Peer reviewed **ORIGINAL ARTICLE**

CAN TERTIARY-BUTYL HYDROPEROXIDE CAUSE CARDIVASCULAR DISEASE?

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ABSTRACT

Long term exposure to tertiary-butyl hydroperoxide (TBHP: initiator of oxidation) in the manufacturing of polyester resin coatings might be a cardiovascular risk for workers. The aim of this study was to investigate the effects of TBHP in the serum of rats by assessing the lipid profile and lipid oxidation as biomarkers of cardiovascular disease. Male Wistar rats (n = 6) aged 10 to 12 weeks were randomly placed in two groups (control and treatment) and fed *ad lib* with standard rat chow and water. Rats in the control group received an intraperitoneal injection of saline while the treatment group received TBHP (20 µM, 70% aqueous, Sigma Chemical Co, South Africa). Six rats were included in each group and injections (0.5 ml) were administered on 5 consecutive days per week for a period of 8 weeks. Cardiovascular biomarkers in the serum was determined by measuring the total cholesterol (TC), high density lipoprotein (HDL), triglycerides (TG) and C-reactive protein (CRP), while the lipid peroxidation in serum was determined by the conjugated diene (CD) measurement, lipid hydroperoxide (LOOH) and by thiobarbituric acid resistance substance (TBARS) assay. The serum of rats injected with 20 µM TBHP had significantly increased the levels of cardiovascular biomarkers and lipid peroxidation. The findings of the study indicate that TBHP injection in rats may increase the susceptibility of the serum to lipid peroxidation which could lead to cardiovascular disease.

KEYWORDS

High density lipoprotein, lipid oxidation, low density lipoprotein, tertiary-butyl hydroperoxide, triglycerides.

INTRODUCTION

Oxidative stress (OS) is a condition associated with an imbalance between the production of free radicals, mainly reactive oxygen/nitrogen species (ROS/RNS), and their scavenging capacity by antioxidants. When the production of ROS/RNS exceed the available antioxidant defence, significant oxidative damage occurs to many endothelial cells and cellular organelles by damaging lipids, proteins, and DNA, ultimately leading to cell death [1]. Exposure of endothelial cells to hydroperoxides, particularly tertiary-butyl hydroperoxide (TBHP), can result in the production of free radicals which may lead to a number of membrane changes including lipid oxidation [2-4], protein polymerization [5] and protein amino acid degradation [6]. TBHP (lipid-soluble oxidant) can be absorbed by the skin through direct contact or evaporation [7]. To date no occupational exposure limits have been established for TBHP. Studies have shown that exogenous ROS/RNS [8] and other trace metals [9, 10] present in the environment may contribute towards the oxidative stress phenomenon, which may lead to the present epidemic of cardiovascular disease. The ROS and RNS have been shown to play a contributing role in the development of cardiovascular diseases [11]. Cardiovascular disease is the most common cause of mortality in the Western world and uprising in the developing counties which accounts for up to a third of all deaths worldwide. Also, cardiovascular disease is multifactorial and involves a complex interplay between lifestyle (diet, smoking, exercise, alcohol consumption) and fixed (genotype, age, menopausal status, gender) causative factors. Although atherosclerosis was first considered to be a simple disease involving arterial lipid accumulation, it is now known to involve a defined cascade of inflammatory processes [12, 13]. In cardiovascular disease, the damage to endothelium [14] is the initiation step, which exposes these cells and the underlying cell layers to a deleterious inflammatory process which ultimately leads to the formation of atherosclerotic lesions [15]. In a healthy individual and prior to the onset of cardiovascular disease the endothelium plays a homeostatic role in maintaining vascular tone and blood flow [15]. In the early stages of cardiovascular disease progression, endothelial dysfunction triggers a chronic inflammatory process in the vessel wall. Induction of adhesion molecules in endothelial cells facilitates the attachment of monocytic leukocytes, trapping them and allowing their transmigration through the endothelial layer into the underlying intima. They become tissue macrophages and subsequently form cells following the uptake of oxidatively-modified lipids [13]. Other exogenous factors such as smoking and the existence of other disease states such as diabetes have also been reported to contribute to oxidative stress and are strong risk factors for cardiovascular disease [14].

