School of Life Sciences, Lanzhou University[1], The First Affiliated Hospital of Lanzhou Medical College[2], Medical College of Northwest Minorities University[3], Lanzhou, China

Oxidative stress in Graves' disease patients and antioxidant protection against lymphocytes DNA damage *in vitro*

Xu-Lei Tang[1, 2], Xiao-Ju Liu[2], Wei-Ming Sun[2], Jin Zhao[1, 3], Rong-Liang Zheng[1]

Received August 25, 2004, accepted October 28, 2004

Rong-Liang Zheng, School of Life Sciences, Lanzhou University, Lanzhou 730000, P.R. China
zhengrl@lzu.edu.cn

Pharmazie 60: 696–700 (2005)

DNA damage to peripheral blood lymphocytes of patients with Graves' disease (GD) was studied *in vitro* before and after treatment with antioxidants, melatonin, quercetin, *N*-acetylcysteine (NAC) and vitamin C. DNA damage (comet %) was remarkably higher in patients ($23.7 \pm 5.5\%$) than that in healthy persons ($9.8 \pm 3.2\%$, $p < 0.01$). Plasma malondialdehyde (MDA) content (7.90 ± 1.77 μM) of patients was significantly higher than that of healthy persons (4.71 ± 1.19 μM, $p < 0.01$). Also, the plasma total antioxidant capacity (TAC) (7.53 ± 1.35 U/ml) in GD patients was significantly lower than that in healthy persons (10.56 ± 2.21 U/ml, $p < 0.01$). Negative correlations were observed between plasma TAC and DNA damage in lymphocytes ($r = -0.599$, $p < 0.01$), and between plasma TAC and MDA ($r = -0.40$, $p < 0.05$) in GD patients. After treatment with 100 μM melatonin, quercetin or NAC for 4 h *in vitro*, DNA damage in lymphocytes in GD patients declined significantly (from $23.8 \pm 4.4\%$ to $14.4 \pm 4.0\%$, $p < 0.001$ for melatonin, from $23.4 \pm 4.7\%$ to $18.1 \pm 4.3\%$, $p < 0.01$ for quercetin, from $23.7 \pm 4.0\%$ to $18.7 \pm 5.7\%$, $p < 0.05$ for NAC), while there was little change with concentrations of 1–100 μM of vitamin C. However, 1000 μM vitamin C enhanced DNA damage significantly (from $23.8 \pm 2.3\%$ to $30.3 \pm 3.9\%$, $p < 0.05$). Our results showed that oxidative stress existed in GD patients and the antioxidants melatonin, quercetin and NAC are beneficial for DNA damage in lymphocytes of GD patients *in vitro*.

1. Introduction

Graves' disease (GD), the most common form of autoimmune thyroid disease, is characterized by hyperthyroidism due to overproduction of thyroid hormones. There have been some reports of oxidative stress in patients with hyperthyroidism. In the course of hyperthyroidism, oxidative stress and the peroxidation of lipids generated have been found (Venditti et al. 1997). Enhanced generation of reactive oxygen species (ROS) and impairment of cellular and extracellular antioxidant system potential have been found in patients with Graves' disease (Bianchi et al. 1999; Komosinska-Vassev et al. 2000). GD pathogenesis may impose a potential genotoxic hazard for patients with insufficient antioxidant defence and reduced DNA repair capacity. There is a paucity of information concerning oxidative DNA damage in GD patients, while no report of the effect of antioxidants on oxidative DNA damage in lymphocytes of GD patients has been found, so we considered that it would be of interest to explore the effect of typical antioxidants on damaged DNA in lymphocytes of GD patients *in vitro*.

Melatonin (*N*-acetyl-5-methoxytryptamine), quercetin (3,3′,4′,5,7- pentahydroxyflavone), *N*-acetylcysteine (NAC) and vitamin C are all typical antioxidants. Previous studies from our laboratory showed that these compounds have activities as antioxidants and in repair of oxidative DNA damage. Quercetin can not only protect against DNA damage in mononuclear cells of umbilical blood and human lymphocytes (Zhao et al. 2004; Liu et al. 2002) but can also fast repair DNA damage (Zhao et al. 2001, 2002, 2003a, 2003b). Melatonin can repair DNA damage of tumor associated lymphocytes in cancer patients (Liu et al. 2003). Therefore we selected these typical antioxidants for this study.

