REVIEW

Role of Oxidative Stress in Male Reproductive Dysfunctions with Reference to Phthalate Compounds

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Purpose: A wide variety of environmental chemicals/xenobiotics including phthalates have been shown to cause oxidative stress targeting the endocrine system and cause reproductive anomalies. The present review describes various issues by oxidative stress causing male reproductive dysfunctions. Here in this review, the importance and role of phthalate compounds in male reproductive dysfunction has been well documented.

Materials and Methods: One class of environmental endocrine disruptors is phthalates. Phthalate compounds are mostly used as plasticizers, which increase the flexibility, durability, longevity, and etc. of the plastics. Large-scale use of plastic products in our daily life as well as thousands of workers engaged in the manufacture of plastic and plastic products and recycling plastic industry are potentially exposed to these chemicals. Further, general population as well as vulnerable groups i.e. children and pregnant women are also exposed to these chemicals. Phthalates are among wide variety of environmental toxicants capable of compromising male fertility by inducing a state of oxidative stress in the testes. They may also generate reactive oxygen species (ROS) that may affect various physiological and reproductive functions.

Results: The available data points out that phthalate compounds may also induce oxidative stress in the male reproductive organs mainly testis and epididymis. They impair spermatogenic process by inducing oxidative stress and apoptosis in germ cells or target sertoli cells and thereby hamper spermatogenesis. They also impair the Leydig cell function by inducing ROS, thereby decreasing the levels of steroidogenic enzymes.

Conclusion: Thus in utero and postnatal exposure to phthalate compounds might lead to decreased sperm count and various other reproductive anomalies in the young male.

Keywords: reproduction; spermatozoa; oxidative stress; prenatal exposure delayed effects; oxidative stress; physiology.

1. OVERVIEW

Humans have always been exposed to certain persistent chemicals, some pesticides, heavy metals, solvents especially organic solvents, illicit drugs, tobacco smoking, and etc. which are reported to have reproductive toxic potential for both sexes depending upon the dose and duration of the exposure of the toxicants. Naturally occurring environmental chemicals (e.g., phytoestrogens and estrogenic mycotoxins) induce infertility in domestic animal species and may alter human reproductive function.[1] Exposure to synthetic chemicals such as dichlorodiphenyltrichloroethane (DDT) and its metabolites, alkylphenol ethoxylates, polychlorinated biphenyls (PCBs) and dioxins, produces reproductive problems in a variety of vertebrate species via endocrine mechanisms.[2,3] Concern regarding the adverse effects of various environmental contaminants on the male reproductive system has been growing nowadays. A wide variety of environmental chemicals/xenobiotics including phthalates have been shown to cause oxidative stress, which potentially target the endocrine system and cause reproductive anomalies.

Free radicals such as reactive oxygen species (ROS) are generated by exogenous agents (e.g., radiations, chemicals, and hyperoxia) or via endogenous processes such as in normal cellular metabolism. A number of studies indicate that ROS are a double-edged sword: they have a role in pathological processes but can also serve as key signal molecules in physiological processes. When ROS are present at certain levels, they greatly overwhelm the capacity of endogenous cellular antioxidant defense system, thus cause oxidative stress. The resulting damage to cells and organs may induce and/or accelerate disease processes. Oxidative stress has been implicated in cancer, aging, atherosclerosis, ischemic injury, inflammation, and neurodegenerative diseases.[4,5]

A major factor in the etiology of male infertility is oxidative stress. ROS attack can induce lipid peroxidation and DNA fragmentation disrupting both the motility of the cells and their ability to support normal embry-

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onic development at spermatozoon level. At the level of the testes, oxidative stress is capable of disrupting the steroidogenic capacity of Leydig cells as well as the capacity of the germinal epithelium to differentiate normal spermatozoa. Although experimental data in animal models demonstrating a causal relationship between the induction of oxidative stress in the testes and the impairment of male reproductive functions are available, the causative mechanism remains unresolved. There are so many factors (physical, chemical, and pathological) capable of inducing oxidative stress in the testes strongly suggesting that this is a vulnerable tissue which is both highly dependent on oxygen to drive spermatogenesis and yet highly susceptible to the toxic effects of reactive oxygen metabolites.[6] Phthalates are one of the classes of environmental endocrine disruptors (EDs), which are used as plasticizers for polyvinyl chloride plastics. Recently, attention has been paid to these chemicals because the prenatal phthalate exposure to rats can cause testicular dysgenesis like syndrome (TDS) in male offspring postnatally.[7,8]

The use of plastics has increased several-folds worldwide. Phthalate compounds, which are used as plasticizers for the manufacture of plastics, leach in to the surrounding medium. They are ubiquitously distributed in the environment and might have toxic potential to all the living beings. The physicochemical properties that impart usefulness as plasticizers also permit migration and leaching of phthalates from polymer substrates. This potential for leaching from products manufactured from plastics combined with a well-recognized toxicity profile for some phthalate esters has led to concern over potential health impacts particularly from use in consumer products where widespread public exposure to phthalate esters is possible. Large-scale use of plastic products in our daily life as well as thousands of workers engaged in the manufacture of plastic and plastic products and recycling plastic industry are potentially exposed to these chemicals. Further, general population as well as vulnerable groups i.e., children and pregnant women are also exposed to these chemicals.

Structurally, phthalate esters are characterized by a diester structure consisting of a benzenedicarboxylic acid head group linked to two ester side chains. The Phthalate Esters Panel High Production Volume (HPV) Testing Group (2001) derived three categories of phthalates based on their use, physicochemical and toxicological properties.[9] They are low molecular weight phthalates, transitional phthalates and high molecular weight phthalates. Low molecular weight phthalates were defined as those produced from alcohols with straight-chain carbon backbones of ≤ C3 [e.g., dimethyl phthalate (DMP), diethyl phthalate (DEP), dialkyl phthalate (DAP), dimethylethyl phthalate (DMEP), and diisobutyl phthalate (DIBP)]. Whereas high molecular weight phthalates were defined as those produced from alcohols with straight-chain carbon backbones of ≥ C7 or ring structure [e.g., diisononyl phthalate (DINP), dinonyl phthalate (DNP), and diisodocyl phthalate (DIDP)]. On the other hand, transitional phthalates are defined as those produced from alcohols with straight-chain carbon backbones of C4-6 [e.g., dibutyl phthalate (DBP), benzyl butyl phthalate (BBP) and di (2-ethylhexyl) phthalate (DEHP)]. Lower molecular weight phthalates are often associated with uses as solvents, whilst the higher molecular weight phthalates are associated with uses as plasticizers. The transitional phthalates are used as both plasticizers and solvents.[10]

