UNIVERSITY OF SOUTHAMPTON

STUDIES ON THE ANTIOXIDANT ACTIVITY AND IMMUNOMODULATORY PROPERTIES OF BLACK TEA

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ABSTRACT

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Black tea components are reported to have many biological activities. These include anticarcinogenic properties and reduction of the risk of cardiovascular diseases. Antioxidant activity of black tea components has been suggested as the reason for these properties. Although antioxidant activity of black tea components has been demonstrated in many *in vitro* studies, evidence for their bioavailability and *in vivo* antioxidant activity are limited. Therefore the effect of black tea ingestion on serum antioxidant capacity was investigated in Wistar rats. Serum total radical trapping antioxidant parameter (TRAP) values increased two hours after the ingestion of a tea extract and remained elevated after four hours and decreased to baseline level after eight hours, indicating that black tea components are absorbed through the digestive tract and they have antioxidant activity *in vivo*.

Large amounts of reactive oxygen species and free radicals are produced during the inflammatory response. These oxidant species enhance the production of cytokines, the mediators of the inflammatory response. Synthetic antioxidants are known to alter the inflammatory response by their action on reactive oxygen species and free radicals. Therefore the effect of black tea components on inflammatory response induced by endotoxin (lipopolysaccharide from E.coli strain) was studied in Wistar rats. When the antioxidant defences of animals were compromised by feeding a vitamin E deficient diet plasma concentrations of the cytokine interleukin-6 and caeruloplasmin were decreased and liver glutathione concentration increased indicating the antioxidant activity of black tea components during inflammatory response. Paradoxically black tea solids (5g/kg diet) increased the number of neutrophils and myeloperoxidase activity in lung after endotoxin treatment. This may be due to increased recruitment of neutrophils to the lung by increased secretion of neutrophil chemotactic factor by alveolar macrophages under the influence of components of black tea during inflammatory response. Thus black tea solids may have two important properties which modulate the action of the immune system. Firstly in modifying the extent of the inflammatory response when antioxidant defences are compromised and secondly by enhancing neutrophil recruitment to lung when defences are normal.

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ABBREVIATIONS

DGT	Decaffeinated green tea
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EPR	Electron paramagnetic resonance
GR	Glutathione reductase
GSH	Reduced glutathione
GSHPx	Glutathione peroxidase
GSSG	Oxidised glutathione
IFN	Interferon
IL 1-12	Interleukin 1-12
MDA	Malondialdehyde
NCF	Neutrophil chemotactic factor
NDEA	N-nitrosodiethylamine
NFkB	Nuclear factor kappa B
NMBzA	N-nitrosomethylbenzylamine
NNK	4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanone
ROS	Reactive oxygen species
SOD	Super oxide dismutase
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
TF	Theaflavin
TNF	Tumour necrosis factor
TR	Thearubigin
TRAP	Total radical trapping antioxidant parameter

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CHAPTER 1

GENERAL INTRODUCTION

Tea *(Camellia sinensis)* drinking originated in China. Legend has it that tea was discovered by the Chinese emperor Shen Nung, in 2700 BC. Its earliest mention is in a Chinese dictionary of 350 BC. From China the habit of tea drinking spread to Japan around 1000 AD. However it was only by the middle of the 17th century that tea was introduced to Europe. Today it is probably, after water, the world's most popular beverage (Nathaniel, 1986).

Tea is mainly grown in the Asian region and in East African countries. An estimated 2.5 million metric tons of tea are manufactured annually. India (0.72 million metric tons), China (0.54), Sri Lanka (0.23) and Kenya (0.20) are the major producing countries. When per capita consumption is considered Ireland (3.04 kg per capita) and United Kingdom (2.81) heads the list, while Asian countries have a per capita consumption of around 1 kg (Graham, 1992).

In the processing of tea, tender shoots with two leaves and a bud are plucked from the plant. The most abundant chemical component in these fresh shoots are polyphenols (25-30% by dry weight) which includes catechins and other flavonoids. Caffeine accounts for 3-5% of dry weight. The chemical composition of young tea shoots is given in table 1.1 (Hilton, 1986)

There are three main types of processed teas produced in the world. i.e. Green tea,

Oolong tea and Black tea. About 78% of tea produced in the world are black tea. India, Sri Lanka and Kenya are the major producers and exporters of black tea to Europe, Middle East, U.S.A. and other countries. Black tea is the main type of tea consumed world wide.

Green tea accounts for about 20% of the world production. China and Japan are the major producers of green tea and consumption is also mainly confined to that region of the world. Oolong tea is produced in some regions of China and Taiwan and consumed mainly in the producing regions. Oolong tea accounts for only 2% of the world tea production (Mulky, 1993).

There are four stages in black tea processing. i.e. Withering, Rolling, Fermentation and Drying. Plucked shoots are allowed to wither in withering troughs for 12-16 hours to bring down the moisture level by about 50%. Then in the rolling process leaves are macerated into small particles inside the rollers. Then macerated leaves are laid on fermenting tables for 1 to 2 1/2 hours for fermentation. Major chemical changes occur during this stage of processing. The most important change is the enzymic oxidation of catechins (Fig.1.1) to their dimers theaflavins (TF, Fig.1.2) and further reaction produces thearubigins (TR). TR are a group of compounds which are polymeric and complex in nature. The exact structure of TR is not known yet. TR are the major components in black tea beverage accounting for 12-18% of extracted solids in the beverage. Principle components in black tea beverage are given in table 1.2. The fermentation reactions that take place in the fermenting stage of processing are brought about by the enzyme polyphenol oxidase which comes into contact with the substrate (catechins) when the leaf is macerated. Finally the fermented leaves are dried in a drier by a stream of hot air to bring down the moisture level to 3-4% and the enzymic reactions are halted by the high temperature in the drier (Samaraweera, 1986, Wickremasinghe, 1978).

Table 1.1 Chemical composition of young tea shoots: (Hilton, 1986)

Soluble in cold water	**(g/100g dry weight)**
Flavanoids:epigallocatechin gallate	9 - 13
epigallocatechin	3 - 6
epicatechingallate	3 - 6
epicatechin	1 - 3
gallocatechin	1 - 2
catechin	1 - 2
Flavonoids and their glycosides	3 - 4
Leucoanthocyanins	2 - 4
Phenolic acids	4
Caffeine	3 - 4
Amino acids: theanin	2
others	2
Carbohydrates	4
Organic acids	0.5
Volatile substances	0.01
Partially soluble in hot water	
Polysaccharides: starch	2 - 5
other	12
Protein	15
Ash (inorganic material)	5
Insoluble in water	
Cellulose	7
Lignin	6
Lipids	3

Table 1.2 Principal components of black tea beverage.

Catechins	3 - 10
Theaflavins	3 - 6
Thearubigins	12 - 18
Flavonols	6 - 8
Phenolic acids and depsides	10 - 12
Amino acids	13 - 15
Methylxanthins	8 - 11
Carbohydrates	15
Protein	1
Mineral matter	10
Volatiles	<0.1

Components measured as weight (g) per 100g of extracted solids (Graham, 1992).

In the green tea processing the leaves are heated (usually by steam) before maceration to make the enzymes inactive. Therefore fermentation reactions do not take place and the chemical composition of the end product is more or less similar to that of the fresh shoots (see fig.1.0 for schematic representation of black tea and green tea processing). Oolong teas are partially fermented teas. Their chemical composition depend on the degree of fermentation and it lies between black tea and green tea (Samaraweera, 1986).

It has been assumed, for a long time, that tea drinking is beneficial for health. Ancient Chinese medical chronicals have recorded that tea drinking is beneficial to health. However

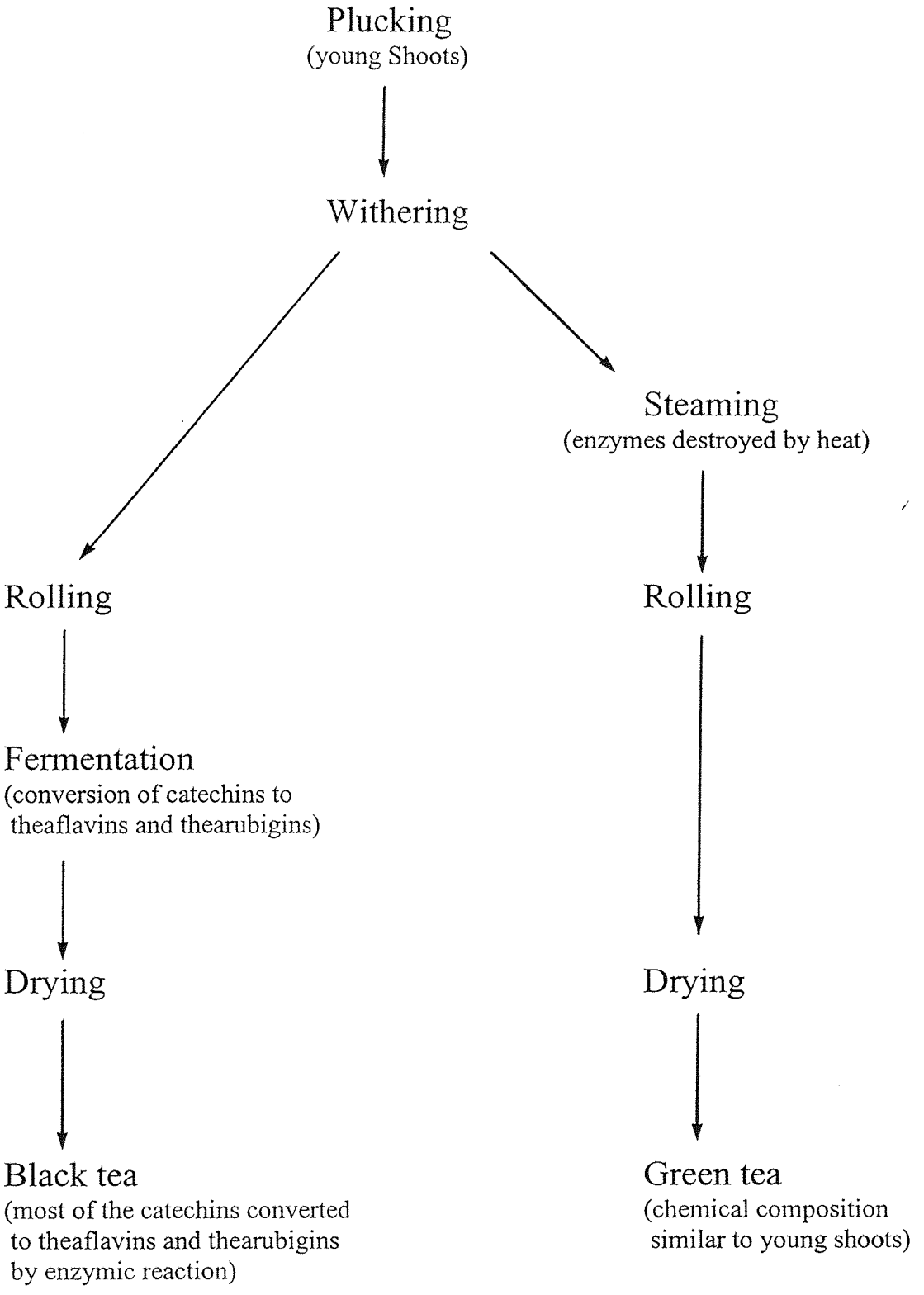
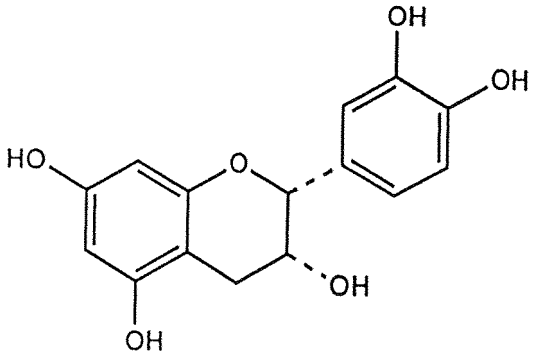
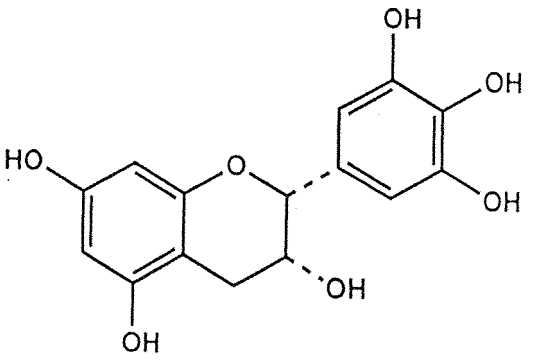


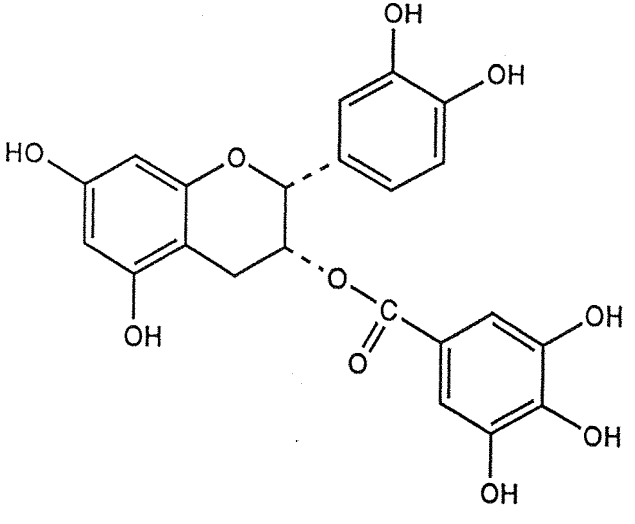
Figure 1.0 Schematic representation of black tea and green tea processing.



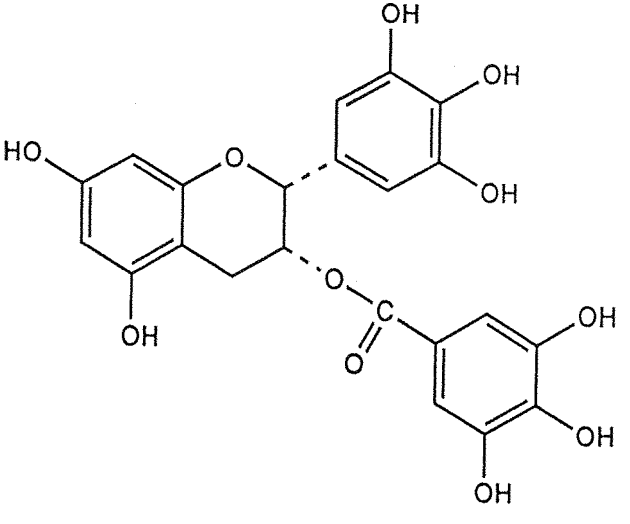
(-)-Epicatechin (EC)



(-)-Epigallocatechin (EGC)



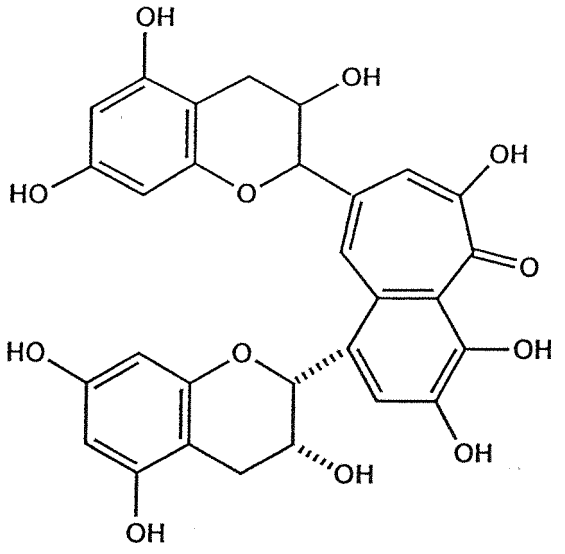
(-)-Epicatechin gallate (ECg)



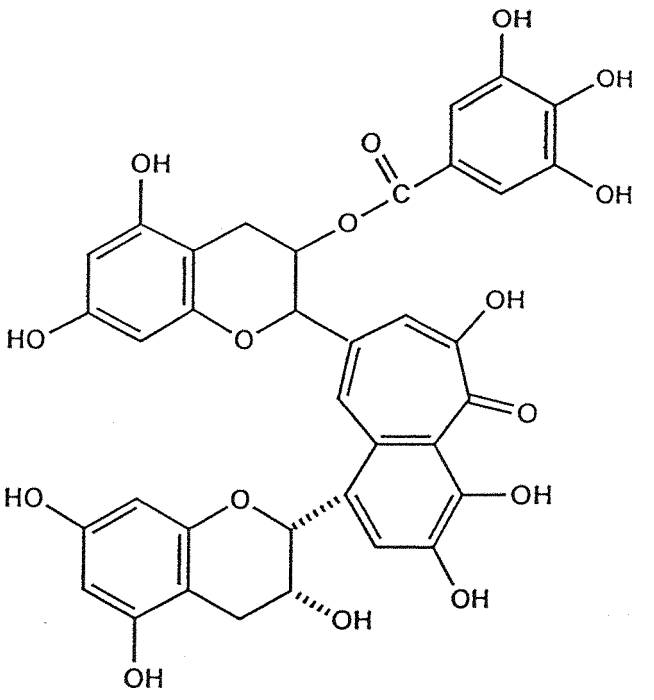
(-)-Epigallocatechin gallate (EGCg)

Figure 1.1

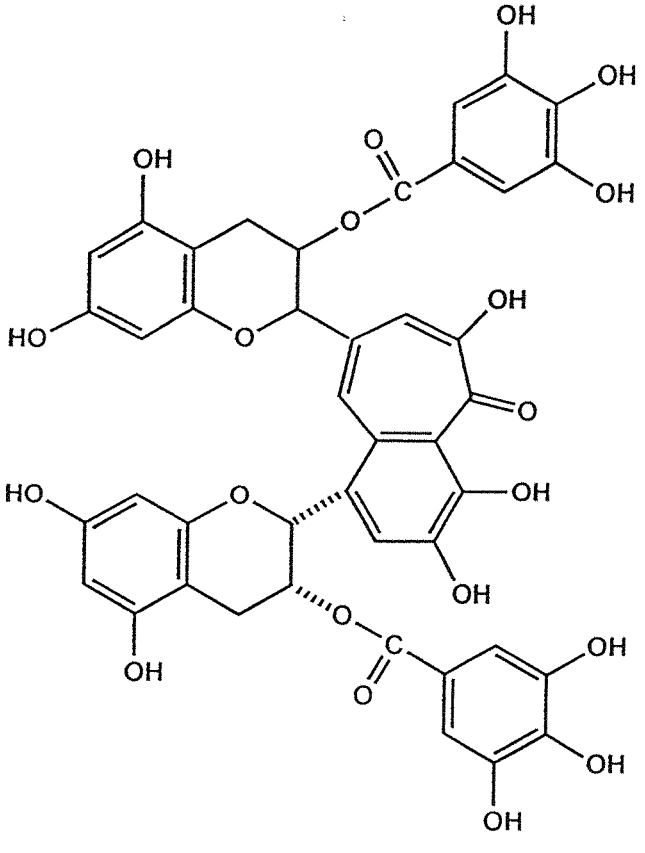
Structural formulae of catechins



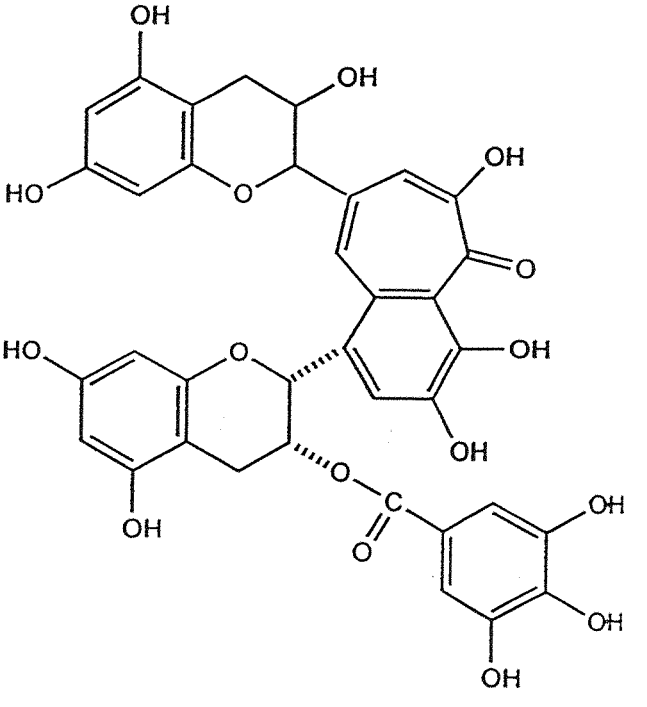
Theaflavin (TF1)



Theaflavin monogallate A (TF2A)



Theaflavin digallate (TF3)



Theaflavin monogallate B (TF2B)

Structural formulae of theaflavins

Figure 1.2

proper scientific investigations on the biological effects of tea consumption were started only in the recent past. The majority of research on biological effects was done either on green tea extracts, or on compounds isolated from green tea. During the last few years more and more attention has been directed towards black tea as 80% of tea consumed in the world is black tea.

In the literature, attention has been focused on three main areas. They are, prevention of carcinogenesis, prevention of cardiovascular diseases and improvement of oral health. In addition to this, antibacterial, antifungal and antiviral activities are also reported (Marks, 1992).

A number of authors have suggested that the anticarcinogenic property and reduction of the risk of cardiovascular diseases by tea are due to the antioxidative activity of flavanoid components found in tea (discussed in sections 1.1, 1.2 and 1.4). Activation of the immune system results in the production of large amounts of free radicals and reactive oxygen species which play an important role in combating the invading pathogens (discussed in section 1.6). The immune system may be involved in the removal of malignant cells and play a part in pathogenesis of atherosclerosis (Alexander, 1994). However, despite these facts no attention has been focused on the influence of tea components on immune function.

The work covered in this thesis, describe studies on the effect of black tea components on some aspects of immune activity and systems associated with immune function and antioxidant defences. A discussion of the known anticarcinogenic, antioxidant and cardiovascular disease prevention effects of tea and aspects of immune function and the

associated systems, relevant to the thesis, are set out below.

1.1 Anticarcinogenic activity of tea

Many laboratory and epidemiological studies have demonstrated inhibitory effects of tea against carcinogenesis. The effectiveness of tea extracts against tumorigenesis in different sites has been tested. Skin, lung, liver, kidney, pancreas, mammary gland, oesophagus, fore stomach, duodenum, small intestine and colon are the sites which had been tested for the effectiveness of tea extracts (Yang and Wang, 1993).

Carcinogenesis is a multistage process. Three sequential stages have been identified in this process. They are the initiation, promotion and progression stages. Initiation involves the permanent, irreversible, induction of genetic damage by a chemical agent, or other factors such as radiation. These factors result in the formation of an initiated cell. Though the initiated cell is genetically altered it may not proceed to become a carcinoma. Subsequent changes in gene expression and additional mutations appear to be necessary for this process. This stage is called the promotion stage. The progression stage involves additional genetic changes and the formation of benign and malignant carcinomas (Ito and Imaida, 1992). Tea extracts and compounds isolated from tea extracts, have been tested in prevention of carcinogenesis during these stages, using laboratory animal models. However the majority of the studies were done on the prevention of the initiation and promotional stages caused by chemical carcinogens.

In one such experiment female A/J mice were used to study the effect of green tea and

black tea extract on the lung and forestomach tumours induced by N-Nitrosodiethylamine (NDEA) and 4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK). NDEA administration by gavage (10mg/kg body weight), once a week for 8 weeks, produced forestomach and lung tumours in 90% of animals, 16 weeks after the last dose of NDEA. The animals had an average of 8.3 forestomach and 2.5 lung tumours per mouse. 1.25% green tea infusion (12.5g green tea infused with 1l boiling water) and 0.63% green tea infusion (diluting the 1.25% infusion 1:2) as the sole source of drinking water for the whole experimental period reduced lung tumour incidence by 44% and 18% respectively. It also reduced the forestomach tumour incidence by 26% and 18% respectively. Treatment of A/J mice with a single dose of NNK (103mg/kg) resulted in lung tumours in almost all the animals with average 9.3 tumours per mouse after 16 weeks. 0.6% decaffeinated green tea or black tea (0.6g tea solids in 100ml water) as drinking fluid reduced tumour incidence by 67% and 65% respectively. It is suggested by the authors that antioxidant properties of tea components may be important in the inhibition of tumorigenesis. However there is a possibility that tea components may have interacted directly with the carcinogens as both were administered through the same route(orally). This may have reduced the carcinogenicity of NDEA and NNK (Wang *et al*, 1992).

Wang and others have shown that N-nitrosomethylbenzylamine (NMBzA) induced tumorigenesis in male Sprague-Dawley rats was inhibited by green tea and black tea extracts. Treatment of rats with NMBzA (2.5mg/kg, s.c., twice weekly) for 5 weeks resulted in 65% of rats having oesophageal tumours with an average of 1.4 tumours per rat 39 weeks after the initial treatment of NMBzA. Both decaffeinated green tea(DGT) or decaffeinated black tea (6mg tea solids/ml) as the sole source of drinking fluid during the NMBzA treatment period

reduced tumour incidence by 70%. When the tea preparations were given after the NMBzA treatment period the tumour incidence was reduced by 50%. Results obtained in this study provide the important information that the reduction of tumorigenesis is not by the direct action of components in tea with the carcinogen as tea and NMBzA were administered by two routes. i.e. Tea is given orally and NMBzA given subcutaneously. The authors suggest that the activity of tea solutions may be through the inhibition of carcinogen activation or by prevention of oxidative damage of DNA due to reactive oxygen species produced during carcinogen metabolism (Wang *et al*, 1995a).

Several other authors have reported the anticarcinogenic activity of both green and black tea extracts on different sites induced by chemical carcinogens. Tea extracts have shown anticarcinogenic activity in both initiation and promotion stages of carcinogenesis (Reviewed by Mukhtar, Katiyar and Agarwal, 1994, Yang and Wang, 1993).

Although the laboratory animal model experiments have produced favourable results on the anticarcinogenic activity of tea, epidemiological studies have shown mixed results. Epidemiological studies conducted in China revealed that most of the areas with higher oesophageal and gastric cancer mortality rates are in the areas where tea is infrequently consumed. Exposure to endogenously and exogenously formed N-Nitroso compounds is associated with this increased risk of cancers. Ingestion of secondary amines and nitrite could react *in vivo* to produce carcinogenic nitrosamines. Xu and others have carried out a study in Moping County, China (a high risk area) to study the effect of green tea on endogenous formation of N-nitrosoproline. Eighty six subjects were used for the study. Subjects were given a controlled diet and their urinary base line levels of N-nitrosoproline were established.

Then a daily dose of 300mg L-Proline increased the urinary excretion of N-nitrosoproline. In the next stage 1.55g of green tea solids (Prepared by adding boiling water to green tea, then filtering it to remove solid particles and freeze drying the extract to a powder, 4.65g of green tea solids consumed is equivalent to 6-8 cups per day) were given three times a day with the daily dose of 300mg L-Proline. This significantly decreased the urinary excretion of N-nitrosoproline (Xu *et al*, 1993). Wu and others also had shown that different types of Chinese teas and its polyphenol extractions inhibit N-Nitrosation *in vitro* and *in vivo* (Wu *et al*, 1993). A case control study carried out in Shanghi, China using 902 patients and 1552 controls also had revealed that green tea consumption has a protective effect on oesophageal cancer (Gao *et al*, 1994). Studies by Oguni and others in Shizuoka Prefecture in Japan show that stomach cancer death rate in this tea producing and consuming area was lower than the national average (Oguni *et al*, 1992).

A recent study carried out in Netherlands on diet and cancer, using 58,279 men and 62,573 women, has shown that black tea consumption had no relationship to cancer. Three types of cancer were studied, that is colorectal, lung and breast cancers. Black tea consumption among the subjects ranged from no cups (13%), 1-2 cups (37%), 3 - 4 cups (34%) and 5 or more cups (16%) per day (Goldbohm *et al*, 1996).

In the literature the mechanisms suggested for the anticarcinogenic action of tea components include the direct action of tea components with the chemical carcinogen. Thereby preventing the formation of the active form of the carcinogen. The quenching of reactive oxygen species and other free radicals produced by the active carcinogens play an important role in cancer initiation by damaging the DNA strands of the target cells. The

antioxidative activity of tea components are discussed in section 1.4.

1.2 Effect of tea on cardiovascular diseases

Epidemiological studies to find out the relationship between tea consumption and heart diseases also have given mixed results and are inconclusive.

A study carried out in Netherlands, using 805 elderly men, has shown that daily flavonoid intake has an inverse relationship to the incidence of myocardial infarction. Black tea (61%), onions (13%) and apples (10%) were identified as the major sources of flavonoid intake. The incidence of myocardial infarction for three levels of flavonoid intake, 0 - 19.0mg/day (low), 19.1 - 29.9mg/day (middle) and more than 29.9mg/day (high) were 16.2 (per 1000 persons), 13.8, 7.6 respectively. For black tea consumption, 0 - 2 cups (low), 2 - 4 cups (middle) and more than 4 cups (high) was 17.1%, 8.1% and 9.5% respectively. The authors concluded that flavonoids, in regularly consumed foods, may reduce the risk of death from coronary heart disease. However the power of the study was limited due to the small number of incidence cases in the subjects studied (Hertog *et al*, 1993a).

A cross sectional study was performed in Japan to find out the relationship between green tea consumption and serum cholesterol and triglyceride concentrations. 1371 men in Yoshimi, Japan were used for the study. Increased green tea consumption was associated with decreased concentrations of total serum cholesterol and triglyceride, and an increase in high density lipoprotein cholesterol, with a decreased proportion of low density lipoprotein cholesterol. Serum measurements for three levels (0 - 3 cups, 4 - 9 cups, more than 9 cups) of

tea consumption are, total cholesterol (mmol/l) 4.85, 4.76, 4.58, triglyceride (nmol/l) 1.65, 1.60, 1.45, High density lipoprotein cholesterol (as % of total) 36.4, 36.5, 37.4, Low density lipoprotein cholesterol(as % of total) 62.5, 62.6, 61.7 respectively. Because of these alterations in serum markers the authors of the paper suggest that green tea may act protectively against cardiovascular disease. Though these alterations are statistically significant it could be argued that with the small change of cholesterol level (4.85mmol/l to 4.58mmol/l) any clinical significance is unclear (Imai and Nakachi, 1995).

In a similar study carried out in Northern Kyushu, Japan, Kono and others found that serum cholesterol levels were inversely related to the green tea consumption. However no association was found with triglyceride or high density lipoprotein cholesterol levels. Total serum cholesterol(mg/dl) for four levels of green tea consumption (0 - 2 cups per day, 3 - 5 cups, 6 - 8 cups, more than 9 cups) were 193, 190, 187, 185 respectively (Kono *et al*, 1992).

Another study, performed in Norway, also showed that total serum cholesterol and systolic blood pressure were inversely related to black tea consumption. 9856 men and 10,233 women were used for the study. Serum total cholesterol (mmol/l) in men for four levels of tea consumption (0 - 1 cups/day, 1 - 2 cups, 3 - 4 cups, more than 5 cups) was 6.24, 6.20, 5.96, 6.19 respectively and systolic blood pressure (mm) was 136.2, 135.0, 135.9, 133.1 respectively. For women total serum cholesterol was 6.11, 5.96, 5.89, 5.92 respectively and systolic blood pressure was 131.7, 130.2, 127.9, 127.2 respectively (Stensvold *et al*, 1992).

However a similar study carried out in Israel found no significant association between black tea consumption and serum cholesterol levels. 3858 men and 1511 women were used in

the study. However results of this study may have been affected by the fact that a relatively small percentage of subjects were in the high levels of tea consumption. Of the five levels of tea consumption used (0 cups/day, 1 - 2 cups, 3 - 4 cups, 5 cups, more than 5 cups) 23.4%, 61.1%, 11.9%, 1.5%, 1.9% men were in each level respectively and 27.4%, 58.9%, 11.3%, 1.3%, 0.9% women were in each level (Green and Harari, 1992).

Results from the Scottish Heart Health Study found no relationship between black tea consumption and diagnosis of coronary heart disease or serum cholesterol concentration. A total of 10,359 people in twenty two Scottish districts were used for this study. Subjects in the study had a mean consumption of 4 cups per day (Brown *et al*, 1993).

It is worth drawing attention to a study done in Austria on coffee and tea consumption and lifestyle. A total of 2400 people were in this study. Lifestyle factors were related to four realms of behaviour, that is smoking, eating, drinking and physical activity. It was found that drinking coffee was positively associated with factors that promote coronary heart disease (such as increased smoking, fat consumption) while tea drinking was associated with a preventive lifestyle (such as increase of fresh fruit intake, physical activity). Therefore investigations on health effects of coffee and tea consumption must be carefully controlled for confounding behavioral factors (Schwarz *et al*, 1994). In the above mentioned studies those parameters had been taken into account and results are adjusted for these confounding parameters.

Several authors have reported the cholesterol lowering effects of tea components in animal studies. A study done with Wistar rats showed that addition of 10g and 20g of crude

tea catechins (isolated from green tea extract) to 1kg of diet (1% and 2%) had significantly decreased the total cholesterol concentration in rats consuming a cholesterol rich diet. Addition of 1% cholesterol to the diet had significantly increased the cholesterol concentration when compared with the group not receiving cholesterol in their diet. Addition of 1% tea catechins decreased the cholesterol concentration to a level slightly above the baseline level, while 2% addition of tea catechins brought down the cholesterol levels to the base line value (Muramatsu *et al*, 1986). Other authors also have reported similar observations (Yamaguchi *et al*, 1991, Ali *et al*, 1988)

This reduction of serum cholesterol levels may occur due to the decreased absorption of cholesterol from gastro-intestinal tract under the influence of tea polyphenols. This has been demonstrated using animals with cannulated thoracic duct. Both cholesterol and triglyceride absorption were decreased in the presence of tea catechins. The reduction of absorption may be due to the decreased micellar solubility of cholesterol. In the same experiment it was observed that tea polyphenols precipitated cholesterol solubilised in mixed bile salt micelles (Ikeda *et al*, 1992).

In addition to the alterations in serum lipids Mitane and others have studied the inhibitory effects of tea catechins on platelet aggregation which plays a central role in thrombus formation. In an *in vitro* study using rabbit platelets it was found that 0.025mg/ml, 0.05, 0.1 and 0.2 epigallocatechin (EGCG) isolated from green tea had inhibited the platelet aggregation in a dose dependant manner. At 0.2mg/ml level aggregation was completely inhibited (Mitane *et al*, 1990). However the *in vivo* effects of tea components on platelet aggregation are yet to be studied.

Free radicals and reactive oxygen species are believed to be responsible for the aetiology of cardiovascular diseases and carcinogenesis. Hence antioxidants may be important in preventing these diseases. Therefore attention has been drawn to the antioxidant activity of tea components. In addition free radicals and reactive oxygen species are released during the immune response. The likelihood of damage to the host from these molecular species would be reduced by an increase in some endogenous components of antioxidant defence. The intake of antioxidant substances from the diet will also exert an influence on the strength of the antioxidant defences. The antioxidant defences and the antioxidant activity of tea components are discussed below.

1.3 Antioxidant defence system of the body

An antioxidant could be defined as any substance which can delay or prevent the oxidation of a substrate when it is present in small amounts relative to the amount of the substrate (Diplock, 1994). Antioxidants may act at several different levels of the oxidation sequence. Halliwell and Gutteridge considered that five mechanisms of action may be taking place in the process (Halliwell and Gutteridge, 1989). They are

i) Decreasing localised oxygen concentration.

ii) Preventing chain initiation by scavenging initiating radicals.

iii) Binding catalysts such as metal ions to prevent initiating radical generation.

iv) Decomposing peroxides so that they cannot be converted to initiating radicals.

v) Chain breaking, to prevent continued hydrogen abstraction by active radicals.

The body has an array of extra and intracellular antioxidant systems. The action of these systems are interrelated and they produce a concerted effort in protecting the host cells by the potential oxidising species. The first level of defence, which is largely enzymic, involves enzymes which control the formation of primary radicals derived from molecular oxygen such as super oxide radical, hydrogen peroxide and other hydroperoxides which could generate much more reactive hydroxyl and peroxyl radicals. Super oxide dismutase (SOD), catalase and glutathione enzyme system fall into this category (Diplock, 1994). The second group of antioxidants are involved in the control of the proliferation of chain reactions such as in lipid peroxidation. The vitamins C and E and probably carotenoids are involved in this (Palozza *et al*, 1992). Molecules such as caeruloplasmin and transferrin act as antioxidants through their ability to bind metal ions (Plonka and Metodeiwa, 1979). In addition, several other molecules such as uric acid and albumin are also involved in the control of oxidation process *in vivo*, which help the functions of the above system (Halliwell, 1988). Furthermore some molecules such as flavanoids, derived from diet, may also act as antioxidants *in vivo* by supplying hydrogen for abstraction, metal ion chelation and binding with activated molecules such as chemical carcinogens which could initiate chain reactions. The altered flavanoid molecules will subsequently be excreted from the body (Rice-Evans, 1995a).

The primary reduced species derived from molecular dioxygen by electron acceptance are the super oxide radical and hydrogen peroxide. Further proliferation of these molecular species will lead to the formation of other more reactive radicals and chain reactions. Therefore containment of these species is the first level of defense. Enzymic systems superoxide dismutase, catalase and glutathione peroxidase are the main antioxidant defense systems which act at this level.

1.3.1 Super Oxide Dismutase

The identification of superoxide dismutase(SOD), which has no other action other than to reduce super oxide radicals, drew the attention of scientists to the importance of free radicals and antioxidant defences in living systems. The universal distribution of SOD in animal tissues implies an important functional role of the enzyme. Liver kidney and adrenal gland contain relatively large amounts of the enzyme. Two different SODs are found in mammalian systems. Cu/Zn containing enzyme is found in the cytoplasm of most cells and a Mn containing enzyme is present within the mitochondria. Both catalyse the conversion of super oxide to hydrogen peroxide and molecular oxygen (Harris, 1992).

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

Both catalase and glutathione peroxidase system integrate with SOD by removing hydrogen peroxide. This is important as hydrogen peroxide could generate more reactive hydroxyl radicals and ions through Fenton reaction in the presence of free metal ions.

eg. $H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^{\cdot} + OH^-$

$H_2O_2 + Cu^{2+} \longrightarrow Cu^{3+} + OH^{\cdot} + OH^-$

1.3.2 Catalase

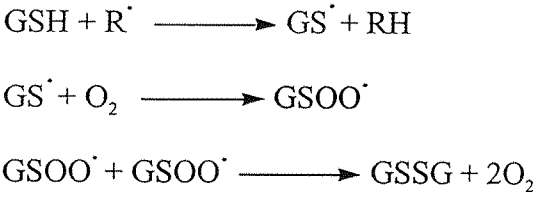
Catalase is a haemoprotein which is present in most aerobic cells. In most plant and

animal cells catalase is found in peroxisomes. In human tissues relatively high concentrations are found in liver and erythrocytes. It removes hydrogen peroxide from cells after SOD has converted superoxide to hydrogen peroxide.

$$H_2O_2 \longrightarrow 2H_2O + O_2$$

1.3.3 Glutathione enzyme system

Glutathione (GSH) plays an important role in defense against xenobiotic compounds, reactive oxygen species and free radicals (Meister and Anderson, 1983). GSH could react with radicals to give GSSG.



$$GSH + R^{\cdot} \longrightarrow GS^{\cdot} + RH$$

$$GS^{\cdot} + O_2 \longrightarrow GSOO^{\cdot}$$

$$GSOO^{\cdot} + GSOO^{\cdot} \longrightarrow GSSG + 2O_2$$

Glutathione can function as an antioxidant alone as shown above or as a part of the larger antioxidant enzyme system.

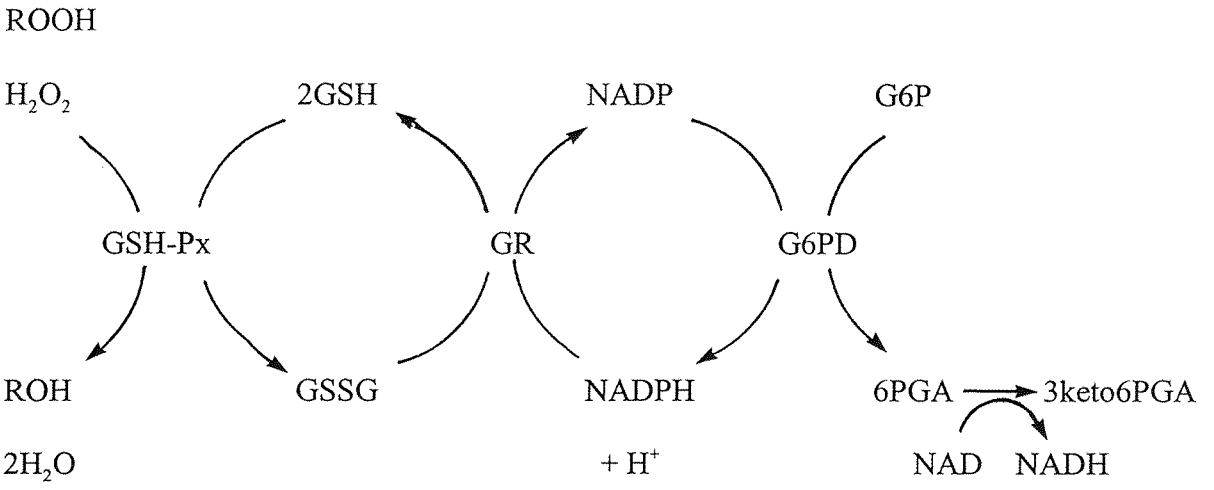


Figure 1.3 Glutathione redox cycle. GR - Glutathione reductase, GSH-Px - Glutathione peroxidase, G6P(D) - Glucose 6 Phosphate (Dehydrogenase), PGA - Phosphoglucuronic acid

Glutathione peroxidase (GSH-Px) catalyses the reduction of peroxides and hydrogen peroxide to ROH and water. There are two types of GSH-Px. Selenium dependant and selenium independent glutathione peroxidases. The dependant GSH-Px reduces hydrogen peroxide and organic hydroperoxides (ROOH) in a similar way to catalase. The selenium independent GSH-Px reduce only hydroperoxides (McCay *et al*, 1976). Oxidised GSSG is regenerated by the action of glutathione reductase (GR) where NADPH is involved.

The oxygen derived radicals can attack other molecules and form secondary radicals and initiate chain reactions. (eg. formation of peroxyl radicals from polyunsaturated fatty acids in membranes and LDL). Molecules such as vitamin E, C and carotenoids act against these radicals.

