

Antioxidative properties of traditional herbal medicines and the application of comet assay on antioxidative study

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ABSTRACT

Traditional Chinese medicine (TCM) in single herb or formula prescription has been used for thousands of years. Many of them possess antioxidant activity and the activity may contribute the therapeutic effect. This paper would review the relationship of traditional herbal medicine and antioxidant with particular reference to ginseng. This medicinal herb has been used worldwide with extensive tonic effect. The comet assay, a technique for DNA protecting and damaging investigation would be introduced and the application of comet assay on TCM would be discussed.

Keywords ginseng, antioxidant, DNA, herbs, comet assay

INTRODUCTION

Antioxidants are thought to protect our body from oxidative stress. Certain traditional herbal medicines are good at anti-aging and enhance body to against cancer. Both aging and cancer are somehow associated to oxidative stress. Ginseng is a very famous and popular herbal medicine and is rich in antioxidants. In this review, the properties of ginseng and its therapeutic action will be discussed. In addition, current updates and applications of comet assay (single cell gel electrophoresis assay) which is an important tool in antioxidant and DNA damage studies will also be reviewed.

DNA damage, oxidative stress and antioxidants

DNA damage

DNA damage has emerged as a major cause of cancer and many diseases related to aging (Hoeijmakers, 2009). The main source of damage is reactive oxygen species (ROS) produced by mitochondria. Normally, mitochondria have an efficient antioxidant defence system. When the mitochondrial generation of ROS exceeds its antioxidant capacity, mitochondrial proteins, lipids and nucleic acids become targets of oxidation. The oxidation of mitochondrial membrane proteins leads to mitochondrial permeability transition which can contribute to the accumulation of oxidative damage in mitochondria and subsequent DNA damage (Costa et al., 2011). ROS can be produced from endogenous sources of mitochondria, peroxisomes and inflammatory cell activation, and exogenous sources including environmental agents, pharmaceuticals and industrial chemicals. This oxidative stress may cause DNA protein damage leading to changes in chromosome instability, genetic mutation, and altered gene expression that may result in cancer (Klaunig et al., 2010). DNA damage interferes molecular function and cellular response that drive aging

indirectly. When damage is high, replication of damaged templates or imperfect repair can generate DNA mutation. Damaged molecules, ROS, and mutated DNA are accumulated with age (Campisi and Vijg, 2009).

Mitochondrial DNA (mtDNA) is highly susceptible to oxidative damage. The reason is that mtDNA is located close to the inner mitochondrial membrane where reactive oxygen species are generated. Besides, it is small in size and is not protected by histone proteins. Mitochondrial DNA fragmentation seems to be closely related to cellular damage associated with aging. Because mtDNA encodes for essential proteins involved in the processes of oxidative phosphorylation, this fragmentation leads to dysfunction of the mitochondrial respiratory chain and stimulation of mitochondrial ROS, RNS (reactive nitrogen species) generation and oxidative mtDNA damage. Mitochondrial DNA damage has been suggested to be responsible for several diseases (Hwang and Bowen, 2007).

Oxidative stress

DNA damage is caused by the interaction of DNA with ROS. Free radicals such as $\cdot\text{OH}$ and $\text{H}\cdot$ react with DNA by addition to bases or abstractions of hydrogen atoms from the sugar moiety. The C4-C5 double bond of pyrimidine is sensitive to attack by $\cdot\text{OH}$ and generates damage products such as thymine glycol, uracil glycol, urea residue, 5-hydroxy-deoxyuridine and 5-hydroxy-deoxycytidine. Interaction of $\cdot\text{OH}$ with purines generates 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines and other purine oxidative products. Accumulation of damaged DNA in different organs causes a decline in functional capacity which is recognized as aging (Nisha and Deshwal, 2011). Among the damage products, 8-OHdG has been implicated in carcinogenesis and is generally used as a biomarker for oxidative DNA damage. The urinary 8-OHdG was also found to be significantly higher in exposure to toxic metals (Lin et al., 2011).

A study on the antioxidant status and oxidative DNA damage of Bangladeshi dockyard laborers measured the plasma levels of malondialdehyde (MDA) which is a quantitative marker of lipid peroxidation by ROS, antioxidant levels (retinol,

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α -tocopherol, ascorbic acid) and extent of damaged DNA to evaluate the oxidative stress by comparing with control laborers (Sayeed et al., 2011). The plasma MDA level and the extent of DNA damage were significantly higher while the retinol and ascorbic acid levels were significantly lower in dockyard laborers than in the control group. It showed positive correlation between oxidative stress and DNA damage, so DNA damage may be interpreted as the result of oxidative stress. Oxidative DNA damage is associated with deregulation of cell homeostasis leading to malignant diseases and the decreased efficacy of DNA repair mechanism which causes further upregulation of pro-oxidant induced DNA damage.

Since DNA damage is essential for cancer genesis and aging, reducing DNA damage is essential for preventing the development of cancer and the process of aging. Load of DNA damage can be delayed by reducing exposure to exogenous genotoxins and by suppressing metabolism to produce fewer reactive species (Hoeijmakers, 2009). Strong interest in identifying antioxidants that diminish oxidative DNA damage in the area of nutrition is present.

