EFFECTS OF TUALANG HONEY SUPPLEMENTATION ON OXIDATIVE STRESS STATUS, INFLAMMATION AND LIPID PROFILES IN CHRONIC SMOKERS

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by

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LIST OF ABBREVIATIONS

ATP	adenisone triphosphate
BMI	body mass index
B cells	B lymphocytes
CAT	catalase
Cu/Zn-SOD	copper zinc-containing superoxide dismutase
Cu^{+}	cuprous ion
Cu^{2+}	cupric ion
CRP	C-reactive protein
CS	cigarette smoke
CO	carbon monoxide
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC-SOD	extracellular copper containing superoxide dismutase
EDTA	ethylenediaminetetraacetic acid
FAMA	Federal Agricultural Marketing Authority
FRAP	ferric reducing antioxidant power
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
FFA	free fatty acid
GPx	gluthathione peroxidase
GR	glutathione reductase
GSH	glutathione

GSSG	glutathione disulfide
GST	glutathione S-transferase
HDL	high density lipoprotein
H_2O	water
H_2O_2	hydrogen peroxide
HNO_2	nitrous acid
$HO^{2\cdot}$	hydroperoxyl
HOCL	hypochlorous acid
hsCRP	high sensitivity C-reactive protein
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
kg	kilogram
kg/m^2	kilogram per square meter
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAP	mean arterial pressure
MDA	malonaldehyde
mL	mililiter
mM	milimolar
mg	miligram
min	minute
mmoles	milimoles
Mn-SOD	manganese-containing superoxide dismutase
MS	mainstream smoke

n	sample size
nm	nanometer
ng	nanogram
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NADP+	nicotinamide adenine dinucleotide phosphate (oxidized)
NaOH	sodium hydroxide
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cell
$NO^{\cdot}$	nitric oxide
$NO^{2\cdot}$	nitrogen dioxide
N_2O_3	dinitrogen trioxide
N_2O_4	dinitrogen tetroxide
NO_2^+	nitronium cation
$^1O_2$	singlet oxygen
O^2	oxygen
O_2^-	superoxide
O_3	ozone
$OH^{\cdot}$	hydroxyl
$ONOO^-$	peroxynitrite
ONOOH	peroxynitrous acid
PASW	predictive analytics software
pg	picogram
pNPP	para-nitrophenyl phosphate
RNS	reactive nitrogen species

ROS	reactive oxygen species
$RO^{2\cdot}$	peroxyl
$RO^{\cdot}$	alkoxyl
ROONO	alkyl peroxynitrites
SEM	standard error mean
SOD	superoxide dismutase
SS	sidestram smoke
SPE	solid-phase extraction
TAS	total antioxidant status
TBARS	thiobarbituric acids reactive substances
T cells	T lympocytes
TC	total cholesterol
TLR	Toll-like receptor
TG	triglycerides
THS	third hand smoke
TNF-α	tumour necrosis-alpha
U/L	units/liter
U/mg	units/miligram
U/ml	units/mililiter
USM	Universiti Sains Malaysia
USA	United States of America
VLDL	very low density lipoprotein
%	percent
°C	celcius
µM	micromolar

µL	microliter
µg	microgram
µmole	micromole

KESAN SUPLEMEN MADU TUALANG KE ATAS STATUS STRES OKSIDATIF, KERADANGAN DAN PROFIL LIPID DALAM KALANGAN PEROKOK KRONIK

ABSTRAK

Peningkatan stres oksidatif, keradangan dan perubahan dalam profil lipid perokok telah dikaitkan sebagai faktor utama kaitan merokok dengan penyakit kardiovaskular. Walaubagaimanapun, kesan kebaikan madu sebagai antioksidan, antiradang dan antilipid dalam kalangan perokok masih belum dilaporkan. Oleh itu, kajian ini adalah untuk menentukan faedah suplemen Madu Tualang ke atas stres oksidatif, keradangan dan profil lipid dalam kalangan perokok. Seramai 36 bukan perokok dan 72 perokok telah dipilih. Kumpulan perokok kemudiannya dibahagikan secara rawak kepada dua kumpulan iaitu perokok tanpa suplemen dan perokok dengan suplemen madu sebanyak 20 gram setiap hari selama 12 minggu. Sampel darah telah diperolehi bagi menentukan tahap stres oksidatif, keradangan dan profil lipid daripada semua kumpulan di peringkat pra-intervensi dan daripada kumpulan perokok di peringkat pos-intervensi. Pada peringkat pra-intervensi, perokok secara signifikan mempunyai nilai F_2-isoprostanes, jumlah status antioksidan (TAS) dan katalase (CAT) yang lebih tinggi berbanding dengan bukan perokok. Aktiviti superoksida dismutase (SOD) dan glutation peroksidase (GPx) secara signifikan adalah lebih rendah dalam kalangan perokok berbanding dengan bukan perokok. Nilai tahap sensitiviti protein C-reaktif (hsCRP) secara signifikan adalah lebih tinggi dalam kalangan perokok

berbanding dengan bukan perokok. Walaubagaimanapun, tiada perbezaan yang signifikan ditunjukkan dalam nilai factor tumor necrosis alfa (TNF-α) dan interleukin-6 (IL-6). Selain itu, perokok pada peringkat pra-intervensi menunjukkan secara signifikan tahap yang lebih rendah bagi nilai lipoprotein ketumpatan tinggi (HDL) dan tahap yang lebih tinggi bagi jumlah kolesterol (TC), lipoprotein ketumpatan rendah (LDL) dan trigliserida (TG) berbanding dengan bukan perokok. Dalam kalangan perokok, Madu Tualang secara signifikan menurunkan nilai F_2-isoprostanes dan juga meningkatkan nilai/aktiviti TAS, CAT, dan GPx. Madu Tualang juga secara signifikan meningkatkan nilai TNF-α dan menurunkan nilai hsCRP. Selain itu, Madu Tualang secara signifikan menurunkan nilai TC dan LDL. Penemuan ini mencadangkan faedah Madu Tualang terhadap stress oksidatif, keradangan dan juga perubahan dalam profil lipid dalam kalangan perokok yang mungkin boleh mengurangkan pembentukan ateroma dan seterusnya risiko penyakit jantung. Sebahagian daripada kesan ini boleh dikaitkan dengan kepelbagaian komponen Madu Tualang termasuklah asid fenolik dan sebatian flavonoid yang memiliki sifat antioksidan, antiradang dan antilipid. Namun, kajian lebih lanjut diperlukan untuk menjelaskan kesan mekanisme yang tepat ke atas faedah Madu Tualang.

