

Epigenetic Effects of Endocrine Disrupting Chemicals

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Abstract

Recently, there is increasing evidences supported by animal studies emphasizing that exposure to environmental factors, nutritional conditions, xenobiotic chemicals, reproductive factors and low dose exposure to radiations can alter epigenetic programming and concomitantly enhance the risk of developing diseases. Endocrine disrupting chemicals (EDCs) can influence organization of developing brain, which may appear in expression of species typical social behaviors. Environmental exposures to EDCs are shown to increase the susceptibility against many kind of diseases. Currently, there are already several articles, books and journals about the epigenetic effects of EDCs available in media and internet. The aim of this thesis is to review these articles and to evaluate the possible consequences of being exposed to these agents in our daily life for the process of risk assessment of these chemicals. Epigenetic effects do not change the gene sequence. They affect the gene expression and therefore identification of expression profile in sensitive genes to epigenetic effects may be used as a biomarker for disease and environmental exposure. In the future, these epigenetic biomarkers may help for early diagnosis of sensitivity of an individual for adult onset disease or maybe used to prevent the disease before the disease's symptoms develop. When an organism grows and develops, precisely organized chemical reactions activate or deactivate parts of the genome at specific time and locations. The study of these chemical reactions and factors influencing them is called epigenetics. It has been observed that, trans-generationally modified progenies in their life are more prone to different kinds of diseases, such as mammary tumors, prostate disease, kidney disease, testis abnormalities and immune abnormalities. Our health depends on our normal development and reproductive ability. Healthy endocrine system is needed to have normal development and normal reproduction. Few years ago we thought that our life starts with the DNA we receive from our parents, but current studies have shown that we receive more than just DNA from our parents. Famine, stress, fear and even drug use could all leave chemical marks on parent's genetic material. Environmental exposure to EDCs during early development and pregnancy can modify epigenomes and induce trans-generationally asthma, autism, cancer, cardiovascular dysfunctions, diabetes, obesity, schizophrenia, infertility, reproductive diseases and dysfunction later in life. There is evidence showing that EDCs can induce epigenetic gene alterations by which these altered genes can be transferred into subsequent generations.

Keywords: Environment; Epigenetics; EDCs; DNA methylation; Histone modification; miRNA

Abbreviations: Abiotic factor: In biology and ecology, abiotic components or, abiotic factors, are non-living chemical and physical parts of the environment that affect living organisms and the functioning of ecosystems; ADI: acceptable daily intake; AGD: anogenital distance is the distance from anus to genitalia, the base of penis or vagina; AGI: anogenital index, is defined as; AGD/weight (mm/kg); AhR: aryl hydrocarbon receptor; AHRR: aryl hydrocarbon receptor repressor; AVP: vasopressin is a neurohypophysial hormone found in most mammals; Biotic component: biotic components are the living things that shape an ecosystem; BPA: bisphenol-A; BW: body weight; Caspase: essential enzymes for apoptosis; Cdyn: dynamic compliance; CM: chromatin modifiers; COMT: catechol-O-methyltransferase is one of several enzymes that degrade catecholamines such as dopamine, epinephrine, and norepinephrine; Catechol-O-methyltransferase protein is encoded by the COMT gene; CNS: central nervous system; CpG: the site or CG region of DNA where a cytosine nucleotide occurs next to guanine nucleotide in the linear sequence of bases along its length; DBP: dibutyl phthalate (DBP) is a commonly used plasticizer; DEHP: bis(2-ethylhexyl) phthalate (di-2-ethylhexyl phthalate, diethylhexyl phthalate) is the commonly used plasticizer; DES: diethylstilbestrol; DMR: differential DNA methylation regions; DNA methylation: a biochemical process whereby a methyl group is added to cytosine or adenine DNA nucleotides; DNMTs: DNA methyltransferases; EC: European commission; EDM: environmentally induced DNA methylation region; Epstein: European food safety authority; EGF: epidermal growth factor or EGF is a growth factor that stimulates cell growth, proliferation, and differentiation by binding to its receptor

EGFR; Endocrine disrupter: An endocrine disrupter is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary (consequent) to changes in endocrine function; Environmental Epigenetic: focuses on how cell or organism responds to environmental factors or insults to create altered phenotypes or disease; Epigenetic: the study of changes in organisms caused by modification of gene expression rather than alteration of the genetic code itself; Epimutation: heritable change in gene expression that does not change the actual base pair sequence of DNA; ESTR mutation: the abbreviation of expanded simple tandem repeat mutation; Ezh2: a histone-lysine N-methyltransferase enzyme encoded by EZH2 gene that participates in DNA methylation and ultimately transcriptional repression; F₁: the F₁ generation is the generation resulting immediately from a cross of the first set of parents (parental generation); F₃: for crosses which have parents (P) and offspring (Filial generations) F₁=children of parents, F₂=grandchildren, F₃=great grandchildren, etc.; FAS: fetal Alcohol Syndrome (the largest cause of mental retardation in Western world); FDA: US, food and drug administration; Genetic toxicology: a

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branch of toxicology that assesses the effects of chemical and physical agents on hereditary material (DNA) and on genetic process of living cells; Genotoxic carcinogens: they initiate carcinogenesis by direct interaction with DNA, resulting in DNA damage or chromosomal aberrations; HATs: histone acetyl transferases; Hypospadias: a male birth defect in which the urethral orifice is abnormally located on the ventral side of the penis; H3K4me3: histone 3 lysine 4 trimethylation; LOAEL: lowest observed adverse effect level; Mutagenic: capable of giving rise to mutations; Mutation: a permanent structural alteration of DNA; ncRNA: a non-coding RNA (ncRNA) is a functional RNA molecule that is not translated into a protein; NOAEL: no observed adverse effect level; Obesogen: a chemical compound that disrupts normal development and balance of lipid metabolism and some cases leads to obesity; Oxytocin: a mammalian neurohypophysial hormone, secreted by pituitary gland and plays neuromodulatory role in the brain; PCAH: polycyclic aromatic hydrocarbon; Piwi-interacting RNA (piRNA): the largest class of small non-coding RNA molecules expressed in animal cells; Play soliciting behavior in animal tests: like crawling over or under other mouse, pushing others, approaching the other mouse head-on and following others; PND: postnatal day; POF: premature ovarian failure; PVC: polyvinyl chloride; ROS: reactive oxygen species; RR: relative risk; Rr: respiratory resistance; Social behavior in animal tests: side-by-side sitting, grooming their partners and side-by-side interactions; SRY: known as sex-determining region Y (SRY) protein, is a DNA-binding protein (also known as gene-regulatory protein/transcription factor) encoded by the SRY gene that is responsible for the initiation of male sex determination in humans; Nonsocial behavior in animal tests: consists of exploring, self-grooming and sitting alone; TDI: tolerable daily intake; TDS: testicular digenesis syndrome; TFs: transcription factors; TG: transgenerational; Transgenic: an experimentally produced organism in which DNA has been artificially introduced and incorporated into the organism's germline, usually by injecting the foreign DNA into the nucleus of a fertilized embryo; Transposon movement: a movement in which a DNA sequence changes its position within the genome, sometimes it may create or reverse mutations and alters the cell's genome size; USEPA: environmental protection agency in USA; Vasopressin: a neurohypophysial hormone in most mammals; It retains water in our body and constricts our blood vessels; WBCs: white blood cells; XREs: xenobiotic response elements.

Introduction

Health is usually defined as variety of factors. Physical environment, genetics, biological and social factors, economic and cultural factors are all having significant role in human health. Environmental factors play a major role in determining the health and wellbeing of human being, especially among the susceptible populations of young children and aged ones [1].

We live in a world in which the chemicals are non-separating part of our everyday life. Some of these chemicals that we are exposed to can intentionally or unintentionally affect our hormonal system and some of them may interfere with the developmental process of human and wildlife. Exposure to EDCs during early life can affect the developmental process of an individual. Our health depends on our normal development and reproductive ability. Healthy endocrine system is needed for normal development and normal reproduction.

During last few years, there has been an increasing attention to the role of epigenetic changes in production of altered phenotypes and diseases. The European Commission has been active in taking actions on endocrine disrupters since 1995. In 1999, both the European

parliament and the European council started to support a community strategy (COM (1999) 706 final) on endocrine disrupters which is divided in to three different phases. Short term strategy focuses on the establishment of list of substances for further evaluation of their role in endocrine disruption and on the use of existing legislation to control the risk. Medium term actions focus on the identification and assessment of endocrine disrupters as well as further research needed for better understanding of endocrine disruption phenomenon. Long term actions focus on legislative actions to protect human health and the surrounding environment. We are all exposed to the vast majority of environmental chemicals which are affecting our somatic cells, but only environmental chemicals which may affect our germ line cells are able to make epigenetic inheriting changes. In future, there is a hope that epigenetic testing could inform whether there is a potential risk of disease susceptibility in a person.

Small variations in genes can lead to production of a slightly different protein. Most of the mistakes and changes take place when DNA molecule copies itself just before its division. Normally, there is 1 mistake in 100 million base copies. Human DNA consists of 3 billion bases, so approximately 30 mistakes build up each time when the DNA is copied. Some environmental and dietary factors can affect negatively our genetics and increase the risk of this copy machinery mistakes even though, only 2% of the human DNA is specified for protein coding and most of the mutations are occurring in noncoding regions from which no proteins are produced [2].

When an organism grows and develops, carefully organized chemical reactions activate or deactivate parts of the genome at strategic times in specific locations. The study of these chemical reactions and factors that are influencing them is called epigenetics. In other words, epigenetics is defined as heritable changes in gene expression. It can be associated with gene activations or deactivations and does not contain changes in the underlying DNA sequence. It can cause a change in phenotype but no changes in genotype. Several factors can influence the epigenetic changes in the body including the age, the environment we live in, the lifestyle and the disease state [2].

Over the past several decades, pubertal abnormalities have showed a soaring increase in human population. Recently, human sperm count is decreasing gradually in most of countries. It is estimated that human male infertility is increasing and at the moment to be about 10% of the whole male population of the world. This increase in testicular diseases and sperm count deficiency is suspected to be at least partly due to environmental chemicals that we are exposed to environmental exposure to some chemicals during early development and pregnancy can modify epigenomes and by trans-generationally induce asthma, autism, cancer, cardiovascular dysfunctions, diabetes, obesity and schizophrenia later in life. It has been observed that, trans-generationally modified progenies are more prone to different kinds of diseases such as mammary tumors, prostate disease, kidney disease, testis abnormalities and immune abnormalities [3].

Dietary supplements can play an important role in epigenetics, for instance hyper-methylating dietary supplements can provide DNA hyper-methylation and by that, they can regulate the gene expression or alter the transcription and form epigenomes. Methionine supplementation may increase hyper-methylation in specific genomic region of the DNA [4].

There are some bioactive food components which can modulate the DNA methylation and make an individual more susceptible to cancer. Nutritional condition can influence the DNA methylation. Vitamin B6, Vitamin B12, folate, methionine and choline can interact with DNA

methylation process. Animal studies have demonstrated that DNA hypo-methylation can be a result of folate deficiency and may lead to tumor development. Many nutrients, such as phytoestrogen genistein, zinc, selenium and vitamin A can affect the DNA methylation and cause cancer susceptibility [5].

Experimental studies in animal models indicate that, gestational exposure to endocrine disruptors can have trans generational effects and may transfer to the next generation although most of the animal studies have determined these effects only in F₁ and F₂ generations [6].

Environmental factors

When we study environmental science, an environmental factor or eco-factor can be abiotic or biotic factor. The endocrine system regulates the function of different organs and tries to maintain homeostasis in human body. Recently, the scientific community has recognized that exposure to environmental factors such as environmental chemicals can caused adverse effects on the developing organisms and result in lifetime risks of having chronic diseases in adulthood. Many other sort of health problems also incurred by Environmental EDCs activity in exposed men and women. Environmental EDCs can act as a hormone mimic whether they are synthetic or natural EDCs, they can disturb the developing organ systems and cause abnormalities which may not apparent until much later in life [7].

Endocrine disrupting chemicals

Endocrine system contains different glands and the hormones produced by those glands. The activity of glands is controlled directly by the nervous system, pituitary hormones and sometimes hormones produced by other glands. The endocrine system regulates the function of different organs and tries to maintain homeostasis in our body. For instance, cellular metabolism, reproduction, sexual development, sugar and mineral homeostasis, heart rate and digestion are some of the processes controlled by endocrine system [8].

According to the European Workshop in Weybridge, UK, 1996, Endocrine disruption is defined as; "An exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" [9].

There are two different types of endocrine disruption mechanisms, the most specific type of endocrine disruption and non-specific endocrine mechanisms. The primary type mechanisms, include disruption in receptor binding, disruption in hormone synthesis and hormonal metabolism or disruptions in hypothalamus-pituitary-gonadal axis. The non-specific type of endocrine disruption mechanisms include general toxicity or manipulated or altered food consumption. Endocrine disruption is a secondary outcome of toxic effects. In this review, the focus is on endocrine disruption which is a specific type of disruption in hormone synthesis or hormone metabolism. An "endocrine disruptor" has been broadly defined as "an exogenous chemical that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental process or more recently, an endocrine disruptor is described as a compound, either natural or synthetic, which through environmental or inappropriate developmental exposure, alters the hormonal and homeostatic systems that enable the organism to communicate with and respond to its environment.

EDCs have also been defined as chemicals that may interfere with the body's endocrine system and produce adverse developmental, reproductive, neurological and immune effects in both human and wildlife [10].

People are exposed to EDCs passively, e.g., to plasticizers which are present in our daily life and to pesticides which are used for cropping plants in agriculture to produce fruits and vegetables and actively, e.g., in form of medical drugs, like vinclozolin. In many areas of the world burning trash and plastic is still one way to get rid of them. However, this is one of the major sources of dioxin production. People who are living or working close by those burning places may get highly exposed to dioxin. Moreover, in occupations like military forces and airport workers there is a higher risk for being exposed to jet fuels and hydrocarbons.

EDCs may act directly or indirectly on hormone receptors. For example, bisphenol-A can act directly as an estrogen receptor agonist, and DDE and vinclozolin as androgen receptor antagonists. Some EDCs, such as dioxins, may influence indirectly the estrogen receptors [10].

In an indirect disruption, EDCs may disturb the hormone biosynthesis like fungicides and dioxins. They can also disturb the hormonal transportation in blood such as PCBs or even affect the hormonal metabolism or elimination, like dioxins. Some EDCs, like dioxins, can also disturb the feedback controlling process in endocrine system [10].

Estrogen agonists are the best known EDCs. The first evidence on EDCs chemicals was their effects on wildlife in areas contaminated with EDCs. In these contaminated areas, the food chain top species like white tail eagles, showed the first signs of EDCs effects. Their egg shells were thinning by exposure to EDCs. Different kinds of endocrine and morphological changes were observed in Baltic seals and alligators of the lake Apopka in Florida which were feminized by hormonal disruptions caused by exposure to EDCs [10].

The suspected effects of EDC exposure in human populations is decreased sperm quality and increased incidence of testicular cancer. However, the evidence is not yet very strong. In animal models there is a link between exposure to EDC and sperm quality loss or other reproductive disorders. These chemicals do not directly change the genetic codes, but they may alter the way genes are expressed and induce long-lasting changes in the endocrine balance and other functions of the body including brain functions and behavior [10].

The incidence of altered pubertal onset has increased over the past several decades. Early onset of puberty is associated with altered development and growth of the body, and may ultimately lead to increased disease susceptibility [11]. Steroid hormones, particularly estrogens and androgens are regulating the transcription of many other genes and hormones like vasopressin (Avp) and oxytocin (Oxt).

Table 1 describes the classifications of endocrine disrupting chemicals which is mentioned in the cooperative agreement among United Nations Environmental Programme (UNEP) and World Health Organization (WHO). In this table, EDCs are grouped into 11 different categories.

As can be seen in the table, EDCs are originated from very different chemical groups with different characteristics and mechanisms.

Epigenetic effects

The environmental factors, we are exposed to via the air we breathe, the water we drink and the food we eat, may affect the next generations by transgenerational alterations of gene expression. During the process of growth and development of an organism, exposure to ECDs may activate or deactivate some part of the genome in specific time and locations, to cause epigenetic effects. An altered epigenome will transfer to the

Classification	Specific Examples of EDCs'
Persistent and bioaccumulative halogenated chemicals	
Persistent Organic Pollutants (POPs) (Stockholm Convention) (section 3.1.1.1)	PCDDs/PCDFs, PCBs, HCB, PFOS, PBDEs, PBBs, Chlordane, Mirex, Toxaphene, DDT/DDE, Lindane, Endosulfan
Other Persistent and Bioaccumulative Chemicals (section 3.1.1.2)	HBCDD, SCCP, PFCAs (e.g., PF0A), Octachlorostyrene, PCB methyl sulfones
Less persistent and less bioaccumulative chemicals	
Plasticizers and Other Additives in Materials and Goods (section 3.1.1.3)	Phthalate esters (DEHP, BBP, DBP, DiNP), Triphenyl phosphate, Bis(2ethylhexyloxadipate, n-Butylbenzene, Triclocarban, Butylated hydroxyanisole
Polycyclic Aromatic Chemicals (PACs) including PAHs (section 3.1.1.4)	Benzo(a)pyrene, Benzo(a)anthracene, Pyrene, Anthracene
Halogenated Phenolic Chemicals (HPCs) (section 3.1.1.5)	2,4-Dichlorophenol, Pentachlorophenol, Hydroxy-PCBs, HydroxyPBDEs, Tetrabromobisphenol A, 2,4,6-Tribromophenol, Tridosan
Non-halogenated Phenolic Chemicals (Non-HPCs) (section 3.1.15)	Bisphenol A, Bisphenol F, Bisphenol S, Nonylphenol, Octylphenol, Resorcinol
Pesticides, pharmaceuticals and personal care product ingredients	
Current-use Pesticides (section 3.1.1.6)	Atrazine, Carbaryl, Malathion, Mancozeb, Vindoxolin, Prochloraz, Procymidone, Chlorpyrifos, Fenitrothion, Linuron
Pharmaceuticals, Growth Promoters, and Personal Care Product Ingredients (section 3.1.1.7)	Endocrine active (e.g., Diethylstilbestrol, Ethinylestradiol, Tamoxifen, Levonorgestrel), Selective serotonin reuptake inhibitors (SSRIs; e.g., Fluoxetine), Flutamide, 4-Methylbenzylidene camphor, Octyl-methoxycinnamate, Parabens, Cyclic methyl siloxanes (D4, D5, D6), Galaxolide, 3-Benzylidene camphor
Other chemicals	
Metals and Organometallic Chemicals (section 3.1.14)	Arsenic, Cadmium, lead, Mercury, Methylmercury, Tributyltin, Triphenyltin
Natural Hormones (section 3.1.1.9)	1713-Estradiol, Estrone, Testosterone
Phytoestrogens (section 3.1.1.9)	Isoflavones (e.g., Genistein, Daidzein), Coumestans (e.g., Coumestrol), Mycotoxins (e.g., Zearalenone), Prenylflavonoids (e.g., 8-prenylnaringerin)

Table 1: Classification of EDCs.

subsequent generations by germline in the process called; “epigenetic transgenerational inheritance”. Currently, the importance of epi-genotype of disease susceptibility is becoming more and more highlighted.

Epigenetics was described in 1942 by Waddington. He defined epigenetics as “physical nature of genes”. He explained the epigenetics as an interaction between genes and their environment which results as a phenotype. The idea was that, physical arrangement of DNA in nucleus can exert additional control on gene activation, regardless of basic genetic sequences. Later in 1975, Hollyday and Pugh described the covalent chemical DNA modification like DNA methylation of cytosine-guanine (CpG). The epigenetics was introduced in 1990s as the study of changes in gene expression that occurs by modifying DNA methylation and remodeling chromatin without changing the DNA sequence [12].

An epigenetic effect basically means the effects of chemical exposure that is transferred to next generations. When the F₀ generation female is exposed during her pregnancy, the fetus and the germline of the fetus of F₁ generation and also the cells in the gonads of the F₁ fetus that will become F₂ generation are all directly exposed to the chemicals (Figure 2). The first generation for epigenetic studies which is not directly exposed to the chemical is the F₃ generation [2].

Epigenetic effects do not change the gene sequence. They affect the gene expression and therefore identification of expression profile in sensitive genes to epigenetic effects may be used as a biomarker for disease and environmental exposure. In the future, these epigenetic biomarkers may help human for early diagnosis of sensitivity of an individual for adult onset disease or used to prevent the disease before the disease's symptoms develop. The DNA methylation region of sperm can provide an epigenetic biomarker for environmental exposure linked to transgenerational diseases [2].

Epigenetic modifications are good signs to prove that there is a plausible link between environmental exposures and alteration in gene

expression that might end up to disease. Animal studies support the role of environmental epigenetics in disease susceptibility. Recent studies proved the relation between disease susceptibility, environmental exposure and germline mutations on two specific parts of the genes which are coding and promoter regions [2].

