92***
Overcrowding	63.98 ± 2.01	22.08 ± 1.19	48.28 ± 1.23***	19.99 ± 1.20
Isolation	64.08 ± 2.32	21.03 ± 0.89	49.97 ± 2.61	19.18 ± 0.35
Alcohol	31.08 ± 1.28*	21.08 ± 0.99	38.33 ± 1.04*	10.03 ± 0.51*
Cigarette smoke	87.32 ± 2.93*	39.98 ± 1.90*	50.01 ± 2.01	24.36 ± 0.32*

Values represents mean ± SD of 6 rats in each group (n=6)
*P<0.001, **P<0.01, ***P<0.05
Control group is compared with FWS, similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposed group respectively.

FWS: Fresh water swimming stress.
CWS: Cold water swimming stress.

Table 5c

Value of reduced glutathione (nmoles/mg protein) in the liver, lungs, heart and kidney of rats subjected to different types of stress

Group	Liver	Lungs	Heart	Kidney
Control	10.98 ± 0.192	7.81 ± 0.50	16.08 ± 0.191	6.09 ± 0.210
F W S	08.39 ± 0.210*	7.23 ± 0.48	13.01 ± 0.102*	4.89 ± 0.230*
C W S	08.10 ± 0.187*	7.39 ± 0.41	12.99 ± 0.081*	4.77 ± 0.180*
Over crowding	10.79 ± 0.099	7.76 ± 0.29	15.89 ± 0.102	6.02 ± 0.192
Isolation	10.70 ± 0.278	7.83 ± 0.51	15.99 ± 0.070	5.99 ± 0.291
Alcohol	06.08 ± 0.190*	7.01 ± 0.32***	10.29 ± 0.060*	3.09 ± 0.301*
Cigarette smoke	05.32 ± 0.010*	4.98 ± 0.32*	15.99 ± 0.131	3.41 ± 0.032*

Value represents mean ±SD of six rats in each group
*P<0.001 ***P<0.05.
Control group is compared with FWS, similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposed group respectively

FWS: Fresh water swimming
CWS: Cold water swimming

Table: 5d

Values of glutathione peroxidase (U/mg protein) in the liver, lungs, heart and kidney of rats subjected to different types of stress

Stress	Liver	Heart	Kidney
Control	0.18 ± 0.06	0.21 ± 0.04	0.10 ± 0.08
F W S	0.07 ± 0.01***	0.14 ± 0.10***	0.07 ± 0.10
C W S	0.09 ± 0.04***	0.15 ± 0.03***	0.09 ± 0.09
Overcrowding	0.18 ± 0.03	0.20 ± 0.05	0.10 ± 0.03
Isolation	0.17 ± 0.07	0.22 ± 0.03	0.08 ± 0.09
Alcohol	0.47 ± 0.09*	0.29 ± 0.02*	2.19 ± 0.02 *
Smoke	0.58± 0.07*	0.22 ± 0.02	3.01 ± 0.01 *

Values represent mean ±SD of six rats in each group
*P<0.001 **P<0.01 ***P<0.05
Control group is compared with FWS, similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposed group respectively

FWS: Fresh water swimming stress
CWS: Cold water swimming stress

Table: 5e

Value of glutathione-S- transferase (U/mg protein) in the liver, lungs, heart and kidney of rats subjected to different types of stress

Stress	Liver	Heart	Kidney
Control	10.01 ± 0.13	16.08 ± 0.09	0.90 ± 0.08
F W S	09.01 ± 0.09	16.10 ± 0.07	0.89 ± 0.09
C W S	08.20 ± 0.17*	15.99 ± 0.08	0.91 ± 0.10
Overcrowding	10.01 ± 0.17	16.31 ± 0.07	0.92 ± 0.07
Isolation	10.08 ± 0.17	16.12 ± 0.09	0.90 ± 0.05
Cigarette smoke	15.32 ± 0.30 *	20.99 ± 0.01*	2.10 ±0.09*
Alcohol	16.08 ± 0.29*	18.29 ± 0.03*	2.60 ± 0.01*

Values represent mean ±SD (n=6)
*P<0.001 **P<0.01.
Control group is compared with FWS, similarly with CWS, overcrowding, isolation, alcohol administration and cigarette smoke exposed group respectively.

FWS: Fresh water swimming stress
CWS: Cold water swimming stress

EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT FOR THE DEGREEE OF

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CHAPTER 6

COMBINED EFFECT OF ALCOHOL AND CIGARETTE SMOKE ON LIPID METABOLISM, LIPID PEROXIDATION AND ANTIOXIDANT STATUS

The study of the effect of ethanol on lipid metabolism is relevant because the effect of alcohol consumption on body weight and body composition, the pathogenesis of alcoholic fatty liver and hyperlipidemia, and to atherosclerosis. Man and woman who drink alcohol tend to have a stable body weight over a decade of observation compared their non-drinking counterparts, whose weight increases (Liu, et al, 1994). Energy wastage when ethanol is metabolised by the microsomal ethanol oxidizing system is one reason for relatively low body weight (Pirola et al, 1975). The pathogenesis of alcoholic fatty liver and alcoholic hyperlipidemia has been known for a long time to be mainly due to a combination of decreased fatty acid oxidation in mitochondria. Alcohol consumption in humans is a serious health hazard and liver is the major organ susceptible to it (Lieber et al,1984). Administration of ethanol results in a variety of changes in the liver and ethanol ingestion causes fatty liver and hypertriglyceridemia (Porta et al). It may also alter normal function and composition of lipid membrane (Rubin et al, 1968). Hepatic cirrhosis is a major cause of death in chronic alcoholics (Lieber et al,1999)

Cigarette smoke produced by incomplete combustion of tobacco generates a high free radical load in vivo (Fridovich , 1979). It contains various chemical compounds including biphenyl and polycyclic aromatic hydrocarbons, which could initiate and promote oxidative damage (Ansari ,1997 and Chow,1993). The oxidant present in cigarette smoke is involved in the pathogenesis of diseases of lungs and vascular system (Harman et al, 1989). Serum levels of high-density lipoproteins (HDL) are generally lower in smokers, compared to those of non-smokers (Garrison et al 1978). Prolonged exposure to cigarette smoke besides decreasing HDL- cholesterol increase low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) level (Latha et al, 1978). LDL can alter by diet and certain reactive chemicals like malondialdehyde (MDA). Plasma MDA level is high in smokers since cigarette smoke contains reactive oxygen radicals (Pryor et al, 983) and acetaldehyde (Geokas, 1984), which

can increase lipid peroxidation. Cigarette smoking is an exogenous factor, which is reported to cause a 3 fold increase in the incidence of myocardial infarction (MI).

Reactive oxygen species (ROS) formed in the body as a result of normal metabolic reactions, exposure to ionizing radiation, cigarette smoke, environmental pollution, and by the influence of several xenobiotics, etc, are implicated in several diseases, including cancer (Gracy et al, 1999). ROS damage DNA, proteins, carbohydrates and lipids, affect enzyme activity and the genetic machinery. Biological system possesses a number of mechanisms to remove free radicals. The integrated antioxidant system scavenges free radicals (Sun,1990). In the present study, we have examined the combined effect of alcohol and cigarette smoke on lipid metabolism, lipid peroxidation and antioxidant status in albino rats.

6.1. Materials and methods

6.1.2. Animal model

Male albino rats of Sprague Dawley strain weighing 200-250g were maintained under environmentally controlled conditions with free access to standard food (Lipton, India) and water.

Rats were divided in to four groups:

Group I: Normal diet (Control group)

Group II: Normal diet +18% alcohol (4g alcohol/kg body weight/day) orally---
(alcohol treated group)

Group III: Normal diet + Cigarette smoke (Cigarette smoke exposed group)

Group IV: Normal diet + 18% alcohol (4g alcohol/kg body weight/ day) + Cigarette smoke exposed.

