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21

Protection Studies by Antioxidants Using Single Cell Gel Electrophoresis (Comet Assay)

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1. Introduction

Oxidation-reduction reactions, simply referred as "redox" reactions, describe all the chemical reactions in which atoms have their oxidation state changed. This can either be a simple redox process like the oxidation of carbon (C) to carbon dioxide (CO_2) or the reduction of C by hydrogen (H) to yield methane (CH_4). However, in biology redox reactions are rather complex and 'redox biology' is fundamental to aerobic life **(Peters et al., 2008; Baliga et al., 2007)**. The simplest example to give is the oxidation of glucose to CO_2 and water in photosynthesis **(Halliwell, 2006)**.

Aerobes are constantly subject to free radicals, but modulate their actions by synthesizing antioxidants. Free radicals are atoms, molecules, or ions with one or more unpaired electrons on an open shell configuration **(Gutteridge & Halliwell, 2000)**. The simplest form is the atomic H. There are many types of free radicals in living systems, but both nitrogen (N) and oxygen (O) radicals are the main concern for the researchers of several fields as they are suspected to be the underlying factors of several conditions and diseases **(Halliwell, 2006)**. O_2 toxicity was suggested to be due to the inactivation of a variety of enzymes (particularly of antioxidant enzymes) by targeting the thiol group of cysteine residues. In the last decades, molecular biology techniques established that the toxic effects of O_2 are directly linked to its reactive forms, the reactive oxygen species (ROS), acting on cellular components. Oxidative stress is a serious imbalance between the generation of ROS and antioxidant protection in favor of the former, causing excessive oxidative damage **(Dröge, 2002; Halliwell, 2011)**. Oxidative stress and ROS can account for changes that may be detrimental to the cells **(Dröge, 2002)**. ROS are shown to contribute to cellular damage, apoptosis and cell death **(Dalton et al., 1999; Finkel, 1998)**. The link between O_2 toxicity and many pathologies, e.g. pulmonary diseases, **(Frankl, 1991)**, and its effect on swelling of the blood–gas barrier **(Drath et al., 1981)**, retina defects **(Geller et al., 2006)**, bowel disease **(Grisham, 1994)** neurodegeneration **(Wang et al., 2006)**, cancer **(Cerutti, 1994)**, diabetes **(Seet et al., 2010)** and ageing **(Irminger-Finger, 2007)** is very well-established. Besides, in the last decade a relationship between obesity and ROS was demonstrated **(Seet et al., 2010; Halliwell, 2011)**.

Antioxidant is a molecule that protects a biological target against oxidative damage **(Halliwell, 2011)**. Accumulating data implicate that both low antioxidant status and genetics may contribute to the risk of several types of malignancies **(Peters et al., 2008;**

Baliga et al., 2007). The field of antioxidants and free radicals is often perceived as focusing around the use of antioxidant supplements to prevent human disease. Currently, there is a growing interest in environmental chemicals that can cause oxidative stress. The genotoxic effects of some compounds are of particular interest for researchers as humans are exposed to these chemicals abundantly. Exposure to such chemicals may result in disturbances of several physiological processes and may lead to wide variety of degenerative diseases including cancer **(Soory, 2009)**.

First described by **Östling & Johanson (1984)**, and then modified by **Singh et al. in 1988,** the single cell gel electrophoresis assay (also known as Comet assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It has since gained in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing **(Singh et al., 1988)**.

2. Why comet assay is a suitable tool for antioxidant research?

Comet assay can easily detect the *in vitro* toxicity of environmental chemicals on different cell types, as well as *in vivo* toxicity in tissue samples obtained from animals. Besides, it is also a valid technique to evaluate whether antioxidants/micronutrients are able to protect the integrity of the genetic material **(Anderson et al., 1997; Heaton et al., 2002; Novotna et al., 2007)**.

The benefits of Comet assay can be summarized as below:

- Sensitivity for detecting low levels of DNA damage: The limit of sensitivity is approximately 50 strand breaks per diploid mammalian cell and will lose sensitivity above about 10,000 breaks per cell **(Olive & Banáth, 2006)**.
- Requirement for small number of cells per sample: <10,000 cells are enough to perform the assay.
- Flexibility: Comet assay is applicable to virtually any type of cell, as long as a single cell suspension is obtained. Besides, different combinations of unwinding and electrophoresis conditions and lesion-specific enzymes can be used to detect different types and levels of DNA damage **(Wong et al., 2005)**.
- Low cost and ease of application **(Anderson et al., 1997).**
- Studies can be conducted using relatively small amounts of a test substance **(Anderson et al., 1997)**
- A relatively short time is needed to complete an experiment.

The advantages and disadvantages of Comet assay are shown in **Figure 1**.

3. Technical information on comet assay

DNA damage can simply be evaluated using Comet assay that allows the measurement of DNA single- and double-strand breaks (frank strand breaks and incomplete excision repair sites) together with alkali labile sites and crosslinking. By choosing different pH conditions for electrophoresis and the preceding incubation, different levels of damage can be assessed. The degree of DNA migration can be correlated to the extent of DNA damage occurring in each single cell. *In vitro* studies can be performed on virtually with any cell type; however, the cell-type-of-choice in biomonitoring is mostly the lymphocyte because blood is easily

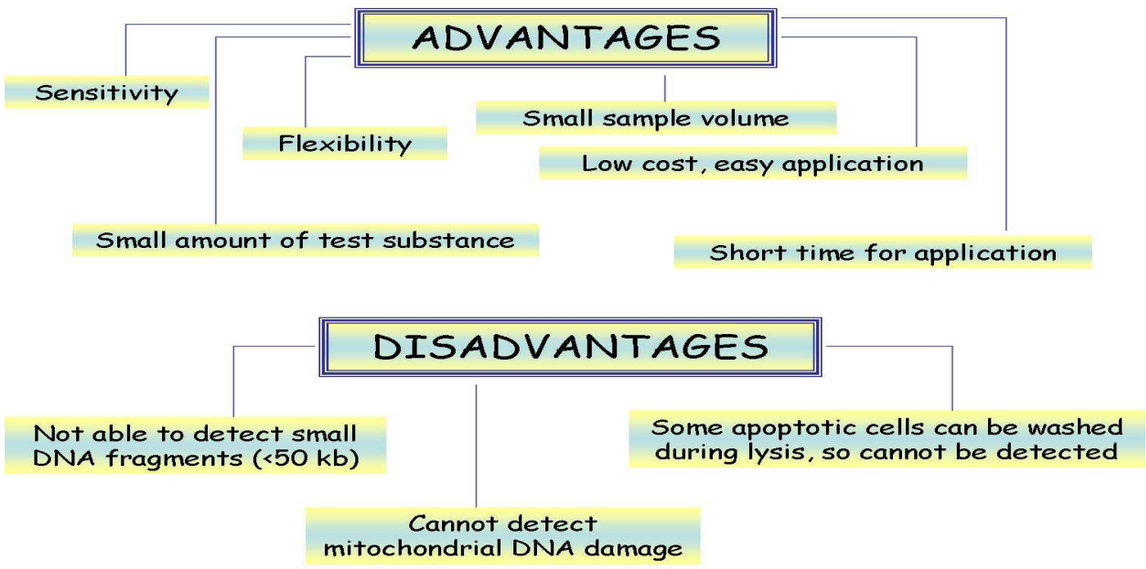

Fig. 1. Advantages and disadvantages of Comet assay

collected and lymphocytes have proved to be good surrogate cells. For example, lymphocytes exhibited genotoxicity caused by anticancer agents targeting several different organs **(Faust et al., 2004)**.

There are differences between laboratories in the isolation of lymphocytes, cells from organs/tissues or other specimens, or in the solutions used for electrophoresis. A simple alkaline Comet assay protocol can be performed in the following steps:

a. The slides that will be used in the study should be covered with agarose (1%) the day before the experiment.
b. In the basic alkaline Comet assay, for primary and other cell cultures, after exposing small number of cells to a physical or chemical agent, the cells are trypsinized, centrifuged, washed, and resuspended in PBS. Because of the flexible application of the technique, the cells used can be isolated lymphocytes, cells isolated from bone marrow, cells isolated from solid organs or tissues or cells from primary or other cell cultures. Lymphocytes can be isolated from whole blood using different isolation solutions and centrifugation. Cells from bone marrow can be obtained by perfusing femur in cold mincing solutions and centrifugation. Solid organs or tissues must be minced into fine pieces, later be suspended in cold mincing solutions and centrifugated. Blood-rich organs like liver and kidney have to minced into larger pieces, the mincing solution can be aspirated and fresh mincing solution should be added. Mincing solution can be Hank's Buffered Salt Solution (HBSS, with 20 mM EDTA and 10% DMSO).
c. Usually 50 µl of the cells obtained from either cell cultures blood or organs/tissues should be mixed with 450 µl solution of low melting point agarose (0.6% in PBS), and 100 µl of the solution is spread on microscope slides covered with agarose.
d. Cells are lysed (in 2.5 M NaCl, 0.5 MNa_2-EDTA, 10 mM Tris, 1% sodium lauryl sulfate, 1% Triton X-100, 10% DMSO, pH 10) at 4°C in dark for 1 h. After lysis, cells were

immersed in freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA, pH 13) for 30 min to allow DNA unwinding.

e. Electrophoresis is then performed at 25 V/300 mA for 30 min.

f. After electrophoresis, slides are rinsed three times for 5 min with neutralization buffer (0.4 M Tris-HCl, pH 7.4), and stained with ethidium bromide (20 µg/ml) in PBS. Ethidium bromide is an intercalating agent commonly used as a fluorescent nucleic acid stain in molecular biology. There are a number of alternative stains to ethidium bromide, including acridine orange, propidium iodide, YOYO-1 iodide stain, SYBR Gold nucleic acid gel stain, SbYR Green I stain, TOTO-3 stain and silver (for non-fluorescent staining).

g. For quantification, a fluorescence microscope can be used which can be connected to a charge-coupled device (CDC) and a computer-based analysis system.

h. The extent of DNA damage was determined after electrophoretic migration of DNA fragments in the agarose gel.

i. For each condition randomly selected comets (50/100/200) on each slide can be scored, and % head DNA, % tail DNA, tail length, tail moment and comet length can be determined. Usually, % tail DNA and tail moment are preferred for assessing the DNA damage.

