Taylor & Francis
Taylor & Francis Group

Toxicology Mechanisms and Methods

ISSN: 1537-6516 (Print) 1537-6524 (Online) Journal homepage: http://www.tandfonline.com/loi/itxm20

Genotoxicity of phthalates

Pınar Erkekoglu & Belma Kocer-Gumusel

To cite this article: Pınar Erkekoglu & Belma Kocer-Gumusel (2014) Genotoxicity of phthalates, Toxicology Mechanisms and Methods, 24:9, 616-626, DOI: 10.3109/15376516.2014.960987

To link to this article: https://doi.org/10.3109/15376516.2014.960987

Accepted author version posted online: 01 Sep 2014.
Published online: 23 Sep 2014.

Submit your article to this journal

Article views: 449

View related articles

View Crossmark data

Citing articles: 10 View citing articles

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=itxm20

Toxicology Mechanisms and Methods

http://informahealthcare.com/txm
ISSN: 1537-6516 (print), 1537-6524 (electronic)

Toxicol Mech Methods, 2014; 24(9): 616–626
© 2014 Informa Healthcare USA, Inc.
DOI: 10.3109/15376516.2014.960987

informa healthcare

REVIEW ARTICLE

Genotoxicity of phthalates

Pınar Erkekoglu and Belma Kocer-Gumusel

Department of Toxicology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey

Abstract

Many of the environmental, occupational and industrial chemicals are able to generate reactive oxygen species (ROS) and cause oxidative stress. ROS may lead to genotoxicity, which is suggested to contribute to the pathophysiology of many human diseases, including inflammatory diseases and cancer. Phthalates are ubiquitous environmental chemicals and are well-known peroxisome proliferators (PPs) and endocrine disruptors. Several *in vivo* and *in vitro* studies have been conducted concerning the carcinogenic and mutagenic effects of phthalates. Di(2-ethylhexyl)-phthalate (DEHP) and several other phthalates are shown to be hepatocarcinogenic in rodents. The underlying factor in the hepatocarcinogenesis is suggested to be their ability to generate ROS and cause genotoxicity. Several methods, including chromosomal aberration test, Ames test, micronucleus assay and hypoxanthine guanine phosphoribosyl transferase (HPRT) mutation test and Comet assay, have been used to determine genotoxic properties of phthalates. Comet assay has been an important tool in the measurement of the genotoxic potential of many chemicals, including phthalates. In this review, we will mainly focus on the studies, which were conducted on the DNA damage caused by different phthalate esters and protection studies against the genotoxicity of these chemicals.

Keywords

Comet assay, di(2-ethylhexyl)phthalate, genotoxicity, phthalate, reactive oxygen species

History

Received 1 August 2014
Revised 27 August 2014
Accepted 27 August 2014
Published online 23 September 2014

Introduction

The term ''free radical'' refers to any molecular species; characterized by the presence of one or more unpaired electrons. The ''unstable'' chemical nature of these radicals endows them their property of being highly reactive to major macromolecules like DNA, RNA, proteins and lipids and thus lead to cellular toxicity and genotoxicity (Franco et al., 2008).

Free radicals include both reactive oxygen (ROS) and nitrogen (RNS) species. When free radical production exceeds the cell's ability to metabolize and detoxify, the state of oxidative stress arises (Halliwell, 2012). ROS production can be of endogenous (mitochondrial oxidative metabolism, cytochrome P450 [CYP450] metabolism and inflammatory cell activation) as well as exogenous (radiation, industrial chemicals and several xenobiotics including drugs) origin. In fact, many of the environmental, occupational and industrial chemicals, including phthalates, are able to generate ROS and/or RNS. These free radicals may cause genotoxicity that contribute to the pathophysiology of many human diseases including inflammatory diseases and cancer (Gruber et al., 2008; Halliwell & Cross, 1994). In this review, we will mainly focus on the genotoxic properties of phthalate plasticizers, which are the most widely used industrial chemicals through the globe.

Address for correspondence: Dr Pinar Erkekoglu, Assoc. Prof., Department of Toxicology, Faculty of Pharmacy, Hacettepe University, Ankara 06100, Turkey. E-mail: erkekp@yahoo.com

Phthalates

Phthalate esters have attracted substantial attention due to their high production volume and use in a variety of polyvinyl chloride (PVC)-based consumer products (Akingbemi & Hardy, 2001; Andrade et al., 2006). The molecular formulas of different phthalates are given in Figure 1.

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 (footwearand raincoats), food packaging, children products (toysand grip bumpers) and medical devices (Heudorf et al., 2007), while relatively lower MW phthalates like dimethyl phthalate (DMP), diethyl phthalate (DEP) and dibutyl phthalate (DBP) are mainly used as odor/color fixatives or as solvents and in cosmetics, insecticides and pharmaceuticals (Heudorf et al., 2007). The uses of different phthalates in industry are summarized in Table 1.

Increasing number of studies on human blood and urine reveals the ubiquitous phthalate exposure of consumers in industrialized countries (Durmaz et al., 2010; Frederiksen et al., 2012, 2014; Just et al., 2012; Teitelbaum et al., 2012; Wormuth et al., 2006). Phthalates migrate out PVC-containing items into food, air, dust, water and soils and create human exposure in various ways (Waring & Harris, 2005). Diet, particularly fatty food (e.g. fish and oils), is the main source of phthalate exposure in the general public (Meek & Chan, 1994; Wormuth et al., 2006). Other sources

Figure 1. The molecular formulas of different phthalates. DEHP, di(2-ethylhexyl)-phthalate; MECMHP, mono(2-carboxymethylhexyl) phthalate; MECPP, mono(2-ethyl-5-carboxypentyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEHP, monoethylhexylphthalate and MEOHP, mono(2-ethyl-5-oxohexyl)phthalate.

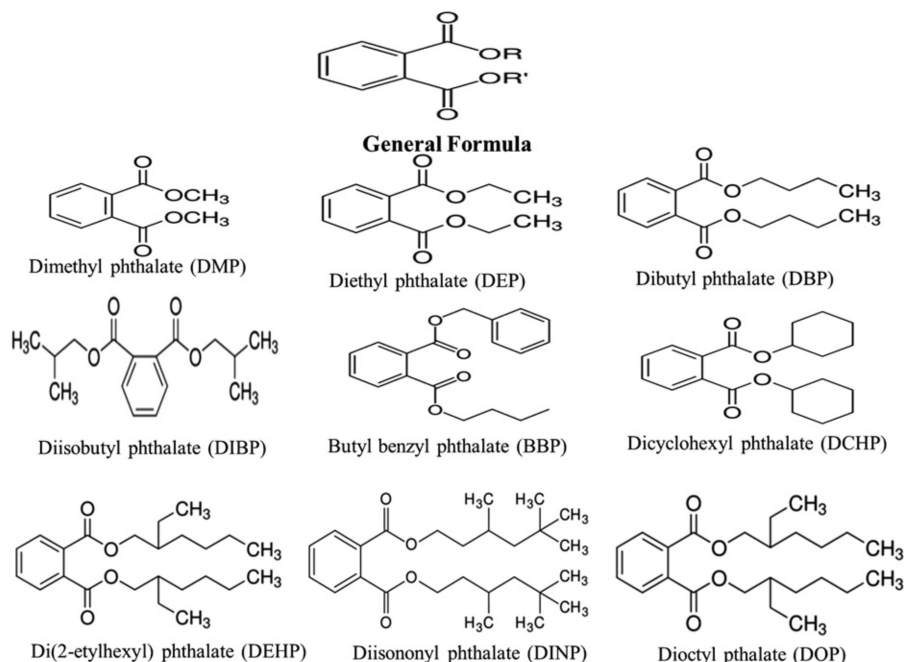

Table 1. The use of different phthalates in industry.