Detailed experimental procedure is given in the chapter 2. At the end of the experimental period animals were sacrificed and their blood and tissues were collected for following biochemical analysis.

6.1.3. Methods

Serum cholesterol was estimated by the CHOD- PAP method (Duncan et al, 1982). Serum HDL cholesterol was determined by precipitation method followed by CHOD-PAP (Harris et al 1996). Triglycerides in serum were determined by GPO-PAP (NCEP, 1995) method. Lipid peroxidation in serum was estimated by the thiobarbituric acid method as modified by Yoshioka (Yoshioka et al, 1979), using TCA and TBA. Serum ALT was estimated by the method of IFCC (IFCC, 1976). Serum AST by the method of IFCC (IFCC,1976) . Tissue MDA was assayed (Ohkawa et al, 1979) in terms of thiobarbituric acid reactive substances (TBARS), using 1,1,3,3- tetramethoxy propane as the standard and expressed as nanomoles of MDA formed/mg protein. Hydroperoxides and conjugated dienes were estimated by the method described earlier (Brien, 1969 and Nair et al 1971). Catalase was assayed by the method of Aebi (Aebi,1947) and superoxide dismutase (SOD) was determined by the method of Mc Cord and Beauchamp (Mc Cord et al,1969). Reduced glutathione (GSH) content was determined by the method of Moron (Moron et al, 1974), glutathione peroxidase by the method of Hafemann (Hafemann et al,1974), glutathione-S – transferase by the method of Habig (Habig et al,1974) and glutathione reductase by the method of Racker (Racker et al,1995) and protein in the sample estimated by the method of Lowry (Lowry et al,1951). The detailed experimental procedure is given in the chapter 2.

Statistical analysis was carried out using students't' test. Values expressed are mean ±SD of six rats in each group.

6.2. Results and Discussion

6.2.1. Lipid profiles

Table 6a represents the concentration of total cholesterol, triglycerides and HDL cholesterol in the serum of control, alcohol administered, cigarette smoke exposed and the combination group of rats (alcohol and smoke together). Concentration of total cholesterol, triglycerides and LDL cholesterol significantly increased (P<0.001) in the serum of alcohol administered, cigarette smoke exposed and the combination group when compared to that of normal control rats. The concentration of HDL cholesterol was found to be decreased significantly (P<0.001) in the serum of alcohol administered, cigarette smoke exposed and in combination group when compared to that of normal control rats.

6.2.2. Malondialdehyde in blood

Table 6b represents the concentration of malondialdehyde in the serum of control, alcohol administered, cigarette smoke exposed and combination group. Concentration of malondialdehyde was increased significantly (P<0.001) in the serum of alcohol-administered rats, cigarette smoke exposed and in combination group of rats when compared to those of normal control rats.

6.2.3. Tissue lipid peroxides

Table 6c represents the concentration of malondialdehyde, hydroperoxides and conjugated dienes in the liver, lungs, heart and kidney of rats exposed to cigarette smoke alcohol and in combination group. Concentration of malondialdehyde significantly increased (P<0.001) in the liver and kidney of rats exposed to cigarette smoke, alcohol administered and in combination group when compared to that of normal control rats. Concentration of malondialdehyde in the lung tissue was significantly increased (P<0.001) only in the cigarette smoke exposed group compared to that that of normal control rats. No significant change was

observed in the lungs of rats administered with alcohol and heart tissue of rats exposed to cigarette smoke. A significant increase was observed in the heart tissue of rats administered with alcohol and in combination group. The values of conjugated dienes and hydroperoxides were significantly increased in the liver and kidney of rats administered with alcohol, and in combination group compared to that of normal control rats. No significant change was observed in the heart tissue of rats exposed to cigarette smoke. Values of hydroperoxides and conjugated dienes were significantly increased ($P<0.001$) in the lung tissue of rats exposed to cigarette smoke and both in combination. No significant change was observed in the lung tissue of rats administered with alcohol.

6.2.4. SOD and CAT

Table 6d represents the values of SOD and CAT in the liver, lungs, heart and kidney of rats exposed to cigarette smoke, alcohol administered and in combination group. The values of SOD and CAT were significantly decreased ($P<0.001$) in the liver and kidney of alcohol administered and in combination group when compared to that of normal control rats. A significant increase ($P<0.001$) in the activity of SOD and CAT was observed in the liver, lungs and kidney of alcohol administered and in combination group when compared to that of normal control rats A significant increase ($P<0.001$) was observed in the lung tissue of rats exposed to cigarette smoke and in combination group when compared to that of normal control rats. A significant decrease ($P<0.001$) in the values was observed in the heart tissue of rats exposed to alcohol and in combination when compared to that of normal control rats. No significant change was observed in the heart tissue of rats exposed to cigarette smoke.

6.2.4. GST, GPX and GSH

Table 6e represents the values of GST, GPx and GSH in the liver, lungs, heart and kidney of rats exposed to cigarette smoke, alcohol administered and in combination group. The values of GST and GPx significantly increased ($P<0.001$) in the liver tissue of rats

exposed to cigarette smoke, alcohol and in combination group when compared to that of normal control rats. A significant increase ($P<0.001$) was observed in the lung tissue of rats exposed to cigarette smoke, and in combination when compared to that of normal control rats. A significant increase ($P<0.01$) was observed in the kidney of rats administered with alcohol , exposed to cigarette smoke and in combination group when compared to normal control rats. No significant change was observed in the lung tissue of rats exposed to alcohol. The values of reduced glutathione content were significantly decreased ($P<0.001$) in liver and kidney of rats exposed to cigarette smoke, alcohol administered and in combination group. No significant change was observed in the lung tissue of rats administered with alcohol. A significant decrease ($P<0.001$) was observed in the lung tissue of rats exposed to cigarette smoke and in combination group. No significant change was observed in the lung tissue of rats administered with alcohol. A significant decrease ($P<0.001$) was observed in the heart tissue of rats administered with alcohol and in combination group. No significant change was observed in the heart tissue of rats exposed to cigarette smoke.

6.2.5. Transaminases

Table 6f represents the values of glutamate oxaloacetate transaminase(AST) and pyruvate transaminase (ALT) in the serum of rats administered with alcohol, exposed to cigarette smoke and both in combination. The values of AST and ALT were found to be significantly ($P<0.001$) increased in the serum of rats administered with alcohol, exposed to cigarette smoke and in combination group.

From the present study, it was observed that ethanol feeding enhanced the endogenous synthesis of triglycerides and reduced the utilization of dietary lipids resulting their accumulation in the liver and plasma (Lieber,1994). Alcohol enhances liver cirrhosis and thereby enhances the activities of serum transaminases. Ethanol has been found to enhance the generation of oxygen free radicals during its oxidation in the liver and these free

radicals are responsible for causing lipid peroxidation in the serum of alcohol-administered rats. The prevalence of hypercholesterolemia and triglyceridemia in heavy smokers were reported earlier (Yang et al, 1993). The elevated levels of cholesterol and triglycerides by smoke exposure may be due to cadmium a metallic element present in the cigarette smoke. The increased cholesterol synthesis is due to increased activity of HMG CoA reductase, which is the key enzyme involved in cholesterol synthesis. Cadmium and nicotine content present in cigarette smoke may decrease the activity of lipoprotein lipase resulting in elevated level of triglycerides. We observed that stress induced by alcohol, smoke and both in combination enhanced lipid peroxidation. Ethanol is said to enhance the generation of oxygen free radicals during its oxidation in the liver. The free radicals and oxidants in cigarette smoke may be responsible for lipid peroxidation in different tissues of rats. The massive surface area makes lung a target organ for oxidative stress due to cigarette smoke (Cross et al, 1994). Decreased activities of antioxidant enzymes, SOD and CAT, in alcohol administered rats could be due to the harmful effects of alcohol or direct effect of acetaldehyde formed from the oxidation of alcohol on these enzymes. SOD is responsible for the dismutation of highly and potentially active oxy-radicals, thus leading to an increased level of lipid peroxidation products. An increase in activity of GST and GPx could be a defense mechanism against the free radicals. The concentration of GSH is of clinical importance in tissue injury caused by toxic substances, which is a naturally occurring antioxidant. Binding of acetaldehyde, a metabolite of ethanol with glutathione may contribute to the reduction in the levels of GSH or it may be due to enhanced utilization of GSH by antioxidant enzymes GST and GPx.