Rather than making use of the cell's own repair enzymes to reveal damage, we can achieve greater specificity and higher sensitivity by treating the DNA with purified repair enzymes which will convert particular lesions into breaks. Thus, Comet assay protocol can also be performed using different base or nucleotide excision repair enzymes **(Collins et al., 1997)**. The most commonly used repair enyme is formamidopyrimidine DNA glycosylase (Fpg) which recognizes and removes 8-oxodeoxyguanosine (8-oxoGua) and other oxidized purines. 8-oxoguanine glycosylase (OGG1) also recognizes 8-oxoGua. Endonuclease III (Endo III) deals with oxidized pyrimidines; and T4 endonuclease V is able to incise at sites of pyrimidine dimers. Digestion with these enzymes is carried out after the initial lysis step. The excision repair pathways act more slowly than strand break rejoining **(Collins & Horvathova, 2001)**, and samples should be taken over a period of a few hours.

Different versions of Comet assay are also used for different puposes. Neutral Comet assay is usually used for assessing double strand DNA breaks in sperm cells. On the other hand, a "Comet Chip" protocol, first introduced by Massachusetts Institute of Technology (MIT) Engelward Lab**,** is nowadays gaining significant importance as a high throughput DNA damage analysis platform. This new method is also used for evaluating DNA strand breaks, sites of DNA modification and interstrand crosslinks. A limitation of the traditional assay is that each sample requires a separate glass slide and image analysis is laborious and data is intensive, thus reducing throughput. This new technique uses microfabrication technologies to enable analysis of cells within a defined array, resulting in a >200 fold reduction in the area required per condition. Each well of a 96-well plate contains patterned microwells for single cell capture and DNA damage quantification. The "CometChip" can be used to analyze dozens of conditions on a single chip. The newly developed automated image analysis software is used for detection of DNA damage, thus greatly reducing analysis time. This new technology will enable the researchers to conduct both large scale epidemiological and clinical studies **(Engelward Lab, 2011)**.

A new technique "Comet fluorescence in situ hybridization (Comet FISH)" combines two well-established methods. The Comet assay comprises the basis of Comet-FISH and allows separation of fragmented from nonfragmented DNA and quantification of DNA damage and repair. FISH enables detection of specifically labeled DNA sequences of interest, including whole chromosomes. The combined technique of Comet-FISH is a modification of the Comet assay that inserts a hybridization step after unwinding and electrophoresis and permits the labeling of specific gene sequences or telomeres. Comet-FISH has been applied for detection of site-specific breaks in DNA regions that are relevant for development of various diseases, and has also been used to study the distribution of DNA damage and repair in the complete genome. Moreover, DNA sequence modifications can be detected in individual cells using Comet-FISH. The results from the Comet assay alone are only reflections of overall DNA damage. However, the addition of the FISH technique allows the assignment of the probed sequences to the damaged or undamaged part of the comet (tail or head, respectively) **(Schlörmann & Glei, 2009)**.

A spesific illustration for alkaline Comet assay methodology is shown in **Figure 2**. Different protocols of Comet assay in research field are given in **Figure 3**.

In this chapter, I will mainly focus on the genotoxicity of different environmental chemicals and both *in vivo* and *in vitro* protection studies by several selenocompounds, vitamins, and isothiocyanates (ITCs) against the toxicity of these compounds.

4. Protection studies using comet assay

4.1 Prevention of genotoxicity by selenocompounds

There is considerable interest in developing strategies that prevent genotoxicity and cancer with minimal risk or toxicity. Trace elements like selenium (Se) are of particular interest as it is the key component of antioxidant enzyme systems.

The requirement for Se and its beneficial role in human health have been known for several decades. Se is an essential trace element commonly found in grains, nuts, and meats and many years of research showed that that low, non-toxic supplementation with either organic and inorganic forms could reduce cancer incidence following exposure to a wide variety of carcinogens **(El-Bayoumy, 2004)**.

Along with its important role for the cellular antioxidant defense, Se is also essential for the production of normal spermatozoa and thus plays a critical role in testis, sperm, and reproduction **(Flohé, 2007)**. In the physiological dosage range, Se appears to function as an antimutagenic agent, preventing the malignant transformation of normal cells and the activation of oncogenes (**Schrauzer, 2000**). Although most of its chemopreventive mechanisms still remain unclear, the protective effects of Se seem to be primarily associated with its presence in the glutathione peroxidases (GPxs), which are known to protect DNA and other cellular components from damage by oxygen radicals **(Negro, 2008)**. Low activity of another important peroxidase, GPx4, can lead to reduction in reproduction **(Flohé, 2007)**.

Selenoenzymes are known to play roles in carcinogen metabolism, in the control of cell division, oxygen metabolism, detoxification processes, apoptosis induction and the functioning of the immune system oncogenes (**Schrauzer, 2000**). Several studies have determined the low activity of Se-containing cytosolic GPx, known as GPx1, as a substantial