Antioxidants

Antioxidants are substances that neutralize free radicals while pro-oxidants are substances that can generate free radicals. In normal cell, there is a balance between them. Oxidative stress results when levels of pro-oxidants are higher than levels of antioxidants. Naturally occurring antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase, and thioredoxin (Hwang and Bowen, 2007). SODs catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. CAT further converts hydrogen peroxide into harmless water molecule. They are one of important antioxidant defenses in nearly all cells (Doonan et al., 2008). There are different types of SODs. Copper-zinc-SOD is present in cytosol especially in lysosomes and the nucleus while manganese-SOD mostly in mitochondria (Cemeli et al., 2009).

Interesting findings have been reported about the relation between antioxidant level and DNA damage. Researches about the relationship between DNA damage, total antioxidant capacity (TAC) and coronary artery disease had been made. In coronary artery disease, level of DNA damage was increased while TAC was decreased (Demirbag et al., 2005). A negative correlation was also found between antioxidant level and DNA damage. Another study investigated the relationship between antioxidant and DNA damage in type 2 diabetes (Song et al., 2007). Diabetic patients had a higher MDA, but a lower TAC level in plasma as well as lower erythrocyte SOD activity. A positive association was found between insulin resistance and MDA concentration, but TAC status and SOD activity was negatively correlated. DNA damage revealed by comet assay showed significant correlations between DNA damage and level of hyperglycemia. The study suggested that imbalance between oxidative stress and antioxidative defense systems enhanced oxidative DNA damage which possibly led to more unfavourable hyperglycemia status. As researches showed negative correlation between antioxidant activity and DNA damage, it can be concluded that antioxidant activity is essential for providing DNA protection.

Food and antioxidants

Natural antioxidants are present in diet, and some foods are found to have various antioxidant components such as vitamin C, β -carotene and polyphenols. Food products such as fennel, red cabbage, strawberries, and buckwheat grouts are good sources of polyphenols which are positively related to the

antioxidant activity (Zujko and Witkowska, 2011). In general, a balanced diet rich in various antioxidants instead of single antioxidant is essential for increasing the antioxidant status of the organism (Limberaki et al., 2012). The effects of dietary antioxidants on DNA damage have been tested previously (Ames et al., 1993). Antioxidants ameliorate oxidative stress which is associated with DNA damage, and this oxidative change to key biomolecules is believed to be involved in the aging process and increased risk of cancer. Pretreatment with some antioxidants on human lymphocytes increases DNA resistance to oxidant damage (Szeto and Benzie, 2002).

Ascorbic acid (vitamin C) is an important dietary derived antioxidant. Increased intake and subsequent high plasma levels of ascorbic acid are associated with lower risk of chronic diseases such as cancer and coronary heart disease (Chung et al., 2001). Vegetarian diets based on fruit and vegetables are believed to contain plant-based antioxidant compounds and ascorbic acid. Vegetarians for 5 to 55 years were found to have higher plasma ascorbic acid concentration but lower triacylglycerol, uric acid and high-sensitivity C-reactive protein (hsCRP) concentration (Szeto et al., 2004). It was suggested that long term vegetarians had a better antioxidant status and coronary heart disease risk profile than apparently healthy subjects. Methods of pre-cooking treatment could influence the antioxidative effect of vegetables. Good antioxidant capacity was found in vegetables with minimal processing. There was no significant reduction of antioxidant activity or scavenging capacity compared with fresh vegetables (Murcia et al., 2009). The cooking treatments that maintain the antioxidant activity of vegetables are microwaving, sautéing and baking while the steaming, boiling and frying methods caused the highest loss of antioxidant activity. Higher antioxidant activity was seen in dehydrated condiments than the ready-to-eat soup. Loss of phenolic-related antioxidant power in vegetables is likely to occur with crushing, chopping or pureeing. Cellular disruption resulted in less ascorbic acid and other antioxidants than expected, and they may remain in the food matrix which may affect their apparent bioavailability (Szeto et al., 2002). Furthermore, rapid loss of antioxidants following fragmentation of some vegetables was noted and this was prevented by mild acidification.

Another example of dietary antioxidant is green tea (Benzie et al., 1999). Study confirmed that green tea contains polyphenolic antioxidant compounds that are absorbed into the systemic circulation rapidly after ingestion that results in increase in antioxidant power. Comparison among different kinds of teas was studied (Benzie and Szeto, 1999). Green teas have the highest antioxidant power and black teas have the lowest values. The antioxidant power of oolong teas is expected to be intermediate between green and black teas. Stability of antioxidants in tea was found to be at least 48 hours when stored at 4°C. The total antioxidant power was correlated strongly with the total phenolic content of tea of all types. Nevertheless, the potential for all types of tea to contribute significantly to the dietary intake of antioxidant power is high. Caffeine is a major component of teas and tea is known to contain a high level of antioxidant. The antioxidant effect of green tea makes it to be extensively used in cosmetics. A modified comet assay for detecting DNA crosslinks was used to assess the photoprotective effect of caffeine to reduce 5-methoxypsoralen (5-MOP) induced phototoxicity with ultraviolet C (UVC) exposure (Szeto and Tong, 2010). Presence of 5-MOP in combination with irradiation UVC can induce DNA crosslinks, which are a form of phototoxicity. In this study, low level of caffeine (at a concentration of 5mmol/L) demonstrated a significant reduction in DNA crosslinks.