Cigarette smoke induces lipid peroxidation in all the tissues except heart. It has been suggested that metabolism of toxic compounds including free radicals, occurs mainly in the liver and metabolites from the liver diffuses in to various extra hepatic tissues causing lipid

peroxidation and cell injury. Smoke does not induce lipid peroxidation in heart. It has also been reported that the myocardium utilizes glucose in preference to fatty acids during stress for its energy requirement and decreased the utilization of fatty acids by the myocardial tissue may be one of the reason for no change in lipid peroxide level in the heart. Increased SOD and CAT activities due to smoke is suggestive of the possibility of an attempt made by the antioxidant defense to minimize the oxidative stress caused by increased free radical burden. GSH is an important member of non-enzymatic defense system and disturbance in its levels may have serious implications. It is the substrate for GST and GPx. The activities of these enzymes are increased, thereby reducing the concentration of GSH. Increase in the activities of GST and GPx in the liver of alcohol treated rats and liver and lungs of smoke exposed rats might be a compensatory mechanism by the body against these oxidants. The decrease in the concentration of GSH in the kidney of rats exposed to cigarette smoke was reported earlier (Anand et al, 1996). The decrease in glutathione was associated with the increased utilization of GSH by antoxidant enzymes GST and GPx , could be a defense mechanism against free radicals.

The combined treatment of alcohol and smoke enhances the lipid levels and lipid peroxidation and serum transaminases. In conclusion combined exposure to alcohol and smoke enhanced the oxidative stress and thus adversely affecting the antioxidant system and thus is likely to be more harmful than the effect of smoke or alcohol alone.

Table: 6a

Concentration of total cholesterol, triglycerides and HDL- cholesterol and LDL cholesterol in the serum of control, alcohol administered and smoke exposed rats (Concentration as mg/dl)

Group	Cholesterol	Triglycerides	HDL-cholesterol	LDL cholesterol
Control	60.02 ± 3.92	11.28 ± 0.30	36.89 ± 1.02	20.92 ± 0.38
Alcohol administration	82.70 ± 2.82*	28.28 ± 0.32*	30.58 ± 0.57*	46.20 ± 0.35*
Cigarette smoke	98.23 ± 2.10*	33.12 ± 0.56*	25.38 ± 0.78*	65.80 ± 0.23*
Alcohol+ Cig smoke	100.8 ± 2.08*	40.12 ± 0.52*	12.17 ± 0.67*	80.70 ± 0.56*

Values represent mean ±SD (n=6)
*P<0.001
Control is compared with alcohol-administered rats, cigarette smoke exposed rats and with combined group.

Table: 6b

Concentration of malodialdehyde in the serum of rats, administered with alcohol, cigarette smoke and both in combination.
Malondialdehyde (nmoles/mg protein)

Group	Lipid peroxidation products (nmol/ml of serum)
Control	1.25 ± 0.012
Alcohol administration	4.50 ± 0.051*
Cigarette smoke	4.90 ± 0.016*
Alcohol+ Cigarette smoke	7.99 ± 0.029*

Values represent mean ±SD (n=6)
*P<0.001
Control is compared with alcohol-administered rats, cigarette smoke exposed rats and with combined group.

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Table: 6c

Concentration of malodialdehyde, hydroperoxides and conjugated dienes in the tissues of rats administered with alcohol, cigarette smoke and both in combination.

Malondialdehyde (nmoles/mg protein)

Group	Liver	Lungs	Heart	Kidney
Control	0.59 ± 0.02	0.53 ± 0.03	0.30 ± 0.10	1.27 ± 0.12
Alcohol	3.89 ± 0.09*	0.55 ± 0.02	1.94 ± 0.08*	2.98 ± 0.09*
Cigarette smoke	4.98 ± 0.08*	3.97 ± 0.06*	0.34 ± 0.03	2.67 ± 0.10*
Alcohol+Cig smoke	6.02 ± 0.19*	4.13 ± 0.05*	2.99 ± 0.07*	3.56 ± 0.03*

Hydroperoxides (μmoles/100g wet tissue)

Group	Liver	Lungs	Heart	Kidney
Control	14.85 ± 0.04	14.85 ± 0.24	09.92 ± 0.15	17.01 ± 1.08
Alcohol	20.33 ± 0.09*	14.68 ± 0.26	15.14 ± 1.05*	25.07 ± 1.00*
Cigarette smoke	24.36 ± 0.21*	30.06 ± 0.39*	10.74 ± 0.21	31.07 ± 1.00*
Alcohol+ Cig smoke	30.39 ± 0.72*	40.29 ± 0.17*	20.01 ± 0.19*	30.07 ± 0.93*

Conjugated dienes (μmoles/100g wet tissue)

Group	Liver	Lungs	Heart	Kidney
Control	46.09 ± 2.02	10.36 ± 0.21	08.99 ± 0.36	17.01 ± 0.19
Alcohol	55.50 ± 1.59*	10.56 ± 0.20	18.18 ± 0.58*	23.01 ± 0.10*
Cigarette smoke	66.98 ± 2.70*	30.26 ± 0.39*	09.08 ± 0.39	30.01 ± 0.60*
Alcohol + Cig smoke	78.01 ± 3.01*	40.06 ± 0.99*	22.80 ± 0.99*	36.08 ± 0.80*

Values represent mean ±SD of six rats in each group

*P<0.001 Control is compared with alcohol-administered group, cigarette smoke exposed group and with combined group

Table 6d

Activities of superoxide dismutase (SOD), catalase (CAT), in the tissues of rats administered with alcohol, cigarette smoke and both in combination.

Superoxide dismutase (U/mg protein)

Group	Liver	Lungs	Heart	Kidney
Control	10.35 ± 0.57	08.09 ± 0.10	4.99 ± 0.31	16.01 ± 0.13
Alcohol administration	05.30 ± 0.23*	07.99 ± 0.09	2.01 ± 0.01*	10.03 ± 0.51*
Cigarette smoke	14.20 ± 0.33*	012.3 ± 0.13*	4.69 ± 0.18***	19.01 ± 0.31*
Alcohol + Cig smoke	04.33 ± 0.87*	10.08 ± 0.18**	1.99 ± 0.19*	08.01 ± 0.03*

Catalase (U/mg protein)

Group	Liver	Lungs	Heart	Kidney
Control	66.61 ± 3.57	22.99 ± 1.10	50.20 ± 3.80	20.20 ± 1.30
Alcohol administration	31.08 ± 2.28*	21.78 ± 0.99	38.33 ± 1.04*	14.36 ± 0.92*
Cigarette smoke	87.32 ± 2.93*	39.98 ±1.90*	50.01 ± 2.01	26.12 ± 0.72*
Alcohol + Cig smoke	30.30 ± 1.98*	25.18 ± 0.91*	40.10 ± 2.10*	10.38 ± 0.63*

Values represent ± SD of six rats in each group
*P<0.001 **P<0.01 ***P<0.05
Control is compared with alcohol-administered group, cigarette smoke exposed group and with combined group

Table: 6e

Activities of glutathione-s-transferase, glutathione peroxidase and concentration of reduced glutathione in the tissues of rats administered with alcohol, cigarette smoke and both in combination.