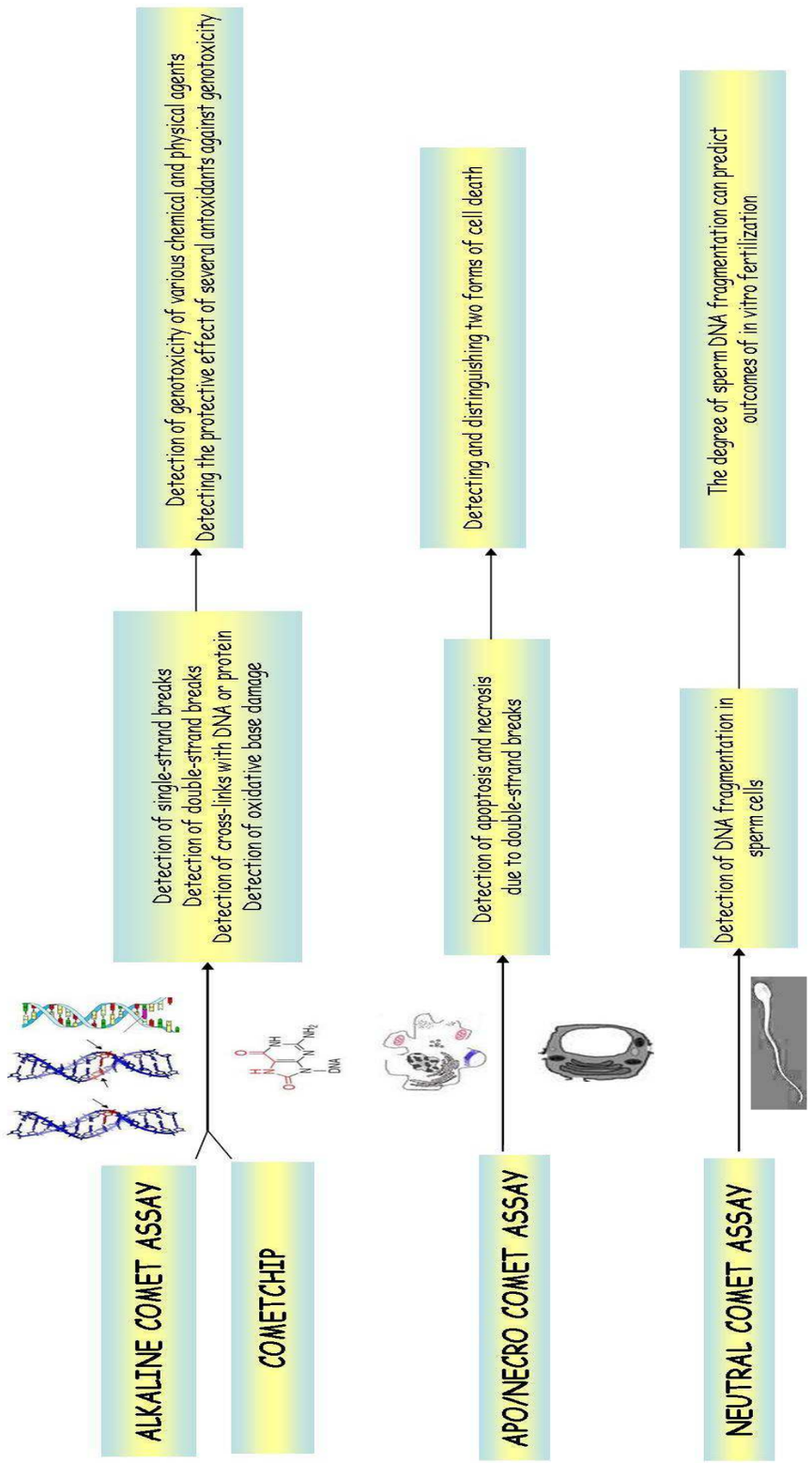

Fig. 2. Different protocols of Comet assay in research field

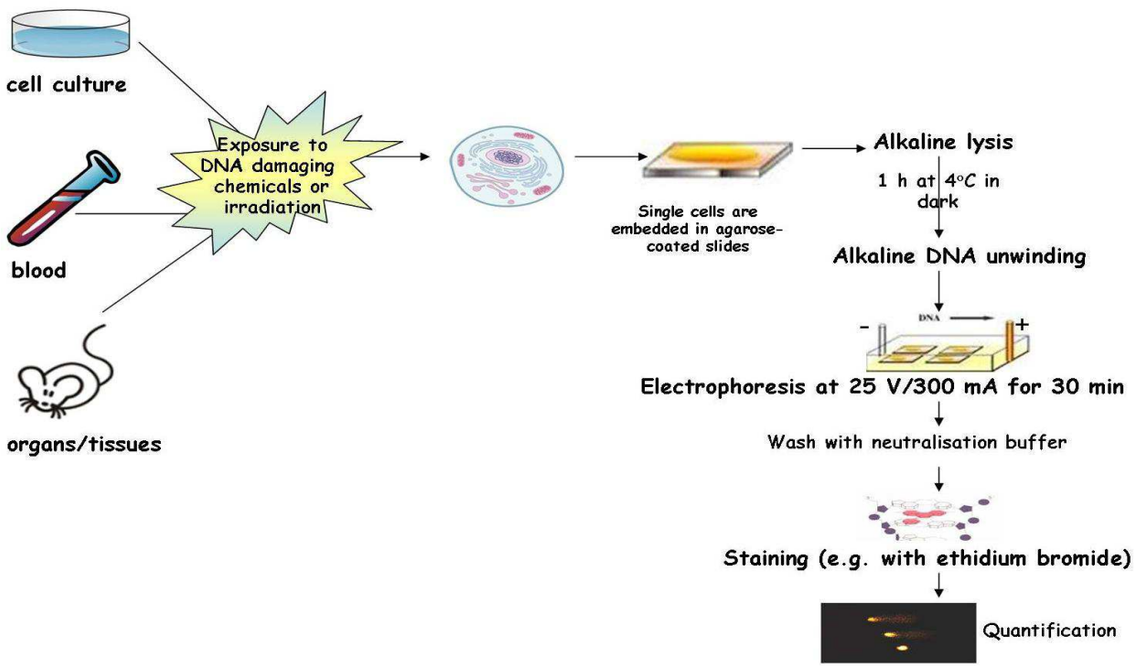

Fig. 3. Alkaline Comet assay methodology

factor in cancer risk **(Esworthy et al., 1985)**. Other modes of action, either direct or indirect, may also be operative, such as the partial retransformation of tumor cells and the inactivation of oncogenes. However, the effects of Se in the physiological dosage range are not attributable to cytotoxicity, allowing Se to be defined as a genuine nutritional cancer-protecting agent **(Yu et al., 1990)**. On the other hand, selenocompounds such as selenodiglutathione, methylselenol, selenomethionine (SM), and Se-methylselenocysteine might affect the metabolism of carcinogens, thus preventing initiation of carcinogenesis **(Gopalakrishna & Gundimeda, 2001)**. These compounds might also restrict cell proliferation by inhibiting protein kinases and by halting phases of the cell cycle that play a central part in cell growth, tumor promotion, and differentiation **(Brinkman et al., 2006)**. A further possible mechanism of action is enhancement of the immune system by stimulating the cytotoxic activities of natural killer cells and lymphokine activated killer cells to act against cancer cells **(Combs, 1998)**. The anticarcinogenic effects of Se are counteracted by Se-antagonistic compounds, and elements (**Schrauzer, 2000)**.

For maximal utilization of its cancer-protective potential, Se supplementation should start early in life and be maintained over the entire lifespan **(Schrauzer & White, 1978; Persson-Moschos et al., 1998; Schrauzer, 2000**). In addition, exposure to Se antagonists and carcinogenic risk factors should be minimized by appropriate dietary and lifestyle changes **(Schrauzer, 1976; Schrauzer, 1977)**. Because geographical studies done in the 1970s reported a possible inverse association between Se and cancer mortality, epidemiological studies have focused on investigating the anticarcinogenic properties of this nutrient **(Brinkman et al., 2006)**. Two key findings that emerged from these early studies were the inverse association between Se and cancer seemed to be both sex and organ specific **(Li et al., 2004)**.

A larger difference in the reduced death rates was reported for men than for women in regions with high levels of Se, and mortality was significantly lower for some types of cancer **(Shamberger et al., 1976; Clark et al., 1991)**. Higher blood levels of Se have been associated with a lower risk of many types of neoplasia, including prostate, lung, colorectal, and possibly bladder, although the data are inconsistent. A significant 39% decreased risk of bladder cancer associated with high levels of Se by combining results from seven epidemiologic studies, conducted in different populations, which applied individual levels of Se measured in serum or toenails **(Brinkman et al., 2006)**.

Supra-physiological levels of sodium selenite (SS) in the presence of polythiols have oxidative properties that might have an anticancer effect by increasing the vulnerability of cancer cells to destruction. It was stated that Se, independent of type (organic/inorganic), can alter several genes to prevent cancer. High doses of Se might upregulate phase II detoxification enzymes, some Se-binding proteins, and some apoptotic genes, and downregulate phase I activating enzymes and cell proliferation genes **(El Bayoumy & Sinha, 2005)**. Inhibition of carcinogen–DNA adducts formation and induction of apoptosis by high doses of Se suggests that protection occurs at both the initiation and post-initiation phases of carcinogenesis **(El Bayoumy & Sinha, 2005)**. However, at lower physiological doses, Se prevents apoptosis, and induces DNA repair **(Longtin, 2003)**.

The literature agrees on the protective effect of Se evaluated with the Comet assay towards a variety of chemical or physical toxic agents. However, it remains inconclusive which is/are the most suitable Se compound/s to prevent DNA damage and which doses should be used to observe protection. In this chapter, the protective effects of both inorganic and organic selenocompounds, against phthalate and radiation toxicity will be discussed.

4.1.1 Prevention of phthalate genotoxicity by selenocompounds

Phthalate esters are a widespread class of peroxisome proliferators (PPs) and endocrine disruptors. They have attracted substantial attention due to their high production volume and use in a variety of polyvinyl chloride (PVC)-based consumer products **(Akingbemi et al., 2001; Grande et al., 2006)**.

Uses of the various phthalates mainly depend on their molecular weight (MW). Higher MW phthalates, such as di(2-ethylhexyl) phthalate, (DEHP), are used in construction materials and in numerous PVC products including clothing (footwear, raincoats), food packaging, children products (toys, grip bumpers), and medical devices **(Heudorf et al., 2007)**, while relatively lower MW phthalates like di-methyl phthalate (DMP), di-ethyl phthalate (DEP), and di-n-butyl phthalate (DBP) are mainly used as odor/color fixatives or as solvents and in cosmetics, insecticides and pharmaceuticals, but are also used in PVC **(Heudorf et al., 2007)**.

Phthalate migrate out from PVC-containing items into food, air, dust, water, and soils and create human exposure in various ways **(Clark et al., 2003)**. Increasing number of studies on human blood and urine have revealed the ubiquitous phthalate exposure of consumers in industrialized countries **(Wormuth et al., 2006, Frederiksen et al., 2008; Frederiksen et al., 2010; Janjua et al., 2011 , Durmaz et al., 2010)**.

DEHP is the most important phthalate derivative with its high production, use and occurrence in the environment. It is mainly used in PVC plastics in the form of numerous