TBHP is widely used in the chemical industry as initiators of oxidation for the production of polymers and fibre-reinforced plastics, in the production of polyester resin coatings and in the pharmaceutical industries. Exposure of workers to such environments is call for concern. TBHP has extensively been used as model prooxidants to induce OS in various *in vitro* systems [16-20]. However, little studies have been done on living organisms [19, 21]. The aim of this study was to investigate the relationship between lipid profile parameters and certain lipid oxidation biomarkers in OS induced rats (using TBHP) as a possible indicator of cardiovascular disease.

MATERIALS AND METHODS

Animals and treatments

This study received institutional review board clearance. Male Wistar rats (165 to 190 g), individually housed in polypropylene cages in an ethically approved animal facility, received water

and standard rat chow (SRC) *ad libitum*. Rats were randomly allocated into either a placebo group injected intraperitoneally with 0.5 ml saline (Sigma Chemical Co, South Africa) or a treatment group injected with TBHP solution (0.5 ml, 20 µM, 70% aqueous, Sigma Chemical Co, South Africa) for five consecutive days a week over a period of eight weeks.

Serum sample preparation

Animals were subsequently sacrificed, blood was obtained by heart puncture and directly transferred into tubes, thereafter centrifuged (4000 rpm, 8 min, 4°C) and the serum removed by aspiration and frozen (-80°C) for further analyses.

Lipid profiles assays

Lipid profile parameters were measured using Medica Easy Random Access (EasyRA™, Bedford, Massachusetts, USA). The analyser manages the utilisation and protocols for up to 24 on-board reagents by utilising a new wireless radio frequency identification technology. Assays were automatically analysed at their respective absorbance by using the absorbance photometry for the chemistry tests, with all reagents packaged in liquid form and in single or dual reagent wedges ready to use. Thawed serum samples (100 µl) were analysed and parameters measured were total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) and triglycerides (TG). The assays were conducted according to the protocol provided by the manufacturer (EasyRA™, Bedford, Massachusetts, USA).

Total cholesterol

Total cholesterol (TC) was determined using an enzymatic tinder end-point reaction, based on the work of Allain and co-workers [22]. Serum (100 µl) was used to measure TC in triplicate. The indicator, quinoneimine, is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase which turns red, the intensity of which is read at 520 nm with 600 nm as a blanking wavelength which is directly proportional to the concentration of cholesterol in the serum sample.

High-density lipoprotein cholesterol

The direct assay method for measuring high-density lipoprotein cholesterol (HDL-C) requires 100 µl of serum. This method of measuring HDL-C involves the removal of other non-HDL lipoproteins via a selective preparation reaction. The detergent solubilises the HDL-C, which then reacts with a chromagen to develop a colour. The intensity of the colour at the maximum absorbance of 600 nm is proportional to the concentration of HDL-C in the sample. After completion of the assay, the Medica EasyRA Chemistry Analyzer determines the HDL concentration from the ratio of the corrected unknown sample's absorbance to the corrected absorbance of the calibrator multiplied by the calibrator value.

Triglycerides

The assay method described by Fossati & Prencipe for triglycerides (TG) measurement includes several sequential enzymatic reactions [23]. Triglycerides were determined after the enzymatic hydrolysis with lipoprotein lipases using 100 µl of serum. When the reaction takes place, the indicator, red quinine, is formed from hydrogen peroxide, 4-aminoantipyrine and 4-chlorophenol under the catalytic influence of peroxidase. The intensity of the red colour at the maximum absorbance at 520 nm is proportional to the triglycerides concentration in the sample.

Low-density lipoprotein cholesterol

Low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald formula [LDL-C] = (Total cholesterol – HDL-C) – (TG / 2.2) [24]. Calculations were performed using Microsoft Excel® 2007 and results obtained were expressed in mmol/l.

Lipid oxidation biomarkers

While one single assay of lipid oxidation is probably not sufficient to serve as a marker for cardiovascular risk, this study involves the measurements of several markers [25]. In the present study, conjugated dienes, lipid hydroperoxides and thiobarbituric acid reactive substances were measured as oxidative stress biomarkers. All the lipid oxidation assays had the intra assays coefficient variation of 5%.