1.3.4 Vitamin E

Vitamin E consists of eight chemically similar tocopherols and each may exist in a number of different steric forms. The most active tocopherol in antioxidant activity is α-tocopherol. Tocopherols are lipid soluble and they are widely distributed in membranes and lipoproteins and play an important role in preventing damage to these structures by peroxidation of unsaturated fatty acids in theses structures (Diplock, 1985).

The αTOC· radical is fairly stable due to the delocalisation of its unpaired electron. However, it could undergo further oxidation. Only small amounts of these further oxidised products are detected in cells. It is now believed that α-tocopherol is regenerated by vitamin C and glutathione (Englard and Seifler, 1986). Other than peroxyl radicals tocopherol also can react with hydroxyl and superoxide radicals.

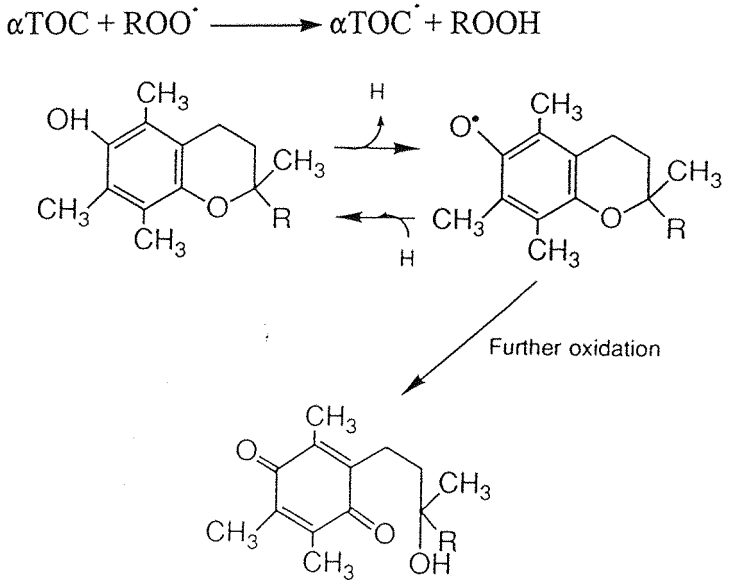


Figure 1.4 Radical trapping action of vitamin E. (-TOC - α-Tocopherol, α-TOC - α-Tocopheroxyl radical)

1.3.5 Vitamin C

Vitamin C (ascorbic acid) is an important water soluble antioxidant. In its antioxidant action donation of one electron to radicals results in the production of semihydroascorbate radical with electrons delocalised between three oxygen atoms. Further oxidation gives dehydroxyascorbate.

Dehydroxyascorbate could break down to further products. However there is an efficient mechanism by which it is reconverted to ascorbic acid which involves glutathione (Wefers *et al*, 1983).

Dehydroxy ascorbate + 2GSH ⟶ Ascorbate + GSSG

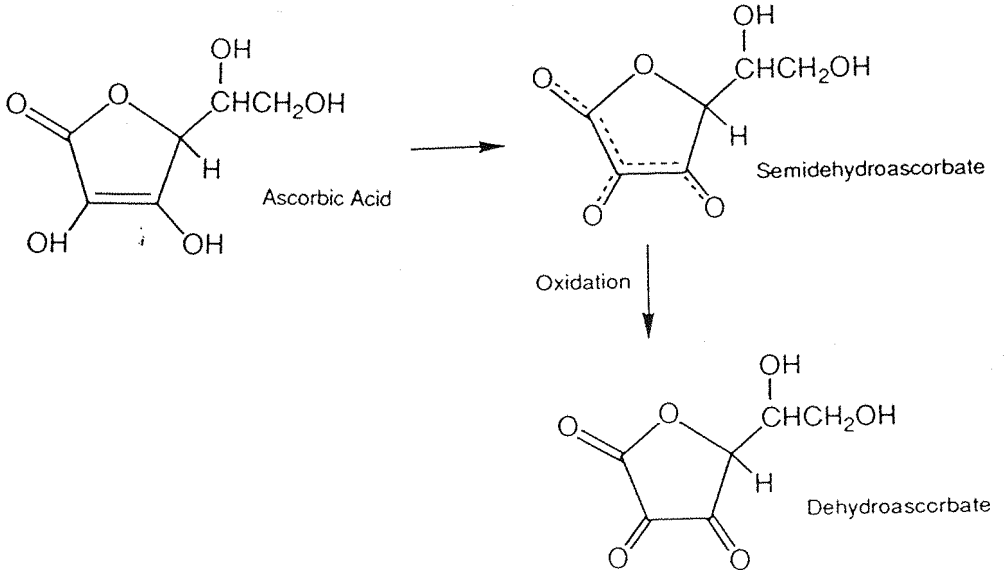


Figure 1.5 Radical trapping action of vitamin C

In addition to this function it is believed that vitamin C may be involved in regenerating vitamin E (Packer *et al*, 1979).

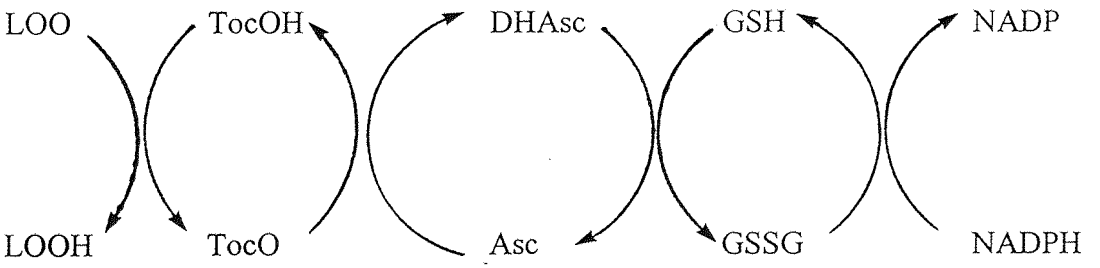


Figure 1.6 Regeneration of vitamin E

LOO - Lipid peroxyl radical, LOOH - Lipid hydroperoxide, TocOH - Tocopherol, TocO - Tocopheroxyl radical, DHAsc - Dehydroxyascorbate, Asc - Ascorbate, GSH - Reduced glutathione, GSSG - Oxidised glutathione.

1.3.6 Carotenoids

In plant chloroplasts carotenoids scavenge singlet oxygen generated by excited chlorophylls. This takes place by energy transfer from singlet oxygen to carotenoid which absorbs and dissipates this energy with out chemical change. Although the mechanism is not fully understood it is believed that carotenoids act as an antioxidant in animal tissues. It has become known that carotenoids are of significance in the prevention of certain forms of cancer (Krinsky, 1991) and in prevention of formation of atheromatous plaques (Gey *et al*, 1987). Palozza and others have found that a combination of ß-carotene and α-tocopherol was

much more effective than the effect of the sum of two compounds in preventing lipid peroxidation in membrane lipids. ß-Carotene may be acting synergistically with α-tocopherol as an effective radical trapping antioxidant (Palozza *et al*, 1992).

1.3.7 Caeruloplasmin

Caeruloplasmin is an α2 globulin protein containing 6-8 copper atoms. It is the major copper transport protein. Caeruloplasmin is a positive acute phase protein and concentrations in plasma are known to increase by the action of interleukin-6 during the acute phase response (Fleck, 1989). Caeruloplasmin also catalyses the conversion of Ferrous (Fe^{2+}) ion to Ferric (Fe^{3+}) ion. This reaction reduces the available ferrous ions for the fenton reactions. It also binds free copper ions which are involved in Fenton reactions where more harmful hydroxyl radicals are produced from hydrogen peroxide. It has been reported that caeruloplasmin is also able to dismutate the super oxide radicals (Plonka and Metodiewa, 1980).

1.3.8 Transferrin

Transferrin is a ß globulin protein containing two iron atoms. It is the major iron transport protein in serum. The antioxidant function of transferrin is through binding of free iron in an acute phase response. There is a concomitant decrease of the iron transport protein transferring and increase in iron storage protein ferritin during inflammation. These actions reduce the availability of ferric ions which is required in Fenton reactions which produce more reactive hydroxyl radicals from hydrogen peroxide (Bullen and Griffith, 1987).

In addition to the above mentioned mechanisms other molecules such as albumin and uric acid may also be acting as antioxidants *in vivo.*

1.3.9 Albumin

Albumin is a large protein of molecular weight 68,000. 50% of the serum total protein is accounted by albumin (Davis and Pacht, 1991). Albumin has been recognised as an antioxidant. The concentration of albumin decrease in acute phase response. Halliwell (1988) had suggested that this happens through the role that albumin play as an sacrificial antioxidant. Albumin binds copper and ferrous ions and as a consequence free radicals such as $OH^{\cdot}$ react with the albumin molecule leading to the catabolism of albumin molecule. This prevents these radicals attacking more important molecules such as DNA. As albumin is present in high concentrations in plasma this destruction and decrease in concentration has little consequence in normal situations.

1.3.10 Uric acid

Uric acid is found in high concentrations in plasma (400-600μM)(Ames *et al*, 1981). The double ring structure with an hydroxyl group provides the capability to scavenge free radicals and it had been reported to be a chain breaking antioxidant (Simic and Jovanovic, 1989).

In addition to the above mentioned natural antioxidants in the body, other non nutrient

substances in the diet also act as antioxidants *in vivo* (eg. flavanoids). They may enhance and improve the antioxidant defences of the body.

1.4 Antioxidative activity of tea

Flavanoids are the major component of (25-30% by dry weight basis) young tea shoots, which is used for processing of tea. These flavanoids are extracted into the solution when tea is brewed and they are the major component in the brew (see table 1.1 and 1.2 for the chemical composition of young tea shoots and tea brew). Therefore tea is an important dietary source of flavanoids and makes an important contribution to the daily intake of flavanoids (Hertog, 1993b, Ho, 1992)

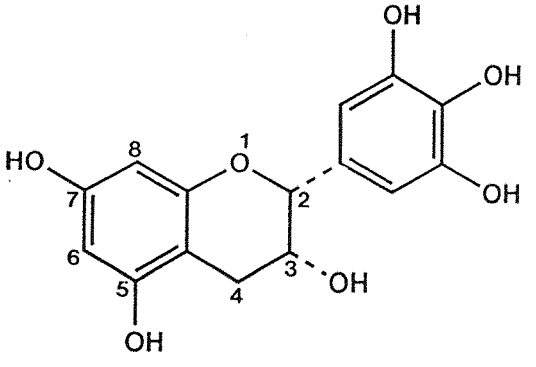


Figure 1.7 Common skeleton of flavanoids found in tea

Catechins and other flavonoids found in both green and black tea have a common carbon skeleton (Fig. 1.7). Theaflavins and thearubigins formed in the fermenting stage of black processing also retain the above structure. These compounds are able to act as antioxidants by virtue of the hydrogen donating capacity of their phenolic groups. In addition to this, in the structure of these molecules 5 and 7 hydroxyl groups and 1-Oxygen makes the carbons at position 6 and 8 strongly nucleophilic. This makes it possible for these molecules to form C-O or C-C bonds with oxidising species and undergo oxidative polymerisation. In addition to this the presence of two adjacent hydroxyl groups on the aromatic ring makes them strong metal ion chelators. They can bind with cellular free ferric, ferrous and other metal ions and thus decrease the free cellular metal ions which are required for the generation of reactive oxygen radicals (see section 1.3.1) in cellular reactions (Yang and Wang, 1993).

Many studies have been done to demonstrate the antioxidative action of both green and black tea and its components in *in vitro* studies.

In one such study radical scavenging ability of tea polyphenols on super oxide anions had been compared with that of vitamin C and E. Superoxide anions were produced by a irradiation of a system which contained riboflavin and 5,5-dimethyl-1-pyrroline-1-oxide. Formation of superoxide radicals were measured by electron spin resonance. Scavenging rate of tea polyphenols were compared with vitamin C and E. A 200μg/ml of green tea polyphenol extract, vitamin C and vitamin E were added separately to measure the scavenging ability on the formation of super oxide anions. The scavenging rates were 72%, 96% and 23% for tea polyphenols, vitamin C and vitamin E respectively (Zhao *et al*, 1989).

Yen and others have tested various tea extracts (green tea, oolong tea and black tea) for its antioxidative activity and scavenging effect on superoxide, hydroxyl and α,α-diphenyl-ß-picrylhydrazyl (DPPH) radicals in *in vitro* systems. Antioxidative action was measured by placing 500μg of tea samples with linoleic acid in buffer at 37°C and then measuring the oxidation by spectrometry after colour generation with ferric chloride and thiocyanate and comparing with the blank. Oolong tea had shown 73% inhibition of linoleic acid peroxidation and both green and black tea extracts had an inhibition of 40%. To measure the scavenging action on superoxide anion, it was generated by a nonenzymic system. Generation of superoxide was measured by means of spectrophotometric measurement of the product on reduction of nitro blue tetrazolium (NBT). 500μg of Oolong, green and black tea had 75%, 58% and 52% scavenging effects respectively. Hydroxyl radicals were produced by the reaction of hydrogen peroxide with ferrous ions. Hydroxyl radicals formed were trapped with 5,5-dimethyl-pyrroline-N-oxide (DMPO) and DMPO-OH adducts formed were measured by electron paramagnetic resonance spectrometry (EPR). 2mg of oolong, green and black tea reduced the EPR signal by 92%, 91% 77% indicating the scavenging activity on hydroxyl radical. The carcinogenic activity of polyaromatic hydrocarbons may be due to the in situ generation of their free radicals. Therefore the scavenging effect of tea extracts on DPPH radicals was also measured. 2mg of oolong, green and black tea had 54.6%, 59.4% and 49.0% scavenging activity respectively (Yen and Chen, 1995).

Scott and others also had investigated the action of catechin and epicatechin on hydroxyl, superoxide, trichloromethyl peroxyl radicals and hypochlorous acid. They also have found that catechin and epicatechin have scavenging action on above species (Scott *et al*, 1993).

Rice-Evans and others have measured the antioxidative activity of several plant derived polyphenols. Quercetin, myricetin, epicatechin and catechin were included in the study which are found in tea. The total antioxidant potential against aqueous phase radicals were measured and compared with water soluble analog of tocopherol, trolox. Results are given as trolox equivalent antioxidant capacity (TEAC)(see chapter 2 for method). The TEAC values for quercetin, myricetin, epicatechin and catechin (which are components in tea) were 4.7, 3.1, 2.5 and 2.2 respectively. Indicating that they had more antioxidant potential than trolox (Rice-Evans *et al*, 1995b).

Katiyar and co workers have investigated the inhibition of lipid peroxidation in mouse epidermal microsomes by epicatechin derivatives from green tea. Epidermal microsomal protein (2.0mg) was incubated for 1 hour at 37°C in the presence of ferrous ions and ADP. After one hour the reaction was terminated by addition of trichloroacetic acid and the production of malondialdehyde (MDA) was employed as a marker of lipid peroxidation. MDA levels were measured by the reaction with thiobarbituric acid followed by spectrophotometry. 50, 100, 200μmol of epicatechin(EC), epigallocatechin(EGC), epicatechingallate(ECG) and epigallocatechingallate(EGCG) were added separately to the reaction mixture to measure the inhibition against a control. The control had a value of 10.69 (nmol MDA equivalent). For EC (50, 100, 200μmol doses) 10.51, 10.30, 7.65, for EGC 9.55, 8.95, 6.81, for ECG 8.47, 7.11, 5.10 and for EGCG 6.79, 5.25, 3.15 nmol MDA equivalents were observed. This indicates the inhibition of lipid peroxidation under biological conditions by epicatechin derivatives found in tea (Katiyar *et al*, 1994).

In a similar experiment Hong and others have demonstrated that catechin derivatives

obtained from tea inhibit the lipid peroxidation of rat heart mitochondria (Hong *et al*, 1994).

Miura and others had carried out an experiment to study the effect of tea polyphenols on lipid peroxidation in low density lipoprotein(LDL). LDL fraction was separated from porcine serum by ultra centrifugation. LDL was incubated with 5μM copper sulphate for 2 hours at 37°C with flavanols(0.5μM) ECG, EGCG, EC, C, and EGC. Antioxidant dibutyl hydroxy toluene (BHT) was used as a standard compound. Peroxidation was estimated by measuring Thiobarbituric Acid Reactive Substances (TBARS). TBARS values were ECG (0.95), EGCG (1.03), EC (1.13), C (1.36), EGC (2.74) and for BHT (3.09). This indicates that inhibition of LDL lipid peroxidation by catechins found in tea are better than BHT (Miura *et al*, 1994) .

Most of the catechin derivatives found in the green tea are converted to theaflavins (TF) and thearubigins (TR) when green leaves are processed into black tea. Yosino and co workers have drawn the attention to the ability of TF and TR to inhibit lipid peroxidation. In this study also, a method similar to the above was used. Lipid peroxidation in a rat liver homogenate was initiated by adding t-butyl hydroperoxide and the peroxidation was measured by thiobarbituric acid reactive substances (TBARS). The inhibition potential of TF and TR was more than catechin and epicatechin. While ECG, EGC and EGCG had more potential than TF and TR. All tea derivatives were more effective than glutathione (GSH) and α-tocopherol (Yoshino *et al*, 1994).

In a similar experiment it has been shown that theaflavins (TF) isolated from black tea inhibited the lipid peroxidation in rabbit erythrocyte membrane, initiated by t-butyl

hydroperoxide. It was also shown that lipid peroxidation in rat liver microsomes is inhibited by TF. Further to that they have investigated the effect of TF on DNA single strand cleavage by hydrogen peroxide. PhageϕX174 RFI DNA was used for the experiment. RFI DNA is a super coiled double-strand circular DNA. Single strand cleavage was detected by observing the change of RFI form to RFII form, a relaxed circular form. RFI DNA (75ng/10μl) was treated with 300μM hydrogen peroxide, 19.5μM cytochrome c (Fe^{2+}), 20μM diethylenetriaminepentaacetate to induce the cleavage. The mixture was incubated at 37°C for 1 hour and electrophoresed on agarose gel to separate and quantify RFI and RFII forms. Addition of 10μM TF reduced the strand cleavage by 60%. α-tocopherol had a 30% reduction (Shiraki *et al*, 1994).

The effect of catechins, isolated from green tea, on serum α-tocopherol levels and lipid peroxidation have been investigated in Wistar rats. They were divided into four groups and two groups each received 30% palm oil and 30% perilla oil. {Palm oil is rich in saturated and monounsaturated fatty acids 16:0(46%), 18:1(37%), 18:2(10%) and Perilla oil (obtained from the seeds of *Perilla frutescens* found in eastern Asia which is used as an edible oil) is rich in unsaturated fatty acids 18:3(53%), 18:2(16%), 18:1(18%), 16:0(7%)}. One of the palm oil and perilla oil groups received 1% crude catechin mixture isolated from green tea. α-tocopherol was added to each diet to give a final concentration of 6mg/100g in each diet. All other ingredients in the diet were similar in all four groups. After feeding on these diets for a month animals were sacrificed and plasma samples were obtained. Plasma α-tocopherol and thiobabituric acid reactive substances (TBARS, to measure the level of lipid peroxidation) were measured. α-tocopherol concentrations (μg/dl) in each groups were palm oil (433), palm oil+catechin (493), perilla oil (55), and perilla oil+catechin (134). α-tocopherol in perilla oil groups were reduced by acting as antioxidants. Addition of catechins to both palm oil and

perilla oil diets had increased the plasma α-tocopherol levels suggesting that tea catechins may be counteracting the decrease in α-tocopherol by acting as an antioxidant *in vivo*. Plasma TBARS (nmol/ml) for different groups were palm oil (1.18), palm oil+catechin (1.16), perilla oil (3.86), and perilla oil+catechin (3.36). Reduction of TBARS in the catechin groups suggest that tea catechins may be acting to inhibit lipid peroxidation *in vivo* (Nanjo *et al*, 1993).

In another study the effect of green tea and black tea powder in the diet, on lipid peroxidation in rat liver was investigated. The rats were divided into 3 groups and one group was kept as the control while other two groups received 3%(w/w) green tea and black tea powder in their diet. They were kept on these diets for 50 days and then liver slices were incubated at 37°C for two hours. Lipid peroxidation was induced by t-butyl hydroperoxide. Lipid peroxidation was estimated by measuring TBARS. Green tea and black tea in the diet had reduced the lipid peroxidation by 23% and 38% respectively. However in this experiment powdered green tea and black tea were used in the diet. Some of the components found in green tea and black tea does not come into the infusion when they are brewed for drinking. Therefore results of this experiment may not reflect the effects of green tea or black tea drinking (Sano *et al*, 1995).

Results mentioned in the above studies provide evidence for the antioxidative action of tea components under *in vitro* conditions and under biological conditions and in limited experiments under *in vivo* conditions. This antioxidative action of tea may help to prevent carcinogenesis and cardiovascular diseases.

1.5 Adverse effects associated with tea consumption

Processed tea contains 3-5% caffeine on a dry weight basis. Caffeine had been implicated in many adverse effects in humans. A cup of tea contains 60-75mg of caffeine. In adults more than 90% of ingested caffeine is quickly absorbed by the gastrointestinal tract and distributed to tissues and organs. Peak plasma levels are reached within 15-45 minutes after ingestion. The plasma half life varies among individuals and ranges from 2.5 to 7.5 hours. Caffeine has an stimulant effect on the central nervous system. It increases psychomotor coordination resulting in decreased motor time and increased vigilance (Curatolo, and Robertson, 1983).

Leonard and others have reviewed the effect of caffeine on various body systems. In addition to the above mentioned stimulation of the central nervous system caffeine has been found to increase the secretion of gastric acid and pepsin. Caffeine also have a diuretic action on the kidneys, increasing urinary volume and sodium excretion. It is known to have broncodilator effect (Leonard *et al*, 1987). Caffeine is also known to increases the blood pressure and thus increase the risk of stroke (Pincomb, *et al*, 1985). Caffeine ingestion is also believed to have an atherogenic effect. A Japanese study on rats had shown that caffeine added to a diet induced hypercholesterolemia. However polyphenols in tea acts in the other direction and overall effect of tea ingestion was observed to be hypocholesterolemic (Yokogoshi, *et al*, 1983).

Caffeine ingested by drinking normal amounts of tea (4-5 cups a day) is around 300 -

375mg per day. This is well below the level of 600mg advocated by many for those with ischaemic heart disease, hypercholesterolaemia and in pregnancy (Ashton, 1987). However one has to consider the intake from other sources with tea as coffee, cola beverages chocolate, over the counter stimulants, analgesics and cold preparations contain considerable amounts of caffeine (Leonard *et al*, 1987).

There is evidence found in literature that tea consumption has an effect on iron absorption from the gastrointestinal tract. It significantly reduce the non haem iron absorption when taken with a meal. This reduction in absorption may be due to the chelation of iron with the polyphenols in tea. It has been observed that iron absorbed from bread was reduced by 35% in a study carried out with nine adult Chilean woman (Pena, *et al*, 1991). A study done with 15 women in UK had shown that serum ferritin levels had a negative relationship with meal time tea consumption (Razagui *et al*, 1991). This reduction of iron absorption may have a significant effect on people with limited supplies in their diet and when majority of iron intake is from non haem form. In a study done in Tunisia it was observed that tea reduced iron absorption by 36-61% from a typical Tunisian 'Couscous' meal. Meal time tea drinking had been implicated in iron deficiency anaemia found through out Tunisia (Hamdaoui, *et al*, 1994).

The tea plant and specially its leaves are known to accumulate aluminium. Therefore there was a school of thought that tea consumption contributes to an excessive intake of aluminium. However it has been found that aluminium accumulation is several fold more in mature tea leaves than in tender parts of the plant which is used to process for drinking. Anathakumaraswamy and Sivapalan had reported that tea brews prepared from various Sri

Lankan, Indian and Chinese teas (prepared by adding 125ml of boiling deionised water to 2.5g of tea) had a range of aluminium from 1.6 to 7.1mg/l. The average value was 4.2mg/l (Anathakumarswamy and Sivapalan, 1990). The concentration of aluminium in Japanese tea infusions ranged from 1.49-5.58 mg/l (Matsushima *et al*, 1993). A study carried out in U.K. with 13 brands of commercially available teas have shown that aluminium content ranged from 2.2 - 4.5mg/ml. The brews for the study was prepared by adding 150ml of boiling deionised water to 3.0g of tea and allowing to stand for 4 minutes (Baxter *et al*, 1990). A study done with twelve healthy volunteers has revealed that consumption of up to 1.6mg of aluminium from tea does not influence serum aluminium levels when compared with consumption of 0.001mg aluminium from water. This suggests that tea drinking does not significantly contribute to increase total body aluminium. (Drewitt *et al*, 1993). Studies on bioavailability of Al from tea done by Powell and others also have shown that only 5% of Al from tea is available for absorption from small intestine (Powell *et al*, 1993). This supports the findings of the above mentioned study.

Though evidence is found in literature that intake of fluoride from tea improves oral health (Rosen *et al*, 1984, Onisi *et al*, 1980, Mann *et al*, 1985), under some conditions it may lead to fluorosis. Fluorosis is caused by intake of over doses of fluoride for a long time. Initial symptoms are dental fluorosis and may spread to the bone joints, muscle and nervous system. Fluorosis had been observed in Tibetan region and in some regions of China where fluoride levels in water are high. Drinking large amounts of `brick type' tea in these areas had contributed to increase the fluoride intake. Unlike in normal tea processing, in brick tea processing, mature parts of the shoots are also used. In `brick tea' processing macerated wet leaves are moulded into brick shapes and dried and hence the name. As mature parts of tea

shoot contain more fluoride than in tender parts, brick tea drinking may positively contribute towards fluorosis (Wang and Huang, 1995, Han, *et al*, 1995).

High incidence of oesophageal cancer was observed in a geographical belt extending from Himalayan region to Northern China. Consumption of high amounts of tea was implicated as a contributing factor for this high incidence of oesophageal cancer(Dhar, *et al*, 1993). However drinking boiling hot salted tea is a common practice in this region. This drinking of tea at high temperature may be an more important contributory factor than components in tea. Kumar and others have suggested that this, which leads to the irritation of oesophagus may be an important contributory factor in the etiology of oesophageal cancer in this region (Kumar, *et al*, 1992). Recent studies also have revealed that another contributory factor for the high incidence of oesophageal cancer may be the high dietary intake of amines and nitrates in this region which results in nitrosation reactions(Siddiqi, *et al*, 1992).

1.6 Infection and the response of the immune system

Invasion of the body by infectious agents such as bacteria, virus and fungi in the environment can cause severe damage to the host. The immune system of the body acts to destroy these invading pathogens and to continue the normal metabolic processes of the body. The exterior of the body presents an effective barrier to most agents; in particular most infectious agents cannot penetrate intact skin. Most exogenous body secretions have lysozymes in them which act against the invading pathogens. e.g. mucus, tears, sebaceous gland secretions (Male and Roitt, 1993).

When an organism penetrates this passive component it will trigger the immune system of the host and an immune response will be initiated. The responses may be directly due to the invading pathogen or as a result of the toxins released by the pathogen. The immune response could be divided into two components. Non specific or the inflammatory response and the specific immune response. The inflammatory response is mediated via the phagocytic cells which respond in a general way to any foreign body by releasing cytokines and free radicals and initiating an acute phase response. Activation of the complement system is also termed as part of this response. Specific immunity can be divided into humoral and cell mediated immunity. Humoral immunity revolves around antibodies produced by B lymphocytes and cellular immunity involves a wide range of different cell interactions and is mediated by T lymphocytes. Although grouped separately, considerable interactions take place between all aspects of the immune system (Raska and Ponzio, 1994).

For the purpose of this thesis, only the details of the inflammatory response will be discussed. In addition, the response in lung receive particular attention as lung tissue is quickly subjected to inflammation and is easily accessible for study.

1.7 Inflammation

Inflammation occurs when body is invaded by an infectious agent, exposed to inflammatory agents or due to tissue damage. Production of oxidant molecules occurs during the process and represents a challenge to the antioxidant defences of the body. Three major changes occur during the inflammatory response. The blood supply is increased to the infected area. Capillary permeability is increased thus allowing the soluble mediators of the

inflammatory response to reach the site of infection. Leucocytes and macrophages will migrate out of the capillaries to the site by chemotaxis. These events are collectively known as inflammation (Male and Roitt, 1993).

1.8 Acute phase response

Dramatic changes in metabolic parameters which occur during early infection are called as the `acute phase response'. This response can result not only from infection but also from tissue injury, neoplastic growth, persistent diseases such as Crohns disease and rheumatoid arthritis and immunological disorders. (Dinarello, 1984a)

This response is non specific and will occur with any causative agent mentioned above regardless of the localised or systemic nature of the disease. Changes in metabolic, endocrinologic, neurologic and immunologic functions occur during acute phase response. Most changes are observed after a few hours of the infection or injury. Some changes occur after a few days (Andus *et al*, 1991).

The development and effects of acute phase response are shown on figure 1.8.

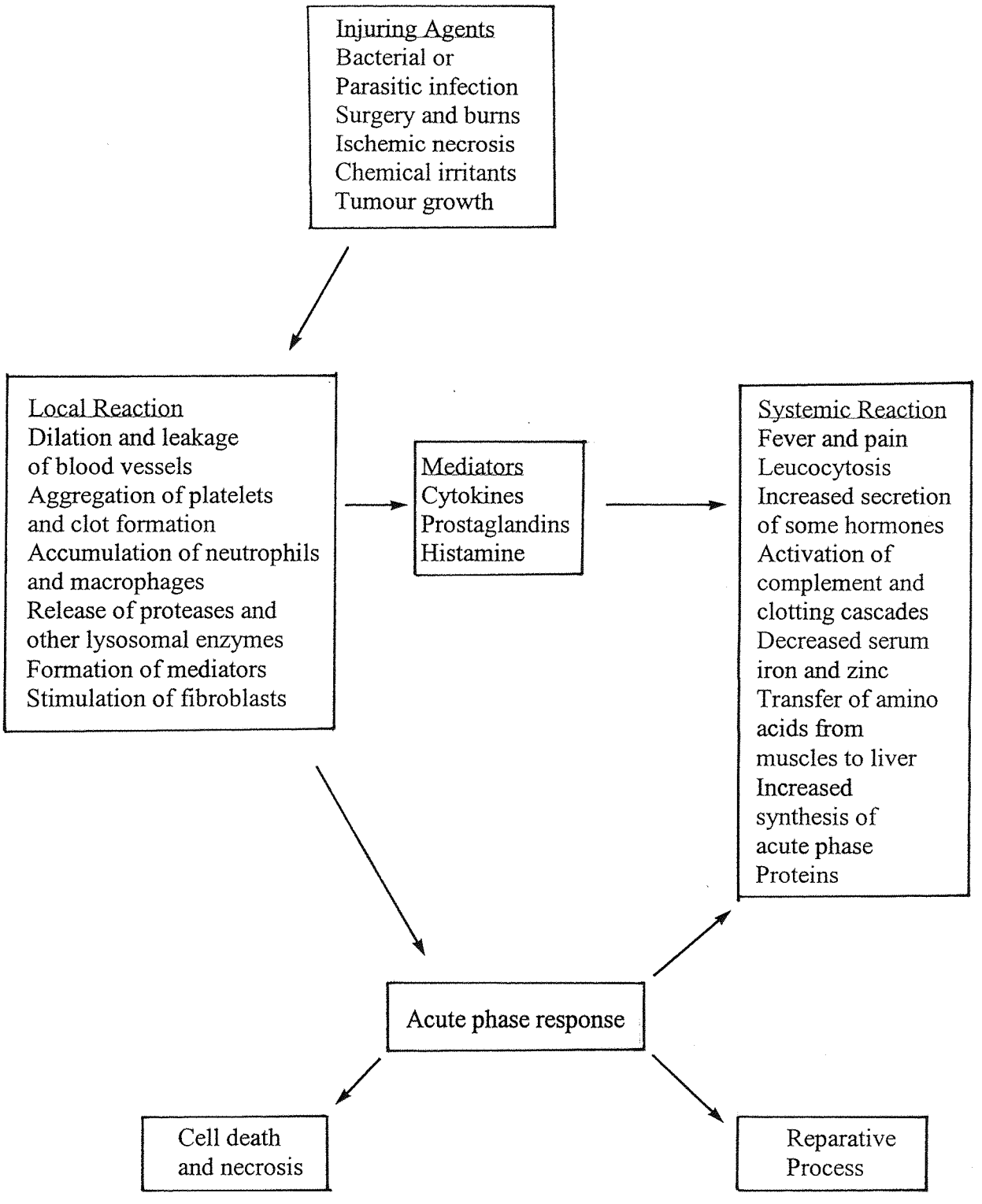


Figure 1.8 Development and effects of the acute phase response (Sigal, 1994)

One aspect of acute phase response is the change in concentrations of a number of plasma proteins. These proteins are called as acute phase proteins. Two types of acute phase proteins have been identified. Positive acute phase proteins are those that increase in concentration after injury and negative acute phase proteins are the ones which decrease in concentration after injury. Positive acute phase proteins include caeruloplasmin, α-Acid glycoprotein (Orosomucoid), C-reactive protein and haptoglobin. Compliment components B, C3 and C4 (ß-globulins) are also known to increase during the acute phase reaction. Negative acute phase proteins include albumin and transferrin. Cytokines have been found to bring about changes in the synthesis of both the positive and negative acute phase proteins (Kushner and Mackiewicz, 1986).

Caeruloplasmin (an α-2-globulin) is an important component of antioxidant defences as described in section 1.3.7. Thus possibly preventing tissue damage due to the increased production of free radicals during the inflammatory response by macrophages and other immune cells. The ability of caeruloplasmin to change ferrous ions to ferric ions is important in containing pro-inflammatory effects of iron metabolism during inflammation. Ferrous ion is released from haemoglobin and ferric ion is the form bound by transferrin, the iron transport protein. Thus caeruloplasmin may play a role in the removal of iron released by haemoglobin from the red blood cells at the site of an inflammatory process (Frieden and Hsieh, 1976).

α-Acid glycoprotein (Orosomucoid) is also known to increase up to four fold during inflammation. The polysaccharide chains of the molecule may account for some of its physiologic functions. eg. Its ability to interact with a variety of cellular membranes. It is

known to block the interaction of malaria parasites (Plasmodium species) with red blood cells. It also inhibit the platelet aggregation and lymphocyte response (Sigal, 1994).

C-reactive protein (CRP) is also a positive acute phase reactant. The name comes from its ability to bind and precipitate the C polysaccharide of *S. pneumoniae*. CRP can bind to a large number of microorganisms. The result of binding of CRP to bacteria is capsular swelling, precipitation and agglutination of the microorganism. Binding of CRP increases the opsonisation and clearance of the bound particle. CRP also binds to necrotic tissue and help speed phagocytic removal (Pepys and Balz, 1982).

Negative acute phase proteins include albumin and transferrin. Albumin binds many drugs, bilirubin, amino acids and some lipids. It also transports serum zinc. Though it accounts for about 50% of the serum proteins albumin serves no defined unique function. As described earlier it has been suggested that it acts as an antioxidant (Halliwell, 1988). Transferrin is the major iron transport protein. Iron is a requirement for growth of mammalian cells and for bacteria. The concomitant decrease in circulating transferrin and increase in intrahepatic iron storing ferritin decreases the availability of iron at the site of infection (Fleck, 1989). Iron is involved in the formation of hydroxyl radicals from hydrogen peroxide in Fenton reactions (see section 1.3.1). Although the hydroxyl radical is important in destroying the invading pathogens, the presence of excessive amounts for a long time also could damage the host cells. Therefore reduction of transferrin during acute phase reaction may help prevent host damage.

1.9 Cytokines

Cytokines are a group of polypeptides produced by the cells of the immune system under stimulation by invasion of pathogens or tissue damage. Phagocytes, T lymphocytes, fibroblasts and various endothelial and bone marrow cells are capable of producing cytokines under stimulation (Beutler & Cerami, 1986). These include interleukins, tumour necrosis factor and interferons.

Phagocytes and T lymphocytes produce the majority of cytokines. They act as the messengers during the inflammatory response. Production of these will result in stimulation and enhancement of the immune system and in metabolic effects on other tissues. (Fig 1.9)

Interleukin-6 (IL-6) is an important mediator of the acute phase response along with interleukin-1 (IL-1) and tumour necrosis factor (TNF). IL-6 plays an important role in stimulating the hepatocytes. It has now been found to be identical to hepatocyte-stimulating factor (HSF) discovered in 1983, which was produced by activated macrophages (Gauldie *et al*, 1987). When recombinant human IL-6 is injected into rats there is an increase in blood concentrations of all the acute phase proteins and this increase was rapid and dose dependent. At the same time IL-6 caused dose dependent decrease in the amount of albumin synthesised by the liver (Geiger *et al*, 1988). IL-1 and TNF also have been found to stimulate the hepatic acute phase protein production. However IL-1 is known to induce the production of IL-6 in several cell types and TNF also can stimulate the production of IL-1 (Ramadori *et al*, 1988). Therefore the effect of both IL-1 and TNF may be mediated via the production of IL-6. As IL-6 has a more pronounced and direct effect on hepatocyte stimulation and acute phase

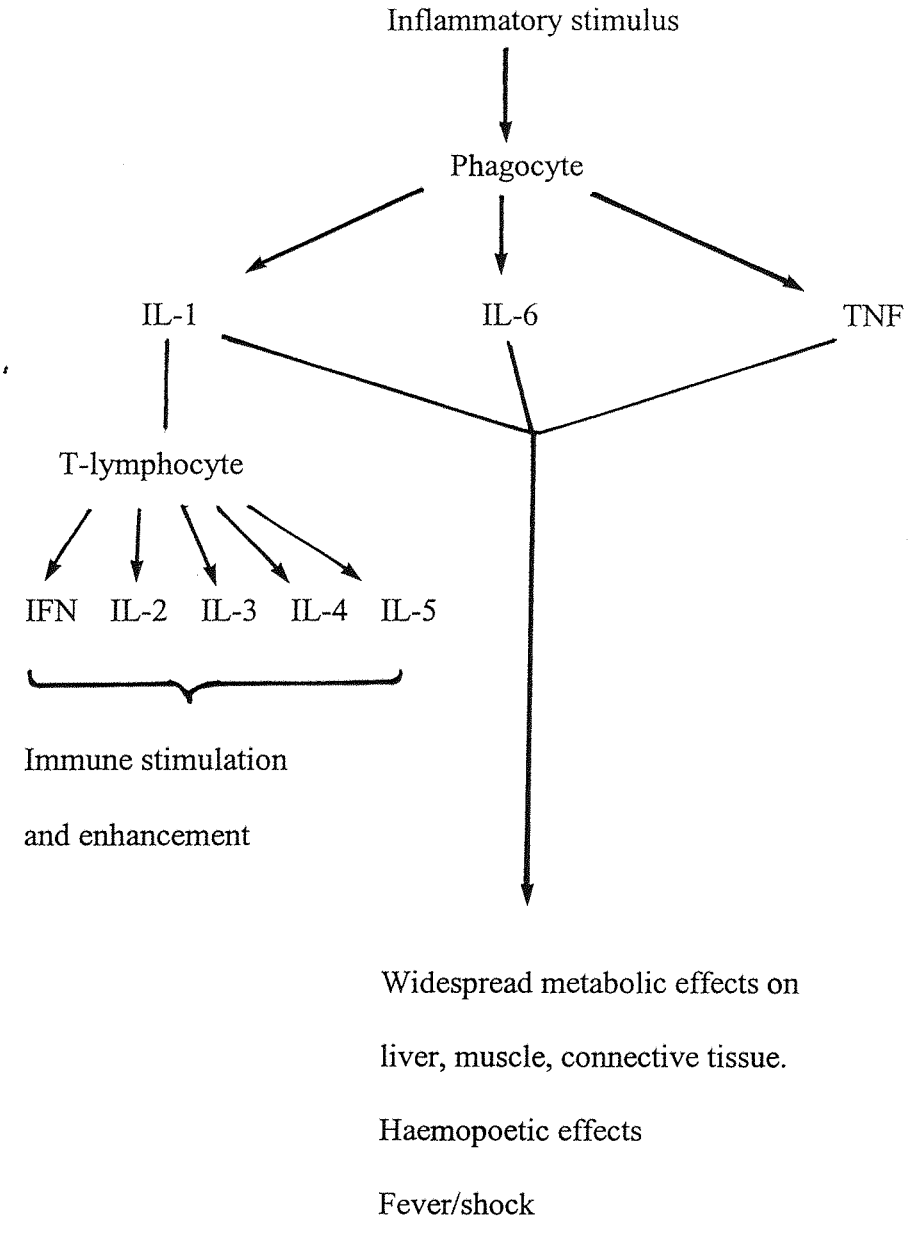


Fig 1.9: Metabolic effects of cytokines. IL 1 - 6: Interleukin 1 - 6, τIFN: τinterferon, TNF: tumour necrosis factor, T lym: T lymphocyte. (adapted from Grimble -1989)

protein production; for the purpose of this report only the actions of IL-6 will be discussed in detail.

Interleukin 6 (IL6)

IL6 is produced by T and B lymphocytes, tumour cells, fibroblasts, monocytes and other cell types such as endothelial cells and keratinocytes (Tosato et al, 1986).

IL6 is an important mediator of the acute phase response (Gauldie et al, 1987). It has been shown to stimulate increased synthesis of acute phase proteins including ß-fibrinogen, caeruloplasmin, α-acid glycoprotein and haptoglobin by stimulating the hepatocytes. When recombinant human IL-6 is injected into rats there is an increase in the blood concentrations of all the positive acute phase proteins. The onset of the increase is rapid and dose dependant. At the same time IL-6 causes a dose dependant decrease in the amount of albumin synthesised by liver. IL-6 induce the synthesis of all the major acute phase proteins and this can be inhibited by a monoclonal antibody to IL-6 (Geiger *et al*, 1988).

It also influences the activation, growth and differentiation of both T & B cells. It has been shown that it acts on B cells enhancing significantly the production of immunoglobulin G and A (Kishimoto and Hirano, 1988a). IL6 acts synergistically with IL1 in T cell proliferation. Thus it may also be important in the development of cell mediated immunity (Mizel, 1989). IL-6 also support the growth of granulocyte and monocyte precursors from mouse bone marrow (Ikebuchi *et al*, 1987).

The altered regulation of IL-6 production has been seen in several pathological situations. However it is difficult to determine whether this a cause rather than the effect of the disease. Elevated IL-6 levels have been found in infections with retroviruses such as

human immuno deficiency virus (HIV) (Makajima *et al*, 1989), human lympotropic virus-I (HTLV-I) ((Yoshida and Seike, 1987), some autoimmune diseases such as rheumatoid arthritis (Kishimoto and Hirano, 1988b), and in patients with certain types of benign or malignant tumours where production of IL-6 by the tumour is associated with autoimmune manifestations (Hirano *et al*, 1987).