Although dietary intake is a natural form of antioxidant supplement, studies indicated that the best health and nutrition results can be achieved not only from the consumption of fruits and vegetables with high antioxidant capacities, but also from medicinal herbs and plants as some Chinese medicinal plants possess more potent antioxidant activity than fruits and vegetables (Cai et al., 2004).

Antioxidants and traditional medicines

Traditional medicines

Numerous studies using different traditional Chinese medicines have been conducted for their antioxidant activities. Some Chinese medicinal seeds and fruits are well known for their antioxidant properties (Chan et al., 2008). Studies have found that some traditional Chinese medicine decoctions significantly increase SOD and GPx activity (Guo et al., 2011). SOD and GPx catalyze free radical breakdown and prevent the formation of reactive radicals.

A study showed that water extracts of di yu, xian he cao and da huang had the highest growth inhibitory effect on human lung adenocarcinoma A549 and human breast cancer MCF-7 cells (Li et al., 2007). Therefore, the antioxidant effects of some herbs might contribute to their anticancer activity. They also had the highest phenolic content. Positive correlations between antioxidant capacity and the phenolic content suggest that the antioxidant activities of the medicinal plants can be mainly determined by their phenol content (Song et al., 2010). One of the anti-cancer and anti-tumor herbs, saffron, apart from its antioxidant activity, has also played a role in reducing the in vivo genotoxicity of anti-tumor agent (Premkumar et al., 2006). It could be used as an adjuvant in chemotherapy. Lingzhi, a widely used Chinese herb for promoting health, has been tested for antioxidant activity (Wachtel-Galor et al., 2004). A significant post-ingestion increase in plasma antioxidant capacity was seen with slightly increased SOD and GPx activities. Plasma uric acid and lipid concentration tended to decrease while increase in ascorbic acid concentration.

Addition of ginseng, du-zhong or both to corn oil increased resistance to oxidation (Cheung et al., 2007). Shelf-life and usage time of cooking oils will be increased with addition of herbs via increasing resistance to oxidation. Many of the herbs have been shown to possess antioxidative or anti-carcinogenic effects in food model. This study suggested the possibility to use traditional Chinese medicine for food preservation as well as tonic supplement.

Different Chinese herbs have different antioxidant activity. Oxygen radical absorbance capacity (ORAC) assay had been used on wide range of Chinese herbs for testing TAC (Liao et al., 2008). The result showed that there was a 50-fold difference in antioxidant capacity between *Spatholobus suberectus* vine (1990 mmol TE/g) and *Ziziphus jujuba* fruit (40 mmol TE/g). Interesting relationship was found between the flavor of herbs and the antioxidant activity. Flavor of herbs is distinguished by yin/yang nature of herbs. Bitter flavor herbs belong to yin character while sweet flavor to yang. The average ORAC value of bitter flavor herbs was higher than that of sweet flavor herbs. That implied yin property herbs should generally have higher antioxidant activity than yang property herbs. However, this concept was not supported conclusively. Another study of yin/yang nature of Chinese herbs using the comet assay did not support the hypothesis (Szeto and Benzie, 2006). Twelve yin-tonic and 13 yang-tonic herbs were tested for their DNA protective effect on human lymphocytes. Results showed that more yang-tonic herbs provided DNA protection than yin-tonic herbs. Therefore, in terms of DNA protection

against oxidative damage, the yin-tonic herbs may not have superior antioxidant effect than yang-tonic herbs.

Further support of higher antioxidant effect of yang-tonic herbs can be found. Long-term treatment of a yang-tonic Chinese herbal formula showed improvement in generalized tissue protection with increase in activities of mitochondrial antioxidant components and red cell copper-zinc-SOD levels (Chiu et al., 2008). Wu-Zi-Yan-Zong-Wan extract which is another yang-tonic Chinese herbal formula protected against ethanol-induced toxicity in E47 cells (Chen et al., 2010). It was proposed the functional ability was mediated via the enhancement of the mitochondrial antioxidant status.

Ginseng

Ginseng can be referred to the species of the genus *Panax* (Korean, Chinese, Japanese or American ginseng) as well as to *Eleutherococcus senticosus* (Siberian or Russian ginseng) according to Aramwit and Wirotsaengthong (2012). Asian, American and Siberian ginseng were found to possess a wide range of antioxidant potency. Asian ginseng was the most potent to remove free radical reactions generated by hypochlorite while Siberian ginseng was the most potent against peroxynitrite (Chen et al., 2010). Comparison between *Panax ginseng* and *Panax quinquefolium* (American ginseng) had been done (Chen et al., 2008). *Panax ginseng* is superior than *Panax quinquefolium* in enhancing central nervous system activity, wound healing and hypoglycemic effects while *Panax quinquefolium* is better in anti-cancer. About 200 components such as amino acids, peptides, polysaccharides, polyacetylenes and ginsenosides have been isolated from *Panax ginseng*, and over 100 substances have also been found in *Panax quinquefolium* (Kim, 2012). The number of ginsenoside types contained in *Panax ginseng* (38 ginsenosides) is substantially more than that contained in *Panax quinquefolium* (19 ginsenosides). Furthermore, Korean ginseng (*Panax ginseng*) has been identified to contain more main non-saponin compounds, phenol compounds, acid polysaccharides and polyethylene compounds than *Panax quinquefolium* (Choi, 2008).