Epigenetic findings and follow-up studies on animals are important in a sense that, they suggest the idea of maternal exposure to EDCs like DES. It can alter directly the reproductive system of exposed person and also affect the fetus and subsequent generations. The mechanisms through which the transmission of the disease takes place from generation to another are not fully known but, likely persistent epigenetic changes induced on some genes, in a way that the fate of the tissue or organ is altered can involve in this transmission [7].

Cells in our body have the same DNA, but our body has many different cells such as: neurons, inflammatory, pancreatic and many other types of cells. These cells are differentiated from pluripotent stem cells because of epigenetic silencing or activation of specific genes. Epigenetic changes have the ability to switch genes on or off, they can also determine which proteins could be transcribed [13].

Our DNA contains the instructions for building all parts of the body. DNA is wrapped around proteins, called histones. Together, both DNA and histones are covered with chemical tags which are called epigenome. Histones tightly wrap genes, making them unreadable and thus inactive. It relaxes activated genes, making them easily accessible. Different types of genes are active in different cell types. The DNA code remains same for life, but the epigenome is flexible. Epigenetic tags react to the signals from the outside world, such as diet, stress and changes in living environment. Epigenetic changes are the response of our body to signals coming from the neighboring cells or from the outside environment [2].

Several different types of epigenetic modifications, modify DNA and histones, remodel nucleosomes and incorporate variant histones.

They are thought to involve with mitotic memory. DNA alterations and histone modifications may have a controlling role on the gene expression by forming epigenetic changes although they do not change the DNA sequence. Epigenetic changes are classified as irreversible cell changes if they influence the transcription during cell division process. Methylation of DNA is recognized as long term effect and modification of histone proteins are short term epigenetic effect. First documented epigenetic effects were demonstrated in worms, flies, plants and mammals [14].

Transgenerational epigenetic inheritance is changes in biological traits that are not mediated by DNA sequence and may pass through the next generation. Like all other forms of inheritance, the transgenerational inheritance also must be coded in genome or epigenome of the primordial germ cells. The expression of traits has to be persistent enough to be transmitted through at least 3 subsequent generations after the initial exposure to the environmental EDCs which is called transgenerational inheritance change [15].

Figure 1 shows the transgenerational inheritance in multiple generations. A pregnant woman or P_0 carries epigenetic information at least up to F_2 generations. The fetus and its germ cells of F_2 generation can be also affected by exposure to environmental EDCs.

DNA methylation, histone modification and noncoding RNA are the most important epigenetic mechanisms and they play an important role in transgenerational phenomena. DNA methylation caused by environmental exposure in developing germ cells may become fixed or trans-generationally transmitted. These epigenetic changes in the resulting somatic cells may lead to aberrant gene expression and cause disease susceptibility.

The general understanding is that, in mammals, in the formation of sperm and eggs and also in early embryos, cells are going through a reprogramming stage which wipes away all the methylation marks on genes, except on few genes which are very crucial to early development of embryos. Current studies suggested that methylation marks on additional genes could escape the reprogramming even in the F_3 generations which are not exposed directly to the chemicals. EDCs have the potential to reprogram the germ line and promote trans-generational diseases [3].

Figure 2 shows a general view in which EDCs are binding to steroid receptors and TFs and change the local chromatin state or the expression of various chromatin modifiers such as: DNA modifiers, histone modifiers or ncRNAs. EDCs may change the composition and activity of epigenetic chromatin regulators and by that, they will affect directly the epigenome.

TF refers to transcription factor or sequence specific DNA binding factor and it is a protein which can bind to the specific sequences and thereby it can control the rate of transcription of the genetic information from DNA into messenger RNA. CM refers to chromatin modifiers which are important in key developmental transitions, such as the segregation of embryonic and extra-embryonic lineage in blastocyst which is a stage of embryos development or they play an important role in formation of three germ layers differentiation of adult stem cells and gastrulation. EDCs can bind to steroid receptors or other TFs [15].

Aims of the Work

The aim of this thesis is to review data in articles, books and journals about the epigenetic effects of endocrine disruptors available in media and internet for evaluation of the possible consequences of being exposed to these agents in our daily life. The aims are also to describe

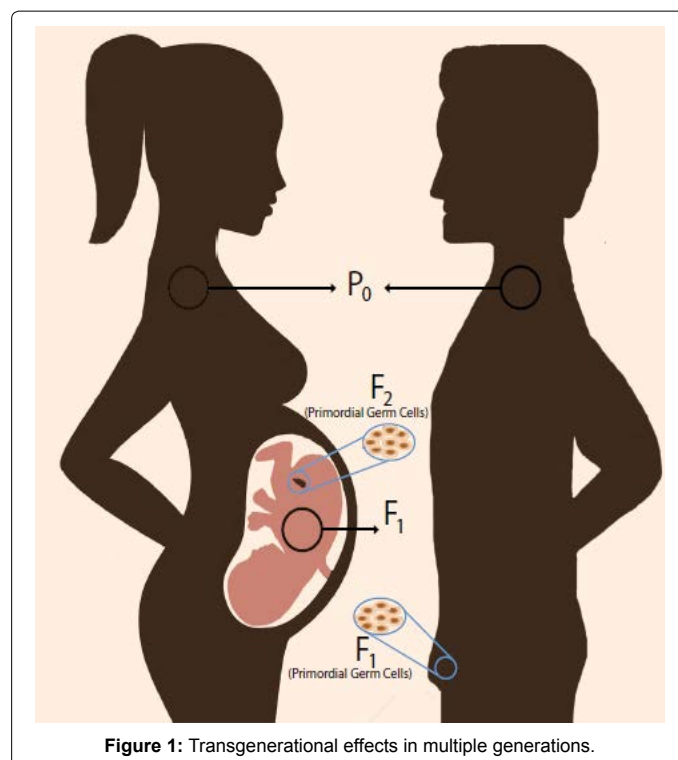


Figure 1: Transgenerational effects in multiple generations.

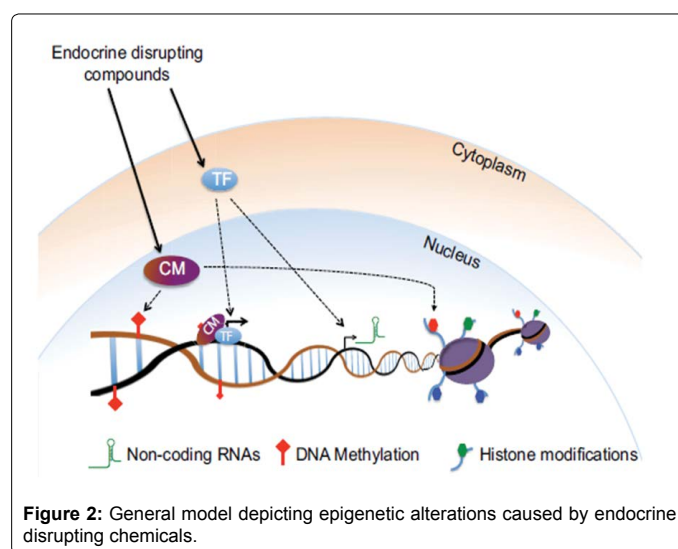


Figure 2: General model depicting epigenetic alterations caused by endocrine disrupting chemicals.

- The concept of epigenetic effects and the mechanisms of epigenetic effects.
- The role and contribution of epigenetic effects of chemicals in adverse developmental and reproductive effects and cancer development.
- The adverse effects of the best known groups of chemicals known to cause epigenetic effects and to give examples on such effects, both in animals and humans.

Features of Epigenetic Programming

Epigenetic transgenerational inheritance is the condition in which all the epigenetic information is transmitted between generations

without any direct environmental manipulations. There are two major types of epigenetic manipulations. First, epigenetic changes are done at DNA level by DNA methylation, which is a long-term epigenetic modification and will remain through next generations. Second, the changes are done on proteins or histones. They are called short-term epigenetic modifications and are typically reversible [15].

Alterations of DNA, modifications in histone proteins and non-coding RNAs (ncRNAs) are three major epigenetic mechanisms. These mechanisms can regulate the way DNA sequence information is being used. We still do not know the exact mechanisms of transmission for epigenetic information done by mitotic or meiotic division in the cell cycle. Histone modifiers such as enzymes that catalyze the modification are also transmitted and can stay bounded into DNA when it is replicating. They can increase the reestablishment of histone marks on sister chromatid. When it's about the germline dependent transmission of epigenetic information, this epigenetic information should be transmitted through the meiosis process [15].

There are some regions of DNA where a guanine nucleotide in the linear sequence is next to cytosine nucleotide, these regions are called CpG or CG sites of the DNA. These two nucleotides are separated by one phosphate and look like “—C—phosphate—G—” but shortly shown as CpG site. The phosphate links cytosine and guanine nucleotides together. The difference between CpG and CG is only existence of phosphate in between. The cytosine in CpG dinucleotides can be methylated and form methyl-cytosine and methylated cytosine can change the DNA expression [16].

The process of replication dependent passive DNA demethylation happens in maternal genome and is completed by blastocyst stage. The genome of inner cell mass is hyper-methylated for about 20% of the CpG sites but in sperm 90% and in oocyte 40% of CpG sites are methylated. In the process of embryonic development, during embryonic day of 6.5 in mice, the genome will undergo de novo methylation for about 70%. The second reprogramming occurs during development of primordial germline cells. These two global epigenomic reprogramming, one during the preimplantation development and the other one during primordial germline cell specification are very well organized processes. There are extensive reprogramming of DNA methylations and histone modifications during these two processes of epi-genomic reprogramming [15].

Several environmental EDCs are known to have the ability to disrupt the normal imprinting process during fertilization or even the germ cell migratory periods after exposure occurs. For instance, low dose exposure of mice from gestational day 12.5 with diethylhexyl phthalate or bisphenol-A, caused hypo-methylation in germ cells and the genes are hypo-methylated until postnatal day 21 when the oocytes were compared with control animals. Moreover, the hypo-methylation persists also in F₂ generations germ cells. EDCs can alter the epigenetic state when they regulate the activity of several (transcription factors) TFs and manipulate the chromatin functional regulators, such as DNA transferase, histone methyl transferase or even ncRNAs. They can also recruit epigenetic regulators and directly change the local chromatin states [15].

During spermatogenesis, the chromatin undergoes several remodeling processes. Mapping studies have shown that the retained nucleosomes are enriched in CpG rich gene promoters which can lack the DNA methylation in mouse spermatozoa. Epigenetic role of histone and DNA methylation mechanisms and several other mechanisms are to be studied in the future [15].

Some examples of epigenetic effects of endocrine disrupting chemicals

What makes chemicals with epigenetic effects special/important for risk assessment: because there is a transgenerational component in their effects the next generation(s) must also be taken into account. (So far only genotoxic chemicals have been thought to have potential effects on future generations when their effects take place in germ cells.) This could potentially alter the paradigm of risk assessment of chemicals.

A good example of multigenerational epigenetic in human is the diethylstilbestrol catastrophe. Children and grandchildren of women treated with DES during pregnancy to prevent stillbirth, still are showing abnormalities and susceptibility to disease. In animal models, these abnormalities are associated with epigenetic changes [17].

Insecticides DDT and permethrin, insect repellent DEET, jet fuel- jp8 (hydrocarbon mixture), plastic additives or phthalates and bisphenol A and dioxin (TCDD) are known to have the potential to trigger transgenerational diseases, such as obesity and ovarian diseases in rats. Manmade steroid like substances for instance; vinclozolin, diethylstilbestrol, methoxychlor and chromium may cause persistent defects through generations.

Reproductive system as a target of EDC effects

Reproductive systems of both male and female are very important target in epigenetics. Exposed individuals can transfer the altered genes into the next generations during their childbearing period. During last few years, human sperm count has had a significant decline and male infertility rate has increased in some areas of the world. Environmentally induced epigenetic transgenerational effects have been suggested to be the main causal effects for these changes. In majority of developed countries, the amount of early pubertal onsets has significantly increased among girls during past several decades [18].

Early onset of puberty may induce behavioral, mental and endocrine related physiological effects on females and increase the incidence of adulthood onset diseases. Changes in primordial follicle pool size and total number of the follicle are decreased in among the female population. The primordial follicle pool develops early in fetal life and the follicle pool size is the ovarian reserve for oocyte production. Environmental exposure to EDCs affects trans-generationally both the total number of the follicles and the size of the primordial follicle pool size. Premature ovarian failure (POF), is the condition of premature follicle loss is one important reason for infertility all around the world. As yet a few years ago was thought that genetic origin is the only fact which may induce POF in female but new studies suggest that environmental exposure to EDCs may also induce female infertility and premature onset of menopause. Epidemiological and animal studies are both emphasizing that, there is a link between in utero and neonatal exposure to EDCs and the adult female reproductive disorders [18].

Androgens are important steroid hormones in the male reproductive system. They are male steroid hormones such as testosterone. Testosterone stimulates the development and maintenance of the male reproductive system and sexual characteristics. Early onset puberty and decreased sperm count are observed in EDCs exposed animals in comparison with control group so EDCs are suspected to cause reproductive and sexual changes in both males and females [18].

Sexual differentiation

The gonads are derived from urogenital ridges. Urogenital ridges themselves are the derivatives of intermediate mesoderm. Wolffian

duct in male and Müllerian duct in female are formed by urogenital ridges. Male sex is determined by Y chromosome, but the gonadal sex differentiation is initiated by activation of the sex determining region of Y chromosome (SRY). SRY activation initiates the testes to transform pre-sertoli cells into sertoli cells. In absence of SRY in females, SRY-related HMG BOX gene 9 or SOX9 gene remains silent and sertoli cells are not formed [19].

Testosterone is metabolized to di-hydro-testosterone (DHT) by 5 α -reductase in prostate and outer part of genitalia. Di-hydro-testosterone is essential for development of scrotum, penis and prostate in a developing male fetus. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) are secreted from pituitary gland to control Sertoli cell proliferation in testis. Secretion of both LH and FSH are stimulated by hypothalamic gonadotropin releasing hormone (GnRH). Testosterone, dihydrotestosterone and the expression of androgen receptors by target cells are known determining factors in male sexual differentiation. Both endogenous and exogenous factors can cause disturbances in the balance of endocrine system which can lead to hypospadias [19].

In the process of sexual differentiation, specific genes induce gonadal differences and these gonadal differences produce hormonal differences and lead to anatomic differences between male and female. Sexual differentiation of the brain is associated with permanent changes in structure and functional potencies of the brain during early development. Epigenetic regulations can be involved in sexual differentiation control of the brain. Sexual differentiation is critical because in the preoptic area histones are associated with estrogen receptors to aromatase the steroid hormones which can lead to male sexual behavior. Estrogen receptor α and Arom are two very essential genes in masculinization of the brain. During the early postnatal period, histone deacetylation is involved in masculinization of the brain. Some EDCs have the potential to play a role as an agonist or antagonist of the estrogen receptors and can influence the brain and hormonal statue during the critical period of sexual differentiation. EDC exposure can cause gonadal and hormonal production and differentiation during development and behavioral changes. For example, males can behave like females [20].

Epigenetic effects of endocrine disruptors on reproductive system

Environmental exposure to endocrine disrupting chemicals may disturb the testis and ovary function and influence fertility. Gradual decline in spermatogenesis, sperm count and sperm motility is observed in exposed populations. The estimated incidence of human male infertility is up to 10% in different populations [21]. This is even worst in female populations. Developing premature ovarian failure which is associated with the loss of follicle pool, promotes female infertility up to 15% of different populations. Part of all these infertilities among men and women may be related to the environmental exposure to endocrine disrupting agents. The basic molecular mechanisms are still unknown. In F₂ generations, exposure to plastics and dioxin promote early puberty onset in male rats [14].

In females, puberty is a developmental process involving hypothalamic-pituitary-gonadal axis which is initiated during fetal development and will mature in adolescence. Plastics induces delayed female pubertal onset in F₁ rats but in F₂ generation, plastics, dioxin and jet fuel promote early onset of puberty in female rats. Vast majority of transgenerational actions on the ovary are showing up as reduction in primordial follicle numbers [14].

By keeping in mind that both testis and ovary are hormone regulated organs and both are producing steroids, one may analyze the hormone level to determine the transgenerational respond of endocrine system in male and female. Anogenital distance (AGD), is regulated by dihydrotestosterone which can be disrupted by some above mentioned endocrine disrupters. Changes in anogenital distance of male F₃ generations was observed by exposure of F₁ pregnant Sprague Dawley female rats to endocrine disrupting agents like; BPA, DEHP and DBP by analyzing AGI in F₃ male progenies [14].

In the study of Manikkam et al. ancestral F₀ female rat generation, exposed to plastics, promoted transgenerational adverse effects such as altered onset of puberty, testicular spermatogenic function and ovarian follicular development in F₃ generation progenies. Onset of female puberty was changed by exposure to plastics, dioxin and jet fuel [14]. (Figure 3A), but not in males (Figure 3B). However, apoptotic spermatogenic cells per testis section were increased when F₀ female rats were exposed to jet fuels (Figure 3C). The total number of ovarian follicles per section was also decreased after all exposure in females (Figure 3D).

During embryonic sex determination, EDCs may re-program the epigenome of developing germ cell. They may also cause alterations in transcriptomes of testis and other organs Figure 2. Shows the schematic mechanisms through which EDCs may cause spermatogenic defects (Figure 4).

Mechanisms of Epigenetic Alterations

Environmentally induced gene expression changes may affect the genome through three different susceptible genomic targets: the promoter region of some housekeeping genes, the transposable elements that lie adjacent to genes with metastable epi-alleles and the regulatory elements of imprinted genes. These genomic targets are recognized as the most susceptible targets for gene expression changes. Their susceptibility is due to their high amount of CpG dinucleotide sequences that are possibly methylated, un-methylated or differentially methylated.

Epigenetic alterations can regulate the gene activity by silencing or activating the gene. These regulations can happen through three different mechanisms: changes in DNA level (DNA methylation), changes in histone or protein level (histone modification) and RNA associated silencing. Figure 5, demonstrates the different epigenetic alterations (DNA methylation, histone modification and miRNAs).

Endocrine disrupting chemicals (EDCs) do not directly change the genetic codes. They do alter the way genes are expressed and help drive long-lasting changes in brain functions and behavior. In addition, they affect endocrine organ systems and growth which increase the disease susceptibility.

DNA methylation

This process is the most studied epigenetic modification as a biochemical mechanism [15]. The methylation of DNA is a chemical process in which methyl group is covalently attached to the DNA [22]. DNA methylation typically happens in the region where cytosine nucleotide is located next to guanine and linked by phosphate. Methyl group is added to the cytosine or adenine. DNA methylation usually takes place at carbon-5 position of cytosine in CpG dinucleotides. This region is called CpG site. CpG site is methylated by DNA methyltransferase (DNMTs). Three DNA methyltransferases are involved in DNA methylation. DNA methylation is a covalent type of DNA modification and it is heritable in cell division. Methylation turns the gene off and inhibits the transcription process but demethylation, turns the gene on [22].

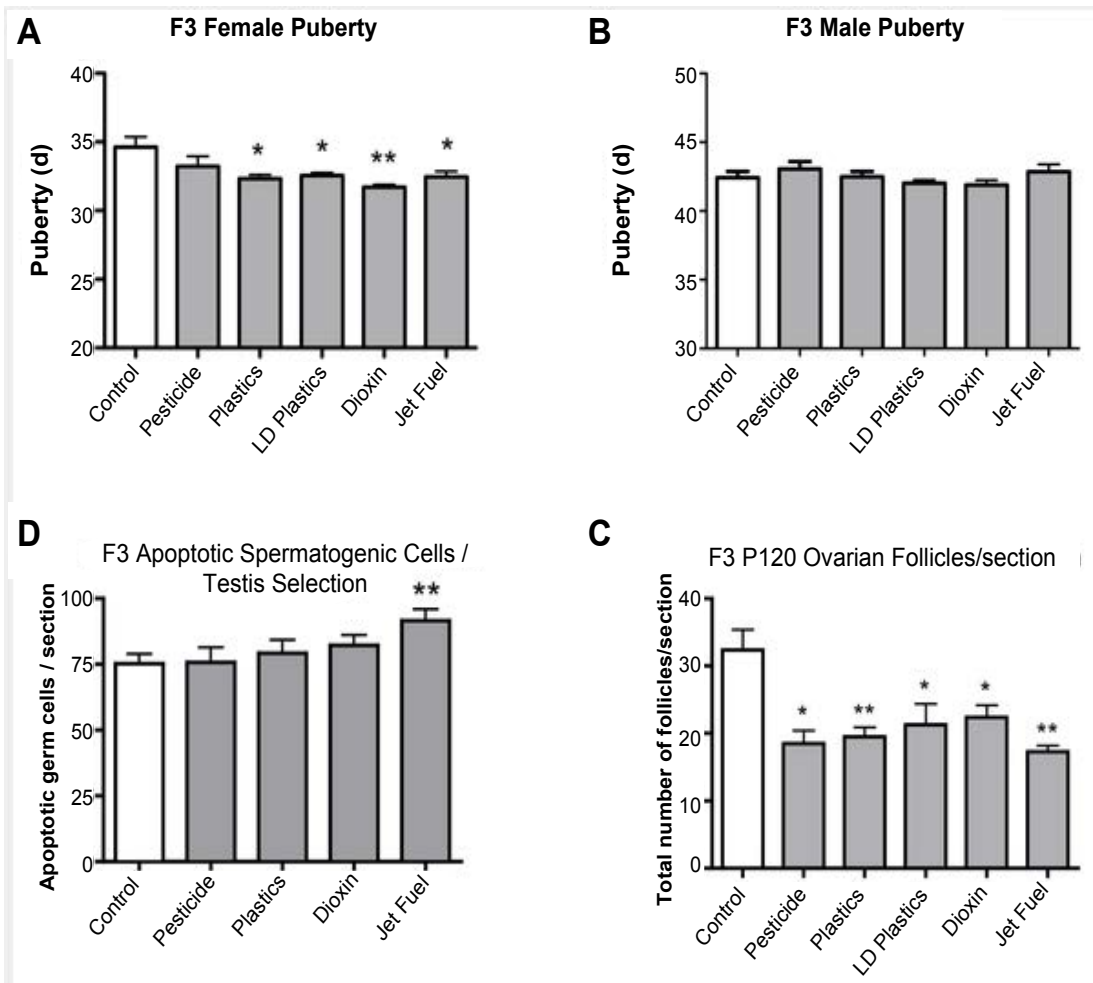


Figure 3: F₀ female rats were exposed to environmental chemicals and the effects were monitored in F₃ progenies.