Determination of conjugated dienes

Conjugated dienes (CDs) concentrations in serum samples were determined as described by Recknagel & Glende [26]. Thawed serum samples (100 µl) were mixed with 405 µl of a chloroform/ethanol mixture (2:1) and kept on ice. Solutions were vortexed (1 min) and microcentrifuged (8000 rpm; 15 min; 4°C). The bottom organic chloroform layers were dried under nitrogen (N_2) gas in a Techne sample concentrator (Labotec, South Africa). To each of the dried residues, cyclohexane (1 ml) was added and vortexed (for 1 min). Thereafter, 300 µl of the solution transferred into micro-well plates and the absorbance was determined at 234 nm spectrophotometrically. All CD calculations were performed according to Equation 1 and expressed as nmol CD/ml serum. Cyclohexane was used as the blank.

$$\frac{A_{234}\,(\text{sample}) - A_{234}\,(\text{blank})}{0.265} \times 10$$

Equation 1: Calculation formula of conjugated dienes (CD)

Determination of lipid hydroperoxides

Lipid hydroperoxides (LOOH) in the serum of rats were determined using the modified ferrous oxidation-xylenol orange version I (FOX-1) assay as described by Fossati and Prencipe [20]. Briefly, the modified FOX-1 assay was performed by taking 90 µl of the serum sample in two test tubes. In one tube, 10 µl of methanol solution-1, containing 400 mmol/l butylated hydroxytoluene (BHT) was added, while in the other tube, 10 µl of methanol solution-2, containing 10 mmol/l triphenylphosphine (TPP) and 400 mmol/l BHT) was added. Both the tubes were vortexed and incubated at room temperature (15 min). Thereafter, in both tubes, 900 µl of the working FOX-1 reagent was added. Both tubes were vortexed and incubated at room temperature for a further 30 min. The absorbance of the solution in the two tubes was measured at 560 nm. Serum hydroperoxide was calculated from the absorbance difference of the two test tubes (with and without TPP) from a standard curve and expressed as µmol/l.

Determination of thiobarbituric acid-reactive substance

Lipid oxidation (LPO) was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) and expressed as nmol malondialdehyde (MDA) formed/l of serum [27]. In short, 50 µl of serum were added to 6.25 µl cold butylated hydroxyl toluene/ethanol (4 nM) and 50 µl of ortho-phosphoric acid (0.2 M) in an eppendorf tube. After mixing for 10 seconds,

6.25 µl of thiobarbituric acid reagent (0.11 M), was added and then heated to 90°C (45 min). Samples were first cooled on ice (2 min) and thereafter at room temperature (5 min) before the addition of n-butanol (500 µl) and saturated NaCl (50 µl). Eppendorfs were centrifuged (12000 rpm, 2 min, 4°C) and 300 µl of the supernatants (top butanol) was transferred to a 96 well plate. Absorbance was measured (532 and 572 nm) in a Multiskan plate reader (Multiskan spectrum, Thermo Electron Corporation – Vantaa, Finland).

RESULTS AND DISCUSSION

A number of internal and external sources can form reactive oxygen species (ROS); these include metabolic reactions, dietary intake, infections, cigarette smoking and chemical substances [28]. The detoxification reaction involving glutathione peroxidase and its substrate (glutathione: GSH), which gives rise to glutathione disulfide (GSSG) that in turn alters Ca^{2+} homeostasis and increases physiological formation of ROS [29-31]. However, endogenous and exogenous defense mechanisms, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione, transferrin, vitamins C and E, flavonoids, may alleviate the damaging effects of ROS [30].