EFFECTS OF TUALANG HONEY SUPPLEMENTATION ON OXIDATIVE STRESS STATUS, INFLAMMATION AND LIPID PROFILES IN CHRONIC SMOKERS

ABSTRACT

Elevated oxidative stress, inflammation and alteration in lipid profiles in smokers have been proposed as among the major factors that contribute to the development of smoking-related cardiovascular disease. However, the possible beneficial effect of Tualang honey that has antioxidant, anti-inflammatory and antilipid properties among smokers has yet been reported. The aims of this study, therefore, to determine the effects of Tualang Honey supplementation on oxidative stress markers, inflammatory markers and lipid profiles in smokers. A total of 36 non smokers and 72 smokers were recruited. The smokers were then randomly divided into 2 groups namely smokers without supplementation and smokers with honey supplementation at the dose of 20 gram/day for 12 weeks. Blood was obtained for determination of oxidative stress markers, inflammatory markers and lipid profiles from all groups at pre-intervention and from smokers group at post-intervention. At pre-intervention, smokers had significantly higher levels of F_2-isoprostanes, total antioxidant status (TAS) and catalase (CAT) activity compared to non smokers. Meanwhile, the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were significantly lower in smokers than non smokers. A significantly higher level of high sensitivity C-reactive protein (hsCRP) was

found in smokers compared to non smokers. However, no significant differences were found for the levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). Furthermore, significantly lower high-density lipoprotein (HDL) as well as higher total cholesterol (TC), low-density lipoprotein (LDL) and triglyceride (TG) levels were observed in smokers compared to non smokers. In smokers, Tualang honey supplementation significantly decreased the level of F_2-isoprostanes as well as increased the levels/activities of TAS, CAT and GPx. Tualang honey also significantly increased the level of TNF-α and decreased the level of hsCRP. Apart from that, Tualang honey significantly decreased the levels of TC and LDL. These observations might suggest a beneficial effect of Tualang honey against cigarette smoke-induced oxidative stress, inflammation as well as alteration in lipid profiles among smokers which may provide an effect in reducing the atherothrombosis formation and subsequent risk of cardiovascular disease. These beneficial effects could be, partly attributed to its various components which include phenolic acids and flavonoid compounds that have antioxidant, anti-inflammatory and antilipid properties. However, further studies are required to elucidate the exact mechanism of action on these benefical effects of Tualang honey

CHAPTER 1

INTRODUCTION

Cigarette smoking has been reported as one of the major risk factor for the development of cardiovascular diseases. The other factors include hypertension, coronary artery disease, atherosclerotic vascular disease, myocardial infarct and stroke (Ockene and Miller, 1997). The related major factors in initiating and accelerating the atherothrombotic process which contribute to the development of cardiovascular disease are oxidant-antioxidant imbalance, inflammation, alteration in lipid profiles as well as endothelial dysfunction.

Several studies reported that cigarette smoke (CS) exposure is associated with increased lipid peroxidation (Garg *et al.*, 2006; Bloomer, 2007; Pasupathi *et al.*, 2009), an alteration in the levels of erythrocyte antioxidant enzymes, (Pannuru *et al.*, 2011; Tonguc *et al.*, 2011; Aziz *et al.*, 2013), decreased total antioxidant status (TAS) (Bloomer, 2007; Jha *et al.*, 2007; Aziz *et al.*, 2013), increased levels of serum inflammatory markers such as tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP) (Petrescu *et al.*, 2010) as well as decreased level of plasma antioxidant such as ascorbic acid, the reduced form of vitamin C (Ayaori *et al.*, 2000). In addition, as smoking is associated with increased lipolysis, it has been reported that the levels of total cholesterol (TC), low density lipoprotein (LDL) and triglycerides (TG) are

significantly higher while the level of high density lipoprotein (HDL) is significantly lower in smokers compared to non smokers (Rao and Emmanuel, 2012).

Although quit smoking is the best method to reduce the risk of cardiovascular diseases, there is still a need to reduce this risk among chronic smokers who fail to quit smoking by any means. This has led to increased interest to investigate the possible beneficial effect of natural products in reducing this risk among smokers. A study has reported that 6-week supplementation of green algae namely *Chlorella vulgaris,* which has antioxidant activity, among smokers significantly increases the levels of erythrocyte antioxidant enzymes activities such as catalase (CAT) and superoxide dismutase (SOD). The levels of plasma antioxidants such as vitamin C and α-tocopherol are also significantly increased (Lee *et al.,* 2010). Meanwhile, Leelarungrayub and colleague have observed that 8-week supplementation of another herb namely *Vernonia cinerea Less* among active smokers significantly decreases malonaldehyde (MDA) level and increases TAS (Leelarungrayub *et al.*, 2010). Apart from that, supplementation of camu-camu juice (with high vitamin C content), which has anti-inflammatory activity, among male smokers for 7 days significantly reduces the levels of serum high sensitivity C-reactive protein (hsCRP), interleukin-6 (IL-6) and interleukin-8 (IL-8) (Inoue *et al.*, 2008). Meanwhile, 2-month supplementation of vitamin C significantly reduces the levels of plasma F_2-isoprostanes as well as C-reactive protein (CRP) in passive smokers (Dietrich *et al.*, 2003). In addition, supplementation of vitamin C-rich fruit juices for 26 days (Alvarez-Parrilla *et al.*, 2010) as well as Quercitin-rich supplement for 10 weeks (Lee *et al.*, 2011) significantly decreases TC and LDL levels in smokers.