Ginseng as antioxidant

Chinese medicine ginseng has been used extensively for curative and restorative functions. The antioxidant activity of ginseng has been widely studied. A double-blinded randomized control trial was prepared for testing the effect of ginseng to serum level of free radicals and antioxidant activity (Kim et al., 2011). Ginseng was found to cause decreased generation of ROS and effectively decreased serum MDA levels. Although ginseng did not increase the activities of several antioxidant enzymes, it significantly increased the total glutathione content and glutathione reductase. Study on the effect of ginseng extract on cell death responses in peroxynitrite-treated keratinocytes found that ginseng extract increases cell viability by protecting cells against peroxynitrite-induced genotoxicity through modulating the expression of p53 (Kim et al., 2010). The effect of ginseng extract on lipid metabolism in humans was studied by measuring cholesterol, MDA, CAT and SOD (Kim and Park, 2003). Administration of ginseng extract for 8 weeks decreased serum total cholesterol, triglyceride, low density lipoprotein and plasma MDA levels while high density lipoprotein was increased. These findings support the fact that ginseng has hypolipidemic potential. Lipid lowering effect of ginseng could be induced by the ginseng's antioxidant activity. One of action mechanisms includes increased SOD/CAT activities and decreased MDA level.

Protection against oxidative damage of chemicals by

ginseng was demonstrated (Shukla and Kumar, 2009). Ginseng was found to have antioxidant effect against cadmium chloride challenge on liver cells. Subjects with combined treatment of ginseng and cadmium chloride showed significant decrease in lipid peroxidation, aspartate transaminase, alanine transaminase and elevation in glutathione (GSH) and alkaline phosphatase as compared to cadmium chloride treated group. Another example of toxic chemical is carbon tetrachloride (CCl₄). CCl₄ exerts its toxic effects by activation of liver cytochromes P450 to generate methyltrichloride radical (CCl₃) which leads to the production of chloroform and lipid radicals (Boll et al., 2001). Ginseng elicited protective effects evidenced by decrease of tissue concentrations of oxidative stress markers and the reduction of CCl₄-induced antioxidant depletion (Karadeniz et al., 2009). CCl₄-induced liver injury was attenuated as reflected by histology. These results showed the beneficial antioxidant effects of ginseng against toxic effect of CCl₄. Sodium nitroprusside, a nitric oxide (NO) donor, presents in the roots of ginseng and it caused reduction of excess copper-induced toxicity by modulation in the activities of antioxidant enzymes involved in H₂O₂ detoxification and in the maintenance of cellular redox couples (glutathione reductase), and contents of molecular antioxidants (Tewari et al., 2008). Exogenous NO supply also improved the activity of SOD and NADPH oxidase in excess copper poisoned ginseng. It could be concluded that reduction of copper toxicity-induced oxidative damage can be provided by NO supplied in the adventitious roots of ginseng.

Ginseng has the ability to protect liver cells against oxidative damage by diminishing ROS and hepatic lipid peroxidation with inducing activity and expression of cellular antioxidant enzymes (Bak et al., 2012). Ginseng also inhibited the phosphorylation of upstream mitogen-activated protein kinase. Therefore, ginseng might be considered as a useful source of cellular defense agent in liver cells.

Ginseng was found to have supportive effect on exhaustive exercise. Subjects with 8 week administration of ginseng extract showed prolonged exercise duration until exhaustion when compared with those without administration as control group (Kim et al., 2005). Increase in serum MDA level was significantly lower in subjects with ginseng administration than the control group. Scavenger enzyme level of CAT and SOD in the control group was much lower than that of subjects with ginseng administration. This suggested that CAT and SOD activities were up-regulated by ginseng intake which resulted in lowered MDA level. The effect of ginseng contributed to the decrease in MDA level during prolonged duration of exercise until exhaustion.

The effect of ginseng on protection of heart failure had been studied in rat model (You et al., 2005). Adriamycin which would induce cardiomyopathy was administered to rats with addition of ginseng for the study group. The rates of DNA, RNA and protein synthesis, myocardial antioxidants and lipid peroxidation in the hearts of the rats were determined at the end of treatment. The administration of ginseng significantly lowered the myocardial effects, mortality and lipid peroxidation, while increased in myocardial GPx, synthesis of macromolecular and SOD activities induced by Adriamycin in animal model. These findings indicated that ginseng may be partially protective against adriamycin-induced heart failure by maintaining the antioxidant status. Another study (Naval et al., 2007) used the primary cultures of rat astrocytes to assess the effect of ginseng extract to counteract the oxidant stress generated by hydrogen peroxide (H₂O₂). H₂O₂ decreased the activities of antioxidants and increased ROS generation. A significant effect of ginseng extract was found on the reduction

of astrocytic death induced by H₂O₂. Dose-response experiments revealed that ginseng extract increased cell viability at a wide range of concentrations.

Ginsenosides

Ginsenosides are responsible for the pharmacological actions of ginseng and are named as 'Rx', where the 'R' stands for the root and the 'x' describes the chromatographic polarity in an alphabetical order. Ginsenosides have been identified and classified into two categories: (1) the 20(S)-protopanaxadiol (PPD; Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, Rs1) and (2) the 20(S)-protopanaxatriol (PPT; Re, Rf, Rg1, Rg2, Rh1) (Leung and Wong, 2010).