1.10 Inflammation of the lung

The lung comprise of a unique interface between the body and the environment. It has a large alveolar surface area and only a minimal barrier between the alveolar space and the extensive vascular network. While this configuration is ideal for gas exchange, it also increases the vulnerability to noxious stimuli and pathogens. Therefore pulmonary tissue has the ability to generate brisk inflammatory responses against both inhaled and haematogenous challenges. This provides the means to rapidly clear the offending agents and thus to avoid compromise of essential gas exchange function (Strieter and Kunkel, 1994).

The rapid inflammatory response by the lung tissues is initiated by the rapid infiltration of the tissue by neutrophils. The recruitment of neutrophils to the lung tissue is dependent on a series of events which include the following steps. Initially the endothelial cells are activated which leads to the expression of the endothelial cell adhesion factors and result in the adhesion of neutrophils to the endothelial cells. The adhesion of neutrophils is mediated by a group of molecules called selectins. Three members of the selectin family are important in neutrophil-endothelial cell adhesion. On the neutrophil, the major selectin expressed is L-selectin. This molecule is actively expressed on mature neutrophils. The E-

selectins are expressed on the endothelial cell surfaces. The expression of E-selectins must be induced for the adhesion of neutrophils. E-selectins are known to be activated by endotoxin, tumour necrosis factor (TNF) and interleukin-1 (IL-1) (Springer, 1990). P-selectins from activated platelets also help in neutrophil-endothelial cell adhesion.

After adhesion to the endothelial cells, neutrophils pass through gaps between adjacent endothelial cells by diapedesis. Neutrophils then migrate through the lung tissue up the chemoattractant gradient by chemotaxis. Interleukin-8 (IL-8) and neutrophil chemotactic factor (NCF) secreted by alveolar macrophages help the neutrophils to move through the lung tissue by chemotaxis (Strieter *et al*, 1993). On reaching the target neutrophils destroy it by phagocytosis.

1.11 Free radicals and reactive oxygen species and their effect on cell components.

Cells of the immune system such as macrophages produce large amounts of free radicals and reactive oxygen species (ROS) under stimulation as part of the inflammatory response. This is an essential part in action against the invading pathogens. They act to destroy the pathogens in phagocytosis at the site of infection (Babior, 1978). Free radicals and reactive oxygen species are also produced in the normal aerobic metabolism (Rice-Evans, 1994). In addition radiation exposure, smoke and pollutants in the air also stimulate the production of free radicals. The cytochrome p450 system produces large amounts of oxygen radicals in xenobiotic metabolism (Archakov and Bachmanova, 1989). Ischaemia and reperfusion likewise produces reactive oxygen radicals in tissues (McCord, 1985). Though the production of free radicals is an essential part of energy production in aerobic metabolism

and in immune reactions their over production and prolonged presence leads to pathological conditions. Lipid peroxidation in low density lipoprotein(LDL) by free radicals is known to play an important role in the initiation and formation of atheromatous plaques in atherogenesis (Steinberg *et al*, 1989). DNA strand breakage by free radicals are known to initiate carcinogenesis process (Loeb *et al*, 1988).

A free radical is a molecule that contains one or more unpaired electrons. The presence of these unpaired electrons make these molecules very reactive. They are capable of accepting electrons and hydrogen ions from other molecules that they come into contact with. Removal of hydrogen ions can cause damage to a molecule. This could be beneficial to the host as it leads to the destruction of invading pathogens. However if the production of free radicals is not checked these will attack the host cells and will bring damage to the host it self (Watanabe et al, 1988, Halliwell et al ,1989, Barnes,1990). Adequate antioxidant defences are therefore of importance in prevention of such diseases and keeping a correct balance of the oxidant molecules in the body.

Free radicals are very reactive and will react with other molecules in a variety of ways. With another radical it will form a covalent bond and a stable molecule will be produced. However it could attack a non radical molecule and make it a free radical, thus initiating a chain reaction. Most commonly these chain reactions will result in the damage to lipids(lipid peroxidation). When this occurs at the cell membrane loss of integrity of the membrane will occur. This chain of damage will occur until the chain is broken by an antioxidant (Halliwel and Gutteridge,1989).

Reactive oxygen species and other free radicals mainly cause damage to the host by reacting with three important classes of molecules in the body. Those are lipids, DNA and proteins.

Lipid peroxidation

Polyunsaturated lipids found in membranes and lipoproteins contain double bonds which are susceptible to hydrogen abstraction by free radicals. This produces an relatively stable lipid free radical. However rearrangement of these radicals forms conjugated dienes with alternating single and double bonds. Molecular oxygen could react with these to yield lipid peroxides. Hydrogen abstraction from lipid peroxides produce highly reactive peroxyl radicals. Formation of peroxyl radicals lead into chain reaction.

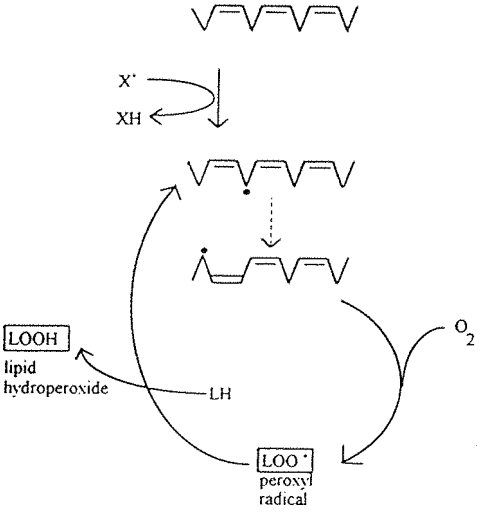


Fig 1.10 Mechanism of polyunsaturated fatty acid oxidation by free radicals

When this occur at cell membrane loss of integrity of the membrane occurs and further damage to the membrane is done by reacting with membrane proteins (Davies and Goldberg, 1987). In addition to the damage done to membranes another important way of damage is the peroxidation of lipids in the low density lipoprotein fraction(LDL). This leads to the deposition of them in the intima of blood vessels which plays an important role in the aetiology of atherosclerosis (Dean *et al*, 1986).

DNA oxidation

Oxidation of DNA by free radicals causes single and double strand breakage. This could lead to mutations in these cells. This could also lead to the changes and activation of oncogenes and thus to the initiation of carcinogenesis (Kasai *et al*, 1986).

Protein oxidation

Oxidation of protein by free radicals leads to the inactivation of proteins. As mentioned earlier this could lead to the damage of membranes. In patients with rheumatoid arthritis a large proportion of α_1-antitrypsin in the rheumatoid joint has been shown to be inactivated by the action of free radicals (Wong and Travis, 1980).

1.12 Interaction between free radicals, reactive oxygen species and cytokine production.

As is apparent from the earlier section stimulation of the immune system by infection or injury results in the production of cytokines and oxidant molecules by macrophages and other immune cells.

Recent studies have indicated that these two groups of molecules can enhance the production of each other. TNF and IL-1 increase reactive oxygen species production (Farante *et al*, 1988) and nitric oxide production (Lamas *et al*, 1991) from macrophages. On the other hand free radicals also can stimulate the production of cytokines. Chaudhri and Clark had shown that TNF production by endotoxin was enhanced by alloxan, a prooxidant drug. They also found that the antioxidant butylated hydroxy anisole(BHA) and iron chelating agent desferrioxamine had decreased the effect of alloxan (Chaudhri and Clark, 1989). Ku and others had shown that probucol, a drug with antioxidant properties can suppress the IL-1 production (Ku *et al*, 1990).

The mechanism whereby reactive oxygen species enhance cytokine production has recently been identified. It involves transcription factors such as nuclear transcription factor kappa B (NFkB). NFkB influence the production of IL-1, IL-6, TNF and acute phase proteins. Under normal conditions the factor is present in the cytoplasm in an inactive form due to the attachment of an inhibitory sub unit. Reactive oxygen species trigger reactions that lead to the degradation of this bound sub unit. Loss of the inhibitory sub unit results in the migration of activated factor to the nucleus and binding to DNA, which enhance the transcription of RNA that relate to cytokine and acute phase production (Sen and Packer, 1996). Therefore the mechanism by which antioxidants reduce the production of cytokines may be by reducing the effect of oxidants on NFkB by decreasing the amounts of oxidant species. Suzuki and Packer have found that catechol derivatives with antioxidant properties were able to inhibit TNF induced NFkB activation (Suzuki and Packer, 1994).

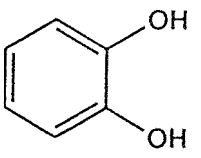


Fig. 1.11 Structure of catechol

Therefore antioxidants derived from diet such as flavanoids found in tea which have similar structures may have an effect on inflammatory response via the alterations in cytokine production.

1.13. Influence of tea components upon the immune system

As described earlier (section 1.4) tea components are known to act as antioxidants *in vitro*. Limited studies also indicate an antioxidant activity *in vivo* also. Large number of studies done on carcinogenesis and atherogenesis where free radicals are involved, also indicate a protective effect from tea components. As described earlier free radicals are also involved in the immune response and alterations are brought about by antioxidants. Despite the above observations attention had not been drawn to the effect of tea consumption on the immune system.

1.14 Aims of study:

Antioxidant activity of black tea components have been demonstrated in a number of *in vitro* studies (section 1.4). *In vivo* antioxidant activity has been suggested as the reason for anticarcinogenic activities and in reducing the risk of cardiovascular diseases. However evidence for the antioxidative activity of black tea components *in vivo* are limited. Therefore studies were conducted to investigate whether black tea components act as antioxidants *in vivo*.

Infection or injury (or endotoxin treatment) impose an oxidant stress on the body by stimulation of production of cytokines and free radicals by macrophages and other immune cells. Cytokines, which are the mediators of the inflammatory response, and free radicals and reactive oxygen species, enhance the production of each other. Free radicals and reactive oxygen species activate the nuclear transcription factor kappa B (NFkB). Activated NFkB enhance cytokine production. Therefore antioxidants may decrease the production of cytokines probably via inhibiting the activation of NFkB by oxidant species (discussed in section 1.12). Therefore whether black tea components have an effect on the inflammatory response through their antioxidant activity was studied.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated all reagents were of analytical grade and obtained from Sigma Chemical Company Limited, Poole, Dorset, England.

Exceptions were:

Poly Sciences, Warrington, UK.

2-azobis(2-aminopropane)hydrochloride (ABAP)

Aldrich, Gillingham, UK.

Trolox

Special Diet Services, Witham, England.

Vitamin mix

Mineral mix

Cellulose powder(Solkafloc)

Maize flour(Snowflake)

Maize oil (Mazola)

St.Coombs Estate, Talawakelle, Sri Lnaka.

Black tea (BOP grade, Broken Orange Pekoe - one of the main and widely used grades of tea. Teas are graded according to particle size by sifting through a sieve. A typical BOP grade contains particles which pass through a 1.40mm mesh and retained on a 0.85mm mesh. Samaraweera, 1986).

2.2 Animals and animal maintenance protocol

The animals (weanling male Wistar rats) were obtained from the Southampton University Medical School colony. They were housed in wire bottomed plastic cages. Rats were maintained in a 12 hour light/dark cycle and under controlled temperature (23±2^{0}C) and humidity. Animals were fed and watered *ad libitum* for the dietary period. The composition of the diets are varied in different experiments and composition of those are given in the relevant chapters.

Weight change and food intake for all animals were recorded throughout the study.

2.3 Culling Procedure

On decapitation blood was collected into heparinised tubes via heparinised funnels. Liver, lung, kidney, spleen and muscle (tibialis) were dissected and weighed. Small portions of blood, liver and lung were used immediately for the glutathione and haemoglobin assay. Tissues were frozen in liquid nitrogen and later stored at -70^{0}C. Blood was stored on ice until culling was complete and then spun at 2000rpm for 15 minutes at 4^{0}C. Plasma was removed and stored at -70^{0}C.

2.4 Tissue, blood and plasma assays

The following techniques were used for the analysis of liver, lung, spleen, muscle and plasma.

2.4.1 Haemoglobin assay

The principle of the technique used was that in the presence of potassium ferricyanide, haemoglobin and its derivatives, except sulphaemoglobin are oxidised to methaemoglobin. Sulphaemoglobin will react with potassium cyanide to form cyanmethaemoglobin. The maximum absorbance for this is at 540nm. Colour intensity at this wave length is proportional to total haemoglobin content. (Dacie and Lewis, 1975)

Drabkin Reagent

One vial was made up to 1 litre with distilled water and stored in an amber bottle at room temperature. Each vial contained Sodium bicarbonate (100 parts), Potassium ferricyanide (20 parts) and Potassium cyanide (5 parts).

Haemoglobin standards

Standard haemoglobin was diluted in Drabkin reagent to give standards over the concentration range 0 - 18 g Hb/dl.

Method

20µl of blood was added to 5.0ml of drabkin reagent. The solution was mixed and the absorbance was measured at 540nm on a standard spectrophotometer after allowing it to stand for at least 15 minutes.

Haemoglobin concentration was (g/dl) obtained directly from the calibration curve.

2.4.2 Glutathione Assay

In this assay after precipitation of protein from blood (or tissue), glutathione in the supernatant will react with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) to give a coloured

compound. The absorbance of this could be measured at 412nm. (Beutler *et al*,1963)

Reagents:

Precipitating solution:

Metaphosphoric acid	16.7g
Sodium EDTA	2.0g
Sodium chloride	300.0g

Solution was made up to 1 litre with distilled water. This reagent is stable for 3 weeks at 4^0C.

Phosphate solution:

42.588g of Disodium hydrogen orthophosphate was dissolved in 1 litre of water and stored at 4^0C. If any crystals formed, it was heated to 37^0C in a water bath.

5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) solution:

5,5-dithiobis-(2-nitrobenzoic acid)	40mg
Sodium citrate	1.0g

Made up to 100ml with distilled water. This reagent is stable for 13 weeks at 4^0C.

Glutathione standards:

25mg of reduced glutathione was dissolved in 10.0ml of phosphate solution. This stock solution was diluted to give standards over concentration range from 0 - 0.5mg/ml.

Method:

Blood assay:

200µl of blood(standard) was added to 800µl of distilled water. Solution was vortex mixed and 1.5ml ice cold precipitating solution was added. The solution was mixed again and

allowed to stand on ice for 5 minutes. Then the solution was vortex mixed and filtered. 1.0ml of the filtrate was added to 4.0ml of phosphate solution and 1.0ml of DTNB was also added. Solution was then vortex mixed and absorbance at 412nm was read within 5 minutes of the addition of DTNB.

Tissue assay:

A weighed portion of liver (0.08 - 0.12g) or lung (0.12 - 0.15g) was homogenised in 2.5ml of ice cold precipitation solution. A further 2.5ml of precipitating solution was added to this and allowed to stand on ice for 5 minutes. Then the solution was mixed and filtered. 1.0ml of the filtrate was added to 4.0ml of phosphate solution, followed by 1.0ml of DTNB. Solution was vortex mixed and absorbance at 412nm was measured within 5 minutes of the addition of DTNB solution.

Glutathione concentration was directly obtained from the standard curve as mg/ml. Blood glutathione expressed per g Hb was obtained from

$$\text{mg GSH/g Hb} = \frac{\text{Blood (mg/ml)} \times 100}{\text{Hb (g/dl)}}$$

2.4.3 Caeruloplasmin assay

This assay is based on the oxidase activity of caeruloplasmin and is a colorimetric assay. Fresh plasma should be used to get the maximum oxidase activity. (Schosinsky et al, 1974)

Reagents:

Acetate buffer:

Sodium acetate	13.608g
Glacial acetic acid	2.6ml

The solution was made up to 1 litre with distilled water and corrected to pH 5.0.

O-Dianisidine dihydrochloride solution:

250mg of O-Dianisidine dihydrochloride was dissolved in 100ml of distilled water.

9M sulphuric acid:

Stock sulphuric acid	486.0ml
Distilled water	514.0ml.

Method:

0.75ml of acetate buffer was added to two test tubes labelled A and B respectively. To each tube 0.05ml plasma was added. The tubes were kept on a water bath at 37°C for 5 minutes. Then 0.2ml of O-Dianisidine dihydrochloride was added to each tube. Exactly 5 minutes after the addition of O-Dianisidine dihydrochloride 2.0ml of 9M sulphuric was added to tube A. Exactly 15 minutes after the addition of O-Dianisidine dihydrochloride 2.0ml of 9M sulphuric was added to test tube B. The absorbance of these solutions were measured at 540nm against a distilled water blank. Caeruloplasmin oxidase activity was calculated from,

Caeruloplasmin oxidised (units/ml plasma) = (Abs B - Abs A) x 0.625

(0.625 = Molar absorptivity x Dilution factors)

2.4.4 Albumin assay

Bromocresol green method was used to measure the plasma albumin concentration.

(McPherson and Everard,1972)

Reagents:

Bromocresol green reagent:

1M Glycine	94.5ml
1M Hydrochloric acid	5.5ml
0.02M Alkaline bromocresol green	3.0ml
30% Brij 35	4.0ml

The solution was made up to 1 litre with distilled water and corrected to pH 3.8.

Standards:

Standards were prepared from bovine serum albumin over the range

0 - 50mg/ml

Method:

To 25µl of plasma (standard) 5.0ml of bromocresol green reagent was added and mixed. The absorbance was read immediately at 635nm.

Concentration of albumin was obtained directly from the standard curve.

2.4.5 Zinc assay

After acid digestion of the tissue, Zn was measured by atomic absorbance spectrometry. (Tocco-Bradley and Kluger, 1984)

Reagents:

Concentrated Nitric acid

Zn standards:

Standards were prepared over the range of 0 - 1.0µg/ml zinc.

Method:

Accurately, approximately 0.5g of liver (0.1g lung, 0.25g kidney, 0.1g tibialis, 0.1g spleen) was weighed and a 2.0ml (0.4ml, 1.0ml, 0.4ml, 0.4ml) aliquot of concentrated nitric acid was added. Samples were incubated at 40°C for 12 hours. Then the samples were diluted 1:4 with deionised water. A 200µl of this solution was then diluted to 1.0ml with deionised water.The samples were mixed well before assaying for Zn on a Perkin-Elmer atomic absorption spectrometer at 214nm.

The standard curve was used to obtain zinc concentration as µg/200µl. Tissue Zn concentration and tissue Zn content were calculated from,

$$\text{Tissue Zn } (\mu g/g) = \frac{\mu g/200\mu l \times \text{Dilution factor}}{\text{tissue sample weight}}$$

Zinc content of tissue (µg) = µg/g x tissue weight

2.4.6 Protein assay

Principle of this assay is that proteins will reduce copper(II) to copper (I) in a concentration dependent manner. Bicinchoninic acid (BCA) is highly specific for copper (I)

and forms a complex with maximum absorbance at 562nm and the absorbance is directly proportional to the protein concentration. (Smith *et al*, 1985)

Reagents:

Bicinchoninic acid solution

4% copper sulphate solution

1.0g of copper sulphate pentahydrate was dissolved in 25.0ml of water.

Protein standards:

Standards were prepared from bovine serum albumin over the concentration range 0 - 1.0mg/ml

Method:

100mg of liver (100mg kidney, 100mg tibialis, 60mg spleen) was homogenised in 10.0ml (10.0ml, 5.0ml, 3.0ml) of distilled water. A 1.0ml of homogenate was diluted 1/10 (1/6.5, 1/10, 1/10). A 10µl aliquot of sample (standard) was added to microelisa plate in triplicate.

Protein determinant solution was prepared immediately before the assay by adding 1 part of 4% copper sulphate solution to 50 parts of BCA solution. A 200µl of this was added to each well and the plate was incubated at 37^{0}C for 30 minutes. The plate was then cooled and the absorbance of the samples (standards) were read at 562nm using micro elisa plate reader.

The protein concentration in mg/ml was obtained directly from the standard curve. Tissue protein concentration and content was calculated from,

$$\text{Protein concentration (mg/g)} = \frac{\text{mg/ml x dilution factor}}{\text{tissue sample weight}}$$

Protein content of tissue (mg) = mg/g x tissue weight.

2.4.7. Interleukin 6 (IL6) assay

This assay was kindly carried out by Dr.P.S.Tappia, Department of Human Nutrition, University of Southampton.

This method utilises the proliferative effect of this cytokine on IL6-dependent murine hybridoma cell-lines such as B9 as described by Wadhwa et al (1991).

Reagents:

3-(4,5-dimethylthiazol-2-ys)-2,5-diphenyl tetrazolium bromide (MTT)

5mg of MTT was made up to 1ml in phosphate buffered saline. The resulting reagent was filter sterilised and stored in the dark.

Acid sodium dodecyl sulphate solution (SDS)

1g of SDS was made up to 10ml with 0.02M hydrochloric acid.

Interleukin 6 standards

Standards were prepared over the concentration range of 0-30U/ml.

Method:

Maintenance of B9 cell line

Cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum and approximately 1 U/ml of IL6. Cultures were split 1:5 to 1:10 every 2-3 days, and were re fed with IL6 when the cell density reached approximately 5 x 10^5 cell/ml. Cultures were

maintained at 37°C in a humidified CO_2 incubator.

Bioassay for IL6

Cells were prepared by washing twice in RPMI 640 medium and centrifuging the cells at 250g for 10min. The viability of the washed cells were checked and the cells were resuspended to a concentration of 5 x 10^4 cell/ml in RPMI 640 medium supplemented with 5% fetal calf serum. A 100µl aliquot of cell suspension was applied to each well of a sterile microtitre plate and incubated for 24 hours at 37°C in a humidified CO_2 incubator.

Standards were applied in triplicate in 100µl volume to the cells. A 100µl aliquot of each sample was applied undiluted and in duplicate to the cells. The plate was incubated for approximately 72 hours at 37°C in a humidified CO_2 incubator. To each well 10µl of MTT was added and the plates returned to the incubator for a further 4.5hours.

After incubation, 25µl of acid SDS was added to each well and carefully mixed. The plate was left in dark for one hour at room temperature. The absorbance was then determined at 630nm using a microelisa reader which is directly propotional to number of viable cells. Activity of IL6 (per 100µl) was determined directly from the standard curve. Amount of IL6 was determined from

98 U/ml IL6 = 1 ng/ml

2.4.8. Antioxidant Capacity assay (TEAC)

The antioxidant capacity measurement method by Miller and others was slightly modified. Instead of analysis on the Roche Cobas biocentrifugal analyzer, a plate reader was used. The change in absorbance of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was followed every minute for 6 minutes at 636nm in each of the plate wells. The

plasma samples were diluted 1:25 in 5mM phosphate buffered isotonic saline (pH 7.4) and 25µl of the diluted sample was added to a well. A 200µl assay mix of ABTS and metmyoglobin was added and the reaction was started with the addition of 25µl of H_2O_2. The final concentrations in the well were 170µM ABTS, 2.4µM metmyoglobin and 75µM H_2O_2. The absorbance value of the blank at 6 minutes minus that for the sample, divided by the blank value, was the fractional inhibition of the sample. This inhibition was then compared with the inhibition of the standard. Trolox, a water soluble vitamin E analogue was used as the standard (6-Hydroxy 2,5,7,8 tetramethylchroman-2-carboxylic acid, made water soluble by addition of a carboxylic group to phytol chain). The unit of activity is the Trolox equivalent antioxidant capacity (TEAC). Which is the concentration (mmol/l) of Trolox having the equivalent antioxidant capacity to the sample (Miller *et al*, 1993).

2.4.9 Cytochrome p450 assay

Preparation of liver microsomal fractions

Livers were removed immediately after the decapitation, weighed and each liver placed in 3 volumes of ice cold 0.1M potassium phosphate buffer (pH 7.4, containing 1mM EDTA). All subsequent operations were done at 0-4^{0}C. Livers were homogenised in the buffer using a glass/teflon motor driven homogenizer and centrifuged at 10,000g for 20 minutes. The supernatant was removed and recentrifuged at 96,000g for 60 minutes to obtain the initial microsomal pellet. The pellets were resuspended in 50mM tris-HCl buffer (pH 7.4) and the homogenate recentrifuged at 100,000g for 60 minutes to obtain the washed microsomal fraction.

Enzyme assay

The content of cytochrome p450 in the liver preparations were determined by scanning the optical density spectrum (400-500nm) using a Beckman dual beam spectrometer. The washed microsomal suspension (2ml, containing 1-2mg protein/ml) was placed in each of two matching cuvettes and a similar quantity (a few milligrams) of sodium dithionite was added to each tube and the baseline was recorded. Carbon monoxide was then bubbled through the sample cuvette for 30 seconds and the spectrum recorded. The amount of cytochrome p450 present in each sample was calculated from the optical density difference (450-480nm) and the extinction coefficient $91mM^{-1}cm^{-1}$. The amounts of cytochrome p450 was expressed as per mg of protein (Omura and Sato, 1964).

2.4.10 Glutathione enzyme assay

Reagents

Homogenising Buffer

30mM Potassium chloride

10mM dipotassium phosphate

Made up to 1 litre with distilled water and corrected to pH 7.4.

Method

Approximately 400mg of liver tissue was homogenised in homogenising buffer, using an Ultraturrax homogeniser to give a final concentration of exactly 100mg tissue/ml. The homogenate was sonicated for 1 minute on a cycle of 6 x 10 seconds with 5 second rests (amplitude 18). Samples were centrifuged for 5 minutes in a microcentrifuge and frozen in 0.5ml aliquots at $-70^{0}C$ until use.

Glutathione peroxidase

This assay is based on the ability of glutathione peroxidase to oxidise glutathione in the presence of a peroxide (Wendel, 1981).

Reagents

*t-Butyl Hydroperoxide (TBHP)

Stock TBHP diluted 1:10000 in a glass vessel.

* 0.1M Tris/EDTA

0.1M Tris buffer

5mM EDTA

pH corrected to 8.0.

* Substrate

20mg NADPH

61.4mg glutathione

100 unit glutathione reductase

Made up to 100ml with tris/EDTA.

Method

To 20µl of sample or blank 210µl of substrate was added. The reaction was started with 20µl of TBHP and the absorbance at 340 nm was followed for 1 minute.

The activity of glutathione peroxidase was calculated from

U/ml = (sample-blank) x 2.01

Glutathione reductase

This assay utilises the reduction of oxidised glutathione to reduced glutathione by the enzyme (Carlberg and Mannervik, 1975).

Reagents

Buffered reagent

0.1mM NADPH

0.5mM EDTA (disodium salt)

1.0mM Oxidised glutathione

Made up to 100ml with 0.1M sodium phosphate buffer (pH 7.6)

Method

In a microtitre plate, 200µl of buffered reagent was added to 30µl of sample or blank. The absorbance at 340nm was followed for 1 minute at 30°C.

The activity of glutathione reductase was calculated from

U/ml = (sample - blank) x 1.233

2.4.11 Myeloperoxidase activity assay

Chemicals

Potassium dihydrogen orthophosphate

di-Potassium hydrogen orthophosphate(anhydrous)

O-Dianisidine dihydrochloride

Hexadecyltrimethylammonium Bromide (HTAB)

Hydrogen peroxide (30%)

Reagents

Solution A - 3.4g of Potassium dihydrogen orthophosphate in 500ml of distilled water.

Solution B - 4.35g of di-Potassium Hydrogen Orthophosphate in 500ml of distilled water.

Potassium phosphate buffer - Add solution B to solution A until pH is 6.0.

HTAB Buffer - 2.5g of HTAB in 500ml of potassium phosphate buffer (warm if necessary).

1% Hydrogen Peroxide - 1ml of 30% hydrogen peroxide + 29ml of distilled water.

O-dianisidine reagent - 16.7mg of O-dianisidine dihydrochloride was dissolved in 90ml of distilled water. 10ml of potassium phosphate buffer and 50µl of 1% hydrogen peroxide was added. This solution was made fresh each time.

Method

50mg of tissue was weighed. 1ml of HTAB buffer was added and homogenised using an ultratraux homogeniser. Tissue homogenate was centrifuged in a microcentrifuge for 2 minutes. Supernatant was taken and diluted 1:1 with distilled water. 10µl of this was added to a plate reader well and 200µl of O-dianisidine reagent was added. Absorbance was read at 490nm. Myeloperoxidase activity was calculated according to the following equation.

X = Absorption value/1.13×10^{-1} (Molar absorptivity)

Y ={Weight of tissue (50mg) x amount in a well(10µl)}/2000(Dilution factor)

Myeloperoxidase activity (U/mg) = X/Y.

2.4.12 Total Radical Trapping Antioxidant Parameter (TRAP) assay.

Chemicals

Linoleic acid

2-azobis(2-aminopropane) hydrochloride (ABAP)(polysciences, Warrington, UK)

Trolox (Aldrich, Gillingham, UK)

Method

100µl of plasma was mixed with 10µl of linoleic acid in a glass vial by vortex mixing for approximately 30 seconds. 50µl of this mixture was added to a perspex oxygen electrode cell(Rank Brothers, Bottisham, UK) containing 3ml of phosphate buffered saline(PBS, pH 7.5) and 30µl of 4.0M ABAP (1.08g in 10ml of distilled water). Temperature of the cell was maintained at 37°C by passing water at 37°C through the outer jacket of the cell. Light was excluded from the cell to prevent photodecomposition of ABAP. Oxygen loss from the cell representing oxygen uptake during the peroxidation of the linoleic acid was followed until the rate of loss was maximal. At this point, 25µl of 0.4mM Trolox was added.

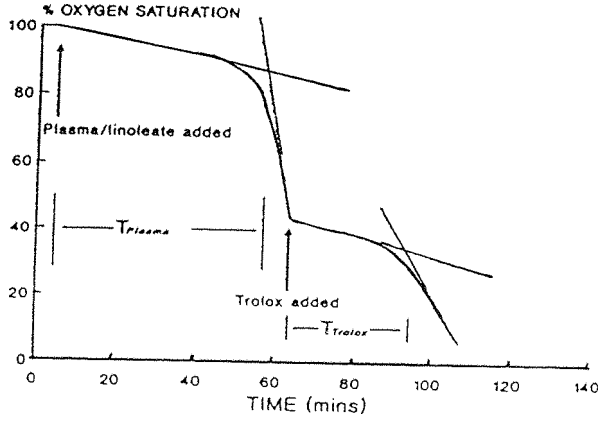


Figure 2.1 Schematic representation of TRAP assay.

The TRAP value was calculated from the equation

$$TRAP = Ri \times T_{plasma}$$

$$Ri = 2[Trolox]/T_{Trolox}$$

Ri is the rate of peroxyl radical generation from the decomposition of ABAP at 37^{0}C and T_{Plasma} and T_{Trolox} are the respective lengths of the induction periods during which the plasma antioxidants or Trolox buffers against the maximal rate of oxygen loss (Fig 2.1) (Wayner *et al*, 1985).

2.4.13 Lung neutrophils

The number of neutrophils in the lungs was determined by counting the fraction of neutrophils as a percentage of the total number of cells in a small section of the lung.

Reagents

Neutral buffered formalin (pH 7.4)

6.5g of disodium hydrogen orthophosphate

4.0g of sodium dihydrogen orthophosphate

100ml of 37% formalin

Made up to 1 litre with distilled water.

Method

Immediately after dissection a small sample of lung tissue was fixed in neutral buffered formalin to prevent autolysis of cells. Slides of these samples were kindly prepared by Mr R. Lee of Southampton General Hospital using Haematoxylin stain and Eosin counter stain (sections were 4 microns thick).

Slides were observed using a magnification of x1000. For each lung slice 16 uniform

planes were closely examined for the presence of neutrophils and other cells. The number of neutrophils were expressed as a percentage of the total number of cells.

2.5 Preparation of tea extract

To 100g of black tea (BOP grade from St.Coombs Estate, Talawakelle, Sri Lanka) 1L of boiling distilled water was added and allowed to stand for a period of 5 min. The solution was filtered through a muslin cloth to remove solid particles and allowed to cool. Caffeine was removed by chloroform extraction (2x 500ml washes). The extract was freeze dried. An average yield of 25g was obtained.

2.6 Statistical analysis

All data are expressed as mean values ± SD. Mean values of the measurements made were examined for significant differences by one way analysis of variance (ANOVA) or two way ANOVA. Differences in mean values with $p<0.05$ were considered significant. Differences in mean values with $p>0.05$ were considered as not significant. Where significant differences were found differences between groups were examined by an unpaired student t-test.

2.7 Validation of methods

Reproducibility and reliability of methods used in the experiments were evaluated by measurement of coefficient of variation. Intra assay and inter assay variations were measured

where possible. Intra assay variation was determined by measuring same sample up to ten times. Inter assay variation was determined by measuring the same sample on ten different days. Coefficient of variation was calculated by;

$$CV = (SD \times 100)/Mean$$

CV = Coefficient of variation

SD = Standard deviation.

Table 2.1 Coefficient of variation for Caeruloplasmin, Albumin and Haemoglobin assays - Intra assay

	Caeruloplasmin(u/ml)	Albumin(mg/ml)	Haemoglobin(g/dl)
1	0.146	43.38	13.18
2	0.152	40.19	13.13
3	0.148	42.95	14.21
4	0.147	42.76	13.07
5	0.162	39.55	13.28
6	0.156	41.85	14.19
7	0.142	41.62	13.85
8	0.145	40.19	14.00
9	0.153	42.21	13.28
10	0.140	38.77	13.56
Mean	0.149	41.35	13.58
CV	4.4%	3.8%	3.3%

Table 2.2 Coefficient of variation for Zinc assay. Intra and inter assays.

	Zinc(μg/g liver) Intra assay	Inter assay
1	39.55	40.36
2	40.34	42.85
3	44.83	38.21
4	42.24	50.62
5	40.14	41.67
6	45.35	48.17
7	42.37	43.88
8	43.42	45.51
9	48.31	36.71
10	38.15	52.10
Mean	42.45	44.00
CV	7.3%	11.6%

Table 2.3 Coefficient of variation for Protein assay. Intra and inter assays.

	Protein(mg/g liver) Intra assay	Inter assay
1	175	179
2	180	175
3	165	188
4	193	183
5	178	204
6	174	161
7	201	172
8	169	181
9	174	169
10	176	172
Mean	179	178
CV	6.0%	6.6%

Table 2.4 Coefficient of variation for TEAC assay. Intra and inter assays.

	TEAC(mmol/l) Intra assay	Inter assay
1	0.19	0.21
2	0.19	0.24
3	0.21	0.23
4	0.23	0.18
5	0.22	0.28
6	0.20	0.25
7	0.24	0.17
8	0.23	0.22
9	0.25	0.21
10	0.22	0.26
Mean	0.22	0.23
CV	9.0%	13.0%

Table 2.5 Coefficient of variation for Myeloperoxidase assay. Intra and inter assays.

	Myeloperoxidase(U/g) Intra assay	Inter assay
1	3.24	3.51
2	3.04	4.21
3	4.70	3.72
4	3.63	4.20
5	4.20	5.12
6	3.63	2.34
7	3.25	3.57
8	3.27	4.10
9	3.78	3.32
10	3.77	3.46
Mean	3.65	3.76
CV	13.8%	19.4%

Table 2.6 Coefficient of variation for TRAP assay. Intra assays.

	TRAP(mmol/l) Intra assay
1	1048
2	1038
3	1069
4	1055
5	1085
6	1021
Mean	1053
CV	2.2%

CHAPTER 3

INFLUENCE OF TEA SOLIDS ON THE RESPONSE TO ENDOTOXIN

3.1 Introduction

Stimulation of the immune system by invading pathogens, injury or chronic inflammatory diseases produce highly potent molecules such as reactive oxygen species (ROS) and free radicals by the system. The objectives of the production of these molecules are to kill the pathogens that cause the immune stimulation and to destroy damaged cells, thereby restoring normal functions of tissues.

Cytokines are released by the immune cells as part of the immune response. These include interleukins (IL) 1 to 12, tumour necrosis factor(TNF) and interferons (IFN) (Male and Roitt, 1993). In addition to cytokines, free radicals such as super oxide, hydroxyl and nitric oxide radicals and other oxidant products like hypochloric acid and hydrogen peroxide are also produced by immune cells (Halliwell, 1991). Cytokines can enhance production of free radicals and other oxidant molecules (Farante *et al*, 1988). On the other hand free radicals also can enhance the production of cytokines (Chaudhri and Clark, 1989).

These oxidant molecules, produced by the immune system, although aimed at protecting the host by destroying the invading pathogen, could be a potential threat to the host tissues. However there are natural antioxidant defence systems in the body which provide protection against oxidants and maintain a correct balance between the oxidant and

antioxidant system (discussed in chapter 1, section 1.3). Some of the antioxidant substances in this system such as vitamin E, vitamin C and carotenoids are derived from the diet.

Components in black tea extracts are known to act as free radical scavengers and antioxidants *in vitro*. Claims had been made that they act as antioxidants in vivo also (Discussed in chapter 1, section 1.4). Though prooxidants and antioxidants are known to have an effect on immune response (Chaudhri and Clark, 1989, Ku *et al*, 1990, De Forge *et al*, 1992, Roth and Droge, 1987) studies had not been carried out to find the effect of tea components on the immune response. Therefore in this experiment the influence of addition of black tea solids to the diet, on the response to endotoxin were studied in an animal model. An endotoxin challenge results in an acute inflammatory response and will results in the production of large amounts of reactive oxygen species and free radicals by macrophages and other immune cells as part of the immune response. The body will then be subjected to an oxidative stress. This process will facilitate investigation of the antioxidant properties of tea components *in vivo* under oxidative stress. In the study a wide range of metabolic parameters are studied, including those associated with antioxidant defence, to observe the influence of tea solids.

3.2 Experimental Protocol

Twenty weanling male Wistar rats, weighing 56(±2)g (mean ± S.D.), were divided into two groups. They were put in separate cages. Group one was fed with a normal diet (Normal) and the second group was fed with a diet which had 5g of decaffeinated tea solids per kg of diet in addition to the other components(Tea). They were fed these diets for 21 days. Then they were sub divided into two groups. Animals in sub group one were given an

endotoxin injection (Lipopolysacchride from E.coli, i.p., 200μg/kg body weight). All the rats were fed *ad libitum* for further 24 hours and endotoxin injected animals were sacrificed by decapitation. Then sub group two animals were injected with sterile non pyrogenic saline (0.9g in 100ml water, i.p, 1ml/kg body weight). They were pair fed (with food intake of endotoxin injected animals during the last 24 hours) for further 24 hours and sacrificed by decapitation.

Table 3.1 Composition of diets:

Component	**Normal (g/kg diet)**	**Tea(g/kg diet)**
Casein	180	180
L-methionine	3	3
Premix (Vit E def)	50	50
Vitamin E	0.05	0.05
Corn Oil	30	30
Cellulose	100	100
Sugar	318	316
Starch	318	316
Decaffeinated Tea Solids	-	5

3.3 Results

3.3.1 Food intake and weight gain

Both groups started with the same mean body weight and there was no significant difference in the body weight gain after 21 days. There was no difference in the food intake also. No differences in physical appearance were observed in the tea solids fed group when compared with the normally fed group. However there was a significant difference in post injection food intake. The loss of appetite in the endotoxin challenged and tea solids fed animals were less than that of endotoxin challenged and normally fed animals (Table 3.2).

Table 3.2 Initial and final body weights, growth and food intake of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Initial body weight(g)			
Endo	55(3)a	56(2)a	NS
Saline	57(2)a	58(2)a	
Growth (pre injection) g/day			
Endo	6.6(0.36)a	6.9(0.36)a	NS
Saline	6.5(0.14)a	6.8(0.22)a	
Food intake (pre injection)(g/day)			
Endo	24.1(1.0)a	23.2(0.6)a	NS
Saline	23.8(0.7)a	24.2(0.8)a	
Food intake Post inj. (g/24 hours)			
Endo	13.4(10.0)a	23.8(6.4)b	p_d=.00019, F=23.03
Saline	13.4(10.0)a	23.8(6.4)b	

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from other dietary group by two way ANOVA ($p<0.05$). p_d= differences due to diet.

3.3.2 Body composition:

The results made from liver, lung, kidney, spleen, muscle(tibialis) and plasma analysis

are given below.

Table 3.3 Effect of endotoxin injection on liver composition of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	5.17(0.18)*	5.52(0.31)*	p_t<0.0001 F=35.98
Saline	4.61(0.20)	4.66(0.34)	
Protein mg/g			
Endo	203(13)	189(33)	NS
Saline	190(16)	202(25)	
Zinc µg/g			
Endo	49(8)	45(8)	NS
Saline	47(3)	49(3)	
GSH mg/g			
Endo	1.18(0.15)*	1.05(0.12)	p_t=0.0013, F=15.08
Saline	0.85(0.11)	0.94(0.13)	

Results expressed as mean (± SD). n = 5. Values with * are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

3.3.3 Liver composition

Relative liver weights (as a % of body weight) of endotoxin injected animals were significantly higher in both tea fed and normally fed animals when compared with saline injected animals. There were no statistically significant differences between the groups in protein concentrations. The same observation was also made for the zinc concentration. Liver glutathione concentration in endotoxin injected normally fed animals were significantly higher than that of normally fed saline injected animals. However there was no difference between tea solids fed, endotoxin injected and tea solids fed, saline injected animals. Inclusion of tea solids in the diet had no influence on glutathione concentration in either the endotoxin or saline injected animals (Table 3.3).

Table 3.4. Effect of endotoxin injection on lung composition of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	0.63(0.04)a	0.65(0.05)a	NS
Saline	0.62(0.02)a	0.62(0.03)a	
Zinc µg/g			
Endo	32(6)a	31(5)a	NS
Saline	31(1)a	34(7)a	
GSH mg/g			
Endo	0.28(0.05)a*	0.38(0.03)b*	p_t=0.011,F=8.03
Saline	0.23(0.02)a	0.33(0.05)b	p_d<0.001,F=30.18

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from the other dietary group and values with '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t=differences due to treatment and p_d=differences due to diet.

3.3.4 Lung composition

Although relative lung weights of endotoxin injected animals were slightly higher than that of saline injected animals, this was not statistically significant. No differences were observed for the zinc concentration of the lung also. Glutathione concentrations of animals

fed tea solids were significantly higher when compared with corresponding normally fed group. Also the glutathione concentrations of endotoxin treated animals were significantly higher than the corresponding saline treated group (Table 3.4).

Table 3.5 Effect of endotoxin injection on spleen composition of rats fed a normal diet or a diet supplemented with tea solids.

Diet	**Normal**	**Tea**	**Two way ANOVA p & F ratios**
Weight as % body weight			
Endo	0.41(0.03)	0.46(0.01)*	$p_t<0.001$,F=25.66
Saline	0.38(0.03)	0.37(0.03)	
Zinc µg/g			
Endo	29(3)	29(4)	NS
Saline	29(1)	30(3)	
Protein mg/g			
Endo	183(8)	184(9)	NS
Saline	180(13)	200(14)	

Results expressed as mean (± SD). Values with a '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$) p_t= differences due to treatment.