Glutathione-s-transferase (U/mg protein)

Group	Liver	Lungs	Kidney
Control	1.02 ± 0.06	1.90 ± 0.01	0.90 ± 0.08
Alcohol administration	3.82 ± 0.17*	1.99 ± 0.07	2.10 ± 0.02**
Cigarette smoke	3.81 ± 0.16*	4.89 ± 0.23*	2.60 ± 0.01**
Alcohol + Cig smoke	5.09 ± 0.03*	6.00 ± 0.09*	3.81 ± 0.08*

Glutathione- peroxidase (U/mg protein)

Group	Liver	Lungs	Heart	Kidney
Control	0.18 ± 0.06	0.14 ± 0.07	0.21 ± 0.09	0.10 ± 0.05
Alcohol administration	0.47 ± 0.08*	0.13 ± 0.10	0.32 ± 0.02*	0.28 ± 0.02**
Cigarette smoke	0.59 ± 0.32*	0.39 ± 0.06*	0.20 ± 0.07	0.27 ± 0.01**
Alcohol + Cig smoke	0.66 ± 0.08*	0.42 ± 0.07*	0.34 ± 0.02*	0.39 ± 0.03*

Reduced glutathione (nmoles/mg protein)

Group	Liver	Lungs	Heart	Kidney
Control	10.38 ± 0.19	7.81 ± 0.50	16.08 ± 0.091	6.02 ± 0.21
Alcohol administration	06.08 ± 0.19*	7.01 ± 0.22***	13.29 ± 0.031*	4.29 ± 0.30*
Cigarette smoke	05.32 ± 0.01*	4.98 ± 0.32*	15.99 ± 0.012	3.41 ± 0.03*
Alcohol + Cig smoke	3.02 0± 0.32*	4.02 ± 0.18*	11.09 ± 0.080*	4.02 ± 0.18*

Values represent ± SD of six rats in each group

*P<0.001 **P<0.01 ***P<0.05

Control is compared with alcohol-administered group, cigarette smoke exposed group and with combined group

Table: 6f

Values of serum transaminases AST and ALT (U/L)

Group	AST	ALT
Control	118.49 ± 2.70	61.18 ± 1.70
Alcohol	180.38 ± 2.87*	92.32 ± 3.23*
Cigarette smoke	176.38 ± 2.29*	96.34 ± 2.99*
Alcohol+Cig smoke	201.43 ± 3.01*	99.02 ± 3.99*

Values represents ± SD of six rats in each group
*P<0.001
Control is compared with alcohol-administered group, cigarette smoke exposed group and with combined group

EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT FOR THE DEGREEE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY (FACULTY OF SCIENCE)

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CHAPTER 7

EFFECT OF S-ALLYL CYSTEINE SULPHOXIDE ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALCOHOL FED RATS

Alcohol consumption is increasing steadily in humans the world over and it has one of the serious health hazards. Several harmful effects of alcohol is known, but liver is the major organ that is most susceptible to the toxic effects of alcohol (Lieber , 1991). Ethanol can also alter the normal function and composition of membranes, which may ultimately lead to serious cellular impairment. Ethanol administration causes alcoholic fatty liver and hyperlipidemia (Ramakrishnan, et al 1974). Hepatic cirrhosis is a major cause of death in young and middle aged individuals, who are chronic alcoholics. Partial protection against alcoholic fatty liver has been observed on administration of antioxidants (Di Luzio,1958).

Garlic (Allium sativum linn) and compounds derived from it are found to have many therapeutic effects (Augusti,1990). There are several reports on the effect of garlic extract on lipid metabolism, lipid peroxidation and antioxidant status (Adamu et al , 1982, Lieber et al, 1982). Members of allium family are reported to have antimutgenic properties and therapeutic values and possess diuretic properties, aid in digestion and act as a heart stimulant alleviate rheumatic problems (Fenwick Jr et al, 1985). These properties of garlic can be attributed to the presence of sulphur compounds present in it. S-allyl cysteine sulphoxide commonly called allin is an amino acid isolated from garlic (Sathyavadi et al, 1988) and is found to have a major effect on lipid metabolism.

7.1. Materials and methods

Garlic was purchased from local market. SACS was extracted from fresh garlic according to the method of Itokawa (Itokawa et al,1973) with some modifications using ion exchange resins washed with IN HCl, 1N NaOH or deionized water. Fresh garlic was boiled in water to inactivate the enzyme allinase. It was then ground and extracted with 80% methanol, filtered and passed through a column of amberlite IR-120 (Strong cation exchanger) so as to absorb the amino acids. The column was washed with deionised water to remove the impurities and the amino acids were eluted with NH_4OH (2N). Ammonia was

removed by concentration of the eluates in rotary evaporator at 40-43^{0}C and the concentrate was loaded on to a column of amberlite CG-120 (strong cation exchanger). The column was washed with deionised water and the amino acids eluted with 0.1N NH_4OH. Fractions of the effluent were tested for the presence of SACS by thin layer chromatography (TLC) as described below. The SACS containing fractions were pooled and passed through a column of amberlite IRA-45 (weakly basic anion) so that unwanted amino acids and ammonia were absorbed on to the resin and removed. The effluent was collected and concentrated. Pure SACS was obtained from it after three crystallization steps from 80% ethanol. It had a melting point of 164^{0}C. The yield of SACS was 1.06g/kg garlic. In TLC on silica gel G using butanol: acetic acid: water (12:3:5), pure SACS gave an R_f value of 0.24, similar to that of an authentic sample obtained from the Biochemical Institute, Helsinki.

Male albino rats (Sprague Dawley strain) weighing 100-120g were used for the study. They were maintained under environmentally controlled conditions with free access to standard food (Lipton, India) and water. They were divided in to 3 groups of six rats each. A dose of 18% alcohol was administered orally for a period of 50 days. The animals were grouped as follows for the experiment.

GroupI - Control (Normal diet + ad lib)

Group II - Normal diet ad lib and 18% alcohol (4gm alcohol/kg body weight/ day) orally;

Group III - Normal diet ad lib, 18% alcohol (4gm alcohol/kg body weight/ day) along with S- allyl cysteine sulphoxide (SACS 500mg /kg body weight/ day) orally.

The rats were maintained on respective diets for a period of 50 days. The normal control group showed a gradual increase in body weight during the experimental period. The alcohol treated group showed an increase in body weight during the initial period of two weeks and later on showed a gradual decrease in body weight up to the end of the

experimental period. On the other hand SACS-treated group showed a regular increase in body weight during the entire experimental period. At the end of the experimental period the animals were decapitated and blood and tissues were quickly collected for various biochemical studies. Ethanol was obtained from E-Merck and thiobarbituric acid was obtained from BDH laboratories. BSA and tris were obtained from Sigma chemical company USA. All other chemicals and reagents used were of analytical grade.

Cholesterol in the serum was determined by CHOD - PAP (Duncan et al, 1982) method. Triglycerides in the serum were determined by GPO-PAP (NCEP,1995) method. Phospholipids in the sample were estimated by Trinders method (Trinder,1969). Tissue lipid peroxidation was estimated by thiobarbituric acid assay method (Ohkawa et al,1979) .Hydroperoxides was estimated by (Nair,1971). Conjugated dienes was estimated by the method of Brein, (Brein, 1966). Catalase was assayed by the method described by Aebi (Aebi, 1947). Superoxide dismutase activity estimated by the method of Fridovic and Beauchamp (Mc Cord et al, 1969). HMG CoA reductase activity was estimated by the method described by Rao and Ramakrishnan(Venugopala Rao et al,1975). Protein in the sample was quantitated by the method of Lowry (Lowry et al, 1951). Statistical analysis was carried out using student's t test. Values represent mean ±SD of six rats.