2. Investigations and results

2.1. Indices of thyroid function

Serum total thyroxine (TT_4) and total triiodothyronine (TT_3) are markedly higher, and thyroid-stimulating hormone (TSH) level is significantly reduced in GD patients ($n = 33$) compared with those of controls ($n = 31$, Table 1). These results accorded with a diagnosis of hyperthyroidism.

2.2. DNA damage of lymphocytes and lipid peroxidation total antioxidant capacity (TAC)

DNA Damage in lymphocytes (comet %) was 2.4-fold higher in GD patients than in healthy persons. Malondialdehyde (MDA) content of plasma of GD patients was 1.7-fold higher than that of healthy persons. TAC of plasma

Table 1: Serum TT_3, TT_4 and TSH in GD patients

Group	n	TT_3 (ng/dl)	TT_4 (μg/dl)	TSH (μI U/ml)
Healthy persons	31	162.46 ± 29.59	10.01 ± 1.74	3.42 ± 1.66
GD patients	33	453.17 ± 236.04**	25.56 ± 15.11**	0.04 ± 0.07**

** $p < 0.01$, compared with healthy persons

Table 2. Plasma TAC, MDA and comet % of lymphocytes in GD patients

Group	n	TAC (U/ml)	MDA (μM)	comet (%)
Healthy persons	31	10.56 ± 2.21	4.71 ± 1.19	9.8 ± 3.2
GD patients	33	7.53 ± 1.35**	7.90 ± 1.77**	23.7 ± 5.5**

** $p < 0.01$, compared with healthy persons

of GD patients was 28.7% lower than that of healthy persons (Table 2). Negative correlations were observed between TAC and DNA damage ($r = -0.599$, $p < 0.01$, Fig. 1), and between TAC and MDA in GD patients ($r = -0.40$, $p < 0.05$, Fig. 2).

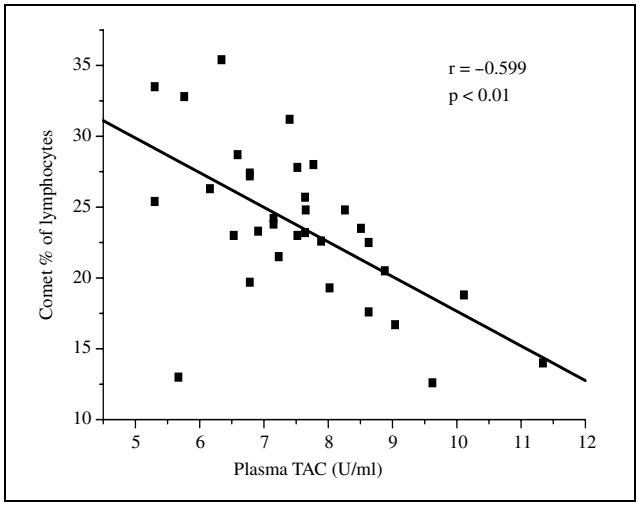

Fig. 1: Negative correlation between plasma TAC and comet % of lymphocytes in GD patients ($n = 33$, $r = -0.599$, $p < 0.01$)

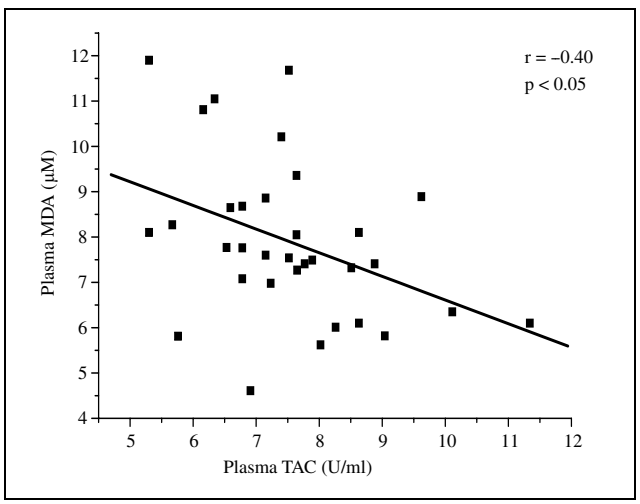

Fig. 2: Negative correlation between plasma TAC and MDA in GD patients ($n = 33$, $r = -0.40$ $p < 0.05$)

2.3. Protection by antioxidants against DNA damage in lymphocytes

DNA damage in lymphocytes (comet %) decreased with increasing concentrations (10–1000 μM) of melatonin, quercetin or NAC. However, there was no effect of vitamin C in the range 1–100 μM, and enhanced DNA damage at 1000 μM (Figs. 3a–d).