Some of the transitional phthalates such as dibutyl phthalate (DBP), benzyl butyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEHP) are known to interfere with male reproductive development in rats, and due to their reproductive toxicity, they have been banned for use in toys and other consumable products. DIBP has similar properties as DBP however less toxic in nature, hence used in place of DBP wherever required.[11] While DINP is replacing DEHP nowadays due to its process ability, durability and availability.[12]

Recently Wang and colleagues determined whether phthalate levels in semen were associated with infertility.[13] Using semen samples from 107 infertile and 94 fertile men, the presence and quantity of five phthalate esters were measured using high-performance liquid chromatography (HPLC).[13] The cumulative levels of the measured phthalate esters were significantly higher in the infertility group as compared to the control group ($P < .05$). Concentrations of the five phthalate esters in men varied by age with older men showing higher cumulative levels. The presence of phthalates may contribute to male infertility.[13] Dadkhah and colleagues determined the relation of semen parameters in processed and unprocessed semen samples with pregnancy rate in intrauterine insemination (IUI) in the treatment of male factor infertility and found that there was an inverse relationship between pregnancy rate and duration of infertility.[14] IUI is a valuable method for the treatment of male factor infertility. The higher number of sperms, total motile sperms and IUI sessions, and lower duration of infertility, all have a positive relationship with pregnancy rate.[14] Nikoobakht and colleagues evaluated the effect of hypothyroidism on erectile function using International Index of Erectile Function (IIEF-5) and sperm parameters and observed that hypothyroidism adversely affects erectile function and sperm parameters, including sperm count, morphology, and motility.[15] In patients with sperm abnormalities and erectile dysfunction, measurement of thyroid hormones is recommended.[15]

The literature was collected through searching various databases such as PubMed, Medline, Toxline, Google, and certain other web sites and consulting relevant reproductive toxicity journals. This review is divided into different sections such as the route of exposure and metabolism of phthalates, mode of action of phthalates, oxidative stress, genotoxic, and reproductive potential as well as developmental toxicity due to phthalate exposure.

2. ROUTE OF EXPOSURE AND METABOLISM

Phthalates can easily leach out of products to contaminate the external environment because they are not chemically bound to the plastic matrix or to other chemicals in formulations.[16] As a result of the ubiquitous use of phthalates in personal care and consumer products, human exposure is widespread. Exposure through ingestion, inhalation and dermal contact are considered important routes of exposure for the general population.[17,18] Ingestion exposure includes household goods (food packaging such as meat, fish, eggs, baby milk and milk products), phthalate-contaminated water, child soft toys (teethers and rattles), and certain medical procedures.

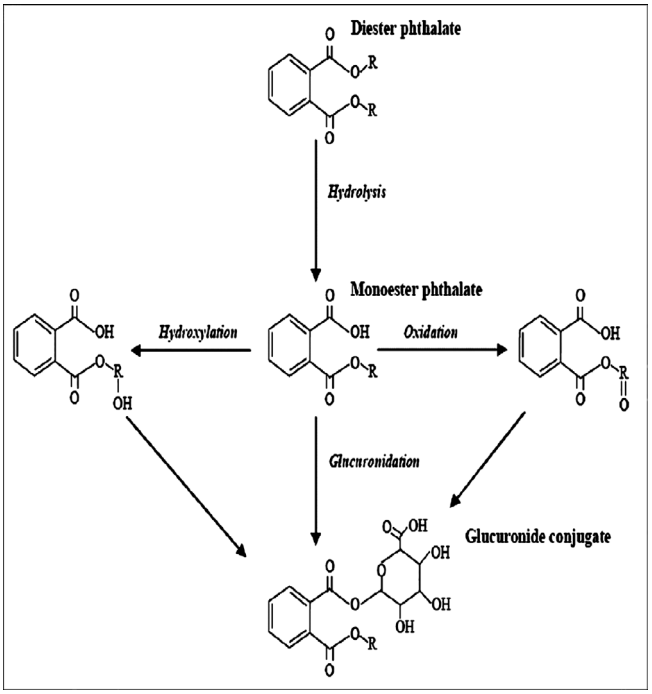

Figure 1. General metabolic pathway for phthalates (adopted from Frederiksen et al. 2007).

Whilst inhalation exposure may occur via household goods/dust [off-gassing of Polyvinyl chloride (PVC) flooring], medical inhalation therapy (tubing inserted into throat for forced-air ventilation), and occupational exposure (heated/melted products that can emit phthalate vapors). On the other hand, dermal exposure may occur via clothing (raincoats and gloves), personal care products (soap, shampoo, nail polish, fragrance bases for perfumery and cosmetic products) including direct injection exposure through the use of medical bags and tubes-products used in hospitals made of PVC.

After exposure, they are rapidly metabolized and excreted in the urine and feces. They undergo phase I biotransformation, that is, the diesters are primary metabolized into their hydrolytic monoesters by hydrolysis of one of their ester bonds plus free alcoholic group. Then the second ester bond gets further hydrolyzed to phthalic acid. Further enzymatic oxidation of the alkyl chain occurring in some of the phthalates, results in more hydrophilic oxidative metabolites. Monoesters and the oxidative metabolites of phthalates may continue to undergo phase II biotransformation to produce glucuronide conjugates with increased water solubility.[19] General metabolic pathway of phthalates is shown in **Figure 1**.