Phthalate	Abbreviation	Usage
Dimethyl phthalate	DMP	Repellentand plasticiser
Diethyl phthalate	DEP	Cosmetics (shampoos, perfumes, soaps, lotions), industrial solvents and drugs (tablet coating, capsule production)
Dibutyl phthalate	DBP	Adhesives, cosmetics, industrial solvents, drugs (tablet coating, capsule production), glues and cosmetics
Diisobutyl phthalate	DiBP	Adhesives, cosmetics, industrial solvents and glues
Butyl benzyl phthalate	BBP	Adhesives, cosmetics, industrial solvents, vinyl coatings and seals
Dicyclohexyl phthalate	DCHP	Stabilizer in rubber and polymer industry
Di(2-etylhexyl) phthalate	DEHP	Plasticiser in soft plastics (IV bags, toys, home products, food packaging), paper industry, electric capasitors, paints/pigments, resins, textile products, rubber and industry
Dioctyl phthalate	DOP	Plasticiser in soft plastics
Diisononyl phthalate	DINP	Plasticiser in soft plastics (instead of DEHP)

include blood storage bags (Labow et al., 1986; Shintani, 1985), kidney dialysis and blood transfusion equipment (Mettang et al., 1999; Nassberger et al., 1987; Sjoberg & Bondesson, 1985).

Phthalate esters are well-known ‘‘peroxisome proliferators (PPs)’’. Several studies suggest an intimate association of ROS with the mechanism of tumorigenesis by peroxisome proliferator-activated receptor α (PPARα) agonists. An increase in the number and size of peroxisomes leads to hepatomegaly, higher formation of H_2O_2 and other oxidants. In addition, ROS play a signaling role in a rapid increase of parenchymal cell proliferation caused by PPs (Rusyn et al., 2000a,b). Besides, activation of metabolizing enzymes and enhanced activity of peroxisomal marker enzymes such as catalase (CAT), CYP450s (e.g. CYP4A1 and CYP4A3) and acyl coenzyme A (CoA) oxidase might be other substantial factors leading to high intracellular ROS production after phthalate exposure (Gazouli et al., 2002; Klaunig et al., 2003; O’Brien et al., 2005). It has been hypothesized that such overproduction of oxidants might cause DNA damage and can cause mutations and cancer (Reddy & Rao, 1989; Yeldani et al., 1989). Rodents are the most sensitive species to the carcinogenic effects of phthalates; while monkey, and human are weakly responsive at dose levels that produce a marked response in rats and mice (Ashby & Tennant, 1994; Bentley et al., 1993; Cattley et al., 1998; Seo et al., 2004). At the molecular level, the activation of the three isoforms of PPAR (α, β and γ) by phthalate metabolites and the resulting metabolic consequences have been extensively documented (Bility et al., 2004). Several studies suggested PPARα-dependent mechanisms leading to hepatocarcinogenesis are low relevance to humans (Melnick, 2001; Willhite, 2001). Such discrepancy necessitates more investigations to elucidate the difference in the mechanism of action of phthalate in rodent and human livers.

Di(2-ethylhexyl)-phthalate

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). Upon ingestion, pancreatic lipases present in the intestine convert DEHP to its monoester, monoethylhexylphthalate (MEHP), which is preferentially absorbed (Huber et al., 1996). In addition, MEHP can also be produced by plasmatic and hepatic lipases, which transform

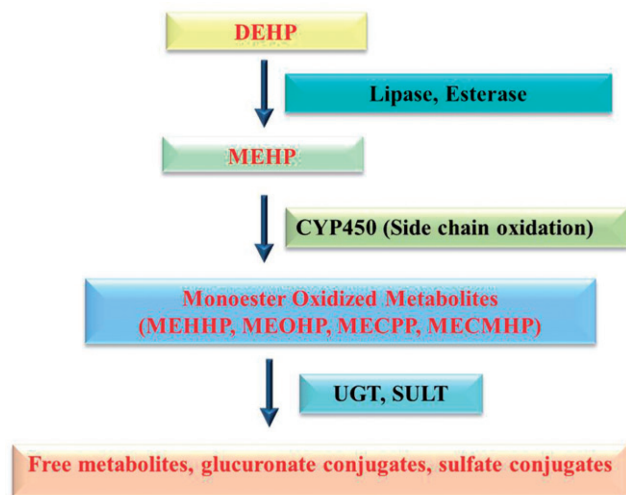

Figure 2. The biotransformation of di(2-ethylhexyl)-phthalate. CYP450, cytochrome P450 enzymes; DEHP, di(2-ethylhexyl)-phthalate; MEHP, monoethylhexylphthalate; SULT, sulfone transferase and UGT, Uridine 5′-diphospho-glucuronosyltransferase.

DEHP directly reaching the blood through absorption or medical contamination (Bility et al., 2004). MEHP is further metabolized to mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), which is then metabolized to mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP). Another metabolite for MEHP is mono-2-(1-hydroxyethyl) hexylphthalate (MHEHP), which is metabolized to mono-2-(1-oxyethyl)hexylphthalate (MOEHP). MEHP can also be metabolized to structural isomers of MEHHP, which also have further metabolism to different monoethylphthalate structures (Silva et al., 2006). The biotransformation of DEHP is summarized in Figure 2.

DEHP and other phthalates, such as di-(2-ethylhexyl) adipate (DEHA), diisononyl phthalate (DINP) or 2-ethylhexanol (2-EH) are shown to be hepatocarcinogenic in both sexes in mice and rats, causing both hepatocellular carcinomas and adenomas (Astill et al., 1996; Kaufmann et al., 2002; Kluwe et al., 1985). Collectively, it appears that oxidant-related molecular events could interact with other pathways activated by PPs *in vivo* in rodent liver, and thus it is critically important to understand their precise mechanism of action (Rusyn et al., 2006).

Exposure to phthalates, particularly to DEHP resulted in decreased testicular testosterone production in rodents (Jones et al., 1993) and most of the reprotoxic effects are suggested to related to their antiandrogenic potential (Ge et al., 2007). This indicates that Leydig, along with Sertoli, cells are their targets. In several studies, it was shown that DEHP caused disruption in the function of both cell types. In fact, Richburg & 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) (Richburg & Boekelheide, 1996). 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 (Tay et al., 2007). We have also reported that in DEHP-treated pre-pubertal rats, significant disruption and collapse of vimentin filaments and disruption of seminiferous epithelium in Sertoli cells was observed. Moreover, DEHP was shown to cause apoptotic germ cell death (Erkekoglu et al., 2012a).

Genotoxicity of phthalates

Effects of phthalates on DNA breaks and chromosomal aberations

Several environmental chemicals, including phthalates, may cause cytogenetic damage to animals and humans. The biological effects of phthalates are of major concern but so far elusive. For years, phthalates were classified as epigenetic carcinogens. However, recent evidences suggest that these chemicals also possess genotoxic properties (Anderson et al., 1999; Erkekoglu et al., 2010a,b; Kleinsasser et al., 2000, 2001).

Several *in vivo* and *in vitro* tests including chromosomal aberration test, unscheduled DNA synthesis (UDS), Ames test, micronucleus test and hypoxanthine guanine phosphoribosyl transferase (HGPRT) mutation test are used to determine the genotoxic potentials of phthalates. In the short-term *in vitro* mammalian cell chromosome aberration test, cultured mammalian cells are exposed to a test substance, harvested and the frequency of asymmetrical structural chromosome aberrations is measured (Clare, 2012). The UDS test with mammalian liver cells *in vivo* identifies substances that induce DNA repair after excision. The test is usually based on the incorporation of tritium-labelled thymidine, 3H-TdR, (during 3–8 h) into the DNA of hepatocytes which are low frequent in the S-phase of the cell cycle. The uptake of 3H-TdR is usually determined by autoradiography (Painter & Cleaver, 1969).

The Ames test uses several strains of the bacterium *Salmonella typhimurium* that carry mutations in genes involved in histidine synthesis. These strains are auxotrophic mutants, requiring histidine for growth. The method depends on the test substance's ability to cause mutations that result in a reversion back to a ''prototrophic'' state, so that the cells can grow on a histidine-free medium. To determine frameshift mutations, strains TA-1537, TA-1538, TA97a or TA98 are used. For point mutations, strains TA-1531, TA100, TA102 or TA104 are used (Mortelmans & Zeiger, 2000).