Honey is a natural product of bees produced from nectar of blossoms and honeydew. The honeydew is formed from plant-sucking insects or secretions of plants (Alimentarius, 2001). The flavour, colour and contents of honey vary depending on botanical origin of honey (Oddo *et al.*, 2004). The primary contents of honey are carbohydrates such as fructose and glucose as well as organic acids, proteins, minerals and aromatic compounds (Bogdanov *et al.*, 2008). Apart from that, honey also contains antioxidants such as vitamins A and E (Al-Waili, 2003), enzymes such as glucose oxidase and catalase (Gheldof *et al.*, 2002), phenolic acids (Michalkiewicz *et al.*, 2008; Pyrzynska and Biesaga, 2009), flavonoids (Yao *et al.*, 2004; Khalil *et al.*, 2011a), as well as has antioxidant properties (Mohamed *et al.*, 2010a).

Earlier studies have reported the effectiveness of honey in treating wounds, burns, ulcers and other inflammatory diseases in both human (Cavanagh *et al.*, 1970; Blomfield, 1973; Zumla and Lulat, 1989; Subrahmanyam, 1991; Pieper, 2009) and animal subjects (Bergman *et al.*, 1983; Oryan and Zaker, 1998). Meanwhile, scientific studies have demonstrated that honey possesses numerous important biological properties which include antioxidant (Beretta *et al.*, 2005; Perez *et al.*, 2006; Korkmaz and Kolankaya, 2009; Mohamed *et al.*, 2010a; Tartibian *et al.*, 2011), anti-inflammatory (Prakash *et al.*, 2008; Bashkaran *et al.*, 2011; Tartibian *et al.*, 2011; Ahmad *et al.*, 2012; Mohd Effendy *et al.*, 2012), immunomodulatory (Timm *et al.*, 2008), antimicrobial (Molan, 2006; Tan *et al.*, 2009; Khoo *et al.*, 2010; Al-Waili *et al.*, 2011; Sukur *et al.*, 2011) and anticancer properties (Swellam *et al.*, 2003; Ghashm *et al.*, 2010). In addition, supplementation of honey in rats exposed to CS for 13 weeks has been reported to improve testicular functions (Mahaneem *et al.*, 2011) as well as to reduce damage and

oxidative stress in the testis partly by reducing lipid peroxidation and restoring antioxidant system (Mohamed *et al.*, 2010b).

However, to date, no study has been reported to determine whether honey supplementation is able to protect against oxidative stress and inflammation as well as to improve lipid profiles among chronic smokers. Therefore, the aim of this study was to determine the possible role of honey in protecting against or reducing the oxidative stress and inflammation as well as in improving the lipid profiles among chronic smokers.

The specific objectives of this study were:

1) To determine the effects of honey supplementation on oxidative stress markers such as plasma F_2-isoprostanes, erythrocyte antioxidant enzymes [SOD, glutathione peroxidase (GPx), CAT] and TAS in chronic smokers.
2) To determine the effects of honey supplementation on the levels of plasma inflammatory markers such as IL-6, hsCRP and TNF-α in chronic smokers.
3) To determine the effects of honey on serum fasting lipid profiles such as TC, LDL, HDL and TG in chronic smokers.

The hypotheses of this study were:

1) Honey supplementation significantly reduces lipid peroxidation, improves erythrocyte antioxidant enzymes as well as improves TAS in chronic smokers.
2) Honey supplementation significantly reduces inflammation in chronic smokers.
3) Honey supplementation significantly improves lipid profiles in chronic smokers.

CHAPTER 2

LITERATURE REVIEW

2.1. An overview on cigarette smoke

CS can be divided into 2 phases namely tar phase and gas phase. The tar or particulate phase of CS which contains $>10^{17}$ free radicals/gram, is the material that is trapped when the smoke stream is passed through the Cambridge glass-fiber filter that retains 99.9% of all particulate material with a size of >0.1 μm. This phase contributes to most of the tobacco-associated carcinogenicity. Meanwhile, the gas phase which contains $>10^{15}$ free radicals/puff is the material that passes through the filter (Pryor and Stone, 1993).

CS can also be further categorized into mainstream smoke, sidestream smoke, environmental tobacco smoke and third hand smoke. Mainstream smoke (MS) is defined as smoke generated at a burning cigarette's tip when the smoker puffs. It is deeply inhaled by the active smoker and comprises 8% of tar and 92% of gaseous component (Pryor and Stone, 1993; Nelson, 2001). Sidestream smoke (SS) is defined as the smoke emitted from the burning ends of a cigarette and it contains a relatively higher concentration of the toxic gaseous component compared to MS (Glantz and Parmley, 1991). As the production of SS is at lower temperature compared to MS, most of carcinogens and toxicants are produced in greater amounts in SS than in MS. The particle size for SS is 0.01-1.0 μm while the particle size for MS is 0.1-1.0 μm. This discrepancy has implications for the particles to deposit in the various regions of the human respiratory tract. In addition, compared to MS, SS has 2–6 times more

condensate per gram (Swan and Lessov-Schlaggar, 2007; Bernert *et al.*, 2010) and the smokers may be exposed to higher concentrations of carcinogens as a result of incomplete combustion process. Meanwhile, environmental tobacco smoke results from the mixture of SS (85%) and a small fraction of exhaled MS (15%) which may be inhaled by non smoker in a closed or open environment (Taylor *et al.*, 1992).

On the other hand, third hand smoke (THS) is defined as contamination of residual tobacco that remains after the cigarette is extinguished (Winickoff *et al.*, 2009). THS includes the residual tobacco smoke pollutants that either (i) persist in dust and on surfaces after tobacco has been smoked, (ii) are re-radiate back into the gas phase, or (iii) react with oxidants as well as other compounds in the environment forming secondary pollutants (Matt, 2011). Exposure to THS is the outcome from the involuntary inhalation, dermal uptake or ingestion of THS pollutants in the dust, air and on surfaces. In the form of particulate matter, these THS pollutants are accumulated in a layer onto every surface within the house in loose household dust. Apart from that, these toxins also take the form of volatile toxic compounds that 'off gas' into the air over days, weeks and months. Therefore, one day of smoking indoor may lead to THS pollutant exposure to people within that space in the future (Singer *et al.*, 2002; Matt *et al.*, 2004).