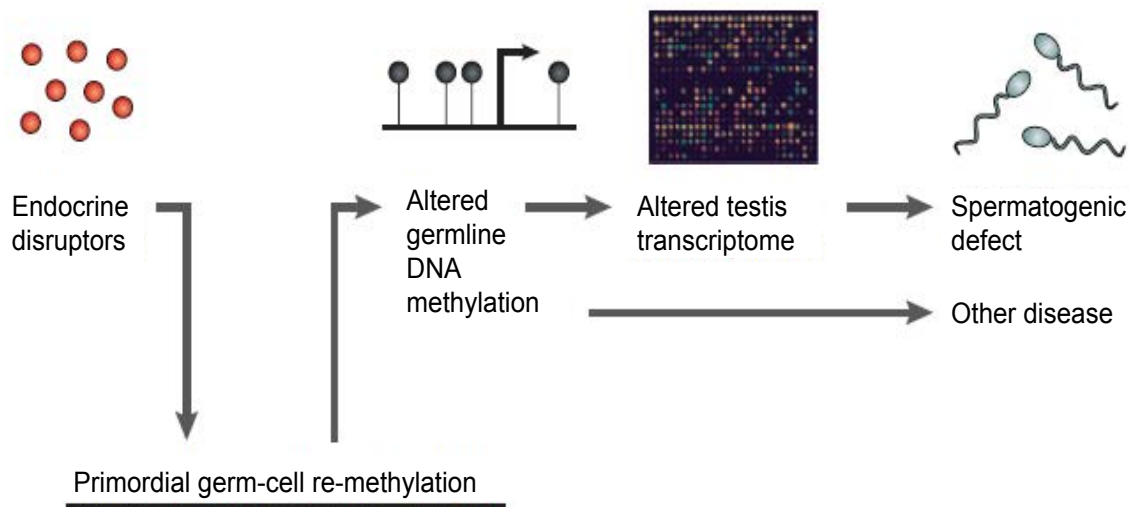


Figure 4: Endocrine disruptors may induce epigenetic transgenerational disease and spermatogenic defects.

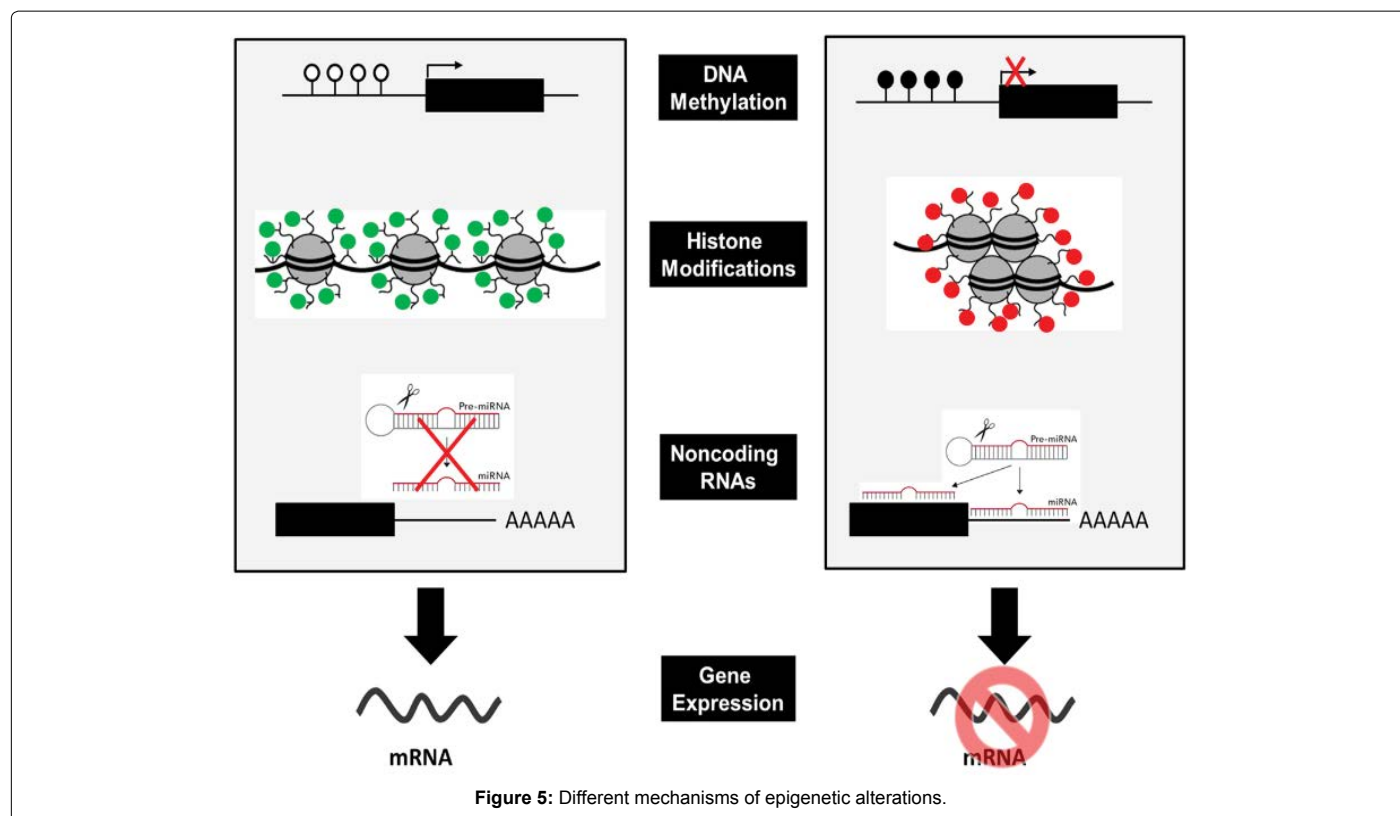


Figure 5: Different mechanisms of epigenetic alterations.

Transgenerational changes in DNA methylation caused by EDCs are globally observed at the specific genome loci [15]. In general, gene promoter hypermethylation is consequently associated with reducing expression of the gene. The result of cytosine methylation is gene silencing and methylated genes cannot be transcribed [22].

The biggest challenge is to understand how the epigenomes are transmitted through cell division. The mechanism involved in transgenerational changes during male sex determination is the reprogramming of germ line or sperm cells. Then the altered sperm epigenome is permanently reprogrammed and escapes the DNA methylation programming at fertilization. These reprogrammed sperm epigenomes allow the transmission of the altered sperm epigenome and produce altered cell or tissue transcriptomes and finally promote transgenerational effects and possible diseases [14].

When a methyl group is bound to cytosine or adenine, this methylated DNA may alter the gene expression as the cell divides and differentiates from embryonic stem cells to specific tissue cells. The results are usually permanent. During zygote formation, DNA methylation is typically removed but some methylation modifications which can regulate the gene expression are heritable and may cause genome imprinting. Global DNA hyper-methylation may cause genomic instability which can increase the risk of cancer. Combustion of the fossil fuels emits different kinds of gases and chemicals. These emitted chemicals may enhance the DNA methylations and stabilize epigenetic modification of mammalian genomes and it is crucial for normal development, proliferation and maintenance of genome stability [23]. Yauk et al. have shown that exposure of mice to air pollutions from industrial and urban environment for 16 weeks caused sperm DNA hyper-methylation. Many of those hyper-methylations still existed after removing the exposure source. DNA methylation caused by environmental exposure in developing germ cells may become fixed or trans-generationally transmitted. DNA hyper-

methylation may be linked to structural changes in chromatin, decreased gene expression and decreased rates of transposon movement. In worst scenarios, DNA methylation pattern disruptions may contribute also to cancer development [23].

Mechanism of cytosine methylation

Cytosine itself is not nucleophilic. Methyltransferase-enzyme is needed to make the cytosine atom more nucleophilic. Then S-adenosyl-l-methionine, which is an electrophilic cofactor, attacks cytosine atom. The cytosine thiolate also attacks cytosine molecules and covalent bonds are formed between cytosine and cytosine sulfur (Figure 6).

Differential methylation region (DMR)

Part of the DNA in an organism's genome that have different DNA methylation pattern is called differential methylated region (DMRs). EDCs can cause differential DNA methylation regions. There are some regions of 2-5 mega-bases size but statistically significantly over presenting of regulated genes in case of epi-mutations and long non-coding RNA. DMRs are on all autosomes and X chromosome. Considering the differential specific regions, there are some short recurring patterns on DNA that are presumed to have more biological functions than others. They indicate sequence specific binding sites for proteins and transcription factors. These short patterns are so called DNA sequence motifs. The DNA sequence motif may promote a region of sensitivity for DMRs to be trans-generationally programmed.

DMRs are considered as epigenetic biomarkers for environmental exposures. When F_0 generation gestating female rats were exposed with dioxin, pesticide, plastics or jet fuel (hydrocarbons) the chromosomal location of DMRs of each specific exposure is shown in Figure 7.

One hypothesis indicates that 5-hydroxymethylcytosine which is a DNA pyrimidine nitrogen base, is associating with active genes and may

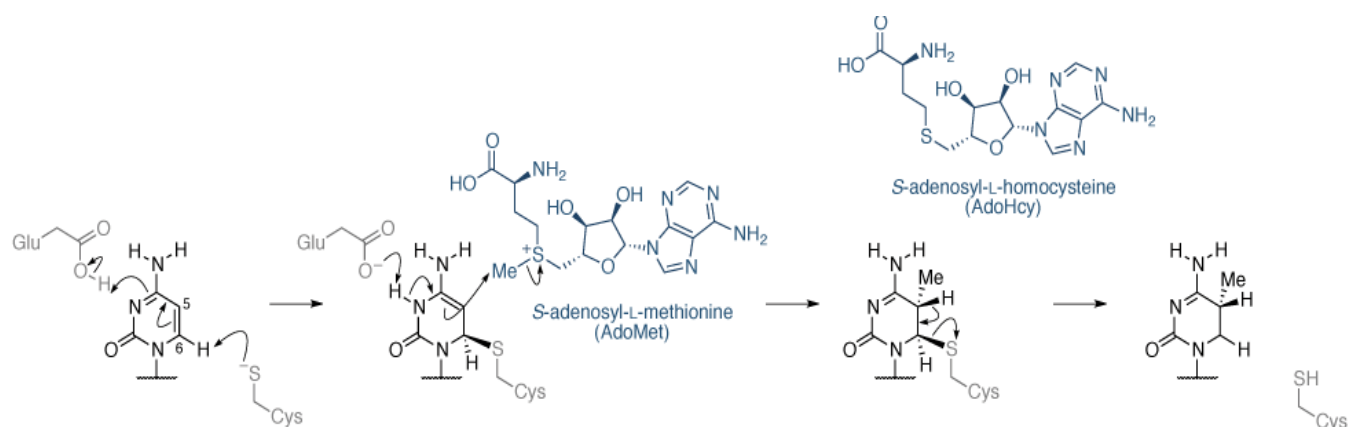
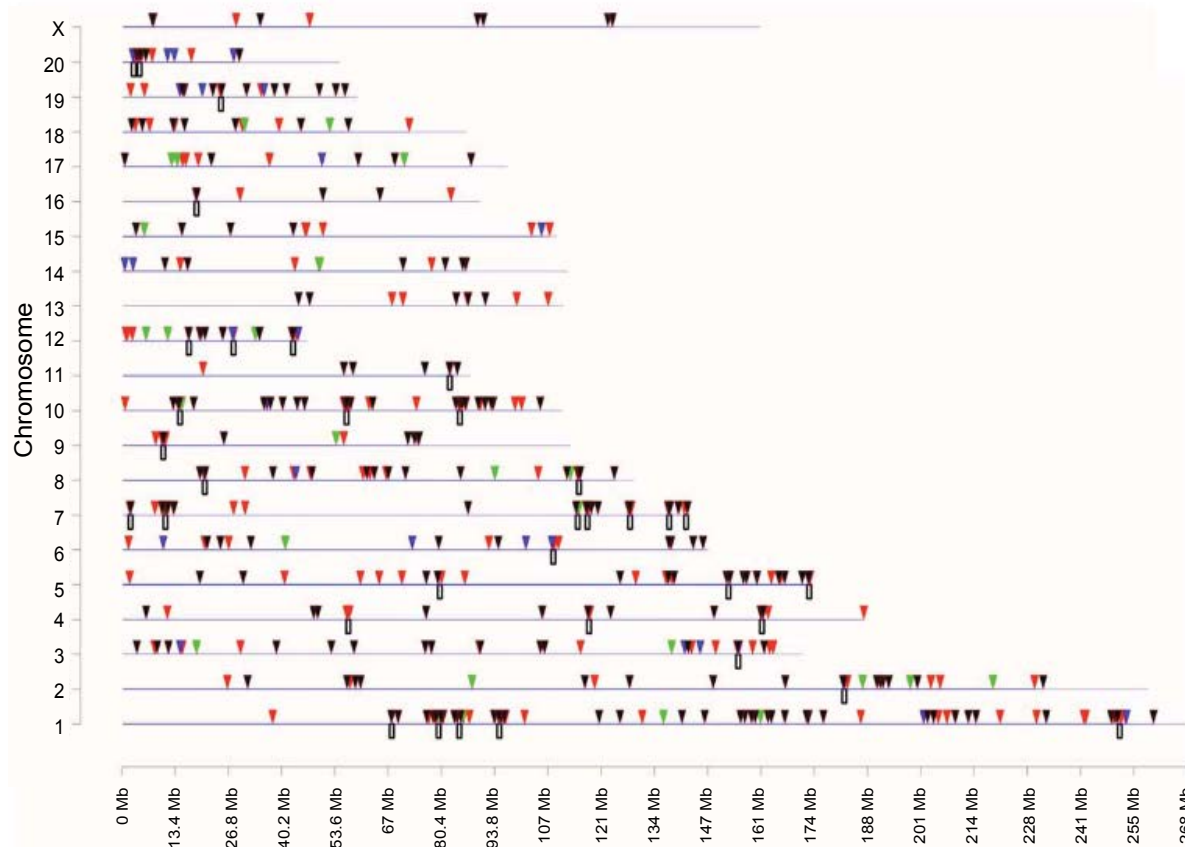


Figure 6: Cytosine methylation mechanism.

Figure 7: Schematic DMRs on different chromosomes in F₃ rat generation after exposure of F₀ generation to plastics (red arrow), dioxin (green arrow), hydrocarbons or jet fuels (blue arrows) and pesticides (black arrows) indicating chromosomal location of each exposure group.

facilitate demethylation at some very important stages of early embryo after fertilization or during the development. Early life environmental factors, for instance therapeutic and behavior conditions may alter the brain programming which affect the brain development and behavior. Exposure of neonates to endocrine disruptors such as BPA, DES with estrogenic potentials, may reprogram estrogen responsive gene and causes uterine tumors [3].

Histone modification

Histones are charged proteins that are surrounded by supercoiled DNA strands with negative charge, wrapped around them. The most complete information about the transgenerational epigenetic inheritance which is caused by histone modification is derived from studies in *Caenorhabditis elegans* [24].

Caenorhabditis elegans is about 1 mm long transparent nematode or roundworm. It does not have DNA methylation [24].

Descendants having mutation in H3K4me3 (Histone 3 lysine 4 tri-methylation) compass or methyltransferase complex, have longer life span. Mutation in lysine specific demethylase or H3K4me2 histone demethylase enzyme can cause small brood sized and sterility in next generations of worms. The same H3K4me2 enzyme is increased in mutant animals and the result is aberrant regulation of spermatogenesis genes and sterility over subsequent generations. GFI1 gene has significant effects on numerous cellular events, such as proliferation, apoptosis, differentiation, lineage decisions and oncogenicity. This gene is part of a gene complex which is able to cause histone modification [15].

Histone modification process is important in the sense that modifications of histones can change the way DNA wraps around them. So it can affect the gene expression. Histone modification is important for action of the cofactors, polymerase binding and maintaining chromatin stability. There are two important histone modifications. Acetylation reaction of a lysine residue and methylation of a lysine and arginine residue. Most of the histone manipulations take place in unstructured, N-terminal tail of the histone (Figure 8).

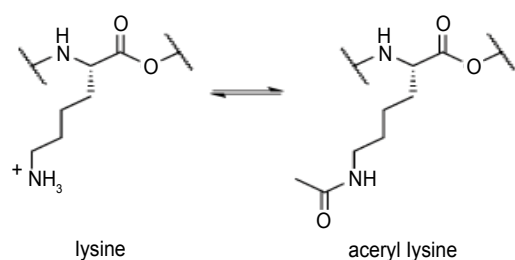
Histone acetylation

This is the most studied epigenetic protein modification, which takes place in alkaline N-terminal tails of the specific lysine residue. Lysine acetylation of histone is usually together with gene activation or increased gene expressions. Histone acetyltransferases (HATs) are the enzymes, which catalyze the lysine acetylation. Positive charge of the lysine residue is neutralized and this charge neutralization is hypothesized to reduce the affinity between DNA and histone and to make the DNA available for polymerases and increase transcription. Acetylation is associated with increase in transcriptional activation while deacetylation is linked with deactivation of transcription process [25].

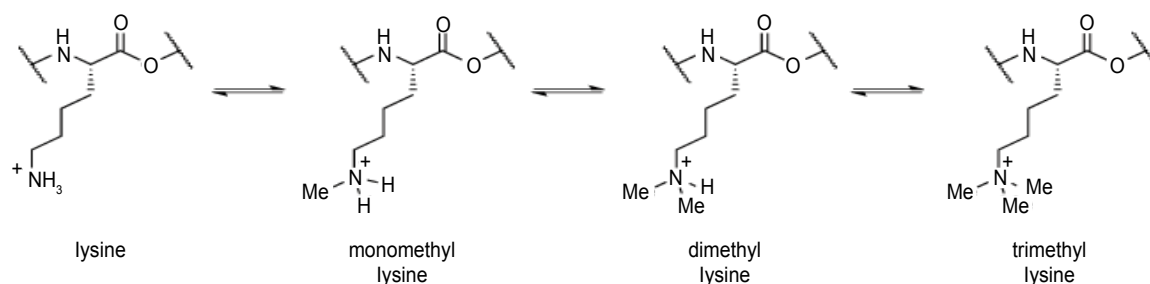
Histone methylation

Histone methylation takes usually place in lysine or arginine residues. Recently histones were recognized as dynamic proteins that are undergoing multiple type of post- translational modifications. Methylation in lysine residue is one of these modifications. There are three different types of methylations in lysine residue: mono-di- and tri-methylation. They are involving in different nuclear functions and transcriptional states.

lysine acetylation



lysine methylation



arginine methylation

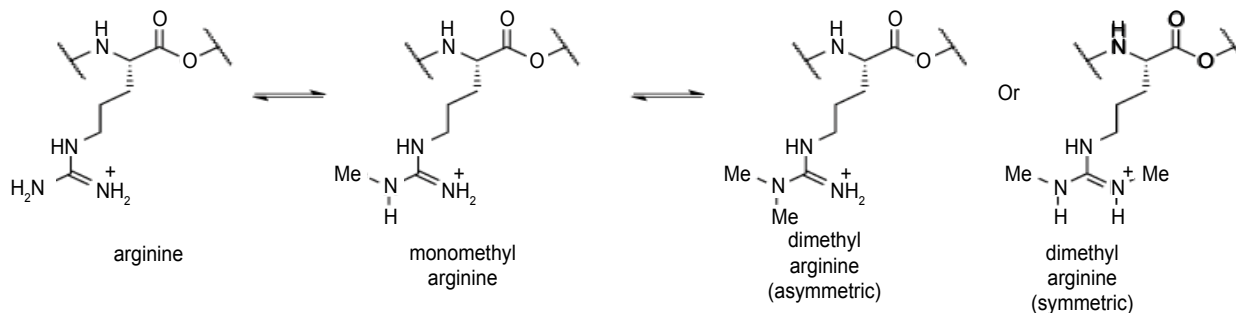


Figure 8: Schematic view of important histone modifications.

There are only few studies done on the effects of environmental chemicals on histone modifications.