3.3.5 Spleen composition

The increase of the relative spleen weight, in endotoxin injected animals of normally fed group, was not statistically significant when compared with normally fed saline injected rats. However there was a significant increase in the weight of spleen of endotoxin injected animals fed tea solids when compared with saline injected animals fed tea solids. There were no significant differences in zinc concentration of the different groups. Statistically significant differences were not observed for the protein concentration in spleen of the different dietary or injection groups (Table 3.5).

Table 3.6 Effect of endotoxin injection on kidney composition of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	0.96(0.04)*	0.94(0.01)	p_t=0.038, F=5.09
Saline	0.91(0.04)	0.90(0.05)	
Zinc µg/g			
Endo	35(3)	36(3)	NS
Saline	39(3)	37(2)	
Protein mg/g			
Endo	136(9)	145(5)	NS
Saline	144(9)	141(6)	

Results expressed as mean (± SD). n = 5. Values with a '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$) p_t= differences due to treatment.

3.3.6 Kidney composition

Relative weight of the kidneys of endotoxin injected and normally fed animals were significantly higher than that of the corresponding saline injected animals. Although the relative weights of the kidneys of endotoxin injected tea solids fed animals were higher than that of the saline injected tea solids fed animals this was statistically not significant. No

differences were observed in protein and zinc concentrations of the kidney in different groups (Table 3.6).

Table 3.7 Effect of endotoxin injection on muscle composition of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	0.21(0.02)a*	0.24(0.02)b	p_t=0.004, F=11.17
Saline	0.25(0.01)a	0.25(0.01)a	p_d=0.034, F=5.36
Zinc µg/g			
Endo	19(2)a	19(3)a	NS
Saline	18(1)a	19(1)a	
Protein mg/g			
Endo	171(19)a	180(22)a	NS
Saline	157(21)a	182(32)a	

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from the other dietary group and values with a '*' are different from the corresponding saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

3.3.7 Muscle composition

Tibialis weight of the endotoxin injected rats in normally fed group was significantly lower than that of saline injected normally fed rats. However there was no difference between the weights of endotoxin injected and saline injected animals in the tea solids fed group. When endotoxin injected animals in normally fed group was compared with endotoxin injected tea fed group a statistically significant increase was observed.

Though increased concentrations of protein were found in animals fed tea solids this was not statistically significant. No differences were observed for zinc concentration (Table 3.7).

Table 3.8 Effect of endotoxin injection on blood composition of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Glutathione mg/g Hb			
Endo	3.36(0.45)a	4.00(0.51)a	NS
Saline	3.08(0.61)a	3.64(0.91)a	
Haemoglobin g/dl			
Endo	13.5(2.5)a	9.5(0.4)b	p_d=0.027, F=5.90
Saline	10.8(2.2)a	10.3(2.6)a	

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from the other dietary group by two way ANOVA ($p<0.05$). p_d= differences due to diet.

3.3.8 Blood composition

Increased concentrations of glutathione was observed for the groups fed tea solids. However this was not statistically significant. Decreased concentrations of haemoglobin was observed for animals fed tea solids, when endotoxin injected groups were compared. This effect was not observed when saline injected groups were compared.

Table 3.9 Effect of endotoxin injection on plasma composition of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Albumin mg/ml			
Endo	37.9(3.4)	35.7(4.0)	NS
Saline	41.4(2.0)	38.0(3.6)	
Caeruloplasmin U/ml			
Endo	0.20(.03)*	0.22(.09)	p_t=0.025, F=6.09
Saline	0.15(.01)	0.17(.02)	

Results expressed as mean (± SD). n = 5.Values with different letters are significantly different from the other dietary group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

3.3.9 Plasma composition

Although statistically not significant, albumin levels were decreased in the endotoxin injected animals. The increase in caeruloplasmin concentration in the endotoxin challenged normally fed animals was significant higher than saline injected normally fed group. Although there was a similar increase among the animals fed tea solids this was not statistically significant (Table 3.9).

3.4 Discussion

During the experimental period animals in both dietary groups have consumed similar amounts of food. Their body weight gains are also similar(Table 3.2). This indicates that addition of tea solids to the diet had not impaired or improved the food consumption or the growth of the animals. No differences in appearance were also observed between two dietary groups. These observations indicate that addition of tea solids to the diet at 5g per kg of diet had no effect on growth of rats. The average amount of tea solids consumed by both groups were 0.12g per rat per day. An average cup of tea prepared by using 2.5g of tea leaves and brewed for five minutes with boiling water contains about 0.60g - 0.75g of tea solids. Therefore the amount of tea solids consumed by rats could be compared to about 1/5 of tea solids found in a cup of tea. No experimental data are available, in the scientific literature, on the tolerance of rats to tea solids in the diet. However Tono-Oka and co workers performed a study on tea catechins (which accounts for about 10% of the solids extracted from black tea) in rats. In a short experiment 2g, 5g, 10g, 20g, 30g and 50g of tea catechins were mixed with 1kg of diet and fed *ad libitum* for one month. Food intakes were low in the first few days for the groups with 30g and 50g tea catechins. The intake increased and levelled off after 7-10 days. At the end of the experiment body weight gains were lower in groups which received 30g and 50g tea catechins. Below this level of addition there was no difference when compared with the control group that received no tea catechins in the diet. No diarrhoea or any other apparent disorder was observed in all groups including the groups which received 30g and 50g tea catechins. In a long term experiment (three months) addition of 10g or 20g to a kg of diet had no effect on weight gain, food intake, appearance or behaviour of rats (Tono-Oka *et al*, 1991). Extrapolation of these results for green tea consumption may be reasonable

as catechins account for more than 70% of extracted solids. However it may not be possible to compare this with black tea consumption (as in the present study) as catechins account for only 10% of the extracted solids, where other catechins are polymerised into theaflavins and thearubigins. From the present study we can say that addition of 5g of black tea solids per kg of diet had no effect on food consumption, weight gain or appearance during the experimental period.

After endotoxin injection food intake was significantly lowered in the normally fed group. Whereas in the tea solids fed group there was no difference in the food intake after endotoxin injection. Endotoxin challenge results in an inflammatory reaction where cytokines and free radicals are released by macrophages and other immune cells. The characteristic anorexia and weight loss after endotoxin challenge may occur via the actions of cytokines IL-1 and TNF (Tracey *et al*, 1988). Production of cytokines are also stimulated by free radicals (Chaudhri and Clark, 1989). Therefore the reduction of anorexia in rats fed tea solids may be through the quenching of free radicals by components of tea solids which leads to a reduction in cytokines which are responsible for the anorexia.

Relative liver weights (liver weight as % of body weight) of endotoxin challenged animals were higher than that of corresponding saline injected rats in both dietary groups. This observation is in agreement with a general acute phase response (Andus *et al*, 1991). An increased water content in liver may contribute to the increase of liver weight in endotoxin treated animals (Pringle, 1994). The liver is known to increase the synthesis of proteins involved in acute phase response induced by IL-1, TNF and IL-6 (Dinarello, 1984b). Increased acute phase protein synthesis, after an endotoxin challenge, may also be mediated

via these cytokines. Liver metallothionein also increases after endotoxin challenge. Zinc is sequestered into metallothionein and a increase in liver zinc content occurs (Di Silvestro and Cousins, 1984). While in the present experiment an increase of protein or zinc concentration were not observed in endotoxin treated animals, the increase in liver weight would result in increase in total liver content of these components.

Liver glutathione increased in the endotoxin injected animals when compared with saline injected animals. This was statistically significant in the normally fed group while it was not significant in animals fed tea solids. Liver synthesises and use glutathione and also exports it to the blood stream to be used in other sites in the body. It acts as an antioxidant and reducing agent. As endotoxin challenge increases free radical generation by macrophages and other immune cells, glutathione production increases to enhance antioxidant defences. Components in tea solids may also be acting to neutralise the oxidant species produced after the endotoxin challenge. Different antioxidant mechanisms in the body act together to alleviate a oxidant stress in the body. A decrease in one antioxidant may be compensated by the increase of another and vice versa. This may be the reason for the observation that there was no significant increase in liver glutathione in animals fed tea solids.

Relative weights of lungs (weight as % of body weight) were not different between dietary groups or injection groups. However lung glutathione concentrations were significantly higher in the animals which received tea solids in their diet when compared with normally fed group. This was observed in both endotoxin injected and saline injected animals (Table 3.4). There was an increase in glutathione concentration of endotoxin injected animals when compared with saline injected animals in both dietary groups. Increased glutathione in

tea solids fed groups may be due to increased synthesis of glutathione in these groups. However this was not apparent in liver glutathione concentrations as the groups fed tea solids did not exhibit an increase in glutathione concentrations. An increase in utilisation of glutathione occur during an inflammatory response due to its involvement in antioxidant and detoxification reactions (Meister ,1984). Increased amounts of glutathione in rats fed tea solids could be better explained by the reduced utilisation of glutathione in these animals. It could be speculated that components in tea solids are also involved in antioxidant reactions and hence the loss of glutathione will be reduced.

Although statistically not significant, there was a slight increase in the whole blood glutathione in the endotoxin injected animals (Table 3.8). Liver glutathione synthesis increase as a result of the endotoxin challenge. Glutathione will be released to the blood stream for utilisation in other sites for antioxidant and detoxification reactions. Therefore an increase of blood glutathione will occur in endotoxin treated animals. In plasma a slight decrease in albumin levels were observed in endotoxin challenged rats in both dietary groups (Table 3.9). However this change was statistically not significant. As albumin is a negative acute phase protein a decrease is expected after endotoxin treatment. Halliwell suggests that albumin may act as a sacrificial antioxidant. That is, it may react with a potential oxidant molecule to neutralise it and in the process albumin molecule it self will be destroyed. This will prevent the oxidant molecule attacking more important targets such as DNA (Halliwell, 1988). Plasma caeruloplasmin concentration showed the opposite response to albumin. That is, concentrations in endotoxin treated animals were higher. As caeruloplasmin is a positive acute phase protein concentrations will increase after the endotoxin treatment. Inclusion of tea solids in the diet had no effect on both albumin and caeruloplasmin concentration in plasma.

Relative spleen weights (weight as % of body weight) were higher in endotoxin injected animals in both dietary groups (Table 3.5). The increase in spleen weight of endotoxin injected animals, when compared with saline injected animals, was statistically significant in the group fed tea solids while it was not statistically significant in the normally fed group. The increase of spleen weight is expected as a large proportion of cells found in spleen are lymphocytes and macrophages.

The relative weights of tibialis muscle (weight as % of body weight) were significantly lower in endotoxin injected normally fed group when compared with saline injected animals of the same dietary group. Such a decrease was not found in the tea solids fed group. Endotoxin treatment brings about changes in liver metabolism through cytokine release. Liver gluconeogensis and protein synthesis are increased by these cytokines. Amino acid substrate for these increased synthesis are provided by the catabolism in skeletal muscles. In addition muscle also release glutamine which is an important nutrient for the immune system (Newsholme *et al*, 1985). *In vitro* studies have found that IL-1 and TNFα cause muscle proteolysis (Goldberg *et al*, 1984). In the present study muscle weight had not been reduced after endotoxin treatment in animals fed tea solids. This may be due to the decreased cytokine production after the endotoxin injection mediated via the antioxidant activity of black tea components.

Observations made in this study suggest that addition of tea solids at 0.5 (5g per kg of diet) level to the diet had no effect on growth or food consumption in rats. However increases in lung glutathione and a decrease in skeletal muscle wasting, after endotoxin treatment in animals fed tea solids support the idea that components in tea solids may act as antioxidants during inflammation.

CHAPTER 4

THE INFLUENCE OF VARIOUS DOSES OF TEA SOLIDS ON RESPONSE TO ENDOTOXIN

4.1 Introduction

In the earlier experiment addition of tea solids to the diet at 0.5% (5g of tea solids in kg of diet) level altered some of the responses to endotoxin challenge. However it is not known whether the amount of tea solids consumed had a maximal effect on these parameters. Therefore in this experiment the influence of different doses of tea solids on responses to endotoxin was investigated. In addition to the measurements made in the earlier experiment, the antioxidant capacity of plasma was also measured.

4.2 Experimental Protocol

50 weanling male Wistar rats weighing 63±5g were randomly divided into five groups. They were kept in separate cages and fed five different diets.

1. Normal Diet (0%)
2. Normal Diet + 0.2% (2g Tea solids/kg of Diet)Tea solids (0.2%)
3. Normal Diet + 0.5% Tea solids (0.5%)
4. Normal Diet + 1% Tea solids (1%)
5. Normal Diet + 2% Tea solids (2%)

Rats were fed these diets for 21 days. Then each dietary group was divided into two. One sub group received an i.p. dose of endotoxin (200μg/kg body weight) and was killed 24 hours after injection. On day 22 the other sub group was injected with saline(i.p, 1ml per kg body weight)and pair fed with the intake of the endotoxin injected animals, for 24 hours prior to sacrifice.

Table 4.1 Dietary composition of different groups

Component (g/kg)	0%	0.2%	0.5%	1%	2%
Casein	180	180	180	180	180
L-methionine	3	3	3	3	3
Vitamin Mixture	20	20	20	20	20
Mineral Mixture	40	40	40	40	40
Calcined magnesite	1	1	1	1	1
Corn Oil	30	30	30	30	30
Cellulose	100	100	100	100	100
Sugar	313	312	310	305	300
Starch(Cornflour)	313	312	310	305	300
Decaffeinated tea solids	-	2	5	10	20

4.3 Results

4.3.1 Food intake and weight gain:

Animals in all dietary groups had no statistically significant differences in initial body weights. There were no significant differences in body weight gain or food intake during the experimental period. After the endotoxin injection food intake was significantly decreased. However unlike the previous study, addition of different levels of tea solids had no effect on the post injection food intake (Table 4.2).

Table 4.2 Initial and final body weights, growth and food intake of rats fed diets containing different amounts of tea solids.

Diet	0%	0.2%	0.5%	1%	2%	Two way ANOVA
Initial body weight (g)						
Endo	65(4)	62(6)	64(4)	66(7)	65(7)	NS
Saline	63(5)	59(5)	64(2)	63(5)	63(5)	
Final body weight (g)						
Endo	191(10)	192(18)	197(24)	209(11)	192(14)	NS
Saline	197(16)	206(10)	210(13)	202(9)	200(8)	
Food intake g/day						
Endo	24(4)	24(2)	26(2)	28(3)	23(4)	NS
Saline	22(1)	24(1)	26(2)	25(5)	23(1)	
Growth rate g/day						
Endo	5.8(0.49)	5.9(0.68)	6.1(1.0)	6.5(0.49)	5.7(0.51)	NS
Saline	5.9(0.63)	6.4(0.35)	6.4(0.59)	6.0(0.51)	6.0(0.39)	
Post injection food intake g/24 Hours						
Endo	17(12)	16(12)	14(10)	18(14)	18(11)	NS
Saline	17(12)	16(12)	14(10)	18(14)	18(11)	

Results expressed as mean (± SD). n = 5.

4.3.2 Body composition

The results obtained from liver, lung, kidney, spleen, muscle(tibialis), blood and plasma analysis are given below.

Table 4.3 Effect of endotoxin injection on liver composition of rats fed diets containing different amounts of tea solids.

Diet	0%	0.2%	0.5%	1%	2%	Two way ANOVA p & F ratios
Weight as % of body weight						
Endo	5.23(.36)	5.09(.45)*	5.66(.95)*	5.44(.36)*	5.30(.53)	p_t<0.001,F=22.7
Saline	4.68(.68)	4.22(.74)	4.47(.74)	4.58(.65)	4.66(.33)	
Glutathione mg/g						
Endo	1.13(.07)	1.08(.10)	1.24(.24)*	1.20(.25)*	1.13(.12)	p_t<0.001,F=22.0
Saline	0.91(.23)	0.86(.20)	0.80(.25)	0.91(.27)	0.96(.17)	
Protein mg/g						
Endo	130(11)*	150(11)	154(17)	153(4)	144(13)	p_t=0.005,F=8.7
Saline	169(16)	142(12)	165(10)	159(14)	163(10)	

Results expressed as mean (± SD). n = 5. Values with '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t = differences due to treatment.

4.3.3 Liver composition

Liver weights (as % of body weight) in endotoxin injected animals of the different dietary groups were not statistically different from each other. A similar observation was also made for the saline injected groups. However statistically significant differences were observed between endotoxin injected and saline injected groups. Relative liver weights of endotoxin injected and 0.2%, 0.5% and 1% Tea solids diet fed groups were higher than those of corresponding saline injected groups.

Endotoxin treatment increased the hepatic GSH concentration in all dietary groups. Among the endotoxin injected animals, the group fed the diet containing 0.5% tea solids had the highest amounts of glutathione. However this was statistically not significant. Among the saline injected animals the group fed the diet containing 0.5% tea solids had the lowest amount of glutathione. This was also not statistically different from other saline injected dietary groups. When endotoxin injected and saline injected groups were compared the groups fed the diets containing 0.5% and 1% tea solids had statistically significant differences in GSH concentration.

Liver protein concentrations in endotoxin injected animals were not significantly different between the dietary groups. This was the same for the saline treated animals. In the dietary group which was not supplemented with tea solids the endotoxin treated group had a significantly lower protein concentration than the saline treated group. This effect was not observed for the dietary groups supplemented with tea solids (Table 4.3).

4.3.4 Lung composition

Relative lung weights of endotoxin injected animals were slightly higher than the relative lung weights of the saline injected animals. This difference was not statistically significant. No statistically significant differences were found among different dietary groups of endotoxin injected or saline injected animals.

No statistically significant differences were observed in lung glutathione concentration among injection groups or dietary groups (Table 4.4).

Table 4.4 Effect of endotoxin injection on lung composition of rats fed diets containing different amounts of tea solids.

Diet	0%	0.2%	0.5%	1%	2%	Two way ANOVA p & F ratios
Weight as % of body weight						
Endo	0.64(.18)	0.65(.19)	0.63(.11)	0.56(.08)	0.67(.13)	NS
Saline	0.57(.03)	0.54(.03)	0.52(.05)	0.55(.02)	0.61(.08)	
Glutathione mg/g						
Endo	0.30(.02)	0.31(.02)	0.30(.02)	0.32(.01)	0.29(.03)	NS
Saline	0.31(.04)	0.31(.03)	0.31(.03)	0.32(.04)	0.30(.03)	

Results expressed as mean (± SD). n = 5.

4.3.5 Blood composition

Addition of tea solids to the diet had increased the haemoglobin concentration. An addition of 0.5% tea solids caused the highest increase in blood haemoglobin both in endotoxin and saline treated groups. In the endotoxin treated group addition of tea solids to the diet had significantly increased the haemoglobin concentration when compared with the dietary group with out tea solids. The peak haemoglobin concentration was in the group consuming the diet with an addition of 0.5% tea solids. In the saline injected group only, an addition of 0.5% of tea solids had significantly increased the haemoglobin concentration when compared with the group without the addition of tea solids. When endotoxin treated and saline treated animals were compared a difference was found only in the group which had received a diet containing 0.5% tea solids, where endotoxin treated group had a higher amount.

Blood glutathione concentrations were not different among the groups when expressed as mg/ml. However, when expressed as per unit of haemoglobin, the reverse of the pattern found for haemoglobin concentration was found (lowest value in the 0.5% tea solids group) (Table 4.5).

Table 4.5 Effect of endotoxin injection on blood composition of rats fed diets containing different amounts of tea solids.

Diet	0%	0.2%	0.5%	1%	2%	Two way ANOVA p & F ratios
GSH mg/ml						
Endo	0.35(.04)a	0.36(.05)a	0.36(.03)a	0.39(.10)a	0.33(.08)a	NS
Saline	0.36(.07)a	0.31(.05)a	0.30(.02)a	0.32(.03)a	0.29(.03)a	
GSH mg/g Hb						
Endo	4.11(.53)a	3.22(.66)b	2.69(.28)b	3.30(1.0)b	2.67(.41)b	p_d<0.001,F=6.7
Saline	3.63(.84)a	3.20(.55)ab	2.54(.27)b	3.08(.87)ab	2.67(.24)b	
Haemoglobin g/dl						
Endo	8.7(1.4)c	11.4(1.3)b	13.4(0.6)a*	12.0(0.7)ab	12.4(1.2)ab	p_d<0.001,F=9.0
Saline	10.1(0.7)b	9.8(1.7)b	11.7(0.7)a	10.7(2.3)ab	11.0(0.5)ab	p_t=0.01,F=6.7

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from other dietary groups and values with '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t = differences due to treatment and p_d = differences due to diet.

4.3.6 Plasma composition

Plasma antioxidant capacity, in endotoxin injected animals, was higher than that of saline injected animals. This difference was statistically significant with the addition of 0.5% and 2% tea solids to the diet. Among the endotoxin injected groups, an increase was observed with increased addition of tea solids to the diet. However this increase was not statistically

significant. Among the saline injected animals such an increase in antioxidant capacity was not observed. Plasma caeruloplasmin concentrations were also high in endotoxin injected animals when compared with corresponding saline injected group. This was statistically significant at 0%, 0.5% and 2% levels. However no significant differences were observed among different dietary groups in endotoxin or saline injected animals (Table 4.6).

Table 4.6 Effect of endotoxin injection on plasma composition of rats fed diets containing different amounts of tea solids.

Diet	0%	0.2%	0.5%	1%	2%	Two way ANOVA p & F ratios
Antioxidant Capacity (TEAC)mmol/l						
Endo	0.19(.04)	0.18(.02)	0.22(.04)*	0.20(.06)	0.24(.04)*	p_t<0.001,F=15.9
Saline	0.18(.03)	0.16(.02)	0.17(.02)	0.18(.01)	0.14(.04)	
Caeruloplasmin U/ml						
Endo	0.18(.06)*	0.18(.04)	0.21(.02)*	0.18(0.2)	0.19(.02)*	p_t<0.001,F=33.0
Saline	0.14(.01)	0.15(.03)	0.14(.01)	0.15(.02)	0.14(.01)	

Results expressed as mean (± SD). n = 5. Values with a '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t = differences due to treatment.

4.3.7 Weights of Kidney, Spleen and Muscle (Tibialis) (as % of body weight).

No statistically significant differences were observed for relative weights of (weight as % of body weight) Kidney, Spleen and Tibialis muscle (Table 4.7).

Table 4.7 Effect of endotoxin injection on weights of kidney, spleen and muscle (tibialis) of rats fed diets containing different amounts of tea solids.

Diet	0%	0.2%	0.5%	1%	2%	Two way ANOVA p & F ratios
Kidney weight as % body weight						
Endo	0.93(.04)	0.91(.02)	0.98(.09)	0.98(.04)	0.90(.07)	NS
Saline	0.92(.02)	0.97(.15)	0.97(.03)	0.93(.05)	0.92(.02)	
Spleen weight as % body weight						
Endo	0.48(.11)	0.44(.07)	0.46(.10)	0.46(.06)	0.46(.09)	NS
Saline	0.41(.10)	0.38(.03)	0.41(.09)	0.41(.03)	0.45(.07)	
Muscle weight as % body weight						
Endo	0.23(.01)	0.21(.02)	0.22(.03)	0.22(.03)	0.23(.03)	NS
Saline	0.23(.04)	0.24(.02)	0.22(.03)	0.22(.02)	0.22(.02)	

Results expressed as mean (± SD). n = 5.

4.4 Discussion

Animals in the different dietary groups had no differences in body weight gain or in average food consumption during the experimental period (Table 4.2). No differences in appearance were observed. This indicates that addition of tea solids to the diet up to 2% (20g in kg of diet) had no effect on growth, food consumption or any other visible effect. The body weight gains observed in this experiment were similar to that in the earlier experiment (Table 3.2). Average food consumption was also similar to that in the earlier experiment. In the present experiment also there was a decrease in food intake after the endotoxin injection, which was caused by the release of cytokines after the endotoxin challenge (discussed in chapter 3, section 3.4). However in the present study addition of tea solids to the diet had not altered food consumption after the endotoxin challenge. In the earlier experiment, where animals which received tea solids in the diet had significantly reduced loss of appetite after the endotoxin treatment when compared with the group which did not receive tea solids in the diet. Food intake after endotoxin injection was similar to the control group (0%, with out tea solids in the diet) in all the other groups where tea solids were added at different levels (Table 4.2). In the previous study food intakes fell from 24.1 to 13.4g/d in response to endotoxin, in the animals given a diet containing no tea solids. In the present study the decrease in appetite was less severe from 24 to 17g/d. This difference in anorexic response may have influenced parameters, which are sensitive to food intake, such as liver glutathione concentration. It is commonly observed that the anorexic response to a particular dose of endotoxin will vary from time to time. Factors such as stage of development of the animal, degree of environmental stress, time of injection, diet, seasonality and ill defined factors influence the strength of the response (Bibby, 1990, Wan, 1987). The first four of these factors were

carefully eliminated in the present study.

Relative liver weights (weight as % body weight) were higher in endotoxin injected animals (Figure 4.1) when compared with saline injected animals as expected (discussed in chapter 3, section 3.4). The relative weights were similar to that in the earlier experiment in both endotoxin and saline injected animals. The differences between endotoxin injected and saline injected animals were largest in animals which received 0.5% (5g of tea solids in 1kg of diet) tea solids in their diet. There was a gradual decrease in the extent of difference in lower and higher levels of addition of tea solids.

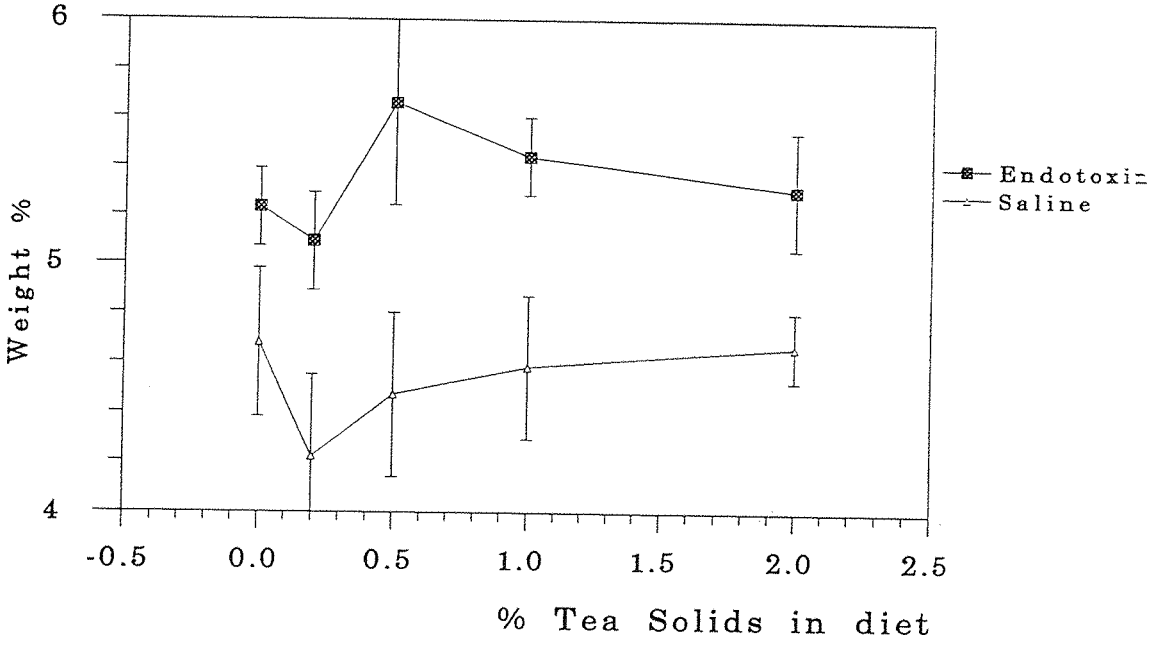


Figure 4.1 The effect of various doses of tea solids and endotoxin injection on relative liver weights.

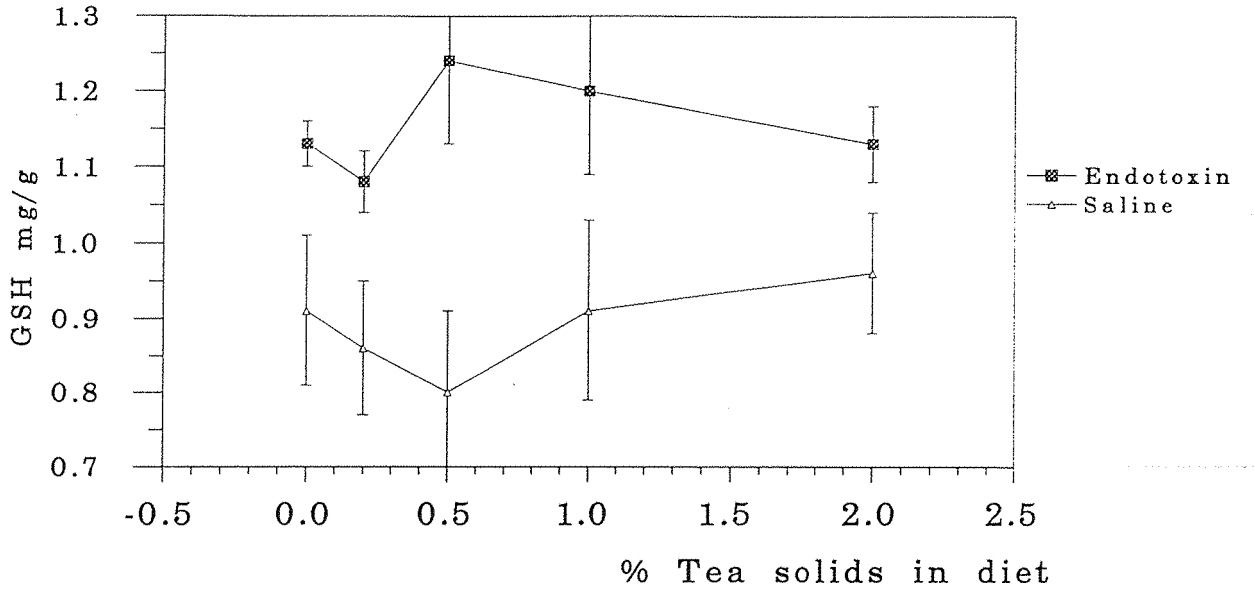


Figure 4.2 The effect of various doses of tea solids and endotoxin injection on liver Glutathione concentration (mg/g).

Liver glutathione concentrations were also higher in endotoxin treated animals when compared with corresponding saline injected group, as expected. The glutathione concentrations in both endotoxin injected and saline injected animals were similar to that in the earlier experiment. Liver glutathione also took the same pattern as relative liver weights, that is differences were largest between endotoxin and saline injected animals in the group where tea solids were added at 0.5% (5g of tea solids per kg of diet) level (Figure 4.2). This indicates that the largest alteration in liver metabolism, in response to endotoxin challenge, had occurred in the group where tea solids were added at the 0.5% level to the diet.

No statistically significant differences were found in relative lung weight or in lung

glutathione concentration among the dietary or injection groups. However differences were observed in lung glutathione concentration. In the earlier experiment an increase in lung glutathione was observed in animals supplemented with tea solids in both endotoxin and saline injected groups (Table 3.4). In that experiment tea solids had prevented the post injection loss of appetite to a certain extent and post injection food intake in tea solids supplemented animals was significantly higher than that of normally fed animals. This higher food intake for the last 24 hours, prior to killing, may be the reason for higher concentration of glutathione in the lung. Whereas in the present experiment post injection loss of appetite is less severe and tea solids had no influence on post injection food intake. This may have contributed to the lack of effect on lung glutathione concentration.

Addition of tea solids to the diet increased the haemoglobin concentrations. This effect was observed for both endotoxin and saline treated animals (Table 4.5). Among the endotoxin injected animals 0.5%, 1% and 2% addition of tea solids to the diet resulted in a significantly higher haemoglobin concentration than in 0% (with out tea solids) and 0.2% addition of tea solids with the peak at 0.5% level. In saline treated groups also 0.5% addition of tea solids to the diet resulted in the highest haemoglobin concentration. The concentration was significantly higher than all the other saline injected groups. Blood glutathione concentrations showed no differences when expressed as mg/ml. However when expressed as mg/g haemoglobin the reverse of the pattern of haemoglobin concentrations was observed. Values obtained for both haemoglobin and glutathione were similar to that obtained for the earlier experiment.

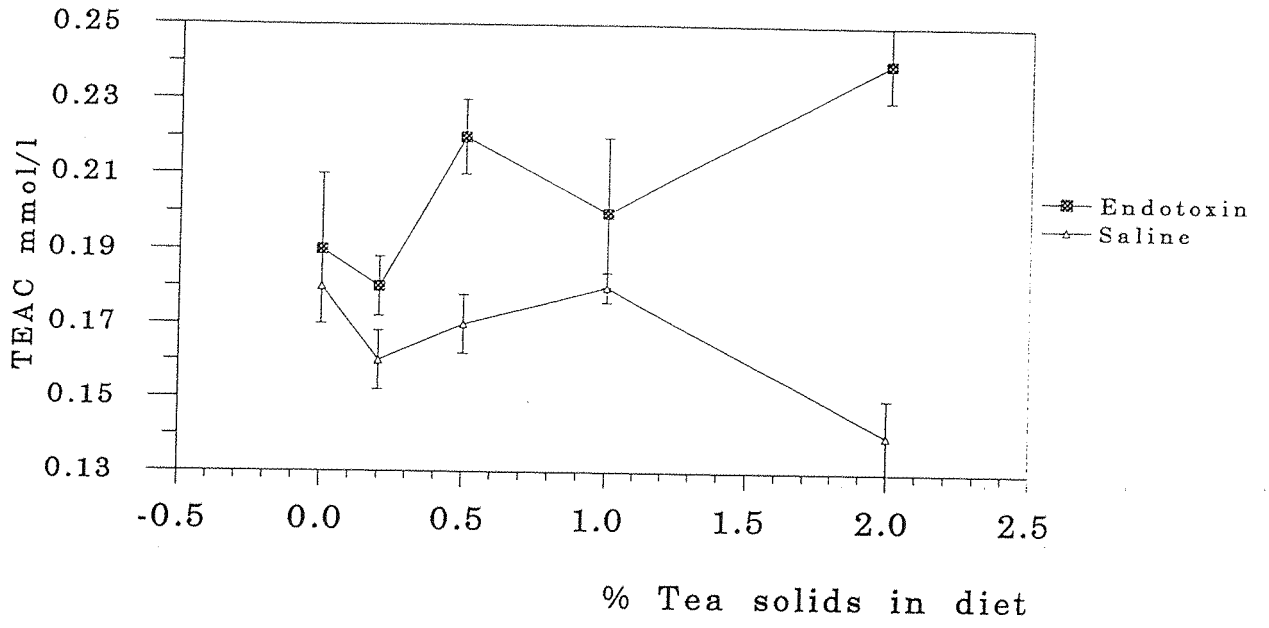


Figure 4.3 The effect of various doses of tea solids and endotoxin injection on Trolox Equivalent Antioxidant Capacity (TEAC) of plasma (mmol/l).

Antioxidant capacity (TEAC) of plasma in endotoxin injected animals were higher than that of corresponding saline injected animals (Figure 4.3). As discussed in chapter 1, this may be due to an enhancement of components of antioxidant defences during the response to the endotoxin challenge. The components would combat the oxidant species that were generated during the inflammatory response. Among the endotoxin challenged animals the antioxidant capacity slightly increased with the increased addition of tea solids to the diet. Rice-Evans and co workers have found that plant derived polyphenols (including catechin, epicatechin, quercetin and kaempferol which are found in tea infusions) have antioxidant potentials four times that of Trolox, a water soluble vitamin E analog (Rice-Evans *et al*, 1995b). However in the present study it appears that the increased antioxidant capacity

observed among endotoxin treated animals, with increasing addition of tea solids to the diet, may be due to the release of endogenous antioxidants into the circulation system in the presence of tea components, rather than the direct antioxidant activity of tea components. This is evident as an increase of antioxidant capacity was not observed among the saline treated animals with increasing addition of tea solids to the diet. Plasma caeruloplasmin concentrations were higher in endotoxin treated animals when compared with saline treated animals as expected. Addition of tea solids had not altered the caeruloplasmin level significantly.

From the results obtained in the present experiment it can be seen that the largest alterations in the response to endotoxin is observed at 0.5% (5g of tea solids to kg of diet) addition of tea solids to the diet except in the antioxidant capacity. Where the largest alterations were found when tea solids were added at 2% (20g of tea solids per kg of diet). Therefore 0.5% level was employed for the remainder of the studies reported in the thesis.

CHAPTER 5

INFLUENCE OF TEA SOLIDS ON GLUTATHIONE PEROXIDASE, GLUTATHIONE REDUCTASE AND CYTOCHROME p450

5.1 Introduction

In the earlier studies it was observed that inclusion of tea solids in the diet altered the concentration of reduced form of glutathione in liver and lung during an inflammatory response (Table 4.3, 3.4). Glutathione concentration in tissues is influenced by number of factors which include

i) the rate of recycling of the oxidised form (GSSG) to reduced form (GSH) of glutathione.

ii) the rate of synthesis of glutathione.

iii) the rate of utilisation of reduced glutathione (GSH) in detoxification reactions.

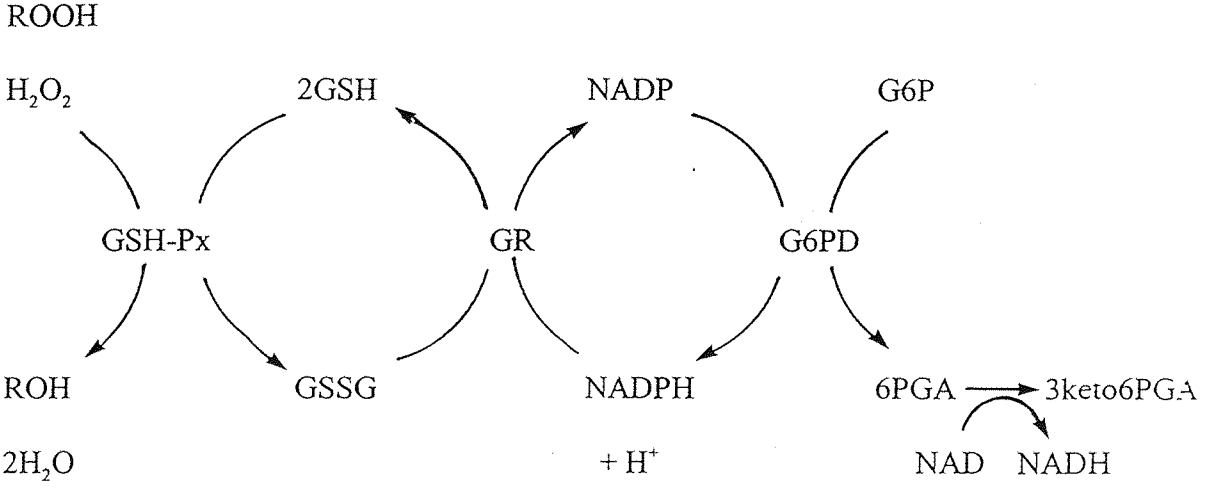


Figure 5.1 Glutathione redox cycle. GR - Glutathione reductase, GSH-Px - Glutathione peroxidase, G6P(D) - Glucose 6 Phosphate (Dehydrogenase), PGA - Phosphoglucoronic acid

Glutathione functions as part of the larger antioxidant enzyme system shown above. Here hydrogen peroxide (H_2O_2) and other organic hydroperoxides (ROOH) become reduced by the action of the enzyme glutathione peroxidase (GSH-Px). In this step reduced glutathione (GSH) becomes oxidised to its oxidised form (GSSG). Oxidised glutathione (GSSG) is again recycled back to reduced glutathione by the enzyme glutathione reductase (GR). Therefore the concentrations of GSH in a tissue will be influenced by the activities of the enzymes glutathione peroxidase (GSH-Px) and glutathione reductase (GR). In this experiment whether tea solids in the diet altered the activities of these enzymes was investigated.

Cytochrome p-450 is a major enzyme system, involved in detoxication reactions. The multiplicity of compounds in tea solids may expose the body to a xenobiotic load. Cytochrome p-450 activity results in formation of oxygenated forms of organic substances and other oxidant species such as hydrogen peroxide and super oxide anions (Figure 5.2). Cytochrome P-450 dependant oxygenations represent the first step in the metabolism of xenobiotics. This process will render the hydrophobic compounds more polar and facilitate solubilisation of these in the aqueous phase. Solubilisation will help further conversion of these compounds by other systems and removal by the body (Jacoby, 1980). The initial oxygenation convert many organic compounds to active forms. This is very important in metabolism of certain classes of drugs. These drugs are initially in inactive form, but they become active form after the oxygenation by cytochrome P-450 system (Levine, 1982). While this is a beneficial effect, evidence has been found that some foreign compounds such as insecticides, food additives and atmospheric pollutants are also converted to their active form by cytochrome P-450. Activation of such compounds will result in allergy, carcinogenesis, mutagenesis and cytotoxicity (Nebert, 1975).