DNA damage levels hardly differed before and after incubation without antioxidant for 4 h (Fig. 4a). With the same concentration (100 μM) of different antioxidants and incubating lymphocytes for 4 h, melatonin, quercetin or NAC significantly decreased DNA damage in lymphocytes (Fig. 4b–d), from 23.8 ± 4.4% to 14.4 ± 4.0% for melatonin ($p < 0.001$), from 23.4 ± 4.7% to 18.1 ± 4.3% for quercetin ($p < 0.01$), from 23.7 ± 4.0% to 18.7 ± 5.7% for NAC ($p < 0.05$), while vitamin C had almost no effect ($p > 0.05$, Fig. 4e). Therefore, the protective effect of melatonin was greatest.

3. Discussion

It has been reported that direct indices of DNA oxidative damage including strand breaks and the number of alkali-label sites, as well as indirect indices of DNA oxidative damage such as the numbers of formamidepyrimidin DNA glycosylase-sensitive sites and endonuclease III-sensitive sites can be assessed globally by single cell gel electrophoretic assay (Pouget et al. 2000; Bruner et al. 2000; Cadet et al. 2000). Our data indicated that DNA damage in lymphocytes was remarkably higher in GD patients than in healthy persons. The TAC reflected on altered redox balance of the affected fluids, tissues, or organs, therefore indirectly reflecting the degree of oxidative stress in some pathological conditions (Benzie and Strain 1996; Leinonen et al. 2000). MDA, the product of peroxidation of lipids, is often used to evaluate the extent of oxidative stress (Ohkawa et al. 1979; Zheng and Slater 1992). We found that plasma TAC decreased and plasma MDA increased in these patients. There were two negative relationships, between plasma TAC and lymphocyte DNA damage, and between plasma TAC and MDA. This indicated that oxidative stress presented in GD patients. Therefore, oxidative stress may be closely correlated with hypermetabolism in hyperthyroidism. The higher endogenous peroxides in hyperthyroid patients may reflect the hypermetabolic state of these patients (Resch et al. 2002). The higher level of thyroxine can induce neutrophil respiratory burst and re-

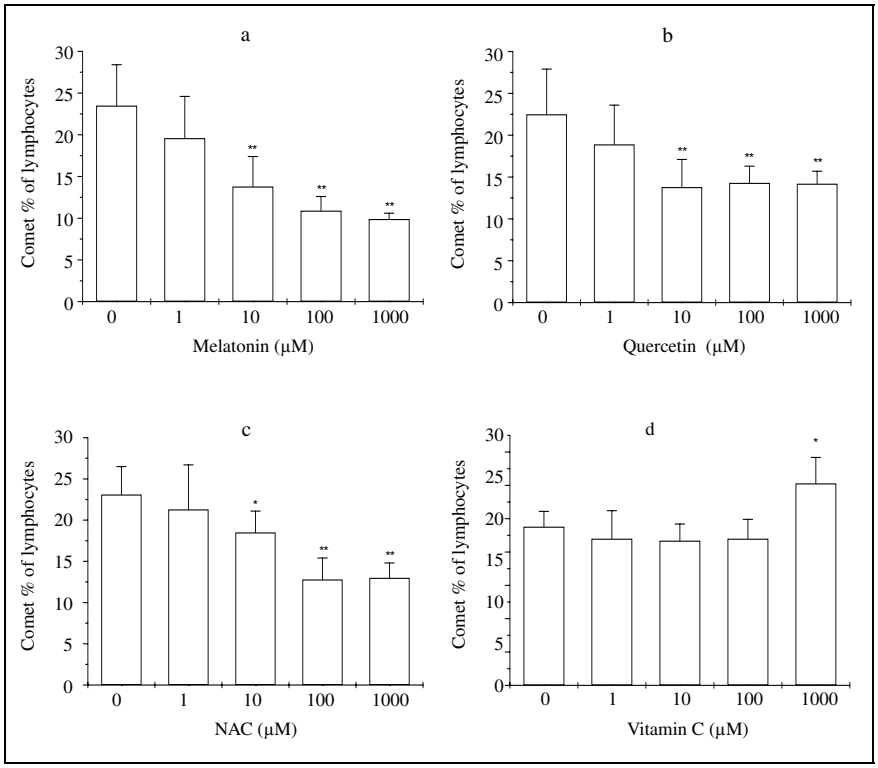

Fig. 3:
Comet % of lymphocytes treated with different concentrations of melatonin (n = 5), quercetin (n = 6), NAC (n = 5) or vitamin C (n = 5) for 4 h *in vitro*, * $p < 0.05$, ** $p < 0.01$, compared with controls