Micronucleus test is one of the most successful and reliable assays for both aneugens and clastogens. A micronucleus is the erratic (third) nucleus formed during cell division. Micronuclei are cytoplasmic bodies having a portion of acentric chromosome or whole chromosome which was not carried to the opposite poles during cell division. Their formation results in the daughter cell lacking a part or all of a chromosome (Fenech, 2000). There are two major versions of this test: *in vivo* and *in vitro*. The *in vivo* test normally uses mouse bone marrow or mouse peripheral blood while in the *in vitro* assay, usually cultured mammalian cells are preferred (Decordier & Kirsch-Volders, 2006, Tinwell & Ashby, 1994).

The HPRT gene is on the X chromosome and is used as a model gene to determine gene mutations in mammalian cell lines. The HPRT assay can detect a broad range of xenobiotics capable of causing DNA damage and mutation. The HPRT

methodology is such that mutations which destroy the functionality of the HPRT gene (and/or protein) are detected by positive selection using a toxic analogue, and HPRT mutants are observed as viable colonies (Johnson, 2012).

Blom et al. (1998) showed phthalates, such as DBP, contribute to high proliferation of human breast-cancer cell lines, which was explained in part by the potency of phthalates in terms of a xenoestrogenic impact (Blom et al., 1998). This impact appeared to be related to a direct estrogen receptor binding of some (Nakai et al., 1999), but not all, phthalates (Zacharewski et al., 1998). On the other hand, phthalates are PPs, which mediate changes in gene expression and the metabolism of xenobiotics. This capability may result in promotion of hepatic carcinogenesis in rodents (Huber et al., 1996).

An earlier study suggested that DEHP induced single chromatid aberrations and sister chromatid exchange (SCE) in human lymphocytes. Besides, DEHP caused lymphatic mitotic inhibition after 4 h of exposure and caused an increase in the doubling time of human lymphocytes (Turner et al., 1974). Later, Stenchever et al. (1976) reported that DEHP caused chromosomal breaks in three of freshly obtained human lymphoctes from donors ($n = 4$, two male and two female) after 4 h of incubation while in the lymphoctes of one female donor there was decrease in chromosomal breaks. Besides, the researchers observed that in the lymphocytes of two donors (one female and one male) there were increases in chromosomal gaps while in other two, the gaps showed decreases. In all the lymphocytes, mitotic rate decreased. In the same study, researchers also observed DEHP caused polyploidy and aneuploidy in human fetal lung cells. Phillips et al. (1982) observed that MEHP caused chromosome damage, without affecting in the SCE and HPRT mutation test in Chinese hamster (CH) cells. However, a study by Astill et al. (1986) did not observe a change in Ames test, mouse lymphoma activation assay, micronucleus test, UDS and cell transformation tests with DEHP treatment in rat hepatocytes. A study performed on both Chinese hamster ovary (CHO) cells and RL4 liver cells showed that MEHP caused chromosomal abberations in both of the cell types. However, S-9 mix, which activates a wide variety of mutagens, had no effect on the chromosome damage produced by MEHP in CHO cells (Phillips et al., 1986). A study using mouse hepatocytes obtained from DEHP and MEHP treated animals evaluated the genotoxicity of these compounds determining the changes in DNA repair or UDS and comparing the results with the percentage of cells undergoing scheduled DNA synthesis (SDS). No changes were observed in DNA repair capacity. However, UDS of the hepatocytes obtained from mice (3.1%) treated with DEHP containing diet (500 mg/kg) showed significantly higher UDS compared to control hepatocytes (0.2%) (Smith-Oliver & Butterworth, 1987). Lindahl-Kiessling et al. (1989) observed that DEHP induced SCE in human lymphocytes which were co-cultured with rat liver cells for 3–6 h. Researchers indicated in lymphocytic cultures, rat hepatocytes were a better source of metabolic activation as S-9 mix cannot replace metabolic activation by hepatocytes because the cofactors (i.e. NADPH, NADP and G-6-P) which are used to prepare S-9 mix inhibit DNA synthesis strongly. This phenomenon can further explain the above-mentioned results of Phillips et al. (1982) which showed that S-9 mix had no effect on the chromosome damage produced by MEHP in CHO cells. On the other hand, Elliott & Elcombe (1987) also did not observe any increase in DNA damage in rat hepatocytes treated with DEHP. In a study performed by Müller-Tegethoff et al. (1995), the researchers showed that PPs like fenofibrate, nafenopin, Wy-14,643 and DEHP (10^{-6}M to 10^{-2}M) did not induce micronucleus formation in rat hepatocytes. Kim et al. (2002) showed that DBP (5000 ppm DBP containing diet for 16, 32 and 52 weeks) caused both chromatid and chromosomal type chromosomal abberations (break and exchange) in the lymphocytes of B6C3F1 mice and this abberations showed marked increases while the week of exposure increased. In supravital micronucleus test, the number of micronucleated reticulocytes showed increases compared to control; however this increase was not time-dependent. Concerning the two tests, the results obtained from mice fed with DBP containing diet for 52 weeks were not significantly different than mice fed with DBP for 16 or 32 weeks. In a study conducted by McKee et al. (2000), DINP was tested in Ames test, *in vitro* cytogenetics assay and mouse micronucleus assay, whereas di(isodecyl) phthalate (DIDP) was evaluated in a mouse micronucleus test. All of the tests produced negative results, i.e. neither phthalate was mutagenic in any of the test systems. Lee & Lee (2007) examined the genotoxic properties of phthalic acid (PA) and terephthalic acid (TPA) using Ames test, chromosome aberration test and micronucleus test. The two agents did not produce any mutagenic responses in the absence or presence of S9 mix on the *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 or TA1537. In the chromosomal abberation test, PA and TPA did not show any significant cytogenetic effect on Chinese hamster ovary (CHO) cells. In the mouse micronucleus test, no significant alterations in occurrence of micronucleated polychromatic erythrocytes was observed in male mice at any of the doses administered (0, 20, 100, 500, 2500 or 12,500 μM/kg, i.p.). The summary of these studies are given in Table 2.

Phthalates and Comet assay

The Comet assay is abundantly used to measure DNA damage as a marker of exposure to genotoxic chemicals or to determine genoprotective effects of different antioxidants in eukaryotic cells or disaggregated tissues (Anderson et al., 1997; Azqueta & Collins, 2013; Collins et al., 2014; Heaton et al., 2002; Novotna et al., 2007). This assay is based on the relaxation of supercoiled DNA in agarose-embedded nucleoids (the residual bodies remaining after lysis of cells in the presence of a detergent and high salt concentration), that allows the DNA to be drawn out towards the anode under electrophoresis and the comet-like images formed are observed under fluorescence microscope (Azqueta & Collins, 2013). 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

Table 2. The effects of phthalates on DNA breaks and chromosomal aberrations.