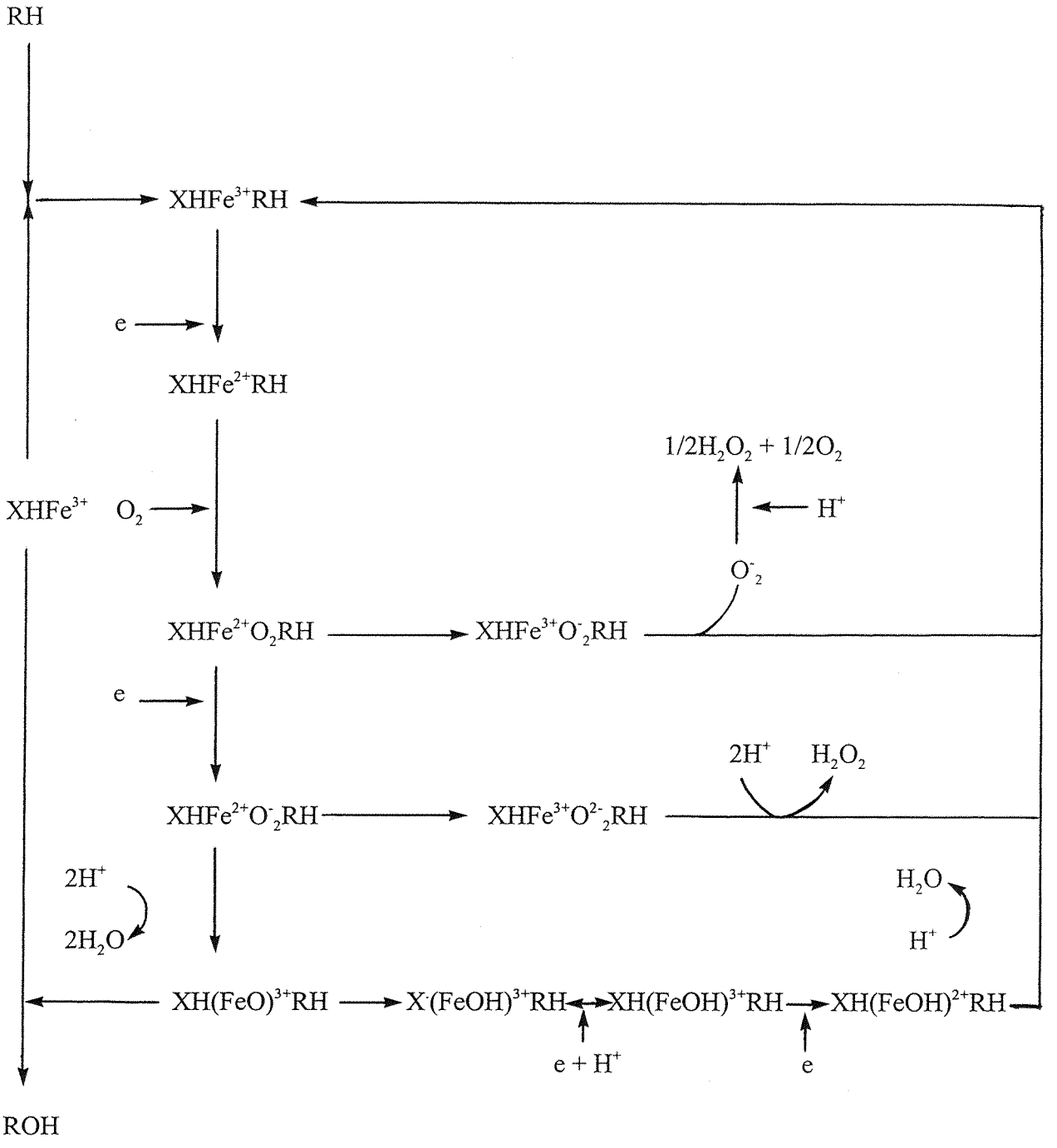


Figure 5.2 General scheme of cytochrome P-450 action.

RH - Organic substrate, Fe^{3+} - Cytochrome P-450 haem iron, X - a ligand to cytochrome P-450 haem iron.(Archakov and Bachmanova, 1989)

Glutathione enzyme systems are involved in the removal of these oxidant species (Figure 5.1). Therefore an alteration in the activity of cytochrome P-450 may result in alterations in tissue glutathione concentrations. In addition, electrons for the conversion of Fe^{3+} cytochrome P-450 haem iron to Fe^{2+} cytochrome P-450 haem iron (Fig.5.2) are provided by $FADH^{-}/FMNH_2$ system. Regeneration of this system involves NADPH/NADP (Figure 5.3). However NADPH is also used in reduction of GSSG to GSH (Figure 5.1). Therefore any change in cytochrome P-450 activity will alter the availability of NADPH for the glutathione recycling and this may also alter the tissue GSH concentrations.

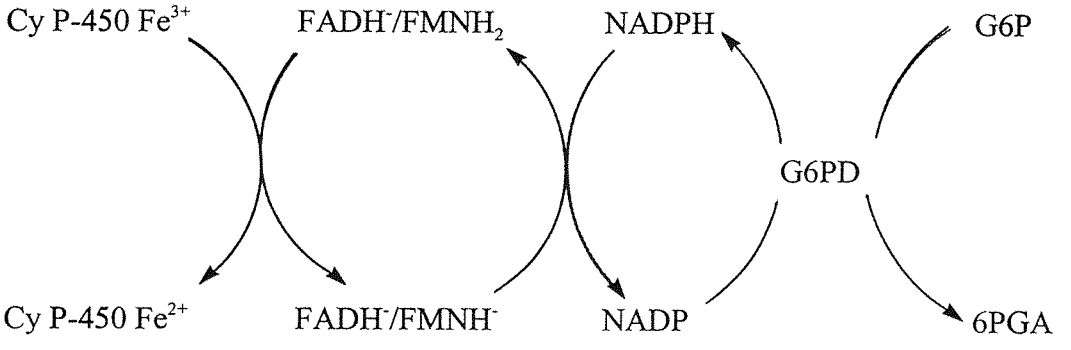


Figure 5.3 Conversion of Cytochrome p450 Fe^{3+} to Fe^{2+}

Several authors have found that plant flavonoids and other polyphenolic compounds can alter hepatic cytochrome P-450 activity (Obermeier *et al*, 1995, Kapitulnik *et al*, 1977). Obermeier and others have shown that catechins in green tea alter the activity of cytochrome P-450 (Obermeier *et al*, 1995). Therefore this study investigated whether black tea solids in the diet altered hepatic cytochrome P-450 activity.

5.2 Experimental protocol

Twenty weanling male Wistar rats weighing 64±4g (mean± S.D.), were randomly divided into two groups. They were put in separate cages. Group one was fed with a normal diet (Normal) and the second group was fed with a diet which had tea solids at 0.5% (5g per kg of diet) in addition (Tea). Animals were fed *ad libitum* on these diets for one month. Then each dietary group was divided into two. One sub group received an i.p. dose of endotoxin (Lipopolysaccharide from E.coli, 200μg/kg of body weight) and was killed 24 hours after injection. Following day, the other sub group was given a i.p. dose of sterile non pyrogenic saline (0.9g/100ml solution, 1ml per kg body weight) and pair fed with the intake of the endotoxin injected animals for further 24 hours prior to sacrifice.

Table 5.1 Composition of diets

Component	Normal (g/kg diet)	Tea(g/kg diet)
Casein	180	180
L-methionine	3	3
Vitamin mixture	20	20
Mineral mixture	40	40
Calcined magnesite	1	1
Corn Oil	30	30
Cellulose	100	100
Sugar	313	310
Starch	313	310
Decaffeinated tea solids	-	5

5.3 Results

5.3.1. Food intake and weight gain

Both groups started with the same mean body weight and there was no significant difference in the body weight gain after 21 days. There was no difference in the food intake also. No differences in appearance was observed in the tea solids fed group when compared with the normally fed group. Post injection food intake in animals fed the diet containing tea

solids were higher than that of normally fed animals. However this difference was statistically not significant (Table 5.2).

Table 5.2 Initial and final body weights, growth and food intake of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA
Initial body weight (g)			
	63.3(3.09)	64.8(5.49)	NS
Final body weight (g)			
	265.2(12.6)	271.1(14.9)	NS
Growth g/day			
	6.85(0.40)	6.99(0.46)	NS
Pre injection food intake g/day			
	31(3)	31(4)	NS
Post injection food intake g/24 hours			
	18(9)	27(10)	NS

Results expressed as mean (± SD). n = 5.

5.3.2 Body composition

The results obtained from the analysis of liver are given below.

5.3.3 Liver composition and enzyme activities

As in the earlier experiments, relative liver weights of endotoxin treated animals were higher than that of saline treated groups. This was observed for both groups receiving a normal diet or a diet supplemented with tea solids. Liver protein measurements revealed that there were no statistically significant differences between any of the groups. Glutathione peroxidase activity and glutathione reductase also showed no differences. Inclusion of tea solids in the diet had no effect on either glutathione peroxidase or on glutathione reductase activity. There were no differences in the cytochrome p450 concentrations between the dietary groups or injection groups (Table 5.3).

Table 5.3 Effect of endotoxin injection on liver composition and enzyme activities of rats fed a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	5.10(0.22)*	5.51(0.18)*	$p_t<0.001$,F=65.3
Saline	4.34(0.10)	4.25(0.47)	
Protein mg/g			
Endo	165(29)	143(20)	NS
Saline	139(12)	156(19)	
Glutathione peroxidase U/g Protein			
Endo	2.48(0.55)	2.52(0.44)	NS
Saline	3.04(0.46)	2.19(0.56)	
Glutathione reductase U/mg Protein			
Endo	43.7(10.0)	51.4(12.0)	NS
Saline	43.2(1.5)	44.6(8.2)	
Cytochrome p450 nmol/mg Protein			
Endo	2.09(0.38)	1.55(0.51)	NS
Saline	1.59(0.40)	1.39(0.29)	

Results expressed as mean (± SD). Values with a '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t = differences due to treatment.

5.4 Discussion

There were no differences in body weight of animals in two dietary groups after the experimental period. There were also no differences between the groups in body weight gain (Table 5.2). No differences in appearance was observed. As in the earlier experiments this again confirm that addition of 5% (5g of tea solids in kg of diet) had no visible effect on the growth of the experimental animals.

Among the saline treated groups, although statistically not significant, glutathione peroxidase activity of the group fed with a diet containing tea solids was lower than that of group fed the normal diet. Glutathione peroxidase activity increases in the presence of higher amounts of hydrogen peroxides and other organic peroxides in an effort to reduce them. Therefore this decreased activity in tea solids may indicate that tea solids may be acting to reduce these species. This decreased activity of glutathione peroxidase in animals supplemented with tea solids may explain the increased amounts of liver glutathione found in earlier experiments. After the endotoxin treatment, an increase in glutathione peroxidase is expected, as increased amounts of hydrogen peroxides are produced by the action of endotoxin. However no statistically significant changes were found in glutathione peroxidase activity after the endotoxin treatment. It appears that tea solids supplementation of the diet also had no effect on glutathione peroxidase activity after the endotoxin treatment.

Tea solids supplementation had no effect on the glutathione reductase activity after the endotoxin treatment or saline treatment. Endotoxin treatment also had no effect on glutathione reductase.

In this study tea solid supplementation of diet had no effect on the cytochrome p450 activity after the endotoxin treatment or saline treatment. It was assumed that large polymeric molecules such as thearubigins (the structure of which is not yet fully understood) may be metabolised through cytochrome p450 action and hence alter the activity of the cytochrome p450 activity. However from the observations made in this experiment it appears that the tea solids in the diet had not altered cytochrome p450 activity and therefore this had not contributed to any alterations in glutathione metabolism.

CHAPTER 6

INFLUENCE OF TEA SOLIDS AND VITAMIN E ON IL-6 AND OTHER RESPONSES TO ENDOTOXIN

6.1 Introduction

When cellular antioxidant defences are low the intensity of the inflammatory response is increased. Studies have shown that a reduction in cellular antioxidants increase the amount of TNF and IL-1 produced in response to an inflammatory stimulus in mice. Antioxidants such as butylated hydroxy anisole (BHA) prevented this increase (Chaudhri and Clark, 1989). Ku and co workers have shown that probucol, which has antioxidant properties inhibited endotoxin induced secretion of IL-1 from peritoneal macrophages in mice (Ku *et al*, 1990). Vitamin E deficiency is also known to exacerbate the inflammatory effects of endotoxin (Troughton and Grimble, 1993).

From our earlier studies it was indicated that tea solids may improve the antioxidant defences in the body. Therefore in the present study the effects of inclusion or exclusion of an established antioxidant (Vitamin E, antioxidant activity of vitamin E is discussed in chapter 1, section 1.3.4) on the response to an inflammatory stimulus induced by endotoxin is examined and compared with the effects of tea solids.

As mentioned above it is known that antioxidants have an effect on IL-1 and TNF production. Interleukin-6 (IL-6) is a major mediator of hepatic aspects of the inflammatory

response. The effects of dietary antioxidants such as Vitamin E and tea solids on IL-6 production are not known. Therefore in the present experiment it was investigated whether addition of tea solids and vitamin E have an effect on the plasma interleukin-6 concentrations after an injection of endotoxin.

6.2 Experimental protocol

Forty eight weanling male Wistar rats (60±10g) were divided into four groups. They were kept in separate cages and fed four different diets *ad libitum*.

1. Basal Diet (-) Vitamin E and (-) Tea solids
2. Basal Diet (-) Vitamin E and (+) Tea solids
3. Basal Diet (+) Vitamin E and (-) Tea solids
4. Basal Diet (+) Vitamin E and (+) Tea solids

Animals were fed these diets for 21 days. Then each dietary group was divided into two sub groups. One sub group received an i.p. dose of endotoxin (200μg/kg body weight) and were killed 24 hours after injection. On the following day the other sub group was injected with 0.9% (0.9g/100ml) sterile non pyrogenic saline(i.p., 1ml per kg of body weight) and pair fed with the intake of endotoxin injected animals for 24 hours prior to sacrifice. In addition to the compositional measurements made in chapter 3 and 4 lung neutrophil number was examined as an index of inflammation in the tissue.

Table 6.1 Composition of diets and dietary groups

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +
Casein	180	180	180	180
L-methionine	3	3	3	3
Premix(Vit E def)	50	50	50	50
Vitamin E	-	-	0.05	0.05
Corn Oil	30	30	30	30
Cellulose	100	100	100	100
Sugar	318	316	318	316
Starch	318	316	318	316
Decaffeinated tea solid	-	5	-	5

+ and - indicate the presence and absence respectively of vitamin E or tea solids.

6.3 Results

6.3.1 Food intake and weight gain

Animals in all dietary groups had no statistically significant differences in initial body weights. There were no significant differences in body weight gain or food intake during the experiment. After endotoxin treatment food intake was significantly reduced for all the

dietary groups. However there was no difference between dietary groups in their post injection food intake (Table 6.2).

Table 6.2 Initial and final body weights, growth and food intake of rats fed diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA
Initial body weight (g)					
	58(11)	62(13)	53(10)	66(8)	NS
Final body weight (g)					
	181(23)	180(23)	163(14)	184(18)	NS
Growth rate g/day					
	6.6(0.8)	6.6(1.4)	5.7(0.9)	6.4(0.8)	NS
Pre injection food intake g/day					
	26(3)	24(2)	24(2)	24(2)	NS
Post injection food intake g/24 Hours					
	8(10)	6(9)	3(2)	4(2)	NS

Results expressed as mean (± SD). n = 12.

6.3.2 Body composition

The results obtained from liver, lung, kidney, spleen, muscle (tibialis), blood and plasma analysis are given below.

Table 6.3 Effect of endotoxin injection on liver composition of rats given diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA p and F ratios
Weight as % body weight					
Endo	5.76(.31)ab*	5.70(.29)b*	6.09(.19)a*	5.60(.18)b*	p_t<0.001,F=35.9
Saline	4.58(.17)a	4.10(.21)b	3.89(.18)b	3.82(.08)b	p_d=0.007,F=4.5
Glutathione mg/g					
Endo	1.11(.06)a*	1.15(.06)ab*	1.20(.09)ab*	1.27(.06)b*	p_t<0.001,F=96.5
Saline	0.98(.11)a	0.89(.08)ab	0.86(.12)ab	0.81(.06)b	p_d=0.04,F=5.1
Protein mg/g					
Endo	162(45)a	194(5)a	191(16)a	197(19)a	NS
Saline	195(21)a	196(23)a	192(17)a	182(13)a	
Zinc µg/g					
Endo	37(7)a	43(7)a	43(6)a	37(4)a	NS
Saline	34(9)a	38(8)a	33(12)a	48(5)a	

Results expressed as mean (± SD). n = 6. Values with different letters are significantly different from other dietary groups and values with '*' are different from saline injected group by two way ANOVA ($p<0.05$). p_t = differences due to treatment and p_d = differences due to diet.

6.3.3 Liver composition

Liver weights (as % of body weight) of endotoxin injected animals was significantly higher than that of saline injected animals in all dietary groups. The highest values were observed when the diet contained vitamin E but not tea solids. Thus addition of tea solids in

the presence of vitamin E reduced the extent of the increase in liver weight.

Endotoxin treatment increased the hepatic GSH concentration in all dietary groups. The lowest concentration was observed in the absence of vitamin E and Tea solids in the diet. Inclusion of these two separately in the diet did not produce any statistically significant effect on the liver GSH concentration. However addition of both vitamin E and Tea solids together produced a significant increase in the liver GSH concentration. In the saline treated groups absence of tea solids, or Vitamin E, in the diet increased GSH concentration. That is, the liver GSH concentration in animals that did not receive Vitamin E or tea solids was significantly higher than that of animals which received Vitamin E and tea solids in their diet.

There were no statistically significant differences of liver Zn concentration between dietary or injection groups (Table 6.3).

Table 6.4 Effect of endotoxin injection on lung composition and neutrophil content in rats fed diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA p & F ratios
Weight as % body weight					
Endo	0.69(.10)a	0.83(.17)a*	0.73(.06)a	0.68(.07)a	p_t=0.001,F=12.5
Saline	0.61(.07)a	0.59(.06)a	0.65(.11)a	0.66(.12)a	
Glutathione mg/g					
Endo	0.57(.02)a*	0.43(.02)b	0.46(.02)b	0.47(.03)b*	p_t<0.001,F=19.0
Saline	0.41(.03)a	0.39(.02)a	0.38(.02)a	0.38(.02)a	p_d=0.04,F=2.84
Zinc µg/g					
Endo	26(3)a	26(3)a	29(2)a	33(2)b*	p_t=0.03,F=5.0
Saline	27(2)ab	24(2)b	28(4)a	27(5)ab	p_d<0.001,F=6.6
Neutrophils as % of total lung cells					
Endo	1.50(.51)a*	1.84(.88)a*	0.74(.28)b	0.77(0.28)b	p_t=0.001,F=11.5
Saline	0.22(.10)a	0.19(0.08)a	0.46(0.24)a	0.18(.14)a	p_d=0.01,F=3.5

Results expressed as mean (± SD). n = 6. Values with different letters are significantly different from other dietary groups and values with '*' are different from saline injected group by two way ANOVA ($p<0.05$). p_t = differences due to treatment and p_d = differences due to diet.

6.3.4 Lung composition

The weight of the lungs of endotoxin injected animals were higher than that of the corresponding saline injected groups.

Lung GSH concentrations were also higher in endotoxin injected animals when

compared with the saline injected animals of the corresponding dietary group. Among the endotoxin treated animals addition of tea solids and vitamin E to the diet significantly lowered the glutathione concentrations. The highest GSH was found in rats which did not receive either vitamin E or tea solids in their diet. Among the saline injected rats such a difference was not found between the dietary groups.

Zn concentration of endotoxin treated animals were highest in the group which received both vitamin E and Tea solids in their diet.

Lung neutrophils in the endotoxin injected groups were higher than their corresponding saline treated groups. However statistically significant differences were found only in the groups which received vitamin E deficient diets. Addition of vitamin E lowered the number of neutrophils among endotoxin treated groups. Among saline treated groups such a difference was not observed. Among the endotoxin treated groups addition of tea solids to the diet (Vit.E - Tea +) increased lung neutrophil numbers when compared with the diet not supplemented with vitamin E and tea solids (Vit.E- Tea-). However this increase with the addition of tea solids was not apparent when the diet had sufficient vitamin E (Table 6.4).

Table 6.5 Effect of endotoxin injection on spleen composition of rats fed diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA p & F ratios
Weight as % body weight					
Endo	0.46(.04)a	0.51(.07)a*	0.52(.03)a*	0.50(.05)a*	p_t<0.001,F=42.5
Saline	0.41(.06)a	0.39(.04)a	0.41(.01)a	0.43(.05)a	
Protein mg/g					
Endo	145(17)a*	130(12)a*	156(9)a	158(13)a	p_t=0.01,F=7.0
Saline	172(22)a	154(17)a	154(25)a	159(15)a	
Zinc µg/g					
Endo	22(1)a*	26(2)b	24(2)b*	25(2)b*	p_t<0.001,F=21.1
Saline	25(2)b	26(2)b	27(1)ab	28(1)a	p_d=0.001,F=6.2

Results expressed as mean (± SD). n = 6. Values with different letters are significantly different from other dietary groups and values with '*' are different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

6.3.5 Spleen composition

Relative spleen weights of endotoxin treated groups were higher than that of the corresponding saline treated groups. This difference was statistically significant in all groups except the group which did not receive either vitamin E or tea solids in their diet. When the relative spleen weights of endotoxin treated animals were compared, addition of tea solids and vitamin E to the diet had increased the weights, however this was statistically not

significant. Among saline treated animals such a difference was not found between the dietary groups. Among the endotoxin injected animals the lowest spleen protein concentration was found in animals which received tea solids in their diet. Such a difference was not found among the saline treated animals (Table 6.5).

6.3.6 Kidney composition

Relative kidney weights of endotoxin injected animals were significantly higher than that of saline injected animals.

In animals receiving diets containing vitamin E, endotoxin caused an increase in protein concentration. However among the saline injected animals protein concentration was raised by the absence of vitamin E from the diet. Endotoxin injection caused no further increase (Table 6.6).

Table 6.6 Effect of endotoxin injection on kidney composition of rats fed diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA p & F ratios
Weight as % body weight					
Endo	1.10(.03)ab*	1.07(.09)ab*	1.12(.07)a	1.02(.07)b	p_t<0.001,F=47.8
Saline	0.97(.07)ab	0.90(.04)b	1.00(.07)a	0.92(.04)b	p_d=0.006,F=4.7
Protein mg/g					
Endo	161(15)a	172(16)a	164(9)a*	175(10)a*	p_t<0.001,F=17.4
Saline	160(10)a	168(18)a	135(29)b	131(11)b	p_d=0.014,F=3.9
Zinc µg/g					
Endo	29(2)b	30(2)b*	24(3)c*	34(1)a	p_t<0.001,F=97.2
Saline	34(5)b	40(4)a	41(3)a	36(1)b	p_d=0.007,F=4.59

Results expressed as mean (± SD). n = 6. Values with different letters are significantly different from other dietary groups and values with '*' are different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

6.3.7 Muscle composition (tibialis)

No significant differences were observed for weight or the protein concentration of muscle (Table 6.7).

Table 6.7 Effect of endotoxin injection on muscle (Tibialis) composition of rats fed diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA
Weight as % body weight					
Endo	0.22(.02)	0.23(.03)	0.23(.01)	0.24(.01)	NS
Saline	0.23(.02)	0.24(.02)	0.25(.02)	0.25(.02)	
Protein mg/g					
Endo	185(21)	185(28)	159(25)	161(22)	NS
Saline	167(14)	164(10)	191(25)	161(21)	

Results expressed as mean (± SD). n = 6.

6.3.8 Blood composition

There were no statistically significant differences in the GSH or haemoglobin concentration of blood (Table 6.8).

Table 6.8 Effect of endotoxin injection on blood composition of rats fed diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA
Glutathione mg/g Hb					
Endo	4.78(0.99)	4.65(1.14)	5.76(0.76)	5.88(.68)	NS
Saline	6.90(3.55)	5.02(1.10)	5.81(0.64)	5.79(0.92)	
Haemoglobin g/dl					
Endo	12(1)	13(1)	13(1)	13(1)	NS
Saline	11(2)	13(1)	13(2)	13(1)	

Results expressed as mean (± SD). n=6.

6.3.9. Plasma composition

Among the saline treated animals the group without vitamin E or tea supplementation had significantly higher concentrations of albumin. Animals supplemented with both tea solids and vitamin E had the lowest concentrations of albumin among the saline treated animals. Endotoxin injection frequently results in a fall in albumin concentration. However a significant decrease in concentration is only observed in animals fed the diet deficient in vitamin E and tea solids. Consequently there were no differences in albumin concentrations among the dietary groups of endotoxin treated animals.

Endotoxin injection resulted in a significant increase in caeruloplasmin concentration in all dietary groups. Among the endotoxin injected animals the group supplemented with

both vitamin E and tea solids had a caeruloplasmin concentration which was significantly lower than those observed in the other dietary groups. In animals receiving saline also, a addition of a combination of tea solids and vitamin E had a similar effect. In animals receiving endotoxin those which consumed the diet without vitamin E or tea solids exhibited the highest concentration of IL-6. Addition of vitamin E or tea solids resulted in a significantly lower IL-6 concentration. Addition of a combination of both produced synergistic effect. In the saline treated groups no differences were observed (Table 6.9).

Table 6.9 Effect of endotoxin injection on plasma composition of rats fed diets containing variable amounts of vitamin E and tea solids.

Diet	Vit.E - Tea -	Vit.E - Tea +	Vit.E + Tea -	Vit.E + Tea +	Two way ANOVA p & F ratios
Albumin mg/ml					
Endo	40.94(4.50)a*	39.87(2.10)a	41.07(3.41)a	41.51(2.01)a	p_t=0.03,F=4.8
Saline	50.62(4.28)a	43.66(3.48)b	41.76(7.73)bc	37.72(2.58)c	p_d=0.005,F=4.87
Caeruloplasmin U/ml					
Endo	0.23(.02)a*	0.23(.03)a*	0.25(.02)a*	0.20(.01)b*	p_t<0.001,F=16.5
Saline	0.17(.02)a	0.17(.02)a	0.16(.01)a	0.13(.02)b	p_d<0.001,F=9.5
Interleukin-6 ng/ml					
Endo	2.05(.22)a*	1.68(.13)b	1.69(.17)b	1.65(.20)b	p_t<0.001,F=12.9
Saline	1.49(.14)a	1.47(.33)a	1.50(.12)a	1.53(.08)a	p_d=0.04,F=2.9

Results expressed as mean (± SD). n = 6. Values with different letters are significantly different from other dietary groups and values with '*' are different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

6.4 Discussion

There were no differences in the food intake or growth of animals between different dietary groups. This is in agreement with earlier experiments. Mean rates of growth and food consumption were similar to that in earlier experiments. As in the earlier experiments this demonstrate that addition of tea solids at 0.5% level to the diet had no visible effect on the experimental rats. Similarly the presence or absence of vitamin E (50mg/ kg diet) produced no visible effect. There was a decrease in food intake after the endotoxin injection as expected. However in the present study the loss of appetite after endotoxin injection was not modified by the presence or absence of neither tea solids nor vitamin E in diets. The presence of tea solids at a concentration of 5g/kg diet had reduced the extent of the loss of appetite following endotoxin injection in an earlier study (Chapter 3, Table 3.2).

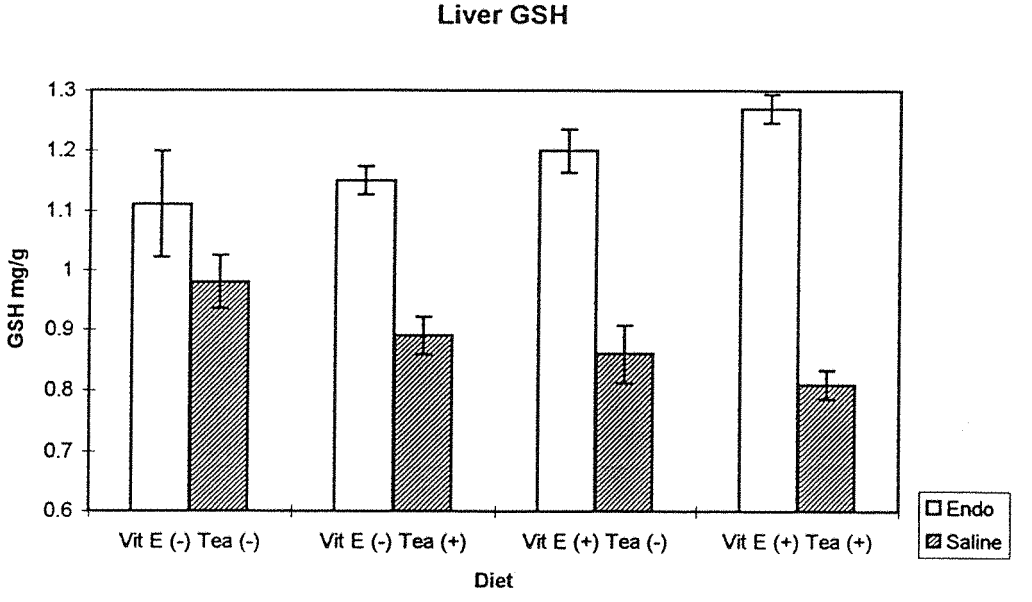


Figure 6.1 Liver glutathione concentration of rats given diets with or without vitamin E and tea solids, and injected with endotoxin or saline.

Relative liver weights of endotoxin injected animals was higher than corresponding saline injected groups. This was in agreement with observations in earlier experiments. Endotoxin treatment increased the hepatic GSH concentration in all dietary groups (Figure 6.1). The lowest concentration was observed in the absence of vitamin E and tea solids in the diet. Inclusion of these two separately increased the concentrations. However this increase was not statistically significant. Addition of both vitamin E and tea solids together produced a significant increase in the liver GSH concentrations among endotoxin treated animals. This may be due to the reduced utilisation of GSH in the presence of vitamin E and substances with antioxidants properties in tea solids. In the saline treated groups a compensatory increase in GSH occurred in the absence of tea solids or vitamin E from the diet. Improvement of antioxidant defences by the addition of a known antioxidant (vitamin E) and a suspected antioxidant dietary component (tea solids) suppressed the compensatory increase in an additive fashion. The compensatory increase in GSH was observed in blood and lung tissue in the absence of vitamin E and tea solids from the diet (Table 6.4 and 6.8). Furthermore the concentration of plasma caeruloplasmin, another component of antioxidant defence, also increased in saline injected animals consuming the diet lacking vitamin E or tea solids (Table 6.9). This phenomenon had been observed in other studies where liver GSH concentrations were raised in experimental animals receiving a vitamin E deficient diet (Grimble, 1994).

In the endotoxin treated animals lung GSH responded in an opposite manner to that observed for liver. That is, vitamin E and tea solids addition had decreased the GSH concentration in lung while increasing it in liver. This differences in the liver and lung was observed in the earlier experiment also (Chapter 5).

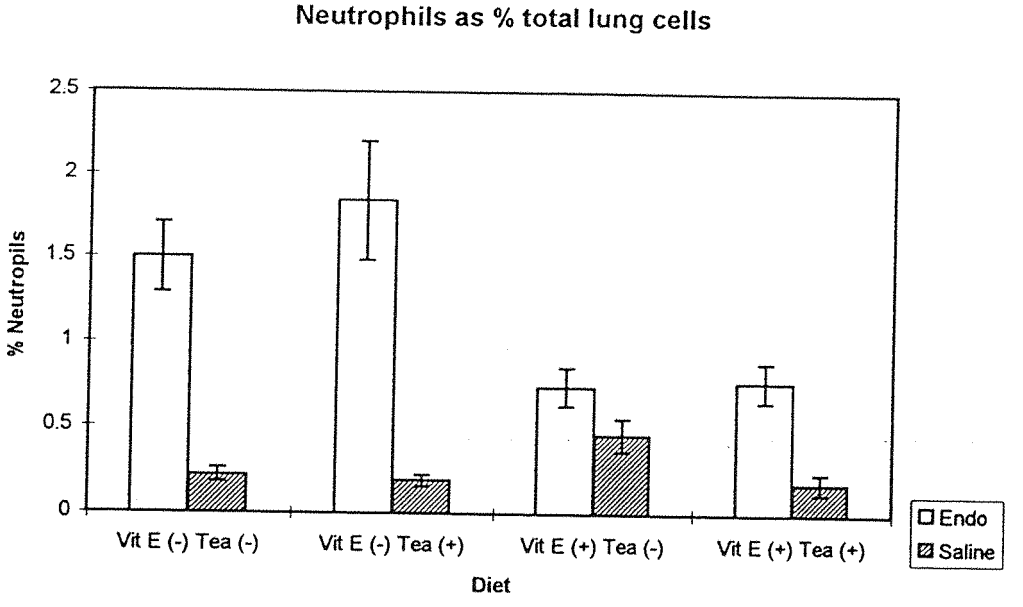


Figure 6.2 Lung neutrophils as % of total lung cells of rats fed diets with or without vitamin E and tea solids, and injected with endotoxin or saline.

Lung neutrophil numbers (Fig 6.2) in the endotoxin injected animals were higher than saline treated animals as expected, indicating the inflammatory response in these animals. Addition of vitamin E to the diet had decreased the number of neutrophils in the endotoxin treated animals as expected. However addition of tea solids to the vitamin E deficient diet did not decrease the lung neutrophils as expected from the results obtained in the earlier studies which suggest that tea solids contain substances with antioxidant properties. An apparent increase in the number of neutrophils was observed in the presence of tea solids in the diet. Thus while tea solids contain substances which have antioxidant properties they or other components act in a different manner to vitamin E. There was no increase of lung neutrophils in animals supplemented with tea solids prior to saline treatment. Therefore it appears that tea

solids do not provide an inflammatory stimulus. It would appear that the presence of an on going inflammatory response is necessary for tea solids to enhance lung neutrophil numbers.

Recruitment of neutrophils to the lung tissue is induced by the chemoattractants released by alveolar macrophages and neutrophils already present in the tissue. The chemoattractants are released by these cells when they encounter pathogenic microorganisms or other foreign stimulants such as endotoxin. Neutrophil migration from capillaries to the lung tissue takes place in stages. Initially they adhere to endothelial cells of the capillaries. They then pass through the endothelial cells by diapedesis. Finally the neutrophils migrate through the lung tissue up the chemoattractant gradient by chemotaxis (discussed in section 1.10). Interleukin-8 (IL-8) and neutrophil chemotactic factor (NCF) released by alveolar macrophages help the neutrophils to move through the lung tissue by chemotaxis. In the scientific literature no information was found on the effect of tea components on recruitment of neutrophils to lung tissue. However Rohrbach and others have shown that cotton condensed tannins increase the secretion of NCF from alveolar macrophages. This resulted in the increased recruitment of neutrophils to lung tissue (Rohrbach, 1989). Cotton condensed tannins are comprised mainly of polymerised products of flavan-3-ols and flavan-3,4-diols (Salunkhe *et al*, 1990).

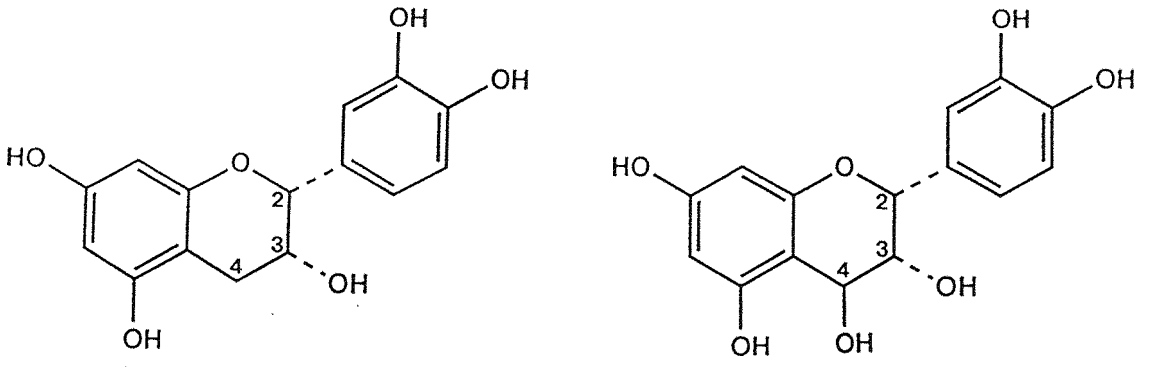


Fig.6.3 Structures of flavan-3-ols and flavan-3,4-diols.

Tea also contain similar compounds in its theaflavin (TF) and thearubigin(TR) fractions (see chapter 1). Therefore tea components may also be acting in a similar way to increase the recruitment of neutrophils to lung tissue.

There were no statistically significant differences in blood glutathione concentrations between the groups. This was in agreement with the results obtained for the earlier experiments. Blood haemoglobin concentrations also does not show any differences between the various supplemental groups.

In plasma, no differences were observed for albumin concentrations among the different dietary groups of endotoxin injected animals. However in the saline injected group there was a increase of albumin in the dietary group which did not receive either vitamin E or tea solids in their diet. This increase may have resulted from the poor antioxidant status of

those animals. Increased albumin might improve antioxidant status by acting as sacrificial antioxidant.

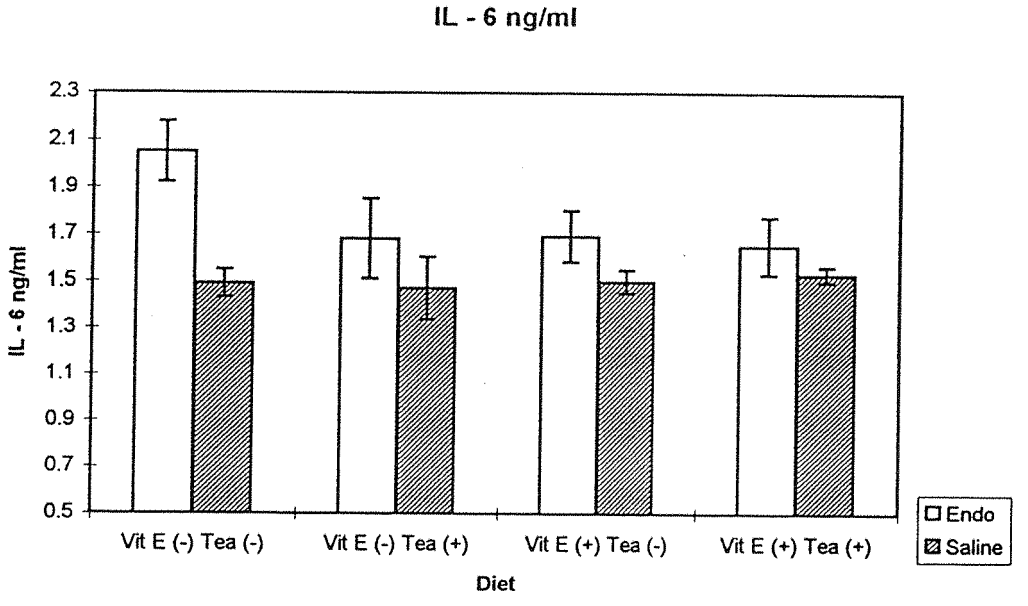


Figure 6.4 Plasma Interleukin-6 concentrations of rats fed diets with or without vitamin E and tea solids, and injected with endotoxin or saline.

Plasma interleukin-6 concentration (Fig 6.4) among the saline injected animals were similar between the different dietary groups. However among the endotoxin injected groups a greater increase was observed when both vitamin E and tea solids were absent from the diet. Addition of either substance to the diet reduced the extent of increase, with lowest levels in the group where both vitamin E and tea solids were added to the diet. A number of studies have shown that synthetic antioxidants, such as butylated hydroxy anisole (BHA) and N-acetyl cysteine (NAC), inhibit IL-1, TNF and IL-8 production (Peristaris *et al*, 1992, Chaudhri and Clark, 1989). Results from present study show that both vitamin E and tea

solids prevent the enhanced IL-6 production which occurs when the antioxidant defences are poor. The effect of tea solids on IL-6 is very similar to that of vitamin E in contrast to the effect of the two dietary components on lung neutrophil numbers in the presence of an inflammatory response. Both suppress the IL-6 production after the endotoxin injection to a similar extent. The similarity in action of vitamin E and tea solids indicate that components in tea solids may be acting as antioxidants.

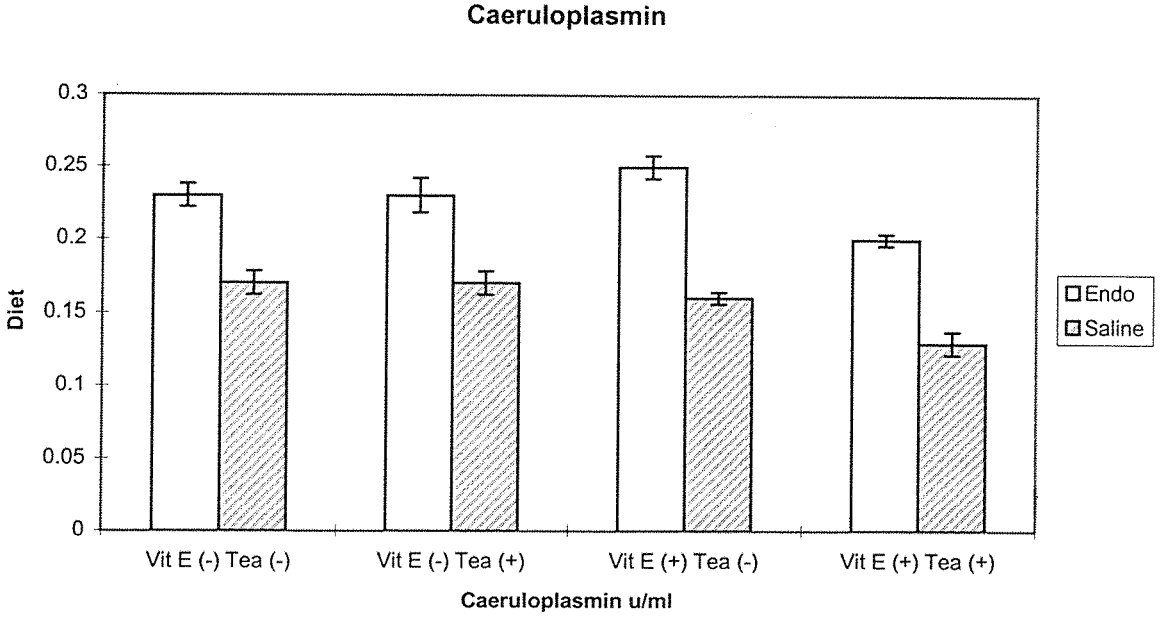


Figure 6.5 Plasma Caeruloplasmin concentration of rats fed diets with or without vitamin E and tea solids, and injected with endotoxin or a saline.

Plasma caeruloplasmin concentrations (Fig 6.5), among the saline injected animals, were highest in the group that did not receive either vitamin E or tea solids in the diet. There was a decrease with the addition of these with the lowest levels in the group where both vitamin E and tea solids were added to the diet. As mentioned earlier this may be to increase

the antioxidant defences when other components of antioxidant defences were reduced in amounts. The concentrations of caeruloplasmin in endotoxin injected animals were significantly higher than that of corresponding saline injected groups. This is expected as increased cytokine production among the endotoxin injected animals will stimulate caeruloplasmin production. Among the endotoxin treated groups the lowest levels were found in the group which received both vitamin E and tea solids in their diet. This is in agreement with the result that IL-6 levels were lowest in this group, as caeruloplasmin production is stimulated by IL-6.

The results obtained for IL-6, caeruloplasmin and hepatic glutathione in this study strongly support the idea that components in tea solids are acting as antioxidants *in vivo*. It is interesting to note that tea solids act in a different manner depending upon whether the animal is undergoing an inflammatory stress or not. In the absence of stress, their presence in the diet prevent the compensatory rise in GSH in liver. In the presence of an inflammatory stress they enhance liver GSH concentration particularly in the presence of vitamin E (Fig. 6.1).

