

Testicular dysgenesis syndrome and phthalate exposure: A review of literature

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Abstract

Endocrine disruptors are chemicals that interfere with the body's endocrine system and cause adverse effects in biological systems. Phthalates are a group of man-made chemicals which are mainly used as plasticizers and classified as endocrine disruptors. They are also used in cosmetic and personal care products as color or smell fixators. Moreover, phthalates are present in inks, adhesives, sealants, automobile parts, tools, toys, carpets, medical tubing and blood storage bags, and food packages. Pathological condition known as "testicular dysgenesis syndrome" (TDS) or "phthalate syndrome" is usually linked to phthalate exposure and is coined to describe the rise in alterations in reproductive health in men, such as reduced semen quality (decrease in sperm counts, sperm motility and increase in abnormal sperms), hypospadias, cryptorchidism, reduced anogenital distance and early-life testicular cancer. Phthalates are suggested to cause direct effect on gonadal and non-gonadal tissues, impair the differentiation and morphogenesis of seminiferous tubules and accessory sex organs and testicular cells (both Sertoli and Leydig cells), alter estradiol and/or testosterone levels, decrease insulin-like 3 (INSL3) peptide production, impair spermatogenesis and lead to epigenetic alterations, all of which may lead to TDS. This review will mainly focus on phthalates as causes of TDS and their mechanisms of action.

Keywords: testicular dysgenesis syndrome, endocrine disruptor chemicals, phthalate, male reproductive system defects

Introduction

Since *Silent Spring* was published on September 27th, 1962, awareness of the public to the exposure to environmental chemicals has been on the rise. *Silent Spring*, written by Rachel Carlson, mainly focused on the environmental toxicity caused by the high usage of different kinds of pesticides. Chemical companies met the book with fierce opposition. However, it brought numerous changes to the United States' national pesticide policy and a nationwide ban on the agricultural uses of dichlorodiphenyltrichloroethane, commonly known as DDT, in 1972. Moreover, this environmental movement provided the establishment of the U.S. Environmental Protection Agency (US EPA) (1-4). The Stockholm Convention on Persistent Organic Pollutants banned most uses of DDT and other organochlorine pesticides, except its use as an anti-malarial agent (5). DDT is now a well-known endocrine disruptor (6,7). Although studies have suggested that it is not genotoxic, DDT is considered to be possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC) (8,9). Its main metabolite dichlorodiphenyldichloroethylene (DDE) is considered to be a potent androgen antagonist. Another metabolite, o,p'-DDT, has weak estrogenic activity (10). *In utero* DDT exposure may lead to a higher risk of breast cancer (11).

Nowadays, life is inevitable without plastics. In order to soften plastics, phthalates are used abundantly and they are highly present in the environment. Other than their use in plastics, they are used in cosmetics and personal care products and as solvents in the industry. They are well-known endocrine disruptors, and they are suggested to be one of the causes of the decrease in fertility throughout the world.

This review will mainly focus on phthalates as causes of testicular dysgenesis syndrome (TDS) and their mechanisms of action. We will review the literature on mechanisms of endocrine disruption, phthalates, their uses and exposure routes, how phthalates may cause TDS and the *in vitro*, *in vivo* and human studies that suggest a link between TDS and phthalate exposure.

Endocrine disruptors

Over the last three decades, several studies have suggested that many natural and man-made chemicals might interfere with the body's endocrine system and cause adverse effects in humans, animals, and wildlife. Scientists often refer to these chemicals as "endocrine disruptors" (12). EPA defines endocrine disrupting chemicals as "chemicals that interfere with the normal functions of the endocrine system and lead to problems with reproduction (i.e. egg and sperm production) and development (i.e. healthy fetal growth) in both humans and wildlife" (13). Today, the phrase "endocrine disruption" is seemingly linked with terms like "environmental estrogens", "environmental anti-androgens", "male infertility" and "falling sperm counts". Although these links help the public understand the effects of endocrine disruption better, they are simply a narrow view of the way in which this phenomenon affects the endocrine system, since its effects include but are not limited to these. Other than effects on gonads and reproduction, endocrine-disrupting

effects also include “effects on other hormone-producing organs/glands like thyroid, parathyroid, anterior and posterior pituitary, pancreas, adrenals and pineal” (14).

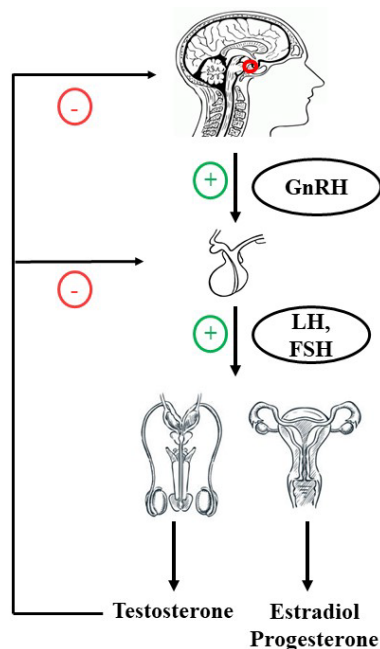


Figure 1. Schematic representation HPG axis HPG: Hypothalamic-Pituitary-Gonad; GnRH: Gonadotrophin-Releasing Hormone; LH: Luteinizing hormone; FSH: Follicle stimulating hormone

Slika 1. Shematski prikaz HPG ose HPG: Hipotalamusno-hipofizno-gonadalna osovina; GnRH: gonadotropin-oslobađajući hormon; LH: luteinizirajući hormon; FSH: folikulostimulirajući hormon

The endocrine system is one of the most complex systems in human body, with many interconnecting and interacting axes. The mature reproductive system is mainly regulated by the hypothalamus–pituitary–gonad (HPG) axis (Figure 1), which functions as a classical negative feedback system. This axis mainly includes the gonadotrophin-releasing hormone (GnRH) neurons in the hypothalamus, which release GnRH into the portal blood supply. GnRH’s main function is to stimulate gonadotrophin release, namely luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the gonadotroph cells localized in the pituitary gland. After LH and FSH are released into systemic circulation, they affect the endocrine active cells of the testis or ovary. Testes have different cell types: under the effect of LH, Leydig cells secrete testosterone and estradiol. On the other hand, FSH mainly affects the Sertoli cells that secrete inhibin B and create a microenvironment for sperm cells to survive. Later, pituitary and hypothalamus receive these gonadal signals by a feedback mechanism and they can regulate the release of GnRH and the gonadotrophins (14). Several endocrine-disrupting chemicals can potentially affect the HPG axis at any level. However, data from different studies mainly support for the idea that these chemicals affect the development and programming of the

axis during fetal life, and *in utero* exposure can be the most sensitive period in which environmental chemicals, including endocrine disruptors, can alter the homeostatic mechanisms of the endocrine system permanently (14).

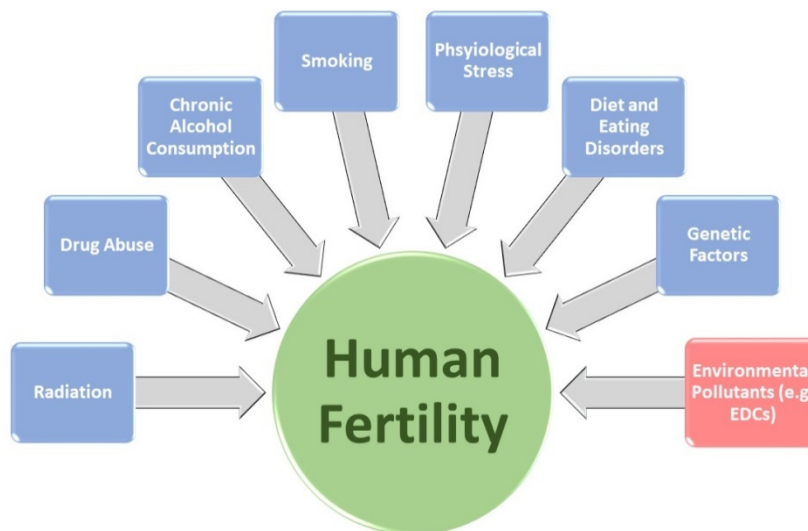


Figure 2. Parameters that affect human fertility EDC: Endocrine disrupting chemicals
Slika 2. Parametri koji utiču na plodnost ljudi EDC: endokrini ometači

Although lifestyle (diet, eating disorder, smoking and/or alcohol consumption, drug abuse, physiological stress) is considered to be the main factor that affects human fertility, literature suggests that endocrine disruptors may also affect reproduction, particularly male, and cause pathologies if the exposure to these chemicals happens in early life (Figure 2) (15). On the other hand, limiting the effects of endocrine disruptors to their “estrogenic mode of action” is too simplistic. Many studies currently suggest that these chemicals can act as estrogens, anti-estrogens, androgens and antiandrogens. Moreover, they can inhibit or induce steroidogenic enzymes, increase or decrease protein expression of certain hormones, they can be inhibitors of thyroid hormones and/or their receptors, or they can affect the synthesis or secretion of pancreatic or adrenal hormones. The current opinion of many scientists is that chemicals that interfere with the action and/or production of steroids may be of more concern to reproductive health. On the other hand, in the last decade, the phenomenon of neuroendocrine disruption by endocrine disruptors is well defined and now it is suggested that many endocrine-disrupting chemicals may also lead to alterations in the HPG or hypothalamus–pituitary–thyroid (HPT) axis (Figure 3). On the other hand, some of these chemicals can also cause immunotoxicity (16).