Changes in lipid profile in rats

Table 1 demonstrates the results of lipid profile parameters, which are markers of cardiovascular disease. The administration of TBHP (20 µM) in the treatment group has shown a significantly ($p < 0.001$) higher level of serum TC than that of the control group. The serum LDL-C and the TG levels in the treatment group were also significantly ($p < 0.01$) higher when compared to the control group. However, the treatment group exhibited significantly lower HDL-C levels ($p < 0.01$) when compared to the control group. Free radicals reaction of lipid peroxidation in cell membranes are responsible for the degradation of unsaturated fatty acids and cholesterol [32]. Oxidative modification of LDL and cellular lipids contributes to foam cell formation, endothelial dysfunction, and destructive inflammatory processes associated with atherosclerosis [33-35]. The association between LDL, HDL-C and increased risk for cardiovascular disease has been well established through epidemiological and clinical studies [36]. This relationship is supported by the potential anti-atherogenic properties of HDL, including its mediation of reverse cholesterol transport, in which cholesterol from peripheral tissues is returned to the liver for excretion in the bile. This study confirmed that TC, LDL-C, HDL-C and TG could be used to predict the risk of cardiovascular disease following exposure to TBHP.

Changes in the levels of lipid oxidation biomarkers in rats

After 8 weeks of TBHP (20 µl) administration, following OS induction, the lipid oxidation biomarkers including CDs, LOOH and TBARS levels were determined (Table 2). The serum concentration of CDs in the treatment group increased significantly ($p < 0.001$) when compared to the control group. Conjugated dienes have been reported to be an important indicator of lipid oxidation and are the initial markers of oxidative stress [37]. The administration of oxidant TBHP for 8 weeks also significantly ($p < 0.001$) increased the level of LOOH in the treatment group when compared to the control group. Lipid hydroperoxides derived from unsaturated phospholipids, glycolipids, and cholesterol are prominent intermediates of peroxidative reactions induced by activated species such as hydroxyl radical, lipid oxyl or peroxyl radicals, singlet oxygen, and peroxynitrite [38, 39]. Once formed, LOOH may undergo reductive degradation which either diminishes or enhances cytotoxic potential, depending on a variety of circumstances [29, 39]. As an intermediate of peroxidative reactions, LOOH propagates lipid peroxidation as a degenerative process that affects cell membranes and other lipid-containing structures under OS conditions [39, 40]. Following the 8 weeks administration of TBHP as oxidant, the TBARS levels in the treatment group were significantly ($p < 0.001$) higher in the serum of rats when compared to the control group (Table 2). TBHP may be metabolized by free iron ions or cytochrome P450, leading to the formation of intermediate free radicals that initiate lipid oxidation to affect the integrity of the cell and form covalent bonds with cellular molecules resulting in cell death [30].

Correlation of lipids parameters and lipid oxidation biomarkers in the control and treatment groups

When comparing the CDs and lipid profile parameters of the control group, a positive correlation was found with TC and the LDL, while a negative correlation was observed when compared with HDL and TG. The LOOH assay was positively correlated with the TC, HDL and LDL, while a negative correlation was observed when compared to TG. A positive, correlation was evident with TBARS and the following lipid parameters TC, LDL and TG, while with HDL, a negative correlation was shown. However, in the treatment group, the CDs correlation with the lipid profile parameters was found to be positive for TC, HDL and TG while a negative correlation was observed with LDL. The LOOH assay, showed a positive correlation with the TC, LDL and TG while with HDL, a negative correlation was observed. TBARS was positively correlated with TC, HDL and TG, but negatively correlated with LDL. Plasma levels of HDL cholesterol are strongly and inversely correlated with atherosclerotic cardiovascular disease. Both clinical and epidemiological studies have reported an inverse and independent association between serum HDL-cholesterol levels and coronary heart disease (CHD) risk [41]. Oxidative modification of lipids associated with LDL and cellular constituents contribute to endothelial dysfunction and inflammatory pathways associated with atherosclerosis and plaque development [33-35, 41]. A correlation between oxidative stress markers, including LOOH, and angiographic measurements had been observed in an earlier study of 1,200 patients [42]. The mechanism of *in vivo* LDL and protein oxidation in human atherosclerotic lesions has been attributed to myeloperoxidase activity [43]. Other enzymatic sources of reactive oxygen species include reduced NAD(P)H, lipoxygenases, xanthine oxidases, uncoupled endothelial nitric oxide synthases, cyclooxygenases, and mitochondria. Approaches that have been used to assess oxidative stress levels include monoclonal antibodies against oxidized LDL, quantitation of protein oxidation markers, and measurement of isoprostanes [44-46]. Studies have shown that exposure to hydroperoxides (intraperitoneal injection) dramatically increases the level of lipid peroxidation and LOOH [19, 20, 47]. In addition, the levels of circulating LOOH have been shown to be significantly elevated in association with myocardial ischemia [48] and cardiovascular risk factors [49-53]. With sustained exposure to reactive oxygen species, LOOH undergoes further decomposition to reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal. Walter and co-workers reported that levels of MDA (measured as thiobarbituric acid reactive substances [TBARS])