Although phthalates do not bio-accumulate in the body like dioxins and other chemicals, their ubiquitous presence in the environment and the size of the population exposed suggest that the potential impact of phthalate exposure could be very large.[20] Phthalates have also been measured in residential indoor environments in both house dust and indoor air as well as in foods, milk, and drinking water. However, the relative contribution from the various sources and routes of exposure to phthalates is unknown.[21,18] Few studies also have investigated the carcinogenic effect of sulfur mustard exposure on the genitourinary system as well as the prevalence of male infertility. Recent studies by Amirzargar and colleagues on Iranian victims 20 years after sulfur mustard (SM) exposure confirmed that infertile SM-exposed men had higher serum levels of FSH than fertile SM victims.[22] Moreover, dramatically low serum values of testosterone were not observed more frequently in infertile versus fertile SM-exposed men in the study of Amirzargar and colleagues.[22] These findings imply the relative resistance of the Leydig cells to SM toxicity along with the seminiferous tubule damage twenty years after SM exposure.[23]

Hauser and colleagues estimated the incidence/prevalence of selected male reproductive disorders/diseases and associated economic costs that can be reasonably attributed to specific endocrine-disrupting chemicals (EDC) such as phthalate compounds exposures in the European Union (EU).[24] In this study, the expert panel identified low epidemiological and strong toxicological evidence for male infertility attributable to phthalate exposure, with a 40-69% probability of causing 618,000 additional assisted reproductive technology procedures, costing €4.71 billion annually.[24] EDCs may contribute substantially to male reproductive disorders and diseases, with nearly €15 billion annual associated costs in the European Union (EU). These estimates represent only a few EDCs for which there were sufficient epidemiological studies and those with the highest probability of causation.

Lagos-Cabré and Moreno, critically discussed the available information regarding the effect of various derivatives of phthalate compounds such as bisphenol A [2,2-bis(4-hydroxyphenyl) propane] (BPA), 4-nonylphenol (NP) and di(2-ethylhexyl) phthalate (DEHP), and its metabolite mono-2-ethylhexyl phthalate (MEHP) upon mammalian spermatogenesis, a major target of endocrine disruptors (EDs) and observed that germ cell sloughing, disruption of the blood-testis-barrier and germ cell apoptosis are the most common effects reported in the available literature.[25]

The output and quality of sperm are useful tools to measure the effect of exogenous compounds on spermatogenesis.[25] A high correlation has been observed between urine BPA (phthalate compound) levels and semen quality in Chinese men (including motility, viability, sperm count and sperm concentration), which also correlated with the educational level and longer employment history; men with better education and a long history of employment had lower levels of BPA since they were not in contact with EDs, unlike men who worked in factories.[26] A recent study demonstrated that DEHP-contaminated air was associated with an increase in sperm DNA fragmentation and a decrease in sperm motility in PVC factory workers.[27] As in the case of DEHP exposed workers, air BPA-exposed workers also show reduced sexual desire, accompanied by erectile dysfunction and ejaculation difficulties.[28] Although high phthalate exposures have been evaluated among susceptible populations such as women of childbearing age, high-risk occupational groups have not been well studied. Potential high-risk occupational groups include workers in PVC plants, massage therapists, and nail and beauty salon employees.[29] Occupational health nurses should be aware of these high risk occupational groups and their potential for adverse reproductive effects. Occupational health nurses can

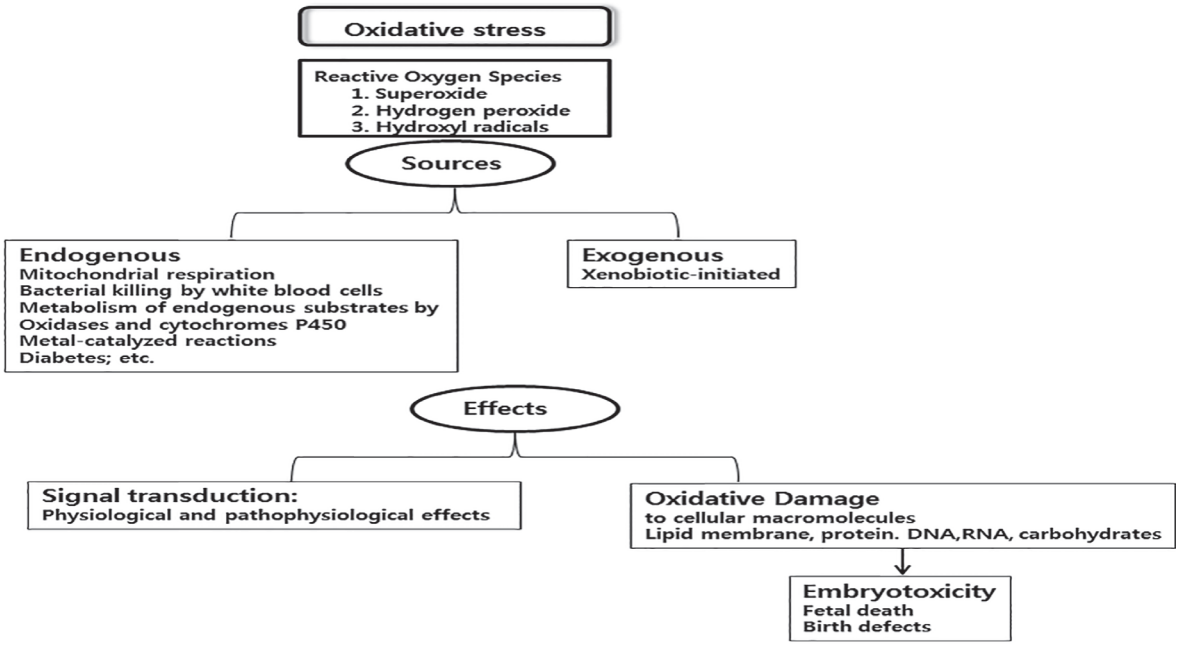

Figure 2. General Mechanism by which oxidative stress alters cellular function (adopted from Wells et al. 2009).

educate high-risk occupational groups to use phthalate-free products, available alternatives found in retail stores. These products are labeled "phthalate-free." Occupational health nurses can advocate for legislation mandating workplace safety inspections among susceptible populations.[30] Once the sources, extent, and routes of phthalate exposures are understood, specific environmental controls may be instituted to reduce them. Workplace accommodation may play a role in preventing adverse effects among workers at high risk for phthalate exposure.[29] Since there is the increasing evidence that exposure to phthalates may affect human health, therefore, the literature on exposure to phthalates, reproductive outcome and children health has been reviewed herein.