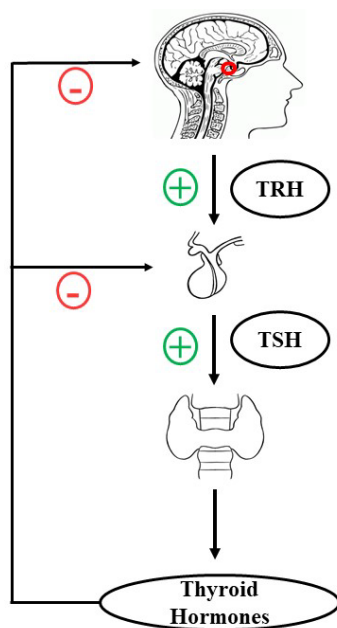


Figure 3. Schematic representation of HPT axis HPT: hypothalamic-pituitary-thyroid; TRH: thyrotropin-releasing hormone; TSH: thyroid-stimulating hormone

Slika 3. Shemataski prikaz HPT osa HPT: Hipotalamusno-hipofizno-tiroidna osovina; TRH: tireotropin oslobađajući hormon; TSH: tireoidni stimulirajući hormon

Testicular dysgenesis syndrome

In the last decades, reproductive disorders in males have frequently been reported by serious reports and epidemiological studies. From different parts of the globe, several researchers are now investigating the effects of endocrine disruption on male reproductive system. Studies conducted by researchers from different disciplines mainly point out that there is a decline in male fertility. Moreover, an increase has been observed in congenital reproductive tract anomalies (such as cryptorchidism, hypospadias and reduced anogenital distance (AGD)) and testicular cancers (between ages 35-45).

The term testicular dysgenesis syndrome (TDS) mainly describes “the rise in alterations in reproductive health in men, such as reduced semen quality (decrease in sperm counts and sperm motility as well as increase in abnormal sperms), hypospadias, cryptorchidism and early-life testicular cancer” (17,18). The TDS hypothesis proposes that a proportion of these pathologies may be symptoms of one underlying developmental disease called TDS, which is most likely a result of disturbed gonadal development in the embryo (19). Although lifestyle, genetics and geographical regions are influential factors, they may not be sufficient to fully explain the increase in TDS. Scientists today suggest

that higher exposure to chemicals in large cities or industrialized areas seem to be effective in this rise. The pathogenesis of TDS is shown schematically in Figure 4.

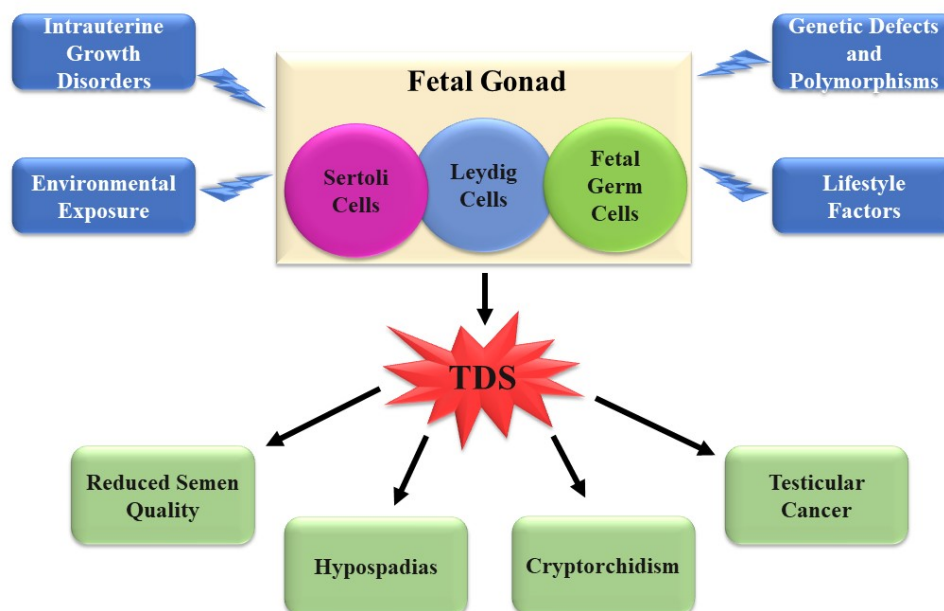


Figure 4. Pathogenesis of testicular dysgenesis syndrome TDS: Testicular dysgenesis syndrome

Slika 4. Patogeneza sindroma testikularne disgeneze TDS: sindrom testikularne disgeneze

Comparing two Nordic countries, Finland and Denmark, can be an interesting example. Finland is the country with the lowest risk of testicular cancer in Europe, whereas the incidence of testicular cancer in Denmark is the highest in Europe. Moreover, Danish men have a lower sperm count in comparison to Finnish men, and Danish boys have a higher prevalence of cryptorchidism and hypospadias when compared to Finnish newborn boys (20,21). However, it is observed that reproductive health has deteriorated in Finland in recent years. The testis cancer incidence is increasing, and sperm concentrations are decreasing due to higher endocrine-disrupting chemical exposure compared to recent decades (22). The lower rates of TDS and early-life testicular cancer can be based on their good genetic background for Finnish boys and men. However, the increasing rates can be dependent on higher exposure to endocrine disruptors today. What is even more interesting is that Finnish men, who migrated to Sweden in their early twenties, follow the Finnish incidence of testis cancer, whereas their sons, who were conceived and born in Sweden, follow the Swedish incidence rate (23). This finding clearly suggests that the hypothesis “*in utero* high endocrine disruptor exposure may be an important factor in TDS’s etiology”.

The scientific evidence that TDS may be caused by a marked decrease in the production of testosterone by the fetal testis is mainly dependent on many studies on phthalates using rodents. Indeed, all of the TDS phenotypes other than testicular cancer were suggested to be in association with gestational exposure of rodents, particularly rats, to high doses (500–750 mg phthalate/kg body weight) of phthalates. These exposures mainly caused significant declines in the production of testosterone by fetal testis. It should also be emphasized that gestational exposure of rats to estradiol or diethylstilbestrol (DES) results in similar alterations, such as alterations in the testis and accessory sex organs and cryptorchidism, caused by phthalates (24,25). The similarity between estradiol/DES exposure and phthalate exposure may be dependent on the androgen–estrogen imbalance, since all of these chemicals may cause such effects during fetal development (26).

There have been several epidemiological studies conducted on the phthalate exposure of the general populations. A study conducted by Centers for Disease Control and Prevention (CDC) measured the urinary levels of 13 phthalate metabolites of over 2600 people (age: 6 years and older) who took part in the National Health and Nutrition Examination Survey (NHANES) in 2003–2004. CDC published these findings in the Fourth National Report on Human Exposure to Environmental Chemicals. This study revealed that the general American population had detectable levels of many phthalate metabolites. Adult women had higher urinary levels of phthalate metabolites compared to men, most probably due to their higher use of cosmetics and/or personal care products like body washes, shampoos, soaps and make-up products. Non-Hispanic Blacks had higher urinary phthalate and their metabolite levels in comparison to Non-Hispanic Whites. The results indicated that the general American population had a widespread exposure to many phthalate derivatives (27).

Phthalates and exposure routes

Life is inconceivable without plastics. Phthalates are (di-alkyl or alkyl-aryl) diesters of phthalic acid. These derivatives are used as plasticizers in order to impart flexibility and durability to plastic products (28,29). Some phthalates in polyvinyl chloride (PVC) plastics are used to make products such as medical tubing, garden hoses and plastic packaging. Some phthalates are used to help dissolve other materials. Phthalates are also present in hundreds of products, such as vinyl flooring, carpets and lubricating oils (30). In addition, they are used as a matrix in cosmetic products and personal-care products (soaps, shampoos, hair sprays, nail polish, etc.) in order to protect the color or the smell for longer periods. Moreover, phthalates are present in medical tubing, packaging films, inks, adhesives, sealants, plastics of personal care products (i.e. toothbrushes), automobile parts, tools, toys, carpet-back coating and blood storage bags, floor tile, wire, cables, and food packaging (28,31,32).