Non-coding RNAs

Recently, ncRNAs (non-coding RNAs) have been increasingly recognized to have a major role in epigenetic gene regulations and in global chromatin organization. Thousands of ncRNAs are discovered with different lengths, but the molecular function of most of them is not known yet. They have significant functional roles in gene regulation and chromatin organization. Small ncRNAs mediate epigenetic gene regulations through DNA methylation. All RNAs such as long and short ncRNAs, microRNAs, small nuclear RNAs and piwi interacting RNAs (piRNAs), which are largest class of non-coding RNAs in animal cells, together are significant regulators of gene expression in different organisms from yeast to human being [15].

MicroRNAs and epigenetics

Micro RNAs (miRNAs) are the non-coding type of RNAs. They are single stranded of about 21-23 nucleotides and they are transcribed from DNA, but they are not translated into proteins. The main function of miRNAs is to down regulate gene expression by interfering with mRNA functions [22].

A gene can be turned off or silenced by miRNA by causing antisense, noncoding RNA or RNA interference during transcription process. miRNA can enhance the rate of histone modifications and DNA methylations. miRNA can produce heterochromatin which can alter the gene expression [13].

Examples of Endocrine Disrupting Chemicals with Epigenetic Effects

ECDs are chemicals, suspected to be associated with reproductive functional alteration in male and female. There are some EDCs which are known to have epigenetic effects. These chemicals are suspected to increase the incidence of breast cancer, abnormal growth patterns and neurological developmental defects in children and changes in immune function. Humans may get exposed to EDCs through ingestion of food and nutrients, dust or even drinking water. In addition to ingestion, inhalation exposure to gases and particles in the air and dermal exposure to EDCs through the skin are the three major exposure routes. These chemicals may be transferred from pregnant mother to the developing fetus by passing through the placenta or via breast milk to the infant [15].

In the year 2000, Andrea Cupp was wondering whether the insecticide methoxychlor with the characteristics of endocrine disruption may interfere with the development of ovaries and testes in developing fetus of rats when it is injected to female rats during pregnancy. She observed that the male offspring had lower sperm counts and less motile sperm and when these treated males were bred with the daughters of other pregnant rats which were not treated with methoxychlor, their offspring showed the same sperm defects [26]. They repeated the experiment for 15 times and every time they came up with the same results. Later on Skinner's team repeated the same experiment with another endocrine disrupting agent vinclozolin for longer period of time and they achieved the same results in F₃ and F₄. The result of all long chain of experiments brought up the idea that; "methyl groups added to some genes can suppress their transcription into protein" [26].

Diethylstilbestrol

Diethylstilbestrol is a synthetic nonsteroidal estrogen. DES is classified as a recognized endocrine disruptor. It was first synthesized

in 1938. It is a non-genotoxic developmental stage specific compound. It can induce persistent epigenetic change in developing organs such as uterus and exposure to this compound is associated with increased risk of breast cancer in adult women exposed in utero [22] (Scheme 1).

It is a known carcinogen used to prevent miscarriages in pregnant women. During 1960s, DES was used in beef and poultry industries as a growth hormone [22].

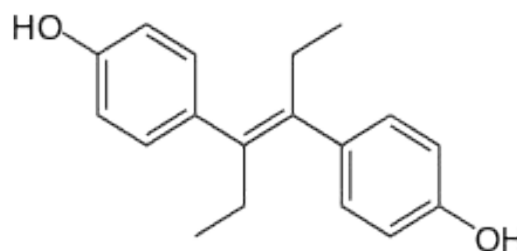
Worldwide estimation about DES exposure is between 2 to 8 million pregnancies treated with this compound during 1938-1971. What we know today about DES is that, this compound is a trans-placental carcinogen, because it can cross the maternal placenta and later in life cause reproductive tract cancer in the progenies. The prevalence of neoplasia caused by DES in exposed population is estimated to be 0.1%, but this is much lower than the prevalence of subfertility and infertility occurred in DES exposed population which is estimated to be >90% and it can occur in both sons and daughters. The reproductive abnormalities that are associated with DES exposure are so called "DES syndrome". Fetus exposure to DES was the first documented EDC-case, which causes long term changes in offspring and these changes are not apparent until later in life and after the onset puberty [7].

The main adverse effect of DES is disturbance in androgenic or estrogenic balance in developing fetuses of humans and animals because it has both estrogenic and anti-androgenic actions. It can compete with natural androgens for binding to androgen receptors in *In vitro* [27]. Exposure to DES produces low birth weight children and the kids are more susceptible to TDS or testicular digenesis syndrome in an *In vivo* test [15].

Animal evidence: When animals are directly exposed to DES, the effects on F₁ and F₂ generations of mice was vast. From rare genital tract cancers, structural and cellular anomalies of vagina, uterus and fallopian tubes to sub-fertility and infertility to menstrual cycle irregularities. Over the last 35 years, rats, mice hamsters or non-human primates were tested with DES and changes were compared with those exposed men and women. Prenatal exposure to DES in mice has raised the possibility of transgenerational effect in occurrence with hormonal factors in critical period of penile and urethral development are associated with hypospadias [28].

Animal studies at the beginning showed increasing transgenerational susceptibility for malignant type of tumors of the female reproductive tract which is probably the result of germ cell alterations and abnormal imprinting. On the other hand, exposure to DES in human beings may cause permanent changes in germ cells [27].

Exposure to estrogenic compounds during critical period of pregnancy can induce teratogenic and carcinogenic lesion in reproductive tissues of human and animals. Studies in female mice have



Scheme 1: Diethylstilbestrol (DES).

indicated that exposure to DES during critical developmental period can end up in persistent epigenetic alterations containing manipulations in gene expression. Primordial germ cells are undergoing demethylation when they are migrating and also during early colonization of embryonic gonad and at the time of sex determination and DNA re-methylation starts in a sex specific manner. Sufficient re-methylation alteration of germ line in male fetus is recognized in exposed pregnant mothers at a time of fetal male sex determination. Permanent reprogramming also happens in imprinted DNA methylation process. Neonatal exposure to DES affects expression of two genes, lactoferrin and epidermal growth factor (EGF) genes. Exposure to DES during neonatal period leads to persistent ovary independent induction of mRNA and protein encoded by these genes in mouse vagina and uterus. DES can cause expression of lactoferrin, EGF or even other estrogen regulating genes and may lead to permanently estrogenized phenotype and abnormal tissue morphogenesis or abnormal tissue function. The same study showed higher incidence of tumors in male and female mice progenies when they are prenatally exposed to DES [29].

Pre and neonatal exposure to DES can cause wide range of gene expression changes in mice. There is an indirect relation between DES and methylation changes. For example DES induces inhibition of catechol-O-methyltransferase (COMT) enzyme which adds a methyl group to the catecholamine [22].

Exposure to DES in the mouse model can cause abnormalities such as oviductal malformation. In molecular scope, cellular abnormalities caused by DES are associated with altered programming of genes such as Hox and Wnt. These two genes are known to play a very important role in reproductive tract differentiation. These findings and follow-up studies are important in the sense that, they suggest the idea of maternal exposure to EDCs like DES can alter directly the reproductive system of exposed person and also affect the fetus and subsequent generations. The mechanisms through which the transmission of the effect from generation to another occurs are not known but, likely persistent epigenetic changes induced in some genes contribute in a way that the fate of the tissue or organ is altered [7].

Prenatal exposure to DES in mice has raised the possibility of transgenerational effect in occurrence of genital malformation in males. During the first trimester of pregnancy, the role of androgens is crucial for male genital development. Changes in androgen or estrogen balance due to exogenous or even endogenous hormonal factors in critical period of penile and urethral development is associating with hypospadias. Incidence of reproductive tract tumors in male and female descents of mice which are developmentally exposed to DES is significantly increased [27].

Human evidence (normal population, highly exposed population and smokers): Humans were exposed to diethylstilbestrol through several different sources, for example as dietary ingestion from supplemented cattle feed and drugs. Many cancers such as breast and prostate cancers are associated with the use of DES. In 1971, DES was recognized definitively to cause clear cell carcinoma in a form of rare vaginal tumor in ladies who were exposed to DES. The drug is nowadays withdrawn from use in pregnant women but due to some anti-carcinogenic characteristics of this compound, it is still in limited use for cancer treatment. The prevalence of different disease starting from cellular and structural anomalies of vagina, uterus and fallopian tube, such as infertility and estrous cycle irregularities to rare genital tract cancers are much higher in daughters of exposed mothers compared with non-exposed daughters of a control group [28].

In 1970s, between 2 and 4 million women were exposed to DES while they were pregnant. After this catastrophe, DES exposed men and women were born from the exposed mothers and they became a model through which delayed effects of gestational exposure to hormones or other chemical compound were observed. Various mechanism underlying cell differentiation and organogenesis may play a major role in promoting the toxic effects of DES during development. In the case of DES exposure, a specific base of the DNA was methylated in specific parts of the genes and regulates the gene expression and underlies cell differentiation [28].

The prevalence of hypospadias was very low in unexposed boys whereas it was very high in the in utero exposed boys (3.57%). Moreover, the prevalence of hypospadias was higher among the third generation boys (8.2%) in comparison with non-exposed control group parents. This can clearly show the transgenerational effects of DES on F₃ generation and it is even higher than previous generations [27].

In the study of Hoover et al. 4653 in utero exposed women and 1927 unexposed women were studied for a continued long-term follow-up. Twelve different adverse outcomes of DES exposure were assessed, with regards to 45 years of age for reproductive outcomes and 55 years of age for other outcomes in subsequent generations and the relationship between exposure and vaginal epithelial structural changes observed. Women with vaginal epithelial changes were at higher risk for adverse outcomes of DES. Table 2 shows the comparisons between exposed and non-exposed group of the study.

As can be seen, high risk of a broad spectrum of adverse health outcomes were associated with *in utero* exposure of DES in women [30].

Exposure of humans to DES may lead to permanently altered germ cells. Primordial germ cells are undergoing demethylation process during their migration and early colonization of the embryonic gonads, which is associated with re-methylation at the time of sexual determination of the fetus. When pregnant mother is exposed to DES at the time of the sex determination of fetus it can lead to altered re-methylation process of the germ line and cause permanent reprogramming and imprinted DNA methylation in male fetus [27].

Exposure to DES in men can cause endocrine disrupting activity on genital development. The risk of reproductive tract abnormalities is increasing in DES case sons and it can manifest in different forms. Hypo-plastic testis, epididymal cysts, cryptorchidism or hypospadias are some examples of these malformations in men [27].

Adverse outcomes of DES exposure	% effects in exposed group	% effects in non-exposed group	Hazard ratio %
Infertility	33.3	15.5	2.37
Spontaneous abortion	50.3	38.6	1.64
Preterm delivery	53.3	17.8	4.68
Loss of second trimester pregnancy	16.4	1.7	3.77
Ectopic pregnancy	14.6	2.9	3.72
Preeclampsia	26.4	13.7	1.42
Stillbirth	8.9	2.6	2.45
Early menopause	5.1	1.7	2.35
Cervical intraepithelial neoplasia	6.9	3.4	2.28
Breast cancer at 40 years or higher	3.9	2.2	1.82

Table 2: Comparison of adverse outcomes of DES exposure between exposed and non-exposed women in a cohort epidemiological study.

Hypospadias is a congenital birth defect in boys characterized by dysplasia of ventral penis and urethra. Often several surgeries are needed to correct the defect. The general incidence rate of hypospadias is 1 in 150 new male births. The increased risk of hypospadias due to mother's exposure to DES in utero was the first suggestion of transgenerational inheritance in humans. When the baby is a girl and the pregnant mother is exposed to DES, the risk of reproductive structural abnormalities, vaginal and cervical cell adenocarcinoma, infertility, miscarriage, ectopic pregnancy and premature birth is much higher than normal [19].

There is no monotonic dose response relationship for DES exposure and effects are varying differently depending on the time of exposure during development of different organisms. Even small maternal occupational exposure to DES during pregnancy which can happen through food or clothing can be contributed to hypospadias malformation of the third generation boys in human [27].

Exposure to DES can cause vaginal cancer in both exposed mothers and their subsequent generations. Furthermore, in the second generation of DES exposed females, menstrual irregularities are reported. Moreover, in third generation daughters, increased incidence of ovarian cancer has been reported [7].

In an epidemiological cohort follow up study on five hundred twenty-nine families hypospadias also on the second and third generation offspring's were studied. Figure 9 details the number of hypospadias cases:

When a mother is exposed to DES, her son is at 4 times more risk of having hypospadias than non-exposed mothers [31].

Exposure to DES in pregnant women caused fertility problems with the prevalence ratio of 21.3 [31]. DES reduced fertility but infertility treatment might intermediate factors in the causal pathway to increase the risk of son with hypospadias. Among the 583 hypospadias cases

retrieved from hospital information system and the 251 non-exposed pregnant women as referent group, the odd ratio (OR) with 95% confidence intervals (CI) for association for DES induced hypospadias was estimated to be OR=2.3 (95% CI 0.7-7.9) [19]. Another study estimated that the prevalence risk of hypospadias among sons of exposed mothers to DES was more than 3% [27].

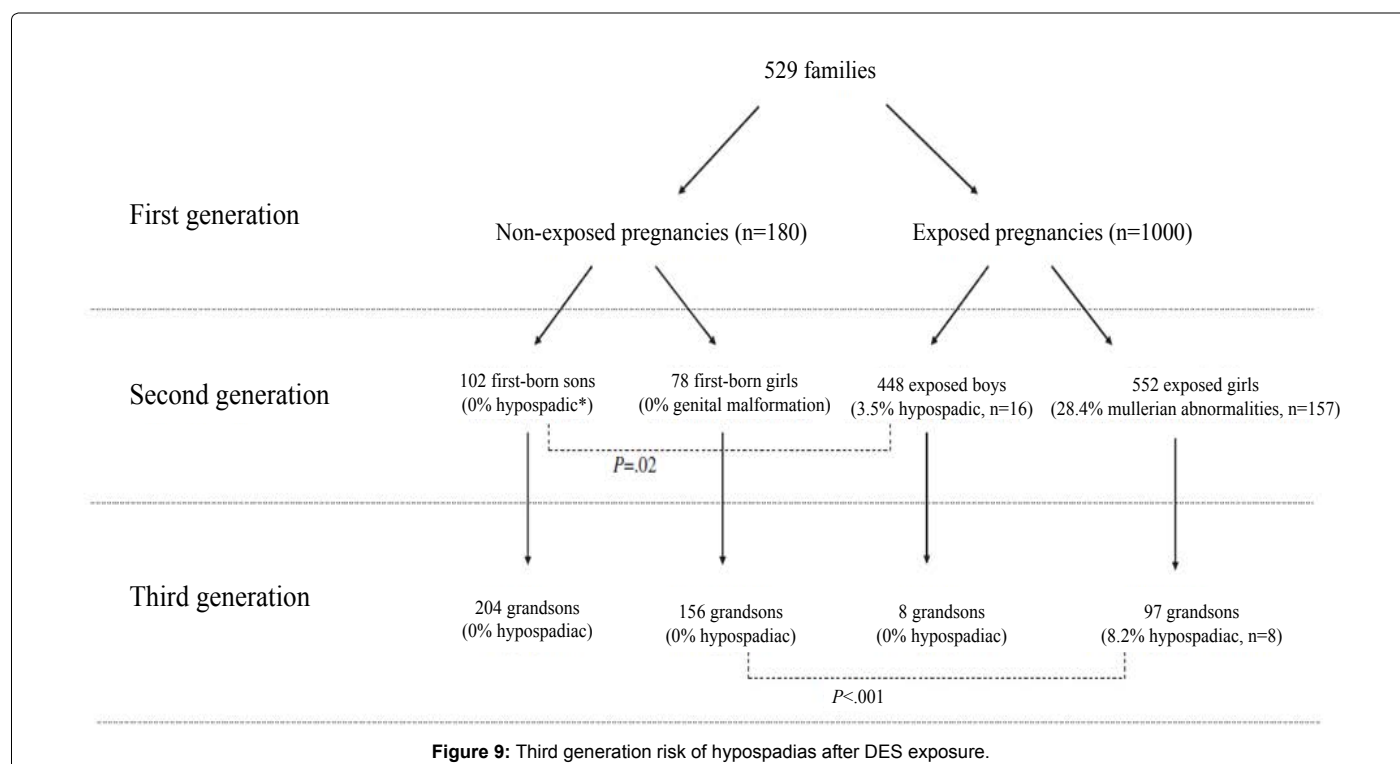
Human exposure to DES during pregnancy increased the relative risk of breast cancer from 1% up to 1.4% among the exposed mothers. The daughters of the exposed women had a relative risk of 2.5 for developing breast cancer when they were more than 40 years old in comparison with unexposed women of the same age [32].

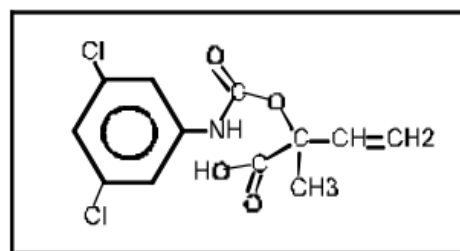
One epidemiological multi-central cohort study investigated the prenatal DES exposure based on the mothers report and self-report of birth defect individuals [29]. 4029 sons and 3808 daughters were born with DES caused birth defects. The follow-up sub cohort study of 793 third generation daughters with birth defects indicated the birth defects and cardiac disease elevations among sons of up to 1.53% and for daughters up to 2.35% in prenatal maternal DES exposed cohort group. Particularly daughter progenies are more susceptible to birth defects. Table 3, indicates the type of malformations caused by DES [29].

The proportion of cardiac defect that were caused by DES exposure among males of the exposed and non-exposed group were the same (0.7 and 0.6 respectively%), but among the exposed females 0.8%, which is significantly higher than non-exposed control females with cardiac defects (0.2%) [29].

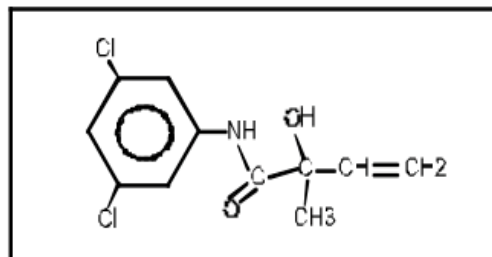
Vinclozolin

Vinclozolin is a common dicarboximide fungicide which is used in vineyards to control plant diseases such as blights, rots and molds. It is a non-systemic fungicide for use on raspberries, lettuce, kiwi, canola, succulent beans and dry bulb onions in the United States (Figure 10). BASF Company is the manufacturer of vinclozolin. It is classified as possible human carcinogen [33] (Scheme 2).



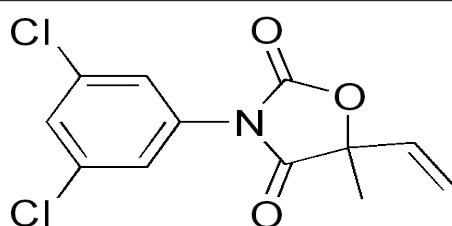


M1 or metabolite B



M2 or metabolite E

Figure 10: Two metabolites of vinclozolin.



Scheme 2: Vinclozolin.

Malformations caused by DES exposure in children when their mother was exposed during pregnancy

Heart defects	Atrial septal defect and ventricular septal defect.
Skeletal anomalies	Hip dysplasia, scoliosis, club foot, missing limbs or extra digits.
Heritable or chromosomal defect	Down's syndrome, Noonan's syndrome or Williams's syndrome.
Neurological anomalies	Cerebral palsy and anencephaly.
Genitourinary anomalies	Kidney, renal agenesis and dysgenesis or even born with one kidney or double kidney, horseshoe shaped kidney.
Skin anomalies	hemangioma

Miscellaneous conditions, benign tumors, cysts, cleft palate, eye and vision anomalies or ear and hearing anomalies, learning disabilities, blood disorders, muscle or musculoskeletal anomalies and gastrointestinal anomalies.

Table 3: The association between mothers DES exposure during pregnancy and presence of a birth defect in children of exposed mothers.

There are two different metabolites produced after vinclozolin exposure. These metabolites are known as M1 or metabolite B which is a reversible hydrolysis product or M2 or metabolite E which is an irreversible product of metabolized vinclozolin. Lethal dose or LD₅₀ for vinclozolin is 10,000 mg/kg which indicates low acute toxicity of this compound [33].

This compound can circulate through the water and air, and can end up to untreated foods and vegetables as well as treated ones. Vinclozolin was one of the first EDC known for having transgenerational action. This compound has been widely used in agriculture since 1984. It is still in limited use for commercial and industrial purposes. This compound can cause both hypo-methylation and hyper-methylation of DNA [15].

Animal evidence: Vinclozolin is known as an endocrine disruptor, which can cause transgenerational pathogenesis in rats and lead to spermatogenic defect, infertility among male rats, breast cancer in female rats, kidney disease, prostate disease in male rats and immune system abnormalities. These effects can be transmitted to majority of their progenies for four generations through male germ line and affect both males and females of subsequent generation. Vinclozolin treated rats were found to have altered epigenome and transcriptome [15].