7.2. Results and discussion

The effect of long- term administration of alcohol and that of SACS on serum lipid levels are given in the table 7a. From the data it is clearly seen that the elevated levels of total cholesterol, LDL-cholesterol, triglycerides and phospholipids in alcohol –fed rats ($P<0.001$) were brought back to near normal values on administration of SACS ($P<0.001$). Concentration of HDL cholesterol significantly decreased by the administration of alcohol is brought back to near normal values on the administration of SACS. The values of lipid

peroxides, hydroperoxides and conjugated dienes in the liver, heart and kidney of alcohol administered and Alcohol and sacs treated group are given in the table 7b, 7c and 7d respectively. It is observed that the levels of lipid peroxides, hydroperoxides and conjugated dienes increased significantly ($P<0.001$) in all the tissues on the administration of alcohol and the values significantly decreased to near normal levels on treatment with SACS. The activities of antioxidant enzymes catalase and superoxide dismutase decreased significantly ($P<0.001$) on administration of alcohol and these values increased significantly ($P<0.001$) to normal levels on treatment with SACS. The values are given in the table 7e. The value of reduced glutathione content significantly decreased ($P<0.001$) by the administration of alcohol and the values increased to near normal values on the administration of SACS. The values are given in the table 7f. The activities of glutathione peroxidase significantly increased ($P<0.001$) on administration of alcohol, and were brought back to near normal values on administration of SACS ($P<0.001$). The values are given in the table 7g. The activity of HMG CoA reductase in the liver significantly decreased ($P<0.001$) on administration of alcohol, which increased to near normal levels on administration of SACS. The values are given in the table 7h. The values of serum glutamate oxaloacetate transaminase and serum glutamate pyruvate transaminase were significantly increased ($P<0.001$) and values were brought back to near normal values on administration of SACS. The values are given in table 7i.

Ethanol feeding has been found to enhance the endogenous synthesis of triglycerides and reduce the utilization of dietary lipids, resulting their accumulation in the liver, heart, kidney and blood plasma. In the present study we found that SACS found in garlic was able to counteract the effect of ethanol. Garlic protein is rich in sulphur-containing aminoacids like methionine, cysteine and SACS. These amino acids in turn define the anti atherogenicity of garlic (Itokawa et al,1973). Another finding is that these sulphur

containing aminoacids and their derivatives could counteract the hyperlipidemic and oxidant effect of alcohol. It is also pointed out that sulphur-containing aminoacids have a special role as hypolipidemic agents.

In the present study we found that the administration of alcohol has considerably increased the serum lipid levels, lipid peroxidation products MDA, hydroperoxides and conjugated dienes, where as antioxidant enzymes such as catalase, superoxide dismutase and reduced glutathione were decreased and glutathione peroxidase and serum glutamate pyruvate transaminase and glutamate oxaloacetate were increased on the administration of alcohol. On administration of alcohol the ratio of HMG CoA to mevalonate is found to be increased ie HMG CoA reductase activity is decreased in alcohol fed rats. This shows that increased serum cholesterol levels in alcohol fed rats may not be due to increased cholesterogenesis in the liver, but due to decreased esterification and utilization (Ginsberg et al,1998).

The decreased value of lipid levels on the administration of SACS is due to hypolipidemic effect of garlic (Chang et al, 1980). The lipid peroxidation products such as MDA, hydroperoxides and conjugated dienes increased in alcohol-administered rats in the present study. The administration of SACS significantly decreased lipid peroxidation. Effect of ethanol administation on lipid peroxidation has been suggested to enhance the generation of oxygen free radicals during its oxidation in the liver (Dianzani, 1988). These free radicals are responsible for the oxidation of LDL molecule causing lipid peroxidation (Diaz, 1997). Antioxidant enzymes such as catalase, superoxide dismutase and concentration of reduced glutathione decreased in alcohol fed rats. These effects are counteracted by the administration of SACS. This is may be due to the antioxidant action of the compound. The mechanism involved may be that SACS has counteracted the oxidant effect of alcohol, as the sulphydril group present in the compound is able to scavenge the

free radicals. Alcohol causes liver damage and enhances blood level of the liver enzymes SGOT and SGPT. SACS counteracts this effect. Increase in the values of glutathione peroxidase and glutathione-S- transferase by the administration of alcohol is a defense mechanism against the free radical produced by the oxidant action of the alcohol. SACS reduces the oxidant action of the alcohol. HMG CoA, that plays a major role in the cholesterol biosynthesis functions as the lipogenic enzymes in the liver tissues. A decrease is observed in the activity of HMG CoA reductase in alcohol fed rats. This may be due to the alcoholic fatty liver. All these effect of alcohol is reversed by the administration of SACS.

Present study indicates that SACS extracted from garlic has antilipidemic, antioxidant and hepatoprotective effects.

Table: 7a

Values of cholesterol, HDL cholesterol, LDL cholesterol, Triglycerides (TG) and phospholipids (mg/dl) in the serum of control, alcohol administered and SACS treated rats

Group	Cholesterol	HDL-chol	LDL-chol	Triglycerides	Phospholipids
Normal	60.52 ± 0.87	30.81 ± 0.64	26.82 ± 0.72	11.28 ± 0.50	109 ± 3.0
Alcohol administered	80.56 ± 1.73*	25.49 ± 0.68*	52.27 ± 1.20*	14.01 ± 0.19*	179 ± 5.6*
Alcohol +SACS	62.87 ± 2.31*	29.59 ± 0.74*	30.88 ± 0.98*	12.03 ± 0.0*	120 ± 2.8*

Values represent mean ±SD n = 6.
* $P<0.001$
Group II is compared with group I and group III is compared with group II

Table: 7b

Values of malondialdehyde (nmoles/mg protein) in the liver, heart and kidney of alcohol administered and SACS treated rats

Group	Liver	Heart	Kidney
Normal	0.75 ± 0.01	1.00 ± 0.12	0.31 ± 0.05
Alcohol administered	0.95 ± 0.08**	1.99 ± 0.18*	0.54 ± 0.02*
Alcohol+SACS	0.77 ± 0.09***	1.08 ± 0.13*	0.33 ± 0.09*

Values represents mean ±SD n=6
* $P<0.001$ **$P<0.01$ $P<0.05$
Group II is compared with group I and group III is compared with group II

Table: 7c

Value of hydroperoxides (mmol/100gm wet tissue) in the liver, heart and kidney of alcohol administered and SACS treated rats

Group	Liver	Heart	Kidney
Normal	11.35 ± 0.39	10.32 ± 0.13	17.01 ± 1.58
Alcohol administered	14.86 ± 0.22*	15.14 ± 1.05*	30.32 ± 3.08*
Alcohol+SACS	12.01 ± 0.35*	10.99 ± 0.99*	18.99 ± 1.68*

Values represent mean ±SD n=6
* P<0.001
Group II is compared with group I and group III is compared with group II

Table 7d

Values of conjugated dienes (μmmol/100g wet tissue) in the liver, heart and kidney of alcohol administered and SACS treated rats

Group	Liver	Heart	Kidney
I Normal	52.5 ± 1.30	8.99 ± 0.36	16.08 ± 2.01
II Alcohol administered	74.5 ± 0.58*	16.1 ± 0.58*	24.87 ± 3.13*
III Alcohol+SACS	53.0 ± 2.73*	10.8 ± 0.91*	20.21 ± 2.09*

Values represent mean ±SD n=6
*P<0.001
Group II is compared with group I and group III is compared with group II