3. ACTIONS OF PHTHALATES ON LEYDIG CELLS

There is a building consensus that phthalates are anti-androgenic. However, phthalates and their mono-phthalate metabolites do not bind to the androgen receptor (AR) in vitro at concentrations of up to 10 µM indicating that phthalates are not direct AR antagonists.[31] In fact, phthalate toxicity toward Leydig cells depends on the dosage and time of exposure during development. Leydig cells are the primary source of testosterone production in males, and differentiation of Leydig cells in the testes is one of the primary events in male sex differentiation, puberty, and fertility.[32] One possible mechanism is that phthalate metabolites bind to peroxisome proliferator-activated receptors (PPARs).[33] The PPAR family contains three subtypes such as peroxisome proliferator-activated receptor α (PPARα), PPARβ and PPARγ.[34] Rat fetal Leydig cells (FLCs) express PPARα and PPARγ.[35] Phthalates have been known to induce the actions of PPARα and PPARγ.[36-38] However, the action of phthalates on Leydig cells cannot be explained entirely by a PPARα-mediated pathway, because PPARα-knockout mice remain sensitive to phthalate-mediated reproductive toxicity. Another signaling pathway in Leydig cells that might be affected

Table. Working actions of various phthalates (adopted from Hu et al. 2009).

Compound Name (Abbreviation)	Metabolite Name (Abbreviation)	Actions of all Listed Phthalates
Di-(2-ethylhexyl) phthalate (DEHP)	Mono-ethyl hexyl phthalate (MEHP)	Agonists of peroxisome
Dibutyl phthalate (DBP)	Mono-butyl phthlalate (MBP)	proliferator-activated receptor α
Butyl Benzyl phthalate (BBP)	Mono-butyl benzyl phthlalate (MBzP)	(PPARα) and PPARγ (MEHP, MBP, MBzP)
Di-isononyl phthalate (DINP)	Mono-isononyl phthalate (MINP)	
Di-isodecyl phthalate (DIDP)	Mono-isodecyl phthalate (MIDP)	Leydig cell aggregation (DEHP, DBP)
Dioctyl phthalate (DOP)	Mono octyl phthlalate (MOP)	
Dihexyl phthalate (DHP)	Mono hexyl phthlalate (MHP)	Inhibition of testosterone and Insl3
Diethyl phthalate (DEP)	Mono ethyl phthlalate (MEP)	production (DEHP, DBP)

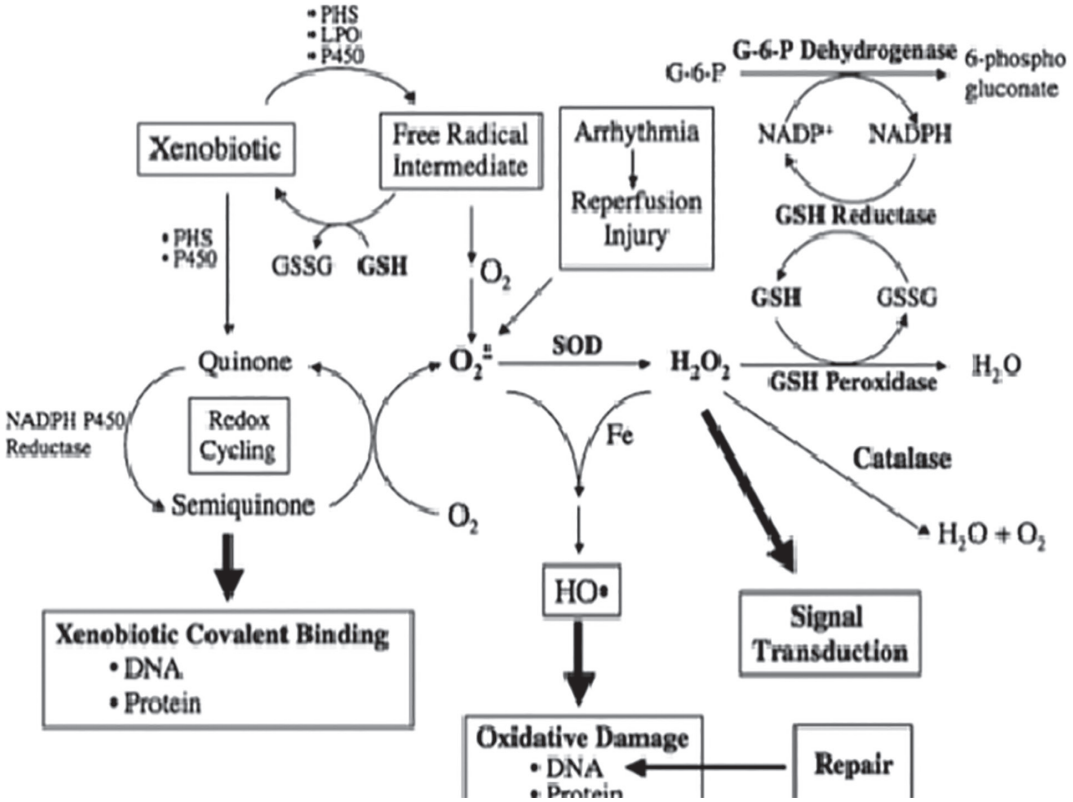

Figure 3. Xenobiotic-enhanced ROS formation (adopted from Wells et al. 2009).

by phthalates is that of the aryl hydrocarbon receptor. In fact, fetal testes from animals treated with phthalates have increased expression levels of aryl hydrocarbon receptor and its downstream gene cytochrome Cyp1b1.[39] Inhibition of the cellular function of the Leydig cells may perturb testosterone and insl3 synthesis resulting in disturbances in the normal development of the male reproductive tract whilst interference with sertoli cells may result in failure to proliferate with subsequent depleted germ cells.[10] Diverse action of various phthalates is shown in the **Table**.