General population is orally exposed to phthalates by foods, which have contacted packages that contain different phthalate derivatives. They are also present in drinking water and ambient air at very low amounts (28,29). In workplaces that produce or use

phthalates, exposure can occur from breathing phthalate particles in the air. On the other hand, the general population can be exposed to phthalates by inhaling the released phthalates from packages, floorings, PVC windows and/or carpets (27). Exposure routes to phthalates are shown in Figure 5.

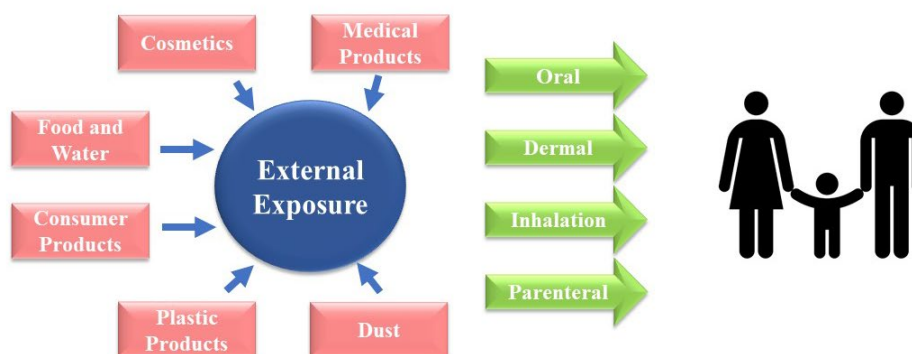


Figure 5. Exposure routes of phthalates
Slika 5. Putevi izloženosti ftalatima

Babies and young children have the habit of touching many things and then put their hands in their mouths. Because of this significant behavior, phthalate particles present in the dust may pose a greater risk for children than for adults. On the other hand, they put their toys in the mouth and therefore, phthalate-containing toys can be an exposure route for children (27). Humans can also be exposed to phthalates through medical procedures (i.e. blood transfusion, dialysis, respirators) and exposure of newborns to different medical equipment in NICU can be an important risk factor for TDS and other reproductive disorders in later life (28,29,33).

The environmental release of plasticizers is unpreventable since they are not covalently bound to the plastic materials (34). Phthalates have been suggested to have endocrine-disrupting properties in several *in vitro* and *in vivo* studies. Most of these studies suggest that phthalates, and particularly di-(2-ethylhexyl)phthalate (DEHP), have anti-androgenic potential (35-39). Phthalates can particularly affect the developing male reproductive system. However, they are also suggested to affect female reproductive system as well. Moreover, these endocrine disruptors may cause developmental anomalies. They can also affect the neuroendocrine system and can be associated with different endocrine diseases and pathological conditions, like precocious puberty (PP), diabetes, gynecomastia, obesity and/or metabolic syndrome, asthma and autism (40-46). Phthalates are also suspected to be one of the underlying factors of the increase in the prevalence of TDS (47).

Biotransformation of phthalates

Upon ingestion, lipases and esterases in the intestine and parenchyma are responsible for the biotransformation of phthalates to monoester metabolites (Figure 6). Although the main tendency of biotransformation is detoxification of substances, this is not the case with phthalate diesters, and more active mono-ester phthalates are produced by Phase I metabolism (48). For instance, in *in vitro* studies mono(2-ethylhexyl)phthalate (MEHP), which is the primary metabolite of di(2-ethylhexyl)phthalate (DEHP) produced higher cytotoxicity and oxidative stress compared to DEHP in different cell lines (37,49,50). *In vitro* and *in vivo* biotransformation studies revealed that short-branched phthalates are mainly excreted as their urinary monoester phthalates. However, long-branched phthalate esters undergo further biotransformation processes. The Phase I biotransformation of phthalates are achieved through hydroxylation and oxidation. Later, the primary compound or the first or secondary oxidized metabolites undergo conjugation (by glucuronidation and by sulfation to a lesser extent) before they are excreted by the kidneys or intestine (48,51-53).

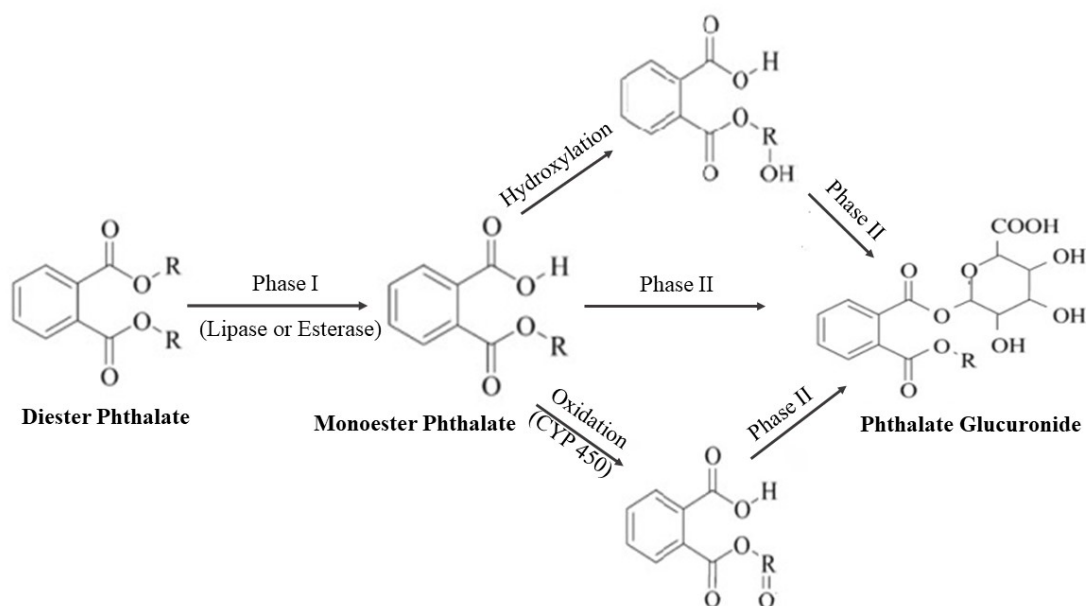


Figure 6. Biotransformation of phthalates

Slika 6. Biotransformacija ftalata

Different phthalate derivatives

Di(2-ethylhexyl)phthalate

Plastics may contain 1% to 40% of DEHP. Almost three billion kilograms of DEHP is produced and used every year worldwide (54). DEHP is the diester of phthalic acid and the branched-chain 2-ethylhexanol. As DEHP is a very good and cheap plasticizer and fixator for color and smell, it is the most widely used phthalate in flexible PVC products and vinyl chloride resins (28,29). It is also used as a hydraulic fluid and as a dielectric fluid in capacitors. DEHP also finds use as a solvent in glow sticks. DEHP is a component of many household items, including PVC windows, tablecloths, floor tiles, shower curtains, furniture upholstery, swimming pool liners and garden hoses. It may also be present in rainwear, dolls, toys and shoes. DEHP was first introduced in the 1940s to blood bags. This phthalate derivative is now present in medical devices like such as dialysis bags and tubing, intravenous tubing and bags, nasogastric tubes, IV catheters, blood bags and transfusion tubing, and air tubes (55). Due to high exposure of ICU and NICU patients, health concerns are now being expressed about the leachates of DEHP transported directly to the bloodstream of the patient, particularly in those who are in need of extensive infusions (56). Moreover, hemophiliacs and kidney dialysis patients are clearly at high risk (55,57). According to the European Commission Scientific Committee on Health and Environmental Risks (SCHER), DEHP exposure may exceed the tolerable daily intake (TDI) in people exposed through medical procedures such as kidney dialysis. The exposure in NICU may increase the risk of developmental abnormalities in premature babies, newborns and neonates. On the other hand, lactating and pregnant women who are in need of medical interventions are at a great risk, as they can pass these chemicals directly to their babies (58). The use of DEHP-containing medical devices is not suggested by The American Academy of Pediatrics. The academy advocates for the use of DEHP-free medical devices (59).

DEHP is metabolized to its hydrolytic monoester MEHP by pancreatic lipases in the lumen of the gut after ingestion (48,51,52). MEHP is further metabolized to oxidative metabolites in both rats, monkeys and humans (60-64). MEHP and its three major oxidative metabolites namely, mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), have been unequivocally identified in humans (48,52,65). MEOHP and MEHHP are now suggested to be the potential DEHP exposure biomarkers (66). These metabolites can be glucuronidated before being excreted by the kidneys and intestine (48,52,65,67). DEHP is classified as “possibly carcinogenic to humans” as there is sufficient evidence in animals for its carcinogenic potential (68).