were highly predictive of cardiovascular events, independent of traditional risk factors [54]. In that study, levels of TBARS did not predict changes in large stenotic lesions. This has led to their hypothesis that primary end products of lipid peroxidation may be useful, and perhaps more sensitive, diagnostic biomarkers of cardiovascular disease (CVD) progression.

Table 1: Changes in the lipid profile parameters.

Variables	Control (n=6)	Treatment (n=6)	Student's t-test *P*
TC	1.84 ± 0.09	2.95 ± 0.08***	<0.001
HDL-C	1.74 ± 0.06	0.61 ± 0.07**	<0.01
LDL-C	0.63 ± 0.07	1.49 ± 0.07**	<0.01
TG	1.71 ± 0.06	2.84 ± 0.11**	<0.01

*Results are presented as mean ± S.E.M., *P<0.01 vs. control, ***P<0.001 vs. control. TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides are all expressed in mmol/l.*

Table 2: Changes in the serum lipid oxidation biomarkers of rats.

Variables	Control (n=6)	Treatment (n=6)	Student's t-test *P*
CDs	10.93 ± 0.52	16.46 ± 0.35***	<0.001
LOOH	5.47 ± 0.28	7.22 ± 0.39***	<0.001
TBARS	1.60 ± 0.36	3.85 ± 0.14***	<0.001

*Results are presented as mean ± S.E.M., ***P<0.001 vs. control. CDs: conjugated dienes expressed as nmol CD/ml; LOOH: lipid hydroperoxides expressed as µmol/l and TBARS: thiobarbituric acid reactive substances expressed in nmol MDA/l plasma.*

Table 3: Linear regression analyses comparing changes in lipid profile parameters with the lipid oxidation biomarkers in the control group.

Variables	TC	HDL-C	LDL-C	TG
CDs	r = 0.295 *(p=0.041)*	r = -0.386 *(p=0.060)*	r = 0.360 *(p=0.036)*	r = -0.267 *(p=0.052)*
LOOH	r = 0.334 *(p=0.037)*	r = 0.424 *(p=0.021)*	r = 0.231 *(p=0.05)*	r = -0.340 *(p=0.043)*
TBARS	r = 0.326 *(p=0.046)*	r = -0.075 *(p=0.662)*	r = 0.507 *(p=0.011)*	r = 0.483 *(p=0.042)*

r: correlation of coefficient, p*: p-values, TC: total cholesterol, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, TG: triglycerides, CDs: conjugated dienes, LOOH: lipid hydroperoxides and TBARS: thiobarbituric acid reactive substances.*

Table 4: Linear regression analyses comparing changes in lipid profile parameters with the lipid oxidation biomarkers in the treatment group.

	TC	HDL-C	LDL-C	TG
CDs	r = 0.690 *(p<0.01)*	r = 0.363 *(p<0.05)*	r = -0.483 *(p<0.001)*	r = 0.078 *(p=0.652)*
LOOH	r = 0.391 *(p<0.05)*	r = -0.191 *(p<0.734)*	r = 7.253 *(p<0.046)*	r = 0.406 *(p<0.038)*
TBARS	r = 0.772 *(p<0.021)*	r = 0.340 *(p=0.014)*	r = -0.812 *(p<0.001)*	r = 0.824 *(p<0.001)*

r: coefficient of correlation; p*: p-values. TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TG: triglycerides; CDs: conjugated dienes; LOOH: lipid hydroperoxides and TBARS: thiobarbituric acid reactive substances.*