Vinclozolin at high dose can reduce male sex organ weight. It can cause the male sex organ malformations such as reduced penis size, ectopic testes, vaginal pouches, hypospadias or some other additional ambiguities of urogenital system, reduced fertility caused by hypospadias, delayed puberty in female rats and kidney stones [15]. They all are related to high dose exposure to vinclozolin. The most androgen sensitive effects are noted at low dose levels (>3 mg/kg/day) of vinclozolin in rats. These androgen sensitive effects in male rats are, reduced prostate weight, reduced weight of sex organs, nipple development and decreased anogenital distance. Vinclozolin can induce a hormonally mediated increase in number of Leydig cell tumors in rats. It is regarded as its threshold response [33].

The acute dietary risk assessment for female of child bearing age has been conducted. No observed adverse effect level (NOAEL) for a single dose, 6 mg/kg/day is assumed to be the safe dose for acute oral exposure. This dose is taken from the oral exposure of rat. Ventral prostate weight loss in rat male offspring is the most sensitive indicator of acute anti-androgenic developmental toxicity. The lowest observed adverse effect level (LOAEL) for rat is estimated to be 11.5 mg/kg/day. Histopathological changes in liver, lesions of the lung, ovaries and eyes are observed at the LOAEL of 2.3 mg/kg/day in rat in a chronic carcinogenicity study. The NOAEL for chronic exposure of rat is determined to be 1.2 mg/kg/day [33].

A number of different genes were expressed in the development of polycystic ovary disease and primary ovarian insufficiency, which is a sign that those genes are somehow involved in disease appearance. Also trans-generationally altered transcriptome and of epigenome were observed in somatic Sertoli cells of the testis, which is the sign of male infertility caused by ancestral exposure to vinclozolin [34].

When male fetuses of rats and mice were exposed to high dose of vinclozolin in utero, the outcomes in adulthood were higher rate of germ cell death, disease like changes in prostate, kidneys and also other tissues for at least three generations. 75% of the exposed animals to vinclozolin developed different kinds of adverse effects in adulthood. Moreover, 34% had two or more diseases caused by daily intraperitoneal injection of 100 mg/kg vinclozolin per day [35].

Effects on rat sperm count and sperm motility were more severe than in mice, when the dose, exposure time, observation time and all other conditions were the same for both species [15].

When gestating female rats were exposed to 100-200 mg/kg vinclozolin by intraperitoneal injection during late embryonic or early postnatal period, effects on sexual differentiation and reproductive function were observed. The offspring of exposed animals had less oocytes and less primary follicles in mature ovaries. These are the result of germ cell death in EDC exposed lineage. Also the behavioral changes

were significant in vinclozolin exposed offspring. Female rat showed more anxiety and behavioral changes in their mating [32].

In the study by Nilsson and Skinner, 11 different adult tissues genes expressed differentially between controls and vinclozolin treated animals. Vinclozolin can affect steroid hormone receptors, such as progesterone, estrogen and androgen receptors. Exposure of rats to vinclozolin resulted in disrupted spermatogenesis, changes in adult mate preference and anxiety behavior [36].

Vinclozolin impairs male rat fertility and promotes transgenerational phenotype of spermatogenic cell apoptosis. In F_3 generation female rats, vinclozolin caused reduction in total follicle numbers. The majority of transgenerational changes in gonadal functions and pubertal onset are relatively between 6-12 months of age [37].

Vinclozolin promoted permanent epigenetic DNA-methylation and lead to changes in sperm in both rat and mouse and causes several different transgenerational diseases in adulthood. Vinclozolin exposure causes testis disease, prostate disease, kidney disease, immune system abnormalities, tumors, and uterine hemorrhage during the pregnancy and poly cyclic ovarian disease in F_3 generation of mice [37].

High incidence of defect which was up to 90% of vinclozolin exposed rats in all three generations and absence of abnormalities among female line brought the concept of gametic epigenetic inheritance for this compound. Exposure of germ cells at specific developmental stage is necessary to produce heritable epigenetic mutations [22].

Findings in human populations: Vinclozolin has low acute toxicity by oral, dermal or even inhalation exposure. It can be a skin sensitizer in case of dermal exposure. The principal toxicity of vinclozolin is related to anti-androgenic capacity of this compound. The US Environmental Protection Agency believes that chronic exposure to vinclozolin can express carcinogenic risk. Vinclozolin is used to treat turf. There are no risk concerns for children or adults playing for example golf on treated sod. However the risk of the direct dermal contact of children with treated sod is of concern [33].

Food-derived vinclozolin derived food cancer risk estimate is about 2.6×10^{-7} compared with non-exposed people. Aggregate acute or chronic exposure to vinclozolin is not of serious concern. Workers who are handling this compound should be equipped and containers should be labeled to prevent the risk of vinclozolin exposure [33].

The first documented transgenerational epigenetic observation of disease susceptibility demonstrated by increase in spermatogenic cell apoptosis after ancestral exposure to vinclozolin. Afterwards, the increased incidence of a wide variety of health abnormality and disease susceptibilities, like; prostate disease, kidney disease, mammary tumor development, immune abnormalities, behavioral effects on reproduction, stress response and obesity have been reported after exposure [33].

The key figures (NOAELs, LOAELs and other reference doses) for health risk assessment are indicated in Table 4. According to Table 4 the overall anti-androgenic effects of vinclozolin has a NOAEL of 1.2 mg/kg/day and if this is divided by 1000=1.2 µg/kg/day can be regarded as a safe dose for chronic exposure of humans.

The United States Environmental Protection Agency (EPA) has reported the non-carcinogenic tolerable daily intake (TDI) value of 0.025 mg/kg/day for oral exposure to vinclozolin (EPA 2007). Acceptable daily intake (ADI) of vinclozolin in food is assumed 0.005 mg/kg/body weight (FSA 2008).

Chronic exposure in food to vinclozolin is assessed as 0.000034 mg/kg/day for female of child bearing age and 0.000078 mg/kg/day for 1-6 year old children [33]. This is only the estimated exposure in food. However, possible exposure to drinking water may result from soil and dermal contamination. Drinking water exposure was found to be 41 µg/day for general population and 11 µg/day for children at the age of 1-6 years old. Non-dietary exposure to vinclozolin has been estimated 5 mg/kg/day for children playing on treated turf [33].

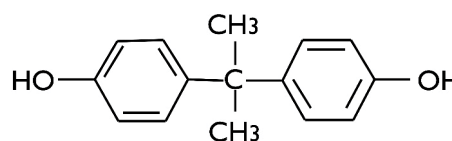
Bisphenol-A

BPA is a known EDC. This monomer is commonly used in the production of several products. Approximately, 6 billion tons of BPA is produced annually [32]. BPA is a plasticizer used in industry to soften and shape plastic materials. It is present in various products for daily use: Polycarbonate plastics, epoxy resins, food containers, papers, dental sealants and many others. Bisphenol-A is detected in human blood and urine, due to ubiquitous use of BPA-containing products (Scheme 3).

Bisphenol-A is an endocrine disruptor which can induce reproductive capacity impairment, obesity promotion and metabolic diseases in general [38].

Exposure	Dose (mg/kg/day)	Endpoint	Study
Acute dietary (female 13+)	NOAEL=6.0 LOAEL=11.5	Decreased ventral prostate weights in offspring observed at 11.5 mg/kg/day.	Perinatal developmental toxicity-rat
Acute Dietary (Adult Males, Infants, and Children)	Acute 0.06	This assessment is not required. There were no toxicological effects applicable to these populations and attributable to a single exposure dose.	Observed in oral toxicity studies including the developmental toxicity studies in mice, rats, and rabbits.
Chronic (Non-cancer) dietary	NOAEL=1.2 LOAEL=2.3	Histopathological lesions in the lungs males, liver males, ovaries females and eyes both sexes at the LOAEL does.	Combined Chronic Toxicity/ Carcinogenicity-Rat
Chronic (Non-cancer) dietary	Chronic 0.012	Carcinogenic dietary risks have been estimated.	
Overall Anti-androgenic Effects (Carcinogenic Dietary)	NOAEL=1.2 LOAEL=2.3	Anti-androgenic mode of action. The chronic NOAEL with an UF of 1,000 is protective of the developmental, reproductive, and carcinogenic effects of vinclozolin's antiandrogenicity.	Combined chronic toxicity/ carcinogenicity-Rat

Table 4: Health risk assessment of vinclozolin.



Scheme 3: Bisphenol-A (BPA).

BPA exposure can influence brain development and can cause some behavioral changes in exposed humans [39]. Bisphenol-A has estrogenic potential. The worst scenario is that a developing fetus is exposed to high amount of BPA through the placenta. This may have severe effects especially on male fetuses, on testes size and Sertoli cells. It may cause testicular dysgenesis syndromes in new born kids, causes higher risk of testicular cancers or fertility dysfunctions due to decreased sperm counts. Typical BPA levels in human plasma are shown in Table 5.

Because polycarbonate plastics and epoxy resins are used in the production of countless plastic items such as water bottles, sport and medical and dental filing equipment and sealants, household electronics or even the eyeglasses, avoidance of exposure is impossible.

This compound can act through a variety of physiological receptors, like genomic estrogen receptors, membrane bound estrogen receptors, androgen receptor and thyroid hormone receptors [40].

BPA exposure may induce pubertal onset, disruption of estrous cycles, prostate diseases, prostate neoplasia, abnormal mammary gland development and presence of intra-ductal hyperplasia and pre-neoplastic lesions in adulthood, uterus alterations such as cystic endometrial hyperplasia and cystic ovary abnormalities. BPA can also induce changes in the brain and behavior, including abnormal development of sexually dimorphic regions, abnormal steroid receptor levels, hyperactivity, aggressiveness, changed socio-sexual behaviors and makes the exposed individuals more prone to different kinds of addiction. BPA disturbs glucose homeostasis and alters body weight and composition. In general, early life exposure to BPA promotes a variety of adult onset diseases states [38].

BPA can promote hypo-methylation and hyper-methylation. It can modify the expression of several estrogen receptors in the brain. BPA can enhance or decrease the rate of DNA methylation depending on its dose. BPA increases the expression of the HSP70 and ecdysone receptor genes and protein for Ezh2, histone methyltransferase in cancer cells, especially in mammary tissues. There are more than one epigenetic mechanism involving in transgenerational actions and caused by BPA [15].

BPA can affect onset of meiosis in both animal and *in vitro* tests. It can interfere with the germ cell nest breakdown in animal experiments. BPA is recognized as an ovarian toxic chemical. BPA accelerates follicle transition in different animal species. In multiple animal models and human females, it can alter steroidogenesis and reduces oocyte quality. This compound can impair uterine endometrium proliferation, decrease uterine receptivity and increases the risk of implantation failure in animals and because of these effects, BPA is a known uterine toxicant. Exposure to BPA is associated with adverse birth outcomes, hyperandrogenism, and sexual dysfunction. In male animals, BPA works as a testicular toxicant and can affect male reproductive system in both human and animals [40].

Animal evidence: Exposure to low doses of BPA during gestation causes immediate and long-lasting transgenerational effects on mRNA of the brain and can affect social behavior and the brain function. *In vivo* perinatal exposure of rodents to BPA may modify their sexual

differentiation of brain. Prenatal exposure of mice to BPA increases anxiety, aggression, decreased novelty seeking and different kinds of cognitive [36].

When rhesus monkeys were exposed to BPA, less social behavior in the form of exploratory behavioral changes were observed among the progenies. At 5 mg of BPA per kilogram of body weight for the period of 7-10 days before pairing and after pairing and 20 µg BPA per day over the last 10 days of gestation mixed in food caused transgenerational changes in mice regarding behavioral and socio-sexual behavior [36].

Gestational exposure to BPA influences the display of juvenile social behaviors and alters DNA methylation. BPA has transgenerational effects on sperm fertility and other reproductive parameters in male rats [36]. In the first generation, exposure to BPA decreased social preference and interactions and increased play soliciting behavior, but in F₂ generation BPA increased social behavior and decreased nonsocial behaviors and these patterns persisted into fourth generation. The fourth generations were never directly exposed to BPA. BPA may decrease the number of estrogen receptor α, vasopressin receptor and vasopressin. Persistent changes in Avp (vasopressin) and Oxt (Oxytocin) levels in F₄ are occurring via heritable epigenetic process. DNA methylation within regulatory regions of Avp can be modified by estrogens. Epigenetic modifications such as alterations of histones, chromatin modifications or noncoding RNA may explain the heritable actions of BPA on gene expression and behavior [36].

In mice, fetal exposure to Bisphenol-A can induce neoplasia and changes in mammary tissue [32]. As an example on behavioral effects of BPA in a 10 min reciprocal social interaction task, BPA exposed mice (5 mg/kg diet) spent more time sitting next to each other than control group mice but during side by side sitting had less interaction with each other than controls. Also anogenital sniffing was reduced in exposed groups but play soliciting behavior significantly increased (Figures 11 and 12).

Bis (2-ethylhexyl) phthalate (DEHP)

DEHP is vastly used as a plasticizer to produce polyvinyl chloride (PVC). Plasticizers such as DEHP can improve the polymer material's workability and flexibility (Scheme 4). There are several different trade names for this compound such as: FLEXOL plasticizer DOP, Essochem DOP, Palatinol AH, Palatinol AH-I (MED), Genomoll 100 and Vestinol AH. Only few data are available about its purity. Usually it is used as a mixture with some other chemicals e.g., with bisphenol-A. DEHP is colorless oily liquid at room temperature. It is poorly soluble in water and easily forms colloidal dispersions in water when the concentration is around 300 µg/l. The colloids are assumed to have less bioavailability in the body. The solubility of DEHP is 15% in well water and 42% in seawater [41].

About 51% of total phthalate used as plasticizer in different products is DEHP. Europe is a big producer of plasticizers by producing less than half of the world's DEHP total production. The production of DEHP in Western Europe was 595,000 tones, in 1997. Flexible PVC which contains DEHP is used in toys, building materials such as flooring, cables, profiles and roofs. It can be used in medical products like blood bags, dialysis equipment etc. The wide use of this compound can cause many possible health problems for human and environment [41].

Products containing DEHP can be recycled and reused, placed in landfill or incinerated but always some fraction of disposal material is remaining in the environment. Occupational exposure to DEHP occurs at the production sites or industrial uses of this compound. Consumer exposure occurs via food or medical products [41].

Exposed subjects	BPA ng/ml in plasma
Non-pregnant women	0-2
Pregnant women	0.3-18.9
Fetal serum	0.2-9
Amniotic fluid	0.55-8.3

Table 5: Human exposure to BPA.

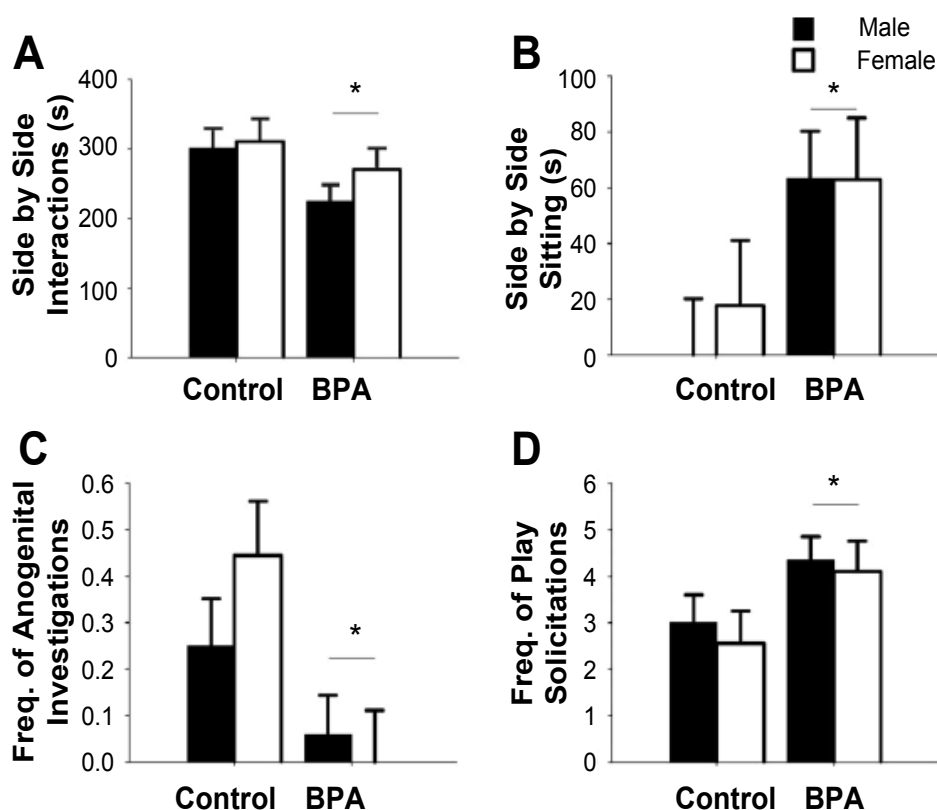
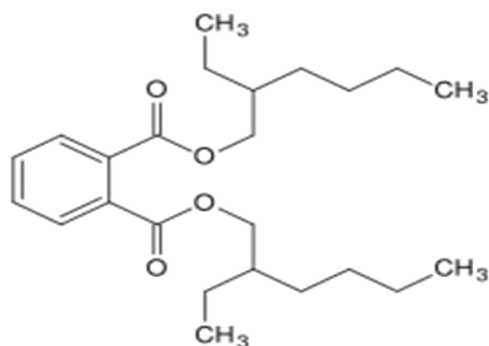


Figure 11: Mouse behavior display during 10 minutes reciprocal social interaction task, demonstrating that, gestational exposure to BPA affects juvenile social interactions in mice.



Scheme 4: Bis (2-ethylhexyl) phthalate (DEHP).

The colloids of this compound may change the physico-chemical property of the water. Concentration up to 3,000 mg/l can cause mortality in fish [41]. DEHP may affect lipid metabolism in birds because low concentration of 25 ppm ingested, increased lipid deposition in birds. Likely by that high concentration of DEHP (10,000-20,000 ppm) lowered plasma lipid concentrations and also lowered cholesterol in birds. Long term exposure of White Leghorn Hen exposed to 5,000 ppm of DEHP for a period of 230 days could stop the egg production and caused abnormalities of ovaries and hypertrophy of liver and kidneys [41].

DEHP is a known reproductive and developmental toxicant in both humans and animals. In mice, exposed to DEHP via oral exposure

in a chronic >90 days toxicity study, 1,000 ppm of DEHP produced a dose dependent and significant decrease in the number of litters as well as the number and proportion of pups born alive [41].

In another chronic study, young male rats were exposed to 500 ppm DEHP in the diet for 13 weeks and the result was minimal to mild sertoli cell vasculisation. Increased dose up to 5,000 ppm reduced testis weight, caused atrophy of seminiferous tubules and complete loss of spermatogenesis but the primary reproductive impairment was shown already at a dose of 500 ppm [41].

Possible Endocrine disrupting characteristics of DEHP has been tested in number of studies. DEHP can interfere with the endocrine function and it can also affect the sexual differentiation in both *In vivo* and *in vitro* studies. They have been addressed in the paragraph Plasticizer mixture.

Occupational exposure to DEHP during handling or intermittent contact is assumed to be 0.1-1 mg/cm²/day. During handling usually both hands are exposed, this corresponds to an exposure area of 420 cm² which results in dermal exposure of 42-420 mg/day. Inhalation exposure in industries may vary between 10-30 mg/m³. Consumer's exposure to DEHP may occur when the substance is released from products. Plasticizers are not chemically bound to the products and they can be released from the products during their life time. Oral exposure is the main route of exposure for small children as they suck, chew and bite their plastic made toys. Daily exposure for a child weighing 8 kg when he/she plays with a 10 cm² toy for 3 hours a day is assumed to be 200 µg/kg/day [41].