Diisononyl phthalate

Diisononyl phthalate (DINP) is a phthalate derivative that is particularly present in food contact materials (69). DINP is also used in various types of plastic consumer products, including gloves, shoes, tubing, PVC flooring, materials used in automobile

interiors, wire and cable insulation, as well as garden hoses. This phthalate is also present in some non-PVC products, such as some adhesives, paints, sealants, inks, pigments, and lacquers. DINP can be gradually released from consumer products into indoor environments. DINP can be detected on floors and other surfaces. DINP can accumulate in dust and air. DINP is on the Proposition 65 (the Safe Drinking Water and Toxic Enforcement Act of 1986) list. When California voters approved it by a 63-37% majority, this proposition was passed as a law in November 1986. DINP was suggested to cause cancer in this proposition (70).

DINP is metabolized in the gastrointestinal tract by hydrolyzed to minor urinary metabolite monoisononyl phthalate (MiNP). MiNP is then oxidized in the liver to form secondary oxidative metabolites with hydroxyl, oxo and carboxyl functional groups [mono(carboxyisooctyl) phthalate (MCiOP), mono(oxoisononyl) phthalate (MOiNP), and mono(hydroxyisononyl) phthalate (MHiNP)] (71-73).

The maximum specific migration limit (SML) from food contact materials for DINP is set as 9 mg/kg (as the sum of diisononyl phthalates and diisodecyl phthalates) in the European Union (EU) (74).

Diethyl phthalate

Diethyl phthalate (DEP) is used as a plasticizer, fixative and solvent in several cosmetic formulations, including bath preparations (oils, soaps, tablets, and salts), make up products, fragrances, hair products, nail products and skin care preparations (75). More specifically, DEP is used in nail polish as a solvent for nitrocellulose and cellulose acetate (75-77).

Diethyl phthalate is also used as a plasticizer in different cellulose ester plastic films/sheets and also in molded/extruded articles. It is also present in tool handles, toys, toothbrushes and automotive components. There is a wide variety of consumer products that contain diethyl phthalate or are covered with diethyl phthalate-containing plastic packaging (75). In addition, this phthalate derivative is used in insecticide sprays and mosquito repellents. DEP is also as an ingredient in drug (e.g. aspirin) coatings, used as a plasticizer in solid rocket propellants, as a dye application agent, as a wetting agent, as a diluent in polysulfide dental impression materials, and in adhesives, plasticizers, and surface lubricants used in food and pharmaceutical packaging. In one study, the concentration of DEP in different medical devices (e.g. dialysis tubing) was found to be <1% of total volatiles. However, in an intestinal tubing sample its concentration reached to <20% of total volatiles (78).

There is little evidence on the chronic toxicity of this phthalate. Studies performed on rats suggested that repeated administration caused germ cell apoptosis in testicular tissue. However, DEHP did not affect male sexual differentiation in rats (71,79-81). The use of DBP in cosmetics (including nail polishes) is banned in EU under Directive 76/768/EEC 1976 (82). In EU, DBP has been restricted in children's toys since 1999 (83).

In the lumen of the gastrointestinal tract or in the intestinal mucosal cells, it is hydrolyzed to its main metabolite, namely monoethyl phthalate (MEP) and ethanol by carboxyl esterase after oral exposure (83).

Dibutyl phthalate

Dibutyl phthalate (DBP) is a widely used phthalate derivative. The primary use for DBP is to soften and increase plastic flexibility. In elastomers, nail polish, lacquers, explosives and solid rocket propellants, DBP can be used as a plasticizer. Raincoats, food wraps, shower curtains and car interiors may also contain DBP. It may also be present in insect repellents and in perfume oil and resins as a solvent. Other uses include perfume fixative, textile lubricating agent, safety glass additive, printing inks, and adhesives. DBP is classified as a 'Group D' carcinogen by the EPA. The EPA stated that no definitive carcinogenic characteristics have been reported (84).

DBP is biotransformed to its main metabolite monobutyl phthalate (MBP) (31,63). It can also be glucuronidated in many organisms including humans, rats, hamsters and guinea pigs. It is also excreted as phthalic acid and unchanged compound. In addition, omega or omega-1 urinary oxidation products of MBP may be detected (84).

Benzylbutyl phthalate

Benzylbutyl phthalate (BBP) is mainly used as a plasticizer in PVC production. In vinyl foams, which are often used as floor tiles, BBP is commonly used as a plasticizer. It is also used in food packaging materials, conveyor belts, traffic cones and artificial leather (85).

BBP is metabolized to monoester metabolites by gut esterases. The major metabolites of BBP are monobutyl phthalate (MBP), mono-benzyl phthalate (MBzP) and mono-n-butyl phthalate monobenzyl phthalate (MnBP) to a lesser extent. The ratio of MBP to MBzP was found to be 5:3. These metabolites can be absorbed and excreted directly. They may also be subject to glucuronidation (85,86).

It is suggested that 70% of BBP is not conjugated, while 30% is conjugated in rats. When BBP exposure is high, relatively less metabolite is conjugated. This suggests that the glucuronidation is saturated at high administrations. Its metabolites are rapidly excreted and 90% of these metabolites are eliminated within 24 h. Therefore, the half-life of BBP in the blood is very low and it is only 10 min (86). However, monoester metabolites of BBP are shown to have a longer half-life (~6 h) (87).

Until now, experimental research has not clearly identify the modes of action of BBP although it is known that this phthalate binds to the rat estrogen receptor (88). In vitro studies indicated the estrogenic potential of BBP. It has an effect on estrogen-mediated gene expression. However, its metabolites are only weakly reactive with the estrogen receptor (89).

Diisobutyl phthalate

Diisobutyl phthalate (DiBP) is another butyl phthalate derivative. DiBP is present in, for instance, floorings, in the production of titanium catalyzers, adhesives, coating products, fillers, putties, plasters, modelling clays, lacquers, inks, hydraulic fluids and lubricants. It has been used as marker in fuels for tax purposes (90). The results of several toxicological studies show that the male reproductive tract is a particularly sensitive target for effects after exposure to phthalates, including DiBP (91,92).

Its major metabolite is mono-isobutyl phthalate (MiBP) (30, 90). In humans, MiBP has a short halftime (3.9 h) while MiBP's oxidized further metabolites [3-OH-monoisobutyl phthalate (3-OH-MiBP) and 2-OH-monoisobutyl phthalate (2-OH-MiBP)] had longer half-lives (4.1 and 4.2 h). These secondary oxidized metabolites can be used as additional and valuable biomarkers of DiBP exposure (93).

Phthalate derivatives and their uses are summarized in Table I.

Table I Phthalate derivatives and their uses.

Tabela I Derivati ftalata i njihove upotrebe

| Phthalate Derivative | Usage |
|---|---|
| Di(2-ethylhexyl)phthalate (DEHP) | PVC products and vinyl chloride resins, household items (PVC Windows, tablecloths, floor tiles, shower curtains, furniture upholstery, swimming pool liners and garden hoses), medical devices (dialysis bags and tubing, intravenous tubing and bags, nasogastric tubes, IV catheters, blood bags, transfusion tubing, and air tubes). |
| Diisononyl phthalate (DINP) | Plastic consumer products (PVC flooring, materials used in automobile interiors, wire and cable insulation, gloves, tubing, garden hoses and shoes), some inks and pigments, adhesives, sealants, paints and lacquers. |
| Diethyl phthalate (DEP) | Bath preparations (oils, tablets, and salts), eye shadow, toilet waters, perfumes and other fragrance preparations, hair sprays, wave sets, nail polish and enamel removers, nail extenders, bath soaps, detergents, aftershave lotions, and skin care preparations. |
| Dibutyl phthalate (DBP) | nitrocellulose lacquers, elastomers, explosives, nail polish, solid rocket propellants, shower curtains, raincoats, food wraps, and car interiors. |
| Benzylbutyl phthalate (BBP) | PVC production, vinyl foams, traffic cones, food conveyor belts, and artificial leather. |
| Diisobutyl phthalate (DiBP) | Titanium catalyzers, adhesives, coating products, fillers, putties, plasters, modelling clays, lacquers, inks, hydraulic fluids and lubricants. |

Mechanisms for testicular dysgenesis syndrome caused by phthalates

Testis formation

Testis formation is driven by the sex-determining region of the Y chromosome (SRY) activation, which leads to Sertoli cell differentiation (94). Formation of Sertoli cells later determines the fate of the indifferent gonad, directing its progression along testis development. Therefore, Sertoli cells are of particular importance for testicular development and they orchestrate dynamic processes such as cellular movement, cellular organization and differentiation of both themselves and all the other testicular cell types (95,96).