Mono-(2-ethylhexyl) phthalate (MEHP) directly alters the expression of Leydig cell genes and CYP17 lyase activity in cultured rat fetal testis.[40] Exposure to phthalates in utero alters fetal rat testis gene expression and testosterone production, however much remains to be done to understand the mechanisms underlying the direct action of phthalates within the fetal testis. Chauvigné and colleagues investigated the direct mechanisms of action of MEHP on the rat fetal testis, particularly focusing on Leydig cell steroidogenesis. Exposure to MEHP led to a dose-dependent decrease in testosterone production.[40] Moreover, the production of 5 alpha-dihydrotestosterone (5α-DHT) (68%) and androstenedione (54%) was also inhibited by 10 μM MEHP. These findings indicate that under in vitro conditions known to support normal differentiation of the fetal rat testis, the exposure to MEHP directly inhibits several important Leydig cell factors involved in testis function and that the Cyp17a1 gene is a specific target to MEHP explaining the MEHP-induced suppression of steroidogenesis observed.[40]

Recently, Savchuk and colleagues reported that MEHP inhibits LH/human chorionic gonadotropin (hCG)-stimulated androgen production by both isolated rat Leydig cells and MA-10 mouse tumor Leydig cells.[41-43] However, a little has been known about the relationship between the steroidogenic potential of Leydig cells and their sensitivity to phthalate exposure. Recently 2 mouse genotypes, CBA/Lac and C57BL/6j, were identified whose Leydig cells showed high and low androgen production potential.[44] Savchuk and colleagues demonstrated for the first time that the sensitivity of mouse Leydig cells to these monophthalates was not associated to their capacity to produce androgens.[41] MEHP was found to be the only phthalate that caused a biological effect on mouse Leydig cell steroidogenesis and mitochondrial function.[41] The mechanism(s) by which MEHP can stimulate steroidogenesis in the Leydig cells is under debate. Recently Svechnikova and colleagues reported that MEHP stimulated basal steroidogenesis associated with increased StAR protein expression in rat progenitor Leydig cells and immature granulosa cells.[45]

In addition, Zhou and colleagues showed that incubating Leydig cells with MEHP resulted in reductions of luteinizing hormone (LH)-stimulated cyclic adenosine monophosphate (cAMP) and progesterone productions. cAMP did not decrease in response to MEHP when the cells were incubated with cholera toxin or forskolin. Incubation of MEHP-treated cells with dibutyryl-cAMP, 22-hydroxycholesterol or pregnenolone inhibited the

reductions in progesterone.[43] Increased levels of reactive oxygen species (ROS) occurred in response to MEHP. These results indicate that MEHP inhibits Leydig cell steroidogenesis by targeting LH-stimulated cAMP production and cholesterol transport, and that a likely mechanism by which MEHP acts is through increased oxidative stress.[43]

4. MECHANISM OF OXIDATIVE STRESS

Oxidative stress is a cellular condition in which damage to cellular macromolecules occurs as a result of excessive amounts of ROS. ROS such as hydrogen peroxide and hydroxyl radicals are formed via a variety of physiological and pathophysiological reactions (**Figure 2**) and ROS formation can be enhanced by radiation and xenobiotics, including drugs and environmental chemicals. These short-lived ROS can play physiological roles in signal transduction, however they can contribute to the mechanisms of disease when produced excessively by dysregulation of signal transduction and/or by oxidative damage to cellular macromolecules (lipids, proteins, DNA, RNA, carbohydrates) that exceeds the cellular capacity for regeneration or repair. This can also lead to embryo toxicity.[46]

At the embryonic level, most antioxidative enzyme activity (**Figure 3**) is around only 5% of maternal activity.[47] Early organogenesis stage embryos are particularly sensitive to toxic insult during the transition phase from anaerobic to aerobic metabolism coinciding with the maturation of mitochondrial structure and function. This may reflect the observations that low levels of antioxidant enzyme activities increase as organogenesis proceeds, and that early in organogenesis the embryo may not be able to respond as effectively to oxidative imbalances.[48,49]

Phthalate-induced oxidative stress results in decreasing the antioxidant capacity, especially in GPX (glutathione peroxidase) and GST (glutathione S-transferase).[50] Moreover, increase in lipid peroxidation, CAT (Catalase) and SOD (Cu/Zn superoxide dismutase) activity was observed.[50] ROS can affect peroxisome proliferator (PP) leading to parenchymal cell proliferation. It is mentioned that Kupffer cells are a potential oxidant in rodent liver.[51] In fact, phthalates induce toxicity not only via affecting the antioxidant enzymes activity but also through gene expression. and colleagues assessed the effect of DEHP on gene expression of antioxidant enzymes. A decrease in SOD1 expression was noted.[52] Also, Wang and colleagues evaluated the effects of MEHP on the gene expression.[52] The results revealed that SOD1 and GPX expression decreased at 100 µg/mL. Decrease of anti-apoptotic factor (Bcl-2) expression and increase of pro-apoptotic factor (Bax) expression were noted at three doses of 1, 10 and 100 µg/mL. Moreover, expression of cell cycle genes decreased at the same doses.[52]

One of the most important mechanisms for phthalates toxicity is defined regarding oxidative stress. The most common ROS are superoxide and H_2O_2 that can be converted into H_2O and O_2 by antioxidant enzymes such as SOD, GPX and CAT.[53] Phthalates are demonstrated to alter the expression and activity of these enzymes leading to disruption of the cell function.[52] Although there are several reports demonstrating the role of oxidative stress in this regard, the presence of a clear relationship between phthalate toxicity in some organs and oxidative stress as the main cause is still under question.[50] However, an additional mechanism of interest which has been understudied is the action of phthalates through induction of oxidative stress. The ability of phthalates to bind and activate peroxisome proliferator activated receptors (PPARs) has been well-characterized.[54] Binding may cause increased intracellular oxidative stress by overly activating certain enzymes involved in ROS generation but only slightly activating those involved in their degradation.[55] This action may lead to systemic increases in oxidative stress which could have a range of downstream effects. For example, this mechanism could lead to altered metabolism, obesity, and development of type II diabetes.[50] These outcomes have been linked to phthalate exposure in a handful of epidemiologic studies.[56] Therefore, the goal of the present study is to systematically review the related reports and papers published in the valid and credible journals to confirm phthalate toxicity and oxidative stress causes infertility.

5. EFFECTS OF PHTHALATE INDUCED OXIDATIVE STRESS

Most environmental chemicals are hormonally active compounds that target the endocrine system and cause reproductive anomalies.[57] An increase in these environmental contaminants impair testicular functions by disturbing the pro-oxidant/ antioxidant balance of testicular cells, thereby activating associated downstream pathways such as apoptosis.[58] For the normal functioning of the testes, physiological levels of ROS and apoptosis are required however an imbalance or pathological levels may cause deleterious effects.