CONCLUSION

TBHP has been used as a prototypic inducer of oxidative stress *in vitro*, but has not yet been widely used to induce oxidative stress related to cardiovascular diseases *in vivo*. However, lipid oxidation is responsible for the degradation of unsaturated fatty acids and cholesterol in lipid bilayers; it has been suggested to play a major role in a number of pathological events. Our results suggest that TBHP administration in rats may induce oxidative stress responses by altering lipid profile and lipid oxidation parameters which could ultimately contribute to cardiovascular disease. Further studies investigating the *in vivo* effects of TBHP would help shed some light on the oxidative stress responses and this could possibly be used to develop strategic prevention strategies for cardiovascular disease.

REFERENCES

1. Murik O, Kaplan A: Paradoxically, prior acquisition of antioxidant activity enhances oxidative stress-induced cell death. *Environmental microbiology* 2009, 11(9):2301-2309.
2. Jain SK, Shohet SB: Calcium potentiates the peroxidation of erythrocyte membrane lipids. *Biochimica et biophysica acta* 1981, 642(1):46-54.
3. Jain SK: The accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes. *The Journal of biological chemistry* 1984, 259(6):3391-3394.
4. Moore RB, Brummitt ML, Mankad VN: Hydroperoxides selectively inhibit human erythrocyte membrane enzymes. *Archives of biochemistry and biophysics* 1989, 273(2):527-534.
5. Hochstein P, Jain SK: Association of lipid peroxidation and polymerization of membrane proteins with erythrocyte aging. *Federation proceedings* 1981, 40(2):183-188.
6. Davies KJ, Goldberg AL: Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *The Journal of biological chemistry* 1987, 262(17):8220-8226.
7. Zhang Q, Seltmann H, Zouboulis CC, Konger RL: Involvement of PPARgamma in oxidative stress-mediated prostaglandin E(2) production in SZ95 human sebaceous gland cells. *The Journal of investigative dermatology* 2006, 126(1):42-48.
8. Turko IV, Murad F: Protein nitration in cardiovascular diseases. *Pharmacological reviews* 2002, 54(4):619-634.
9. Navas-Acien A, Guallar E, Silbergeld EK, Rothenberg SJ: Lead exposure and cardiovascular disease--a systematic review. *Environmental health perspectives* 2007, 115(3):472-482.
10. Tan C, Chen H, Xia C: The prediction of cardiovascular disease based on trace element contents in hair and a classifier of boosting decision stumps. *Biological trace element research* 2009, 129(1-3):9-19.
11. Terashima M, Ohashi Y, Azumi H, Otsui K, Kaneda H, Awano K, Kobayashi S, Honjo T, Suzuki T, Maeda K et al: Impact of NAD(P)H oxidase-derived reactive oxygen species on coronary arterial remodeling: a comparative intravascular ultrasound and histochemical analysis of atherosclerotic lesions. *Circ Cardiovasc Interv* 2009, 2(3):196-204.
12. Ross R: Atherosclerosis--an inflammatory dis-