On day 10 (E10), testis differentiates from the gonadal ridge primordium. Around the primordial germ cells, Sertoli cells form clusters and this phenomenon allow them to enclose germ cells within the seminiferous cords. One theory is that Sertoli cells signal the overlying mesonephros in order to allow cell migration into these cords and later further enabling cord development and expansion of the cell population (97). Eventually, seminiferous cords are surrounded by a basal lamina, which is formed by an interaction between Sertoli cells and peritubular myoid cells. Sertoli cells also induces Leydig cell differentiation is also induced by Sertoli cells (97). Leydig cells then produce testosterone, which is essential for the masculinization of the male reproductive tract. Müllerian duct regression in males is also induced by Sertoli cells by anti-Müllerian hormone (AMH). During testicular development, Sertoli cells secrete an unidentified inhibiting substance leads to the prevention of the germ cells to enter meiosis. This substance also blocks germ cells from entering the oogenic pathway. It is now known that many of genes as well as signaling pathways are involved in testis organogenesis. However, all of those genes/pathways are yet to be identified (95,97).

Between embryogenesis and puberty, two separate generations of Leydig cells successively develop in the testis in rodents. The first are fetal Leydig cells, which differentiate from stem cells during gestation. Stem fetal Leydig cells commit and differentiate into mature fetal Leydig cells by E12. These cells are fully successful for steroidogenesis (98). Peak steroidogenic activity of fetal Leydig cells can be seen on 1 or 2 days prior to birth on gestation day 19 (99). The secretion of testosterone is crucial for male secondary sexual differentiation (i.e., the development of the penis and accessory sex glands) (98). Fetal Leydig cells also produce the protein-structured hormone insulin-like growth factor 3 (INSL3). This hormone binds to its specific receptor the leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8) in the gubernaculum (100). INSL3 and testosterone both induce the scrotal descent of the testis (101). Research has shown that INSL3 is the critical hormone responsible for early stage descent of the testis from the abdominal to inguinal position. Studies show that mutations in humans result in the loss of function of INSL3 and in INSL3 knockout mice, this process is prevented (102). Therefore, it can be stated that any influence including hormone levels and environmental exposures on the development of fetal Leydig cells can lead to cryptorchidism.

Fetal Leydig cells can remain in the testicular interstitial area after birth, although they quickly involute (103). After birth, their fate remains a subject of debate and there is no clear explanation in literature (104). However, research on the luteinizing hormone receptor null (LHRKO) mice stands behind the hypothesis that the cells are lost through attrition in the postnatal period. This point of view is backed by the fact that fetal Leydig cell count and their testosterone production are almost at the same level in null and wild-type mice just after birth, though a progressive failure of steroidogenesis is observed with the increasing age (105-107). This suggests that the fetal postnatal Leydig cell contribution to androgen secretion can be neglected. The cells that stem from adult Leydig cells first become apparent on PD11 as 3β -hydroxysteroid dehydrogenase expressing spindle-shaped cells are observed in the interstitium (108). It has become evident that the process of postnatal development of adult Leydig cells includes: **a.** self-renewal of stem Leydig cells. **b.** their commitment to the adult lineage. **c.** further cytological and biochemical differentiation into fully differentiated and testosterone-producing adult Leydig cells (109). Several studies suggest that the exposures of fetal and adult Leydig cells to environmental chemicals, particularly to phthalates, may cause a wide array of testicular defects, including signs of TDS (15,109,110).

Another important issue that should be discussed in the outcome of TDS are the effects on peritubular myoid cells and seminiferous cord formation. Peritubular myoid cells are positioned on the outer circumference of the seminiferous cord. These cells mainly serve as a visual boundary to separate the cords from the interstitium. The formation of the seminiferous cord has been described after *in utero* DBP administration to rats PD4, where abnormal seminiferous cord formation was observed. Such formation might lead to large and discrete areas in the testis with malformed cords, which contain Sertoli cells and gonocytes. These abnormal foci can become a permanent feature of the testis, which become more separate as the testis is enlarged during postnatal development (111). In human biopsies, malformed and morphologically abnormal seminiferous tubules were seen in the contra-lateral testis of testis cancer, suggesting this may also be a feature of TDS (98).

Sex development disorders

Although sex development disorders were rare in the 1960s and 1970s, they are observed at higher rates today. These pathologies were suggested to be caused by the hormonal disturbance of sexual determination and gonadal differentiation. In such conditions, there is a discrepancy between the phenotypic and the karyotypic sex of an individual and cases where classifying the phenotypic sex of an individual as male or female may be difficult. Sex development disorders that are mostly genetically determined (such as 45,X/46,XY mosaicism) and androgen insensitivity syndrome are considered to be among the risk factors most likely to be the leading causes of severe forms of TDS in humans (112-114). Starting from the end of the 1970s, several studies have verified that the prevalence of carcinoma *in situ* testis (CIS) and gonadal tumors is high in intersex patients with a karyotype including a Y chromosome (115-122). A special

gonadal tumor named gonadoblastoma consists of nests of CIS-like cells and supportive cells resembling Sertoli/granulosa cells. This kind of tumors lead to gonadal neoplasia, arising almost exclusively in individuals with disorders of sex development. In phenotypic males, gonadoblastomas were linked to an increased risk of developing testicular cancer, whereas they were linked to dysgerminoma in phenotypic females (123-125). However, CIS is not commonly seen in children with ambiguous genitalia in general, because the DSD is heterogeneous, and the increased risk of CIS is limited to individuals with the presence of Y chromosome material. In addition, patients with conditions associated with a lack of post-pubertal androgen stimulation (for instance, patients with the complete form of androgen insensitivity syndrome) or patients who have hypo-gonadotrophic hypogonadism rarely develop germ-cell neoplasia. This points out that androgen signaling may be one of the most important factors in the malignant transformation of pre-invasive CIS cells into overt tumors (126,127).

Insulin-like 3 (INSL3) peptide is an insulin-like hormone in protein structure. It is encoded by the INSL3 gene in humans. In both genders, this peptide is mainly produced in gonadal tissues. Several rodent studies suggest that INSL3 can have roles in both urogenital tract development and female fertility. This hormone starts meiotic progression in follicle-enclosed oocytes by mediating a reduction in intra-oocyte cyclic adenosine monophosphate (cAMP) concentration by activating LGR8. It may also regulate the growth and differentiation of gubernaculum, thus mediating intra-abdominal testicular descent. The mutations in this gene may lead to cryptorchidism (128). Gene mutations in INSL3 and LGR8 are the first-described human genetic alterations specifically associated with cryptorchidism. The cumulative frequency of mutations in the INSL3 and LGR8 genes was determined to be 4.2% in a population of men with cryptorchidism. As these mutations represent a relatively small number of patients with cryptorchidism and there are different phenotypes observed in patients with the same mutation, endocrine and/or environmental factors are suggested to play important roles in the development of the phenotypes with cryptorchidism. Depending on the exposure time and dose, an environmental chemical can affect one, two or most of these processes and may cause a defect. Such defects may be one of the underlying factors of TDS (129).

Testicular dysgenesis and phthalates

For the last two decades, public awareness on the reproductive toxicity of different phthalate derivatives has been rising. In humans and rodents, phthalate exposure during testis development is associated with TDS, which is also called “phthalate syndrome”. TDS has mainly endpoints, mostly associated with decreased testicular testosterone and INSL3 peptide levels. These endpoints are (93):

- Decreased fertility (associated with decreased sperm count and motility and increased abnormal sperm morphology)
- Decreased AGD
- Hypospadias
- Cryptorchidism
- Early-life testicular cancer

Some of these endpoints—along with other health effects—have also been observed in epidemiologic studies (93). Some of the mechanisms by which *in utero* phthalate exposure cause TDS are (Figure 7) (130):

1. Direct effect on gonadal and non-gonadal tissues (including adrenals, brain, liver and adipose tissue).
2. Increase in estradiol levels due to alteration in aromatase activity.
3. Changes in testicular proteome.
4. Changes in homeostasis of interstitial fluid and/or vascular damage in the testis both of which leading to hemorrhage and consequent inflammatory changes.
5. Clustering of Leydig cells and decreased function:
 - a. Decreased INSL3 production which may lead to cryptorchidism;
 - b. Androgen insufficiency which causes decreased testosterone production, decrease in AGD and/or hyposphadias.
6. Disturbance in Sertoli cell functions:
 - a. Impaired spermatogenesis;
 - b. Testicular cancer.
7. Alterations in testosterone: estradiol ratio in testis.
8. Impairment of differentiation and morphogenesis of seminiferous tubules and accessory sex organs.
9. Epigenetic alterations.