Spermatogenesis and steroidogenesis occur within the seminiferous tubules and interstitium of the testes. These two compartments are functionally connected however they differ morphologically. Several intra and extra testicular regulatory processes are involved in the regulation of normal spermatogenesis. The ROS that are generated during normal testicular function also play an important role in regulating the function of the testis. Although ROS are known to have damaging effects, controlled and low levels of ROS play a beneficial role in normal testicular function.[57] To overcome the effect of ROS, the testis is equipped with a very potent antioxidant system that protects it from the damaging effects of ROS. The glutathione family of proteins, superoxide dismutase, catalase and several non-enzymatic antioxidants help the testis by counteracting any oxidative impact.[6]

Several environmental toxicants induce apoptosis in germ cells, thereby resulting in defective spermatogenesis. Phthalates are among wide variety of environmental toxicants that are capable of compromising male fertility by inducing a state of oxidative stress in the testes, in addition to endocrine disruption. At the level of testes, oxidative stress is capable of disrupting the steroidogenic capability of Leydig cells as well as the capacity of the germinal epithelium to differentiate normal spermatozoa. Laboratory experiments implicate a role for oxidative stress in phthalate-stimulated liver tumorigenesis, male reproductive toxicity and developmental toxicity.[59-62]

In a recent study, epididymal weight, activities of epididymal alpha-glucosidase and glutathione peroxidase (GSH-Px) were significantly decreased in rats of

500 mg/kg DBP exposure group than control.[60] The activity of superoxide dismutase (SOD) was significantly decreased while the level of malondialdehyde (MDA) was significantly increased in the epididymal tissue of the 250 and 500 mg/kg DBP exposure groups as compared to control group. This showed that DBP exposure alters the epididymal structure and functions by inducing oxidative stress in epididymis of adult rats. DEHP led to a significant decrease in GSH/GSSG redox ratio (> 10-fold) and marked increase in TBARS levels. Thus found to induce oxidative stress in rat testis.[63]

Epidemiologic studies have reported relationships between biomarkers of phthalate exposure and increased levels of the oxidative stress markers malondialdehyde (MDA) and 8- hydroxydeoxyguanosine (8-OHdG).[64,65] In a study, the activities of SOD and GSH-Px in DIBP treated groups were significantly lower while the MDA and 8-OHdG contents were significantly higher than the control group, indicating that oxidative stress induced by diisobutyl phthalate can decrease the activities of antioxidative enzymes and results in oxidative damage of tissues.[66] A study by Hong and colleagues indicated that environmental chemicals, such as polycyclic aromatic hydrocarbons, volatile organic compounds, bisphenol A and phthalates exposure is associated with oxidative stress in urban adult populations.[64]

Nikravesh and Jalali, evaluated the effect of camphor on histopathological changes of reproductive system in young male mice of balb/c racial type.[67] Administration of camphor and its effects on male mice reproductive system may result in significant structural changes, including vascularization and proliferation of sexual cells.[67] This can affect maturation of seminiferous tubules and subsequently reproductive function of testes in mice. Also continuous administration of low doses of camphor can affect the development and differentiation of testicular tissue and reduce its spermatogenesis activity.[67]

Lee and colleagues compared the effects of di (n-butyl) phthalate (DBP) on the oxidative damage and antioxidant enzymatic activity in testes of hyperthyroid rats.[68] Hyperthyroidism was induced in pubertal male rats by intraperitoneal injection of tri-iodothyronine (T3, 10 µg/kg body weight) for 30 days. An oral dose of DBP (750 mg/kg) was administered simultaneously to normal or hyperthyroid (T3) rats over a 30-day period. No changes in body weight were observed in the hyperthyroid groups (T3, T3 + DBP) compared with controls. There were significantly higher serum T3 levels observed in the hyperthyroid rats than in the control, however the serum thyroid stimulating hormone levels were markedly lower in the hyperthyroid rats. DBP significantly decreased the weight of the testes in the normal (DBP) and hyperthyroid (T3 + DBP) groups. The serum testosterone concentrations were significantly lower in only DBP group. DBP significantly increased the 8-hydroxy-2-deoxyguanosine (8-OHdG) level in the testes, whereas the DBP-induced 8-OHdG levels were slightly higher in T3 + DBP group. Superoxide dismutase and glutathione peroxidase activities were significantly higher in the testes of the DBP or T3 + DBP groups. Catalase (CAT) activity was significantly higher in the DBP treatment group, however the T3 + DBP group showed slightly lower DBP-induced CAT activity. The testicular expression of thyroid hormone receptor alpha-1 (TRalpha-1) was significantly higher

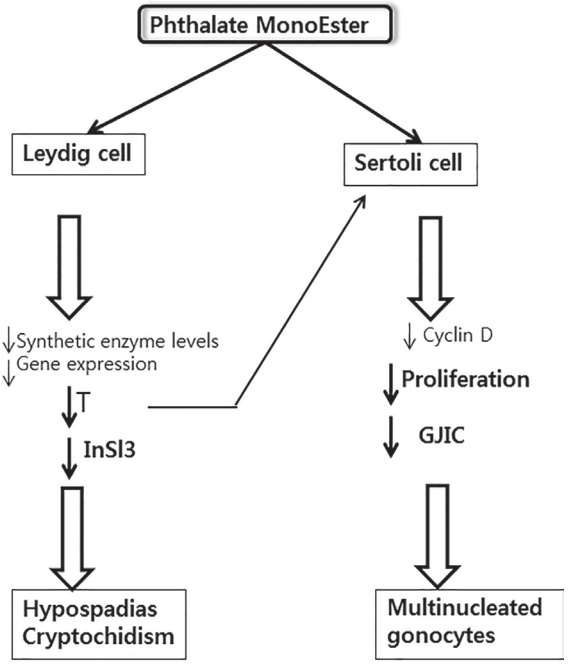

Figure 4. Proposed mode of action of phthalate monoesters in the developing fetal leydig cells [Source: David (2006)].

in the DBP groups, and androgen receptor (AR) expression was not detected in the DBP treatment group. In addition, DBP significantly increased the peroxisome proliferator-activated receptor-r (PPAR-r) levels in the testis. These results suggest that hyperthyroidism can cause a change in the expression level of PPAR-r in testes, and may increase the levels of oxidative damage induced by the metabolic activation of DBP.