Table: 7e

Values of superoxide dismutase (SOD) and catalase (CAT) [(U/mg protein)] in the liver, heart and kidney of alcohol administered and SACS treated rats

	SOD		CAT			
Group	Liver	Heart	Kidney	Liver	Heart	Kidney
I Normal	9.16 ± 0.21	4.99 ± 0.57	12.01 ± 0.57	60.2 ± 0.98	50.2 ± 1.87	10.0 ± 0.3
II Alcohol	5.36 ± 0.28*	3.19 ± 0.51*	9.01 ± 0.32*	46.8 ± 1.30*	38.2 ± 1.04*	7.8 ± 0.03*
III Alcohol +SACS.	8.79 ± 0.30*	4.00 ± 0.43*	11.99 ± 0.45*	56.9 ± 2.01*	45.3 ± 1.98*	9.0 ± 0.09*

Values represent mean ±SD n=6
* $P<0.001$
Group II is compared with group I and group III is compared with group II

Table: 7f

Values of reduced glutathione content (nmoles/mg protein) in the liver, heart and kidney of alcohol administered and SACS treated rats

Group	Liver	Heart	Kidney
I Normal	10.38 ± 0.19	7.86 ± 0.09	16.08 ± 0.09
II Alcohol administered	06.08 ± 0.29*	5.12 ± 0.10*	13.29 ± 0.03*
III Alcohol + SACS	09.48 ± 0.17*	6.89 ± 0.07*	14.71 ± 0.07*

* P<0.001 Values represent mean ±SD of 6 rats in each group.
Group II is compared with group I and group III is compared with group II

Table: 7g

Values of glutathione peroxidase (U/mg protein) in the liver, heart and kidney of alcohol administered and SACS treated rats

Group	Liver	Heart	Kidney
Normal	0.18 ± 0.06	0.21 ± 0.09	0.26 ± 0.05
Alcohol administered	0.47 ± 0.09*	0.30 ± 0.02**	0.25 ± 0.02
Alcohol +SACS	0.20 ± 0.07*	0.23 ± 0.10**	0.26 ± 0.03

Values represent mean ±SD n=6.
* $P<0.001$ ** $P<0.05$
Group II is compared with group I and group III is compared with group II

Table: 7h

HMG. CoA reductase activity (HMG CoA/mevalonate) in the liver, of alcohol administered and SACS treated rats

Group	HMG Co.A/Mevalonate
I Control	1.94 ± 0.31
II Alcohol administered	2.99 ± 0.44*
III Alcohol +SACS	1.80 ± 0.12*

* $P<0.001$ Values represent mean ±SD of six rats in each group
Group II is compared with group I and group III is compared with group II

Table: 7i

Values of AST and ALT (U/L) in the serum of alcohol administered and SACS treated rats

Group	AST	ALT
Normal	123.54 ± 2.9	60.23 ± 1.7
Alcohol administered	180.38 ± 2.8*	92.32 ± 3.3*
Alcohol+ SACS	137.34 ± 3.1*	72.15 ± 1.9*

Values represent mean ±SD n=6
* P<0.001
Group II is compared with group I and group III is compared with group II

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CHAPTER 8

HEPATOPROTECTIVE EFFECTS OF DIALLYL DISULPHIDE ON ETHANOL INDUCED LIVER DAMAGE IN RATS.

Ethanol is currently recognized as the most prevalent known cause of abnormal human development. Alcohol abuse and alcoholism represents one of the major health, social and economic issues facing the world. Liver is the most susceptible organ to the toxic effects of alcohol (Lieber etal, 1981). Acute ethanol administration causes alcohol fatty liver and hypertriglyceridemia (Ramakrishnan et al, 1974).

Alcohol consumption is increasing and it has become one of the serious health hazards. Several harmful effects of alcohol are known and liver is the major organ affected by excess ethanol. Ethanol can also alter the function and composition of membranes that may ultimately lead to serious cellular impairment. Hepatic cirrhosis is a major cause of death in young and middle aged individuals who are chronic alcoholics. Partial protection against alcoholic fatty liver has been reported on administration of antioxidants. Enhanced lipid levels and lipid peroxidation products have been observed in the serum and liver of alcoholics (Lieber et al,1998). Diallyl disulphide is a flavour component of garlic (Allium sativum). The potential source of naturally occurring sulphur are plants of Allium species of which garlic and onion can be placed ahead of the list due to their specific odour. These sulphur compounds are found to be good anticancer agents. Diallyl disulphide (DADS), unsaturated polysulphides found in Allium species inhibit tumour promotion by enhancing glutathione dependent detoxification enzymes (Sparnins et al, 1982; Goldberg et al, 1983). Allium species has antiatherosclerotic, hypocholesterolemic, hypolipidemic, antithrombotic, antioxidant and antidiabetic properties (Koscienly et al, 1990, Augusti et al, 1975, Ali et al, 1990, Yamasaki et al, 1997, Augusti, 1975). Prolonged extraction of fresh garlic at room temperature yields odorless aged garlic extract (AGE), S- allyl cysteine (SAC) and S- allyl mercaptocysteine (SAMC).

8.1. Materials and methods

Male albino rats (Sprague Dawley strain) weighing (150-175g) were used for the study. They were maintained under environmentally controlled conditions with free access to standard food (Lipton, India) and water. They were divided into three groups of six rats. A dose of 18% alcohol administered orally for a period of 30 days. The animals were grouped as follows

Group I. Normal diet

Group II. Normal diet +18% alcohol (4gm alcohol/kg body weight/day)

Group III. Normal diet +18% alcohol (4g alcohol/kg body weight / day) + DADS (10mg in 18% alcohol / kg body weight)

The rats were maintained on respective diet for a period of 30 days and decapitated at the end of the experimental period. Blood and tissues were quickly collected for various biochemical studies. Ethanol was obtained from E-Merck and thiobarbituric acid was obtained from BDH laboratories, DADS and BSA from Sigma Chemical Co, USA. All other chemicals and reagents used were analytical grade.

Cholesterol in the serum was determined by CHOD-PAP method (Duncan et al, 1982). Triglycerides in the serum were determined by GPO-PAP method (NCEP, 1995) Phospholipids in the serum was estimated by Trinders method (Trinder et al,1969). Malondialdehyde was estimated by thiobarbituric acid assay method (Ohakawa et al, 1979). Hydroperoxides was estimated by iodimetric assay (Nair et al, 1971). Conjugated dienes was estimated by the method of Brein (Brein et al, 1996). Catalase was assayed (Aebi, 1947) spectrophotometrically following a decrease in absorbance at 230 nm and superoxide dismutase activity by the method of Fridovich and Beauchamp (Mc Cord et al, 1969).

Reduced glutathione content by the method of Moron et al (Moron et al, 1974), and glutathione peroxidase by Habig et al (Habig et al, 1974). Protein in the sample was quantitated by the method of Lowry et al (Lowry et al, 1951) using bovine serum albumin as standard. Detailed procedure for various biochemical estimations is given in the chapter 2. Statistical analysis was carried out using the students 't' test. Values are expressed as mean ±SD.