Ginsenoside-Rg1's effects on hepatoprotection induced by exhaustive exercise-induced oxidative stress were investigated (Korivi et al., 2012). Forty rats were assigned into control or ginsenoside-Rg1 group for 10-week treatment. Rats were then subjected to exhaustive swimming. Thiobarbituric acid reactive substance (TBARS) and protein carbonyls were the oxidative damage markers. Both parameters elevated after exhaustive exercise but alleviated in ginsenoside-Rg1 pretreatment group. Exhaustive exercise drastically decreased GSH content and also decreased SOD, CAT and GPx activities. These changes were attenuated in Rg1 group. Moreover, increased xanthine oxidase activity and nitric oxide levels after exercise were also diminished by Rg1 supplementation. Strong evidence that ginsenoside-Rg1 can protect the liver against exhaustive exercise-induced oxidative damage was demonstrated. Ginsenoside-Rg1 was found to prevent cell premature senescence induced by chronic oxidative stress such as tert-butyl hydroperoxide through pushing the cells from the G1 into the S phase (Chen et al., 2008). Rg1 also protects mitochondria from oxidative damage by increasing complex IV activity and ATP production. Furthermore, Rg1 has the ability to decrease the level of ROS, increase GSH and decrease the vitality of SOD.

Potential protective effects of Rg3, 20(R)-Rg3 and 20(S)-Rg3 were investigated on cyclophosphamide (Cy) mediated oxidative stress in mice (Wei et al., 2012). Results showed that Rg3 significantly inhibited Cy mediated oxidative stress in mice. CAT, SOD and lysozyme activities were elevated with the decreasing the xanthine oxidase activity and the levels of MDA and nitric oxide. Stereospecific of Rg3 is important in antioxidant activities as R form exhibited stronger antioxidant effects than S form.

Ginsenoside Rh2 may protect the cells from genotoxic damage induced by Cy through modulating the oxidative stress status (Wang et al., 2006). Oral administration of ginsenoside Rh2 showed enhancement on antitumor activity against Cy. Rh2 also decreased the genotoxic effects induced by Cy in vivo by decreasing the micronucleus formation in polychromatic erythrocytes and DNA strand breaks in white blood cells. Rh2 could be recognized as a powerful drug for enhancing the therapeutic effects and decreasing the toxic effects of chemotherapy.

Different sugar moieties attach to triterpene dammarane form a series of ginsenosides derivatives. Various forms of ginsenosides with respect to their intracellular reactive oxygen species scavenging activity had been compared (Chae et al., 2010). Rb2 and Rc demonstrated the strongest antioxidant activity followed by Rg2, Rh2, Rh1, Rf, Rg3, Rg1, Rb1, Re and Rd. Furthermore, the antioxidant activity of the various forms of ginsenosides was influenced by dammarane type as well as the number of sugar moieties and substitutive groups.

Eleven ginsenosides extracted from ginseng fruits were evaluated for structure-activity relationship with their in vitro

cytotoxicity against several human cancer cell lines (Wang et al., 2007). Among eleven compounds, 20(R)-dammarane-3 β ,12 β ,20,25-tetrol (25-OH-PPD), 20(S)-protopanaxadiol (PPD) and 20(S)-ginsenoside-Rh2 (Rh2) were the most effective inhibitors of cancer cell growth and proliferation, and inducers of apoptosis and cell cycle arrest. The results suggested that the type of dammarane, the number of sugar moieties, and differences in the substituent groups affect their anti-cancer activity.

Ginseng processing

Steam treatment decreased the level of most free amino acids with the greatest reduction that was observed in ginseng steamed at 120°C (Cho et al., 2008). In contrast, the level of Maillard reaction products (MRPs) increased with steam treatment. It means that the reduction of amino acids is attributed to the extent of the Maillard reaction. Since MRPs are useful antioxidants, the scavenging activity of them against free radicals was found to increase with steam treatment. This indicated that MRPs are major contributors to antioxidant activity enhanced by steam treatment. The changes in ginsenosides and antioxidant activity of ginseng were measured with heating temperature and pressure (Hwang et al., 2010). The levels of ginsenosides were similar at different pressure exerted. After heating, the phenolic compounds and free radical scavenging activity of ginseng were increased. Changes in ginsenoside compositions were significant after heating. Levels of some components were decreased while others increased. It was interesting that some new ginsenosides which were absent in raw ginseng were detected after heating. A comparison study of the total content of ginsenoside components in the process of steaming has been done (Lee et al., 2012). Steaming of ginsengs was made from 1 to 9 times and the contents of ginsenosides were evaluated each time. The results showed that the contents of ginsenosides peaked in steaming 8 times. Effect of carbon dioxide on ginsenosides and antioxidant activity of ginseng was also studied (Thanh et al., 2006). Ginsenoside concentrations decreased with increasing carbon dioxide concentration. One, 2.5 and 5% carbon dioxide supplied resulted in decreased ginsenosides up to 11.6, 19.5 and 50.6%, respectively. Increases in antioxidant enzymes were noticed with increase of carbon dioxide concentration (Ali et al., 2008). This increase of antioxidant activity may be due to the defense response to the cellular damage induced by carbon dioxide. Influence of cold temperature on ginseng activity and secondary metabolite genes with addition of potassium nitrate showed that the most genes were activated with potassium supply as well as activities of CAT, ascorbate peroxidase, guaiacol peroxidase, and phenylalanine ammonia-lyase (Devi et al., 2012). It was concluded that high level of potassium is needed for ginseng to increase its cold tolerance through activating the antioxidant system as well as to increase ginsenoside-related secondary metabolite transcripts. Ginsenoside production and volumetric yield of ginseng cell culture were found to be stimulated by elevated osmotic pressure with addition of 0.2 - 0.3M sorbitol (Wu et al., 2005). This stimulation of ginsenoside production was part of the cell responses to osmotic stress of ROS created by sorbitol.