Study (year)	Type of experiment	Phthalate	Overview	Conclusion
Blom et al. (1998)	*in vitro*	DBP	DBP contributed to high proliferation of human breast-cancer cell lines.	DBP might have xenoestrogenic impact.
Turner et al. (1974)	*in vitro*	DEHP	DEHP induced single chromatid aberrations and SCE in human lymphocytes.	DEHP had genotoxic effect.
Stenchever et al. (1976)	*in vitro*	DEHP	DEHP caused chromosomal breaks, increases or decreases in chromosomal gaps and decrease in mitotic rate in human lymphocytes. DEHP caused polyploidy and aneuploidy in human fetal lung cells.	DEHP caused both chromosomal damage and alteration in chromosomal numbers.
Phillips et al. (1982)	*in vitro*	MEHP	MEHP caused chromosome damage, without affecting in the SCE and HGPRT mutation test in CH cells.	MEHP caused chromosomal damage; but not mutations.
Astill et al. (1986)		DEHP	DEHP did not cause changes in Ames test, mouse lymphoma activation assay, micronucleus test, UDS, and cell transformation tests in rat hepatocytes.	DEHP did not cause genotoxicity.
Phillips et al. (1986)	*in vitro*	MEHP	MEHP caused chromosomal aberrations in CHO cells and RL4 liver cells. S-9 mix had no effect on the chromosome damage produced by MEHP in CHO cells.	MEHP caused chromosomal aberrations.
Smith-Oliver & Butterworth (1987)	*in vivo*	DEHP and MEHP	Mouse hepatocytes obtained from DEHP-treated animals showed no changes in DNA repair capacity. UDS of the hepatocytes obtained from mice treated with DEHP containing diet showed significantly higher UDS compared to control.	DEHP induced UDS but did not affect DNA repair capacity.
Lindahl-Kiessling et al. (1989)	*in vitro*	DEHP	DEHP induced SCE in human lymphocytes which were co-cultured with rat liver cells.	DEHP induced SCE.
Elliott and Elcombe (1987)	*in vitro*	DEHP	Rat hepatocytes treated with DEHP showed no increase in DNA damage.	DEHP did not induce DNA damage.
Müller-Tegethoff et al. (1995)	*in vitro*	DEHP	Rat hepatocytes treated with DEHP (10^{-6} M to 10^{-2} M) did not show increases in micronucleus formation.	DEHP did not induce micronucleus formation.
McKee et al. (2000)	*in vitro*	DINP and DIDP	DINP was tested in Ames test, *in vitro* cytogenetics assay and mouse micronucleus assay. DIDP was evaluated in a mouse micronucleus test. All of the tests produced negative results.	Neither phthalate was mutagenic in any of the test systems.
Kim et al. (2002)	*in vivo, in vitro*	DBP	DBP containing diet caused both chromatid and chromosomal type chromosomal aberrations (break and exchange) in the lymphocytes of B6C3F1 mice. In micronucleus test, the number of micro-nucleated reticulocytes showed increases compared to control.	DBP caused chromosomal aberrations and genotoxicity.
Lee & Lee (2007)	*in vitro*		The two agents did not produce any mutagenic responses with or without S9 mix on the *Salmonella typhimurium* (TA98, TA100, TA102, TA1535 or TA1537) in Ames test. In the chromosomal abberation test, PA and TPA did not show any significant cytogenetic effect on CHO cells. In the mouse micronucleus test, no significant alterations in occurrence of micronucleated polychromatic erythrocytes at any of the doses administered (0, 20, 100, 500, 2500 or 12 500 μM/kg, i.p.).	PA and TPA did not induce mutatations in *Salmonella* strains, did not show any significant cytogenetic effect on CHO cells and did not induce micronucleus formation in the mouse micronucleus test.

CHO, Chinese Hamster Ovary; DBP, dibutyl phthalate; DEHP, di(2-ethylhexyl)-phthalate; DIDP, di(isodecyl) phthalate; DINP, diisononyl phthalate; MEHP, monoethylhexylphthalate; PA, phthalic acid; TPA, terephthalic acid and UDS, unsceduled DNA synthesis.

be assessed. The degree of DNA migration can be correlated to the extent of DNA damage occurring in each single cell and the relative amount of DNA in the comet tail indicates DNA break frequency. *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 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). The assay has been modified to detect various base alterations, by including digestion of nucleoids with a lesion-specific endonuclease (Azqueta & Collins, 2013; Collins, 2014).

The benefits of Comet assay are (Azqueta & Collins, 2013; Erkekoglu, 2012; Gutzkow et al., 2013):

- Ease of application and a relatively short time to complete an experiment.
- Low cost.

- High sensitivity for detecting low levels of DNA damage.
- Requirement for small number of cells (<10 000) and small amounts of test substance.
- Flexibility.
- Different combinations of unwinding and electrophoresis conditions and lesion-specific enzymes for detecting different types and levels of DNA damage.
- New high through-put assays to run many samples simultaneously.

However, there are also disadvantages (Azqueta & Collins, 2013; Erkekoglu, 2012):

- Not able to detect mitochondrial DNA damage.
- Some apoptotic cells are washed during lysis and cannot be detected.
- Not able to detect small DNA fragments smaller than 50 kb.
- Limited dynamic range (in terms of breaks produced by ionising radiation, between a fraction of a Gy and 10 Gy)
- No standard units for tail moment.
- Special care reqirement when performing on plants.

In the last two decades, several studies are performed on the genotoxicity of phthalates using Comet assay. A study by Anderson et al. (1999) determined that both DEHP and MEHP induced DNA damage in human leukocytes as evidenced by increases in tail moment in Comet assay. Besides, DEHP at high doses caused significant increases in tail moment in human lymphocytes. A study by Kleinsasser et al. (2000) compared susceptibilities to DBP and di-iso-butyl-phthalate (DiBP), in non-tumor patients to those in patients with squamous cell cancer (SCC) of the oropharynx or larynx using Comet assay. Tissue biopsy specimens were used to prepare mucosal cell cultures. DBP and DiBP produced significant DNA damage on mucosal cells in all groups of patients. Rank statistics regarding Olive tail moment (OTM) indicated that DBP and DiBP produced significant differences between oropharynx (TO), larynx (TL), TO plus TL groups and the non-tumor donors even after Bonferroni correction. Another study by the same working group evaluated whether there was a correlation between the genotoxic sensitivities to DBP and its isomer DiBP in either mucosal cells or lymphocytes using Comet assay. The assay was applied to detect DNA strand breaks in human epithelial cells of the upper aerodigestive tract ($n = 132$ specimens). Human mucosa was harvested from the oropharynx in non-tumor patients and patients with squamous cell carcinomas of the oropharynx. Laryngeal mucosa of patients with laryngeal squamous cell carcinomas was harvested as well. Peripheral lymphocytes ($n = 49$ specimens) were separated from peripheral blood. Both DBP and DiBP showed genotoxic effect in both epithelial cells and lymphocytes ($p < 0.001$, all). At equal concentrations, DiBP induced higher genotoxicity compared to DBP in both cell types. In analyzing DBP and DiBP results, genotoxic impacts in mucosal cells showed an intermediate correlation ($r = 0.570$). Correlations between the OTM values for the negative control and the substances were very weak in both mucosal cells ($r = 0.264$ for negative control and DBP, $r = 0.232$ for negative control and DiBP) and lymphocytes ($r = 0.378$ for negative control and DBP, and $r = 0.286$ for negative control and DiBP, respectively) (Kleinsasser et al., 2001). Biscardi et al. (2003) reported that DEHP can leach out of polyethylene terephthalate (PET) bottles in time, especially after 9–10 months of storage and this amount of DEHP (extracted with acetone, found between 0.39 and 3.21 mg/L by GC/MS) can cause increases in both total tail length and number of cells >95th distribution in human leukocytes *in vitro* (Biscardi et al., 2003).

In a study conducted on HeLa cells, DEHP along with bisphenol A, nonylphenol and paraquat dichloride were tested for their genotoxicity potentials. DEHP showed genotoxic effect above 90 μM dose and this was evidenced by significant increases in tail moment (Park & Choi, 2007). In a recent study performed on HepG2 cells exposed to various concentrations of DEHP (0, 2.5, 5, 10, 25, 50, 100 and 250 mM) for 24 or 48 h, the genotoxicity of this particular phthalate was determined using Comet assay. The mean value of the OTM of the control HepG2 cells was 1.06 ± 0.02. After exposure to DEHP for 24 and 48 h, DNA damage increased significantly in a dose-dependent manner (from 2.5 to 250 mM). The mean values of OTMs of HepG2 exposed to the highest concentration of DEHP (250 mM) for 24 and 48 h were 2.50 ± 0.02 ($p < 0.001$ versus control) and 3.47 ± 0.05 ($p < 0.001$ versus control), respectively (Choi et al., 2010).