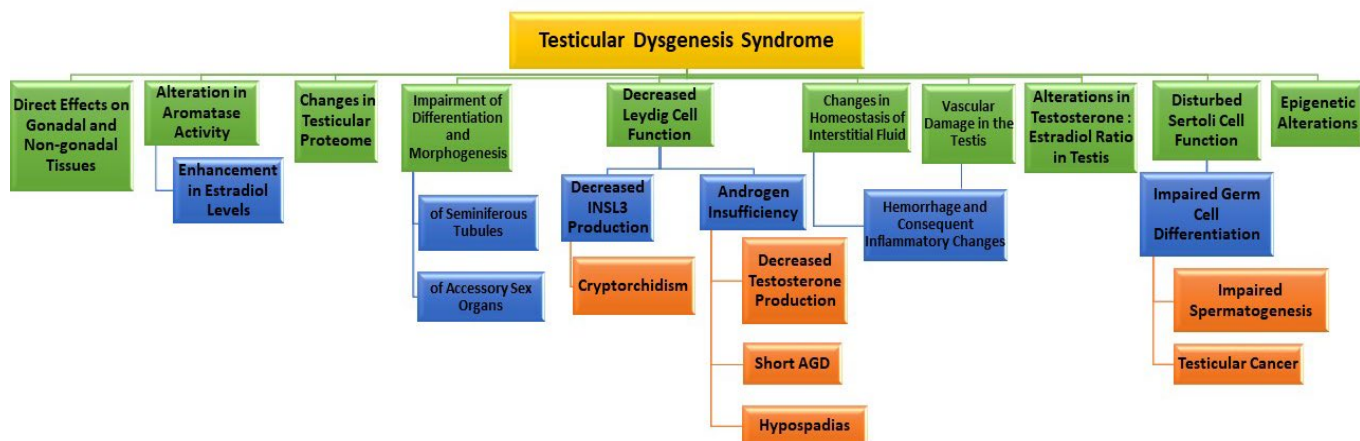


Figure 7. Mechanisms of testicular dysgenesis syndrome
 Slika 7. Mehanizmi nastanka sindroma testikularne disgenoze

In vitro studies

After the administration of DEHP during the co-culture of gonocytes and Sertoli cells, gonocytes showed detachment from the Sertoli cells. This phenomenon suggests problems with cell adhesion (131). In one study, primary Sertoli cells obtained from Sprague-Dawley rats were treated with 1,10 and 100 mg/L DBP. It was shown that DBP could significantly reduce the survival rate of Sertoli cells in a dose-dependent manner. Moreover, Hoechst staining results showed that the Sertoli cells treated with DBP emerged with typical morphological characteristics of apoptosis, nuclear condensation and chromatin condensation. In addition, DBP significantly increased the apoptotic rate of Sertoli cells dose-dependently. Moreover, showed that the expression of Phosphatase and tensin homolog (PTEN) * protein in Sertoli cells was markedly higher than control group after treated with different concentrations of DBP for 24 h, while the expression of the proteins phosphorylated phosphatidylinositol-3-kinase (PI3K1), phosphorylated protein kinase B (AKT), phosphorylated P-70-S6 kinase (70S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) in the PI3K/AKT/mTOR signal pathway were significantly decreased. The authors suggested that PTEN/PI3K/AKT/mTOR signaling pathway played an important role in DBP-induced apoptosis of testicular Sertoli cells in rats (132).

In vivo studies

Starting from the 1990s, studies have focused on the gross changes caused by phthalates on male reproductive tract development, such as reduced AGD, hypospadias, malformed epididymis and, later on, nipple retention (133-137). In the 2000s, researchers started to investigate testicular histopathological changes observed in the testis after *in utero* phthalate exposure and observed that phthalates could directly affect the differentiation of fetal and neonatal testes (111,137,138). Some of these changes could be permanent and lead to testicular dysfunction in adult life. These histological alterations are now known to be present in patients with TDS as well (98,139).

There is now more *in vivo* evidence that is driven by the development of a possible animal model for TDS. It has been suggested that *in utero* phthalate administration during testicular development might cause changes in the function of Sertoli cells. DBP [500 mg/kg, on gestational days (GD) 13-21] was administered to rats by gavage as 500 mg/kg between gestational days (GD) 13-21. At GD19, an abnormal interaction between the Sertoli cells and gonocytes was reported in the fetus. Moreover, in postnatal testis examination, researchers observed evidence of abnormal Sertoli cell maturation, which was suggested to be caused by the lack of expression of the cell cycle marker p27kip in Sertoli cells in dysgenetic areas or Sertoli-cell-only tubules (SCO). In this study, abnormal seminiferous cord formation was also observed. The findings of this study suggested that *in utero* DBP exposure led to a lack of Sertoli cell–germ cell interaction/adhesion in rats and they developed cryptorchidism, hypospadias, infertility and testicular abnormalities (111). In the same year, another study showed that testicular development was disturbed after DBP was administered to pregnant rats by gavage at a

dose of 500 mg/kg between embryonic day (E)13.5-20.5. In addition, prenatal fetal Leydig cell aggregation, which was proposed as a key event in the formation of the postnatally observed focal dysgenetic areas in the testis, was also observed (140). The results of these studies indicated that exposure to phthalates could be a cause of TDS-like symptoms. On the other hand, other agents, including a number of pesticides defined as environmental estrogens, may also have anti-androgenic properties (141).

Aside from the apparent cell adhesion problems between Sertoli cells and gonocytes and abnormal seminiferous cord formation after DBP or DEHP exposure, abnormal germ cell mitosis has also been reported (81,111,137,138). In one report, DBP (500 mg/kg/day) or testosterone antagonist flutamide (100 mg/kg/day) were given to pregnant rats on GD 12-21. DBP caused Leydig cell hyperplasia and many of the Leydig cells were 3 β -hydroxysteroid dehydrogenase- and/or AR-positive. Focal hyperplasia was characterized as the increases in the number of Leydig cells, positive for proliferating cell nuclear antigen (PCNA). Testis atrophy was apparent and seminiferous cords in DBP-exposed fetuses were enlarged and contained multinucleated gonocytes that, unlike controls, were PCNA-positive at GD 21. DBP, but not flutamide, markedly decreased testicular testosterone levels at GD 18 and 21. Fewer epididymal ducts and reduced AR staining in some ducts were evident with DBP treatment. In another study, maternal DEHP treatment (750 mg/kg/day) from GD14 to postnatal day (PND) 3 led to a decrease in testosterone production and reduced testicular and whole-body testosterone levels in fetal and neonatal male rats from GD17 to PND2. As a consequence, AGD was reduced by 36% in the exposed male offspring on PND 2. However, no changes were seen in female offspring. By GD20, DEHP administration also led to significant decreases in testis weight. Histopathological findings showed that testes displayed enhanced 3 β -hydroxysteroid dehydrogenase staining and increased numbers of multifocal areas of Leydig cell hyperplasia, as well as multinucleated gonocytes as compared to controls at GD 20 and PND 3 in the DEHP treatment group. In contrast to the effects of DEHP on testosterone levels *in vivo*, neither DEHP nor its metabolite MEHP displayed affinity for the human AR at concentrations of up to 10 μ M *in vitro*. These data indicate that DEHP disrupts male rat sexual differentiation by reducing testosterone to female levels in the fetal male rat during a critical stage of reproductive tract differentiation. In rodents, the appearance of multinucleated gonocytes (MNGs) was also observed in the fetal testes after phthalate exposure (81,111,137,138).

Other than Sertoli cells, studies suggested that the number and distribution of fetal Leydig cells could be affected after rodents were exposed to altered DEHP or DBP *in utero* (111,137,138). At around GD16, both focal hyperplasia within the interstitium and immuno-localization with the steroidogenic enzyme 3- β hydroxysteroid dehydrogenase of fetal Leydig cells was observed. In addition, when animals were examined in the postnatal period, Leydig cell hyperplasia was still present, especially near dysgenetic areas. Some of these areas were very large and they were classified as “Leydig cell adenomas” (133-135).

Testosterone is the main factor in the normal masculinization of the male reproductive tract. The synthesis of testosterone by the fetal testis can first be at detectable levels on GD15. It reaches to a peak at around GD18/19. Testosterone levels remains high until birth. In different studies, both DEHP and DBP were shown to inhibit testosterone production (60–85%) by the fetal testis during this critical developmental window, as indicated above. The decreased testosterone levels were almost at comparable levels with females of the same age. Testosterone inhibition may be a leading factor in the outcome of hypospadias and cryptorchidism (111,137,138).