sult in oxidative stress (Videla and Fernandez 1994). It is reasonable to assume that lower TAC may be related to a higher degree of lymphocyte DNA damage caused by oxidative stress. Our results are similar to those of others (Abalovich et al. 2003; Isman et al. 2003).

DNA oxidative damage can prevent the replication and transcription of DNA possibly resulting in dysfunction and apoptosis in many kinds of cells including lymphocytes, and it may participate in the pathogenesis of GD. The scavenging of ROS by endogenous and exogenous antioxidants has attracted much attention and seems to be a feasible approach to preventing DNA from being attacked by ROS. Antioxidants protect cells from DNA damage not only by directly removing reactive free radicals, thus reducing the amount of DNA damage that leads to tumorigenesis, but also by indirectly metabolizing ROS, which can result in recovery of cell functions. Melatonin, one of the strongest scavengers of ROS, may activate cellular DNA repair enzymes, facilitating more rapid repair of the damaged DNA (Vijayalaxmi et al. 1995, 1996). It is also devoid of pro-oxidant actions (Reiter et al. 1998), while most antioxidants exhibit pro-oxidation at higher concentrations. All these properties of melatonin may play a role in reducing the extent of DNA damage in lymphocytes. Our previous study showed that melatonin could decreased DNA damage in tumor associated lymphocytes of patients with malignant pleural effusion (Liu et al. 2003). In this study melatonin was the most powerful DNA protector among the four antioxidants at the same concentration (100 μM). Quercetin is a kind of flavonoid, which can inhibit free radical mediated damage of lipid peroxidation and DNA single-strand breakage, and its consumption has been linked to protection against heart disease and cancer (Hertog et al. 1992). In our laboratory, Zhao et al. and Liu et al. respectively showed that quercetin is able to scavenge both ROS and reactive nitrogen species, and is also able to repair DNA oxidative damage (Zhao et al. 2001, 2002, 2003a, 2003b; Liu et al. 2002). Our data showed that quercetin could decrease DNA damage in lymphocytes of GD patients. Its effect on damaged DNA was slightly less than that of melatonin. NAC, a strong antioxidant and precursor of intracellular cysteine and glutathione, has antigenotoxic and anticarcinogenic properties currently being investigated in clinical trials. NAC is deacetylated in many tissues and cells to form cysteine, supporting biosynthesis of glutathione that serves directly as an antioxidant or as a substrate in the glutathione redox cycle (Zandwijk 1995). NAC lowered the concentration of 8-OH-Gua, one of the products of damaged DNA, indicating that NAC reduces the mutagenic potential of oxidized DNA (Malins et al. 2002). All the above reports are consistent with ours. The antioxidant activity of vitamin C is useful for protection of cellular macromolecules, particularly DNA, from oxidative damage induced by different agents (Nefic 2001). Vitamin C also possesses DNA damaging and mutagenic activity inducing DNA strand breakage (Stich et al. 1979), increasing the frequency of sister-chromatid exchanges (SCEs) in cells, and increasing the number of somatic mutations. However, vitamin C is not an active mutagen itself. Its mutagenicity is mediated by oxygen radicals appearing during oxidation of vitamin C by molecular oxygen (Nefic 2001). Our result showed that low concentrations of vitamin C (1–100 μM) could not reduce lymphocyte DNA damage in GD patients *in vitro*, but a high concentration of vitamin C (1000 μM) unexpectedly enhanced lymphocyte DNA damage. This may relate to the prooxidant mechanism of vitamin C.

Taken together, all the indices studied indicated that GD patients are exposed to severe oxidative stress. Three antioxidants, melatonin, quercetin and NAC can remarkably alleviate DNA oxidative damage, so it is possible to recover lymphocyte immune dysfunction and remit the pathogenesis in GD patients. We speculate that antioxidants may benefit by alleviating oxidative stress in GD patients.