CS contains about 5,000 chemicals (Borgerding and Klus, 2005; Thielen *et al.*, 2008) produced following burning of tobacco leaves. The hazardous chemicals present in CS include nicotine, tar, carbon monoxide (CO), cadmium, ammonia, benzene, and 4-aminobiphenyl (Swan and Lessov-Schlaggar, 2007; Bernert *et al.*, 2010). Other compounds are hydrogen cyanide, arsenic, vinyl chloride, acrolein, formaldehyde,

acetaldehyde, catechol, cresol, hydroquinone, lead, methylethyl, nitricoxide, phenol, styrene, tolutene and butane.

CS is a major risk factor for the development of cardiovascular diseases such as atherosclerotic vascular disease, hypertension, coronary artery disease, myocardial infarct and stroke (Ockene and Miller, 1997). Endothelial dysfunction, inflammation, oxidant-antioxidant imbalance or increased lipid peroxidation, activation of leucocytes and platelets as well as an alteration of antithrombotic and prothrombotic factors are among the smoking related major determinants of initiation and acceleration of the atherothrombotic process leading to these cardiovascular diseases. By year 2025, 10 million smokers are expected to die each year if current trends continue (Davis *et al.*, 2007; Hatsukami *et al.*, 2008). In addition, according to World Health Organization, there are estimated 5.4 million premature deaths caused by tobacco smoking worldwide (Organization, 2008).

2.2 Oxidative stress

Oxidative stress is a result from imbalance between free radicals species production and the antioxidant system in cells (Smith *et al.*, 2005). Oxidative stress occurs when there is diminished detoxification or excessive production of free radical species. Prolonged highly oxidized cells predispose the surrounding cells to oxidative damage (Halliwell, 2007).

Free radicals are defined as atoms and molecules with unpaired electrons in their orbital or outer shell (Guetens *et al.*, 2002; Penna *et al.*, 2009). The common property is the presence of unpaired electron shared by most radicals. Most of the radicals are highly reactive and may either extract or donate an electron from other molecules, thus, behaving as reductants or oxidants. A free radical is easily formed when there is broken in a covalent bond between entities and one electron remains with each newly formed atom. These newly formed free radicals are very unstable, highly reactive and only may become stable once they acquire electrons from protein, lipids, carbohydrates, nucleic acids and other nearby molecules which therefore, leading to a cascade of damage and disease (Da Silva *et al.*, 2010).

Free radicals may react with lipids, carbohydrates, proteins and DNAs causing cellular damage. High level of cellular damage may result in cell death through necrosis or apoptosis (Marnett *et al.*, 2003). Lipid peroxidation is due to reaction of free radicals on lipids. For example, hydroxyl radical which is a free radical may act as initiator by extracting a hydrogen atom, preferably from the double bond of a polyunsaturated fatty acid in a lipid membrane. The chain reaction is then propagated forming lipid peroxyl radicals and lipid peroxides. This may lead to degradation of lipid forming products such as F_2-isoprostanes, malondialdehyde (MDA), 4-hydroxynoneal and acrolein.

F_2-isoprostanes are prostaglandin F_2-like compounds, produced *in vivo*, primarily in situ, by nonenzymatic free-radical–catalyzed peroxidation of esterified arachidonic acid (Lynch *et al.*, 1994). It is then cleaved by phospholipase before being released into the circulation. Finally, it is excreted in the urine as free isoprostanes. The plasma levels

of F_2-isoprostanes have been shown as an indicator of *in vivo* oxidative stress (Lynch *et al.*, 1994). In human, F_2-isoprostanes are present in two forms, either esterified to lipids which is being the most abundant and or as free F_2-isoprostanes (Dalle-Donne *et al.*, 2006). Previous study reported that measurement of F_2-isoprostanes is a reliable indicator of oxidative stress in vivo due to several factors: (i) F_2-isoprostanes are stable compounds (ii) specific lipid peroxidation products (iii) formation of F_2-isoprostanes increases dramatically in vivo in number of animal models of oxidant injury (iv) levels of F_2-isoprostanes are not affected by lipid content of the diet (v) formation of F_2-isoprostanes is modulated by antioxidant status (vi) F_2-isoprostanes levels are present in detectable quantities in all normal biological tissues and fluids, thus, allowing the definition of a normal range (Roberts II and Morrow, 2000). In addition, another study observed that F_2-isoprostanes is the 'gold standard' and very specific compared to other lipid peroxidation assays (Hollman *et al.*, 2011).

On the other hand, nitrosative stress is due to reaction between reactive oxygen species (ROS) and reactive nitrogen species (RNS) in damaging the cells. ROS originate from oxygen while RNS originate from the reaction of oxygen with nitrogen. ROS and RNS are collective terms that include both radicals as well as non radicals that are oxidizing agents and/or easily converted into radicals. Example of ROS and RNS are shown in Table 2.1.

2.2.1 Reactive Oxygen Species (ROS)

Primary ROS are the superoxide anion either arising following oxygen 'activation' by physical irradiation or through metabolic processes. The secondary ROS are formed when the primary ROS further interact with other molecules either directly or prevalently through enzyme- or meta-catalyzed processes (Valko *et al.*, 2005). Formation of superoxide anion is by univalent reduction of triplet-state molecular oxygen ($^3O_2$) (Droge, 2002). It is mediated by the enzymes such as xanthine oxidase and NAD(P)H oxidase or nonenzymically by redox-reactive compounds which include semiubiquinone compound of the mitochondrial electron transport chain (Droge, 2002). Production of superoxide anion occurs mostly within the cell of mitochondria (Cadenas and Sies, 1998). Conversion of superoxide anion to hydrogen peroxide (H_2O_2) is by SOD (Deby and Goutier, 1990). Nonenzymically, superoxide anion is converted to nonradical species H_2O_2 and singlet oxygen ($^1O_2$) (Steinbeck *et al.*, 1993). H_2O_2 is converted to hydroxyl radical ($^{\cdot}OH$) which is highly reactive with reduced transition metals such as copper or ferrous ions (Chance *et al.*, 1979). Consumption of oxygen in the peroxisome leads to production of H_2O_2, which is then used to oxidize a variety of molecules (Valko *et al.*, 2007).