Ginseng and DNA protection

As mentioned in the previous section, DNA damage could be resulted from oxidative stress. Many studies have reported medical benefits of ginseng as antioxidant and anticancer actions. Another types of researches concentrated on the effect of ginseng on oxidative stress markers were also performed. Effect of ginseng on lymphocyte DNA damage was widely

investigated.

Some studies about ginseng on DNA protection were done using animals. The effects of Panax ginseng extracts on DNA damage and reproductive toxicity were evaluated in the testes of rats exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Lee et al., 2007). Comet assays were performed to evaluate lymphocyte DNA damage. Histological changes in the seminiferous tubules were determined. The 2,3,7,8-TCDD treated rats showed a reduced seminiferous tubular diameter, an increased number of damaged tubules and a significant increase of DNA damage in testes. A significantly decreased level of DNA damage and reduced pathological effects were observed in the 2,3,7,8-TCDD with ginseng extracts treated groups when compared with the TCDD treated group. Ginseng extract possessed a therapeutic capacity to reduce 2,3,7,8-TCDD effects on pathological and genotoxic damage in rat testes.

DNA damage in rat model was done using aflatoxins as a source of oxidative stress and carcinogen (Abdel-Aziem et al., 2011). Ingestion of aflatoxins significantly increased micronucleated normochromatic erythrocytes in bone marrow, DNA fragmentation and lipid peroxidation. On the other hand, a significant decrease in phospholipid hydroperoxide-glutathione-peroxidase (PHGPx) gene expression and GSH in liver and testis were observed. Treatments with ginseng or whey-protein concentrates or their combination with exposure to an aflatoxins-contaminated diet increased PHGPx gene expression and GSH in testis accompanied with a significant decrease in lipid peroxidation in liver and testis. Although they did not fully reverse the effects of the aflatoxins, the genotoxicity of aflatoxins can be in part prevented by dietary supplementation with ginseng, whey-protein concentrates or their combination.

Comet assay using primary cultured rat hepatocytes was made to investigate the effects of ginseng extract and *Phellinus linteus* on DNA damage to eukaryotic nuclei (Park et al., 2009). Both ginseng and *P.linteus* displayed protective effects against DNA strand breaks singularly. However, the protection against DNA strand breaks was more effective at lower concentrations than in single applications of the extracts singularly. It is likely that the two extracts are able to work synergistically to improve their inhibitory functions against DNA damage induced by oxidative stress.

Radioprotective efficacy of ginseng root extract in the testicular enzymes of Swiss albino mice has been assessed (Kumar et al., 2003). Levels of acid and alkaline phosphatases and lipid peroxidation (LPO) in testes were estimated on ginseng treated, radiation treated and combination groups. The results showed that ginseng extract provides the protection by reflecting a significant decrease in acid phosphatase activity and LPO level but significant increase in alkaline phosphatase activity as compared to irradiated animals. Ginseng extract was found to contain Baicalcein ginsenosides Rf, Baicalin ginsenosides Rbl and Rg1 which were effective to suppress radiation induced lipid peroxidation and reduce the radiation induced cellular damage. It is possible that ginseng reduces DNA damage by antiradical action which may be induced by inhibiting lipid peroxidation with decline of testicular acid phosphatase and LPO level and elevated alkaline phosphatase activities. As a result, administration of ginseng before radiation treatment significantly protected the germ cell population against gamma radiation in mice.

The effects of ginseng on DNA damage were also studied in humans. A randomized, double-blind, placebo-controlled study with three groups of placebo, low-dose (3 g/day) and high-dose (6 g/day) was analyzed for lymphocyte DNA damage, antioxidative enzyme activity, and lipid peroxidation before

and after the 8-week supplementation (Kim et al., 2012). Results assessed by comet assay showed that the DNA tail length and tail movement as indicators of lymphocyte DNA damage significantly decreased after ginseng supplementation comparing to baseline. Antioxidative enzymes of plasma SOD, CAT, GPx and erythrocyte SOD activities were significantly higher in ginseng group than the placebo group. Decreased plasma low-density lipoprotein was also found.

Tool for detecting DNA protection and damage

The comet assay

The comet assay is a simple and sensitive methodology for measuring strand breaks of DNA in single cells. Cells are anchored in a layer of agarose on a microscope slide and membrane is lysed and removed with detergent and high salt solution leaving a nucleoid. The DNA strand breaks, if present, will be relaxed in the supercoiled loops of DNA. A small electrical current is applied through the gel, the DNA loops are then pulled towards the anode forming a comet like structure. Comets can be visualized under fluorescent microscopy stained by ethidium bromide or propidium iodide etc. The amount of DNA in the tail, relative to the head, is proportional to the amount of strand breaks. The comet assay can be applied to nearly all eukaryotic cells with lymphocytes as the most commonly used type of cell in human studies. Various modification of comet assay has been developed to achieve various objectives (Wong et al., 2005).