CHAPTER 7

INFLUENCE OF TEA SOLIDS ON GLUTATHIONE CONCENTRATION OF RATS GIVEN A LOW PROTEIN DIET

7.1 Introduction

A number of studies have shown that low protein diets impair the normal response to inflammatory agents (Bell and Hoffman-Goetz, 1983, Jennings and Elia,1990, Grimble *et al*, 1992). An inflammatory response will result in, among other things, an increase in free radical production by macrophages and other immune cells. Antioxidant defences will also become enhanced during the response to prevent damage to the host. An increase in production of glutathione in liver plays a part in the enhancement of antioxidant defences. Low protein consumption is known to impair the increase in glutathione production during inflammatory response (Grimble, 1992). Inclusion of cysteine in the diet improves the production of glutathione.

Cysteine is one of the constituent amino acids of glutathione. In the production of glutathione, combination of glutamic acid with cysteine under the influence of glutamyl cysteine synthetase is the rate limiting step in the biosynthetic pathway. Cysteine is the rate limiting substrate for the pathway, thus GSH concentrations fall when the diet contains insufficient amounts of the amino acid or its precursor L-methionine.

In the earlier study (Chapter 6) it was observed that tea solids in the diet increased the

hepatic glutathione concentrations following an inflammatory stimulus, when the antioxidant defences are impaired by lack of vitamin E in the diet. From this study it was not clear whether tea solids provide sulphur amino acids which are required in the production of glutathione. Therefore this experiment investigates whether tea solids could increase the production of glutathione when animals consume a low protein diet which is relatively deficient in methionine and cysteine. In the study the response of endotoxin was examined in rats fed a low protein supplemented with L-cysteine or L-alanine or tea solids.

7.2 Experimental Protocol

Thirty weanling male Wistar rats weighing (66±6g, mean±SD), were divided into three groups. They were housed in separate cages. Group one was fed with a low protein diet(8% casein) with the addition of alanine (A) and the second group was fed with a similar diet which had tea solids at 0.5% instead of alanine (T). The third group was also fed a similar diet but here the addition was cysteine (C) (in place of alanine or tea solids). Rats were fed on these diets for one week. Then each dietary group was divided into two. One group received an i.p. dose of endotoxin (200μg/kg body weight) and were killed 24 hours after injection. The other group was injected with 0.9% sterile non pyrogenic saline and pair fed with the intake of the endotoxin animals for 24 hours prior to sacrifice.

Table 7.1 Composition of diets and dietary groups (g/kg)

Diet	Alanine	Tea	Cysteine
Casein	80	80	80
Vitamin mixture	20	20	20
Mineral mixture	40	40	40
Calcined magnesite	1	1	1
Corn Oil	30	30	30
Cellulose	100	100	100
Sugar	358.5	362	360.5
Starch	358.5	362	360.5
Alanine	12	-	-
Decaffeinated tea solids	-	5	-
L-cysteine	-	-	8

7.3 Results

7.3.1 Food intake and weight gain

Animals in all dietary groups had no significant differences in initial body weights. However after feeding for one week the body weights of cysteine fed animals were significantly higher than other groups. Weight gain per day also exhibited the same trend.

However, though the food intake of cysteine fed animals were slightly higher than the other two groups this difference was not significant (Table 7.2).

Table 7.2 Initial weight, final weight, growth and food intake of rats fed a protein deficient diet supplemented with alanine, cysteine or tea solids and injected with endotoxin or saline.

Diet	Protein Def + Alanine	Protein Def + Tea	Protein Def + Cysteine	Two way ANOVA p & F ratios
Initial weight (g)				
Endo	65(6)a	70(6)a	66(8)a	NS
Saline	64(4)a	68(7)a	65(5)a	
Final Weight (g)				
Endo	80(4)a	83(9)a	100(7)b	p_d<0.001,F=10.9
Saline	84(8)a	89(16)a	102(10)b	
Growth g/day				
Endo	2.80(.87)a	2.15(1.07)a	5.17(.44)b	p_d<0.001,F=30.3
Saline	3.32(.98)a	3.00(.67)a	5.29(.68)b	
Pre injection food intake g/day				
Endo	18.7(3.9)a	20.0(1.4)a	20.9(2.2)a	NS
Saline	18.0(1.1)a	18.7(1.7)a	19.3(1.3)a	
Post injection food intake g/day				
Endo	11.2(2.2)a	10.0(5.0)a	13.6(4.0)a	NS
Saline	11.2(2.2)a	10.0(5.0)a	13.6(4.0)a	

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different form other dietary group by two way ANOVA ($p<0.05$). p_d= differences due to diet.

7.3.2 Body composition

The results obtained from liver, lung, blood and plasma analysis are given below.

Table 7.3 Effect of endotoxin injection on liver composition of rats given a protein deficient diet supplemented with alanine, cysteine or tea solids.

Diet	Protein Def + Alanine	Protein Def + Tea	Protein Def + Cysteine	Two way ANOVA p & F ratios
Weight as % body weight				
Endo	4.75(.23)a*	4.90(.47)a*	5.94(.43)b*	p_t<0.001,F=48.4
Saline	3.55(.73)a	3.78(.73)ab	4.28(.33)b	p_d<0.001,F=9.4
GSH mg/g				
Endo	0.39(.05)a	0.42(.06)a	1.09(.13)b*	p_t<0.001,F=129
Saline	0.32(.02)a	0.34(.03)a	0.39(.07)a	p_d<0.001,F=99

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from other dietary groups and values with `*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

7.3.3 Liver composition

Relative liver weights (Liver weight as % of body weight) of endotoxin injected animals were significantly higher than the corresponding saline injected groups. Among the endotoxin injected groups highest relative liver weights were found in the cysteine supplemented group. This was statistically different from the groups with alanine and tea

solids supplementation. Relative liver weights of saline injected also had the same pattern, that is the cysteine supplemented group had the highest weight.

Liver glutathione of saline injected animals was not different among the different dietary groups. Among the endotoxin injected groups cysteine supplemented animals had the highest glutathione concentration which was significantly different from the other dietary groups (Table 7.3).

Table 7.4 Effect of endotoxin injection on lung composition of rats given a protein deficient diet supplemented with alanine, cysteine or tea solids.

Diet	Protein Def + Alanine	Protein Def + Tea	Protein Def + Cysteine	Two way ANOVA p & F ratios
Weight as % body weight				
Endo	0.79(.03)a	0.78(.06)a	0.75(.04)a	NS
Saline	0.74(.12)a	0.71(.07)a	0.77(.04)a	
GSH mg/g				
Endo	0.35(.01)a	0.35(.03)a	0.42(.02)b*	p_t<0.001,F=39.5
Saline	0.33(.01)a	0.33(.02)a	0.32(.03)a	p_d=0.008,F=5.9

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from other dietary groups and values with `*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

7.3.4 Lung composition

There were no differences between the relative lung weight of different groups. Glutathione concentrations of saline injected animals also did not show any differences among dietary groups. However among the endotoxin injected animals the cysteine supplemented group had the highest concentration of glutathione. This difference between cysteine supplemented group and alanine and tea supplemented groups was statistically significant (Table 7.4).

Table 7.5 Effect of endotoxin injection on blood composition of rats given a protein deficient diet supplemented with alanine, cysteine or tea solids.

Diet	Protein Def + Alanine	Protein Def + Tea	Protein Def + Cysteine	Two way ANOVA p & F ratios
Haemoglobin g/dl				
Endo	13.02(1.15)a	14.46(1.46)a	13.46(1.44)a	NS
Saline	13.32(2.11)a	13.34(2.09)a	10.72(2.59)a	
GSH mg/g Hb				
Endo	3.58(.60)a	3.36(.23)a	3.03(.47)a*	p_t=0.002,F=11.2
Saline	3.77(.79)a	3.81(.83)a	4.81(.83)b	

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from other dietary groups and values with '*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

7.3.5 Blood composition

There were no differences in the haemoglobin content between different dietary or injection groups. The glutathione concentration among the endotoxin injected animals were also similar in all dietary groups. However among the saline injected animals the cysteine supplemented group had higher glutathione concentrations than the groups receiving diets supplemented with alanine and tea solids (Table 7.5).

Table 7.6 Effect of endotoxin injection on plasma caeruloplasmin concentration of rats given a protein deficient diet supplemented with alanine, cysteine or tea solids.

Diet	Protein Def + Alanine	Protein Def + Tea	Protein Def + Cysteine	Two way ANOVA p & F ratios
Caeruloplasmin U/ml				
Endo	0.22(.04)a	0.27(.05)b*	0.23(.02)a*	p_t=0.001,F=35.5
Saline	0.19(.02)a	0.18(.03)a	0.13(.01)b	p_d=0.01,F=5.1

Results expressed as mean (± SD). n = 5. Values with different letters are significantly different from other dietary groups and values with `*' are significantly different from saline injected group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

7.3.6 Plasma Composition

Group receiving the diet supplemented with tea solids had the highest caeruloplasmin concentration among the endotoxin treated animals and was significantly different from other dietary groups. Among the saline treated groups, the lowest concentration was found in

cysteine supplemented group which was also significantly different from all the other dietary groups (Table 7.6).

7.4 Discussion

When the growth rate of this study was compared with that of the earlier studies (Table 3.4.1) where the diet contained normal amounts of protein (18% casein) it is evident that the growth of the rats had been impaired when rats receive 8% casein in their diet. However when the cysteine supplemented group is compared with the alanine and tea supplemented groups a marked improvement in growth was observed in the cysteine supplemented group (Figure 7.1). This demonstrates the importance of cysteine in the growth and development of the rat. This beneficial effect of cysteine on the growth of rats receiving a low protein diet had been demonstrated earlier (Grimble *et al*, 1992). However such a marked difference was not observed for the food intake. This demonstrates that efficiency of converting food into body weight is impaired in the alanine and tea solids supplemented group. Further, the growth of tea solids supplemented animals were slightly less than alanine supplemented animals. This may be due to the interaction of proteins in the diet with polyphenols in tea. This would reduce the absorption of proteins and may have a growth impairment effect when the diet is deficient in protein.

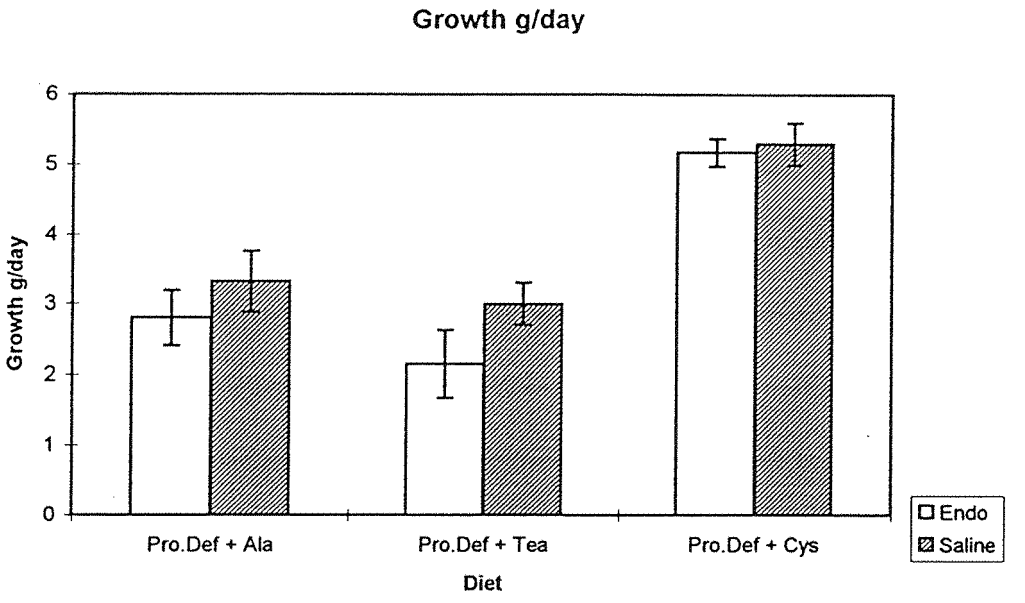


Figure 7.1 Growth of rats given a protein deficient diet supplemented with alanine, cysteine or tea solids, prior to injection of endotoxin or saline.

Relative liver weights also reflected the differences in the body weights. Cysteine supplemented rats had a significantly higher relative liver weights than the other two groups. Liver glutathione concentration in group supplemented with tea solids had a slight increase when compared with alanine supplemented group, however the cysteine supplemented group exhibited a marked increase of hepatic glutathione concentration above the other two groups (Figure 7.2). Decreased glutathione concentration with a protein deficient diet had been observed in earlier studies also (Grimble, 1992). At the levels of protein given in the diet in the present experiment sulphur amino acids will not be provided in adequate amounts for the glutathione synthesis. Therefore a decreased glutathione concentration was observed in the alanine and tea solids supplemented groups as against the cysteine supplemented group. This

demonstrates that tea solids were not able to provide sulphur amino acids for the synthesis of glutathione. This observation provide support for the observation made in the earlier study that tea solids supplementation (in a vitamin E deficient diet, Figure 6.1) may increase the hepatic glutathione concentration by reducing the glutathione utilisation through antioxidant activity of components in tea solids.

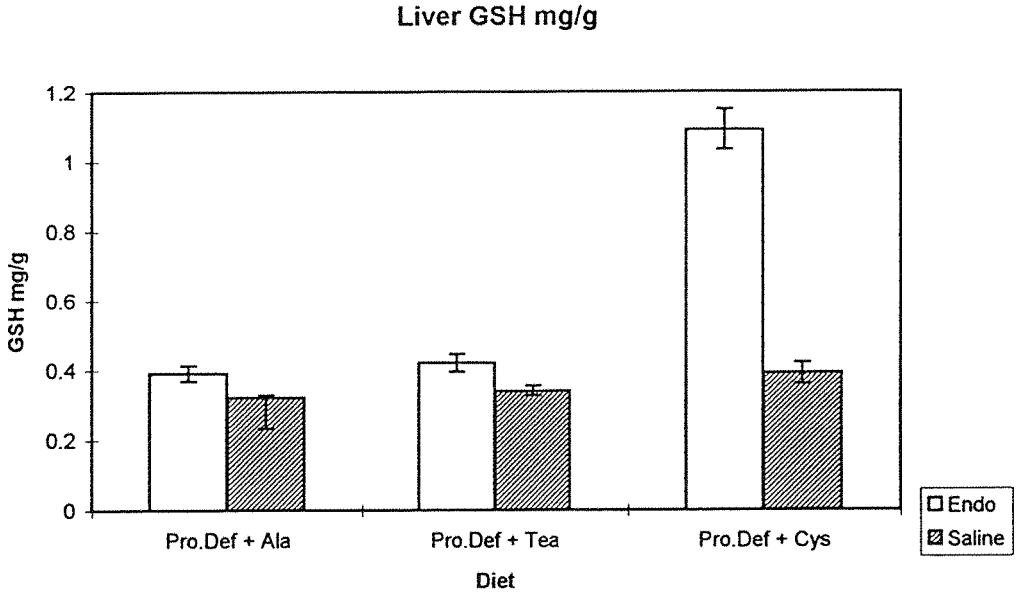


Figure 7.2 Liver glutathione concentration of rats given a protein deficient diet supplemented with alanine, cysteine or tea solids and injected with endotoxin or saline

Plasma caeruloplasmin concentrations were significantly higher in the group supplemented with tea solids when compared with other two groups, after the endotoxin challenge. This was not observed in the earlier studies where the animals received adequate

amounts of protein and sulphur amino acids in their diet. This effect of tea solids was not apparent when antioxidant defences were compromised by a lack of vitamin E (Table 6.9).

The observations made in this study further emphasise the complex nature of the ability of tea solids to improve the antioxidant defences during an inflammatory challenge. While tea solids can enhance hepatic GSH concentrations during an inflammatory response in the presence of an adequate vitamin E and protein intake (Fig. 6.1) they are unable to do so in the presence of an adequate vitamin E intake alone (Fig. 7.2). However tea solids are not enhancing GSH by supplementing the sulphur amino acid intake (Fig. 7.2).

CHAPTER 8

EFFECT OF ADDITION OF TEA SOLIDS TO THE DIET AND POST INJECTION PAIR FEEDING OR *AD LIB* FEEDING ON MYELOPEROXIDASE ACTIVITY AND OTHER RESPONSES TO ENDOTOXIN

8.1 Introduction

In the earlier experiments it was found that tea solids in the diet had altered some responses to endotoxin challenge. In these experiments, during the 24 hours after the endotoxin challenge, food consumption was reduced to a large and variable extent between studies. The control groups in these experiments were given a saline injection to produce the mild stress of injection and they were pair fed with the same amount of food consumed by endotoxin challenged animals. This reduced food consumption 24 hours prior to sacrifice may have an effect on some of the parameters measured, such as glutathione which is altered by dietary factors. Therefore in this experiment in addition to the endotoxin challenged and pair fed groups, another group was fed *ad libitum* 24 hours prior to sacrifice and compared with above two groups.

Histological examination of lung tissues in an earlier experiment had shown that tea solids in the diet had increased the number of neutrophils in the tissue when challenged with endotoxin (Figure 6.2). This indicates that components in tea solids may have further stimulated the production of activated neutrophils under the influence of the endotoxin challenge. Activated neutrophils contain large amounts of myeloperoxidase which is used in

host defence mechanisms. Myeloperoxidase catalyses the formation of hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions.

$$H_2O_2 + Cl^- \xrightarrow[\text{Myeloperoxidase}]{} OCl^- + H_2O$$

Hypochlorous acid is a potent oxidising and chlorinating agent that react rapidly with amines to yield cytotoxic N-chloramines. Production of HOCl by myeloperoxidase activity is important in destroying any invading microorganisms. The amount of myeloperoxidase in a tissue is directly proportional to the number of activated neutrophils present. Therefore in the present experiment myeloperoxidase activity of lung was measured to estimate the number of neutrophils in lung.

8.2 Experimental protocol

Thirty six weanling male Wistar rats weighing 61±6g (mean± S.D.), were divided into two groups. They were put in separate cages. Group one was fed with a normal diet (Normal) and the second group was fed with a diet which had tea solids at 0.5% (5g/kg diet) in addition (Tea). Animals were fed on these diets for 21 days. On day 21 each dietary group was divided into three. One sub group received an i.p. dose of endotoxin (200µg/kg of body weight) and was killed 24 hours after injection. Following day, the other two sub groups were given an i.p. dose of sterile non pyrogenic saline (0.9g in 100ml solution, 1ml per Kg body weight) and one sub group was pair fed with the intake of the endotoxin injected animals for 24 hours,

while the last sub group was fed *ad libitum* for 24 hours prior to sacrifice.

Table 8.1 Composition of diets and dietary groups

Component	Normal (g/kg diet)	Tea(g/kg diet)
Casein	180	180
L-methionine	3	3
Vitamin mixture	20	20
Mineral mixture	40	40
Calcined magnesite	1	1
Corn Oil	30	30
Cellulose	100	100
Sugar	313	310
Starch	313	310
Decaffeinated tea solids	-	5

8.3 Results

8.3.1. Food intake and weight gain

Both groups started with the same mean body weight and there was no significant difference in the body weight gain after 21 days. There was no difference in the food intake also. Any differences in appearance or behaviour was not observed in the tea solids fed group when compared with the normally fed group (Table 8.2).

Table 8.2 Initial and final weights, growth and food intake of rats given a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Initial body weight(g)			
Endo	68.7(7.0)	63.0(14.7)	NS
Ad Lib	65.5(5.3)	55.2(19.8)	
Pair fed	61.5(9.2)	53.0(19.3)	
Final body weight(g)			
Endo	222.3(18.5)	217.7(25.1)	NS
Ad lib	216.8(14.9)	208.2(29.0)	
Pair fed	215.8(26.7)	194.7(24.9)	
Growth g/day			
Endo	7.32(.82)	7.37(.63)	NS
Ad lib	7.21(.49)	7.29(.47)	
Pair fed	7.35(.86)	6.75(.50)	
Pre injection food intake g/day			
Endo	29.8(2.6)	29.7(2.3)	NS
Ad lib	30.7(1.9)	28.8(2.5)	
Pair fed	31.0(5.1)	26.7(1.5)	
Post injection food intake g/24 hours			
Endo	16.5(7.5)	12.8(10.0)	$p_t<0.001, F=41.2$
Ad lib	33.8(2.9)*	30.3(2.9)*	
Pair fed	16.5(7.53)	12.8(10.0)	

Results expressed as mean (± SD). n = 6. Values with '*' are different from corresponding pair fed and endo groups by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

8.3.2 Body composition

Results of liver and plasma analysis are given below.

Table 8.3 Effect of ad libitum or pair feeding after endotoxin injection on liver composition of rats given a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Liver weight as % body weight			
Endo	4.70(.56)a	4.99(.27)a	p_t<0.001,F=21.7
Ad lib	5.04(.39)a	5.09(.25)a	
Pair fed	4.21(.28)a*	4.11(.26)a*	
Protein g/liver			
Endo	1.61(.16)a	1.58(.18)a	NS
Ad lib	1.57(.09)a	1.52(.30)a	
Pair fed	1.45(.16)a	1.31(.21)a	
GSH mg/g			
Endo	1.41(.31)a	1.38(.24)a	NS
Ad Lib	1.31(.19)a	1.28(.18)a	
Pair fed	1.22(.12)a	1.20(.15)a	

Results expressed as mean (± SD). n=6. Values with a '*' are significantly different from other treatment groups by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

8.3.3 Liver composition

Relative liver weights (liver weight as % of body weight) of ad lib fed animals was the highest, while lowest relative weights were found in pair fed groups. Endotoxin treatment increased the relative liver weights when compared with pair fed animals. Addition of tea solids to the diet had no effect on relative liver weights. Although statistically not different, total liver protein, in endotoxin treated groups, was highest and it was lowest in pair fed groups. Liver glutathione concentrations also had the same pattern as liver protein. Tea solids in the diet had no effect on either parameters (Table 8.3).

Table 8.4 Effect of ad libitum or pair feeding after endotoxin injection on lung composition of rats given a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	0.61(.02)a	0.71(.20)a	NS
Ad lib	0.67(.17)a	0.65(.10)a	
Pair fed	0.57(.04)a	0.65(.06)a	
GSH mg/g			
Endo	0.69(.06)a	0.72(.18)a	p_t=0.002,F=7.6
Ad Lib	0.64(.10)a	0.64(.05)a	
Pair fed	0.52(.03)a*	0.58(.06)a*	
Myeloperoxidase U/g			
Endo	6.2(5.6)a	14.9(5.3)b*	p_t<0.001,F=17.5
Ad lib	3.3(1.0)a	4.8(2.1)a	p_d=0.003,F=10.2
Pair fed	3.0(0.5)a	3.5(1.1)a	

Results expressed as mean (± SD). n=6. Values with different letters are significantly different from other dietary group and Values with '*' are significantly different from other treatment groups by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

8.3.4 Lung composition

There were no statistically significant differences between dietary or treatment groups for relative lung weights (lung weight as % body weight). Lung glutathione concentrations of endotoxin treated groups were higher when compared with other corresponding treatment

groups, while the pair fed groups had the lowest concentrations. Addition of tea solids to the diet had no significant effects when compared with the corresponding treatment group fed a normal diet. Myeloperoxidase activity in endotoxin treated animals were higher when compared with corresponding treatment groups in both normally fed and tea solids supplemented animals. However the increase was significantly higher in the group where tea solids were added to the diet (Table 8.4).

Table 8.5 Effect of ad libitum or pair feeding after endotoxin injection on blood composition of rats given a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Haemoglobin g/dl			
Endo	13.8(1.4)	14.0(1.4)	NS
Ad lib	14.0(0.8)	13.0(0.5)	
Pair fed	13.8(1.4)	14.0(1.4)	
GSH mg/g Hb			
Endo	6.72(1.11)	5.75(0.89)*$	$p_t<0.001, F=9.9$
Ad Lib	4.64(0.68)*	4.85(0.43)$	
Pair fed	5.84(1.30)	6.64(1.00)*	

Results expressed as mean (± SD). n=6. Values with different symbols are significantly different from other treatment groups by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

8.3.5 Blood composition

No statistically significant differences were observed for haemoglobin concentrations. Blood glutathione concentrations were highest after endotoxin treatment and lowest for animals in ad lib fed groups receiving a normal diet. While for the groups receiving a tea supplemented diet the highest GSH was for the pair fed group and lowest was for the ad lib group (Table 8.5).

Table 8.6 Effect of ad libitum or pair feeding after endotoxin injection on plasma composition of rats given a normal diet or a diet supplemented with tea solids.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Albumin mg/ml			
Endo	40.4(1.3)a	38.9(1.4)a	p_t<0.001,F=38.5
Ad lib	42.4(0.9)a*	42.4(0.4)a*	
Pair fed	43.0(0.9)a*	43.5(1.2)a*	
Caeruloplasmin U/ml			
Endo	0.25(0.03)a$	0.27(0.01)a	p_t<0.001,F=11.6
Ad Lib	0.20(0.01)a*ǂ	0.19(0.06)a*	
Pair fed	0.22(0.02)aǂ$	0.23(0.02)aǂ	
TEAC mmol/l			
Endo	0.47(0.07)a	0.29(0.05)b	p_t<0.001,F=14.2
Ad Lib	0.25(0.05)a*	0.31(0.04)a	p_d=0.04,F=4.19
Pair fed	0.31(0.03)a*	0.32(0.02)a	

Results expressed as mean (± SD). n=6. Values with different letters are significantly different from other dietary groups and Values with different symbols are significantly different from other treatment groups by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet. TEAC = Trolox equivalent antioxidant capacity.

8.3.6 Plasma composition

Plasma albumin concentrations of endotoxin treated animals were lower in both dietary groups. No differences were found between groups fed tea solids and normally fed groups. Antioxidant capacity of endotoxin treated animals was higher than the ad lib fed and

pair fed animals in the group fed normal diet. However in animals fed tea solids and given endotoxin treatment, lowest TEAC values were observed (Table 8.6).

8.4 Discussion

During the experimental period no differences were observed in growth or in food intake between the groups. No differences were found in final body weights also. This again confirms the observations made in the earlier experiments that addition of tea solids to the diet had no visible effects upon experimental rats.

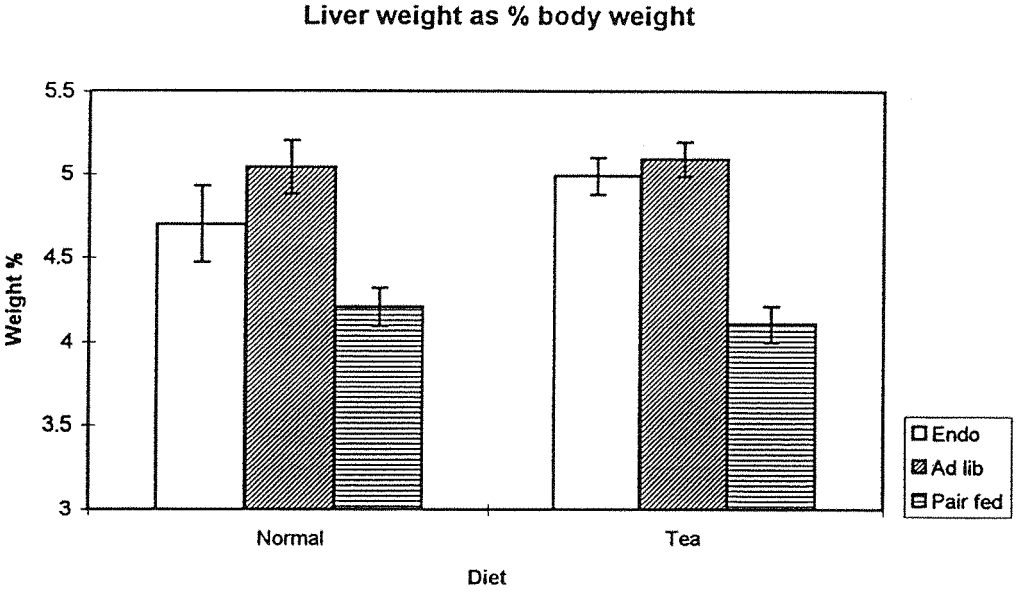


Figure 8.1 Relative liver weights of rats given a normal diet or a diet supplemented with tea solids for 3 weeks and *ad libitum* fed or pair fed after endotoxin injection.

The relative liver weights had decreased in pair fed groups when compared with ad lib fed group. (Fig 8.1) This may be due to the reduced food intake which matched that consumed by the endotoxin treated group. However despite an relatively low food intake during the 24 hours following endotoxin injection relative liver weight in endotoxin injected animals almost reached values in the group fed ad libitum.

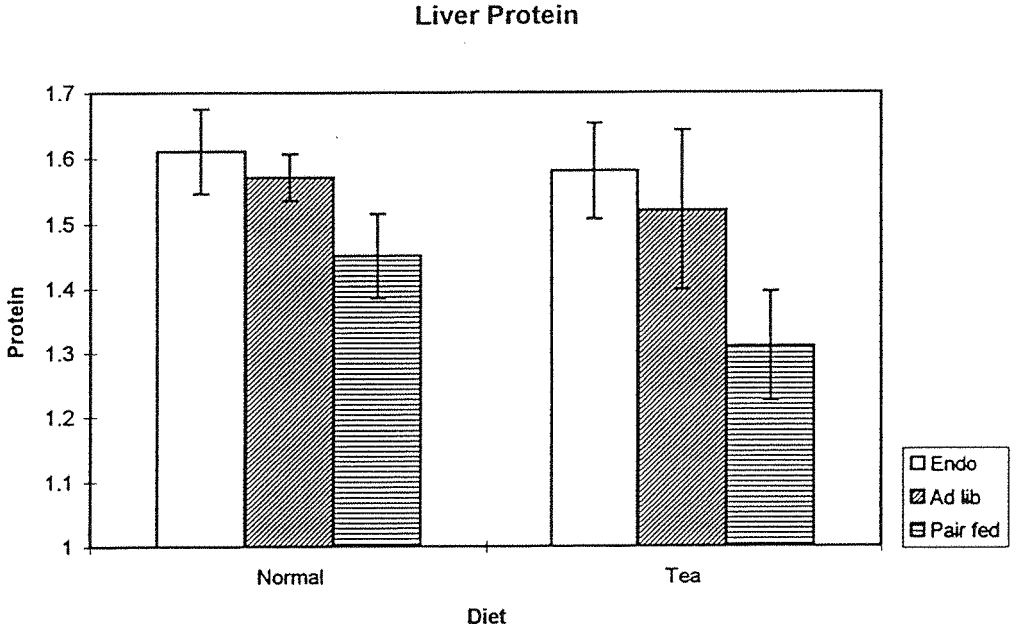


Figure 8.2 Liver Protein (mg/g) of rats given a normal diet or a diet supplemented with tea solids for 3 weeks and *ad libitum* fed or pair fed after endotoxin injection.

This phenomenon is due to the increased hepatic activity in endotoxin injected animals which is reflected in increased protein (Fig.8.2) and glutathione concentrations (Fig.8.3) in endotoxin treated animals. Although statistically not significant, highest liver protein concentrations were found in endotoxin treated animals indicating acute phase protein production. Liver glutathione also had the same pattern as protein. Tea solids in the diet while not showing any effect on these parameters resulted in greater mean liver weights in animals given endotoxin.

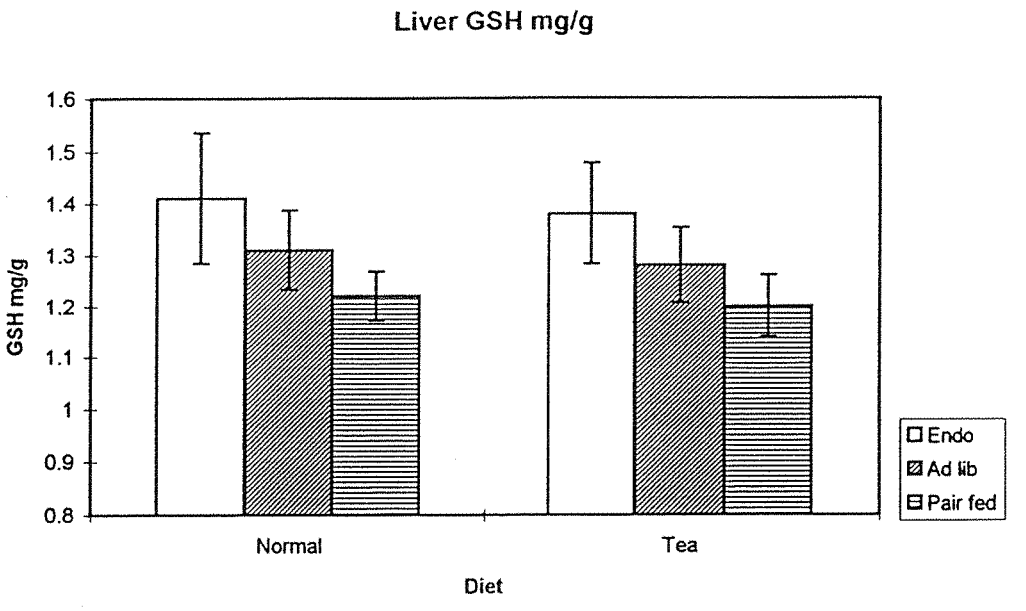


Figure 8.3 Liver Glutathione(mg/g) of rats given a normal diet or a diet supplemented with tea solids for 3 weeks and *ad libitum* fed or pair fed after endotoxin injection.

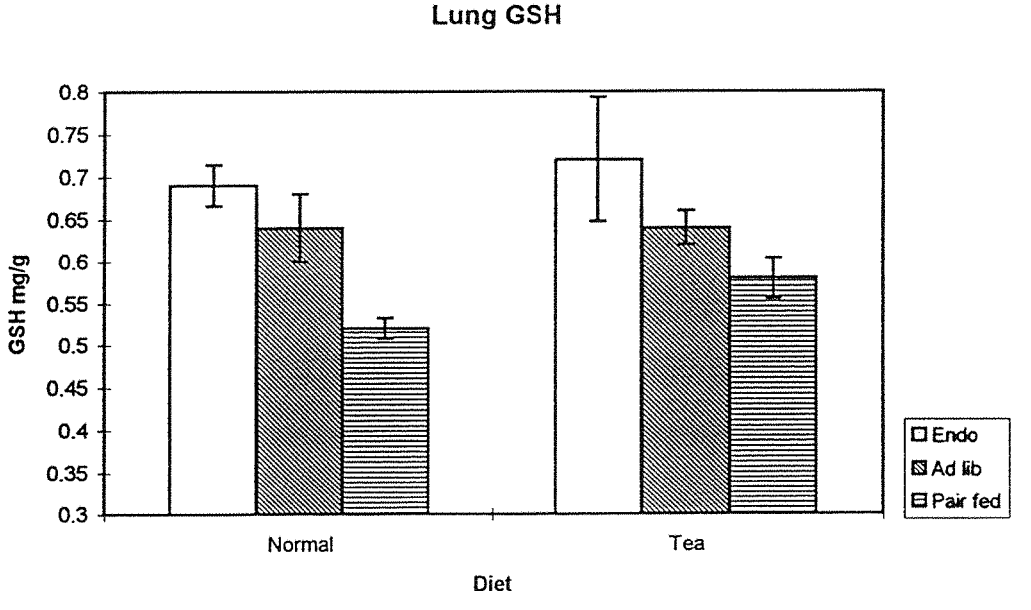


Figure 8.4 Lung glutathione(mg/g) of rats given a normal diet or a diet supplemented with tea solids for 3 weeks and *ad libitum* fed or pair fed after endotoxin injection.

The weights of the lungs do not show any statistically significant differences. Lung glutathione concentration was higher in endotoxin treated animals than in other treatment groups (Fig. 8.4). However tea solids in the diet produced no statistically significant effects although mean values were higher when the diet contained tea solids. Myeloperoxidase activity in the endotoxin treated group was higher than in corresponding treatment groups for both normally fed and tea solids supplemented groups (Fig. 8.5). Endotoxin treatment increase the activated neutrophils through the inflammatory response and this increase is reflected in myeloperoxidase activity in lung. Dietary supplementation with tea solids markedly increased myeloperoxidase activity in endotoxin treated animals. This observation further confirms the observation made in a earlier experiment (Chapter 6) that the percentage of neutrophils in the group supplemented with tea solids is higher than in animals fed a normal diet after the treatment with endotoxin.(Fig.6.2). This implies that tea solids increase neutrophil numbers when the animals are experiencing an inflammatory response. The increase in neutrophils in the group supplemented with tea solids may result in increased production of free radicals. However the increased production of oxidant species is not reflected in lung GSH concentration or on liver and blood glutathione and in plasma caeruloplasmin concentrations. Therefore while acting to increase neutrophils in lung, components in tea solids may be acting as antioxidants simultaneously.

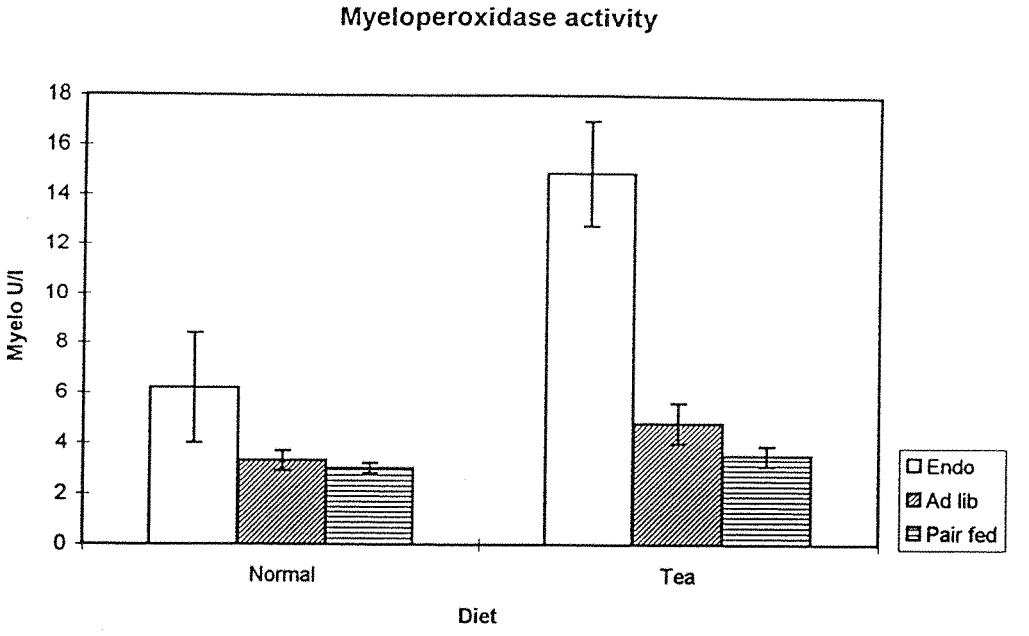


Figure 8.5 Myeloperoxidase activity (u/g) of rats given a normal diet or a diet supplemented with tea solids for 3 weeks and *ad libitum* fed or pair fed after endotoxin injection.

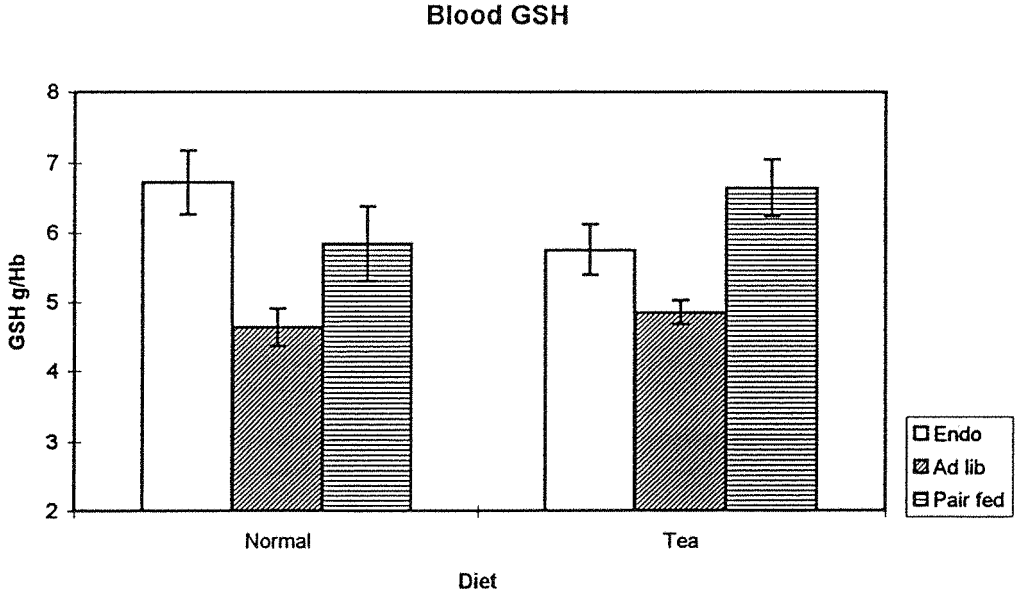


Figure 8.6 Blood glutathione of rats given a normal diet or a diet supplemented with tea solids for 3 weeks and ad libitum fed or pair fed after endotoxin injection.

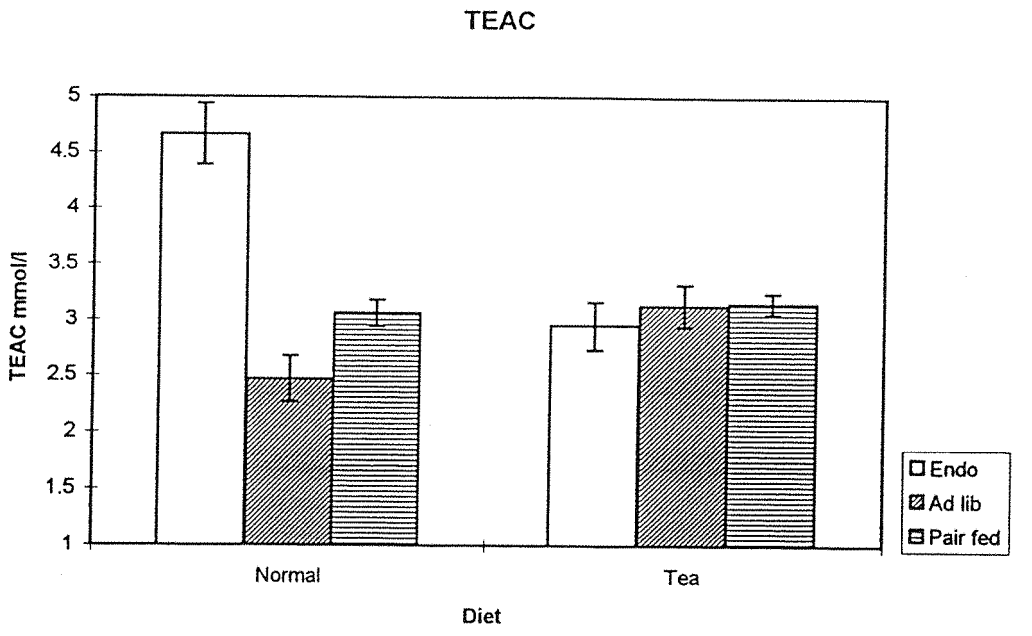


Figure 8.7 Plasma antioxidant capacity (TEAC) of rats given a normal diet or a diet supplemented with tea solids for 3 weeks and ad libitum fed or pair fed after endotoxin injection.