More recently, a study conducted by Li et al. (2015) investigated the association between *in utero* DINP exposure on fetal Leydig cell function and testis development. Female pregnant Sprague Dawley rats orally received different doses of DINP (10, 100, 500, and 1000 mg/kg) from GD12 to GD21. Testosterone production in the testis, testicular gene and protein expression levels and fetal Leydig cell numbers and distribution were mainly at GD21.5. DINP led to a dose-dependent increase of fetal Leydig cell aggregation. The low observed adverse-effect level (LOAEL) was suggested to be 10 mg/kg. For multinucleated gonocyte production, the LOAEL was found to be 100 mg/kg. DINP also significantly increased fetal Leydig cell size, but inhibited INSL3 and 3 β -hydroxysteroid dehydrogenase gene expression and protein levels at 10 mg/kg dose. At the highest dose, this particular phthalate caused the inhibition of testicular testosterone levels. Researchers suggested that *in utero* DINP exposure affected gonocyte multi-nucleation, some fetal Leydig cell steroidogenic gene expressions and caused the aggregation of Leydig cells (142).

Decreases in AGD have been shown to be one of the most sensitive endpoints for prenatal exposure to phthalates in male animals. As a longer AGD is suggested to be the masculinization of the external genitalia, its shortening can be interpreted as a subtle pattern of demasculinization or feminization (136).

Studies in the 2010s suggested a clear association between low sperm counts and motility in adult rats after DEHP exposure. DEHP was suggested to cause abnormal sperm production in rats and lead to disruption and collapse of vimentin filaments, as well as significantly induce the apoptotic death of germ cells (37,143). A recent study aimed to evaluate the effects of single and combined exposures to BPA and/or DEHP in prenatal and lactational period on the male rat reproductive system in later stages of life. Pregnant Sprague-Dawley rats were randomly divided into four groups ($n = 3/\text{group}$): DEHP was applied as 30 mg/kg/day, while BPA was applied as 50 mg/kg/day. In the combined exposure group, BPA+ DEHP were applied as 30 mg/kg/day DEHP and 50 mg/kg/day BPA. Groups were orally exposed to BPA and/or DEHP through GD6-21 and lactation period. Male offspring ($n = 6/\text{group}$) from each mother were fed until adulthood. Later, reproductive hormones, sperm parameters, and oxidative stress parameters were determined. The researchers suggested prenatal and lactational exposure to BPA and DEHP may cause unwanted changes in the male reproductive system in later stages of life, while combined exposure seemed to have more pronounced effects (144). Moreover, another study also suggested that exposure to BPA and DEHP in fetal and neonatal

periods could cause marked testicular histopathological alterations and lead to increases in testicular apoptosis markers (caspase 3 and caspase 8 level increases; increment in TUNEL-positive spermatogonia as well as TUNEL-positive testicular apoptotic cells) and autophagic proteins (as evidenced by increased LC3 and Beclin levels and decreased p62 levels). The researchers proposed that combined exposure to endocrine disrupting chemicals might lead to more significant changes in testicular structure and increase testicular cell death (145).

In a recent study, pregnant CD rats (n = 10) were orally given DBP (0, 250, 500, or 750 mg/kg/day) throughout pregnancy and lactation, until their offspring were at PND20. Although maternal body weights throughout the dosing period were not significantly different, the number of live pups per litter at birth was decreased and maternal effects on pregnancy and post-implantation loss were likely to have occurred at the highest dose group. AGD decreased at birth in the male offspring in both 500 and 750 mg/kg/day-applied groups. The epididymis was absent or underdeveloped in 9, 50, and 71% of adult offspring (100 days old) at 250, 500, and 750 mg/kg/day, respectively. This phenomenon was associated with testicular atrophy and widespread germ cell loss. Hypospadias occurred in 3, 21, and 43% of males and ectopic or absent testes in 3, 6, and 29% of males at 250, 500, and 750 mg/kg/day, respectively. In both 500 and 750 mg/kg/day-applied groups, the absence of the prostate gland and seminal vesicles, as well as small testes and seminal vesicles were also observed. Estrogen-dependent events, namely vaginal opening and estrous cyclicity, were not affected, although low incidences of reproductive tract malformations were observed at 500 and 750 mg/kg/day in the female offspring. DBP produced the same spectrum of effects elicited by the antiandrogen flutamide in the male offspring. The findings indicate that DBP specifically impaired the androgen-dependent development of the male reproductive tract. Therefore, the authors suggested that DBP is not only estrogenic, but anti-androgenic as well at higher dose levels (133). Another recent study on citrate substitutes (such as acetyl triethyl citrate-A TEC and acetyl tributyl citrate-ATBC) of DEHP investigated the anti-androgenic effects and cytotoxicity in castrated male Sprague-Dawley rats employing the *in vivo* Hershberger assay and *in vitro* mouse Leydig (TM3) cells and mouse fibroblast (NIH-3T3) cell lines. In the Hershberger assay, rats were administered testosterone propionate and A TEC or ATBC at 20, 100 or 500 mg/kg b.w./day or DEHP (500 mg/kg b.w./day). Positive controls received flutamide, negative controls received testosterone only and vehicle controls were given corn oil only. Both A TEC and ATBC administrations did not produce significant differences compared with testosterone in 5-androgen-dependent tissue weights, including the ventral prostate, seminal vesicles, levator ani-bulbocavernosus muscle, Cowper's glands, and glans penis. In the highest dose ATBC group, there were marked decreases in liver weight. Cytotoxicity tests showed that cell viabilities of both TM3 and NIH-3T3 cells treated with A TEC were not significantly altered. However, ATBC significantly reduced TM3 and NIH-3T3 cell viability in a concentration-dependent manner. Furthermore, ATBC reduced cell viability to a greater extent in TM3 versus NIH-3T3 cells. Concerning the alterations in reproductive tissue responses and cytotoxicity test results, the authors

suggested that ATEC might be a better alternative to DEHP compared to ATBC for potential commercial uses (146).

As humans are exposed to different endocrine disruptors in mixtures in daily life, studies are now focusing on the toxic effects of different mixtures of these substances. The type of interaction between endocrine disruptors may be antagonistic or synergistic. In order to determine the real alterations in the metabolic, reproductive and endocrine systems, different *in vivo* models are used. The mode of action and biological activities of the mixtures are not well known today. Therefore, more studies are needed in order to make a reliable risk assessment. In a recent study, researchers were able to define interactions between different bisphenol derivatives (namely BPA, BPS, BPF, BADGE, BADGE·2HCl and phthalates (DEP, DBP) in ternary mixtures, at environmentally relevant concentration levels (BPA, BPF, BPS and BADGE·2HCl: 1.89, 1.42, 3.08, and 0.326 μM , respectively) by employing bioluminescent bacteria (Microtox assay). The researchers unexpectedly observed a strong antagonistic impact with DEP, in mixtures containing BPA and BADGE analogues (also confirmed with mathematical models). This study also showed that the impact of BPS and BPF in many mixtures was highly concentration dependent, justifying the necessity of performing mixture studies using wide concentration ranges (147).

A study published in 2021 assessed the synergic, additive or antagonistic effects of oral co-exposure to BPA and DEHP in juvenile rats at environmentally relevant dose levels. Animals were grouped by applying low, middle and high doses of BPA (2, 6, and 18 mg/kg b.w./day) and DEHP (9, 21, and 48 mg/kg b.w./day). The degree of synergism or antagonism was evaluated by synergy score calculation, using present data and results from the single compound individually administered. After certain calculations, the authors stated that co-exposure of BPA and DEHP mainly showed interactions of the antagonistic type (148).

In one recently conducted study, the link between DEHP, DBP and BPA co-exposure and type 2 diabetes mellitus (T2DM), as well as the ability of multi-strained probiotic to reduce DEHP, DBP and BPA mixture-induced oxidative damage in rat pancreas were investigated. For data-mining, the Comparative Toxicogenomics Database, Cytoscape software and ToppGene Suite were used. Animals were sorted into seven groups (n = 6) and were sacrificed after 28 days: (1) Control group: corn oil, (2) P: probiotic: *Saccharomyces boulardii* + *Lactobacillus rhamnosus* + *Lactobacillus plantarum* LP 6595 + *Lactobacillus plantarum* HEAL9; (3) DEHP: 50 mg/kg b.w./day (oral), (4) DBP: 50 mg/kg b.w./day (oral), (5) BPA: 25 mg/kg b.w./day (oral), and (6) MIX: 50 mg/kg b.w./day DEHP + 50 mg/kg b.w./day DBP + 25 mg/kg b.w./day BPA (oral); (7) MIX + P (oral). *In silico* investigation highlighted 44 DEHP, DBP and BPA mutual genes linked to the T2DM, while apoptosis and oxidative stress were highlighted as the main mechanisms of DEHP, DBP and BPA mixture-linked T2DM. *In vivo* assessments confirmed the presence of significant changes in oxidative stress (total antioxidant capacity, superoxide dismutase and -SH groups) only in the MIX group,

indicating possible additive effects, while probiotics ameliorated mixture-induced redox status changes in rat pancreatic tissue (149).