consumer and personal care products and medical devices **(Doull et al., 1999)**. The biological effects of DEHP are hence of major concern but so far elusive. Although, the main mechanism underlying hepatocarcinogenicity of phthalates is not fully elucidated, ROS are thought to be associated with the mechanism of tumorigenesis by PPs, including DEHP. This assumption is based to a fact that various proteins that are induced by DEHP in liver parenchymal cells (peroxisomes, mitochondria and microsomes) are prone to formation of H_2O_2 and other oxidants. Besides, activation of metabolizing enzymes and peroxisome proliferator-activated receptor α (PPARα) might be other substantial factors leading to high intracellular ROS production **(O'Brien et al., 2005; Gazouli et al., 2002)**. However, the mechanisms by which phthalates and particularly DEHP exert toxic effects in reproductive system are not yet fully elucidated. Irreversible and reversible changes in the development of the male reproductive tract like vimentin collapse of Sertoli cells as well as apoptosis of germ cells, effects on sex hormones (mainly on testosterone) as well as follicle stimulating hormone (FSH) and luteinizing hormone (LH), histopathological changes in testis and sperm anomalies were observed with phthalate exposure **(Corton & Lapinskas, 2005; Foster et al., 2001; Erkekoglu et al., 2011a; Erkekoglu et al., 2011b; Kasahara et al, 2002; Noriega et al., 2009)**. Most of the toxic effects were related to its antiandrogenic potential **(Ge et al., 2007)**. A PPARα-mediated pathway based on its peroxisome proliferating (PP) activity **(Gazouli et al., 2002)**, and activation of metabolizing enzymes have also been suggested **(O'Brien et al., 2005)**. While the induction of an oxidative stress may represent a common mechanism in endocrine disruptor–mediated dysfunction, especially on testicular cells **(Latchoumycandane et al., 2002)**, recent studies are also providing supporting evidences for such an effect with DEHP and its major metabolite, mono(2-ethylhexyl)phthalate (MEHP) **(Erkekoglu et al. 2010a; Erkekoglu et al. 2010b; Erkekoglu et al. 2011c; Fan et al. 2010)**. Thus, the primary targets for the DEHP and MEHP are the Sertoli and Leydig cells of testis. In several studies, it was shown that DEHP caused disruption in the function of both cell types. In fact, **Richburg and Boekelheide (1996)** demonstrated histopathological disturbances and alterations of cytoplasmatic distribution of vimentin in Sertoli cells in testis of 28-day-old Fisher rats after a single oral dose of MEHP (2000 mg/kg). Administration of MEHP to Wistar rats at a single oral dose (400 mg/kg bw) was toxic to Sertoli cells and caused detachment of germ cells **(Dalgaard et al., 2000). Tay et al. (2007)** reported vimentin disruption in MEHP-treated C57Bl/6N mice, and gradual disappearance of vimentin in Sertoli cell cultures as time and dose increased. We have also reported that in DEHP-treated rats, significant disruption and collapse of vimentin filaments and disruption of seminiferous epithelium in Sertoli cells was observed **(Erkekoglu et al., 2011b)**. Among several others, an earlier data has demonstrated the increase of ROS generation and depletion in antioxidant defenses by DEHP treatment in rat testis **(Kasahara et al., 2002)**. Our recent studies on MA-10 Leydig **(Erkekoglu et al., 2010b)** and LNCaP human prostate cells **(Erkekoglu et al., 2010a)** have also produced comprehensive data suggesting that at least one of the mechanisms underlying the reproductive toxicity of DEHP is the induction of intracellular ROS. The data of **Fan et al. (2010)** have also suggested oxidative stress as a new mechanism of MEHP action on Leydig cells steroidogenesis *via* CYP1A1-mediated ROS stress. On the other hand, in rats exposed to 1000 mg/kg DEHP for 10 days, we observed that this particular phthalate induced oxidative stress in rat testis, as evidenced by significant decrease in GSH/GSSG redox ratio (>10-fold) and marked increase in TBARS levels **(Erkekoglu et al., 2011d)**.

Several strategies have been attempted to prevent the oxidative stress caused by toxic chemicals and the use of antioxidant vitamins has been the most common approach. **Ishihara et al. (2000)** showed that supplementation of rats with vitamin C and E protected the testes from DEHP-gonadotoxicity. **Fan et al. (2010)** reported that the increase in ROS generation with MEHP exposure in MA-10 cells was inhibited by N-acetylcysteine (NAC). In the above mentioned *in vitro* studies **(Erkekoglu et al., 2010a; Erkekoglu et al., 2010b)**, we demonstrated that Se supplementation in either organic form (SM, 10 μM) or in inorganic form (SS, 30 nM) was highly protective against the cytotoxicity, ROS producing and antioxidant status-modifying effects of DEHP and MEHP in both MA-10 Leydig and LNCaP cells.

Concerning LNCaP cells, we observed that DEHP had a flat dose–cell viability response curve while MEHP showed a very steep dose–response curve and the cytotoxicity of the MEHP was much higher than that of the parent compound. On the other hand, we determined that both organic and inorganic Se supplementation increased resistance to DEHP and MEHP cytotoxicity. From these data, the doses of DEHP and MEHP to be used for the antioxidant status measurements and Comet assay were chosen as close to IC_{50} values and were 3 mM for DEHP and 3 μM for MEHP. We demonstrated that MEHP was the main active form in LnCAP cells with an almost ~1000- fold higher cytotoxicity than the parent compound. Intracellular ROS production showed marked increases with both DEHP and MEHP treatment; however the effect of MEHP was much higher. Both selenocompounds were partially effective in reducing intracellular ROS production. For the antioxidant enzymes, both DEHP and MEHP caused substantial decreases in GPx1 activity (~3-fold, and ~4-fold, respectively) compared to control cells. However, there was no significant difference between the effects of the two phthalate derivatives. Se supplementation with either SS or SM effectively countered the effect of DEHP by completely restoring the activity up to the control level (NT-C) or even higher. In the case of MEHP treatments, both SS and SM supplementations significantly restored the effect of 3 μM MEHP on GPx1 activity, providing ~2-fold increase. For thioredoxin reductase (TrxR) activity, DEHP did not cause a change compared to control; however, MEHP caused a marked increase. Se supplementation in both organic and inorganic forms increased the TrxR activity almost up to the levels of SS and SM supplemented cells alone. However, no changes were observed with both of the phthalates in glutathione S-transferase (GST) activity and total glutathione (GSH) levels. On the other hand, using alkaline Comet assay, we have demonstrated that in LnCAP cells both DEHP and MEHP produced significant DNA damage as evidenced by increased tail % intensity (~2.9-fold and ~3.2-fold, respectively), and tail moment (~2.4-fold and ~2.6-fold, respectively) compared to NT LNCaP cells. The overall difference between the DNA damaging effects of the parent compound and the metabolite was insignificant. Se supplementation itself did not cause any alteration in the steady-state levels of the biomarkers of DNA damage in LNCaP cells, whereas the presence of Se either in SS or SM form reduced the genotoxic effects of DEHP and MEHP as evidenced by significant (~30%) decreases in tail % intensity. These results thus indicated that the Se with the doses and forms used in this study was not genotoxic, but showed antigenotoxic activity against the genotoxicity of DEHP and MEHP. However, the protective effect of Se with the doses used in this study was not complete. Tail intensity remained ~90% and ~80% higher than that of NT-C in SS/DEHP-T and SM/DEHP-T cells, respectively. Similarly, in SS/MEHP-T and SM/ MEHP-T cells, tail intensities were still ~95% and ~120% high compared to NT-C cells. On the other hand, the extent of tail moment increase induced by DEHP was reduced ~30% with SS

and ~18% with SM supplementations, and the tail moment induced by MEHP was reduced ~24% with SS supplementation; however, none of these were statistically significant. Only SM supplementation provided a significant (~34%) reduction in the tail moment induced by MEHP. But again, tail moments remained ~64 and ~95% higher than that of NT-C in SS/DEHP-T and SM/DEHP-T cells, respectively; similarly in SS/MEHP-T and SM/MEHP-T cells, tail moments were still ~94 and ~69% high compared to NT-C cells. In all cases, protective effects of SS and SM were not significantly different than each other **(Erkekoglu et al., 2010a)**.

For Leydig MA-10 cells, The IC_{50} values for DEHP and MEHP were again found to be ~3 mM and ~3 µM, respectively. Se supplementation of the cells with either SS (30 nM) or SM (10 µM) was protective against the cytotoxic effects of DEHP, and MEHP. Intracellular ROS production showed substantial increases with both of the phthalates where the effect of MEHP was much more pronounced. SS and SM showed partial protection against the ROS increment for both the phthalates. In cells exposed to DEHP or MEHP, GPx1 and TrxR activities decreased significantly. Se supplementation either with SS or SM in DEHP-exposed cells was able to enhance the both of the selenoenzyme activities. Moreover, GST activity also decreased significantly with both of the phthalates. However, Se supplementation in both of the forms was not effective in restoring GST activity. GSH levels also decreased significantly in DEHP and MEHP treated Leydig cells while Se supplementation in both forms provided significant restoration in both groups. On the other hand, both DEHP and MEHP produced high level of DNA damage as evidenced by significantly increased tail % intensity (~3.4-fold and ~3.8-fold, respectively), and tail moment (~4.2-fold and ~3.8-fold, respectively) compared to non-treated MA-10 cells. The difference between the DNA damaging effects of the parent compound and the metabolite was insignificant. Se supplementation itself did not cause any alteration on the steady state levels of the DNA damage biomarkers of MA-10 cells. But Se was highly effective to decrease the genotoxic effects of phthalate esters. Increased tail % intensities by DEHP and MEHP exposure were lowered ~50–55% with SS supplementation, whereas SM treatment provided ~30–40% protection. SS decreased the tail moments of the DEHP- or MEHP-exposed cells by ~55–65%, whereas the protective effect of SM on tail moments was significantly lower than SS as being ~45% and ~34% for the effects of DEHP and MEHP, respectively. However, both SS and SM reduced the tail moments of the DEHP- and MEHP-exposed cells down to the levels that were not significantly different than that of control cells **(Erkekoglu et al., 2010b)**.

4.1.2 Prevention of radiation genotoxicity by selenocompounds

Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than X-rays, in the range 10-400 nm, and energies from 3 -124 eV. UV light is found in sunlight, can be emitted by electric arcs and specialized lights such as black lights. It can cause chemical reactions, and it causes many substances to glow or fluoresce. Most UV is classified as non-ionizing radiation **(Müller et al., 1998, Griffiths et al., 1998; Grossman et al., 1988)**.