8.2. RESULT AND DISCUSSION

The effect of long-term administration of alcohol and DADS on serum lipid levels are given in the table 8a. The table 8b shows the values of malondialdehyde, hydroperoxides and conjugated dienes in the liver, heart and kidney of alcohol administered and DADS treated rats. The activities of SOD and catalase in the liver, heart and kidney of control and treated rats are given in the table 8c. The activitis of glutathione peroxidase and value of reduced glutathione are given in the table 8d. The values of serum glutamate pyruvate transaminase and glutamate oxaloacetate transaminase are given in the table 8e. Ethanol fed rats gained less weight as compared to control rats. From this data it is evident that the administration of alcohol has significantly increased ($P<0.001$) the lipid levels. The elevated lipid levels in alcohol fed rats were brought back to near normal values on the administration of DADS. Alcohol significantly increased ($P<0.001$) the lipid peroxidation products such as malondialdehyde, hydroperoxides and conjugated dienes in the liver, heart and kidney. The activities of antioxidant enzymes such as superoxide dismutase, catalase and the level of reduced glutathione in the liver, heart and kidney of rats were significantly decreased ($P<0.001$) by the administration of alcohol. The activities of glutathione peroxidase were significantly increased ($P<0.001$) by the administration of alcohol. The administration of DADS to the alcohol fed rats brought back the lipid levels and lipid peroxidation products to

near normal values. The activities of antioxidant enzymes and level of reduced glutathione and glutathione peroxidase were brought back to near normal values by the administration of DADS to the alcohol fed rats. The values of AST and ALT were returned to near normal values by the administration of DADS. Alcohol has been found to enhance the generation of free radicals during its oxidation in the liver. The free radicals are responsible for the oxidation of LDL molecules. The decreased value of lipid levels on the administration of DADS is due to the hypolipidemic effect of garlic. DADS also restored the level of ethanol induced lipid pertoxidation to that of normal values. The decrease in activity of antioxidant enzymes, superoxide dismutase, catalase and level of reduced glutathione may be due to the damaging effects of free radical produced by the administration of alcohol or alternately due to the direct effect of acetaldehyde formed from the oxidation of alcohol on these enzymes. DADS treatment restored the activity of SOD, catalase, GPx and level of reduced glutathione. This may be due to the antioxidant action of the compound. The possible mechanism involved is that the sulphide group present in DADS is able to scavenge the free radicals.

The binding of acetaldehyde, a metabolite of ethanol, with glutathione may contribute to the reduction in the levels of glutathione in the liver, kidney and heart of alcohol treated group. The results obtained indicate that ethanol induced hepatic damage was prevented by DADS treatment. DADS also restored the level of ethanol induced lipid peroxidation products and reduced glutathione to near normal values. It has been observed that effect of alcohol was counteracted by the addition of DADS. This may be due to the antioxidant action of the compound or that the sulphide group present in the compound is able to scavenge the free radicals. Various biochemical effects, anticarcinogenic action of the compounds (Sparnins et al,1997) suggests that the sulphur and allyl group together make

them more active. DADS, the organosulphur compound found in garlic is found to inhibit chemically induced tumerogenesis in lung (Hong et al,1992), liver (Lau et al,1990) and colon (Wargovich 1987). Our results show that DADS is a hepatoprotective agent.

Table 8a

Values of total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides in the serum of alcohol administered and DADS treated rats(mg%l)

Group	Total cholesterol	HDL-chol	LDL-chol	Triglycerides
I-Normal	61.98 ± 1.03	29.68 ± 0.64	26.03 ± 0.72	109.3 ± 3.0
II-Alcohol	79.99 ± 0.98*	27.38 ± 0.68*	52.98 ± 1.01*	183.8 ± 5.8*
III-DADS	63.31 ± 0.78*	29.09 ± 0.74**	28.32 ± 0.93*	125.6 ± 2.8*

Values represent mean ±SD (n=6)
*P<0.001 ** P<0.01
Group II is compared with group I. Group III is compared with group II

Table: 8b

Values of malondialdehyde (nmoles/mg protein), hydroperoxides (μ mol/100g wet tissue) and conjugated dienes (μ mol/100g wet tissue) in liver, heart and kidney of control, alcohol administered and DADS treated rats

Malondialdehyde

Group	Liver	Heart	Kidney
I.Normal	0.75 ± 0.01	0.28 ± 0.09	1.00 ± 0.12
II.Alcohol	1.08 ± 0.03*	1.98 ± 0.20*	0.63 ± 0.10*
III.Alcohol+DADS	0.91 ± 0.07*	0.30 ± 0.03*	1.50 ± 0.13*

Hydroperoxides

Group	Liver	Heart	Kidney
I.Normal	11.76 ± 0.39	10.08 ± 0.36	16.09±2.06
II.Alcohol	14.56 ± 0.20*	15.14 ± 1.03*	30.32±3.60*
III.Alcohol+DADS	12.01 ± 0.63*	10.99 ± 0.99*	21.12±0.99*

Conjugated dienes

Group	Liver	Heart	Kidney
I.Normal	53.2 ± 1.20	17.08 ± 2.36	08.99 ± 0.36
II.Alcohol	78.5 ± 0.60*	25.78 ± 1.73*	16.80 ± 0.60*
III.Alcohol+DADS	59.0 ± 1.63*	21.12 ± 0.19*	21.12 ± 0.99*

Values represent mean ±SD n=6 *P<0.001
Group II is compared with group I and group III is compared with group II

Table: 8c

Values of superoxide dismutase and catalase in the liver, heart and kidney of control, alcohol administered and DADS treated rats (U/mg protein)

	SOD			Catalase		
Group	Liver	Heart	Kidney	Liver	Heart	Kidney
I.Normal	9.16 ± 0.21	4.99 ± 0.57	12.01 ± 0.5	66.02 ± 0.98	50.80 ± 1.9	10.38 ± 0.05
II.Alcohol	6.05 ± 0.30*	02.9 ± 0.71*	09.31 ± 0.32*	49.80 ± 1.39*	39.50 ± 1.04*	06.51 ± 0.03*
Alcohol+DADS	8.91 ± 0.27*	4.09 ± 0.53*	12.9 ± 0.45*	57.90 ± 2.01*	46.30 ± 1.89*	09.00 ± 0.07*

Values represent mean ±SD n=6

*P<0.001

Group II is compared with group I and group III is compared with group II

Table: 8d

Values of reduced glutathione content (GSH nm / mg protein), glutathione peroxidase (U/mg protein) in the liver, heart and kidney of control alcohol administered and DADS treated rats

Reduced glutathione

Group	Liver	Kidney	Heart
I. Normal	10.38 ± 0.19	8.01 ± 0.05	16.08 ± 0.07
II. Alcohol	05.99 ± 0.30*	5.18 ± 0.10*	13.38 ± 0.05*
III. Alcohol+DADS	09.99 ± 0.28*	7.97 ± 0.07*	15.88 ± 0.19*

Glutathione peroxidase

Group	Liver	Heart	Kidney
I Normal	0.18 ± 0.06	0.20 ± 0.09	0.26 ± 0.05
II Alcohol	0.47 ± 0.09*	0.30 ± 0.02*	0.30 ± 0.06
III Alcohol+DADS	0.23 ± 0.07*	0.25 ± 0.10**	0.27 ± 0.05

Values represent mean ±SD n=6 *P<0.001 **P<0.01
Group II is compared with group I and group III is compared with group II

Table: 8e

Values of SGOT and SGPT in the serum of alcohol administered and DADS treated rats

Group	SGOT (U/L)	SGPT (U/L)
Normal	120.54 ± 3.9	60.23 ± 2.7
Alcohol administered	185.38 ± 3.8*	99.32 ± 3.3*
Alcohol+ SACS	142.34 ± 2.9*	78.15 ± 0.9*

Values represent mean ±SD (n=6)
*P<0.001
Group II is compared with group I and group III is compared with group II

EFFECT OF DIFFERENT TYPES OF STRESS ON LIPID METABOLISM AND ANTIOXIDANT STATUS IN ALBINO RATS

THESIS SUBMITTED TO THE UNIVERSITY OF CALICUT FOR THE DEGREEE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY (FACULTY OF SCIENCE)

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SUMMARY AND CONCLUSION

Stressful stimulus influences the onset and progression of a number of disorders in human beings leading to hypertension, stroke and depression. The stress increases the blood pressure, heart rate, increases plasma cholesterol levels and has adverse effects on coagulation and fibrinolysis. All the systems: the heart, the blood vessels, the immune system, the lungs, the digestive system, the sensory organs and brain are modified to meet the perceived danger. Prolonged exposure to continued stress can be very damaging by decreasing hippocampus function and also by decreasing cell proliferation rates. With the urbanization and westernization of our society stress is increasing day by day which causes an increase in the incidence of psychosomatic, psychiatric and cardiovascular diseases. Stress enhances the release of various hormones. Epinephrine increases blood pressure and heart rate, diverts blood to muscles and speeds reaction time. Cortisol releases sugar from the body reserves. Stress circuit affects systems throughout the body. The hormones of the hypothalamic- pituitary-adrenal axis exert their effect on the autonomic nervous system, which controls such vital functions as heart rate, blood pressure and digestion.