2.2.2 Reactive Nitrogen Species (RNS)

Production of nitric oxide radical is by oxidation of one terminal guanido-nitrogen atoms of L-arginine catalyzed by nitric oxide synthase (Palmer *et al.*, 1988). Meanwhile, nitric oxide can be converted into various RNS namely nitronium cation (NO_2^+) and peroxynitrate ($ONOO^-$).

Table 2.1: Examples of ROS and RNS.

Types	**ROS**	**RNS**
Radicals	Superoxide ($O_2^{\cdot -}$) Hydroxyl ($OH^{\cdot}$) Peroxyl ($RO^{2\cdot}$) Hydroperoxyl ($HO^{2\cdot}$) Alkoxyl ($RO^{\cdot}$)	Nitric oxide ($NO^{\cdot}$) Nitrogen dioxide ($NO^{2\cdot}$)
Nonradicals	Hydrogen peroxide (H_2O_2) Hypochlorous acid (HOCL) Singlet oxygen ($^1O_2$) Ozone (O_3)	Nitrous acid (HNO_2) Dinitrogen trioxide (N_2O_3) Dinitrogen tetroxide (N_2O_4) Peroxynitrite ($ONOO^-$) Peroxynitrous acid (ONOOH) Nitronium cation (NO_2^+) Alkyl peroxynitrites (ROONO)

2.2.3 Antioxidant defense system

Antioxidants that protect the cells against the oxidative damage by free radicals can be divided into two groups namely enzymatic antioxidants and non-enzymatic antioxidants. The enzymatic antioxidants include SOD, CAT, GPx, glutathione reductase (GR) (Smith *et al.*, 2005) as well as glutathione S-transferase (GST) (Hayes *et al.*, 2005). Meanwhile, the non-enzymatic antioxidants are glutathione, vitamin C (ascorbic acid), vitamin E (α-tocopherol), flavonoids (Smith *et al.*, 2005) and metal binding proteins such as ceruloplasmin and transferrin (Halliwell and Gutteridge, 1990). Enzymatic antioxidants convert ROS or free radicals into non-toxic products while non-enzymatic antioxidants terminate free radical chain reactions and prevent free radicals formation (Smith *et al.*, 2005).

(a) Enzymatic antioxidants

Three major antioxidant enzymes that have significant roles in protecting cells from oxidants are SOD, CAT and GPx (Bowen, 2003).

i. Superoxide dismutase

SOD is present in every cell in the body (endogenously produced antioxidant enzyme). There are three SOD isoforms in human which include: copper zinc-containing SOD (Cu/Zn-SOD), manganese-containing SOD (Mn-SOD) and extracellular Copper-zinc containing SOD (EC-SOD). Cu/Zn-SOD is rich in cytoplasm, nucleus and peroxisomes. Cu/Zn-SOD plays an important role in the first line of antioxidant defense system as it contributes most of the total activity of SOD (McCord and Fridovich, 1969). Meanwhile, Mn-SOD is abundantly found in mitochondria but very minimal in

cytoplasm. Within the mitochondria, its central role is catalyzing superoxide anions. On the other hand, EC-SOD is produced by fibroblast, vascular smooth muscle and glial cell and secreted into extracellular space. EC-SOD functions as plasma protein and thus, a protector in the extracellular space.

Role of SOD is to protect harmful effects of superoxide free radical against oxygen-metabolizing cells. It is involved in catalyzing the dismutation of superoxide to H_2O_2 and oxygen. However, in decomposing the H_2O_2, SOD must work in conjunction with other enzymes which include GPx and CAT. If H_2O_2 is left unchallenged, it may unite with iron or copper ions forming a very reactive hydroxyl radical.

ii. Catalase

CAT is widely distributed in the cell. Majority of CAT activity occurs in the peroxisomes and mitochondria (Kirkman and Gaetani, 1984). CAT catalyzes the decomposition of H_2O_2 to water and oxygen molecules (Mates *et al.*, 1999). Of all the enzymes, CAT has one of the highest turnover rates as one molecule of CAT is able to convert millions molecules of H_2O_2 to water and oxygen per second (Switala and Loewen, 2002). Removal of H_2O_2 indirectly detoxifies superoxide radicals which have been converted into H_2O_2 by the action of SOD. CAT is an intracellular antioxidant enzymes mainly present in cellular peroxisomes and to some extent in the intracellular granules and cytosol (Young and Woodside, 2001). Therefore, CAT mainly catalyzes the decomposition of H_2O_2 produced within the peroxisomes. The high level of CAT in these organelles may prevent toxic compounds accumulation. On the other hand, when the level of CAT is insufficient, H_2O_2 produced within the peroxisomes will be delivered

into the cytosol which subsequently predisposed to oxidative stress (Kirkman and Gaetani, 1984).

iii. Glutathione peroxidase

Six different isoforms of GPx protein have been identified which referred to GPx1 to GPx6. GPx requires selenium at the active site for its catalytic activity. GPx activity is dependent on the constant GSH availability. GPx involves in catalyzing the reduction of H_2O_2 to water as well as the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) (Forstrom *et al.*, 1978).

(b) Non-enzymatic antioxidants

Non-enzymatic antioxidants can be further divided into two categories which include chain breaking antioxidants and transition metal binding proteins.

i. Chain breaking antioxidants

Chain breaking antioxidants can be classified into lipid phase and aqueous phase antioxidants. The most common lipid phase antioxidants include flavonoids, Vitamin E, carotenoids and ubiquinol-10 while the most common aqueous phase are vitamin C and protein bound thiols group such as thioreduxin and glutathione (Powers *et al.*, 2004). When there is an interaction between two molecules of free radicals, this may lead to generation of secondary molecule of radicals and subsequently, this molecule may react with other targets to produce more radical species. The reaction may continue until the radicals are neutralized by a chain breaking antioxidants or two radicals may combine to form stable products (Young and Woodside, 2001).