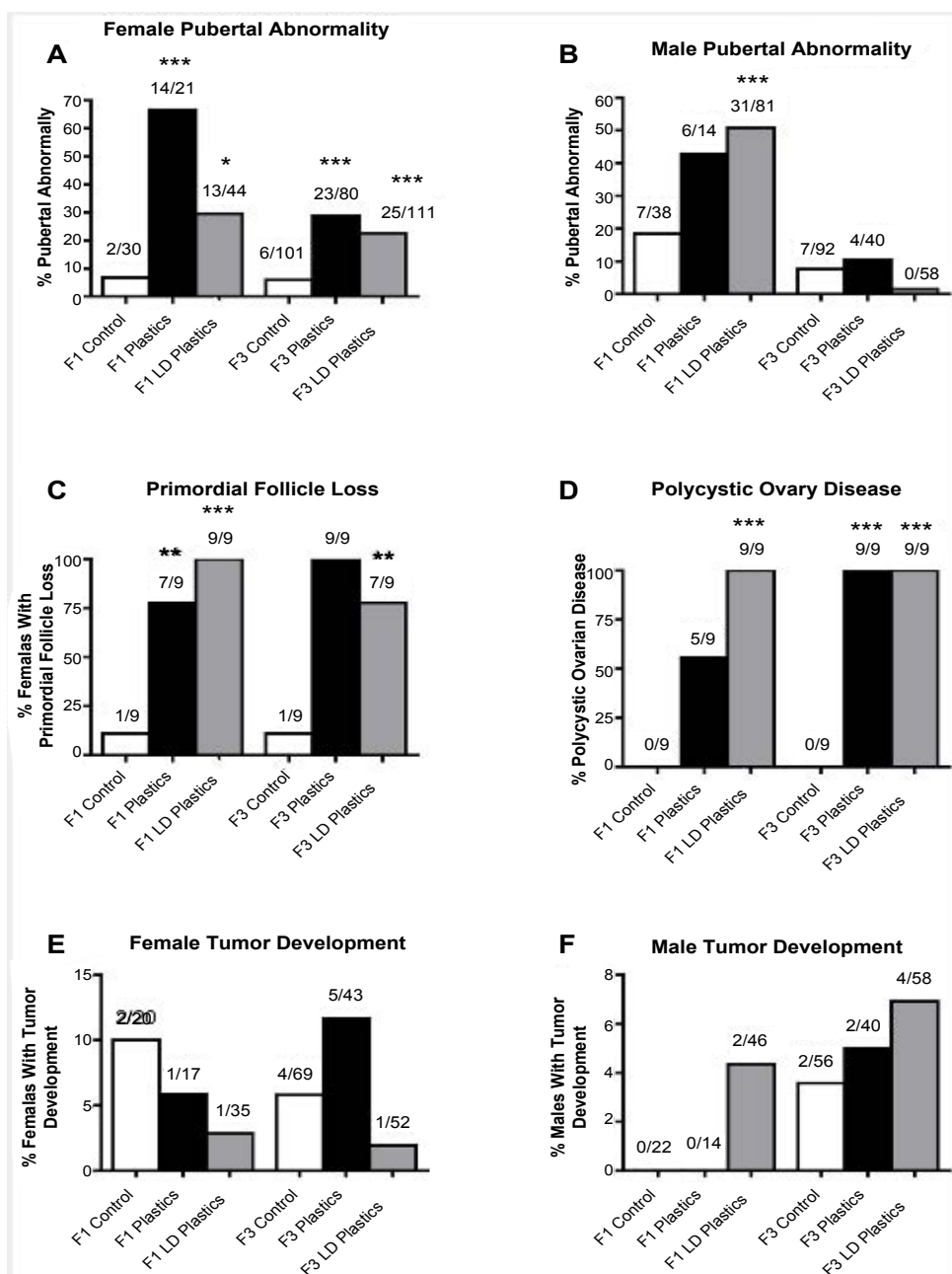


Figure 12: Incidence of different effects in F₁ and F₃ rat generation exposed to plastics (BPA, DEHP and DBP).

Exposure to DEHP in medical processes may be high. Table 6, demonstrates the amount or daily exposure to DEHP during medical processes:

Total DEHP intake for children is about 0.0194 mg/kg/day and for adults 0.00193 mg/kg/day. Urinary elimination half-life was estimated to be 12 hours for DEHP [41].

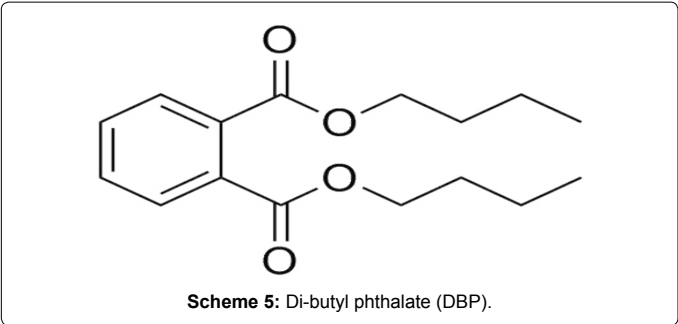
Di-butyl phthalate

DBP or di-butyl phthalate is used to give flexibility to plastics such as latex, adhesives, cosmetics, cellulose plastics and the solvent used in dyes. It is known to be very toxic for aquatic life and dangerous for environment. DBP may cause harm to unborn child and it is a known

risk factor for impaired fertility. In 1998 the production of DBP in Europe was estimated to be 26,000 tones [42]. At PH 9.0 temperature of 50°C, DBP has the half- life of 65.8 hours. This substance has high potential for bioaccumulation [43] (Scheme 5).

Air concentration of 10 µg/m³ DBP can cause visual injury on a variety of plant species, chlorosis, necrosis, leaf deformation and total loss of color in leaves and needles [42].

DBP concentration of 100 µg/l or more can cause harmful effects on aquatic life. The concentration of 10 mg/kg body weight per day in bird population can change the egg shell thickness, breaking strength, permeability of egg. DBP has estrogenic receptor affinity *in vitro* [42].



Scenario	Average daily Exposure (µg/kg BW/day)
Lond-term haemodialysis (adults)	3,100
Lond-term blood transfusion (adults) Haemophiliacs	30
Lond-term blood transfusion (children)	75
Transfusions (neonates)	1,700
Lifetime average daily dose (LADD) Infusion of platelets Haemodialysis Autopheresis	0.001 0.099 0.00058

Table 6: Daily exposure to DEHP during medical processes.

Exposure of a pregnant females to high dose of DBP, which is assumed to be more than 500,000 µg/kg body weight per day, enhances stillbirth, reduces birth weight, skeletal malformation and reproductive dysfunctions associated with infertility in both male and female progenies [44,45].

Human exposure to DBP can occur via workplace, consumer use of products or indirectly via environmental factors. There are three main routes of exposure, inhalation, ingestion or dermal contact with the substance. Workers who are handling DBP could be exposed to 0.1-1 mg/cm²/day of this substance. The area of the exposed skin in this case is around 420 cm², the exposure will be 40-400 mg/day [42].

The consumer inhalation exposure is assumed to be 2 × 10⁻⁹ mg/kg of the body weight per day. Children playing with the DBP containing toys will ingest 0.81 µg/kg body weight of this substance. Breast milk may contain 10-51 µg/kg DBP [42].

The epigenetic effects of DBP are addressed below, in chapter Plasticizer mixture.

Plasticizer mixture

Animal evidence: Exposure to plastics can induce transgenerational sperm epigenome manipulation. Interestingly, the effect of DNA methylation and its associations with changes in coat color of the exposed animals could be prevented by maternal dietary supplementation with sources of methyl group, for instance with folic acid with the phytoestrogen genistein [22].

In the study shown below, Harlan Sprague Dawley female rats were exposed to a mixture of plastic derived compounds BPA, EDHP and DBP by intraperitoneal daily injection during day 8/14 of gestation. F₁ generation rat offspring were bred to differently exposed females to obtain F₂ generation. Non-littermate females and males of F₂ generation were again bred to obtain F₃ generation.

Animals were exposed to the mixture of three different plasticizers. BPA, 50 mg per kg body weight per day, DEHP 750 mg per kg of the body weight per day and DBP 66 mg per kg body weight per day. Low dose plastics are half of the dose of the plastics exposed group [38].

The study revealed that, exposure of gestating female rodents during the fetal gonadal sex determination time to BPA, DEHP or DBP can produce transgenerational inheritance effects in F₃, such as testis, prostate, kidney and ovary effects, tumors and may induce obesity in subsequent generation [38].

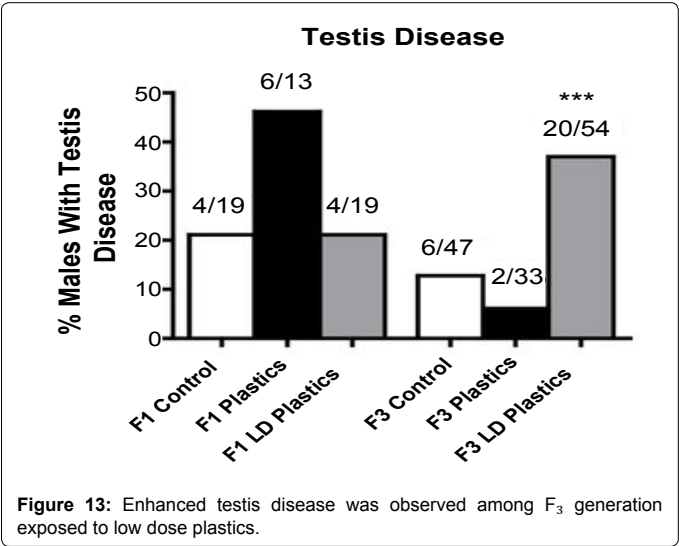
The testicular abnormality rate was much higher among F₃ litters of rat especially at the age of one year. Spermatogenic cell apoptosis was increased in F₃ male rats at the same age. All F₃ female rats were sentenced to ovarian polycystic disease [38].

The low dose exposure to plastics did not change the phenotype in F₃ generation significantly and only a few changes were seen in tissue weights (decreased uterine weight, decreased seminal vesicle weight), several transgenerational changes were seen on testes. Testis histopathology was abnormal (Figure 14). Seminiferous tubules were azoospermic and atretic and contained vacuoles which are usually filled with water containing inorganic and organic materials. Spermatogenic cells were gathered around each other and formed a mass at the center of seminiferous tubules and blocked the seminiferous tubules lumen. Spermatogenic cell apoptosis rate was increased and germ cell apoptosis reduced. The rat male testis disease incidence observations are shown in Figure 13 [38].

The histopathological results of F₃ Harlan Sprague Dawley rats when F₀ gestating females were treated daily with intraperitoneal injection of a mixture of plasticizers (BPA, DEHP and DBP) during days 8 to 14 of gestation. C and F groups in Figure 14 are control non-exposed F₃ rat groups, D and G are F₃ plastic exposed group and E and H are lower dose (one half of the dose as plastic exposed group). Testis and prostate disease caused by plastics are shown in D and G lineage. Histopathological changes leading to testis and prostate diseases caused by lower dose exposure to plasticizers (BPA, DEHP and DBP) are E and H lineage, in comparison with the control group or C and F lineage [38] (Figure 14).

Ancestral exposure to low dose plastics induced also obesity among the F₃ progenies (Figure 15). In the obese animals, abdominal adipose tissue was observed in most organs. Majority of F₃ females of plastic lineage developed both obesity and polycystic ovarian disease [38].

In F₃ females, effects on ovaries were seen, follicle loss and ovarian polycystic disease and pubertal abnormalities. Incidence of some other



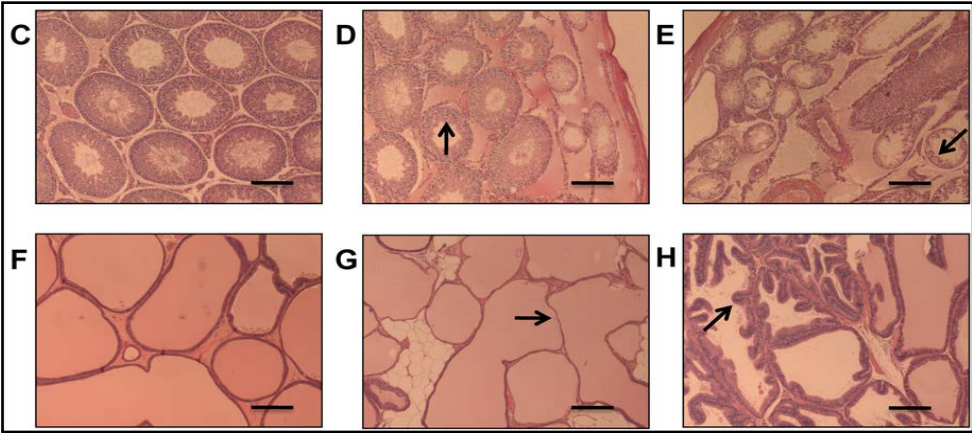


Figure 14: Representative micrograph showing histopathology images of adult onset transgenerational testis (upper row) and prostate disease (lower row) caused by low dose plastics (LD plastics) C and F controls, D and G the Plastics groups, E and H the LD plastics groups.

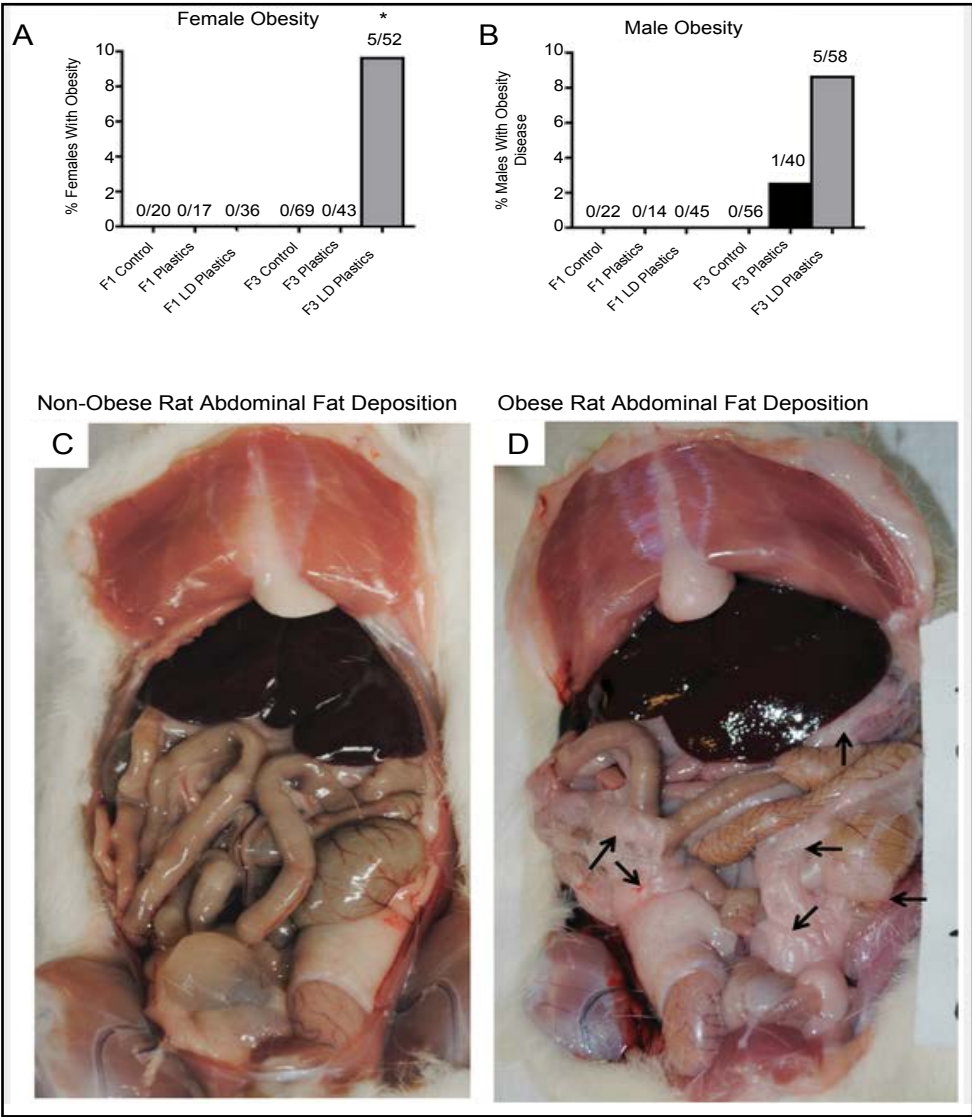


Figure 15: Obesity developed in plastic exposed rats (D). Picture C is control group.

diseases such as; cataract of the eyes, focal fat necrosis, interstitial pneumonia, liver degeneration, sinusitis, seizures, tremor, liver cirrhosis, swollen epididymis, blindness and vulvar abscess were higher in F₃ generation of the low dose plastic exposure [38].

Even though, there was no significant observable toxicity in F₁ generation of the exposed animals in the study of Manikkam et al. the phthalates DEHP and DBP are known to have reproductive and developmental functional disrupting characteristics. They may induce male reproductive tract abnormalities which are androgen dependent for development [38]. They can cause testicular functional disruptions in males. Fetal exposure to phthalates (DEHP and DBP) causes phenotypical changes in sex organs, testicular injuries, reduced daily sperm production, retention of nipples and decreased anogenital distance. Fetal exposure to DEHP and DBP reduce testosterone secretion into blood circulation and also increase the diameter of seminiferous cords and produce gonocyte multi-nucleation in rat fetal testis. In female rat's prolonged estrous cycle, estradiol deficiency and in adult female rats absent of ovulation are associated with phthalate exposure. Exposed female rats also have shown decrease in their fertility, disruption in pregnancy, abortion, fetal teratogenic abnormalities, skeletal and malformations, delayed age of pubertal onset, and altered number of ovarian follicles are all and all observed in experimental tests [38].

Plasticizers can cause changes in animal's behavior. They may affect the steroid aromatization process in male hypothalamus and cause behavioral changes seen as [36].

F₃ female rat low dose exposure to plasticizers showed increased proportion of early onset of puberty while among the F₃ male rats increased incidence of delayed onset puberty was observed [38].

In rhesus monkey, prenatal exposure to plasticizers can also cause endocrine disruption and androgenize the fetus [36].

Human evidence: Since early 1980s, there has been a lot of concern about the use of plasticizers and their effect on human health. By 1990s, many other issues regarding the use of plasticizers and their effect on human reproductive system and function of hormones were raised. One other major concern about phthalates was the exposure of children via breast milk, toys and medical equipment. As a result, the European Commission permanently banned the use of 4 plasticizers in all children equipment (EC 2015).

Plasticizers can express EZH2 gene and induce hyper-methylation at lysine residue in human breast cancer cells. Human placental cell lines when they are exposed to plasticizers has been shown miRNA expression manipulations [32]. Table 7 shows the estimation of normal daily human exposure to different plasticizers.

Breast feeding is the main pathway of exposure of fetuses and young infants to BPA [32].

Exposure to plasticizers during gestation is associated with hyperactivity and aggression in 2 years old children. In older children, plasticizers can cause anxiety and depression [36].

Exposure of a pregnant female to high dose of DBP which is assumed to be more than 500,000 µg/kg of her body weight per day may enhance stillbirth, reduces birth weight, skeletal malformation and reproductive dysfunctions and these are usually associated with infertility in both male and female progenies [38].

Jet fuel (jet propellant 8- JP8)

Jet fuel or aviation turbine fuel, is either colorless or straw colored type aviation fuel. It is designed for air craft which are powered by gas

turbine engines. Jet fuel is usually mixture of different hydrocarbons.

There are limited data on epigenetic effects of Jet fuel (one animal study) but they suggest that Jet fuel may have epigenetic effects.

Animal evidence: Tracey et al. have conducted a study on epigenetic transgenerational inheritance of reproductive disease and sperm epi-mutations after exposure of rats to jet fuel JP-8. Gestating female outbred Harlan Sprague Dawley rats were intraperitoneally injected during days 8-14 of fetal development. F₁ generation animals at 90 days of age were mated to the same lineage and F₂ generation animals were also mated in the same way to generate F₃ generation [21]. Alteration of sperm epigenome was studied for DNA methylation.

JP-8 promoted early onset of puberty in F₂ and F₃ female rats. In F₃ male rat testis, the jet fuel was able to increase spermatogenic cell apoptosis. Testosterone levels were reduced in F₃ generation of male rat. Males were more susceptible to transgenerational changes than females [21].

Permethrin

Permethrin is a pyrethroid which was produced and being used in 1973. It is a yellow brown to brown liquid or crystalline material. It is a common synthetic chemical, used as an insecticide, acaricide and insect repellent and it belongs to the family of pyrethroids. Annual production of permethrin is approximately 600 tones worldwide. It has a half-life ranging between 4 to 5 days in human brain and adipose tissues, 30-38 days in soil and 51-71 days in environment.

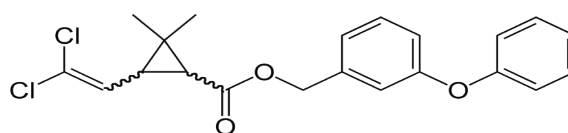
General populations are mainly exposed to permethrin via food products and dietary residues. Permethrin is neurotoxic [46]. It affects neuron membranes by prolonging sodium channel activation. It is highly toxic to cats and fish [47] (Scheme 6).

Animal evidence: In several animal studies, permethrin has not shown any effects of fertility or teratogenicity. No data exist in humans on this point. Permethrin is extremely toxic to cats, fleas and aquatic life, especially fish. In high doses it has tangible neurotoxic effects on birds and mammals. Permethrin has had no effects on reproduction of hens when administered in diet at a dose of 40 mg/kg. It is highly toxic for honey bees. It is estimated that 0.22 µg of permethrin can kill honey bees [48].

Permethrin has low acute toxicity to laboratory animals such as rats, mice, rabbits or even guinea-pigs. Common symptoms observed in animals are tremor, incoordination, hyperactivity, prostration and paralysis. In a sub-chronic study with 10,000 mg/kg permethrin administration to rats and mice for 26 weeks, increased liver weight, and liver hypertrophy and with signs of neurotoxicity, such as tremor, were observed. The NOAEL in rats have been defined to be between 20-150 mg/kg [48].

Bisphenol-A (BPA)	1 µg/kg/day
Bis (2-ethylhexyl) Phthalate (DEHP)	52 µg/kg/day
Dibutyl Phthalate (DBP)	5 µg/kg/day

Table 7: Estimated human exposure to different plasticizers.



Scheme 6: Permethrin.