ease. *The New England journal of medicine* 1999, 340(2):115-126.
13. Rader DJ, Daugherty A: Translating molecular discoveries into new therapies for atherosclerosis. *Nature* 2008, 451(7181):904-913.
14. Fearon IM, Faux SP: Oxidative stress and cardiovascular disease: novel tools give (free) radical insight. *Journal of molecular and cellular cardiology* 2009, 47(3):372-381.
15. Hadi HA, Carr CS, Al Suwaidi J: Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vascular health and risk management* 2005, 1(3):183-198.
16. Latour I, Demoulin JB, Buc-Calderon P: Oxidative DNA damage by t-butyl hydroperoxide causes DNA single strand breaks which is not linked to cell lysis. A mechanistic study in freshly isolated rat hepatocytes. *FEBS letters* 1995, 373(3):299-302.
17. Rajesh Kumar T, Doreswamy K, Shrilatha B, Muralidhara: Oxidative stress associated DNA damage in testis of mice: induction of abnormal sperms and effects on fertility. *Mutation research* 2002, 513(1-2):103-111.
18. Chen HW, Chiang T, Wang CY, Lii CK: Inhibition of tert-butyl hydroperoxide-induced cell membrane bleb formation by alpha-tocopherol and glutathione. *Food Chem Toxicol* 2000, 38(12):1089-1096.
19. Kumar TR, Muralidhara: Induction of oxidative stress by organic hydroperoxides in testis and epididymal sperm of rats in vivo. *Journal of andrology* 2007, 28(1):77-85.
20. Kaur P, Kaur G, Bansal MP: Tertiary-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice: role of transcription factor NF-kappaB and testicular antioxidant enzymes. *Reproductive toxicology (Elmsford, NY)* 2006, 22(3):479-484.
21. Aboua YG, Du Plessis SS, Brooks N: The impact of organic hydroperoxides on rat testicular tissue and epididymal sperm. *African Journal of Biotechnology* 2009, 8(22):6416-6424.
22. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC: Enzymatic determination of total serum cholesterol. *Clinical chemistry* 1974, 20(4):470-475.
23. Fossati P, Prencipe L: Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. *Clinical chemistry* 1982, 28(10):2077-2080.
24. Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clinical chemistry* 1972, 18(6):499-502.
25. Gocmen AY, Gumuslu S, Semiz E: Association between paraoxonase-1 activity and lipid peroxidation indicator levels in people living in the Antalya region with angiographically documented coronary artery disease. *Clinical cardiology* 2004, 27(7):426-430.
26. Recknagel RO, Glende EA, Jr.: Spectrophotometric detection of lipid conjugated dienes. *Methods in enzymology* 1984, 105:331-337.
27. Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, Hadley M: A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. *Free radical biology & medicine* 1993, 15(4):353-363.
28. Inyang F, Ramesh A, Kopsombut P, Niaz MS, Hood DB, Nyanda AM, Archibong AE: Disruption of testicular steroidogenesis and epididymal function by inhaled benzo(a)pyrene. *Reproductive toxicology (Elmsford, NY)* 2003, 17(5):527-537.
29. Lin F, Girotti AW: Photodynamic action of merocyanine 540 on leukemia cells: iron-stimulated lipid peroxidation and cell killing. *Archives of biochemistry and biophysics* 1993, 300(2):714-723.
30. Lin WL, Wang CJ, Tsai YY, Liu CL, Hwang JM, Tseng TH: Inhibitory effect of esculetin on oxidative damage induced by t-butyl hydroperoxide in rat liver. *Archives of toxicology* 2000, 74(8):467-472.
31. Hwang JM, Wang CJ, Chou FP, Tseng TH, Hsieh YS, Lin WL, Chu CY: Inhibitory effect of berberine on tert-butyl hydroperoxide-induced oxidative damage in rat liver. *Archives of toxicology* 2002, 76(11):664-670.
32. Salonen JT: Markers of oxidative damage and antioxidant protection: assessment of LDL oxidation. *Free radical research* 2000, 33 Suppl:S41-46.
33. Witztum JL, Berliner JA: Oxidized phospholipids and isoprostanes in atherosclerosis. *Current opinion in lipidology* 1998, 9(5):441-448.
34. Chisolm GM, Steinberg D: The oxidative modification hypothesis of atherogenesis: an overview. *Free radical biology & medicine* 2000, 28(12):1815-1826.
35. Steinberg D: Low density lipoprotein oxidation and its pathobiological significance. *The Journal of biological chemistry* 1997, 272(34):20963-20966.
36. Gordon DJ, Rifkind BM: High-density lipoprotein--the clinical implications of recent studies. *The New England journal of medicine* 1989, 321(19):1311-1316.
37. Gumuslu S, Serteser M, Ozben T, Balkan S, Balkan E: Inhibitory role of N omega-nitro-L-arginine methyl ester (L-NAME), a potent nitric oxide synthase inhibitor, on brain malondialdehyde and conjugated diene levels during focal cerebral ischemia in rats. *Clinica chimica acta; international journal of clinical chemistry* 1997, 267(2):213-223.
38. Girotti AW: Mechanisms of lipid peroxidation. *Journal of free radicals in biology & medicine* 1985, 1(2):87-95.
39. Halliwell B, Gutteridge JM: Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in enzymology* 1990, 186:1-85.
40. Kappus H (ed.): Lipid peroxidation: mechanism, enzymology, and biological relevance New York: H.Sies; 1985.
41. Tabet F, Rye KA: High-density lipoproteins, inflammation and oxidative stress. *Clin Sci (Lond)* 2009, 116(2):87-98.
42. Kummerow FA, Olinescu RM, Fleischer L, Handler B, Shinkareva SV: The relationship of oxidized lipids to coronary artery stenosis. *Atherosclerosis* 2000, 149(1):181-190.
43. Hazen SL, Gaut JP, Crowley JR, Hsu FF, Heinecke JW: Elevated levels of protein-bound p-hydroxyphenylacetaldehyde, an amino-acid-derived aldehyde generated by myeloperoxidase, are present in human fatty streaks, intermediate lesions and advanced atherosclerotic lesions. *The Biochemical journal* 2000, 352 Pt 3:693-699.
44. Hulthe J, Fagerberg B: Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR Study). *Arteriosclerosis, thrombosis, and vascular biology* 2002, 22(7):1162-1167.
45. Pratico D: F(2)-isoprostanes: sensitive and specific non-invasive indices of lipid peroxidation *in vivo*. *Atherosclerosis* 1999, 147(1):1-10.
46. Shishehbor MH, Brennan ML, Aviles RJ, Fu X, Penn MS, Sprecher DL, Hazen SL: Statins promote potent systemic antioxidant effects through specific inflammatory pathways. *Circulation* 2003, 108(4):426-431.
47. Kumar A, Vajpayee P, Ali MB, Tripathi RD, Singh N, Rai UN, Singh SN: Biochemical responses of Cassia siamea Lamk.