The toxic effects of UV from natural sunlight and therapeutic artificial lamps are a major concern for human health. The major acute effects of UV irradiation on normal human skin

comprise sunburn inflammation erythema, tanning, and local or systemic immunosuppression. On the other hand, UV irradiation present in sunlight is an environmental human carcinogen. There is considerable evidence that UV is implicated in skin carcinogenesis and the risk of cutaneous cancers has increased during the last decade due to increase of sun exposure. For a long time, ultraviolet B radiation (UVB: 290-320 nm) have been considered to be the more efficient wavelength in eliciting carcinogenesis in human skin. It is today clear that ultraviolet A (UVA, 320-400 nm), especially UVA_1 (340-400 nm) also participate to photo-carcinogenesis. It penetrates deeply, but it does not cause sunburn. One of molecular mechanisms in the biological effects of UV is the induction of ROS directly or through endogenous photosensitization reactions. UVA radiation mainly acts *via* this production of ROS and the subsequent oxidative stress seems to play a crucial role in the deleterious effects of UVA. UVA does not damage DNA directly like UVB and UVC, but it can generate highly reactive chemical intermediates, such as hydroxyl and oxygen radicals, which in turn can damage DNA and lead to the formation of 8-oxoGua **(Ridley et al., 2009)**. UVB light can cause direct DNA damage. The radiation excites DNA molecules in skin cells, causing aberrant covalent bonds to form between adjacent cytosine bases, producing a dimer. When DNA polymerase comes along to replicate this strand of DNA, it reads the dimer as "AA" and not the original "CC". This causes the DNA replication mechanism to add a "TT" on the growing strand. This mutation can result in cancerous growths, and is known as a "classical C-T mutation". The mutations caused by the direct DNA damage carry a UV signature mutation that is commonly seen in skin cancers. The mutagenicity of UV radiation can be easily observed in bacterial cultures. This cancer connection is one reason for concern about ozone depletion and the ozone hole. UVB causes some damage to collagen, but at a very much slower rate than UVA. Fortunately, the skin possesses a wide range of inter-linked antioxidant defense mechanisms to protect itself from damage by UV-induced ROS. However, the capacity of these systems is not unlimited; they can be overwhelmed by excessive exposure to UV and then ROS can reach damaging levels. An interesting strategy to provide photoprotection would be to support or enhance one or more of these endogenous systems **(Béani, 2001)**.

There is limited number of studies in literature concerning the protective effect of selenocompounds on UV-caused genotoxicity. In a study by **Emonet-Piccardi et al. (1998)**, the researchers determined the protective effects of NAC (5 mM), SS (0.6 μM) or zinc chloride ($ZnCl_2$, 100 μM) against UVA radiation in human skin fibroblasts using Comet assay. The cells were incubated with NAC, SS or $ZnCl_2$ and then UVA was applied as 1 to 6 J/cm2 to the cells. The tail moment increased by 45% (1 J/cm^2) to 89% (6 J/cm^2) in non-supplemented cells ($p<0.01$). DNA damage was significantly prevented by NAC, SS and $ZnCl_2$, with similar efficiency from 1 to 4 J/cm^2. For the highest UVA dose (6 J/cm^2), SS and $ZnCl_2$ were more effective than NAC.

In a study assessing the effects of pretreatment of primary human keratinocytes with Se on UV-induced DNA damage, cells were irradiated with UVB from FS-20 lamps and were subjected to Comet assay. Comet tail length due to UVB-induced T4 endonuclease V-sensitive sites (caused by cyclopyrimidine dimers, CPDs) increased to 100% immediately after irradiation (time 0). After 4 h, 68% of the damage remained and after 24 h, 23% of the damage was still present. Treatment with up to 200 nM SM or 50 nM SS had no effect on CPD formation or rates of repair, or on the number of excision repair sites as measured by cytosine arabino furanoside and hydroxyurea treatment. However, both SS and SM

protected against oxidative damage to DNA as measured by formation of formamidopyrimidine (FaPy) glycosylase-sensitive sites, which are indicative of 8-oxoGua photoproduct formation. Preincubation for 18 h with 50 nM SS or with 200 nM SM abolished the UVB-induced increase in comet length. The researchers concluded that both of selenocompounds were protective against UVB-induced oxidative damage in human keratinocytes; however they did not protect from formation of UVB-induced excision repair sites **(Rafferty et al., 2003)**.

Diphenyl diselenide (DPDS) is an electrophilic reagent used in the synthesis of a variety of pharmacologically active organic Se compounds. Studies have shown its antioxidant, hepatoprotective, neuroprotective, anti-inflammatory, and antinociceptive effects. In a study by **Rosa et al. (2007)**, the researchers used a permanent lung fibroblast cell line derived from Chinese hamsters and investigated the antigenotoxic and antimutagenic properties of DPDS. In the clonal survival assay, at concentrations ranging from 1.62 to 12.5 µM, DPDS was not cytotoxic, while at concentrations up to 25 µM, it significantly decreased survival. The treatment with this DPDS at non-cytotoxic dose range increased cell survival after challenge with H_2O_2, methyl-methanesulphonate, and UVC radiation, but did not protect against 8-methoxypsoralen plus UVA-induced cytotoxicity. In addition, the treatment prevented induced DNA damage, as verified in the Comet assay. The mutagenic effect of these genotoxic agents, as measured by the micronucleus test, similarly attenuated or prevented cytotoxicity and DNA damage. Treatment with DPDS also decreased lipid peroxidation levels after exposure to H_2O_2, MMS, and UVC radiation, and increased GPx1 activity in the cells. The results of this study demonstrated that DPDS at low concentrations presents antimutagenic properties, which are most probably due to its antioxidant properties **(Rosa et al., 2007)**.

4.2 Prevention of genotoxicity by vitamins

4.2.1 Ascorbic acid

Diet should include components such as vitamins and flavonoids and the antioxidant capacity of body is directly linked to the diet. Vitamins like ascorbic acid (vitamin C, AA) are important antioxidants. About 90% of AA in the average diet comes from fruits and vegetables **(Vallejo et al., 2002)**.

AA is a water soluble dietary antioxidant that plays an important role in controlling oxidative stress **(Vallejo et al., 2002)**. Most importantly, AA is a mild reducing agent. For this reason, it degrades upon exposure to oxygen, especially in the presence of metal ions and light. It can be oxidized by one electron to a radical state or doubly oxidized to the stable form called “dehydroascorbic acid”. Typically it reacts with oxidants such as ROS, such as the •OH formed from H_2O_2. Hydroxyl radical is the most detrimental species, due to its high interaction with nucleic acids, proteins, and lipids. AA can terminate these chain radical reactions by electron transfer. AA is special because it can transfer a single electron, owing to the stability of its own radical ion called "semidehydroascorbate". The oxidized forms of AA are relatively unreactive, and do not cause cellular damage. However, being a good electron donor, high concentrations of AA in the presence of free metal ions can not only promote, but also initiate free radical reactions, thus making it a potentially dangerous pro-oxidative compound in certain metabolic contexts **(Choe and Min, 2006; Blokhina et al., 2003)**.

AA is able to suppress ROS efficiently *in vivo*; thus, reducing DNA damage to tumor suppressor genes which might explain its anticancer properties **(Crott et al., 1999)**. *In vitro*, AA acts in conjunction with vitamin E, present in lipid membranes, to quench free radicals and prevent lipid peroxidation **(Niki et al., 1995)**.

In the Comet assay, evidence of protection was seen against the effects of H_2O_2 when AA was present at low concentrations (up to 1 mM); by contrast, there was exacerbation at higher doses (>5 mM) **(Harréus et al., 2005; Anderson et al., 1994; Anderson and Phillips, 1999)**. After 2–4 h after intake, AA provided significant protection to the DNA of isolated lymphocytes when challenged with H_2O_2 **(Panayiotidis and Collins, 1997)**. Besides, AA was found to be protective against H_2O_2-induced DNA damage (DNA strand breaks and oxidized purines/pyrimidines) in human hepatoma cells (HepG2 cells) **(Arranz et al., 2007a, Arranz et al., 2007b)**. In intervention studies, supplementation of 100 mg/day to 50–59 year-old men led to a decrease in oxidative base damage and enhanced resistance against oxidative damage **(Duthie et al., 1996)**. In a long-term study, the antioxidant effect of AA was studied by measuring oxidative DNA damage and DNA repair in blood cells with the Comet assay. Male smokers were given AA (2 × 250 mg) daily in the form of plain or slow release tablets combined with plain release vitamin E (2 × 91 mg), or placebo for 4 weeks. The results of this study suggested that long-term AA supplementation at a high dose, i.e. 500 mg, together with vitamin E in moderate dose, i.e. 182 mg, decreased the steady-state level of oxidative DNA damage in lymphocytes of smokers **(Møller et al., 2004)**. In a study performed on gastric epithelial cells SGC-7901, both AA and SS were found to be protective against Helicobacter pylori-induced oxidative stress and genotoxicity **(Shi and Zheng, 2006)**.