Manikkam et al. conducted a study to find out whether the pesticide mixture of permethrin and the insect repellent N, N-diethyl-meta-toluamide (DEET) induces epigenetic transgenerational inheritance of disease sperm epi-mutations. F₀ gestating (day 8-14 of gestation) outbred Harlan Sprague Dawley female rats were exposed to the mixture of permethrin (150 mg/kg/BW/day) and DEET (40 mg/kg/BW/day) by intraperitoneal injection. Total disease incidence was evaluated in F₁ and F₃ generations [14].

The total testis disease incidence increased in F₁ and F₃ generations (Figure 16A) but no significant increase in prostate diseases (Figure 16B) was observed.

Figure 17D shows the histopathology images of adult onset transgenerational testis disease. Azoospermic and atretic seminiferous tubules were seen (marked by asterisk in the pictures). Vacuoles in basal regions of the seminiferous tubules are presented, gathered spermatogenic cells in the center of seminiferous tubules and lack of lumen. In prostate hyperplastic ductular epithelium was observed.

In contrast, the pesticide mixture (permethrin+DEET) did not promote a significant adult onset transgenerational kidney diseases in exposed male or female rats (Figure 18).

Total amount of adult onset transgenerational diseases in F₁ and F₃ rat generations by the permethrin+DEET increased statistically both in female (Figure 19A) and males (Figure 19B).

Altogether, 363 DMRs were identified in F₃ generation sperm epigenome. Observations strengthen the concept that this pesticide mixture (permethrin and DEET) can induce epigenetic transgenerational inheritance of adult onset disease [14].

Human evidence: No data exist at present on possible epigenetic effects of permethrin in humans. However, there are some information about low dose exposure to permethrin and its chronic effects on human.

Permethrin has quite low mammalian toxicity because it is poorly absorbed through the skin. It is also rapidly inactivated in the body. High dose exposure may cause nausea, headache, muscle weakness, excessive salivation, shortness of breath and seizures.

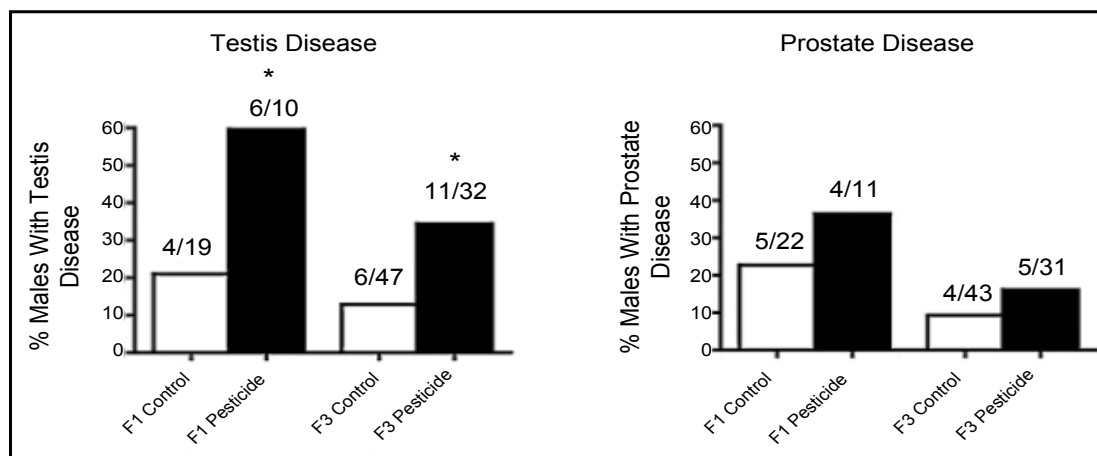


Figure 16: Effects of ancestral exposure to the permethrin and DEET mixture on testis and prostate diseases in F₁ and F₃ generation.

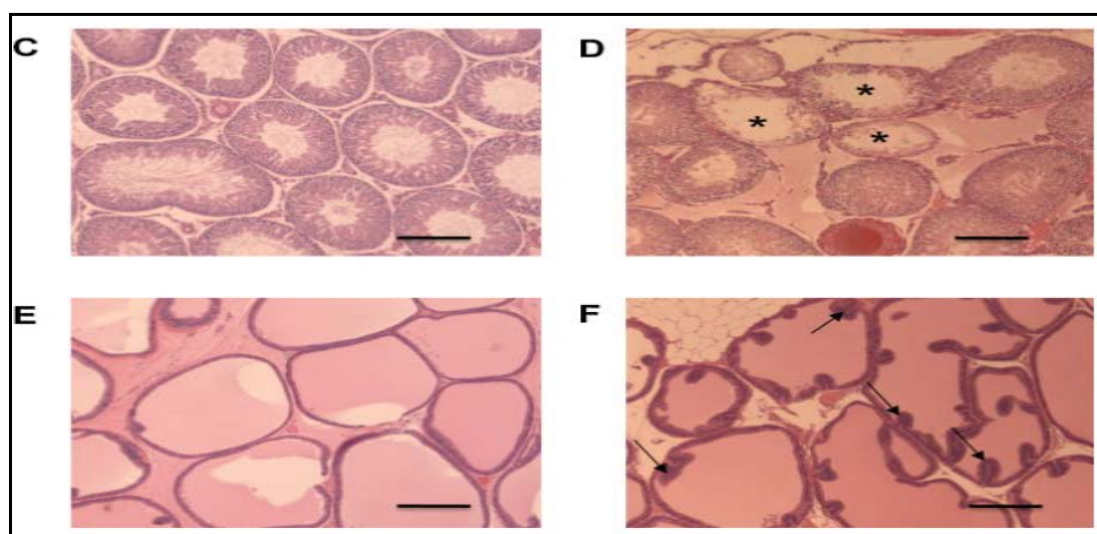


Figure 17: Histopathology images of adult-onset transgenerational testis (upper panel) and prostate disease (lower panel) in permethrin and DEET treated lineage. C and E controls, D and F pesticide mixture-treated animals.

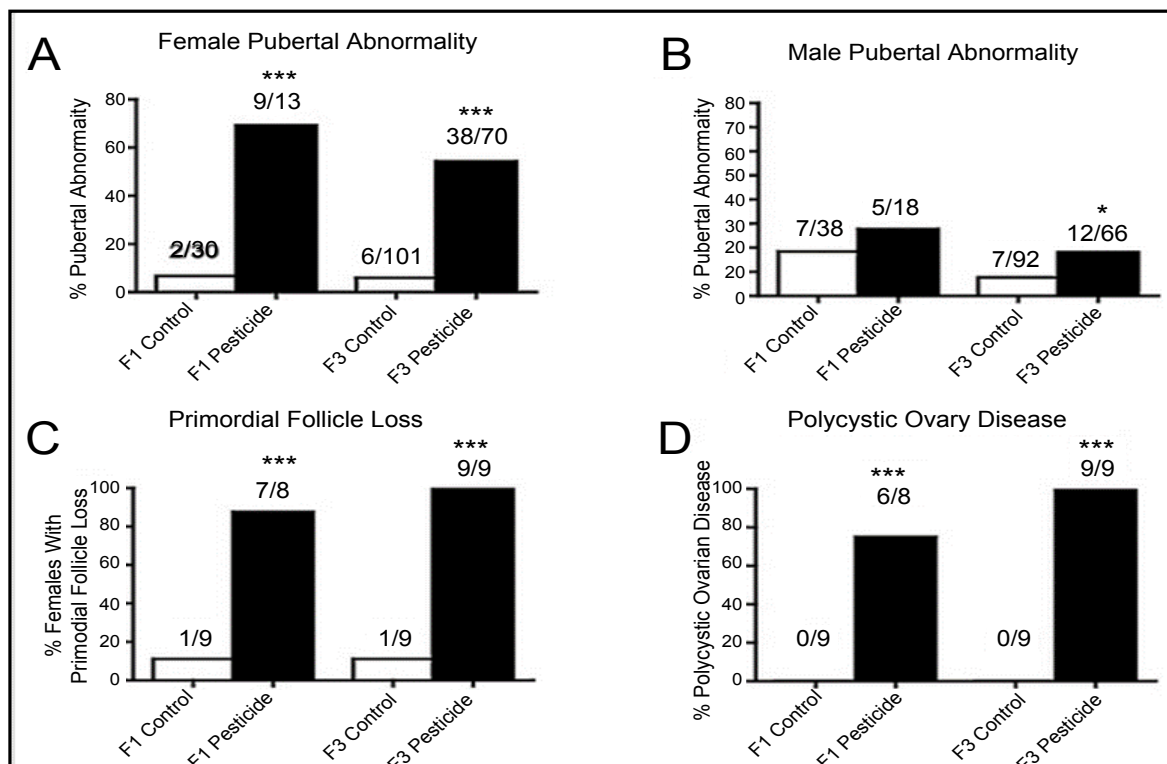
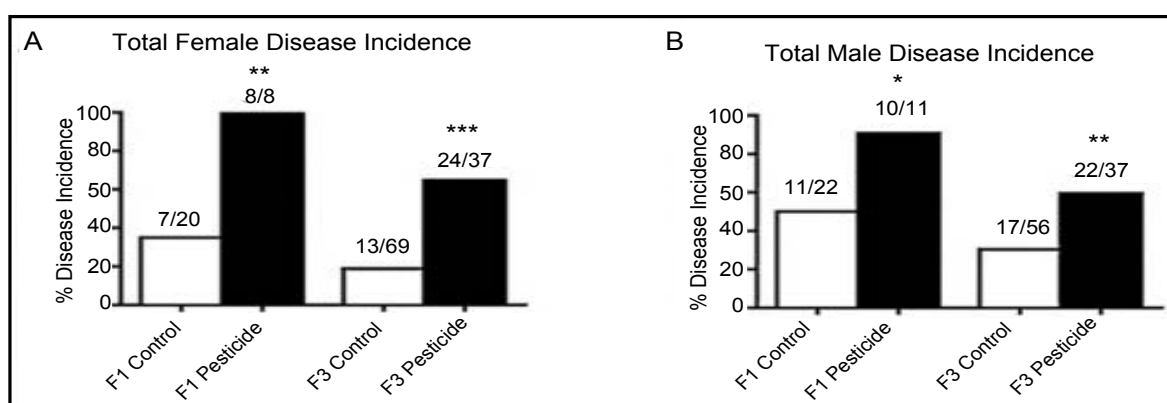


Figure 18: Exposure to pesticide mixture causes reproductive tract abnormalities.

Figure 19: Total amount of adult onset transgenerational diseases in F₁ and F₃ generation of pesticide mixture exposed rats.

In medicine, permethrin is used to treat some sort of skin infections because of its poor absorbance from skin and it is available as cream productions with different names. In US and British military, to protect soldiers from biting insects, new uniforms are treated with permethrin, so it is possible to imagine that the route of exposure is usually dermal [48].

“Environmental Protection Agency” (EPA) in December 2009 classified permethrin as a likely human carcinogen but the International Agency for research on Cancer (IARC) classified permethrin in 1991 as “not classified as to its carcinogenicity to human”.

There are few reproductive and developmental toxic effects of high doses of permethrin after oral administration available but not in human. There are also disagreements between the studies in which permethrin toxicity have been observed. No reproductive and

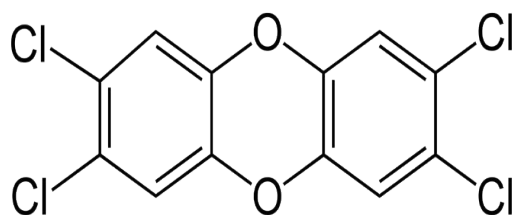
developmental toxicity data are still available from dermal exposure to permethrin.

Dioxin

Dioxin (TCDD) is an environmental contaminant and a known toxic compound present as a contaminant e.g., in the herbicide Agent Orange. Dioxin has the potential to promote epigenetic transgenerational inheritance diseases (Scheme 7).

Dioxins consist of three groups of chemicals:

- Polychlorinated dibenzo-p-dioxins (PCDDs)
- Polychlorinated dibenzofurans (PCDFs)
- Polychlorinated biphenyls (PCBs)



Scheme 7: Dioxins [Dioxin (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, TCDD)].

This group of chemicals is very resistant to environmental degradation, which usually happens as chemical, biological and photolytic processes. So they tempt to stay in the environment as persistent pollutants. Moreover, they bio-accumulate in human and animal tissues and can bio-magnify in the food chain.

Agent Orange is an herbicide that containing 2-4-5-trichlorophenoxyacetic acid and low concentration of TCDD impurities. During the Vietnam War, the United States of America used Agent Orange on Vietnam's forests in 1961-1971 as herbicide (antifoliant agent) to abolish hiding places of Vietnamese soldiers. The activity exposed some American soldiers and a large number of Vietnamese people and civilians to Agent Orange. The Agent Orange catastrophe in Vietnam killed 400,000 people and up to now, there are 500,000 maimed kids born with serious health defects in contaminated area [49].

A number of toxic effects of dioxin exposure have been observed in people who were exposed to Agent Orange. They include developmental defects, liver damage, weight loss, thymic atrophy and immune suppression and long-term effects such as lymphoma and leukemia. The list of diseases caused by Agent Orange is still growing. The transgenerational effects are still observable in new born children of that war [49].

In Taiwan, China, Japan and Seveso (Italy) also almost similar effects were observed after accidents where people were exposed to TCDD or dioxin-like compounds. Dioxin toxicity is largely mediated by Aryl-hydrocarbon receptor (AhR). Dioxin toxicity alters the transcription of target genes [22].

Animal evidence: Few studies have been done on transgenerational effects of dioxins. The first animal study was conducted to demonstrate the transgenerational potential of dioxin on mouse fertility. Nephrotoxicity in rats observed in shape of creatinine, urea and nitrogen level increase in blood which lead to kidney histopathological alterations [50].

In the study conducted by Manikkam et al. transgenerational inheritance of dioxin toxicity was studied in rats. F₀ gestating rat females were exposed to dioxin during fetal days 8-14. The females were administered daily intraperitoneal injections of dioxin (TCDD 100 ng/kg BW/day) [21].

In F₁ generation 13-% of the female animals showed the pubertal abnormality, half of this 13-% had early pubertal onset and the other half had a delayed puberty. In the control group only 7-% of females had the pubertal abnormalities and all of them had a delayed pubertal onset. The amount of pubertal abnormalities was increased up to 47-% in F₃ female rats and all of them showed early onset of puberty. In the control group of F₃ females, only 6-% of the females had pubertal abnormalities and the majority of them had early pubertal onset [21].

Effects of TCDD exposure on ovarian function and steroid level of the female rats were also demonstrated [21]. Ovarian weights and the

corpora lutea and follicles numbers were reduced. Ovarian primordial pool size was decreased. Spermatogenic cell apoptosis was affected trans-generationally. TCDD also caused reduction of testosterone level in F₃ male generation. No changes in female hormonal levels was observed. Therefore males are more sensitive to TCDD-induced transgenerational hormonal changes.

In animal experiments, dioxin has caused cleft palate and kidney malformations in new born mice. Dioxin also causes developmental neurobehavioral cognitive effects, endometriosis or the condition in which endometrium cells of uterus are flourished outside the uterine cavity, and other developmental reproductive effects, such as a decrease in sperm counts, female urogenital malformations plus immune-toxic effects [50].

Human evidence: Humans are exposed to dioxins mainly through contaminated foods, consumer products, and direct contacts via inhalation, dermal contact or ingestion. Developing fetuses and children, elderly, people with immune system dysfunctions like immunosuppression and women in child bearing age are the most susceptible to dioxin exposure.

The estimated half-life of dioxin is about 10 years in humans but it may still affect pregnant women even after 20 years of exposure. Serum or adipose tissues are used for dioxin intoxication measurements. Few epidemiological cohort studies are conducted mostly on carcinogenicity of dioxin. All these studies are done for the people who were exposed to the highest recorded amounts of dioxin. Some neurodevelopmental and endocrine disruptions such as thyroid hormonal level disruptions are associated with dioxin exposure.

In many areas of the world burning trash and plastic causes exposure to dioxin. During the last few years, human sperm count has a significantly declined and male infertility rate increased in some areas of the world. Environmentally induced epigenetic transgenerational effects are suspected as the main causal effects for these effects.

Exposure to environmental EDCs such as dioxin, can cause endometriosis, fibroids, subfertility or infertility and breast cancer in women and in men. They can decrease sperm quality and increase the incidence of subfertility or infertility or abnormalities such as cryptorchidism [7].

Several other adverse effects have been associated with dioxins: Prostate cancers, respiratory and lung cancers, multiple myeloma, type II diabetes, Hodgkin's disease, non-Hodgkin's lymphoma, soft tissue sarcoma, chloracne, porphyria cutanea tarda, peripheral neuropathy or damages of peripheral nervous system, chronic lymphocytic leukemia, spina bifida, hairy B-cell leukemia, Parkinson's disease and ischemic heart disease are significantly increased in Vietnam, especially in areas contaminated with Agent Orange. Many of these effects are transferred into the next generations. However, the contribution of epigenetic mechanisms as causative mechanisms to cause these transgenerational effects are not yet known [49].

High dioxin level exposure in human may induce chronic kidney disease. Prenatal exposure to dioxins may enhance the risk of immune-toxicity, glomerulonephritis and mesangial proliferation in human. Early onset of menarche and puberty in girls which is the result of dioxin exposure may lead to the disruption of brain development, may cause endocrine disruption and increase disease susceptibility [51].

After the Seveso accident (in Italy 1976), leading to wide dioxin exposure of inhabitants, different effects on reproduction has been observed: ovarian tumors, slow follicular maturation, morphological changes of ovary, abnormal cyclicity with disruption of estrus cycle,

reduced ovulation and early menopause among the female population [49]. The reason is assumed to be the ancestral exposure of gestating females to dioxin, which promoted altered fetal gonadal development and the alterations of the male germline by epigenetic reprogramming of the germline and epigenome production, which can transfer the ovarian diseases trans-generationally.

At present, the lowest exposure level to cause specific epigenetic changes in humans are not known.

Cigarette smoke

Cigarettes, cigars and pipe are all produced from dried tobacco leaves. Some other ingredients are also added to tobacco to add flavor and to make smoking more pleasant for smokers. The cigarette smoke is a mixture of chemical compounds which are produced by burning tobacco and its additives. Cigarette smoke contains 7,000 different chemical compounds and at least 70 of them are known carcinogens. Some other chemicals in tobacco smoke can cause heart and lung diseases or even sudden death. Tobacco smoke contains e.g.:

- Benzene
- Formaldehyde
- Methanol
- Cyanide
- Acetylene
- Ammonia
- Polycyclic Aromatic Hydrocarbons

In addition, it contains poisonous gasses like carbon monoxide and nitrogen oxide, tar and nicotine. Nicotine is the addictive chemical that produces a variety of effects in human body. Tobacco leaves may contain some radioactive materials depending on the soil that they are cultivated on [52]. Smoking in the United States causes at least 30-% of all cancer deaths. It has been estimated that among men 87-% and among women 70-% of all lung cancers are caused by cigarette smoke. Cancer of nasopharynx or upper throat, nasal cavity, paranasal sinus, lip, larynx or throat, esophagus or swallowing tube and also bladder cancer are associated with tobacco smoke in smokers [52].

It has been estimated that 300 million people are suffering from asthma worldwide. This number will increase up to 400 million by the year 2025. Maternal smoking is a well-known contributor for childhood asthma [6].

Animal evidence: *Intrauterine* exposure to nicotine can cause asthma like phenotype in rats and it is inherited to the next generations of rat for at least two subsequent generations [6]. The potential mechanism of airway hyper-responsiveness to nicotine challenge was analyzed by determination of the expression of the airway contractility and differentiation markers by lung fibroblasts. Activated γ factor and peroxisome proliferation are the molecular functional markers in fibroblast. Fibroblasts were isolated from the F_3 generation rat lungs. The numbers of fibroblast were significantly decreased among the F_3 pups. Abnormal pulmonary function occurred in upper airways especially among males. This was considered as the first signs discovered on transgenerational transmission of asthma disease phenotype in the third generation offspring, following the prenatal nicotine exposure of the F_0 generation rats [6].

In this study, pregnant F_0 generation Sprague Dawley rats was exposed to 1 mg/kg nicotine from embryonic day 6 of gestation until

the PND 21. Then the follow up continued through delivery and F_1 , F_2 and F_3 generations. The F_1 and F_3 generation pups were subjected to pulmonary function, airway contractility, mesenchymal differentiation mRNA and protein level and tracheal tension tests at PND 21.

Figure 20 shows the effects of the perinatal exposure to nicotine on total respiratory system resistance (RrS) and total compliance (Cdyn) in F_1 and F_3 rat generations in comparison with non-exposed group. There was a highly significant increase in total airway resistance (Rrs) and a huge decrease in total compliance (Cdyn) with challenge of methacholine in both F_1 and F_3 generations.

Transgenerational (TG) transmission of perinatal nicotine exposure caused asthma in both male and female rats but male rats were more susceptible for asthma induced by nicotine: The specific tracheal contractility response occurred only in F_3 male offspring. Thus, lung functional effects of perinatal exposure to nicotine can be transferred to next generations at least up to the F_3 generation. Asthma caused by perinatal nicotine exposure can transmit into subsequent generations [6].