Neutral comet assay

The comet assay was first introduced by Ostling and Johanson (1984) which employed pH of 9.5 for lysis and electrophoresis. Single stranded breaks are detected in the neutral or mildly alkaline comet assay with the same limit of detection of DNA damage as the alkaline comet assay but the comet tails are less pronounced and this is less sensitive than using alkaline condition (Angelis et al., 1999). This can be applicable when a less sensitive method is needed such as when investigating cells that have a large amount of background or induced damage is high (Wong et al., 2005). A different kind of neutral comet assay employed extended treatment of lysed cells in agarose at 50°C to facilitate detection of double-stranded breaks without interference from single-strand breaks. It is likely that the behavior of double-stranded pieces of DNA is being concerned (Collins, 2004).

Alkaline comet assay

Singh and co-workers (1988) presented the alkaline version of the comet assay in which DNA is allowed to unwind at pH >13. The alkaline comet assay is capable of detecting a wide variety of DNA damage such as DNA single-strand breaks (SSBs), double-strand breaks or DNA-protein cross-links. SSBs are formed from alkali-labile sites at pH >13, revealing otherwise hidden damage. Employing milder alkaline (pH=12.3) conditions prevents conversion of alkaline labile sites into breaks. Therefore, by modifying the pH of the comet assay, different sensitivity towards certain types of induced DNA damage can be applied (Baumgartner et al., 2009).

Lysed cell comet assay

The original version of the comet assay treated cells with the potential genoprotective agent of interest before the lysis step. The cell membrane would prevent entry of certain types of molecule into cells affecting assessment of the direct effects of such agents on DNA. Besides, genoprotection in the original version of comet assay might be exerted indirectly. Lysing embedded cells before treatment with antioxidant pre-treatment

or oxidative challenge agents allows direct contact of 'naked' nuclear DNA with the agent under study, and removes the possibility of cellular response or adaptation (Szeto et al., 2002). Another modification is the placement of slides into cold lysing solution for 1 hour to solubilize cellular proteins by leaving the DNA as nucleoids. This dissolves the cell membrane but not the nuclear membrane (Sham et al., 2003).

Advantages and usage of comet assay

Advantages of comet assay are numerous and it has been widely used in various studies. Results are obtained by counting individual cells that makes convenient and reliable statistical analysis. Besides, small number of cells is needed for each sample. The test is relatively rapid, sensitive and simple. Moreover, high variety types of cells such as human population, aquatic organisms or even plant cells can be used in vitro or in vivo (Kumaravel et al., 2009). Furthermore, high flexibility of comet assay allows various combinations of unwinding and electrophoresis conditions, and can be used to detect different types and levels of DNA damage. Comet assay has been applied in various types of studies, including genotoxicity studies, DNA repair studies, supplementation trials and environmental biomonitoring (Wong et al., 2005).

The importance of comet assay has been shown in several studies. In genotoxicity studies, the assay is used in its simple form to measure strand breaks (Collins, 2004). Mechanisms of action were accrued from inclusion of repair endonucleases to measure specific types of lesions. There is also tendency to replace traditional assay in regulatory genotoxicological studies in vivo (Kumaravel et al., 2009). Inflicting DNA damage on cells and monitoring the speed with which they remove the lesions are for the measurement of DNA repair capacity. Lymphocytes can be treated with ionizing radiation or other pre-oxidants, followed by treatment with base damaging chemicals to cause rejoining of breaks. The remaining lesions can be assayed by use of an appropriate endonuclease on the gel (Collins, 2004).

The comet assay has been used to determine oxidative DNA damage in several health conditions and also has been used to show different protective effects of dietary supplementation on antioxidant activity (Park et al., 2009). Environmental biomonitoring applications include monitoring occupational exposure to genotoxic chemicals or radiation, assessment of oxidative stress associated with various human diseases and detection of DNA damage (Collins, 2004). Protective effects of several diets on chemo-protective studies could be used. In translational research this assay has been used to assess tumor-radio sensitivity and chemo-sensitivity that allows clinicians to effectively manage cancer patients. Administration of toxic therapy to patients can be avoided (Kumaravel et al., 2009).

Applications of comet assay

Comet assay has been widely used for genotoxicological studies. One example of the investigation was to examine whether ephedrine, a natural alkaloid from plants of the genus *Ephedra*, can exhibit genotoxic effects on isolated human lymphocytes in the comet assay (Radakovi et al., 2011). Co-treatment of the negative control with DNA repair inhibitors (cytosine arabinoside and hydroxyurea) caused an increase of DNA damage while cells treated with ephedrine and DNA repair inhibitors did not express increased DNA damage. The obtained results showed that ephedrine did not induce DNA damage in isolated human lymphocytes.