Recent studies on phthalates are mainly focusing on their reproductive toxicity potential. Several studies have been conducted on the correlation between urinary phthalate levels and sperm DNA damage in the last decade. In a study by Duty et al. (2003) semen and urine samples of 141 subjects were analyzed for 5 phthalate metabolites [monoethyl phthalate (MEP), monobenzyl phthalate (MBzP), mono *n*-butyl phthalate (MBP), MEHP, monomethyl phthalate (MMP)] and sperm DNA damage was assessed by neutral Comet assay. The comet extent was only correlated with MEP levels, while there was no correlation with other phthalate metabolites. In a study by Hauser et al. (2007), the researchers measured the urinary concentrations of phthalate metabolites among 379 men who administered to infertility clinic. The metabolites determined for DEHP were MEHP, MEHHP and MEOHP while other phthalate metabolites were MEP, MBP, MBzP and monomethyl phthalate (MMP). Sperm DNA damage was associated with MEP and MEHP after adjusting for DEHP oxidative metabolites, which may serve as phenotypic markers of DEHP metabolism. The urinary levels of phthalate metabolites among these men were similar to those reported for the US general population, suggesting that exposure to some phthalates may affect the population distribution of sperm DNA damage. We have also reported that DEHP caused increases in Comet assay parameters (tail intensity, tail moment), cytotoxicity and oxidant/antioxidant status in LnCAP (human prostatic cell line) and MA-10 Leydig cells (mouse Leydig cells). The details of these studies are given below (Erkekoglu et al., 2010a,b).

Recently, Ahbab et al. (2014) evaluated possible genotoxicity of di-n-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) at different concentrations using Comet assay in male rat pups. The researchers administered DCHP and DHP to the pregnant rats by gavage at the doses of 0 (vehicle), 20, 100 and 500 mg/kg/day from gestational day 6 (GD6) to GD19. Male rats were allowed to grow till different ages (prepubertal, pubertal and adulthood) after delivery.

The testicular apoptosis was detected using terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end-labeling (TUNEL) assay. The Comet assay was performed on blood lymphocytes and testes samples of adult male rats and the results showed that tail length, tail intensity, OTM and percentage of DNA present in tail increased in a dose-dependent manner after DHP was administered. DHP significantly induced genotoxicity at doses of 100 and 500 mg/kg/day versus control; however DCHP did not show the same effect. Increases in TUNEL-positive cells of prepubertal, pubertal and adult testicular cells were observed in the DHP-treated groups. DCHP also induced testicular apoptosis dose-dependently in prepubertal and pubertal rats. The researchers concluded that prenatal exposure to DHP and DCHP might cause apoptosis in rat testicular cells at different stages of development and DHP caused a dose-dependent genotoxicity in both lymphocytes and testicular cells. The summary of these studies are given in Table 3.

Protection against phthalate genotoxicity

Different strategies have been used to prevent the oxidative stress caused by phthalates in limited number of studies and the use of antioxidants has been one of the most common approaches. Fan et al. (2010) reported that the increase in ROS generation with MEHP exposure in MA-10 cells was inhibited by N-acetylcysteine. Ishihara et al. (2000) showed that supplementation of rats with vitamin C and E protected the testes from DEHP-gonadotoxicity.

Throughout our studies on DEHP and MEHP, we used Comet assay in determining the genotoxicity of phthalates. Comet assay is known as a valid technique to evaluate whether antioxidant/micronutrients are able to protect the integrity of the genetic material against variety of environmental physical or chemical agents (Anderson et al., 1997; Erkekoglu, 2012). We demonstrated that Se supplementation in either organic form (selenomethionine, SM at 10 μM) or inorganic form (sodium selenite, SS at 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 (Erkekoglu et al., 2010a,b).

Concerning LNCaP cells, we observed that DEHP had a flat dose–cell viability response curve while MEHP showed a very steep dose–response curve. MEHP was highly cytotoxic even at μM doses (showing ~1000-fold higher cytotoxicity than the parent compound), while DEHP was cytotoxic at mM doses. On the other hand, we observed that both SM and SS 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. Intracellular ROS production showed marked increases with both DEHP and MEHP treatment where the effect of MEHP was much more pronounced. Both selenocompounds were partially effective in reducing intracellular ROS production and increasing antioxidant enzyme activities and total glutathione levels. On the other hand, by 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 control cells. The overall difference between the DNA damaging effects of the parent compound and the metabolite was insignificant. Selenium supplementation in both forms did not cause any alteration in the steady-state levels of the biomarkers of DNA damage in LNCaP cells, whereas the presence of selenium either in SS or SM form reduced the genotoxic effects of DEHP and MEHP as evidenced by significant (≥30%) decreases in tail % intensity. Thus, the results of Comet assay suggested that the both forms of selenium used in this study was not genotoxic, but rather showed antigenotoxic activity against the genotoxicity of the parent phthalate and its metabolite. However, the protective effect of selenium with the doses used in this study was not complete. Tail intensity remained ~90 and ~80% higher than that of control cells treated with both SS + DEHP and SM + DEHP, respectively. Similarly, in SS + MEHP and SM + MEHP-treated cells, tail intensities were still ~95 and ~120% high versus control 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 versus DEHP- or MEHP-treated cells. Only SM supplementation provided a significant (~34%) reduction in the tail moment induced by MEHP. Tail moments remained ~64 and ~95% higher than that of control in SS + DEHP and SM + DEHP cells, respectively; similarly in SS + MEHP and SM + MEHP cells, tail moments were still ~94 and ~69% higher when compared to control cells. In all cases, protective effects of SS and SM were not found to be markedly different from 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. Selenium 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 more evident. SS and SM showed partial protection against the ROS increment for both the phthalates. Both selenocompounds were partially effective in increasing antioxidant enzyme activities and total glutathione levels. 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. Selenium supplementation in both forms did not cause any alteration on the steady state levels of the DNA damage biomarkers of MA-10 cells. However, both of the selenocompounds were effective in providing decreases in the genotoxicity of the two phthlates. 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

Table 3. The effects phthalates on DNA damage using Comet assay.

Study (year)	Type of experiment	Phthalate	Overview	Conclusion
Anderson et al. (1999)	*in vitro*	DEHP	DEHP and MEHP were tested for their genotoxicity potential in human leukocytes. Both of these phthalates caused increases in tail moment in Comet assay. Besides, DEHP at high doses caused significant increases in tail moment in human lymphocytes.	Both DEHP and MEHP were genotoxic in human leukocytes.
Kleinsasser et al. (2000)	*in vitro*	DBP and DiBP	Susceptibilities to DBP and DiBP in non-tumor patients to those in patients with SCC of the oropharynx or larynx were compared by using Comet assay. DBP and DiBP produced significant DNA damage on mucosal cells in all groups of patients.	Both DBP and DiBP were genotoxic in human mucosal cells.
Kleinsasser et al. (2001)	*in vitro*	DBP and DiBP	DNA strand breaks in human epithelial cells of the upper aerodigestive tract ($n = 132$ specimens) and in peripheral lymphocytes ($n = 49$ specimens) after exposure to DBP and DiBP were evaluated. were separated from peripheral blood. Both of the phthalates showed genotoxic effects in both of the cell types.	Both DBP and DiBP showed genotoxic effect in both epithelial cells and lymphocytes. DiBP induced higher genotoxicity compared to DBP in both cell types at equal concentrations. The genotoxic impacts of these phthalates in mucosal cells showed an intermediate correlation ($r = 0.570$).
Park & Choi (2007)	*in vitro*		DEHP was tested for their genotoxicity potentials. DEHP showed genotoxic effect (>90 μM) on HeLa cells.	DEHP was genotoxic as evidenced by significant increases in tail moment.
Choi et al. (2010)	*in vitro*	DEHP	HepG2 cells exposed to various concentrations of DEHP (0, 2.5, 5, 10, 25, 50, 100 and 250 mM) for 24 or 48 h. After exposure to DEHP for 24 and 48 h, DEHP caused increases in DNA damage.	DEHP was genotoxic in HepG2 cells in dose-dependent manner (from 2.5 to 250 mM).
Duty et al. (2003)	*in vivo*	MEHP	Semen and urine samples ($n = 141$) were analyzed for five phthalate metabolites (MEP, MBzP, MBP, MEHP, MMP) and sperm DNA damage was assessed by Comet assay.	The comet extent was only correlated with MEP levels, while there was no correlation with other phthalate metabolites.
Hauser et al. (2007)	*in vivo*	DEHP, MEHP, MEHHP MEP, MBP and MBzP	Urinary concentrations of phthalate metabolites were measured among men ($n = 379$) who applied to infertility clinic. Sperm DNA damage was associated with MEP and MEHP after adjusting for DEHP oxidative metabolites.	Exposure to some phthalates may affect the population distribution of sperm DNA damage.
Erkekoglu et al. (2010a)	*in vitro*	DEHP and MEHP	DEHP and MEHP were evaluated for their genotoxic potential in LnCAP cells. DEHP was genotoxic at very higher doses compared to MEHP.	Both DEHP and MEHP were genotoxic in LnCAP cells.
Erkekoglu et al. (2010b)	*in vitro*	DEHP and MEHP	DEHP and MEHP were evaluated for their genotoxic potential in MA-10 cells. DEHP was genotoxic at very higher doses compared to MEHP.	Both DEHP and MEHP were genotoxic in MA-10 cells.
Ahbab et al. (2014)	*in vivo*	DHP and DCHP	Possible genotoxicity of DHP and DCHP were tested by using Comet assay in male rat pups. DCHP and DHP were administered to the pregnant rats by gavage at different doses from GD6 to GD19. Male rats were allowed to grow till different ages (prepubertal, pubertal, and adulthood) after delivery. The Comet assay was performed on blood lymphocytes and testes samples of adult male rats. DHP significantly induced genotoxicity at doses of 100 and 500 mg/kg/day versus control; however DCHP did not show the same effect.	Prenatal exposure to DHP caused a dose-dependent genotoxicity in both rat lymphocytes and testicular cells.