Haemoglobin concentrations showed no significant differences among any groups. This indicates that tea supplementation had no effect on haemoglobin concentration. Tea supplementation had no significant effects on blood GSH also.(Fig.8.6)

Plasma albumin levels were lowered in endotoxin treated animals as expected. The presence of tea solids in the diet did not modify the effects. Plasma caeruloplasmin levels were raised in endotoxin treated animals as expected from its function as a positive acute phase protein. Again tea solids had no effect on the response. Plasma antioxidant capacity measurements(TEAC) indicate a slight increase with tea solids consumption in both ad lib and pair fed groups(Fig.8.7). This may be due to the antioxidant components in tea solids.

However in endotoxin treated groups, tea solids supplementation decreased the antioxidant capacity. This may be due to the increased free radical production by the increased inflammatory cells in this group as indicated by the increased myeloperoxidase activity. Antioxidant utilisation in this group would therefore increase and hence TEAC decrease.

The studies described so far in this thesis have revealed that tea solids produce apparently paradoxical effects which are most prominent in animals undergoing an inflammatory response. Antioxidant and potentially pro-inflammatory effects have been identified. During an inflammatory response, when food intake is low, components derived from tea solids would appear to be released from endogenous sources. This implies absorption of substances and tissue storage. In the studies described in the next two chapters an attempt was made to identify the components absorbed from tea solids, to examine the acute effects of ingestion of tea solids on antioxidant components of the blood and to examine the period over which tea solids need to be consumed to exert their biological effects.

CHAPTER 9

ABSORPTION OF TEA COMPONENTS FROM THE DIGESTIVE TRACT OF RAT INTO THE CIRCULATORY SYSTEM

9.1 Introduction

Tea extracts exhibit various pharmacological and physiological properties (discussed in Chapter 1). A number of biological effects of tea solids have also been found in the studies conducted in the present research programme. In these studies assumption has been made that components of the tea are absorbed and exert biological effects *in vivo*. Therefore it is important to see this is indeed the case.

As discussed in chapter one most of the biological activities exerted by consumption of tea are believed to be caused by its polyphenolic flavanoids. In black tea two types of flavanoids are present. That is catechins and the dimeric and polymeric forms of catechins formed during the fermentation process of black tea processing (Theaflavins and thearubigins). However there is a lack of quantitative data on the absorption and bioavailability of these compounds. It is not known whether the dimeric (theaflavins) and polymeric forms(thearubigins) are absorbed intact or whether they are broken down to catechins by the digestive system prior to absorption. Therefore a study was carried out using Wistar rats to see whether polyphenolic compounds are absorbed into the circulatory system through digestive system. Serum obtained from the rats that were given a tea extract, was analysed for catechins (specifically for epigallocatechin gallate, (EGCG) and the dimeric

forms (theaflavins) using high pressure liquid chromatography (HPLC).

9.2 Materials and Methods

Male Wistar rats weighing 112±14g (34 animals) were used for the study. They were starved for 14 hours before the experiment and 26 animals were given 1ml of tea extract orally. A further 8 animals were given 1ml of water and used as controls. Animals were anaesthetized with diethyl ether and blood was drawn by cardiac puncture after 15, 30, 45, minutes and 1, 2, 3, 4, 5, 6, 8, 12, 20 and 24 hours (Two animals were examined at a point). Control animals were used after 15 minutes, 1, 6 and 24 hours. Serum was obtained and 2ml of serum was vortex mixed with 1ml ethyl acetate to extract the polyphenolic compounds. Aqueous and ethyl acetate layers were separated by centrifugation and the ethyl acetate layer was removed. This was repeated two more times and ethyl acetate fractions were combined and concentrated to 1ml under a stream of nitrogen. The concentrate was filtered through a 0.45μm filter and 20μl was injected to the HPLC.

9.2.1. HPLC conditions (Hoefler and Coggon, 1976)

for Epigallo catechin gallate (EGCG):

Column - C18 μ-Bondapack reverse phase (30cm)

Mobile phase - acetic acid: methanol: dimethylformamide: water, 1:2:40:157.

Detector wave length - 280nm

Flow rate - 2ml/min

for Theaflavins (TF)

Column - C18 μ-Bondapack reverse phase (30cm)

Mobile phase - acetic acid: acetone: water, 1:60:139

Detector wave length - 365nm

Flow rate 2ml/min

9.2.2. Preparation of the tea extract

To 100g of black tea (BOP grade from St.Coombs estate, Talawakelle, Sri Lanka) 500ml of boiling distilled water was added. This was mixed and allowed to stand for five minutes and filtered through a muslin cloth to remove solid particles. The filtrate obtained was concentrated to 100ml under reduced pressure. A 1ml dose was given to a rat orally by gavage. The dose contained 21.5, 22.7 and 32mg of epigallo catechin gallate (EGCG), theaflavin (TF) and caffeine respectively per rat.

9.3 Results and Discussion

In the serum samples obtained neither epigallo catechin gallate (EGCG) nor theaflavin(TF) could be detected in the HPLC chromatograms. However caffeine was detected in the serum samples obtained after the ingestion of tea solution. Amounts of caffeine found are given in the table 9.1.

Table 9.1. Concentration of caffeine in serum

Time after ingestion	caffeine concentration(mg/ml)
15 min	0.19
30 min	0.21
45 min	0.22
60 min	0.22
2 hours	0.37
3 hours	0.62
4 hours	0.61
5 hours	0.67
6 hours	0.83
8 hours	0.05
12 hours	-
20 hours	-
24 hours	-
15 min control	-
1 hour control	-
6 hour control	-
24 hour control	-

In the serum samples obtained neither epigallo catechin gallate (EGCG) nor theaflavin (TF) could be detected in the HPLC chromatograms. However in another study it has been observed that oral administration of pure EGCG (50mg per rat) to Wistar rats resulted in a increase in plasma levels reaching the highest level one hour after dosing (Unno and Takeo, 1995). He and Kies also observed that serum polyphenol content increase after green tea, black tea and decaffeinated black tea consumption in humans (He and Kies, 1994). The amounts of EGCG (21.5mg) and TF (22.7mg) given to the rats in the present experiment may have been insufficient to be detected in the serum. Unno and Takeo gave a dose of 50mg of pure EGCG which is more than double the amount given in the present study. Absorption of

EGCG may be high when given in pure form, as other components in tea solids could hinder the absorption. Recently Lee and others have found that plasma lipids interfere with the ethyl acetate extraction of catechins. This was overcome by a methylene chloride extraction prior to ethyl acetate extraction which removes lipids but not catechins (Lee *et al*, 1995). This could be a further reason for not detecting the catechins in the serum samples of the present study.

However in the present experiment caffeine was detected in the serum samples (Fig 9.1). Caffeine in the serum was identified by injecting a pure sample of caffeine which gave the same retention time. This was further confirmed by addition of pure caffeine to a serum sample which enhanced the peak area.

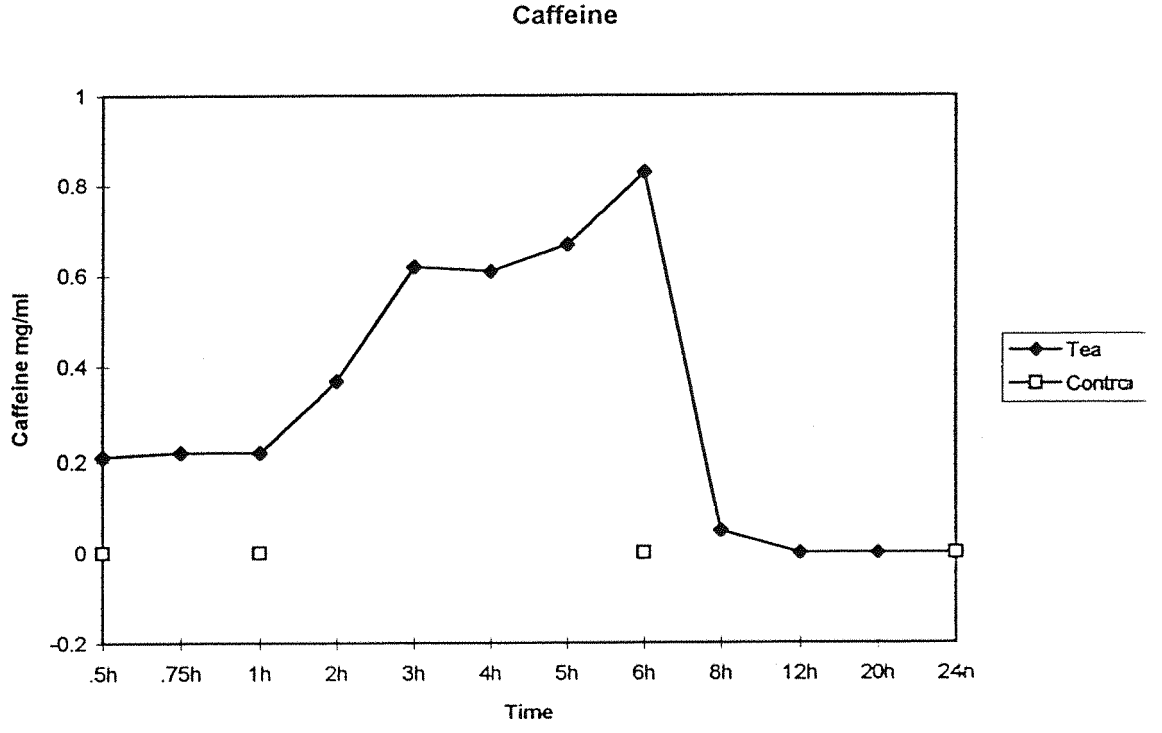


Figure 9.1 Caffeine in plasma after ingestion of tea solution

Caffeine appeared in the very first serum sample obtained 15 minutes after giving the tea extract. It gradually increased to a maximum after 6 hours and decreased rapidly. After 12 hours caffeine was not found in the circulatory system. In the control animals also caffeine was not found.

CHAPTER 10

EFFECT OF BLACK TEA INGESTION ON SERUM ANTIOXIDANT CONTENT OF RATS

10.1 Introduction

Toxicity by oxygen radicals has been suggested as a major cause of cancer, heart disease and in aging process (Discussed in chapter 1). Oxygen radicals and other radicals produced in the normal metabolic processes such as the oxidative phosphorylation in the respiration process, and radicals formed due to environmental conditions such as radicals from smoke, polluted air and other chemical carcinogens leads to toxic reactions *in vivo*. These radicals could initiate chain reactions largely through lipid peroxidation. Lipid peroxidation leads to the generation of highly reactive peroxyl radicals and hydroperoxides. These molecular species have the capability to damage DNA, proteins and cellular membranes. They are also implicated in atherogenesis (discussed in chapter 1). Aerobic organisms have an array of protective mechanisms both for preventing the formation of oxidants and breaking the chain reactions. The protective system include enzymes such as super oxide dismutase, glutathione peroxidase and glutathione reductase and radical scavengers such as α-tocopherol (vitamin E) and ß-carotene in the lipid portion of the cell and glutathione and ascorbic acid in the aqueous phase (discussed in chapter 1).

In addition to the above mentioned antioxidant systems in the body compounds derived from the diet are also known to act as antioxidants *in vivo*. Polyphenolic components

in both green and black tea have shown antioxidant activities in *in vitro* systems. Large number of studies performed on the effect of tea components on heart diseases and cancer also led to the theory that tea components act as antioxidants *in vivo* also. Studies carried out on green tea and compounds isolated from green tea such as epigallo catechin gallate(EGCG) had shown antioxidant activity *in vivo*, in animal studies. Black tea components have shown antioxidant activity under *in vitro* conditions and experiments have shown that they act as antioxidants under biological conditions also (discussed in chapter 1). However studies done to investigate whether ingested black tea exert an antioxidant activity *in vivo* are very limited. Therefore in the present study it was investigated whether black tea consumed enhance the antioxidant properties of serum in the rat.

Two methods were employed for the measurements of antioxidant capacity of serum after the consumption of black tea by rats. Methods used were for measuring the total radical trapping antioxidant parameter (TRAP) (Wayner *et al*, 1985) and the trolox equivalent antioxidant capacity as employed in Chapters 4 and 8 (TEAC) (Miller *et al*, 1993).

10.2 Materials and Methods

Thirty five male Wistar rats, weighing 160±19g, were used for the study. They were given a 1ml solution of black tea solution by oral gavage. The rats were sacrificed by decapitation 0, 1, 2, 4, and 8 hours after giving the tea solution and blood was collected (five rats in each group). A further ten rats were used as controls which were not given the tea solution at 1 and 8 hours. Serum was separated and TRAP and TEAC values were measured as described in chapter 2. Further to this, five dilutions of tea solutions were added to a serum

sample from an animal that had not received the tea solution, to measure the enhancement of TRAP and TEAC values.

10.2.1 Preparation of the tea extract

For administration to rats

Tea extract was prepared as described in section 9.2.2. Caffeine was extracted by chloroform extraction (2 x 500ml). Aqueous layer obtained was concentrated to 100ml under reduced pressure. 1ml of this tea solution was given to a rat.

For addition to serum samples

100ml of boiling distilled water was added to 1.25g of black tea. This was mixed and allowed to stand for five minutes and filtered through a muslin cloth to remove solid particles. Caffeine was removed by chloroform extraction (2 x 100ml). 10µl of this tea solution (T_5) or its diluted solutions with distilled water 1:1 (T_4), 1:2 (T_3), 1:3 (T_2), 1:4 (T_1) were added to a 50µl of serum sample and TRAP and TEAC values were measured and compared with that of pure serum sample from a control rat for the enhancement by the tea solutions.

10.3 Results

Table 10.1 TRAP values of serum after ingestion of tea solution

Sample	TRAP (μmol/l)
0 Hour	1037(25)
1 Hour	1039(55)
2 Hour	1161(20)*
4 Hour	1109(62)*
8 Hour	1031(50)
1 Hour control	1051(89)
8 Hour control	1031(66)

Results expressed as mean (±SD). n = 5. Values with different symbols are significantly different from other groups by one way ANOVA ($p<0.05$).

TRAP values had significantly increased two hours after the ingestion of tea solution. A return to baseline values was observed after eight hours.

Addition of 10µl of tea solution to serum significantly increased the TRAP value. Dilution of this tea solution had decreased the TRAP value (Table 10.2).

Table 10.2 TRAP values of serum after addition of tea solution

Sample	TRAP(µmol/l)
Control (plasma)	1046(31)
T_1	1222(33)*
T_2	1306(36)**
T_3	1397(19)***
T_4	1738(40)****
T_5	2288(35)*****

T_5 - serum+10µl of tea solution prepared with 1.25g of Black tea. T_4, T_3, T_2, T_1 are serum+10µl of 1:1, 1:2, 1:3, 1:4 dilutions of the tea solution prepared with 1.25g of black tea respectively. Results expressed as mean (±SD). n = 3. Values with different symbols are significantly different from other groups by one way ANOVA ($p<0.05$).

Table 10.3 TEAC values of serum after ingestion of tea solution.

Sample	TEAC (mmol/l)
0 Hour	0.23(.12)
1 Hour	0.25(.03)
2 Hour	0.23(.16)
4 Hour	0.24(.10)
8 Hour	0.22(.17)
1 Hour control	0.25(.06)
8 Hour control	0.23(.09)

Results expressed as mean (±SD). n = 5.

In the TEAC values measured a difference was not observed after the ingestion of tea.

Table 10.4 TEAC values of serum after addition of tea solution

Sample	TEAC(mmol/l)
Control(plasma)	0.28(.03)
T_1	0.56(.04)*
T_2	0.64(.03)**
T_3	0.74(.03)***
T_4	0.82(.02)****
T_5	0.86(.05)****

T_5 - serum+10µl of tea solution prepared with 1.25g of Black tea. T_4, T_3, T_2, T_1 are serum+10µl of 1:1, 1:2, 1:3, 1:4 dilutions of the tea solution prepared with 1.25g of black tea respectively. Results expressed as mean (±SD). n = 3. Values with different symbols are significantly different from other groups by one way ANOVA ($p<0.05$).

Addition of different dilutions of tea solutions had increased the TEAC values.

10.4 Discussion

When increasing concentrations of black tea solution were added to a plasma sample, both TRAP and TEAC values significantly increased indicating an *in vitro* antioxidant activity of the black tea solution (Fig 10.1 and Fig. 10.2). In the *in vivo* experiment black tea ingestion had slightly increased the plasma TEAC values after one hour. However this increase of the plasma TEAC values was not significant when compared with the plasma sample at zero hour or the control samples (Fig 10.3). However the TRAP values of the plasma samples significantly increased after two hours of the ingestion (Fig. 10.4). The peak

obtained at two hours decreased after four hours and at eight hours the TRAP values had fallen back to the normal levels. This clearly indicates the antioxidant activity of black tea *in vivo*. The increase in TRAP in the in vivo study clearly demonstrate that substances form tea solids with antioxidant properties are taken rapidly up into the blood stream. The values declined from peak values farely rapidly. However it is not possible to tell whether this is due to metabolism to substances with no antioxidant properties, to storage in tissues or to excretion into urine.

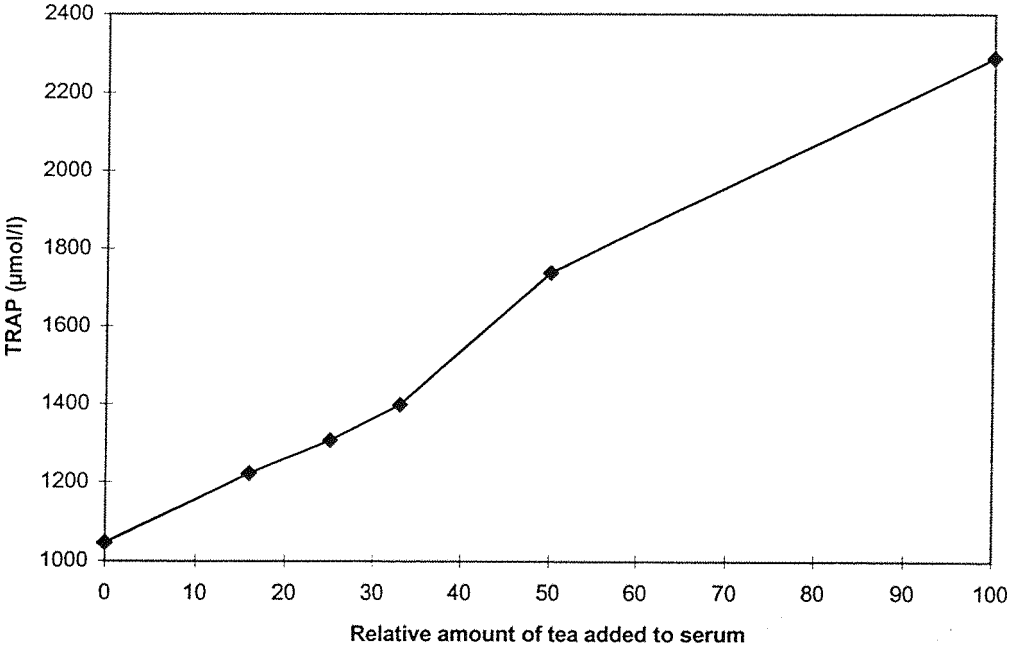


Figure 10.1 TRAP values of serum after addition of increasing amounts of tea solution.

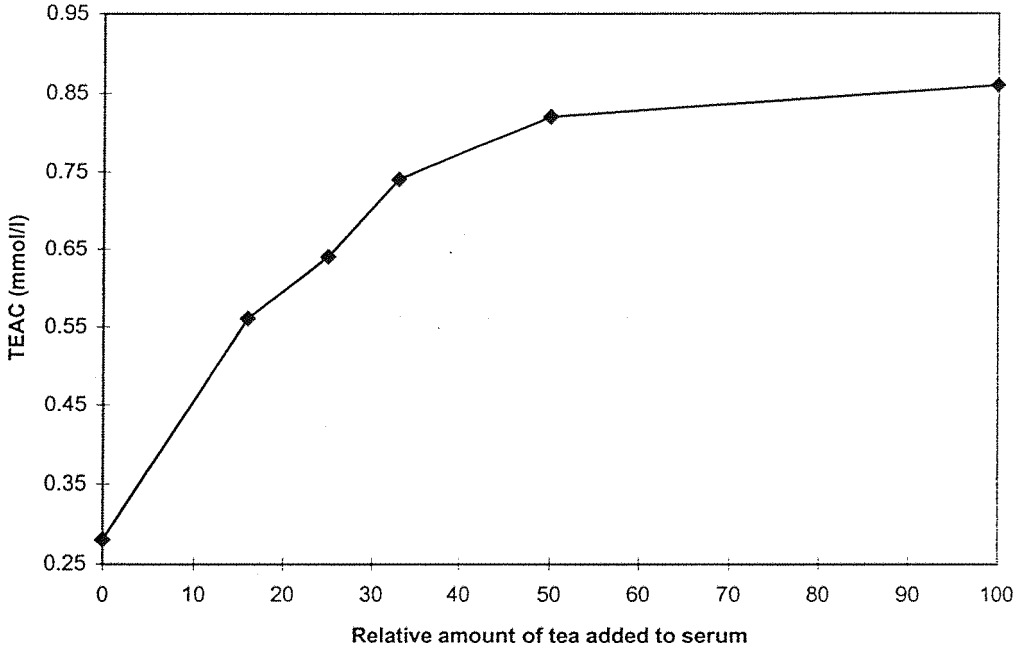


Figure 10.2 TEAC values of serum after addition of increasing amounts of tea solution.

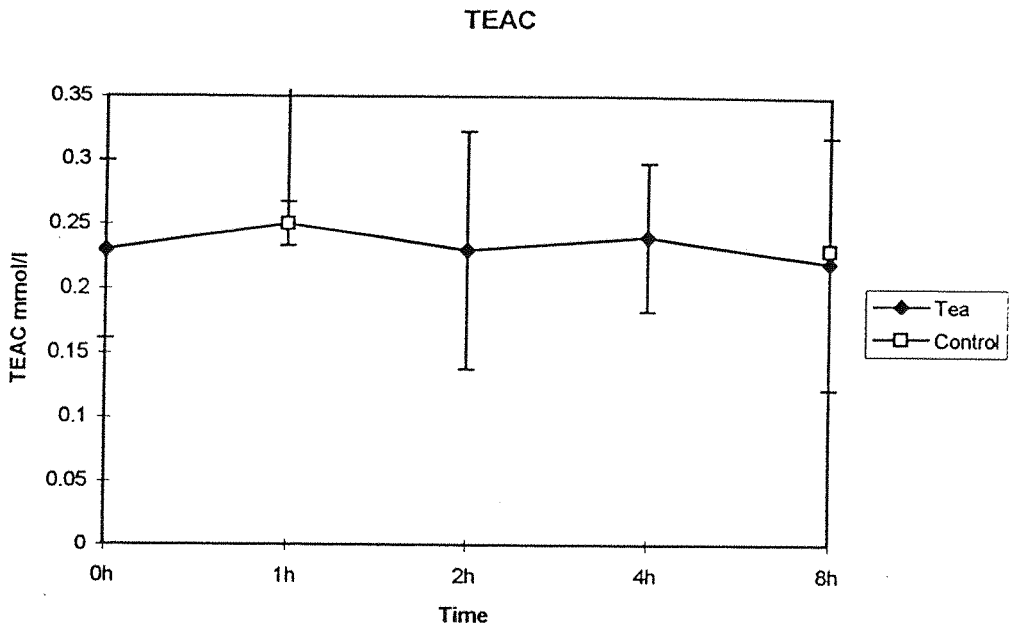


Figure 10.3 TEAC values of serum after ingestion of tea solution

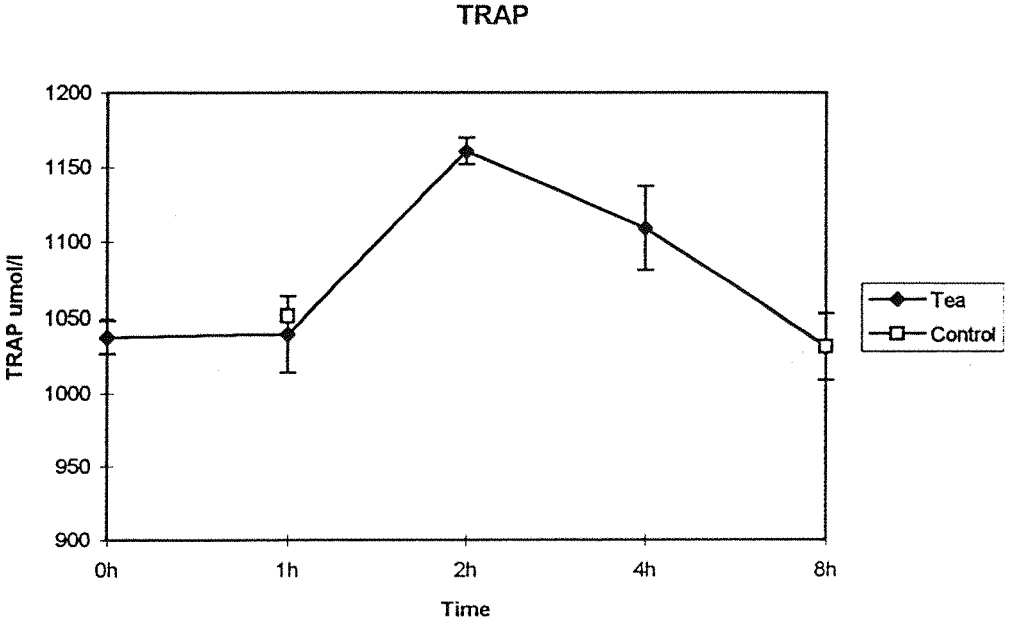


Figure 10.4 TRAP values of serum after ingestion of tea solution

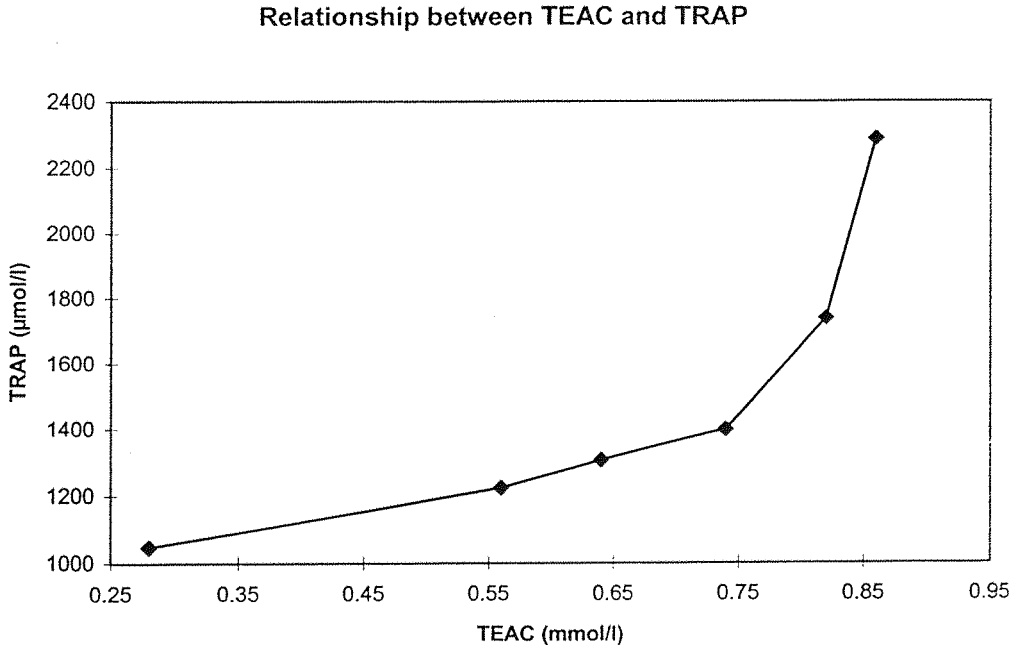


Fig. 10.5 Relationship between TEAC and TRAP

Significant increase in TEAC values were not observed in the *in vivo* study. The relationship between TEAC values and TRAP values *in vitro* is not linear (Fig. 10.5). It appears that TEAC values increase at high magnitude with the increasing concentration of tea solids only when the tea concentrations are relatively low (Fig. 10.2). At higher concentrations of tea the increase is small. However response of TRAP appears to be linear (Fig. 10.1). This could be the reason that an increase in TEAC was not observed in the *in vivo* study.

The increase in plasma antioxidant capacity (TRAP values) which follow tea consumption in the present experiment support the recent studies providing evidence that

there is a epidemiological link between polyphenol intake, antioxidant status and cardiovascular disease. Hertog and others had observed that there is an inverse relationship between dietary intake of flavanoids and cardiovascular disease. They have further observed in that study, that 60% of dietary flavanoid intake of the subjects came from black tea consumption (Hertog *et al*, 1993). Block and others also have observed that there is an inverse relationship between plant-rich diets and mortality from chronic degenerative disease (Block *et al*, 1992).

In a recent study Serafini and others have found that both green tea and black tea (300ml per subject, prepared by adding 100ml of boiling water to 2g of black tea or green tea) increase the plasma radical trapping potential in humans. Peak TRAP values for black tea was fifty minutes after the ingestion and peak values for green tea ingestion are after thirty minutes (Serafini *et al*, 1996). In our study peak values were obtained after two hours and there was a reasonable activity after four hours.

The mechanisms of absorption of tea polyphenols and their bioavailability have not yet been elucidated. Results from the present study indicate that they are absorbed by the gastrointestinal tract and reach the circulatory system and increase the radical trapping potential in plasma.

CHAPTER 11

INFLUENCE OF DURATION OF DIETARY SUPPLEMENTATION WITH TEA SOLIDS ON LUNG NEUTROPHIL NUMBERS AND OTHER RESPONSES TO ENDOTOXIN

11.1 Introduction

In the earlier experiments it was found that tea solids in the diet altered some responses to endotoxin challenge. In these experiments, the most noted effect was on the number of neutrophil cells in the lung tissue. Histological examination of lung tissue in a earlier experiment had shown that supplementation with tea solids(5g/kg diet) of a diet deficient in vitamin E, for a period of three weeks resulted in a marked increase in number of neutrophils in the lung after endotoxin treatment (Table 6.4.2). It was also noted in the same experiment that among the control animals, where a saline injection (i.p. 1ml/kg body weight, 0.9g/100ml solution) was given instead of endotoxin, supplementation with tea solids had no effect on the number of neutrophils in the lung (Fig.6.2). This finding suggested that components in tea solids are not providing an inflammatory stimulus and are only acting to enhance the number of neutrophils in lung tissue in which an inflammatory response is in progress.

In the experiment described in chapter 8 where a normal diet was supplemented with tea solids (5g/kg diet) for a period of three weeks, it was found that tea solids supplementation resulted in increased myeloperoxidase activity in lung tissue of endotoxin

treated (i.p. injection 200μg/kg body weight) animals (Fig.8.6). Activated neutrophils contain large amounts of myeloperoxidase. The enzyme catalyses the formation of hypochlorous acid from hydrogen peroxide and chloride ions, which acts to destroy invading microorganisms (discussed in chapter 8). The myeloperoxidase activity in a tissue is directly proportional to the number of activated neutrophils in the tissue. The finding of increased lung myeloperoxidase activity further emphasise that tea solid supplementation for three weeks result in increased number of neutrophils after endotoxin treatment. Therefore in the present experiment the effect of dietary supplementation with tea solids for a three week period on number of neutrophils, myeloperoxidase activity and other responses to endotoxin were investigated, to confirm earlier findings. Further, the effects of dietary supplementation with tea solids were investigated after one week and three weeks to study the time course for the influence of supplementation on the inflammatory response.

11.2 Experimental protocol

Twelve weanling male Wistar rats weighing 75±5g (mean± S.D.), were divided into two groups. They were put in separate cages. Group one was fed with a normal diet (Normal) and the second group was fed with a diet which had decaffeinated tea solids at 0.5% (5g/kg diet) in addition (Tea). Animals were fed on these diets for one week. After one week each dietary group was divided into two. One sub group received an i.p. dose of endotoxin (200μg/kg of body weight) and was killed 24 hours after injection. Following day, the other sub group was given an i.p. dose of sterile non pyrogenic saline (1ml per Kg body weight, 0.9g/100ml solution) and pair fed with the intake of the endotoxin injected animals for 24 hours prior to sacrifice.

With a another set of twelve weanling Wistar rats weighing 68±9g the same procedure was carried out where the dietary period was three weeks.

Table 11.1 Composition of diets and dietary groups

Component	Normal (g/kg diet)	Tea(g/kg diet)
Casein	180	180
L-methionine	3	3
Vitamin mixture	20	20
Mineral mixture	40	40
Calcined magnesite	1	1
Corn Oil	30	30
Cellulose	100	100
Sugar	313	310
Starch	313	310
Decaffeinated Tea solids	-	5

11.3 Results

11.3.1 Results after one week of dietary supplementation

Results obtained for the set of animals where the dietary supplementation period was one week are given below.

11.3.2 Food intake and weight gain after one week (Table 11.2)

Both groups started with the same mean body weight and there was no significant difference in food intake and body weight gain after one week. No differences in appearance were observed in the group fed tea solids when compared with the normally fed group.

Table 11.2 Initial and final weights, growth and food intake of rats given a normal diet or a diet supplemented with tea solids for one week.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Initial body weight (g)			
Endo	78(2)	74(3)	NS
Saline	76(2)	73(4)	
Final body weight (g)			
Endo	123(5)	120(4)	NS
Saline	119(3)	113(7)	
Growth g/day			
Endo	6.5(0.6)	6.5(0.1)	NS
Saline	6.1(0.2)	5.8(0.2)	
Pre injection food intake g/day			
Endo	18(0.5)	17(0.8)	NS
Saline	17(0.7)	16(1.8)	
Post injection food intake g/24 hours			
Endo	3.0(1.7)	4.0(2.0)	NS
Saline	3.0(1.7)	4.0(2.0)	

Results expressed as mean (± SD). n = 3.

11.3.3 Body composition

Results of liver, lung and plasma analysis of rats fed a normal diet or a diet supplemented with tea solids for one week are given below.

11.3.4 Liver composition (table 11.3)

Table 11.3 Effect of endotoxin injection on liver composition of rats given a normal diet or a diet supplemented with tea solids for one week.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Liver weight as % body weight			
Endo	4.37(.28)a	4.57(.26)a*	p_t=0.005,F=14.2
Saline	3.94(.36)a	3.79(.18)a	
GSH mg/g			
Endo	1.15(.15)a*	1.05(.21)a*	p_t<0.001,F=68.4
Saline	0.46(.06)a	0.48(.04)a	

Results expressed as mean (± SD). n=3. Values with a '*' are significantly different from saline treated group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

Endotoxin treatment increased the relative liver weights (liver weight as % of body weight) when compared with pair fed animals. This difference was statistically significant in the group where tea solids were added to the diet. Liver glutathione concentrations of endotoxin treated animals were significantly higher than saline treated animals in both dietary groups.

11.3.5 Lung composition (Table 11.4)

Table 11.4 Effect of endotoxin injection on lung composition of rats given a normal diet or a diet supplemented with tea solids for one week.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	0.62(.02)a	0.64(.06)a	NS
Saline	0.70(.14)a	0.68(.03)a	
GSH mg/g			
Endo	0.40(.01)a*	0.39(.01)a	p_t=0.01,F=11.1
Saline	0.32(.05)a	0.36(.01)a	
Myeloperoxidase U/g			
Endo	17.8(5.6)a*	24.1(3.4)b*	p_t<0.001,F=65.05
Saline	3.5(1.0)a	4.8(2.1)a	p_d=0.02,F=7.7
Neutrophils as % of total lung cells			
Endo	1.13(.08)a*	1.80(.15)b*	P_t<0.001,F=66.6
Saline	0.32(.05)a	0.38(.03)a	p_d<0.001,F=47.0

Results expressed as mean (± SD). n=3. Values with different letters are significantly different from other dietary group and Values with `*' are significantly different from saline treated group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

Relative lung weight (lung weight as % body weight) had no statistically significant difference between dietary or treatment groups. Lung glutathione concentrations of endotoxin treated groups were higher when compared with saline treated group. This difference was statistically significant among normally fed group. Myeloperoxidase activity in endotoxin

treated animals were higher when compared with corresponding saline treated groups in both normally fed and tea solid supplemented animals. However the increase of activity in the tea solid fed and endotoxin treated group was significantly higher than the corresponding normally fed group. The number of neutrophils in the lung tissue (as % of total lung cells) also showed the same pattern of change as myeloperoxidase.

11.3.6 Blood composition (Table 11.5)

Table 11.5 Effect of endotoxin injection on blood composition of rats given a normal diet or a diet supplemented with tea solids for one week.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Haemoglobin g/dl			
Endo	11.3(.41)a*	11.3(1.0)a*	p_t<0.001,F=43.6
Saline	16.8(.26)a	16.6(0.5)a	
GSH mg/ml			
Endo	0.52(.04)a	0.59(.04)a	NS
Saline	0.55(.03)a	0.47(.02)a	
GSH mg/g Hb			
Endo	4.64(.21)a*	5.22(.12)a*	p_t<0.001,F=39.0
Saline	3.30(.17)a	2.85(.12)a	

Results expressed as mean (± SD). n=3. Values with different symbols are significantly different from saline treated group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

Blood haemoglobin concentrations of endotoxin treated animals were significantly lower than that of saline treated groups in both dietary groups. Blood glutathione concentrations, when expressed as mg/ml showed no differences between dietary or treatment groups. When glutathione concentrations were expressed per g of haemoglobin they showed the reverse of the pattern for haemoglobin concentrations.

11.3.7 Plasma composition (Table 11.6)

Table 11.6 Effect of endotoxin injection on plasma composition of rats given a normal diet or a diet supplemented with tea solids for one week.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Albumin mg/ml			
Endo	34.7(1.1)a*	33.8(2.4)a*	p_t=0.001,F=23.5
Saline	39.2(0.6)a	39.4(2.4)a	
Caeruloplasmin U/ml			
Endo	0.19(0.02)a	0.19(0.01)a	NS
Saline	0.17(0.02)a	0.17(0.01)a	
TRAP µmol/l			
Endo	986(41)a	896(112)a	NS
Saline	872(41)a	856(60)a	

Results expressed as mean (± SD). n=3. Values with different symbols are significantly different from saline treated group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

Plasma albumin concentrations of endotoxin treated animals were significantly lower in both dietary groups. No differences were found for plasma caeruloplasmin concentrations between dietary or treatment groups. For plasma antioxidant potential (Total Radical Trapping Antioxidant Parameter,TRAP) also there were no differences between dietary or treatment groups.

11.3.8 Results after three weeks of dietary supplementation

Results obtained for the set of animals where dietary supplementation period was three weeks are given below.

11.3.9 Food intake and weight gain after three weeks (Table 11.7)

Table 11.7 Initial and final weights, growth and food intake of rats given a normal diet or a diet supplemented with tea solids for three weeks.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Initial body weight (g)			
Endo	67(11)	66(11)	NS
Saline	66(12)	73(5)	
Final body weight (g)			
Endo	168(23)	184(8)	NS
Saline	170(21)	190(4)	
Growth g/day			
Endo	4.7(0.7)	5.6(0.2)	NS
Saline	4.9(0.6)	5.6(0.1)	
Pre injection food intake g/day			
Endo	23(2.3)	25(0.7)	NS
Saline	23(1.1)	25(1.1)	
Post injection food intake g/24 hours			
Endo	11(12.5)	9(8.7)	NS
Saline	11(12.5)	9(8.7)	

Results expressed as mean (± SD). n = 3.

Both groups started with the same mean body weight and there was no significant difference in food intake or in body weight gain after three weeks. No differences in appearance were observed in the group fed tea solids when compared with the normally fed

group.

11.3.10 Body composition

Results of liver lung and plasma analysis of rats fed a normal or a diet supplemented with tea solids for three weeks are given below.

11.3.11 Liver composition (table 11.8)

Table 11.8 Effect of endotoxin injection on liver composition of rats given a normal diet or a diet supplemented with tea solids for three weeks.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Liver weight as % body weight			
Endo	4.78(.58)a	5.17(.77)a	NS
Saline	4.55(.20)a	3.91(.35)a	
GSH mg/g			
Endo	1.32(.14)a	1.26(.05)a*	p_t=0.008,F=12.0
Saline	1.07(.23)a	0.92(.08)a	

Results expressed as mean (± SD). n=3. Values with a `*' are significantly different from saline treated group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

Endotoxin treatment had increased the relative liver weights (liver weight as % of body weight) when compared with saline treated animals. However this difference was not

statistically significant. Liver glutathione concentrations of endotoxin treated animals were also higher than saline treated animals in both dietary groups. However this difference was statistically significant only in the animals given a diet supplemented with tea solids.

11.3.12 Lung composition (Table 11.9)

Table 11.9 Effect of endotoxin injection on lung composition of rats given a normal diet or a diet supplemented with tea solids for three weeks.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Weight as % body weight			
Endo	0.60(.14)a	0.58(.03)a	NS
Saline	0.64(.18)a	0.53(.06)a	
GSH mg/g			
Endo	0.41(.02)a	0.40(.02)a	NS
Saline	0.35(.06)a	0.37(.03)a	
Myeloperoxidase U/g			
Endo	15.7(2.8)a*	21.0(2.7)a*	$p_t<0.001$,F=19.5
Saline	4.5(1.0)a	3.7(2.1)a	
Neutrophils as % of total lung cells			
Endo	0.85(.10)a*	1.36(.18)b*	$p_t<0.001$,F=85.7
Saline	0.43(.05)a	0.47(.12)a	$p_d=0.004$,F=15.6

Results expressed as mean (± SD). n=3. Values with different letters are significantly different from other dietary group and Values with `*' are significantly different from saline treated group by two way ANOVA ($p<0.05$). p_t= differences due to treatment and p_d= differences due to diet.

Relative lung weights (lung weight as % body weight) showed no statistically significant differences between dietary or treatment groups. Lung glutathione concentrations also had no differences. Myeloperoxidase activity in endotoxin treated animals were higher when compared with corresponding saline treated groups in both normally fed and tea solids supplemented animals. Although statistically not significant increase of activity in tea solids fed endotoxin treated group was higher than the corresponding normally fed group. Number of neutrophils in endotoxin treated groups was significantly higher than the corresponding saline treated groups. Increase of neutrophil cells in endotoxin treated tea solids supplemented group was also significantly higher than endotoxin treated normally fed group.