Comet assay for detecting DNA damage had been used in animal studies. DNA damage in Chinese toad tadpoles was

induced by exposing to sublethal concentrations of four herbicides namely acetochlor, butachlor, chlorimuron-ethyl and paraquat (Yin et al., 2008). Significant increase in DNA damage was observed from erythrocytes of tadpoles exposed to sub lethal concentrations in dose-dependent manner. Comet assay had also been used for investigation of oxidative DNA damage of blood mononuclear cells in equine for two potentially increasing oxidative DNA damage conditions namely aging and equine recurrent airway obstruction (Marlin et al., 2004). No difference in endogenous DNA damage was observed between mature and aged ponies. However, mononuclear cells from RAO-affected animals had greater endogenous DNA damage than control animals which may be due to higher ROS with a lower antioxidant capacity.

A study concerning for DNA damage and the subsequent repair was estimated in isolated lymphocytes of healthy donors and patients with head and neck cancer before radiotherapy (Palyvoda et al., 2003). Comet assay was used to assess the background and radiation-induced DNA damage, rate of repair, and residual non-repaired damage. The level of background DNA damage before irradiation was significantly higher in the cancer patient group than in the healthy donors, while repairing rates were widely scattered in both groups. Cancer patient group showed high DNA damage, low repair rate and high non-repaired DNA damage level on irradiated lymphocytes.

Protective study of dietary antioxidant effect on DNA damage had been done for almonds (Li et al., 2007). Almonds were added to the diet of healthy male soldiers who were smokers with comparison with nonsmoking soldiers. Comet assay results were compared between smokers, nonsmokers and smokers with almond diet supplementation. DNA breaks in lymphocytes were higher in smokers than nonsmokers. The magnitude of DNA breaks in smokers after 4 weeks almond supplementation was not significantly different than that of nonsmokers. The results suggested that almond can enhance antioxidant defenses and decrease oxidative lymphocyte DNA damage in smokers.

Environmental biomonitoring by comet assay had been used for testing the occupational exposure to various pesticides and the presence of DNA damage (Kaur et al., 2011). Significant differences in DNA damage were observed between freshly exposed workers and control group as well as freshly exposed and after 5-6 months. However, factors namely age, smoking, drinking and dietary habits which were expected to associate to the damage were found not related to DNA damage in the current study. It can be concluded that pesticides did cause DNA damage irrespective of duration of exposure. Repairing of damage was seen at the follow up cases during null or low period of spraying with significantly lower frequency of DNA damage in comparison to fresh cases.

A study (Yow et al., 2000) had been conducted to assess the effectiveness of photocytotoxic and genotoxic effects of two photosensitizers, temoporfin (meta-tetra-hydroxyl-phenyl-chlorin; mTHPC) and merocyanine 540 (MC540) on nasopharyngeal carcinoma (NPC) cells. Comet assay was used to evaluate the DNA strand break and potential genotoxic effect induced by mTHPC and MC540 on the NPC cells. High phototoxic efficacy of mTHPC for the NPC cells was demonstrated. Both photosensitizers tested exerted a cytotoxic effect but did not cause DNA damage at dark and low overall doses. The selective tumor eradication property of the drugs allowed photodynamic therapy without damaging adjacent normal tissues.

CONCLUSION

The genoprotective effect of American and Asian ginseng on lymphocytic DNA was studied (Szeto et al., 2011). Results showed that aqueous extracts of American and Asian ginseng were able to diminish damage to human DNA induced by hydrogen peroxide. However, there are many rules and beliefs in regard to the use of herbs in the practice of traditional Chinese medicine. There may be existence of incompatibility or antagonism between different herbs or 'functional foods'. This can be demonstrated by the general belief that co-consumption of ginseng and turnip is contraindicated, and the 'qi tonifying' effect of ginseng is diminished by turnip. It means that addition of the juice from turnip counteracted the beneficial effect of ginseng. An in vitro study of turnip juice with ginseng extract on the effect of lymphocyte DNA damage was performed using comet assay (Szeto et al., 2011). Results showed that incubating ginseng along with turnip juice abolished the DNA protective effect of both American and Asian ginseng. Both unboiled and boiled turnip juice gave similar effects in terms of removing the genoprotective effects of ginseng. Enzymes such as glycosidases or others which may hydrolyze or breakdown ginsenosides would have been inactivated by boiling. Therefore, the effect of turnip juice is unlikely to be due to enzyme action.

Limitations of comet assay

Due to variations in procedures and conditions between researchers, comet assay sometimes give an inconsistent result which makes comparison difficult. Owing to different objectives of each study, a wide range of parameters can be used so that standardization of procedures does not seem to be feasible. Besides, it must be noted that in vitro effect resulted from the comet assay does not necessarily represent the in vivo effect and vice versa. Moreover, an effect on DNA does not mean that the same effect will result in other types of biomolecule because protection and damage may be site-specific. Furthermore, the absolute concentration of antioxidant is critical. Cooperation between antioxidants may be crucial, and increasing the level of a single antioxidant may disturb the integrated system and may not be a successful strategy. The standard protocol used is not able to distinguish fragmentation whether it is originated from either necrosis or apoptosis. Modifications to the 'standard' protocol of the comet assay should be made to maximize the application of the assay (Szeto, 2007). Comet assay detects only DNA damage in the form of strand breaks. Specific classes of DNA damage including base oxidation and DNA adduct formation cannot be measured. A possible mechanism of carcinogenicity, aneugenic effect, and epigenetic mechanism of DNA damage cannot be detected either (Dhawan et al., 2009).

CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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