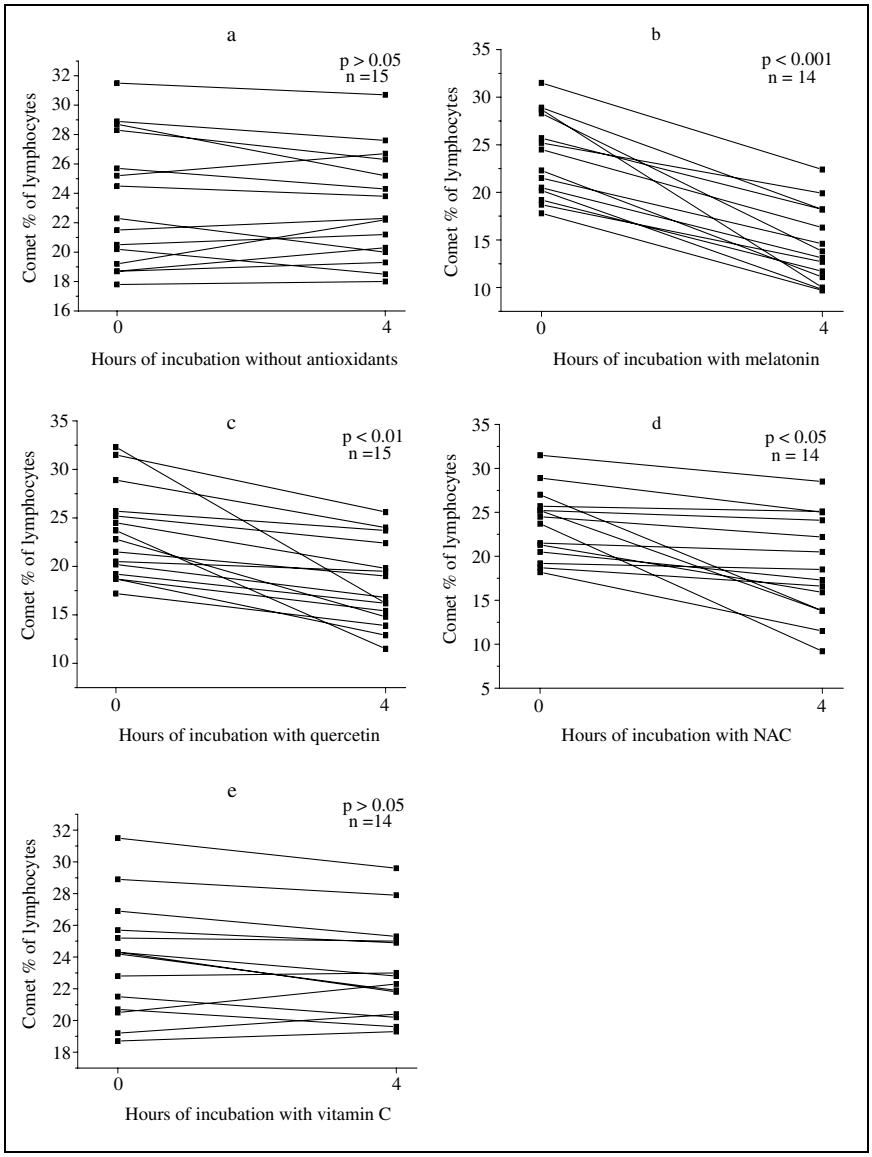

Fig. 4:
Comet % of lymphocytes before and after incubation with or without different antioxidants (100 μM). Each line represented the change of one sample

4. Experimental

4.1. Chemicals and reagents

Melatonin, NAC, quercetin, vitamin C, low and normal melting point agarose, Triton-x-100 and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium lauroyl sarcosine was produced by Amresco. Ficoll-Hypaque was produced by Shanghai Second Reagent Company. All other reagents were of analytical purity.

4.2. Subjects

Blood samples were collected from 33 untreated GD patients (7 males and 26 females, with average age 36.1 ± 12.6 years). The diagnosis was based on physical examination, clinical features of hyperthyroidism and serum hormone levels: TT_4, TT_3 and TSH. Blood samples from 31 age- and sex-matched healthy persons were collected as controls (6 males and 25 females, with average age 32.7 ± 8.4 years, $p > 0.05$ compared to patients). To eliminate the factors which might affect free radical antioxidant activity we excluded smokers and alcoholics, as well as individuals suffering from chronic or acute diseases such as hypertension, diabetes mellitus, hyperlipaemia, diseases of the liver, kidney and endocrine, and immunological disorders, from both GD patients and healthy persons. According to the ethical guidelines of the Helsinki Declaration, informed consent was obtained from all participants and was monitored by the Local Ethics Committee of the Medical College of Lanzhou.