DBP, dibutyl phthalate; DCHP, dicyclohexyl phthalate; DEHP, di(2-ethylhexyl)-phthalate; DHP, *n*-hexyl phthalate; DiBP, diisobutyl phthalate; GD, gestational day; LnCAP, human prostatic cell line; MA-10 cells, Mouse Leydig carcinoma cells; MBP, mono *n*-butyl phthalate; MBP, mono *n*-butyl phthalate; MBzP, monobenzyl phthalate; MEHHP, mono-(2-ethyl-5 hydroxyhexyl) phthalate; MEHP, monoethylhexylphthalate; MEP, monoethyl phthalate; MMP, monomethyl phthalate and SCC, squamous cell cancer.

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).

Conclusion

ROS operate as intracellular signaling molecules, a function that has been widely documented, but is still controversial (D'Autreaux & Toledano, 2007; Nose, 2000; Veal et al., 2007). On the other hand, excessive production of ROS leads to oxidative stress which could subsequently cause loss of cell function and cell death by apoptosis or necrosis, and/or mutagenic and carcinogenic effects (Nose, 2000). In fact, a shift in the prooxidant–antioxidant balance has been proposed as a factor that contributes to carcinogenesis (Saeidnia & Abdollahi, 2013). Though known as non-genotoxic/epigenetic carcinogens, phthalates are shown to be genotoxic in several studies in the last two decades (Anderson et al., 1997; Erkekoglu et al., 2010a,b; Kleinsasser et al., 2000, 2001). Their genotoxic as well as carcinogenic potentials are attributed to their ability to produce ROS both *in vivo* and *in vitro* (Erkekoglu et al., 2011a), and genotoxicity might be suggested as an underlying factor both in their hepatocarcinogenic effect and their reproductive toxicity. Several strategies have been attempted to provide protection from the epigenetic and genotoxic effects of environmental chemicals. Limited numbers of studies are present in literature, concerning the protective strategies against the genotoxicity of phthalates. In our previous studies, we have shown that phthalate genotoxicity can be partially prevented by both organic (SM) and inorganic (SS) selenocompounds *in vitro*. Both SS and SM supplementation in both prostate and Leydig cells provided marked decreases in ROS production and DNA damage evidenced by Comet assay. On the other hand, we have shown that SS supplementation in diet provided significant recruitment in testicular, hepatic, thyroidal and renal oxidant/antioxidant balance in DEHP-exposed rats (Erkekoglu et al., 2011b, 2012b,c, 2014). Finally, we can postulate that fair intake of trace elements and vitamins with diet can be protective against the genotoxic and carcinogenic potentials of environmental chemicals, particularly against phthalates. However, more studies are needed to provide to convincing data on this hypothesis. The molecular mechanisms for the induction of genotoxicity in different cell types and organs by DEHP and other phthalates require further elucidation.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

Ahbab MA, Ündeğer Ü, Barlas N, Başaran N. (2014). In utero exposure to dicyclohexyl and di-n-hexyl phthalate possess genotoxic effects on testicular cells of male rats after birth in the comet and TUNEL assays. Hum Exp Toxicol 33:230–9.

Akingbemi BT, Hardy MP. (2001). Oestrogenic and antiandrogenic chemicals in the environment: effects on male reproductive health. Ann Med 33:391–403.

Anderson D, Dobrzyńska MM, Basaran N. (1997). Effect of various genotoxins and reproductive toxins in human lymphocytes and sperm in the Comet assay. Teratog Carcinog Mutagen 17:29–43.

Anderson D, Yu TW, Hincal F. (1999). Effect of some phthalate esters in human cells in the Comet assay. Teratog Carcinog Mutagen 19: 275–80.

Andrade AJ, Grande SW, Talsness CE, et al. (2006). A dose response study following in utero and lactational exposure to di-(2-ethylhexyl) phthalate (DEHP): reproductive effects on adult male offspring rats. Toxicology 228:85–97.

Ashby J, Tennant RW. (1994). Prediction of rodent carcinogenicity for 44 chemicals: results. Mutagenesis 9:7–15.

Astill B, Barber E, Lington A, et al. (1986). Chemical industry voluntary test program for phthalate esters: health effects studies. Environ Health Perspect 65:329–36.

Astill BD, Gingell R, Guest D, et al. (1996). Oncogenicity testing of 2-ethylhexanol in Fischer 344 rats and B6C3F1 mice. Fundam Appl Toxicol 31:29–41.

Azqueta A, Collins AR. (2013). The essential Comet assay: a comprehensive guide to measuring DNA damage and repair. Arch Toxicol 87: 949–68.

Bentley P, Calder I, Elcombe C, et al. (1993). Hepatic peroxisome proliferation in rodents and its significance for humans. Food Chem Toxicol 31:857–907.

Bility MT, Thompson JT, McKee RH, et al. (2004). Activation of mouse and human peroxisome proliferator-activated receptors (PPARs) by phthalate monoesters. Toxicol Sci 82:170–82.

Biscardi D, Monarca S, De Fusco R, et al. (2003). Evaluation of the migration of mutagens/carcinogens from PET bottles into mineral water by Tradescantia/micronuclei test, Comet assay on leukocytes and GC/MS. Sci Total Environ 302:101–8.

Blom A, Ekman E, Johannisson A, et al. (1998). Effects of xenoestrogenic environmental pollutants on the proliferation of a human breast cancer cell line (MCF-7). Arch Environ Contam Toxicol 34:306–10.

Cattley RC, DeLuca J, Elcombe C, et al. (1998). Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans? Regul Toxicol Pharmacol 27:47–60.

Choi S, Park SY, Jeong J, et al. (2010). Identification of toxicological biomarkers of di(2-ethylhexyl) phthalate in proteins secreted by HepG2 cells using proteomic analysis. Proteomics 10: 1831–46.

Clare G. (2012). The *in vitro* mammalian chromosome aberration test. Method Mol Biol 817:69–91.

Collins A, Koppen G, Valdiglesias V, et al. (2014). The Comet assay as a tool for human biomonitoring studies: the ComNet project. Mutat Res Rev Mutat Res 759:27–39.

Dalgaard M, Ostergaard G, Lam HR, et al. (2000). Toxicity study of di(2-ethylhexyl) phthalate (DEHP) in combination with acetone in rats. Pharmacol Toxicol 86:92–100.