AA was also tested for its protective effects against the genotoxicity of several toxic chemicals, drugs and metals. Using peripheral blood lymphocytes, AA as well as vitamin E were found to be protective against benzo(a)pyrene [B(a)P]-induced DNA damage **(Gajecka et al., 1999)**. In rats, using Comet assay, the genotoxicity of p-dimethylaminoazobenzene (DAB), a hepatocarcinogen, was found to be decreased by AA administration. Besides, vitamin A, vitamin E and combination of these three vitamins were also found be effective against the toxicity **(Velanganni et al., 2007)**. A significant increase in the levels of protein oxidation, DNA strand breaks, and DNA-protein cross-links was observed in blood, liver, and kidney of rats exposed to arsenic (100 ppm in drinking water) for 30 days. Co-administration of AA and vitamin E in the form of α-tocopherol to arsenic-exposed rats showed a substantial reduction in the levels of arsenic-induced oxidative products of protein and DNA **(Kadirvel et al., 2007)**. For anti-cancer drugs there are inconclusive results. AA was protective against epirubicin- and adriamycin-induced genotoxicity in cancer patients **(Mousseau et al., 2005; Shimpo et al., 1991)**. However, there was no evidence of a protective effect of AA against the damage caused by bleomycin **(Anderson & Phillips, 1999)**. Moreover, results were also inconclusive when oestrogenic compounds were co-incubated with AA (0.5 and 1 mM) in isolated lymphocytes showing no common pattern in the responses **(Anderson et al., 2003)**.

Nitrosamines (NOCs) can be formed endogenously from nitrate and nitrite and secondary amines under certain conditions such as strongly acidic pHs of the human stomach **(Jakszyn and Gonzalez, 2006; Bofetta et al., 2008; Tricker, 1997)**. Humans are exposed to a wide range of NOCs from diet (cured meat products, fried food, smoked preserved foods, foods subjected to drying, pickled and salty preserved foods), tobacco smoking, work place and

drinking water **(Bartsch and Spiegelhalder, 1996; Bofetta et al., 2008; Jakszyn & Gonzalez, 2006; Tricker, 1997)**.

In several studies, AA was found to be protective against NOC-induced genotoxicity using Comet assay. In a study by **Robichová et al. (2004)**, the researchers used three cell lines (HepG2, V79 and VH10) to determine the genotoxic effect of N-Nitrosomorpholine (NMOR). NMOR was found to induce DNA damage in a dose-dependent manner but the extent of DNA migration in the electric field was unequal in the different cell lines. Although the results obtained by Comet assay confirmed the genotoxicity of NMOR in all cell lines studied, the number of chromosomal aberrations was significantly increased only in HepG2 and V79 cells, while no changes were observed in VH10 cells. In HepG2 cells pre-treated with vitamin A, vitamin E and AA the researchers found a significant decrease of % tail DNA induced by NMOR. The reduction of the clastogenic effects of NMOR was observed only after pretreatment with Vitamins A and E**.** AA did not alter the frequency of NMOR-induced chromosomal aberrations under the experimental conditions of this study. In a study by **Arranz et al. (2007)**, HepG2 cells were simultaneously treated with AA and the genotoxic effects of the N-nitrosamines, namely, N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR), N-nitrosodibutylamine (NDBA) or N-nitrosopiperidine (NPIP) were reduced in a dose-dependent manner. At concentrations of 1-5 μM AA, the protective effect was higher towards NPYR-induced oxidative DNA damage (78-79%) than against NDMA (39-55%), NDBA (12-14%) and NPIP (3-55%), in presence of Fpg enzyme. However, a concentration of 10 μM AA led to a maximum reduction in NDBA (94%), NPYR (81%), NPIP (80%) and NDMA (61%)-induced oxidative DNA damage, in presence of Fpg enzyme. The greatest protective effect of AA (10 μM) was higher towards NDBA-induced oxidative DNA damage. The authors concluded that one feasible mechanism by which AA exerted its protective effect could be that it might interact with the enzyme systems catalyzing the metabolic activation of the N-nitrosamines, blocking the production of genotoxic intermediates.

In our previous studies performed using Comet assay, we have shown that AA was highly protective in HepG2 cells against the genotoxicity of both nitrite and three important NOC, namely NDMA, Nitrosodiethylamine (NDEA) and NMOR **(Erkekoglu et al., 2010c)**. Nitrite was added as 20 μM, NDMA as 10 mM, NDEA as 10 mM and NMOR as 3 mM to the medium for 30 min with or without AA (10 μM). When compared to untreated cells, nitrite ($p>0.05$), NDMA ($p<0.05$), NDEA ($p<0.05$), and NMOR ($p<0.05$) raised the tail intensity up to 1.18-, 3.79-, 4.24-, and 4.16-fold, respectively. AA was able to reduce the tail intensity caused by nitrite, NDMA, NDEA, and NMOR to 34%, 59%, 44%, and 44%, respectively, and these reductions were statistically significant when compared to each individual toxic compound applied group (all, $p<0.05$). Besides, nitrite, NDMA, NDEA, and NMOR increased the tail moment up to 1.94, 6.04, 6.05, and 5.70, respectively. AA (10 μM) enabled a reduction of 27%, 30%, 23%, and 22% in the tail moment in nitrite, NDMA, NDEA, and NMOR-treated cells, respectively, and these reductions were statistically significant when compared to each individual toxic compound applied group (all, $p<0.05$) **(Erkekoglu et al., 2010c)**.

In an experiment performed on multiple organs of mice, the genotoxicity of endogenously formed N-nitrosamines from secondary amines and sodium nitrite was evaluated in, using Comet assay. Dimethylamine, proline, and morpholine were simultaneously with sodium

nitrite and the stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow were sampled 3 and 24 h after these compounds had been ingested. DNA damage was observed mainly in the liver following simultaneous oral ingestion of these compounds **(Ohsawa et al., 2003)**.

4.2.2 Vitamin E

Vitamin E refers to a group of fat-soluble compounds that include both tocopherols and tocotrienols **(Brigelius- Flohé and Traber, 1999)**. Naturally occurring vitamin E exists in eight chemical forms (alpha-, beta-, gamma-, and delta-tocopherol and alpha-, beta-, gamma-, and delta-tocotrienol) that have varying levels of biological activity. Alpha- (or α-) tocopherol is the only form that is recognized to meet human requirements. γ-tocopherol is the most common in the North American diet **(Traber, 1998)**. γ-tocopherol can be found in corn oil, soybean oil, margarine and dressings **(Bieri and Evarts, 1974; Brigelius-Flohé & Traber, 1999)**. The most biologically active form of vitamin E, α-tocopherol, is the second most common form of vitamin E in the North American diet and perhaps the common form in European and Mediterranean diet. This variant of vitamin E can be found most abundantly in wheat germ oil, sunflower, and safflower oils **(Reboul et al., 2006)**. Serum concentrations of α-tocopherol depend on the liver, which takes up the nutrient after the various forms are absorbed from the small intestine. The liver preferentially resecretes only α-tocopherol *via* the hepatic α-tocopherol transfer protein **(Traber, 2006)**. As a result, blood and cellular concentrations of other forms of vitamin E are lower than those of α -tocopherol and have been the subjects of less research **(Sen et al., 2006; Dietrich et al., 2006)**.

Vitamin E is an important vitamin for preventing lipid peroxidation and it has many reported health effects and is recognized as the most important lipid-soluble, chain-breaking antioxidant in the body **(Fenech & Ferguson, 2001)**. This vitamin might have a protective role against chromosomal damage, DNA oxidation and DNA damage. Vitamin E has also been reported to play a regulatory role in cell signaling and gene expression. Epidemiological studies showed that high blood concentrations of vitamin E were associated with a decreased risk of certain cancers. This effect might emerge in part, by enhancing immune function **(Frank, 2005; Claycombe & Meydani, 2001, Salobir et al., 2010)**. Vitamin E might also block the formation of carcinogenic NOCs formed in the stomach from nitrite and secondary amines **(Weitberg and Corvese, 1997)**.

Vitamin E was shown to prevent the genotoxicity of several environmetal chemicals and several drugs. Nitrosamine toxicity was shown to be protected by vitamin E. Hepatocytes freshly isolated from rats fed with a common diet or a vitamin A- or vitamin E-supplemented diet were assayed for sensitivity to DNA breakage and cytogenetic changes induced by several carcinogens including NMOR. NMOR was the only agent that induced DNA breaks, chromosomal aberrations, and micronuclei. Both vitamin A and vitamin E were able to reduce these effects, and the protection by vitamin A was more pronounced **(Slamenová, 2001)**. On the other hand, vitamin E was also found to be protective against the genotoxic properties of one of the most commonly used herbicides, atrazine, in male rats. Atrazine caused a significant increase in tail length of comets from blood and liver cells compared to controls. Co-administration of vitamin E (100 mg/kg bw) along with atrazine resulted in decrease in tail length of comets as compared to the group treated with atrazine alone. Besides, micronucleus assay revealed a significant increase in the frequency of micro-

nucleated cells (MNCs) following atrazine administration. In the animals administrated vitamin E along with atrazine**,** there was a significant decrease in percentage of micronuclei as compared to atrazine treated rats. The increase in frequency of micronuclei in liver cells and tail length of comets confirm genotoxicity induced by atrazine in blood and liver cells. In addition, the findings clearly demonstrated protective effect of vitamin E in attenuating atrazine-induced DNA damage **(Singh et al., 2008)**. In mouse retina, both vitamin E and AA were shown to markedly reduce the cell apoptosis, lipid peroxidation and DNA damage caused by the organophosphorus insecticide chlorpyrifos **(Yu et al, 2008)**. Vitamin E supplementation was also protective against pyrethroid (both cypermethrin and permethrin), induced lymphocyte DNA damage **(Gabbianelli et al., 2004)**.