4.3. Methods

4.3.1. Preparation of peripheral blood lymphocytes

The blood was layered on Ficoll-Hypaque cushions. After centrifugation at 1000 × g for 20 min, lymphocytes were collected from the interface, washed with phosphate buffered saline (PBS) twice and suspended in PBS at a concentration of 500,000 cells/ml. The viability of lymphocytes was tested using the trypan blue dye exclusion method. Plasma was frozen at −20 °C for detection of TAC and MDA.

4.3.2. The comet assay in lymphocytes

Single cell micro gel electrophoresis assay, or comet assay as a synonym, is a rapid, simple and sensitive technique for measuring and analysing DNA breakage in single mammalian cells. The comet assay has been used in various studies to investigate the effect of ROS on DNA (Singh et al. 1988; Pouget et al. 2000; Bruner et al. 2000; Cadet et al. 2000). Therefore, the comet assay was used in this study to determine DNA damage.
The comet assay was performed as described by Singh et al. (1988) with minor modification, as described in our previous study (Liu et al. 2002). After electrophoresis, cells were stained with ethidium bromide and viewed at 200 magnification with a fluorescent microscope (BH-2 OLYMPUS, excited by green light 546 nm and barrier filter of 590 nm) within 24 h and photomicrographs were taken. For each sample, three slides were scored, with at least 100 cells counted randomly on each slide. DNA with strand breaks in cells migrates towards the anode during electrophoresis, the cell appearing as a comet, while with intact DNA in cells they retain a circular appearance. Cells were graded by eye into two categories according to Everett (2000). Thus the comet percentage reflected the level of damaged DNA in cells.

4.3.3. Treatment of lymphocytes with melatonin, quercetin, NAC and vitamin C

A kinetic study of the effect of four different antioxidants on damaged DNA was made. Separated lymphocytes were resuspended in Hank's solution and adjusted to 1×10^6/ml, and were then treated with 0, 1, 10, 100

and 1000 μM of melatonin, quercetin, NAC or vitamin C at 37 °C in a humidified atmosphere with 5% CO_2 for 4 h. A comet assay was then carried out.

The optimal concentration (100 μM) of antioxidants was determined by the kinetic study. Lymphocytes from GD patients were then incubated with 100 μM melatonin, quercetin, NAC and vitamin C at 37 °C in a humidified atmosphere with 5% CO_2 for 4 h, and a comet assay was then performed.

4.3.4. Assay of plasma TAC

TAC was measured using a test kit (Nanjing Jiancheng Bioengineering Institute) based on the generation of the Fe^{2+}-*o*-phenanthroline complex due to the total reducing agents in plasma or pleural effusion reducing Fe^{3+} to Fe^{2+}, which reacts with substrate o-phenanthroline. The stable color of the Fe^{2+}-*o*-phenanthroline complex was measured at 520 nm. The final result for TAC was expressed as units per milliliter (U/ml), defined as an absorbency ($OD_{520\,nm}$) value increase 0.01 per minute at 37 °C one milliliter of plasma (Liu et al. 2003; 2000; Benzie and Strain 1996).

4.3.5. Assay of plasma MDA

The generation of free radicals and peroxidation of lipids are extremely fast reactions, which are generally measured by their endproducts, mostly thiobarbituric acid reactive substances (TBARs), of which MDA is the most usual. Plasma MDA was assayed with a human MDA kit (Jiancheng Biological Technical Institute, Nanjing, China), using the TBARs test, as described in the literature (Ohkawa et al. 1979; Zheng and Slater 1992).

4.3.6. Tests of serum TT_4, TT_3 and TSH

Serum TT_3, TT_4 and TSH were tested using a chemiluminescent immunoassay kit (Diagnostic Products Corporation, USA).

4.3.7. Statistical methods

The data are presented as means ±S.D. Statistical analyses were conducted using Student's t test. The differences of comet % before and after antioxidant treatment were calculated by the paired t test. Differences were considered significant at $p < 0.05$.

Acknowledgement: This project was supported by the Doctoral Programme of the Ministry of Education of China.

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