Farombi and colleagues carried out a study to evaluate the ameliorative effects of kolaviron (a biflavonoid from the seeds of Garcinia kola) and curcumin (from the rhizome, Curcuma longa) on the di-n-butylphthalate (DBP)-induced testicular damage in rats.[69] Administration of DBP to rats at a dose of 2 g/kg for 9 days significantly decreased the relative testicular weights compared to the controls, while the weights of other organs remained unaffected. Curcumin or kolaviron did not affect all the organ weights of the animals. While only DBP treatment significantly increased the testicular malondialdehyde level and gamma-glutamyl transferase activity (gamma-GT), whereas markedly decreased glutathione level, the testicular catalase, glucose-6-phosphate dehydrogenase, superoxide dismutase, sperm gamma-GT activities and serum testosterone level compared to the control group. Data on cauda epididymal sperm count and live/dead ratio were not significantly affected in the DBP-treated rats. Alone, DBP treatment resulted in a 66% decrease in spermatozoa motility and a 77% increase in abnormal spermatozoa in comparison to control. DBP-treated rats showed marked degeneration of the seminiferous tubules with necrosis and defoliation of spermatocytes. The DBP-induced injuries in biochemical, spermatological parameters, and histological structure of testis were recovered by treatment with kolaviron or curcumin. The pattern in the behavior of these compounds might be correlated with their struc-

tural variations. Their results indicate that kolaviron and curcumin protect against testicular oxidative damage induced by DBP. The chemoprotective effects of these compounds may be due to their intrinsic antioxidant properties and as such may prove useful in combating phthalate-induced reproductive toxicity.

DBP exposure may affect the sperm motility and the anti-oxidative systems. The testis is a vital target organ influenced by DBP since DBP showed inhibitory effect on SOD activities in the testis, and it was significant in the highest exposure group i.e. 1000 mg/kg in peanut oil compared with the control ($P < .05$).[69] In another study, GSHPx activities in the serum and GSH levels in the testis homogenate showed a decreasing tendency, however GSHPx activities increased markedly in the testis homogenate ($P < .05$), after 2-week DBP exposure at dosages of 0, 250, 500 and 1000 mg/kg.[70,71] After 4-week DBP exposure, alkaline phosphatase (ALP) activities in the serum revealed an increasing tendency; sorbitol dehydrogenase (SDH) activities were inhibited significantly in both the serum and the testis homogenate at the dosage of 1000 mg/kg compared with the control group ($P < .01$). Furthermore, GSH contents in the serum were also affected at this dose ($P < .05$).

A study by Kasahara and colleagues indicated that administration of DEHP increased the generation of ROS and selectively decreased GSH and ascorbic acid in the testes leading to apoptosis of spermatocytes to cause testicular atrophy.[68] Results of So and colleagues. indicated that DBP and MBuP induced developmental toxicity in rat embryonic limb bud cells and suggested that this effect of DBP might be exerted through oxidative stress.[72] From the above studies, it can be concluded that phthalates may induce oxidative stress by reducing the levels of anti-oxidative enzymes, testosterone and increasing the levels of MDA and 8-OHdG in the male reproductive tissues such as testes and epididymis. Thus they interfere with the normal spermatogenesis process leading to testicular atrophy and oxidative stress.

Several other studies have demonstrated that ROS involve in neurological and psychiatric disorders, especially in depression.[73] Likewise, phthalates could induce these disorders as a result of imbalance in antioxidants and ROS levels.[74] Moreover, the accumulation of ROS has been related to a variety of neurodegenerative diseases.[75] On the other hand, ROS cause impairment in learning behavior and reduce motor activity.[76] Furthermore, oxidative stress might be considered as a main factor in neurotoxicity of phthalates, a factor by which disruption of neuronal systems may lead to neurobehavioral abnormalities.[77]

Zhang and colleagues. observed that Di-(2-ethylhexyl) phthalate (DEHP) is the most widely used plastizer in the world and can suppress testosterone production via activation of oxidative stress.[78] Genistein (GEN) is one of the isoflavones ingredients exhibiting weak estrogenic and potentially antioxidative effects.[78] In this study, DEHP and GEN were administrated to pre-pubertal male Sprague-Dawley rats by gavage from postnatal day 22 (PND22) to PND35 with vehicle control, GEN at 50 mg/kg body weight (bw)/day (G), DEHP at 50, 150, 450 mg/kg bw/day (D50, D150, D450) and their mixture (G + D50, G + D150, G + D450). On PND90, general morphometry (body weight, AGD, organ weight, and organ coefficient), testicular redox state, and testicular histology were studied. Results indicated that DEHP could significantly decrease sex organs weight, organ coefficient, and testicular antioxidative ability, which largely depended on the dose of DEHP.[78] However, co-administration of GEN could partially alleviate DEHP-induced reproductive injuries via enhancement of testicular antioxidative enzymes activities, which indicates that GEN has protective effects on DEHP-induced male reproductive system damage after pre-pubertal exposure and GEN may have promising future in its curative antioxidative role for reproductive disorders caused by other environmental endocrine disruptors.[78]

6. PHTHALATE INDUCED TESTICULAR DYSGENESIS IS ALSO A RESULT OF OXIDATIVE STRESS

Developmentally toxic phthalate esters target multiple pathways in the developing fetal testis. In fetal Leydig cells, molecular pathways associated with lipid and cholesterol synthesis and transport and steroidogenesis are reduced, resulting in a dramatic reduction in testosterone (T) synthesis. Insulin-like 3 (Insl3) production by fetal Leydig cells is also reduced and this reduction is likely involved in phthalate-induced cryptorchidism.[79] Insl3 is involved in testicular descend. A reduction in alpha-inhibin production likely plays a role in altered sertoli cell maturation and function; this altered maturation together with phthalate-induced disruption in sertoli-gonocyte interaction likely plays a role in the development of multinucleated gonocytes (**Figure 4**). Free radical formation is a normal occurrence during steroidogenesis and it is likely that the reduction in expression of genes associated with protecting the cell from oxidative stress such as glutathione transferase and superoxide dismutase is due to a reduction in oxidative stress following reduction of testosterone synthesis leading to reduced fertility.[80]