Flavonoids which are one of phenolic compounds present in the plant-derived food have been demonstrated to act as antioxidants. They may (i) inhibit enzymes such as xanthine oxidase which is responsible for formation of superoxide anion (ii) prevent the involvement of copper and ferrous in the Fenton reaction to form hydroxyl radical by chelating these metals and (iii) form a complex with the free radicals to stabilize them by donating the electrons to superoxide or lipid peroxyl radicals (Smith *et al.,* 2005).

Vitamin E such as α-tocopherol is involved in protection against lipid peroxidation in the cell membranes. It terminates free radical chain reaction by donating an electron to lipid peroxyl radical and then another electron to another lipid peroxyl radical. These two steps of reactions may lead to formation of more stable lipid peroxides. This is followed by conversion of Vitamin E itself to fully oxidized form. On the other hand, vitamin C which circulates unbound in extracellular fluid and blood is a water soluble antioxidant. It is also involved in terminating the free radical chain reaction by donating electrons to free radicals in two steps reactions and becomes oxidized. Apart from that, by donating electrons in a redox cycle, vitamin C may regenerate the reduced form of vitamin E (Smith *et al.,* 2005).

Glutathione (GSH) or γ-L-glutamyl-L-cysteinyl glycine is a water soluble tripeptide. It is the most prominent low molecular weight thiol in animal cells. GSH is produced within the cell from cysteine, glycine and glutamine and catalyzed by two cytosolic enzymes which include GSH synthase and γ-glutamylcysteine synthase (Wu *et al.,* 2004). Most of cellular GSH is mainly found in the cytosol (85-90%) while the remainder present in the organelles such as peroxisomes, mitochondria and nuclear

matrix (Lu, 2009). GSH in cells is utilized by GPx. During glutathione redox cycle, oxidized glutathione (GSSG) or glutathione disulfide forms in the mitochondria are reduced to GSH by the action of GR or it may accumulate within the mitochondria. Therefore, compared to cytosol, GSSG level is found higher in mitochondria. It is suggested that the degradation of H_2O_2 inside the mitochondria is done by GPx and GSH (Lu, 2009).

ii. Transition metal binding proteins

The most common transition metal binding proteins include transferrin, ferritin, ceruloplasmin and lactoferrin. These proteins act as important components of the non-enzymatic antioxidant by sequestering copper and iron to prevent the occurrence of hydroxyl radical formation. The example of copper binding protein is caeruloplasmin which acts by catalyzing the oxidation of divalent iron ferrous ion (Fe^{2+}) to form ferric ion (Fe^{3+}). The less reactive Fe^{3+} form resulting from rapid oxidation of Fe^{2+} is thus an antioxidant effect of caeruloplasmin (Young and Woodside, 2001). The actions of these enzymatic and non-enzymatic are illustrated in Figure 2.1.