D'Autréaux B, Toledano MB. (2007). ROS as signaling molecules: mechanisms that generate specificity in ROS homeostasis. Nat Rev Mol Cell Biol 8:813–24.

Decordier I, Kirsch-Volders M. (2006). The *in vitro* micronucleus test: from past to future. Mutat Res 607:2–4.

Doull J, Cattley R, Elcombe C, et al. (1999). A cancer risk assessment of di(2-ethylhexyl) phthalate: application of the new US EPA risk assessment guidelines. Regul Toxicol Pharmacol 29:327–57.

Durmaz E, Ozmert EN, Erkekoglu P, et al. (2010). Plasma phthalate levels in pubertal gynecomastia. Pediatrics 125:e122–9.

Duty SM, Singh NP, Silva MJ, et al. (2003). The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral Comet assay. Environ Health Perspect 111: 1164–9.

Elliott BM, Elcombe CR. (1987). Lack of DNA damage or lipid peroxidation measured *in vivo* in the rat liver following treatment with peroxisomal proliferators. Carcinogenesis 8:1213–18.

Erkekoglu P, Rachidi W, De Rosa V, et al. (2010a). Protective effect of selenium supplementation on the genotoxicity of di(2-ethylhexyl) phthalate and mono(2-ethylhexyl) phthalate treatment in LNCaP cells. Free Radic Biol Med 49:559–66.

Erkekoglu P, Rachidi W, Yuzugullu OG, et al. (2010b). Evaluation of cytotoxicity and oxidative DNA damaging effects of di(2-ethylhexyl)-phthalate (DEHP) and mono(2-ethylhexyl)-phthalate (MEHP) on MA-10 Leydig cells and protection by selenium. Toxicol Appl Pharmacol 248:52–62.

Erkekoglu P, Rachidi W, Yüzügüllü OG, et al. (2011a). Induction of ROS, p53, p21 in DEHP- and MEHP-exposed LNCaP cells-protection by selenium compounds. Food Chem Toxicol 49:1565–71.

Erkekoglu P, Zeybek ND, Giray B, et al. (2011b). Reproductive toxicity of di(2-ethylhexyl) phthalate in selenium-supplemented and selenium-deficient rats. Drug Chem Toxicol 34:379–89.

Erkekoglu P. (2012). Protection studies by antioxidants using single cell gel electrophoresis Comet assay. In: Magdeldin S, ed. Gel electrophoresis – advanced techniques. Rijeka: InTech, 413–46.

Erkekoglu P, Zeybek ND, Giray B, et al. (2012a). The effects of di(2-ethylhexyl) phthalate exposure and selenium nutrition on sertoli cell vimentin structure and germ-cell apoptosis in rat testis. Arch Environ Contam Toxicol 62:539–47.

Erkekoglu P, Giray BK, Kızilgün M, et al. (2012b). Di(2-ethylhexyl) phthalate-induced renal oxidative stress in rats and protective effect of selenium. Toxicol Mech Method 22:415–23.

Erkekoglu P, Giray BK, Kizilgun M, et al. (2012c). Thyroidal effects of di-(2-ethylhexyl) phthalate in rats of different selenium status. J Environ Pathol Toxicol Oncol 31:143–53.

Erkekoglu P, Zeybek ND, Giray BK, et al. (2014). The effects of di(2-ethylhexyl) phthalate on rat liver in relation to selenium status. Int J Exp Pathol 95:64–77.

Fan J, Traore K, Li W, et al. (2010). Molecular mechanisms mediating the effect of mono-(2-ethylhexyl) phthalate on hormone-stimulated steroidogenesis in MA-10 mouse tumor Leydig cells. Endocrinology 151:3348–62.

Faust F, Kassie F, Knasmüller S, et al. (2004). The use of the alkaline Comet assay with lymphocytes in human biomonitoring studies. Mutat Res 566:209–29.

Fenech M. (2000). The *in vitro* micronucleus technique. Mutat Res 455: 81–95.

Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI. (2008). Oxidative stress, DNA methylation and carcinogenesis. Cancer Lett 266:6–11.

Frederiksen H, Jensen TK, Jørgensen N, et al. (2014). Human urinary excretion of non-persistent environmental chemicals: an overview of Danish data collected between 2006 and 2012. Reproduction 147: 555–65.

Frederiksen H, Sørensen K, Mouritsen A, et al. (2012). High urinary phthalate concentration associated with delayed pubarche in girls. Int J Androl 35:216–26.

Gazouli M, Yao ZX, Boujrad N, et al. (2002). Effect of peroxisome proliferators on Leydig cell peripheral-type benzodiazepine receptor gene expression, hormone-stimulated cholesterol transport and steroidogenesis: role of the peroxisome proliferator-activator receptor alpha. Endocrinology 143:2571–83.

Ge RS, Chen GR, Tanrikut C, Hardy MP. (2007). Phthalate ester toxicity in Leydig cells: developmental timing and dosage considerations. Reprod Toxicol 23:366–73.

Gruber J, Schaffer S, Halliwell B. (2008). The mitochondrial free radical theory of ageing–where do we stand? Front Biosci 13:6554–79.

Gutzkow KB, Langleite TM, Meier S, et al. (2013). High-throughput Comet assay using 96 minigels. Mutagenesis 28:333–40.

Halliwell B, Cross CE. (1994). Oxygen-derived species: their relation to human disease and environmental stress. Environ Health Perspect 102:5–12.

Halliwell B. (2012). Free radicals and antioxidants: updating a personal view. Nutr Rev 70:257–65.

Hauser R, Meeker JD, Singh NP, et al. (2007). DNA damage in human sperm is related to urinary levels of phthalate monoester and oxidative metabolites. Hum Reprod 22:688–95.

Heaton PR, Reed CF, Mann SJ, et al. (2002). Role of dietary antioxidants to protect against DNA damage in adult dogs. J Nutr 132:1720S–4S.

Heudorf U, Mersch-Sundermann V, Angerer J. (2007). Phthalates: toxicology and exposure. Int J Hyg Environ Health 210:623–34.

Huber WW, Grasl-Kraupp B, Schulte-Hermann R. (1996). Hepatocarcinogenic potential of di(2-ethylhexyl)phthalate in rodents and its implications on human risk. Crit Rev Toxicol 26: 365–481.

Ishihara M, Itoh M, Miyamoto K, et al. (2000). Spermatogenic disturbance induced by di-(2-ethylhexyl) phthalate is significantly prevented by treatment with antioxidant vitamins in the rat. Int J Androl 23:85–94.

Johnson GE. (2012). Mammalian cell HPRT gene mutation assay: test methods. Method Mol Biol 817:55–67.

Jones HB, Garside DA, Liu R, Roberts JC. (1993). The influence of phthalate esters on Leydig cell structure and function *in vitro* and *in vivo*. Exp Mol Pathol 58:179–93.

Just AC, Whyatt RM, Miller RL, et al. (2012). Children's urinary phthalate metabolites and fractional exhaled nitric oxide in an urban cohort. Am J Respir Crit Care Med 186:830–7.

Kaufmann W, Deckardt K, McKee RH, et al. (2002). Tumor induction in mouse liver: di-isononyl phthalate acts via peroxisome proliferation. Regul Toxicol Pharmacol 36:175–83.

Kim MY, Kim YC, Cho MH. (2002). Combined treatment with 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone and dibutyl phthalate enhances ozone-induced genotoxicity in B6C3F1 mice. Mutagenesis 17:331–6.

Klaunig JE, Babich MA, Baetcke KP, et al. (2003). PPARalpha agonist-induced rodent tumors: modes of action and human relevance. Crit Rev Toxicol 33:655–780.

Kleinsasser NH, Kastenbauer ER, Weissacher H, et al. (2000). Phthalates demonstrate genotoxicity on human mucosa of the upper aerodigestive tract. Environ Mol Mutagen 35:9–12.

Kleinsasser NH, Wallner BC, Kastenbauer ER, et al. (2001). Genotoxicity of di-butyl-phthalate and di-iso-butyl-phthalate in human lymphocytes and mucosal cells. Teratog Carcinog Mutagen 21:189–96.