Low molecular weight phthalates such as DMP and DEP have no developmental effects, however DIBP has some developmental effects. Endocrine endpoints were studied in offspring at gestation day (GD) 19 or 21. DIBP, butylparaben and rosiglitazone reduced plasma leptin levels in male and female offspring. DIBP and rosiglitazone additionally reduced fetal plasma insulin levels. In males, DIBP reduced anogenital distance, testosterone production and testicular expression of Insl-3 (Insulin-Like Factor 3) and genes related to steroidogenesis. PPARα mRNA levels were reduced by DIBP at GD 19 in testis and liver.[81]

Pregnant Wistar rats were gavage feeded from GD7 to GD 19 or 20/21 with either vehicle (corn oil) or 600 mg/kg bw/day of DIBP.[82] Administration of DIBP resulted in significant reduction in anogenital distance (AGD) in male pups (and increased AGD in female pups) at GD 20/21 together with reduction in body weights of male and female fetuses and reductions in testicular testosterone production and testicular testosterone content in the male offspring. Testicular pathological changes such as clustering of small Leydig cells on GD19 or GD20/21 and vacuolization of sertoli cells on GD20/21 were also noted. In another study, pregnant Wistar rats were exposed from gestation day (GD) 7–21 to di-isobutyl phthalate (DIBP), butylparaben, perfluorooctanoate, or rosiglitazone (600, 100, 20, or 1 mg/kg bw/day, respectively). DIBP decreased fetal weight and increased the incidence of undescended testes at 500 mg/kg dose.[83]

Also, in male fetuses at term, DIBP decreased testicular testosterone production ex vivo and testosterone levels in testes and plasma, decreased AGD and induced pathological changes in the testes including clustering of small Leydig cells and vacuolization of sertoli cells.[84]

Transitional phthalates produce antiandrogen effects by inhibiting fetal testosterone production and insl3. Gestational exposure to BBP, DBP or DEHP induced a decrease in expression of insl3 in rat fetal testes[79] perhaps explaining the increased incidence of cryptorchidism. Serum testosterone was reduced following gestational treatment with BBP, DEHP or DBP.[85-87,31] Thus, the above studies indicate that phthalates might be inducing oxidative stress by disrupting the steroidogenic pathways leading to reduced testosterone synthesis and Insl3 production by the fetal Leydig cells which is likely to cause cryptorchidism.

The molecular mechanism of DEHP toxicity has been attributed to the toxicological properties of its metabolite mono (2-ethylhexyl) phthalate (MEHP), formed by cytochrome P450 oxidation.[88] There is a general agreement that male reproductive organs are particularly susceptible to the deleterious effects of ROS and lipid peroxidation (LPO) which ultimately lead to impaired fertility.[89] In utero exposure to DEHP exerts both short-term and long-lasting suppressive effects on testosterone production in the rat.[90] Recently, it was stated that MEHP primary metabolite of DEHP affects the steroidogenesis in rat Leydig cells by provoking ROS perturbation.[91] Sekaran and colleagues tried to find out the impact of lactational exposure of DEHP in testes of first filial generation (F1) progeny male rat postnatal day (PND)-60. The results of the study showed that lactational exposure of DEHP caused dose-dependent changes in testicular sertoli cells (SC) of male offspring through ROS-induced apoptosis and perturbation of the tight junctional proteins.[88]

7. GENOTOXICITY

The data on genotoxicity of individual phthalate vary widely i.e., some have no data on genotoxicity, some have only in vitro genotoxic potential while some have both in vitro and in vivo genotoxic potential. Data on the genotoxic potential of DIBP indicate that it has low genotoxic potential since it exhibited genotoxic activities in one or more in vitro assays or in vivo dominant lethal assays.[10] Moreover, oxidative stress has been suggested as the etiologic link for reported relationships between urinary phthalate metabolites of DEHP and DEP with increased DNA damage in human sperm.[92,93] Kleinsasser and colleagues used the alkaline micro gel electrophoresis assay to detect single-strand breaks in the DNA following incubation with di-butyl phthalate (DBP) and di-isobutyl phthalate (DIBP). They reported that both DBP and DIBP induce the DNA damage in oropharyngeal and nasal mucosa, though the effect of DIBP was more pronounced than that of DBP.[94]

Recently, Ahbab and colleagues evaluated possible genotoxicity of di-n-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) at different concentrations using Comet assay in male rat pups.[95] 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 (pre-pubertal, pubertal and adulthood) after delivery. Erkekoglu and Belma Kocer-Gumusel, 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.[96]

In addition, the results demonstrated genotoxic effects of phthalates on human mucosal cells of the upper aerodigestive tract, in contrast to earlier findings in animal models. Later Ma and colleagues investigated the oxidative damage induced by di-isobutyl phthalate (DIBP) in mice treated with DIBP (0, 50, 250, 500 and 1000 mg/kg).[66] By the end of the 8th week, the comet assay of blood was tested. The comet assay showed that the oxidative damage of DNA in DIBP groups was significant in comparison to the control group. It was investigated that DIBP induced oxidative damage in mice treated with DIBP. While DINP was reported to be non-genotoxic in a battery of bacterial and mammalian cell assays.[97] Thus, due to insufficient available data, less is known about the in vivo genotoxic potential of phthalate compounds.[29,30] These studies suggest that some phthalates have the potential to induce DNA damage however; further research is needed to fully characterize whether the genotoxic effect of phthalates is due to oxidative stress or not.

8. CONCLUSIONS

Based on the available data, it can be inferred that exposure to phthalates might lead to oxidative stress in the male reproductive organs mainly testis and epididymis and causes disruption of the normal spermatogenesis and steroidogenesis. They impair spermatogenic process by inducing oxidative stress and apoptosis in germ cells or target sertoli cells and thereby hamper spermatogenesis. Phthalates also decrease the Leydig cell function by inducing ROS, thereby decreasing the levels of steroidogenic enzymes. Thus in utero and postnatal exposure to phthalate compounds might lead to decreased sperm count and various other reproductive anomalies in the young ones.

CONFLICTS IF INTEREST

None declared.

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