grown on coal combustion residue (fly-ash). *Bulletin of environmental contamination and toxicology* 2002, 68(5):675-683.
48. Buffon A, Santini SA, Ramazzotti V, Rigattieri S, Liuzzo G, Biasucci LM, Crea F, Giardina B, Maseri A: Large, sustained cardiac lipid peroxidation and reduced antioxidant capacity in the coronary circulation after brief episodes of myocardial ischemia. *Journal of the American College of Cardiology* 2000, 35(3):633-639.
49. Singh RB, Ghosh S, Niaz MA, Singh R, Beegum R, Chibo H, Shoumin Z, Postiglione A: Dietary intake, plasma levels of antioxidant vitamins, and oxidative stress in relation to coronary artery disease in elderly subjects. *The American journal of cardiology* 1995, 76(17):1233-1238.
50. Sanderson KJ, van Rij AM, Wade CR, Sutherland WH: Lipid peroxidation of circulating low density lipoproteins with age, smoking and in peripheral vascular disease. *Atherosclerosis* 1995, 118(1):45-51.
51. Mosca L, Rubenfire M, Tarshis T, Tsai A, Pearson T: Clinical predictors of oxidized low-density lipoprotein in patients with coronary artery disease. *The American journal of cardiology* 1997, 80(7):825-830.
52. Santini SA, Marra G, Giardina B, Cotroneo P, Mordente A, Martorana GE, Manto A, Ghirlanda G: Defective plasma antioxidant defenses and enhanced susceptibility to lipid peroxidation in uncomplicated IDDM. *Diabetes* 1997, 46(11):1853-1858.
53. Marra G, Cotroneo P, Pitocco D, Manto A, Di Leo MA, Ruotolo V, Caputo S, Giardina B, Ghirlanda G, Santini SA: Early increase of oxidative stress and reduced antioxidant defenses in patients with uncomplicated type 1 diabetes: a case for gender difference. *Diabetes care* 2002, 25(2):370-375.
54. Walter MF, Jacob RF, Jeffers B, Ghadanfar MM, Preston GM, Buch J, Mason RP: Serum levels of thiobarbituric acid reactive substances predict cardiovascular events in patients with stable coronary artery disease: a longitudinal analysis of the PREVENT study. *Journal of the American College of Cardiology* 2004, 44(10):1996-2002.