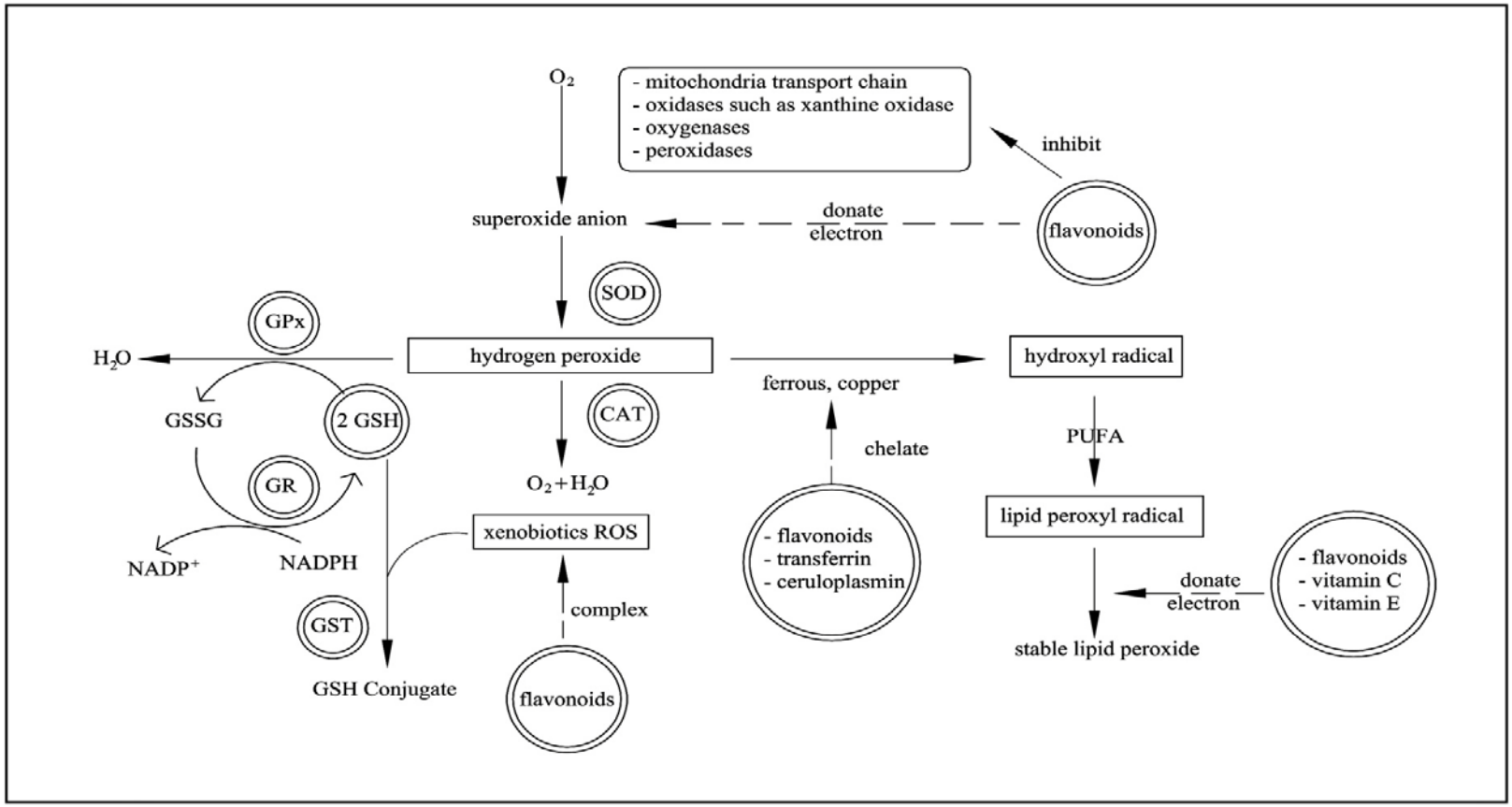

Figure 2.1: Actions of enzymatic and non-enzymatic antioxidants: O_2, oxygen; SOD, superoxide dismutase; GPx, glutathione peroxidase; H_2O, water; PUFA, polyunsaturated fatty acid; CAT, catalase; GSH, reduced glutathione; GSSG, glutathione disulphide or oxidised glutathione; GR, glutathione reductase; ROS, reactive oxygen species; GST, glutathione-S-transferase; $NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of $NADP^+$.
[Adapted from Mohamed, M. (2012)].

2.3. Cigarette smoke and oxidative stress

Although the precise mechanism is not clearly determined, the free radical-mediated oxidative stress appears to play a major role in smoking-mediated cardiovascular diseases (Tanriverdi *et al.,* 2006). CS has been demonstrated to have free radicals which include quinone, semiquinone, hydroquinone, nitric oxide as well as carbon-centered radicals such as acyl- and alkylaminocarbonyl radicals (Church and Pryor, 1985; Bartalis *et al.*, 2009). Quinone, semiquinone, and hydroquinone can be oxidized to form superoxide anion which finally form H_2O_2 and hydroxyl radical. Meanwhile, nitric oxide can be oxidized forming nitrogen dioxide (Church and Pryor, 1985)**.**

RNS and ROS are produced when there is interaction between mainstream smoke with physiological fluids or aqueous media. Some CS components are involved in oxidative stress only after they are chemically modified by metabolic process *in vivo*. For example, benzo[a]pyrene can be metabolized via a redox cycling mechanism to its corresponding quinone, which may produce ROS (Winston *et al.*, 1993; Briede *et al.*, 2004).

Several studies reported that aqueos dimethyl sulfoxide solutions of aqueous extract of cigarette tar saturated with air and buffered at pH 9 contain superoxide radical anion which is one of the ROS involved in oxidative stress. The suggested mechanism for the superoxide formation in aqueous extract of cigarette tar was the autooxidation of the hydroquinone anion (and related anions) in air to give superoxide and benzosemiquinone radical (Brunmark and Cadenas, 1989; Zang *et al.*, 1995). In

addition, another important stress-related ROS hydroxyl radical, is identified in aqueous extract of cigarette tar. The suggested mechanism for the hydroxyl radical formation was the catalytic disproportionation of H_2O_2 by transition metal ions, known as Fenton reaction (Cosgrove *et al.*, 1985).

H_2O_2 is a by-product of oxidative stress. It is formed in living organisms during normal respiration by catalytic disproportionation of superoxide radicals by SOD. CAT, another enzyme, is efficient at converting H_2O_2 to water and oxygen. If this cellular defense mechanism is overwhelmed, the excess H_2O_2 may undergo disproportionation via Fenton reaction forming hydroxyl radicals. The hydroxyl radicals derived from H_2O_2 are well known to cause oxidative damage to essential biomoleculecules including DNA and highly oxidixing species (Halliwell and Gutteridge, 1999). H_2O_2 that forms by the physiological response to smoke components and exogenous H_2O_2 found in cigarette are known to be a source of oxidative stress in smokers (Wooten *et al.*, 2006).

Meanwhile, the toxicity of quinones is reported to occur by two mechanisms namely the formation of covalent bonds with essential biological molecules (especially molecules containing thiol groups) and the redox cycling mechanisms which generates excess ROS as byproducts (Seung *et al.*, 1998; Rodriguez *et al.*, 2004). Both mechanisms may lead to the onset of oxidative stress. Quinones derived from CS components undergo redox cycling by entering into the NADPH reductase pathway in living organisms (Squadrito *et al.*, 2001; Hirakawa *et al.*, 2002). The reduction of quinones by ascorbate or NADPH may contribute to generation of the parent quinols, thus creating the redox cycle. Redox cycling of xenobiotic quinones may significantly

increase the cellular burden of ROS as well as deplete their antioxidant defences (Roginsky *et al.*, 1999).

In CS, redox inactive metals such as lead, cadmium, arsenic and mercury may deplete cells of thiol-containing antioxidants and reduce the activity of antioxidant enzymes. Meanwhile, redox active metals such as iron, copper, nickel and chromium may undergo redox cycling with concomitant ROS formation in oxygenated aqueous solutions. Heavy metals may exert other molecular effects such as activation of cellular signalling and inhibition of DNA repair (Kasprzak, 2002; Barchowsky and O' Hara, 2003). Therefore, both redox-inactive and redox-active metals may potentially lead to an increase in ROS in smokers. Transition metals in the tar of CS have a capacity to promote hydroxyl radicals formation both in aqueous extracts of CS and in living tissues via the Fenton reaction. Both cuprous ion (Cu^{+}) and ferrous ion (Fe^{2+}) are suggested to be active in the formation of hydroxyl radicals as these ions may form complexes with many organic molecules which include those that undergo redox cycling (Stohs and Bagchi, 1995; Bagchi, 1997). Cupric ion (Cu^{2+}) has been reported to oxidize hydroquinone and catechol to their respective quinones. However, ferric ion ($Fe^{3+)}$ does not significantly enhance the rate of hydroquinone oxidation (Li *et al.*, 1995; Hirakawa *et al.*, 2002).