11.3.13 Blood composition (Table 11.10)

Table 11.10 Effect of endotoxin injection on blood composition of rats given a normal diet or a diet supplemented with tea solids for three weeks.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Haemoglobin g/dl			
Endo	15.4(.53)a	14.4(.89)a	NS
Saline	14.0(.66)a	14.3(0.2)a	
GSH mg/ml			
Endo	0.63(.07)a	0.67(.06)a	NS
Saline	0.49(.08)a	0.57(.09)a	
GSH mg/g Hb			
Endo	4.13(.54)a	4.65(.50)a	NS
Saline	3.47(.40)a	4.00(.57)a	

Results expressed as mean (± SD). n=3.

No differences were found for blood haemoglobin concentrations or blood glutathione concentrations.

11.3.14 Plasma composition (Table 11.11)

Table 11.11 Effect of endotoxin injection on plasma composition of rats given a normal diet or a diet supplemented with tea solids for three weeks.

Diet	Normal	Tea	Two way ANOVA p & F ratios
Albumin mg/ml			
Endo	31.4(0.3)a*	34.3(1.44)b	$p_t<0.001$,F=42.2
Saline	36.5(1.22)a	36.6(0.3)a	$p_d=0.02$,F=7.0
Caeruloplasmin U/ml			
Endo	0.27(0.07)a	0.27(0.01)a	NS
Saline	0.23(0.01)a	0.25(0.02)a	
TRAP µmol/l			
Endo	1035(42)a*	1068(18)a	$p_t=0.008$,F=11.9
Saline	925(54)a	987(67)a	

Results expressed as mean (± SD). n=3. Values with different symbols are significantly different from saline treated group by two way ANOVA ($p<0.05$). p_t= differences due to treatment.

Plasma albumin concentrations of the endotoxin treated group were significantly lower when compared with saline treated group in normally fed animals. Such a decrease was not found in endotoxin treated group which received a diet supplemented with tea solids. No differences were found for plasma caeruloplasmin concentrations between dietary or treatment groups. Plasma antioxidant potential (Total Radical Trapping Antioxidant Parameter,TRAP) of endotoxin treated animals were higher than saline treated animals in both dietary groups. This difference was statistically significant in normally fed groups.

11.5 Discussion

Influence of tea solid supplementation for one week on responses to endotoxin

Tea solids supplementation (5g/kg diet) for one week had no effect on food intake or growth of animals after one week. After endotoxin injection food intake was significantly reduced. This may be due to the anorexic effects produced by cytokines (Tracey *et al*, 1988) during the inflammatory response after endotoxin injection. However addition of tea solids to the diet did not alter the post injection food intake (Table 11.2). The finding was in agreement with the lack of effect of tea solids reported in chapter 4, 6, 7 but at variance with the inhibitory effect on endotoxin induced anorexia (see chapter 3, 5).

Relative liver weights and liver glutathione concentrations in endotoxin injected animals were significantly higher than the saline treated animals. These observations are in agreement with a general acute phase response (Andus *et al*, 1991). However supplementation of diet with tea solids did not altered those parameters (Table 11.3).

In the lung, myeloperoxidase activity in endotoxin treated animals were significantly higher than in the corresponding saline treated animals. This increase in the group supplemented with tea solids was significantly higher than in normally fed group. Addition of tea solids to the diet resulted in no increase in saline treated animals (Table 11.4). This result is in agreement with observations made in a earlier experiment (Fig.8.6). The number of neutrophils, as a % of total lung cells, showed the same pattern as myeloperoxidase activity. As neutrophils contain large amounts of myeloperoxidase, it is to be expected that myeloperoxidase activity in a tissue would reflect the number of activated neutrophils in

lung. Results obtained for lung myeloperoxidase activity and % neutrophils in the present study further support the observations made in the earlier study. That is, components in tea solids are acting to further enhance recruitment of neutrophils to lung tissue after endotoxin treatment (discussed in Chapter 6, section 6.5).

Haemoglobin concentrations were lowered in both dietary groups by endotoxin treatment (Table 11.5). This may be due to limited availability of glycine for haemoglobin synthesis. It had been shown that glycine availability may become limited under traumatised conditions (Pui and Fisher, 1979, Jackson *et al*, 1987).

Plasma Total Radical Trapping Antioxidant Parameter (TRAP) in endotoxin treated animals were slightly higher in both dietary groups (Table 11.6). This may be due to increased endogenous antioxidant defences to control increased amounts of free radicals and reactive oxygen species produced during the inflammatory response. Supplementation of diet with tea solids had no effect on TRAP values.

Influence of tea solid supplementation for three weeks on responses to endotoxin

After a three week period of tea solid supplementation (5g/kg diet) no effect on growth, food intake or appearance of animals was found. This is in agreement with observations made in all experiments reported in this thesis. Therefore tea solids supplementation to the diet at 5g/kg diet did not have any adverse or beneficial effect on growth of rats.

The most notable effect of inclusion of tea solids in the diet after three weeks was the enhancement of number of neutrophils in lung tissue after endotoxin injection (Table 11.9). The effect was apparent as early as after one week of inclusion of tea solids in the diet. This effect may be due to increased secretion of Neutrophil Chemotactic Factor (NCF) from alveolar macrophages by the influence of flavanoids in tea solids (discussed in Chapter 6).

There was a increase in plasma TRAP values after endotoxin treatment in both dietary groups. There was a slight increase in TRAP values of tea solids supplemented groups when compared with corresponding normally fed groups (Table 11.11). This may be due to contribution from antioxidant substances in tea solids.

Number of neutrophils and myeloperoxidase activity in lung after endotoxin treatment.

Increased neutrophil numbers were observed even after one week of tea solids supplementation (Fig.11.1). It appears that tea solids supplementation for longer periods (three weeks) had no enhancing effect on the above. In fact after three weeks % of neutrophils in lung tissue had decreased when compared with one week.

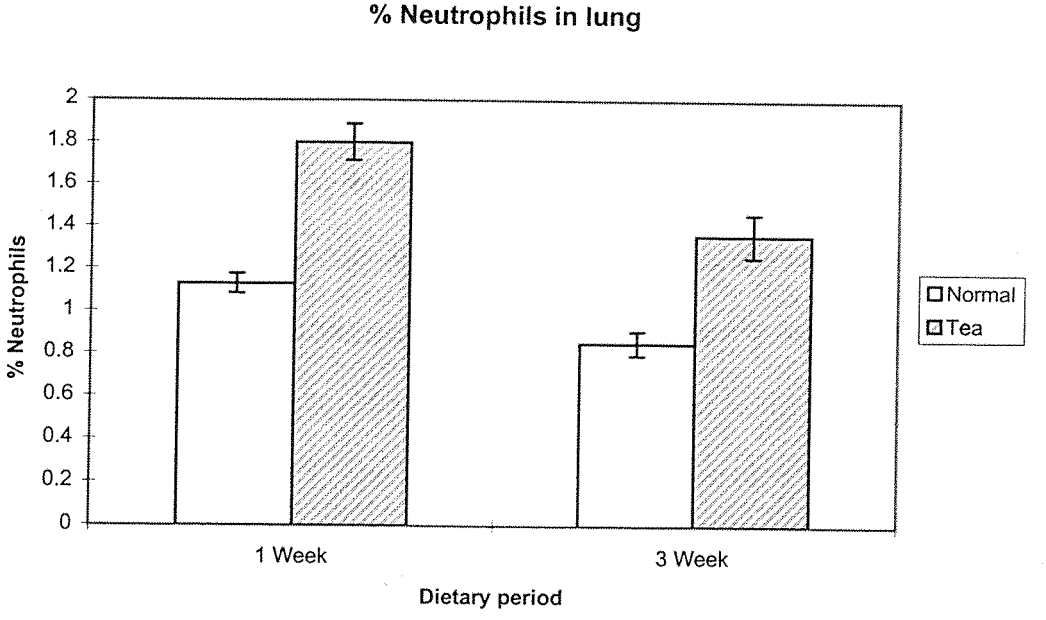


Fig.11.1. % of neutrophils in lung of rats receiving a normal diet or a diet supplemented with tea solids (5g/kg diet) for one or three weeks and given an endotoxin injection (LPS, 200μg/kg body weight)

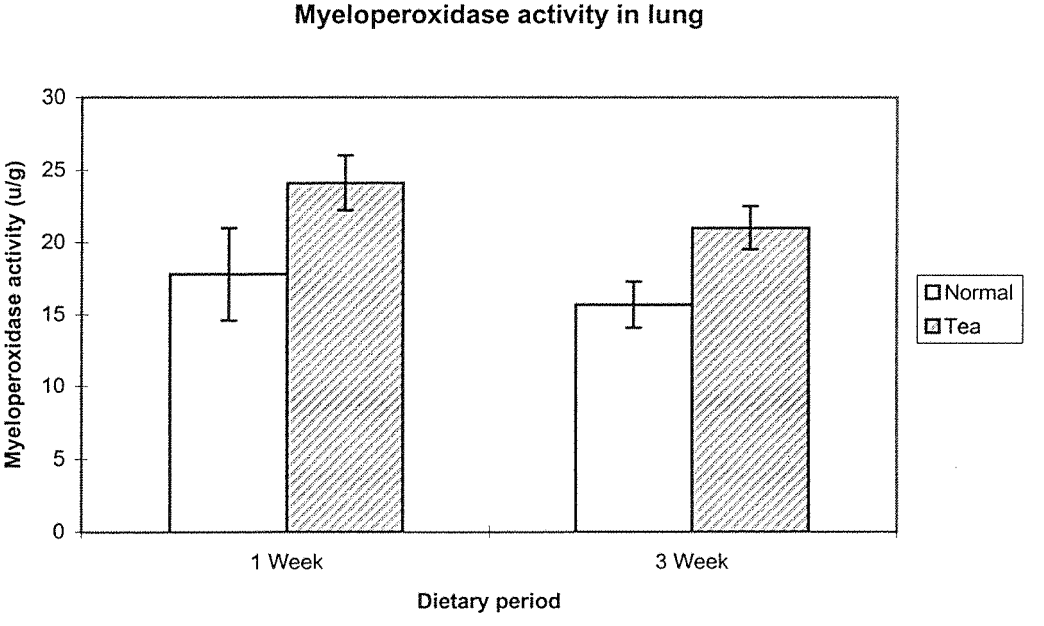


Fig.11.2. Myeloperoxidase activity in lung of rats receiving a normal diet or a diet supplemented with tea solids (5g/kg diet) for one or three weeks and given an endotoxin injection (LPS, 200μg/kg body weight)

Lung myeloperoxidase activity after endotoxin treatment was significantly higher in the animals whose diet was supplemented with tea solids when compared with animals receiving a normal diet. This was observed even after a dietary period of one week. After three weeks dietary periods also, the groups which received tea solids in their diets had higher myeloperoxidase activity (fig.11.2). Myeloperoxidase activity had the same pattern as that for number of neutrophils in lung. There was a good correlation between the number of neutrophils and myeloperoxidase activity in lung of endotoxin treated animals ($r=0.778$, $p=0.0147$).

Results obtained for % neutrophils and myeloperoxidase activity in lung tissue confirm the earlier observations (Chapter 6) that tea solids increase the number of neutrophils in lung after endotoxin treatment.

CHAPTER 12

GENERAL DISCUSSION

Black tea is widely consumed in many countries and is therefore an important component of the human diet, for sociological reasons. Consumption of tea in normal amounts results in the ingestion of gram quantities of tea solids. Much interest and attention had focussed upon caffeine, the pharmacological component of black tea. However as stated in the introduction, polyphenolic compounds in tea have recently attracted attention and health giving properties have been proposed for these substances. The majority of work which has led to these claims has been epidemiological in nature. The strongest claims in this field have been made for protective effect of black tea solids against atherosclerosis.

Oxidant damage to DNA, protein and lipid has been identified as a major contributory factor for many diseases such as cancer, atherosclerosis and heart disease, cataract and in aging process (Ames, 1983, Halliwell and Gutteridge, 1989, Scott, 1995, Robertson, 1991). Cancer initiation involves the damage to DNA by free radicals. This genetic alteration in a cell may lead to carcinogenesis through promotional and progression stages (discussed in Chapter 1). Oxidation of unsaturated fatty acids in low density lipoprotein(LDL) by free radicals increase the ability of its adhesion to the artery walls and thus initiate the atheromatous plaque formation. The body has an array of antioxidant defence mechanisms to inhibit free radical damage. These defences include enzyme systems such as superoxide dismutase(SOD), Catalase, Glutathione peroxidase and other molecules such as glutathione and uric acid (discussed in chapter 1). In addition to these, antioxidants derived from the diet

also act as antioxidants *in vivo*. Earlier research were focused on Vitamin E (tocopherols), Vitamin C (ascorbic acid) and ß-Carotene which had now been established as antioxidants *in vivo* (discussed in chapter 1).

Recently attention has been focused on the antioxidant activity of polyphenols. Polyphenols are secondary plant metabolites which occur widely in plants (Harbone, 1989). The *in vitro* antioxidant activity of plant polyphenols has been widely studied and there are ample evidence that they act as antioxidants by scavenging reactive oxygen species and free radicals (Rafat *et al*, 1987, Robak and Gryglewski, 1988, Hanasaki *et al*, 1994). These also acts as secondary antioxidants through sequestration of metal ions (Morel *et al*, 1994). However their action *in vivo* and their bioavailability and metabolic fate are not yet completely understood.

Both green tea and black tea contain a high proportion of polyphenols. In green tea 25 - 30% of extracted solids are catechins (a group of polyphenols). 25 - 30% of extracted solids in black tea also contain catechins and its dimeric and polymeric products theaflavin (TF) and thearubigins (TR). Therefore attention had been drawn to the antioxidant activity of both green tea and black tea and their antioxidant activity had been demonstrated under *in vitro* conditions (discussed in Chapter 1). Further to this, attention had been drawn to anticarcinogenic activity and in prevention of coronary heart diseases. Studies done on these properties using laboratory animal models suggest that tea may have preventive action through its antioxidant activity (discussed in chapter 1). However the results from epidemiological studies show mixed results and therefore are inconclusive. Evidence for *in vivo* antioxidant activity of black tea are also limited.

The aims of these studies were to establish whether black tea extracts act as an antioxidant *in vivo*. Further it was investigated whether black tea has an effect on the inflammatory response through its antioxidant activity

In the present study experiments studied both *in vitro* and *in vivo* antioxidant effect of black tea (Chapter 10). For the *in vivo* study Wistar rats were used. The method used to measure the antioxidant activity was total radical trapping antioxidant parameter (TRAP). It was found in the *in vitro* study that black tea extract acted as an antioxidant in a concentration dependant manner (Figure 12.1). In the *in vivo* study it was found that serum TRAP value of the rats increased after the ingestion of black tea extract (Figure 12.2). Indicating that antioxidant components in black extract are absorbed in to the circulatory system. In a recent study Serafini and others also have found that ingestion of both black tea and green tea extracts increase the serum TRAP values in humans (Serafini *et al*, 1996)

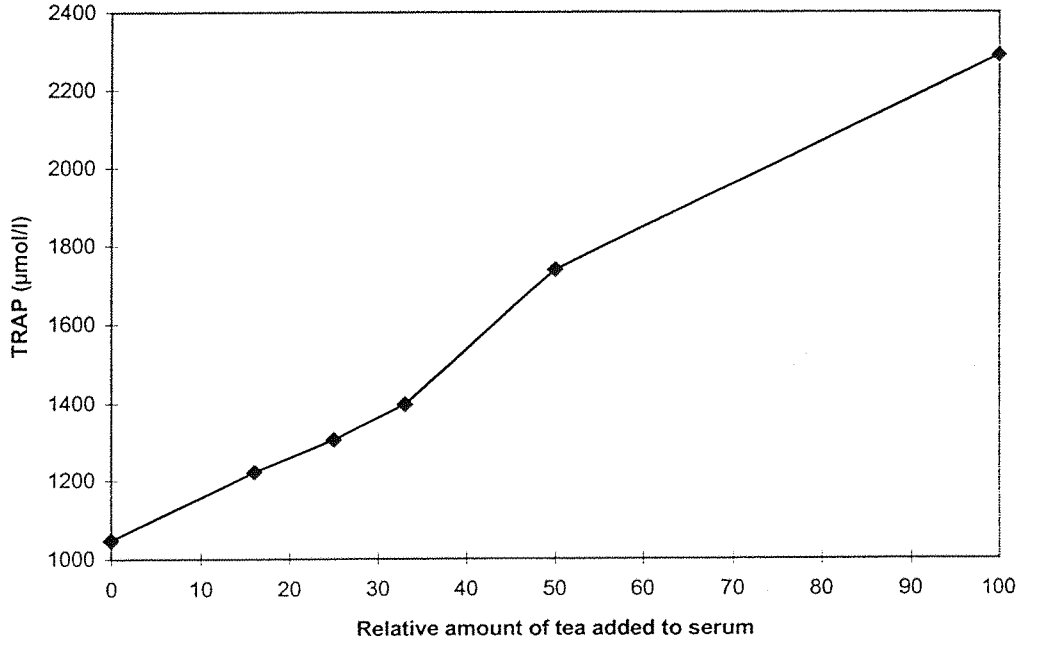


Figure 12.1 TRAP values of rat serum after addition of tea solution.

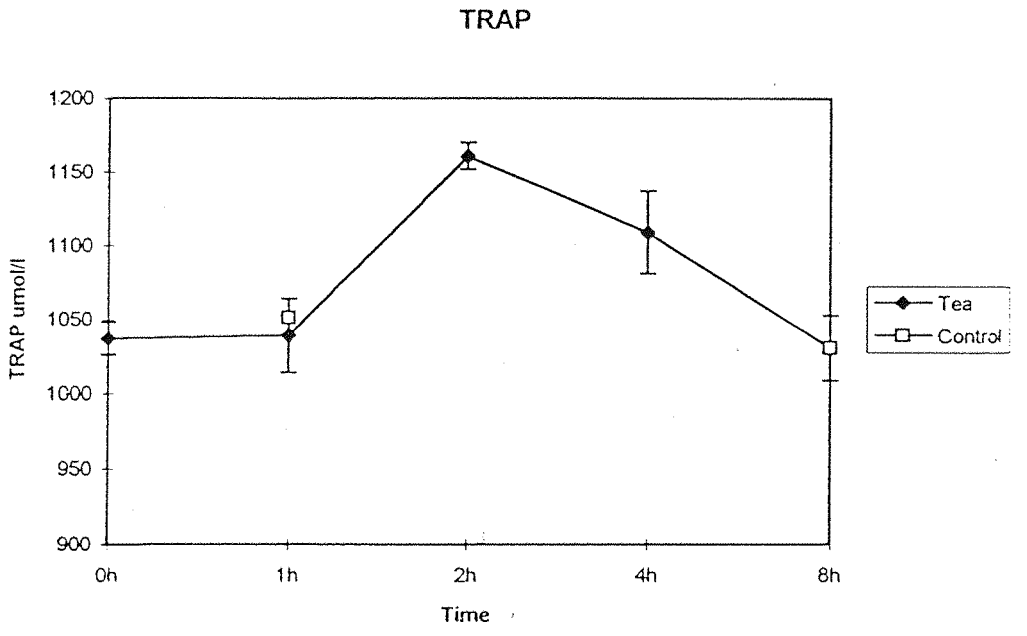


Figure 12.2 TRAP value of serum after ingestion of tea solution by rats.

Having found that aqueous decaffeinated extracts of black tea had antioxidant properties *in vivo* it was also investigated whether they influenced the inflammatory response through antioxidant activity.

It was postulated that the mode of action of the extract might be in the following manner. An inflammatory stimulus results in the release of cytokines and free radicals from the cells of the immune system. The inflammatory response is mediated through the action of cytokines. Both cytokines and the free radicals produced by the system enhance the production of each other (Figure 12.3).

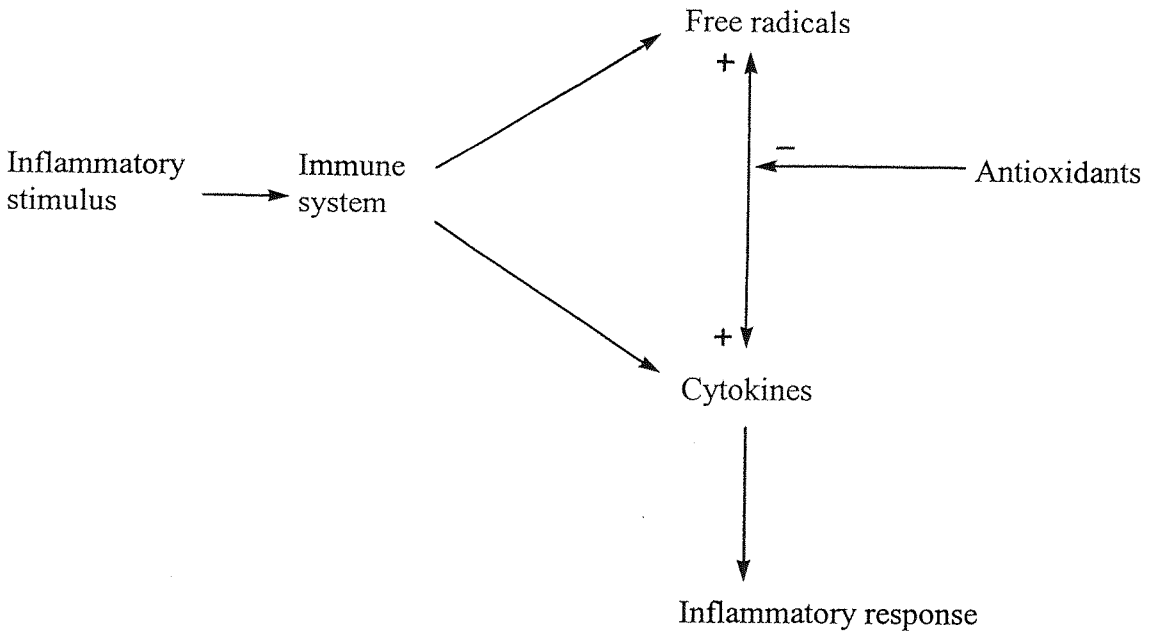


Figure 12.3 Influence of antioxidants on cytokine production

Although essential in destroying the invading pathogens and in restoration of normal metabolic process, prolonged production of cytokines and free radicals could be harmful to the host cells also. Therefore the body has an array of antioxidant mechanisms to keep a correct balance between oxidant/antioxidant status. The antioxidants derived from food may also have an effect on inflammatory response through reducing the cytokine production.

In the present study the influence of black tea solids on the response to inflammatory stimulus was investigated in the presence and absence of dietary Vitamin E (Chapter 6). It might be expected that a weakening in antioxidant defences by reduction in cellular Vitamin E would increase the inflammatory response to endotoxin via enhanced activation of NFkB. Thus if consumption of tea solids exert any influence on antioxidant defence, it would be expected to influence the extent of the increased inflammatory response in Vitamin E

deficient animals. It was observed in this study that elevated concentrations of interleukin-6 (IL-6) after the endotoxin treatment were brought down by the addition of Vitamin E and tea solids separately to a diet deficient in vitamin E. The effect of tea solids was similar to that of Vitamin E in magnitude (Figure 12.4)

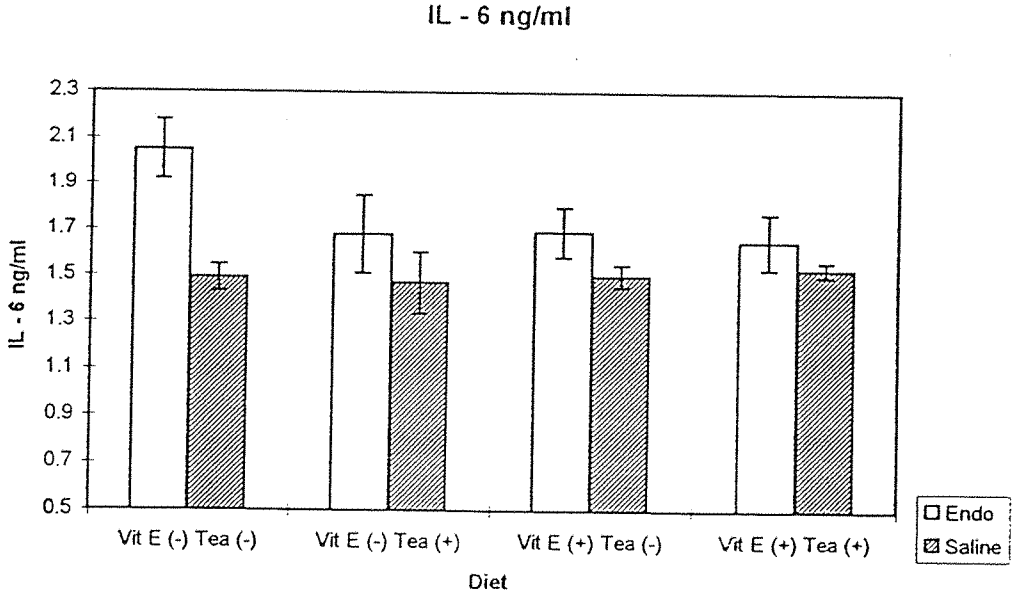


Figure 12.4 Influence of presence or absence of Vitamin E and tea solids on plasma IL-6 after endotoxin injection.

Nuclear transcription factor kappa B (NFkB) influences the production of IL-1, IL-6, and TNF production. Reactive oxygen species and free radicals contribute to the activation of NFkB (discussed in section 1.12). Therefore antioxidants can reduce the production of above cytokines by decreasing the amounts of oxidant species present in the cells producing cytokines. Therefore components in black tea solids may be reducing IL-6 production after an

inflammatory stimulus by acting as antioxidants *in vivo*. It has been found that catechol derivatives with antioxidant properties were able to inhibit TNF induced NFkB activation (Suzuki and Packer, 1994). Tea solids contain catechins, theaflavins and thearubigins which have similar structures. Therefore these components in tea solids also may act in a similar way to reduce cytokine production after an inflammatory stimulus (See Fig 1.1and 1.2 for structures).

Interleukin-6 stimulates the production of acute phase proteins in liver. Therefore plasma caeruloplasmin would act as an indirect measure of the cytokine mediated aspects of inflammation. The plasma caeruloplasmin levels, were not affected by addition of Vitamin E or tea solids separately to the diet. However addition of both together had reduced the extent of the increase of plasma caeruloplasmin concentrations following endotoxin injection(Figure 12.5). This group, where both Vitamin E and tea solids were added to diet, had the lowest concentration of IL-6 following endotoxin injection. Therefore the reduction of caeruloplasmin concentration may be brought about through the down regulation of IL-6 production.

Liver glutathione (GSH) concentration also increased with the addition of Vitamin E and tea solids to the diet. Addition of both together resulted in the greatest increase. This may have resulted by the lower utilisation of GSH in the presence of Vitamin E and tea solids (Figure 12.6)

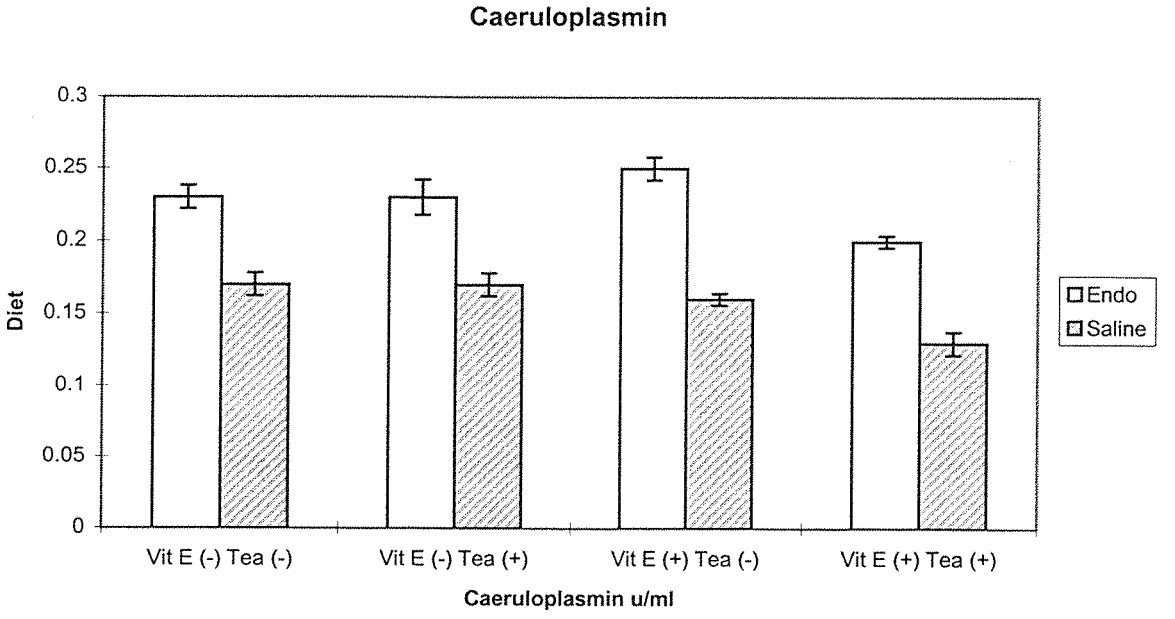


Figure 12.5 Plasma caeruloplasmin concentration of rats after endotoxin injection, receiving a diet with or without Vitamin E and tea solids.

Results mentioned so far in this section indicate that some antioxidant components in tea solids are absorbed through gastro-intestinal tract and they act as antioxidants *in vivo.* Further they are acting as antioxidants under inflammatory conditions and that they could mimic the action of Vitamin E. Although not specifically tested, it was generally assumed in literature that tea has antiinflammatory properties due to its antioxidant activity. Results of the present study mentioned above also indicate this possibility. However in the same experiment an unexpected result was observed. The lung neutrophil cell count had shown that while addition of Vitamin E to the diet had decreased their number, addition of tea solids to the diet had actually increased the number of neutrophils in the lung after endotoxin treatment (Figure 12.7).

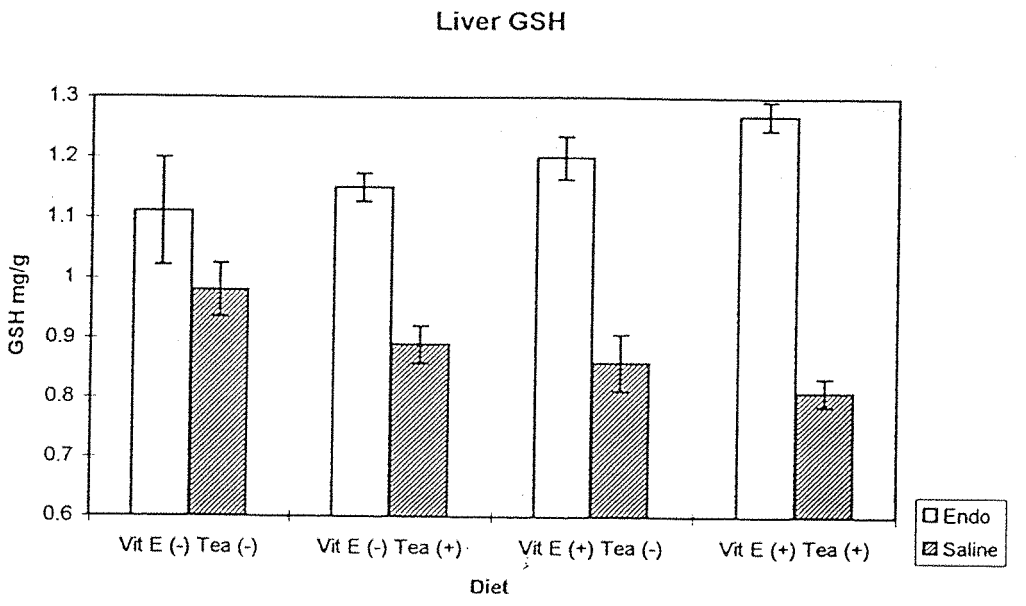


Figure 12.6 Liver glutathione concentrations of rats after endotoxin injection, receiving a diet with or without Vitamin E and tea solids.

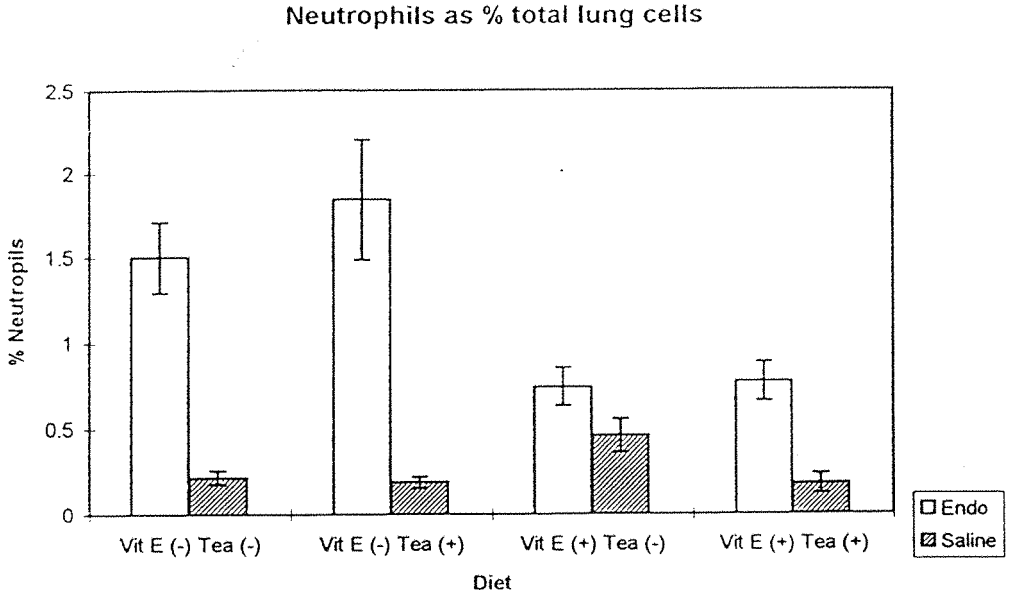


Figure 12.7 % Lung neutrophil cells of rats receiving a diet with or without Vitamin E and tea solids after endotoxin injection.

This observation was further confirmed by the measurement of myeloperoxidase activity in the lung in a later study (Chapter 8). Myeloperoxidase activity is directly proportional to the number of activated neutrophils in the tissue. Addition of tea solids to the diet had significantly increased the myeloperoxidase activity after endotoxin treatment (Figure 12.8).

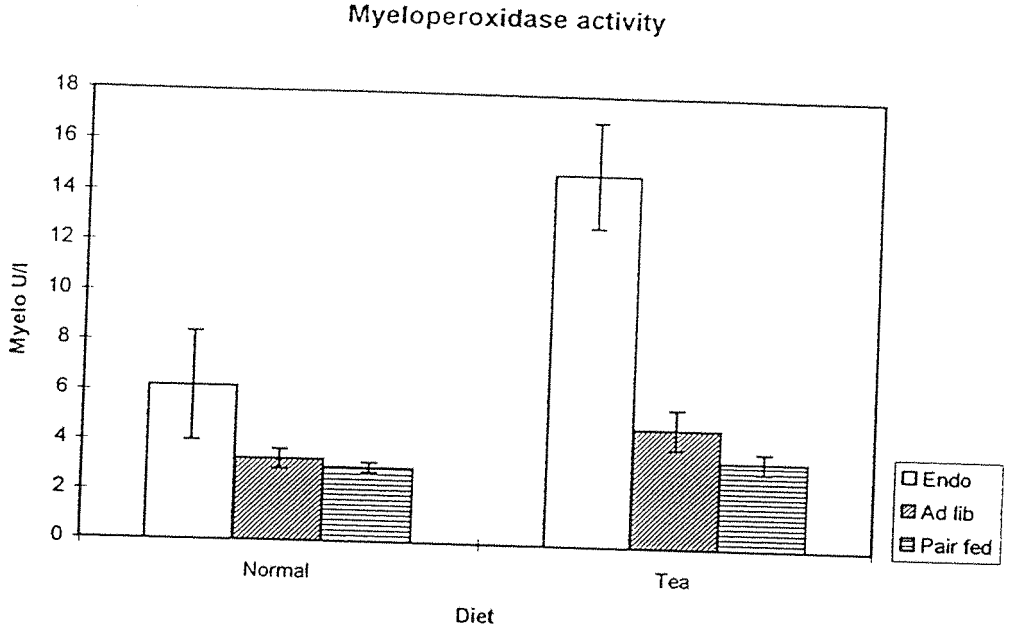


Figure 12.8 Lung myeloperoxidase activity of rats given a normal diet or a diet supplemented with tea solids for three weeks. After the dietary period, Endo= Endotoxin injected (200μg/kg body weight) and *ad libitum* fed for 24 hours, Ad lib= *Ad libitum* fed for last 24 hours, Pair fed= Pair fed for last 24 hours with the intake of endotoxin injected rats for last 24 hours.

Another study carried out to confirm the above findings also gave similar results (Chapter 11). This indicates that although tea solids act as an antioxidant *in vivo* they have proinflammatory properties by increasing the number of activated neutrophils under an inflammatory stimulus, at least in lung.

However in these studies it was noted that tea components increase the number of neutrophils in lung only after endotoxin treatment. In control groups where endotoxin injection was not given an increase of lung neutrophils was not found. Therefore it could be ruled out that tea components are providing an inflammatory stimulus and thus increase the neutrophils in lung. There are two possible mechanisms by which number of neutrophils in lung could increase. That is either by increased activation and proliferation of neutrophils or by increased recruitment of neutrophils to the lung tissue under the influence of some component in tea solids after endotoxin injection.

In an *in vitro* study Hu and co workers have found that epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and theaflavin digallate (TFDG) (which are components in tea solids) enhanced the spontaneous and endotoxin induced proliferation of mouse splenic B-cells. However there was no effect on T-cells. They have found that other catechins(Catechin and epicatechin) found in tea which does not contain galloyl groups (Fig 12.9) had no effect on both B-cell and T-cell proliferation (see fig 1.1 and 1.2 for structures of catechins and theaflavins).

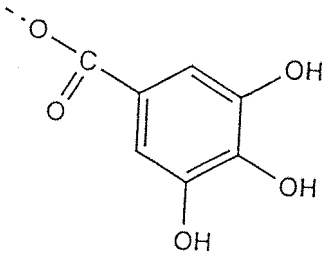


Figure 12.9 Structure of galloyl group.

However the mechanism by which these compounds enhance the proliferation of B-cells could not be explained (Hu *et al*, 1992). No information is available however in the literature on the effect of tea components on neutrophil function.

Recruitment of neutrophils to the lung tissue is induced by the chemoattractants which are released by these cells when encounter pathogenic microorganisms or other foreign stimulants such as endotoxin. Neutrophil migration from capillaries to lung tissue takes place by initially adhering to endothelial cells of the capillaries. They then pass through the endothelial cells by diapedesis and migrate through the lung tissue by chemotaxis. Interleukin-8 and Neutrophil Chemotactic Factor (NCF) released by alveolar macrophages help in the recruitment of neutrophils to lung tissue (discussed in section 1.10).

Rohrbach and others have found that cotton condensed tannins increase the secretion of NCF from alveolar macrophages. This resulted in increased recruitment of neutrophils to lung tissue (Rohrbach *et al*, 1989). Cotton condensed tannins are comprised mainly of polymerised products of flavan-3-ols and flavan-3,4-diols (Salunkhe *et al*, 1990).

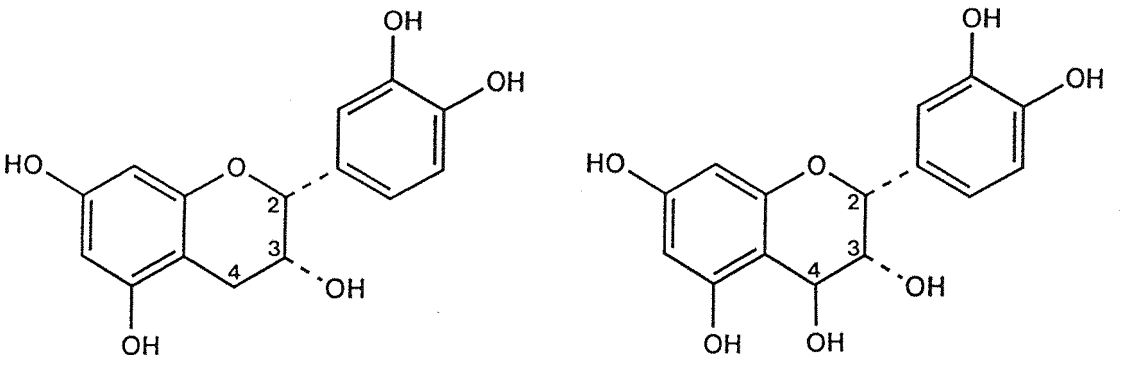


Fig 12.10 Structures of flavan-3-ols and flavan-3,4-diols.

Tea also contain similar flavanoid components (catechins, theaflavins and thearubigins, see chapter 1 for structures). Therefore these components may also be acting in a similar way to increase the recruitment of neutrophils to lung tissue.

In summary, from the results obtained in the present research programme, antioxidant components in black tea are absorbed through the gastro-intestinal tract and they exert antioxidant activity *in vivo*. It is also found that components in black tea influence to increase the number of neutrophils in lung after an inflammatory stimulus. The effect of tea solids on other populations of immune cells in the body is unknown.

There are dangers in extrapolating results from rat studies tohumans. It could be argued that the dose of tea solids used in the present studies was substantial (1/5 cup per rat or 1 cup/kg body weight). Nevertheless tea clearly contains substances with the potential for

enhancing antioxidant defence during inflammation and with immunomodulatory properties. Future studies on these effects would seem to be of potential benefit to human health and wellbeing.

Despite the presence of a variety of antioxidants in the body, oxidative stress exerted by free radicals and reactive oxygen species has been implicated in the aetiology of a wide variety of diseases. These include mutagenesis, cancer, atherosclerosis and chronic inflammatory diseases.

Environmental exposure to free radicals has increased in recent times due to the increased production of exhaust gases and other pollutants, thereby increasing the incidence of above diseases. Therefore a regular intake of antioxidants from a source like tea could be considered as a part of a healthy life style.

The metabolic fate and bioavailability of polyphenolic components in tea are poorly understood yet. Therefore studies on absorption and tissue distribution of these components and their antioxidant potential in different body tissues will provide important information on their effectiveness in the prevention of oxidant mediated diseases.

Present studies have shown that tea components have immunomodulatory properties by decreasing interleukin-6 concentration and by increasing number of neutrophil cells in lung after an inflammatory stimulus. However, effect of tea components on other cytokines and the effect on neutrophils in other tissues are not known. Further studies on these will provide a better understanding of clinical importance of above findings.

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