Vitamin E was also shown to reduce the genotoxic effects of the anti-HIV drug stavudine (**Kaur & Singh**, **2007)** and the antibiotic, ciprofloxacin **(Gürbay et al., 2006)**. In a study performed on primary culture of rat astrocytes, the researchers incubated the cultured cells with various concentrations of ciprofloxacin, and DNA damage was monitored by Comet assay. The results showed a concentration-dependent induction of DNA damage by ciprofloxacin. Pretreatment of cells with Vitamin E for 4 h provided partial protection against this effect **(Gürbay et al., 2006)**.

Vitamin E was also found to be protective against the toxicity of anesthesics. In a study performed with sevoflurane on rabbits, vitamin E and SS were administered 15 days before the anesthesia treatment and blood samples were collected after 5 days of treatment with sevoflurane. Both vitamin E and SS administration prevented the sevoflurane induced genotoxicity in the lymphocytes **(Kaymak et al., 2004)**.

Several supplementation studies have also been performed both vitamin E and AA. Supplementation of the diet for 12 weeks with AA and vitamin E resulted in a significant decrease in the DNA damage in diabetic patients **(Sardaş et al., 2001)**. Vitamin E supplementation was also shown to reduce oxidative DNA damage in both hemodialysis and peritoneal dialysis patients **(Domenici et al., 2005)**. In another study performed on 26 healthy subjects, a daily drink including 1.8 mg vitamin E was administered for 26 days and blood samples were obtained. The DNA damage was measured in the lymphocytes subjected to oxidative stress and genotoxicity was found to be significantly lower (42%, $p<0.0001$) **(Porrini et al., 2005)**.

There are few protection studies with vitamin E against radiation toxicity using Comet assay. An *in vitro* study on dermal microvascular endothelial cells by the same research group, gamma- irradiated cells at 3 and 10 Gy, and 0.5 mM of pentoxifylline (PTX) and trolox (Tx, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E), were added either before (15 min) or after (30 min or 24 h) irradiation. ROS measured by the dichlorodihydrofluorescein diacetate assay, and DNA damage, assessed by the Comet and micronucleus assays, were measured at different times after exposure (0 - 21 days). The PTX/Tx treatment decreased the early and delayed peak of ROS production by a factor of 2.8 in 10 Gy-irradiated cells immediately after irradiation and the basal level by a factor of 2 in non-irradiated control cells. Moreover, the level of DNA strand breaks, as measured by the comet assay, was shown to be reduced by half immediately after irradiation when the PTX/Tx treatment was added 15 min before irradiation. However, unexpectedly, DNA strand breaks was decreased to a similar extent when the drugs were added 30 min after radiation exposure. This reduction

was accompanied by a 2.2- and 3.6-fold higher yield in the micronuclei frequency observed on days 10 and 14 post-irradiation, respectively. These results suggest that oxidative stress and DNA damage induced in dermal microvascular endothelial cells by radiation can be modulated by early PTX/Tx treatment. These drugs acted not only as radical scavengers, but they were also responsible for the increased micronuclei frequency in 10 Gy-irradiated cells. Thus, these drugs may possibly interfere with DNA repair processes **(Laurent et al., 2006)**.

In another study, the effects of vitamin E supplementation were evaluated in cultured primary human normal fibroblasts exposed to UVA. Cells were incubated in medium containing α-tocopherol, α-tocopherol acetate or the synthetic analog Trolox for 24 h prior to UVA exposure. DNA damage in the form of frank breaks and alkali-labile sites, collectively termed single-strand breaks (SSB), was assayed by Comet assay, immediately following irradiation or after different repair periods. The generation of H_2O_2 and superoxide ion was measured by flow cytometry through the oxidation of indicators into fluorescent dyes. Pretreatment of cells with any form of vitamin E resulted in an increased susceptibility to the photo-induction of DNA SSB and in a longer persistence of damage, whereas no significant change was observed in the production of H_2O_2 and superoxide, compared to controls. The researchers indicated that in human normal fibroblasts, exogenously added vitamin E exerted a promoting activity on DNA damage upon UVA irradiation and might lead to increased cytotoxic and mutagenic risks **(Nocentini et al., 2001)**.

In an *in vivo* study by **Konopacka at al. (1998),** the modifying effects of treatment with vitamin E, AA and vitamin A in the form of β-carotene on the clastogenic activity of gamma rays were investigated in mice. Damage *in vivo* was measured by the micronucleus assay in bone marrow polychromatic erythrocytes and exfoliated bladder cells. The vitamins were administered orally, either for five consecutive days before or immediately after irradiation with 2 Gy of gamma rays. The results showed that pretreatment with vitamin E (100-200 mg/kg/day) and β-carotene (3-12 mg/kg/day) were effective in protecting against micronucleus induction by gamma rays. AA depending on its concentration enhanced the radiation effect (400 mg/kg/day), or reduced the number of micro-nucleated polychromatic erythrocytes (50-100 mg/kg/day). Such effect was weekly observed in exfoliated bladder cells. The most effective protection in both tissues was noted when a mixture of these vitamins was used as a pretreatment. Administration of the all antioxidant vitamins to mice immediately after irradiation was also effective in reducing the radiation-induced micronucleus frequency. The data from the *in vitro* experiments based on the Comet assay show that the presence of the vitamins in culture medium influences the kinetic of repair of radiation-induced DNA damage in mouse leukocytes.

4.3 Prevention of genotoxicity by thiocyanates

Human cancer can be prevented by changing the dietary habits **(Kelloff , 2000; Vallejo et al., 2002; Hecht, 1996; Milner , 2004; Davis & Milner, 2006)**. Studies show that antioxidant-rich diets are associated with low risk of cancer and whole diet plays a more important role than the individual components. The protective effects of vegetables and fruits may be attributed to the combined effect of various phytochemicals, vitamins, fibers, and allium compounds rather than the effect of a single component **(Lee et al., 2003)**. There is powerful

evidence in literature for a cancer-protective effect of the vegetables of the family *Cruciferae* that includes broccoli, watercress, cabbage, kale, horseradish, radish, turnip, and garden cress **(Verhoeven et al., 1996; Hecht, 1999)**. This effect is attributed to ITCs, which occur naturally as thioglucoside conjugates (glucosinolates). They are hydrolysis products of glucosinolates and are generated through catalytic mediation of myrosinase, which is released upon processing (cutting or chewing) of cruciferous vegetables from a compartment separated from glucosinolates. Evidence exists for conversion of glucosinolates to ITCs in the gut. At least 120 different glucosinolates have been identified. ITCs have a common basic skeleton but differ in their terminal R group, which can be an alkyl, an alkenyl, an alkylthioalkyl, an aryl, a β-hydroxyalkyl, or an indolylmethyl group. The widely studied ITCs include phenethyl isothiocyanate (PEITC), benzyl isothiocyanate (BITC), indole-3-carbinol (I_3C) and allyl isothiocyanate (AITC) **(Fahey et al., 2001; Arranz et al., 2006)**.

The most important biological property discovered about ITCs is their ability to inhibit carcinogenesis, induced by several chemicals including nitrosamines in the lung, stomach, colon, liver, esophagus, bladder and mammary glands in animal models **(Hecht, 1999; Zhang et al., 2003; Zhang and Talalay, 1994; Hecht et al., 1995; Munday et al., 2003)**. Two mechanisms can be suggested for the protective effect of ITCs against nitrosamine-induced DNA damage:

a. Blocking the production of genotoxic intermediates by inhibiting Phase I enzymes: PEITC was shown to reduce p-nitrophenol hdroxylase (CYP2E1), ethoxyresorufin O-deethylase (CYP1A1) and coumarin hdroxylase (CYP2A6) activities **(García et al., 2008)**.
b. Enhancement of detoxification pathways through the induction of Phase II enzymes **(Arranz et al., 2006)**.

Furthermore, ITCs may have ROS scavenging capacity, alter cell proliferation, stimulate DNA-repair, and induce NAD(P)H: quinine oxidoreductase activity as also mentioned for AA before **(Gamet-Payrastre et al., 2000; Chaudière and Ferrari-Iliou et al., 1999; Surh, 2002; Surh et al., 2001; Roomi et al., 1998)**.

ITCs were shown to be effective in the inhibition of lung tumorigenesis in mice and rats induced by the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Because NNK is believed to play a significant role as a cause of lung cancer in smokers, PEITC is being developed as a chemopreventive agent, which is presently in Phase I a clinical trial in healthy smokers **(Hecht, 1996; Stoner et al., 1991)**. PEITC is a potent inhibitor of rat esophageal tumorigenesis induced by NBMA **(Stoner et al., 1991)**. A comparative study demonstrates that phenylpropyl isothiocyanate (PPITC) is even more potent, whereas BITC and 4-phenylbutyl isothiocyanate (PBITC) have little effect on tumorigenesis **(Wilkinson et al., 1995)**. However, phenylhdroxyl isothiocyanate (PHITC) enhances tumorigenesis in the same model **(Stoner et al., 1995)**. Mechanistic studies clearly show that PEITC inhibits the metabolic activation of NBMA in the rat esophagus, probably through inhibition of a cytochrome P450 (CYP450) enzyme **(Morse et al., 1997)**. Concomitant with this inhibition, inhibition of O^6-methylguanine formation in rat esophageal DNA was observed. The inhibitory effects on tumorigenicity correlate with their inhibitory effects on O^6-methylguanine formation **(Wilkinson et al., 1995; Stoner & Morse, 1997)**. Inhibition of

N'- nitrosonornicotine (NNN) tumorigenicity in the rat esophagus by PEITC also appears to be due to inhibition of its metabolic activation **(Stoner et al., 1998)**.