CS consists of abundant oxidizing agents that are formed in the gas vapor phase (Church and Pryor, 1985; Pyror, 1992). Nitric oxide is a radical which combines slowly with molecular oxygen forming toxic oxidant and nitrating agent which is nitrogen dioxide. Pryor *et al.* (1983) reported that nitrogen dioxide reacts immediately with other

smoke components which include butadiene and isoprene forming nitroso carbon-centered radicals which is highly reactive species. The gas phase carbon-centered radicals in smoke react rapidly with molecular oxygen forming peroxyl radicals that react with smoke gas phase nitric oxide to form nitrogen dioxide and alkoxyl radicals, thus, generating a continuous cycle. At physiological concentrations, nitric oxide itself is relatively unreactive with non radical molecules (Halliwell and Gutteridge, 1999). However, it may react with tyrosyl radical, which is located at the active sites of some enzymes namely ribonucleotide reductase (Kwon *et al.*, 1991; Lepoivre *et al.*, 1994). Nitric oxide may be converted to more reactive substances known as RNS. Nitration of tyrosine and DNA damage in cells exposed to the gas phase of CS has been reported due to the action of RNS (Eiserich *et al.*, 1994; Spencer *et al.*, 1995). Nitric oxide is demonstrated to enhance the phenolic compounds toxicity by oxidation to their respective quinones (Urios *et al.*, 2003).

The simultaneous reaction between nitric oxide and superoxide may form peroxynitrite. Although peroxynitrite is being derived from two free radicals, it is itself not a free radical. However, in physiological media, it is a powerful oxidant that has been reported to induce damage to essential biomolecules (Halliwell and Gutteridge, 1999; Denicola and Radi, 2005). Peroxynitrite is known as an oxidative stress-inducing compound of aqueous CS fractions (Muller and Gebel, 1994; Muller and Gebel, 1998). Peroxynitrite interferes with specific target molecules after depletion of intracellular GSH content by electrophilic aldehydes in cigarette smoking-treated cells *in vitro* which results in the activation of stress related signal transduction and gene expression (Muller and Gebel, 1994).

The free radicals from components of CS such as superoxide may react with nitric oxide causing decrease in nitric oxide bioavaibility (Kojda and Harrison, 1999) which subsequently, may lead to endothelial dysfunction. Endothelial dysfunction is a major factor in contributing for acute cardiovascular events as well as initiating atherogenesis events (Neunteufl *et al.*, 2002). Previous studies have reported that CS exposure is associated with a decrease in vasodilatory function in both animal models (Ota *et al.*, 1997) and humans (Barua *et al.*, 2001; Ijzerman *et al.*, 2003). In smokers, an impairment of arterial vasodilatation appears to be as a result of impaired release of endothelium-derived relaxing factor which is suggested to be nitric oxide. Exposure to serum of smokers decreases nitric oxide availability from both human coronary artery endothelial cells and human umbilical vein endothelial cells by altering the endothelial nitric oxide synthase enzyme expression and activity (Higman *et al.*, 1993). Normal release of nitric oxide has beneficial cardiovascular role in regulating the adhesion of leukocyte to the endothelium, activation of platelet and thrombosis (Napoli and Ignarro, 2001). Therefore, impairment of nitric oxide release in cigarette smokers may predispose to acute cardiovascular events and acceleration of atherogenesis (Puranik and Celermajer, 2003).

On the other hand, nitric oxide may both promote and inhibit lipid peroxidation. Nitric oxide by itself acts as a potent lipid peroxidation chain reaction inhibitor by scavenging propagatory lipid peroxyl radicals. Furthermore, nitric oxide may inhibit potential initiator of lipid peroxidation such as peroxidase enzymes. However, in the presence of superoxide, nitric oxide may form peroxynitrite, a strong oxidant which is

able to initiate lipid peroxidation as well as oxidize lipid soluble antioxidants (Hogg and Kalyanaraman, 1999).

The prothrombotic effects following CS exposure have been reported to cause alterations in platelet function, antithrombotic/prothrombotic factors and fibrinolytic factors. Previous studies reported that platelets isolated from smokers demonstrated spontaneous aggregation (Rival *et al.*, 1987; Fusegawa *et al.*, 1999). Apart from that, platelets isolated from non smokers shows hyperaggregability after exposure to serum of smokers (Blache, 1995). CS may decrease the sensitivity of platelet to exogenous nitric oxide and decrease the availability of platelet-derived nitric oxide which subsequently, causes increased activation and adhesion (Sawada *et al.*, 2002). In addition, several studies have also reported the effects of smoking on thrombo-hemostatic factors (tissue factor and tissue factor pathway inhibitor). Barua and colleague have reported that serum from chronic smokers incubated with human umbilical vein endothelial cells significantly decreases tissue factor pathway inhibitor-1 level and relatively increases tissue factor level in tissue culture (Barua *et al.*, 2002). Furthermore, an increase in activity of circulating tissue factor in smokers has been observed in human plasma 2 hours folowing smoking two cigarettes (Sambola *et al.*, 2003). In addition, higher hematocrits, red blood cell counts, blood viscosity and an ongoing inflammatory process, in turn, will enhance the prothrombotic process associated with smoke exposure (Glantz and Parmley, 1991; Smith and Fischer, 2001).

Apart from affecting thrombo-hemostatic factors, CS may also cause alteration in fibrinolytic factors (tissue plasminogen activator and plasminogen activator inhibitor-1).

Serum of chronic smokers incubated with human umbilical vein endothelial cells shows a significant decrease in release of both basal and substance-P-stimulated tissue plasminogen activator as well as a significant alteration in tissue plasminogen activator / plasminogen activator inhibitor-1 molar ratio (Barua *et al.*, 2002). Furthermore, decreased plasma tissue plasminogen activator antigen and activity have been reported in samples isolated from brachial and coronary arteries following pharmacologic stimulation in smokers (Newby *et al.*, 1999; Pretorius *et al.*, 2002). Thus, smoking associated with dysfunctional thrombohemostatic mechanisms may initiate and accelerate the atherothrombotic process.The effects of CS on oxidative stress and acceleration of atherothrombotic process are summarized in figure 2.2.