Human evidence: Maternal smoking increases risk of adverse health outcomes like low birth weight, childhood cancers, reduced lung function, high blood pressure, obesity and early respiratory diseases in children [53].

In majority of developed countries, the amount of early pubertal onsets has significantly increased among girls during past several decades. Early onset of puberty may induce; behavioral, mental and endocrine physiological effects and increase the incidence of adulthood onset diseases. Changes in primordial follicle pool size and total number of the follicle are decreased among the female population. The primordial follicle pool develops early in fetal life and the follicle pool size is the ovarian reserve for oocyte production. Environmental exposure to tobacco smoke has transgenerational effect on both total number of the follicles and the size of the primordial follicle pool size [54]. Premature ovarian failure (POF, premature follicle loss) is one important reason for infertility all around the world. Few years ago people thought genetic origin is the only fact which may induce POF but new studies suggest that environmental exposure to tobacco smoke may also induce female infertility and premature onset of menopause [54-56].

In the study of Joubert et al. the role of in utero exposure to tobacco smoke was studied in early programming for childhood and adulthood illnesses. Multiple adverse health outcomes in children were linked to maternal smoking but the mechanisms are still unclear. Pregnant women who participated in the study were categorized according to their blood cotinine level into 4 different groups; nonsmokers with the cotinine level of less than 0 nmol/L, low cotinine more than 0-56.8 nmol/L, moderate >56.8-388 nmol/L and high >388 nmol/L cotinine groups. The cotinine level more than 56.8 was considered to indicate maternal active smoking. 1,062 infant cord blood samples were also taken after birth. A significant association was recognized between the maternal plasma cotinine and cord blood DNA methylation of the new born children [53].

One group of the chemical compounds which exist in tobacco smoke is polycyclic aromatic hydrocarbons (PAHs). PAHs in tobacco smoke bind to the AhR and translocate them into nucleus to form a heterodimer plus AhR nuclear transporter which can bind to DNA sequence. Maternal smoking in pregnancy has been shown to increase CYP1A1. Xenobiotic response elements start to initiate CYP1A1 expression to detoxify and excrete PAHs. In pregnant female smokers, lower methylation of AHRR and higher methylation of CYP1A1 has been observed in comparison with non-smoking pregnant women [53].

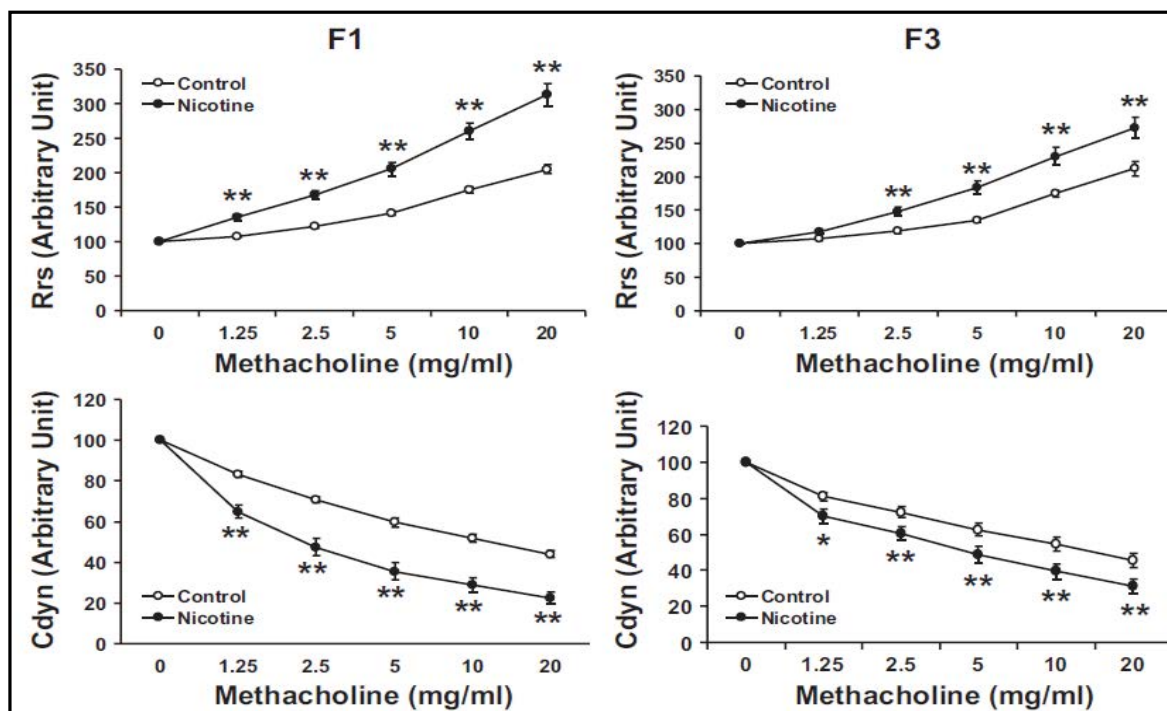


Figure 20: The effects of perinatal nicotine exposure on total airway resistance (Rrs) and total compliance Cdyn.

In the study by Shenker et al. about 520000 individuals with standardized life style from Italy participated in cancer and nutrition cohort study. Methylation level in DNA extracted from peripheral circulating mononuclear cells of the lung tissue at two different areas of AHRR gene was significantly decreased in smokers in comparison with none or previous smokers measured between 4.6 and 7 years in average. AHRR knockdown has been shown to increase tumor cell production and increase the invasiveness of tumor cells in a range of tissue types. It also increases breast, colon, lung and ovarian cancers. Even if a smoker quits smoking, the expression of some genes, including AHRR will never return to their previous levels [57].

AHRs receptors are important receptors in a sense that, they are tight to a broad range of biological compounds and synthetic environmental pollutants. Increase in AHRR, will lead to the activity of AHR pathway, on the other hand, a decrease in AHR pathway mediates the formation of tumors or activation of carcinogenesis [57].

The cohort study of “Avon longitudinal study of parents and children”, done during 1991_1992 by the University of Bristol, 14,000 pregnant women participated in the study. The study shows that toxic substances that are present in cigarette smoke enter the circulation of smokers from alveolar capillary system and these toxic substances can directly affect the epigenetic profile of circulating white blood cells (WBCs) of smoker. Increase in mortality rates, cardiovascular diseases, diabetes and obesity were associated with transgenerational effects of paternal childhood smoking to their offspring's [3].

Epidemiological and experimental studies have found associations between the effects of prenatal tobacco smoke exposure and disorders such as allergies, diabetes, neurodegenerative diseases and cardiovascular diseases. It has been suggested that the fetal and early postnatal exposure to tobacco smoke can influence developmental plasticity and result in altered programming. The programming process

is responsible for lasting functional change of organs that can end up in variety of complex diseases. Epidemiological studies have e.g., shown the transgenerational inheritance transmission of asthma [6,50].

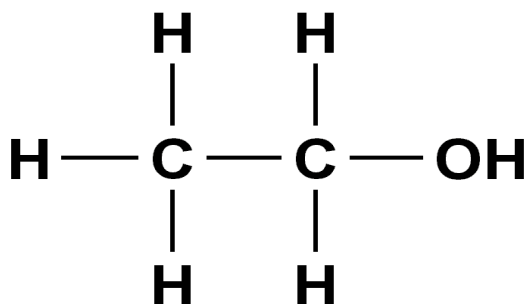
It has been implicated that there are specific time periods in the life when individuals are more susceptible to asthma triggers such as tobacco smoke. Epigenetic modifications can occur mostly during these susceptible periods. Several epidemiologic studies suggested that the risk of asthma transmission caused by maternal exposure to tobacco smoke may continue across subsequent generations [50]. Moreover, some *in vitro* experimental studies support this idea by demonstrating that DNA methylation of genes are critical to T-helper differentiation and can induce polarization toward or away from the allergic phenotype [50].

As epigenetic mechanisms to increase the asthma risk genomic imprinting, histone modification, altered DNA methylation of regulatory sequences in some genes and regulation by microRNA have been suggested also to tobacco smoke [50].

Ethanol

Ethanol is a flammable and colorless chemical compound. It has a strong odor and has been used as an antiseptic, solvent, fuel or even as an active fluid in thermometers because of low freezing point. It is a constituent of alcoholic beverages. Ethanol is a neurotoxic psychoactive compound and one of the oldest recreational drugs used by ancient humans (Scheme 8).

When alcoholic beverage is swallowed, it goes through the stomach and passes through small intestine. In the small intestine ethanol is rapidly absorbed and distributed throughout the body. Water content of tissues contribute to its tissue distribution. Ethanol is found more in blood and brain tissues than in muscles or fat tissues. Blood alcohol levels are used to determine the degree of ethanol intoxication. The



Scheme 8: Ethanol.

majority of people show the measurable mental impairments already at 0.05% of blood alcohol. At 0.10%, mental impairments is associated with some physical signs such as unsteady walk. Un-consciousness ensues at 0.4-% and above 0.5-% it is lethal [58].

There are some evidences in experimental animals showing epigenetic effects of ethanol on gestational programming of adult phenotype [59].

Animal evidence: Several studies in animal models suggest that ethanol is capable to induce a wide range of developmental abnormalities. There are three particularly important developmental periods that are peak periods of epigenetic reprogramming: preconception, preimplantation, and gastrulation. Ethanol has been shown to interfere with carbon metabolism, DNA methylation, histone modifications, and noncoding RNA. Many of these effects have important roles for epigenetic mechanisms in the etiology of fetal alcohol spectrum disorders [60].

Animal models show that early alcohol use may have detrimental effects on the developing brain leading to cognition problems later in life [61].

In vitro exposure of ethanol or acetaldehyde may impair the murine embryo development [62]. In a study where mice received 10-% ethanol in drinking water during gestational period of days 0.5-8.5. Changes in expression of an epigenetically sensitive alleles (Avy), which controls coat color, were observed [59]. The does used for mice was about 5 ml of alcohol mixed with 50 ml of its drinking water per day. Variable expression of the sensitive allele caused predictable range of coat colors in mice. Ethanol exposure can influence the Avy expression early in development and increase the risk of transcriptional silencing at this specific locus [59].

In an adult female rhesus macaque monkeys receiving the increasing ethanol dose in drinking water (from 0.75 g/kg / day to 1.5 g/kg/day for 6 months) altered follicle cell gene expressions were observed, associated with oocyte follicle loss and reduced preimplantation embryonic development [63]. Manipulations of oocyte and cumulus cell gene expression were observed. Spontaneous abortion risk was raised during early gestation. The ethanol consumption resulted also in elevated rate of abortion among animals. The average amount of administered alcohol in this study was assumed to be equivalent with 4-5 drinks in young human females [63].

Human evidences: There are increasing evidences emphasizing that ethanol modifies several epigenetic parameters in liver and GI tract. Ethanol can influence the gene expression [64]. Ethanol can bind to the transcription factors and modify chromatin structure. Most of the mechanism behind most of these epigenetic effects is DNA

methylation. Binge ethanol consumption can affect oocytes just before the ovulation [63].

Alcohol consumption during adolescence period which is a period of rapid growth and physical changes can disrupt the development to cause long term consequences. Adolescents who are heavily drinking alcohol may have liver, bone, growth and endocrine developmental problems [61].

Fetal Alcohol Syndrome (FAS) is the largest cause of mental retardation in Western world. This birth defect may develop in children whose mother has been drinking alcohol during pregnancy. FAS is characterized by facial anomalies, microcephaly, mental handicaps, low birth weight, learning disabilities, central nervous system dysfunction, hyperactivity, poor coordination, attention problems, etc. It can be diagnosed when growth retardation, CNS damage or head and facial abnormalities are recognized either in utero or postnatally.

Facial malformations caused by maternal alcohol use are shown in Figure 21. Acetaldehyde is toxic metabolite of ethanol. Average chronic exposure to acetaldehyde from alcoholic beverages is estimated to be 0.112 mg/kg/body weight/day. This amount of acetaldehyde can increase the cancer risk of an exposed individual up to 7.6% [65].

Food and Nutrition

All nutrients are extracted from food, enter the metabolic pathways, and are then manipulated, modified and molded into molecules that our body can use them. This is one of the most important pathways responsible for making methyl groups and can result in epigenetic changes and silencing a gene. Environmental factors, such as stress or mal nutrition, especially during childhood can cause epigenetic changes that last in adulthood or into the subsequent generation [26].

Diet can change epigenetic phenomena such as DNA methylation and histone modification. Modifying the expression of some important genes will associate with physiologic and pathologic processes including embryonic development, aging and carcinogenesis. Nutrients and bioactive food components can affect the epigenetic phenomena either by direct inhibition of enzymes that catalyze DNA methylation or histone modifications. They can even alter the availability of necessary substrates for those enzymatic reactions. Nutritional epigenetics can be a good tool to prevent pediatric developmental diseases and cancer [66].

Dietary supplements can play an important role in epigenetics, for instance; hypo-methylating dietary supplements can provide DNA hypo-methylation and by that, they can protect the epigenomes. High soy diet may increase the DNA methylation [12].

Recently, there are some studies done to indicate that, in utero exposure to some nutritional supplements and chemicals can affect the developing embryo which can result in adulthood diseases [59].

Environmental factors such as food and nutrition, can promote epigenetic effects and disease susceptibility [14]. During childhood cause adult disease in offspring [3].

There are some evidences available showing that increased morbidity and mortality are associated with parental or even grandparental nutritional status. This can introduce the possible role of epigenetic mechanisms and transgenerational effects on fetal programming [6].

Animal evidence

Food and nutritional supplements can affect the genome of developing mouse embryo and lead to adult disease. Dietary methyl

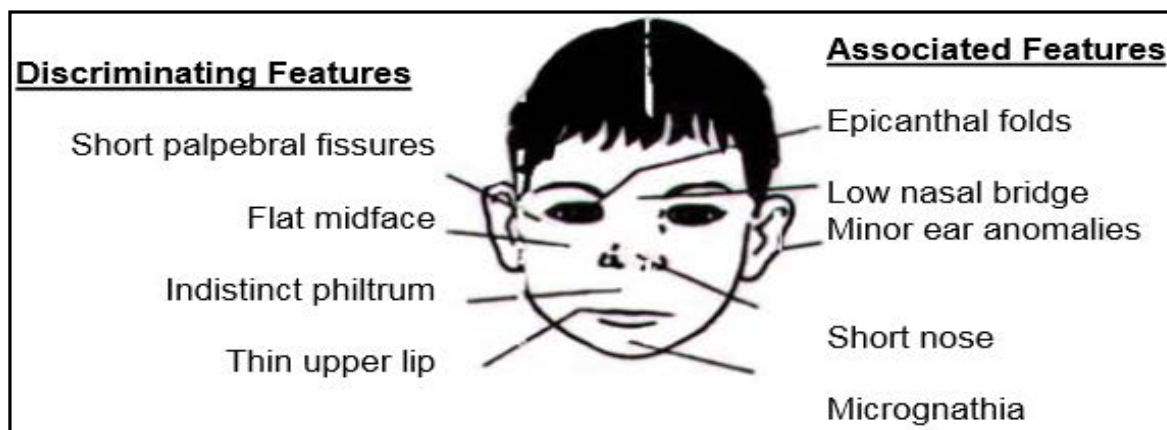


Figure 21: The possible facial malformation caused by Fetal Alcohol Syndrome.

supplementation with folic acid, Vitamin B₁₂ and other nutritional supplements can influence the heritable phenotype of the agouti mice offspring via increased CpG methylation. The result of complete unmethylation of agouti gene in mouse is a yellow coat and obesity and the animal is prone for diabetes and cancer. When the agouti gene is methylated in normal mice, the coat color is brown and the disease incidence is significantly lower. When pregnant yellow mice are fed with methyl rich diet, most of the pups became brown and healthier. The result of this experiment shows that our health is not only dependent on what we eat, but it also depends on what our parents have eaten [12]. Early postnatal diet can manipulate the epigenetic regulation of imprinted gene *Igf2* and result in several human cancers in murine model studies [67].

Human evidence

Environmental factors such as food and nutrition, can promote epigenetic effects and disease susceptibility [37]. Famine in China during 1959-1960, enhanced the rate of schizophrenia in that area and there are some suspicions that epigenetic effects caused by malnutrition might have a role in enhanced schizophrenia diseases there [3].

Swedish scientists have investigated effects of nutrition on death due to cardiovascular diseases and diabetes. The study aimed to find out, whether cardiovascular disease and diabetes caused by malnutrition, can transfer to the next generations. They estimated the access of each individual to food by examining records of annual harvests and food prices in Sweden across three generations by starting as far back as 1890s. The Swedish scientists demonstrated that; if the father did not have enough nutrition especially during the critical period in his development just before the puberty, his sons were less likely to die from cardiovascular diseases. On the other hand, diabetes related death in grandchildren was significantly increased when their grandfathers had over eating or used a lot of nutrition. This study brought up the idea that diet may cause changes in genes and these changes can pass through the next generations. These changes in genes can make individuals more susceptible to certain diseases [13].

When the diet during pregnancy contains no meat and fish, the risk of hypospadias increases among offsprings. Increasing risk of hypospadias for up to 4-fold is reported [68].

To have a better understanding of the relation between diet and epigenome, one chance is mapping the human gene variations. This can give a window into each person's medical needs. There is a possibility

that in the future of nutrigenomic science, the nutritionists will take a look at patients methylation pattern and then design a personalized nutrition plan for that individual [69].

Conclusions

Environmental exposure to EDCs may disturb the testis and ovary function and influence fertility. Women, during first trimester of their pregnancy are the most sensitive population to epigenetic changes.

Even little maternal occupational exposure to endocrine disrupting chemicals during pregnancy e.g., through food or clothing, may contribute to hypospadias malformation of the third generation boys in human [27]. Human being, during years of living on this planet adapted with many environmental changes by becoming more resistance or more weak to them. These adaptive changes might have been the results of epigenetic changes during our history. This is true also for other living organisms such as plants, worms, flies, rats, mice. Epigenetic effect may answer some of these adaptability and susceptibilities.

In human female, epigenetic mechanisms are suspected to contribute to several different effects. Early onset of puberty accelerates bone mineralization and short height in adulthood and it can also predispose those females for breast cancers. Early onset of puberty in girls, caused by environmental exposure to endocrine disrupting agents can disrupt their health by affecting their brain development, endocrine organ function and growth and enhance susceptibility to disease. Obesity can also be one of the results of transgenerational effect. Obesity which can be induced by epigenetic effects in F₃ or indirectly exposed generation is usually associated with some other diseases such as cardiovascular disease, type 2 diabetes and decreased in average life expectancy of human. Obesity in women can cause amenorrhea and infertility. Majority of obese women have polycystic ovarian diseases [44].

Primary transgenerational diseases caused by epigenetic changes are observed in several different organs. Testis diseases, ovary diseases obesity and pubertal abnormalities are linked to epigenetic effect.

After the recognition of vinclozolin's transgenerational effects which raised the hypothesis that, estrogenic EDCs may cause the early pubertal onsets, Nilsson, Skinner, Manikkam and many other researcher groups have progressed the idea and step by step shown that not only direct exposure of the females may cause the onset puberty but also ancestral exposures of the previous generations may cause

the precocious puberty in their progenies. Several important and well known laboratories have come up with the same results and confirmed this hypothesis. Many follow up studies are done and many are still going on all around the world. There is a hope that scientists can determine the exposure levels and margins of exposure for such effects for EDCs in the future.

Compounds that have been considered safe can also cause epigenetic effects and endocrine disruption when they are mixed with other chemicals and we are usually exposed to the mixture of compounds. The data produced from guideline studies which are normally used to check the adverse effects of EDCs in high doses, are gathering mostly the information needed for the current EDC policy. The classical risk assessment paradigm is that a substance must show evidence of adverse effects that are increasing proportionally with the increase in dose to be considered as a dangerous substance. However, for most of the EDCs the dose response is not well known, especially in humans, and there is at present an ongoing debate among scientists, whether or not the effects of EDCs are “threshold effects” or “non-threshold” ones. The hormone or hormone mimic may overwhelm or down-regulate the responsible of endocrine system. Regulatory community may exclude crucial evidence of harmful EDC actions if the low dose studies from policy considerations are eliminated. It is also difficult to create a cause-effect relationships among environmental factors, epigenetic modifications and the occurrence of diseases [22].

The major unanswered question is: How the transgenerational changes can be detected? This and several other questions are to be answered in the future yet. The whole genome mapping of both histone modifications and DNA methylation can reveal in the chromatin whether they are normal or malignant at specific loci during development. Also utilization of genomic and epi-genomic approaches in controlled experimental settings can allow the identification of both short and long term effects of EDCs [15].

There are also other mechanistic questions still to be answered such as, the maintenance of the epi-genomic changes at specific loci via the germline and how it would be possible to determine the duration of transgenerational epigenetic traits. Again to find the answer, epigenome mapping of the germline cells is essential [15].

Many of EDCs such as DES were used as a drug, and the adverse effects resulted at therapeutic doses, no safety margins may be defined for the exposure. Animal studies support the role of environmental epigenetics in disease susceptibility and for several chemicals such effects have already been demonstrated.

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