The involvement of reactive oxygen species (ROS) has been identified in the pathogenesis of various diseases. ROS are continuously generated in cells exposed to an aerobic environment during the course of normal metabolism. ROS damage proteins, DNA and other biomolecules. ROS are short-lived species and are generated in normal cells under pathological conditions, metabolism of different xenobiotics, exposure to ionizing radiations and different stress conditions.

Stress both physical and psychological has a major role to play in the pathogenesis of a number of diseases. Our study is aimed to study the effect of various types of stress on lipid metabolism and antioxidant status. It is considered relevant to study the levels of lipid parameters - total cholesterol, triglycerides, HDL-cholesterol and LDL- cholesterol and scavenger enzymes of reactive oxygen species- superoxide dismutase (SOD), catalase (CAT),

glutathione peroxidase (GPx), glutathione-S- transferase (GST) and reduced glutathione (GSH) content in various stress conditions with a view to asses the role played by these stress conditions in the incidence of cardiovascular diseases.

Hence the present study is aimed to understand

1. Whether physical and psychological stress and stress caused by external agents produce any changes in the lipid metabolism and antioxidant status in experimental animals and also to understand whether this information is useful in understanding the risk of getting coronary artery disease in these stress condition.

Materials and Methods

In present study Male albino rats (Sprague-Dawley strain) weighing (150-175g), were divided into seven groups of 6 rats each. The groups were

Group I - Normal control rats

Group II - Fresh water swimming (F W S)

Group III - Cold water swimming (C W S)

Group IV - Overcrowding

Group V - Isolation

Group VI - Exposure to cigarette smoke

Group VII - Alcohol administered

Animals were subjected to the above-mentioned stress for a period of one month. At the end of the experimental period the animals were sacrificed and their blood, liver, heart, kidney and aorta were collected for various biochemical estimations. The biochemical investigations such as lipid profiles- total cholesterol, LDL-Cholesterol, triglycerides and HDL- cholesterol and markers of oxidative stress such as superoxide dismutase, catalase, glutathione peroxidase, glutathione-S- tranferase and reduced glutathione content and lipogenic enzymes and transaminases (serum glutamate oxaloacetate and pyruvate

transaminase) were also estimated in the tissues of stress exposed and normal animals. The following findings were obtained.

1. The lipid profile parameters such as cholesterol, triglycerides and LDL- cholesterol and transaminases were also found to be significantly elevated in isolation, overcrowding and alcohol administration and cigarette smoke exposure when compared to those of normal control rats. HDL-cholesterol was significantly decreased when compared to that of the normal control rats. Swimming stress showed a significant decrease in cholesterol, triglycerides and LDL-cholesterol and an increase in HDL - cholesterol were obtained.
2. The markers of oxidative stress such as superoxide dismutase, catalase, glutathione peroxidase and glutathione- S- transferrase significantly decreased in all stress condition except cigarette smoke exposure. Cigarette smoke exposure significantly enhanced the activities of all the antioxidant enzymes. Alcohol significantly increases the activities of glutathione -S- transferrase and glutathione peroxidase.
3. The values of reduced glutathione significantly decreased in all the tissues of rats exposed to all types of stress. The values of lipid peroxidation significantly increased in the serum and tissues of rats exposed to all types of stress.
4. The activities of HMG CoA reductase was significantly increased in the swimming, isolation and cigarette smoke exposure and a significant decrease in HMG CoA reductase activity was observed in the alcohol administered group. Activities of glucose 6 phosphate dehydrogenase and malic enzyme significantly increased in Fresh water swimming , cold water swimming , alcohol administration and cigarette smoke exposed group.
5. Combined action of alcohol and cigarette smoke lead to a significant increase in lipid parameters, lipid peroxides and serum transaminases. Combined exposure to alcohol and cigarette smoke enhanced the oxidative stress and thus adversely affecting the antioxidant system.

All the types of stress studied: swimming, isolation, overcrowding, alcohol administration and cigarette smoke exposure produces significant changes in the lipid metabolism. Alcohol administration and exposure to cigarette smoke produces more damage to tissues, followed by fresh water swimming and cold-water swimming stress. Overcrowding and isolation stress cause least damage to tissues compared with other stresses. It is also clear that stress - physical, oxidative and psychological increases lipid peroxidation in rats when compared with normal control rats. The alcohol administration, smoke exposure and induces more lipid peroxidation compared with control rats. The fresh water swimming and cold-water swimming stress induces less lipid peroxidation compared with oxidative stress, and isolation and overcrowding stress induces very little elevation in the lipid peroxidation products. The stress induces the production of free radicals, which reduces the activities of antioxidant enzymes. Alcohol administration and exposure to cigarette smoke produces more oxidative damage to the tissues followed by fresh water swimming and cold-water swimming stress. Over crowding and isolation stress cause least damage to the tissues compared with other stress conditions.

The combined treatment of alcohol and cigarette smoke was found to enhance the lipid parameterss and lipid peroxidation and serum transaminases. In conclusion combined exposure to alcohol and smoke enhanced the oxidative stress and thus adversely affect the antioxidant system and thus is likely to be more harmful than the effect of smoke or alcohol alone. The study was extended to evaluate the effect of S-allyl cysteine sulphoxide (SACS) and diallyl disulphide (DADS) on various parameters in alcohol administered experimental rats. It was observed that the oxidative stress induced by alcohol administration may be partially neutralized bys the administration of SACS and DADS.

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ABBREVIATIONS

ALT	Glutamate pyruvate transaminase
AST	Glutamate oxaloacetate transaminase
CAT	Catalase
CDNB	1-Chloro 2,4 dinitro benzene (CDNB)
CWS	Cold water swimming stress
DADS	Diallyl disulphide
DTNB	5 5' dithio-bis 2 nitrobenzoic acid
FWS	Fresh water Swimming
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S- transferase
HDL	High-density lipoprotein
HMG. CoA	Hydroxy methyl glutaryl CoA
HPA	Hypothalamic- Pituitary- adrenal
LDL	Low-density lipoprotein
MDA	Malondialdehyde
NADPH	Nicotinamide,adenine dinucleotide phosphate reduced
NBT	Nitroblue tetrazolium (NBT)

POD	Peroxidase
ROS	Reactive oxygen species
SACS	S-allyl cysteine sulphoxide
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloro acetic acid
TG	Triglyceride
TLC	Thin layer chromatography
VLDL	Very low-density lipoprotein
ATP	Adenosine tri phosphate
SAS	Sympathetic adrenomedullary system
I-R	Ischemia reperfusion
CRH	Corticotropin Releasing Hormone
ACTH	Adrenocorticotropic hormone
PTSD	Post Traumatic Stress Syndrom
GnRH	Gonodotropin Releasing Hormone
MR	Mineralocorticoid Receptor
GR	Glucocorticoid receptor
IL-6	Interlukein-6
APR	Acute Phase Reactants
CRP	C-Reactive Proteins
VP	Vasopressin

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