The antimutagenic properties of ITCs have been reported towards NDMA and NPYR-induced oxidative stress before. In studies performed by **Knasmüller et al. (1996, 2003)** using PEITC as a chemopreventive agent, the researchers observed a reduction in NDMA- and NPYR-induced DNA damage in *Escherichia coli* K-12 and a considerable reduction in NDMA-induced micronuclei in HepG2 cells. The results of several studies demonstrated that ITCs exhibited strong antimutagenic effects against NDMA and NPYR in a dose dependent manner. In a study by **Smerák at al. (2009)**, the researchers investigated the effect of PEITC on the mutagenic activity of indirect-acting mutagens and carcinogens like aflatoxin B1 (AFB1) and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) using the *Ames* bacterial mutagenicity test, the Comet assay, an *in vivo* micronucleus test, and direct-acting mutagen and carcinogen N-nitroso-N-methylurea (MNU). In the Ames test, the antimutagenic activity of PEITC was studied in the concentration range 0.3-300 μg/plate. PEITC at concentrations of 0.3, 3 and 30 μg/plate reduced dose-dependently mutagenicity of AFB1 and IQ in both *Salmonella typhimurium* TA98 and TA100 strains. In the case of the direct mutagen MNU, the antimutagenic effect of PEITC was detected only at concentration of 30 μg/plate in the strain TA100. The PEITC concentration 300 μg/plate was toxic in the Ames test. The 24 h pre-treatment of HepG2 cells with PEITC at concentration 0.15 μg/ml resulted in a significant decrease of DNA breaks induced by MNU at concentrations 0.25 and 0.5 mM. Although a trend towards reduced strand break level were determined also at PEITC concentrations 0.035 and 0.07 μg/ml**,** it did not reach the statistical significance. No effect, however, of PEITC on IQ-induced DNA breaks was observed. Chemopreventive effect of PEITC was revealed also *in vivo*. Pretreatment of mice with PEITC concentrations of 25 and 12.5 mg/kg bw administered to mice in three daily doses resulted in reduction of micronucleus formation in mice exposed to all three mutagens under study, with statistically significant effect at concentration of 25 mg/kg. Results of this study indicated that the strong PEITC antimutagenic properties may have an important role in the prevention of carcinogenesis and other chronic degenerative diseases that share some common pathogenetic mechanisms. In a recent study by **Tang et al. (2011),** PEITC was shown to induce a dose-dependent decrease in cell viability through induction of cell apoptosis and cell cycle arrest in the G_2/M phase of DU 145 human prostate cells. Besides, PEITC induced morphological changes and DNA damage in DU 145 cells. The induction of G_2/M phase arrest was mediated by the increase of p53 and Wee1 and it reduced the level of M-phase inducer phosphatase 3 (CDC25C) protein. The induction of apoptosis was mediated by the activation of caspase-8-, caspase-9- and caspase-3-depedent pathways. Results of this study also demonstrated that PEITC caused mitochondrial dysfunction, increasing the release of cytochrome c and Endo G from mitochondria, and led cell apoptosis through a mitochondria-dependent signaling pathway. The researchers concluded that PEITC might exhibit anticancer activity and become a potent agent for human prostate cancer cells in the future.

There are a few studies on ITCs against nitrosamine-induced genotoxicity in literature. In a study by **Arranz et al. (2006)**, the protective effect of three ITCs was tested. ITC were highly protective against NPYR-induced oxidative DNA damage than against NDMA. The greatest protective effect towards NPYR-induced oxidative DNA damage was shown by I_3C (1 μM,

79%) and by PEITC (1 μM, 67%) and I_3C (1 μM, 61%) towards NDMA (in presence of Fpg enzyme). However, in absence of Fpg enzyme, AITC (1 μM, 72%) exerted the most drastic reduction towards NPYR-induced oxidative DNA damage, and PEITC (1 μM, 55%) towards NDMA. These results indicated that ITCs protect human-derived cells against the DNA damaging effect of NPYR and NDMA, two carcinogenic compounds that occur in the environment. Another study performed by **García et al. (2008)** aimed to investigate the protective effect of ITCs alone or in combination with AA towards NDBA or NPIP-induced oxidative DNA damage in HepG2 cells by Comet assay. PEITC and I_3C alone showed a weak protective effect towards NDBA (0.1 μM, 26-27%, respectively) or NPIP (1 μM, 26-28%, respectively)-induced oxidative DNA damage. AITC alone did not attenuate the genotoxic effect provoked by NDBA or NPIP. In contrast, HepG2 cells simultaneously treated with PEITC, I_3C and AITC in combination with AA showed a stronger inhibition of oxidative DNA-damage induced by NDBA (0.1 μM, 67%, 42%, 32%, respectively) or NPIP (1 μM, 50%, 73%, 63%, respectively) than ITCs alone. One feasible mechanism by which ITCs alone or in combination with AA exert their protective effects towards N-nitrosamine-induced oxidative DNA damage could be by the inhibition of their CYP450 dependent bioactivation. PEITC and I_3C strongly inhibited the p-nitrophenol hydroxylation (CYP2E1) activity (0.1 μM, 66-50%, respectively), while the coumarin hydroxylase (CYP2A6) activity was slightly reduced (0.1 μM, 25-37%, respectively). However, the ethoxyresorufin O-deethylation (CYP1A1) activity was only inhibited by PEITC (1 μM, 55%). The results indicated that PEITC and I_3C alone or PEITC, I_3C and AITC in combination with AA protect human-derived cells against the oxidative DNA damaging effects of NDBA and NPIP.

In our study performed on HepG2 cells, we tested AITC (0.5 μM) against the nitrite and nitrosamine toxicity. Nitrite was added as 20 μM, NDMA as 10 mM, NDEA as 10 mM and NMOR as 3 mM to the medium for 30 min with or without AITC. When compared to untreated cells, nitrite, NDMA, NDEA and NMOR raised the tail intensity up to 17 %, 279 %, 324 % and 288 %, respectively (all, $p<0.05$). AITC was able to reduce the tail intensity caused by nitrite 36 %, by NDMA 36 %, by NDEA 49 % and by NMOR 32 %, respectively. These reductions were statistically significant when compared to each individual toxic compound applied group (all, $p<0.05$). Besides, when compared to untreated cells, nitrite, NDMA, NDEA and NMOR raised the tail intensity up to 94%, 126%, 157% and 207%, respectively (all, $p<0.05$). AITC was able to reduce the tail moment caused by nitrite 16 %, by NDMA 32 %, by NDEA 41 % and by NMOR 19 %, respectively and these reductions were statistically significant when compared to each individual toxic compound applied group **(Erkekoglu & Baydar, 2010d)**.

5. Conclusion

The protective effect of antioxidants is universally accepted. However, as also seen in AA, the mode of action of antioxidants particularly with dual behavior (prooxidant and antioxidant) remain unclear and more research must be conducted on these compounds. For instance, the elucidation of how antioxidant properties operate *in vitro* can provide a better understanding of the *in vivo* situation. On the other hand, Comet assay can be an important tool for the determining of the genotoxic effect of several environmental chemicals, as well as the antioxidant properties of several compounds.

Most of these chemicals exert their toxicity over their ability of producing ROS. ROS can be balanced by the antioxidant action of non-enzymatic antioxidants as well as antioxidant enzymes and it was shown that the genotoxicity of several environmental chemicals can be reversed by proper doses of antioxidants *in vitro*. More *in vitro* studies are needed to prove the beneficial antioxidant effects of trace elements and vitamins. Medicine might benefit from current investigations demonstrating the properties of a vast number of antioxidants as well as studying the effects of different diets. Modest antioxidant supplementation might help prevent chemical-induced carcinogenesis in healthy individuals. On the other hand, antioxidant applications might be beneficial in individuals who may have polymorphisms in genes, including those for antioxidant enzyme. Additionally, populations deficient in several trace elements and vitamins might exhibit modest DNA-repair defects that could be functionally rescued by dietary antioxidants. The future interest of several researchers as well as ours is to understand the pathways underlying the genotoxicity of several agents, particularly phthalates and to determine the antioxidant effect of trace elements and vitamins against the toxic effects of such agents *in vitro* and *in vivo* systems.

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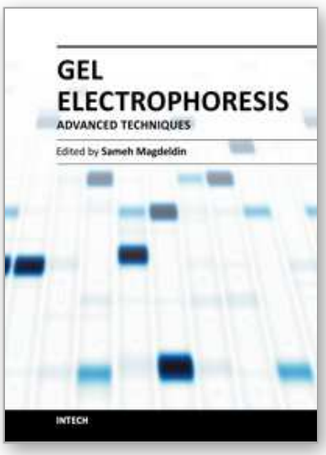

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As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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