Kluwe WM, Huff JE, Matthews HB, et al. (1985). Comparative chronic toxicities and carcinogenic potentials of 2-ethylhexyl-containing compounds in rats and mice. Carcinogenesis 6:1577–83.

Labow RS, Tocchi M, Rock G. (1986). Contamination of platelet storage bags by phthalate esters. J Toxicol Environ Health 19:591–8.

Lee KH, Lee BM. (2007). Study of mutagenicities of phthalic acid and terephthalic acid using *in vitro* and *in vivo* genotoxicity tests. J Toxicol Environ Health A 70:1329–35.

Lindahl-Kiessling K, Karlberg I, Olofsson AM. (1989). Induction of sister-chromatid exchanges by direct and indirect mutagens in human lymphocytes, co-cultured with intact rat liver cells. Effect of enzyme induction and preservation of the liver cells by freezing in liquid nitrogen. Mutat Res 211:77–87.

McKee RH, Przygoda RT, Chirdon MA, et al. (2000). Di(isononyl) phthalate (DINP) and di(isodecyl) phthalate (DIDP) are not mutagenic. J Appl Toxicol 20:491–7.

Meek M, Chan P. (1994). Bis(2-ethylhexyl)phthalate: evaluation of risks to health from environmental exposure in Canada. J Environ Sci Health Part C 12:179–94.

Melnick RL. (2001). Is peroxisome proliferation an obligatory precursor step in the carcinogenicity of di(2-ethylhexyl)phthalate (DEHP)? Environ Health Perspect 109:437–42.

Mettang T, Alscher DM, Pauli-Magnus C, et al. (1999). Phthalic acid is the main metabolite of the plasticizer di(2-ethylhexyl)phthalate in peritoneal dialysis patients. Adv Perit Dial 15:229–33.

Mortelmans K, Zeiger E. (2000). The Ames Salmonella/microsome mutagenicity assay. Mutat Res 455:29–60.

Müller-Tegethoff K, Kasper P, Müller L. (1995). Evaluation studies on the *in vitro* rat hepatocyte micronucleus assay. Mutat Res 335: 293–307.

Nakai M, Tabira Y, Asai D, et al. (1999). Binding characteristics of dialkyl phthalates for the estrogen receptor. Biochem Biophys Res Commun 254:311–14.

Nässberger L, Arbin A, Ostelius J. (1987). Exposure of patients to phthalates from polyvinyl chloride tubes and bags during dialysis. Nephron 45:286–90.

Nose K. (2000). Role of reactive oxygen species in the regulation of physiological functions. Biol Pharm Bull 23:897–903.

Novotna B, Topinka J, Solansky I, et al. (2007). Impact of air pollution and genotype variability on DNA damage in Prague policemen. Toxicol Lett 172:37–47.

O'Brien ML, Spear BT, Glauert HP. (2005). Role of oxidative stress in peroxisome proliferator-mediated carcinogenesis. Crit Rev Toxicol 35:61–88.

Painter RB, Cleaver JE. (1969). Repair replication, unscheduled DNA synthesis, and the repair of mammalian DNA. Radiat Res 37: 451–66.

Park SY, Choi J. (2007). Cytotoxicity, genotoxicity and ecotoxicity assay using human cell and environmental species for the screening of the risk from pollutant exposure. Environ Int 33:817–22.

Phillips BJ, James TE, Gangolli SD. (1982). Genotoxicity studies of di(2-ethylhexyl)phthalate and its metabolites in CHO cells. Mutat Res 102:297–304.

Phillips BJ, Anderson D, Gangolli SD. (1986). Studies on the genetic effects of phthalic acid esters on cells in culture. Environ Health Perspect 65:263–6.

Reddy JK, Rao MS. (1989). Oxidative DNA damage caused by persistent peroxisome proliferation: its role in hepatocarcinogenesis. Mutat Res 214:63–8.

Richburg JH, Boekelheide K. (1996). Mono-(2-ethylhexyl) phthalate rapidly alters both Sertoli cell vimentin filaments and germ cell apoptosis in young rat testes. Toxicol Appl Pharmacol 137: 42–50.

Rusyn I, Rose ML, Bojes HK, Thurman RG. (2000a). Novel role of oxidants in the molecular mechanism of action of peroxisome proliferators. Antioxid Redox Signal 2:607–21.

Rusyn I, Denissenko MF, Wong VA, et al. (2000b). Expression of base excision repair enzymes in rat and mouse liver is induced by peroxisome proliferators and is dependent upon carcinogenic potency. Carcinogenesis 21:2141–5.

Rusyn I, Peters JM, Cunningham ML. (2006). Modes of action and species-specific effects of di-(2-ethylhexyl)phthalate in the liver. Crit Rev Toxicol 36:459–79.

Saeidnia S, Abdollahi M. (2013). Toxicological and pharmacological concerns on oxidative stress and related diseases. Toxicol Appl Pharmacol 273:442–55.

Seo KW, Kim KB, Kim YJ, et al. (2004). Comparison of oxidative stress and changes of xenobiotic metabolizing enzymes induced by phthalates in rats. Food Chem Toxicol 42:107–14.

Shintani H. (1985). Determination of phthalic acid, mono-(2-ethylhexyl) phthalate and di-(2-ethylhexyl) phthalate in human plasma and in blood products. J Chromatogr 337:279–90.

Silva MJ, Samandar E, Preau Jr JL, et al. (2006). Urinary oxidative metabolites of di(2-ethylhexyl) phthalate in humans. Toxicology 219: 22–32.

Sjöberg P, Bondesson U. (1985). Determination of di(2-ethylhexyl) phthalate and four of its metabolites in blood plasma by gas chromatography-mass spectrometry. J Chromatogr 344:167–75.

Smith-Oliver T, Butterworth BE. (1987). Correlation of the carcinogenic potential of di(2-ethylhexyl)phthalate (DEHP) with induced hyperplasia rather than with genotoxic activity. Mutat Res 188:21–8.

Stenchever MA, Allen MA, Jerominski L, Petersen RV. (1976). Effects of bis(2-ethylhexyl) phthalate on chromosomes of human leukocytes and human fetal lung cells. J Pharm Sci 65:1648–51.

Tay TW, Andriana BB, Ishii M, et al. (2007). Disappearance of vimentin in Sertoli cells: a mono(2-ethylhexyl) phthalate effect. Int J Toxicol 26:289–95.

Teitelbaum SL, Mervish N, Moshier EL, et al. (2012). Associations between phthalate metabolite urinary concentrations and body size measures in New York City children. Environ Res 112:186–93.

Tinwell H, Ashby J. (1994). Comparative activity of human carcinogens and NTP rodent carcinogens in the mouse bone marrow micronucleus assay: an integrative approach to genetic toxicity data assessment. Environ Health Perspect 102:758–62.

Turner JH, Petricciani JC, Crouch ML, Wenger S. (1974). An evaluation of the effects of diethylhexyl phthalate (DEHP) on mitotically capable cells in blood packs. Transfusion 14:560–6.

Veal EA, Day AM, Morgan BA. (2007). Hydrogen peroxide sensing and signaling. Mol Cell 26:1–14.

Waring RH, Harris RM. (2005). Endocrine disrupters: a human risk? Mol Cell Endocrinol 244:2–9.

Willhite CC. (2001). Weight-of-evidence versus strength-of-evidence in toxicologic hazard identification: di(2-ethylhexyl)phthalate (DEHP). Toxicology 160:219–26.

Wormuth M, Scheringer M, Vollenweider M, et al. (2006). What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? Risk Anal 26:803–24.

Yeldani AV, Milano M, Subbarao V, et al. (1989). Evaluation of liver cell proliferation during ciprofibrate-induced hepatocarcinogenesis. Cancer Lett 47:21–7.

Zacharewski TR, Meek MD, Clemons JH, et al. (1998). Examination of the *in vitro* and *in vivo* estrogenic activities of eight commercial phthalate esters. Toxicol Sci 46:282–93.