The A study conducted by Baralić et al. (2021) aimed to determine and compare the capacity of DEHP (50 mg/kg b.w./day), DBP (50 mg/kg b.w./day), BPA(25 mg/kg b.w./day) and their mixture (MIX: 50 mg/kg b.w./day DEHP+50 mg/kg b.w./day DBP+25 mg/kg b.w./day BPA) to produce testicular toxicity after subacute exposure (28 days) in male rats. The authors also explored the mechanisms behind the changes in the testis by using the *in silico* toxicogenomic approach. Histopathological investigations revealed that testicular toxicity observed in MIX group was more pronounced. Desquamated germinal epithelium cells, enlarged cells with hyperchromatic nuclei, multinucleated cell forms and intracytoplasmic vacuoles were observed more frequently when compared to DEHP or BPA groups. The alterations in the redox status parameters were either more prominent, or present only in the MIX group. *In silico* toxicogenomic analysis revealed that 20 genes linked to male reproductive disorders were altered both in individual exposure groups and in the MIX group. The effects on the metabolism, aryl hydrocarbon receptor (AhR) pathway, apoptosis and oxidative stress could be the most probable mechanisms involved in the subacute testicular toxicity of the mixture of DEHP+DBP+BPA (150).

The combined effects of *in utero* exposure to cadmium (Cd, 0.25 mg/kg/once) and/or DBP (250 mg/kg/day) on fetal testis development in rats were investigated in an *in vivo* study performed on pregnant Sprague-Dawley rats. Cd and/or DBP were applied and the dams were randomly divided into four groups: control, Cd, DBP (250 mg/kg/day), and Cd + DBP. Cd was intraperitoneally injected to the dams on gestational day 12 and DBP was daily gavaged to the dams on gestational day 12 for 10 days. The researchers suggested that Cd, DBP, and Cd + DBP lowered serum testosterone levels in male fetuses. However, Cd and DBP did not alter fetal Leydig cell (FLC) number, but the combined exposure led to a decreased FLC number. Cd did not affect FLC aggregation, while DBP caused FLC aggregation and the combined exposure worsened FLC aggregation. Cd lowered the FLC mRNA levels of certain genes (Lhcgr, Star, Cyp11a1, and Insl3) and DBP lowered the mRNA levels of Lhcgr, Star, Insl3, and Nr5a1. Cd and DBP and their mixture did not lead to decreases in Sertoli cell numbers and gene (Amh, Fshr, and Sox9) expressions. It was suggested that the combined exposure of Cd and DBP exerts antiandrogenic effects synergically *via* targeting FLC development (151).

Human studies

Among men, lower sperm concentration and motility have been reported after higher DnBP exposure in both infertility clinics (152-154) and the general population (155). A population-based study has suggested a weak relationship between maturation timing and urinary low molecular weight phthalates (e.g. DnBP and DiBP) and their monoester metabolites (156). Some studies also showed an increased risk of certain gynecological conditions when humans are exposed to high levels of DnBP (157, 158). Epidemiologic studies that have focused on neurodevelopment in infants (159,160) and

children (45,161-163) also suggest a link between neurodevelopmental disorders and phthalate exposure. On the other hand, Leydig cell hyperplasia ('micronodules') is common in testicular biopsies from men with impaired spermatogenesis and patients with testicular cancer, suggesting this is a feature of TDS (98,164). An American study has examined this new endpoint in a group of 134 boys (2–30 months) and the urinary concentrations of 9 phthalate monoesters were correlated with the AGD. After adjusting for age, the researchers found a marked inverse correlation between AGD and 4 of phthalate metabolites (165). A recent study conducted on 100 umbilical cord blood samples analyzed the levels of bisphenol A (BPA), DEHP, MEHP and sex hormones. Male newborns were physically examined for AGD, stretched penile length (SPL), and penile width after birth. All endocrine disruptor levels were at detectable levels in ~99% of cord blood samples. In covariate-adjusted models, cord blood BPA levels were inversely associated with the SPL of newborns and positively associated with cord blood estradiol levels. Moreover, a marked inverse relationship was present between DEHP levels and the AGD index. The results of this study suggested that *in utero* BPA and DEHP had significant effects on both fetal male reproductive development and cord blood estradiol levels (166).

Conclusion

It is impossible to avoid exposure to endocrine disruptors, as they are abundantly present in the environment. Phthalates are among the most common chemicals in the environment, and they affect and will continue to affect both human and environmental health negatively. Although exposure to phthalates is associated with many pathological conditions, TDS has been underestimated and overlooked. Today, the decline in fertility is a threat for future generations. Exposure to endocrine disruptors, especially different phthalate derivatives, is now recognized as one of the main causes of male fertility decline. Although some of the mechanisms by which phthalates cause TDS are understood, there are some mechanisms yet to be discovered. Scientists must clearly focus on these mechanisms through different *in vitro* and *in vivo* studies. Moreover, comprehensive epidemiological studies should be conducted, and the data of these studies should be well-evaluated in order to inform the society about the harmful effects of phthalates and their relationship with TDS.

Scientists must also suggest new regulations for phthalates. Due to the results of several studies on the toxic effects of phthalates, restriction proposal of four phthalates DEHP, BBP, DBP and DIBP in articles was prepared in 2016 by the European Chemicals Agency (ECHA) and Denmark. The assessment of the proposal by two ECHA Committees confirmed an inadequately controlled risk to human health from the exposure to four phthalates DEHP, BBP, DBP and DIBP in articles. The restriction proposal took into account the cumulative effects and combined exposure to these 4 phthalates from different articles. The restriction on placing articles containing the four phthalates in a concentration equal to or above 0.1% by weight individually or in any combination in any plasticized material on the market is set in a revised entry 51 of Annex XVII to the

REACH Regulation (167). Turkey has also accepted that childcare supplies (like plastic parts of pencils and notebooks, erasers, connectors, scissors, sharpeners, rulers, parcels, tapes, sticky note pads, strips, liquid correctors, crayons, paint supplies, lunch bags, school bags, backpacks, painting bags), food imitation products, swimming aids, gym mats, sun beds, sea beds, plastic prints and accessories in textile design (raincoats, sports soles, plastic slippers) can only contain 0.1% phthalate by their weight (168).

In conclusion, we can suggest that new and less toxic alternatives to phthalates should be synthesized and used in the industry. The decrease in fertility and TDS will pose a significant problem in the near future, as the world's population is getting older and public health services will not be able to overcome this burden. New regulations should be implemented and governments should work together to build a greener and less toxic environment for future generations.

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Sindrom testikularne disgeneze i izloženost ftalatima: Pregled literature

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Kratak sadržaj

Endokrini ometači su hemikalije koje ometaju rad endokrinog sistema i dovode do štetnih posledica u biološkim sistemima. Ftalati su grupa veštačkih hemikalija koje se uglavnom koriste kao plastifikatori i ubrajaju se u endokrine ometače. Takođe se upotrebljavaju kao fiksatori boje i mirisa u kozmetičkim proizvodima i proizvodima za ličnu negu. Pored toga, ftalati su pristuni u mastilima, lepkovima, zaptivačima, automobilskim delovima, alatima, igračkama, tepisima, medicinskim cevčicama i kesama za čuvanje krvi, kao i pakovanjima za hranu. Patološko stanje poznato kao „sindrom testikularne disgeneze“ (TDS) ili „ftalatni sindrom“ se obično dovodi u vezu sa izloženošću ftalatima. Ovaj termin je uveden kako bi se opisao porast promena u reproduktivnom zdravlju muškaraca, poput slabijeg kvaliteta semena (sniženi broj i pokretljivost spermatozoida, a povećan broj abnormalnih spermatozoida), hipospadije, kriptorhizma, smanjenja anogenitalne udaljenosti i raka testisa u ranoj životnoj dobi. Pretpostavlja se da ftalati izazivaju direktne posledice na gonadnim i negonadnim tkivima, ometaju diferencijaciju i morfogenezu seminifernih tubula i pomoćnih reproduktivnih organa i ćelija testisa (kako Sertolijevih, tako i Lejdigovih ćelija), dovode do promena u nivou estradiola i/ili testosterona, smanjuju proizvodnju insulinu-sličnih 3 (INSL3) peptida, narušavaju spermatogenezu i dovode do epigenetskih promena, što sve može prouzrokovati TDS. Ovaj pregled će se pretežno usredsrediti na ftalate kao uzročnike TDS i mehanizme njihovog delovanja.

Ključne reči: sindrom testikularne disgeneze, endokrini ometači, ftalat, poremećaji muškog reproduktivnog sistema
